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

Cord M Brundage and **Barbara E Taylor***

Institute of Arctic Biology, Department of Biology and Wildlife, University of Alaska Fairbanks

Abstract

The ability for air-breathing vertebrates to adjust ventilation in response to increased CO_2 (hypercapnia) is fundamental to maintaining pH homeostasis. Developmental nicotine exposure has been shown to impair tadpole neuroventilatory responses to hypercapnia following 8-12 wk of exposure. It is not clear, however, to what extent the timing of exposure during development and/or the duration over which the exposure takes place contribute to this impairment. Here tadpoles were exposed to 30 μg/L of nicotine for 3- or 10-wk durations, 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 of isolated brainstems. Preparations derived from early metamorphic tadpoles failed to increase lung neuroventilation in response to hypercapnia whether they had been exposed to nicotine for 3 or 10 wk. Preparations derived from late metamorphic tadpoles failed to respond to hypercapnia after being exposed to nicotine for 10 wk. These results suggest that both the developmental timing and duration of exposure are important when considering nicotine's effect on the hypercapnic neuroventilatory response.

Keywords

control of breathing; neuroteratogen; neurotoxin

INTRODUCTION

Hypercapnic acidosis stimulates respiratory output, a modulation chiefly mediated by central CO_2/pH sensitive neurons (O'Regan and Majcherczyk, 1982; Milsom, 1995; Nattie, 1999; Putnam et al., 2004), which have been demonstrated in all tetrapod vertebrates (Milsom, 2002). CO_2/pH-sensitive neurons provide feedback to brainstem respiratory control regions on the adequacy of ventilation relative to metabolism (Nattie, 1999).Ventilatory responses to hypercapnia in mammals are low in newborns and subsequently increase to adult levels with development (Putnam et al., 2005). 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 post

*corresponding author 902 N Koyukuk Drive, Fairbanks, AK, 99775, USA telephone: 907-474-2487 fax: 907-474-6050 ffbet@uaf.edu.

metamorphic stages (Smatresk and Smits, 1991; Torgerson et al., 1997; Taylor et al., 2003b; Gheshmy et al., 2006). These hypercapnic neuroventilatory responses (HCnVRs) 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 HCnVRs may be shared among all vertebrates, and consist of an orchestrated transition in the relative role of excitatory and inhibitory synaptic transmission (Putnam et al., 2005).

Nicotine is a developmental neurotoxin, altering the replication, formation and differentiation of brain tissue (Slotkin, 2004). The neuroteratogenic nature of prenatal nicotine exposure has been well established and chronic exposure may impair ventilatory responses to exogenous stressors such as hypoxia or hypercapnia (Hafstrom et al., 2005). An attenuated response to hypoxic ventilatory stress following prenatal nicotine exposure has been demonstrated in rat pups (St-John and Leiter, 1999; Simakajornboon et al., 2004). Early metamorphic tadpoles exposed to 8-12 weeks of 30 μg/L nicotine demonstrated a diminished ventilatory response to hypercapnia *in vivo* and in *in vitro* brainstem preparations (Taylor et al., 2008).

Despite evidence that nicotine impairs tadpole HCnVRs (Taylor et al., 2008), it is not clear whether a minimum 8-wk duration of nicotine exposure or exposure at the time of early tadpole development is necessary to create an impairment. Both early and late times in tadpole development may be particularly sensitive to the effects of nicotine exposure. Early in development tadpoles undergo considerable neurogenesis and synaptogenesis (Horn, 1991; Stofer and Horn, 1993). 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 (Burggren and West, 1982; Taylor et al., 2003a). Nicotine exposure during either of these developmental time points may underlie impairments in the HCnVR.

In the present study we differentiate how developmental timing and duration of chronic nicotine exposure affect tadpole HCnVR. Our experiments specifically targeted early and late time points of tadpole metamorphosis and nicotine exposure durations of 3 and 10 weeks. We hypothesized that early metamorphic tadpoles would be more susceptible to nicotine exposure than late metamorphic tadpoles; we anticipated impairments of the HCnVR in early but not late metamorphic tadpoles after both 3 and 10 weeks of nicotine exposure. This hypothesis was based on the observation that tadpoles have a robust, fully developed HCnVR by late metamorphosis (Taylor et al., 2003a; 2003b), suggesting that the neural mechanisms underlying the hypercapnic response exist by late metamorphosis and may be less vulnerable than early metamorphic tadpoles to the neuroteratogenic effects of nicotine.

METHODS

Animals

Studies were performed on *Lithobates* (formerly *Rana*) *catesbeiana* tadpoles (n = 80) purchased from a commercial supplier (Sullivan Co. Inc., www.researchamphibians.com).

