NICOTINE EXPOSURE IN THE DEVELOPING BULLFROG:

INFLUENCES ON NEUROVENTILATORY RESPONSES TO CO_2

By

Cord M. Brundage

RECOMMENDED:

Advisory Committee Chair

Richard D. Boone

Chair, Department of Biology and Wildlife

APPROVED:

Dean, College of Natural Science and Mathematics

Dean of the Graduate School

July 25, 2008

Date

NICOTINE EXPOSURE IN THE DEVELOPING BULLFROG: INFLUENCES ON NEUROVENTILATORY RESPONSES TO CO_2

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Cord M. Brundage, B.S.

Fairbanks, Alaska

August 2008

ABSTRACT

Developmental exposure to the neuroteratogen nicotine may affect ventilatory responses to hypercapnia. Developmental changes in normocapnic and hypercapnic neuroventilation of the isolated bullfrog brainstem preparation have been previously characterized. I investigated the effect of 3- and 10-wk chronic nicotine (30 µg/L) exposure on lung burst frequency exhibited by early and late metamorphic bullfrog tadpoles during normocapnia (1.5 % CO_2) and hypercapnia (5.0 % CO_2). Chronic nicotine exposure impairs the hypercapnic neuroventilatory response of early metamorphic tadpoles following both 3- and 10-wk exposure. Late metamorphic tadpoles demonstrated an impaired hypercapnic neuroventilatory response only after 10-wk exposure. Chronic nicotine exposure had no effect on normocapnic neuroventilation. Brainstem preparations from early and late metamorphic tadpoles and juvenile bullfrogs were exposed acutely to 18 µg/L nicotine. Acute nicotine had no effect on normocapnic or hypercapnic neuroventilation of early metamorphic tadpoles. Late metamorphic tadpoles and juvenile bullfrogs demonstrated depressed normocapnic neuroventilation in response to acute nicotine exposure, while late metamorphic tadpole brainstems responded significantly to hypercapnia during acute exposure. This suggests that bullfrogs have a differential response to acute nicotine exposure that increases with development. Collectively these data suggest that the consequences of developmental nicotine exposure differ between acute and chronic exposure and throughout bullfrog development.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I must first and foremost acknowledge the support and dedication of my advisor and mentor Dr. Barbara E. Taylor. I am not sure if it was fortuitousness or happenstance that brought me to your lab. What I do know is that I will be forever grateful for this opportunity and the wisdom you instilled. Your steadfast dedication, boundless competency, and eloquent word-smithing are truly awe inspiring. I know that your modesty often negates such praise, but know that your confidence, commitment and knowledge motivate me daily to be a better scientist and human being.

Special thanks must be given to my graduate committee; their support perhaps could best be described as a complex series of interdependencies. Dr. Michael Harris who's bountiful acumen and cunning mastery of electrophysiology were always quick to my aid. You are truly the "brain whisperer". Dr. Jonathan Runstadler, thank you for your support and mentorship; I know that putting up with us "head cases" wasn't the grandest experience. Your insight has been a blessing and I remain in your debt. Dr. Kelly Drew, thank you for always raising the bar of excellence and for your encouragement that always pushed me to achieve more.

I fervently acknowledge the other members of Dr. Barbara Taylor's Comparative Neurophysiology Laboratory and the Electrophysiology Core. They had to put up

with my meddling and managed to do so (thus far) without major incident, and that says a lot.

I would like to thank my family and the score of individuals that have touched my life however briefly. I am no more than the product of those I have known. I can only hope to give as much to others as you have to me.

I was funded in part under a teaching assistantship through the University of Alaska Fairbanks (UAF) Department of Biology and Wildlife and through a research assistantship provided by the UAF Institute of Arctic Biology. My program was supported by the Alaska Basic Neuroscience Program, which is funded by a Specialized Neuroscience Research Program grant. My research was paid for by a National Institute of Health grant through the National Institute of Neurological Disorders and Stroke. Grant # 2U54NS041069-601A

All procedures outlined in this thesis were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC; approval # 04-04).

GENERAL INTRODUCTION

The isolated *in vitro* brainstem prepared from the bullfrog (*Lithobates catesbeiana*) has been used previously as a model to investigate the development, evolution and central mechanisms of neuroventilatory control (8, 26). My research investigated the acute and long-term developmental effects of nicotine exposure on the neuroventilation exhibited by the bullfrog brainstem at discrete points in development. I focused primarily on the CO_2-induced increase in lung burst frequency, which is designated as the hypercapnic response.

The ventilatory-related motor output from the isolated bullfrog brainstem was first identified in the early 1970s (36), but was later developed independently and further characterized in the early to middle 1990s (7, 23, 25, 34). Since the middle 1990s the isolated bullfrog brainstem preparation has been used to elucidate the central mechanisms underlying vertebrate respiratory rhythmogenesis (7, 9, 28, 48, 56, 58, 59). Arguably the most extensively investigated ventilatory-related phenomenon in these preparations is the neuroventilatory response of bullfrogs to changes in pH/CO_2 (9, 12, 19, 30, 49-54). Hypercapnia (5 % CO_2) causes an increase in the occurrence/frequency of neural discharge associated with lung ventilation. The magnitude of the CO_2-induced lung burst frequency changes increases with bullfrog development; early metamorphic tadpoles exhibit only moderate increases in lung burst frequency

while late metamorphic tadpoles and fully developed bullfrogs are highly responsive.

Mammals, similar to bullfrogs, exhibit a developmental increase in their hypercapnic neuroventilatory response. Mammalian neonates respond to hypercapnia but lack the robust increase in ventilatory frequency that accompanies development (35). Human infants with diminished capacity to respond to hypercapnia or other ventilatory stressors, during the critical developmental period when ventilatory control is first maturing, may be particularly vulnerable to sudden infant death syndrome (SIDS) (4, 10, 16). Developmental exposure to nicotine, an alkaloid found in tobacco that targets nicotinic acetylcholine receptors, has been identified as a risk factor for SIDS (5, 17, 32).

Nicotine, through its activation of nicotinic acetylcholine receptors, can have profound effects on neural development. Nicotinic acetylcholine receptors (nAChRs), which are heteropentameric ligand-gated ion channels assembled by the combination of $\alpha(\alpha 1\text{-}10)$, $\beta(\beta 1\text{-}4)$, γ, δ, and ε subunits (22, 39), are widely distributed across the central nervous system. nAChRs play a critical role in development as early as gastrulation (21). nAChRs have been detected in developing vertebrate brains during early neurulation (1, 37). The cholinergic system helps orchestrate the transition in neuronal development from an

emphasis on replication to an emphasis on terminal differentiation (42, 43, 46), as well as serving as a modulator of both synaptogenesis and axonogenesis (40, 41). The cholinergic system remains important as development continues. The cholinergic system is important in maintaining the integrity and assembly of brain regions regulating learning, memory and behavior (14, 15). nAChRs are also expressed in brainstem regions associated with respiratory control. nAChRs are implicated in both ventilatory regulation and CO_2 sensitivity (6, 29, 38, 39, 57).

Chronic prenatal exposure to nicotine and persistent nAChR activation affects neuronal cell replication and differentiation, impairs synaptic function and elicits several behavioral deficits (41, 42, 44). Despite the evidence of nicotine's neuroteratogenic action, the causal link between nicotine and ventilatory pathologies such as SIDS has not been revealed (2, 32, 45). This may be partly due to the enigmatic nature of SIDS diagnosis, which is simply defined as the death of an infant under 1 yr of age that remains unexplained after a full investigation (4, 10, 20). Another difficulty is in the development of models that potentially exhibit SIDS or SIDS-associated pathologies.

An impaired hypercapnia ventilatory response has been demonstrated in early metamorphic bullfrogs *in vivo* and their isolated brainstems *in vitro* following experiments involving chronic developmental exposure to nicotine (49). This pathology is similar to that hypothesized to occur in some SIDS deaths (11).

Comparable work has been done in mammals with similar results (3, 47), but assessing the impact of nicotine on ventilation throughout development while maintaining or controlling the amount of nicotine exposure is difficult in mammals owing to their *in utero* development, which hinders controlled developmental exposure (49). The free living larval forms of the bullfrog can be reared in tanks with a constant level of nicotine exposure, and any resulting neuroventilatory deficits can be assessed easily at any stage of development.

Use of the isolated bullfrog brainstem preparation to understand the ventilatory consequences of nicotine exposure is relevant to understanding the general impact of developmental nicotine exposure on vertebrate neuroventilation. The neural network involved in the sensation of hypercapnia and the resulting compensatory increase in ventilation is poorly understood among vertebrates (26, 27). Understanding how developmental nicotine exposure impairs the bullfrog neuroventilatory response to hypercapnia may offer insights into the mechanisms involved in CO_2-sensitive ventilatory modulation among vertebrates in general.

My aim was to determine how developmental nicotine exposure would impact neuroventilatory function. My overarching hypothesis was that developmental nicotine exposure would elicit an impairment in the maturation of early metamorphic tadpoles' hypercapnic neuroventilatory response, an impairment

distinguishable from that of acute nicotine exposure. I manipulated the duration and timing of chronic developmental nicotine exposure and compared the effects of varied developmental exposures to the effects of acute exposure.

I varied the duration of nicotine exposure because previous work addressing the hypercapnic neuroventilatory response of early metamorphic tadpoles exposed animals to nicotine for 8-10 wk before assessing their neuroventilation (49). It was not clear whether a full 8-10 wk of exposure was necessary to impair the tadpole hypercapnic neuroventilatory response. I hypothesized that a shorter duration of nicotine exposure would impair the modest hypercapnic responses of early metamorphic tadpole brainstems. To test this hypothesis, in Chapter 1, I compared the hypercapnic neuroventilatory responses of tadpoles following 3- and 10-wk nicotine exposure with those of control tadpoles not exposed to nicotine.

The robust nature of the late metamorphic tadpole response to hypercapnia is similar to that of the mature bullfrog (50, 51). I theorized that this was because maturation of the bullfrog respiratory control network is complete by late metamorphosis. I hypothesized that the relative immaturity of the early metamorphic tadpoles would result in an increased sensitivity to the neuroteratogenic effects of nicotine, and therefore, a greater deficit in the neuroventilatory response to hypercapnia. To test this hypothesis on the effect of

the timing of chronic developmental nicotine exposure (see Chapter 1) I investigated the hypercapnic neuroventilatory response of early and late metamorphic tadpoles following both the 3- and 10-wk chronic developmental exposures to nicotine. I compared these responses to the hypercapnic neuroventilatory responses of control tadpoles of the same stages.

To demonstrate that chronic nicotine exposure during development elicits a duration-dependent functional deficit, I had to distinguish its effects from those of acute nicotine exposure. Little work has been done on amphibians to investigate the role of the cholinergic system in ventilatory control. The effect of central acute nicotine exposure has been investigated in the intact adult cane toad and was found to increase episodic breathing frequency during normal isocapnic conditions (18). No change in normocapnic ventilation was evident in chronically exposed early metamorphic tadpoles (49). Together these studies suggest that chronic developmental nicotine exposure and acute nicotine exposure affect tadpole ventilation differently. To test this hypothesis (see Chapter 2), I applied acute nicotine during normocapnia and then subsequent hypercapnia in isolated brainstem preparations of early and late metamorphic tadpoles, and juvenile frogs. These responses were then compared to those generated by isolated brainstems that were not acutely exposed to nicotine.

It is my contention that that the ontogeny of the hypercapnic neuroventilatory response is highly vulnerable early in vertebrate development and that a chronic nicotine insult during this sensitive time contributes to functional neuroventilatory deficits, which may underlie disorders such as SIDS.

CHAPTER 1

TIMING AND DURATION OF DEVELOPMENTAL NICOTINE EXPOSURE CONTRIBUTE TO ATTENUATION OF THE TADPOLE HYPERCAPNIC NEUROVENTILATORY RESPONSE[1]

1.1 ABSTRACT

The ability of air-breathing vertebrates to adjust ventilation in response to increased CO_2 (hypercapnia) is fundamental to maintaining physiologically appropriate pH levels. Developmental nicotine exposure has been shown to impair tadpole neuroventilatory responses to hypercapnia following 10-wk exposure. It is not clear, however, whether the timing of exposure during development and/or the duration over which the exposure takes place are critical to this impairment. Here tadpoles were exposed to 30 µg/L of nicotine for 3- or 10-wk periods, either early or late in tadpole development. Correlates of tadpole lung neuroventilation were monitored during normocapnic (1.5 % CO_2) and hypercapnic (5 % CO_2) conditions. Preparations derived from early metamorphic tadpoles failed to increase lung neuroventilation in response to hypercapnia whether they received 3- or 10-wk nicotine exposure. Preparations derived from

[1] C.M. Brundage and B.E. Taylor. 2008. Timing and duration of developmental nicotine exposure contribute to attenuation of the tadpole hypercapnic response (prepared for submission in) American Journal of Physiology: Integrative and Comparative Physiology.

late metamorphic tadpoles failed to respond to hypercapnia if they had been exposed to nicotine for 10 wk. These results suggest that both the stage of development and duration of exposure are important when considering nicotine's effect on the hypercapnic neuroventilatory response.

1.2 INTRODUCTION

Hypercapnic acidosis stimulates respiratory output, a modulation chiefly mediated by central CO_2/pH-sensitive neurons (11, 15, 17, 19). CO_2/pH-sensitive neurons provide feedback on the adequacy of ventilation relative to metabolism to brainstem respiratory control regions (15). The existence of central CO_2/pH receptors has been demonstrated in all tetrapod vertebrates (10). Peripheral CO_2/pH receptors are known to exist in the carotid bodies. Ventilatory responses to hypercapnia in mammals are low in newborns and subsequently increase to adult levels with development (18). This hypercapnic response pattern is mirrored in anuran amphibians where the proportional increase in lung frequency in response to hypercapnia becomes greater as metamorphosis progresses from early tadpole stages to postmetamorphic stages (3, 28, 36, 37). These hypercapnic neuroventilatory responses (HCnVR) are characterized by an increase in lung burst frequency in response to high concentrations of CO_2.The specific mechanism responsible for the development of these hypercapnic response patterns may be shared among all vertebrates, and it may consist of an

orchestrated transition in the relative role of excitatory and inhibitory synaptic transmission (18).

Nicotine is a developmental neurotoxin that alters the replication, formation and differentiation of brain tissue (23). The neuroteratogenic nature of prenatal nicotine exposure has been well established and may impair ventilatory responses to exogenous stressors such as hypoxia or hypercapnia (4). An attenuated response to hypoxic ventilatory stress following prenatal nicotine exposure has been demonstrated in rat pups (21, 29). Early metamorphic tadpoles exposed to 8-12 wk of 30 μg/L nicotine demonstrated a diminished ventilatory response to hypercapnia *in vivo* and in *in vitro* brainstem preparations (34).

Despite evidence that nicotine impairs tadpole HCnVRs (34), it is not clear whether a minimum of 8 wk of nicotine exposure is necessary to inhibit hypercapnia-induced increases in tadpole lung ventilation. There may be one or more specific periods of vulnerability in tadpole development when nicotine exposure impairs hypercapnic response mechanisms. Both early and late periods in tadpole development may be particularly sensitive to the effects of nicotine exposure. Early in development tadpoles undergo considerable neurogenesis and synaptogenesis (5, 31). Late in development tadpoles are at the peak of metamorphosis and are undergoing major transitions in anatomy and

physiology. These include an increased dependence on lungs for gas exchange, which is coupled with an increase in lung-related neural activity (2, 36). Either of these developmental phenomena may underlie an impairment of the HCnVR.

In the present study I attempted to differentiate how developmental timing versus duration of chronic nicotine exposure might affect impairments of the tadpole HCnVR. My experiments specifically targeted early and late periods of tadpole development and nicotine exposure durations of 3 and 10 wk. I hypothesized that early metamorphic tadpoles would be more susceptible to nicotine exposure than late metamorphic tadpoles; I anticipated impairments of the HCnVR in early but not late metamorphic tadpoles after both 3- and 10-wk nicotine exposure. This hypothesis was based on the observation that tadpoles have a robust, more fully developed HCnVR by late metamorphosis (35, 36) making them potentially less vulnerable to the neuroteratogenic effects of nicotine.

1.3 MATERIALS AND METHODS

Animals

Studies were performed on *Lithobates* (formerly *Rana*) *catesbeiana* tadpoles (n = 80) purchased from a commercial supplier (Sullivan Co. Inc., www.researchamphibians.com). The tadpoles were designated as "early metamorphic" (forelimbs absent, hind limbs paddle-like without joints or

separated toes) or "late metamorphic" (forelimbs and hind limbs present, tail being resorbed), corresponding to developmental stages 1-10 or 18-25, respectively, in the classification scheme of Taylor and Köllros (33). All tadpoles were maintained at 23 °C and fed goldfish food daily.

