Nicotinic Receptor Activation Occludes Purinergic Control of Central Cardiorespiratory Network Responses to Hypoxia/Hypercapnia

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Abstract

Prenatal nicotine exposure alters the cardiorespiratory network responses to hypoxia/hypercapnia; however the mechanism(s) responsible for these cardiorespiratory network responses and their alteration by prenatal nicotine exposure are unknown. We used an in vitro medullary slice that allows simultaneous examination of rhythmic respiratory-related activity and excitatory synaptic neurotransmission to cardioinhibitory vagal neurons (CVNs). Respiratory related increases in glutamatergic neurotransmission only occurred upon recovery from hypoxia/hypercapnia in unexposed animals. These responses were not altered by nicotinic antagonists but were mediated in part by activation of P2 purinergic receptors. Prenatal nicotine exposure transformed central cardiorespiratory responses to hypoxia/hypercapnia; CVNs received a respiratory related glutamatergic neurotransmission during periods of hypoxia and hypercapnia, while increases in glutamatergic neurotransmission during recovery were absent. The excitatory neurotransmission to CVNs during hypoxia/hypercapnia in prenatal nicotine exposed animals were wholly dependent upon nicotinic receptor activation. In the presence of nicotinic antagonists the responses in prenatal nicotine animals reverted to the pattern of responses in unexposed animals in which an increase in glutamatergic neurotransmission occurred not during, but only upon recovery from hypoxia/hypercapnia, and this recruited excitatory pathway was blocked by P2 receptor antagonists. These data identify a new functional role for purinergic receptors in the cardiorespiratory responses to hypoxia/hypercapnia and their role in occluding nicotinic receptor activation with prenatal nicotine exposure.

Key Words: Nucleus Ambiguus; ATP; Nicotine
Introduction

Heart rate is principally controlled by the activity of premotor parasympathetic cardioinhibitory vagal neurons (CVNs) within the medulla (Loewy and Spyer 1990), and the activity of CVNs is intricately linked to respiratory function. For example, respiratory sinus arrhythmia, in which heart rate increases during each inspiration to optimize blood flow to the lungs, is mediated by increases in inhibitory GABAergic and glycinergic neurotransmission to CVNs during inspiration (Neff et al. 2003). The increase in GABAergic, but not glycinergic neurotransmission to CVNs during inspiratory activity is dependent upon the activation of nicotinic receptors (Neff et al. 2003).

Hypoxia elicits a parasympathetically mediated bradycardia, which decreases metabolic demands on the heart and increases survival (Guntheroth and Kawabori 1975). The biphasic respiratory and heart rate response to hypoxia is paralleled by a biphasic change in inhibitory neurotransmission to CVNs (Neff et al. 2004). However, exposure to prenatal nicotine can alter the nicotinic modulation of CVN activity, and significantly alter the responses of the cardiorespiratory network to physiological challenges such as hypoxia and/or hypercapnia (Evans et al. 2005; Huang et al. 2005; Neff et al. 2004).

The purine nucleotide adenosine 5’-triphosphate (ATP) is a clearly identified neurotransmitter within the central nervous system. Purinergic receptors are present in key central cardiovascular and respiratory control centers such as the nucleus of the solitary tract (NTS), rostroventrolateral medulla, ventral respiratory group, and hypoglossal nucleus (Collo et al. 1996; Gourine and Spyer 2003). ATP is released synaptically to mediate both pre- and postsynaptic effects at
ionotropic P2X and/or metabotropic P2Y cell surface receptors (Jo and Schlichter 1999; Ralevic and Burnstock 1998; Robertson and Edwards 1998; Zhang et al. 2000). Purinergic signaling is important in respiratory network interactions; ATP release in the ventral medulla during hypoxia and hypercapnia facilitates central chemoreception and respiratory network plasticity (Gourine et al. 2005a, b).

Although purinergic receptors are present within the medulla and contribute to respiratory control, very little is known regarding the purinergic contribution to respiratory modulation of parasympathetic cardiac neurons or their role in the responses to hypoxia/hypercapnia in brainstem parasympathetic cardiac neurons. Recent work also indicates P2X and nicotinic acetylcholine receptors (nicotinic receptor) exhibit mutual occlusion, and suggests P2X and nicotinic receptors form heterooligomers within the plasma membrane, effecting cross-inhibition when one or both receptors are activated (Khakh et al. 2005; Khakh et al. 2000). In this study we examined the cardiorespiratory network responses in an in-vitro brainstem preparation to a mimicked apneic event by decreasing oxygen and increasing carbon dioxide levels in the perfusate, and tested whether P2X and nicotinic receptors play an opposing role in the recruitment of excitatory synaptic neurotransmission to cardiac vagal neurons in response to hypoxia/hypercapnia, and whether prenatal nicotine exposure alters this competition.
Materials and Methods

