Prenatal Nicotine Exposure Alters the Types of Nicotinic Receptors That Facilitate Excitatory Inputs to Cardiac Vagal Neurons

Zheng-Gui Huang, Xin Wang, Cory Evans, Allison Gold, Evguenia Bouairi, and David Mendelowitz

Department of Pharmacology and Physiology, George Washington University, Washington, DC 20037

Submitted 12 May 2004; accepted in final form 19 June 2004

Huang, Zheng-Gui, Xin Wang, Cory Evans, Allison Gold, Evguenia Bouairi, and David Mendelowitz. Prenatal nicotine exposure alters the types of nicotinic receptors that facilitate excitatory inputs to cardiac vagal neurons. J Neurophysiol 92: 2548–2554, 2004. First published June 22, 2004; 10.1152/jn.00500.2004. Nicotinic receptors play an important role in modulating the activity of parasympathetic cardiac vagal neurons in the medulla. Previous work has shown nicotine acts via at least three mechanisms to excite brain stem premotor cardiac vagal neurons. Nicotine evokes a direct increase in holding current and facilitates both the frequency and amplitude of glutamatergic neurotransmission to cardiac vagal neurons. This study tests whether these nicotinic receptor–mediated responses are endogenously active, whether α4β2 and α7 nicotinic receptors are involved, and whether prenatal exposure to nicotine alters the magnitude of these responses and the types of nicotinic receptors involved. Application of neostigmine (10 μM) significantly increased the holding current, amplitude, and frequency of miniature excitatory postsynaptic current (mEPSC) glutamatergic events in cardiac vagal neurons. In unexposed animals, the nicotine-evoked facilitation of mEPSC frequency, but not mEPSC amplitude or holding current, was blocked by α-bungarotoxin (100 nM). Prenatal nicotine exposure significantly exaggerated and altered the types of nicotinic receptors involved in these responses. In prenatal nicotine-exposed animals, α-bungarotoxin only partially reduced the increase in mEPSC frequency. In addition, in prenatal nicotine-exposed animals, the increase in holding current was partially dependent on α-7 subunit–containing nicotinic receptors, in contrast to unexposed animals in which α-bungarotoxin had no effect. These results indicate prenatal nicotine exposure, one of the highest risk factors for sudden infant death syndrome (SIDS), exaggerates the responses and changes the types of nicotinic receptors involved in exciting premotor cardiac vagal neurons. These alterations could be responsible for the pronounced bradycardia that occurs during apnea in SIDS victims.

INTRODUCTION

Nicotinic receptors have been shown to be important modulators of parasympathetic cardiac neuron activity originating from the brain stem (Mendelowitz 1998, 1999; Neff et al. 1998, 2003; Wang et al. 2001a,b, 2003b). Nicotine, but not agonists of muscarinic receptors, activates postsynaptic receptors and evokes depolarizing inward currents in premotor cardiac vagal neurons (Neff et al. 1998; Wang et al. 2001a). In addition, nicotine acts at different presynaptic and postsynaptic sites to facilitate glutamatergic neurotransmission to these neurons in the brain stem (Neff et al. 1998; Wang et al. 2001a). Presynaptic nicotinic receptors increase the frequency of glutamatergic transmitter release and are blocked by α-bungarotoxin (α-BgTX), an antagonist selective for α-7 subunit–containing nicotinic receptors (Neff et al. 1998; Wang et al. 2001a). Nicotine has also been shown to elicit an augmentation of postsynaptic non-N-methyl-D-aspartate (NMDA) currents in cardiac vagal neurons (Neff et al. 1998; Wang et al. 2001a).

