A Critical Period for Nicotine-Induced Disruption of Synaptic Development in Rat Auditory Cortex

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Cholinergic markers in the middle layers of rat auditory cortex are transiently upregulated during the second postnatal week, at which time α7 nicotinic acetylcholine receptors (nAChRs) selectively regulate NMDA receptor (NMDAR)-mediated EPSPs. To investigate the developmental role of this regulation, we determined whether manipulating nAChR function at specific times during the first 4 weeks after birth could alter subsequent neuronal function. Rat pups were injected twice daily with nicotine (1 or 2 mg/kg) or saline during approximately the first, second, or fourth postnatal week (i.e., before, during, or after the peak upregulation of nAChRs). Glutamate EPSPs and intrinsic membrane properties were measured during whole-cell recordings from visually identified pyramidal neurons in layers II-IV of brain slices prepared at least 15 hr after the last injection. Chronic nicotine exposure (CNE) had little effect on intrinsic membrane properties and during week 1 or 4 did not affect synaptic function. However, CNE during week 2 resulted in EPSPs with long durations, multiple peaks, and enhanced NMDAR components. These changes remained significant even 10 d after CNE. Rapid application of nicotine, which in control neurons selectively enhances NMDAR EPSPs during week 2, produced only weak effects after CNE. Receptor binding studies showed that CNE-induced EPSP alterations occurred in the absence of altered α7 nAChR numbers or agonist binding affinity. Thus, altered stimulation of nAChRs by CNE during week 2, but not before or after, disrupts the development of glutamate synapses in rat auditory cortex.

Key words: acetylcholine; auditory; cortex; development; EPSP; glutamate; nicotine; NMDA; intrinsic properties; synaptic

Nicotine, the neuroactive ingredient in tobacco, is a highly addictive substance that has multiple effects on the nervous system (for review, see Benowitz, 1996; Dani and Heinemann, 1996; Role and Berg, 1996). The effects of nicotine extend to nonsmokers by way of secondhand smoke exposure, and both infants and fetuses are particularly vulnerable. Children born to mothers who smoked during pregnancy are often born prematurely and exhibit decreased body weight and size, increased body tremor, and impaired respiratory function (for review, see Nash, 1988). Perinatal nicotine exposure may also produce sensory deficits. For example, infants exposed to nicotine either in utero or after birth show decreased ability to habituate to sounds and to orient toward an auditory stimulus (Saxton, 1978; Picone et al., 1982) and continue to show auditory and auditory-related cognitive impairments as they mature (Sexton et al., 1990; McCartney et al., 1994; Fried et al., 1997). Thus, early exposure to nicotine can adversely affect nervous system development, including that of the auditory system. During the second postnatal week in rats, primary sensory cortex displays a dramatic increase in expression of the cholinergic hydrolytic enzyme, acetylcholinesterase (AChE) (Kristt, 1979; Prusky et al., 1988; Robertson et al., 1991), and α7 nicotinic acetylcholine receptors (nAChRs) (Fuchs, 1989; Broide et al., 1995, 1996) in the middle cortical layers. This period corresponds to the third trimester of human development, when thalamic fibers innervate auditory neocortex and columns of AChE appear transiently in its middle layers (Krmpotic-Nemanic et al., 1980, 1983). Recently, we demonstrated that during the period of enhanced cholinergic expression in rat auditory cortex, rapid application of nicotine to pyramidal neurons selectively enhances the NMDAR component of glutamate EPSPs (Aramakis and Metherae, 1998). This effect is most prominent during postnatal days (P) 8–12 (>80% of neurons affected) and virtually disappears after P19 (<6% of neurons). These findings suggest that nAChR regulation of NMDAR activity during the second postnatal week in the rat may be important for auditory cortex development.

The goal of this study was to determine whether manipulation of nAChRs via chronic nicotine exposure (CNE) could affect the functional development of auditory cortex. We report that CNE during the second postnatal week, but not during the first or fourth weeks, disrupts synaptic development by selectively altering NMDAR-mediated synaptic transmission. Thus, postnatal week 2 appears to be a sensitive period for the disruptive actions of nicotine that may lead to long-term impairment of auditory cortex function.

MATERIALS AND METHODS

CNE treatment. Pups from timed-pregnant Sprague Dawley rats (Charles River, Wilmington, MA) were maintained on a 12 hr light/dark cycle. The date of birth was recorded as P0. Litters ranged in size from 10 to 16 pups, which were randomly assigned to saline or CNE treatment groups. Nicotine hydrogen tartrate was injected subcutaneously at a dose of either 1 or 2 mg/kg (0.35 or 0.7 mg/kg free base) in a 2 ml/kg volume using a 50 or 100 μl Hamilton syringe. Saline-treated littersmates received an equivalent volume of 0.9% sterile saline. Injections were made twice daily, at 8:00–9:00 A.M. and 4:00–5:00 P.M. After injections, pups were placed individually in an open container to monitor behavior for 2–15 min and then returned to their home cage and litter. Nicotine- and saline-treated pups gained weight at the same rate (p > 0.10).

