SEROTONERGIC MODULATION OF RESPIRATORY RHYTHMOGENESIS AND CENTRAL CHEMORECEPTION

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In addition to evidence supporting serotonergic modulation of respiratory rhythmogenesis, serotonergic mechanisms play a role in central respiratory chemoreception. We examined the role of serotonin 5HT1A receptors in respiratory rhythmicity and central respiratory chemosensitivity in *in vitro* brainstem preparations of the bullfrog tadpole, *Rana catesbeiana*. Spontaneous respiratory motor output was recorded from cranial nerve 7 at control bath pH (7.8) and hypercapnic bath pH (7.4) as bath concentrations of a 5HT1A receptor agonist were steadily increased from 0.5 to 25 μM. Activation of the 5HT1A receptor significantly altered the respiratory burst cycle. Significant increases in both gill and lung burst cycle were observed in response to bath application of 8-OH-DPAT; gill burst cycle in response to 8-OH-DPAT was influenced by bath pH, as gill burst cycle at bath pH 7.8 was not significantly increased at 0.5 or 5.0 μM 8-OH-DPAT. However, when the pH was reduced to 7.4 gill burst cycle was significantly increased at these same bath concentrations of 8-OH-DPAT. Gill burst amplitude was not altered in response to bath application of 8-OH-DPAT; however, lung burst amplitude was significantly decreased at 25.0 μM 8-OH-DPAT at bath pH 7.8. These data indicate that 5HT1A receptors are involved in neural respiratory rhythmogenic and chemoreceptive circuits in the bullfrog tadpole, and support the hypothesis that abnormalities in serotonergic systems may be an underlying component of Sudden Infant Death Syndrome. (*Ethn Dis*. 2010;20[Suppl 1]:S1-39–S1-44)

Key Words: Serotonin, Respiration, Carbon Dioxide, Chemoreceptors

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INTRODUCTION

Serotonergic modulation of respiratory rhythm generation has been described recently in mammalian,[1–3] cat,[4–7] turtle[8] and amphibian preparations.[9] Although respiratory rhythm generation and central chemoreception are currently being studied in reduced mammalian preparations, the inaccessibility of the mammalian fetus makes ontogenetic studies in mammals difficult. In addition, mammalian brain slice preparations that exhibit respiratory burst activity must be relatively thin to facilitate adequate oxygenation of the tissue. Reducing the mammalian brainstem to a thin slice in order to maintain oxygenation eliminates the ability to simultaneously study rhythm generation and central chemoreception given that respiratory chemoreceptors are widely distributed throughout the brainstem,[2] but are absent from these slices. The *in vitro* tadpole brainstem preparation lets us investigate the ontogeny of both respiratory rhythmogenesis and central chemoreception simultaneously, as synaptic connectivity of their central control networks is intact, and the developmental emergence of these functions is experimentally accessible throughout metamorphosis. For example, we have been able to identify chemosensitive regions while simultaneously recording respiratory motor output; something that has not been possible with current mammalian preparations. The development of both respiratory rhythm generation and central respiratory chemoreception are of considerable interest in that disruption in the normal development of rhythm generation or chemoreception has been implicated in the pathophysiology of Sudden Infant Death Syndrome (SIDS).