Activation of nicotinic receptors also facilitates inhibitory GABAergic and glycinergic neurotransmission to cardiac vagal neurons, and this cholinergic facilitation is endogenously active (Wang et al. 2003b). The physiological importance of nicotinic modulation of inhibitory neurotransmission to cardiac vagal neurons is apparent in the interactions between the cardiovascular and respiratory systems. During inspiration, cardiac vagal neurons are strongly inhibited by both GABAergic and glycinergic synaptic events (Neff et al. 2003). This inspiratory-related inhibition of cardioinhibitory cardiac vagal neurons likely mediates respiratory sinus arrhythmia, in which heart rate increases during each inspiration. Focal application of the nicotinic antagonist dihydro-β-erythroidine (DHβE) in an α4β2 selective concentration (3 μM) abolishes the respiratory-evoked increase in GABAergic frequency but not the increase in glycinergic frequency during inspiration. Presynaptic α4β2 nicotinic receptors on GABAergic, but not glycinergic, neurons likely mediate the cardiorespiratory interactions in the brain stem responsible for respiratory sinus arrhythmia (Neff et al. 2003).

The involvement of nicotinic receptors in mediating respiratory sinus arrhythmia is particularly interesting because prenatal nicotine exposure is among the highest risk factors for sudden infant death syndrome (SIDS) (Meny et al. 1994; Taylor and Sanderson 1995). Infants that succumb to SIDS often experience a sustained bradycardia, presumably due to increased activity of cardiac vagal neurons, which is preceded or accompanied by a life-threatening apnea (Cote et al. 1998; Meny et al. 1994). These life-threatening events in SIDS victims are thought to be caused by exaggerated central cardiorespiratory responses in response to a challenge, such as hypoxia or apnea (Meny et al. 1994; Slotkin et al. 1997). Nicotinic modulation of GABAergic neurotransmission has been shown to be exaggerated with prenatal exposure to nicotine, and this increased inhibition of cardiac vagal neurons may be responsible for the increased heart rate observed in SIDS victims (Neff et al. 2003).

This study has three goals: 1) test whether the nicotinic activation of cardiac vagal neurons and facilitation of glutamatergic neurotransmission to cardiac vagal neurons is endogenously active; 2) determine if α4β2 nicotinic receptors are involved; and 3) examine whether prenatal exposure to nicotine...
nicotine alters the types of nicotinic receptors responsible for facilitating glutamatergic neurotransmission to cardiac vagal neurons.

**METHODS**

In an initial surgery, 2- to 6-day-old rats were anesthetized with ketamine/xylazine and exposed to hyperthermia during the surgery (10–20 min) to slow the heart and aid in recovery. A right thoracotomy was used to expose the heart, and rhodamine (XRTIC, Molecular Probes, 2% solution, 20–40 μL) was injected into the pericardial sac. Control injections of rhodamine either into the chest cavity but outside the pericardial sac or intravenous injections failed to label any neurons in the medulla, except for rare labeling of area postrema neurons observed with intraventricular injections.

One group of animals was studied after prenatal nicotine exposure. Adult female rats were anesthetized with ketamine/xylazine on the third day of gestation and implanted with Alzet osmotic minipumps (Durect, Cupertino, CA) containing (–nicotine (56.1 mg/ml bacte- rioriostatic water; Sigma, St. Louis, MO). Pumps delivered 2.1 mg nicotine/day (a dosage that produces blood nicotine levels approximately equivalent to those that occur in moderate to heavy smokers) to the pregnant dams throughout the pregnancy and prenatal period (Slotkin et al. 1997). These pups were not only exposed to nicotine prenatally but continued to be exposed to nicotine via maternal nursing until death.

On the day of the experiment (1–3 days after injection of the fluorescent tracer), the animals were anesthetized deeply with halothane and killed by cervical dislocation. The brain was submerged in cold (4°C) buffer of the following composition (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 5 glucose, and 10 HEPES (continually gassed with 100% O₂). Under a dissection microscope, the cerebellum was re-examine spontaneous TTX-independent miniature excitatory synaptic events. Nicotine (100 μM) was used to block α7, β2, and α4β2 nicotinic receptors, and DHβE was utilized at a concentration of 100 μM to block all nicotinic receptors (Alkondon and Albuquerque 1993). These antagonists were added cumulatively during the course of each experiment by inclusion in the perfusate. All drugs were purchased from Sigma Aldrich (St. Louis, MO). Analysis of TTX-insensitive mEPSCs were performed using MiniAnalysis (Synaptosoft, version 4.3.1) with minimal acceptable amplitude of 8 pA. Results are presented as means ± SE. Statistical comparisons were performed using ANOVA with repeated measures to examine the responses throughout the time course of the experiment, paired Student’s t-tests when comparing the data from control periods to during nicotine application, and unpaired Student’s t-tests when comparing the results from control animals to animals exposed to nicotine prenatally. Significant difference was set at P < 0.05.

