

Neonatal nicotine exposure impairs nicotinic enhancement of central auditory processing and auditory learning in adult rats

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Abstract

Children of women who smoke cigarettes during pregnancy display cognitive deficits in the auditory–verbal domain. Clinical studies have implicated developmental exposure to nicotine, the main psychoactive ingredient of tobacco, as a probable cause of subsequent auditory deficits. To test for a causal link, we have developed an animal model to determine how neonatal nicotine exposure affects adult auditory function. In adult control rats, nicotine administered systemically (0.7 mg/kg, s.c.) enhanced the sensitivity to sound of neural responses recorded in primary auditory cortex. The effect was strongest in cortical layers 3 and 4, where there is a dense concentration of nicotinic acetylcholine receptors (nAChRs) that has been hypothesized to regulate thalamocortical inputs. In support of the hypothesis, microinjection into layer 4 of the nonspecific nAChR antagonist mecamylamine (10 μ M) strongly reduced sound-evoked responses. In contrast to the effects of acute nicotine and mecamylamine in adult control animals, neither drug was as effective in adult animals that had been treated with 5 days of chronic nicotine exposure (CNE) shortly after birth. Neonatal CNE also impaired performance on an auditory-cued active avoidance task, while having little effect on basic auditory or motor functions. Thus, neonatal CNE impairs nicotinic regulation of cortical function, and auditory learning, in the adult. Our results provide evidence that developmental nicotine exposure is responsible for auditory–cognitive deficits in the offspring of women who smoke during pregnancy, and suggest a potential underlying mechanism, namely diminished function of cortical nAChRs.

Introduction

The harmful effects of nicotine on human development are an important public health issue, given that most women who smoke cigarettes continue to do so during pregnancy (Severson *et al.*, 1995; Carmichael & Ahluwalia, 2000). Maternal smoking is associated with reduced fetal growth and brain impairment in offspring, and animal studies have confirmed a causal role for nicotine in the harmful effects of tobacco (Simpson, 1957; Li *et al.*, 1993; Slotkin, 1998; Oncken & Kranzler, 2003). Recent evidence has associated maternal smoking with cognitive deficits in offspring, notably in the auditory–verbal domain (Saxton, 1978; Picone *et al.*, 1982; Sexton *et al.*, 1990; McCartney *et al.*, 1994; Fried *et al.*, 1997, 2003). To date, however, a causal link between developmental nicotine exposure and subsequent auditory–cognitive deficits has not been confirmed in an animal model, which also could provide valuable insight into possible underlying mechanisms.

Findings from animal studies suggest a mechanism by which developmental chronic nicotine exposure (CNE) could affect higher auditory function. For primary auditory cortex (A1), important developmental events that occur postnatally in the rat (and during the third trimester in humans) include the growth of auditory inputs

from the thalamus, the onset of hearing in postnatal week 2, and a transient up-regulation of cholinergic markers – acetylcholinesterase and $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) – that peaks in week 2 and declines by the end of week 3 (Kramovic-Nemanic *et al.*, 1980, 1983; Iwasa & Potsic, 1982; Fuchs, 1989; Robertson *et al.*, 1991; Broide *et al.*, 1995). During weeks 2–3, presynaptic $\alpha 7$ nAChRs regulate excitatory synaptic transmission in auditory cortex (Aramakis & Metherate, 1998). The coincidence of this regulation with the onset of hearing led to the suggestion that the transient expression of nAChRs may define a critical period for auditory development (Metherate & Hsieh, 2003). Consistent with this proposal, CNE during week 2 alters synaptic development in auditory cortex (Aramakis *et al.*, 2000; Hsieh *et al.*, 2002). Thus, the transient expression of nAChRs may not only define a critical period, but may also confer heightened vulnerability to the deleterious effects of nicotine.

Developmental CNE also could affect auditory function by altering nAChR function in adults (Miao *et al.*, 1998). It is well known that acutely delivered nicotine in adults can enhance sensory and cognitive function (Levin & Simon, 1998; Mancuso *et al.*, 1999; Harkrider & Champlin, 2001a; Domino & Kishimoto, 2002; Harkrider & Hedrick, 2005). The effects of nicotine on the cerebral cortex may involve a dense concentration of $\alpha 4\beta 2$ nAChRs that are well positioned in cortical layers 3–4 to regulate thalamocortical inputs (Parkinson *et al.*, 1988; Gil *et al.*, 1997; Lavine *et al.*, 1997; Clarke, 2004). Evidence that nAChRs are associated with thalamocortical regulation includes

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the effects of thalamic lesions, which reduce the density of nAChRs in the corresponding ipsilateral cortex (Parkinson *et al.*, 1988; Sahin *et al.*, 1992; Lavine *et al.*, 1997), and nicotinic enhancement of cortical responses to thalamic stimulation in brain slice preparations (Gil *et al.*, 1997; Kawai & Metherate, 2004). These findings also imply different functions for nAChRs in sensory cortex during development and in the adult (Metherate, 2004), but it is unknown whether developmental CNE alters adult nAChR function.

In this study, we sought to determine if neonatal CNE in rats could affect adult function, and thereby provide a model to understand the deficits produced by developmental nicotine exposure in humans. To this end, we exposed pups to nicotine for 5 days during postnatal week 2, around the peak expression of nAChRs in A1, and after 2 months tested auditory and nAChR function in adult animals.

Materials and methods

Chronic nicotine exposure

All animal procedures were conducted in accordance with NIH guidelines, and were approved by the University of California, Irvine IACUC. Sprague–Dawley rat pups from timed-pregnant dams (Charles River, Wilmington, MA, USA) were randomly assigned to control or CNE groups on postnatal day 6 (P6) (day of birth is P0). From P8 to P12, pups were weighed and injected with sterile saline or nicotine hydrogen tartrate (2 mg/kg, reported as 0.7 mg/kg nicotine base, *s.c.*; Sigma, St. Louis, MO, USA) in 2 mL/kg saline (pH = 7) using a 50- μ L Hamilton syringe. Rats were injected twice per day, at 09:00 and 17:00 h. Each nicotine injection delivered a dose similar to those commonly used in CNE studies (Abdulla *et al.*, 1996; Rowell & Li, 1997) to approximate blood levels of nicotine in smokers (Isaac & Rand, 1972; Murrin *et al.*, 1987; Henningfield *et al.*, 1993). Pups were returned to their mother 10 min after the injection. Pups were weaned at P21 and housed in pairs until testing, which took place after P60.

