Neuropsychopharmacology (2007) 32, 1082–1097

www.neuropsychopharmacology.org

Permanent, Sex-Selective Effects of Prenatal or Adolescent Nicotine Exposure, Separately or Sequentially, in Rat Brain Regions: Indices of Cholinergic and Serotonergic Synaptic Function, Cell Signaling, and Neural Cell Number and Size at 6 Months of Age

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Nicotine is a neuroteratogen that disrupts neurodevelopment and synaptic function, with vulnerability extending into adolescence. We assessed the permanence of effects in rats on indices of neural cell number and size, and on acetylcholine and serotonin (5HT) systems, conducting assessments at 6 months of age, after prenatal nicotine exposure, adolescent exposure, or sequential exposure in both periods. For prenatal nicotine, indices of cell number and size showed few abnormalities by 6 months, but there were persistent deficits in cerebrocortical choline acetyltransferase activity and hemicholinium-3 binding to the presynaptic choline transporter, a pattern consistent with cholinergic hypoactivity; these effects were more prominent in males than females. The expression of 5HT receptors also showed permanent effects in males, with suppression of the $5HT_{1A}$ subtype and upregulation of $5HT_2$ receptors. In addition, cell signaling through adenylyl cyclase showed heterologous uncoupling of neurotransmitter responses. Nicotine exposure in adolescence produced lasting effects that were similar to those of prenatal nicotine. However, when animals were exposed to prenatal nicotine and received nicotine subsequently in adolescence, the adverse effects then extended to females, whereas the net effect in males was similar to that of prenatal nicotine by itself. Our results indicate that prenatal or adolescent nicotine exposure evoke permanent changes in synaptic function that transcend the recovery of less-sensitive indices of structural damage; further, prenatal exposure sensitizes females to the subsequent adverse effects of adolescent nicotine, thus creating a population that may be especially vulnerable to the lasting behavioral consequences of nicotine intake in adolescence.

Neuropsychopharmacology (2007) **32,** 1082–1097. doi:10.1038/sj.npp.1301231; published online 18 October 2006

Keywords: acetylcholine; adolescence; neural cell development; nicotine; serotonin; sex differences

INTRODUCTION

There is substantial agreement that nicotine is a neuroteratogen that alters patterns of neural cell replication, differentiation, synaptogenesis, and synaptic function in the developing brain (Levin and Slotkin, 1998; Slikker *et al*, 2005; Slotkin, 1998, 1999, 2004), thus contributing in a major way to the long-term liabilities associated with maternal smoking during pregnancy (Ernst *et al*, 2001; Hellstrom-Lindahl and Nordberg, 2002; Wakschlag *et al*, 2002; Weissman *et al*, 1999; Weitzman *et al*, 2002). Brain development continues into adolescence and recent work indicates that many of the mechanisms by which nicotine adversely affects the fetus still operate in the adolescent brain, producing similar (albeit lesser) functional anomalies and even outright neurotoxicity (Slotkin, 2002). In addition, the adolescent brain is much more responsive to nicotine than is the adult, enhancing the synaptic and behavioral responses that contribute to dependence and addiction (Abreu-Villaça *et al*, 2003a–c; Collins *et al*, 2004; Elliott *et al*, 2005; Faraday *et al*, 2001; Slotkin, 2002), echoing observations made in adolescent smokers (DiFranza *et al*, 2000, 2002a, b). Furthermore, the synaptic alterations evoked by fetal nicotine exposure affect the response to nicotine in adolescence (Abreu-Villaça *et al*, 2004a, b; Jacobsen *et al*,

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Received 26 June 2006; revised 8 August 2006; accepted 11 August 2006
Online publication: 15 September 2006 at http://www.acnp.org/citations/Npp091506060422/default.pdf

2006; Slotkin *et al*, 2006), likely contributing to the subpopulation in the offspring of smokers that are especially vulnerable to nicotine dependence (Jacobsen *et al*, 2006; Kandel *et al*, 1994; Niaura *et al*, 2001; Porath and Fried, 2005; Roberts *et al*, 2005).

The neurochemical and morphological substrates that underlie the developmental neurotoxicity of nicotine in the fetus and adolescent have been defined primarily over a course spanning the period of nicotine exposure and withdrawal, ranging up to young adulthood (Levin and Slotkin, 1998; Slikker *et al*, 2005; Slotkin, 1998, 1999, 2004). Nevertheless, it is now evident that neurogenesis and neural plasticity persist into adulthood (Altman and Bayer, 1990; Bayer, 1983; Bayer *et al*, 1982; Huttenlocher, 1990; McWilliams and Lynch, 1983; Scheetz and Constantine-Paton, 1994) and drugs can elicit 'developmental' effects even at this late stage (Grote *et al*, 2005). Accordingly, the current study addresses the permanence of the cellular and synaptic anomalies evoked in rats given prenatal or adolescent nicotine exposure, or given sequential exposure in both prenatal and adolescent stages, as reflected by measurements at 6 months of age. We utilized established treatment regimens that deliver nicotine continuously throughout pregnancy or adolescence, achieving plasma nicotine concentrations comparable to that in smokers (Slikker *et al*, 2005; Slotkin, 1992, 1998, 2002, 2004). Our evaluations made use of families of biomarkers that typify cell number and size, acetylcholine (ACh) and serotonin (5HT) synaptic function and transsynaptic cell signaling, as established in earlier work with prenatal or adolescent nicotine exposure (Abreu-Villaça *et al*, 2003c, 2004b; Muneoka *et al*, 2001; Navarro *et al*, 1989; Slotkin *et al*, 1987; Trauth *et al*, 2000a, b; Xu *et al*, 2001, 2002; Zahalka *et al*, 1992; Zhu *et al*, 2000). First, we assessed the impact on neural cell number and size by measurements of DNA and cell protein fractions. As each neural cell contains only a single nucleus (Winick and Noble, 1965), the DNA content (amount of DNA in each brain region) reflects the total number of cells and the DNA concentration (DNA per unit tissue weight) reflects the cell-packing density (Bell *et al*, 1987; Slotkin *et al*, 1984; Winick and Noble, 1965). The ratio of total protein/DNA rises with growth and thus reflects average cell size (Qiao *et al*, 2003, 2004; Slotkin *et al*, 2005), whereas the relative proportion of membrane/total protein reflects membrane complexity associated with neural connectivity, which climbs with the increase in formation of the neuropil.

For ACh systems, we assessed choline acetyltransferase activity (ChAT) and the binding of [^{3}H]hemicholinium-3 (HC3) to the high-affinity presynaptic choline transporter. ChAT is a constitutive marker for ACh nerve terminals, whereas HC3 binding is responsive to neuronal activity (Aubert *et al*, 1996; Happe and Murrin, 1992; Klemm and Kuhar, 1979; Navarro *et al*, 1989; Simon *et al*, 1976; Slotkin *et al*, 1990; Zahalka *et al*, 1992, 1993), so that comparative changes in the two markers permit distinction between effects on synaptic outgrowth as distinct from synaptic activity. We also evaluated effects on nicotinic ACh receptors (nAChRs), focusing on the $\alpha4\beta2$ nAChR, the predominant subtype in mammalian brain (Flores *et al*, 1992) and one which shows differential sensitivity to nicotine in the adolescent *vs* the adult (Trauth *et al*, 1999a). For 5HT synapses, we assessed ligand binding for $5HT_{1A}$ and $5HT_2$ receptors and the presynaptic 5HT transporter. The two receptors converge on common end points in 5HT cell signaling (Barnes and Sharp, 1999; Morin *et al*, 1992; Rovescalli *et al*, 1993) and are key players in 5HT-related mental disorders (Arango *et al*, 2001; Fujita *et al*, 2000; Yatham *et al*, 1999, 2000); the 5HT transporter regulates the synaptic concentration of 5HT and is the major target for antidepressant drugs (Maes and Meltzer, 1995; Nemeroff, 1998; Nutt, 2002). These three proteins have been evaluated previously for short-term changes evoked by adolescent nicotine treatment (Xu *et al*, 2001, 2002). Finally, we evaluated cell signaling mediated through adenylyl cyclase (AC), a pathway known to be targeted by both prenatal and adolescent nicotine treatments (Slotkin *et al*, 1992, 2006; Xu *et al*, 2002). We focused on assessments of basal AC activity, the maximal response capability evoked by a direct AC stimulant, forskolin, and the ability of a neurotransmitter receptor coupled to AC activation, the β-adrenergic receptor (βAR) to stimulate AC.