Preparation

CVNs were identified in a thick medullary slice that generates rhythmic respiratory-related motor discharge in hypoglossal cranial nerves by injecting rhodamine (XRITC, Invitrogen) into the fat pads at the base of the heart 1-5 days prior to sacrifice as described previously (Bouairi et al. 2006; Evans et al. 2005; Huang et al. 2006; Huang et al. 2005; Neff et al. 2004; Neff et al. 2003; Wang et al. 2003a). This slice was placed in a recording chamber that allowed perfusion (4 mL/min) with artificial cerebrospinal fluid (in mM: 125 NaCl, 3 KCl, 2 CaCl$_2$, 26 NaHCO$_3$, 5 glucose, 5 HEPES, equilibrated with 95% O$_2$, 5% CO$_2$, pH 7.35-7.4). Slices were maintained at 21°C. Spontaneous respiratory-related activity was recorded by monitoring motoneuron population activity from hypoglossal nerve rootlets using a suction electrode. Hypoglossal rootlet activity was amplified 50,000 times and filtered (10-300 Hz bandpass; CWE, Ardmore, PA) and electronically integrated ($\tau$ = 50 msec; CWE). All animal procedures were performed with the approval of the Animal Care and Use Committee of The George Washington University in accordance with the recommendations of the panel on euthanasia of the American Veterinary Medical Association and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patch-clamp Techniques

CVNs in the external formation of the nucleus ambiguus were identified by the presence of the fluorescent tracer and studied using patch clamp techniques as described in detail previously (Bouairi et al. 2006). Pipettes were filled with a solution containing (in mM) 135 K-
gluconic acid, 10 HEPES, 10 EGTA, 1 CaCl₂, and 1 MgCl₂, at a pH of 7.35–7.4. All synaptic activity in CVNs was recorded at -80 mV. Only one experiment was performed per preparation.

Focal drug application was performed using a pneumatic picopump pressure delivery system (WPI, Sarasota, FL). Drugs were continuously ejected from a patch pipette positioned within 30 μm of the patched CVN. The maximum range of drug application has been previously determined to be 100 to 120 μm downstream from the drug pipette and considerably less behind the drug pipette. Glutamatergic neurotransmission was isolated by focal application of strychnine hydrochloride (1μM) and gabazine (25 μM) to block glycine and GABA receptors, respectively. In some experiments, α-bungarotoxin (100 nM), dihydro-beta-erythroidine (DHβE) 3 or 100 μM were included to block α7 nicotinic receptors, α4β2 nicotinic receptors, or all nicotinic receptors, respectively (Alkondon and Albuquerque 1993). In some experiments the broad P2 purinergic receptor antagonist suramin (100 μM) or pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS, 100 μM) were included in the drug pipette. All drugs were obtained from Sigma.

**ATP Application**

To examine miniature EPSCs, gabazine (25 μM) and strychnine (1 μM) were included in the perfusate to block GABA and glycine receptors. In addition, TTX (1 μM) was added to the perfusate to block voltage-gated sodium channels. ATP-Na⁺ salt (1 mM) was dissolved in aCSF and focally applied to whole-cell patch clamped CVNs. Nicotine (10 μM) was subsequently added to the perfusate.
**Hypoxia/Hypercapnia**

Rhythmic inspiratory-related activity glutamatergic EPSCs in a single CVN were recorded simultaneously for four minutes in control artificial cerebrospinal fluid (in mM: 125 NaCl, 3 KCl, 2 CaCl\(_2\), 26 NaHCO\(_3\), 5 glucose, 5 HEPES, equilibrated with 95% O\(_2\), 5% CO\(_2\), pH 7.35-7.4). Hypoxia/hypercapnia was induced by changing the control perfusate to an identical solution bubbled with 9% CO\(_2\), 6% O\(_2\), 85% N\(_2\), and readjusted back to 7.35-7.4 immediately prior to use. Slices were exposed to hypoxia/hypercapnia for ten minutes, and then returned to the original perfusate for thirty minutes. At the end of each experiment, glutamatergic synaptic activity was reversibly inhibited using D-2-amino-5-phosphonovalerate (AP5, 50 \(\mu\)M) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 50 \(\mu\)M) to block N-methyl-D-Aspartate (NMDA) and non-NMDA receptors.

