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Developmental nicotine exposure alters cholinergic control of respiratory frequency in neonatal rats

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Abstract

Prenatal nicotine exposure with continued exposure through breast milk over the first week of life (developmental nicotine exposure, DNE) alters the development of brainstem circuits that control breathing. Here, we test the hypothesis that DNE alters the respiratory motor response to endogenous and exogenous acetylcholine (ACh) in neonatal rats. We used the brainstem-spinal cord preparation in the split-bath configuration, and applied drugs to the brainstem compartment while measuring the frequency and amplitude of the fourth cervical ventral nerve roots (C4VR), which contain the axons of phrenic motoneurons. We applied ACh alone; the nicotinic acetylcholine receptor (nAChR) antagonist curare, either alone or in the presence of ACh; and the muscarinic acetylcholine receptor (mAChR) antagonist atropine, either alone or in the presence of ACh. The main findings include: 1) atropine reduced frequency similarly in controls and DNE animals, while curare caused modest slowing in controls but no consistent change in DNE animals; 2) DNE greatly attenuated the increase in C4VR frequency mediated by exogenous ACh; 4) stimulation of nAChRs with ACh in the presence of atropine increased frequency markedly in controls, but not DNE animals; 5) stimulation of mAChRs with ACh in the presence of curare caused a modest increase in frequency, with no treatment group differences. DNE blunts the response of the respiratory central pattern generator to exogenous ACh, consistent with reduced availability of functionally competent nAChRs; DNE did not alter the muscarinic control of respiratory motor output.

Keywords

acetylcholine; brainstem-spinal cord preparation; cholinergic; control of breathing; muscarinic receptor; nicotinic receptor

INTRODUCTION

A thorough understanding of the influence of nicotine exposure on cholinergic neurotransmission in the brain is important given the widespread practice of prescribing nicotine patches to pregnant smokers (Berlin et al., 2014), and the burgeoning use of e-

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cigarettes (Kralikova et al., 2013). Although in both cases the intent is to curb the craving for nicotine while avoiding the other toxins in tobacco smoke, some evidence suggests that use of the nicotine patch in pregnancy does not change smoking cessation rates (Berlin et al., 2014), implying that this practice may in fact increase nicotine intake. This is troubling inasmuch as nicotine exposure *in utero* alters cardiorespiratory control in infants and neonatal animals (Hafstrom et al., 2005). Recent studies in animal models show that exposure to nicotine *in utero*, with continued exposure through breast milk over the first week of life (developmental nicotine exposure, DNE), desensitizes nicotinic acetylcholine receptors (nAChRs) on hypoglossal motoneurons (Pilarski et al., 2012) and on the presynaptic terminals of excitatory, inhibitory and modulatory interneurons in the brain (Wonnacott, 1997; Gentry and Lukas, 2002; Fregosi and Pilarski, 2008). Thus, the effects of chronic nicotine exposure and the subsequent desensitization of nAChRs are widespread and complex. Acetylcholine signals through both nAChRs and mAChRs in the mammal brain, and it has long been recognized that both nicotinic and muscarinic receptors are involved in the control of breathing (Kase and Borison, 1958; Gillis et al., 1988; Nattie and Li, 1990; Kubin and Fenik, 2004; Shao and Feldman, 2009). Importantly, it has long been known that activation of nAChRs on the presynaptic terminals of cholinergic neurons increases the release of ACh (Koelle, 1961; Wonnacott et al., 1990). If long-term desensitization of nAChRs by chronic nicotine exposure reduces endogenous ACh release, the density and/or function of both nAChRs and muscarinic ACh receptors (mAChRs) could be altered (Pauly et al., 1991; Xiao et al., 2009).

