Central cholinergic regulation of respiration: nicotinic receptors

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Nicotinic acetylcholine receptors (nAChRs) are expressed in brainstem and spinal cord regions involved in the control of breathing. These receptors mediate central cholinergic regulation of respiration and effects of the exogenous ligand nicotine on respiratory pattern. Activation of α4* nAChRs in the preBötzinger Complex (preBötC), an essential site for normal respiratory rhythm generation in mammals, modulates excitatory glutamatergic neurotransmission and depolarizes preBötC inspiratory neurons, leading to increases in respiratory frequency. nAChRs are also present in motor nuclei innervating respiratory muscles. Activation of post- and/or extra-synaptic α4* nAChRs on hypoglossal (XII) motoneurons depolarizes these neurons, potentiating tonic and respiratory-related rhythmic activity. As perinatal nicotine exposure may contribute to the pathogenesis of sudden infant death syndrome (SIDS), we discuss the effects of perinatal nicotine exposure on development of the cholinergic and other neurotransmitter systems involved in control of breathing. Advances in understanding of the mechanisms underlying central cholinergic/nicotinic modulation of respiration provide a pharmacological basis for exploiting nAChRs as therapeutic targets for neurological disorders related to neural control of breathing such as sleep apnea and SIDS.

Keywords: respiratory control; nicotinic acetylcholine receptors; preBötzinger complex; inspiratory neuron; hypoglossal nucleus; phrenic nucleus; motoneuron; perinatal nicotine exposure

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Introduction

Respiration is a vital behavior, essentially continuous from birth to death. Respiratory rhythm is generated within the central nervous systems (CNS). The coordinated motor patterns of the respiratory muscles including the pump, eg, diaphragm, intercostal and abdominal, muscles as well as the upper airway, eg, genioglossal, muscles involve multilevel and diverse neurotransmitter regulation.

ACh plays an important role in the neural control of respiration[1-14], including in central chemosensitivity, ie, the ability of the brain to sense CO₂ (and/or pH) to regulate ventilation[15-21]. Impairments in central cholinergic system are implicated in pathophysiology of some prevalent neurological disorders involving respiratory control such as SIDS and sleep apnea[13, 22-26]. Studies of central cholinergic regulation of respiration date to the early 1930s. Intraventricular injection of ACh into the lateral ventricle or the third ventricle of the brain in anesthetized, vagotomized cats, usually induces temporary depression of breathing, but in some cases, produces acceleration[1]. Cerebral arterial injection or intraventricular application to the fourth ventricle of ACh increases the frequency and amplitude of respiration in dogs. These effects are potentiated by the acetylcholinesterase inhibitor physostigmine and persist after denervation of the carotid and aortic chemosensors, confirming central components of these ACh effects[2]. Perturbations of ACh synthesis, release, degradation, or activation of ACh receptors, in the medulla result in perturbations of respiratory pattern both in vivo[7, 8, 27] and in vitro[9, 10, 12, 19, 28, 29].

Pontine cholinergic mechanisms associated with state-dependent modulation of respiratory control have been recently reviewed[30, 31]. In this review, we focus on the role of nAChRs in the preBötC, an essential site for respiratory rhythm generation[32-34], and in brainstem and spinal cord respiratory motor nuclei in regulation of respiration.

nAChRs have attracted wide research interests since, in addition to mediating endogenous cholinergic regulation of respiratory pattern, nAChRs mediate the effects of nicotine from tobacco smoke, a matter of considerable public health significance. We also outline recent research on the effects of

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perinatal exposure to nicotine on neonatal control of breathing. In the interest of focus, we do not discuss the role of nAChRs in central chemoreception.