Behavioral experiments

Adult (> P60) male rats were tested for avoidance learning using a shuttle box (83 cm long, 27 cm wide and 33 cm high, model E10-16SC, Coulbourn Instruments, Allentown, PA, USA) in an acoustic chamber (IAC, New York, USA) with dim infrared illumination for an overhead video camera that recorded all trials. During testing, the experimenter was blind to the treatment group. A black plastic barrier (2.5 cm high) divided the shuttle box in half, with each half having a separate shock grid floor. The location of subjects was detected by infrared photobeams. A Coulbourn model #E69-20 speaker was located at one end wall and a Coulbourn model #H11-01R 6-W house lamp directed upwards was on the opposite end wall (pilot studies revealed no behavioral bias toward either side due to speaker or lamp placement). Animals were required to cross to the opposite side of the box before the end of a 5-s tone [8 kHz, 70 dB SPL (sound pressure level)] in order to avoid a shock (40-Hz scrambled bipolar pulses, 0.8–1.0 mA, Coulbourn model #E13-14 shock generator), with intertrial intervals averaging 45 s (range 30–60 s). ‘Avoidance’ behavior indicates crossing during the tone, prior to the shock, whereas ‘escape’ behavior indicates crossing during the shock. Avoidance training (tone paired with shock) took place over four consecutive days (days 1–4) preceded by 1 day of escape training (shock only) for 50 trials. Shock levels were gradually increased during the first 5–10 trials until consistent escape behavior was obtained. There was no difference in shock levels for CNE and control animals (unpaired

t-test, $n = 8$ control and 8 CNE animals, $t = 0.59$, $P > 0.05$). During escape training both CNE and control animals learned to escape the shock, and both groups exhibited progressively shorter latencies across five-trial blocks ($F_{7,21} = 10.79$, $P < 0.001$). Day 1 of avoidance training began with ten escape trials, to ensure memory for escape learning, followed by 50 avoidance trials where the shock was paired with a preceding tone. Days 2–4 consisted of 50 avoidance trials.

In vivo electrophysiology

Electrophysiology experiments were performed on adult (> P60) male rats using procedures for surgery, acoustic stimulation and neurophysiological recording similar to those described previously (Kaur *et al.*, 2004). During testing, the experimenter was blind to the treatment group. Briefly, rats were anesthetized with urethane (1.5 g/kg *i.p.*, Sigma) and xylazine (10 mg/kg *i.p.*, Phoenix Pharmaceuticals, St. Joseph, MO, USA), placed in a sound-attenuating chamber (IAC), and maintained at 36–37 °C. Note that urethane anesthesia slightly enhances the function of some nAChRs (Hara & Harris, 2002), but this effect was preferable to the suppression of nAChR function produced by most anesthetics, including barbiturates and ketamine (Tassonyi *et al.*, 2002). A craniotomy was performed and A1 was identified physiologically (Kaur *et al.*, 2005). For current source density (CSD) measurements, a 16-channel silicon multiprobe (recording site impedance 2–3 M Ω , 100- μ m separation; University of Michigan Center for Neural Communication, Ann Arbor, MI, USA) was used to record local field potentials (LFPs) throughout the cortical depth. Acoustic stimuli were pure tones (100-ms duration, 10-ms rise/fall ramps) presented to the contralateral ear (open field). One-dimensional CSD analysis was performed off-line (Kaur *et al.*, 2005). For LFP recordings in layer 4 alone, glass microelectrodes (1 M Ω at 1 kHz) were placed at a depth of 600 μ m below the pia. Frequency receptive fields were determined using tones of 1.25–40 kHz in one-octave steps at intensities from below threshold to 70 dB SPL in 5- or 10-dB steps. To set a criterion for a threshold response, we first averaged baseline fluctuations over a 100-ms period in eight animals (75 trials per animal). Baseline variability, expressed as one standard deviation of the mean, was ~ 35 μ V, and we therefore established 40 μ V as the response threshold, with the added provision that a response must occur within 8–20 ms of the stimulus onset. Visual evaluation of near-threshold responses indicated that this method for determining thresholds was reasonable. Characteristic frequency (CF, the frequency with the lowest threshold) was determined for each recording site. The effects of nicotine were determined on responses to CF stimuli and stimuli three octaves below CF (‘nonCF’).

Systemic nicotine (0.7 mg/kg nicotine base) or saline was delivered subcutaneously. Intracortical microinjections were delivered using a 10- μ L Hamilton syringe and pipette backfilled with either saline or mecamylamine and inserted into layer 4, ~ 200 μ m from the recording electrode (a glass micropipette in layer 4; the recording and drug pipettes were aligned outside the brain so that when lowered into layer 4 their tips would be within ~ 200 μ m). A minipump (SP1001, WPI, Sarasota, FL, USA) was used to infuse 1 μ L of solution at 0.2 μ L/min. After drug administration, the pipette remained in place for at least 5 min and was then removed before recording of acoustic-evoked responses began. Effects of nicotine or mecamylamine were always compared with preceding saline controls in the same animal, and were delivered within 10–15 min after the saline injection. At the end of each experiment, animals were killed with a lethal dose of anesthesia.

Autoradiography

Adult rats were killed by CO₂ asphyxiation, and their brains extracted and frozen in isopentane at -20 °C. Coronal sections (20 µm) were cut on a cryostat at -20 °C, then labeled with [¹²⁵I]epibatidine for measurements of α4β2 nAChR binding sites (Perry *et al.*, 2002) or [¹²⁵I]α-bungarotoxin for measurements of α7 nAChR binding (Ospina *et al.*, 1998). For [¹²⁵I]epibatidine binding, slides were preincubated at room temperature for 10 min in buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂; pH 7.4), then incubated with 0.08 nM of the radioligand at room temperature for 90 min in the absence, and presence, of nicotine (300 µM) to define nonspecific binding. Additional sections were incubated with cytosine (200 nM) to define binding to α4β2 sites (Perry *et al.*, 2002). After incubation, slides were rinsed twice for 10 min in ice-cold buffer, dipped briefly in ice-cold water and dried with a stream of cool air. For [¹²⁵I]α-bungarotoxin binding, a similar method was used except that the buffer consisted of 50 mM Tris, pH 7.4, with 120 mM NaCl. Slides were incubated at room temperature for 2 h with [¹²⁵I]α-bungarotoxin (5 nM) in the absence, and presence, of α-cobratoxin (10 µM) to define nonspecific binding. Slides were apposed to Kodak Biomax MR film for 24 h, and the resulting autoradiograms were developed and fixed. Slides were then fixed with paraformaldehyde and stained with cresyl violet for identification of cortical layers.

Autoradiograms were quantified with a computer-based image analysis system (MCID, Imaging Research, St. Catherine, Ontario, Canada) using calibrated standards of reference (Ospina *et al.*, 1998). A calibration curve of optical density against radioligand concentration (fmol/mg tissue) was constructed using values of known radioactivity. Layer 4 of A1 was identified in the autoradiograms by comparison with the corresponding Nissl-stained sections, optical densities were measured and corresponding values of radioactivity were determined by interpolation from the calibration curve. Specific binding values in each region were determined by subtracting residual binding in the presence of excess inhibitor from the total binding values.