Tadpoles were maintained at 23 °C and fed goldfish food daily. Tadpoles were maintained in aquaria for 10 wk 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 (Taylor et al., 2008) and is within the concentration range of nicotine found in the body fluids of an average smoker (Moyer et al., 2002). 3-wk nicotine-exposed tadpoles were maintained in dechlorinated water for 7 wk before being transferred to nicotine-containing water for 3 wk. Therefore all animals were maintained in the laboratory for 10 wk regardless of the duration of nicotine exposure. We chose 3- and 10-wk exposure durations because the Taylor et al. (2008) reported impairment of hypercapnic response after 8 to 12 wk of nicotine exposure. We wanted reproduce those findings and determine if that impaired HCnVR was stage dependant.10-wk exposure, the mid-range of that previous study, was selected. 3-wk nicotine exposure represents one third to one quarter the previous exposure duration and was chosen to determine whether a significantly shorter exposure could lead to a similar impairment.

Tadpoles' developmental stages were determined at the start of treatment and at the time of dissection to insure developmental homogeneity. At the time of dissection each tadpole was either "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 7-12 or 20-25, respectively, in the classification scheme of Taylor and Köllros (1946). Tadpoles included in early metamorphic groups were stages 7-9 at the start of their 10 wk laboratory maintenance, while those included in late metamorphic groups were stages 18-20. This was true for animals that received either 3 or 10 wk of chronic nicotine exposure, as they were all maintained for 10 wk. Animals that did not remain within these stage ranges for 3 or 10 wk were excluded from the data sets.

We established 6 experimental groups of early and late metamorphic non-nicotine exposed treatment-control tadpoles (n = 14 and n = 14 respectively), early and late metamorphic 3-wk nicotine-exposed tadpoles (n = 16 and n = 16 respectively), and early and late metamorphic 10-wk nicotine-exposed tadpoles (n = 10 and n = 10 respectively). A subset of each experimental group was used as time-controls to determine the robustness of neuroventilation. These preparations remained normocapnic throughout the experiment (early and late metamorphic treatment controls, n = 6 of each, early and late metamorphic 3-wk nicotine-exposed, n = 6 of each, and early and late metamorphic 10-wk nicotine-exposed, n = 4 of each). All care and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Alaska Fairbanks, and complied with all state and federal ethical guidelines.

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 resected and

the fourth ventricle opened by removing the choroid plexus. The remaining brainstem and spinal cord were removed *en bloc* and further trimmed 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 that the HCO_3^- concentration in plasma from early metamorphic tadpoles (Just et al., 1973). These methods have been used in previous tadpole studies (Taylor et al., 2003a; 2003b; 2008); Concentration of aCSF was selected to insure comparability between experiments on animals of different metamorphic stages.

The isolated brainstem-spinal cord was transferred *en bloc* to a 2.5-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 each time-control group. 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 putative ventilatory rhythm at baseline, the HCnVR and the return to baseline activity were recorded. The treatment-control group received the same sequence of normocapnia followed by hypercapnia followed by normocapnia. For time-control groups, neurograms were recorded during 90 min of normocapnia (the total duration of the hypercapnic treatments) to quantify changes in neuroventilation would occur over the time course of the experiment.

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), as previously described (Torgerson et al., 1998; Taylor et al., 2003a; 2003b). Putative gill breaths had lower integrated burst amplitude on 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. (Taylor et al., 2003a; 2003b) 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, we quantified only the frequency of putative lung bursts, which was done by comparing the number of lung bursts per minute in 3 consecutive minutes (at the end of the 30-minute neurogram) of each CO_2/pH condition (normocapnia, hypercapnia and return to normocapnia).

Frequencies of lung neuroventilation were compared using repeated-measures analysis of variance (RM-ANOVA; 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 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 always mean ± SE.

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 normocapnia in early metamorphic tadpoles are illustrated in Fig. 2. There was no significant difference between the frequency of putative lung breaths exhibited during normocapnia by the isolated brainstems of all control (0.6 ± 0.2) and 3- or 10-week early metamorphic nicotine-exposed tadpoles (1.0 ± 0.2 P = 0.24 and 2.0 ± 0.4 P = 0.30, respectively). Normocapnia was maintained in time-controls for 90 min. Early metamorphic control, 3- and 10-week chronic nicotine-exposed tadpoles exhibited no significant change in lung burst frequency over the 90-min protocol (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 either early metamorphic tadpole brainstems.

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. 3. Early metamorphic tadpoles failed to increase lung burst frequency in response to hypercapnia following 3-wk chronic nicotine exposure (from 1.2 ± 0.5 to 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. Consistent with the findings of Taylor et al. (2008) 10-wk of chronic nicotine exposure also inhibited a hypercapnia-induced increase in lung burst frequency in early metamorphic tadpoles (from 0.7 ± 0.3 to 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.

