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Development of central respiratory control in anurans: the role of neurochemicals in the emergence of air-breathing and the hypoxic response

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Abstract

Physiological and environmental factors impacting respiratory homeostasis vary throughout the course of an animal's lifespan from embryo to adult and can shape respiratory development. The developmental emergence of complex neural networks for aerial breathing dates back to ancestral vertebrates, and represents the most important process for respiratory development in extant taxa ranging from fish to mammals. While substantial progress has been made towards elucidating the anatomical and physiological underpinnings of functional respiratory control networks for air-breathing, much less is known about the mechanisms establishing these networks during early neurodevelopment. This is especially true of the complex neurochemical ensembles key to the development of air-breathing. One approach to this issue has been to utilize comparative models such as anuran amphibians, which offer a unique perspective into early neurodevelopment. Here, we review the developmental emergence of respiratory behaviours in anuran amphibians with emphasis on contributions of neurochemicals to this process and highlight opportunities for future research.

1. Introduction

Respiratory behaviours observed at adulthood often differ in their expression from those of early developmental stages. These disparities result, in part, from anatomical changes to the respiratory apparatus such as body wall and lung compliance, and muscle development. In addition, the connectivity and functionality of respiratory neural networks may change with age as a consequence of use-dependent maturation, changes in gene expression or, in the case of amphibians, to accommodate changing respiratory substrate (water vs. air). Mechanisms of rhythm generation, breathing pattern (episodic, continuous), ventilatory responses to chemoreceptive stimuli, motoneuron activity and neuromodulatory inputs can undergo substantial modifications as development proceeds, the nature of which can differ between (and within) vertebrate classes.

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Despite decades of research, the evolutionary origins of vertebrate air-breathing structures and underlying neural control mechanisms remain enigmatic. Central respiratory neural networks are often assumed to be homologous in vertebrates but data exist both in support of this view and against it. Anatomical evidence argues for homology between central respiratory oscillators of amphibians and mammals (Baghdadwala et al., 2016), and mechanistic studies have shown respiratory-related oscillators sensitive to CO_2/pH to be present in lamprey and amphibians (Hoffman et al., 2016). However, recent data suggest that the tetrapod lineage may be polyphyletic (Clack, 2012) and that "transitional" forms utilizing both water and air-breathing may have multiple evolutionary origins. Conceivably, this phenotypic plasticity could have driven evolution of non-homologous neural substrates sub-serving the highly adapted respiratory systems of extant vertebrates (Wright and Turko, 2016). Resolution of this debate will require explicit testing by complimentary methods. Comparative studies are desirable but must be interpreted cautiously as respiratory-related traits have been gained and lost through vertebrate evolution (e.g. air-breathing in fish). Phylogenetic analyses have been used historically but outcomes can depend on the substrate considered (anatomy vs. genes). Fate mapping of central respiratory neurons across vertebrate classes also offers promise. This debate notwithstanding, the pursuit of comparative respiratory physiology is still valuable as it informs on the fundamental principles governing respiratory neurophysiology, despite phenotypic differences. Anurans are an attractive model because species undergo emergence of obligate air-breathing due to developmental programs and as a consequence of transitioning between aquatic tadpoles and terrestrial (or semi-terrestrial) adults, permitting the study of complex neural mechanisms facilitating the development of adult respiratory behaviours. Other comparative models, such as lamprey and turtle, have also proven instrumental to our understanding of diverse respiratory control mechanisms; however, most investigations have been performed on mature animals (Bongianni et al., 2016; Cinelli et al., 2017; Hoffman et al., 2016; Johnson et al., 2016, 2007).

Respiratory development is brought about in part by genetically programmed and activity-dependent maturation of neurochemical pathways and by changing receptor/enzyme expression profiles. It should be emphasized that environmental factors also contribute substantially to maturation of respiratory behaviours in vertebrates (Baldy et al., 2017; Bavis and Mitchell, 2008; Ferner and Mortola, 2009; McDonald et al., 2015; Rousseau et al., 2017). Yet, the contribution of various neurochemical systems to the emergence adult respiratory behaviours remains poorly defined. Many neurochemicals are involved in the respiratory control of anurans including: glutamate, GABA, glycine, serotonin, noradrenaline, nitric oxide, sex hormones and thyroid hormones; age-related differences in their actions contribute to the production and regulation of a respiratory command that is appropriate for the animal's developmental stage (Belzile et al., 2002; Broch et al., 2002; Chen and Hedrick, 2008; Denver, 2013; Gargaglioni et al., 2008; Harris et al., 2002; Hedrick et al., 1998; Rousseau et al., 2016b; Wilson et al., 2002).

In this review, we will focus on neurochemical systems for which there is compelling evidence of contributions to the developmental emergence of air-breathing in anurans. The emergence of air-breathing in anuran tadpoles is introduced and development of multiple central oscillators underlying adult ventilation discussed. Next, data are presented on

potential contributions of neurochemical systems for the establishment of adult respiratory characteristics, including episodic breathing, motoneuron activity and the central hypoxic chemoreflex. Finally, we introduce the concept of thyroid hormones acting as master regulators of metamorphosis, a process that includes maturation of the respiratory control system.

2. Emergence of air-breathing during anuran development

2.1. Anuran developmental stages

Amphibians typically develop though a series of progressively changing larval stages before metamorphosing into a juvenile form, which grows and matures into a reproductive adult. Development of Ranidae (true frogs) is best characterized as progression through specific stages (I through XXV), originally described for *Lithobates pipiens* (Taylor and Kollros, 1946). This classification is based on anatomical characteristics of the foot, tail and head, and anatomic transformations associated with metamorphic development. Although progressive anatomic changes lend themselves to categorizing distinct developmental phases, physiological changes do not always align in a similar progression. This has led to the use of equivocal terminology by different research groups to describe Ranid tadpole stages (for example, “pre-metamorphic” may refer to different stages depending on the authors). Moreover, a categorization of *Xenopus laevis* stages by Nieuwkoop and Faber (1956) includes categories that differ from those of Taylor and Kollros. While the staging criteria put forward by Taylor and Kollros and Nieuwkoop and Faber remains the gold standard, ambiguous terminology complicates meaningful comparisons across different studies. In this regard, we have attempted to harmonize the terminology applied to Ranid stage groups to provide consistency. Table 1 illustrates our strategy for grouping developmental stages based on a few key physiologic and anatomic distinctions specific to respiratory development. This system, based on data from several studies, is meant to give the reader a general sense of the developmental state of the animal and contribute to a more standardized nomenclature in the literature.

Tadpoles in a period of early development subsequent to embryogenesis have been distinguished from the next developmental stages. These groupings roughly conform to the Taylor and Kollros “limb bud” or “foot paddle” stages, or “foot” stages. For clarity, we simply refer to these groupings as “early staged” (TK IV-IX) and “middle staged” (TK X-XVIII), respectively. Tadpoles in developmental stages more immediately prior to, during, and subsequent to metamorphic climax are designated as “late staged”. When necessary, late staged tadpoles may be subcategorized as “metamorphic” (TK XIX-XXII), or as “froglets” (TK XXIII-XIV). Metamorphosis is complete when animals enter the “juvenile” stage (TK XXV), and subsequent maturation and reproductive competency progress juveniles to the adult stage.

2.2. Developmental emergence of lung ventilation and breathing mechanics

Breathing water vs. air presents unique challenges for gas exchange mechanics, motor control and chemoreception. The development of aquatic, water-breathing tadpoles into semi-aquatic or terrestrial air-breathing frogs necessitates dramatic metamorphosis of the

respiratory structures and associated musculature, as well as modifications to the neural motor command and reflexive responses to respiratory stimuli. The developmental changes likely to facilitate adult respiration are discussed in later sections and summarized in Table 2.

Studies of anuran respiratory development have mostly utilized *Lithobates catesbeianus* (American bullfrog), *L. pipiens* (northern leopard frog) and *Xenopus levis* (African clawed toad), with contributions from a few other species including Bufonid toads (Cane toad, American toad) and tree frogs (various families). It should be stressed that anurans are diverse; their morphology, respiratory behaviours and developmental trajectories reflect adaptions specific to their environment and evolutionary history. Studies of a relatively few species should not be taken as absolutely representative of the entire class but instructive in the context of species biology. Generally, early-staged tadpoles exchange O_2 and CO_2 using gill ventilation driven by a continuous, low-force motor rhythm, with substantial contributions from cutaneous gas exchange (Fig. 1; Burggren and West, 1982). In later stages, developmental and environmental cues trigger metamorphosis and transformation of respiratory muscles and organs, including gill regression, lung maturation and blood flow redistribution (Alley and Barnes, 1983; Burggren and Infantino, 1994; Denver, 2009; Just et al., 1973). The O_2 requirements of juvenile and adult anurans are met largely by lung ventilation with contributions from cutaneous gas exchange (Fig. 1; Burggren and West, 1982). Up to 80% of CO_2 excretion occurs cutaneously in adults of *L. catesbeianus*. The degree of involvement of specific gas exchange surfaces in respiratory homeostasis through development also depends on factors such as temperature, metabolic demand and inter-species variability.

Morphological development does not necessarily precede the emergence of air-breathing in some anuran species (i.e. brain development is actually ahead of the body). Considering that time spent at the water surface exposes tadpoles to significant predation risks and requires extra energy expenditure, there must be clear advantages for air-breathing in young tadpoles. In the aquatic species *Xenopus*, lung ventilation accounts for 17% of total O_2 uptake after hatching (NF stages 35–36; Feder and Wassersug, 1984). In contrast, the un-vascularized lungs of young Ranid tadpoles contribute negligibly to basal gas exchange (Just et al., 1973). In these species, air-breathing at early stages is likely adaptive as it contributes to lung development (Crowder et al., 1998; Pronych and Wassersug, 1994; Rose and James, 2013), buoyancy control (Bruce et al., 1994; Fejtek et al., 1998; Milsom, 1990) and may serve a respiratory purpose under conditions of aquatic hypoxia (Feder and Wassersug, 1984).