Statistical analysis

All statistical analyses are ANOVAs unless otherwise noted. Variability is indicated by standard error of the mean.

Results

Newborn littermates were assigned randomly to control or CNE groups, and injected systemically for 5 days from P8 to P12 with saline or nicotine (0.7 mg/kg base, s.c.), respectively. Controls and CNE pups gained weight at the same rate over P8–P12 ($F_{1,14} = 1.46$, $P > 0.5$). All testing was done in adult animals, after P60. Testing consisted of measuring (i) behavioral performance in an auditory-cued task, (ii) physiological responses in anesthetized animals or (iii) nAChR density.

Behavioral experiments

To determine if neonatal CNE impaired behavioral function, we tested adult animals in an auditory-cued active avoidance task. Active avoidance tasks in general depend on intact cortical function (Scharlock & Miller, 1964; Massopust *et al.*, 1965, 1966, 1967, 1971; Delay & Rudolph, 1994; Duvel *et al.*, 2001), and the task we selected is also one on which performance is enhanced by systemic

administration of nicotine (see Discussion; Evangelista *et al.*, 1970; Erickson, 1971; Orsingher & Fulginiti, 1971; Yilmaz *et al.*, 1997).

To perform the task, rats placed in one chamber of a two-chamber shuttle box learned to cross to the other chamber before the end of a 5-s tone, in order to avoid a shock. (Prior to testing, all rats quickly learned to 'escape' during the shock by crossing to the other chamber; during testing they learned to 'avoid' the shock by crossing during the tone that preceded the shock; see Materials and methods.) Control rats learned to avoid the shock over 4 days of training (50 trials/day), reaching a performance plateau of 50–60% avoidance (Fig. 1A; $n = 12$ control animals). Animals tended to avoid the shock well before the end of the 5-s tone (Fig. 1B), and a closer look at individual performance on day 4 showed that avoidance latency was correlated strongly with percentage avoidance, i.e. animals that avoided on more trials did so at shorter latencies (Fig. 1C, left; correlation coefficient $r = 0.84$, $P < 0.001$). This close correlation of avoidance latency and percentage avoidance developed gradually over the 4 days of training (Fig. 1D, left).

By contrast, neonatal CNE resulted in adult animals that avoided on significantly fewer trials than did controls (Fig. 1A; $n = 11$ CNE littermates). Note that CNE animals oriented to the tone cue (as observed on videotape of trials) and were capable of hearing the tone and avoiding the shock well before the end of the 5-s tone (Fig. 1B), indicating that they still possessed some basic auditory and motor functions. However, the close relationship between avoidance latency and percentage avoidance that occurred in control animals did not develop after CNE (Fig. 1C, right). Rather, avoidance latency and percentage avoidance remained uncorrelated over the 4 days of training (Fig. 1D, right). We conclude that neonatal CNE impairs auditory-cued behavior in adult animals.

Neonatal CNE also impaired learning as reflected in the number of animals that were never able to learn the task. In the control group, one animal (8% of 12 animals) never avoided the shock on more than ~10% of trials each day, i.e. that animal never learned the task (note that this animal's performance is included in the group data in Fig. 1, as the inclusion criterion is that animals learned to escape the shock, not that they learned to avoid the shock). By contrast, five CNE animals (45% of 11 animals) avoided on 10% or fewer trials each day, i.e. significantly more CNE animals failed to learn the task (chi-squared test, $P < 0.05$). Note that the remaining CNE animals – those that did learn the task – remained impaired relative to control animals that learned the task ($n = 6$ CNE animals, 11 controls; comparison of learning curves as in Fig. 1A; $F_{1,15} = 4.44$, $P < 0.05$). Thus, the effect of CNE on auditory learning reflects not just a few severely affected animals, but rather a wider range of moderate to severe impairments.

In vivo auditory physiology: systemic administration of nicotine

To determine how nicotine affects neural responses in auditory cortex, we recorded tone-evoked LFPs in A1 of anesthetized control animals (Kaur *et al.*, 2004). Using a 16-channel multi-electrode probe (100-µm electrode separation) to record neural activity in all layers of A1, we determined that pure tone stimuli at CF elicited LFPs in all cortical layers (Fig. 2B; $n = 6$ control animals). CSD analysis demonstrated a major current sink in layers 3–4, which reflects synaptic inputs from the auditory thalamus (Cruikshank *et al.*, 2002; Kaur *et al.*, 2005). Systemic administration of nicotine enhanced tone-evoked LFPs relative to the effects of saline injections (Fig. 2C). The effect could be observed in all layers, but was most prominent in layers 3–4.

In subsequent physiological experiments we focused on neural responses in layer 4 by placing a single recording pipette at that depth

