

Short-Term Adolescent Nicotine Exposure has Immediate and Persistent Effects on Cholinergic Systems: Critical Periods, Patterns of Exposure, Dose Thresholds

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In adolescents, the symptoms of nicotine dependence can appear well before the onset of habitual smoking. We investigated short-term nicotine exposure in adolescent rats for corresponding cholinergic alterations. Beginning on postnatal day 30, rats were given a 1-week regimen of nicotine infusions or twice-daily injections, at doses (0.6, 2, and 6 mg/kg/day) set to achieve plasma levels found in occasional to regular smokers. In the cerebral cortex, midbrain, and hippocampus, we assessed nicotinic cholinergic receptor (nAChR) binding, choline acetyltransferase (ChAT) activity, a constitutive marker for cholinergic nerve terminals, and [³H]hemicholinium-3 (HC-3) binding to the high-affinity choline transporter, which responds to cholinergic synaptic stimulation. nAChR upregulation was observed with either administration route, even at the lowest dose; in the hippocampus, increases could be detected with as little as 2 days' treatment at 0.6 mg/kg/day. In the midbrain, upregulation was still significant even 1 month post-treatment. Adolescent nicotine treatment also produced lasting decrements in HC-3 binding that were separable from effects on ChAT, suggesting cholinergic synaptic impairment. Again, these effects were obtained at the lowest dose and remained significant 1 month post-treatment. Our results indicate that in adolescence, even a brief period of continuous or intermittent nicotine exposure, elicits lasting alterations in cholinergic systems in brain regions associated with nicotine dependence. As the effects are detected at exposures that produce plasma concentrations as little as one-tenth of those in regular smokers, the exquisite sensitivity of the adolescent brain to nicotine may contribute to the onset of nicotine dependence even in occasional smokers.

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INTRODUCTION

Adolescence is associated with increases in novelty-seeking and risk-taking behaviors that assist in acquiring adult skills (Adriani *et al*, 2002; Spear, 2000), but at the same time contribute to experimentation with tobacco, alcohol, and illicit drugs (Spear, 2000). In recent years, nearly one-third of US high school students reported smoking cigarettes and about three thousand teenagers begin smoking every day (Centers for Disease Control and Prevention, 1998, 2000; National Institute on Drug Abuse, 1998). Of these, three-quarters become daily smokers by the age of 20 (National

Institute on Drug Abuse, 1998; Nelson *et al*, 1995), comprising a cohort for whom smoking will be a long-term addiction (Pierce and Gilpin, 1996), with high daily consumption and a low probability of quitting (Chen and Millar, 1998). Accordingly, the number of deaths attributable to tobacco is expected to more than double worldwide by 2020 (Murray and Lopez, 1997).

Classically, the onset of nicotine dependence is considered to be a slow, sequential process requiring prolonged, daily smoking (DiFranza *et al*, 2000; Jackson, 1998; Lamkin and Houston, 1998; Leventhal and Cleary, 1980). However, recent studies indicate that subsets of adolescent smokers exhibit symptoms of nicotine dependence before the onset of daily smoking, often with only a few days of occasional tobacco use (DiFranza *et al*, 2000, 2002a, b; O'Loughlin *et al*, 2002). This suggests that the adolescent brain is inherently more susceptible to nicotine dependence, in keeping with the idea that responses to psychoactive substances are innately different during this developmental period (Cirulli

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and Laviola, 2000; Markwiese *et al*, 1998; Spear, 2000; Spear and Brake, 1983). In spite of these facts, only recently has basic research focused on the biological substrates that may underlie the susceptibility of the adolescent brain to nicotine dependence (Slotkin, 2002). It is well established that nicotine exposure during prenatal development induces cognitive and behavioral deficits that reflect deleterious actions of nicotine that alter cell proliferation and differentiation, synaptic maturation, and cell signaling (Levin and Slotkin, 1998; Slotkin, 1992, 1998, 1999). Brain development continues into adolescence, specifically encompassing neuroproliferation, apoptosis, and synaptic rearrangement (Bayer, 1983; Bayer *et al*, 1982; Huttenlocher, 1990). More specifically, the maturation of central cholinergic systems involved in learning, memory, and psychostimulant responses, is consolidated during the periadolescent period (Matthews *et al*, 1974; Nadler *et al*, 1974; Zahalka *et al*, 1993). We recently developed a rat model of adolescent nicotine exposure that produces plasma nicotine levels comparable to those in regular smokers (25 ng/ml) (Slotkin, 2002). The salient features of this model include more profound and persistent upregulation of nicotinic acetylcholine receptors (nAChRs) as compared to adults, as well as prolonged suppression of cholinergic activity, and desensitization of nAChRs after nicotine withdrawal (Trauth *et al*, 1999, 2000a, 2001). These effects are accompanied by unique patterns of behavioral alterations that distinguish the effects of adolescent nicotine administration and withdrawal from those seen in adults (Levin, 1999; Trauth *et al*, 2000c); the lasting desensitization of cholinergic inputs to monoaminergic systems (Trauth *et al*, 2001) likely contributes to long-term loss of psychostimulant reward responses (Kelley and Middaugh, 1999), as well as persistent changes in EEG and locomotor activity that have been characterized as a uniquely adolescent 'nicotine abstinence syndrome' (Slawecki and Ehlers, 2002).

