

Effects of Prenatal Nicotine Exposure on Primate Brain Development and Attempted Amelioration with Supplemental Choline or Vitamin C: Neurotransmitter Receptors, Cell Signaling and Cell Development Biomarkers in Fetal Brain Regions of Rhesus Monkeys

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Studies in developing rodents indicate that nicotine is a neuroteratogen that disrupts brain development by stimulating nicotinic acetylcholine receptors (nAChRs) that control neural cell replication and differentiation. We administered nicotine to pregnant Rhesus monkeys from gestational day 30 through 160 by continuous infusion, achieving maternal plasma levels comparable to those in smokers (30 ng/ml). Fetal brain regions and peripheral tissues were examined for nAChR subtypes, other neurotransmitter receptors, and indices of cell signaling and cell damage. Nicotine evoked nAChR upregulation, but with distinct regional disparities indicative of selective stimulatory responses. Similarly, indices of cell loss (reduced DNA), cell size and neuritic outgrowth (protein/DNA and membrane/total protein ratios) were distinct for each region and did not necessarily follow the rank order of nAChR upregulation, suggesting the involvement of additional mechanisms such as oxidative stress. We then attempted to offset the adverse effects of nicotine with standard dietary supplements known to interact with nicotine. By itself, choline elicited nicotine-like actions commensurate with its promotion of cholinergic neurotransmission. When given in combination with nicotine, choline protected some regions from damage but worsened nicotine's effects in other regions. Similarly, Vitamin C supplementation had mixed effects, increasing nAChR responses while providing protection from cell damage in the caudate, the brain region most susceptible to oxidative stress. Our results indicate that nicotine elicits neurodevelopmental damage that is highly selective for different brain regions, and that dietary supplements ordinarily thought to be neuroprotectant may actually worsen some of the adverse effects of nicotine on the fetal brain.

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INTRODUCTION

Maternal smoking during pregnancy accounts for thousands of perinatal and neonatal deaths in the US each year, along with a wide spectrum of neurodevelopmental disorders as the offspring progress through childhood and adolescence (DiFranza and Lew, 1995; Ernst *et al*, 2001;

Frank *et al*, 2001; Wakschlag *et al*, 2002; Weitzman *et al*, 2002). Animal models, chiefly in rodents, have shown that nicotine is a neuroteratogen that targets neural replication and differentiation, synaptic development and synaptic activity, culminating in functional deficits and cognitive impairment that parallel the findings in offspring of smokers (Levin and Slotkin, 1998; Slotkin, 1992, 1999, 2004). Thus, although tobacco smoke contains thousands of bioactive compounds, nicotine by itself contributes significantly to fetal brain damage and its functional consequences, a factor that must be taken into account in smoking cessation strategies that utilize nicotine replacement therapy (Benowitz *et al*, 2000). Nicotine affects brain development because it stimulates nicotinic acetylcholine

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receptors (nAChRs); in the immature brain, acetylcholine, among other neurotransmitters, plays a critical neurotrophic role in determining the patterns of cell development (Lauder and Schambra, 1999; Weiss *et al*, 1998). Essentially, by providing excessive cholinergic stimulation throughout fetal life, nicotine discoordinates the numerous events in cell replication, differentiation and synaptic development that are necessary to the proper assembly of the mammalian brain.

Nevertheless, there are key questions about the developmental neurotoxicity of nicotine for which models based on rats or mice are particularly problematic. First, because these species are altricial, birth occurs at neurodevelopmental stages analogous to the end of the second trimester of human pregnancy, so that adverse effects originating in late gestation would be better modeled in nonhuman primates such as the monkey. Second, the larger amounts of tissue available from fetal monkey brain enables determinations to be carried out more readily in specified regions and subregions. Third, and most obviously, brain development in primates more closely mimics the same processes in human brain development. To our knowledge, however, only scant information is available in primates concerning the effects of nicotine on neurodevelopment, limited to examination of nAChRs in the lung and with environmental tobacco smoke exposure (rather than just nicotine) in one broadly defined brain region (Sekhon *et al*, 1999; Slotkin *et al*, 2002). In contrast, there is far more information on the developmental neurotoxicity of cocaine in monkeys, information that has given a much more comprehensive view of mechanisms and consequences of exposure to that drug of abuse (Choi and Ronnekleiv, 1996; Fang and Ronnekleiv, 1999; Lidow and Song, 2001).

In the current study, we administered nicotine to pregnant Rhesus macaque monkeys throughout pregnancy until just before term, using continuous infusions of nicotine via osmotic minipumps, so as to avoid confounding effects of fluctuating drug levels and the associated incidence of fetal hypoxia/ischemia (Levin and Slotkin, 1998; Slotkin, 1992, 1999, 2004), carefully matching the dose rate so as to simulate the plasma levels of nicotine commonly associated with maternal smoking or with use of transdermal nicotine patches (Benowitz *et al*, 1990; Fredrickson *et al*, 1995; Isaac and Rand, 1972; Lichtensteiger *et al*, 1988; Luck *et al*, 1985; Murrin *et al*, 1987). Fetal brain regions, heart and lung were then examined for four families of biomarkers known to characterize neurodevelopmental deficits associated with nicotine exposure. First, we assessed radioligand binding to two nAChR subtypes that play defined roles in neural development, $\alpha 4\beta 2$ and $\alpha 7$. The $\alpha 4\beta 2$ nAChR is the most abundant form in the mammalian brain (Flores *et al*, 1992; Happe *et al*, 1994; Whiting and Lindstrom, 1987; Whiting and Lindstrom, 1988) and its upregulation by nicotine in the fetus is mechanistically involved in the adverse effects of nicotine on cell development, as well as providing a biomarker for neural cell stimulation by nicotine (Levin and Slotkin, 1998; Slotkin, 1992, 1999, 2004). Although present in lower concentrations, the $\alpha 7$ nAChR is closely associated with the response to brain injury (Verbois *et al*, 2002) and with the effects of nicotine on neuritic outgrowth (Chan and Quik, 1993; Quik *et al*, 1994). Second, we focused on the effects on

neurotransmitter receptors and cell signaling proteins that control the generation of the second messenger, cyclic AMP. In the developing brain, cyclic AMP plays a key role in the coordination of cell replication and differentiation, synaptic outgrowth, and apoptosis (Shaywitz and Greenberg, 1999; Stachowiak *et al*, 2003) and is a common site for disruption of brain development by neuroteratogens (Levitt *et al*, 1997; Slotkin *et al*, 2003a; Whitaker-Azmitia, 1991; Yanai *et al*, 2002). We performed a battery of tests designed to evaluate the impact of fetal nicotine exposure on the signaling cascade converging on adenylyl cyclase (AC) the enzyme that generates cyclic AMP (Figure 1). First, we assessed radioligand binding to two receptors that exert opposing actions on AC: β -adrenoceptors (β ARs), which stimulate the enzyme through participation of the stimulatory G-protein (G_s), and type 2 muscarinic cholinergic receptors (m_2 AChRs), which suppress AC activity through the inhibitory G-protein (G_i). We then tested the ability of β AR and m_2 AChR receptor stimulants to affect AC activity, and contrasted the effects with alterations in basal AC activity or in the response of AC to direct stimulants (forskolin, Mn^{2+}) that bypass the need for receptor activation. Forskolin and Mn^{2+} act at different epitopes on the AC molecule, so the preference for one over the other reflects shifts in molecular conformation, primarily influenced by the AC isoform (Zeiders *et al*, 1999b). Effects mediated at the level of AC are 'heterologous,' that is, they influence the cellular response to multiple neurotransmitter and hormonal inputs that converge on cyclic AMP as a second messenger (Slotkin *et al*, 2003a).

The third battery of measurements focused on more general indices of cell development that have been shown to detect nicotine-induced neural cell injury (Levin and Slotkin, 1998; Slotkin, 1992, 1999, 2004). As each neural cell contains only a single nucleus (Winick and Noble, 1965), the DNA concentration reflects the cell packing density (Bell *et al*, 1987; Slotkin *et al*, 1984; Winick and Noble, 1965). We also assessed the complement of cell

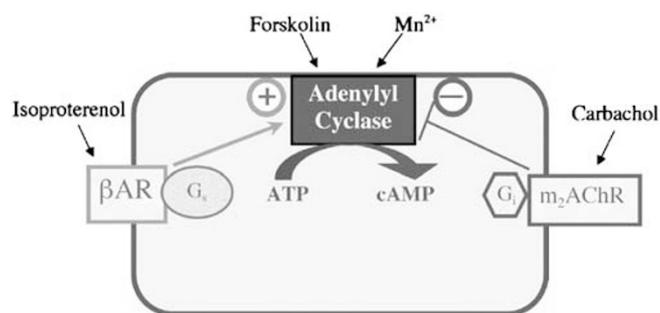


Figure 1 Stimulatory and inhibitory inputs controlling adenylyl cyclase (AC) activity. β -Adrenoceptors (β ARs) enhance AC activity through the stimulatory G-protein, G_s , whereas type 2 muscarinic cholinergic receptors (m_2 AChRs) diminish AC activity through the inhibitory protein, G_i . Each step in the pathway can be probed with the appropriate stimulant: isoproterenol for β ARs, carbachol for the m_2 AChR, and forskolin or Mn^{2+} for AC itself. The attachment of G_s to AC enhances the response to forskolin while suppressing the response to Mn^{2+} and in addition, different AC isoforms show preferential responses to forskolin vs Mn^{2+} . When alterations involve signaling elements downstream from the receptors (G_s , AC), effects will be shared by multiple receptors, and will therefore be 'heterologous.'