Four CNE schedules (CNE groups I–IV) were used. These protocols were designed to expose pups at different developmental times relative to the α7 nAChR upregulation in cortical layer IV, which begins shortly after birth, peaks at ~P10, and ends after P15 (Broide et al., 1996). CNE group 1 pups were injected with nicotine (2 mg/kg) from P1 through P8 (~9 treatments were maintained up to the day before electrophysiological recordings; slices were prepared the morning after the last injection). CNE group II rats were injected with either 1 or 2 mg/kg nicotine from P8 through P14, and slices were again prepared the next morning. CNE group III pups received 2 mg/kg nicotine from P8 through P16 and were allowed to survive an additional 7–10 d, and slices were made on P23 or P26. CNE group IV received 2 mg/kg nicotine from P20 through P23–25, and slices were prepared the morning after the last injection.
The majority of experiments used a nicotine dose of 2 mg/kg (0.7 mg/kg free base, CNE groups I–IV). This dose is commonly used in CNE studies (Abdulla et al., 1996; Rowell and Li, 1997) to approximate blood levels of nicotine in smokers (Isaac and Rand, 1972; Murrin et al., 1987; Henningfield et al., 1993). Some animals in CNE group II received a lower dose of 1 mg/kg nicotine (0.35 mg/kg free base).

Slices were placed in a submersion recording chamber on the fixed brain slices, nicotine was dissolved in ACSF (25 mM) and applied by pressure ejection from a pipette with a 5 μm tip, using a picospritzer (General Valve, Fairfield, NJ). The nicotine-filled pipette was placed within 30 μm of the neuron’s apical dendrite.

Electrophysiological recording and stimulation. Neurons were visualized with infrared differential interference contrast (IR-DIC) optics with a video camera (Hamamatsu Photonics, Tokyo, Japan) and monitor (Stuart et al., 1993). Whole-cell recordings were made from the somas of layer II–IV neurons with clear ascending apical dendrites (i.e., presumed pyramidal neurons). Patch pipettes were made from filamented glass capillary tubes (1.5 mm outer diameter, A-M Systems, Seattle, WA), with tip diameters of ~2–3 μm and DC resistances of 5–8 MΩ. Brains were blocked, and coronal sections (300 – 400 μm) were filled with (in mM): 125.0 K-MeSO₄, 0.05 CaCl₂, 0.5 NaCl, 1.6 EGTA, 0.5 Na-GTP, and 10 HEPES. The pH was adjusted to 7.3 with KOH (1 M), and final osmolarity was 270–280 mOsm/kg.

ACSF-filled micropipettes with tip diameters of ~5 μm were used for electrical stimulation of afferent fibers. The electrode was placed a distance of 50–125 μm (mean 74 ± 2 μm, n = 106) lateral to the recording site, i.e., within the same cortical layer. Stimulation consisted of monophasic constant current pulses (200 μsec, 5–100 μA).

Recordings were obtained using an intracellular amplifier (Axoclamp 2B, Axon Instruments, Foster City, CA). Seal resistance was 5–12 GΩ. Series resistance ranged from 3 to 25 MΩ, but most often was ~<12 MΩ, and was compensated using the bridge balance. Membrane potential (V_m) was not corrected for liquid junction potential estimated to be approximately ~13 mV (Metherate and Aramakis, 1999). V_m was monitored on an oscilloscope, digitized at 5.5 kHz, and stored on computer. Software (Axodata, Axograph, Axon Instruments) controlled data acquisition and analysis.

Pharmacological agents. Pharmacological agents included (±)-2-amino-5-phosphonovaleric acid (APV; RBI, Natick, MA) and (–)-nicotine hydrodrene tartrate (nicotine; Sigma, St. Louis, MO). APV was prepared as a concentrated stock solution in purified H₂O and diluted to its final concentration with ACSF. Nicotine for subcutaneous injection was prepared weekly as a 0.5 or 1 mg/ml solution in 0.9% sterile saline, adjusted to pH 7.0–7.5 (1 M NaOH), and refrigerated. For rapid application to neurons in brain slices, nicotine was dissolved in ACSF (25 μM) and applied by pressure ejection from a pipette with a 5 μm tip, using a picospritzer (General Valve, Fairfield, NJ). The nicotine-filled pipette was placed within 30 μm of the neuron’s apical dendrite.

Electrophysiological analysis. For each cell, afferent stimulus intensities ranged from below the threshold for eliciting an EPSP to above the threshold for eliciting an action potential. For all ages and treatments, EPSP measurements were obtained using stimulus intensities that were just subthreshold for generating spikes ("maximum subthreshold EPSPs"). EPSP amplitude was measured at the absolute peak, and latency to peak was measured from stimulus onset. EPSP duration was measured at one-third, one-half, and two-thirds peak amplitude. Measurements were made

![Figure 1](image-url)
from averages of two to six responses taken at 10 sec intervals; however, traces used in Figures are single responses, not averages, except as noted in Figure 7. Mean data are presented ± 1 SE. Because synaptic and membrane properties develop rapidly during early postnatal life (Metherate and Aramakis, 1999; present study), a factorial ANOVA was used for age-matched comparisons between treatment groups. For CNE groups I and II, data were grouped by individual days, whereas for CNE groups III and IV data were pooled within two age ranges, P22–24 and P25–26, because of the small number of cells at each age and the relatively slow rate of change in neuronal properties by P22. Except where noted, additional within-cell and between-group comparisons used t tests for paired or unpaired samples, respectively. Differences were considered significant at P < 0.05.