Adult anurans lack a diaphragm and possess structurally ‘simple’ lung cavities (Burggren and Robert L. Infantino, 1994), yet, their respiratory behaviours are diverse and show a high degree of motor complexity (Gargaglioni and Milsom, 2007; Sanders and Milsom, 2001). Low-force buccal pumping results in airflow through the nares that refreshes air within the oral cavity and contributes to olfaction. Importantly, high-force lung ventilation utilizes the same buccal force pump to drive positive-pressure inflation of the lungs (see Fig. 2 for examples of buccal vs. lung motor output; Gans et al., 1969; West and Jones, 1975). Multi-phasic lung ventilation involves buccal and lung ventilation cycles, and requires coordination with the nares and glottis musculature acting as valves to trap (inhalation) or release (exhalation) pulmonary air (Gargaglioni and Milsom, 2007). Lung ventilation in many adult

ectothermic species occurs episodically, although the developmental timing when breathing episodes emerge is not well understood in anurans (Kinkead, 1997; Milsom and Vitalis, 1984; Milsom, 1991; Taylor et al., 2010). Early stages of *L. catesbeianus* do not produce air-breathing episodes or lung inflation cycles, but both are observed in adults under basal conditions. In adults, episode duration lengthens with increased respiratory drive until becoming continuous at high levels of drive (e.g. elevated CO_2; Taylor et al., 2003a, 2003b). Clustering of lung breaths has been attributed to medullary and pontine structures (Gargaglioni et al., 2007; Kinkead et al., 1997; Milsom et al., 1999; Reid et al., 2000), sensory feedback (Kinkead and Milsom, 1997) and neurochemicals (Harris et al., 2002; Straus et al., 2000a). The frequency and pattern of qualitatively distinct buccal and lung ventilation depends on many factors including the level of respiratory drive, behavioral state, species, and environmental conditions making it difficult to tease apart physiological considerations from purely developmental aspects. To fully understand the dynamic and varied respiratory behaviours of anurans, it will be necessary to continue researching the basic principles underlying neural control of respiratory motor output.

2.3. Non-respiratory functions of adult ventilation

Buccal and lung ventilation makes additional contributions to important non-respiratory behaviours in adult anurans. Olfaction and prey capture behaviours utilize the buccal musculature and must be coordinated with ventilation. Adult vocalizations are dependent on buccal and lung ventilation in terrestrial species. An exception is the aquatic *Xenopus*, which has developed a novel mechanism of underwater vocal production utilizing laryngeal dilators independent of breathing (Zornik and Kelley, 2007). Lung ventilation also plays a specific role in blood volume regulation in adult anurans. Lymph production is high in anurans due to their “leaky” vasculature and tends to pool in the limbs and vertical lymphatic sacs. Lung inflation has been shown to transmit pressure to the surrounding lymph sacs forcing fluid vertically towards the dorsal lymph hearts (Hedrick et al., 2007). Therefore, the developmental emergence of lung ventilation has implications for other important adult behaviours and cardiovascular control.

3. Development of central generators for lung ventilation

3.1. Utility of brainstem preparations in the study of anuran respiratory control

The progressive emergence of air-breathing during anuran development and eventual prominence of lung over buccal ventilation alludes to causal changes within the essential respiratory control networks. Studies of the central neural correlates of anuran ventilation have largely utilized isolated brainstem preparations comprised of intact regions of the rostral spinal cord, medulla and midbrain. The desirability of these preparations stems from their ability to recapitulate *in vivo* motor activity in terms of pattern and timing, without requiring peripheral sensory input. These preparations are robust and the neural correlates of breathing can be recorded at physiologically relevant temperatures (Gdovin et al., 1998; Kinkead et al., 1994). Moreover, recordings of respiratory motor output can be made at developmental stages ranging from post-hatching to adulthood.

Recordings of motor output arising from the cranial and spinal nerves (CNs V,VII, X; SNs I, II) of brainstem preparations have characterized distinct motor outputs driving muscle activation for buccal and lung ventilation (Fig. 2). Low-amplitude, high-frequency buccal bursts dominate CN V motor output of early-staged tadpoles but are periodically interrupted by high-amplitude bursts sub serving lung ventilation. Efferents of CN X projecting to the glottis are also engaged during lung ventilation (Fig. 2A). “Priming bursts” are a more capricious component of the motor output which, when present, precedes lung bursts but is only observed on CN VII and SN II (Fig. 2B). Comparison of motor activity from early-staged tadpoles with that of adults reveals increased lung burst frequency, clustering of lung bursts into episodes and “ramping” of lung burst amplitude (Fig. 2C, D). The source of these distinct motor outputs is presumed to be central oscillators located in each medullary hemisphere capable of producing rhythmic motor output (Fig. 2E).

3.2. Central neural networks generating respiratory motor output in anurans

Beginning in the mid 1990’s, research spanning 20 years identified two spatially distinct central oscillators in late-staged anurans, located bilaterally in the brainstem (Fig. 2E; Kogo et al., 1994; McLean et al., 1995; Wilson et al., 2002; Hedrick, 2005). The caudal site straddles rhombomeres 7/8 immediately lateral to the motor nucleus of CN X and is necessary for buccal burst generation. The rostral site, necessary for lung bursts, is located in rhombomere 5 of the medulla immediately caudal to CN’s VII/VIII and lateral to the CN IX motor nucleus (Baghdadwala et al., 2015). Although rhombomeric organization is transient in most vertebrates (Straka et al., 2006), it is maintained through anuran development into adulthood. The buccal and lung oscillators have also been identified in the same anatomical locations in early-staged tadpoles (Duchcherer et al., 2013; Reed et al., 2018).

Many studies support the existence of caudal buccal and rostral lung oscillators in the anuran brainstem but there is uncertainty surrounding the existence of additional central oscillators, their interconnectivity and evolutionary origins (Kinkead, 2009). Recent data provide evidence of a third, diffuse oscillator in late-staged tadpoles driving the priming bursts that sometimes precedes lung bursts. This “priming oscillator,” extending rostro-caudally through rhombomeres 4–6, encompasses the previously described lung burst generator (Fig. 2E; Baghdadwala et al., 2015). The presence of a priming oscillator in early-stages has not been ascertained; however, “priming-like” events can be observed from CN VII in brainstems derived from middle-staged tadpoles (see Fig. 2B). This view of multiple oscillators that each contribute to different phases of the anuran respiratory cycle (buccal, priming, lung powerstroke) is similar to that proposed for mammals (Del Negro et al., 2018), offering support for conservation of respiratory control substrates. The most parsimonious scenario is that rhythm generators for aerial respiration evolved in a common ancestor to tetrapods and are homologous amongst amphibians, reptiles, mammals and birds. However, the oscillator-homology theory is not without its caveats. For example, although the rhombomeric location of key respiratory rhythm generators is similar in amphibians and mammals, the rhythmogenic mechanisms of proposed homologous sites differ (Wilson et al., 2006). Moreover, the ability of the putative anuran priming area to act as an oscillator *per se* (i.e. to function without phasic input) has not been demonstrated unequivocally. Anuran lung bursts do not require, nor always show, a precedent priming burst engaging only a subset of

muscles (innervated by CN VII and SN II) comprising the buccal pump and glottis. Since lung ventilation engages motoneurons that control levator and depressor muscles, the priming and lung areas may work as one oscillator to engage of different muscle groups and not distinct phases of lung ventilation. Future studies in semi-intact and intact animals providing good spatial-temporal resolution of neuronal activity would help resolve the functional significance priming bursts.

An alternative scenario was put forward by Klingler and Hedrick (2013) in which multiple rhythmogenic sites reside within each brainstem rhombomere. The author's transection data show that, in addition to caudal and rostral rhythmogenic areas, isolated brainstem regions encompassing CN X (rhombomeres 6/7, i.e. lying midway between the buccal and lung oscillators) also produced respiratory-like motor output in ~18–27% of preparations derived from early- and middle-staged tadpoles and juvenile frogs. These data agree with those from fish showing that a diffuse rostral-caudal rhythmogenic network underlies buccal pumping (Duchcherer et al., 2010). Similarly, rhythmogenic areas initially reside within each embryonic brainstem rhombomere of chicks and mice that are later recruited into larger networks (Champagnat and Fortin, 1997). Rhombomeric organization of rhythmogenic areas opens the possibility that a diffuse, rostral-caudal network of brainstem rhythm generators may have become re-organized and specialized multiple times during tetrapod evolution to support lung ventilation in different vertebrate classes. This view is attractive given that the essential motor nuclei recruited by the respiratory oscillators for lung ventilation differ among vertebrate classes (e.g. amphibians: CNs V, VII, X; mammals: phrenic and intercostal nerves). It is possible that evolving respiratory oscillators may have originally been associated with different motor nuclei.

3.3. Development of rhythmogenic mechanisms for anuran ventilation

3.3.1. Buccal oscillator rhythmogenesis—The buccal oscillator produces robust motor output similar to that seen *in vivo* in early to juvenile stages, even when physically or chemically isolated from other brainstem regions indicating strong intrinsic rhythmogenic mechanisms (Gdovin et al., 1999; Pack et al., 1993). Presently, there are no data to suggest that these mechanisms change through development. Glutamate likely contributes to buccal rhythmogenesis during post-embryonic stages; in brainstem preparations, microinjection of glutamate agonists into the buccal oscillator, or superfusion with glutamate receptor antagonists elicits similar effects on buccal motor output in early-, middle- and late-staged tadpoles and juvenile frogs (Chen and Hedrick, 2008; McLean et al., 1995; Wilson et al., 2002). Agonizing or antagonizing ionotropic GABA receptors in brainstems from middle- and late-staged tadpoles and juvenile frogs also diminishes buccal motor output (Broch et al., 2002; Galante et al., 1996; Gdovin et al., 2006; Wilson et al., 2002), and Cl^--free media mimics these responses. These results have been replicated in related species of *Pelophylax esculenta* and *L. pipiens* (Leclère et al., 2012; Vasilakos et al., 2006) and point to synaptic inhibition as a mechanism for buccal rhythm generation. Buccal oscillator activity may also depend on extracellular $[K^+]$, hinting that voltage-dependent mechanisms generating endogenous activity could function concurrently with synaptic inhibition (Winmill and Hedrick, 2003; Hedrick, 2005).