Tadpoles were maintained in aquaria with either dechlorinated water only or dechlorinated water containing nicotine (30 μg/L). This concentration of nicotine is consistent with that of a previous study using bullfrogs (34) and is within the concentration range of nicotine found in the body fluids of an average smoker (14). Four treatment groups were chronically exposed to nicotine: groups of early and late metamorphic tadpoles were maintained for either 3 or 10 wk (n = 16, 10, 16 and 10, respectively) in separate aquaria containing dechlorinated water with nicotine. Data collected from these treatment groups were compared with data from a group of treatment-control tadpoles (those that experienced hypercapnia but no nicotine exposure; n = 15), as well as time-control tadpoles (those that remained normocapnic for duration of the experiment; n = 12); both were maintained in an aquarium with dechlorinated water only. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Alaska Fairbanks.

Surgical preparation

Each tadpole was anesthetized by immersion for 1-2 min in cold (4 ° C) 0.2 mM tricaine methanesulfate (MS222; Sigma, www.sigmaaldrich.com) in dechlorinated water buffered to pH 7.8 with $NaHCO_3$. Using a razor blade, the front of the head rostral to the nares and the back of the body (hind limbs and tail, if present) were removed. Under a dissecting microscope, the dorsal cranium and forebrain rostral to the diencephalon were removed and the fourth ventricle opened by removing the choroid plexus. The remaining brainstem and spinal cord were removed *en bloc*, further trimmed rostral to the optic tectum and at the brachial nerve, and the dura mater was removed. During dissection, exposed tissues were superfused with cold artificial cerebral spinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 10 D-glucose, 25 $NaHCO_3$ and 2.4 $CaCl_2$ equilibrated with 100 % O_2. The aCSF HCO_3^- concentration is similar to that of plasma from late metamorphic tadpoles and frogs, but higher than the HCO_3^- concentration in plasma from early metamorphic tadpoles. These methods have been used in previous tadpole studies (34-36); to ensure comparability between experiments on animals of different metamorphic stages the aCSF composition remained consistent.

The isolated brainstem/spinal cord was transferred *en bloc* to a 0.5-ml, Plexiglas, flow-through recording chamber and was supported, ventral side up, between coarse nylon mesh such that all surfaces were bathed with aCSF flowing from

rostral to caudal at a rate of 5 ml/min. A supply of aCSF, equilibrated with O_2-CO_2 mixtures that produced the desired pH, flowed through plastic tubing to the recording chamber and bathed the isolated brainstem. The pH of the aCSF was maintained at either pH 7.8 (1.5 % CO_2: 98.5 % O_2; normocapnia) or pH 7.4 (5.0 % CO_2: 95.0 % O_2; hypercapnia) by adjusting the fractional concentrations of O_2 and CO_2 in the equilibration gas. CO_2 was monitored with a CO_2 analyzer (Capstar 100; CWE, www.cwe-inc.com). After isolation the brainstem was allowed to stabilize for ~1 h while superfused at 23 °C, with aCSF of pH 7.8 and ~9 torr P_{CO_2}.

Nerve recording

Roots of the facial and hypoglossal nerves were drawn into glass suction electrodes pulled from 1-mm-diameter capillary glass to tip diameters of 30-60 μm. Whole-nerve discharge was amplified (X100 by DAM 50 amplifiers, World Precision Instruments, www.wpiinc.com; X1000 by a four-channel model 1700 amplifier, A-M Systems, www.a-msystems.com) and filtered (100 Hz high pass to 1 kHz low pass). The amplified and filtered output was sent to a data acquisition system (Powerlab, AD Instruments, www.adinstruments.com), which sampled at 1 kHz; data were archived as whole-nerve discharge, and duplicate integrated (full-wave rectified and averaged over 200 ms) neurograms were acquired simultaneously. Such recordings were made from brainstems that were either 3- and 10-week chronically exposed to nicotine as well as brainstems derived from

tadpoles in the treatment-control and time-control groups. Neurograms were recorded during 30 min of normocapnia, followed by 30 min of hypercapnia, followed by 30 min of normocapnia for all hypercapnia-treated brainstems. In this way I recorded putative ventilatory rhythm at baseline, during hypercapnia and during the return to baseline. The treatment-control group received the same sequence of normocapnia followed by hypercapnia followed by normocapnia. For the time-control group, neurograms were recorded during 90 min of normocapnia (the total duration of the hypercapnic treatments) to determine whether changes in neuroventilation would occur over the time course of the experiments. Time-control experiments were conducted on brainstems of tadpoles maintained in dechlorinated water, as well as on brainstems from all nicotine-exposed groups.

Data analyses and statistics

Burst activity patterns in the neurograms recorded from the isolated brainstems were designated as either putative gill or putative lung breaths on the basis of the amplitude of the integrated nerve activity and the presence or absence of coincident firing in both the facial and hypoglossal nerves (Fig. 1.1), as previously described (35, 36, 38). Putative gill breaths had lower integrated burst amplitude in the facial nerve than putative lung breaths, and little or no coincident burst activity in the hypoglossal nerve. Putative lung breaths had higher integrated burst amplitude in the facial nerve and coincident burst activity in the hypoglossal

nerve. Taylor et al. (35, 36) demonstrated that changes in the frequency of putative lung breaths are the primary manifestation of the HCnVR; the duration and amplitude of putative lung breaths and the frequency, duration and amplitude of putative gill breaths are unresponsive to hypercapnia. Thus, I quantified only the frequency of putative lung bursts, which was done by comparing the number of lung bursts per min in 3 consecutive min (at the end of the 30-min neurogram) of each CO_2/pH condition (normocapnia, hypercapnia and return to normocapnia).

Frequencies of lung neuroventilation were analyzed using repeated-measures analysis of variance (RM-ANOVA) between each treatment group (RM-ANOVA procedure, SigmaStat, www.systat.com). When RM-ANOVA indicated that significant differences existed between the treatment groups, multiple comparisons were made using the Holm-Sidak multiple comparison test. Hypercapnia-induced relative changes in lung burst frequency were analyzed with T-test comparisons of the average percent change from normocapnic ventilatory frequency between each nicotine-exposed treatment group and treatment-controls. Values reported in the text are mean ± SE bursts/min unless stated otherwise.

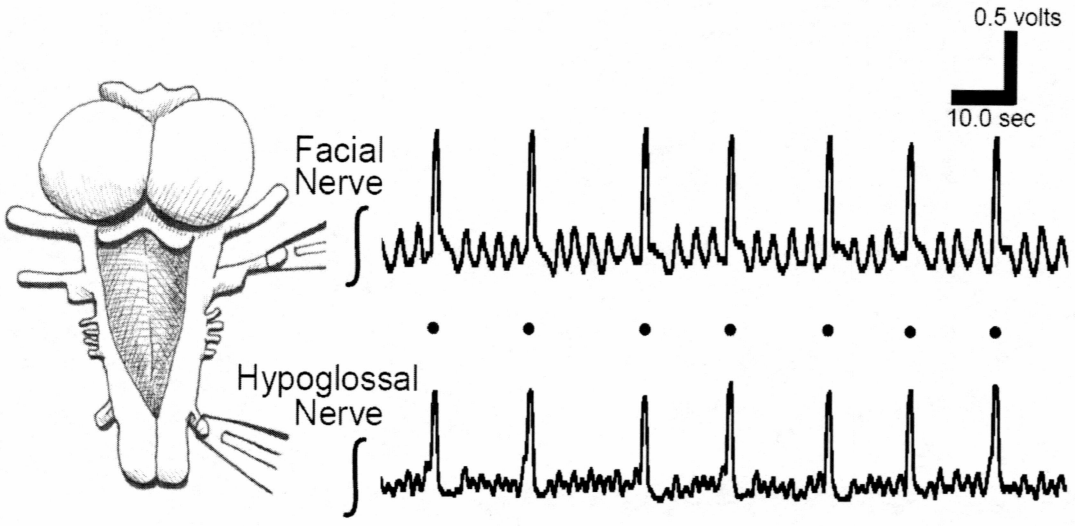

Fig. 1.1. Neuroventilatory motor output produced by the isolated tadpole brainstem. Drawing of the *in vitro* tadpole brainstem with integrated neural burst activity recorded by suction electrodes attached to the facial and hypoglossal nerve rootlets. Putative lung bursts (•) designated by presence of high amplitude nerve output occurring concomitantly on both facial and hypoglossal nerves.

1.4 RESULTS

Effect of developmental nicotine exposure on the neuroventilation of early metamorphic tadpoles.

The effects of developmental exposure to 30 µg/L nicotine on the frequency of lung bursts over 90 min of normocapnia in early metamorphic tadpoles are illustrated in Fig. 1.2. There was no significant difference between the frequency of putative lung breaths exhibited by the isolated brainstems during normocapnia of all control (0.6 ± 0.2) and 3- or 10-wk early metamorphic nicotine-exposed tadpoles ($P = 0.24$ and $P = 0.30$). Normocapnia was maintained in time-controls for 90 min. Early metamorphic control, 3- and 10-wk chronic nicotine-exposed

tadpoles exhibited no significant change in lung burst frequency over the 90-min period (P = 0.41, P = 0.43 and P = 0.42). Thus, neither 3- nor 10-wk of chronic nicotine exposure had an effect on the normocapnic lung burst frequency of early metamorphic tadpole brainstems.

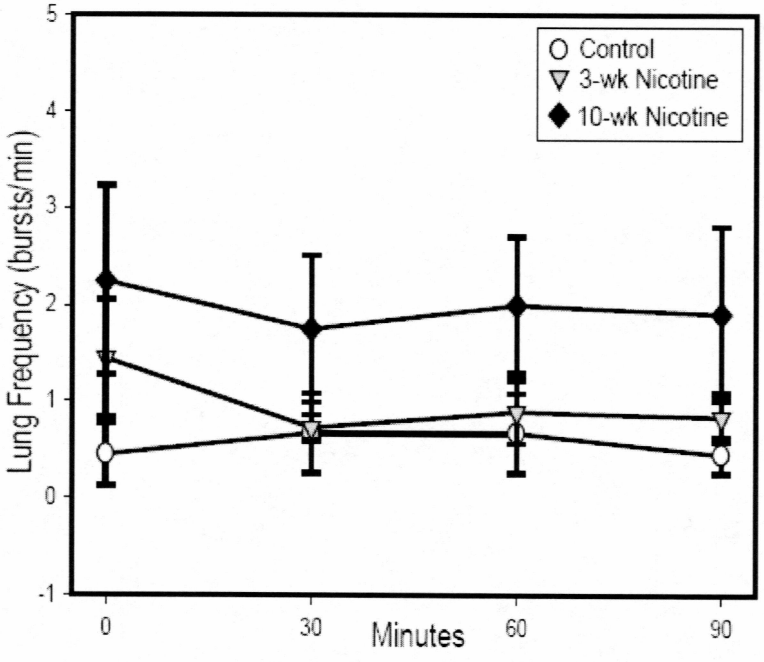

Fig. 1.2. Time has no effect on the normocapnic lung burst frequency of early metamorphic chronic nicotine-exposed tadpoles. The mean lung burst frequency over the last 3 min of every 30 min was compared in all time-control treatment groups (Control n = 6; 3-wk nicotine n = 6; 10-wk nicotine n = 4). 90 min of normocapnia had no effect on the lung burst frequency of early metamorphic tadpoles following exposure to either 3 or 10 wk of 30 µg/L nicotine, compared to control animals (P < 0.05). Data shown are mean values ± standard error.

The effects of chronic developmental exposure to 30 µg/L nicotine on the frequency of lung bursts during hypercapnia in early metamorphic tadpole brainstems are illustrated in Fig. 1.3. Early metamorphic tadpoles failed to increase their lung burst frequency in response to hypercapnia following 3-wk

chronic nicotine exposure (0.8 ± 0.2; P = 0.75). Returning CO_2 levels to normocapnia also had no effect on putative lung ventilation (0.8 ± 0.2; P = 0.71) of these animals.

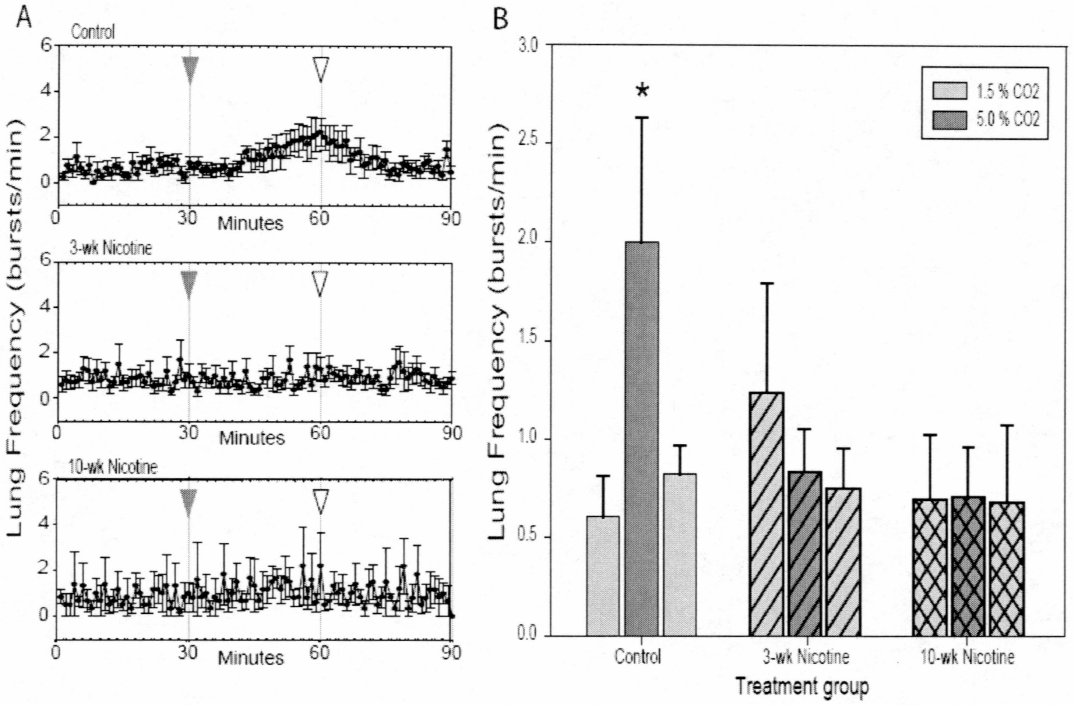

Fig. 1.3. Impact of chronic nicotine on the hypercapnic neuroventilatory response of early metamorphic tadpoles. A: Average lung burst frequency for each treatment group over an initial 30 min of normocapnia (1.5 % CO_2) followed by a switch to hypercapnic aCSF perfusate (5 % CO_2; shaded triangle) for 30 min and then returned to normocapnia (open triangle) for 30 min in treatment-control (n=8), 3-wk (n=10) and 10-wk (n=6) nicotine-exposed preparations. B: Average lung burst frequency over the last 3 min of normocapnia, hypercapnia and subsequent normocapnia in control and chronic nicotine treatment groups. Treatment-controls demonstrate significant increases in lung burst frequency during hypercapnia, (✱ $P < 0.05$) compared to normocapnia. Neither 3- nor 10-wk 30 µg/L nicotine-exposed tadpoles responded significantly to changes in P_{CO_2} ($P > 0.05$). Data shown are mean values ± standard error.

Consistent with the findings of Taylor et al. (34), 10-wk of chronic nicotine exposure also inhibited a hypercapnia-induced increase in lung burst frequency in early metamorphic tadpoles (0.7 ± 0.3; P = 0.96). There was no significant

change in lung burst frequency following the return of CO_2 levels to baseline (0.7 ± 0.4; P = 1.00). Thus, both 3- and 10-wk chronic nicotine exposures eliminated the hypercapnia-induced increase in putative lung ventilation in early metamorphic tadpoles.

To compare the magnitude of the hypercapnic response we compared the relative increase in lung burst frequency induced by hypercapnia in early metamorphic treatment-control tadpoles to that of the 3- and 10-wk nicotine-exposed animals (Fig. 1.4). Control tadpoles increased lung burst frequency by 417 ± 210 % (relative to normocapnia) in response to superfusion with 5 % CO_2. Following 3-wk nicotine exposure, tadpole brainstems exhibited a hypercapnia-induced relative increase in lung burst frequency that was significantly lower than treatment-controls (71 ± 64 %; P = 0.04).

In the 10-wk nicotine-exposed early metamorphic tadpoles, relative increase in lung burst frequency compared with their normocapnic lung burst frequency was also significantly lower than treatment-control animals (62 ± 27 %; P = 0.03). Therefore, 3- and 10-wk chronic nicotine exposure inhibited the neuroventilatory response of early metamorphic tadpoles to hypercapnia and both 3- and 10-wk exposed tadpoles exhibited a hypercapnia-induced relative change in lung neuroventilation that was lower than controls.