**RESULTS**

Effects of CNE on electrophysiological measures of auditory cortex development were determined using slices from nicotine-injected, saline-injected, and untreated rats. Data were obtained from 190 pyramidal neurons in layers II–IV of slices from 68 pups ranging in age from P8 to P26. Of these, 45 neurons were from animals that had been injected with nicotine, and 145 neurons were from controls, comprising an equal number of untreated rats (p > 0.10). These data were therefore combined in the analysis, and are referred to as controls. An additional 16 animals injected with nicotine or saline were used for receptor binding and in situ hybridization studies.

| Table 1. EPSPs in layers II–IV pyramidal neurons of rat auditory cortex during normal development and after CNE<sup>a</sup> |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Age/condition   | Stimulus intensity (μA) | Latency to peak (msec) | Peak (mV) | EPSP width @ one-third peak | EPSP width @ one-half peak | EPSP width @ two-thirds peak | n |
| P8–12           | 26.5 ± 7.0       | 16.8 ± 4.7       | 12.1 ± 1.3    | 107.8 ± 12.3    | 73.0 ± 8.5       | 50.6 ± 6.6        | 12 |
| P13–16          | 22.7 ± 3.6       | 9.2 ± 0.7        | 15.3 ± 1.2    | 66.5 ± 7.7      | 48.3 ± 6.9       | 28.4 ± 1.9        | 17 |
| P20–22          | 15.5 ± 5.0       | 5.7 ± 0.4        | 17.7 ± 1.0    | 34.5 ± 1.8      | 24.5 ± 1.4       | 16.4 ± 1.1        | 13 |
| P24–26          | 12.4 ± 3.5       | 4.9 ± 0.5        | 23.0 ± 2.5    | 27.1 ± 2.4      | 18.3 ± 2.2       | 12.8 ± 1.6        | 5  |
| Control         | 38.8 ± 12.4      | 20.9 ± 8.7       | 11.0 ± 1.7    | 102.9 ± 16.7    | 72.7 ± 14.6      | 52.4 ± 12.1       | 6  |
| CNE group I     | 50.0 ± 15.8      | 20.0 ± 5.5       | 7.9 ± 2.2     | 115.5 ± 14.7    | 80.6 ± 10.2      | 53.6 ± 6.7        | 5  |
| CNE group II    | 20.7 ± 2.9       | 10.2 ± 1.2       | 14.9 ± 1.1    | 76.3 ± 8.6      | 53.4 ± 6.2       | 32.8 ± 2.9        | 22 |
| 1 mg/kg         | 19.0 ± 2.6       | 14.5 ± 2.9       | 13.5 ± 1.3    | 112.3 ± 11.8<sup>b</sup> | 82.9 ± 10.4<sup>b</sup> | 61.9 ± 8.9<sup>b</sup> | 13 |
| 2 mg/kg         | 17.5 ± 1.4       | 17.5 ± 4.1       | 12.9 ± 1.2    | 118.5 ± 14.9<sup>b</sup> | 82.4 ± 11.7<sup>b</sup> | 60.8 ± 10.0<sup>b</sup> | 9  |
| Control         | 10.1 ± 2.5       | 4.7 ± 0.3        | 21.2 ± 2.0    | 27.3 ± 1.9      | 18.5 ± 1.5       | 12.7 ± 1.1        | 8  |
| CNE group III   | 9.5 ± 1.8        | 14.8 ± 4.6       | 16.3 ± 2.0    | 46.6 ± 5.9<sup>b</sup> | 36.6 ± 5.5<sup>b</sup> | 28.5 ± 5.1<sup>b</sup> | 7  |
| Control         | 10.1 ± 2.5       | 4.7 ± 0.3        | 21.2 ± 2.0    | 27.3 ± 1.9      | 18.5 ± 1.5       | 12.7 ± 1.1        | 8  |
| CNE group IV    | 12.1 ± 2.6       | 5.6 ± 1.2        | 20.3 ± 1.6    | 33.7 ± 2.2      | 24.3 ± 2.4       | 17.1 ± 2.5        | 9  |

<sup>a</sup>Measurements from maximal subthreshold EPSPs.

<sup>b</sup>Significantly different from control; ANOVA, P < 0.05.
Postnatal development of EPSPs in auditory cortex

To illustrate synaptic changes during normal postnatal development, data on maximal subthreshold EPSPs were grouped into four age ranges: P8–12, P13–16, P20–22, and P24–26. At the youngest ages, EPSPs were of relatively small amplitude, long duration (measured at one-third, one-half, and two-thirds peak amplitude), and long latency to peak, and required higher stimulus intensities to be elicited (Fig. 1, Table 1). EPSPs from the oldest age range had the largest amplitude, shortest duration, and shortest latency to peak, and required the lowest stimulus intensities. A continuum in these parameters was observed over intermediate ages. Distances from the stimulating electrode to the recorded neuron averaged 72 ± 2 μm and did not differ between age groups (p > 0.05). These data on postnatal development of synaptic properties served as age-matched controls for the effects of CNE.

Effects of CNE on synaptic development

As described above (Materials and Methods), four groups of rat pups were exposed chronically to nicotine during different phases of the postnatal nAChR upregulation (CNE groups I–IV). A qualitative assessment of the behavioral effects of nicotine treatments indicated that within ~30 sec of the subcutaneous nicotine injection (1 or 2 mg/kg; 0.35 or 0.7 mg/kg free base), young animals (<P10) displayed increased resting tremor and performed “swimming” movements. In older pups, nicotine produced increased locomotion, often resulting in circling movements. Such behavior persisted for up to 15 min, after which time nicotine-injected animals could not be distinguished behaviorally from controls.