**Cholinergic innervation and nAChRs in brainstem and spinal cord regions involved in respiratory control**

Respiratory rhythm is generated in the brainstem. Our current view of respiratory rhythm generation in mammals is that there are two distinct, normally coupled, rhythm generators. The primary site, the preBötC, is ventral to the nucleus ambiguus, midway between the facial nucleus and the obex, caudal to the Bötzingen Complex and rostral to the ventral respiratory group (VRG) in the rostroventrolateral medulla[32,33]. The preBötC is postulated to generate inspiratory rhythmic activity that dominates breathing in mammals at rest[34,35]. A second site that is postulated to drive active expiratory activity is close to the facial nucleus in the region of the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG)[34,36]. Respiratory rhythm is transmitted via medullary premotoneurons[37–39] to respiratory motoneurons in the ventral horn of the spinal cord, including the phrenic nucleus, and medullary cranial motoneuclei such as the XII nucleus; these motoneurons drive the muscles of the respiratory pump and those regulating airway resistance.

In the brainstem, the principal cholinergic projection system originates in the pontomesencephalic tegmental cholinergic complex that includes the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus (PPT/LDT)[40,41]. Cholinergic neurons in the PPT/LDT have descending projections to the medullary reticular nuclei and the lateral reticular nucleus[40,41], probably including the preBötC. Cholinergic neurons in the PPT/LDT also project to motor nuclei of cranial nerves including XII nucleus[40–42]. Numerous cholinergic neurons are found in the medullary reticular formation and near the ventral medullary surface[43–45]. The preBötC probably also receives cholinergic input from these local sources[44].

XII motoneurons have cholinergic innervation. The terminals that make synaptic contact with XII motoneurons contain both choline acetyltransferase (ChAT) and acetylcholinesterases (AChEs)[46,47]. The soma and proximal dendrites of XII motoneurons are covered by a plexus of large puncta expressing a vesicular acetylcholine transporter (VACHT)[45,48–50] and a high-affinity choline transporter[51]. Premotor neurons in the caudal medullary intermed-iate reticular (IRt) region provide inspiratory drive to XII motoneurons[37–39,52]. Although inspiratory drive is mainly glutamatergic, over half of these premotor neurons retro-

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atropine\(^{[69]}\). This area of the rabbit is equivalent to the region we now define as the preBötC in the rat\(^{[32, 33]}\), the cat\(^{[70, 71]}\) and the rabbit\(^{[72, 73]}\), suggesting that the preBötC is a critical area where ACh acts to affect respiratory rhythm.

Bath application of ACh in the en bloc brainstem-spinal cord preparation from neonatal rat in vitro increases the frequency of respiratory rhythmic activity recorded from the 4th or 5th cervical spinal nerve (C4 or C5) ventral roots; this effect is diminished, but not completely abolished, by atropine. The effect of ACh can be completely abolished by further addition of the nicotinic antagonist dihydro-β-erythroidine (DHβE)\(^{[6]}\).

Unilateral microinjection of nicotine into the preBötC in the medullary slice preparation from neonatal rats that generates respiratory rhythm in vitro\(^{[32]}\) increases respiratory frequency and decreases the amplitude of rhythmic motor activity recorded from hypoglossal nerve root (XII). In contrast, nicotine injected into the XII nucleus induces tonic activity and an increase in amplitude but not in frequency of inspiratory bursts from XII. These effects can be blocked by the nicotinic antagonist mecamylamine (Figure 1). The results suggest that nicotine acts on the preBötC modulating respiratory frequency and on the XII nucleus to modulate the amplitude of inspiratory bursts\(^{[28]}\).