METHODS

Animals and Nicotine Infusions

All studies were carried out with the approval of the Duke University Institutional Animal Care and Use Committee, in accordance with the declaration of Helsinki principle and with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. Timed-pregnant Sprague–Dawley rats were shipped on gestational day 2 by climate-controlled truck (total transit time <1 h), housed individually and allowed free access to food and water. There were four treatment groups: controls (prenatal vehicle + adolescent vehicle), prenatal nicotine exposure (prenatal nicotine + adolescent vehicle), adolescent nicotine exposure (prenatal vehicle + adolescent nicotine), and those receiving the combined treatment (prenatal nicotine + adolescent nicotine). On gestational day 4, before implantation of the embryo in the uterine wall, each animal was quickly anesthetized with ether, a 3×3 cm area on the back was shaved, and an incision made to permit s.c. insertion of type 2ML2 Alzet osmotic minipumps. Pumps were prepared with nicotine bitartrate dissolved in bacteriostatic water, to deliver an initial dose rate of 6 mg/kg of nicotine (calculated as free base) per day. The incision was closed with wound clips and the animals were permitted to recover in their home cages. Control animals were implanted with minipumps containing only the water and an equivalent concentration of sodium bitartrate, adjusted to the same pH (6.0) as the nicotine bitartrate solution. It should be noted that the pump, marketed as a 2-week infusion device, actually takes 17.5 days to be exhausted completely (information supplied by the manufacturer) and thus the nicotine infusion terminates during gestational day 21. Maternal plasma nicotine levels achieved with this administration model resemble those seen in heavy smokers (25–60 ng/ml) as characterized previously (Isaac and Rand, 1972; Levin and Slotkin, 1998; Lichtensteiger *et al*, 1988; Murrin *et al*, 1985; Slotkin, 1992, 1998, 1999, 2004). This animal model also resembles the effects of maternal smoking in that fetal nicotine levels

exceed those found in maternal plasma (Luck *et al*, 1985; Sarasin *et al*, 2003).

Parturition occurred during gestational day 22, which was also taken as postnatal day 0. After birth, pups were randomized within treatment groups and litter sizes were culled to 10 (five males and five females) to ensure standard nutrition. Randomization was repeated every few days to distribute differential effects of maternal caretaking equally among all litters; cross-fostering, by itself, has no impact on neurochemical or behavioral effects of nicotine exposure (Ribary and Lichtensteiger, 1989). Animals were weaned on postnatal day 21.

On postnatal day 30, each animal was implanted with a minipump (Alzet type 1002) as already described, again set to deliver either vehicle or nicotine at an initial dose rate of 6 mg/kg/day, with the infusion terminating during postnatal day 47 (Slotkin, 1998; Trauth *et al*, 1999b, 2000b). The nicotine exposure period thus spans the recognized boundaries of adolescence in the rat, as typified by endocrine, pubertal and behavioral parameters (Spear, 2000). In the adolescent rat, this paradigm produces plasma nicotine levels of 25 ng/ml, similar to that in typical smokers (Lichtensteiger *et al*, 1988; Trauth *et al*, 2000b). Studies were then conducted at 6 months of age (postnatal day 180), more than 4 months after the termination of adolescent nicotine treatment. For each treatment group, 12 animals were examined, equally divided into males and females, with each litter of origin contributing no more than one male and one female to any of the determinations.

Animals were decapitated and the brain was dissected into cerebral cortex, striatum, hippocampus, midbrain, brainstem, and cerebellum (Trauth *et al*, 2000b), and the cerebral cortex was then divided down the midline to separate left and right hemispheres. Tissues were flash-frozen in liquid nitrogen and stored at -45°C until assayed. The hippocampus, striatum, midbrain, and left half of the cerebral cortex were used for biomarkers of cell number and size and for ACh biomarkers, whereas the brainstem and right half of the cerebral cortex were evaluated for 5HT and AC biomarkers. Because the cerebellum is sparse in both ACh and 5HT projections but has a high norepinephrine concentration, this region was used for assessment of AC signaling and also for βAR binding.

Biomarkers of Neural Cell Number and Size

Each tissue was thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in ice-cold 10 mM sodium–potassium phosphate buffer (pH 7.4) and aliquots of the homogenate were withdrawn for measurement of DNA and total protein. DNA was assessed with a modified (Trauth *et al*, 2000b) fluorescent dye-binding method (Labarca and Piagen, 1980). Aliquots were diluted in 50 mM sodium phosphate, 2 M NaCl, 2 mM EDTA (pH 7.4) and sonicated briefly (Virsonic Cell Disrupter, Virtis, Gardiner, NY). Hoechst 33258 was added to a final concentration of 1 μg/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 membrane preparations used for radioligand binding, as described below.

ACh Biomarkers

Aliquots of the same homogenate used for DNA determinations were assayed in duplicate for ChAT using established procedures (Lau *et al*, 1988; Qiao *et al*, 2003, 2004). Each tube contained final concentrations of 60 mM sodium phosphate (pH 7.9), 200 mM NaCl, 20 mM choline chloride, 17 mM MgCl2, 1 mM EDTA, 0.2% Triton X-100, 0.12 mM physostigmine, 0.6 mg/ml bovine serum albumin, and 50 μM [^{14}C]acetyl-coenzyme A. Blanks contained homogenization buffer instead of the tissue homogenate. Samples were preincubated for 15 min on ice, transferred to a 37°C water bath for 30 min, and the reaction terminated by placing the samples on ice. Labeled ACh was then extracted, counted and the activity determined relative to tissue protein (Smith *et al*, 1985). Preliminary determinations established that enzyme activity was linear with time and tissue concentration under these conditions.

For measurements of HC3 binding, an aliquot of the same tissue homogenate was sedimented at 40 000*g* for 15 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 using a smooth glass homogenizer fitted with a Teflon pestle, in 10 mM sodium–potassium phosphate buffer (pH 7.4) containing 150 mM NaCl. An aliquot was withdrawn for the determination of membrane protein (Smith *et al*, 1985) and radioligand binding was evaluated with 2 nM [^{3}H]HC3 (Vickroy *et al*, 1984), with incubation for 20 min at room temperature, followed by rapid vacuum filtration onto glass fiber filters (presoaked for 30 min with 0.15% polyethyleneimine in buffer). The nonspecific component was defined as radioligand binding in the presence of an excess concentration of unlabeled HC3 (10 μM) and binding values were expressed relative to membrane protein. For nAChR binding, each assay contained a final concentration of 1 nM [^{3}H]cytisine in a total volume of 250 μl of a buffer consisting of 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM Tris (pH 7.4). Incubations lasted 75 min at 4°C, with or without 10 μM nicotine to displace specific binding.

5HT Biomarkers

Brain regions used for 5HT biomarkers were thawed and homogenized (Polytron) in ice-cold 50 mM Tris (pH 7.4) and the cell membrane fraction was prepared by differential sedimentation as already described, with final resuspension in Tris buffer. As before, an aliquot was withdrawn for the determination of membrane protein (Smith *et al*, 1985). Two radioligands were used to determine 5HT receptor binding (Xu *et al*, 2002): 1 nM [^{3}H]8-hydroxy-2-(di-n-propylamino)tetralin for $5HT_{1A}$ receptors (Park *et al*, 1999; Stockmeier *et al*, 1998), and 0.4 nM [^{3}H]ketanserin for $5HT_2$ receptors (Leysen *et al*, 1982; Park *et al*, 1999). For $5HT_{1A}$ receptors, incubations lasted for 30 min at 25°C in a buffer consisting of 50 mM Tris (pH 8), 2 mM $MgCl_2$, and

2 mM sodium ascorbate; 100 μM 5HT was used to displace specific binding. For $5HT_2$ receptors, incubations lasted 15 min at 37°C in 50 mM Tris (pH 7.4) and specific binding was displaced with 10 μM methylsergide. For binding to the presynaptic 5HT transporter (Moret and Briley, 1991; Slotkin *et al*, 1997, 1999, 2000; Xu *et al*, 2001), the membrane suspension was incubated with 85 pM [^{3}H]paroxetine with or without addition of 100 μM 5HT to displace specific binding, and incubations lasted 120 min at 20°C.