The hypercapnia-induced increase in lung burst frequency exhibited by early metamorphic treatment-control tadpoles was compared to that exhibited by the 3- and 10-wk nicotine-exposed animals (Fig. 4). Control tadpoles increased lung burst frequency by 317 ± 210 % from normocapnia in response to superfusion with 5 % CO_2. Following 3-wk nicotine exposure, tadpole brainstems exhibited a hypercapnia-induced change in lung burst frequency that was significantly lower than treatment-controls (−29 ± 64 %; P = 0.04) and was not significantly different from normocapnia (P = 0.30).

In the 10-wk nicotine-exposed early metamorphic tadpoles, relative change in lung burst frequency compared with their normocapnic lung burst frequency was also significantly lower than treatment-control animals (−38 ± 27 %; P = 0.03) and was not a significant change from normocapnic ventilation (P = 0.07). 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. 5A).

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

Figure 5 depicts representative neurograms for both early and late metamorphic tadpoles. The effects of developmental exposure to 30 μg/L nicotine on the frequency of normocapnic and hypercapnic lung bursts are illustrated in Fig. 5B. Late metamorphic tadpoles have been shown to exhibit significantly more lung breaths per minute *in vivo* (Burggren and West, 1982; 1986) and *in vitro* (Torgerson et al., 1997) than early metamorphic tadpoles, our results are consistent with those observations (6.8 ± 1.9 compared to 0.6 ± 0.2; P = 0.003, data not shown).

Lung burst frequency over 90 minutes of normocapnia was assessed in time-controls following 3- and 10-wk nicotine exposure (Fig. 6.) 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 protocol (P = 0.53, P = 0.74 and P = 0.58). Thus, neither 3- nor 10-wk of 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 protocol 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. 7. 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 (from 6.2 ± 1.7 to 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 (from 11.3 ± 4.2 to 16.3 ± 4.2; P = 0.19). However, it is important to point out that this may be reflective in part by the somewhat elevated normocapnic ventilation in this 10-wk nicotine exposed treatment group as the average frequency evoked by CO_2 was similar for all late metamorphic treatment groups (15.1 ± 4.4, 12.86 ± 3.0, 16.3 ± 4.2 for control 3- and 10-wk nicotine exposed tadpoles respectively). Consequently, late metamorphic tadpoles failed to respond to hypercapnia following 10- but not 3-wk nicotine exposure.

The hypercapnia-induced increase in lung burst frequency exhibited by late metamorphic treatment-control tadpoles was compared to that of the 3- and 10-wk nicotine-exposed tadpoles (Fig. 8). Late metamorphic treatment-control tadpoles exhibited a considerable hypercapnia-induced increase in putative lung ventilation (162 ± 29 %). Following 3-wk nicotine exposure late metamorphic tadpoles demonstrated a more modest, but statistically significant hypercapnia-induced increase in lung burst frequency when exposed to hypercapnia (87 ± 38 %; P = 0.05), this increase was not significantly different than treatment-controls (P = 0.15).

The 10-wk nicotine-exposed late metamorphic tadpoles demonstrated a markedly smaller, hypercapnia-induced change in lung burst frequency that was not significantly different than their modestly elevated normocapnic ventilation (62 ± 27 %; P = 0.07) Also, while the peak frequency evoked by hypercapnia was similar in each age group, the average hypercapnia-induced change demonstrated by 10-wk nicotine-exposed late metamorphic tadpoles was significantly lower than that of controls (P = 0.03). Thus 10-wk, but not 3-wk, nicotine-exposed late metamorphic tadpoles failed to respond significantly to hypercapnia and exhibited a hypercapnia-induced change in neuroventilation that was significantly lower in magnitude than that of treatment-control animals.

DISCUSSION

Tadpole lung burst frequency increases in response to hypercapnia (Taylor et al., 2003a; 2003b). 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 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 developmental timing of 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 our protocol. There was no significant change in putative lung ventilation over the 90-min experimental protocol for any treatment group. Thus, impairments in hypercapnic neuroventilation are not the result of nicotine impacting the *in vitro* preparation's 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 affect 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 (Monteau et al., 1990). Prenatal nicotine exposure has adverse impact on development of the cholinergic system (Navarro et al., 1989). It remains to be determined where in the brainstem and at what step in the CO_2 chemoreflex pathway nicotine is affecting the HCnVR, This determination could not be made with the current data; thus, further study of the effects of nicotine on the generation of respiratory rhythm, and pattern in warranted. Nicotine exposure may affect non-cholinergic pathways implicated in respiratory modulation, including but not limited to, the serotonergic, purinergic and gabaergic systems (Xu et al., 2001; Mihailescu et al., 2002; Huang et al., 2007; Luo et al., 2007; Slotkin et al., 2007). 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.