Recent studies in the mouse brainstem-spinal cord preparation show that augmenting endogenous ACh levels with neostigmine increased the frequency of respiratory motor output similarly in both control and DNE mice (Coddou et al., 2009). However, the response to exogenous ACh was not examined. Obtaining these data is important because most of the major sources of ACh in the basal forebrain and pons are removed in the brainstem-spinal cord preparation. Accordingly, increasing ACh availability by blocking acetylcholinesterase activity may not raise ACh concentration enough to occupy all available cholinergic receptors in this model. Here, we test the hypothesis that DNE alters the respiratory motor response to endogenous and exogenous ACh in young neonatal rats. We used the brainstem-spinal cord preparation in the split-bath configuration, where drugs could be applied to the brainstem compartment without exposing phrenic motoneurons. We applied ACh alone; the nicotinic acetylcholine receptor (nAChR) antagonist curare, either alone or in the presence of ACh; and the muscarinic acetylcholine receptor (mAChR) antagonist atropine, either alone or in the presence of ACh. The measured output variables were the frequency and amplitude of the fourth cervical ventral nerve roots (C4VR), which contain the phrenic motoneurons. The main finding is that DNE markedly reduced the respiratory frequency response to ACh, with no significant changes in nerve burst amplitude. The data suggest that this is due to a failure of ACh to stimulate nAChRs, as DNE did not alter muscarinic responses. Thus, DNE impairs the ability of the respiratory system to respond to an increase in ACh release, as occurs with arousal and changes in central nervous system state.

METHODS

Animals

All data were obtained from experiments approved by the Institutional Animal Care and Use Committee at The University of Arizona. The data reported here were derived from neonatal rats of either sex, born via spontaneous vaginal delivery from Sprague–Dawley dams (Harlan Laboratories). We used 49 control pups and 50 DNE pups (Table 1), ranging in age from birth through the first 6 days of life, i.e., postnatal day zero (P0) through postnatal day 5 (P5). Neonates were housed together with their mothers and siblings until the day of study. Dams had unrestricted access to food and water, and were kept in a quiet room controlled at a temperature of 21–23 °C, 20–30% relative humidity, and a 12/12-h light/dark cycle.

As described previously (Luo et al., 2004; Luo et al., 2007; Huang et al., 2010), subcutaneous osmotic mini-pumps (Alzet, Cupertino, CA) were implanted into the pregnant dams on gestational day 5. The pumps were loaded to administer either nicotine bitartrate at a dose of 6mg/kg/day or physiologic saline (sham control). We have previously shown that the same dosing regimen used here results in plasma cotinine levels ranging from 60–92 ng/ml in P2–P4 neonates (Powell et al., 2015); this is similar to average cotinine levels of 88 ng/ml found in the umbilical cord blood of newborns from mothers who smoked an average of 95 cigarettes per week (Berlin et al., 2010). Since gestation in the dams is 21 days, neonates were exposed to nicotine or saline through the placenta on gestational days 5–21, and via breast milk during the postnatal period (Matta et al., 2007).

In addition to the saline and nicotine exposed group, we included four additional pups from a mother that did not undergo any surgery or pump implantation (Table 1). Since there were no differences in the frequency or amplitude of respiratory motor output in baseline conditions or in response to drugs, the data were combined into a single control group, and will be referred to as such throughout the rest of this manuscript.

Brainstem-spinal cord preparation

Neonatal rats were anesthetized with hypothermia, and the brain stem and spinal cord, extending from the pontomedullary junction to below the fourth cervical nerve roots, was removed *en bloc*. The preparation was placed inside a recording chamber perfused with modified Krebs solution [(in mM) 123 NaCl, 26 NaHCO₃, 30 glucose, 1 MgSO₄, 3 KCl, 1.25 NaH₂PO₄ and 1.2 CaCl], gassed with 95% O₂/5% CO₂, and a pH of 7.45–7.5. The recording chamber was partitioned in such a way that the brainstem was separated from the spinal cord, and perfusion of each compartment was controlled independently, as described previously (Fregosi et al., 2004; Luo et al., 2004; Luo et al., 2007; Fregosi and Pilarski, 2008).

Electrophysiology

The brainstem-spinal cord preparation produces a spontaneous inspiratory motor outflow in the C4VRs, representing the periodic bursting of phrenic motoneurons, as originally described by Suzue (Suzue, 1984). This motor outflow was recorded with suction electrodes to obtain an index of systems-level respiratory-related activity. The output was amplified

(gain = 100,000) and band pass filtered (10 Hz–3 kHz, Grass Instruments P 511, Quincy, MA). Analog signals were digitized at 5 kHz using Spike II software (Cambridge Electronic Design, Cambridge, UK) and stored for subsequent offline data analysis.