Recent findings indicate that the nearly immediate loss of autonomy over cigarette smoking in adolescents (DiFranza *et al*, 2000, 2002a,b) is paralleled in the rodent by rapid sensitization to nicotine's behavioral effects, centered around early adolescence (Adriani *et al*, 2002; Faraday *et al*, 2001). The current study was undertaken to identify the biological components that render the adolescent brain especially susceptible to nicotine, focusing on a specific set of questions. *First*, how long does the exposure need to be in order to elicit nAChR upregulation or to produce persistent reductions in cholinergic activity after nicotine withdrawal? *Second*, can these effects be elicited at plasma nicotine levels lower than those found in regular, active smokers, down to the exposures typical of occasional smoking or exposure to environmental tobacco smoke (ETS)? *Third*, does the pattern of exposure matter? Regular smokers tend to maintain a constant blood level of nicotine during the daytime, whereas occasional users and those subjected to workplace ETS show episodic exposures. This issue is particularly important in trying to understand biological substrates that might underlie the loss of autonomy in adolescent humans who smoke only a few cigarettes (DiFranza *et al*, 2000, 2002a,b). *Fourth*, what is the role of secondary effects of nicotine or tobacco smoke mediated through weight loss and anorexia, or through the episodic

hypoxic/ischemic burden associated with smoking (Bush *et al*, 2000; Slotkin, 1992)? *Fifth*, to what extent do the effects of adolescent nicotine treatment on cholinergic systems reflect underlying neurotoxicity?

To answer these questions, we used the adolescent rat model to examine the dose-response and time-response relations for effects of nicotine involving exposures of only 1 week's duration (and in one situation, as little as 2 days), with studies carried out during nicotine exposure and for up to 1 month after the initiation of withdrawal. To evaluate the role of different patterns of nicotine exposure (continuous vs intermittent), we contrasted two different routes of administration: infusions via implanted osmotic minipumps and twice-daily subcutaneous injections. Nicotine was administered at three different doses (0.6, 2, and 6 mg/kg/day) that, with the infusion model, elicit plasma levels ranging from as little as 2.5–25 ng/ml (Lichtensteiger *et al*, 1988; Trauth *et al*, 2000b). In light of the unique behavioral sensitization seen in early adolescence (Adriani *et al*, 2002), we concentrated on exposure beginning on postnatal day (PN) 30, which, on the basis of brain development, onset of puberty, and patterns of drug reactivity, represents the early adolescent stage in the rat (Spear, 2000). Three cholinergic biomarkers were considered. For evaluation of effects on nAChRs, we utilized [³H]cytisine, a ligand that binds selectively to the $\alpha 4\beta 2$ nAChR, the predominant subtype in mammalian brain; this receptor subtype is robustly upregulated by nicotine administration (Flores *et al*, 1992; Happe *et al*, 1994; Whiting and Lindstrom, 1987, 1988), and shows preferential upregulation in the adolescent (Trauth *et al*, 1999). We also assessed choline acetyltransferase (ChAT) activity and the binding of [³H]hemicholinium-3 (HC-3) to the high-affinity presynaptic choline transporter. ChAT, the enzyme responsible for acetylcholine biosynthesis, is a constitutive marker for cholinergic nerve terminals and serves as an archetypal measure of cholinergic innervation, but its activity does not respond to changes in impulse flow. Accordingly, ChAT increases during cholinergic synaptogenesis but does not change in response to stimuli that alter cholinergic neuronal activity (Aubert *et al*, 1996; Happe and Murrin, 1992; Navarro *et al*, 1989; Slotkin *et al*, 1990; Zahalka *et al*, 1992, 1993). In contrast, high-affinity choline uptake, as assessed with the binding of HC-3 to the presynaptic high-affinity choline transporter, is responsive to neuronal activity (Klemm and Kuhar, 1979; Simon *et al*, 1976), and the comparative changes in ChAT and HC-3 binding or transporter function permit distinction between effects on synaptic outgrowth as distinct from synaptic activity (Aubert *et al*, 1996; Happe and Murrin, 1992; Navarro *et al*, 1989; Slotkin *et al*, 1990; Zahalka *et al*, 1992, 1993). Both ChAT and HC-3 binding have been used previously to characterize the differences between adolescent and adult responses to nicotine (Slotkin, 2002; Trauth *et al*, 2000a).