**Prenatal Nicotine Exposure**

Adult female rats were anesthetized with ketamine-xylazine (87/13 mg/kg, i.p.; Phoenix Pharmaceuticals, St. Joseph, MO) on the third day of gestation and implanted subcutaneously with Alzet osmotic minipumps (Durect, Cupertino, CA) containing (−) nicotine (56.1 mg/ml bacteriostatic saline; Sigma). Osmotic minipumps were chosen to avoid the high plasma nicotine concentrations and subsequent episodic fetal hypoxia-ischemia that can be produced by nicotine injections (Slotkin 1998). Pumps delivered nicotine at 6 mg/kg/day to produce a blood nicotine concentration approximately equivalent to those that occur in moderate to heavy smokers (ie 30-40 ng/ml), for 28 days (Benowitz et al. 1982; Isaac and Rand 1972; Slotkin 1998).
Data Analysis

Analysis of spontaneous synaptic currents was performed using MiniAnalysis (version 5.6.12, Synaptosoft) with minimal acceptable amplitude set at the amplitude at which AP5 and CNQX blocked all synaptic events. The frequency of EPSCs that occurred in CVNs was grouped into 1-s bins and cross-correlated with the onset of inspiratory–related hypoglossal activity. Data were analyzed from all bursts during the last two minutes of the control period, the last two minutes of hypoxia/hypercapnia, and from ten to twelve minutes during the recovery. These periods were chosen for analysis because during these periods, synaptic activities were consistent and any hypoxia/hypercapnia-evoked changes reached a steady state. Burst duration, frequency and amplitude were measured using pClamp 7 software (Axon Instruments, Union City, CA) and from the filtered (10-300 Hz band pass) and electronically integrated hypoglossal rootlet activity. mEPSC frequency was analyzed for 10s before and after ATP application and for 10s one minute following ATP application. Results are presented as mean +/- SEM. Statistical comparisons were performed using ANOVA with repeated measures to examine the responses throughout the time course of the experiments and two-way ANOVA when comparing the results from different series of experiments, such as between control animals and animals that were exposed to nicotine prenatally. Significant difference was set at p<0.05.
Results

Effect of Hypoxia/Hypercapnia on Respiratory Activity

We employed an in vitro brainstem slice that retains spontaneous respiratory-related rhythms and allows simultaneous whole cell recordings of CVNs, the dominant control of heart rate (Blitz and Ramirez 2002; Feldman and Gray 2000; Smith et al. 1991). As previously reported (Huang et al. 2005) hypoxia/hypercapnia evoked a biphasic change in central respiratory activity. Hypoxia/hypercapnia elicited a transient increase, followed by a decrease in respiratory frequency, see fig 1. In addition, hypoxia/hypercapnia significantly depressed (p < 0.01) respiratory burst duration. Hypoxia and hypercapnia induced a small but significant increase in the amplitude of respiratory bursts, (p < 0.01). The responses in animals exposed to prenatal nicotine closely mimicked and were not significantly different from the responses in unexposed animals, see Figure 1.

Effect of Nicotinic Acetylcholine Receptor Antagonists during Hypoxia/Hypercapnia

CVNs exhibit no respiratory modulation of excitatory neurotransmission under control conditions (Fig 2; n = 7; p > 0.05), nor during hypoxia/hypercapnia (p > 0.05). However, upon recovery from hypoxia/hypercapnia there is a significant respiratory-related increase in excitatory neurotransmission (Fig. 2; p < 0.01). These responses persisted for at least 48 minutes post hypoxia-hypercapnia.

Endogenous activation of nicotinic acetylcholine receptors (nicotinic receptor) modulates CVN activity by enhancing inhibitory and excitatory neurotransmission both pre- and
postsynaptically (Neff et al. 1998a; Neff et al. 2003). To determine if nicotinic acetylcholine receptors (nicotinic receptor) also mediate excitatory synaptic neurotransmission to CVNs following hypoxia/hypercapnia, nicotinic antagonists were focally applied to CVNs throughout the entire experiments. Application of \(\alpha\)-bungarotoxin (100 nM) had no effect on excitatory inputs to CVNs during hypoxia/hypercapnia, nor during the recovery period, see figure 3 (p > 0.05; n = 7). Similarly, 3 \(\mu\)M DH\(\beta\)E, a concentration selective for \(\alpha4\beta2^*\) nicotinic receptors, failed to alter glutamatergic neurotransmission in CVNs throughout hypoxia/hypercapnia or during recovery (n = 9; p > 0.05), see figure 3D. Inhibition of all nicotinic receptors with 100 \(\mu\)M DH\(\beta\)E (n = 11) likewise had no effect on excitatory inputs to CVNs during hypoxia/hypercapnia administration, nor during recovery (figure 3E; p > 0.05).