Central chemosensitive neurons are thought to provide tonic drive for respiratory rhythm generation in part through cholinergic mechanisms (Loeschcke, 1982; Eugenin and Nicholls, 1997; Eugenin et al., 2001; Okada et al., 2001). Chemosensitive neurons are excited by acetylcholine (Ach) and depressed by cholinergic inhibition (Fukuda and Loeschcke, 1979; Kennedy et al., 2001). Application of nicotine to brainstem chemosensitive sites induces hyperventilation, and intravenous injection of hexamethonium, a nicotinic Ach receptor antagonist, diminishes nicotine-induced hyperventilation in anesthetized rats (Dev and Loeschcke, 1979). Thus, a clear relationship between endogenous cholinergic mechanisms and central chemoreception exists.

It remains to be demonstrated whether the attenuation of the HCnVR is 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. (Kennedy et al., 2001) found that adult cane toad brainstems increased their lung burst frequency following acute nicotine exposure. We saw no such increase; suggesting that the effect of chronic exposure to nicotine is fundamentally different from that of acute exposure. This is further supported by the fact that late metamorphic tadpoles respond normally to hypercapnia following 3-wk but- not 10-wk nicotine exposure, therefore at least in the case of late metamorphic tadpoles more than 3-wk nicotine exposure is needed to impair HCnVRs. Future studies that investigate the HCnVR of early tadpoles

during even shorter nicotine exposure and after a nicotine-free recovery interval may offer additional insight.

The impaired HCnVR in early metamorphic tadpoles occurs at a point in development when tadpoles are undergoing significant synaptogenesis (Horn, 1991; Moody et al., 1996). Concomitantly, that time is one of increased dependence on lung ventilation and increased chemosenistivity (Torgerson et al., 1997; Taylor et al., 2003a; 2003b). 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.

The ventilatory response to chronic nicotine in mammals is highly variable. This has been attributed to inconsistencies in nicotine exposure duration and animal age (Hafstrom et al., 2005). Here we offer evidence to support the conclusion that both developmental timing and duration are factors that affect nicotine's impact on hypercapnic neuroventilation in anurans and perhaps all vertebrates.

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 neuroventilatory 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 neuroventilation during hypercapnia and illustrates the important role of developmental timing and duration of exposure in determining the neuropathology of nicotine exposure.

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REFERENCES

Burggren W, Doyle M. Ontogeny of regulation of gill and lung ventilation in the bullfrog, Rana catesbeiana. Respir Physiol. 1986; 66:279–291. [PubMed: 3492018]

Burggren WW, West NH. Changing respiratory importance of gills, lungs and skin during metamorphosis in the bullfrog Rana catesbeiana. Respir Physiol. 1982; 47:151–164. [PubMed: 6803316]

Dev NB, Loeschcke HH. A cholinergic mechanism involved in the respiratory chemosensitivity of the medulla oblongata in the cat. Pflugers Arch. 1979; 379:29–36. [PubMed: 571102]

Eugenin J, Llona I, Infante CD, Ampuero E. In vitro approach to the chemical drive of breathing. Biol Res. 2001; 34:117–122. [PubMed: 11715203]

Eugenin J, Nicholls JG. Chemosensory and cholinergic stimulation of fictive respiration in isolated CNS of neonatal opossum. J Physiol. 1997; 501(Pt 2):425–437. [PubMed: 9192313]

Fukuda Y, Loeschcke HH. A cholinergic mechanism involved in the neuronal excitation by H^+ in the respiratory chemosensitive structures of the ventral medulla oblongata of rats in vitro. Pflugers Arch. 1979; 379:125–135. [PubMed: 34826]

Gheshmy A, Vukelich R, Noronha A, Reid SG. Chronic hypercapnia modulates respiratory-related central pH/CO_2 chemoreception in an amphibian, Bufo marinus. J Exp Biol. 2006; 209:1135–1146. [PubMed: 16513940]

Hafstrom O, Milerad J, Sandberg KL, Sundell HW. Cardiorespiratory effects of nicotine exposure during development. Respir Physiol Neurobiol. 2005; 149:325–341. [PubMed: 15970470]

Horn JP. Development of fast synaptic transmission in bullfrog sympathetic ganglia. J Auton Nerv Syst. 1991; 32:107–119. [PubMed: 1851505]

Huang ZG, Griffioen KJ, Wang X, Dergacheva O, Kamendi H, Gorini C, Mendelowitz D. Nicotinic receptor activation occludes purinergic control of central cardiorespiratory network responses to hypoxia/hypercapnia. J Neurophysiol. 2007; 98:2429–2438. [PubMed: 17699693]

Just JJ, Gatz RN, Crawford EC Jr. Changes in respiratory functions during metamorphosis of the bullfrog, Rana catesbeiana. Respir Physiol. 1973; 17:276–282. [PubMed: 4540837]

Kennedy LL, Aguwa CC, Rives JE, 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. 2001; 128:837–849. [PubMed: 11282326]

Loeschcke HH. Central chemosensitivity and the reaction theory. J Physiol. 1982; 332:1–24. [PubMed: 6818338]