Drugs and Protocols

Drugs were obtained from Sigma (St. Louis, MO) or Tocris Bioscience (Ellisville, MO) and mixed daily from stock solutions. Based on data showing that 10 and 30 μ M ACh resulted in an approximately 46 and 60% increase in C4 ventral root frequency in the brainstem spinal cord preparation of the neonatal rat (Murakoshi et al., 1985), we did pilot studies using 30, 60 and 120 μ M ACh (N=3 preparations at each dose). In all cases, ACh was applied only to the brainstem using the split bath approach as reported here. On average, the frequency of C4VR nerve bursts increased by 55, 63 and 74 % of baseline at 30, 60 and 120 μ M ACh, respectively. Thus, quadrupling the dose resulted in only a 19% increase in frequency, suggesting that these are near saturating doses. Therefore, we settled on the midpoint dose of 60 μ M. Antagonist concentrations were determined in initial studies by determining the dose that reversed the frequency stimulating effects of ACh.

All experiments reported here were preceded by at least 30 min of equilibration in Krebs solution until baseline rhythm was stable. Experiments then commenced with a 10-min baseline period in Krebs solution, followed by 15–40 min of drug application. All drugs were applied to the brainstem compartment only. In all but one series of experiments we recorded the response to drug washout, though the time required for C4VR activity to return to baseline values was prolonged and varied widely. Accordingly the focus of our analysis is the difference between baseline responses, and the responses recorded throughout the duration of drug application. At the end of each experiment, the rostral bath was drained and monitored to determine if aCSF was leaking in from the caudal chamber; if such a leak was identified the data were discarded.

The number of animals and drug doses used in each series of experiments are given in Table 1. In the first series of experiments, 25 controls and 26 DNE preparations were exposed to bath applied ACh (60 μ M) for 15 min, following a 10 min baseline period. The second experimental protocol (in 6 control and 6 DNE pups) included 10 min of baseline recording, bath application of atropine (10 μ M) for 15 min to block mAChRs, and a mixture of atropine (10 μ M) and ACh (60 μ M) for 15 min, to stimulate nAChRs. The third series of experiments, also in 6 control and 6 DNE pups, were the same as the second except here we bath applied curare (10 μ M) for 15 min to block nAChRs, followed by a mixture of curare (10 μ M) and ACh (60 μ M), to stimulate mAChRs. In the last series of experiments we bath applied either atropine alone (N = 6 control, 6 DNE) or curare alone (N = 6 control, 6 DNE) for 40 min as a time control.

Data Analysis

The C4VR ENG activity was rectified and digitally integrated with Spike II software (Cambridge Electronics Design) to obtain a moving time average of the activity within each burst (i.e., the burst amplitude), as described previously (Fregosi et al., 2004; Luo et al., 2004; Luo et al., 2007; Fregosi and Pilarski, 2008; Pilarski and Fregosi, 2009). These

processed recordings were then used to compute the mean C4VR burst frequency and amplitude within each 1-min epoch during baseline, drug application and washout periods. The data were analyzed statistically with mixed-model, two-way repeated measures ANOVA, with treatment (saline exposed control vs. DNE) and time (baseline or drug application) as the main factors, and C4VR burst frequency and amplitude the dependent variables. When appropriate, pairwise post-hoc comparisons were made using unpaired t-tests. For the atropine and curare time control experiments, we also compared the average C4VR frequency and amplitude for each min of drug application, and compared these values to the average baseline data with one-way ANOVA's, followed by paired t-tests. In every case, t-scores were subjected to the Bonferroni correction to remove the bias associated with multiple t-tests. Significance was assumed if $P \leq 0.05$. We did not observe any significant effects of drug application or DNE on the amplitude of C4VR bursting in any of the conditions studied. Accordingly, the remainder of the manuscript addresses changes in the frequency of C4VR nerve bursts.