Serotonergic neurons are widely distributed throughout the mammalian central nervous system, including the cerebral cortex,[1] hippocampus and brainstem.[2] Serotonergic neurons have been implicated in synaptic plasticity and long-term potentiation in the hippocampus[10] and neocortex.[11] Serotonergic neurons located in the midline raphe nuclei send projections throughout the medulla and spinal cord to influence respiration, cardiovascular function, and autonomic output.[2] Experiments performed in rats also indicate that serotonin-dependent plasticity of respiratory responses is elicited by simulated apnea,[12] intermittent,[9] and chronic intermittent hypoxia,[13] indicating that serotonin is a modulator of respiratory rhythm generation. While many investigators report serotonergic excitation of respiration, others report biphasic responses to serotonin. Serotonin applied to the bath of the neonatal rat brainstem/spinal cord preparation elicited an initial increase in respiratory motor output, followed by inhibition.[14] Richerson[2] states that caution should be used in the interpretation of studies involving the neonatal rat brainstem/spinal cord preparation, as this preparation "has a poorly oxygenated core." Moreover, with the large variety of serotonin receptor subtypes that exist, some of the ambiguity in responses to serotonin may originate from simultaneous activation of different receptor subtypes. For example, the 5-HT1A receptor is a pre-synaptic autoreceptor that inhibits serotonergic neurons, while 5HT2, 4, 6, and 7 receptor subtypes are excitatory. A role for the 5HT1A receptor in respiratory control has been demonstrated in the stimulation of ventilation following spinal cord injury in rats[3] and in normal cats.[4–6] In

addition, iontophoresis of the 5HT1A agonist 8-OH-DPAT inhibits medullary raphe respiratory neurons.[7,15]

Developmental stage-dependent changes in fictive gill and lung ventilation caused by bath application of 5HT to tadpole brainstem preparations from pre- and post-metamorphic developmental stages have been reported.[16] The motor output recording related to gill and lung ventilation showed that 5HT elicited a dose-dependent depression of gill burst frequency in both early and late stage groups. However, the effect on lung burst frequency was stage dependent; lung burst frequency increased at 0.05 μM 5HT concentrations in the post-metamorphic group, and decreased as 5HT concentrations were increased to 25.0 μM at all developmental stages studied. Therefore, it was concluded that serotonergic modulation of respiratory motor output 1) changes during tadpole development and 2) is distinct for gill and lung ventilation.[16] Also, the differences between gill- and lung-related activities indicate that the neural mechanisms involved in these two modes of breathing are different, and responses derived from 5HT1A receptors contribute to the group differences in 5HT responsiveness for specific components of respiratory motor output. This work by Kinkead et al,[16] documented that serotonergic alteration of respiratory rhythmogenesis was present in the tadpole and has served as a foundation for our study, which expands the scope of serotonergic modulation to central respiratory chemoreception.

In addition to the evidence supporting serotonergic modulation of respiratory rhythmogenesis, serotonergic mechanisms also play a role in central respiratory chemoreception. Carbon dioxide sensitive neurons in the medullary raphe of cats are immunoreactive for 5HT,[17] and some rat medullary raphe neurons were stimulated by increases in CO_2 and inhibited by decreases in CO_2.[18] In addition, lesions of the medullary raphe[19] in the rat, or pharmacological inhibition of serotonergic neurons in the newborn piglet,[20,21] caused depression of the respiratory response to inhalation of CO_2.

Recent studies implicate abnormalities in the serotonin system, specifically the 5-HT1A receptor, as an underlying cause of Sudden Infant Death Syndrome (SIDS).[22,23] Although the incidence of SIDS has decreased dramatically since the inception of the American Academy of Pediatrics 1992 "Back to Sleep Program," the incidence of SIDS is significantly higher in Native American (1.25/1000 live births) and African American (1.18/1000 live births) populations. While research has clearly implicated an important neuromodulatory role for serotonin on respiration and disruption of respiratory rhythm generation,[24] no studies to date have described serotonin's role in modulating the development of respiratory rhythmogenesis and central chemoreception. We utilized the *in vitro* tadpole brainstem preparation to explore the effect of serotonin acting through 5-HT1A receptors on respiratory central pattern generators and central chemosensory responses in the isolated tadpole brainstem preparation.

METHODS

Experiments were performed using the larval bullfrog, *Rana catesbeiana*, purchased from a commercial supplier (Charles Sullivan Co. Inc., Nashville, TN) and housed in several 40-gallon aquaria at 20–23°C. Each aquarium was fitted with a filtration system that filtered water from an external reservoir so that all aquaria received the same water. Tadpoles in each tank were fed an appropriate amount of food (0.3–0.5 g, Fish Flakes, Wardley, AZ, USA) daily so that food was not a limiting resource. All tadpoles were exposed to a 12:12 light-dark cycle. Tadpoles were studied in developmental stages 8 to 14 as described by the Taylor and Kollros[25] staging system.