**Effect of Purinergic Antagonists on Hypercapnia/Hypoxia-evoked responses**

Because P2 receptors facilitate central cardiorespiratory network responses to hypoxia, we hypothesized that respiratory-related glutamatergic neurotransmission to CVNs upon recovery from hypoxia/hypercapnia is mediated by ATP receptors. To test this hypothesis we focally applied the broad purinergic antagonist, suramin (25 or 100 \(\mu\)M) throughout the experiments, or in the case of PPADS (100 \(\mu\)M) after a delay in the hypoxia/hypercapnia period. 25 \(\mu\)M suramin significantly depressed the respiratory-related glutamatergic inputs to CVNs during recovery from hypoxia, see fig 4B (n = 8, p < 0.05, from a peak of 8.9 ± 0.4 Hz, to 7.1 ± 0.3 Hz, a 19.8% decrease from vehicle control). 100 \(\mu\)M suramin further reduced respiratory-related EPSCs in CVNs during recovery from hypoxia/hypercapnia (figure 4C; n = 7; p < 0.05, from a peak of 8.9 ± 0.4 Hz, to 6.2 ± 0.3 Hz, a 30.4% decrease from vehicle control).
In addition to using the broad purinergic antagonist, suramin, we also examined the responses to continuous and focal application of the P2 receptor antagonist PPADS (100 µM). As shown in figure 5, during recovery from hypoxia/hypercapnia there is a significant respiratory-related increase in excitatory neurotransmission to cardiac vagal neurons. This respiratory related increase is diminished, but not abolished, by subsequent focal application of PPADS (figure 5, n=7, PPADS attenuated the response by 30.6 ± 5.2 %, p<0.005).

**Purinergic Facilitation of mEPSCs in CVNs**

Nicotinic and purinergic receptors can exhibit cross-inhibition (Khakh et al. 2000). To test whether nicotinic receptor activation blocks P2 receptor facilitation of glutamatergic neurotransmission, miniature EPSCs (mEPSC) were isolated by inclusion of gabazine (25 µM), strychnine (1 µM) and TTX (1 µM) in the perfusate. Focal application of ATP (1 mM) significantly increased mEPSC frequency in CVNs (Fig. 6; n = 9; p < 0.05). However in the presence of nicotine (10 µM) ATP failed to significantly alter mEPSC frequency (p > 0.05). In animals exposed to prenatal nicotine, ATP also significantly increased mEPSC frequency (n = 7; p < 0.05), which was abolished in the presence of nicotine (10 µM; p > 0.05).

**Contribution of nicotinic receptors to Hypoxia/Hypercapnia-evoked responses in Animals Exposed to Nicotine Prenatally**

In contrast to unexposed animals, in prenatal nicotine exposed animals, CVNs receive a respiratory-related glutamatergic neurotransmission during hypoxia/hypercapnia but not upon recovery from hypoxia/hypercapnia, see figure 7A. To test whether this transformation results from nicotinic receptor activation, we focally applied nicotinic receptor antagonists. Application
of 3 µM DHβE to block α4β2* receptors (n=8) did not alter respiratory-evoked excitatory inputs to CVNs during, nor upon recovery from hypoxia/hypercapnia (Fig. 7B; p>0.05). Focal application of the α7 nicotinic receptor antagonist α-bungarotoxin (100 nM) partially depressed respiratory-related excitatory inputs evoked during hypoxia/hypercapnia (n = 11), without altering glutamatergic neurotransmission during recovery (Fig. 7C). Application of 100 µM DHβE to block all nicotinic receptors (n = 11) ablated respiratory-related excitatory neurotransmission during hypoxia/hypercapnia (Fig. 8A,B) and restored a respiratory-evoked glutamatergic neurotransmission during recovery as in unexposed animals.

Because in unexposed animals respiratory-related glutamatergic inputs are partially modulated by P2 receptors, and nAch and P2 receptors can exhibit cross-inhibition, we reasoned that prenatal nicotine-evoked modifications in nicotinic receptor activity preclude purinergic facilitation of glutamatergic neurotransmission to CVNs. To test whether restored respiratory-related excitatory inputs to CVNs were facilitated by P2 receptors, we coapplied DHβE (100 µM) and suramin (100 µM) in preparations from prenatal nicotine-exposed pups (n=7). Suramin blocked the restored glutamatergic neurotransmission to CVNs during recovery from hypoxia/hypercapnia (Fig. 8C).

Nearly identical results were obtained with continuous and focal application of the P2 receptor antagonist PPADS (100 µM). As shown in figure 9, in prenatal nicotine exposed animals continuous application of DHβE (100 µM) ablated respiratory-related excitatory neurotransmission during hypoxia/hypercapnia (Fig. 9 A,B) and restored a respiratory-evoked glutamatergic neurotransmission during recovery as in unexposed animals. This respiratory-
evoked glutamatergic neurotransmission during recovery was abolished by focal application of the P2 receptor antagonist PPADS (100 µM, n=7).
Discussion

This study has two main conclusions: (1) In unexposed animals respiratory-related excitatory neurotransmission to CVNs recruited during recovery from hypoxia/hypercapnia is not mediated by nicotinic acetylcholine receptors, but is mediated in part by P2 purinergic receptor activation. (2) Prenatal nicotine exposure exaggerates respiratory-related excitation of CVNs by recruiting a nicotinic receptor dependant excitation of CVNs during hypoxia/hypercapnia and repressing respiratory-related increases in glutamatergic neurotransmission during recovery from hypoxia/hypercapnia. This transformation occurs by precluding normal activation of P2 purinergic receptors by nicotinic receptor activation.