MATERIALS AND METHODS

Animals

All the studies were carried out with the approval of the Duke University Institutional Animal Care and Use Committee, in accordance with the declaration of Helsinki

and the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. Sprague-Dawley rats were shipped by a climate-controlled truck (total transit time less than 12 h), housed individually, and allowed free access to food and water.

Nicotine Infusions

For minipump infusions, on PN30, each animal was anesthetized lightly with ether, a 3×4 cm area on the back was shaved and an incision made to permit s.c. insertion of osmotic minipumps (Alzet type 1007D). Pumps were prepared with nicotine bitartrate dissolved in bacteriostatic water, to deliver an initial dose rate of 0.6, 2, or 6 mg/kg of nicotine (calculated as free base) per day (Slotkin, 1998; Trauth *et al*, 1999, 2000b). The incision was closed with wound clips and the animals were permitted to recover in their home cages. The control animals were implanted with minipumps containing bacteriostatic water and sodium bitartrate equivalent to the highest nicotine bitartrate concentration. It should be noted that the pump, marketed as a 1-week infusion device, actually takes just under 8 days to be exhausted completely (information supplied by the manufacturer) and thus the nicotine infusion terminates during PN38. At the highest dose rate, this paradigm produces plasma nicotine levels similar to those in typical smokers, approximately 25 ng/ml (Trauth *et al*, 2000b). All the treatments were within the range over which the plasma nicotine level is linear with the dose rate (Lichtensteiger *et al*, 1988), so that at the intermediate and lower doses, concentrations were about 8 and 2.5 ng/ml, respectively. The lowest concentration is thus ten times below that found in regular smokers, and well within the range of those with exposure to ETS (Eliopoulos *et al*, 1996; Fried *et al*, 1995; Jauniaux *et al*, 1999; Kohler *et al*, 1999; Ostrea *et al*, 1994).

Studies were conducted at three time points, one toward the end of the nicotine administration period (PN37), and the other two at 1 and 4 weeks after the end of nicotine exposure (PN45 and PN65, respectively). Animals were decapitated and the cerebral cortex, hippocampus, and midbrain were dissected (Trauth *et al*, 1999, 2000a, b, 2001), frozen in liquid nitrogen, and stored at -45°C until assay. For some animals, we also removed the spleen for a separate study of lymphocyte mitogenic responses (Navarro *et al*, 2003).

Nicotine Injections

Experiments utilizing subcutaneous nicotine injections were designed to deliver the daily dose of nicotine in a bolus, thus providing a maximum pharmacokinetic contrast to the continuous infusion model. We administered nicotine for 7 days beginning on PN30, at the same total daily doses, but with the minimal number of daily injections. In preliminary experiments, we established that 3 mg/kg represented the maximum tolerated dose, thus requiring two injections each day, administered 12 h apart, so as to deliver the total dose of 6 mg/kg/day for the high-dose group; similarly, the lower dose groups received two daily injections of 1 or 0.3 mg/kg, for total doses of 2 and 0.6 mg/kg/day, respectively. Control animals received injections of bacteriostatic water containing sodium bitartrate in a concentration equivalent to that

of the highest dose of nicotine. For the first few injections, the animals receiving the lower doses of injected nicotine showed increased motor activity for about 5–10 min, followed by an equivalent period of depressed activity. The highest dose elicited rapid respiration and suppressed motor activity lasting 10–15 min, and some animals showed characteristics of systemic hypoxia, such as blanching of the skin and cyanosis (McFarland *et al*, 1991; Slotkin, 1992); of the 36 rats receiving high-dose nicotine injections, four animals (two males, two females) exhibited seizures and died. In the remainder, tolerance developed to all these effects by the second day. As the group receiving the highest dose of injected nicotine experienced systemic toxicity and corresponding weight loss, this group was also used to contrast the secondary toxic effects of nicotine with those mediated at lower doses of injected nicotine, or with nicotine infusions, both of which were devoid of overt systemic toxicity. Studies were conducted on PN37 (12 h after the last nicotine injection), PN45, and PN65, just as for the infusion regimens.