AC Signaling

For AC determinations, the cell membrane fraction was resuspended in a buffer consisting of 125 mM sucrose, 6 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.5). AC assessments were conducted by standard techniques published previously (Auman *et al*, 2000, 2001; Zeiders *et al*, 1997, 1999). Briefly, aliquots were incubated for 10 min at 30°C with final concentrations of 100 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM ATP, 2 mM $MgCl_2$, 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 μM GTP in a total volume of 250 μl. The enzymatic reaction was stopped by placing the samples in a 90–100°C water bath for 5 min, followed by sedimentation at 3000 *g* for 15 min, and the supernatant solution was assayed for cAMP using radioimmunoassay kits. 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.

AC activity was evaluated in several different ways. First, we measured basal AC activity without addition of any stimulants. Next, we assessed the maximal response to a direct AC stimulant, forskolin (100 μM) (Limbird and Macmillan, 1981; Seamon and Daly, 1986). Finally, in the cerebellum (where we measured βAR binding, we determined the AC response to a βAR agonist, 100 μM isoproterenol, as both absolute AC activity and as a ratio to total activity (isoproterenol/forskolin ratio). The chosen concentrations of each stimulant produce maximal responses, as assessed in earlier studies (Auman *et al*, 2000, 2001; Zeiders *et al*, 1997, 1999).

In the cerebellum, the membrane preparation obtained for AC determinations was also used to evaluate βAR binding. Aliquots were incubated with 67 pM [^{125}I]iodopindolol in 145 mM NaCl, 2 mM $MgCl_2$, 1 mM Na ascorbate, 20 mM Tris (pH 7.5), for 20 min at room temperature in a total volume of 250 μl. Displacement of nonspecific binding was evaluated with 100 μM *d,l*-isoproterenol.

Data Analysis

Data are presented as means and standard errors. Differences between groups were first assessed by a global ANOVA (data log-transformed because of heterogeneous variance), incorporating all factors: prenatal treatment, adolescent treatment, brain region, and sex. This initial test was conducted across related measurements (considered as repeated measures, as they were all derived from the same homogenate) corresponding to each index class: indices of cell number (DNA concentration and content) and size (total protein/DNA, membrane/total protein), ACh biomarkers (ChAT, HC3 binding, HC3/ChAT ratio, nAChR binding), 5HT biomarkers ($5HT_{1A}$ and $5HT_2$ receptors, and 5HT transporter binding), and the multiple AC measures. Depending on the treatment interactions obtained in the global tests, data were then subdivided for lower order ANOVAs, followed where appropriate, by Fisher's protected least significant difference to establish effects comparing individual groups. However, where treatment effects were not interactive with other variables, only the main effects are shown without lower-order tests of individual differences. Significance for main treatment effects was assumed at $p < 0.05$, but 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). For convenience, some data are presented as the percentage change from control values, however, statistical evaluations were always carried out on the original data. For reference, control values appear in Table 1.

The study design required two different ways of regarding treatment variables. To compare the effects of prenatal exposure alone, adolescent exposure, or the combined exposure to controls or to each other, the four treatment groups were considered as a one-dimensional factor in the statistical design. To determine whether the effects of prenatal exposure and adolescent exposure 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.

Materials

Animals were obtained from Charles River (Raleigh, NC) and Alzet minipumps from Durect Corp. (Cupertino, CA). Bacteriostatic water used for the minipump solutions came from Abbott Laboratories (N Chicago, IL) and methylsergide was purchased from Sandoz Pharmaceuticals, E Hanover, NJ). PerkinElmer Life Sciences (Boston, MA) was the source for radioisotopically-labeled compounds: [^{14}C]acetyl-coenzyme A (specific activity 60 mCi/mmol, diluted with unlabeled compound to 6.7 mCi/mmol), [^{3}H]HC3 (125 Ci/mmol), [^{3}H]8-hydroxy-2-(di-n-propylamino)tetralin (135 Ci/mmol), [^{3}H]ketanserin (63 Ci/mmol), [^{3}H]paroxetine (19.4 Ci/mmol), [^{3}H]cytisine (35 Ci/mmol), and [^{125}I]iodopindolol (2200 Ci/mmol). Radioimmunoassay kits for the determination of cAMP were obtained from Amersham Biosciences (Piscataway, NJ). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO).

RESULTS

Biomarkers of Neural Cell Number and Size

Neither prenatal nor adolescent nicotine exposure, separately or in combination, elicited significant deficits in body weight or brain region weights (data not shown).

Table 1 Control Values

	Cerebral cortex		Hippocampus		Striatum		Midbrain		Brainstem		Cerebellum	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Cell number and size												
DNA concentration (μg/g tissue)	717±12	737±22	582±7	648±13*	557±14	602±39	720±11	753±13				
DNA content (μg/region)	344±14	320±7	74±3	77±1	66±2	64±2	258±6	243±6				
Total protein/DNA (μg/μg)	121±3	119±2	150±2	142±3	148±5	150±4	124±4	110±3*				
% Membrane/total protein (μg/μg)	44±1	42±1	41±1	39±2	46±1	45±1	44±2	48±1				
ACh markers												
Choline acetyltransferase (pmol/min/mg protein)	1050±25	1055±24	1026±13	1068±33	2666±49	2865±74*	845±32	949±13*				
HC3 binding (fmol/mg protein)	23.8±2.4	20.6±0.6	13.5±0.6	14.6±0.4	51±2	58±2	12.4±0.3	12.2±0.4				
HC3/ChAT (ratio × 1000)	23.6±2.2	19.5±0.7	13.3±0.8	13.3±0.5	19.1±0.9	20.2±0.9	14.9±0.7	12.9±0.2*				
nAChR binding (fmol/mg protein)	62±2	65±1	28±1	29±1	77±1	77±2	66±1	69±1				
5HT markers												
$5HT_{1A}$ binding (fmol/mg protein)	60±1	68±2*							28±2	28±1		
$5HT_2$ binding (fmol/mg protein)	109±7	131±4*							21±1	21±1		
5HT transporter binding (fmol/mg protein)	523±10	588±14*							344±18	371±10		
Adenylyl cyclase												
Basal (pmol/min/mg protein)	197±2	202±3							156±6	135±8	269±14	248±6
Forskolin (pmol/min/mg protein)	1236±60	1236±54							453±14	470±13	1425±42	1471±36
Isoproterenol (pmol/min/mg protein)											317±16	287±9
% Isoproterenol/forskolin											22.6±0.7	19.8±1.1

*Significant difference between males and females.