Similar to studies both *in vivo* and *in vitro*, our data indicate hypoxia/hypercapnia evokes a biphasic response in the central respiratory network; respiratory bursts exhibited a transient increase followed by a secondary decrease in frequency and acquired a gasp-like quality characterized by high amplitude bursts of short duration. Prenatal nicotine exposure elicited no significant changes in the central respiratory responses to hypoxia/hypercapnia. However, some other studies have reported significant changes in cardiorespiratory integrity following prenatal nicotine exposure. Maternal cigarette smoking has been reported to have no effect on ventilatory responses to hypoxia or hypercapnia (Lewis and Bosque 1995) whereas in other work prenatal nicotine exposure diminished the hypoxic ventilatory responses and respiratory drive (Ueda et al. 1999). Similarly it is reported that prenatal nicotine exposure both does not alter (Bamford et al. 1996; Schuen et al. 1997; Slotkin et al. 1997) and diminishes the ventilatory response to hypoxia or hypercapnia (Hafstrom et al. 2002a, b; Simakajornboon et al. 2004). The results from this study indicate that prenatal nicotine exposure does not alter central respiratory responses to hypercapnia and suggests any potential changes in the ventilatory responses to prenatal nicotine
in vivo may depend on changes in the activity of peripheral chemoreceptors rather than within the medulla (Bamford et al. 1999; Holgert et al. 1995; Simakajornboon et al. 2004).

It is somewhat surprising that nicotinic receptors do not mediate respiratory-related excitatory neurotransmission to CVNs in unexposed animals. CVNs receive tonic endogenously active cholinergic input that mediates both excitatory and inhibitory neurotransmission (Neff et al. 1998a; Wang et al. 2003b). Furthermore, \( \alpha 4 \beta 2^* \) nicotinic receptors mediate inspiratory-evoked GABAergic neurotransmission to CVNs responsible for respiratory sinus arrhythmia (Neff et al. 2003). However, excitatory neurotransmission to CVNs was not altered by nicotinic antagonists under control conditions, hypoxia/hypercapnia, nor during recovery. Although activation of nicotinic receptors is one mechanism by which glutamatergic neurotransmission is facilitated to CVNs, this input does not mediate the respiratory-related enhancement of excitatory neurotransmission during recovery from hypoxia/hypercapnia.

Whereas nicotinic receptor antagonists did not alter excitatory neurotransmission to CVNs, both of the P2 receptor antagonists suramin and PPADS inhibited respiratory-related increases in glutamatergic neurotransmission during recovery from hypoxia/hypercapnia. Because suramin and PPADS were focally applied to CVNs, ATP is acting on local P2 receptors to enhance the release of glutamate. Recent work has shown P2X, but not P2Y receptor agonists can facilitate glutamatergic neurotransmission to cardiac vagal neurons (Griffioen et al. 2007). Focal application of the selective P2X agonist \( \alpha, \beta \)-methylene ATP but not the P2Y agonists UTP and adenosine 5'-0-(Z-thiodiphosphate) facilitates glutamatergic EPSCs in CVNs, demonstrating P2X but not P2Y receptors are localized on glutamatergic synaptic terminals on CVNs and can enhance excitatory neurotransmission to CVNs (Griffioen et al. 2007).
However, whether P2X, as well as P2Y receptor activation at distant sites also contributes to glutamatergic neurotransmission to CVNs is unknown. Purinergic neurotransmission plays a role in respiratory neuron activity during hypoxia and hypercapnia (Gourine et al. 2005b), hypoglossal motoneuron output and NTS activation (Funk et al. 1997; Shigetomi and Kato 2004). Therefore, in addition to local P2 receptor facilitation of glutamatergic neurotransmission, P2 receptor activation in other cardiorespiratory control sites outside of the influence of the applied drug in this study may also contribute to the enhancement of excitatory neurotransmission to CVNs.