Tissue Preparation

Tissues were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in 20–40 volumes of ice-cold 50 mM Tris HCl (pH 7.4) and aliquots were withdrawn for measurements of ChAT activity (Lau *et al*, 1988) and total protein (Smith *et al*, 1985). To prepare the cell membrane fraction, the homogenates were sedimented at 40 000g 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 the same buffer using a smooth glass homogenizer fitted with a Teflon pestle. Aliquots of this resuspension were withdrawn for measurements of [^3H]cytisine and [^3H]HC-3 binding, and for membrane protein.

Assays

For ChAT activity, assays contained 30 μl of diluted homogenate (corresponding to 125 μg of original tissue weight) in a total volume of 60 μl with final concentrations of 60 mM sodium phosphate (pH 7.9), 200 mM NaCl, 20 mM choline chloride, 17 mM MgCl_2 , 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. Triplicate samples from each homogenate 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 acetylcholine was then extracted, counted and the activity determined relative to tissue protein. Preliminary determinations established that enzyme activity was linear with time and tissue concentration under these conditions.

[^3H]Cytisine binding to nAChRs was assessed in quadruplicate aliquots of the membrane suspension, corresponding to 5 mg of original wet weight of tissue (two aliquots for total binding and two for nonspecific binding). Each assay contained a final concentration of 1 nM [^3H]cytisine in a total volume of 250 μl of 120 mM NaCl, 5 mM KCl, 2.5 mM

CaCl₂, 1 mM MgCl₂, and 50 mM Tris HCl (pH 7.4). Incubations were conducted for 75 min at 4°C, after which 3 ml of ice-cold buffer were added and the membranes trapped by filtration on glass fiber filters. Labeled membranes were washed three times with 4 ml of buffer and the filters were counted by liquid scintillation spectrometry. Nonspecific binding was assessed by displacement with 20 μM nicotine, and was about 10% of the total binding in the cerebral cortex and midbrain, and about 20% in the hippocampus. Values were then calculated as specific binding per mg of membrane protein. The selection of a single concentration of radioligand for the receptor analysis enables the detection of changes in binding, but does not permit distinction of whether the effects are exerted on receptor affinity (K_d) or capacity (B_{max}). This strategy was necessitated by tissue and technical limitations implicit in the requirement to conduct over 500 assays simultaneously in each individual experiment, entailing every sample for a given age point, region, and route of administration, comprising preparations from 48 animals (six males and six females from each of four treatment groups): triplicate samples of ChAT, quadruplicates for [³H]cytisine binding (two each for total and nonspecific binding), and quadruplicates for [³H]HC-3 binding (two total, two nonspecific). The overall study thus involved nearly 10 000 assays. Nevertheless, it should be noted that, in earlier work, we demonstrated that the upregulation of nAChRs caused by adolescent nicotine represents an increase in the number of receptors and not a change in receptor affinity (Trauth *et al*, 1999).

For measurements of [³H]HC-3 binding, aliquots of the same membrane preparation, corresponding to 10 mg of original tissue weight, were incubated in 10 mM sodium-potassium phosphate buffer (pH 7.4) and 150 mM NaCl, for 10 min at 20°C, using a ligand concentration of 2 nM [³H]HC-3 (Vickroy *et al*, 1984). Membranes were trapped and washed as already described, except that the filters were presoaked for 30 min in a buffer containing 0.1% polyethyleneimine. The nonspecific component was defined as binding in the presence of an excess concentration (10 μM) of unlabeled HC-3 and again, specific binding was

expressed relative to membrane protein. Previous work has shown that developmental changes in HC-3 binding reflect almost exclusively a change in the total concentration of transporter sites (Zahalka *et al*, 1993); however, the interpretation of results of the present study, which relate to HC-3 binding as an index of synaptic activity (Cheney *et al*, 1989; Jope, 1979; Murrin, 1980; Navarro *et al*, 1989; Shelton *et al*, 1979; Simon *et al*, 1976; Zahalka *et al*, 1992, 1993), does not depend upon whether the change is specific to concentration or affinity.