proteins related to differentiation as opposed to cell numbers. As neurons specialize, they enlarge and develop axonal and dendritic projections. The ratio of total protein/DNA thus rises with the expansion of the cell (Bell *et al*, 1987; Slotkin *et al*, 1984). In cells that do not develop projections, the membrane surface-to-volume ratio falls as the cell enlarges, so that the membrane protein concentration falls with hypertrophy (Thai *et al*, 1996); however, for neural cells, the development of neuritic projections necessitates a rise in the contribution of membrane proteins relative to other cell proteins. Accordingly, we also assessed the ratio of membrane protein to total cell protein. As measurements of DNA and protein subfractions integrate values across different cell types (neurons, glia) and across different subregions, the magnitude of changes induced by neurotoxicants tends to be small; that is, a robust effect on a specific neuron population is diluted with unaffected components. However, as these values are tightly controlled, even small differences are readily detectable (Bell *et al*, 1987; Qiao *et al*, 2002, 2003, 2004; Slotkin *et al*, 1984; Winick and Noble, 1965).

Finally, in light of the fact that nicotine may induce oxidative stress, we assayed thiobarbituric acid-reactive species (TBARS) in brain regions, peripheral tissues, and the placenta. Lipid peroxidation, one of the main endpoints of oxidative stress, generates malondialdehyde, which is then readily detected with the TBARS assay (Guan *et al*, 2003).

In addition to evaluating the effects of fetal nicotine exposure, we also assessed the potential amelioration of nicotine's effects with choline or Vitamin C. In a number of brain regions, perinatal dietary choline supplementation has been found to increase synaptic plasticity and performance, including protection from neurotoxicants like ethanol (Meck and Williams, 1999; Montoya *et al*, 2000; Thomas *et al*, 2000; Williams *et al*, 1998). At the same time, earlier work with lecithin dietary supplements suggested that other regions might show adverse developmental effects closely resembling those of nicotine (Bell and Lundberg, 1985; Bell and Slotkin, 1985; Bell *et al*, 1986a,b; Levin and Slotkin, 1998; Slotkin, 1992, 1999, 2004). In fact, choline supplementation enhances acetylcholine synthesis and release (Wurtman, 1988), and choline acts as an agonist at the $\alpha 7nAChR$ (Mike *et al*, 2000), thus potentially producing additive or synergistic effects with nicotine. Accordingly, we evaluated the effects of dietary choline supplementation, alone or in combination with nicotine exposure, on fetal brain development. Similarly, we evaluated whether the antioxidant properties of Vitamin C might be effective in preventing those aspects of nicotine's developmental neurotoxicity that are potentially related to oxidative stress (Guan *et al*, 2003).

MATERIALS AND METHODS

Animal Treatments and Tissue Dissections

All studies were carried out with the approval of the Oregon National Primate Research Center's Institutional Animal Care and Use Committee, in accordance with the declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the

National Institutes of Health. In total, 34 pregnant Rhesus macaque monkeys were divided into five treatment groups that received treatments beginning on day 30 of pregnancy, continuing through day 160: six controls, and seven each in groups receiving nicotine, choline supplementation, nicotine + choline, or nicotine + Vitamin C supplementation. Nicotine was delivered by continuous infusion using subcutaneously implanted osmotic minipumps with a 4-week capacity (Alzet type 2ML4) containing nicotine bitartrate dissolved in bacteriostatic water, set to deliver 2 mg/kg/day (equivalent to 0.7 mg/kg/day of nicotine-free base), whereas groups not receiving nicotine (control, choline) had pumps containing only bacteriostatic water. Pumps were implanted under ketamine anesthesia and were changed every 3 weeks to maintain a steady-state infusion throughout pregnancy; minipump changes were carried out under the direction of a veterinary surgeon following strict sterile protocols. Choline supplementation was supplied by inclusion of choline in apple juice so as to raise the intake five-fold above the normal dietary level, with controls receiving approximately 3 mmol/day and drinking unsupplemented apple juice, whereas the supplemented groups received 15 mmol/day. Ascorbic acid supplementation was delivered orally at a daily 250 mg dose in the form of a chewable vitamin pill.

On the 160th day of pregnancy, animals were anesthetized with ketamine and isoflurane and the fetuses delivered by Caesarean section as described previously (Sekhon *et al*, 2001). Samples of maternal blood and amniotic fluid were analyzed for nicotine and cotinine in the laboratory of Dr Neal Benowitz (University of California, San Francisco), using gas chromatography with nitrogen-phosphorus detection (Jacob *et al*, 1981), modified for simultaneous extraction of nicotine and cotinine (Jacob *et al*, 1991). The limit of quantitation was 1 ng/ml for nicotine and 10 ng/ml for cotinine. Internal standards, 5-methylnicotine and ortho-cotinine, were obtained from Dr Peyton Jacob III (University of California, San Francisco).

A section of the placenta from the periphery of the placental disc was dissected and frozen with liquid nitrogen. Similarly, the fetal cardiac lobe of the left lung and a section across the fetal left and right ventricle were taken from the heart, being careful to use exactly the same location in every animal (Figure 2). Fetal brain dissections were carried out in the cold, in each case using the tissue from the right hemisphere. A cut was made through the cerebral cortex to obtain the frontal lobes with a second cut just caudal to the infundibulum. The section in between the cuts was flipped over and the right temporal cortex and hippocampus were then removed and separated from each other. A coronal cut was made at the level of the optic chiasm and the caudate was separated from the hypothalamus. The parietal cortex and occipital cortex were dissected and separated from each other and the right occipital cortex was frozen for analysis. The midbrain and brainstem were divided just below the pons and the right half of the brainstem was frozen. All tissue samples were shipped on dry ice by overnight express to Duke University and were stored at -80°C until analyzed. Owing to the small amounts of material available, in some tissues we were able to perform only selected determinations from the set of assays described below.

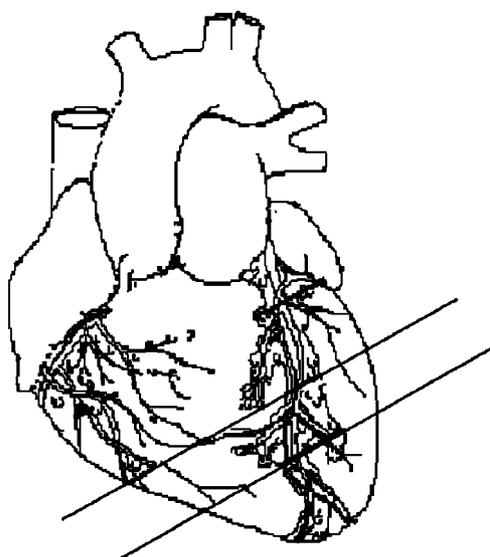


Figure 2 Schematic showing the segment of fetal cardiac left and right ventricle used for analysis, between the indicated cut-lines.

Membrane Preparations

Tissues were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY, USA) in 20–30 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and where necessary to remove connective tissue, strained through several layers of cheesecloth. Aliquots were withdrawn for the measurements of DNA and total protein. To prepare the cell membrane fraction, the homogenates were sedimented at $40\,000 \times g$ for 10 min and the supernatant solution was discarded. The membrane pellet was resuspended (Polytron) in the original volume of buffer, resedimented, and the resultant pellet was resuspended in one-quarter of the original volume of the same buffer, using a smooth glass homogenizer fitted with a Teflon pestle. Aliquots of this resuspension were withdrawn for measurements of ligand binding, and for membrane protein.

The techniques for binding of each of the radioligands have been described in detail previously (Auman *et al*, 2001; Slotkin *et al*, 1999, 2002, 2003b; Trauth *et al*, 1999; Zeiders *et al*, 1997). Briefly, [125 I] α -bungarotoxin binding was assessed in quadruplicate aliquots of the membrane suspension (two aliquots for total binding and two for nonspecific binding). Each assay contained a final concentration of 1 nM radioligand in a total volume of 250 μ l of a buffer consisting of 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂ and 50 mM Tris (pH 7.4). Incubations were conducted for 120 min at 25°C, after which 4 ml of ice-cold buffer were added and the membranes trapped by filtration on glass fiber filters that were presoaked with 0.05% polyethyleneimine. Labeled membranes were washed three times with 4 ml of buffer and the filters were counted for radioactivity. Nonspecific binding was assessed by displacement with 10 μ M nicotine. [3 H]Cytisine binding was evaluated similarly, except that the incubations occurred at 4°C for 75 min. To evaluate β AR binding, membrane aliquots were incubated with [125 I]iodopindolol (final concentration 67 pM), in 145 mM NaCl, 2 mM MgCl₂, 1 mM sodium ascorbate and 20 mM Tris (pH 7.5), for 20 min at room temperature in a total volume of 250 μ l;

nonspecific binding was assessed by displacement with 100 μ M isoproterenol. For m₂AChR binding, membrane aliquots were diluted in 10 mM sodium–potassium phosphate buffer (pH 7.4) and incubated for 60 min at room temperature, using 1 nM [3 H]AFDX384 with or without 1 μ M atropine to displace specific binding.