In vitro Brainstem Preparation

Tadpoles were weighed and anesthetized via immersion in tricaine methanesulfonate (MS-222; 1:10,000) in distilled water until unresponsive to touch. The brainstem was dissected under continuous superfusion of oxygenated artificial CSF (in mM: NaCl, 104; KCl, 4; $MgCl_2$, 1.4; D-glucose, 10; $NaHCO_3$, 25 and $CaCl_2$, 2.4).[26–28] After removal of the dura and arachnoid, the brainstem was transferred to a superfusion chamber for electrophysiological recordings. Efferent recordings from the roots of cranial nerve 7 (CN 7) were made using thin-walled borosilicate glass suction electrodes. Efferent activity was amplified (AM Systems 1700, Sequim, Wash., USA), filtered (100 Hz to 1 kHz), digitized (2000 Hz), passed through a moving time average (CWE MA-821, time constant = 100 msec) and recorded on a Pentium computer (DataPak2K2, Run Technologies, CA, USA) for data analysis.

Protocol

The 5-HT1A serotonin receptor agonist 8-OH-DPAT was bath applied to the *in vitro* tadpole brainstem while simultaneously recording respiratory motor output from cranial nerve 7. Ten-minute recordings of respiratory motor output were performed during control (bath pH = 7.8; approximately 5% CO_2, balance O_2) and hypercapnic acidosis (bath pH = 7.4). Decreases in bath pH were attained by increasing the partial pressure of CO_2 in the superfusate. The pH of the superfusate was continuously monitored using a pH electrode (Thermo Orion 420, Waltham, Mass., USA). The following concentrations of 8-OH-DPAT were tested with and without hypercapnia: 0.5, 1.0, 5.0, 10.0, and 25.0 μM. Ten minutes of the gill and lung respiratory bursts were recorded at bath pH of 7.8 and then the bath pH was changed to

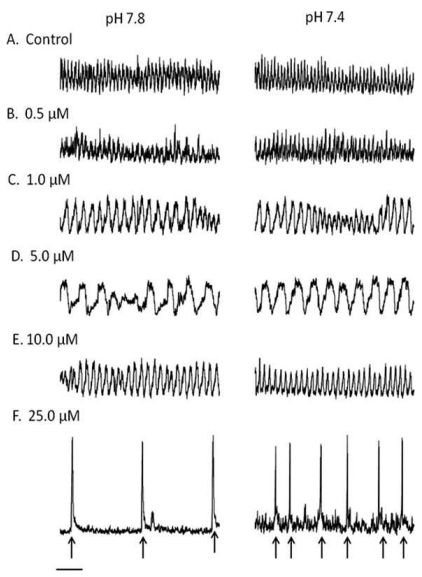

Fig 1. Respiratory burst activity recorded from cranial nerve 7 from a developmental stage 12 tadpole brainstem preparation during control (bath pH = 7.8) and hypercapnia (bath pH = 7.4) as the bath concentration of the 5HT1A receptor agonist 8-OH-DPAT was increased. All neural activity illustrated in panels A–E represent the relatively high frequency gill burst activities. The bath application of 25.0 μM 8-OH-DPAT (Panel F) resulted in a reduction of gill burst activity and the emergence of the pH sensitive large amplitude lung burst indicated by the upward arrow (scale bar = 10 sec)