CNE group I: CNE from P1 to P8–9 does not affect synaptic development

Neurons from pups injected with 2 mg/kg nicotine from P1 to P8–9 had maximal subthreshold EPSPs similar to those from age-matched controls (Fig. 2, Table 1). Nicotine-treated animals had similar EPSP peak amplitudes, peak latencies, and durations as control EPSPs and were elicited by stimuli of similar intensity (Table 1) [distances from the stimulating electrode to the recorded neuron did not differ between any CNE group (CNE I–IV) and its control group; p > 0.05]. Thus CNE for 8–9 d, including the entire first postnatal week, did not affect the measurements of synaptic development used in this study.

CNE group II: CNE from P8 to P11–14 disrupts synaptic development

Nicotine injections (1 or 2 mg/kg) for 4–7 d from P8 through P11–14 dramatically altered EPSPs in two ways (Fig. 3, Table 1). First, EPSPs from nicotine-treated animals had longer durations, measured at one-third, one-half, or two-thirds peak amplitude (Fig. 3A,B,C). Second, multiple, discrete fluctuations resembling miniature EPSPs appeared prominently on the descending phase of EPSPs (Fig. 3Aii, asterisks). These miniature events occurred spontaneously, but their frequency increased markedly for tens of milliseconds after an afferent stimulus (Fig. 3Bi) shows total number of events for 22 cells; comparison of events 50 msec before and after the afferent stimulus demonstrates a statistically significant increase; paired t test, p < 0.001). Similar miniature events occurred at all stimulus intensities (data not shown). Few if any miniature events occurred in control cells (Fig. 3Bi shows total number of events in 22 control cells; small individual events can be seen in traces from P8 and P20 cells in Fig. 1A), nor did control cells show an increase in miniature frequency after an afferent stimulus (p > 0.3). Nicotine doses of 1 and 2 mg/kg were both effective in altering EPSP characteristics (Fig. 3A,C) (comparison of EPSP durations shown in Fig. 3C; p > 0.1). Nicotine-treated EPSPs also tended to have smaller amplitudes and longer peak latencies than controls, but these differences were not significant (Table 1). Together, these data indicate that chronic exposure to nicotine for 4–7 d during the second postnatal week dramatically altered EPSP development in auditory cortex.

CNE group III: long-term effects of CNE

To determine whether the effects of CNE during the second postnatal week were long lasting, rat pups were injected with 2 mg/kg nicotine for 9 d from P8 to P16 and then allowed to survive for an additional 7–10 d before electrophysiological experiments were performed. EPSPs from P23–26 pups in CNE group III were significantly longer than age-matched controls (Fig. 4, Table 1) (p < 0.01), but not as long as CNE group II EPSPs (comparison with CNE group II pups treated with 2 mg/kg, p < 0.05). EPSPs in CNE group III also displayed a small but significant increase in presumed miniature events within the first 20 msec after the stimulus (p < 0.05) (e.g., Fig. 6Bi). Group III EPSPs tended to have smaller amplitudes and longer peak latencies than controls, but these differences were not significant (Table 1). These data indicate that nicotine exposure during the second postnatal week can produce effects that last well into the fourth week. However, the normal developmental trends appear to resume after cessation of CNE.

CNE group IV: CNE from P20 to P23–25 does not affect synaptic development

A final group of animals was injected with 2 mg/kg nicotine for 4–6 d from P20 to P23–25, and slices were made on the morning after the last injection. In seven of nine neurons, EPSPs were indistinguishable from those of age-matched controls (Fig. 5Ai, Aii, com-
pare P26 neurons). In two neurons, maximal subthreshold EPSPs had longer durations that qualitatively resembled EPSPs in CNE group III (P24 neuron in Fig. 5Aii), although unlike group III, lower intensity stimuli elicited EPSPs resembling controls (Fig. 5Aii, see smaller EPSP for P24 neuron). Nevertheless, group data for all nine neurons in CNE group IV showed that EPSP duration, amplitude, latency to peak, and miniature fluctuations did not differ from control values (Fig. 5B, Table 1; \( p > 0.10 \); data for miniature fluctuations not shown). Thus, nicotine exposure during the fourth postnatal week did not significantly affect the measurements of synaptic development used in this study.

**Effects of CNE on NMDAR EPSPs**

Because our previous study provided evidence that nAChRs selectively regulate EPSPs mediated by NMDARs (Aramakis and Mererate, 1998), we might expect that manipulations of nAChR function, such as the CNE treatment used here, would selectively affect NMDAR EPSPs. We therefore compared the contribution of NMDARs to EPSPs in neurons from nicotine-exposed and control pups.

**CNE group II**

As described above (Fig. 3), nicotine treatment from P8 to P11–14 produced long-duration EPSPs with multiple miniature fluctuations. In neurons from pups treated with 2 mg/kg nicotine, bath application of the NMDAR antagonist APV (50 \( \mu \)M) greatly decreased the magnitude and duration of EPSPs (Fig. 6A; \( n = 5 \); note that treatment with 1 mg/kg nicotine produced similar, statistically significant effects in 10 additional neurons, but to facilitate comparisons across CNE groups these data are not shown). Data from APV application allowed comparison of the NMDAR component of the EPSP (obtained by subtracting the EPSP area in APV from the pre-APV area) and the non-NMDAR component (the area in APV) in control and nicotine-treated neurons. As shown in Figure 6Aii, the NMDAR EPSP in CNE group II neurons clearly was enhanced relative to control neurons (102% increase, \( p < 0.01 \)). CNE more than doubled the NMDAR component (164% increase, \( p < 0.05 \))

**CNE group III**

EPSPs recorded 7–10 d after nicotine exposure from P8 to P16 also contained enhanced NMDAR components. APV significantly reduced EPSPs in CNE group III neurons (Fig. 6Bi) (\( n = 5 \)), and explicit comparison of the non-NMDAR and NMDAR components of the EPSP area (Fig. 6Bii) demonstrated that CNE more than doubled the NMDAR component (164% increase, \( p < 0.05 \))
but did not change the non-NMDAR component ($p > 0.10$). These findings are consistent with the data from CNE group II and indicate that CNE during the second postnatal week selectively enhanced the NMDAR component of the EPSP for at least 10 d.