ATP facilitated the presynaptic release of glutamate in both unexposed and prenatal nicotine exposed animals. However this facilitation is prevented in the presence of nicotine. Recent work has suggested cross-talk occurs between nicotinic and purinergic receptors leading to occlusion of purinergic receptor function. As a result of physical receptor coupling, α4β2* nicotinic receptors and P2X2 receptors physically interact and effect mutual cross-inhibition when coactivated (Khakh et al. 2005; Khakh et al. 2000). In the dorsal motor nucleus of the vagus, ATP and nicotinic receptors colocalize (Nabekura et al. 1995). In myenteric neurons P2X and nicotinic receptors are mutually inhibitory (Zhou and Galligan 1998). Further, in sympathetic neurons of the celiac ganglia nicotine occludes ATP currents (Searl et al. 1998). The mechanisms for this cross-inhibition are unknown. In addition to physical receptor coupling another possibility is that the inability of ATP to increase mEPSC frequency following nicotine application is due to nicotine mediated depletion of vesicles. It is possible the prior exposure of nicotine released all or most vesicles in the presynaptic terminal leaving subsequent application of ATP ineffective due to depletion of the vesicle pool.
In addition to physical receptor coupling or vesicle depletion, purinergic and nicotinic receptors could exhibit mutual occlusion by competitively increasing calcium in the presynaptic terminal (Boehm 1999). P2X receptors are nonselective cation channels with equal permeability to potassium and sodium, and a significant permeability to calcium (Evans et al. 1996). Activation of P2X receptors is reported to facilitate neurotransmitter release by direct calcium entry through P2X receptors (Khakh and Henderson 1998), or through activation of voltage gated calcium channels (Gu and MacDermott 1997). Therefore, P2X receptors may facilitate glutamate release by directly mediating presynaptic calcium entry or alternatively, through depolarizing the presynaptic terminal to open voltage gated calcium channels that then elicit glutamate release. Similarly, nicotinic receptor activation facilitates glutamate release onto cardiac vagal neurons, and this increase in glutamatergic neurotransmission occurs via activation of voltage-dependent calcium channels, especially the calcium channels sensitive to agatoxin IVA (Neff et al. 1998b; Wang et al. 2001). The data in this study which shows an increase in glutamatergic release with nicotine prevents any further increase with ATP, in both unexposed and nicotine treated animals, suggest that, as in other systems, nicotinic and purinergic receptors present within the nucleus ambiguus functionally compete, and that nicotinic receptor activation occludes purinergic signaling at the presynaptic glutamatergic synaptic terminal. Further work will be necessary to elucidate the mechanisms, such as direct receptor interactions or competition for calcium channel activation for this cross-inhibition of nicotinic and purinergic receptors.

In unexposed animals there is a burst of GABAergic neurotransmission to CVNs during inspiratory activity, and this respiratory related increase in GABAergic neurotransmission is dependent upon activation of α4β2* nicotinic receptors (Neff et al. 2004). This respiratory related GABAergic neurotransmission to CVNs is exaggerated by prenatal nicotine exposure
(Neff et al. 2004). However, instead of exaggerating an already existing neurotransmission, prenatal nicotine exposure generates a novel excitatory synaptic input not present in unexposed animals. Prenatal nicotine exposure recruits an excitatory neurotransmission to CVNs during hypoxia/hypercapnia and prevents respiratory-related increases in glutamatergic neurotransmission upon recovery from hypoxia/hypercapnia (Huang et al. 2005). Further, nicotinic receptor antagonists restore prenatal nicotine responses to that of unexposed animals. Therefore, prenatal nicotine exposure evokes a dual modification of cardiorespiratory responses to hypoxia/hypercapnia, and causes cardiorespiratory responses not normally under nicotinic control to rely on nicotinic receptor activation.

The changes in glutamatergic neurotransmission evoked by prenatal nicotine exposure likely results from alterations in nicotinic receptor expression and/or activation. The glutamatergic neurotransmission evoked during hypoxia/hypercapnia is mediated by multiple nicotinic receptors, as α-bungarotoxin blunted and 100 µM DHβE completely blocked this input. Previous work has shown that prenatal nicotine exposure alters the types and location of nicotinic receptors mediating excitatory neurotransmission to CVNs (Huang et al. 2004). The results from this study indicate glutamatergic neurotransmission evoked during hypoxia/hypercapnia is mediated in part by α7, but not α4β2* nicotinic receptors. This is consistent with previous work that shows that α4β2* nicotinic receptors are not involved in glutamatergic neurotransmission in CVNs, and that α7 partially mediates responses to nicotine (Huang et al. 2004). Further, while in unexposed animals α7 nicotinic receptors are located presynaptically and non-α4β2* receptors are located postsynaptically on CVNs, prenatal nicotine exposure causes the additional expression of α7 nicotinic receptors postsynaptically (Huang et al. 2004).
Whereas during hypoxia/hypercapnia CVNs receive enhanced respiratory related glutamatergic neurotransmission, prenatal nicotine depresses inspiratory-evoked excitatory neurotransmission during recovery from hypoxia/hypercapnia. Furthermore, application of nicotinic antagonists restored the respiratory-evoked increase in glutamatergic neurotransmission during recovery, thereby converting a nicotine-exposed response to one that resembles an unexposed response. Neither α7 nor α4β2* nicotinic receptors mediate prenatal nicotine-evoked modifications during recovery from hypoxia/hypercapnia; control responses were only restored by 100 µM DHβE. These responses are likely evoked by the conversion of excitatory neurotransmission control by α3β2*/α6βX/α3β4 to solely α3β4 nicotinic receptors (Kamendi et al. 2006).