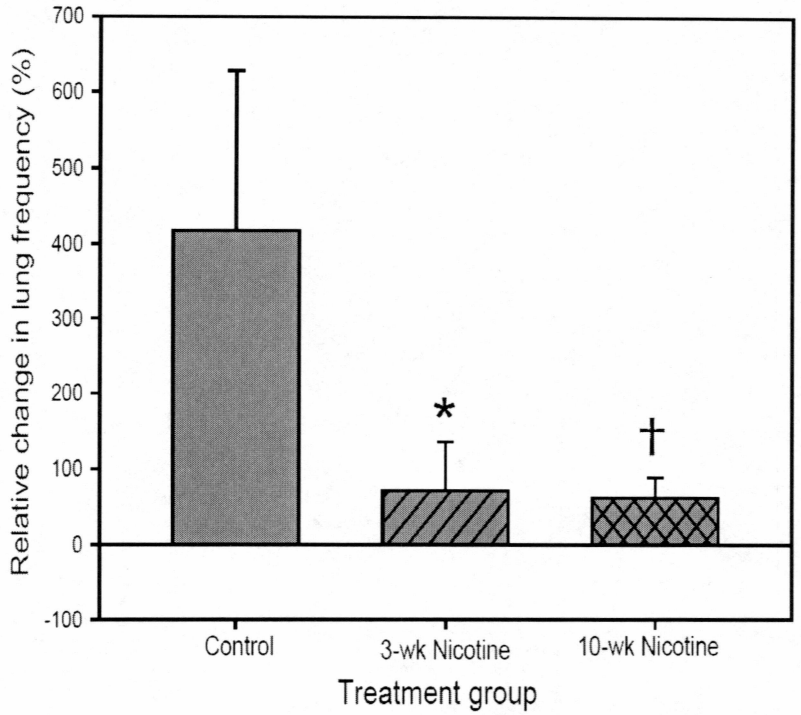

Fig. 1.4. Effect of nicotine on the hypercapnia-induced changes in neuroventilation in early metamorphic tadpole brainstems. The relative change in lung burst frequency between normocapnia and hypercapnia was compared between treatment-controls and 3- and 10-wk nicotine-exposed tadpoles. Early metamorphic tadpoles exposed to 30 µg/L nicotine for either 3 or 10 wk exhibit a significantly lower relative change from normocapnic ventilation than controls (✱ $P < 0.05$; 3-wk nicotine exposed tadpoles compared to treatment-controls, † $P < 0.05$; 10-wk nicotine exposed tadpoles compared to treatment-controls). Data shown are average percent change ± standard error.

Effect of developmental nicotine exposure on the neuroventilation of late metamorphic tadpoles.

The effects of developmental exposure to 30 µg/L nicotine on the frequency of lung bursts in late metamorphic tadpoles over 90 min normocapnia are illustrated in Fig. 1.5. Late metamorphic tadpoles have been shown to exhibit significantly more lung breaths per minute *in vivo* (1, 2) and *in vitro* (37) than early

metamorphic tadpoles; my results are consistent with those observations (6.8 ± 1.9 compared to 0.6 ± 0.2; P = 0.003, data not shown). There was no significant difference in lung burst frequency in the brainstems of 3- or 10-wk nicotine-exposed animals compared to those of late metamorphic control tadpoles (P = 0.89, and P = 0.41). Additionally, control, 3- and 10-wk chronically nicotine-exposed tadpoles exhibited no significant change in normocapnic lung burst frequency over the 90-min time-control period (P = 0.53, P = 0.74 and P = 0.58).

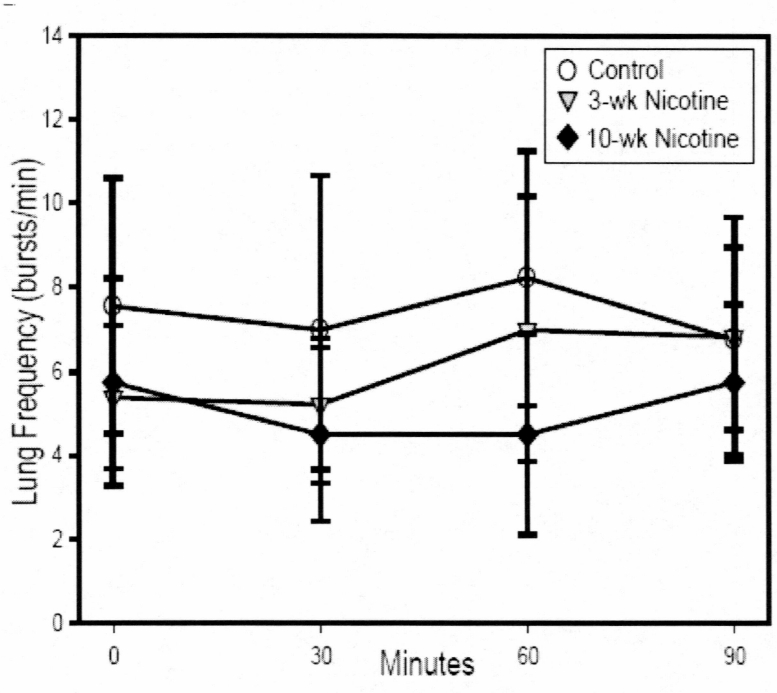

Fig. 1.5. Time has no effect on the normocapnic lung burst frequency of late metamorphic chronic nicotine-exposed tadpoles. The average lung burst frequency over the last 3 min of every 30 min was compared in all time-control treatment groups (Control n = 6; 3-wk nicotine n = 6; 10-wk nicotine n =4). 90 min of normocapnia had no effect on the lung burst frequency of late metamorphic tadpoles following exposure to either 3 or 10 wk of 30 µg/L nicotine, compared to control animals (P > 0.05). Data shown are mean values ± standard error.

Thus, neither 3- nor 10-wk chronic nicotine exposure had an effect on the normocapnic lung burst frequency of late metamorphic tadpole brainstems. There was also no significant change in lung burst frequency over a 90-min period in any of the experimental treatment groups.

The effects of chronic developmental exposure to 30 µg/L nicotine on the frequency of lung bursts during hypercapnia in late metamorphic tadpole brainstems are illustrated in Fig. 1.6. 3-wk nicotine exposure did not impact the ability of late metamorphic tadpoles to respond to hypercapnia with the typical significant increase in lung bust frequency above normocapnic levels (12.9 ± 3.0; $P = 0.002$). 3-wk nicotine-exposed late metamorphic tadpoles also responded to the decrease in CO_2 when returned to normocapnia from hypercapnia; there was a significant decline in their lung burst frequency (5.6 ± 1.4; $P = 0.03$). This was considerably different from the 10-wk nicotine-exposed late metamorphic tadpole brainstems that did not significantly increase neuroventilation in response to hypercapnia (16.3 ± 4.2; $P = 0.19$). Thus, late metamorphic tadpoles failed to respond to hypercapnia following 10- but not 3-wk nicotine exposure.

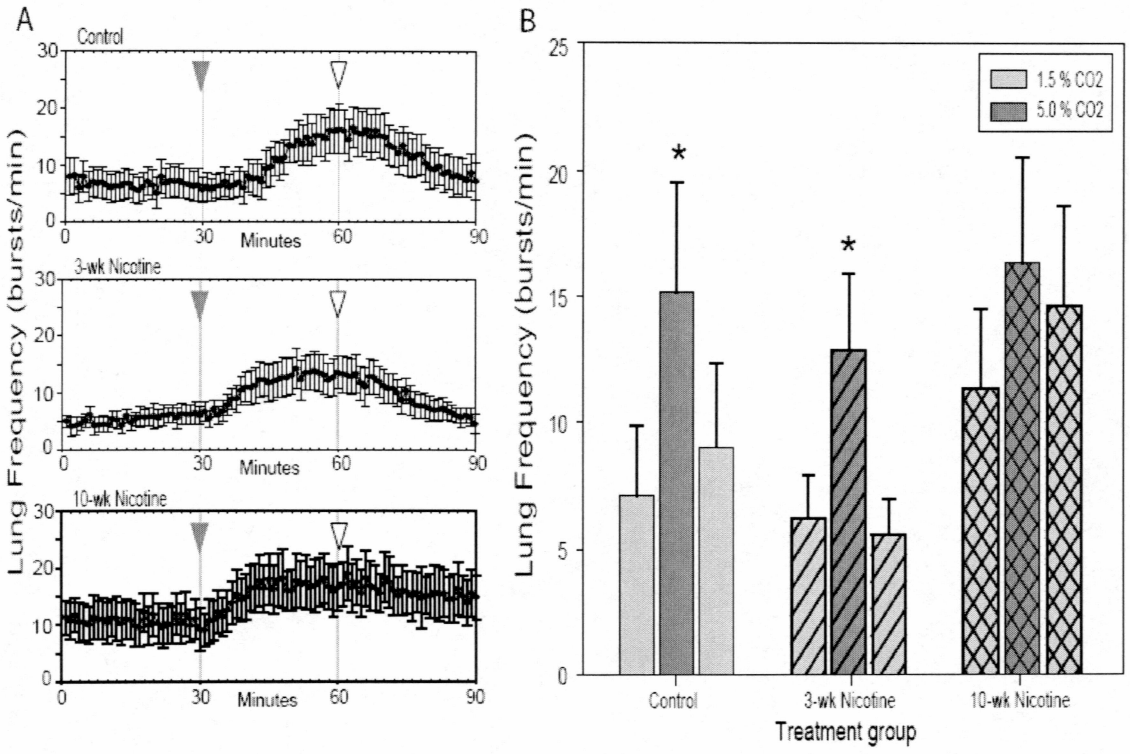

Fig. 1.6. Impact of chronic nicotine on the neuroventilatory hypercapnic response of late metamorphic tadpoles. A: Average lung burst frequency for each treatment group over an initial 30 min of normocapnia (1.5 % CO_2) followed by a switch to hypercapnic aCSF perfusate (5 % CO_2; shaded triangle) for 30 min and then returned to normocapnia (open triangle) for 30 min in treatment-control (n=7), 3-wk (n=10) and 10-wk (n=6) nicotine-exposed preparations. B: Average lung burst frequency over the last 3 min of normocapnia, hypercapnia and subsequent normocapnia in control and chronic nicotine treatment groups. Treatment-controls and 3-wk nicotine exposed late metamorphic tadpoles demonstrated significant increases in lung burst frequency during hypercapnia (✱ $P < 0.05$) compared to initial normocapnia and recovered from hypercapnia with a subsequent return to normocapnia. 10-wk nicotine-exposed tadpoles did not respond significantly to changes in P_{CO_2} ($P > 0.05$). Data shown are mean values ± standard error.

The relative increase in lung burst frequency induced by hypercapnia in late metamorphic treatment-control tadpole brainstems was compared to that of the

3- and 10-wk nicotine-exposed tadpoles (Fig. 1.7). Late metamorphic treatment-control tadpoles exhibited a considerable relative increase in putative lung ventilation in response to hypercapnia (162 ± 29 %). Following 3-wk nicotine exposure late metamorphic tadpoles demonstrated a more modest but statistically significant relative increase in lung bursting when exposed to hypercapnia (87 ± 38 %; P = 0.015).

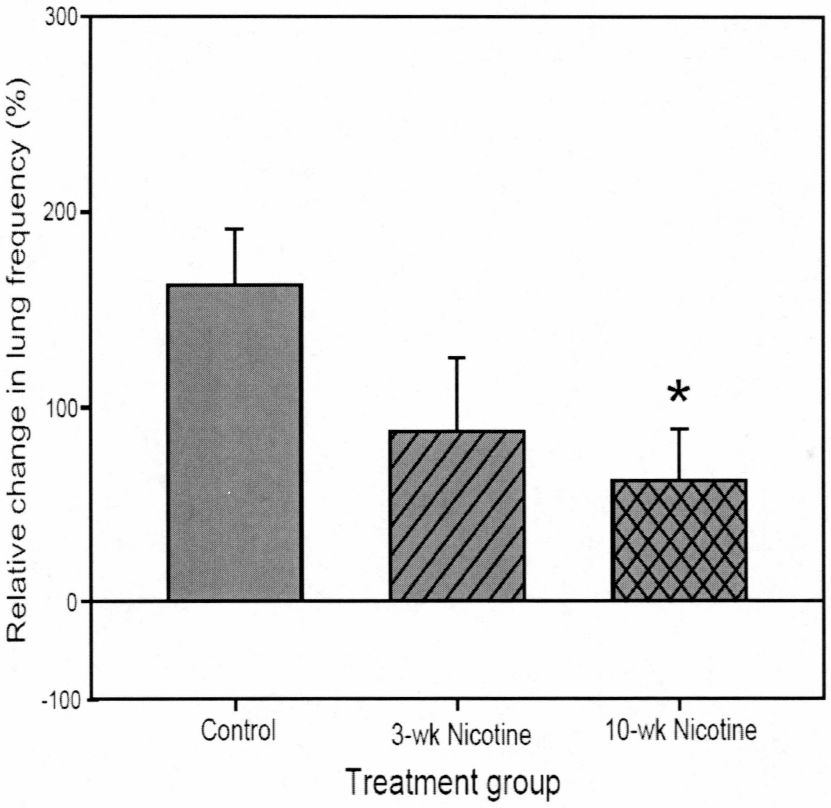

Fig. 1.7. Effect of nicotine exposure on hypercapnia-induced changes in neuroventilation in late metamorphic tadpole brainstems. The relative change in lung burst frequency between normocapnia and hypercapnia was compared between treatment-controls and 3- and 10-wk nicotine-exposed tadpoles. Control late metamorphic tadpoles and those exposed to 30 µg/L nicotine for 3 wk demonstrated similar increases in relative lung burst frequency during hypercapnia. 10-wk nicotine-exposed tadpoles exhibited a significantly lower relative change in lung burst frequency (✱ $P < 0.05$) compared to treatment-controls. Data shown are average percent change ± standard error.

The 10-wk nicotine-exposed late metamorphic tadpoles demonstrated a markedly smaller, but significant, relative increase in lung burst frequency during hypercapnia compared with controls (62 ± 27 %; P = 0.03). 3-wk nicotine-exposed tadpoles exhibited a response to hypercapnia that was not evident in 10-wk nicotine-exposed tadpoles. However, both 3- and 10-wk nicotine-exposed late metamorphic tadpoles were unable to produce a hypercapnia-induced increase in neuroventilation similar to the significant increase produced by treatment-control animals.

1.5 DISCUSSION

Tadpole lung burst frequency increases in response to hypercapnia (35, 36). The present study demonstrates that chronic developmental exposure to 30 μg/L nicotine for 10 wk attenuates that increase in both early and late metamorphic tadpoles. All preparations from 10-wk nicotine exposed tadpoles exhibited significantly lower hypercapnic lung burst frequency changes than treatment-control animals. Early metamorphic tadpoles also failed to respond to hypercapnia following 3-wk of nicotine exposure. This would imply that the 10-wk duration of exposure is not necessary to impair the hypercapnic neuroventilatory response (HCnVR). However, late metamorphic tadpoles responded to hypercapnia following a 3-wk nicotine exposure. This differential response to

hypercapnia following 3-wk nicotine exposure in early and late metamorphic tadpoles suggests that the timing of developmental exposure to nicotine plays a role in its impairment of HCnVR.

Time-controls were used to discern whether the frequency of lung bursts varied throughout the time-span of my protocol. There was no significant change in putative lung ventilation over the 90-min experimental period for any treatment group. Thus, impairments in hypercapnic neuroventilation are not the result of nicotine impacting the *in vitro* preparations' integrity over time. The neuroventilatory lung frequency of treatment-control tadpoles returns to normocapnic levels when CO_2 levels are switched to normocapnia from hypercapnia. This demonstrates that the frequency changes observed during hypercapnia are the result of the hypercapnic treatment alone.

Chronic nicotine had no effect on the normocapnic lung burst frequency of either early or late metamorphic tadpole brainstems. This suggests that nicotine affects either central CO_2/pH chemosensitivity or the ability of central compensatory mechanisms to change ventilatory motor output in response to increases in CO_2/pH. Nicotine binds to nicotinic acetylcholine receptors, which have been shown to affect CO_2 chemosensitivity (12). Prenatal nicotine exposure has adverse impact on development of the cholinergic system (16). Nicotine exposure has also been associated with other pathways implicated in respiratory

modulation, including but not limited to, the serotonergic, purinergic and gabaergic systems (6, 8, 9, 27, 39). The present study was designed to characterize a functional impairment of the hypercapnic response, identifying the mechanism by which nicotine impairs this response is a topic for future research.

It remains to be demonstrated whether the attenuation of the HCnVR due to chronic nicotine exposure is a result of a developmental neural deficit or a pharmacological effect of nicotine. The ventilatory response to acetylcholine in amphibians has not been extensively investigated. Kennedy et al. (7) found that adult cane toad brainstems increased their lung burst frequency following acute nicotine exposure. I saw no such increase, suggesting that the effect of chronic exposure to nicotine is fundamentally different from that of acute exposure. Future studies that investigate the HCnVR of tadpoles during acute nicotine exposure and after a recovery period from chronic nicotine exposure may offer additional insight into the nature of nicotine toxicity.

