Developmental Nicotine Exposure Alters Neurotransmission and Excitability in Hypoglossal Motoneurons

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Pilarski JQ, Wakefield HE, Fuglevand AJ, Levine RB, Fregosi RF. Developmental nicotine exposure alters neurotransmission and excitability in hypoglossal motoneurons. J Neurophysiol 105: 423–433, 2011. First published November 10, 2010; doi:10.1152/jn.00876.2010. Hypoglossal motoneurons (XII MNs) control muscles of the mammalian tongue and are rhythmically active during breathing. Acetylcholine (ACH) modulates XII MN activity by promoting the release of glutamate from neurons that express nicotinic ACh receptors (nAChRs). Chronic nicotine exposure alters nAChRs on neurons throughout the brain, including brain stem respiratory neurons. Here we test the hypothesis that developmental nicotine exposure (DNE) reduces excitatory synaptic input to XII MNs. Voltage-clamp experiments in rhythmically active medullary slices showed that the frequency of excitatory postsynaptic currents (EPSCs) onto XII MNs from DNE animals is reduced by 61% (DNE = 1.7 ± 0.4 events/s; control = 4.4 ± 0.6 events/s; P < 0.002). We also examine the intrinsic excitability of XII MNs to test whether cells from DNE animals have altered membrane properties. Current-clamp experiments showed XII MNs from DNE animals had higher intrinsic excitability, as evaluated by measuring their response to injected current. DNE cells had high-input resistances (DNE = 131.9 ± 13.7 MΩ, control = 78.6 ± 9.7 MΩ, P < 0.008), began firing at lower current levels (DNE = 144 ± 22 pA, control = 351 ± 45 pA, P < 0.003), and exhibited higher frequency–current gain values (DNE = 0.087 ± 0.012 Hz/pA, control = 0.050 ± 0.004 Hz/pA, P < 0.02). Taken together, our data show previously unreported effects of DNE on XII MN function and may also help to explain the association between DNE and the incidence of central and obstructive apneas.

INTRODUCTION

In human infants, chronic nicotine exposure during development is correlated with impaired cardiac function (Franco et al. 2000), autonomic nervous system disorders (Weese-Mayer et al. 2007), sleep disorders (Mennella et al. 2007), and alterations in breathing pattern (Gaultier 2000; Hafstrom et al. 2005). The latter include an increase in the incidence of both central and obstructive apneas (Carroll 2003; Sawani et al. 2004; Toubas et al. 1986), suggesting that exposure to nicotine in utero impairs the development of respiratory neurons. Although many factors can lead to obstructive apnea, inadequate neural drive to the tongue muscles during sleep is a major cause (Gastaut et al. 1969; Horner 2008, 2009; Remmers et al. 1978). In healthy individuals, tongue muscles contract during inspiration, serving to dilate and stiffen the upper airway (Bailey et al. 2006; Fuller et al. 1999; Horner 1996). All tongue muscles are driven by hypoglossal motoneurons (XII MNs), and it is well known that nicotinic acetylcholine receptors (nAChRs) are expressed on these cells. For example, brief exposures to ACh or nicotine evoke fast ionotropic cation influx and membrane depolarization in XII MNs (Chamberlin et al. 2002; Quitadamo et al. 2005; Robinson et al. 2002; Zaninetti et al. 1999). In addition, nAChRs are also expressed on glutamatergic neurons that are the dominant source of excitatory synaptic input to these cells (Dekhordi et al. 2005; Quitadamo et al. 2005). Activation of nAChRs on glutamatergic neurons facilitates glutamate release, providing a mechanism that allows ACh to indirectly modulate XII MN excitability (Quitadamo et al. 2005). Importantly, nAChRs in many regions of the mammalian brain are functionally down-regulated following chronic nicotine exposure, reducing the depolarizing currents passed by these ion channels (reviewed in Gentry and Lukas 2002; Wonnacott 1997). Evidence that chronic nicotine exposure may lead to a functional down-regulation of nAChRs on neurons involved in the control of breathing comes from our recent study showing that the nicotine-mediated increase in central ventilatory output is significantly blunted in developmentally nicotine-exposed animals (Pilarski and Fregosi 2009).

Here we test the hypothesis that developmental nicotine exposure (DNE), defined as the chronic exposure to nicotine during embryonic development and during the first week of life, decreases glutamatergic excitatory synaptic input onto XII MNs. Furthermore, we also examine the effect of DNE on intrinsic excitability of XII MNs. The results of voltage-clamp experiments show that XII MNs from DNE animals receive significantly less excitatory synaptic input compared with control animals, whereas current-clamp experiments demonstrate that XII MNs from DNE animals exhibit increased intrinsic excitability. DNE cells had higher input resistances, began firing at lower current levels, and exhibited higher frequency–gain values. Some of these data have been presented previously in abstract form (Pilarski et al. 2010; Wakefield et al. 2010).