CNE group IV

APV also reduced the area of EPSPs in neurons from pups exposed to nicotine from P20 to P23–25 (Fig. 6C). However, non-NMDAR and NMDAR components of the EPSP were similar in control and nicotine-treated neurons (Fig. 6Cii) ($p > 0.05$; note that although group differences were not significant, the two CNE group IV neurons mentioned above with prominent APV-sensitive components). On average, therefore, nicotine exposure in the fourth postnatal week did not affect the NMDAR component of the EPSP.

Effects of acute (pressure-ejected) nicotine on EPSPs after CNE

Previously we had demonstrated that rapid application of nicotine directly to neurons selectively enhances NMDAR-mediated synaptic transmission in neurons from untreated animals (Aramakis and Metherate, 1998). Because CNE group II neurons in the present study had enhanced EPSPs (Fig. 3) caused by selective enhancement of NMDAR components (Fig. 6), we sought to determine whether these EPSPs could be enhanced further by acute nicotine application (pressure-pulse application from a nicotine-containing micropipette placed adjacent to the apical dendrite of the neuron). Only P13 neurons were used, to examine untreated neurons close to their peak nicotine sensitivity (at ~P10) (Aramakis and Metherate, 1998), as well as CNE neurons that had...
Figure 6. NMDARs mediate the enhanced EPSPs and stimulus-evoked miniature events produced by CNE during week 2. Ai, CNE group II; APV (50 μM) reduced the long-duration and multiple-peaked EPSP in a P13 neuron; \( V_m \) = −70 mV. Aii, The area of the non-NMDAR EPSP (the EPSP in APV) in CNE group II neurons did not differ from control (control, \( 619 \pm 104 \) mV · msec; \( n = 7 \); CNE II, \( 557 \pm 99 \) mV · msec; \( n = 15 \); \( p > 0.10 \)). In contrast, the area of the NMDAR EPSP (pre-APV area minus area in APV) was twice as large in CNE group II neurons as in controls (control, \( 493 \pm 102 \) mV · msec; CNE II, \( 994 \pm 117 \) mV · msec; \( p < 0.01 \)). Aiii, APV reduced the stimulus-evoked increase in miniature events (\( p < 0.01, n = 11 \) neurons; for each neuron, miniature events were counted in three consecutive EPSPs, 10 sec interstimulus interval). Bi, CNE group III: APV decreased EPSP area in a P23 neuron; \( V_m \) = −68 mV. Bii, The non-NMDAR EPSP area was similar in control and CNE group III neurons (control, \( 344 \pm 44 \) mV · msec; \( n = 7 \); CNE III, \( 258 \pm 69 \) mV · msec; \( n = 5 \); \( p > 0.10 \)). However, the NMDAR EPSP area was more than twice as large in CNE group III neurons as in control neurons (control, \( 180 \pm 48 \) mV · msec; CNE III, \( 476 \pm 131 \) mV · msec; \( p < 0.05 \)). Ci, CNE group IV: APV produced a small decrease in the late EPSP; P25 neuron, \( V_m \) = −79 mV. Cii, The non-NMDAR EPSP area was similar in control and CNE group IV neurons (control, \( 344 \pm 44 \) mV · msec; \( n = 7 \); CNE IV, \( 360 \pm 49 \) mV · msec; \( n = 9 \); \( p > 0.10 \)). Similarly, the NMDAR EPSP area did not differ between control and CNE group IV neurons (control, \( 180 \pm 48 \) mV · msec; CNE IV, \( 306 \pm 40 \) mV · msec; \( p > 0.10 \)).

Effects of CNE on the development of intrinsic membrane properties

Intrinsic membrane properties of layer II–IV pyramidal neurons change dramatically during early postnatal life (Metherate and Aramakis, 1999). Data from control neurons indicate that from P8 to P26 resting potential becomes more negative, input resistance and time constant decrease, spike amplitude increases, spike width decreases, and spike threshold shows no change (Table 2). These data raise the possibility that CNE-induced increases in NMDAR EPSPs (Figs. 3, 6) may have important effects on membrane properties, against which the effects of CNE can be compared.

CNE during the first postnatal week altered some intrinsic properties, whereas nicotine exposure during the second and fourth weeks had little effect. CNE group I neurons had lower spike amplitudes, smaller spike amplitudes, and longer spike widths than age-matched controls, whereas resting potential, input resistance, and time constant were not affected (Table 2). Intrinsic properties for CNE group II neurons were not affected by nicotine exposure except for a small reduction in spike width, and CNE groups III and IV showed no effects on any measured parameter. Thus, of the membrane properties measured, CNE during the first postnatal week altered those related to spike generation but not passive membrane characteristics. CNE in subsequent weeks exerted little effect on developing membrane properties.