The impaired HCnVR in early metamorphic tadpoles occurs at a point in development when tadpoles are undergoing significant synaptogenesis (5, 13). Concomitantly, that time is one of increased dependence on lung ventilation and increased chemosenistivity (35-37). The immature configuration of the early metamorphic tadpole brainstems, may account for their susceptibility to nicotine

following the 3-wk exposure. It remains to be determined whether nicotine has any impact on other aspects of tadpole development.

Tadpoles in each experimental group were housed collectively in nicotine treatment tanks, and I did not monitor the effect of nicotine on individual development. Noticeable morphological development took place in all treatment groups, although, tadpoles were staged only at their entry to the treatment tanks and at the time of dissection. The normocapnic neuroventilation of treatment animals was not significantly different from control animals suggesting that any diminished hypercapnic responses were not due to an attenuation of overall tadpole development. The infrequency of early metamorphic tadpole lung neuroventilation has typically made it difficult to identify changes in putative lung ventilatory frequency. Torgerson et al. (37) did not identify a significant lung burst response to hypercapnia in early metamorphic (Taylor-Köllros stages 3-9) *in vitro* brainstem preparations. I, consistently, have been able to demonstrate increases in lung burst frequency in these stages. Discrepancies may originate from levels of CO_2 investigated or other deviations in experimental methodology. Nonetheless, the weight of evidence suggests that the HCnVR slowly matures throughout tadpole development and 10-wk chronic nicotine exposure, regardless of timing, impairs the manifestation of that response.

The lack of hypercapnic neuroventilatory impairment in 3-wk nicotine-exposed late metamorphic tadpoles may be due to the increased myelination and neural organization seen as metamorphosis progresses (20, 22, 30, 32). Chronic nicotine impacts a variety of sites in the central nervous system with a series of deleterious results throughout development (25, 26). There may also be threshold levels of nicotine or periods of sensitivity that change with size and maturity (24, 40). Nicotine may only slowly impact CO_2/pH-sensing mechanisms reaching significance after 10 wk in late metamorphic tadpoles, but may easily overwhelm the weaker chemosensitivity of early metamorphic tadpoles with 3-wk exposure.

The ventilatory response to chronic nicotine in mammals is highly variable. This has been attributed to inconsistencies in nicotine exposure duration and animal age (4). Here I offer evidence to support the conclusion that both timing and duration are factors that affect nicotine's impact on hypercapnic neuroventilation and CO_2/pH homeostasis in anurans and perhaps all vertebrates.

1.6 CONCLUSIONS

3 wk of chronic exposure to 30 µg/L nicotine is sufficient to impair the hypercapnic lung burst response of early metamorphic tadpole brainstems. 10- but not 3-wk nicotine exposure attenuates the hypercapnic response of late

metamorphic tadpoles. Neither 3 nor 10 wk of chronic nicotine exposure causes a change in the normocapnic lung neuroventilatory frequency of either early or late metamorphic tadpole brainstems. This substantiates a deleterious impact of developmental nicotine exposure on the ability of tadpoles to increase lung neuroventilition during hypercapnia and illustrates the important role of timing and duration of developmental exposure when assessing the neuropathology of nicotine exposure.

1.7 ACKNOWLEDGMENTS

I would like to thank Dr. Barbara E. Taylor who served as my advisor and was the principal investigator for the lab overseeing my research. I would also like to thank Dr. Michael B. Harris for his recommendations on the content and structure of this project, and Mr. Justin C. Buehner who contributed to the dataset used in this manuscript. This work was supported by NIH-NINDS grant # 2U54NS041069-601A

1.8 LITERATURE CITED

1. **Burggren W and Doyle M.** Ontogeny of regulation of gill and lung ventilation in the bullfrog, Rana catesbeiana. *Respir Physiol* 66: 279-291, 1986.

2. **Burggren WW and West NH.** Changing respiratory importance of gills, lungs and skin during metamorphosis in the bullfrog Rana catesbeiana. *Respir Physiol* 47: 151-164, 1982.

3. **Gheshmy A, Vukelich R, Noronha A, and Reid SG.** Chronic hypercapnia modulates respiratory-related central pH/CO_2 chemoreception in an amphibian, Bufo marinus. *J Exp Biol* 209: 1135-1146, 2006.

4. **Hafstrom O, Milerad J, Sandberg KL, and Sundell HW.** Cardiorespiratory effects of nicotine exposure during development. *Respir Physiol Neurobiol* 149: 325-341, 2005.

5. **Horn JP.** Development of fast synaptic transmission in bullfrog sympathetic ganglia. *J Auton Nerv Syst* 32: 107-119, 1991.

6. **Huang ZG, Griffioen KJ, Wang X, Dergacheva O, Kamendi H, Gorini C, and Mendelowitz D.** Nicotinic receptor activation occludes purinergic control of central cardiorespiratory network responses to hypoxia/hypercapnia. *J Neurophysiol* 98: 2429-2438, 2007.

7. **Kennedy LL, Aguwa CC, Rives JE, and Bernard DG.** Involvement of cholinergic mechanisms in the central control of respiration in the cane toad, Bufo marinus. *Comp Biochem Physiol A Mol Integr Physiol* 128: 837-849, 2001.

8. **Luo Z, McMullen NT, Costy-Bennett S, and Fregosi RF.** Prenatal nicotine exposure alters glycinergic and GABAergic control of respiratory frequency in the neonatal rat brainstem-spinal cord preparation. *Respir Physiol Neurobiol* 157: 226-234, 2007.

9. **Mihailescu S, Guzman-Marin R, Dominguez Mdel C, and Drucker-Colin R.** Mechanisms of nicotine actions on dorsal raphe serotoninergic neurons. *Eur J Pharmacol* 452: 77-82, 2002.

10. **Milsom WK.** Phylogeny of CO_2/H+ chemoreception in vertebrates. *Respir Physiol Neurobiol* 131: 29-41, 2002.

11. **Milsom WK.** The role of CO_2/pH chemoreceptors in ventilatory control. *Braz J Med Biol Res* 28: 1147-1160, 1995.

12. **Monteau R, Morin D, and Hilaire G.** Acetylcholine and central chemosensitivity: *in vitro* study in the newborn rat. *Respir Physiol* 81: 241-253, 1990.

13. **Moody SA, Miller V, Spanos A, and Frankfurter A.** Developmental expression of a neuron-specific beta-tubulin in frog (Xenopus laevis): a marker for growing axons during the embryonic period. *J Comp Neurol* 364: 219-230, 1996.

14. **Moyer TP, Charlson JR, Enger RJ, Dale LC, Ebbert JO, Schroeder DR, and Hurt RD.** Simultaneous analysis of nicotine, nicotine metabolites, and tobacco alkaloids in serum or urine by tandem mass spectrometry, with clinically relevant metabolic profiles. *Clin Chem* 48: 1460-1471, 2002.

15. **Nattie E.** CO_2, brainstem chemoreceptors and breathing. *Prog Neurobiol* 59: 299-331, 1999.

16. **Navarro HA, Seidler FJ, Eylers JP, Baker FE, Dobbins SS, Lappi SE, and Slotkin TA.** Effects of prenatal nicotine exposure on development of central

and peripheral cholinergic neurotransmitter systems. Evidence for cholinergic trophic influences in developing brain. *J Pharmacol Exp Ther* 251: 894-900, 1989.

17. **O'Regan RG and Majcherczyk S.** Role of peripheral chemoreceptors and central chemosensitivity in the regulation of respiration and circulation. *J Exp Biol* 100: 23-40, 1982.

18. **Putnam RW, Conrad SC, Gdovin MJ, Erlichman JS, and Leiter JC.** Neonatal maturation of the hypercapnic ventilatory response and central neural CO_2 chemosensitivity. *Respir Physiol Neurobiol* 149: 165-179, 2005.

19. **Putnam RW, Filosa JA, and Ritucci NA.** Cellular mechanisms involved in CO_2 and acid signaling in chemosensitive neurons. *Am J Physiol Cell Physiol* 287: C1493-1526, 2004.

20. **Raucci F, Di Fiore MM, Pinelli C, D'Aniello B, Luongo L, Polese G, and Rastogi RK.** Proliferative activity in the frog brain: a PCNA-immunohistochemistry analysis. *J Chem Neuroanat* 32: 127-142, 2006.

21. **Simakajornboon N, Vlasic V, Li H, and Sawnani H.** Effect of prenatal nicotine exposure on biphasic hypoxic ventilatory response and protein kinase C expression in caudal brain stem of developing rats. *J Appl Physiol* 96: 2213-2219, 2004.

22. **Simmons AM, Horowitz SS, and Brown RA.** Cell proliferation in the forebrain and midbrain of the adult bullfrog, Rana catesbeiana. *Brain Behav Evol* 71: 41-53, 2008.

23. **Slotkin TA.** Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. *Toxicol Appl Pharmacol* 198: 132-151, 2004.

24. **Slotkin TA.** Developmental cholinotoxicants: nicotine and chlorpyrifos. *Environ Health Perspect* 107 Suppl 1: 71-80, 1999.

25. **Slotkin TA, Cousins MM, and Seidler FJ.** Administration of nicotine to adolescent rats evokes regionally selective upregulation of CNS alpha 7 nicotinic acetylcholine receptors. *Brain Res* 1030: 159-163, 2004.

26. **Slotkin TA, McCook EC, Lappi SE, and Seidler FJ.** Altered development of basal and forskolin-stimulated adenylate cyclase activity in brain regions of rats exposed to nicotine prenatally. *Brain Res Dev Brain Res* 68: 233-239, 1992.

27. **Slotkin TA, Ryde IT, Tate CA, and Seidler FJ.** Lasting effects of nicotine treatment and withdrawal on serotonergic systems and cell signaling in rat brain regions: separate or sequential exposure during fetal development and adulthood. *Brain Res Bull* 73: 259-272, 2007.

28. **Smatresk NJ and Smits AW.** Effects of central and peripheral chemoreceptor stimulation on ventilation in the marine toad, Bufo marinus. *Respir Physiol* 83: 223-238, 1991.

29. **St-John WM and Leiter JC.** Maternal nicotine depresses eupneic ventilation of neonatal rats. *Neurosci Lett* 267: 206-208, 1999.

30. **Stofer WD and Horn JP.** Expression of neuropeptide-Y-like immunoreactivity begins after adrenergic differentiation and ganglionic synaptogenesis in developing bullfrog sympathetic neurons. *J Neurosci* 10: 3305-3312, 1990.

31. **Stofer WD and Horn JP.** Neurogenesis and differentiation of sympathetic B and C cells in the bullfrog tadpole. *J Neurosci* 13: 801-807, 1993.

32. **Tamai Y, Kojima H, Saito S, Uchida K, Kitajima R, Komatsu H, and Moriya T.** Metamorphic changes in glycolipids and myelin proteins and 2',3'-cyclic nucleotide 3'-phosphohydrolase in bullfrog and axolotl brains. *J Neurochem* 60: 1854-1863, 1993.

33. **Taylor AC and Kollros JJ.** Stages in the normal development of *Rana pipiens* larvae. *Anat Rec* 94: 7-24, 1946.

34. **Taylor BE, Croll AE, Drucker ML, and Wilson AL.** Developmental exposure to ethanol or nicotine inhibits the hypercapnic ventilatory response in tadpoles. *Respir Physiol Neurobiol* 160: 83-90, 2008.

35. **Taylor BE, Harris MB, Coates EL, Gdovin MJ, and Leiter JC.** Central CO_2 chemoreception in developing bullfrogs: anomalous response to acetazolamide. *J Appl Physiol* 94: 1204-1212, 2003.

36. **Taylor BE, Harris MB, Leiter JC, and Gdovin MJ.** Ontogeny of central CO_2 chemoreception: chemosensitivity in the ventral medulla of developing bullfrogs. *Am J Physiol Regul Integr Comp Physiol* 285: R1461-1472, 2003.

37. **Torgerson C, Gdovin M, and Remmers J.** Ontogeny of central chemoreception during fictive gill and lung ventilation in an *in vitro* brainstem preparation of Rana catesbeiana. *J Exp Biol* 200: 2063-2072, 1997.

38. **Torgerson CS, Gdovin MJ, and Remmers JE.** Fictive gill and lung ventilation in the pre- and postmetamorphic tadpole brain stem. *J Neurophysiol* 80: 2015-2022, 1998.

39. **Xu Z, Seidler FJ, Ali SF, Slikker W, Jr., and Slotkin TA.** Fetal and adolescent nicotine administration: effects on CNS serotonergic systems. *Brain Res* 914: 166-178, 2001.

40. **Zahalka EA, Seidler FJ, Lappi SE, McCook EC, Yanai J, and Slotkin TA.** Deficits in development of central cholinergic pathways caused by fetal nicotine exposure: differential effects on choline acetyltransferase activity and [3H]hemicholinium-3 binding. *Neurotoxicol Teratol* 14: 375-382, 1992.

CHAPTER 2

NICOTINE AFFECTS THE NORMOCAPNIC AND HYPERCAPNIC NEUROVENTILATION OF BULLFROGS IN A DEVELOPMENTAL STAGE DEPENDENT MANNER[2]

2.1 ABSTRACT

The nicotinic acetylcholine receptor agonist nicotine is associated with numerous neurodevelopmental aberrations, including attenuation of the neuroventilatory response of bullfrog tadpoles to hypercapnia following prolonged exposure to nicotine during development (chronic exposure). It is unclear, however, how the neuroventilatory response, during acute nicotine exposure, might differ from those following chronic exposure. In this study the neural correlates of ventilation were recorded from *in vitro* brainstem preparations derived from early and late metamorphic tadpoles and juvenile bullfrogs. Lung burst frequencies were compared during control and 18 µg/L-nicotine conditions, during normocapnia (1.5 % CO_2) and in response to hypercapnia (5.0 % CO_2). Early metamorphic tadpoles exhibited no significant neuroventilatory response to nicotine. Late metamorphic tadpoles decreased normocapnic lung burst frequency with acute nicotine exposure, but responded normally to hypercapnia. Juvenile bullfrogs

[1] C.M. Brundage and B.E. Taylor. 2008. Nicotine affects the normocapnic and hypercapnic neuroventilation of bullfrogs in a developmental stage dependent manner. (prepared for submission in) American Journal of Physiology: Integrative and Comparative Physiology.

decreased both normocapnic and hypercapnic lung burst frequency, suggesting both a role of nicotinic acetylcholine receptors in neuroventilation and an impact of nicotine exposure increase with bullfrog development.

2.2 INTRODUCTION

The neurological consequences associated with exposure to nicotine (an agonist for nicotinic acetylcholine receptors) have been extensively investigated. It is widely accepted that the breadth of long-term neural developmental aberrations linked to nicotine exposure classifies it as a neuroteratogen (7, 30, 33, 34). Prenatal exposure is associated with an increase in human infant mortality and morbidity (13, 23). Vulnerability to nicotine is not limited to prenatal exposure; neurological deficits have been identified following neonatal and adolescent nicotine exposure (1, 11, 29). Both the timing and duration of developmental nicotine exposure play key roles in the severity of nicotine's neurophysiological impact ((32) & see Chapter 1).

Nicotinic acetylcholine receptors are widespread across the central nervous system (19, 28), including areas of the brainstem involved in the control of breathing (5, 44). Their activation is considered fundamental to respiratory rhythm generation (17, 26, 27) and may play a role in both central and peripheral chemosensitivity (4, 16, 22). The effect of nicotine exposure on respiratory

function and central chemosensitivity, therefore, becomes of particular importance in understanding the development of its associated pathologies.

The isolated *in vitro* tadpole brainstem preparation has been used to model developmental changes in chemosensitivity (18, 38, 41, 42), network inhibition (2, 9) and respiratory rhythm generation (10, 14, 21). Spontaneous patterns of nerve discharge in cranial and spinal nerves (motor correlates of ventilation; neuroventilation) have been characterized across bullfrog ontogeny (35, 40, 43). Like mammals, bullfrog ventilatory responses to hypercapnic acidosis increase throughout their development (25, 39). Prolonged exposure to nicotine during development (chronic exposure) has been found to impair these responses ((37) & see Chapter 1). It is not clear, however, how this functional deficit might differ from one in response to acute nicotine exposure.

In the present study I assessed the lung neuroventilatory responses of bullfrog brainstems to hypercapnia during acute exposure to 18 µg/L nicotine. I looked specifically at three points in tadpole development testing brainstems from, 1) early metamorphic tadpoles with developed gills used for aquatic respiration, 2) late metamorphic tadpoles with involuted gills and maturing lungs used for aerial respiration and 3) fully metamorphosed air-breathing juvenile frogs. The neural mechanisms underlying control and integration of each of these ventilatory

strategies may differ and offer unique insight into the affect of acute nicotine on the developing bullfrog brainstem.