METHODS

Animals and prenatal nicotine exposure

Wherever possible we minimized the number of animals necessary to assess treatment effects. All procedures were approved and in accordance with guidelines provided by the Institutional Animal Care and Use Committee at the University of Arizona. We studied Sprague–Dawley rats of either sex from birth through the first 6 days of life, i.e., postnatal day zero (P0) through postnatal day 4 (P5) (mean age: control = 3.58 ± 0.22 days, DNE = 3.68 ± 0.24 days, P > 0.77). We studied a total of 72 XII MNs from 19 neonatal animals (10

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control; 9 nicotine) derived from 13 dams. On average two animals per dam were used. All neonates were born via spontaneous vaginal delivery from pregnant dams purchased from Harlan Laboratories. Neonates were housed together with their mothers and siblings until the day of surgery. Dams had unrestricted access to food and water. Animals were kept in a quiet room at 21–23°C, 20–30% relative humidity, on a 12/12-h light/dark cycle.

DNE was achieved by subcutaneous implantation of an Alzet 1007D miniosmotic pump (Alzet, Cupertino, CA) into the pregnant dams. Implantation was performed on gestational day 5 under aseptic conditions, as described previously (Fregosi and Pilarski 2008; Fregosi et al. 2004; Huang et al. 2004; Luo et al. 2007). The osmotic pumps infused fluid into the subcutaneous space at a rate of 2.5 μl·h⁻¹ for 28 days, which includes the first week following parturition when neonatal animals are suckling. Based on this infusion rate, the mean nicotine bitartrate delivery was 6 mg·kg⁻¹·day⁻¹. This dosing regimen produces prenatal plasma levels of free-base nicotine of about 24 ng/ml (150 nM) in the neonates and 18 ng/ml (111 nM) in the dam (Chen et al. 2005) and had no effect on body weight for age-matched neonates (data not shown), consistent with previous reports (Fregosi and Pilarski 2008). These nicotine concentrations are within the range (15–45 ng/ml) found in the blood plasma of pregnant human mothers described as moderate smokers (Benowitz and Jacob 3rd 1984) and in the amniotic fluid from human fetuses in smoke-exposed mothers (Luck and Nau 1985).

**Medullary slice preparation**

Neonatal rats (P0–P5) were cooled rapidly on ice and, following hypothermic analgesia, the brain stem and spinal cord, extending from the pontomedullary border to just below the second cervical nerve roots, were removed en bloc. The cerebellum was severed and the preparation was mounted on a paraffin-coated cutting block placed into a vibrating blade microtome (Vibratome, VT 100; Technical Products International, St. Louis, MO). Serial transverse slices of 100–200 μm were taken in a rostral to caudal direction until the compact division of the nucleus ambiguus and the rostral portion of the inferior olive were visualized in the trim slice. Subsequently, a single 600- to 700-μm slice that included the semicompact division of the nucleus ambiguus, the pre-Bötzinger complex (preBötC), the nucleus tractus solitarius (SCs), the superfusate always contained bicuculline methiodide (10 μM), respectively (see Pilarski and Fregosi 2009). In other studies we used nicotine bitartrate (0.5 mM), to stimulate the Aδ-afferent input, with the purpose of blocking the presynaptic inhibition (Greer et al. 1991). All drugs listed here were bath applied to achieve effective concentrations in the superfusate. In the rhythmic or choreiform activity (Smith et al. 1991). Elevated K⁺ has been shown to potentiate glutamatergic neurotransmission in similar in vitro preparations (Ishibashi et al. 2005) as well as potentiate the frequency and magnitude of synaptic input onto respiratory neurons (Okada et al. 2005). Both thick and thin medullary slices were allowed to equilibrate in the oxygenated aCSF for about 30 min prior to recording.