Nonspecific binding varied widely according to the radioligand and brain region or tissue. For α 7nAChRs, values ranged from 60% in the brainstem to 80% in cerebrocortical regions, whereas nonspecific binding for the α 4 β 2 subtype was much lower (20–30%). The ligands used for β AR and m₂AChR evaluations showed the lowest proportions of nonspecific binding, <10%.

Adenylyl Cyclase

For assessment of AC enzymatic activity, aliquots of the same membrane preparation were incubated for 10 min (brain regions) or 30 min (peripheral tissues) at 30°C with final concentrations of 100 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM ATP, 2 mM MgCl₂, 1 mg/ml bovine serum albumin, and a creatine phosphokinase-ATP-regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU/ml phosphocreatine kinase, with 10 μ M GTP in a total volume of 250 μ l. The enzymatic reaction was stopped by placing the samples in a 90–100°C water bath for 5 min, followed by sedimentation at $3000 \times g$ for 15 min, and the supernatant solution was assayed for cyclic AMP using radioimmunoassay. Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay period and was linear with membrane protein concentration; concentrations of cofactors were optimal and, in particular, higher concentrations of GTP produced no further augmentation of activity. In addition to measuring basal AC activity, we assessed the response to the direct AC stimulants, forskolin (100 μ M) and Mn²⁺ (10 mM), as well as the response to β AR stimulation (100 μ M isoproterenol), or in the caudate, dopamine stimulation (100 μ M). Effects mediated through muscarinic cholinergic receptors were evaluated with 100 μ M carbachol in the presence of GTP and forskolin. As m₂AChRs can evoke inhibition of AC through the inhibitory G-protein, G_i, the effects of carbachol were evaluated in the presence of forskolin so as to stimulate AC, which makes inhibitory responses more readily detectable (Chow *et al*, 2000; Garofolo *et al*, 2002; Slotkin *et al*, 1999). The chosen concentrations of each stimulant elicit maximal responses, as assessed in earlier studies (Auman *et al*, 2000, 2001; Garofolo *et al*, 2002; Zeiders *et al*, 1997, 1999a).

Macromolecules

DNA was determined in aliquots of the same original tissue homogenates used for receptor binding, using a modified (Trauth *et al*, 2000) fluorescent dye-binding method (Labarca and Piagen, 1980). Aliquots were diluted with 10 mM Tris, 0.1 M NaCl, 1 mM EDTA (pH 7.4) and sonicated briefly (Virsonic Cell Disrupter, Virtis, Gardiner, NY, USA). Hoechst 33258 was added to a final concentration of 0.1 μ g/ml. Samples were then read in a spectrofluorometer using an excitation wavelength of 356 nm and an emission wavelength of 458 nm, and were quantitated using standards of purified DNA. The total concentration of

tissue proteins was assayed from the original homogenate spectrophotometrically with bicinchoninic acid (Smith *et al*, 1985); in addition, we assessed the concentration of membrane proteins from the preparations used for radioligand binding.

TBARS

Lipid peroxidation was evaluated by assessment of thiobarbituric acid-reactive species, conducted similarly to published procedures (Guan *et al*, 2003). Aliquots of the original tissue homogenate were diluted with 2% sodium dodecylsulfate, 1 mM diethylenedinitrilotetraacetic acid, and 5% trichloroacetic acid, followed by addition of two volumes of thiobarbituric acid reagent (0.45% 2-thiobarbituric acid dissolved in 1 M NaOH, followed by the addition of acetic acid to a final concentration of 12%). Samples were incubated for 1 h at 95°C, cooled to ambient temperature, sedimented at 3000 × *g* for 15 min, and the absorbance of the supernatant solution was determined at 532 nm. Standard curves were constructed with known concentrations of malondialdehyde that had been run through the same reaction.

Data Analysis

To reduce the likelihood of type 1 statistical errors that might result from repeated testing of the data set, we first performed a global analysis of variance (ANOVA) (with log-transformed data because of heterogeneous variance) incorporating all the variables and measures: prenatal treatment, brain region, and the different biomarkers. The latter were treated as repeated measures, since the various assays all originated from the same tissue homogenate in a given animal. This initial test indicated treatment effects that differed among the different biomarkers, so data were

then examined separately for each measure, again using a multivariate ANOVA. Where appropriate, this was followed by *post hoc* evaluations of each treatment group compared to the controls or to each other, using Fisher's Protected Least Significant Difference; however, where treatment effects did not interact with other variables only the main effect was recorded without testing of individual differences. Significance was assumed at the level of $p < 0.05$ for main effects; however, for interactions at $p < 0.1$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran, 1967).

The study design required two different ways of regarding nicotine and choline treatment variables. To compare the effects *vs* the controls for nicotine or choline exposure alone, or the combined exposure, the four treatment groups were considered as a one-dimensional factor in the statistical design. To determine whether the effects of nicotine and choline were interactive, the treatment factors were changed to a two-dimensional design. In this formulation, more-than-additive (synergistic) and less-than-additive effects appear as significant interactions between the two treatment dimensions, whereas simple, additive effects do not show significant interactions.

Data are presented as means and standard errors. To facilitate comparisons across multiple tissues and variables, the effects of each treatment are given as the percentage change from the corresponding control group, but statistical comparisons were made on the original data. For reference, control values appear in Table 1.

Materials

Osmotic minipumps were obtained from Durect Corp. (Cupertino, CA, USA). Bacteriostatic water was obtained

Table 1 Biomarkers in Control Fetuses

| Measure | Frontal cortex | Occipital cortex | Caudate | Hippocampus | Brainstem | Heart | Lung |
|---|----------------|------------------|-------------|-------------|------------|-------------|-------------|
| <i>Receptor binding (fmol/mg protein)</i> | | | | | | | |
| $\alpha 4\beta 2$ nAChR | 22 ± 1 | 18 ± 2 | 29 ± 1 | 18 ± 2 | 14 ± 1 | ND | ND |
| $\alpha 7$ nAChR | 8.3 ± 0.7 | 5.2 ± 0.5 | 6.6 ± 0.5 | ND | 10.1 ± 0.6 | ND | ND |
| m_2 AChR | 306 ± 24 | 645 ± 49 | 413 ± 31 | ND | 160 ± 8 | 150 ± 16 | ND |
| β AR | 18.6 ± 0.7 | 15.1 ± 0.5 | 13.6 ± 0.5 | ND | ND | 20 ± 1 | 73 ± 5 |
| <i>AC activity (pmol/min/mg protein)</i> | | | | | | | |
| Basal | 440 ± 18 | 506 ± 23 | 435 ± 25 | ND | ND | 24 ± 2 | 24 ± 2 |
| Isoproterenol | 485 ± 26 | 601 ± 35 | ND | ND | ND | 101 ± 8 | 36 ± 3 |
| Dopamine | ND | ND | 559 ± 44 | ND | ND | ND | ND |
| Mn^{2+} | 2685 ± 156 | 3607 ± 150 | 3658 ± 167 | ND | ND | 472 ± 39 | 88 ± 6 |
| Forskolin | 1492 ± 61 | 1831 ± 104 | 2961 ± 175 | ND | ND | 922 ± 64 | 55 ± 4 |
| Carbachol+Forskolin | 1485 ± 58 | 1920 ± 85 | 2899 ± 203 | ND | ND | 1408 ± 119 | ND |
| DNA (mg/g) | 0.93 ± 0.02 | 1.10 ± 0.14 | 0.84 ± 0.01 | ND | ND | 1.27 ± 0.03 | 3.13 ± 0.07 |
| Total protein/DNA | 81 ± 2 | 66 ± 1 | 84 ± 1 | ND | ND | 41 ± 2 | 17.6 ± 0.5 |
| Membrane/total protein | 0.31 ± 0.01 | 0.37 ± 0.01 | 0.34 ± 0.01 | ND | ND | 0.41 ± 0.02 | 0.43 ± 0.02 |

ND = not done.

from Abbott Laboratories (N. Chicago, IL, USA). Radioisotopically-labeled compounds came from PerkinElmer Life Sciences (Boston, MA, USA): [^3H]cytisine (specific activity, 35 Ci/mmol), [^{125}I] α -bungarotoxin (specific activity, 136 Ci/mmol), [^3H]AFDX384 (specific activity, 133 Ci/mmol), and [^{125}I]iodopindolol (specific activity 2200 Ci/mmol). Cyclic AMP radioimmunoassay kits were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Sigma Chemical Co. (St Louis, MO, USA) was the source for all other reagents.