Acute nicotine exposure has been reported to evoke both excitatory and depressant effects on respiration in mammals (8, 13). These differences may relate to differences in the models used to investigate acute nicotine exposure, and may be based on differences in the number of intact peripheral afferents (8). The only published study documenting respiratory effects of acute nicotine in anurans was conducted in an *in situ* cane toad preparation. Nicotine applied to the ventral surface of the brainstem generated an overall increase in episodic breath frequency (17). Although *in vitro* brainstem preparations lack the peripheral afferents present in *in situ* preparations, I hypothesized that acute exposure to nicotine would have a similar overall excitatory effect on lung burst frequency in the isolated brainstem of the bullfrog tadpole. I further hypothesized that these responses to acute nicotine would not directly affect bullfrog central CO_2/pH sensitivity.

2.3 MATERIALS AND METHODS

Animals

Experiments were performed on *Lithobates* (formerly *Rana*) *catesbeiana* juvenile bullfrogs and tadpoles purchased from a commercial supplier (Sullivan Co. Inc.,

www.researchamphibians.com). All animals were maintained at room temperature (21-23 °C) in glass aquaria with dechlorinated water. Animals were fed daily: tadpoles were fed goldfish flakes (TetraFin Goldfish Flakes; Tetra Holding Inc., www.tetra-fish.com), and juvenile frogs were fed crickets purchased from a commercial supplier (Fishtopia, Fairbanks, AK). Tadpoles were designated at the time of experimentation as early metamorphic (forelimbs absent, hind limbs lacking toe pad, and pigment-free metatarso-phalangeal joint patches) or late metamorphic (forelimbs and hind limbs present, tail being resorbed), corresponding to developmental stages 1-13 or 19-24, respectively, in the classification scheme of Taylor and Köllros (36). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Alaska Fairbanks.

Surgical preparation

Both juvenile frogs and tadpoles were anesthetized by immersion for 1-2 min in cold (4 °C) 0.2 mM solution of tricaine methanesulfate (MS222; Sigma, www.sigmaaldrich.com) in dechlorinated water buffered to pH 7.8 with $NaHCO_3$. Using a razor blade I removed the front of the head rostral to the nares and the back of the body (hind limbs and tail, if present). Under a dissecting microscope, the dorsal cranium was removed and the forebrain rostral to the diencephalon resected and the fourth ventricle opened by removing the choroid plexus. The brainstem and spinal cord were removed *en bloc* from the cranium and spinal

canal. The dura mater was removed and the brainstem/spinal cord was transected rostral to the optic tectum and at the brachial nerve. During dissection, exposed tissues were superfused with cold artificial cerebral spinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 10 D-glucose, 25 $NaHCO_3$ and 2.4 $CaCl_2$ equilibrated with 100 % O_2. The aCSF HCO_3^- concentration is similar to that of plasma from late metamorphic tadpoles and frogs, but higher than the HCO_3^- concentration in plasma from early metamorphic tadpoles. These methods have been used in previous anuran studies (38, 39), aCSF composition remained constant to ensure comparability between experiments on animals of different metamorphic stages.

The isolated brainstem was transferred to a 0.5-ml, Plexiglas, flow-through recording chamber and supported ventral side up between coarse nylon mesh such that all surfaces were bathed with aCSF flowing from rostral to caudal at a rate of 5 ml/min. A supply of aCSF, equilibrated with O_2-CO_2 mixtures that produced the desired pH, flowed through plastic tubing to the recording chamber. The pH of the aCSF was maintained at either pH 7.8 (1.5 % CO_2: 98.5 % O_2; normocapnia) or pH 7.4 (5.0 % CO_2: 95.0 % O_2; hypercapnia) by adjusting the fractional concentrations of O_2 and CO_2 in the equilibration gas. CO_2 was monitored with a CO_2 analyzer (Capstar 100; CWE, www.cwe-inc.com). After surgical preparation, the isolated brainstem was allowed to stabilize for ~1 h while superfused with aCSF at 23 °C, pH 7.8 and ~9 torr P_{CO_2}.

Nerve recording

Roots of the facial and hypoglossal nerves were drawn into glass suction electrodes pulled from 1-mm-diameter capillary glass to tip diameters of 30-60 μm. Whole-nerve discharge was amplified (X100 by DAM 50 amplifiers, World Precision Instruments, www.wpiinc.com; X1000 by a four-channel model 1700 amplifier, A-M Systems, www.a-msystems.com) and filtered (100 Hz high pass to 1 kHz low pass). The amplified and filtered output was sent to a data acquisition system (Powerlab, AD Instruments, www.adinstruments.com), which sampled data at 1 kHz, integrated data (full-wave rectified and averaged over 200 ms), as well as recorded and archived the whole-nerve discharges as neurograms.

Treatment protocols

After the initial 1 h of stabilization, neurograms from each brainstem (n = 17) were recorded during 30 min of normocapnia followed by 30 min of hypercapnia, these preparations were then returned to normocapnia for a 60-min washout restabilization period. Following the washout period the aCSF perfusate was switched to one containing 18 μg/L nicotine ((-) – nicotine hydrogen tartrate salt; Sigma, www.sigmaaldrich.com). Recordings continued for 30 min at normocapnic conditions and for 30 min of hypercapnia before being returned to control aCSF and normocapnic conditions for a final 30-min washout period. The level of nicotine chosen corresponds with the human maternal plasma level

needed to impact fetal hemodynamics (13, 20) and approximates the nicotine levels in the body fluids of a 20-cigarette per-day smoker (15, 24).

Additional brainstems (n = 12) received a treatment-control protocol. Preparations were exposed to the same initial hypercapnic treatment as the nicotine protocol; however, the subsequent normocapnic washout restabilization period ran for 90 min before being increased to hypercapnia for 30 min, and then followed by a 30-min final washout period. Treatment-control preparations thus experienced the same P_{CO_2} levels and durations as the nicotine-protocol preparations without exposure to nicotine.

The stability of the *in vitro* tadpole preparation has not been established over a 210-min period such as required for our experimental protocol. These time-control protocol preparations (n =12) established any variation in lung burst frequency that may have occurred over the time course of the nicotine protocol. Recordings were made of preparations that remained isocapnic at normocapnic conditions for 210 min following their initial stabilization period.

Data analyses and statistics

Burst activity patterns in the neurograms recorded from the isolated brainstems were designated as either putative gill/ buccal breaths or putative lung breaths on the basis of the amplitude of the integrated nerve activity and the presence or

absence of coincident firing in both the facial and hypoglossal nerves, as previously described (38, 39, 43). Putative gill breaths had lower burst amplitude in the facial nerve than putative lung breaths and little or no coincident burst activity in the hypoglossal nerve (Fig. 2.1). Putative lung breaths had higher burst amplitude in the facial nerve and coincident burst activity on the hypoglossal nerve (Fig 2.1). Taylor et al. (38, 39) demonstrated that changes in the frequency of putative lung breaths are the primary manifestation of the hypercapnic neuroventilatory response.

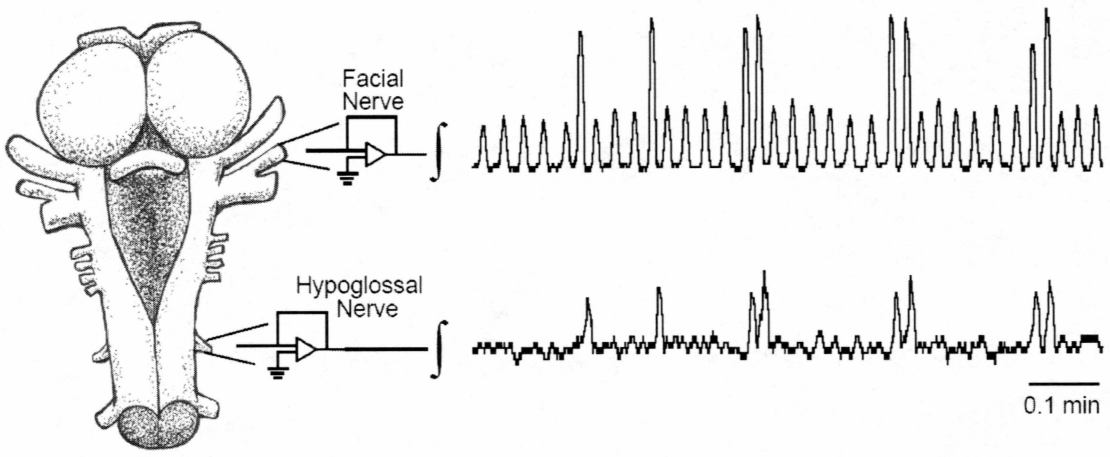

Fig. 2.1. Isolated bullfrog brainstem neuroventilation. Drawing of an *in vitro* bullfrog brainstem with integrated neural burst pattern recorded by suction electrodes attached to the facial and hypoglossal nerve rootlets. Putative lung bursts were designated by presence of coincident high amplitude nerve output on both the facial and hypoglossal nerves.

The duration and amplitude of putative lung breaths and the frequency, duration and amplitude of putative gill breaths are unresponsive to hypercapnia. Thus, only the frequency of putative lung breaths was quantified, by counting the number of lung bursts in 3 consecutive min (at the end of the 30-min record) of each CO_2/pH and nicotine aCSF condition (normocapnia, hypercapnia, initial washout, acute nicotine normocapnia, acute nicotine hypercapnia, final washout).

Frequencies of putative lung ventilation were analyzed using one-way and two-way repeated-measures analysis of variance (RM-ANOVA) for each developmental stage (RM-ANOVA procedure, SigmaStat, www.systat.com). When an RM-ANOVA indicated that significant differences existed between the treatment groups, multiple comparisons were made using the Holm-Sidak multiple comparison tests. One-way RM-ANOVAs determined whether normocapnic lung burst frequencies recovered from hypercapnia and were consistent throughout the experiment; however, they were not able to distinguish whether nicotine treatment impacted responses to CO_2 or *vice versa*. Two-way RM-ANOVAs were used to make that distinction. T-test comparisons were used to compare responses between different developmental stages when applicable. Values reported in the text are means ± SE in lung bursts/min.

2.4 RESULTS

Effect of experimental duration and multiple hypercapnic challenges on bullfrog lung neuroventilation

The effect of the experimental time course as well as multiple bouts of hypercapnia on lung bursts exhibited by the isolated bullfrog brainstem are illustrated in Fig. 2.2. There was no significant difference between the lung burst frequencies of late metamorphic tadpoles and juvenile bullfrogs ($P = 0.68$). The lung burst frequency exhibited by the brainstems of early metamorphic tadpoles was, however, significantly different from that of late metamorphic tadpole brainstems ($P = 0.01$) and juvenile bullfrog brainstems ($P = 0.03$). For that reason I combined control data for late metamorphic tadpoles and juvenile bullfrogs into one group for comparison with early metamorphic tadpoles. In time-control experiments the lung burst frequency exhibited by isolated brainstems did not change significantly over the 210-min experiment duration within either group ($P = 0.64$ and $P = 0.42$, respectively, for early and later developmental groups).

In treatment-control preparations there was no significant difference in the normocapnic lung burst frequency exhibited by the isolated brainstems preceding or following the first or second hypercapnic challenge for early either group ($P = 0.98$ and $P = 0.34$, respectively, for early and later developmental groups).

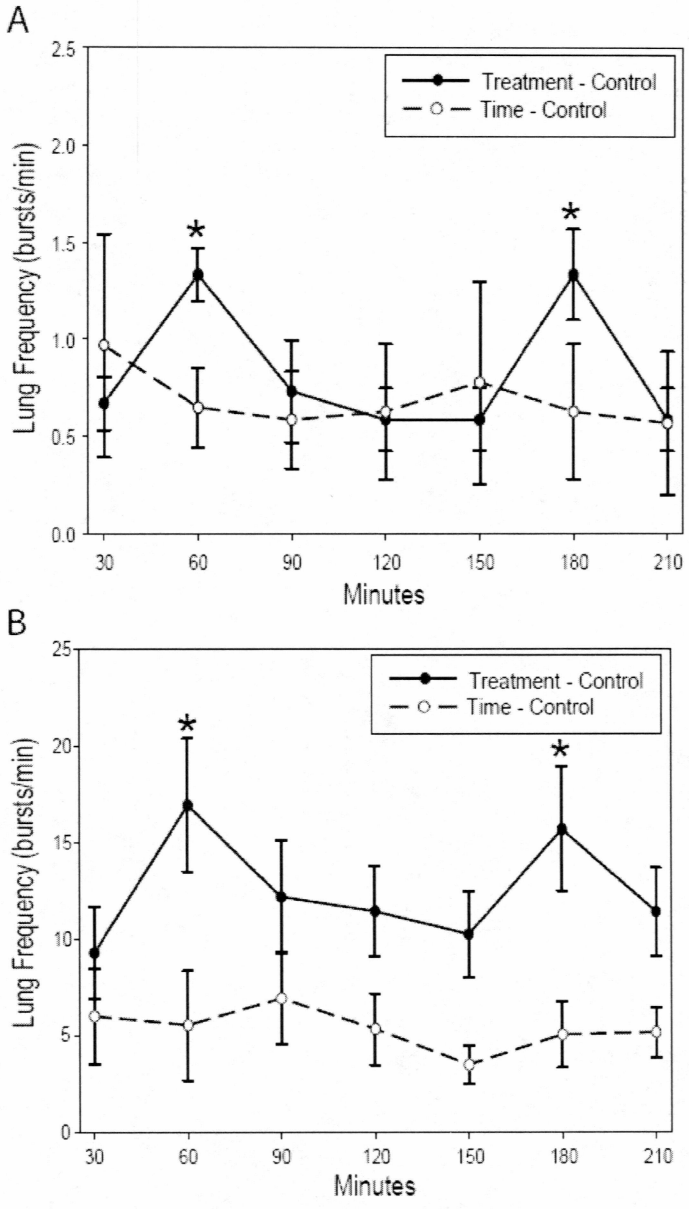

Fig. 2.2. Effect of time and a dual hypercapnic challenge on the lung burst frequency exhibited by the isolated brainstems of bullfrogs. The average lung burst frequency over the last 3 min of every 30 min was compared in all treatment-control (solid line) and time-control (dashed line) groups. 210 min of normocapnia had no effect on the neuroventilatory lung frequency of early metamorphic tadpoles (A; n = 4) or late metamorphic tadpoles and juvenile bullfrogs (B; n = 8). All treatment-controls responded significantly to changes in P_{CO_2} (✱ = $P < 0.05$). There was no significant difference in lung burst frequency between the first or second hypercapnic challenge. Data shown are mean values ± standard error.

All treatment-control brainstem preparations responded significantly to both hypercapnic challenges (all $P < 0.05$). There was no significant difference between the lung burst frequencies exhibited by the isolated brainstems during the first and second hypercapnic challenges either group ($P= 1.00$ and $P = 0.66$, respectively, for early and later developmental groups). Therefore, neither the time-course of the experiment nor the response to a previous hypercapnic challenge altered normocapnic lung burst frequency or the brainstems' ability to respond to a subsequent hypercapnic challenge.

Effect of acute nicotine exposure on lung neuroventilation of the early metamorphic tadpole brainstem

The effects of acute exposure to 18 µg/L nicotine on the frequency of lung bursts in the early metamorphic tadpole brainstem are illustrated in Fig. 2.3. Under normal aCSF conditions early metamorphic tadpoles significantly increased lung burst frequency in response to hypercapnia, from 0.9 ± 0.2 to 2.1 ± 0.5, $P = 0.02$. Superfusion with aCSF containing 18 µg/L nicotine did not significantly alter early metamorphic normocapnic lung burst frequency (1.0 ± 0.4, $P = 0.64$) or the ability of early metamorphic tadpoles to significantly increase their lung burst frequency in response to hypercapnia (2.0 ± 0.6, $P = 0.04$). There was no significant difference between the hypercapnic lung burst frequency under control and nicotine aCSF conditions ($P = 0.94$).