**Electrophysiology**

Thick medullary slices that include the preBötC produce spontaneous inspiratory motor outflow in branches of the XII motor nerve. This motor outflow was recorded with suction electrodes (OD: 1.5 mm; ID: 1.12 mm; World Precision Instruments, Sarasota, FL) to obtain an index of systems-level respiratory-related activity (Suzue 1984), as well as an ensemble recording of the hypoglossal motoneuron population ipsilateral to the recording electrode. Extracellular recordings were amplified 20- to 5,000-fold using a Grass high-impedance probe and AC-coupled differential amplifier (P511; Grass Technologies, Rockland, MA), band-pass filtered (100 to 3,000 Hz), and digitized at 20 kHz. XII nerve output was observed in real time during the experiment and stored on a computer hard drive (Gateway, Irvine, CA). Individual XII MNs (20–60 μm) were visualized using an Olympus BX-50WI fixed-stage microscope (×40, 0.75 NA water-immersion objective) equipped with differential interference contrast (DIC) optics and an infrared videocamera (C25400-07; Hamamatsu, Schüpfen, Switzerland) (Fig. 1A). We only studied XII MNs with large inspiratory-modulated inward currents coincident with rhythmic XII nerve bursts, confirming that the cell was receiving input from the respiratory central pattern generator (Feldman and Del Negro 2006; Rekling and Feldman 2001). XII MNs with a resting membrane potential more positive than −40 mV were not studied. Junction potentials were zeroed prior to gigaseal formation.

Whole cell recordings of XII MNs were made with glass pipettes (3–8 MΩ) pulled from thick-walled borosilicate glass capillary tubes (OD: 1.5 mm; ID: 0.75 mm; Sutter Instrument, Novato, CA). The patch pipettes were loaded with a solution containing (in mM): 135 K-gluconate, 4 KCl, 12.5 disodium phosphate, 10 HEPES, 0.375 Na-GTP, and 5 ATP (Mg²⁺ salt), with pH adjusted to 7.3 and osmolarity adjusted to 250–275 mOsm. XII MNs were approached visually in voltage-clamp mode and tight seals were made using the method of Stuart et al. (1993), in which positive pressure is maintained as the pipette is moved through the tissue toward the XII MN target. The swift transition from positive pressure to light negative pressure following contact makes gigaseal (≥1 GΩ) formation possible. Applying additional short pulses of negative pressure against the cell membrane produced intracellular access and a “whole cell” recording configuration. Current and voltage signals were amplified and acquired using the Axoclamp 1D system, pCLAMP software, and a Digidata 1202 AD/DA data acquisition system (Axon Instruments). Signals were low-pass filtered at 1.0 kHz, digitized at 20 kHz, observed during the experiment in real time, and stored on a computer hard drive (Gateway).

**Drugs and protocols**

Drugs were obtained from Sigma (St. Louis, MO) or Tocris Bioscience (Ellisville, MO) and mixed daily from stock solutions to achieve effective concentrations in the superfusate. In the rhythmic slice used to quantitate excitatory postsynaptic currents (EPSCs), the superfusate usually contained bicuculline methiodide (10 μM) and strychnine (0.4 μM) to block γ-aminobutyric acid type A (GABA_A) receptors and glycine-mediated inhibitory neurotransmission, similar to that reported by Quitadamo et al. (2005). In some experiments, we used nicotine bitartrate (0.5 mM), to stimulate nACHRs, and DH-β-E (0.2 μM) and mecamylamine (1 μM) to block nAChRs, respectively (see Pilarski and Fregosi 2009). In other studies we used N-methyl-D-aspartate (NMDA) and non-NMDA antagonists, (−)-5-methyl-10,11-dihydroxy-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801, 20 μM), and 6-cyano-7-nitroquinolin-2,3-dione (CNQX, 20 μM), respectively, to block glutamatergic neurotransmission (see Greer et al. 1991). All drugs listed here were bath applied to the superfusate. When agonists were added to the superfusate only a single XII MN from each slice was studied to avoid pharmacological problems associated with drug washout and/or receptor sensitization.

In the first set of experiments, our protocol was as follows: system-level whole XII nerve activity and single XII MN synaptic output were monitored using extracellular recordings (see Greer et al. 1991). Both thick and thin medullary slices were allowed to equilibrate in the oxygenated aCSF for about 30 min prior to recording.
activity were recorded for 2 min in the rhythmic slice with the membrane potential held at $-70\,\text{mV}$. We analyzed the synaptic activity during the inspiratory phase (i.e., phasic activity), as well as the period between inspiratory bursts (i.e., tonic activity). This allowed us to quantify the system-level output recorded from a XII nerve rootlet, as well as the amplitude and frequency of EPSCs in XII MNs from control and DNE animals.