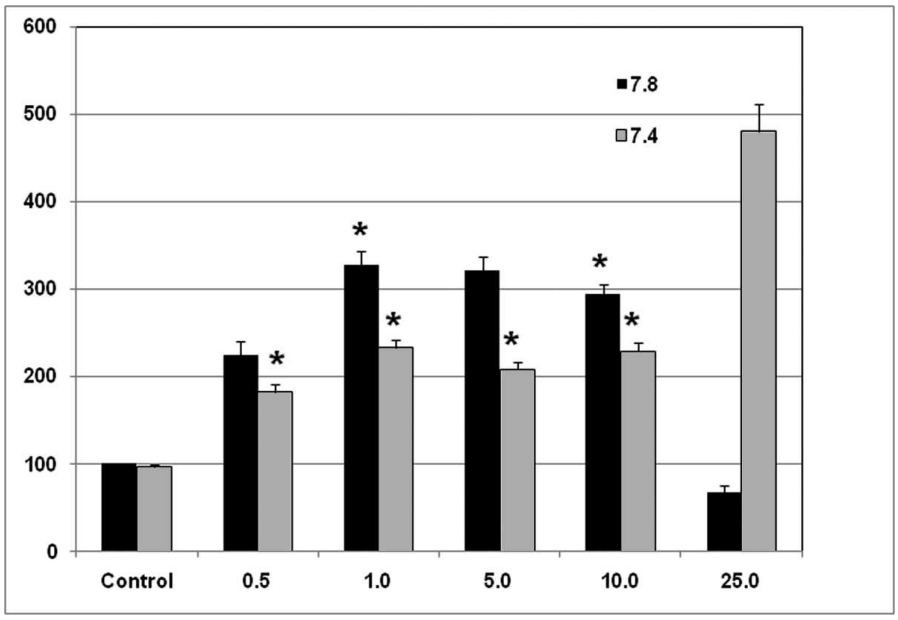

Fig 2. Mean gill cycle (± S.E.M.) during control and increasing bath concentrations of 8-OH-DPAT during normocapnia (bath pH = 7.8) and hypercapnia (bath pH = 7.4) normalized as a percent of the control pH 7.8 value. * indicate significant difference ($P<.05$) from the same pH during control

bath pH 7.4. We observed respiratory activities attain a steady state in response to bath changes in pH within 10 minutes. Upon reaching a steady state of respiratory motor activities, the pH 7.4 recording was performed. This process was repeated for each sequential increase in the concentration of 8-OH-DPAT from 0.5 to 25.0 μM (Figure 1). We recorded all data upon changing drug concentration; however, only steady state respiratory activities were used for data analysis. The tadpole brainstem is a robust preparation and is capable of producing reliable, spontaneous respiratory motor output for durations well beyond the time required to conduct our protocol.

Data Analysis and Statistics

Gill and lung burst amplitudes and cycle were calculated from the CN 7 neurogram and were analyzed during each control and test condition as previously described.[26–28] The significance of drug effects on respiratory activities within each pH condition were examined using a one-way ANOVA with multiple pairwise comparisons. Mean gill and lung burst activities were normalized to the percent of the control pH 7.8 value. Mean ± 1 standard error of the mean are reported; significant effects are reported for a $P<.05$.

RESULTS

Increasing bath concentrations of the 5HT1A receptor agonist 8-OH-DPAT significantly altered respiratory burst activities as the concentration of 8-OH-DPAT was increased through the range of 0.5 to 25.0 μM. Values reported below are normalized as a percent of the control 7.8 value.

Gill Respiratory Motor Activities

Gill burst cycle at control bath pH of 7.8 was not significantly different when the brainstem was exposed to the hypercapnic bath pH of 7.4 (96.0 ± 3.7). Increasing 8-OH-DPAT at bath pH of 7.8 caused significant increases in gill cycle at 1.0 μM (328.1 ± 86.5) and 10.0 μM (293.6 ± 54.7). Bath application of 5HT1A agonist at a bath pH of 7.4 produced significant increases in gill cycle at 0.5 μM (181.7 ± 9.6), 1.0 μM (232.3 ± 8.7), 5.0 μM (207.9 ± 8.7), and 10.0 μM (229.0 ± 10.3). Mean gill burst cycle in response to changes in bath pH and 8-OH-DPAT concentration is illustrated in Figure 2. Within each drug concentration, we found no significant differences in gill cycle between bath pH 7.8 and 7.4. No significant changes were found in the gill amplitude with either changes in bath pH or the presence of 8-OH-DPAT.