Effects of CNE on \( \alpha 7 \) nAChRs

To determine whether nicotine-induced changes in synaptic development resulted from changes in \( \alpha 7 \) nAChRs, we determined levels of \( \alpha 7 \) mRNA and \( [^{125}I] \) α-BTX binding (which reflects levels of \( \alpha 7 \) nAChR protein) (Seguela et al., 1993; Drisdel and Green, 2000) in nicotine- and saline-injected littersmates. As CNE groups II and III, nicotine or saline injections were performed from P8 to P12, and brains were removed on either P13 or P23. Table 3 shows that nicotine exposure produced a trend toward increased \( \alpha 7 \) mRNA levels and \( [^{125}I] \) α-BTX binding at P13 and P23; however, only the increase in \( [^{125}I] \) α-BTX binding at P23 reached statistical significance (repeated measures ANOVA, \( p < 0.001 \)). Thus, the CNE-induced alteration of glutamate EPSPs during postnatal week 2 took place without significant changes in \( \alpha 7 \) nAChR levels.

Despite the apparent lack of change in total \( \alpha 7 \) nAChRs in week 2 (P13 data), it is possible that CNE altered their functional state. We therefore determined the agonist binding affinity of \( \alpha 7 \) nAChRs in nicotine- and saline-injected pups. Nicotine inhibition of α-BTX binding was no different in the two groups (Table 4), either 1 d after P8–12 injections (at P13) or 10 d later (P23), although agonist binding affinity increased over this time (compare P13 and P23 data in Table 4). Thus CNE did not alter the agonist binding affinity of \( \alpha 7 \) nAChR sites, at least not beyond the CNE period itself.

DISCUSSION

To investigate whether nAChR function plays a role in auditory cortex development, we exposed rats chronically to nicotine during
postnatal weeks 1, 2, or 4, and subsequently examined physiological and anatomical development. CNE during week 1 or 4 had little measurable effect (CNE groups I or IV). However, exposure during week 2 (CNE group II) produced long-duration, multi-peaked EPSPs, without changing the levels of α7 nAChRs. The physiological effects were seen even 10 d after the nicotine treatment (CNE group III), at which time an ~15% increase in α7 nAChR levels was observed. Pharmacological manipulations indicated that CNE selectively enhanced the NMDAR component of EPSPs. CNE-enhanced EPSPs were only weakly increased further by acute nicotine application, in contrast to the robust enhancement observed in control neurons. These data identify postnatal week 2 as a period during which CNE can alter synaptic development in rat auditory cortex.

Previously, we have shown in normal animals that rapid activation of α7 nAChRs during postnatal week 2 selectively enhances NMDAR EPSPs (Aramakis and Metherate, 1998). The results implied that presynaptic α7 nAChRs regulated glutamate release at synapses that had only NMDARs postsynaptically, i.e., pure-NMDAR (“silent”) synapses. Our present results showing that CNE selectively affects NMDAR EPSPs is consistent with this hypothesis (see below). Although CNE will affect any nAChR, our previous result serves to focus our interpretation and the subsequent discussion on α7 nAChRs.

**nAChR function during and after CNE**

Nicotinic AChRs are ionotropic receptors that gate cation currents, and nAChRs containing α7 subunits are noteworthy for their high Ca\(^{2+}\) permeability and rapid desensitization (Seguela et al., 1993; Zhang et al., 1994; Castro and Albuquerque, 1995; for review, see Role and Berg, 1996). Presynaptic and postsynaptic α7 nAChRs are found throughout the brain where they can enhance neurotransmitter release and mediate fast synaptic transmission, respectively (for review, see Role and Berg, 1996; Wonnacott, 1997; Jones et al., 1999). During development, α7 nAChRs function in neurite retraction and synapse formation (Pugh and Berg, 1994; Zheng et al., 1994) and regulate glutamate release in sensory cortex (Gil et al., 1997; Aramakis and Metherate, 1998). Other nAChRs may

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**Table 2. Intrinsic membrane properties of layers II-IV pyramidal neurons in rat auditory cortex during normal development and after CNE**