Bath application of low concentrations of nicotine (0.2–0.5 µmol/L, as low as the arterial blood nicotine concentration after smoking a cigarette\(^{[74]}\)) to rhythmic slices increases respiratory frequency in a concentration-dependent manner. In voltage-clamped preBötC inspiratory neurons, nicotine induces a tonic inward current associated with an increase in membrane noise, increases the frequency and amplitude of spontaneous EPSCs during the expiratory period as well as decreases the amplitude of phasic inspiratory drive currents. These effects can be blocked by mecamylamine. These results indicate that nicotine differentially modulates excitatory neurotransmission in the preBötC: it enhances tonic excitatory input to, and inhibits excitatory coupling between, preBötC inspiratory neurons\(^{[28]}\). Based on a computational model of rhythmogenesis\(^{[75]}\), these cellular mechanisms can account for the nicotinic effects on respiratory frequency and pattern\(^{[28]}\). Since miniature EPSC analyses have not been performed, pre- and/or post synaptic mechanisms of nicotinic modulation of glutamatergic transmission and modulation of the excitability of preBötC inspiratory neurons remain to be determined. In addition to the classical synaptic transmission, cholinergic modulation of the excitability of preBötC inspiratory neurons and respiratory pattern may act via volume transmission mechanisms\(^{[76]}\).

The α7 nAChR antagonists α-bungarotoxin or methyllycaconitine (MLA) have little effect on the actions of low concentrations of nicotine on preBötC inspiratory neurons and respiratory pattern in the medullary slice preparation. In contrast, DHβE or hexamethonium completely reverse these nicotinic actions, which are also reduced by d-tubocurarine.

![Figure 1. Unilateral microinjection of nicotine into the preBötC increases frequency and decreases amplitude of respiratory-related rhythmic activity. Rhythmic activity was recorded from hypoglossal nerve roots (XII) in the medullary slice preparation in vitro and the signal was integrated. Left panel: Microinjection (↑) of 10 nL 20 µmol/L nicotine into: 1, ipsilateral preBötC; 2, contralateral preBötC; 3, ipsilateral hypoglossal (XII) nucleus and 4, contralateral XII nucleus respectively. Injection pipettes were inserted into the loci 100–200 µm below the surface of the slice. Right panel: bath applied 1 µmol/L mecamylamine (Meca) blocked nicotine-induced responses. Reproduced from reference [28].](Image)
Comparable concentrations of RJR-2403, an agonist selective for α4β2 nAChRs\textsuperscript{[77]}, have effects similar to those of nicotine. MLA has little effect on RJR-2403 actions, while DHβE completely reverses them\textsuperscript{[29]}. These pharmacological characteristics suggest that the predominant subtype of preBötC nAChRs that mediates the modulation of respiratory pattern by low concentrations of nicotine is an α4β2* combination and not an α7 subunit homomer. Results of pharmacological studies\textsuperscript{[78]} using the \textit{en bloc} brainstem-spinal cord preparation from neonatal rats where respiratory motor activity can be recorded from C4 nerve roots are basically consistent with those of slice preparations. DHβE hyperpolarizes and decreases intraburst firing frequency in both inspiratory and preinspiratory (pre-I) neurons in the rostral ventrolateral medulla. MLA has no effects on the membrane potential of inspiratory neurons but hyperpolarizes and decreases intraburst firing frequency in pre-inspiratory neurons\textsuperscript{[78]}. These authors conclude that α4β2 nAChRs mediate cholinergic modulation of both inspiratory and preinspiratory neurons whereas α7 nAChRs are only involved in cholinergic modulation of pre-inspiratory neurons. However, the concentrations of DHβE and MLA (20 µmol/L) used in this study were too high to be pharmacologically specific for nAChR subtypes. Such high concentrations may produce non-specific effects, calling into question their conclusions regarding the role of specific nAChR subtypes.