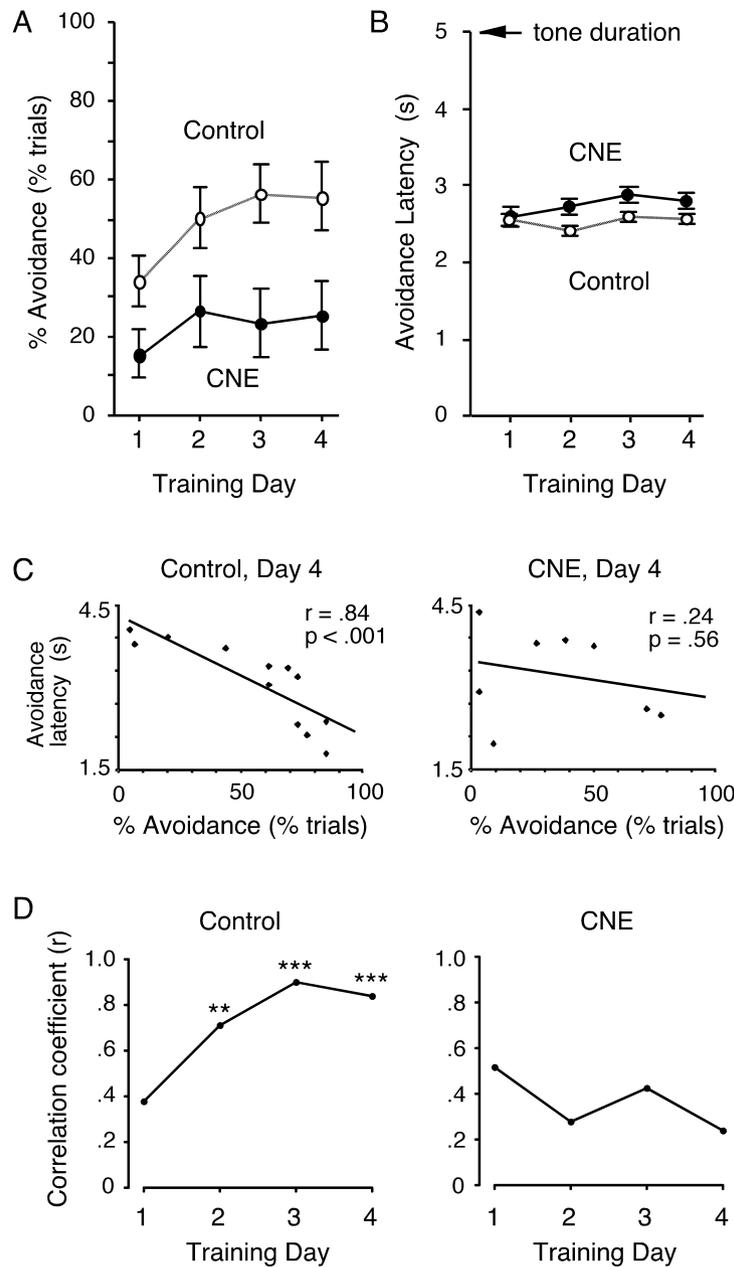


FIG. 1. Neonatal CNE impairs adult auditory learning. Data indicate performance on an auditory-cued active avoidance task. (A) CNE animals avoided on fewer trials than did controls over 4 days of training ($F_{1,21} = 21.3$, $P < 0.001$). (B) Successful avoidances by both control and CNE animals occurred well before the end of the 5-s tone (8 kHz, 70 dB SPL). (C) On Day 4 of testing in control animals (left), avoidance latency was correlated strongly with avoidance percentage (correlation coefficient, $r = 0.84$, $P < 0.001$), whereas CNE animals (right) did not show this correlation ($r = 0.24$, $P > 0.05$). (D) The correlation of avoidance latency and percentage developed over the 4 days of training in control animals (left, $**P < 0.02$; $***P < 0.001$), but not in CNE animals (right).

(Fig. 3). In six control animals, we determined the effect of systemic nicotine on responses to a suprathreshold stimulus at CF (60 dB SPL). Nicotine increased the CF tone-evoked response duration by nearly 50% and decreased onset latency by 15% (Fig. 3A). Nicotine sometimes increased or decreased the peak amplitude of the response, but on average had no consistent effect. In another nine control animals, we varied stimulus intensity at CF and at a second frequency three octaves below CF (referred to as 'nonCF'). Stimulus intensity ranged from 5–10 dB below threshold at CF to 60–70 dB above. Remarkably, nicotine decreased the response threshold for CF stimuli by up to 10 dB, but had no effect on threshold for nonCF stimuli (Fig. 3B; $n = 9$ animals; only data around '0 dB' are shown, to emphasize changes in threshold, but at suprathreshold intensities

nicotine produced no consistent effect on amplitude, as in Fig. 3A). Note that the decreased response threshold for CF stimuli was not the result of simply increasing response amplitudes from just below the criterion voltage (40 μ V) to just above; in most cases stimuli below threshold elicited no discernible response in the control condition (inset traces in Fig. 3B). The time-course of nicotine effects was not explicitly measured, but the effects generally dissipated after ~ 1 h. These findings show that systemic nicotine increases cortical responsiveness and sensitivity to acoustic stimuli, selectively at CF.

Neonatal CNE dramatically diminished nicotinic regulation of auditory responses in adult animals. In recordings from layer 4 of A1, we found that systemic nicotine had no effect on response duration (Fig. 3C), or on response threshold at CF (Fig. 3D), in contrast to its

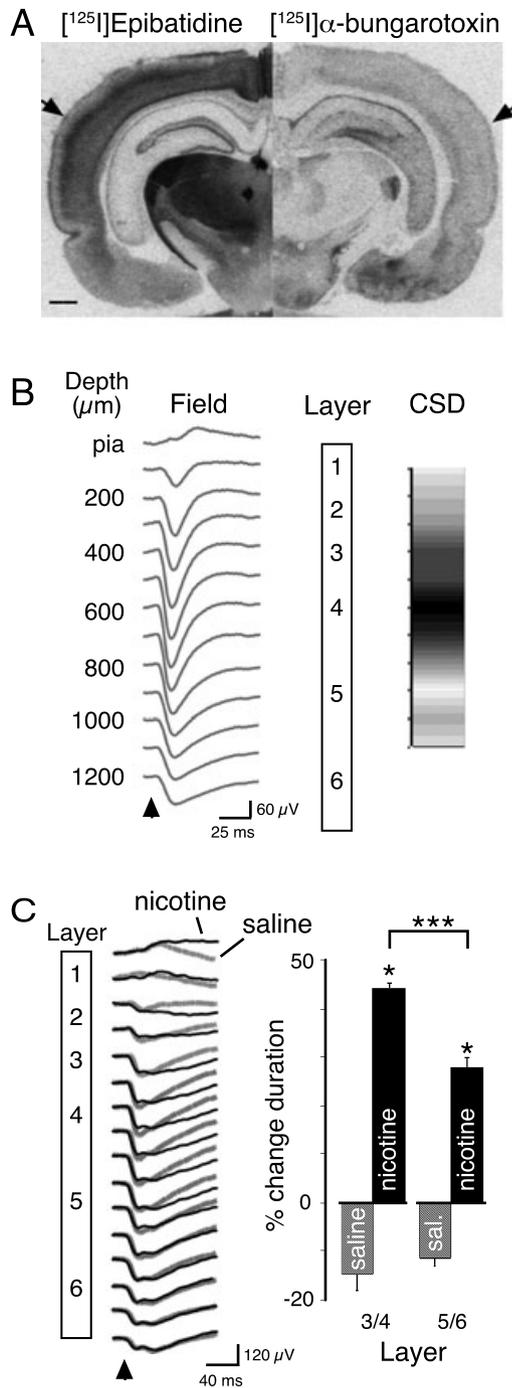


FIG. 2. Cortical nAChRs and the effects of systemic nicotine on auditory processing in adult A1. (A) Cytisine-sensitive binding of [¹²⁵I]epibatidine (left) indicating presence of α4β2 nAChRs is prominent throughout middle layers of neocortex, including auditory cortex (arrow). Binding of [¹²⁵I]α-bungarotoxin (right), indicating the presence of α7 nAChRs, is more evenly distributed throughout neocortex. (B) Laminar profile of tone-evoked LFPs (left) and CSD (right; maximum sink is black, maximum source is white) obtained with a 16-channel probe. Arrowhead indicates onset of 100-ms CF stimulus (10 kHz, 60 dB SPL) delivered to the contralateral ear. (C) Nicotine (0.7 mg/kg, s.c.) enhanced the response duration (black traces and histogram bars) relative to a saline control injection (grey traces and bars; CF stimulus 10 kHz, 60 dB; single asterisks indicate comparison with saline control, paired *t*-test, *n* = 4, **P* < 0.05). The enhancement for layers 5–6 (average for lowest six recording sites) was less than that for layers 3–4 (average of next six sites; *F*_{1,3} = 23.03, ****P* < 0.001). Upper layer effects appeared qualitatively similar but were not quantified due to small LFPs. Scale bar in A, 1 mm.