**RESULTS**

Brief (60 s) applications of nicotine (100 μM) increased both the frequency and amplitude of glutamatergic mEPSCs in premotor cardiac vagal neurons (Fig. 1). Nicotine also evoked a transient inward current (Fig. 1). To determine if repetitive applications of nicotine (100 μM) could evoke consistent repeatable responses, the results from four sequential applications of nicotine, with 10 min between applications, were compared. Each of the three subsequent applications of nicotine (100 μM) evoked significant increases in mEPSC frequency and amplitude and an inward current, which were indistinguishable and not statistically different from the initial responses (P > 0.05; Fig. 1).

To identify the nicotinic receptors responsible for the nicotine-evoked inward current and increase in mEPSC frequency and amplitude, nicotine (100 μM) was applied in the presence of the selective α7 nicotinic receptor antagonist α-BgtX (100 nM) and DHβE at two concentrations: at 3 μM for α4β2 nicotinic receptors and 100 μM which blocks all nicotinic receptors (Alkondon and Albuquerque 1993). These antagonists were added cumulatively during the course of these experiments. As shown in Fig. 2, α-BgtX blocked the increase in mEPSC frequency, but did not significantly alter the increase in mEPSC amplitude or inward current. Application of DHβE at a concentration of 3 μM did not change the responses and had no significant effect on the nicotine-evoked increase in mEPSC amplitude, inward current, or mEPSC frequency. Only DHβE at a concentration of 100 μM blocked the nicotine-mediated inward current and mEPSC amplitude, which were significantly different (P < 0.01) from the responses with DHβE at concentration of 3 μM and α-BgtX (100 nM; Fig. 2).

In animals exposed to nicotine during the prenatal period brief (60 s), applications of nicotine (100 μM) also evoked an inward current, as well as increases in mEPSC frequency and amplitude (Fig. 3). Surprisingly, however, unlike unexposed animals, application of the selective α7 nicotinic receptor antagonist α-BgtX (100 nM), significantly inhibited (P < 0.01), but failed to completely block, the nicotine-evoked increase in EPSC frequency (Fig. 3). Application of α-BgtX (100 nM) also significantly inhibited the inward current compared with the initial responses (P < 0.01), but...
these responses were still significantly different. These results are in contrast with the lack of inhibition of inward current with α-BgTX in unexposed animals. Application of DHβE, at a concentration of 3 μM, failed to abolish the increase in mEPSC frequency in prenatal nicotine-exposed animals, and the responses with 3 μM DHβE and α-BgTX (100 nM) were not significantly different from responses only in the presence of α-BgTX (100 nM). Only DHβE at a
concentration of 100 μM blocked the nicotine-mediated increases in mEPSC frequency, as well as the inward current and mEPSC amplitude (Fig. 3).

Prenatal nicotine exposure not only altered the types of nicotinic receptors involved in these responses, but also increased the magnitude of the responses. As shown in Fig. 4, prenatal nicotine-exposed animals had exaggerated nicotine-evoked inward currents and increases in mEPSC frequency and mEPSC amplitude compared with the responses in unexposed animals. In prenatal nicotine-exposed animals, but not in unexposed animals, the augmented increase in mEPSC frequency persisted in the presence of α-BgTX (100 nM), as well as DHβE (3 μM). Also in prenatal nicotine animals, but not in unexposed animals, the exaggerated inward current responses were significantly diminished by α-BgTX (100 nM). DHβE, at a concentration of 100 μM, blocked all of the responses in both prenatal nicotine and unexposed animals.