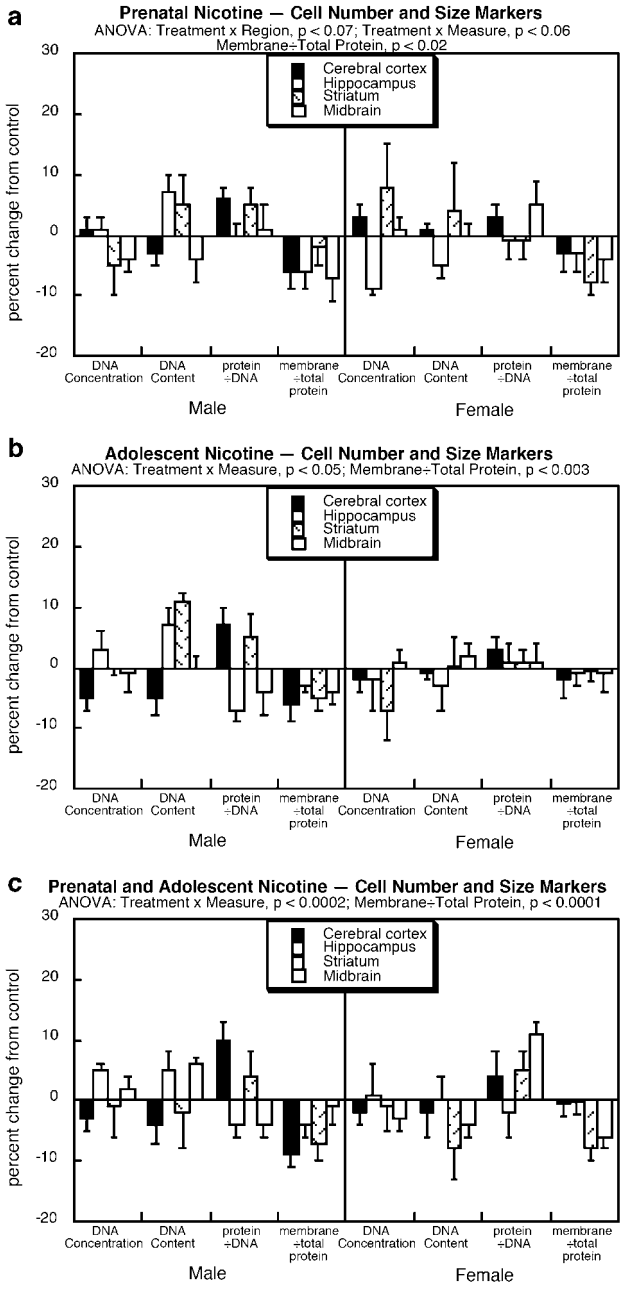

Figure 1 Effects of prenatal nicotine exposure (a), adolescent nicotine exposure (b), or prenatal nicotine followed by adolescent nicotine (c) on biomarkers of cell number and size. Data are shown as the percent change from the corresponding control values (Table 1). ANOVA across all variables appears at the top of each panel. For each treatment paradigm, because of the absence of a treatment × sex interaction, separate statistical analyses were not conducted for males and females; the only measure showing a significant overall effect was the membrane/total protein ratio.

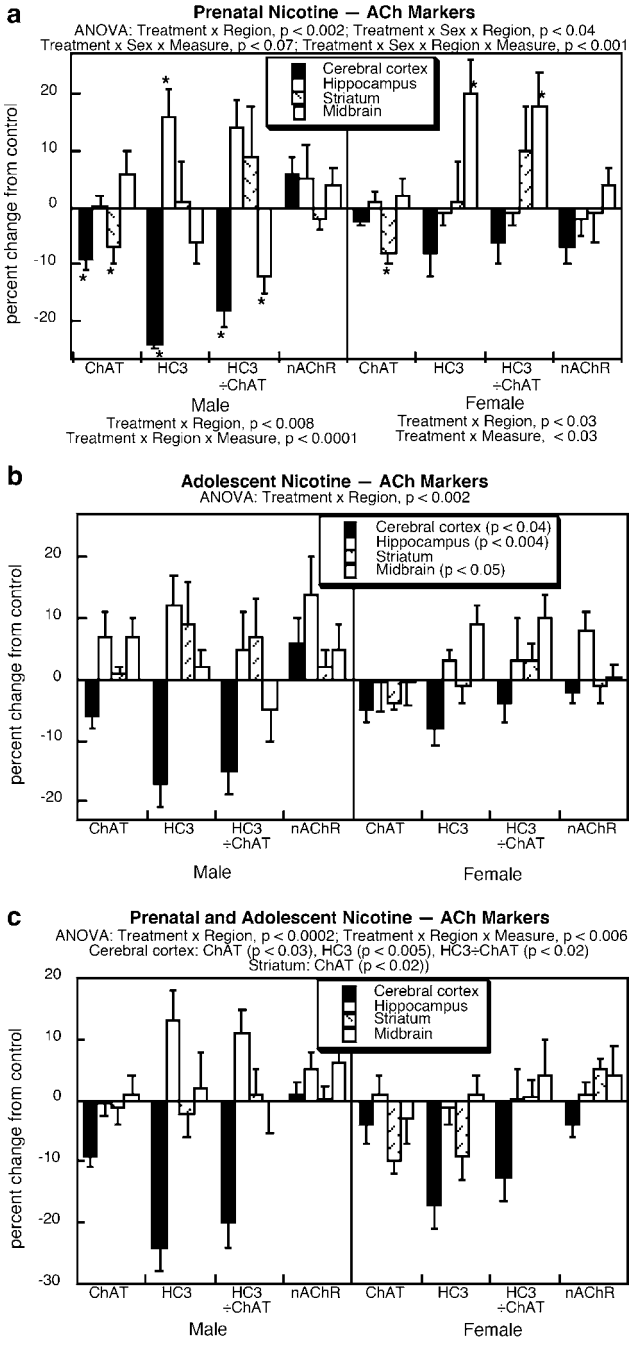

Figure 2 Effects of prenatal nicotine exposure (a), adolescent nicotine exposure (b), or prenatal nicotine followed by adolescent nicotine (c) on ACh biomarkers. Data are shown as the percent change from the corresponding control values (Table 1). ANOVA across all variables appears at the top of each panel. For prenatal nicotine, because of the treatment × sex interaction, separate statistical analyses were conducted for males and females, shown at the bottom of (a); asterisks denote individual brain regions for which the specific measures in prenatal nicotine group differ from controls, as justified by the treatment × region and treatment × measure interactions for each sex. For adolescent nicotine, there was only a treatment × region interaction so that separate analyses for each sex could not be carried out; significance for each region is shown within the panel. For the combined prenatal and adolescent treatment, the lower-order tests for each measure and region are shown at the top of the panel, without separation by sex, as justified by the interaction of treatment with those two covariables.

Nevertheless, global ANOVA across all biomarkers of neural cell number and size indicated regionally-selective effects (treatment × region, $p<0.05$) for specific biomarkers (treatment × measure, $p<0.03$). Regarding prenatal and adolescent treatments as two separate dimensions in the ANOVA design, we also found a significant prenatal nicotine × adolescent nicotine × region interaction ($p<0.02$), indicat-

ing that some of the effects of the dual treatment were not simply reflective of the summation of the two individual effects. Results were therefore subdivided into the three different types of treatments (prenatal nicotine, adolescent nicotine, prenatal + adolescent nicotine) for presentation.

Prenatal nicotine exposure had small and inconsistent effects on DNA concentration, DNA content, and the total protein/DNA ratio, but did cause a significant overall reduction in membrane/total protein, amounting to a net effect of 5–10% (Figure 1a). Adolescent nicotine showed a similar but smaller effect (Figure 1b) and the combination of prenatal and adolescent nicotine exposure also showed a significant reduction in the ratio that was indistinguishable from that of the prenatal treatment alone (Figure 1c). The effects of the combined exposure were significantly lower than expected from the summation of the two individual effects ($p<0.05$ for the interaction of prenatal nicotine × adolescent nicotine).

ACh Biomarkers

Global ANOVA across all ACh-related measures indicated a main treatment effect of nicotine ($p<0.05$) as well as regionally-selective effects (treatment × region, $p<0.002$) that differed among the various measurements (treatment × region × measure, $p<0.005$). Regarding the prenatal and adolescent treatments as two separate dimensions in the ANOVA further indicated non-additive interactions between prenatal and adolescent nicotine exposure that varied according to region, sex and the specific ACh biomarker being measured: $p<0.02$ for prenatal nicotine × adolescent nicotine × sex × measure; $p<0.03$ for prenatal nicotine × adolescent nicotine × region × sex × measure. The sex differences reflected greater effects on the cerebral cortex and hippocampus in males, and lesser effects in the midbrain ($p<0.05$ for treatment × sex for each region); however, the treatment × sex interactions were not always sustained in the lower order tests, as discussed below.

Prenatal nicotine exposure led to sex-selective deficits in ACh biomarkers in the cerebral cortex (Figure 2a). In males, there were significant deficits in ChAT activity but substantially larger deficits in the biomarker of neuronal activity (HC3 binding), and accordingly, the HC3/ChAT ratio declined by nearly 20%. In contrast, there was little or no change for the same parameters in the cerebral cortex of females. In the other regions, prenatal nicotine exposure showed activation of ACh pathways, evidenced by increases in HC3 binding in the hippocampus (males) or midbrain (females). There was little or no effect on nAChR binding in any of the regions.