Luo Z, McMullen NT, Costy-Bennett S, Fregosi RF. Prenatal nicotine exposure alters glycinergic and GABAergic control of respiratory frequency in the neonatal rat brainstem-spinal cord preparation. Respir Physiol Neurobiol. 2007; 157:226–234. [PubMed: 17321805]

Mihailescu S, Guzman-Marin R, Dominguez Mdel C, Drucker-Colin R. Mechanisms of nicotine actions on dorsal raphe serotoninergic neurons. Eur J Pharmacol. 2002; 452:77–82. [PubMed: 12323387]

Milsom WK. The role of CO_2/pH chemoreceptors in ventilatory control. Braz J Med Biol Res. 1995; 28:1147–1160. [PubMed: 8728842]

Milsom WK. Phylogeny of CO_2/H^+ chemoreception in vertebrates. Respir Physiol Neurobiol. 2002; 131:29–41. [PubMed: 12106993]

Monteau R, Morin D, Hilaire G. Acetylcholine and central chemosensitivity: in vitro study in the newborn rat. Respir Physiol. 1990; 81:241–253. [PubMed: 2263784]

Moody SA, Miller V, Spanos A, 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. 1996; 364:219–230. [PubMed: 8788246]

Moyer TP, Charlson JR, Enger RJ, Dale LC, Ebbert JO, Schroeder DR, 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. 2002; 48:1460–1471. [PubMed: 12194923]

Nattie E. CO_2, brainstem chemoreceptors and breathing. Prog Neurobiol. 1999; 59:299–331. [PubMed: 10501632]

Navarro HA, Seidler FJ, Eylers JP, Baker FE, Dobbins SS, Lappi SE, 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. 1989; 251:894–900. [PubMed: 2600820]

O'Regan RG, Majcherczyk S. Role of peripheral chemoreceptors and central chemosensitivity in the regulation of respiration and circulation. J Exp Biol. 1982; 100:23–40. [PubMed: 6816893]

Okada M, Osumi Y, Okuma Y, Ueno H. Nitric oxide inhibits the release of acetylcholine in the isolated retina. Graefes Arch Clin Exp Ophthalmol. 2001; 239:217–221. [PubMed: 11405071]

Putnam RW, Conrad SC, Gdovin MJ, Erlichman JS, Leiter JC. Neonatal maturation of the hypercapnic ventilatory response and central neural CO_2 chemosensitivity. Respir Physiol Neurobiol. 2005; 149:165–179. [PubMed: 15876557]

Putnam RW, Filosa JA, Ritucci NA. Cellular mechanisms involved in CO_2 and acid signaling in chemosensitive neurons. Am J Physiol Cell Physiol. 2004; 287:C1493–1526. [PubMed: 15525685]

Simakajornboon N, Vlasic V, Li H, 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. 2004; 96:2213–2219. [PubMed: 14752122]

Slotkin TA. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. Toxicol Appl Pharmacol. 2004; 198:132–151. [PubMed: 15236950]

Slotkin TA, Ryde IT, Tate CA, 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. 2007; 73:259–272. [PubMed: 17562392]

Smatresk NJ, Smits AW. Effects of central and peripheral chemoreceptor stimulation on ventilation in the marine toad, Bufo marinus. Respir Physiol. 1991; 83:223–238. [PubMed: 1906195]

St-John WM, Leiter JC. Maternal nicotine depresses eupneic ventilation of neonatal rats. Neurosci Lett. 1999; 267:206–208. [PubMed: 10381012]

Stofer WD, Horn JP. Neurogenesis and differentiation of sympathetic B and C cells in the bullfrog tadpole. J Neurosci. 1993; 13:801–807. [PubMed: 8426237]

Taylor AC, Kollros JJ. Stages in the normal development of Rana pipiens larvae. Anat Rec. 1946; 94:7–24. [PubMed: 21013391]

Taylor BE, Croll AE, Drucker ML, Wilson AL. Developmental exposure to ethanol or nicotine inhibits the hypercapnic ventilatory response in tadpoles. Respir Physiol Neurobiol. 2008; 160:83–90. [PubMed: 17974508]

Taylor BE, Harris MB, Coates EL, Gdovin MJ, Leiter JC. Central CO_2 chemoreception in developing bullfrogs: anomalous response to acetazolamide. J Appl Physiol. 2003; 94:1204–1212. [PubMed: 12571143]

Taylor BE, Harris MB, Leiter JC, Gdovin MJ. Ontogeny of central CO_2 chemoreception: chemosensitivity in the ventral medulla of developing bullfrogs. Am J Physiol Regul Integr Comp Physiol. 2003; 285:R1461–1472. [PubMed: 14615406]

Torgerson C, Gdovin M, Remmers J. Ontogeny of central chemoreception during fictive gill and lung ventilation in an in vitro brainstem preparation of Rana catesbeiana. J Exp Biol. 1997; 200:2063–2072. [PubMed: 9319973]