RESULTS

Nicotine administration to pregnant monkeys, alone or in combination with dietary supplementation with choline or vitamin C, produced nicotine levels in maternal plasma of about 30 ng/ml, comparable to those associated with maternal smoking in humans or with the use of transdermal nicotine patches (Benowitz *et al*, 1990; Fredrickson *et al*, 1995; Isaac and Rand, 1972; Lichtensteiger *et al*, 1988; Luck *et al*, 1985; Murrin *et al*, 1987); cotinine averaged 120 ng/ml. The nicotine concentrations in amniotic fluid were substantially lower, in the range of 10–15 ng/ml, and the cotinine levels were 70–100 ng/ml. Surprisingly, coadministration of either choline or Vitamin C substantially increased the nicotine concentration in the amniotic fluid (20–25 ng/ml), and cotinine showed a less notable increase (90–110 ng/ml). The results will be provided in detail in a separate publication containing an analysis of nicotine pharmacokinetics in this model.

In control fetal brain, there were wide regional disparities in the concentrations of neurotransmitter receptors (Table 1). For $\alpha 4\beta 2$ nAChRs, values were highest in the caudate and lowest in the brainstem, with intermediate values in the frontal and occipital cortex and in the hippocampus. A very different pattern was evident for the $\alpha 7$ nAChR subtype, which showed highest values in the brainstem. The concentrations of m_2 AChRs were much greater than those of nAChRs and followed the rank order, occipital cortex > caudate > frontal cortex > brainstem. In the heart, m_2 AChR values were in the same range as the lowest of the brain regions but in contrast, the concentration of β ARs was much higher in peripheral tissues than in any of the brain regions.

Either isoproterenol (frontal cortex, occipital cortex) or dopamine (caudate) evoked significant AC stimulation, as did isoproterenol in the membrane preparations from peripheral tissues (Table 1; $p < 0.0001$ for each region or tissue). However, the degree of stimulation was unrelated to the receptor concentration, as the isoproterenol AC response was 10% above basal values in cortical regions, 50% in the lung, and 300% in the heart, despite the fact that β AR binding was similar in heart and cortical regions, and highest in the lung. This result is in keeping with previous observations that the degree of receptor coupling to downstream signaling elements is the key determinant of the net response (Gao *et al*, 1998, 1999; Navarro *et al*, 1991). Similarly, carbachol failed to inhibit AC despite high m_2 AChR concentrations, implying either that there is relatively poor coupling of these receptors to G_i in the fetal brain or, alternatively, that stimulation by other mAChR

subtypes offsets the inhibitory effect of m_2 AChRs (Gurwitz *et al*, 1994; Mangels and Gnegy, 1992; Migeon *et al*, 1995; Olanas and Onali, 1994); in keeping with the latter interpretation, there was significant stimulation by carbachol in the heart ($p < 0.0001$). The direct AC stimulants, forskolin and Mn^{2+} , produced massive responses in all regions and tissues, but again the magnitude was highly tissue-specific: stimulation by forskolin was 40-fold in the heart but only three- to seven-fold in the lung or brain regions, and stimulation by Mn^{2+} was 20-fold in the heart and only four- to eight-fold elsewhere. In addition, AC isoform differences were apparent from the disparities in the response ratio of forskolin/ Mn^{2+} , which was about 2 in the heart but < 1 in the lung and in brain regions.

Global ANOVA across all treatments and all measurements in the three brain regions for which all parameters could be measured (frontal cortex, occipital cortex, caudate) indicated a main treatment effect ($p < 0.0001$), which differed among the individual measurements and the brain regions ($p < 0.0001$ for treatment \times measure, $p < 0.08$ for treatment \times region \times measure). Accordingly, we separated the evaluations into separate groups of measurements (receptors, cell signaling, cell development biomarkers, TBARS for lipid peroxidation) and then reexamined the treatment effects separately in each region.

Effects on Receptor Binding

In the frontal cortex, nicotine treatment upregulated $\alpha 4\beta 2$ nAChR binding without significant effects on $\alpha 7$ nAChRs, m_2 AChRs, or β ARs (Figure 3a). Choline (Figure 3b) had about half the effect of nicotine on $\alpha 4\beta 2$ nAChRs and thus the increase did not achieve statistical significance. The combined treatment with

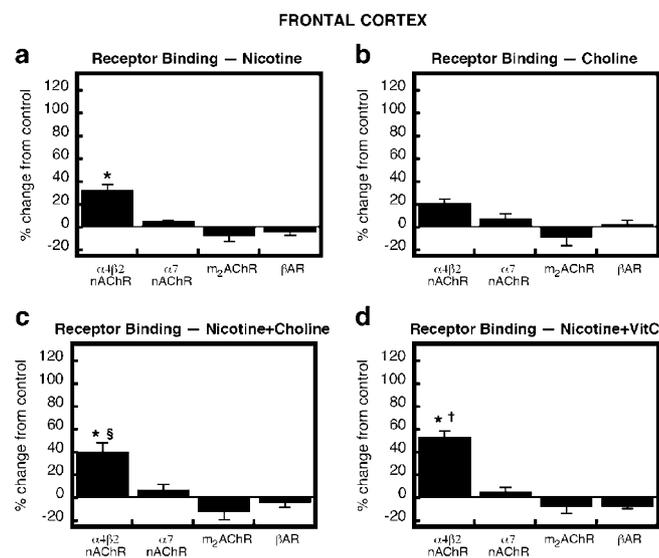


Figure 3 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on receptor binding in the frontal cortex. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). *Significantly different from control values. †Nicotine + VitC group significantly different from nicotine alone. §Nicotine + Choline group significantly different from choline alone.

nicotine and choline produced $\alpha 4\beta 2$ nAChR upregulation corresponding to the summation of the two separate effects (Figure 3c), so that whereas the group receiving combined treatment showed significant elevations over both control and choline groups, there was no significant interaction of nicotine \times choline when the treatments were considered as separate factors in a two-factor ANOVA. Vitamin C supplementation augmented the effect of nicotine, producing significantly greater $\alpha 4\beta 2$ nAChR upregulation than was seen with nicotine alone (Figure 3d).

The effects of nicotine on receptor binding were much greater in the occipital cortex than in the frontal cortex. By itself, nicotine doubled $\alpha 4\beta 2$ nAChR binding and also evoked smaller, but significant increases in the $\alpha 7$ subtype (Figure 4a). Again, choline had about half the effect of nicotine, so that in this region, the upregulation of the $\alpha 4\beta 2$ receptors was robust (50% increase) and statistically significant (Figure 4b); the same trend was seen for $\alpha 7$ nAChRs, namely about half the effect as obtained with nicotine. The combination of nicotine + choline (Figure 4c) produced upregulation of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs comparable to that seen with nicotine alone, and for the latter, two-factor treatment analysis indicated that the combined effect was less than would have been expected from additive effects of nicotine and choline ($p < 0.04$ for the interaction of nicotine \times choline). As in the frontal cortex, Vitamin C supplementation augmented the effect of nicotine on nAChR binding (Figure 4d); although the difference from nicotine alone did not achieve statistical significance, this was primarily because of the large magnitude of nicotine's effect, and the Vitamin C-induced enhancement was statistically indistinguishable from the significant difference seen in the frontal cortex (no significant treatment \times region interaction). The same 'ceiling effect' may be responsible for limiting the total response to nicotine + choline.

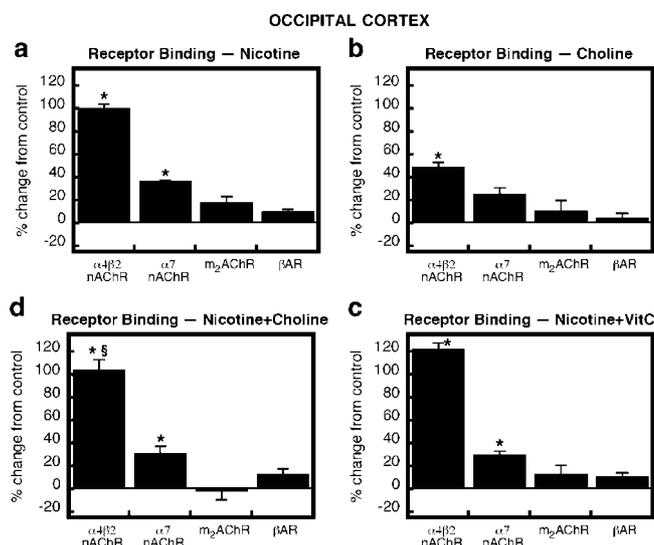


Figure 4 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on receptor binding in the occipital cortex. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). *Significantly different from control values. §Nicotine + Choline group significantly different from choline alone.

The effects of nicotine on receptor binding were least evident in the caudate, where a small ($< 20\%$) but significant upregulation of $\alpha 4\beta 2$ nAChRs was obtained (Figure 5a). Choline was ineffective (Figure 5b) but nevertheless augmented the effect of nicotine (Figure 5c); again, the interaction was indistinguishable from simple additivity of the effects of the individual agents (no interaction of nicotine \times choline treatment in a two-factor ANOVA). The effect of nicotine was slightly larger when animals received Vitamin C supplementation (Figure 5d), but in light of the small overall effect, the difference from the group receiving nicotine alone was not statistically significant.