<table>
<thead>
<tr>
<th>Age/condition</th>
<th>RMP (mV)</th>
<th>Ri (MΩ)</th>
<th>τ (msec)</th>
<th>Spike threshold (mV)</th>
<th>Spike height (mV)</th>
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<td>Development</td>
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<td>P8-12</td>
<td>−64.3 ± 0.7</td>
<td>282.2 ± 10.6</td>
<td>22.9 ± 0.7</td>
<td>−38.7 ± 0.5</td>
<td>65.6 ± 1.0</td>
<td>1.2 ± 0.3</td>
<td>53</td>
</tr>
<tr>
<td>P13-16</td>
<td>−68.4 ± 0.6</td>
<td>195.6 ± 7.7</td>
<td>18.1 ± 0.4</td>
<td>−41.1 ± 0.4</td>
<td>72.8 ± 0.8</td>
<td>1.1 ± 0.3</td>
<td>70</td>
</tr>
<tr>
<td>P20-22</td>
<td>−72.9 ± 1.6</td>
<td>72.5 ± 7.2</td>
<td>11.9 ± 0.6</td>
<td>−40.9 ± 0.6</td>
<td>74.3 ± 1.1</td>
<td>0.74 ± 0.03</td>
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</tr>
<tr>
<td>P24-26</td>
<td>−76.8 ± 1.5</td>
<td>49.8 ± 6.1</td>
<td>8.2 ± 0.5</td>
<td>−38.9 ± 1.8</td>
<td>76.4 ± 2.6</td>
<td>0.68 ± 0.03</td>
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<td>−64.8 ± 1.1</td>
<td>305.2 ± 19.6</td>
<td>21.6 ± 1.0</td>
<td>−38.2 ± 0.8</td>
<td>63.8 ± 1.5</td>
<td>1.1 ± 0.04</td>
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</tr>
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<td>CNE Group I</td>
<td>−60.8 ± 1.2</td>
<td>300.0 ± 24.2</td>
<td>23.3 ± 2.3</td>
<td>−33.1 ± 2.4(^*)</td>
<td>50.8 ± 3.4(^*)</td>
<td>1.5 ± 1.2(^*)</td>
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<tr>
<td>Control</td>
<td>−67.5 ± 0.6</td>
<td>220.5 ± 7.5</td>
<td>19.6 ± 0.5</td>
<td>−40.6 ± 0.4</td>
<td>71.3 ± 0.9</td>
<td>1.1 ± 0.02</td>
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<tr>
<td>CNE Group II</td>
<td>−64.3 ± 1.3</td>
<td>200.4 ± 11.0</td>
<td>20.2 ± 0.9</td>
<td>−40.0 ± 0.5</td>
<td>67.3 ± 1.2</td>
<td>0.95 ± 0.02(^*)</td>
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<tr>
<td>Control</td>
<td>−75.1 ± 1.9</td>
<td>59.0 ± 10.4</td>
<td>8.3 ± 0.3</td>
<td>−39.7 ± 1.3</td>
<td>75.4 ± 2.2</td>
<td>0.66 ± 0.03</td>
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<tr>
<td>CNE Group III</td>
<td>−75.7 ± 2.1</td>
<td>60.9 ± 9.0</td>
<td>8.6 ± 0.8</td>
<td>−42.0 ± 1.7</td>
<td>75.8 ± 2.1</td>
<td>0.75 ± 0.06</td>
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<tr>
<td>Control</td>
<td>−75.1 ± 1.9</td>
<td>59.0 ± 10.4</td>
<td>8.3 ± 0.3</td>
<td>−39.7 ± 1.3</td>
<td>75.4 ± 2.2</td>
<td>0.66 ± 0.03</td>
<td>10</td>
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<tr>
<td>CNE Group IV</td>
<td>−76.7 ± 1.2</td>
<td>53.3 ± 4.3</td>
<td>8.3 ± 0.3</td>
<td>−43.0 ± 1.4</td>
<td>79.3 ± 2.4</td>
<td>0.72 ± 0.03</td>
<td>9</td>
</tr>
</tbody>
</table>

\(^*\)Significantly different from control; ANOVA, \(p < 0.05\).

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**Figure 7.** Effect on EPSPs of acute, rapid nicotine application to control and CNE group II neurons. All data are from P13 neurons. A, In a control neuron, pressure-ejected nicotine enhanced the late portion of the EPSP; \(V_m = 70 \text{ mV}\). In a CNE group II neuron, nicotine increased the EPSP magnitude only slightly; \(V_m = 62 \text{ mV}\). Each trace is an average of five responses at 10 sec interstimulus intervals. B, Rapid application of nicotine (25 μM, 10–40 msec, 20 psi) significantly enhanced EPSPs in control neurons \((n = 17; \text{paired } t \text{ test}, p < 0.02)\), but not CNE group II neurons \((n = 8; p > 0.2)\). C, EPSP enhancement for the 25 neurons whose responses contribute to the histograms in B is shown for neurons that had either an increase (●) or no effect (○) in response to rapid nicotine application. For control neurons, nicotine increased EPSP areas 34–289% (mean 114%). In contrast, increases for CNE group II neurons were only 30–34% (mean 32%). Dashed lines indicate statistical significance at the 0.05 level (see Materials and Methods). Arrows indicate measurements for responses shown in A.
mediate synaptic transmission in developing sensory cortex (Roerig et al., 1997). Thus CNE can impact cortical development directly via nAChRs.

The CNE protocols used in the present study mimic nicotine blood levels during smoking, which may reach ~0.6 μM and remain above baseline levels for hours (Isaac and Rand, 1972; Henningfield et al., 1993; for review, see Benowitz, 1996; Dani and Heinemann, 1996). Exposure to 0.3–0.5 μM nicotine for several minutes will prevent regulation of glutamate EPSPs by α7 nAChRs in auditory cortex (Aramakis and Metherate, 1998), presumably by way of receptor desensitization. Thus, our original intent for the present study was to maintain nAChRs in a desensitized state using the CNE protocol. However, it is not clear what level of desensitization actually occurs and whether it is maintained for the full period between nicotine injections. Low doses of nicotine may not significantly desensitize α7 nAChRs (Fenster et al., 1997), and although chronic exposure can induce prolonged (hours or days) desensitization of nAChRs expressed in oocytes and cell lines (Lukas, 1991; Peng et al., 1994), α7 nAChRs in cultured rat cortical cells can recover within minutes (Kawai and Berg, 1999). Further studies will be needed to characterize nAChR function during the CNE protocol.

Understanding changes in nAChR function after CNE is complicated by developmental regulation of nAChRs. In mouse, CNE from P10 to P16 decreases the total number of nicotine binding sites in P17 mice; this effect is associated with decreased low-affinity and increased high-affinity nicotine binding sites (Nordberg et al., 1991). In rat, CNE from P8 to P16 increases [3H]nicotine binding in adult cortex, with a shift in agonist binding from a low- to high-affinity state (Miao et al., 1998). However, mRNA levels for several nAChR subunits, including α7, remain unchanged (Miao et al., 1998). These studies conclude that CNE in young rodents can produce long-lasting changes in the numbers and agonist binding affinity of nAChRs, and that postnatal week 2 is a critical period for CNE effects.