Study Design and Data Analysis

The study was conducted in two separate cohorts of animals, one for the infusion regimen and one for the injection regimen, with each cohort consisting of 36 animals. Six males and six females were used for each of the four treatment groups and for each of the three age points; the only exception was the group receiving the highest dose of injected nicotine, where five animals for each sex were used at PN45 and PN65. Statistical comparisons of the effects of nicotine were conducted solely on the appropriately matched control group (ie infusion or injection).

Data are presented as means and standard errors. To facilitate comparison across the different measurements, the effects of nicotine were compiled as the percent change from control values, but statistical evaluations involved only the original data; for reference, control values for all variables (combined across the two cohorts) appear in Table 1. The effects of nicotine were first evaluated with a global analysis of variance (ANOVA) incorporating all the variables and measures in a single test, and utilizing log-transformation because of the heterogeneous variance among the different measures: treatment group, route of administration, brain region, age, sex, and the three neurochemical variables (nAChR binding, ChAT, and HC-3 binding). The three assays were treated as repeated measures, since each was obtained from the same tissue homogenate. Since the initial test (see Results) found that treatment effects were dependent upon the type of

Table 1 Control Values for Weights and Cholinergic Biomarkers

Measure	Region	PN37		PN45		PN65	
		Male	Female	Male	Female	Male	Female
Body weight (g)		189 ± 3	162 ± 5*	275 ± 7	225 ± 8*	431 ± 9	275 ± 8*
Region weight (mg)	Midbrain	266 ± 4	258 ± 2	284 ± 4	276 ± 4	295 ± 7	278 ± 5
	Cerebral cortex	842 ± 16	794 ± 14*	892 ± 22	836 ± 14*	902 ± 20	850 ± 14*
	Hippocampus	109 ± 2	104 ± 2*	117 ± 5	105 ± 2	118 ± 2	108 ± 3*
[³ H]Cytisine binding (fmol/mg protein)	Midbrain	89 ± 3	88 ± 4	85 ± 2	83 ± 2	75 ± 2	78 ± 2
	Cerebral cortex	63 ± 2	68 ± 3	64 ± 3	65 ± 3	56 ± 2	58 ± 2
	Hippocampus	42 ± 2	46 ± 3	39 ± 2	37 ± 3	36 ± 2	36 ± 1
ChAT (pmol/min/mg protein)	Midbrain	522 ± 23	537 ± 23	512 ± 12	522 ± 13	479 ± 10	531 ± 21*
	Cerebral cortex	595 ± 15	578 ± 8	546 ± 9	541 ± 11	549 ± 14	539 ± 8
	Hippocampus	565 ± 16	580 ± 16	536 ± 12	597 ± 11*	582 ± 19	669 ± 27*
[³ H]HC-3 binding (fmol/mg protein)	Midbrain	7.8 ± 0.6	8.5 ± 0.9	10.4 ± 0.6	10.4 ± 0.5	10.0 ± 0.6	9.5 ± 0.7
	Cerebral cortex	9.6 ± 0.5	9.9 ± 0.4	10.7 ± 0.7	11.2 ± 0.8	11.8 ± 0.5	13.1 ± 0.6
	Hippocampus	13.2 ± 0.9	15.4 ± 1.2	13.9 ± 1.1	14.9 ± 1.2	16.9 ± 0.6	17.6 ± 1.0

Values were combined across both cohorts of control groups (those receiving vehicle administration by infusion and by injection). However, statistical comparisons of the effects of nicotine were made only with the appropriately matched control cohort. The asterisks denote control values for which females differ significantly from males.

measurement (interactions of treatment \times measure, treatment \times measure \times other variables), the route of administration (interactions of treatment \times route, treatment \times route \times other variables), and the specific brain region in which the measurement was made (interactions of treatment \times region, treatment \times region \times other variables), the data were subdivided into the individual measurements, routes, and regions. Lower-order ANOVAs were then conducted, and where interactions of treatment with the remaining variables were still detected, comparisons for each individual values were carried out using Fisher's Protected Least Significant Difference. However, where treatment effects did not interact with other variables, only the main effect was interpreted. In cases where an interaction of treatment \times sex remained detectable in the lower-order tests, separate analyses were carried out for males and females; otherwise, values were compiled for both sexes combined.