To examine the effects of DNE on intrinsic XII MN excitability, we initially studied cells from rhythmic slices (the same cells from which synaptic activity was quantified) and injected current during the interburst period (see Fig. 1A). This was done in 17 cells from control animals and 20 cells from DNE animals. Although we found a significant difference in the frequency–current ($f–I$) gain in XII MNs of DNE compared with control ($DNE/\text{control} = 0.061 \pm 0.004\,\text{Hz/pA}, \text{control} = 0.048 \pm 0.003\,\text{Hz/pA}, P < 0.03$), the heightened excitability in the rhythmic slice made it difficult to study intrinsic membrane properties. Therefore we measured and present only the $f–I$ relationship in XII MNs from caudal, nonrhythmic medullary slices in both treatment groups using a modified superfusate 3 mM $[\text{K}^{+}]$ and 1.2 mM $[\text{Ca}^{2+}]$. The current injection protocol consisted of 1-s square current pulses beginning with a single hyperpolarizing step, followed by 19 depolarizing steps. The magnitude of each current step was 50 pA. Current injection was initiated from resting membrane potential, which was not significantly different between treatment groups (Table 1).

**Data analysis and statistics**

The peak amplitude, area, and duration of rectified and integrated XII nerve rootlet activity and simultaneously recorded phasic inward currents in XII MNs were analyzed with a custom software program written with Spike2 software (CED, Cambridge, UK). We analyzed each single XII MN and XII nerve rootlet burst that occurred in the 2-min recording period, consisting of about 10–20 bursts per cell. We also measured the frequency and amplitude of EPSCs that occurred during each of the interburst intervals over a period of 2 min. This resulted in the collection and analysis of 60–1,384 EPSCs for each cell studied and these events were quantified and analyzed using a
template recognition algorithm (Mini-Analysis Program V5; Synapto
tosoft, Fort Lee, NJ). EPSC detection was characterized by the
following parameters: amplitude, area or charge transfer, rise time,
decay time, and the duration of the interevent interval. Individual
EPSCs selected by the software were subsequently crosschecked and
spurious events were rejected.

To analyze intrinsic membrane properties of XII MNs we measured
rheobase current, input resistance, and the steady-state frequency–
spurious events were rejected.

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<th>TABLE 1 Effects of developmental nicotine exposure on whole XII nerve motor output in rhythmic medullary slices and on the intrinsic properties of XII motoneurons in caudal, nonrhythmic medullary slices</th>
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<td><strong>A. XII nerve motor output</strong></td>
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<td>Inspiratory burst frequency, min⁻¹</td>
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Values are means ± SE. n = number of animals, with control before the forward slash and DNE after it. XII, hypoglossal. Inspiratory frequency and the amplitude and duration of XII motor nerve bursts represent average values taken during a 2-min recording period. The definition of the intrinsic cellular properties measured in XII MNs is given in METHODS. *Significant difference between control and DNE.

RESULTS

Identification of XII MNs and the role of nAChRs in rhythmic medullary slices

XII MNs were located by their anatomy and their neurophysiology, which has been well described (Bellingham and Berger 1996). XII MNs are large (20–60 µM) multipolar neurons that reside in the XII motor nucleus (Fig. 1A) and innervate all tongue muscles. Figure 1A shows a schematic of a rhythmic medullary slice with key landmarks identified; the image shows several dextran rhodamine-filled XII MNs for clarity. Sample traces of simultaneously recorded XII nerve motor outflow and voltage-clamp responses (I_m) from a XII MN are also shown in Fig. 1A. Note that the cell is receiving large inspiratory drive currents coincident with XII nerve discharge, as well as sporadic excitatory synaptic events during the interburst period (Fig. 1A, top trace). In Fig. 1B bath application of nicotine (0.5 µM) causes a slow inward current, as well as an increase in the frequency of respiratory drive currents and the rate of excitatory postsynaptic potentials (EPSPs) between the inspiratory bursts. Thus the effect of acute nicotine exposure on XII motoneuron activity is clearly excitatory in our preparation. Figure 1C shows that excitatory, respiratory-related synaptic drive to XII MNs is abolished by glutamate antagonists (MK-801 and CNQX), demonstrating that the major source of excitatory input to this system is glutamatergic, consistent with previous observations in the medullary slice preparation (Wang et al. 2002). Finally, Fig. 1D shows a voltage-clamp recording obtained from an untreated XII MN before and after blocking nAChRs via bath application of DH-β-E (0.2 µM), mecamylamine (Meca; 1 µM), and α-bungarotoxin (α-Bgtx; 1 µM). Note that blocking nAChRs decreased the rate of phasic inspiratory drive currents and the rate and amplitude of EPSCs between bursts, thereby implicating the presence of endogenous acetylcholine release in the rhythmic slice. Endogenous ACh neurotransmission has also been demonstrated by others using similar preparations (Quitadamo et al. 2005) and also when blocking acetylcholine esterase (Shao and Feldman 2005). The same procedures depicted in Fig. 1D were carried out in three different rhythmic slices with similar results.