Torgerson CS, Gdovin MJ, Remmers JE. Fictive gill and lung ventilation in the pre- and postmetamorphic tadpole brain stem. J Neurophysiol. 1998; 80:2015–2022. [PubMed: 9772257]

Xu Z, Seidler FJ, Ali SF, Slikker W Jr. Slotkin TA. Fetal and adolescent nicotine administration: effects on CNS serotonergic systems. Brain Res. 2001; 914:166–178. [PubMed: 11578609]

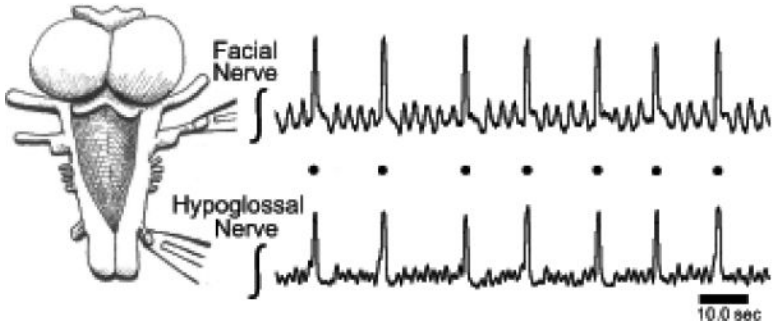

Fig. 1.

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 breaths (•) designated by presence of high amplitude nerve output occurring concomitantly on both facial and hypoglossal nerves.

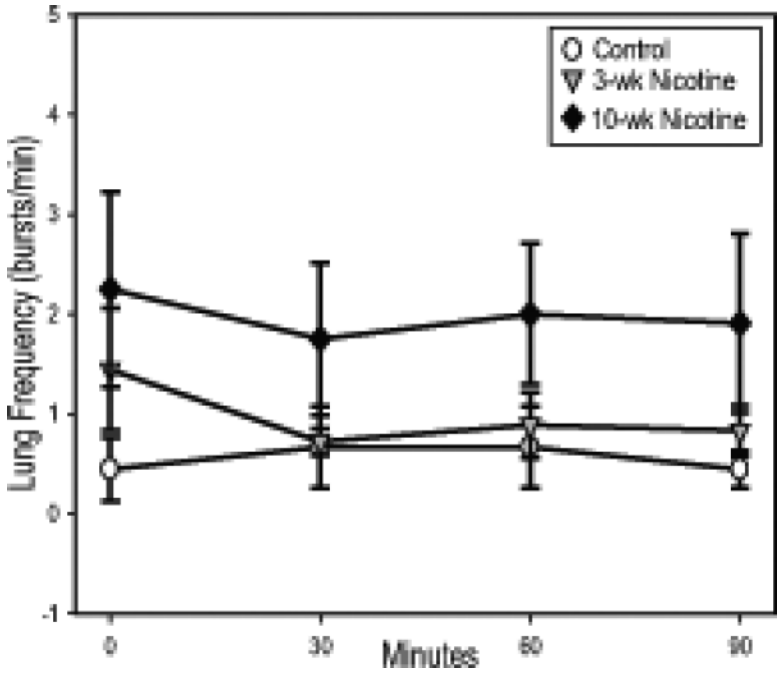

Fig. 2.

Effect of time 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. 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 $P > 0.05$.

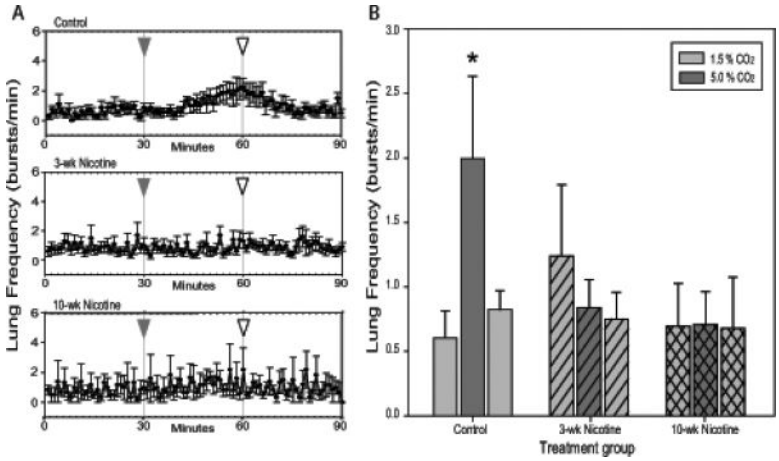

Fig. 3.

Impact of chronic nicotine on the hypercapnic neuroventilatory response of early metamorphic tadpoles. A: Mean 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: Mean lung burst frequency over the last 3 min of normocapnia, hypercapnia and subsequent normocapnia in control and chronic nicotine treatment groups. Treatment-controls demonstrated 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 respond significantly to changes in P_{CO_2} $P > 0.05$.