Adolescent nicotine exposure (Figure 2b) elicited long-term changes in ACh biomarkers very similar to those of prenatal nicotine, although in this case the effects were slightly smaller and the interactions of treatment with sex and measure did not reach statistical significance in the overall ANOVA. Cerebrocortical values were significantly

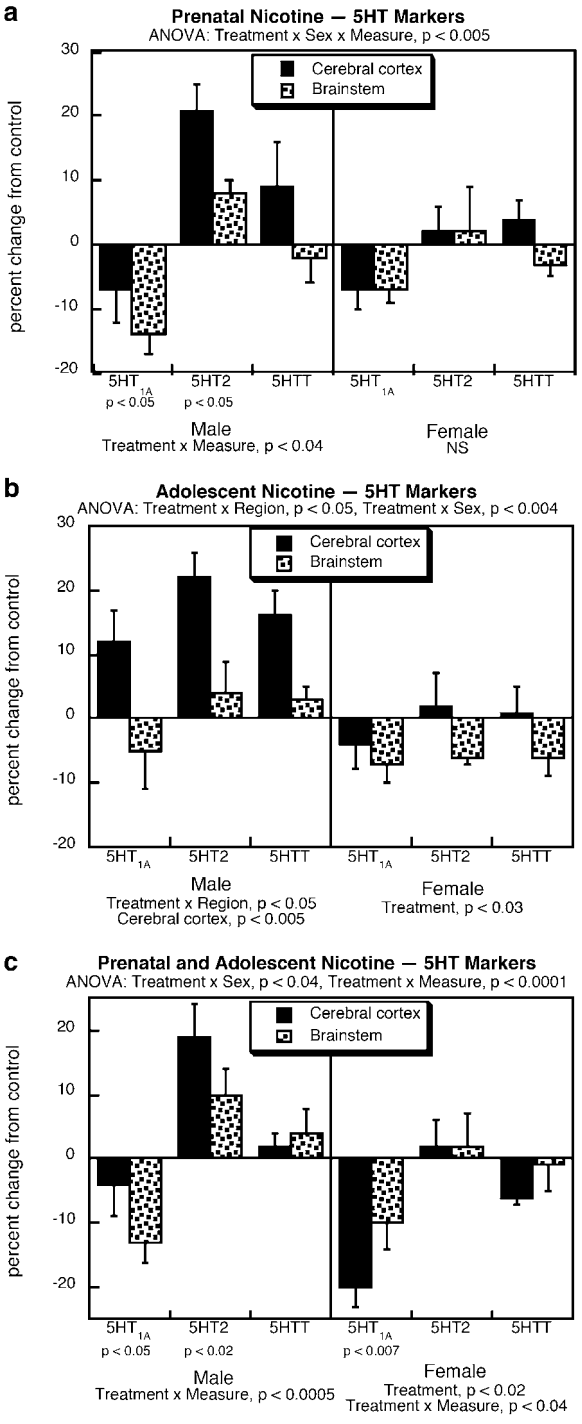

Figure 3 Effects of prenatal nicotine exposure (a), adolescent nicotine exposure (b), or prenatal nicotine followed by adolescent nicotine (c) on 5HT biomarkers. Data are shown as the percent change from the corresponding control values (Table 1). ANOVA across all variables appears at the top of each panel. For prenatal nicotine, because of the treatment × sex interaction, separate statistical analyses were conducted for males and females, shown at the bottom of (a); separate statistical evaluations were conducted for each measure but not for each region, in accordance with the appropriate interaction terms. For adolescent nicotine, lower order analysis for each region appears at the bottom of the panel, without separation of the different measures, in keeping with the treatment interactions with sex and region but not measure. Similarly, for the combined prenatal and adolescent treatment, the lower-order tests for each measure are shown at the bottom of the panel, as justified by the interaction of treatment with sex and measure but not region. Abbreviations: 5HTT, 5HT transporter; NS, not significant.

lower, whereas those in the hippocampus and midbrain were higher than in controls, showing the same trend for larger effects in males as seen with prenatal nicotine; statistical comparisons across the two treatment paradigms showed comparable sex effects, that is, the significant sex difference for prenatal nicotine was not distinguishable from the similar but nonsignificant sex difference for adolescent nicotine.

When animals exposed to prenatal nicotine were then given nicotine in adolescence, the long-term changes in cerebrocortical ACh biomarkers were the largest of all (Figure 2c), and again the same pattern was seen, characterized by suppression of ChAT and HC3, with a greater effect on the latter. To evaluate whether the net effects of the combined treatment were distinguishable from simple additivity of the two individual effects, we performed ANOVA with the two treatment paradigms considered as separate dimensions, and found a significant prenatal nicotine × adolescent nicotine × sex interaction, reflecting less-than-additive effects in males but more-than-additive effects in females. Accordingly, although males showed a significantly larger effect of separate exposure to prenatal or adolescent nicotine on cerebrocortical values, with the combined treatment, both males and females displayed pronounced deficits.

5HT Biomarkers

In the global ANOVA, treatment effects on 5HT biomarkers were both sex-selective (treatment × sex, $p<0.02$) and regionally disparate (treatment × region, $p<0.05$) and also differed among the three different 5HT measures (treatment × measure, $p<0.0001$). Again, regarding prenatal and adolescent exposures as separate ANOVA dimensions revealed non-additive interactions of the two treatments (prenatal nicotine × adolescent nicotine × region, $p<0.007$).

Prenatal nicotine exposure elicited long-term changes in 5HT biomarkers in males but not females (Figure 3a). Whereas $5HT_{1A}$ receptor binding was significantly reduced, $5HT_2$ receptors showed significant overall increases. Adolescent nicotine treatment in males elicited significant elevations in cerebrocortical values across all three 5HT biomarkers but had little effect in the brainstem (Figure 3b); again, these effects were not seen in females, who instead showed a small but statistically significant overall reduction. When animals were exposed to prenatal nicotine and then given nicotine in adolescence, the long-term effects in males were quite similar to those seen with prenatal treatment alone (Figure 3c): reductions in $5HT_{1A}$ receptor binding and increases in $5HT_2$ receptor binding. Using the two treatment paradigms as separate ANOVA dimensions, we found a significant prenatal nicotine × adolescent nicotine interaction ($p<0.02$), indicative of less-than-additive effects that reflected the restriction of the net effect of combined treatment to values no greater than those seen with the prenatal treatment. For females, however, the effects on $5HT_{1A}$ receptors were significantly enhanced by the double treatment ($p<0.05$ for prenatal nicotine × adolescent nicotine), so that whereas each of the individual treatments produced only small changes, the combined exposures produced deficits of up to 20%.