3.3.2. Lung oscillator rhythmogenesis—The mechanisms of lung oscillator rhythmogenesis likely change with development, at least for *L. catesbeianus* in which lung motor output becomes more robust with development (Table 2). In this species, the lung oscillator is presumed to be "functional" at early-stages as lung motor output is induced precociously by microinjection of glutamate, or its receptor agonist (McLean et al., 1995; Wilson et al., 2002). Exogenous glutamate exerts comparable, dose-dependent effects on lung motor output across developmental stages (Chen and Hedrick, 2008; McLean et al., 1995; Wilson et al., 2002) arguing against a significant contribution of glutamatergic development to the emergence of robust lung motor output in late-staged tadpoles.

The intrinsic rhythmogenic capacity of lung oscillators may decrease with development forcing a greater reliance on network properties in later stages. Lung burst frequency depends on extracellular $[K^+]$ in brainstems of early- and middle-staged tadpoles, but not adult frogs, suggesting a decreased capacity for intrinsic "pacemaker" activity (Winmill and Hedrick, 2003). Over the last few decades, many lines of evidence have identified developmental changes in ionotropic GABA and glycine signaling as major candidates in the emergence of lung ventilation. Superfusion of brainstems from early-staged *L. catesbeianus* and stage 51–56 *Xenopus* with GABA (1 mM and 0.5 nM, respectively) increases lung burst frequency (Fournier et al., 2012; Janes et al., *unpublished data*). However, brainstems from middle-staged and metamorphic *L. catesbeinus* tadpoles respond only to relatively low GABA or glycine concentrations (≤ 0.5 mM) with moderate increases in lung burst frequency, while higher concentrations depress lung burst frequency and amplitude (Broch et al., 2002; Galante et al., 1996). Biphasic, dose-dependent effects of GABA have been reported previously and may reflect concentration-dependent changes in GABA receptor conductance (Song et al., 2011). However, Cl^--free media has variable effects on lung burst frequency at this stage (Broch et al., 2002; Galante et al., 1996) suggesting that while GABA/glycine signaling excites the lung motor rhythm, it is not strictly necessary for rhythmogenesis. In brainstems derived from adults, GABA and glycine concentrations as low as 0.5 mM suppress lung burst frequency, as does superfusion with Cl^--free media, bicuculline or strychnine (Broch et al., 2002; Fournier et al., 2007; Gdovin et al., 2006). These data suggest increased lung oscillator sensitivity to GABA and glycine with development and a shift from receptor-mediated excitation to inhibition consistent with increased reliance on synaptic inhibition for rhythmogenesis in late-stages and adults. It is worth noting that in late-staged and adult *L. pipiens* lung burst frequency is increased when superfusate $[Cl^-]$ is reduced (Kimura et al., 1997; Vasilakos et al., 2006). Inconsistent responses to Cl^- manipulation in closely related species highlights the need for more research into rhythmogenic mechanisms.

Metabotropic GABA receptors have also been implicated in the emergence of lung motor output. In brainstems derived from early and middle staged tadpoles, the $GABA_B$ antagonist 2-hydroxy-saclofen (1.5–2.5 mM) increases lung burst frequency in a dose-dependent manner (Straus et al., 2000a). Importantly, lung burst duration and amplitude are unaffected by this treatment, nor is buccal motor output altered indicating that $GABA_B$ receptor antagonism acts specifically on lung motor output. It was therefore proposed that endogenous GABA, acting via metabotropic receptors, inhibits lung oscillator output until

the appropriate developmental time ("developmental dis-inhibition"). The source of GABAergic inhibition remains unresolved, but based on transection and chemical isolation experiments appears to reside within the caudal brainstem (Duchcherer et al., 2013).

3.3.3. Putative oscillators—Current data suggest the possibility that additional, diffuse respiratory oscillators exist within the brainstems of early and late-staged anurans but almost nothing is known of potential rhythmogenic mechanisms at these sites. Scant pharmacological data on rhythmogenic mechanisms for the recently described priming oscillator provide some evidence for Cl^--mediated synaptic inhibition in adults (Baghdadwala et al., 2016; Vasilakos et al., 2006). Since there is also evidence for this mechanism in the apposed adult lung oscillator, both the priming and lung oscillators could theoretically represent a single, diffuse region of rhythmogenesis.

3.4. Developmental changes in connectivity between respiratory rhythm generators

Coupling between functionally related rhythm generators is a common mechanism to ensure appropriate phasing and timing of motor output. Evidence exists for coupling between the respiratory circuits of many vertebrates (Quenet et al., *unpublished*), yet, the precise nature of anuran respiratory oscillator connectivity remains uncertain. Since the juvenile and adult breathing requires the coordination of distinct motor outputs for buccal and lung ventilation, coupling between oscillators may be required to entrain the timing and phasing of motor output, and could also contribute to rhythmogenesis.

In brainstems derived from early- to middle-staged *L. catesbeianus*, physical isolation of lung oscillators via transection at the level of CN IX increases lung burst frequency (Reed et al., 2018). These results confirm those of earlier chemical isolation studies (Duchcherer et al., 2013) and support the contention that caudal brainstem sites inhibit motor output from the lung oscillator during early development. Complementary data from Klingler and Hedrick (2013) showed no change in lung burst frequency following transection rostral to CN X in brainstems of similar staged tadpoles. Therefore, the lung oscillator does not seem to rely on input from caudal sites for rhythm generation during early development. However, contradictory data from Torgerson et al., (2001) show that transections at the level of CN IX abolished lung bursting in the same stage groups. This discrepancy may arise from the fact that brainstem preparations in the latter study were devoid of all sites rostral to the cerebellum, including the locus coeruleus and nucleus isthmi. Pontine sites providing descending excitatory drive (that were kept intact in Duchcherer et al., 2013 and Klingler and Hedrick, 2013) could contribute to maintaining lung oscillator activity following transection, but these pathways remain unresolved.

Trask et al. (2018) recently began addressing the nature of oscillator connectivity by mathematically modeling the functional coupling between buccal and lung oscillators through *L. catesbeianus* development. The data suggest that lung and buccal oscillators are "uncoupled" during early-stages while mature levels of coupling appear at metamorphosis, measured as a decrease in the variation of lung-buccal burst offset times. A developmental increase in oscillator connectivity is supported by observations that focal injection of a glutamate agonist (AMPA) into the buccal oscillator abolishes lung motor output in

brainstems derived from late-staged tadpoles or juvenile bullfrogs. Conversely, focal activation of the lung oscillator increases buccal burst frequency (Wilson et al., 2002). Although reciprocal buccal-lung oscillator connections may exist as development proceeds, the nature (inhibitory/excitatory) and function of these connections remains to be determined. In late-staged tadpoles and juvenile frogs, isolation of the lung oscillator via transection decreases lung burst frequency (Klingler and Hedrick, 2013; Reed et al., 2018; Torgerson et al., 2001). Lung bursting is, however, maintained during chemical isolation of the rostral brainstem, as long as the priming and powerstroke areas are both uninhibited (Baghdadwala et al., 2015). Moreover, abolishing buccal or lung oscillator activity with focally applied GABA agonists in brainstem preparations has no effect on motor output produced by the other oscillator (Wilson et al., 2002). In sexually mature bullfrogs buccal activity is often absent from the respiratory motor output indicating that an active buccal oscillator is not prerequisite for lung motor activity. Taken together, these data may indicate that physical disruption of pathways between the rostral and caudal brainstem segments disrupts lung oscillator rhythmogenesis in later stages and adults, but seems unlikely to occur by interrupting direct connectivity between the buccal and lung oscillators *per se*. Instead, adult oscillator coupling may be an emergent property stemming from the influence of distinct modulatory sites and likely to involve sensory feedback in the intact animal.

4. Emergence of air-breathing episodes

Episodic breathing in intact animals emerges from interplay between multiple mechanisms including those intrinsic to the respiratory oscillators, descending pontine influences and sensory feedback. In *L. catesbeianus*, episodes of successive lung bursts are observed in brainstems derived from metamorphic and juvenile/adult frogs, suggesting that the patterning of lung bursts into episodes first appears close to metamorphosis (Fig. 2D). However, specific manipulations, such as elevated thyroid hormones, can elicit episodic lung output in early-staged tadpoles.

At the medullary level, reciprocal connectivity between respiratory oscillators has been proposed to underlie high frequency episodes of adult lung motor output in modeling studies (Baghdadwala et al., 2016; Table 2). GABA and glycine signaling has also been implicated in episodic lung bursting. In brainstem preparations derived from adult *L. catesbeianus*, reducing superfusate [Cl^-] transforms episodic lung bursting into patterns of periodic single breaths, with no net change in burst frequency (Vasilakos et al., 2006). Similarly, low concentrations of the $GABA_B$ receptor agonist baclofen (0.25 to 0.5μM) applied to brainstems of froglets and juvenile frogs reduces the number of lung bursts per episode, and transforms episodic lung bursting into patterns of periodic single breaths, with no net change in burst frequency (Straus et al. 2000a). Whether GABA and glycine exert their effects via connectivity between the oscillators or from other modulatory sites is not known.