The amount of hippocampal tissue available for analysis limited the determinations to the $\alpha 4\beta 2$ subtype and, similarly, in the brainstem we had to forgo measurement of β ARs. Nicotine treatment caused significant elevation of $\alpha 4\beta 2$ nAChRs in both regions (Figure 6a). Choline treatment caused smaller, nonsignificant increases (Figure 6b) but the combination of nicotine and choline elicited much bigger effects than with either agent alone (Figure 6c). As was true in the other regions, dietary supplementation with Vitamin C augmented the effect of nicotine, significantly increasing $\alpha 4\beta 2$ nAChR binding above the level achieved with nicotine alone (Figure 6d). Vitamin C also augmented the increase in brainstem m_2 AChRs so that the effect, although of small magnitude, achieved statistical significance.

Unlike the brain regions, nicotine treatment elicited robust m_2 AChR upregulation in the heart, while at the same time suppressing the expression of β ARs (Figure 7a). The latter effect was tissue-selective, as no β AR downregulation was seen in the lung. As before, choline elicited about half the effect of nicotine, thus not achieving statistical significance by itself (Figure 7b). However, in this case, choline treatment reduced the effect of nicotine, so that the combined exposure elicited no significant change in cardiac receptor profiles (Figure 7c). Similarly,

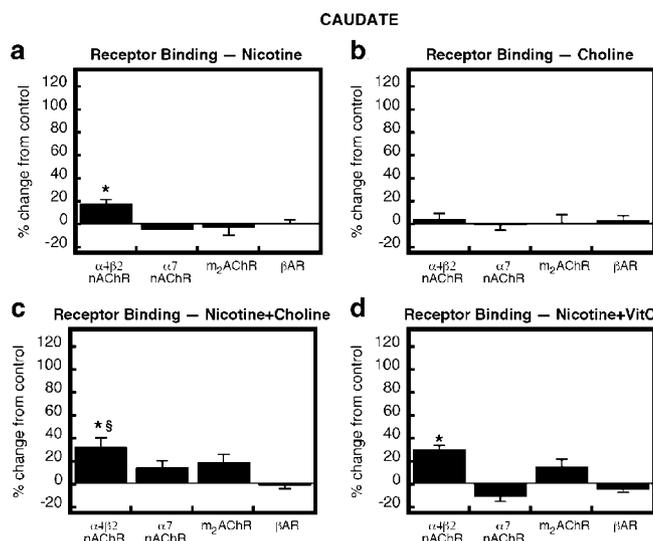


Figure 5 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on receptor binding in the caudate. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). *Significantly different from control values. §Nicotine + Choline group significantly different from choline alone.

Vitamin C supplementation reduced the net effect of nicotine so that neither of the cardiac receptors showed significant differences from the control group (Figure 7d). At the same time, however, combining nicotine either with choline (Figure 7c) or Vitamin C (Figure 7a) elicited significant reductions in lung β ARs.

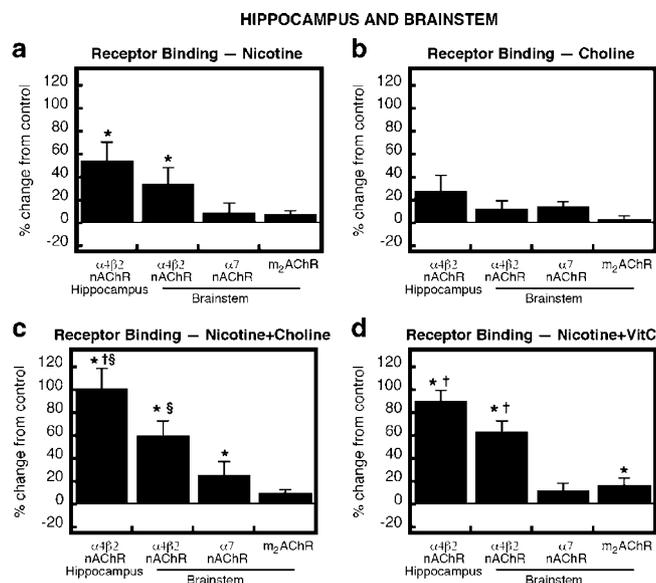


Figure 6 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on receptor binding in the hippocampus and brainstem. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). *Significantly different from control values. †Nicotine + Choline group or Nicotine + VitC group significantly different from nicotine alone. §Nicotine + Choline group significantly different from choline alone.

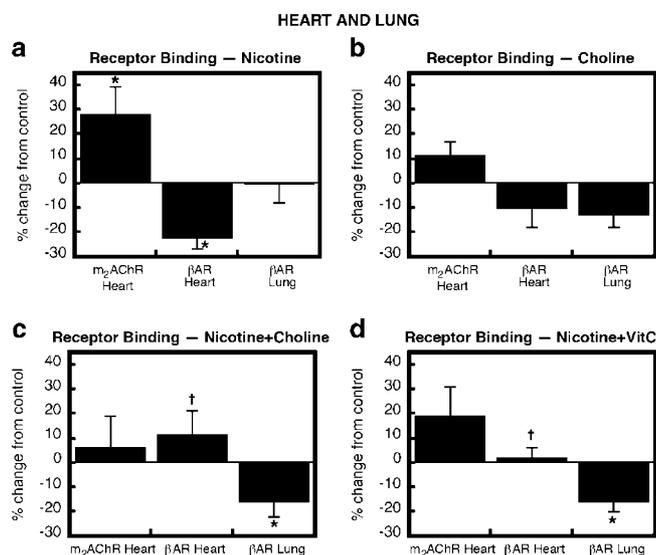


Figure 7 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on receptor binding in the heart and lung. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). *Significantly different from control values. †Nicotine + Choline group or Nicotine + VitC group significantly different from nicotine alone.

Effects on AC Signaling

Just as was true for receptor binding, there were strong regional disparities in the effects on cell signaling mediated through AC. In the frontal cortex, nicotine elicited little or no change in response to any of the AC stimulants (Figure 8a), but choline produced robust activation of the responses (Figure 8b). The pattern reflected heterologous activation of signaling proteins downstream from the receptors, as there was parallel activation of basal AC, receptor-mediated responses, and responses to direct AC stimulants. Cotreatment with nicotine and choline produced an effect midway between those of nicotine and choline alone, with smaller, but still significant, enhancement of AC responses (Figure 8c). On the other hand, Vitamin C supplementation augmented the effect above that seen with nicotine alone (Figure 8d).

Unlike the situation in the frontal cortex, none of the treatments elicited a significant alteration in cell signaling in the occipital cortex (Figure 9). In the caudate, nicotine (Figure 10a) or choline (Figure 10b) alone elicited slight increases in AC responses, but only the choline effect on the dopamine response achieved statistical significance. In contrast, combined exposure to both nicotine and choline elicited robust activation of all AC responses (Figure 10c), representing the summation of the two individual effects (no interaction of nicotine \times choline treatment in a two-factor ANOVA). Vitamin C supplementation augmented the effect of nicotine, producing 20–30% elevations in the AC responses (Figure 10d). In all cases, the effects on AC were heterologous, with parallel changes in basal AC, receptor-mediated responses, and responses to direct AC stimulants

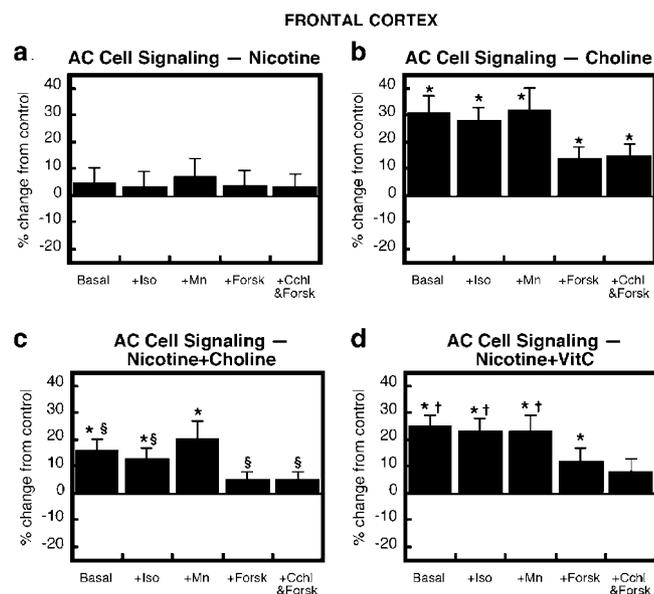


Figure 8 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on AC signaling in the frontal cortex. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). *Significantly different from control values. †Nicotine + VitC group significantly different from nicotine alone. §Nicotine + Choline group significantly different from choline alone. Abbreviations: Iso = isoproterenol, Forsk = forskolin, Cchl = carbachol.