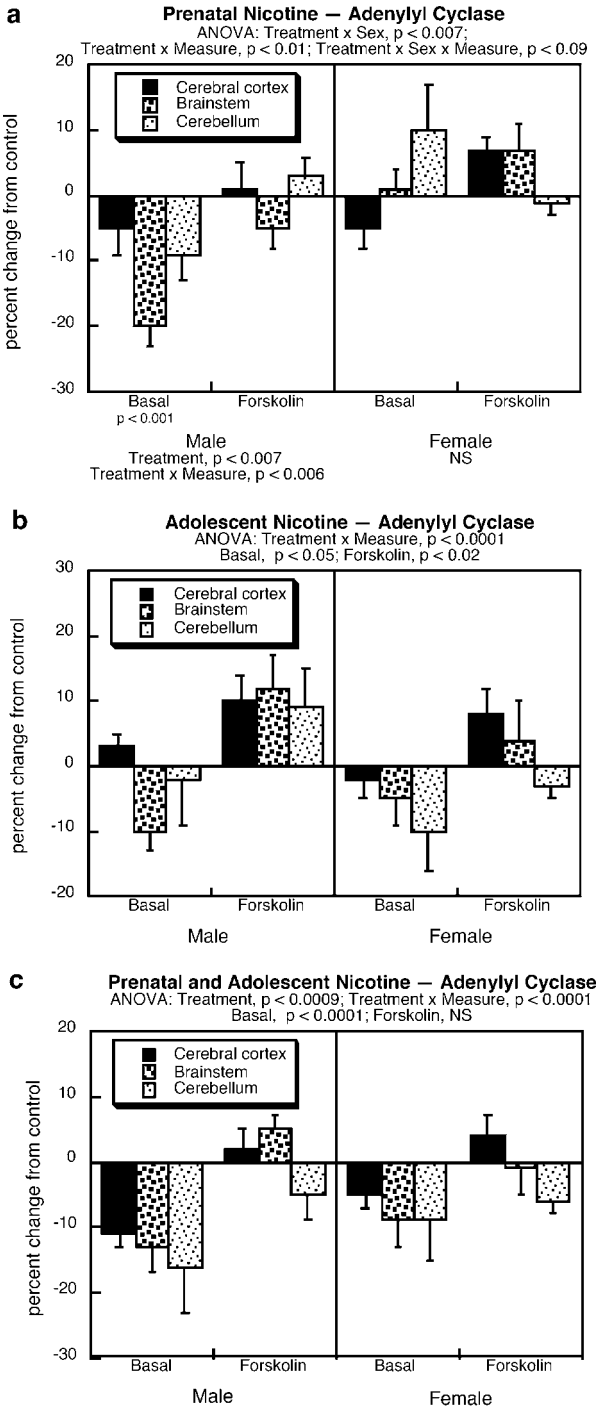

Figure 4 Effects of prenatal nicotine exposure (a), adolescent nicotine exposure (b), or prenatal nicotine followed by adolescent nicotine (c) on AC signaling. Data are shown as the percent change from the corresponding control values (Table 1). ANOVA across all variables appears at the top of each panel. For prenatal nicotine, because of the treatment × sex interaction, separate statistical analyses were conducted for males and females, shown at the bottom of (a); separate statistical evaluations were conducted for each measure but not for each region, in accordance with the appropriate interaction terms. For adolescent nicotine, lower order analysis for each measure appears at the top of the panel, without separation by sex or region, in keeping with the restriction of effects to a treatment × measure interaction. Similarly, for the combined prenatal and adolescent treatment, the lower-order tests for each measure are shown at the top of the panel, as justified by the interaction of treatment measure but not sex or region. Abbreviation: NS, not significant.

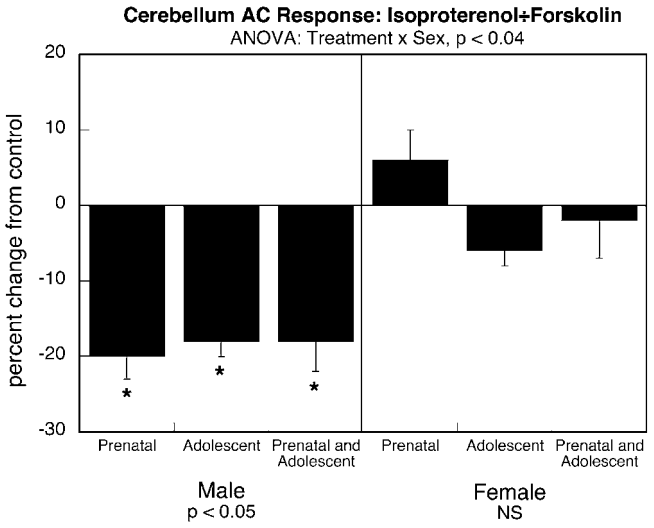

Figure 5 Effects of prenatal nicotine exposure, adolescent nicotine exposure, or prenatal nicotine followed by adolescent nicotine, on the response of cerebellar AC activity to βAR stimulation, evaluated as the ratio of activity in response to isoproterenol *vs* forskolin. Data are shown as the percent change from the corresponding control value (Table 1). ANOVA across all variables appears at the top of the panel and asterisks denote individual values that differ significantly from the control. Abbreviation: NS, not significant.

AC Signaling

Across all variables, nicotine treatment elicited global, sex-dependent changes in AC activity (treatment $\times$ sex, $p<0.004$) that differed between basal and forskolin-stimulated activity (treatment $\times$ measure, $p<0.0001$). With a two-dimensional treatment design, the ANOVA also indicated the need to examine potentially non-additive interactions (prenatal nicotine $\times$ adolescent nicotine, $p<0.06$; prenatal nicotine $\times$ adolescent nicotine $\times$ measure, $p<0.1$).

Prenatal nicotine exposure led to an overall decrease in basal AC activity without a corresponding change in maximal activity evoked by forskolin (Figure 4a). As was true for ACh and 5HT biomarkers, males were affected significantly more than females. The long-term effects of adolescent nicotine exposure were less notable, with small but significant reductions in basal activity and elevations of the forskolin response, without sex preference (Figure 4b). Again, with combined prenatal and adolescent treatment, the effects looked quite similar to those of prenatal nicotine alone, with a significant reduction in basal activity without a corresponding change in the AC response to forskolin (Figure 4c).

To probe the mechanisms underlying the differences between the inhibitory effects on basal AC and the neutral or stimulatory effects on the forskolin response, we evaluated the ability of a βAR agonist, isoproterenol, to stimulate AC in the cerebellum. In control animals, isoproterenol was able to stimulate AC to approximately 20% of its maximal value, as assessed by the isoproterenol/forskolin activity ratio (Table 1). Each of the three nicotine treatment paradigms interfered with the βAR-mediated response, reducing the stimulation by about 20% relative to controls (Figure 5). Again, as with the basal AC measurements, the effects were restricted to males. To determine whether the loss of AC responsiveness reflected a decline in βARs as compared to uncoupling of the receptors from the response, we also evaluated βAR binding. There were no significant differences: males, 30 ± 1 fmol/mg protein for controls, 31 ± 1 for prenatal nicotine, 29 ± 1 for adolescent nicotine, 30 ± 1 for combined treatment; females, 29 ± 1, 31 ± 1, 28 ± 2, and 29 ± 1, respectively.

DISCUSSION

Results of this study indicate that the effects of prenatal or adolescent nicotine exposure on brain development are indeed permanent, with a much more notable impact on synaptic activity and function than on indices of neural cell morphology. To relate our findings to earlier work, each of the treatments will be dealt with in turn.

Prenatal Nicotine Exposure

During the period of fetal nicotine exposure and continuing into early neonatal life, maternal nicotine treatment produces immediate deficits in the number of neural cells in a wide variety of brain regions, accompanied by perikaryal enlargement (swelling) that is typical of cell injury (Levin and Slotkin, 1998; Roy *et al*, 1998, 2002; Slotkin, 1992, 1998; Slotkin *et al*, 1987). Nevertheless, the number of neural cells eventually recovers and even becomes supranormal, with an elevated cell-packing density, largely because of reactive gliosis (Abdel-Rahman *et al*, 2003, 2004, 2005; Roy and Sabherwal, 1994, 1998; Roy *et al*, 2002); the same phenomenon occurs in the brains of children whose mothers smoked during pregnancy (Storm *et al*, 1999). Accordingly, by adolescence, many of the biochemical features of neuronal loss are absent, although detailed morphological examination reveals the underlying imbalances in neuron/glia ratios and in the density of the neuropil (Roy and Sabherwal, 1994, 1998; Roy *et al*, 2002). In keeping with this sequence, in the current study, we found only inconsistent changes in DNA-related biomarkers of cell number and packing density at 6 months of age, but nevertheless found a significant reduction in membrane complexity (subnormal membrane/total protein ratio), as would be expected from the deficiencies in neural connections as are apparent from morphologic examinations of the specific layers of the hippocampus and cerebral cortex (Roy and Sabherwal, 1994, 1998; Roy *et al*, 2002). Here, the biochemical approach is limited by the fact that each brain region is heterogeneous, so that relatively large changes in specific layers or subregions may be 'washed out' by the inclusion of larger amounts of unaffected areas. Obviously, detailed morphologic examination, such as that conducted in younger animals (Abdel-Rahman *et al*, 2003, 2004, 2005; Roy and Sabherwal, 1994, 1998; Roy *et al*, 2002), can resolve these issues. Nevertheless, our findings indicate that the permanence of the adverse effects of prenatal nicotine exposure are more readily demonstrated through indices of synaptic function than of neural cell number and size.