Vertebrate studies have long suggested that pontine areas are also necessary for episodic breathing (Milsom et al., 1997). One candidate structure identified in amphibians is the nucleus isthmi (NI), a cholinergic region located in the caudal midbrain (Gargaglioni and Branco, 2004; Kinkead et al., 1997). The NI has traditionally been ascribed to visual processing; however, extensive metamorphic changes in this region do not correlate well

with the emergence of binocular vision (Udin and Fisher, 1985). Given its size and reciprocal connections with the reticular formation, regional differences in NI function are likely. In intact, decerebrate adults of *L. catesbeianus*, brainstem transections caudal to the optic tectum (eliminating the NI), transform episodic lung ventilation into a pattern of individual breaths, without associated decreases in lung ventilation frequency (Gargaglioni et al., 2007). Moreover, NI lesion by microinjections of kainic acid (4.7 mM, glutamate agonist), or glutamate (10 mM) into the structure of adults also transforms episodic breathing into single breaths (Gargaglioni et al., 2007; Kinkead et al., 1997). Physiological data are supported by the finding that, in *Xenopus*, the number and intensity of neurons immunopositive for choline acetyltransferase increases strikingly at metamorphic climax (López et al., 2002). Therefore, development of NI cholinergic signaling appears to parallel the emergence of episodic breathing at metamorphosis.

5. Transmission of the motor command to motoneurons

Increased glutamatergic connectivity between central oscillators and motoneurons may support the emergence of high frequency lung bursting in later developmental stages (Table 2). Metamorphosis does not involve neurogenesis of trigeminal motoneurons, rather the dendritic field of existing motoneurons expands dramatically in the dorsomedial plane during this stage, indicating changes in network connectivity (Rosenthal and Alley, 1988). While the synaptic pathways relaying central oscillator output to respiratory motoneurons are not resolved in amphibians, AMPA and NMDA receptor activation is necessary for transmission of lung and buccal motor output to the trigeminal and hypoglossal motor nuclei in adults of *L. catesbeianus* (Kottick et al., 2013). A more recent study expanded on these findings using whole cell recordings of *L. catesbeianus* trigeminal motoneurons (early/middle-stages vs. adults) to characterize developmental changes in excitatory post-synaptic currents (EPSC's; Fig. 3A,B) and other intrinsic electrical properties. Two motoneurons subtypes were defined: compared to type I motoneurons, type II have a smaller input resistance, higher rheobase and negligible depolarizing sag amplitude (i.e. hyperpolarization-activated cation currents are absent). It is conceivable that type II motoneurons are generally less excitable and likely recruited by stronger synaptic drives underlying lung ventilation, or non-respiratory behaviours requiring forcible muscle contractions (e.g. feeding, vocalization). Notably, EPSC amplitude increases following metamorphosis only in type II motoneurons (Fig. 3C; Janes et al., 2019). These data lend support to the idea that increased glutamatergic synaptic input onto respiratory motoneurons, acting in concert with other development mechanisms, enhances the expression of lung bursting in adults.

6. Development of the central hypoxic chemoreflex

6.1. The hypoxic ventilatory response through anuran development

Mild to moderate levels of hypoxia typically drive increased ventilation in intact anurans, however, the motor pattern most sensitive to this stimulus changes from buccal to lung with development. Upon hatching, tadpoles of *L. catesbeianus* and *Xenopus* respond to aquatic hypoxia (4–12% O_2) by increasing gill ventilation frequency. Beginning at TK stage IV in

bullfrogs (*Xenopus*: NF stage 48), hypoxia can elicit air-breathing. As development continues, hypoxia-induced air-breathing becomes more robust as baseline lung ventilation increases and is concurrent with a weakening of gill chemoresponsiveness (Burggren and Doyle, 1986; Pan and Burggren, 2010; West and Burggren, 1982). In adult *L. catesbeianus*, *Rhinella schneideri* (Schneider's toad) and *R. marina* (Cane toad), exposure to 3–10% inspired O_2 (25°C, 30 min) increases lung ventilation frequency and tidal volume (Boutilier and Toews, 1977; Gargaglioni and Branco, 2000; Kruhøffer et al., 1987; Rocha and Branco, 1998; Santin and Hartzler, 2016). Severe hypoxia (≤ 3%) blunts and eventually abolishes ventilation in adult *R. marina* and *L. pipiens* (Boutilier and Toews, 1977; Rose and Drotman, 1967).

6.2. The central hypoxic response of anurans

Ventilatory responses to hypoxia are shaped by signal integration from peripheral and central chemoreceptors, and the hypoxic response can differ in reduced preparations lacking peripheral feedback from the oxygen-sensitive carotid labyrinth. Interesting data exist on peripheral chemoreception in amphibians, but are beyond the scope of this review (see: Jonz and Nurse, 2006; Reid, 2006; Van Vliet and West, 1992). One key study performed on freely-behaving *L. catesbeianus* early- and middle-staged tadpoles reported that following peripheral chemoreceptor denervation, hypoxia (7% O_2, 50 sec) elicited delayed increases in gill ventilation, suggesting central O_2-sensing mechanisms (Jia and Burggren, 1997). Building on previous work (Winmill et al., 2005), we and others have sought to resolve maturation of the central hypoxic response by recording respiratory motor output from *L. catesbeianus* brainstems during hypoxic superfusion. Representative traces showing the stage-dependent effects of hypoxia on lung and buccal burst frequency are illustrated in Fig. 4A. As buccal burst frequency is not consistently affected by central hypoxia in any stage group, this stimulus may act primarily on the lung oscillators (Winmill et al., 2005; Taylor et al., 2013). During prolonged hypoxia (4–6% O_2; pH ~7.8; 180 min), lung burst frequency is depressed after 60 min in brainstems derived from late-stages and adults (reversible upon re-oxygenation; Taylor et al., 2013; Fournier et al., 2007, 2008). In contrast, pooled data from brainstems of early- and middle-stages showed non-significant increases in lung burst frequency apparent after 20 min of hypoxia followed by significant depression after 170 min (Taylor et al., 2013). In a follow-up study, we attempted to delineate stage-dependent effects of central hypoxia by comparing results from brainstems derived from early-staged tadpoles vs. middle-stages (Janes and Kinkead, 2018). In brainstems from early-stages, lung burst frequency increased in response to 20 min of hypoxia (5–10% O_2; pH ~7.9), but no significant effects were resolved for middle-staged tadpoles. The central hypoxic chemoreflex thus has a stimulatory effect on lung burst frequency only during early-stages of tadpole development.

The central hypoxic response is also severity- and time-dependent in brainstems derived from early-stages. Lung burst frequency is unaffected by mild hypoxia (15–20%, 25 min), increases during moderate hypoxia (5–10%), and is progressively depressed by anoxia (Fournier et al., 2007; Janes and Kinkead, 2018; Taylor et al., 2013; Winmill et al., 2005). The enhanced lung burst frequency is initially reversible upon tissue re-oxygenation. However, during extended periods of re-oxygenation (2h) previously hypoxic brainstems

show a progressive augmentation of lung burst frequency. While the underlying mechanisms and physiological relevance of these observations remain to be discovered, they suggest that hypoxic exposure at critical developmental stages enhances long-term lung motor output (Janes and Kinkead, 2018).

At present we can only speculate on the adaptive advantage whereby central hypoxia enhances lung burst frequency during early development, but results in depression at adulthood. Although the lungs of early-staged *L. catesbeianus* are poorly vascularized, breathing oxygen-rich air may contribute to respiratory homeostasis during aquatic hypoxia (a strategy employed by many fish species). Furthermore, long-term enhancement of lung ventilation in response to hypoxia may accelerate development of the lungs and associated neural networks, allowing the animal to utilize lung ventilation at earlier stages to escape the constraints of hypoxic water. In the brainstems of adults, hypoxic depression of lung burst frequency could simply reflect adaptive metabolic depression of central neurons by mechanisms not operating in early stages. Unlike tadpoles, froglets and juveniles do not utilize anaerobic metabolism to maintain respiratory motor output during severe hypoxia (Winmill et al., 2005). Central respiratory depression could also function during moderate hypoxia to limit "over-excitation" of respiratory neurons by peripheral O_2 chemoreceptor afferents, and to prevent net oxygen loss from the blood when breathing severely hypoxic air.

6.3. GABAergic signaling contributes to development of the central hypoxic response

The ability of central hypoxia to elicit respiratory excitation at early stages, but inhibition at adulthood alludes to the shift in ionotropic GABAergic signaling from excitatory-inhibitory that occurs developmentally (Ben-Ari et al., 2007). To test this hypothesis, we analyzed lung burst frequency in 5 min increments during central hypoxia (5% O_2, 15 min) for early-, middle- and metamorphic-staged tadpoles, and adults. The ability of GABA to mimic stage- and time-dependent hypoxic responses was tested by superfusing GABA onto brainstems obtained from equivalent stages (0.25 mM, 30 min, 98% O_2 + 2% CO_2). In all stages, GABA reversibly abolished buccal output, likely due to synaptic inhibition operating within the oscillator. Hypoxia progressively suppressed lung burst frequency in brainstems of adults and GABA mimicked this effect in brainstems from late-staged tadpoles (Fig. 4B). In brainstems derived from middle-stages, neither hypoxia nor GABA affected lung burst frequency (Fig. 4C). Brainstems from early-staged tadpoles had a more complex time-dependent hypoxic response: lung burst frequency was augmented during the initial 5 min of hypoxia, gradually returned to baseline over the next 10 min, then showed a second frequency increase at 15–20 min (Fig. 4D). Interestingly, brainstems from this stage group also responded to GABA with increased lung burst frequency during the first 5 min of exposure; this augmentation occurred following abolishment of the buccal rhythm. Subsequent to this transient increase, lung burst frequency was suppressed relative to baseline for the duration of GABA exposure (Fig. 4D). Thus, GABA only partially mimicked the central hypoxic response of brainstems from early staged tadpoles (i.e. the hypoxia-induced frequency increase at 0–5 min, but not 15–20 min). It is possible that buccal oscillator inhibition led to transient increases in lung bursting during the initial 5 min, however, connectivity between oscillators at early-stages has yet to be demonstrated. An

alternative explanation for the slow-onset inhibition of lung bursting in early-stages is activation of $GABA_B$ receptors mediating tonic inhibition (Straus et al., 2000b). Future experiments using a selective $GABA_A$ receptor agonist would help resolve this possibility. The ability of GABA (under basal O_2 conditions) to replicate some aspects of the anuran central hypoxic response provides correlative evidence of this transmitter's involvement in O_2-chemoreflex pathways. A role for ionotropic receptors is further supported by observations that brainstem superfusion with antagonists of $GABA_A$/glycine receptors (bicuculline 1.25μM / strychnine 1.5μM) prevents hypoxia-induced changes in lung burst frequency in early- and middle-staged tadpoles and adult frogs (Fournier et al., 2007). It should be stressed that rather than being the sole factor, GABA likely plays a key role in a larger central O_2-chemoreflex network that includes contributions from noradrenaline (Table 2).