effects in control animals. However, nicotine still reduced onset latency (Fig. 3C). CNE did not affect pre-nicotine (saline) responses to auditory stimuli in terms of response duration and amplitude (cf. Fig. 3A and C), receptive field bandwidth (bandwidth, in octaves, 30 dB above threshold: control 3.2 ± 0.3 , CNE 3.3 ± 0.4 ; unpaired *t*-test, *n* = 6 control, *n* = 6 CNE animals, *t* = 0.32, *P* > 0.05) and absolute response threshold at CF (control 2.5 ± 2.1 dB SPL, CNE 5 ± 2.5 dB SPL; unpaired *t*-test, *t* = 0.76, *P* > 0.05). However, it is clear that neonatal CNE largely prevented, in adult animals, the nicotine-mediated increase in cortical responsiveness and sensitivity to sound.

We also assessed the ability of systemic nicotine to activate (desynchronize) cortical electroencephalogram (EEG) (Harkrider & Champlin, 2001b), as measured by the LFP recording in layer 4. In control animals, the EEG power spectrum (fast Fourier transform) peaked in the frequency range below 3 Hz (i.e. delta range). Nicotine reduced power in this range by $34 \pm 7\%$ (*n* = 6) vs. $11 \pm 12\%$ (*n* = 6) in CNE animals; however, neither effect achieved statistical significance (control, *F*_{1,5} = 1.39, *P* > 0.05; CNE, *F*_{1,5} = 1.67, *P* > 0.05). Thus, nicotine exerted only weak, if any, effects on the EEG.

Taken together, the physiological results indicate that brief, neonatal CNE can produce long-lasting deficits in function for nAChRs that regulate sensory-evoked responses in A1.

In vivo auditory physiology: intracortical microinjection of an nAChR antagonist

As the use of systemically administered nicotine provides little information on the location of relevant nAChRs, we microinjected saline or the nonspecific nAChR antagonist mecamylamine directly into layer 4 of auditory cortex and determined its effect on tone-evoked responses. Because pilot studies showed that bolus injection of 1 μL saline could reduce tone-evoked responses temporarily, infusions were made slowly over 5 min using a minipump, and data collection was begun only after another 5–10 min. In control animals, mecamylamine (10 μM, 1 μL) strongly reduced the amplitude of CF stimulus-evoked responses and increased response threshold in layer 4 (Fig. 4A and B; *n* = 8 animals). When response onsets could still be determined (at higher intensities), we found that mecamylamine had little effect on onset latencies despite its striking effect on amplitude (Fig. 4B, left). Partial to full recovery from the effects of mecamylamine was observed after ~1 h (*n* = 4). Importantly, as mecamylamine was delivered alone – no nicotine was administered – its effect apparently results from blocked action of the endogenous neurotransmitter acetylcholine (ACh). Thus, in apparent similarity to the effects of systemic nicotine (Fig. 3), endogenous ACh acts at cortical nAChRs to enhance sensitivity and responsiveness to sound in adult animals. In fact, release of endogenous ACh in A1 appears to be essential for near-threshold responses.

In contrast to its effects in control animals, mecamylamine infused intracortically after neonatal CNE had less effect on CF stimulus-evoked responses, especially near threshold [Fig. 4B and C; *n* = 5 CNE littermates; neither suprathreshold (B, right) nor near-threshold (C) responses were altered significantly]. These data are consistent with the effects of CNE on responses to systemic nicotine (Fig. 3C and D), and indicate that decreased function of nAChRs located in A1 contributes to the long-lasting deficits produced by neonatal CNE.

Radioligand binding to nAChRs in A1

As CNE impaired the function of cortical nAChRs, we looked for changes in the density of nAChRs in adult A1. We measured