In earlier work, we showed that maternal nicotine administration results in biphasic deficits in ACh synaptic function in the offspring (Navarro *et al*, 1989; Slotkin, 1992, 1998, 1999, 2004; Zahalka *et al*, 1992): initial deficits in the

early postnatal period are made up by weaning, only to reemerge in adolescence and young adulthood. In the current study, cerebrocortical ACh projections showed a persistence of these abnormalities at 6 months of age, evidenced by significant reductions in ChAT and HC3 binding; furthermore, synaptic activity, as monitored by the HC3/ChAT ratio, was severely subnormal. There are two noteworthy features to this pattern. First, the effects were not sustained in the hippocampus, a region in which deficits are prominent in adolescence and young adulthood (Zahalka *et al*, 1992); indeed, if anything, ACh biomarkers were increased in the hippocampus at 6 months. Second, the effects were far more substantial in males, a difference that first emerges in adolescence (Abreu-Villaça *et al*, 2004b). Both of these findings likely reflect the long-term plasticity of the brain, a feature that is particularly prominent for the hippocampus, a region in which neurogenesis continues throughout life (Brezun and Daszuta, 1999; Jacobs *et al*, 2000; McEwen, 2001). In this regard, it is especially important that both hippocampal neurogenesis and synaptic plasticity are controlled in large measure by estrogen receptors (McEwen, 2002; Tanapat *et al*, 1999), thus permitting greater adaptation in females. Nevertheless, the fact that there are ACh-related abnormalities in other brain regions of females is indicative that they are not totally spared the adverse, long-term effects of prenatal nicotine exposure, and as will be discussed later, prenatal nicotine sets the stage for enhancing the damage resulting from adolescent nicotine exposure in females. Finally, it is interesting to note that nAChR binding sites, the immediate target for nicotine, were largely unaffected at 6 months of age, echoing our earlier findings in adolescence and young adulthood (Abreu-Villaça *et al*, 2004b). Here again, these negative results do not rule out the possibility of significant changes in smaller subregions or for different nAChR subtypes (Chen *et al*, 2005; Tizabi and Perry, 2000), so that further examinations are warranted to see if these also persist throughout the lifespan.

The long-term effects of prenatal nicotine exposure on 5HT systems share many of the features noted for ACh pathways. Alterations in the expression of 5HT receptors were far more prominent in males than in females, and the constitutive marker for the integrity of 5HT terminals (5HT transporter) showed less effect than did the 5HT receptors. Here too, we found evidence for continuing plasticity in the response to the damage evoked by prenatal nicotine exposure: in adolescence, there are elevations in both cerebrocortical $5HT_{1A}$ and $5HT_2$ receptors with the same pattern of sex preference (greater effects in males) (Slotkin *et al*, 2006), whereas at 6 months of age, we found reversal of the effects on the $5HT_{1A}$ subtype. Again, although the maintained effect on $5HT_2$ receptors points out the permanence of 5HT abnormalities after prenatal nicotine exposure, the change in $5HT_{1A}$ binding shows that specific indices and direction of change are not always sustained throughout the lifespan.

In our earlier work on AC signaling, we found transient elevations in maximal, forskolin-stimulated activity during adolescence, with a comparative deficiency in other AC measures, reflected either as smaller increases than for maximal activity, or as shifts to suppression of activity in response to neurotransmitter receptor activation (Slotkin *et al*, 1992, 2006). In the current study, by 6 months of age the transient elevation of forskolin-stimulated activity was no longer apparent, but the deficiencies in basal and neurotransmitter-stimulated activity remained. As with the other measures, males were affected more than females. Given the fact that AC is a signaling pathway common to multiple neurotransmitter and hormonal inputs, it is unlikely that the deficiencies seen here reflect a specific response to the abnormalities in ACh or 5HT input but rather represent a separable impact of prenatal nicotine exposure on the 'programming' of cellular responses. Indeed, our finding of deficient βAR-mediated responses without a corresponding decrease in βAR binding reinforces the idea that these effects on cell signaling are mediated downstream from specific receptor inputs and are thus likely to be shared by a wider variety of receptors (ie 'heterologous' desensitization). Our findings suggest that the adverse effects will extend to far more neurotransmitter systems and circuits than those examined in the present study and future work needs to address the potential ubiquity of nicotine-induced anomalies of synaptic signaling and their behavioral correlates.

Adolescent Nicotine Exposure

Because many neurodevelopmental processes continue into young adulthood, the immediate effects of adolescent nicotine administration on biomarkers of neural cell number and size resemble those of fetal exposure, but with a much reduced impact (Abreu-Villaça *et al*, 2003c; Slotkin, 2002; Trauth *et al*, 2000b). Accordingly, in the present study, the only significant difference in these indices that remained detectable at 6 months of age was a decrease in the membrane/total protein ratio, just as was seen for prenatal nicotine. Nevertheless, the alterations for indices of synaptic function remained robust. Again, ACh systems showed persistent reductions in activity, as evidenced by reduced cerebrocortical ChAT and even greater deficits in HC3 binding. In fact, the effects were quite similar to those of prenatal nicotine exposure, including greater effects in males and elevations in other regions (hippocampus, midbrain), as with the prenatal nicotine regimen, there were no sustained changes in nAChR binding, which is not surprising, given that values return to normal within the first month of termination of adolescent nicotine exposure (Abreu-Villaça *et al*, 2003a, 2004b; Trauth *et al*, 2000a). These findings thus reinforce our earlier conclusions that the vulnerability of ACh systems to long-term deficits evoked by nicotine exposure persists from fetal stages all the way through adolescence, and is readily discernible as a deficiency in the indices of presynaptic activity (Abreu-Villaça *et al*, 2003a, 2004b; Trauth *et al*, 2000a).

In our earlier work for the short-term effects of adolescent nicotine exposure, we identified transient changes in the expression of 5HT receptors that were related to the immediate impact of nicotine treatment and subsequent withdrawal (Slotkin *et al*, 2006; Xu *et al*, 2001, 2002). Here, we examined the long-term consequences of the same treatment at 6 months of age and found significant increases in cerebrocortical $5HT_{1A}$ and $5HT_2$ receptors, as well as for the 5HT transporter. The pattern bears similarities to the effects of prenatal nicotine in terms of

regional selectivity (cerebral cortex > brainstem) and sex preference (male > female) but the persistent changes in $5HT_{1A}$ receptors were opposite, decreased by prenatal nicotine but increased by adolescent nicotine. This suggests either a different regulatory adjustment from the two treatment paradigms, or alternatively that the changes with the adolescent exposure model might reflect a different underlying event, namely reactive sprouting consequent to the demonstrable 5HT nerve terminal damage (Xu *et al*, 2001). Although prenatal nicotine administration also damages 5HT projections to the cerebral cortex (Xu *et al*, 2001), the superior plasticity of the fetal/neonatal brain may permit greater subsequent recovery and therefore a different pattern of receptor and transporter changes. Here, detailed morphologic examinations would be required to establish the underlying events, but in any case, we would predict different behavioral consequences and/or 5HT drug reactivities of animals exposed to prenatal *vs* adolescent nicotine.

Withdrawal after adolescent nicotine exposure is associated with an increase in forskolin-stimulated AC activity and a deficiency in basal or receptor-mediated activity relative to the maximum (Abreu-Villaça *et al*, 2003b; Slotkin *et al*, 2006; Xu *et al*, 2002). At 6 months, we found persistence of the same pattern: an elevated forskolin response unaccompanied by a corresponding increase in basal activity or in the response to βAR stimulation. In fact, the latter two showed significant decreases relative to control. The net effect on cell signaling for adolescent nicotine exposure thus resembles that of prenatal nicotine and these alterations appear to be sustained throughout the lifespan. Again, we anticipate that these heterologous changes in cell signaling will be shared by multiple receptor inputs.

Prenatal Nicotine Followed by Adolescent Nicotine

Prenatal nicotine exposure has a far-reaching impact on the response to nicotine administered subsequently in adolescence (Abreu-Villaça *et al*, 2004a, b; Seidler *et al*, 1992; Slotkin *et al*, 2006). Although the prenatal treatment desensitizes nAChRs to the immediate effects of adolescent nicotine treatment, at the level of neural cell damage and loss and for indices of ACh synaptic activity, the effects are enhanced by the double treatment. In the current study, we found reductions in the membrane/total protein ratio at 6 months of age that were essentially indistinguishable from those seen with prenatal nicotine alone but the same was not the case for ACh biomarkers, where there was a clear enhancement by the combined exposure of adverse effects in females. In our earlier work on 5HT systems, we found that the immediate effects of adolescent nicotine administration and withdrawal were obtunded by prior, prenatal nicotine exposure, so that the net effects of the combined treatment looked quite similar to those seen in animals that had received the prenatal treatment alone (Slotkin *et al*, 2006). Here, at 6 months of age, we found the same pattern for 5HT systems in males, namely a virtually identical pattern for the combined exposure and for prenatal exposure alone. However, just as for ACh systems, females given the sequential treatment with prenatal and adolescent nicotine now showed enhanced vulnerability, evidenced by a substantial decrease in $5HT_{1A}$ receptor binding. At the level of AC signaling, we again saw an impairment of basal activity and receptor-mediated responses relative to maximal activity, of the same magnitude as that seen with either prenatal or adolescent nicotine exposure alone, with preferential effects in males; as with the other synaptic markers, the net effect in males was not enhanced by the double treatment, so that essentially, either treatment alone produces the greatest possible deficit.