In the present study on rat auditory cortex, CNE from P8 to P12 did not significantly alter α7 nAChR levels ([125I]-α-BTX binding sites) at P13, but it increased levels ~15% after 10 d. CNE also did not affect agonist binding affinity of α7 nAChRs, although affinity increased during development. Although we focused on α7 nAChRs in rat auditory cortex because of our earlier study (Aramakis and Metherate, 1998), effects of CNE on other nAChR subtypes and in other cortical areas may differ (previous paragraph), given the differential distribution of nAChRs among cortical areas and layers (Fuchs, 1989; Naef et al., 1992; Broide et al., 1996). However, these data indicate that the striking changes in glutamate-mediated EPSPs produced 1 d after CNE are not associated with changes in either the numbers or agonist binding affinity of α7 nAChRs. Altered EPSPs more likely result from altered nAChR stimulation during the CNE protocol than from changes in receptor function per se.

### Postnatal week 2 is a "critical period" in rat auditory cortex development

Several postnatal events are particularly relevant to the present discussion. First, the dramatic upregulation of AChE and α7 nAChRs in the middle layers of rat auditory cortex begins early in week 1, peaks during week 2, and then declines to low (adult) levels by the end of week 3 (Fuchs, 1989; Robertson et al., 1991; Broide et al., 1995). Second, the composition of NMDARs and their contribution to glutamate EPSPs change markedly over the first postnatal weeks, in part driven by sensory experience (Agmon and O'Dowd, 1992; Burgard and Hablitz, 1993; Monyer et al., 1994; Quinlan et al., 1999; for review, see Fox et al., 1999). Some early glutamate synapses are pure NMDAR synapses that are converted, possibly in an experience-dependent process, to mixed AMPA/NMDAR synapses (Liao et al., 1995; Wu et al., 1996; Isaac et al., 1997). A large body of evidence indicates that cholinergic and glutamatergic functions regulate cortical development and may contribute to postnatal critical periods (Bear and Singer, 1986; Hohmann et al., 1991; Robertson et al., 1998; for review, see Hohmann and Berger-Sweeney, 1998; Fox et al., 1999; Yuste and Sur, 1999). For the auditory system, early week 2 is further distinguished by the onset of hearing and the subsequent, rapid maturation of the cortical auditory evoked potential (Jewett and Romano, 1972; Iwasa and Potsic, 1982). Thus, α7 nAChR regulation of NMDAR synapses occurs during a period of intense synaptogenesis and neural circuit formation.

The present study indicates that the second postnatal week may be critical for auditory cortex development, in that CNE produces a dramatic and long-lasting alteration of glutamatergic synaptic transmission. The degree to which this alteration affects acoustic information processing, and hence the designation of week 2 as a critical or sensitive period, remains to be determined. Several mechanisms may underlie the CNE effects. Desensitization of α7 nAChRs would prevent regulation of NMDAR EPSPs (Aramakis and Metherate, 1998), which in turn could prevent or delay the maturation of NMDAR synapses. A delay would produce EPSPs with larger NMDAR components than in age-matched controls, as
seen in the present study, because NMDAR components normally decrease with age (Agmon and O’Dowd, 1992; Burgard and Hablitz, 1993). Our results suggest a delay in development rather than a permanent halt, because the magnitudes of NMDAR EPSPs resume their decline after cessation of CNE.

An additional finding that is not consistent with a simple delay of maturation, however, is the CNE-induced appearance of stimulus-evoked miniature events. These events, which are not prominent in normal development, imply a presynaptic effect of CNE to prolong glutamate release (note also the lack of CNE effect on postsynaptic membrane properties). Nicotine-induced Ca\(^{2+}\) fluxes in presynaptic terminals (McGehee et al., 1995; Gray et al., 1996) may enhance the slow (asynchronous) component of evoked glutamate release (Goda and Stevens, 1994), leading to longer EPSPs and discrete miniature events. Evidence consistent with this suggestion has been obtained in hippocampal neurons, where repeated stimulation of presynaptic a7 nAChRs can produce a long-lasting increase in the frequency of miniature postsynaptic events (Radcliffe and Dani, 1998). Obviously, this scenario is inconsistent with the notion of maintained nAChR desensitization during CNE. An alternative explanation is that nAChRs recover between nicotine injections, such that repeated injections lead to repeated, potent stimulation of a7 nAChRs and long-lasting enhancement of glutamate release. This could also explain the finding that, after CNE, the effect of acute nicotine (i.e., rapid application directly to neurons) appeared to be reduced. That is, glutamate release already enhanced by CNE might not be enhanced further by acute nicotine application. Future studies must distinguish among these alternatives.

**Implications for human auditory development**

If a7 nAChR-mediated regulation of NMDARs contributes to the maturation of cortical circuits, then CNE could disrupt subsequent auditory function. Supportive evidence exists in studies of human development where the analogous developmental period, including transient cholinergic upregulation in auditory cortex, occurs during the third trimester (Krmptovic-Nemanic et al., 1980, 1983). Children exposed prenatally to nicotine via secondhand smoke show decreased auditory responsiveness (habituation or orientation) and auditory-related cognitive deficits (see introductory remarks). Future studies should determine the developmental role of nAChRs in shaping auditory function and the effects of perinatal CNE on auditory dysfunction.

**REFERENCES**


Bear MF, Singer W (1989) Presynaptic regulation of NMDARs contributes to the facilitation of fast excitatory synaptic transmission in CNS by presynaptic a7 nAChRs in shaping auditory function and the effects of perinatal CNE on auditory dysfunction.

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