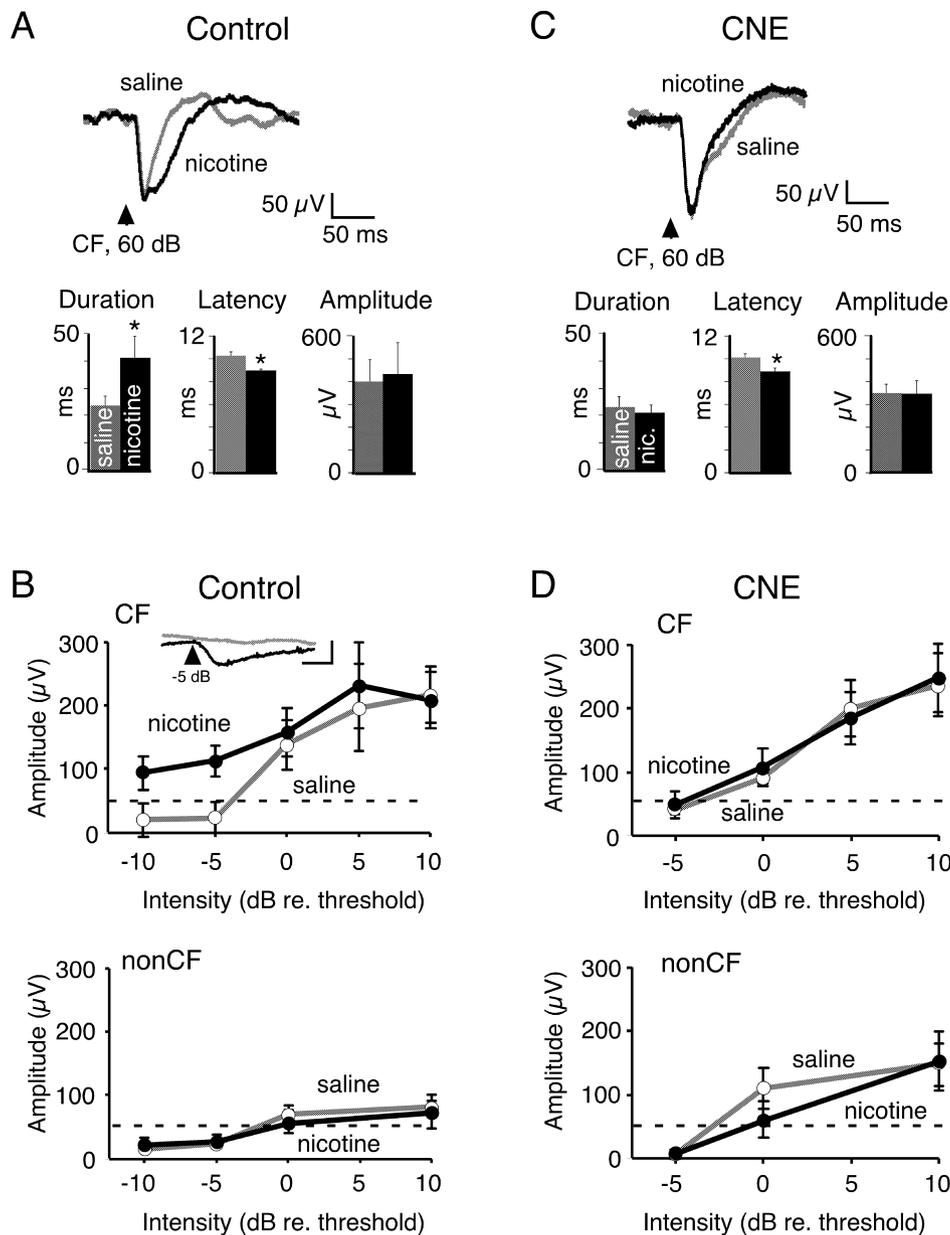


Fig. 3. Nicotine enhances sensitivity and responsiveness to CF stimuli in control adult A1, but not after neonatal CNE. Data are tone-evoked LFPs recorded in layer 4. (A) Systemic injection of nicotine (0.7 mg/kg, s.c.) increased duration and decreased onset latency of response to a 60-dB SPL stimulus at CF (5 kHz in example). Bar graphs in (A) and (C) show average data from six control and six CNE animals; asterisks indicate paired *t*-test, $P < 0.05$ (control duration $t = -3.41$, latency $t = 6.01$, amplitude $t = -0.66$; CNE duration $t = 1.19$, latency $t = 4.65$, amplitude $t = 0.88$). (B) In separate experiments ($n = 9$ animals), stimulus intensity was varied at CF and three octaves below CF (nonCF). Nicotine decreased response threshold to CF stimuli in each animal by at least 5 or 10 dB (sign test, $n = 8$, $P < 0.01$; one animal could not be tested for decreased threshold because of very low pre-nicotine threshold). Inset in B shows response to 20-kHz CF stimulus at -5 dB after saline (grey trace) and nicotine (black trace; scale 100 μ V, 100 ms). (C) After neonatal CNE, systemic nicotine only affected onset latency of response to CF stimulus (60 dB SPL; CF is 10 kHz in example). Asterisk indicates paired *t*-test, $n = 6$ animals, $P < 0.05$. (D) In separate experiments, nicotine did not affect response threshold after CNE ($n = 14$ animals, $P > 0.05$). Experiments on acoustic thresholds (B, D) were performed on animals also used for behavioral studies. Dashed lines in graphs indicate 40- μ V criterion for response threshold. In A and C, arrowheads indicate stimulus onset.

cytisine-sensitive binding of [125 I]epibatidine to assess $\alpha 4\beta 2$ nAChRs (Perry *et al.*, 2002), and [125 I] α -bungarotoxin binding to assess $\alpha 7$ nAChRs (Ospina *et al.*, 1998). Putative $\alpha 4\beta 2$ nAChRs were most dense in layers 3–4 of A1 (Fig. 2A, left), whereas $\alpha 7$ nAChRs were distributed more evenly and weakly across layers (Fig. 2A, right). However, neonatal CNE did not change the density of either nAChR subtype in adult A1 (Table 1). The laminar profile of $\alpha 4\beta 2$ nAChRs is consistent with the profile of nicotine effects on tone-evoked responses (Fig. 2C) and the effects of mecamylamine injections into layer 4 of A1

(Fig. 4A). However, the diminished function of nAChRs after neonatal CNE cannot be explained by changes in nAChR density in A1.

Discussion

The results show that nAChRs in adult A1 normally function to enhance responsiveness and sensitivity to CF stimuli. However, neonatal CNE strongly reduced nicotinic enhancement in adult

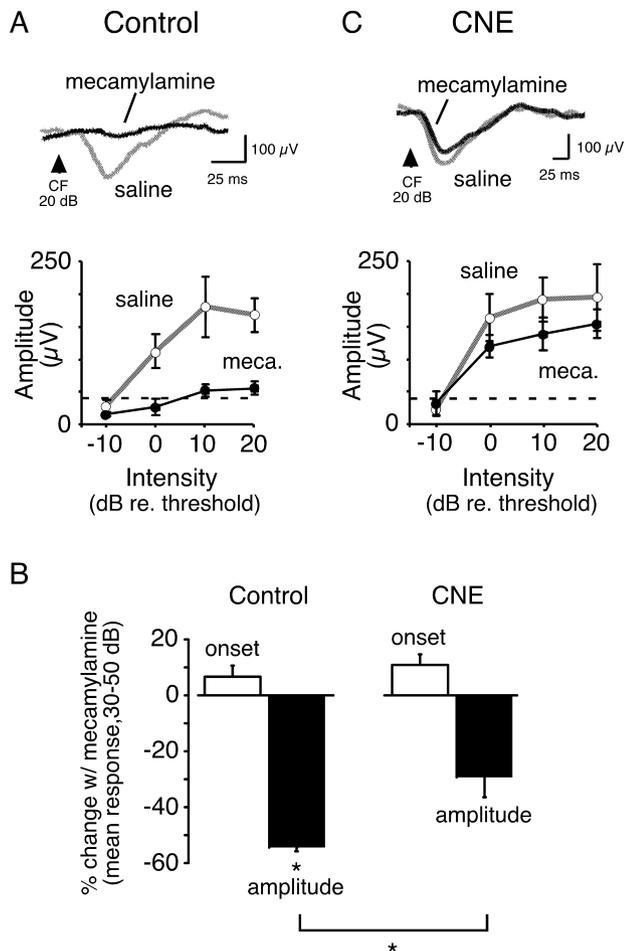


FIG. 4. Neonatal CNE reduces the effect of intracortical mecamylamine on sensory-evoked response in adult animals. (A) Microinjection of mecamylamine (10 μM, 1 μL) directly into layer 4 of A1 reduced responses to CF stimuli relative to responses following saline injections ($F_{1,6} = 28.71$, $P < 0.001$). Example on top (20-dB SPL stimulus) and group data for peri-threshold responses on bottom. (B) At higher intensities (30–50 dB re. threshold) in the same animals as A, mecamylamine reduced response amplitude ($F_{1,6} = 15.189$, $P < 0.05$) without affecting onset latencies (graph on left; onset and amplitude expressed as a percentage of saline value and averaged across intensities; $F_{1,6} = 1.138$, $P > 0.05$). Asterisk indicates $P < 0.05$ (ANOVA performed on raw data, not percentages averaged across intensities). However, after CNE, mecamylamine did not change onset ($F_{1,3} = 3.6$, $P > 0.05$) or amplitude ($F_{1,3} = 3.8$, $P > 0.05$) significantly (graph on right), and the effect of mecamylamine was greater in controls than in CNE animals (asterisk, $F_{1,11} = 7.26$, $P < 0.05$). (C) After CNE (same animals as in B, right), mecamylamine had little effect on peri-threshold response to CF stimuli ($F_{1,3} = 1.98$, $P > 0.05$).