Putting these patterns together, we would anticipate that the net effects of combined treatment on synaptic function and related behaviors will likely have a greater impact on females than on males, even though males are more targeted by separate prenatal or adolescent nicotine exposure. Whereas females are largely spared the long-term neurochemical consequences of the individual effects, the sequential prenatal and adolescent exposure overcomes the sex-selective protective adaptations and neural plasticity that otherwise limit the consequences in females. Accordingly, although males are likely to show neurobehavioral deficits after any of the three exposure scenarios, in females, we expect that the combined exposure will produce greater effects than would be anticipated from the two separate exposures.

Conclusion

Our results indicate that prenatal nicotine exposure evokes permanent changes in indices of ACh and 5HT synaptic function that transcend the apparent recovery of less-sensitive indices of structural neural damage. Equally important, virtually the same effects occur with nicotine exposure in adolescence at plasma nicotine levels found in typical smokers, indicating that the vulnerability of the developing brain to nicotine extends into the stage where most smokers begin tobacco use. There are important ramifications to these findings. First, the fact that abnormalities extend beyond ACh systems, the immediate target of nicotine, to involve 5HT function provides a mechanistic underpinning for the epidemiological findings of greater susceptibility to affective, appetitive and sleep disorders in the offspring of women who smoke during pregnancy as well as in adolescent smokers (Oken *et al*, 2005; Patten *et al*, 2000; Salsberry and Reagan, 2005; Slikker and Schwetz, 2003; Toschke *et al*, 2003; Upadhyaya *et al*, 2002; Wu and Anthony, 1999). Notably, the fact that abnormalities extend to cell signaling cascades shared by multiple neurotransmitter inputs, also suggests that there will be an even wider spectrum of behavioral disorders as outcomes of either prenatal or adolescent nicotine exposure. Second, the greater effects of prenatal nicotine exposure in males correspond to their higher incidence of psychosocial sequelae such as learning and conduct disorders (Wakschlag and Hans, 2002; Wakschlag *et al*, 1997). At the same time, the fact that the relative protection of females disappears with the combined, sequential prenatal, and adolescent exposure points to a biological origin for the greater association for the effects of maternal smoking on the uptake and progression of adolescent tobacco use by their daughters (Kandel *et al*, 1994; Oncken *et al*, 2004; Roberts *et al*, 2005). Indeed, if our findings for combined prenatal and adolescent nicotine exposure in rats extend to

adolescent smokers whose mothers smoked during pregnancy, there are a number of predictions that can be made that represent extensions of recent findings. First, there are individuals who are prone to the rapid onset of nicotine dependence, often after smoking only a few cigarettes, with a higher incidence of vulnerability in females (DiFranza *et al*, 2002a); we anticipate that this association will be even stronger for those whose mothers smoked during pregnancy. Second, we expect that the persistent changes seen here would render prenatally exposed adolescents especially vulnerable to relapse after attempts at smoking cessation (DiFranza and Wellman, 2005) and again, we predict this relationship will be selectively greater for women. Third, when adolescent smokers are abstinent, they show both cognitive impairment and depression (Colby *et al*, 2000; Goodman and Capitman, 2000; Hurt *et al*, 2000; Martini *et al*, 2002; Patten *et al*, 2000; Salin-Pascual *et al*, 1995; Tsoh *et al*, 2000; Wu and Anthony, 1999), problems that are worse in individuals who were exposed to nicotine *in utero* via maternal smoking (Oncken *et al*, 2004). Indeed, whereas adolescent smokers whose mothers did not smoke show cognitive improvement upon smoking cessation, those whose mothers did smoke show worsening of cognitive function during withdrawal (Jacobsen *et al*, 2005, 2006). Again, we expect that an examination of sex differences for this relationship will reveal a greater effect in females than males. Finally, given the targeting of 5HT systems for the lasting effects of prenatal or adolescent nicotine, alone or in combination, we would predict that treatments aimed at restoring 5HT function, such as serotonin-specific reuptake inhibitors, may be particularly useful in smoking cessation therapy for adolescent smokers, with the greatest effect seen in females whose mothers smoked during pregnancy.

Finally, our results address key issues in the persistence of nicotine addictability after both prenatal or adolescent nicotine exposure, either separately or together, and also account for the transgenerational nature of nicotine addiction, without requirement for underlying heritable characteristics. Nicotine exposure during either developmental period produced permanent changes in synaptic function, even after prolonged abstinence. These observations are entirely consistent with the view that the brain adaptations to addictive stimuli do not simply regress to normal after discontinuing drug exposure, but rather are kept in balance through lasting adjustments in synaptic activity, as postulated by the *sensitization-homeostasis* theory of nicotine addiction (DiFranza and Wellman, 2005). Accordingly, there are long-term sequelae that persist beyond the stage of smoking or abstinence, including enhanced susceptibility to relapse, and as found recently, emergence of depression as a consequence of adolescent smoking (Steuber and Danner, 2006). The current results, combined with earlier work on the prenatal + adolescent exposure model, permit the formulation of a hypothesis as to how smoking behaviors can be transmitted across generations (Figure 6). Prenatal nicotine exposure produces dysfunction in multiple neurotransmitter pathways, with the changes in cholinergic and serotonergic function contributing in major ways to abnormalities of cognition, reward, and affect (Slotkin, 1992, 1998, 1999, 2004). Importantly, many of these features emerge in adolescence (Navarro *et al*, 1989; Slotkin, 1992, 1998, 1999, 2004; Zahalka *et al*, 1992), when access to tobacco products becomes available. At that stage, nicotine intake partially relieves the deficiencies; although the adolescent brain is highly responsive to nicotine (Abreu-Villaça *et al*, 2003a–c; Collins *et al*, 2004; Elliott *et al*, 2005; Faraday *et al*, 2001; Slotkin, 2002), prenatal nicotine exposure produces lasting desensitization (Abreu-Villaça *et al*, 2004b; Seidler *et al*, 1992), so that the offspring of smokers will tend toward much higher consumption to obtain the desired effect, which in turn extends and expands the degree of damage and reprogramming of neural circuits (Abreu-Villaça *et al*, 2004a), augmenting and cementing the permanent changes in synaptic function and behavioral performance. When these individuals attempt to quit, they are consequently thrown into a worsened degree of cognitive impairment, depression, and loss of reward than if they had never smoked at all, as revealed in recent findings (Jacobsen *et al*, 2006). These factors then render quitting smoking far less likely, enhancing the probability that this individual, too, will smoke through pregnancy, thus ensuring that the addictive cycle extends to the next generation. These conclusions make it all the more critical that public health focus on prevention of tobacco use during developmental stages ranging from pregnancy through adolescence, over and above efforts toward smoking cessation in current smokers.

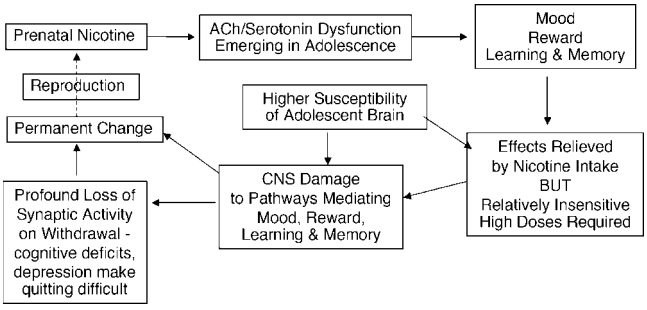

Figure 6 How prenatal nicotine exposure predisposes the brain to nicotine addiction in adolescence and leads to transgenerational effects (see text). Abbreviation: ACh, acetylcholine.

ACKNOWLEDGEMENTS

This research was supported by a grant from Philip Morris, USA and Philip Morris International. The grantors did not have input into the experimental design or interpretation. The authors state that they have no conflicts of interest.

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