Results

Body weight, age and baseline values

There were no significant treatment group differences for age or body mass, but the baseline frequency of C4VR nerve bursts was slightly but significantly higher in DNE pups compared to controls (Fig. 1). Since the DNE animals tended to be slightly older and heavier than the control animals, we tested the hypothesis that the difference in baseline frequency was dependent on age or body mass. Although the slope of the frequency-body mass relation in DNE pups was significantly different than zero, it was not different than the control slope (unpaired t-test on slopes, $t=1.09$, $P=0.28$, $df=68$). Similarly, although there is a trend for baseline frequency to rise with age and mass, the correlations were not significant in either group. Nevertheless, because the baseline frequency varies both within and between treatment groups (Fig. 1C), in the remainder of the manuscript we express frequency in terms of the fold change from the average baseline value. Statistical analysis of both absolute and normalized frequency values gave qualitatively identical results. Thus, reporting normalized data is well justified and makes it easier to appreciate the within group and between group influences of drug application on C4VR frequency.

Response to ACh

Figure 2A shows representative C4VR recordings from a control (upper traces) and a DNE preparation (lower traces). Sixty μM ACh increased C4VR frequency above baseline levels in the control preparation, but not in the DNE animal. The average data in 25 control and 26 DNE animals is shown in Fig. 2B. ACh evoked an initial increase followed by a drop in frequency that remained significantly above baseline in control animals. In DNE animals the initial increase in frequency was quickly followed by an abrupt return to baseline. Two-way ANOVA revealed a significant treatment effect (Table 2, $P=0.0053$), and there were also significant time and interaction effects (Table 2) consistent with the markedly different responses in the two treatment groups.

Response to atropine and atropine plus ACh

We used a second group of animals to examine cholinergic stimulation of nAChRs by applying atropine followed by a mixture of atropine and ACh (Fig. 3). Fig. 3A shows representative recordings under baseline conditions, near the end of 15 min of atropine application, and near the end of 15-min of application of a mixture of atropine and ACh. In control animals (upper traces), atropine alone caused a decrease in C4VR frequency, but frequency increased when ACh was applied together with atropine (Fig. 3A). In DNE animals (Fig. 3A, lower traces), atropine alone decreased burst frequency, while ACh plus atropine failed to increase frequency above baseline levels. Two-way ANOVA (Table 2) revealed a significant time ($P<0.0001$) and treatment-time interaction ($P<0.0001$), indicating that the influence of treatment depended importantly on time, and thus drug application. Post hoc comparisons revealed significant reductions in frequency in response to atropine in both groups (Fig. 3B), whereas ACh combined with atropine increased frequency in control animals while the DNE preparations did not respond, with frequency remaining below baseline levels at the beginning of ACh application. This resulted in significant treatment effects over the first 6 min of atropine + ACh application (Fig. 3B). Although the treatment effects were not statistically significant over the last 9-min of ACh + atropine administration (Fig. 3B), this was the result of a time-dependent decline in the frequency response in the control animals.

Response to curare and curare plus ACh

We used a third group of animals to examine changes in C4VR nerve bursts in response to cholinergic stimulation of mAChRs. Representative recordings are shown in Fig. 4A, and the average data in Fig. 4B. In the control animal (Fig. 4A, upper traces), curare alone reduced C4VR frequency, and curare plus ACh tended to increase frequency back to baseline levels, or slightly higher. In contrast, neither curare alone nor curare + ACh altered C4VR burst frequency in the DNE animal (Fig. 4A, lower traces). Two-way ANOVA (Table 2) revealed a significant time ($P<0.0001$) and treatment-time interaction ($P<0.0001$). Post-hoc analyses showed significant, transient reductions in frequency in response to curare in control animals, but not in DNE preparations (Fig. 4B). ACh in the presence of curare was associated with a very small increase in frequency in control animals, though the changes were not significant with the exception of a single time point just after ACh application (Fig. 4B). In contrast, neither curare nor curare + ACh evoked significant changes in C4VR frequency in DNE preparations. Interestingly, although C4VR frequency slowed during the drug washout period, frequency in control preparations returned to baseline levels while DNE preparations showed a frank decline in C4VR frequency, that was significantly below baseline levels, and also significantly lower than C4VR frequency in control preparations at mins 45, 48 and 50 (Fig. 4B).

Response to prolonged application of atropine or curare

In 24 additional animals (Table 1) we applied atropine alone ($N=6$ control, 6 DNE) or curare alone ($N=6$ control, 6 DNE) for 30 min, as a time control for the experiments summarized in Figs. 3 and 4. As shown in Fig. 5, atropine was associated with modest slowing of the C4VR burst frequency in both control (Fig. 5A) and DNE preparations (Fig.