Effects of nicotine on weight variables were conducted similarly. For body weight, we used a four-factor ANOVA (treatment, route, age, and sex), and five factors (additional factor of region) for brain region weight, both with log-transformed values.

Significance was assumed at the level of $p < 0.05$ for main effects. 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).

Materials

Animals were purchased from Zivic Laboratories (Pittsburgh, PA) and osmotic minipumps from Durect Corp. (Cupertino, CA). Bacteriostatic water was obtained from Abbott Laboratories (N. Chicago, IL). Radioisotopically labeled compounds came from Perkin-Elmer Life Sciences (Boston, MA): [^{14}C]acetyl-CoA (specific activity 44 mCi/mmol, diluted with unlabeled compound to 6.7 mCi/mmol), [^3H]HC-3 (specific activity 161 Ci/mmol), and [^3H]cytisine (specific activity, 32 Ci/mmol). Sigma Chemical Co. (St Louis, MO) was the source for all other reagents.

RESULTS

Effects on Body and Tissue Weights

ANOVA across both routes of nicotine administration indicated an overall effect on body weight only in the group receiving the highest dose ($p < 0.002$ for the main treatment effect; $p < 0.0001$ for control vs nicotine 6 mg/kg/day; other groups not significant vs control). The effect of nicotine was interactive with the route of administration ($p < 0.03$), so body weights were re-evaluated after separation of the groups into the infusion and injection paradigms (Figure 1). In keeping with an earlier study (Trauth *et al*, 1999), nicotine infusions had little or no effect on body weights (not statistically significant). However, for injected nicotine, significant differences remained detectable; again, the reductions were confined to the group receiving the highest dose, amounting to approximately a 5–10% deficit throughout the test period. The deficits arose very early in the treatment period, as examination of body weights on PN32,

after just 2 days of injections, indicated a significant ($p < 0.002$) deficit of the same magnitude (data not shown).

Nicotine-induced differences in body weight did not correlate with differences in brain region weights (Figure 1). Across all treatments, regions and ages, and both routes and sexes, we detected only an interaction of treatment \times age ($p < 0.04$) and a marginal interaction of treatment \times age \times sex ($p < 0.09$). These differences reflected inconsistent effects of small magnitude. After separation of the values by the more robust interactive term (age), we found significant, sex-dependent effects on PN37 (treatment \times sex, $p < 0.03$) and a main effect of nicotine on PN65 ($p < 0.006$). The effect on PN37 did not maintain statistical significance when the values were separated by sex. On PN65, the effect, a 4% increase over control values ($p < 0.0007$), was confined to the group receiving the lowest dose of nicotine (0.6 mg/kg/day).

Overall Statistical Analysis of Cholinergic Biomarkers

In order to reduce the likelihood of type 1 statistical errors, all the values obtained for the three neurochemical measures were first evaluated in a single multivariate ANOVA (Table 2), combining all contributing variables and all three measures. Across both routes of administration, we found significant interactions of treatment \times measure, treatment \times route of administration, and treatment \times measure \times brain region, indicating the need to separate the data according to the two different treatment regimens and the three individual brain regions. To ensure that interactions with measure and region were maintained for each route of administration, we repeated the global ANOVA for each route separately, and again found interactions of treatment \times measure and treatment \times measure \times region. Nicotine treatment was also interactive with age and sex, so after subdividing the data by route, region and measure, we retained these variables in the analysis to see if treatment interactions remained detectable. Thus, despite the absence of significant deficits in brain region weights, there were robust differences in cholinergic biomarkers.

Effects on [^3H]Cytisine Binding

Across all doses, ages, regions, both routes, and both sexes, adolescent nicotine treatment elicited a significant main effect ($p < 0.0001$) as well as interactions of treatment \times route of administration ($p < 0.07$), treatment \times age ($p < 0.0001$), treatment \times route \times age ($p < 0.002$), treatment \times route \times region ($p < 0.02$), treatment \times age \times sex ($p < 0.04$), treatment \times age \times region ($p < 0.1$), treatment \times sex \times region ($p < 0.002$), treatment \times route \times age \times region ($p < 0.006$), and treatment \times route \times sex \times region ($p < 0.0006$). Accordingly, the data were subdivided into separate regions and routes of administration for further analysis.