Transgenic nAChR subunit knock-out and knock-in mice provide a powerful approach to determine the molecular composition of nAChRs and their role in CNS functions. A knock-in mouse strain with a leucine to alanine mutation in the M2 pore-lining region (L9'A) of the nAChR α4 subunit renders α4-containing receptors hypersensitive to agonists\textsuperscript{[79]}. In homozygous L9'A knock-in mice compared with wild-type mice, nicotine affects respiratory rhythm at ~100-fold lower concentrations. Figure 2 shows that nicotine at low nanomolar concentrations (5 nmol/L, bath applied) depolarizes preBötC inspiratory neurons and increases respiratory frequency in L9'A mouse slices; these effects are blocked by DHβE (0.2 µmol/L). Responses of preBötC inspiratory neurons of L9'A mice to nicotine at low nanomolar concentrations are similar to those in wild-type mice at micromolar concentrations. These responses include: i) tonic inward current associated with an increase in membrane noise; ii) decrease in phasic inspiratory drive current; iii) increase in frequency and amplitude of spontaneous EPSCs, and; iv) increase in respiratory frequency and tonic/seizure-like activity in XIl nerve output. These effects can be blocked by the α4* nAChR selective antagonist DHβE. These data showing nicotine hypersensitivity of nAChRs in preBötC inspiratory neuron in L9'A mouse indicate that these nAChRs contain α4 subunits. Nicotine facilitates glutamatergic neurotransmission and increases the excitability of preBötC inspiratory neurons via α4* nAChRs\textsuperscript{[80]}. In resting conditions (without nicotine), the respiratory frequency of L9'A mouse slices is higher than that of wild-type slices suggesting that the preBötC nAChRs are hypersensitive to endogenously released ACh\textsuperscript{[81]}. Unilateral microinjection of 50 nmol/L nicotine into the preBötC increases respiratory frequency. Microinjection of nicotine into the XII nucleus induces tonic/seizure-like activity in the ipsilateral XIl. Bath application of DHβE blocks these effects. These results suggest that functional α4* nAChRs are

![Figure 2](https://www.nature.com/aps/shao_et_al)
present in both the preBötC and the XII nucleus. However, the effects of activating these receptors differ: α4* nAChRs in the preBötC mediate ACh/nicotinic modulation of respiratory rhythm, and α4* nAChRs in the XII nucleus mediate ACh/nicotine modulation of tonic activity in XIIIn[80].

Inhibition of AChEs by physostigmine produces both muscarinic and nicotinic effects in the medullary slice preparation. Physostigmine enhances the excitability of preBötC inspiratory neurons, induces tonic activity and an increase in frequency, amplitude and duration of inspiratory bursts of XIIIn motor output. This suggests that endogenous ACh modulates excitatory neurotransmission and the excitability of preBötC respiratory neurons that, in turn, regulates respiratory frequency and pattern[82].

Activation of nAChRs in respiratory motor nuclei modulates the pattern of rhythmic and tonic activity of motor output

Respiratory motoneurons are cholinergic. They release ACh at neuromuscular junctions. Respiratory drive transmitted to these motoneurons is glutamatergic, mediated primarily by non-NMDA receptors[83, 84]. Respiratory motoneurons also receive cholinergic inputs[40–42, 45, 48–51] that play modulatory role.

Motoneurons in the XII nucleus innervate the tongue and upper airway muscles[85, 86]. They have respiratory-related rhythmic activity and play an important role in regulating upper airway resistance and patency[87]. Activation of nAChRs in XII motoneurons excites these neurons[28, 88–90]. In the medullary slice preparation from neonatal rats in vitro, the nicotinic agonist, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) induces an inward current associated with an increase in membrane conductance in voltage-clamped XII motoneurons in the presence of TTX and atropine. This current is concentration-dependent (Figure 3) and blocked by DHβE. These results suggest that cholinergic/nicotinic modulation of the excitability of XII motoneurons is via post- and/or extra-synaptic nAChRs that do not contain the α7 subunit[89]. Microinjection of low nanomolar concentrations of nicotine into the XII nucleus in medullary slices from the mutant nAChR a4 subunit L9’ A mice induces tonic/seizure-like activity in the XIIIn. These effects are blocked by DHβE[80]. These results are consistent with Zaninetti et al (1999)[88] and Chamberlin et al (2002)[89] and suggest that the nAChRs in the XII nucleus contain α4 subunits.