5B), with no treatment effect or treatment-time interaction (Table 2). The atropine-mediated decline in C4VR burst frequency was transient in both groups, with frequency returning to baseline levels in about 15 min (see Methodological Considerations, in Discussion).

Curare evoked modest slowing of C4VR frequency in control animals (Fig. 5C), with the slowing a bit more pronounced later in the period of drug application than earlier. The influence of curare on C4VR frequency in DNE preparations was erratic, with no significant changes except a small, transient reduction in frequency 13 min after curare was applied to the brainstem.

Discussion

The main finding is that DNE markedly reduced the respiratory motor response to ACh, which was bath-applied to the medulla of neonatal rat brainstem-spinal cord preparations. Additional experiments showed that this is due to the failure of ACh to stimulate nAChRs in the DNE preparations, consistent with the hypothesis that DNE is associated with long-term, functional desensitization of brainstem nAChRs (Pilarski and Fregosi, 2009; Pilarski et al., 2012). In contrast, muscarinic control of respiratory motor output was unaltered. The evidence for, and significance of, these conclusions is summarized and discussed below.

DNE and cholinergic activation of nAChRs

Our conclusion that the marked reduction in the C4VR frequency response to ACh (Fig. 2) is due to a functional desensitization of nAChRs is based on the results of two experiments. In the first, atropine was bath applied to the medulla for 15 min to block mAChRs, and this was followed by application of a cocktail containing atropine and ACh (Fig. 3). The idea was to selectively stimulate nAChRs, while also mimicking the surge in ACh from cholinergic neurons, such as occurs with arousal (Phillis, 2005), upon the transition from sleep to wakefulness (Jones, 2005; Gall et al., 2007), in states of vigilance (Lai and Siegel, 1988; Gall et al., 2007; Roman-Liu et al., 2013) and during exercise (Nakajima et al., 2003). In control animals, addition of ACh in the presence of atropine caused a rapid increase in frequency that peaked in 6-7 min and then began a slow decline towards baseline levels, with a further reduction during drug washout (Fig. 3). In contrast, ACh in the presence of atropine did not change frequency in the DNE preparations. In the second series of experiments, we applied the nAChR antagonist curare for 30 min to examine the temporal changes in C4VR activity in response to nAChR blockade (Fig. 5). In control preparations, curare initially evoked erratic changes in C4VR frequency. However, this was followed by a monotonic, time-dependent decline in frequency and a return to baseline levels with washout (Fig. 5C). In contrast to control preparations, C4VR frequency was not altered significantly in DNE preparations (Fig. 5D).

Taken together, these data are consistent with recent work from our laboratory showing that DNE blunts the increase in C4VR frequency that is normally evoked by exogenous nicotine (Pilarski and Fregosi, 2009) consistent with a functional down regulation of nAChRs that is commonly observed in neurons chronically exposed to nicotine (Gentry and Lukas, 2002). The brainstem region that was exposed to drugs in our split-bath preparation includes the pre-Bötzinger complex (Smith et al., 1991) and other regions implicated in the control of

respiratory frequency, such as the para-facial respiratory group (Onimaru and Homma, 2003; Champagnat et al., 2011). This, however, does not necessarily imply desensitization of nAChRs expressed on para-facial or preBötzing complex interneurons. It is well known that activation of nicotinic AChRs located on the presynaptic terminals of excitatory, inhibitory and modulatory interneurons can increase neurotransmitter release (Wonnacott, 1997). It is therefore possible that the release of excitatory neurotransmitters was reduced in the DNE animals, secondary to desensitization of presynaptic nAChRs. Previous work has shown that DNE desensitizes nAChRs on hypoglossal motoneurons (Pilarski et al., 2012), reduces the frequency of excitatory postsynaptic currents in hypoglossal motoneurons (Pilarski et al., 2011), enhances the sensitivity of frequency generating mechanisms to both excitatory and inhibitory neurotransmitters (Luo et al., 2004; Luo et al., 2007; Pilarski and Fregosi, 2009; Jaiswal et al., 2013) and reduces the ventilatory response to low pH and high CO₂ (Eugenin et al., 2008; Huang et al., 2010). The most parsimonious explanation for the present findings is that the failure of DNE preparations to increase C4VR frequency upon stimulation of nAChRs is due to a functional desensitization of nAChRs that mediate excitation of para-facial and preBotzinger complex interneurons by both postsynaptic and presynaptic mechanisms (Shao and Feldman, 2001; Shao and Feldman, 2009).