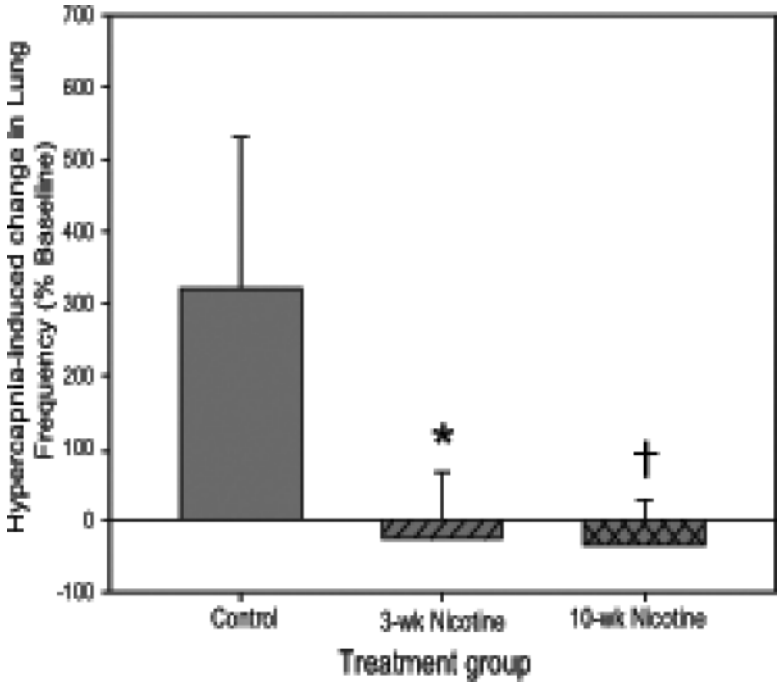

Fig. 4.

Effect of nicotine on the hypercapnia-induced changes in neuroventilation in early metamorphic tadpole brainstems. The hypercapnia-induced change in lung burst frequency, measured as percent increase from lung burst frequency during normocapnia, 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 hypercapnia-induced change in ventilation compared to 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.

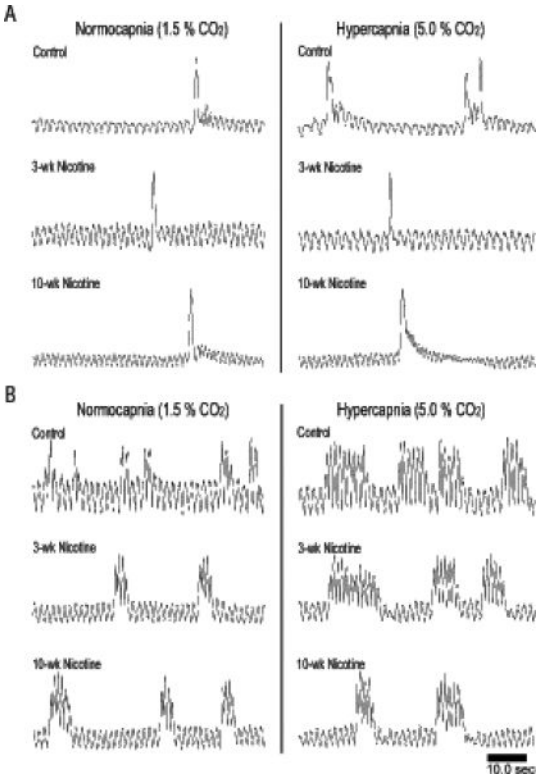

Fig. 5.

Representative neurograms of 1-min integrated output recorded from the facial nerve during normocapnia and hypercapnia for control tadpoles and following either 3- or 10-wk nicotine exposure. A: Early metamorphic control tadpoles increased lung burst frequency during hypercapnia; early metamorphic tadpoles did not increase lung burst frequency following either 3- or 10-wk nicotine exposure. B: Late metamorphic control and 3-wk nicotine-exposed tadpoles increased lung burst frequency in response to hypercapnia. Hypercapnic neuroventilation was significantly attenuated in 10-wk nicotine-exposed tadpoles.