Lung Respiratory Motor Activities

Lung burst cycle at control bath pH of 7.8 was not significantly different

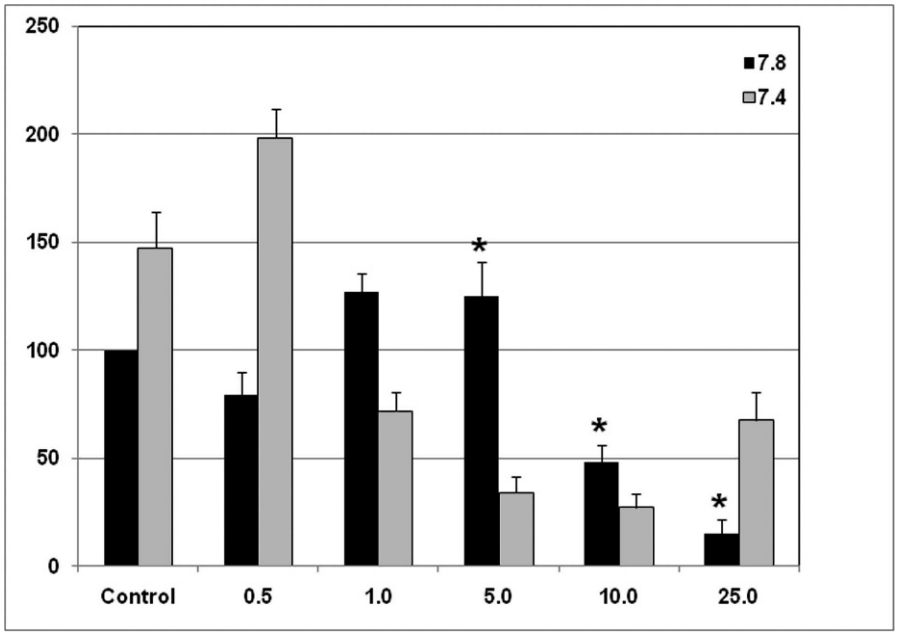

Fig 3. Mean lung cycle (± S.E.M.) during control and increasing bath concentrations of 8-OH-DPAT during normocapnia (bath pH = 7.8) and hypercapnia (bath pH = 7.4) normalized as a percent of the control pH 7.8 value. * indicate significant difference ($P<.05$) from the same pH during control