DNE and cholinergic activation of mAChRs

Although studies consistently show that exogenous nicotine increases the frequency of respiratory motor output via actions on the nAChR (reviewed in) (Fregosi and Pilarski, 2008), stimulation of mAChRs evokes more variable effects. Some investigators find that stimulation of mAChRs decreases frequency (Zanella et al., 2007; Muere et al., 2013) and/or reduces the excitability of neurons in the nucleus of the solitary tract (Endoh, 2007) and hypoglossal motor nucleus (Bellingham and Berger, 1996; Liu et al., 2005), while others report an increase in frequency (Bradley and Lucy, 1983; Monteau et al., 1990; Shao and Feldman, 2000), and an excitation of hypoglossal motoneurons (Ireland et al., 2012). The reason for the discrepant findings is difficult to discern, as there are many factors to consider, such as multiple mAChR subtypes, the use of reduced vs. intact preparations, the dosage and precise action of the drugs utilized, species differences and the fact that muscarinic receptors, like nAChRs, are expressed not only postsynaptically, but also on the presynaptic terminals of excitatory and inhibitory interneurons (Buno et al., 2006; Xiao et al., 2009; Gorini et al., 2010; Ye et al., 2010), wherein receptor activation modulates neurotransmitter release.

The influence of DNE on the muscarinic control of respiratory motor output was also evaluated with two series of experiments. In the first we applied curare for 15 min, followed by 15 min of a mixture of curare and ACh to selectively stimulate mAChRs (Fig. 4). Neither control nor DNE animals responded significantly to ACh in the presence of curare (Fig. 4B). In the second set of studies, the temporal effects of mAChR blockade were evaluated by bath applying atropine for 30 min while C4VR activity was recorded (Fig. 5 A & B). Both groups of animals responded to atropine with a decrease in frequency that was sustained for about 15 min. Similar observations were made in the experiments where atropine was applied before the atropine-ACh cocktail (Fig. 3). These observations are consistent with findings in the neonatal mouse brainstem-spinal cord preparation (Coddou et al., 2009), and the

neonatal rat rhythmic brainstem slice preparation (Shao and Feldman, 2000), with the effects in the slice preparation mediated by M3 mAChRs (Shao and Feldman, 2000). Importantly, the C4VR response to blocking or stimulating medullary mAChRs was the same in control and DNE preparations, confirming recent findings in nicotine-exposed mice (Coddou et al., 2009) and suggesting that DNE does not alter development of muscarinic synaptic transmission. However, others have shown that prenatal nicotine exposure decreases M2, M3, and M4 mAChR mRNA in the hindbrain of fetal rats (Mao et al., 2008), and changes the expression of M3 mAChRs in the medulla oblongata of fetal humans (Falk et al., 2005). These data demonstrate marked and highly complex alterations in cholinergic signaling by exposure to nicotine *in utero*. In the light of these findings, and in acknowledgment of the diversity of mAChRs and their responses to ACh and DNE, this topic warrants detailed investigation in a reduced preparation.