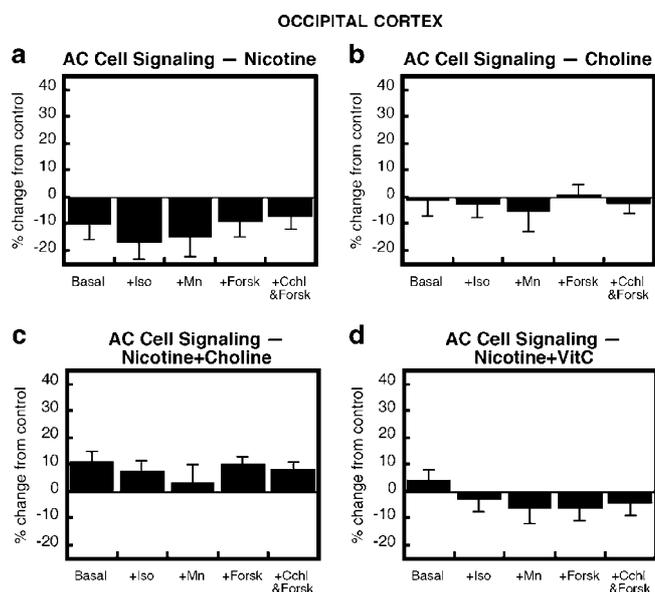


Figure 9 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on AC signaling in the occipital cortex. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). None of the effects is statistically significant. Abbreviations: Iso = isoproterenol, Forsk = forskolin, Cchl = carbachol.

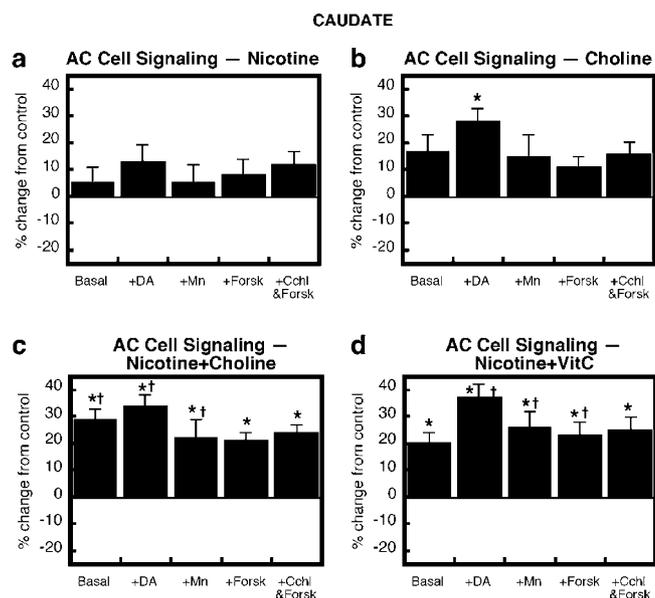


Figure 10 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on AC signaling in the caudate. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). *Significantly different from control values. †Nicotine + Choline group or Nicotine + VitC group significantly different from nicotine alone. Abbreviations: DA = dopamine, Forsk = forskolin, Cchl = carbachol.

(main effects of treatment without significant treatment \times measure interactions).

As was true for the brain regions, nicotine administration by itself had only minor effects on cell signaling in the heart and lung (Figure 11a); similarly, choline alone evoked little or no change (Figure 11b). However, the combined

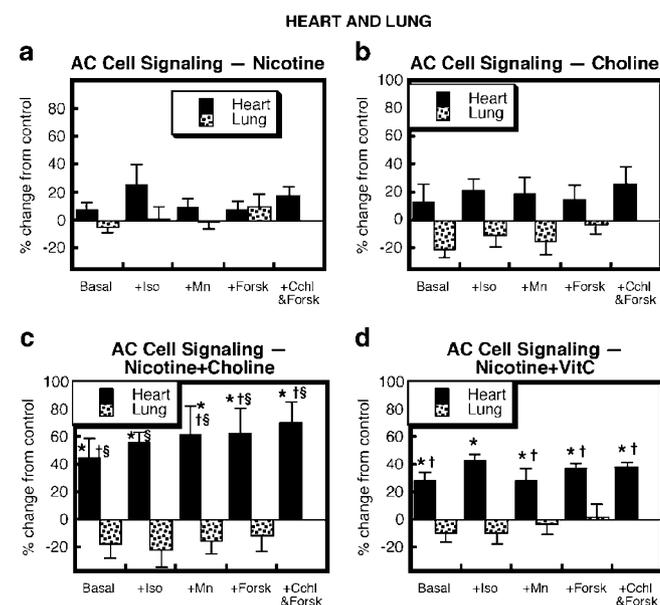


Figure 11 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on AC signaling in the heart and lung. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). *Significantly different from control values. †Nicotine + Choline group or Nicotine + VitC group significantly different from nicotine alone. §Nicotine + Choline group significantly different from choline alone. Abbreviations: Iso = isoproterenol, roterenol, Forsk = forskolin, Cchl = carbachol.

treatment with nicotine + choline elicited massive, heterologous enhancement of all AC signaling parameters in the heart, while leaving signaling in the lung largely unaffected (Figure 11c). Vitamin C also heterologously enhanced the ability of nicotine to stimulate cardiac AC signaling (Figure 11d).

Effects on Cell Development Biomarkers

In the frontal cortex, either nicotine (Figure 12a) or choline (Figure 12b) alone elicited small, nonsignificant changes in DNA concentration and in the ratios of total protein to DNA and membrane to total protein. However, with combined treatment (Figure 12c), significant differences emerged, characterized by a decline in the index of cell size (total protein/DNA) and a corresponding rise in the relative membrane surface area (membrane/total protein). These effects represented the summation of the nonsignificant alterations wrought by nicotine and choline alone, as there was no significant nicotine \times choline interaction in a two-factor analysis of treatment effects. When nicotine was combined with Vitamin C supplementation, there was a substantially larger decrement in the total protein/DNA ratio (Figure 12d).

In the occipital cortex, nicotine exposure had an adverse effect on the membrane/total protein ratio, while leaving indices of cell number and cell size unchanged (Figure 13a). By itself, choline had a similar, albeit lesser effect (Figure 13b) and in this case, choline coadministration lessened the effect of nicotine (Figure 13c). Two-factor analysis of the combined treatment indicated a significant nicotine \times

choline interaction ($p < 0.01$), indicating that the effect was significantly smaller than that predicted by the summation of the two separate effects. Vitamin C had no further effect when superimposed on the actions of nicotine alone (Figure 13d).

Nicotine exerted profound effects on cell development biomarkers in the caudate, evoking about a 10% deficit in the number of cells (reduced DNA concentration), with a corresponding rise in the total protein/DNA ratio

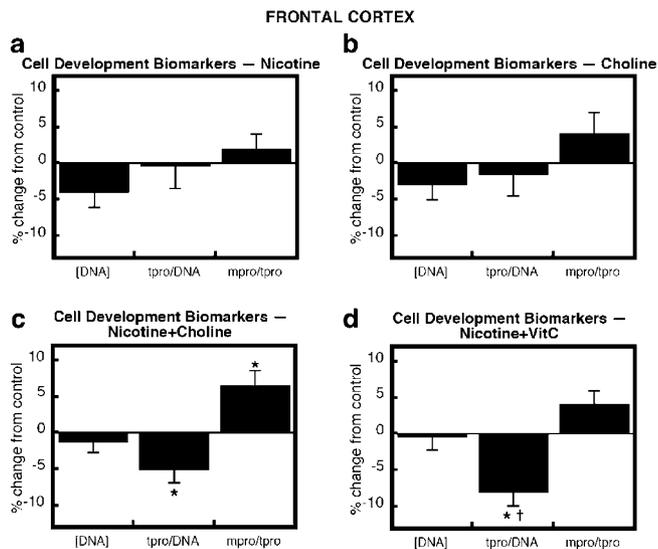


Figure 12 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on cell development biomarkers in the frontal cortex. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). *Significantly different from control values. †Nicotine + VitC group significantly different from nicotine alone. Abbreviations: tpro = total protein, mpro = membrane protein.

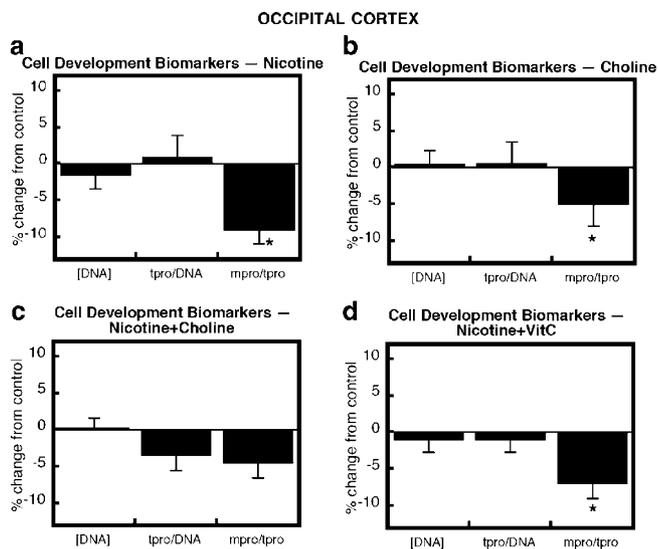


Figure 13 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on cell development biomarkers in the occipital cortex. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). *Significantly different from control values. Abbreviations: tpro = total protein; mpro = membrane protein.