Effect of DNE on respiratory motor output and excitatory synaptic activity in XII MNs in rhythmic medullary slices

Rectified and integrated whole XII nerve discharge was used as an index of XII MN population activity. The frequency of
these whole XII nerve bursts provides an index of the inspiratory drive coming from the respiratory central pattern generator, hypothesized to reside in the preBötC (Funk et al. 1993; Gray et al. 1999; Smith et al. 1991). The frequency and waveform characteristics of XII nerve discharge that we recorded are comparable to those previously reported for similar preparations (Chamberlin et al. 2002; Robinson et al. 2002). We found no differences in XII nerve discharge frequency or amplitude between DNE and control animals (Table 1), consistent with other studies (Pilarski and Fregosi 2009; Robinson et al. 2002).

We then compared the frequency and amplitude of phasic and tonic EPSCs in XII MNs from DNE and control animals. No significant differences were found for the frequency, peak amplitude, or charge carried by the phasic inspiratory drive current in XII MNs (Fig. 2, A–E), consistent with Robinson et al. (2002), although the duration of the drive current was briefer in DNE animals (0.58 ± 0.05 s) than that in controls (0.80 ± 0.08 s; P < 0.006; Fig. 2, B and F). In contrast, the frequency of tonic EPSCs recorded during the interburst interval was markedly reduced in DNE animals (Figs. 3 and 4). Figure 3, A and B shows a side-by-side comparison of XII MN voltage-clamp recordings taken during the interburst interval, in five control and five DNE animals. In every comparison, a reduced EPSC frequency is evident in the cells from DNE animals compared with controls. Figure 4A shows that the cumulative probability distribution of EPSC interevent interval during the interburst period was rightward shifted for DNE compared with the control group, but there was no change in the EPSC amplitude distribution between the two groups (Fig. 4B). Figure 4C shows that the mean EPSC frequency measured in the interburst period was significantly lower in DNE (1.7 ± 0.4 events/s) compared with control animals (4.4 ± 0.6 events/s; P < 0.002), but EPSC amplitude was unaltered by DNE (Fig. 4D; P > 0.261). These data suggest a DNE-
induced reduction in the rate of glutamatergic inputs onto XII MNs during the interburst period.

Influence of prenatal nicotine exposure (DNE) on intrinsic excitability of XII MNs nonrhythmic medullary slices

When a series of square-pulse current injections were applied to XII MNs in nonrhythmic, caudal medullary slices (Fig. 5A), XII MNs from DNE animals exhibited an enhanced $f$–$I$ gain (DNE $0.087 \pm 0.012$ Hz/pA, control $0.050 \pm 0.004$ Hz/pA, $P < 0.02$, Fig. 5B; Table 1), a lower rheobase current (DNE $143 \pm 22$ pA, control $309 \pm 34$ pA, $P < 0.002$; Table 1), a lower threshold current (DNE $144 \pm 22$ pA, control $351 \pm 45$ pA, $P < 0.003$; Fig. 5B and Table 1), and a lower maximum current (DNE $594 \pm 38$ pA, control $1,479 \pm 122$ pA, $P < 0.0002$; Fig. 5B and Table 1) compared with controls. There were no significant differences in resting membrane potential (DNE $-54.4 \pm 1.4$ mV, control $-53.2 \pm 1.8$ mV, $P > 0.601$), spike threshold (DNE $-31.4 \pm 1.5$ mV, control $-27.4 \pm 1.8$ mV, $P > 0.125$), or minimum (DNE $8.2 \pm 1.0$ Hz, control $10.4 \pm 1.4$ Hz, $P > 0.230$) or maximum (DNE $35.4 \pm 1.7$ Hz, control $48.0 \pm 5.6$ Hz, $P > 0.067$) firing frequencies (Table 1). Finally, XII MNs from DNE animals had significantly higher input resistances (DNE $131.9 \pm 13.7$ MΩ, control $78.6 \pm 9.7$ MΩ, $P < 0.008$). Collectively, these findings indicate that XII MNs from DNE animals were intrinsically more excitable than those from control animals.