In a medullary slice preparation, bath application of 0.5 µmol/L nicotine decreases the frequency, but not the amplitude, of glutamatergic miniature EPSCs in XII motoneurons. Further time course analysis suggests that activation of presynaptic nAChRs usually facilitates glutamatergic synaptic transmission to XII motoneurons and then depresses it probably via receptor desensitization[60].

In anesthetized, vagotomized adult rats in vivo, microdialysis of the nicotinic receptor agonist DMPP into the XII nucleus increases tonic and respiratory-related activity of the genioglossus muscle[91]. These studies suggest that nAChRs can be a potential therapeutic target for treatment of obstructive sleep apnea[92, 93] that involves sleep-related loss of tone in the genioglossus muscle.

Studies on cholinergic regulation of phrenic motoneurons are scarce. Intrathecal injection of ACh at the C4 spinal segment increases the inspiratory activity of the phrenic nerve in anesthetized, immobilized and vagotomized rabbits[94]. However, microinjection of carbachol into the phrenic nucleus decreases the inspiratory activity of the

Figure 3. 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP, a nicotinic agonist) produces inward currents that are dose-dependent and associated with an increase in membrane conductance in XII motoneurons. (A) An inward current in a voltage-clamped (-60 mV) XII motoneuron induced by bath-applied 25 µmol/L DMPP (bar). *I/V curve was measured during the break in record. (B) Peak amplitudes of DMPP-induced current (I_{DMPP}) as a function of concentration. Atropine (1 µmol/L) was presence in the bath solution for (A) and (B). Reproduced from reference [89].
phrenic nerve in anesthetized, immobilized rats\textsuperscript{[95]}. These apparently contradictory observations may be due to species differences. Both the excitatory effects\textsuperscript{[94]} and inhibitory effects\textsuperscript{[95]} can be blocked by muscarinic ACh receptor antagonists. The functional significance of the nAChRs in the phrenic nucleus revealed by anatomical approaches\textsuperscript{[58]} awaits investigation.

**Effects of perinatal nicotine exposure on neonatal respiratory control**

SIDS is a leading cause of infant death between one month and one year of age, resulting in 0.55 deaths per 1000 live births in the United States\textsuperscript{[96]}. The cause of SIDS is unknown. Impaired cardiorespiratory control and arousal responsiveness are hypothesized to be important mechanisms\textsuperscript{[97, 98]}. Prone sleeping position and perinatal exposure to cigarette smoke are the leading risk factors for SIDS. Since the successful “back to sleep” campaign, cigarette smoke exposure during pre- and postnatal life has become the principal risk factor for SIDS\textsuperscript{[99, 100]}.

Maternal smoking is associated with a trend of increased nicotine receptor binding in the brain in control groups of infants. However in SIDS infants (infants that died of SIDS) who were exposed to maternal smoking in utero, upregulation of nAChRs is absent in three brainstem nuclei related to arousal and cardiorespiratory control: the Nucleus Parabrachialis Lateralis, Locus Coeruleus and Nucleus Pontis Oralis\textsuperscript{[101]}. These results suggest that altered development of nAChRs in brainstem cardiorespiratory and/or arousal circuits put some infants, i.e. those exposed to cigarette smoke in utero, at risk for SIDS.

In animal studies, prenatal exposure to nicotine affects neuronal development and upregulates nAChRs in the brain\textsuperscript{[102–105]}. Prenatal exposure to nicotine impairs protective responses to hypoxia in an age-dependent manner in rat pups\textsuperscript{[106]}. Prenatal nicotine exposure alters the postnatal development of the ventilatory pattern and increases frequency of apneas\textsuperscript{[107]}. Newborn mice prenatally exposed to nicotine exhibit unstable breathing and impaired arousal. Remarkably similar deficits are detected in pups lacking β2-containing nAChRs. Loss-of-function of these nAChRs reproduces many of the abnormailities caused by perinatal nicotine exposure suggesting that nicotine’s detrimental side effects on a range of crucial defensive reflexes involve loss of function of nAChR subtypes, possibly via activity-dependent desensitization\textsuperscript{[108]}.