Adolescents given nicotine infusions showed upregulation of nAChRs in all the three brain regions (Figure 2, left-hand panels). On PN37, during the period of nicotine administration, there was significant induction even at the lowest dose of nicotine ($p < 0.0004$ across all regions), rising to a 30–40% increase at the highest dose ($p < 0.0001$ across all regions). In the midbrain, values declined to normal

within 1 week after termination of the infusion but there was a secondary increase seen on PN65, 1 month after the end of nicotine exposure; the lack of effect on PN45 was statistically distinguishable from the delayed induction on

PN65 (treatment \times age, $p < 0.05$). In contrast to the effects in the midbrain, nAChR binding in the cerebral cortex and hippocampus remained significantly elevated on PN45 and returned to normal by PN65.

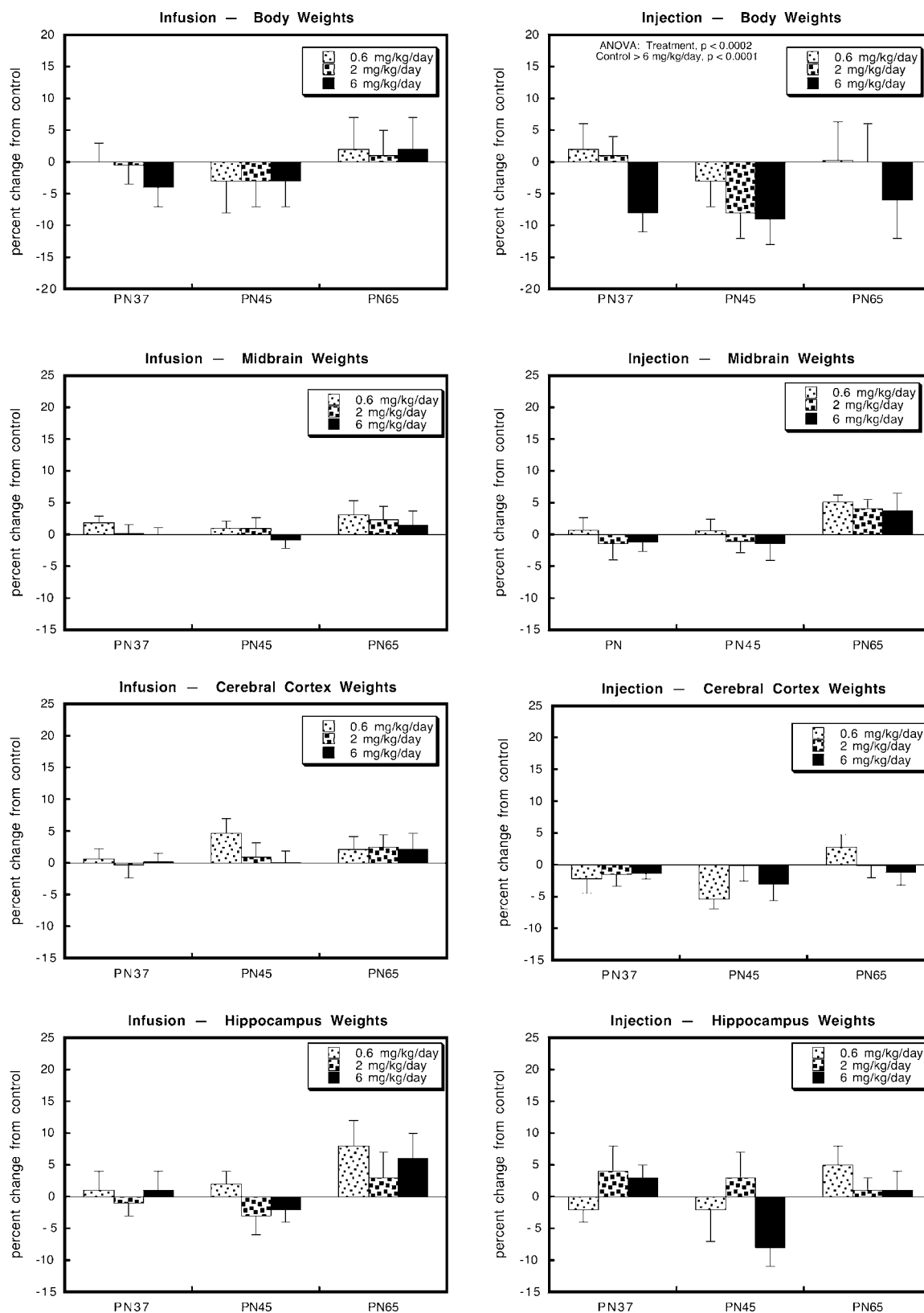


Table 2 Overall Analysis of Cholinergic Biomarkers

Effect	Both routes	Infusion	Injection
Treatment × measure	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Treatment × region	NS	NS	$p < 0.07$
Treatment × measure × region	$p < 0.006$	NS	$p < 0.0005$
Treatment × measure × route	$p < 0.02$	—	—
Treatment × measure × age	$p < 0.0001$	$p < 0.0004$	$p < 0.003$
Treatment × region × age	NS	$p < 0.06$	NS
Treatment × region × sex	$p < 0.08$	$p < 0.02$	NS
Treatment × measure × region × sex	$p < 0.02$	NS	$p < 0.05$
Treatment × measure × route × age	$p < 0.03$	—	—
Treatment × measure × age × sex	NS	$p < 0.04$	NS
Treatment × measure × region × sex	NS	$p < 0.03$	NS
Treatment × route × region × age	$p < 0.1$	—	—
Treatment × measure × region × route × age	$p < 0.06$	—	—
Treatment × measure × region × route × sex	$p < 0.06$	—	—
Treatment × measure × route × age × sex	$p < 0.04$	—	—

'Measure' refers to the three cholinergic neurochemical determinations (nAChR binding, ChAT, HC-3 binding). The main effects and interaction terms which showed no significant differences are not shown in the table. NS = not significant.

Nicotine administration via the injection route also elicited significant nAChR upregulation across the three brain regions at all doses: $p < 0.0003$ at 0.6 mg/kg/day and $p < 0.0001$ at the two higher doses (Figure 2, right-hand panels). However, the effects were generally smaller than those seen with nicotine infusions. On PN 37, just at the end of the treatment period, the highest dose of nicotine elicited only a 15–20% increase in nAChR binding, substantially less than that seen with the infusion route. In the midbrain, the two lower doses showed no significant increase with injected nicotine. By PN65, significant elevations remained detectable in the midbrain ($p < 0.008$), but not in the cerebral cortex. In the hippocampus, values declined nearly to normal by PN45, but re-emerged by PN65 ($p < 0.02$).