The location of GABA neurons participating in the central hypoxic response is uncertain but in *L. catesbeianus*, ablation of caudal pontine structures prevents lung motor responses to central hypoxia in early- and middle-staged tadpoles and adults (Fig. 5A,B; Fournier and Kinkead, 2008). One candidate structure is the NI: chemical lesions of this site in freely-behaving *Rhinella schneideri* result in increased lung ventilation during hypoxia and points to a role for the NI in providing inhibitory modulation of the hypoxic chemoreflex (Gargaglioni et al., 2002). No data are available for the role of this structure in the chemoreflex of early-staged tadpoles. Another candidate structure is the locus coeruleus (LC), a noradrenergic nucleus located in the dorsal pons, ventral to the NI. LC neurons are subject to GABA modulation in mammals (Jin et al., 2016) and exhibit chemosensitivity in anurans (Noronha-de-Souza et al., 2006; Santin and Hartzler, 2013). Expression of the Cl^- pump NKCC1 in LC neurons of early-staged tadpoles suggests excitatory actions of GABA, and decreased NKCC1 expression at metamorphosis is concurrent with emergence of hypoxic inhibition of lung burst frequency. Antagonizing NKCC1 activity with bumetanide partially blocks changes in lung burst frequency during hypoxia in brainstems from early- and middle-staged tadpoles (Fig. 5B; Fournier and Kinkead, 2008). These data suggest that the general excitability of LC neurons could be regulated by $GABA_A$ receptor activation, with the nature of this modulation shifting from promoting to reducing excitability through development. Alternatively, noradrenaline release from LC neurons during hypoxia could modulate downstream GABAergic neurons (Merrywest et al., 2002). Expression of KCC2 (the dominant Cl^- pump of adults) increases in the dorsomedial medulla and respiratory motor nuclei following metamorphosis (Fig. 5C,D). Determining overlap between regions of KCC2 and GABA receptor expression and the respiratory oscillators would support GABAergic inhibition of lung oscillator neurons in adults.

7. Thyroid hormones

The most dramatic changes in respiratory behaviours occur at metamorphosis and begs the question: what global factor(s) incite and coordinate the development of relevant neurochemical pathways at this time? It has long been recognized that thyroid hormones (THs) are central to the process of metamorphosis and act as a transduction mechanism by which environmental signals can influence physiological processes (Denver, 2013; Table 2).

As such, they are critical for brain development and regulate various neurochemical systems such as glutamate, GABA and acetylcholine (Smith et al., 2002; Wiens and Trudeau, 2006).

7.1. Role of thyroid hormones in anuran metamorphosis

Amphibian metamorphosis results from a neuroendocrine signalling cascade, orchestrated by the hypothalamo-pituitary-thyroid axis (HPT; Fig. 6A). The hypothalamus acts as the master regulator, secreting corticotropin-releasing factor (CRF) in response to intrinsic developmental timing cues (Etkin, 1968). Since the hypothalamus also participates in stress responses, environmental factors such as temperature, photoperiod, water level, food supply, competition and predation also induce CRF release (Denver et al., 2002). In this way, the hypothalamus matches the timing of the aquatic-terrestrial transition to genetic programs and ecological factors.

Secretion of CRF stimulates the pituitary gland to secrete thyroid stimulating hormone (TSH), which in turn acts on the thyroid gland to induce the production and release of THs (Fig 6A; Fort et al., 2007). Low levels of THs are present in early- and middle-staged tadpoles, but circulating levels increase up to 20x at metamorphic climax as the thyroid gland matures (Fort et al., 2007). Circulating THs are the primary factor orchestrating the entire suite of molecular, morphological and biochemical changes occurring at metamorphosis; thyroid gland excision is sufficient to prevent these processes (Allen, 1925; Denver, 2013). The thyroid gland primarily releases thyroxine (T_4; 3.5.3’.5’-tetraiodothyronine) and small amounts of triiodothyronine (T_3; 3.5.3’-triiodothyronine). Of these two, T_3 has a higher affinity for TH receptors (TRs) and is considered the biologically active compound (Hulbert, 2000). The actions of T_3 are exerted through the TR, an evolutionarily conserved superfamily of ligand-inducible nuclear transcription factors which interact with genes via thyroid response elements in their promoters (Tata, 1999). Ultimately, gene expression changes lead to cell differentiation, proliferation, migration and death (Brown and Cai, 2007). TR expression increases at metamorphosis, due in part to enhanced transcription by THs (Fort et al., 2007). To the best of our knowledge, neural TRs have not been identified in brain structures that generate or regulate breathing.

Secretion of CRF also promotes pituitary release of adrenocorticotrophic hormone (ACTH), which induces corticosteroid production and release from the interrenal glands (Fig. 6A; corticosterone = CORT; Fort et al., 2007). TH and corticosteroids (corticosterone and aldosterone) act synergistically: TH increases glucocorticoid receptor expression, while corticosterones increase TR expression and ligand affinity, TH production and cellular conversion of T_4 to T_3 (Denver, 2009; Fort et al., 2007; Rose, 2005).

THs play a major role in the remodelling the anuran brain during metamorphosis (Denver, 1998), and their central effects contribute to the emergence of air-breathing. Corticosterone treatment (100 nM; 1 h) produced a 2-fold increase in lung burst frequency in brainstems derived from pre-and pro-metamorphic *Xenopus* (Fournier et al., 2012). In brainstems derived from early- and middle-staged *L. catesbeianus*, lung burst frequency was increased by 1 h of aldosterone (100 nM), but the effect was modest (Fig. 6B; Rousseau et al., 2016a). Lung burst frequency was significantly increased in response to 24 h of exposure to T3 or corticosteroids (Fig. 6C), indicating that genomic effects may take longer to manifest at the

physiological level in young *L. catesbeianus*. When T3 was combined with aldosterone over 24 h, the resulting lung burst frequency was higher than in response to either hormone alone, and was on par with frequencies observed in adult brainstems (Rousseau et al., 2016a). Collectively, these results demonstrate that elevation of T3 or corticosteroids (as occurs during metamorphosis) is sufficient to induce emergence of high frequency lung bursting; T_3 and corticosteroids also function synergistically in young stages. Hormone exposure over 24 h also reduced gill burst frequency, which is intriguingly reminiscent of the blunting of buccal motor activity frequently observed in adults.

To confirm the relevance of these results for intact animals, early and middle staged tadpoles were fitted with a buccal pressure catheter, and injected saline (control) or a combination of T3 and aldosterone (100 nM). After 24 h, the frequency of air-breathing episodes was increased in hormone injected tadpoles (Fig. 7A–C). This effect was specific to lung motor activity, as gill ventilation frequency and oxygen consumption (μL/h/g) were not altered (Fig. 7D,E). Although 24 h of hormone exposure does not mimic true metamorphosis (occurring over many days), these data demonstrate that TH's regulating metamorphosis are sufficient to initiate maturation of brainstem networks that produce air-breathing.

8. Perspectives

The diversity of neurochemicals involved in the development, generation, modulation and plasticity of respiratory behaviours is staggering. This degree of complexity is hardly surprising given the profound changes in brain function and connectivity occurring across an animal's lifespan from embryonic and immature stages, into adulthood and advancing age. It is important to recognize that a multitude of signaling pathways must interact to bring about breathing, and that ventilatory control requires a fine balance between excitation, inhibition and neuromodulation acting in concert. There is good evidence that neurochemicals exhibit complex interactions and a major challenge facing neurophysiology is elucidating how ensembles of neurochemicals act together to establish mature behaviours. Known examples include neurotrophic effects of GABA on developing brainstem monoaminergic neurons (Lauder et al., 1998) and state-dependent modulation of respiratory rhythm generation by interacting modulatory neuropeptides and transmitters (Doi and Ramirez, 2010). Hormones also execute widespread modulation of neurotransmitters: T_3 inhibits GABA-gated Cl^- currents in *Xenopus* oocytes (Chapell et al., 1998) and sex hormones (estradiol, progesterone, testosterone) indirectly modulate breathing through their effects on serotonin, glutamate, GABA/glycine and catecholamines (Behan and Kinkead, 2011). Deciphering sex hormone modulation of developing neurochemical systems is particularly important to advance our understanding of the basic biological principles of sex hormone signalling in the brain. Accumulating evidence suggests that early environmental "experiences" alter the developmental trajectory of neurochemical systems involved in respiratory control, yet little is understand of the underlying mechanisms or consequences (Bavis and MacFarlane, 2017).

While the data described herein constitute support for the involvement of various neurochemicals in the maturation of air-breathing, the specific site(s) of action remain unresolved. One confounding experimental factor is that neurochemicals have many broad actions and superfusion of brainstems with pharmacological agonists/antagonists, although

providing a wealth of important data, does not resolve the site of action. In this regard additional information on receptor expression profiles through development within the brainstem would provide better context important for the interpretation of pharmacological experiments.