(Figure 14a). In contrast, choline alone had little or no effect (Figure 14b) but, when combined with nicotine administration, it completely prevented the adverse effect of nicotine (Figure 14c). Similarly, Vitamin C supplementation protected the caudate from the nicotine-induced deficits (Figure 14d). In the heart and lung, there were no significant alterations of any of the treatments, but high variability of

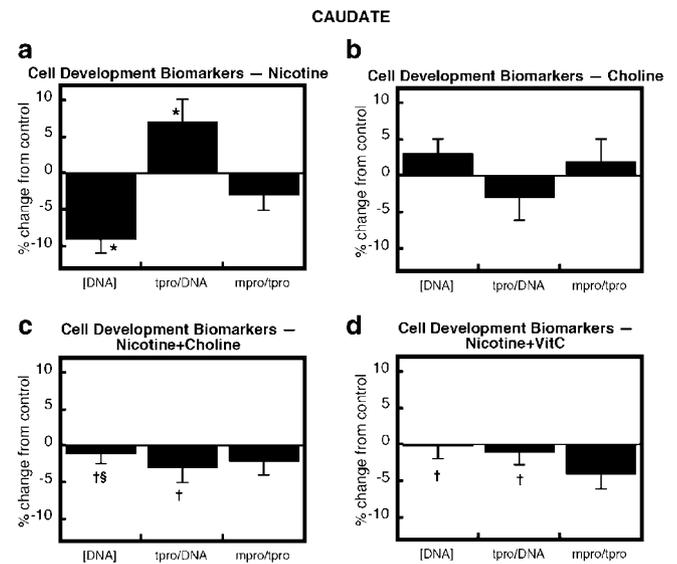


Figure 14 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on cell development biomarkers in the caudate. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). *Significantly different from control values. †Nicotine + Choline group or Nicotine + VitC group significantly different from nicotine alone. ‡Nicotine + Choline group significantly different from choline alone. Abbreviations: tpro = total protein; mpro = membrane protein.

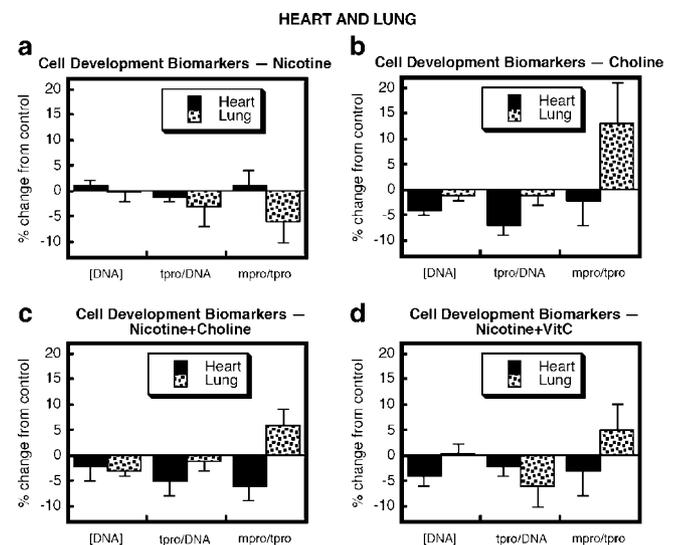


Figure 15 Effects of (a) nicotine, (b) choline, (c) nicotine + choline, and (d) nicotine + Vitamin C (VitC) treatment on cell development biomarkers in the heart and lung. Data are means and standard errors, presented as the percent change from corresponding control values (Table 1). None of the differences is statistically significant. Abbreviations: tpro = total protein; mpro = membrane protein.

some of the measures precluded making conclusions about biologic mechanisms from the negative findings (Figure 15); indeed, many of the nonsignificant changes were of the same magnitude as the significant alterations seen in the brain regions. Accordingly, we relied on multivariate analysis rather than individual effects, that is, looking for abnormal 'patterns' of values across the three measures. Using that approach, the decrease in cardiac values in animals given choline or nicotine + choline was significant ($p < 0.05$ and < 0.04 , respectively). In the lung, the small reductions caused by nicotine achieved significance when grouped together ($p < 0.05$) and the effect of choline was distinguishably opposite ($p < 0.006$ comparing nicotine and choline to each other), primarily reflecting the differences in membrane/total protein. In combination, nicotine + choline gave values midway between the two individual effects in the lung. It should be noted that mature cardiac cells can be multinucleated so that DNA assessments are unreliable in determining the actual number of cells; however, fetal myocytes generally do not show multinucleation (Soonpaa *et al*, 1996).

Effects on TBARS

We did not observe any treatment-related effects on lipid peroxidation as evaluated by TBARS (Table 2). However, there were highly significant differences among the brain regions and peripheral tissues, indicating that treatment effects would have been detectable if present. For the brain regions, the values were much higher in the caudate than in the cortical regions, in agreement with the recognized, greater susceptibility of this dopamine-rich region to the production of oxidative species (Junn and Mouradian, 2001; Karen *et al*, 2001).

DISCUSSION

The results of this study indicate that maternal nicotine administration in primates, at concentrations commensurate with cigarette smoking in humans, elicits the hallmark changes of nicotine-induced fetal brain damage that have been noted previously in rodent models (Levin and Slotkin, 1998; Slotkin, 1992, 1998, 1999, 2004). Nicotine exposure evoked significant increments in $\alpha 4\beta 2$ nAChRs in all regions, verifying that sufficient nicotine reached the fetal brain to evoke chronic nicotinic stimulation of the target cells. Indeed, the degree of upregulation was similar to that seen in comparable brain regions of adult smokers (Breese

et al, 1997) or in rodent models of prenatal nicotine exposure (Levin and Slotkin, 1998; Slotkin, 1992, 1998, 1999, 2004). Nevertheless, there were strong regional disparities in the response, with the greatest increase seen in the occipital cortex, less in the hippocampus, brainstem and frontal cortex, and least in the caudate, suggesting differential susceptibility to nicotine-induced cellular effects. That conclusion was reinforced first by the finding that only the occipital cortex showed upregulation of $\alpha 7$ nAChRs, the receptor subpopulation specifically associated with responses to cell injury and neuritic development (Chan and Quik, 1993; Quik *et al*, 1994; Verbois *et al*, 2002), and this same region showed a deficit in the membrane/total protein ratio, a biomarker for neuronal projections. Nicotine also had an adverse effect on cell number, as indicated by a significant overall decrement in the DNA concentration. By far the greatest effect was in the caudate, where the loss of cells was accompanied by cell enlargement (increased total protein/DNA ratio), which may either be compensatory or could also represent perikaryal swelling as a cytotoxic reaction. However, the caudate was the region showing the least upregulation of nAChRs, implying a different underlying mechanism for cell damage and loss in this region. As will be discussed below, the protective action of Vitamin C in the caudate implies that the additional, adverse cellular effects in this region are likely to involve its high susceptibility to oxidative stress.

In contrast to the robust and significant effects of nicotine on nAChRs and cell injury biomarkers, there was little or no effect on cell signaling parameters or on expression of CNS β ARs or m_2 AChRs. However, studies in rodent models suggest that these emerge at subsequent, postnatal stages (Slotkin *et al*, 1990, 1992) and thus are not likely to be detected in the late gestational period sampled here. In contrast, in the periphery we found a significant decrement in cardiac β ARs and elevation in m_2 AChRs, effects that also have been found with prenatal nicotine exposure in rats (Navarro *et al*, 1990; Slotkin *et al*, 1999). The former effect was specific to the heart rather than representing global interference with β AR expression, since β ARs were unaffected in the CNS or lung. Similarly, the receptor effects were not secondary to more generalized alterations in cardiac cell development or signaling parameters, which were largely unaffected by nicotine exposure. Studies in rodents show changes in cardiac responsiveness that parallel the alterations in β ARs and m_2 AChRs (Navarro *et al*, 1990; Slotkin *et al*, 1997, 1999), and therefore the shifts seen here are likely to be of key importance. β ARs provide

Table 2 Effects on TBARS^a

| Treatment | Frontal cortex | Occipital cortex | Caudate | Heart | Lung | Placenta |
|--------------------|----------------|------------------|----------|--------|--------|----------|
| Control | 25 ± 2 | 47 ± 1 | 232 ± 16 | 42 ± 1 | 42 ± 1 | 131 ± 10 |
| Nicotine | 25 ± 1 | 43 ± 3 | 220 ± 10 | 43 ± 1 | 41 ± 3 | 131 ± 6 |
| Choline | 24 ± 1 | 49 ± 5 | 247 ± 12 | 44 ± 1 | 42 ± 3 | 124 ± 5 |
| Nicotine+Choline | 24 ± 1 | 48 ± 2 | 256 ± 18 | 45 ± 1 | 45 ± 2 | 125 ± 6 |
| Nicotine+Vitamin C | 25 ± 1 | 46 ± 4 | 248 ± 14 | 43 ± 1 | 43 ± 4 | 133 ± 11 |

^anmols per g tissue. None of the differences among treatments is statistically significant. However, differences among tissues are highly significant ($p < 0.0001$).

stimulatory input and m_2 AChRs inhibitory input, so that the imbalance evoked by nicotine would render the heart more susceptible to arrest during challenges such as hypoxia (Slotkin *et al*, 1995, 1997). The fetal environment is hypoxic, and hypoxia is ineluctable during labor (Hanson *et al*, 1993; Lagercrantz and Slotkin, 1986; Southall *et al*, 1993; Valdes-Dapena, 1981). Accordingly, the fact that nicotine exposure upsets the careful balance of cardiac cellular excitation and inhibition is likely to contribute to the association of maternal smoking with increased perinatal morbidity and mortality (DiFranza and Lew, 1995), findings that have their parallel in previous results with rats (Slotkin *et al*, 1995, 1997, 1999; Tolson *et al*, 1995).