To determine if the nicotinic facilitation of glutamatergic neurotransmission to cardiac vagal neurons in both unexposed animals and in animals exposed to nicotine in the prenatal period. In unexposed animals, the nicotine-elicited facilitation of mEPSC frequency, but not mEPSC amplitude or inward current, is completely dependent on activation of α7 subunit-containing nicotinic receptors, since the nicotine-evoked increase in mEPSC frequency can be blocked by α7-BgTX. The nicotine-mediated inward current and increase in mEPSC amplitude do not involve α4β2 nicotinic receptors, since DHβE at a concentration of 3 μM, which selectively blocks α4β2 nicotinic receptors, had no effect (Alkondon and Albuquerque 1993).

Prenatal nicotine exposure significantly increases the endogenous activation of nicotinic receptors responsible for an inward current and augmentation of mEPSC frequency and amplitude in cardiac vagal neurons. In addition, prenatal nicotine exposure evoked both an exaggeration and change in nicotinic receptors responsible for the nicotine-evoked responses that consist of an inward current and increases in mEPSC frequency and amplitude. In prenatal nicotine-exposed animals, the increase in holding current was partially dependent on α7 subunit-containing nicotinic receptors, whereas in unexposed animals, α-BgTX had no effect on the holding current responses. Furthermore, whereas in control animals α-BgTX abolished the increase in mEPSC frequency, in prenatal nicotine-exposed animals, α-BgTX only partially reduced

**DISCUSSION**

This work shows that nicotinic activation of cardiac vagal neurons and facilitation of glutamatergic neurotransmission to cardiac vagal neurons are endogenously active in both unexposed animals and in animals exposed to nicotine in the prenatal period. In unexposed animals, the nicotine-elicited facilitation of mEPSC frequency, but not mEPSC amplitude or inward current, is completely dependent on activation of α7 subunit-containing nicotinic receptors, since the nicotine-evoked increase in mEPSC frequency can be blocked by α7-BgTX. The nicotine-mediated inward current and increase in mEPSC amplitude do not involve α4β2 nicotinic receptors, since DHβE at a concentration of 3 μM, which selectively blocks α4β2 nicotinic receptors, had no effect (Alkondon and Albuquerque 1993).

Prenatal nicotine exposure significantly increases the endogenous activation of nicotinic receptors responsible for an inward current and augmentation of mEPSC frequency and amplitude in cardiac vagal neurons. In addition, prenatal nicotine exposure evoked both an exaggeration and change in nicotinic receptors responsible for the nicotine-evoked responses that consist of an inward current and increases in mEPSC frequency and amplitude. In prenatal nicotine-exposed animals, the increase in holding current was partially dependent on α7 subunit-containing nicotinic receptors, whereas in unexposed animals, α-BgTX had no effect on the holding current responses. Furthermore, whereas in control animals α-BgTX abolished the increase in mEPSC frequency, in prenatal nicotine-exposed animals, α-BgTX only partially reduced
examined in this study, cooperativity could occur between different nicotinic receptors, including between homomeric \( \alpha-7 \) and heteromeric nicotinic receptors. Recent work has shown cooperativity between nicotinic receptors in which the open state of a nicotinic receptor increases the probability of opening neighboring nicotinic channels (Keleshian et al. 2000).

There is considerable evidence that \( \alpha-7 \) subunit–containing nicotinic receptors are expressed at presynaptic glutamatergic terminals in other neuronal pathways (Berg and Conroy 2002; Dani 2001; McGhee et al. 1995). However, evidence that \( \alpha-7 \) subunit–containing nicotinic receptors are involved in postsynaptic synaptic neurotransmission in the CNS is less common. However, some recent studies have shown synaptic currents generated by \( \alpha-7 \) subunit–containing nicotinic receptors, such as in the nigral dopaminergic neurons, interneurons of the hippocampus, and lamina X of the spinal cord (Alkondon et al. 1998; Bradaia and Trouslard 2002; Frazier et al. 1998; Matsubayashi et al. 2004; Zhang et al. 1996).