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Highlights

- Amphibians transition from aquatic to aerial breathing during metamorphosis
- Diverse neurochemicals orchestrate development of respiratory control networks
- Maturation of the central hypoxic ventilatory response depends on GABA development
- Thyroid hormones are master regulators of metamorphosis and neurochemical signaling
- We propose an updated classification of developmental stages based on current data

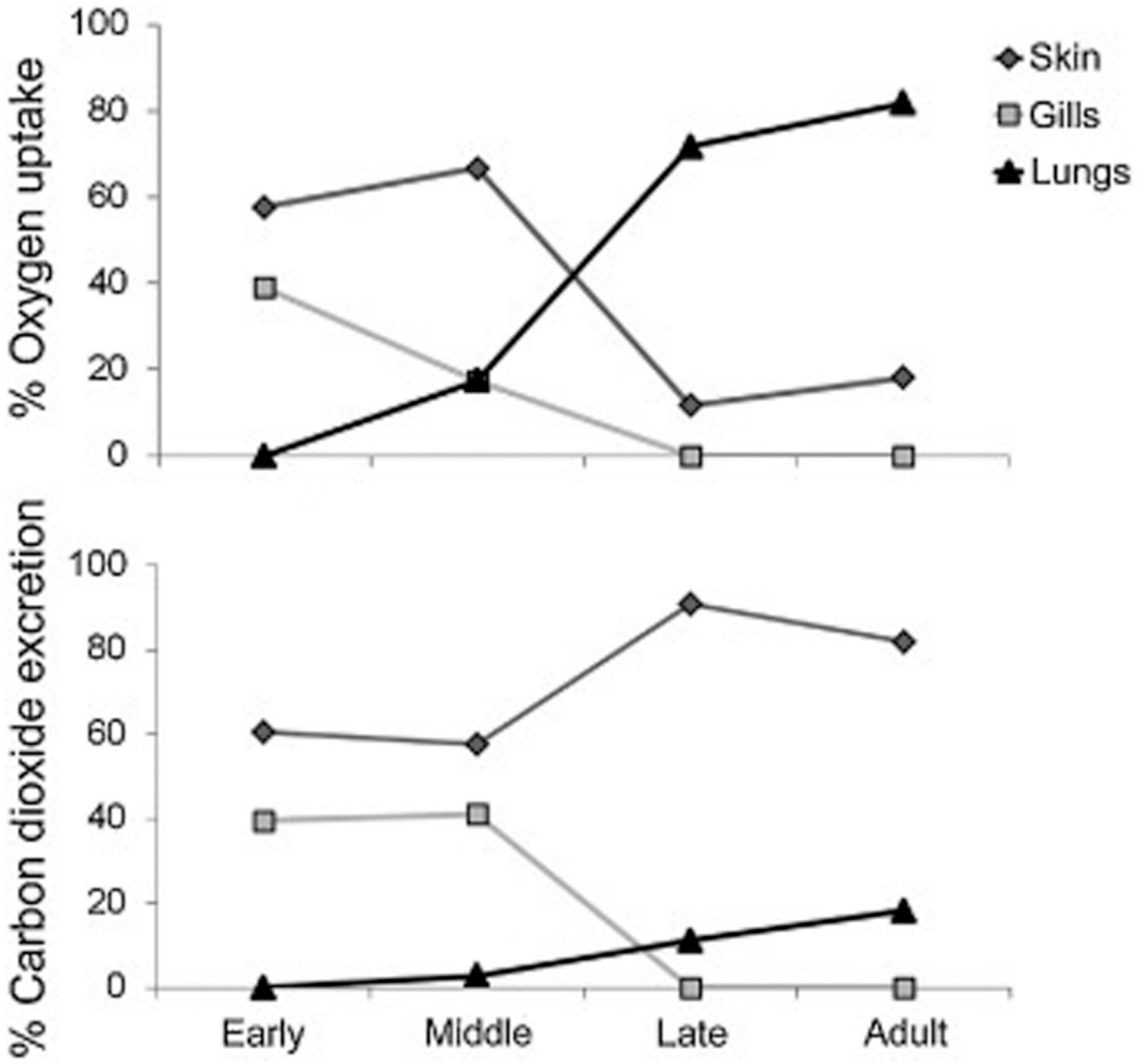

Figure 1.

Gas exchange allocation between the skin, gills and lungs shows developmental changes in *L. catesbeianus*. Gills and skin are a major surface for gas exchange (O_2 and CO_2) in early- and middle-staged tadpoles. At metamorphosis, the lungs mature and become the primary site for O_2 exchange in late tadpole stages and adults. Cutaneous respiration contributes to gas exchange at all stages, and is especially important for CO_2 exchange. *Adapted from* Burggren and West, 1982.

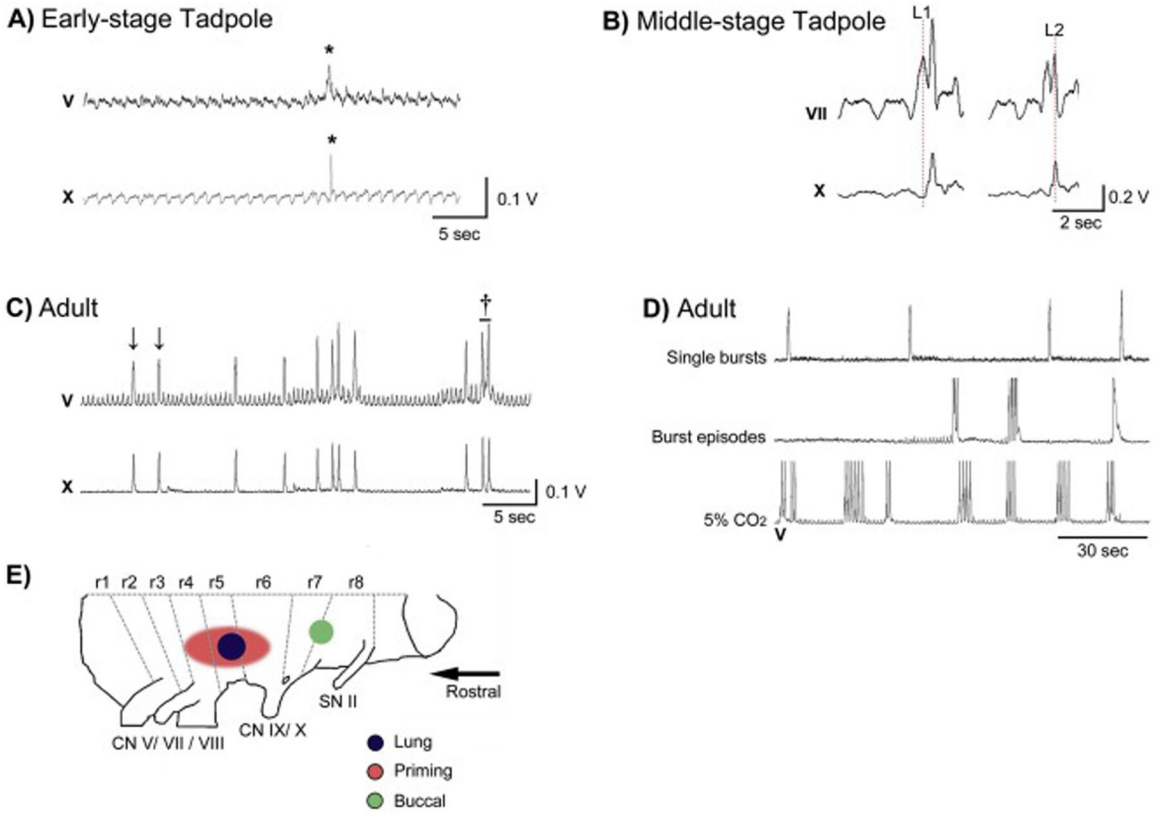

Figure 2.

Representative traces of integrated motor output recorded from the cranial nerves (CNs) of *L. catesbeianus* brainstem preparations. Output from the trigeminal (V), facial (VII) and vagus (X) nerves are shown. A) Motor output from early-staged tadpoles is dominated by low amplitude, high frequency buccal bursting subserving gill ventilation. Occasional high amplitude lung bursts (CN V; *) "push" air into the lungs and are associated with glottis activation (CN X; *). Lung bursting is rare in early stages but its frequency increases with development. B) Motor activity resembling "priming" is evident on CN VII from a middle-staged tadpole. Priming (L1) precedes the lung burst (L2) and is not associated with bursting on CN X. C) In adults, high-amplitude lung bursts occur as single events (↓) or are clustered into distinct episodes (†). Buccal bursts are not always evident in adults (present in this trace on CN V but not CN X). D) Adult lung bursts can occur singly (top), or clustered into episodes (middle). Episode number and lung bursts per episode increases with drive (bottom, 5% CO_2). E) Model brainstem showing putative rhombomeric (r) locations of the buccal, priming and lung oscillators relative to the cranial and spinal nerves (CN V-X, SN II). *Panel E reproduced from* Baghdadwala et al., 2015.

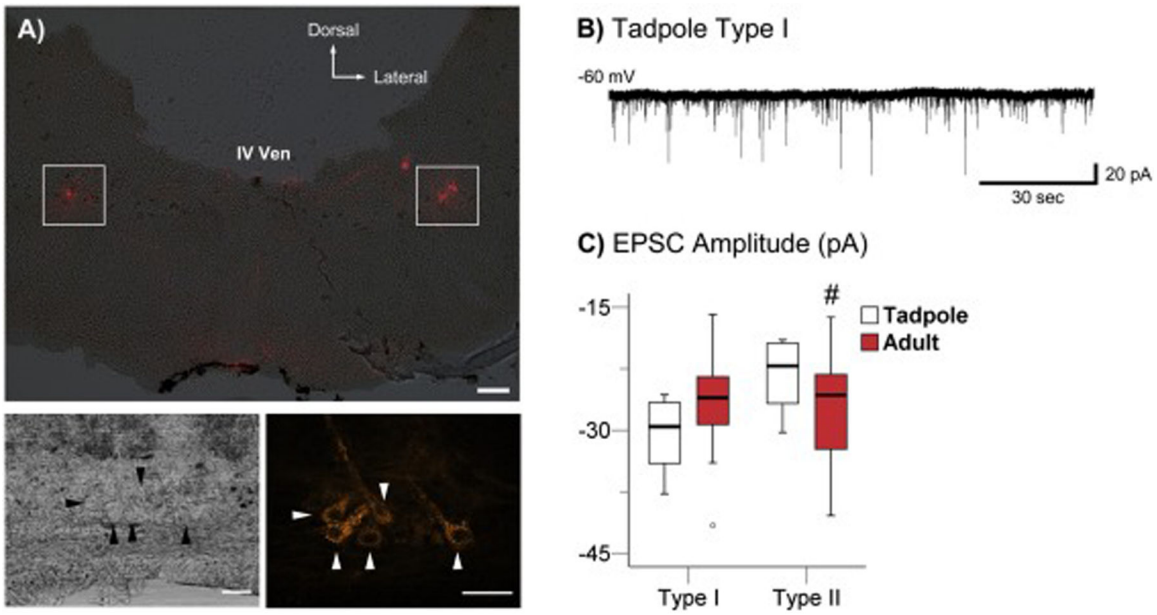

Figure 3.