Choline supplementation is mandated for infant formulas marketed in the US and choline promotes membrane formation necessary for brain development and plasticity, while protecting the brain from injurious effects of neurotoxicants such as ethanol (Meck and Williams, 1999; Montoya *et al*, 2000; Thomas *et al*, 2000; Williams *et al*, 1998). On the other hand, choline supplementation also augments cholinergic effects (Mike *et al*, 2000; Wurtman, 1988), which in the developing brain might contribute to nicotine-like actions that produce neurobehavioral defects (Bell and Lundberg, 1985; Bell and Slotkin, 1985; Bell *et al*, 1986a, b; Levin and Slotkin, 1998; Slotkin, 1992, 1999, 2004). Accordingly, we assessed the effects of choline alone and in combination with nicotine. By itself, choline administration elicited nicotine-like effects, albeit at a lower level than those achieved with nicotine. Upregulation of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs was significant across all the brain regions (ANOVA main effects, $p < 0.0007$ and < 0.04 , respectively), with the greatest response in the occipital cortex, just as found for nicotine. As was true for nicotine, choline elicited a shortfall in the membrane/total protein ratio in the occipital cortex, suggestive of impaired neurite formation, a result in keeping with nicotinic actions predominating over any promotional effects on membrane formation. In the caudate, choline did not evoke the shortfall in cell numbers that were seen with nicotine, an expected result given the likelihood of a different mechanism (oxidative stress) for that particular effect of nicotine.

However, the best illustration of the dual actions of choline is provided by the results for cell signaling. In the frontal cortex and caudate, choline evoked robust increases in all aspects of AC signaling, indicative of induction of the signaling proteins themselves, as would be expected from augmented membrane formation. In contrast, this effect was notably absent in the occipital cortex, the region showing nicotine-like nAChR upregulation and cell damage. Accordingly, it might be expected that, whereas choline could protect the developing brain from the adverse effects of nicotine, it may also augment cholinergic effects contributing to nicotine-induced damage. Indeed, with the combination of nicotine + choline we found higher nAChR upregulation in all brain regions as compared to either treatment alone, representing the additive cholinergic effects of the two treatments. Neuroprotection was evident from the reduced effect on cell damage biomarkers in the occipital cortex and caudate, where the effects of choline offset the adverse changes elicited by nicotine. At the same time, though, the frontal cortex showed additive deficits in the index of cell size: although neither nicotine nor choline

produced significant decrements alone, the combined treatment elicited a significant reduction in total protein/DNA and a concomitant rise in membrane/total protein that would connote an increase in the surface/volume ratio associated with smaller cells. Effects of the combination of nicotine + choline similarly gave a regionally selective pattern of effects on cell signaling. In the frontal cortex, the promotional effect of choline was lessened by the combination with nicotine whereas effects in the caudate appeared to be additive. The most dramatic interaction for effects on signaling was seen in the heart, where the combined treatment elicited massive heterologous stimulation of all inputs to AC activity, effects far exceeding the summation of the modest changes seen with either agent alone. The effect on cardiac signaling was associated with a complete reversal of nicotine's adverse effects on the balance of β ARs and m_2 AChRs. Nevertheless, considering that the AC pathway is shared by numerous neuronal and hormonal inputs, a global increase in activity may be associated with abnormal responsiveness to trophic inputs that are vital to cardiac cell development, and future work will need to address the issue of whether these changes have otherwise unanticipated drawbacks. In the same vein, the effect of combined nicotine + choline treatment worsened the deficits in lung β ARs, so that the two individually nonsignificant effects achieved significance for the combined treatment.

Nicotine has the ability to both evoke and prevent oxidative stress, depending on its concentration and the neurodevelopmental stage at which exposure occurs (Guan *et al*, 2003). Accordingly, we hypothesized that Vitamin C supplementation might offset oxidative-related adverse effects of nicotine on the developing brain. To our surprise, Vitamin C enhanced nAChR upregulation in response to nicotine. Furthermore, in brain regions and tissues where nicotine evoked nonsignificant increases in AC signaling (frontal cortex, caudate, heart), the addition of Vitamin C rendered all these changes robust and significant. For the cell damage biomarkers, rather than providing uniform protection from the adverse effects of nicotine, Vitamin C supplementation had mixed actions. In the frontal cortex, combined treatment worsened the deficits in total protein/DNA, and in the occipital cortex, Vitamin C failed to protect the cells from the nicotine-induced reduction in membrane/total protein. However, there was significant protection from cell damage in the caudate, where Vitamin C completely prevented the cell loss evoked by nicotine. One possible explanation for dichotomous effects of Vitamin C is that in high doses, it can actually be prooxidant (Song *et al*, 1999), so that regional targeting might depend on the underlying oxidative status of each region. The caudate is especially susceptible to oxidative damage in part because of the high concentration of dopamine, an oxidative neurotransmitter that is released by nicotine (Blum *et al*, 2001; El-Bizri and Clarke, 1994; Karen *et al*, 2001; Rowell, 1995), so the protective effect of Vitamin C in this region is likely to represent the different underlying mechanism of neural cell damage from those operating in the frontal or occipital cortex, where nicotine stimulation and upregulation of nAChRs were the predominant events. When we examined each region for lipid peroxidation, we found far higher values in the caudate, in keeping with its prooxidant

status. However, nicotine administration did not elevate TBARS, nor did choline or Vitamin C supplementation affect the values. A strict assignment of a role for oxidative stress or protection by antioxidant effects is thus problematic; since we evaluated TBARS at only one time point, it is conceivable that the oxidative damage occurs earlier and is thus not evident as lipid peroxidation products at the time of sampling.

The effects of Vitamin C supplementation on peripheral tissues were also decidedly mixed. Vitamin C prevented the decline in cardiac β ARs evoked by nicotine but did not alter the upregulation of m_2 AChRs and worsened the decline in β ARs in the lung. Thus, regardless of the underlying mechanism, the point remains that Vitamin C, like choline, exerts dichotomous actions, and rather than producing uniform protection, may worsen some of the adverse effects of nicotine.

Unexpectedly, we found that nicotine in the amniotic fluid was significantly increased by supplementation with either choline or Vitamin C. Although our results do not explicitly identify the mechanisms responsible for these differences, a number of inferences can be drawn from the relative changes in nicotine and cotinine. First, the fact that maternal levels were unchanged suggests that the supplements do not alter maternal distribution, metabolism or clearance of nicotine or cotinine, but rather that the effects are exerted in the fetal compartment itself. In the amniotic fluid, nicotine levels were augmented by the supplements more than were those for cotinine, a result in keeping with impaired nicotine clearance and/or catabolism in the fetal compartment. The mechanisms underlying these effects are thus worth exploring, since higher concentrations of nicotine and cotinine in the fetal environment may be one of the major reasons for the worsening of many of the effects of nicotine on brain development. Similarly, the infusion model used here is an appropriate simulation of continuous nicotine delivery as is typical of transdermal nicotine patches, and although smokers tend to maintain a steady-state nicotine plasma level, these are likely to decay somewhat overnight when smoking ceases. A recent study showed that continuous nicotine delivery in rodents results in more than a two-fold higher fetal nicotine brain level as compared to maternal blood (Sarasin *et al*, 2003). Accordingly, future studies should address the impact of different modes of nicotine delivery, with and without dietary supplements, on the actual levels of nicotine achieved in fetal brain.

In conclusion, our results show that prenatal nicotine exposure in a primate model, at maternal nicotine levels commensurate with human smoking during pregnancy, elicits changes in indices of cholinergic function, cell signaling, and cell damage, indicative of disrupted brain cell development. These effects show discrete regional selectivities that, in part, reflect differences in nAChR responses to prolonged nicotine administration, but that also are influenced by the disparate neurotransmitter and cell populations within each region. Although choline or Vitamin C supplementation protects the developing brain from some aspects of nicotine-induced neurotoxicity, other aspects are worsened, reflecting both pharmacodynamic and pharmacokinetic parameters that augment the cellular responses to nicotine as well as the concentration of

nicotine in the fetal compartment. One critical conclusion from our results is that dietary supplements that may be beneficial in normative populations, or that may protect the developing brain from a defined set of neurotoxicants, may contribute to worsened outcomes when the fetus is exposed to nicotine as in the case of maternal smoking. Future studies need to consider the concept that smoking may thus create vulnerable subpopulations in whom dietary supplements evoke untoward effects in combination with nicotine exposure.

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