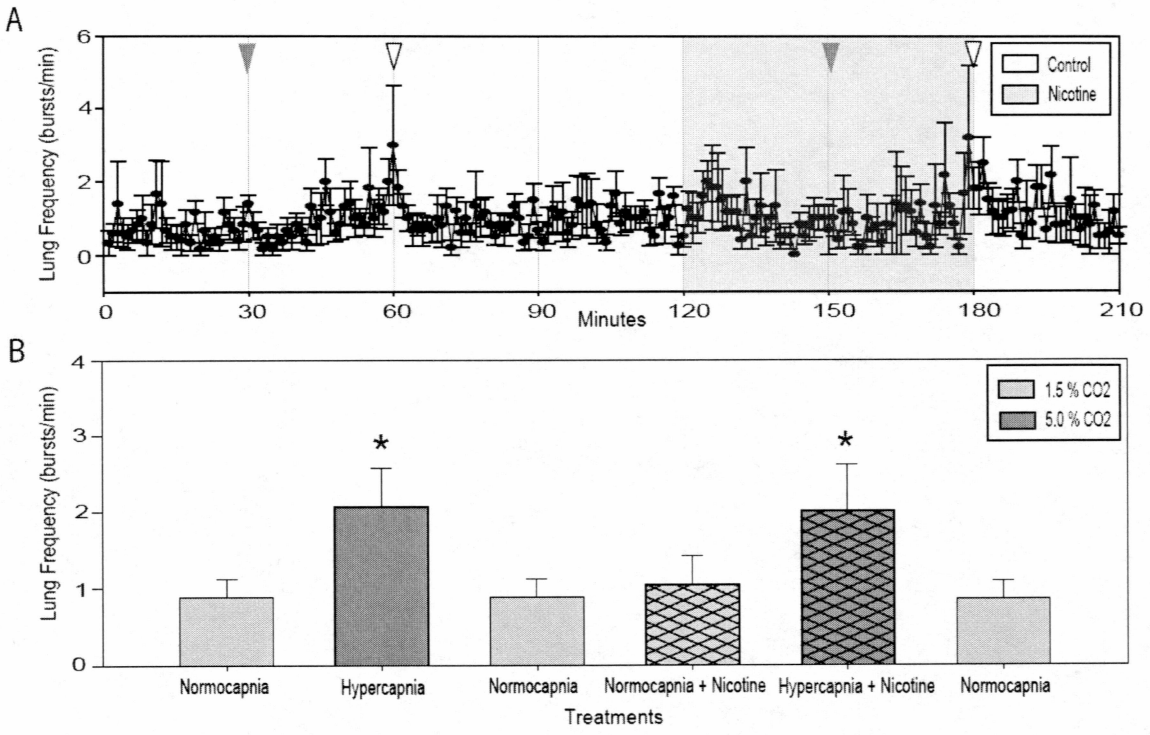

Fig. 2.3. Effect of acute nicotine exposure on the hypercapnic neuroventilatory response of early metamorphic tadpoles. A: Mean lung burst frequency per min over the 210-min treatment protocol (n = 6). Shaded triangles represent a switch to hypercapnia (5 % CO_2); open triangles represent a return to normocapnia (1.5 % CO_2). Shaded region highlights the period of exposure to 18 µg/L nicotine in the aCSF. B: Mean lung burst frequency over the last 3 min for each treatment within the protocol. Early metamorphic tadpole brainstems responded significantly to changes in P_{CO_2} (✱ = $P < 0.05$); there was no significant response to nicotine exposure.

Two-way RM-ANOVA on lung burst frequency exhibited by early metamorphic nicotine-treated tadpole brainstems, with P_{CO_2} as factor 1 and presence or absence of nicotine as factor 2, indicated that P_{CO_2} had a significant impact on lung burst frequency ($P = 0.01$) regardless of acute nicotine exposure ($P = 0.93$). Therefore, acute exposure to 18 µg/L nicotine had no effect on the lung burst frequency or response to hypercapnia of early metamorphic tadpoles.

Effect of acute nicotine exposure on lung neuroventilation of the late metamorphic tadpole brainstem

The effects of acute exposure to 18 µg/L nicotine on the frequency of lung bursts exhibited by late metamorphic tadpole brainstems are illustrated in Fig. 2.4. Under normal aCSF conditions late metamorphic tadpoles significantly increased lung burst frequency in response to hypercapnia, from normocapnic levels of 5.8 ± 3.3 to hypercapnic levels of 10.2 ± 4.4, P = 0.02. Superfusion with aCSF containing 18 µg/L nicotine significantly decreased late metamorphic normocapnic lung burst frequency (3.0 ± 1.7, P = 0.04), but did not impact the ability of late metamorphic tadpole brainstems to increase their lung burst frequency in response to hypercapnia (8.4 ± 1.6, P = 0.01). There was no significant difference between the hypercapnic lung burst frequency of late metamorphic tadpole brainstems under control and acute nicotine conditions (P = 0.69).

Two-way RM-ANOVA on lung burst frequency of late metamorphic nicotine-treated tadpole brainstems, with P_{CO_2} as factor 1 and the presence or absence of nicotine as factor 2, indicated that P_{CO_2} had a significant impact on lung burst frequency (P < 0.001) regardless of acute nicotine exposure (P = 0.46). Therefore, although acute exposure to 18 µg/L nicotine did decrease

normocapnic lung burst frequency it had no effect on the hypercapnic response of late metamorphic tadpole brainstems.

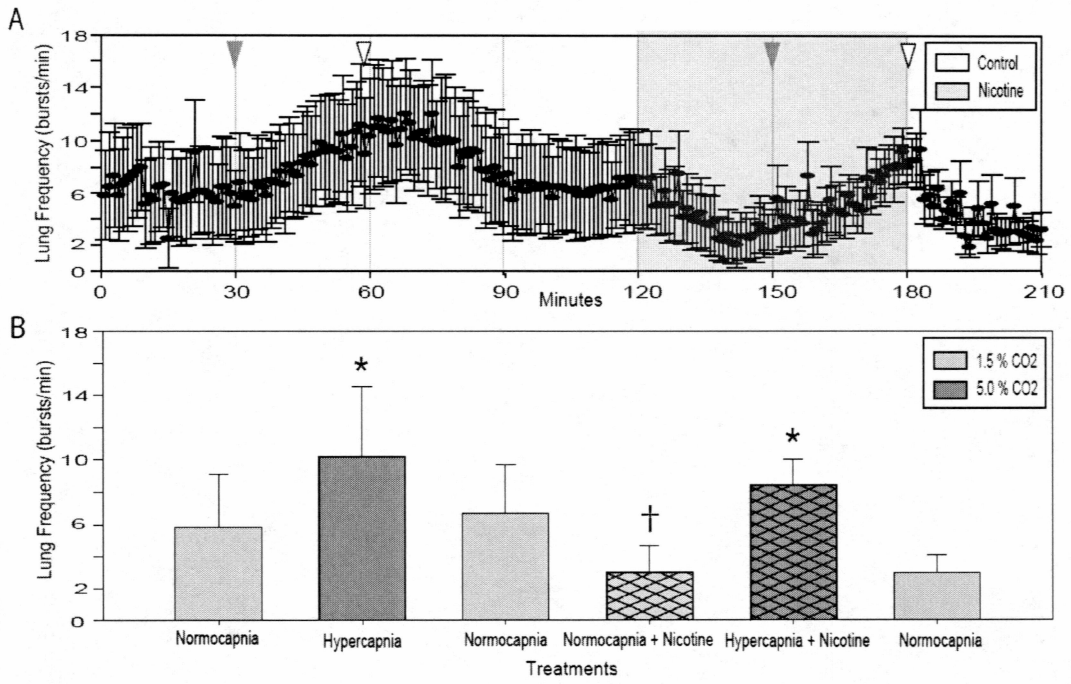

Fig. 2.4. Effect of acute nicotine exposure on the hypercapnic neuroventilatory response of late metamorphic tadpoles. A: Mean lung burst frequency per min over the 210-min nicotine protocol (n = 6). Shaded triangles represent a switch to hypercapnia (5 % CO_2); open triangles represent a return to normocapnia (1.5 % CO_2). Shaded region highlights the period of exposure to 18 µg/L nicotine in the aCSF. B: Mean lung burst frequency over the last 3 min for each treatment in the protocol. Late metamorphic tadpole brainstems responded significantly to changes in P_{CO_2} (✱ = $P < 0.05$). Nicotine significantly decreased normocapnic lung burst frequency († = $P < 0.05$).

Effect of acute nicotine exposure on lung neuroventilation of the juvenile bullfrog brainstem

The effects of acute exposure to 18 µg/L nicotine on the frequency of lung bursts exhibited by juvenile bullfrog brainstems are illustrated in Fig. 2.5. Under normal

aCSF conditions juvenile bullfrogs significantly increased lung burst frequency in response to hypercapnia, from normocapnic levels of 6.7± 2.6 to hypercapnic levels of 14.3 ± 3.2, P = 0.04). Superfusion with aCSF containing 18 µg/L nicotine significantly decreased normocapnic lung burst frequency (3.8 ± 1.4, P = 0.04) in juvenile bullfrog brainstems and inhibited the typical hypercapnia-induced increase in lung burst frequency. Lung burst frequency during hypercapnia was not significantly different from that during normocapnia (2.6 ± 1.1, P = 0.39). There was a significant difference between the hypercapnic lung burst frequency of juvenile bullfrogs under control and nicotine exposure conditions (P = 0.04).

Two-way repeated measures ANOVA on lung burst frequency exhibited by nicotine-treated juvenile bullfrog brainstems, with P_{CO_2} as factor 1 and presence or absence of nicotine as factor 2, indicated that P_{CO_2} had a significant impact on lung burst frequency within control aCSF conditions (P = 0.006), but not within nicotine aCSF conditions (P = 0.57). Thus, there was a significant effect of acute nicotine exposure on changes in P_{CO_2} (P = 0.04). Acute exposure to 18 µg/L nicotine decreased both normocapnic lung burst frequency and the response to hypercapnia in juvenile bullfrogs.

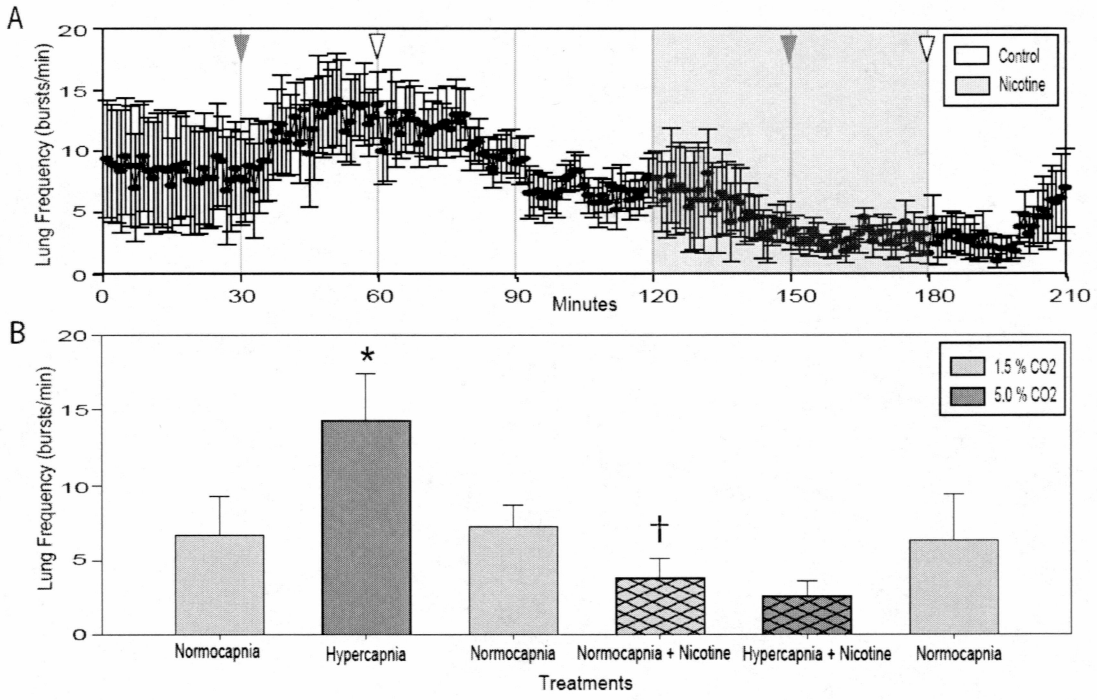

Fig. 2.5.Effect of acute nicotine on the hypercapnic neuroventilatory response of juvenile bullfrogs. A: Mean lung burst frequency per min over the 210-min protocol (n = 5). Shaded triangles represent a switch to hypercapnia (5 % CO_2); open triangles represent a return to normocapnia (1.5 % CO_2). Shaded region highlights the period of exposure to 18 µg/L nicotine aCSF. B: Mean lung burst frequency over the last 3 min for each treatment condition. Juvenile bullfrogs responded significantly to changes in P_{CO_2} (✱ = $P < 0.05$) only under conditions without nicotine. Nicotine significantly decreased normocapnic lung burst frequency († = $P < 0.05$) and blocked the typical hypercapnic response of increased lung burst frequency.

2.5 DISCUSSION

My results indicate that the bullfrog's central respiratory drive is vulnerable to acute nicotine exposure, and this vulnerability becomes more acute with development. Nicotine (18 µg/L aCSF), when bath applied, impeded the lung burst frequency as exhibited by the *in vitro* bullfrog brainstem preparation in a

manner that increased with development. Early metamorphic tadpole brainstems exhibited no significant change in lung burst frequency under normocapnic or hypercapnic conditions when treated with nicotine. Late metamorphic tadpole brainstems exposed to nicotine decreased lung burst frequency under normocapnic conditions but exhibited the typical increase in lung burst frequency in response to hypercapnia. Acute nicotine exposure decreased both the normocapnic and hypercapnic lung burst frequency of juvenile bullfrog brainstems.

The results of the present study differ considerably from the findings of Kennedy et al. (17). Kennedy and colleagues applied filter paper soaked with 62 mM nicotine to the ventral surface of the cane toad brainstem *in situ*, and this resulted in an increase in the frequency of cane toad lung episodes during normal isocapnic conditions. The higher dose of nicotine employed in the toad study is one possible explanation for the differing results. I used a smaller, more physiologically relevant, dose and bath applied it to the entire isolated brainstem. Additionally, Kennedy et al. (16) investigated acute effects of nicotine in a semi-intact toad, a preparation that included sensory afferent inputs to the brainstem respiratory control network. The influence of these afferent inputs may have contributed to the differences in findings of the toad study and my bullfrog brainstem study.

Brainstems from early metamorphic tadpoles that had been chronically exposed to nicotine for 3 or 10 wk demonstrate an attenuated hypercapnic neuroventilatory response ((37) and see Chapter 1). Acute nicotine exposure, however, failed to affect putative lung ventilation and the hypercapnic response at this early stage of development. This indicates that the mechanism by which chronic nicotine exposure impairs the hypercapnic response is not vulnerable to acute nicotine exposure. Chronic nicotine exposure has been shown to affect the proliferation of the central serotonergic neurons, which have been implicated in modulating ventilatory drive during hypercapnia (6, 12, 33, 45). Thus, chronic exposure to nicotine may adversely affect neuronal development within the respiratory control network of early metamorphic tadpoles. Faulty neuronal development may be the mechanism underlying the lack of a central response to hypercapnia rather than a chemical or receptor-based mechanism, which would be the only means for acutely applied nicotine to have an effect.

Acute nicotine exposure had no impact on the hypercapnic neuroventilatory response of late metamorphic tadpoles. The lack of an acute effect is in contrast with 10-wk chronic nicotine exposure, which has been found to impair hypercapnic ventilation of late metamorphic tadpoles ((37) and see Chapter 1). This further supports the conclusion that the neuroventilatory consequences of chronic nicotine exposure are different from those of acute exposure. It remains to been determined whether the tadpole respiratory control network can recover

from the deleterious effect of chronic nicotine exposure, i.e. whether central hypercapnic sensitivity is reinstated if tadpoles experience a period of development in a nicotine-free environment following the chronic exposure. Making this determination may define chronic nicotine effects on early metamorphic tadpoles as developmental effects, those that result in persistent impairment of CO_2/pH chemosensitivity, or pharmacological effects, those that result in impairment of CO_2/pH chemosensitivity only when nicotine is present.

To my knowledge, the effect of chronic nicotine exposure has not been investigated in the juvenile bullfrog. Acute exposure to nicotine, however, is shown here to have a profound effect on juvenile bullfrog brainstems; central respiratory drive was reduced during normocapnia, and the hypercapnia-induced increase in neuroventilatory lung bursts was eliminated. Depressed normocapnic lung burst frequency is either the product of active down-regulation of neuroventilation or a decrease in CO_2/pH sensitivity. If lung burst frequency was down-regulated then one would expect that responses to hypercapnia would be blunted but evident. The lack of any increase in neuroventilatory drive during hypercapnia suggests that nicotine may be directly impacting CO_2/pH sensitivity rather than compensatory mechanisms that increase respiratory drive in response to rising CO_2. An alternative explanation is that nicotine impacts multiple neuroventilatory-related mechanisms at one time.