Trigeminal motoneuron (MN) excitatory post-synaptic current (EPSC) amplitude increases with development. A) Top panel: trigeminal MNs, retrogradely labeled with the fluorescent marker DiI, are visible ventro-lateral to the fourth ventricle (IV Ven) and caudal to the cerebellum in brainstem slices derived from early-staged tadpoles. Confocal images taken at 10x (DIC; bottom left) and 20x (bottom right) show DiI-positive MNs (arrowheads; scale bars = 50 nm). B) Voltage-clamp recording of a trigeminal MN showing glutamatergic EPSC's as downward deflections in trace activity. C) Trigeminal MNs could be classified into Type I vs. II based on intrinsic electrical properties. We speculate that Type II MNs may be recruited during more forceful buccal contractions (e.g. lung ventilation); the EPSC amplitude recorded from Type II MNs increases following metamorphosis. # Different from tadpole Type II and all Type I MNs at $P < 0.05$. *Reproduced from* Janes et al., 2019.

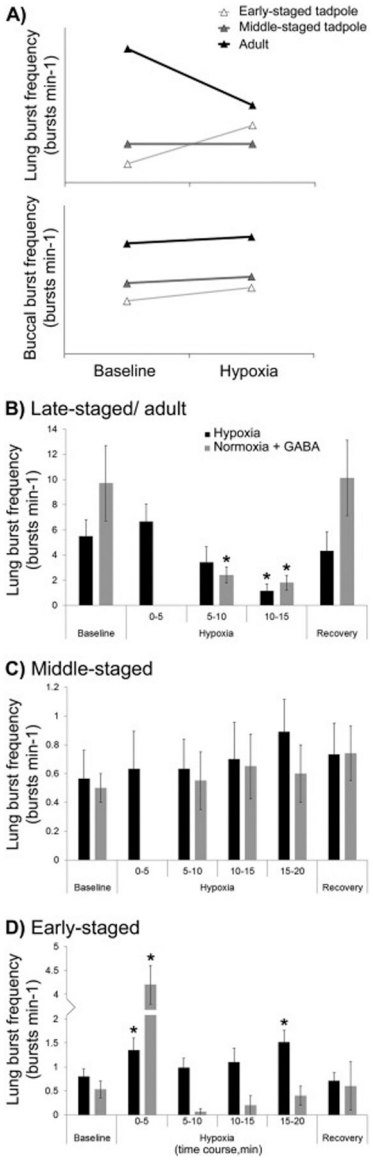

Figure 4.

The anuran central hypoxic response switches from excitatory to inhibitory with development. A) Representative traces showing the effect of hypoxia on lung (top traces) and buccal (bottom traces) burst frequencies in brainstems of *L. catesbeianus*. Hypoxia increases lung burst frequency in brainstems of early-staged tadpoles but has no significant effect on middle stages. Lung burst frequency is suppressed by hypoxia in adults. Buccal burst frequency is not altered by hypoxia at any stage. B-D) Time course of lung burst frequency (5-min increments) in response to hypoxia (5% O_2) or GABA superfusion during normoxia (0.25 mM, 98% O_2) and following a 10–30 min recovery from each treatment. C) In brainstems of adults (n = 6), hypoxia progressively suppressed lung burst frequency and this effect was mimicked by GABA in late-staged tadpoles (n = 9; GABA data not shown for 0–5 min). D) Neither hypoxia (n = 6) nor GABA (n = 9) affected lung burst frequency in brainstems of middle-staged tadpoles (GABA data not shown for 0–5 min). E) In brainstems

derived from early-staged tadpoles, hypoxia increased lung burst frequency during 0–5 min and 15–20 min (n = 12); GABA increased lung burst frequency only during 0–5 min (n = 3). Effects of hypoxia and GABA were reversible upon washout. * Different from corresponding baseline at $P < 0.05$; Repeated-measures ANOVA.

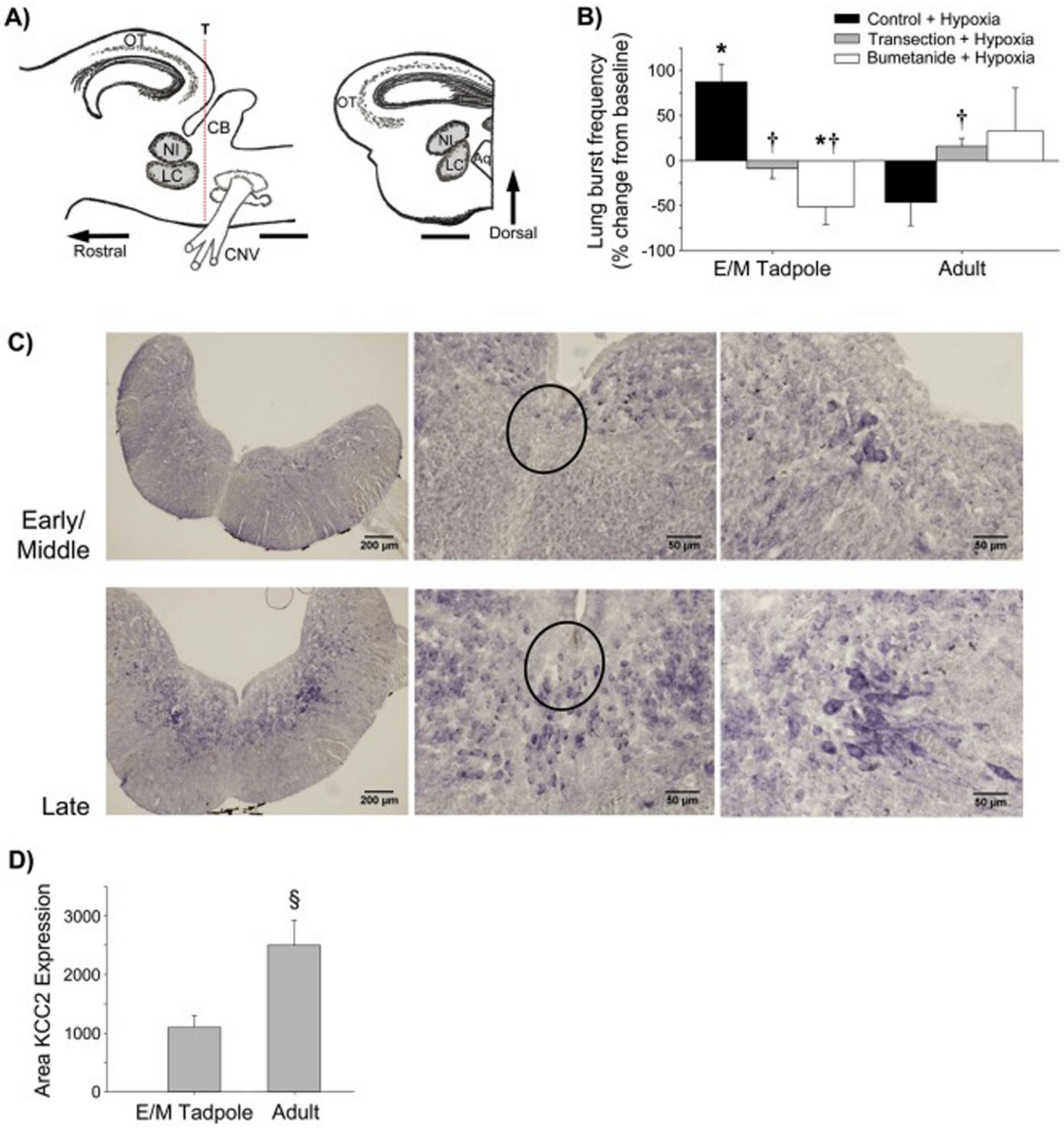

Figure 5.

A) Illustration of an adult brainstem in sagittal (left) and frontal (right) section showing approximate locations of the locus coerules (LC) and nucleus isthmi (NI). The site of transection for data in (B) is marked by "T." Scale bars = 500 µM. Aq, aqueduct of Sylvius; CB, cerebellum; CNV, trigeminal nerve; OT, optic tectum. B) Transection caudal to the LC and NI prevents the lung burst frequency response to hypoxia (5% O_2 + 2% CO_2, bal N_2) in early- and middle-staged tadpoles (E/M Tadpole; control: n=7; transection: n=6) and adults (control: n=7; transection: n=8). Inhibiting NKCC1 activity with bumetanide (1 µM) reverses hypoxia-induced changes in lung burst frequency for early/middle-staged tadpoles (n=6) and blunts the response for adults (n=7). E) KCC2 staining in 18-µm brainstem sections derived from early/middle- (top) and late-staged (bottom) tadpoles. *Left panels*: representative images showing KCC2 staining in the brainstem. *Middle panels*: 20x images

of dorso-medial brainstem sections. Black circles demarcate regions analyzed for semi-quantitative analysis. The area showing KCC2 staining increases with development. *Right panels*: 20x images of putative facial motor nuclei; KCC2 staining increased in sections from late-stages. F) Mean area (± SEM) of KCC2 staining in the midline adjacent to the fourth ventricle increased in metamorphic/adult (n=4) brainstem sections compared to early- and middle-staged tadpoles (n=3). KCC2 staining was defined as square pixels that were 5% darker on 8-bit scale than background intensity. * Different from corresponding baseline; † different from corresponding control value; § different from early/middle-staged value at $P < 0.05$. *Panel B modified from* Fournier et al., 2008.