Methodological considerations

Though several methodological concerns have already been addressed, there are four issues that were not. First, it is not clear where in the medulla the drugs were acting, though the only changes that we observed with drug application and/or DNE were on the frequency of respiratory-related motor output, implicating effects on central pattern generating mechanisms. In the brainstem-spinal cord preparation, the preBötzinger complex (Smith et al., 1991; Feldman and Del Negro, 2006) and the parafacial respiratory group (Onimaru and Homma, 2003; Champagnat et al., 2011) are the most likely candidates. Second, though our drug doses were well within the range used by others in this model (Murakoshi et al., 1985; Monteau et al., 1990; Shao and Feldman, 2005; Zanella et al., 2007), they are higher than the extracellular values observed in the brainstem of rats (15-30 μ M) (Nordberg and Wahlstrom, 1977; Kakihana et al., 1984). However, since there is no blood flow in this preparation drugs must diffuse through the tissue block, requiring higher doses to exert pharmacological effects, especially on neurons that are located far from the surface. Our time control studies with atropine and curare, and the studies using prolonged exposure to ACh, were all characterized by rapid changes in C4VR frequency suggesting that drug penetration was adequate. Third, the atropine time control experiments (Fig. 5) showed that C4VR frequency returned to baseline within 15 min despite continued superfusion of the drug. Thus, in the studies where atropine was given prior to ACh (Fig. 3) it is possible that the effects of atropine had worn off before the ACh was delivered. This is unlikely, given that atropine has long been notorious for its prolonged effectiveness (Clark, 1926; Dascal and Landau, 1982) and its resistance to washout (Clark, 1926). It is more likely that compensatory mechanisms overcame the atropine-induced inhibition of muscarinic signaling, or that atropine may have partially antagonized both $\alpha 7$ and $\alpha 4\beta 2$ nAChRs (Zwart and Vijverberg, 1997), which are the dominant subtypes in the neonatal rodent brainstem. Fourth, atropine alone reduced C4VR frequency in both treatment groups (Figs. 3 & 5), consistent with muscarinic excitation of rhythm generating mechanisms. However, stimulation of mAChRs with a mixture of curare and ACh did not increase frequency in either treatment group. Though we cannot explain this phenomenon we entertain two possibilities: 1) all functionally viable mAChRs are active at low, baseline levels of ACh, and increasing ACh levels fails to augment mAChR-mediated modulation of frequency; 2)

inhibitory mAChR subtypes, which exist in the neonatal rodent brainstem (Gorini et al., 2010), are activated by high doses of ACh, but desensitize rapidly (Mubagwa et al., 1994).

Physiological significance

Nicotine is a highly addictive drug that is widely abused throughout the world, with 10-43% of women worldwide continuing to smoke during pregnancy (Nelson and Taylor, 2001; Al-Sahab et al., 2010). Despite the deleterious effects of DNE on brain development and cardiorespiratory control in animal models discussed above, obstetricians routinely prescribe nicotine patches in pregnant, nicotine-addicted mothers (Coleman et al., 2012), with the intention of avoiding the other chemicals in tobacco smoke. However, whether or not nicotine patches increase smoking cessation in pregnancy or improve birth outcomes is unclear (Coleman et al., 2011; Coleman et al., 2012; Coleman et al., 2013). Moreover, there are no data on the effects of nicotine patches on cardiorespiratory control in human neonates. By showing that DNE alters ACh-mediated regulation of the central pattern generator for breathing, the present results add additional rationale for conducting studies in nicotine-exposed human infants. Specifically, DNE abolished the frequency response to ACh, which is mediated largely by nAChR stimulation. This alteration may help to explain the greater incidence of sleep disordered breathing in neonates born to smoking mothers (Kahn et al., 1994; Calhoun et al., 2010).

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Figure 1. Postnatal age (Panel A), body mass (B) and baseline C4 ventral root nerve burst (C4VR) frequency (C) in neonatal rats from control and DNE litters. Although the DNE animals were on average slightly older and heavier, neither difference was significant. In contrast, baseline C4VR burst frequency was higher in the DNE animals. *, Difference between control and DNE animals ($P = 0.0044$).

Figure 2.

Influence of ACh on C4VR nerve bursts under baseline conditions and during bath application of 60 μ M ACh, which was applied to the brainstem compartment of the split-bath chamber, as described in Methods. *Top panel.* Representative recordings obtained under baseline conditions, and during bath application of 60 μ M ACh in a control animal and a DNE animal. In all sample recordings, the upper traces are the unprocessed nerve bursts, with the rectified and integrated version below ($\int$ C4VR). ACh increased the frequency of nerve bursts in the control pup, with little change in the DNE pup. *Bottom panel.* The frequency of C4 ventral root nerve bursts under baseline conditions and in response to 60 μ M ACh, in control and DNE animals. The data points represent the average frequency each min, expressed as the fold change from baseline (mean at point n / mean baseline). Ten min of baseline recording was followed by 15 min ACh application (*horizontal line*). Note that there was a significant difference between control and DNE animals ($P=0.0053$). * Indicates differences from the average baseline value within each of the treatment groups. Details of the statistical analysis are given in Methods, and the results of ANOVA in Table 2.