DISCUSSION

In the present study we found that: 1) DNE decreased the duration of phasic inspiratory modulated EPSCs and the frequency of tonic EPSCs recorded in rhythmically active XII MNs; 2) XII MNs from DNE animals showed enhanced intrinsic excitability, including increased frequency–current ($f$–$I$) gain, lower rheobase current, lower maximum current, and increased input resistance; and 3) the frequency and amplitude of the XII motoneuron population activity (as measured from XII nerve rootlets) were not different between treatment and control groups. Although it is tempting to reason that XII MNs increase their excitability as a homeostatic response to lower excitatory input, the simplest and most supported explanation for the increase in XII MN excitability is related to the higher input resistances in the DNE animals, perhaps resulting from morphological differences or changes in specific membrane resistivity associated with altered neural development. We think it is likely that the DNE-induced decrease in spontaneous excitatory synaptic input on one hand and the increase in XII MN intrinsic excitability on the other hand represent independent effects of DNE on this breathing-related circuit. Consistent with this idea, nicotine has been linked to a variety of serious alterations in neuronal maturation, including reductions in soma size, cell density, dendritic ar-
borization, and cytoarchitecture (Abdel-Rahman et al. 2005; Roy and Sabherwal 1994; Roy et al. 2002; Slotkin et al. 1986), as well as direct and indirect effects on ion-channel conductances (Griguoli et al. 2010; Hayashida et al. 2005).

Effects of DNE on presynaptic input to XII MNs

Although nicotinic cholinergic neurotransmission is clearly involved in neuromodulatory mechanisms of the pharyngeal airway (Shao and Feldman 2001, 2002, 2005), it is still unclear where and how nAChRs modulate glutamate release from individual neurons. The DNE model provides an excellent opportunity to address some of these questions in a reduced preparation. In the rhythmically active slice, a significant pathway for glutamatergic input to the XII MN is from premotor neurons that translate phasic inspiratory output from the preBötC to XII MN (for a recent review see Feldman and Del Negro 2006). In the present study, we show a small DNE-induced decrease on the duration of phasic XII MN input, but not on the amplitude or the frequency of the inspiratory modulated inward current. Interestingly, in similar preparations, exogenous nicotine injected acutely into the XII motor nucleus potentiates the duration of inspiratory modulated currents in XII MNs (Shao and Feldman 2005), the opposite of our findings. These data suggest that DNE may blunt the normal function of nAChR modulation of glutamatergic input to XII MN, but are not required for the control of phasic burst cycling in itself, at least during baseline conditions. Although more data are needed to address the mechanisms behind this result, we infer that DNE is altering some aspect of glutamate release downstream of the preBötC but upstream of the XII motor nucleus, possible in the intermediate reticular formation (Quitadamo et al. 2005; Shao and Feldman 2005), which provide an area of integration and processing for many inputs to respiratory neurons in the brain stem.

In contrast to the small reduction in phasic input to XII MN following DNE, we show a profound decrease in the frequency of tonic excitatory synaptic input measured in between inspiratory activity. Tonic excitation of the XII motor nucleus may arise from several sources, including chemosensitive regions that project to this level of the medulla, and caudal raphe projections, which provide tonic excitatory input to the intermediate reticular formation (Dobbins and Feldman 1994; Smith et al. 1989) and the XII motor nucleus (Ptak et al. 2009). Because blocking glutamatergic receptors eliminates all EPSCs in XII MNs (Fig. 1C and Funk et al. 2000), even in the presence of acetylcholine (Quitadamo et al. 2005) or nicotine (unpublished observation), we think the EPSC activity that we measured, whether phasic or tonic, reflects network glutamatergic neurotransmission upstream from the XII MNs, as mentioned earlier. Moreover, since it is known that nAChRs are located on presynaptic glutamatergic neurons projecting to the XII nucleus (Dehkordi et al. 2005; Quitadamo et al. 2005) and, since acute stimulation of these receptors with nicotine or ACh enhances glutamate release (Quitadamo et al. 2005), the reduction in EPSC frequency in XII MNs is likely due to a functional down-regulation of nAChRs on these glutamatergic neurons following long-term nicotine exposure (Gentry and Lukas 2002). This thesis is supported by recent findings showing that
DNE markedly reduced the respiratory burst frequency evoked by bath application of nicotine to the neonatal rat brain stem–spinal cord preparation (Pilarski and Fregosi 2009). Others have also shown that chronic nicotine exposure functionally down-regulates nAChRs and neurotransmitter release in neurons throughout the brain (Benwell and Balfour 1997; Bordia et al. 2010; Slotkin et al. 2008), suggesting that this may be a systemic response to chronic nicotine exposure.