TABLE 1. Radioligand binding in layer 4 of auditory cortex ($n = 8$ control and $n = 9$ CNE brains; groups were balanced for male and female animals, but results did not differ by sex and are combined). CNE did not change binding density of [125 I]epibatidine or [125 I] α -bungarotoxin

	Specific binding (fmol/mg)	
	Control	CNE
[125 I]Epibatidine-specific binding		
Epibatidine 0.08 nM	5.45 ± 0.18	5.64 ± 0.22
Epibatidine 0.08 nM + cytosine 200 nM	0.31 ± 0.03	0.31 ± 0.02
[125 I] α BTX-specific binding		
α BTX 5 nM	8.71 ± 0.51	9.13 ± 0.49

α BTX, α -bungarotoxin; CNE, chronic nicotine exposure.

animals, and impaired learning in an auditory-cued behavioral task. The results provide the first evidence in an animal model to link nicotine exposure during development to subsequent auditory-cognitive deficits, and suggest a potential underlying mechanism, namely diminished function of cortical nAChRs.

A developmental period of vulnerability to CNE

The period of CNE lasted only 5 days during postnatal week 2, yet it exerted a profound, and possibly permanent, effect on auditory function in the adult. The brief CNE did not alter weight gain in pups, and therefore avoided the general physical underdevelopment caused by prolonged prenatal nicotine exposure (Slotkin, 1998). Nonetheless, an identical CNE treatment alters synaptic development in auditory cortex, and has been related to the transient expression of $\alpha 7$ nAChRs in A1 that peaks during postnatal week 2. Specifically, CNE delivered during week 2 – but not during weeks 1 or 4 – alters the development of glutamate synapses for at least 2 weeks (Aramakis & Metherate, 1998; Aramakis *et al.*, 2000; Hsieh *et al.*, 2002). The longer-term consequences for glutamate synapses are unclear, given that in the present study CNE did not change auditory-evoked (pre-nicotine) responses in the adult. This latest finding is consistent with the notion that a temporary CNE may only delay, rather than permanently disrupt, glutamate synapse development (Metherate & Hsieh, 2003); however, more detailed studies of receptive field structure may yet reveal evidence for altered synaptic circuitry in the adult. For rat auditory cortex, postnatal week 2 is similar to the third trimester of human development in several ways (see Introduction), and the results of the present study therefore relate to prenatal nicotine exposure in humans. Moreover, the present results suggest that week 2 in the rat may be a ‘critical period’, in that CNE produced long-lasting, profound impairments of function, and support the notion that the transient expression of $\alpha 7$ nAChRs defines the critical period (Aramakis *et al.*, 2000; Metherate & Hsieh, 2004).

The results also raise an important, and unanswered, question: by what mechanism does neonatal CNE produce such long-lasting effects on nAChRs? The question is especially puzzling in light of the finding that nAChR density in the adult does not change. It is possible that CNE alters receptor binding affinity, or cell regulatory mechanisms; for example, CNE may impair receptor function downstream to ligand binding, or may prevent proper insertion of receptors into the cell membrane. It is important to note that chronic exposure in adults does not produce similar deficits in subsequent nAChR function (Levin & Simon, 1998; Mancuso *et al.*, 1999; Harkrider & Champlin, 2001a; Domino & Kishimoto, 2002; Gentry & Lukas, 2002). Thus, neonatal CNE exerts its effects via a mechanism specific to conditions that occur during development, again recalling the transient expression of $\alpha 7$ nAChRs (which, in rats, disappears by the end of the third postnatal week; Broide *et al.*, 1995).

Neonatal CNE impairs auditory learning

We used an auditory-cued active avoidance procedure to test for behavioral deficits in CNE animals for two major reasons: first, this task requires an intact auditory cortex (Scharlock & Miller, 1964; Massopust *et al.*, 1965, 1966, 1967, 1971; Delay & Rudolph, 1994; Duvel *et al.*, 2001), and second, performance on this task is known to be enhanced by systemic nicotine (Evangelista *et al.*, 1970; Erickson, 1971; Orsingher & Fulginiti, 1971; Yilmaz *et al.*, 1997). Thus, given our interest in the function of nAChRs located in auditory cortex, this behavioral test seemed appropriate.

Neonatal CNE had profound effects on adult performance in the behavioral task. CNE animals avoided the shock far less often than controls, although when they did avoid the shock they did so over the same range of latencies as controls (and well before the end of the tone cue). Thus, the avoidance deficit cannot be explained by impairment of basic auditory or motor functions. Moreover, the relationship between avoidance latency and percentage avoidance – a very close relationship in controls, which developed gradually over the 4 days of training – never developed in CNE animals, suggesting a different strategy or mechanism for performing the task. Finally, nearly half of the CNE animals were never able to learn the task at all (and the remaining CNE animals were still impaired relative to controls). These data indicate a profound auditory-behavioral deficit in adult animals following neonatal CNE.

The exact nature of the behavioral deficit is unclear. It is not likely that CNE animals were unable to find their way to the safe compartment, because their avoidance latency on successful trials was near normal. More likely, the deficit is one of information processing prior to the decision to move. Although prenatal stress of various kinds is associated with depression-like 'learned helplessness' in offspring, possibly the result of maternal stress hormones acting on fetal brain development (Weinstock, 2001), postnatal nicotine exposure from P8 to P20 produces a seemingly opposite result, i.e. animals that are less timid and more impulsive (Sobrian *et al.*, 2003). The impaired avoidance ability in the present study may reflect impaired information processing, rather than resignation to an unavoidable aversive stimulus. Although additional behavioral tests will be needed to understand the scope of effects due to neonatal CNE, the present results suggest an auditory learning deficit.