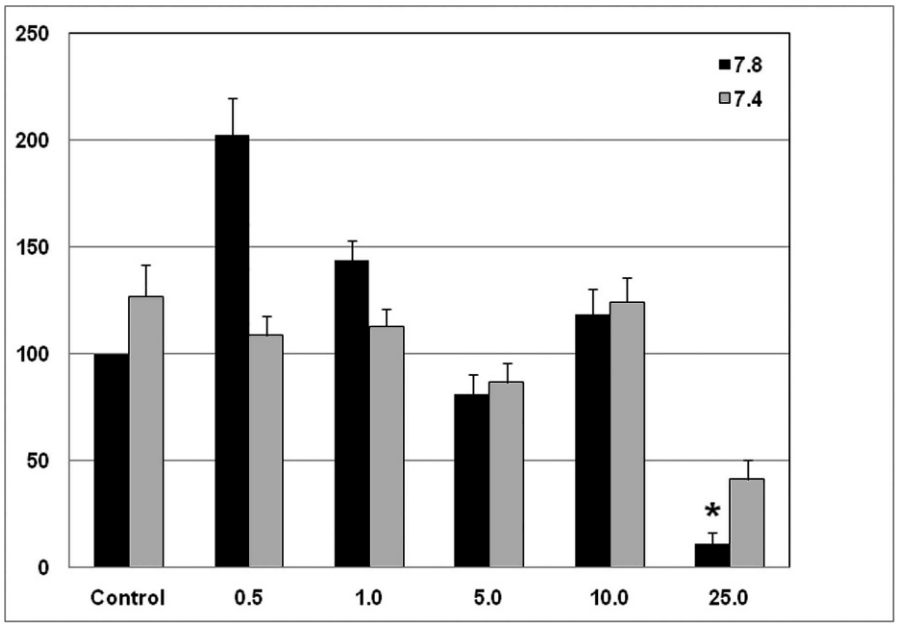

Fig 4. Mean lung amplitude (± S.E.M.) during control and increasing bath concentrations of 8-OH-DPAT during normocapnia (bath pH = 7.8) and hypercapnia (bath pH = 7.4) normalized as a percent of the control pH 7.8 value. * indicate significant difference ($P<.05$) from the same pH during control

when the brainstem was exposed to the hypercapnic bath pH of 7.4 (147.1 ± 104.0). Increasing 8-OH-DPAT at bath pH of 7.8 caused significant increases in lung cycle at 5.0 μM (124.6 ± 96.7). While maintaining bath pH at 7.8, increasing bath concentration of 8-OH-DPAT decreased lung cycle at 10.0 μM (47.8 ± 26.1) and 25.0 μM (15.1 ± 15.1; Figure 3). There were no significant changes in lung burst cycle from control at bath pH 7.4 with the addition of increasing concentrations of 8-OH-DPAT. Within each drug concentration, we found no significant differences in lung cycle between bath pH 7.8 and 7.4. Lung burst amplitude was not significantly different when the brainstem was exposed to the hypercapnic bath pH of 7.4 (127.1 ± 77.8) when compared to control bath pH 7.8 (100 ± 0). Increasing concentrations of 8-OH-DPAT at bath pH 7.8 caused significant decreases in lung amplitude at 25 μM (10.8 ± 10.8) as illustrated in Figure 4. There were no significant differences between lung burst amplitude at control bath pH of 7.4 and increasing 8-OH-DPAT concentrations at bath pH of 7.4. No significant differences were found in lung burst amplitude between bath pH 7.8 and 7.4 at each of the 8-OH-DPAT concentrations.

DISCUSSION

Our study of the activation of the 5HT1A receptor in the tadpole brainstem preparation produced several significant findings. We observed significant increases in gill burst cycle in response to bath application of 8-OH-DPAT at both control bath pH of 7.8 and the hypercapnic bath pH of 7.4. The increase in gill burst cycle at bath pH 7.8 reported in this study is consistent with the findings of Kinkead et al,[16] in which they reported decreases in gill burst frequency in response to bath application of 5HT in similar

developmental stage tadpole brainstem preparations. Whereas significant increases in gill burst cycle at bath pH 7.8 were observed at 1.0 μM 8-OH-DPAT, reducing bath pH to 7.4 via hypercapnia produced significant increases in gill burst cycle at a lower concentration of 0.5 μM 8-OH-DPAT. Because 8-OH-DPAT is an agonist of an autoreceptor, the level of serotonin signaling should decrease as 8-OH-DPAT is increased. Our data therefore suggest that the reduction in serotonin signaling via the 5HT1A receptor mediates the increase in gill burst cycle in the premetamorphic tadpole. In some animals, the gill burst activity was abolished at 25.0 μM 8-OH-DPAT, and this loss of the gill burst rhythm was associated with the emergence of the lung burst pattern (Fig 1, Panel F). The cessation of gill burst activity combined with the emergence of the lung burst pattern recorded at 25.0 μM 8-OH-DPAT is similar to the developmental disinhibition of the lung central pattern generator previously described by Straus et al.[29] These early stage tadpoles have few spontaneous lung bursts during normocapnia, but lung bursting is enhanced by hypercapnia.[26–28] When 8-OH-DPAT treatment suppressed gill bursting sufficiently to allow lung bursts to emerge in these pre-metamorphic animals, the frequency of lung bursts was increased by hypercapnia.