The decrease in lung burst frequency under normocapnic conditions in late metamorphic tadpoles and juvenile frogs is a surprising one. This study was designed to look specifically at responses to hypercapnia, which have been characterized as an increase in lung burst frequency, and one that gains in magnitude over the course of development (36, 37). Focused investigation of other neuroventilatory consequences in developing bullfrogs exposed to acute nicotine could explain the effect of nicotine on normocapnic neuroventilation. However, any attempt at such explanation is beyond the scope of the present study. Factors that were not considered here (lung burst amplitude, duration and changes in buccal neuroventilation) may be uniquely affected by nicotine exposure and provide insight into the role of nicotinic acetylcholine receptors on amphibian rhythmogenesis.

Mammalian nicotinic acetylcholine receptors are up-regulated during development, with a major increase in expression following birth (31). This trend may also exist in amphibians; an increase in nicotinic receptor expression in the midbrain and optic tecta of leopard frogs is seen throughout tadpole development, reaching peak levels only after the culmination of metamorphosis (3). The developmental changes in nicotine responses recorded in my study may reflect developmental or maturational changes in the acetylcholine system of the bullfrog brainstem.

Nicotine exposure has differential impact across bullfrog development. Nicotine exposure alters neuroventilation depending on the duration and timing of exposure. Further investigation is needed to elucidate the specific mechanisms that underlie these responses. The differential effect of nicotine may reflect changes in the role and localization of nicotinic acetylcholine receptors and/or the maturation of ventilatory control.

2.6 CONCLUSIONS

Acute nicotine exposure (18 μg/L aCSF) reduced the respiratory drive generated by the bullfrog brainstem in a development-dependent manner. Lung burst frequency is affected, and late metamorphic tadpoles are the first developmental stage to demonstrate this effect. Late metamorphic tadpoles acutely exposed to nicotine exhibited decreased respiratory drive in the form of decreased normocapnic neuroventilation, but hypercapnic treatment augmented respiratory drive and lung burst frequency increased. Juvenile frogs acutely exposed to nicotine exhibited decreased respiratory drive in the form of decreased normocapnic neuroventilation, and hypercapnic treatment fails to augment respiratory drive because lung burst frequency did not increase. These findings suggest that nicotinic acetylcholine receptors play a vital role in the maturation of the bullfrog respiratory control network.

2.7 ACKNOWLEDGMENTS

Dr. Barbara E. Taylor served as my graduate advisor and was the principal investigator for the lab overseeing my research. Special thanks go to Dr. Michael B. Harris for his contributions on the structure and content of this manuscript, and to Mr. Justin C. Buehner who added in part to the dataset used in this study. This work was supported by NIH-NINDS grant # 2U54NS041069-601A.

2.8 LITERATURE CITED

1. **Abreu-Villaca Y, Seidler FJ, Tate CA, and Slotkin TA.** Nicotine is a neurotoxin in the adolescent brain: critical periods, patterns of exposure, regional selectivity, and dose thresholds for macromolecular alterations. *Brain Res* 979: 114-128, 2003.

2. **Broch L, Morales RD, Sandoval AV, and Hedrick MS.** Regulation of the respiratory central pattern generator by chloride-dependent inhibition during development in the bullfrog (Rana catesbeiana). *J Exp Biol* 205: 1161-1169, 2002.

3. **Butt CM, Pauly JR, and Debski EA.** Distribution and development of nicotinic acetylcholine receptor subtypes in the optic tectum of Rana pipiens. *J Comp Neurol* 423: 603-618, 2000.

4. **Dev NB and Loeschcke HH.** A cholinergic mechanism involved in the respiratory chemosensitivity of the medulla oblongata in the cat. *Pflugers Arch* 379: 29-36, 1979.

5. **Dominguez del Toro E, Juiz JM, Peng X, Lindstrom J, and Criado M.** Immunocytochemical localization of the alpha 7 subunit of the nicotinic acetylcholine receptor in the rat central nervous system. *J Comp Neurol* 349: 325-342, 1994.

6. **Engberg G, Erhardt S, Sharp T, and Hajos M.** Nicotine inhibits firing activity of dorsal raphe 5-HT neurones in vivo. *Naunyn Schmiedebergs Arch Pharmacol* 362: 41-45, 2000.

7. **Ernst M, Moolchan ET, and Robinson ML.** Behavioral and neural consequences of prenatal exposure to nicotine. *J Am Acad Child Adolesc Psychiatry* 40: 630-641, 2001.

8. **Fernandez R, Larrain C, and Zapata P.** Acute ventilatory and circulatory reactions evoked by nicotine: are they excitatory or depressant? *Respir Physiol Neurobiol* 133: 173-182, 2002.

9. **Galante RJ, Kubin L, Fishman AP, and Pack AI.** Role of chloride-mediated inhibition in respiratory rhythmogenesis in an *in vitro* brainstem of tadpole, Rana catesbeiana. *J Physiol* 492 (Pt 2): 545-558, 1996.

10. **Gdovin MJ, Torgerson CS, and Remmers JE.** The fictively breathing tadpole brainstem preparation as a model for the development of respiratory

pattern generation and central chemoreception. *Comp Biochem Physiol A Mol Integr Physiol* 124: 275-286, 1999.

11. **Ginzel KH, Maritz GS, Marks DF, Neuberger M, Pauly JR, Polito JR, Schulte-Hermann R, and Slotkin TA.** Critical review: nicotine for the fetus, the infant and the adolescent? *J Health Psychol* 12: 215-224, 2007.

12. **Guzman-Marin R, Alam MN, Mihailescu S, Szymusiak R, McGinty D, and Drucker-Colin R.** Subcutaneous administration of nicotine changes dorsal raphe serotonergic neurons discharge rate during REM sleep. *Brain Res* 888: 321-325, 2001.

13. **Hafstrom O, Milerad J, Sandberg KL, and Sundell HW.** Cardiorespiratory effects of nicotine exposure during development. *Respir Physiol Neurobiol* 149: 325-341, 2005.

14. **Harris MB, Wilson RJ, Vasilakos K, Taylor BE, and Remmers JE.** Central respiratory activity of the tadpole *in vitro* brain stem is modulated diversely by nitric oxide. *Am J Physiol Regul Integr Comp Physiol* 283: R417-428, 2002.

15. **Hill P, Haley NJ, and Wynder EL.** Cigarette smoking: carboxyhemoglobin, plasma nicotine, cotinine and thiocyanate vs self-reported smoking data and cardiovascular disease. *J Chronic Dis* 36: 439-449, 1983.

16. **Karkos J.** Significance of neurotransmitters and neuromodulators in regulating respiration. A. Central aminergic neurons. *Fortschr Neurol Psychiatr* 56: 69-96, 1988.

17. **Kennedy LL, Aguwa CC, Rives JE, and Bernard DG.** Involvement of cholinergic mechanisms in the central control of respiration in the cane toad, Bufo marinus. *Comp Biochem Physiol A Mol Integr Physiol* 128: 837-849, 2001.

18. **Kinkead R and Milsom WK.** Chemoreceptors and control of episodic breathing in the bullfrog (Rana catesbeiana). *Respir Physiol* 95: 81-98, 1994.

19. **Kinney HC, O'Donnell TJ, Kriger P, and White WF.** Early developmental changes in [3H] nicotine binding in the human brainstem. *Neuroscience* 55: 1127-1138, 1993.

20. **Lindblad A, Marsal K, and Andersson KE.** Effect of nicotine on human fetal blood flow. *Obstet Gynecol* 72: 371-382, 1988.

21. **Milsom WK, Reid SG, Meier JT, and Kinkead R.** Central respiratory pattern generation in the bullfrog, Rana catesbeiana. *Comp Biochem Physiol A Mol Integr Physiol* 124: 253-264, 1999.

22. **Monteau R, Morin D, and Hilaire G.** Acetylcholine and central chemosensitivity: in vitro study in the newborn rat. *Respir Physiol* 81: 241-253, 1990.

23. **Nelson EA and Taylor BJ.** International Child Care Practices Study: infant sleep position and parental smoking. *Early Hum Dev* 64: 7-20, 2001.

24. **Oncken CA, Hardardottir H, Hatsukami DK, Lupo VR, Rodis JF, and Smeltzer JS.** Effects of transdermal nicotine or smoking on nicotine concentrations and maternal-fetal hemodynamics. *Obstet Gynecol* 90: 569-574, 1997.

25. **Putnam RW, Conrad SC, Gdovin MJ, Erlichman JS, and Leiter JC.** Neonatal maturation of the hypercapnic ventilatory response and central neural CO_2 chemosensitivity. *Respiratory Physiology & Neurobiology* 149: 165-179, 2005.

26. **Shao XM and Feldman JL.** Mechanisms underlying regulation of respiratory pattern by nicotine in preBötzinger complex. *J Neurophysiol* 85: 2461-2467, 2001.

27. **Shao XM, Tan W, Xiu J, Puskar N, Fonck C, Lester HA, and Feldman JL.** Alpha4* nicotinic receptors in preBötzinger complex mediate cholinergic/nicotinic modulation of respiratory rhythm. *J Neurosci* 28: 519-528, 2008.

28. **Slotkin TA.** Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. *Toxicol Appl Pharmacol* 198: 132-151, 2004.

29. **Slotkin TA.** Nicotine and the adolescent brain: insights from an animal model. *Neurotoxicol Teratol* 24: 369-384, 2002.

30. **Slotkin TA, MacKillop EA, Rudder CL, Ryde IT, Tate CA, and Seidler FJ.** Permanent, sex-selective effects of prenatal or adolescent nicotine exposure, separately or sequentially, in rat brain regions: indices of cholinergic and serotonergic synaptic function, cell signaling, and neural cell number and size at 6 months of age. *Neuropsychopharmacol* 32: 1082-1097, 2007.

31. **Slotkin TA, Orband-Miller L, and Queen KL.** Development of [3H]nicotine binding sites in brain regions of rats exposed to nicotine prenatally via maternal injections or infusions. *J Pharmacol Exp Ther* 242: 232-237, 1987.

32. **Slotkin TA, Ryde IT, and Seidler FJ.** Separate or sequential exposure to nicotine prenatally and in adulthood: persistent effects on acetylcholine systems in rat brain regions. *Brain Res Bull* 74: 91-103, 2007.

33. **Slotkin TA, Ryde IT, Tate CA, and Seidler FJ.** Lasting effects of nicotine treatment and withdrawal on serotonergic systems and cell signaling in rat brain regions: separate or sequential exposure during fetal development and adulthood. *Brain Res Bull* 73: 259-272, 2007.

34. **Slotkin TA, Southard MC, Adam SJ, Cousins MM, and Seidler FJ.** Alpha7 nicotinic acetylcholine receptors targeted by cholinergic developmental neurotoxicants: nicotine and chlorpyrifos. *Brain Res Bull* 64: 227-235, 2004.

35. **Straus C.** Ontogeny of respiratory muscle control. Evidence from the amphibian model. *Rev Mal Respir* 17: 585-590, 2000.

36. **Taylor AC and Kollros JJ.** Stages in the normal development of *Rana pipiens* larvae. *Anat Rec* 94: 7-24, 1946.

37. **Taylor BE, Croll AE, Drucker ML, and Wilson AL.** Developmental exposure to ethanol or nicotine inhibits the hypercapnic ventilatory response in tadpoles. *Respir Physiol Neurobiol* 160: 83-90, 2008.

38. **Taylor BE, Harris MB, Coates EL, Gdovin MJ, and Leiter JC.** Central CO_2 chemoreception in developing bullfrogs: anomalous response to acetazolamide. *J Appl Physiol* 94: 1204-1212, 2003.

39. **Taylor BE, Harris MB, Leiter JC, and Gdovin MJ.** Ontogeny of central CO_2 chemoreception: chemosensitivity in the ventral medulla of developing bullfrogs. *Am J Physiol Regul Integr Comp Physiol* 285: R1461-1472, 2003.

40. **Torgerson C, Gdovin M, and Remmers J.** Ontogeny of central chemoreception during fictive gill and lung ventilation in an *in vitro* brainstem preparation of Rana catesbeiana. *J Exp Biol* 200: 2063-2072, 1997.

41. **Torgerson CS, Gdovin MJ, Brandt R, and Remmers JE.** Location of central respiratory chemoreceptors in the developing tadpole. *Am J Physiol Regul Integr Comp Physiol* 280: R921-928, 2001.

42. **Torgerson CS, Gdovin MJ, Kogo N, and Remmers JE.** Depth profiles of pH and PO_2 in the *in vitro* brainstem preparation of the tadpole Rana catesbeiana. *Respir Physiol* 108: 205-213, 1997.

43. **Torgerson CS, Gdovin MJ, and Remmers JE.** Fictive gill and lung ventilation in the pre- and postmetamorphic tadpole brain stem. *J Neurophysiol* 80: 2015-2022, 1998.

44. **Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, and Swanson LW.** Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J Comp Neurol* 284: 314-335, 1989.

45. **Xu Z, Seidler FJ, Ali SF, Slikker W, Jr., and Slotkin TA.** Fetal and adolescent nicotine administration: effects on CNS serotonergic systems. *Brain Res* 914: 166-178, 2001.

GENERAL CONCLUSIONS

The neurophysiological consequences of developmental nicotine exposure were investigated in the previous chapters and offer insight into the development of neuroventilation and the consequences of nicotine exposure, be it acute exposure at different stages of development or chronic exposure for different durations during development.

Acute nicotine exposure had no significant impact on the lung burst frequency of early metamorphic tadpoles and did not impact their ability to respond to stimulation with hypercapnia (5 % CO_2). This contrasted with their severely attenuated response to hypercapnia following either 3- or 10-wk chronic nicotine exposure.

Acute nicotine exposure had a different effect on late metamorphic tadpoles; their neuroventilation was depressed during normocapnia but their hypercapnic response was unchanged. Chronic nicotine exposure for 3 wk had no impact on the lung burst frequency exhibited by late metamorphic tadpoles during normocapnia or hypercapnia. However, 10-wk chronic nicotine exposure resulted in neuroventilatory deficits similar to those of 10-wk exposed early metamorphic tadpoles, i.e., an impaired hypercapnic response with no change in normocapnic lung burst frequency.

These results support my hypothesis that chronic nicotine exposure induces a developmental functional impairment in the maturation of early metamorphic tadpoles' hypercapnic neuroventilatory response. The impact of chronic nicotine exposure on the neuroventilatory response of tadpoles is not mirrored during acute exposure. I believe this is a critical distinction, one that suggests a long-term adverse impact of nicotine that alters either central CO_2 chemosensitivity or the central ventilatory compensatory mechanisms that increase lung burst frequency in response to hypercapnia.

Late metamorphic tadpoles demonstrated normal responses to hypercapnia following 3-wk chronic nicotine exposure whereas similarly exposed early metamorphic tadpoles were unable to respond significantly to hypercapnia. This suggests that early metamorphic tadpoles are more vulnerable to chronic nicotine exposure. This enhanced vulnerability could result from either a relative immaturity of their central CO_2 compensatory mechanisms or events in neural development that are unique to early tadpole metamorphosis.

Another topic for future research will be to determine whether the impaired hypercapnic neuroventilatory response exhibited by chronically nicotine-exposed tadpoles can be regained after cessation of nicotine exposure. This could clarify whether an actual developmental aberration or deficit has occurred as a result of

the long-term impact of nicotine. Accordingly, I plan to investigate the hypercapnic neuroventilatory responses of tadpoles allowed to develop in nicotine-free environments for different lengths of time following chronic nicotine exposure.

The lung burst frequency of early metamorphic tadpole brainstems is considerably lower than that of late metamorphic tadpole brainstems, and their hypercapnic response is subtle in comparison (50-52, 55). Chronic developmental nicotine exposure may have been equally deleterious to tadpoles at all developmental stages; however, with such a robust lung burst frequency among late metamorphic tadpoles, the impact of nicotine may have been too small to significantly impair their hypercapnic neuroventilatory response.

The vulnerability of bullfrog neuroventilation to acute nicotine exposure seems to increase with development. Notably, this increase in vulnerability is concomitant with an increase in lung bursting and in the relative magnitude of the hypercapnic neuroventilatory response. Late metamorphic tadpoles were the earliest developmental stage to show an effect of acute nicotine exposure; normocapnic neuroventilation decreased. The effect of acute nicotine exposure was more deleterious in the juvenile frog, which exhibited a decrease in both normocapnic and hypercapnic neuroventilation under the influence of nicotine. These results were surprising and suggest that sensitivity to acute nicotine increases with

development, and supports the work of previous research in mammals suggesting the active role of nAChRs in CO_2 chemosensivity and respiratory regulation (29, 38, 39).

In my protocol, chronic nicotine exposure was accomplished through immersion in a nicotine solution. Juvenile bullfrogs require periods of emersion for rest and feeding. Therefore, I could not have subjected juvenile frogs to identical chronic treatment by immersion. Accordingly, I made no attempt to include juvenile frogs in my chronic nicotine study. There exists, however, previous work on the amphibian response to acute nicotine exposure, done in an *in situ* preparation of adult cane toads (18). To compare my findings to those of Kennedy et al. (18) I conducted acute nicotine exposure studies using the post-metamorphic juvenile bullfrog. Juvenile or adult bullfrogs could be chronically exposed to nicotine with a method that combines aquatic and aerial nicotine administration. This would be an interesting direction for future research.