Prenatal nicotine exposure delays early postnatal changes in breathing pattern in the neonatal mice. During normoxia, neonatal mice exhibit a high frequency of apnea [f(A)] that declines by postnatal day 3 (P3) in control animals, but persists in P3 nicotine-exposed mice. Hypoxia induces a rapid and sustained reduction in f(A). During recovery, f(A) increases above control levels. By P3 this increase is reduced in control but persists in nicotine-exposed mice. Nicotine locally applied over the XII motor nucleus in medullary slices from control neonatal mice *in vitro* increases tonic discharge and potentiates inspiratory burst amplitude in XII. In slices from perinatally nicotine-exposed mice, these effects are significantly reduced (Figure 4) suggesting that prenatal exposure to nicotine reduces cholinergic/nicotinic modulation of XII motoneurons\textsuperscript{[90]}.

Bath application of the GABA\textsubscript{A} receptor agonists muscimol or pentobarbital to the brainstem of a brainstem-spinal cord preparation from neonatal rats decreases the frequency of respiratory activity recorded from the C4 ventral roots. This decrease in frequency is greater in perinatally nicotine-exposed rats compared to control rats. These results suggest that prenatal nicotine exposure potentiates GABA\textsubscript{A} receptor-mediated inhibition of respiratory rhythm in neonatal rats\textsuperscript{[109]}. Microinjection of glycine or muscimol into the

![Figure 4](Image)
preBötC of the brainstem-spinal cord preparation from neonatal rats causes abrupt, reversible apnea. Prenatal nicotine exposure prolongs the apnea duration induced by glycine or muscimol suggesting that prenatal nicotine exposure alters development of GABAergic and glycinergic inhibitory transmission in medullary regions involved in central respiratory control, and that neurons in the preBötC are involved\textsuperscript{[110]}. Prenatal nicotine exposure reduces the nicotine-induced increase in respiratory frequency recorded from the C4 ventral root in the en bloc brainstem-spinal cord preparation in neonatal rats suggesting that prenatal nicotine exposure diminishes nAChR-mediated excitation in medullary regions involved in the control of breathing frequency, particularly the preBötC\textsuperscript{[111]}. In neonatal lambs with prenatal exposure to nicotine, the ventilatory response to hypoxia (10\% O\(_2\)) is similar to controls during wakefulness; however, the ventilatory response to hypoxia during quiet sleep is reduced. Prenatal nicotine exposure also delays arouse from sleep. These results suggest that prenatal nicotine exposure blunts major elements of the cardiorespiratory defense to hypoxia\textsuperscript{[112]}.

**Conclusion**

Our understanding regarding neurochemical control of respiration advanced rapidly in last two decades. Genetically engineered knock-out and knock-in mice provide powerful tools for analyzing the molecular composition of native nAChRs and the role of specific subtypes of receptors in regulation of respiration. Development of in vitro models such as the en bloc brainstem-spinal cord and the medullary slice preparations enables us to bridge gaps in our knowledge between molecular and cellular events and respiratory behavior. Recent studies have led to understanding of the role \(\alpha_4^+\) nAChRs in the preBötC and XII nucleus plays in regulation of respiratory frequency and pattern as well as provided insight into the underlying cellular mechanisms of modulation of neurotransmission and neuronal excitability. There are several prevalent neurological disorders related to central control of breathing such as sleep apnea and SIDS where nAChRs may play a role. Nicotine from maternal smoking (in fact, any environmental tobacco smoke) may predispose newborns to SIDS, and obstructive sleep apnea may involve sleep-related cholinergic modulation of upper airway muscle tone. Insight into the molecular and cellular mechanisms of cholinergic regulation of respiration and of the nicotine actions on development of neonatal central control of breathing will provide a pharmacological basis for prevention and treatment of these disorders.

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