We also observed significant changes in lung burst cycle in response to 8-OH-DPAT application; significant increases in lung burst cycle were observed at 5.0 μM, whereas 10.0 μM reduced lung burst cycle. The increase in lung burst cycle at 5.0 μM followed by decreases in lung burst cycle at 10.0 μM may reflect an inhibition of lung burst activity associated with relatively low 8-OH-DPAT (5.0 μM). However as serotonin signaling continues to decrease with increases of 8-OH-DPAT to 10.0 μM, the lung burst may be released from serotonin-mediated inhibition. Wilson et al,[30] reported evidence of two distinct respiratory oscillators in the tadpole brainstem responsible for gill and lung burst activities. Straus et al,[29] reported that the developmental expression of lung ventilation in the tadpole was due to GABAergic mediated developmental disinhibition of the lung central pattern generator. More recent research and the results of this study also support a developmental neuromodulatory role of serotonin, via the 5HT1A receptor, on gill and lung respiratory central pattern generators.[16,31] Kinkead et al,[16] reported that the decreases in gill burst frequency in response to 5-HT1A receptor activation were less than the gill frequency decreases associated with 5-HT alone. These effects of serotonergic activation on gill burst activity indicate that the depression of gill burst frequency is associated with activation of not only the 5-HT1A receptor, but other serotonin receptor subtypes as well. Belzile et al,[31] eliminated the 5-HT2A/C receptor as a possible mediator of the depressive effects of serotonin on gill burst activities, as the bath application of the selective 5-HT2A/C receptor agonist ([9/]-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; DOI) had no effect on either gill or lung burst activities in the superfused *in vitro* tadpole brainstem preparation.

We have also reported the presence of the lung central pattern generator and the ability to produce lung burst activities *in vitro* via bath application of bicuculline and strychnine in tadpole preparations as young as developmental stage 3.[32] It is not known what the stimuli are for the proposed decrease in GABAergic inhibition of lung burst activities during development; however, the larval bullfrog must be poised to undergo rather rapid development from aquatic to terrestrial life in response to changes in the environment such as availability of resources, water, overcrowding, temperature, and photoperiod.[33] The significant increase in lung activities in response to increases in bath applied 8-OH-DPAT reported in this study support the neuromodulatory role of serotonin on respiratory development. 5-HT inhibits lung bursts activities in the adult turtle brainstem *in vitro*,[8] and it was proposed that 5-HT inhibits respiratory drive of lung ventilation during diving in turtles. While the inhibition of lung bursts in the turtle contrast the excitation of lung activities in the tadpole reported by Kinkead et al,[16] and demonstrated in this study, it is clear that non-mammalian preparations also employ serotonergic transmission to modulate respiration. Further similarities of respiratory control between amphibians and mammals was reported by Sundin et al,[34] as the locus coeruleus is a source of CO_2/pH ventilatory drive in both toads and mammals. Data from the *in vitro* tadpole preparation support a developmentally dependent modulation of respiration via serotonin. The autoinhibition of serotonergic transmission that would occur with 8-OH-DPAT application appears to elicit an increase in the expression of the lung motor pattern, and like the developmental disinhibition of lung burst activities described by Straus et al[29] may serve as an additional mechanism involved in the transition from gill to lung ventilation. The neural development of the lung pattern generator circuitry and the ability to transition to lung burst activities relatively quickly, promotes the ability to quickly adapt to adverse aquatic conditions and move toward a terrestrial life.

ACKNOWLEDGMENTS

This work was supported by the National Centers for Research Resources (NCRR), Research Centers in Minority Institutions (RCMI) grant 5G12 RR013646-08 awarded to MJG.

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