Figure 3.

Top panel. Representative recordings showing C4 ventral root nerve bursts (C4VR) under baseline conditions, and during bath application of 10 μ M atropine, followed by a mixture of 10 μ M atropine + 60 μ M ACh to the brainstem, in a control pup and a DNE pup, as indicated. *Bottom panel.* Same convention as in the *bottom panel* of Fig.2, except here 10 μ M atropine was applied to the brainstem from min 10 through min 40, while a mixture of 10 μ M atropine + 60 μ M ACh was applied from min 25-40, as indicated by the *horizontal lines*. The data points at 40-50 min represent each min of the 10 min drug washout period. * Indicates differences from the average baseline value within each of the treatment groups; τ indicates a significant treatment effect at that time point. For clarity, brackets join adjacent statistically significant data. Details of the statistical analysis are given in Methods, and the results of ANOVA in Table 2.

Figure 4.

Top panel. Representative recordings showing C4 ventral root nerve bursts (C4VR) under baseline conditions, and during bath application of 10 μ M curare, followed by a mixture of 10 μ M curare + 60 μ M ACh to the brainstem, in a control pup and a DNE pup, as indicated. *Bottom panel.* Same convention as in the *bottom panels* of Figs.2 and 3, except here 10 μ M curare was applied to the brainstem from min 10 through min 40, while a mixture of 10 μ M curare + 60 μ M ACh was applied from min 25-40, as indicated by the *horizontal lines*. The data points at 40-50 min represent each min of the 10 min drug washout period. * Indicates differences from the average baseline value within each of the treatment groups; τ indicates a significant treatment effect at that time point. For clarity, brackets join adjacent statistically significant data. Details of the statistical analysis are given in Methods, and the results of ANOVA in Table 2.

Figure 5.

Antagonist time control experiments. Bath application of the mAChR antagonist atropine (*Panels A and B*) was associated with modest, transient slowing in both control (*Panel A*, filled circles) and DNE (*Panel B*, open squares) pups, with recovery complete about halfway through drug application. The nAChR antagonist curare (*Panels C and D*) was associated with erratic frequency changes in both control (*Panel C*, filled circles) and DNE (*Panel D*, open squares) pups, though control animals showed a modest slowing over the final 10 min of curare application. *, Significant Bonferroni post hoc tests for comparisons of drug vs. baseline within a treatment group. There were no between-group differences (Table 2).

Table 1

Number of brainstem-spinal cord preparations used in each experimental series.

Drugs applied	Number of saline exposed/Unexposed animals	Number of nicotine exposed animals
ACh alone (60 μ M)	21/4	26
Atropine (10 μ M), then [10 μ M atropine + 60 μ M ACh]	6	6
Curare (10 μ M), then [10 μ M curare + 60 μ M ACh]	6	6
Atropine time control (10 μ M)	6	6
Curare time control (10 μ M)	6	6
	49	50

Table 2

Results of two-factor ANOVA for the three main pharmacological interventions (also see Figs. 3 & 6).

	Treatment	Time	Treatment × Time
ACh alone	(1, 8.5, 0.0053)	(24, 12.1, <0.0001)	(24, 2.9, <0.0001)
Atropine/Atropine + ACh	(1, 2.13, 0.18)	(49, 4.9, <0.0001)	(49, 3.05, <0.0001)
Curare/Curare + ACh	(1, 0.42, 0.53)	(49, 3.4, <0.0001)	(49, 3.3, <0.0001)
Atropine time control	(1, 0.08, 0.79)	(49, 1.6, 0.0068)	(49, 0.56, 0.99)
Curare time control	(1, 2.2, 0.17)	(49, 1.2, 0.18)	(49, 1.0, 0.49)

The columns represent the two main factors and their interaction, and each cell shows the degrees of freedom (df), the F statistic (F) and the P value (P), displayed as (df, F, P). Values in **bold italics** represent statistically significant results.