The alterations in cardiorespiratory responses to hypoxia/hypercapnia following prenatal nicotine exposure may be of clinical significance. Smoking during pregnancy increases the risk of SIDS 2-4 times (Bultery et al. 1990; Haglund and Cnattingius 1990; Malloy et al. 1988; Schoendorf and Kiely 1992), and nicotine has been proposed to be the link between maternal smoking and SIDS (Bamford and Carroll 1999; Nachmanoff et al. 1998; Slotkin et al. 1997; St-John and Leiter 1999). Some have suggested that SIDS may result from a direct alteration of the development of brainstem sites responsible for cardiorespiratory control and arousal due to prenatal nicotine exposure (Meny et al. 1994; Nachmanoff et al. 1998; Slotkin et al. 1997; St-John and Leiter 1999). During hypercapnia in vivo, the firing rate of CVNs increases during inspiration (Yen et al. 2000), and infants at risk for SIDS exhibit a more pronounced bradycardia during hypercapnia than healthy infants (Edner et al. 2002; Meny et al. 1994; Poets et al. 1999). The recruitment of a glutamatergic neurotransmission to CVNs during hypoxia/hypercapnia
observed in the present study would result in a significant reduction in heart rate and provides a possible mechanism by which severe bradycardia is evoked by hypoxia/hypercapnia in SIDS.

Purinergic neurotransmission is an important component of central cardiovascular and respiratory responses to hypoxia and/or hypercapnia (Gourine 2005; Gourine et al. 2005a, b). Our data suggest novel nicotinic receptor activity following prenatal nicotine exposure prevents purinergic modulation of central cardiorespiratory interactions. When nicotinic activity is blocked, purinergic-mediated cardiorespiratory responses are restored. To our knowledge, this is the first report linking prenatal nicotine exposure to altered purinergic neurotransmission. This hypothesis may provide a cellular mechanism for in vivo studies which report increased parasympathetic outflow following apnea in control infants, but absent parasympathetic increases following apnea in future SIDS victims (Franco et al. 2003). In summary, our study suggests purinergic neurotransmission is a key component of central cardiorespiratory interactions and nicotinic receptor activity modification by prenatal nicotine exposure reversibly precludes purinergic control of cardiorespiratory function.
Sources of Funding

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References


Figure Legends

Figure 1. Hypoxia/hypercapnia evokes biphasic changes in central respiratory activity. (A) Changing the perfusate from aCSF equilibrated with 95% O2/5% CO2 to aCSF equilibrated with 9% CO2, 6% O2, 85% N2 elicited a biphasic change in respiratory activity as shown in a representative example. The examples were taken during the last three minutes of the control period, the last three minutes of hypoxia/hypercapnia, and from ten to thirteen minutes during the recovery. (B) Respiratory burst frequency significantly increased during hypoxia/hypercapnia in both unexposed (squares) and prenatal nicotine exposed (unfilled circles) animals. (C) Hypoxia/hypercapnia significantly depressed burst duration in both unexposed and prenatal nicotine exposed animals. (D) Hypoxia/hypercapnia induced a small but significant increase in burst amplitude in both unexposed and prenatal nicotine exposed animals. There were no statistically significant differences in the respiratory responses between unexposed and prenatal nicotine exposed animals. +/* p<0.05, ++/** p<0.01. In this and all subsequent figures error bars are S.E.M.

Figure 2. Inspiratory-related bursting activity was recorded from the hypoglossal rootlet (XII) and electronically integrated (top). Fluorescently identified CVNs were patch clamped in the whole-cell configuration. Inhibitory GABAergic and glycinergic IPSCs were blocked by focal application of gabazine (25 µM) and strychnine (1 µM), respectively. There was no significant respiratory related glutamatergic neurotransmission to CVNs prior to (control) as well as during the period of hypoxia and hypercapnia (H/H). However during recovery from H/H CVNs received significant increases in glutamatergic synaptic inputs during respiratory bursts. This
excitatory input recruited during recovery persisted for at least 48 minutes post H/H. A typical experiment is shown at top with the average data from 7 cells shown below. Data was analyzed during all bursts in the recovery periods shown.