Cortical nAChRs enhance physiological responsiveness to acoustic stimuli

Our physiological results in control animals showed that nicotine enhanced sensitivity and responsiveness to CF stimuli, but not nonCF stimuli (three octaves below CF). These findings relate to, and link, two recent hypotheses on auditory cortex function. First, nAChRs are hypothesized to regulate synaptic transmission along thalamocortical, but not intracortical, pathways (Parkinson *et al.*, 1988; Sahin *et al.*, 1992; Gil *et al.*, 1997; Lavine *et al.*, 1997; Clarke, 2004). Second, thalamocortical inputs preferentially mediate responses to CF stimuli, whereas information about spectrally distant nonCF stimuli is relayed to the recording site via long-distance ('horizontal') intracortical pathways (Kaur *et al.*, 2004, 2005). These two hypotheses can be integrated to posit a mechanism by which cortical nAChRs could enhance auditory processing selectively for CF stimuli. That is, activation of cortical nAChRs could selectively enhance responses to thalamocortical inputs, which mediate CF information, whereas other cholinergic actions [e.g. muscarinic suppression of intracortical synapses (Hsieh *et al.*, 2000) or nicotinic excitation of inhibitory interneurons (Porter *et al.*, 1999; Christophe *et al.*, 2002; Alkondon & Albuquerque, 2003)] could reduce intracortical transmission relaying information about spectrally distant nonCF stimuli. The result would be a sharpening and threshold reduction for acoustic 'filters' (receptive fields) in A1 to favor processing of CF stimuli (Metherate *et al.*, 2005). Such actions could contribute to the hypothesized role of nAChRs in mediating attention to selected sensory stimuli (Domino & Kishimoto, 2002; Harkrider & Hedrick, 2005; Sarter *et al.*, 2005).

The effects of intracortical mecamylamine show that nAChRs located in A1 powerfully regulate responses to CF stimuli, controlling both response threshold and amplitude. The cortical nAChRs may be

high-affinity, mecamylamine-sensitive $\alpha 4\beta 2$ nAChRs that are prominent throughout layer 4 of adult neocortex and are thought to regulate thalamocortical input (Parkinson *et al.*, 1988; Lavine *et al.*, 1997; Perry *et al.*, 2002; Clarke, 2004). Our data are consistent with this hypothesis, and the concentration of mecamylamine used (10 μM) may be selective for $\alpha 4\beta 2$ over $\alpha 7$ nAChRs (Papke *et al.*, 2001), but due to the difficulty of controlling drug dose *in vivo* we have not attempted to determine the pharmacological profile of the relevant nAChRs. Further studies with more selective antagonists will be needed to determine the exact nature of the nAChRs involved.

Similarly, our results suggest that cortical nAChRs mediate a major portion of the effects produced by acute, systemic nicotine. Although this may seem unlikely given the anatomical evidence for nAChRs throughout the auditory pathway (Morley, 2005), there has been prior physiological support for this possibility. In humans, systemic nicotine (or tobacco smoking) has little effect on latency measures or on most amplitude measures of function in the auditory periphery (otoacoustic emissions) and brainstem auditory pathways (auditory brainstem response), but affects multiple latency and amplitude measures of auditory forebrain function (e.g. middle and long latency potentials) (Knott, 1987; Kishimoto & Domino, 1998; Harkrider & Champlin, 2001a, b; Harkrider *et al.*, 2001). Thus, at least under some conditions, cortical nAChRs may mediate most effects of systemic nicotine measured in A1 (but see text below on regulation of onset latency).

Neonatal CNE impairs nicotinic enhancement of processing in A1

Neonatal CNE greatly reduced most effects of nicotine and mecamylamine on A1 responses to auditory stimuli in the adult, indicating a profound loss of nAChR function. However, CNE did not affect nicotinic regulation of onset latency, and it is possible, given the argument raised in the previous section, that the nAChRs regulating onset latency are not located in A1. Whereas systemic nicotine reduced onset latency in control animals, intracortical injections of mecamylamine did not affect latency, suggesting that the relevant nAChRs are not located in A1. Rather, the nAChRs in subcortical auditory pathways (Morley & Happe, 2000; Morley, 2005) may regulate onset latency of responses recorded in A1. Some of these nAChRs may be insensitive to neonatal CNE or may be expressed only after the CNE period in our study (Morley, 2005), and therefore be unaffected by CNE. These data suggest that cortical nAChRs regulate response threshold and magnitude in A1, but not onset latency, and furthermore, that neonatal CNE affects primarily cortical nAChRs.

A related point is that given the necessity for nAChR activation in order to observe near-threshold acoustic responses in A1 of control animals (i.e. the effects of mecamylamine), it might be expected that the loss of nAChR function after neonatal CNE would render A1 virtually unresponsive to sound. That this did not occur (i.e. pre-nicotine responses to tones were similar in controls and CNE animals) suggests a partial compensation for the loss of nAChR function in A1. However, the loss of nAChR function clearly precluded further enhancement of responsiveness by nicotine or endogenous ACh; as mentioned above, changes in nAChR binding affinity or downstream signal transduction may be responsible.

Relationship between CNE-induced nAChR dysfunction and cognitive deficits

The CNE-induced impairment of nAChR function likely contributes to the behavioral deficit we observed, given the importance of ACh

and nAChRs in a variety of cognitive functions including auditory learning (Evangelista *et al.*, 1970; Erickson, 1971; Orsingher & Fulginiti, 1971; Picciotto *et al.*, 1995; Yilmaz *et al.*, 1997; Levin & Simon, 1998; Mancuso *et al.*, 1999; Weinberger, 2004; Sarter *et al.*, 2005). Developmental CNE in humans (maternal smoking during pregnancy) has been associated with auditory–cognitive deficits, such as an impaired ability to identify degraded signals, competing stimuli or signals in a noisy environment (Saxton, 1978; Picone *et al.*, 1982; Sexton *et al.*, 1990; McCartney *et al.*, 1994; Fried *et al.*, 1997, 2003). Such impairments may result from a deficit in nAChR-mediated attentional mechanisms, possibly involving increased selectivity of auditory filters in A1 during attention (Metherate *et al.*, 2005). Examining nicotinic regulation of auditory-evoked potentials (Harkrider & Champlin, 2001a) in the adult offspring of smokers could help determine if reduced function of nAChR contributes to auditory–cognitive deficits in humans.

Given that the transient expression of $\alpha 7$ nAChRs occurs in each sensory neocortex during development (Fuchs, 1989; Broide *et al.*, 1995), it seems plausible that CNE will impair sensory processing for other modalities. Similarly, nAChR deficits in adults could occur in multiple areas, if the deficits are related to the presence and distribution of nAChRs during the period of neonatal CNE (Slotkin *et al.*, 1987; Morley, 2005). In turn, it would follow that cognitive functions other than those tested here will be affected, for example those that involve nicotinic regulation of processing in the hippocampus (Gray *et al.*, 1996; Giocomo & Hasselmo, 2005). To resolve this issue, it will be necessary to understand the mechanism by which neonatal CNE affects subsequent nAChR function, and the distribution of the relevant nAChRs during neural development.

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Abbreviations

A1, primary auditory cortex; ACh, acetylcholine; CF, characteristic frequency; CNE, chronic nicotine exposure; CSD, current source density; EEG, electroencephalogram; LFP, local field potential; nAChR, nicotinic acetylcholine receptor; P, postnatal day; SPL, sound pressure level.

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