Nevertheless, we want to emphasize that the systemic response to nicotine almost surely extends beyond the desensitization of nAChRs. That is to say despite much data on the direct effects of nicotine on nAChRs and their widespread function as a modulator of neurotransmission, there is a large body of evidence that shows nicotine can also induce several signal transduction pathways, leading to the persistent cellular alterations in the brain (for a review see Barik and Wonnacott 2009). For example, exogenous nicotine can activate intracellular messengers in two main ways. First, nAChRs, which are nonselective cation channels, have an important role in regulating intracellular Ca\(^{2+}\). The ability of nAChRs to influence intracellular concentrations of Ca\(^{2+}\) enables nAChRs agonists, such as nicotine, to evoke gene transcription and plasticity, in addition to its classical role as a modulator of neurotransmitter release. Second, in an interesting recent report, Steiner et al. 2007 demonstrated that cellular responses to nicotine do not always involve fast ligand channel activation. In this study, nicotinic stimulation of cultured cortical neurons activated the extracellular signal-regulated kinase (ERK), leading to the phosphorylation of calcium/calmodulin-dependent protein kinases (CaM kinases), known to influence gene regulation, but did not seem to require the involvement of nAChRs. To summarize, the present study shows that DNE alters synaptic transmission in the rhythmic medullary slice, although future studies need to address where and how nAChRs are involved (see following text), keeping in mind alternate ways in which nicotine can interact with the cellular machinery that ultimately results in the modification of synaptic transmission.

Last, contrary to our expectations, tonic EPSC amplitude was not altered by DNE in the present study (Fig. 4, B and D). Blocking nicotinic neurotransmission with DH-β-E and α-Bgt, which are selective to the α4β2 and α7 nAChR subtypes, respectively, has been shown to decrease both the frequency and amplitude of EPSCs measured from nonrhythmically active XII MNs (Quitadamo et al. 2005). If nAChRs on presynaptic terminals are functionally down-regulated following DNE, as we propose, then EPSC amplitude might be expected to decrease in concert with the frequency because such receptors likely influence the magnitude of neurotransmitter release. Although we cannot explain why EPSC amplitude did not decrease in the DNE animals, it is interesting to note that acute applications of nicotine in nonrhythmically medullary slices increased XII MN EPSC frequency, but EPSC amplitude was unaltered (Quitadamo et al. 2005). Future studies will address this issue directly by recording the frequency of miniature postsynaptic currents in XII MNs isolated from synaptic input with tetrodotoxin. Moreover, we would like to know whether specific nAChR subtypes are unique to presynaptic terminals and preterminal locations in this circuit because nAChR desensitization and recovery vary considerably among the various subtypes (Genty and Lukas 2002; Giniatullin et al. 2005).

Effects of DNE on intrinsic biophysical properties of XII MNs

XII MNs from DNE animals were more sensitive to depolarizing current injection, indicating that DNE evoked changes in intrinsic properties. Although the DNE-induced diminution in the frequency of EPSCs likely involves alterations in nAChRs and reduced glutamate release (explained earlier), it is less clear how DNE alters the intrinsic excitability of XII MNs. The increased excitability certainly seems linked to increased input resistance in DNE-exposed XII MNs (Table 1). Increased input resistance itself can arise because of smaller somadendritic surface area or as a consequence of changes in the specific resistivity of the membrane. Perhaps XII MNs that develop in the presence of nicotine exhibit impaired or delayed maturation. For example, exogenous nicotine is known to alter cell size and density in CA1 pyramidal in the hippocampal and cerebellar Purkinje neurons (Abdel-Rahman et al. 2005), al-

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**FIG. 5.** Effects of DNE on the intrinsic excitability of XII MNs recorded in nonrhythmic medullary slices. A, top: recordings from a representative XII MN from a control and DNE animal. For each MN, the voltage response and instantaneous discharge frequency are shown. The bottom trace in this panel is common to both cells and shows the current steps applied to all XII MNs studied (i.e., 20 square wave pulses lasting 1 s). Current injection began with a single –50-pA hyperpolarizing pulse followed by 19 depolarizing pulses increasing by 50 pA with each step. Note that all cells were driven until depolarization block was produced (indicated by asterisks). Frequency measurements were made over a 0.5-s window during the last 500 ms of each current step. B: frequency–current (f–I) relationships of control (open circles) and DNE (closed circles) cells to increasing steps of injected current. DNE animals had a lower threshold current, a lower maximum current, and an increase in the slope of the f–I relation.
though others found no such changes (Chen et al. 2006). Alternatively, it is also possible that chronic nicotine exposure during development, leading to structural and/or functional changes in ion channels, could also increase specific membrane resistivity and excitability. A recent study by Griguoli et al. (2010) showed that acute nicotine reduces \( I_h \) currents and oscillatory behavior in hippocampal neurons by directly binding to hyperpolarization-activated cyclic nucleotide gated (HCN) channels and, when given chronically, nicotine exposure has been linked to increases in the conducance and expression of L-type high-voltage Ca\(^{2+} \) channels (Hayashida et al. 2005). Although more data are required to assess the mechanisms of DNE-induced changes in XII MN excitability, it is interesting to note the striking similarities between the effects of DNE on these motoneurons and the effect of development on the biophysical characteristics of other respiratory motoneurons. For example, Martin-Caraballo and Greer (1999) showed that phrenic motoneurons studied on embryonic day 16 exhibit higher input resistances, lower rheobase currents, and higher frequency–current gain values compared with animals on postnatal day 0–1 (6–7 days later). In other words, motoneuron immaturity is also associated with increased intrinsic excitability, which suggests that DNE may delay the normal maturation of XII MNs, resulting in the phenotype of a much younger animal.