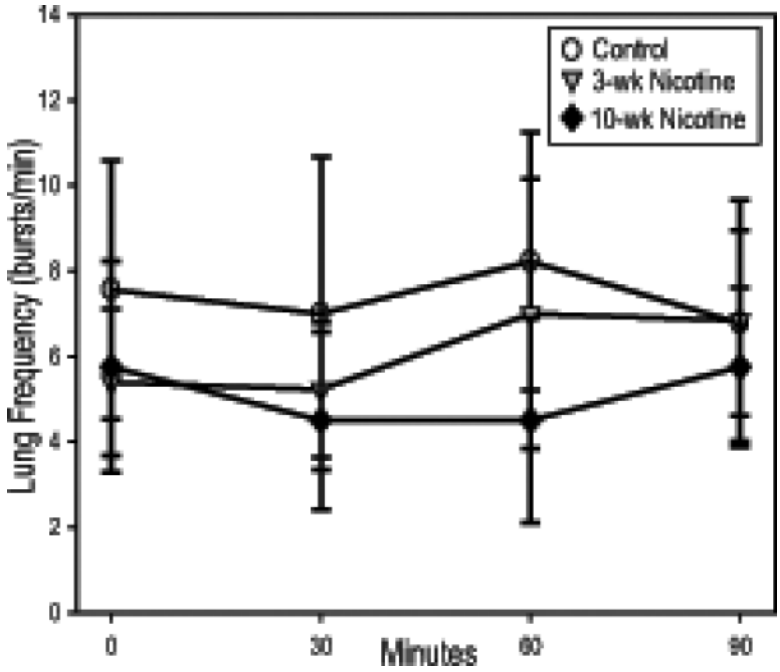

Fig. 6.

Effect of time 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. 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 $P > 0.05$.

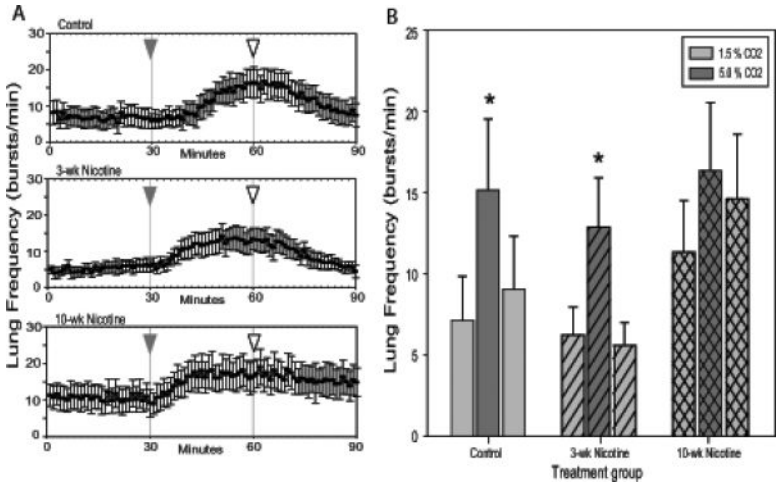

Fig. 7.

Impact of chronic nicotine on the hypercapnic neuroventilatory response of late metamorphic tadpoles. A: Mean 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: Mean 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} $P > 0.05$.

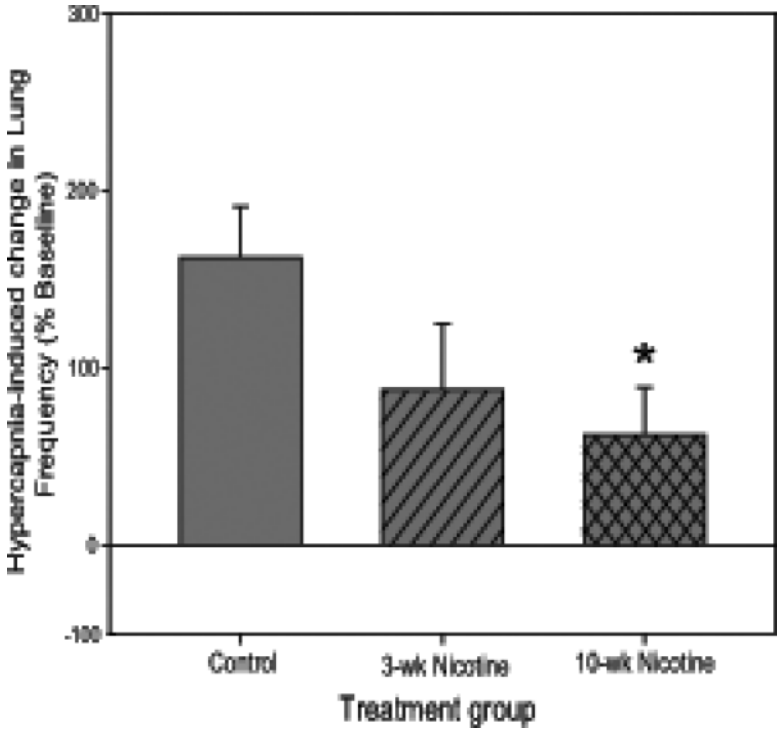

Fig. 8.

Effect of nicotine exposure on hypercapnia-induced changes in neuroventilation in late metamorphic tadpole brainstems. The hypercapnia-induced change in lung burst frequency, measured as percent increase from lung burst frequency during normocapnia, 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 hypercapnia-induced changes in lung burst frequency. 10-wk nicotine-exposed tadpoles exhibited a significantly lower hypercapnia-induced change in lung burst frequency (* $P < 0.05$) compared to treatment-controls.