It is well accepted that tobacco smoking in humans and chronic nicotine exposure in animals increases the number of nicotinic receptors in the brain, but the mechanisms responsible for this up-regulation are controversial (Breese et al. 1997; Marks et al. 1992; Perry et al. 1999). It has been proposed that the up-regulation is an adaptive response to desensitization of nicotinic receptors. However, more recent reports suggest up-regulation of nicotinic receptors is not caused by long-lasting inactivation and may be due to decreased rate of receptor turnover and/or a conversion of a population of low-affinity nicotinic receptors into high-affinity nicotinic receptors (Buisson and Bertrand 2001; Kawai and Berg 2001; Peng et al. 1994). Chronic nicotine exposure has also been shown to differentially up-regulate specific types of nicotinic receptors, particularly \( \alpha 4 \beta 2 \) nicotinic receptors (Buisson and Bertrand 2002; Flores et al. 1992, 1997; Mugnaini et al. 2002; Peng et al. 1994). In addition, \( \alpha 4 \beta 2 \) receptors chronically exposed to nicotine exhibit enhanced responses to acetylcholine and are less sensitive to desensitization (Buisson and Bertrand 2001). The results from this study show that prenatal nicotine exposure initiates both a facilitation of postsynaptic responses involving \( \alpha-7 \) subunit–containing nicotinic receptors in cardiac vagal neurons that are not present in unexposed animals as well as an augmentation of presynaptic glutamatergic neurotransmission by non-\( \alpha-7 \) nicotinic receptors that are not involved in unexposed animals.

The nicotinic facilitation of glutamatergic neurotransmission to cardiac vagal neurons may be beneficial. Increased cardiac parasympathetic activity has been shown to terminate ventricular tachycardia and fibrillation (Vanoli et al. 1991; Waxman and Wald 1977). Recovery of parasympathetic activity after myocardial infarction has been associated with decreased mortality (Lampert et al. 2003). In contrast, patients with low parasympathetic activity have a higher risk for sudden death independent of other risk factors (Algra et al. 1993). A delay in the inhibitory actions of this autonomic motor system is a powerful predictor of overall mortality following exercise (Cole et al. 1999).

Nicotinic receptor–mediated facilitation of glutamatergic neurotransmission and excitation of cardiac vagal neurons is likely involved in cardiorespiratory interactions. Nicotinic receptors are responsible for the increased frequency of
GABAergic inhibitory inputs to cardiac vagal neurons during inspiration, and these responses are exaggerated in prenatal nicotine-exposed animals (Neff et al. 2003). Cardiac vagal neurons do not receive excitatory glutamatergic inputs during the normal respiratory cycle, but do receive increased glutamatergic inputs during respiratory bursts that only occur with an hypoxic challenge (Wang et al. 2003a). The glutamatergic excitation of premotor cardiac vagal neurons associated with hypoxia may contribute to cardiovascular dysfunction in SIDS. SIDS is the leading cause of death in infants between 1 mo and 1 yr of age, yet the mechanisms of SIDS have not been elucidated. Bradycardia is the most prevalent and predictive event in infants monitored for apparent life-threatening events (Cote et al. 1998). Although the cause(s) for SIDS remains unknown, it has been speculated that an abnormality of cardio-respiratory control, and in particular an exaggerated excitation of parasympathetic control of cardiac function to challenges such as hypoxia, may be involved (Divon et al. 1986; Harper and Bandler 1998; Meny et al. 1994; Schechtman et al. 1992; Spyer and Gilbey 1988). This work shows that one of the highest risk factors for SIDS, prenatal nicotine exposure, exaggerates the glutamatergic excitation of cardiac vagal neurons, and these alterations could be responsible for the pronounced bradycardia that occurs in SIDS victims. While an exaggeration of parasympathetic responses to hypoxia may be involved in SIDS, increased parasympathetic activity with nicotinic receptor activation may be cardioprotective in adults.

**GRANTS**

This study was supported by National Heart, Lung and Blood Institute Grants HL-59895 and HL-72006 to D. Mendelowitz.
REFERENCES