**Clinical perspectives**

The present study may shed light onto the increased incidence of obstructive apnea in nicotine-exposed infants and children (Hafstrom et al. 2005; Underner et al. 2006) because airway obstruction often occurs secondary to a reduction in neural drive to the tongue muscles, which are controlled by XII MNs. Interestingly, nicotine exposure has been proposed both as a treatment and as a significant risk factor for obstructive apneas (Gothe et al. 1985). This paradox is due to the opposite effects of acute and chronic nicotine on the control of breathing and the activity of XII MNs. Acute nicotine exposure excites \( \alpha \)-nicotinic acetylcholine receptors and increases respiratory-related motor activity, including drive to XII MNs (see Fig. 1B). Moreover, there is compelling evidence that ACh release helps to maintain respiratory drive when the release of other excitatory neuromodulators (e.g., serotonin, dopamine, norepinephrine) is reduced in rapid eye movement sleep (Bellingham and Funk 2002; Ireland and Bellingham 2005). However, with chronic nicotine exposure, \( \alpha \)-nicotinic acetylcholine receptors on serotonergic, glutamatergic, dopaminergic, and noradrenergic neurons are desensitized (for review see Barik and Wonnacott 2009; Gentry and Lukas 2002), which eventually diminishes the ability of ACh to modulate synaptic transmission. The simultaneous loss of each of these excitatory inputs may diminish neural drive to the tongue musculature, leading to upper airway obstruction as the flaccid tongue falls against the posterior pharyngeal wall.

Our study may also help to explain the difficulty in diagnosing central and obstructive sleep apneas in pediatric populations. As shown here, systems-level motor output in DNE animals is normal under baseline conditions, which may reflect the reciprocal changes in excitatory synaptic input and intrinsic cellular excitability; in other words, the increase in intrinsic excitability in XII MNs from DNE animals allows the cells to reach firing threshold and achieve relative high firing rates despite low levels of excitatory synaptic input. On one hand, this is advantageous for the animal, as breathing and gas exchange are maintained, at least under baseline, nonstressful conditions. On the other hand, any perturbation that changes the balance of inhibitory and excitatory inputs to XII MNs may reduce tongue muscle activity. For example, in previous studies, we showed that DNE markedly enhances the respiratory motor response to agonists of GABA\(_A\) (Luo et al. 2004), glycine (Luo et al. 2007), and AMPA receptors (Pilarski and Fregosi 2009). Thus stressors and/or changes in state that modulate the release of these neurotransmitters, such as hypoxia (Melton et al. 1990), hypercapnia (Bissonnette and Knopp 2004; Kuribayashi et al. 2008), and sleep (Gottesmann 2004; Hehre et al. 2008), may prevent the DNE animal from mounting an effective ventilatory response. This phenomenon was demonstrated in a recent study in awake neonatal animals (Huang et al. 2010), wherein DNE had no effect on baseline breathing, but significantly blunted the response to hypercapnia.

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**DISCLOSURES**

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**REFERENCES**


J Neurophysiol • VOL 105 • JANUARY 2011 • www.jn.org
Feldman JL, Del Negro CA.

Funk GD, Smith JC, Feldman JL.

Prenatal nicotine exposure and development of Fuller DD, Williams JS, Janssen PL, Fregosi RF.

432 PILARSKI, WAKEFIELD, FUGLEVAND, LEVINE, AND FREGOSI Gothe B, Strohl KP, Levin S, Cherniack NS.


Neuromodulation of hypoglossal motoneurons during sleep. Horner RL.

Hehre DA, Devia CJ, Bancalari E, Suguihara C.


Slotkin TA, Ryder IT, Mackillop EA, Bodwell BE, Seidler FJ. Adolescent nicotine administration changes the responses to nicotine given subsequently in adulthood: adenyl cyclase cell signaling in brain regions during