Figure 3. Central cardiorespiratory network responses to hypoxia/hypercapnia are not mediated by nicotinic receptor activation. A typical experiment from an unexposed animal is shown in (A), and the average data from 9 cells is shown in (B). Neither α-bungarotoxin to block α-7 nicotinic receptors (C; n = 7), 3 µM DHβE to block α4β2* nicotinic receptors (D; n = 9), nor 100 µM DHβE to block all nicotinic receptors (E; n = 11) significantly altered respiratory related glutamatergic neurotransmission to CVNs during recovery from hypoxia/hypercapnia.

Figure 4. Activation of P2 purinergic receptors mediates respiratory related glutamatergic neurotransmission to CVNs during recovery from hypoxia/hypercapnia. (A) In the absence of suramin (unfilled bar graphs, n=9) during recovery from H/H CVNs received significant increases in glutamatergic synaptic inputs during respiratory bursts. In the presence of suramin at concentrations of 25 µM (B; n = 8) and 100 µM (C; n = 7, and top representative experiment) the excitatory input to CVNs was significantly decreased by 19.8% and 30.4%, respectively, in unexposed animals.

Figure 5. The P2 receptor antagonist PPADS (100 µM), reduced, but did not abolish the respiratory-related increase in excitatory neurotransmission to cardiac vagal neurons during recovery from hypoxia/hypercapnia. (A) Representative examples of the responses before, during and recovery after hypoxia/hypercapnia (10-12 minutes post hypoxia/hypercapnia)
including recovery during focal application of the P2 receptor antagonist PPADS (100 µM)
(shown at 18-20 minutes post hypoxia/hypercapnia). PPADS decreased by 30.6%, but did not
block, the significant increase in excitatory neurotransmission to CVNs during recovery from
hypoxia/hypercapnia in unexposed animals, B, average data from 7 cardiac vagal neurons.

Figure 6. Nicotinic receptor activation inhibits P2 receptor enhancement of glutamatergic
neurotransmission to CVNs. mEPSCs were isolated with gabazine (25 µM), strychnine (1 µM)
and TTX (1 µM). In both unexposed (n=9, left) and prenatal nicotine exposed (n=7, right)
animals, ATP (1 mM) evoked a significant increase in mEPSC frequency (p<0.05) which was
blocked by nicotine (10 µM; p>0.05). Filled bars denote ATP application.

Figure 7. Central cardiorespiratory network responses to hypoxia/hypercapnia are mediated by
nicotinic receptor activation following prenatal nicotine exposure. (A) Prenatal nicotine exposed
animals exhibit significant respiratory-related increases in excitatory neurotransmission during,
but not upon recovery from, hypoxia/hypercapnia, as shown in a representative cell, top.
Summary data of EPSC frequency without application of nicotinic antagonists (unfilled bar
graphs) is shown below. (B) 3 µM DHβE did not alter hypoxia/hypercapnia responses (n = 8; p
> 0.05). (C) α-bungarotoxin (100 nM) partially depressed respiratory-related excitatory
neurotransmission during hypoxia/hypercapnia (n = 11; p < 0.05).

Figure 8. Prenatal nicotine exposure precludes purinergic facilitation of excitatory
neurotransmission during recovery from hypoxia/hypercapnia. Inhibition of all nicotinic
receptors (100 µM DHβE) restored responses to that observed in unexposed animals, as shown
in a representative example (A). (B) Average data from 11 cells. (C) Coapplication of 100 µM DHβE and suramin (100 µM) blocked the restored respiratory-related glutamatergic neurotransmission during recovery (n = 7).

Figure 9. In prenatal nicotine exposed animals continuous application of DHβE (100 µM) abolished the respiratory-related excitatory neurotransmission that occurs during hypoxia/hypercapnia in prenatal nicotine exposed animals, and restored a respiratory-evoked glutamatergic neurotransmission during recovery as in unexposed animals. Representative examples of the responses before, during and recovery after hypoxia/hypercapnia, including recovery during focal application of the P2 receptor antagonist PPADS (100 µM) are shown in A. PPADS abolished the significant increase in excitatory neurotransmission to CVNs during recovery from hypoxia/hypercapnia in prenatal nicotine exposed animals in the presence of 100 µM DHβE as shown in B which illustrates the average data from 7 cardiac vagal neurons.
Prenatal Nicotine Exposed Animals

A

Control

H/H

Recovery

B

C

3 μM DHβE

α-Bgtx

EPSC Frequency (Hz)
Prenatal Nicotine Exposed Animals

DHBE (100 \mu M)

A

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<th>H/H</th>
<th>Recovery</th>
<th>Recovery + PPADS</th>
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10 pA

5 s

B

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