One of the more unique aspects of my studies was the number and type of controls that I used. There is some inherent variation in the neuroventilation of tadpoles even among those of the same developmental stage. That fact and the generally robust nature of the bullfrog brainstem make it appealing for investigators to use each brainstem as a control for itself. In many protocols isolated tadpole brainstems are subjected to multiple treatments over extended

periods of time. Considerable effort was made in my studies to ensure that the robust nature of the bullfrog brainstem was not comprised by 1 or 2 hypercapnic challenges or by the 3- or 10-wk nicotine exposure. I performed isocapnic time-control experiments for 210 min of neuroventilation to identify any changes in lung burst frequency over time, and I verified that there were no significant differences in lung burst frequency over the time-course of my experiments. This was valuable in assessing the response to a specific treatment. I would advocate that other investigators conduct controls to correct for the time and/or each individual treatment in their studies.

There is some variability in the findings of previous studies that aimed to characterize the hypercapnic neuroventilatory response of the bullfrog (8, 51, 52). This variability may be due to: the level of CO_2/pH in aCSF solution that bathed the preparations, the methodology of stimulus application (focal application or bath application), and the criteria used to group tadpoles into developmental stages. The general consensus is that hypercapnia increases the lung burst frequency of tadpoles, and the percent increase in ventilation also develops with ontogeny (8). For that reason I looked specifically at lung frequency in these studies. I wanted a clear marker to identify a specific functional response after chronic and during acute nicotine exposure. There may be other components of ventilation that differ in response to either nicotine

exposure or hypercapnic treatment. Although not looked at in my studies they may be worthwhile areas for future investigation.

Some of the variation between the results of the chronic and acute nicotine exposure studies may be explained by the choice to use a different dose of nicotine exposure in each study. Both 18 and 30 µg/L nicotine are within the expected body-fluid nicotine concentration of a moderate to heavy smoker (13, 31, 33). I chose to use 30 µg/L nicotine for my chronic exposure studies in order to be consistent with previous work looking at chronic nicotine exposure in early metamorphic tadpoles (49). My hypothesis was that early metamorphic tadpoles that demonstrate the phenotype exhibited in that study were more susceptible to the effect of chronic nicotine than later stage tadpoles. To accurately test this it was reasonable to use the 30 µg/L dose. Hofstrom et al. (11) recently suggested that much of the variability seen in studies on the cardiorespiratory effects of nicotine was based on the many different concentrations that were used. They suggested that 16-20 µg/L was the most appropriate dose to investigate the acute affects of nicotine in the human body (11, 24). For that reason I chose to apply the 18 µg/L dose in my acute nicotine studies to be consistent with the general trend in the literature. Additional work looking at the chronic effect of 18 µg/L nicotine exposure should be done to rule out any inconsistencies.

Clear conclusions can be drawn from my results, despite these comments on methodology and sources of variability. 18 µg/L acute nicotine exposure has an increasing impact on bullfrog lung ventilation from early metamorphosis to juvenile bullfrog, decreasing first normocapnic lung burst frequency and eventually the neuroventilatory hypercapnic response. 30 µg/L chronic nicotine exposure had no effect on normocapnic ventilation at any point in tadpole development, but significantly impaired the neuroventilatory hypercapnic response in early metamorphic tadpoles following 3 wk of nicotine exposure and in both early and late metamorphic tadpoles following a 10-wk exposure. Taken together these results suggest that the activation of nicotinic acetylcholine receptors by nicotine modulates lung burst frequency in developing bullfrogs, and that the effect of prolonged or repeated activation of those receptors during chronic developmental exposure attenuates subsequent lung burst frequency changes in response to hypercapnia.

This work highlights some of the neuroventilatory functional consequences of developmental nicotine exposure on the central control of respiration, and it illustrates how developmental nicotine exposure might contribute to certain ventilatory pathologies in the hypercapnic responses of vertebrates.

LITERATURE CITED

1. **Atluri P, Fleck MW, Shen Q, Mah SJ, Stadfelt D, Barnes W, Goderie SK, Temple S, and Schneider AS.** Functional nicotinic acetylcholine receptor expression in stem and progenitor cells of the early embryonic mouse cerebral cortex. *Dev Biol* 240: 143-156, 2001.

2. **Bamford OS and Carroll JL.** Dynamic ventilatory responses in rats: normal development and effects of prenatal nicotine exposure. *Respir Physiol* 117: 29-40, 1999.

3. **Bamford OS, Schuen JN, and Carroll JL.** Effect of nicotine exposure on postnatal ventilatory responses to hypoxia and hypercapnia. *Respir Physiol* 106: 1-11, 1996.

4. **Caddell JL.** A triple-risk model for the sudden infant death syndrome (SIDS) and the apparent life-threatening episode (ALTE): the stressed magnesium deficient weanling rat. *Magnes Res* 14: 227-238, 2001.

5. **Cheron G and Timsit S.** Smoking and sudden infant death syndrome. *J Gynecol Obstet Biol Reprod (Paris)* 32: 1S33-40, 2003.

6. **Dominguez del Toro E, Juiz JM, Peng X, Lindstrom J, and Criado M.** Immunocytochemical localization of the alpha 7 subunit of the nicotinic acetylcholine receptor in the rat central nervous system. *J Comp Neurol* 349: 325-342, 1994.

7. **Galante RJ, Kubin L, Fishman AP, and Pack AI.** Role of chloride-mediated inhibition in respiratory rhythmogenesis in an in vitro brainstem of tadpole, Rana catesbeiana. *J Physiol* 492 (Pt 2): 545-558, 1996.

8. **Gargaglioni LH and Milsom WK.** Control of breathing in anuran amphibians. *Comp Biochem Physiol A Mol Integr Physiol* 147: 665-684, 2007.

9. **Gdovin MJ, Torgerson CS, and Remmers JE.** The fictively breathing tadpole brainstem preparation as a model for the development of respiratory pattern generation and central chemoreception. *Comp Biochem Physiol A Mol Integr Physiol* 124: 275-286, 1999.

10. **Guntheroth WG and Spiers PS.** The triple risk hypotheses in sudden infant death syndrome. *Pediatrics* 110: e64, 2002.

11. **Hafstrom O, Milerad J, Sandberg KL, and Sundell HW.** Cardiorespiratory effects of nicotine exposure during development. *Respir Physiol Neurobiol* 149: 325-341, 2005.

12. **Hedrick MS and Morales RD.** Nitric oxide as a modulator of central respiratory rhythm in the isolated brainstem of the bullfrog (Rana catesbeiana). *Comp Biochem Physiol A Mol Integr Physiol* 124: 243-251, 1999.

13. **Hill P, Haley NJ, and Wynder EL.** Cigarette smoking: carboxyhemoglobin, plasma nicotine, cotinine and thiocyanate vs self-reported smoking data and cardiovascular disease. *J Chronic Dis* 36: 439-449, 1983.

14. **Hohmann CF and Berger-Sweeney J.** Cholinergic regulation of cortical development and plasticity. New twists to an old story. *Perspect Dev Neurobiol* 5: 401-425, 1998.

15. **Hohmann CF, Brooks AR, and Coyle JT.** Neonatal lesions of the basal forebrain cholinergic neurons result in abnormal cortical development. *Brain Res* 470: 253-264, 1988.

16. **Hunt CE.** Gene-environment interactions: implications for sudden unexpected deaths in infancy. *Arch Dis Child* 90: 48-53, 2005.

17. **Iyasu S, Randall LL, Welty TK, Hsia J, Kinney HC, Mandell F, McClain M, Randall B, Habbe D, Wilson H, and Willinger M.** Risk factors for sudden infant death syndrome among northern plains Indians. *Jama* 288: 2717-2723, 2002.

18. **Kennedy LL, Aguwa CC, Rives JE, and Bernard DG.** Involvement of cholinergic mechanisms in the central control of respiration in the cane toad, Bufo marinus. *Comp Biochem Physiol A Mol Integr Physiol* 128: 837-849, 2001.

19. **Kinkead R, Harris MB, and Milsom WK.** The role of the nucleus isthmi in respiratory pattern formation in bullfrogs. *J Exp Biol* 200: 1781-1793, 1997.

20. **Kinney HC, Filiano JJ, and White WF.** Medullary serotonergic network deficiency in the sudden infant death syndrome: review of a 15-year study of a single dataset. *J Neuropathol Exp Neurol* 60: 228-247, 2001.

21. **Lauder JM and Schambra UB.** Morphogenetic roles of acetylcholine. *Environ Health Perspect* 107 Suppl 1: 65-69, 1999.

22. **Lena C, de Kerchove D'Exaerde A, Cordero-Erausquin M, Le Novere N, del Mar Arroyo-Jimenez M, and Changeux JP.** Diversity and distribution of nicotinic acetylcholine receptors in the locus ceruleus neurons. *Proc Natl Acad Sci USA* 96: 12126-12131, 1999.

23. **Liao GS, Kubin L, Galante RJ, Fishman AP, and Pack AI.** Respiratory activity in the facial nucleus in an in vitro brainstem of tadpole, Rana catesbeiana. *J Physiol* 492 (Pt 2): 529-544, 1996.

24. **Lindblad A, Marsal K, and Andersson KE.** Effect of nicotine on human fetal blood flow. *Obstet Gynecol* 72: 371-382, 1988.

25. **McLean HA, Kimura N, Kogo N, Perry SF, and Remmers JE.** Fictive respiratory rhythm in the isolated brainstem of frogs. *J Comp Physiol [A]* 176: 703-713, 1995.

26. **Milsom WK.** Evolutionary trends in respiratory mechanisms. *Adv Exp Med Biol* 605: 293-298, 2008.

27. **Milsom WK.** Phylogeny of CO_2/H+ chemoreception in vertebrates. *Respir Physiol Neurobiol* 131: 29-41, 2002.

28. **Milsom WK, Reid SG, Meier JT, and Kinkead R.** Central respiratory pattern generation in the bullfrog, Rana catesbeiana. *Comp Biochem Physiol A Mol Integr Physiol* 124: 253-264, 1999.

29. **Monteau R, Morin D, and Hilaire G.** Acetylcholine and central chemosensitivity: in vitro study in the newborn rat. *Respir Physiol* 81: 241-253, 1990.

30. **Morales RD and Hedrick MS.** Temperature and pH/CO_2 modulate respiratory activity in the isolated brainstem of the bullfrog (Rana catesbeiana). *Comp Biochem Physiol A Mol Integr Physiol* 132: 477-487, 2002.

31. **Moyer TP, Charlson JR, Enger RJ, Dale LC, Ebbert JO, Schroeder DR, and Hurt RD.** Simultaneous analysis of nicotine, nicotine metabolites, and tobacco alkaloids in serum or urine by tandem mass spectrometry, with clinically relevant metabolic profiles. *Clin Chem* 48: 1460-1471, 2002.

32. **Nattie E and Kinney H.** Nicotine, serotonin, and sudden infant death syndrome. *Am J Respir Crit Care Med* 166: 1530-1531, 2002.

33. **Oncken CA, Henry KM, Campbell WA, Kuhn CM, Slotkin TA, and Kranzler HR.** Effect of maternal smoking on fetal catecholamine concentrations at birth. *Pediatr Res* 53: 119-124, 2003.

34. **Perry SF, McLean HA, Kogo N, Kimura N, Kawasaki H, Sakurai M, Kabotyanski EA, and Remmers JE.** The frog brainstem preparation as a model for studying the central control of breathing in tetrapods. *Braz J Med Biol Res* 28: 1339-1346, 1995.

35. **Putnam RW, Conrad SC, Gdovin MJ, Erlichman JS, and Leiter JC.** Neonatal maturation of the hypercapnic ventilatory response and central neural CO_2 chemosensitivity. *Respir Physiol Neurobiol* 149: 165-179, 2005.

36. **Schmidt RS.** A model of the central mechanisms of male anuran acoustic behavior. *Behaviour* 39: 288-317, 1971.

37. **Schneider AS, Atluri P, Shen Q, Barnes W, Mah SJ, Stadfelt D, Goderie SK, Temple S, and Fleck MW.** Functional nicotinic acetylcholine receptor expression on stem and progenitor cells of the early embryonic nervous system. *Ann N Y Acad Sci* 971: 135-138, 2002.

38. **Shao XM and Feldman JL.** Mechanisms underlying regulation of respiratory pattern by nicotine in preBötzinger complex. *J Neurophysiol* 85: 2461-2467, 2001.

39. **Shao XM and Feldman JL.** Pharmacology of nicotinic receptors in preBötzinger complex that mediate modulation of respiratory pattern. *J Neurophysiol* 88: 1851-1858, 2002.

40. **Slikker W, Jr., Xu ZA, Levin ED, and Slotkin TA.** Mode of action: disruption of brain cell replication, second messenger, and neurotransmitter systems during development leading to cognitive dysfunction--developmental neurotoxicity of nicotine. *Crit Rev Toxicol* 35: 703-711, 2005.

41. **Slotkin TA.** Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. *Toxicol Appl Pharmacol* 198: 132-151, 2004.

42. **Slotkin TA.** Developmental cholinotoxicants: nicotine and chlorpyrifos. *Environ Health Perspect* 107 Suppl 1: 71-80, 1999.

43. **Slotkin TA.** Fetal nicotine or cocaine exposure: which one is worse? *J Pharmacol Exp Ther* 285: 931-945, 1998.

44. **Slotkin TA.** Nicotine and the adolescent brain: Insights from an animal model. 24: 369, 2002.

45. **Slotkin TA, Lappi SE, McCook EC, Lorber BA, and Seidler FJ.** Loss of neonatal hypoxia tolerance after prenatal nicotine exposure: implications for sudden infant death syndrome. *Brain Res Bull* 38: 69-75, 1995.

46. **Slotkin TA, Ryde IT, and Seidler FJ.** Separate or sequential exposure to nicotine prenatally and in adulthood: persistent effects on acetylcholine systems in rat brain regions. *Brain Res Bull* 74: 91-103, 2007.

47. **St-John WM and Leiter JC.** Maternal nicotine depresses eupneic ventilation of neonatal rats. *Neurosci Lett* 267: 206-208, 1999.

48. **Straus C.** Ontogeny of respiratory muscle control. Evidence from the amphibian model. *Rev Mal Respir* 17: 585-590, 2000.

49. **Taylor BE, Croll AE, Drucker ML, and Wilson AL.** Developmental exposure to ethanol or nicotine inhibits the hypercapnic ventilatory response in tadpoles. *Respir Physiol Neurobiol* 160: 83-90, 2008.

50. **Taylor BE, Harris MB, Coates EL, Gdovin MJ, and Leiter JC.** Central CO_2 chemoreception in developing bullfrogs: anomalous response to acetazolamide. *J Appl Physiol* 94: 1204-1212, 2003.

51. **Taylor BE, Harris MB, Leiter JC, and Gdovin MJ.** Ontogeny of central CO_2 chemoreception: chemosensitivity in the ventral medulla of developing bullfrogs. *Am J Physiol Regul Integr Comp Physiol* 285: R1461-1472, 2003.

52. **Torgerson C, Gdovin M, and Remmers J.** Ontogeny of central chemoreception during fictive gill and lung ventilation in an *in vitro* brainstem preparation of Rana catesbeiana. *J Exp Biol* 200: 2063-2072, 1997.

53. **Torgerson CS, Gdovin MJ, Brandt R, and Remmers JE.** Location of central respiratory chemoreceptors in the developing tadpole. *Am J Physiol Regul Integr Comp Physiol* 280: R921-928, 2001.

54. **Torgerson CS, Gdovin MJ, Kogo N, and Remmers JE.** Depth profiles of pH and PO_2 in the *in vitro* brainstem preparation of the tadpole Rana catesbeiana. *Respir Physiol* 108: 205-213, 1997.

55. **Torgerson CS, Gdovin MJ, and Remmers JE.** Fictive gill and lung ventilation in the pre- and postmetamorphic tadpole brain stem. *J Neurophysiol* 80: 2015-2022, 1998.

56. **Torgerson CS, Gdovin MJ, and Remmers JE.** Sites of respiratory rhythmogenesis during development in the tadpole. *Am J Physiol Regul Integr Comp Physiol* 280: R913-920, 2001.

57. **Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, and Swanson LW.** Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J Comp Neurol* 284: 314-335, 1989.

58. **Wilson RJ, Vasilakos K, Harris MB, Straus C, and Remmers JE.** Evidence that ventilatory rhythmogenesis in the frog involves two distinct neuronal oscillators. *J Physiol* 540: 557-570, 2002.

59. **Winmill RE and Hedrick MS.** Developmental changes in the modulation of respiratory rhythm generation by extracellular K+ in the isolated bullfrog brainstem. *J Neurobiol* 55: 278-287, 2003.