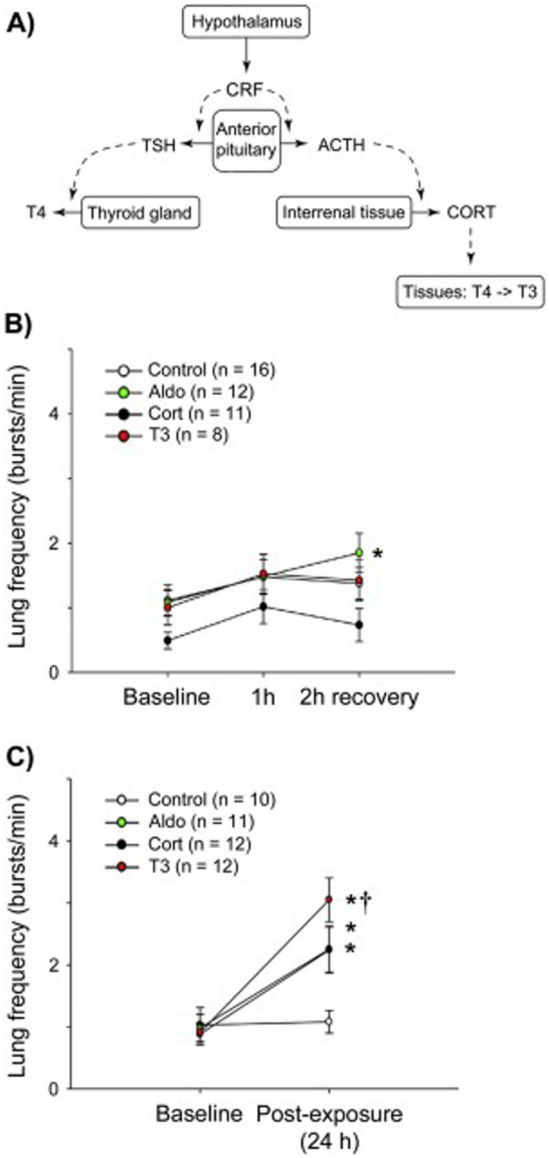

Figure 6.

Thyroid hormones regulate emergence of lung breathing at metamorphosis. A) Signaling pathways involved in the production of thyroid hormones (TH) and corticosteroids. Solid arrows: hormone production and secretion; dashed arrows: stimulatory effect on hormone production or conversion. See text for definitions. B) Acute aldosterone treatment (Aldo, 100 nM, 1 h) results in a long-term increase in lung burst frequency in brainstems from early-staged tadpoles. C) Lung burst frequency is increased following 24 h of exposure to T_3, aldosterone (Aldo) or corticosterone (Cort; each, 100 nM). * Different from corresponding baseline value at $P < 0.05$; † different from control at $P < 0.05$. *Panel A adapted from* Rose, 2005; *panels B and C reproduced from* Rousseau et al., 2016a.

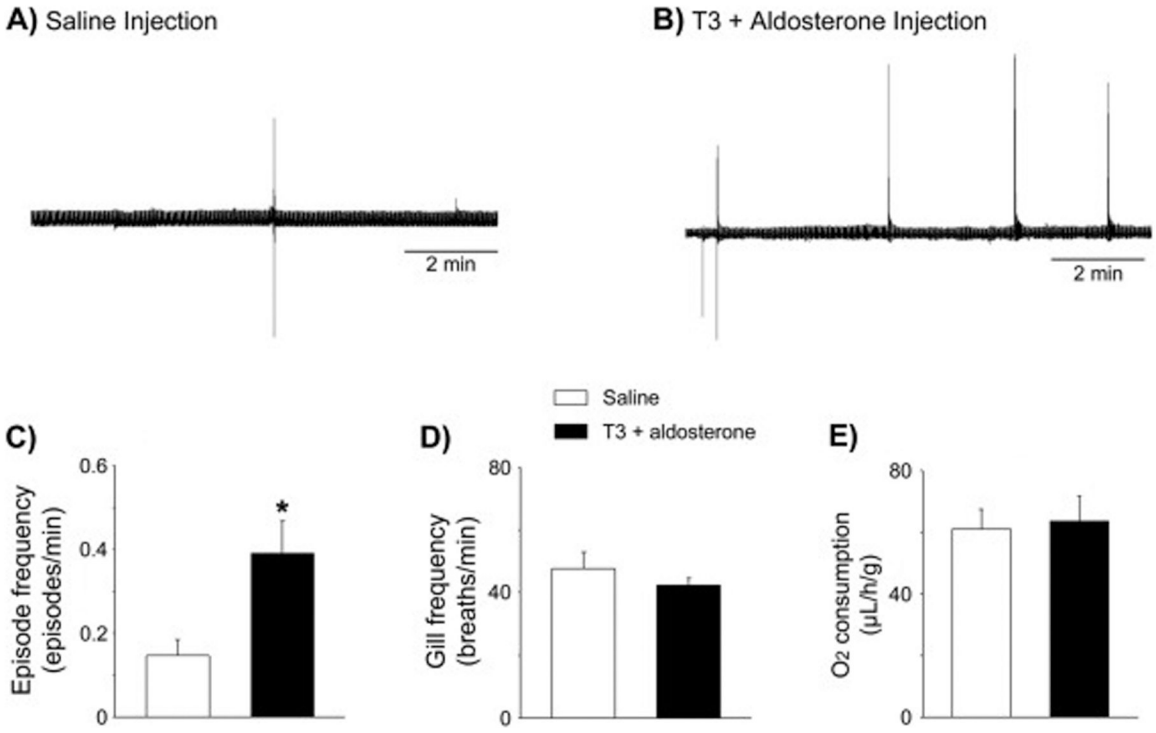

Figure 7.

Metamorphic hormones induce air-breathing in freely behaving early- and middle-staged tadpoles. Buccal pressure catheter recordings of a tadpole A) 24 h after saline injection (n = 17) and B) 24 h after T3 + aldosterone injection (each 100 nM; n = 27). Hormone treatment increased the frequency of air-breathing episodes (C) with no effect on gill ventilation frequency (D) or oxygen consumption (E). * Different from saline controls at $P < 0.05$.

Table 1.

Proposed characterization of Ranidae developmental stages[1].

	Stage		TK Stages[2]	Description
Pre-metamorphosis	Early-staged tadpoles		IV - IX	Hind limbs protrude but are rudimentary; the lungs do not contribute to gas exchange; lung ventilation is absent or occurs with very low frequency; central hypoxia stimulates lung bursting.
	Middle-staged tadpoles		X - XVIII	Hind limbs continue to develop; lungs increase in size, lung ventilation contributes to O_2 uptake (~20%); lung ventilation frequency increases; central hypoxia does not affect lung burst frequency.
Metamorphosis	Late-staged tadpoles	Metamorphic	XIX - XXII	Circulating thyroid hormone levels increase; forelimbs protrude; gills regress and no longer contribute to gas exchange; Blood CO_2 partial pressure (PCO_2) increases associated with compensatory accumulation of bicarbonate ($HCO3^-$) in the blood; central hypoxia depresses lung burst frequency.
		Froglet	XXIII - XXIV	Gross anatomical changes occur in the mouth and pharynx; tail is still present but regressing; PCO_2 is similar to that of adults; adult circulation is established; lungs are developed with septa and contribute ~80% to O_2 uptake.
Postmetamorphosis	Juvenile frog		XXV	Circulating thyroid hormone levels decrease; tail finishes resorbing; body mass is similar to tadpoles.
	Adult frog		N/A	Increased mass and reproductive maturity.

[1]Descriptions based on data from *Lithobates catesbeianus* and *L. pipiens* (Atkinson and Just, 1975; Burggren and West, 1982; Janes and Kinkead, 2018; Just et al., 1973; Mondou and Kaltenbach, 1979; Taylor et al., 2013; Taylor and Kollros, 1946).

[2]TK Stages defined according to Taylor and Kollros (1946).

Table 2:

Summary of developmental changes in respiratory control contributing to adult ventilation.

Characteristics of adult respiratory control		Significance of developmental changes for adult ventilation	Putative neurochemicals and brain regions
Rhythmogenesis	**Buccal oscillator**	Buccal motor output is retained in adults for olfaction, vocalization and lung ventilation. Developmental changes are not documented.	• Glutamate, Cl^--mediated synaptic inhibition • Medulla adjacent to CN X
	Lung oscillator	The frequency and amplitude of lung motor output increases with development; disinhibition may contribute to robust lung ventilation.	• Glutamate, Cl^- -mediated synaptic inhibition, GABA (metabotropic) • Medulla adjacent to CN IX
	Putative oscillators	Contributions of additional putative respiratory oscillators to adult ventilation remain hypothetical.	• Cl^- -mediated synaptic inhibition (?) • Rostral medulla (?)
Connectivity between central respiratory oscillators		Increased connectivity between respiratory oscillators may be important for timing and phasing of adult respiratory motor output.	• Unknown neurochemicals • Central respiratory oscillators
Episodic air-breathing		Under resting conditions, adult air-breaths are often clustered into episodes, separated by periods of respiratory apnea.	• GABA/glycine (ionotropic), GABA (metabotropic), acetylcholine • Medulla; nucleus isthmi (NI)
Motor command to respiratory motoneurons		Increased connectivity between the lung oscillator and motoneurons may facilitate lung ventilation in adults. Central areas generating feeding and vocalization behaviours also engage respiratory motor nuclei.	• Glutamate (AMPA and NMDA receptors) • Trigeminal, facial, vagus and hypoglossal motoneurons
Central hypoxic chemoreflexes		In adults, inhibition of lung ventilation during hypoxia may be metabolically adaptive or serve to counter strong peripheral excitatory inputs.	• GABA (ionotropic); noradrenaline • Nucleus isthmi (NI); locus coeruleus (LC); medulla
Metamorphic remodeling of respiratory organs		Buccal cavity remodeling and lung maturation contributes to adult lung ventilation. Inflation of the lung cavities aids in lymphatic circulation.	• Thyroid hormones: T3 and T4 • Hypothalamo-pituitary-thyroid axis (HPT)