Although some of the effects of nicotine on nAChRs showed sex dependence, in general, the differences were relatively minor and inconsistent. When separate determinations were made in the midbrain of males and females receiving nicotine infusions, the upregulation was significant for both (males, $p < 0.0002$ for main effect, $p < 0.0001$ for treatment × age; females, $p < 0.0001$ for main effect, $p < 0.03$ for treatment × age). In fact, the treatment × sex interaction was confined to one age (PN45, treatment × sex, $p < 0.07$), representing an 11% bigger increase in females given 6 mg/kg/day, as compared to males. Similarly, in animals receiving nicotine via the injection route, effects in the midbrain were significant for both males ($p < 0.002$) and females ($p < 0.0001$) separately, with the females showing a greater overall increase in the group receiving 6 mg/kg/day (average of 20% increase across all three ages, as compared to 10% increase in males). The sex differences in the cerebral cortex of the nicotine injection groups were

significant overall, but were not individually significant when examined at each age point. In the hippocampus, sex differences were detected only for the nicotine infusion groups. Again, the effects were significant in both sexes, with only minor differences in the details of the time course of nAChR upregulation (not shown); on PN37, males had a bigger increase than females at 0.6 mg/kg/day, whereas on PN45, females had a bigger increase than males at 6 mg/kg/day.

In light of the surprisingly sensitive upregulation of nAChR binding at low doses of nicotine, we performed an additional experiment to determine both the rapidity with which receptor increases can first be detected, and the potential threshold for the effect. Using a separate cohort of animals, we administered nicotine or vehicle twice daily by subcutaneous injection for only 2 days beginning on PN30, and examined nAChR binding on PN32, 12 h after the last injection. We examined binding in the hippocampus, a region that showed significant upregulation after 1 week of injections of 0.6 mg/kg/day, only this time, we also examined the effects of an even lower dose, 0.2 mg/kg/day. In the control group (12 males, 12 females), nAChR binding was 35.5 ± 0.9 fmol/mg protein, compared to 33.7 ± 0.9 (six males, six females) in the group receiving 0.2 mg/kg/day, and 38.9 ± 1.5 (six males, six females) in the 0.6 mg/kg/day group. The treatment effects were significant overall ($p < 0.02$) and *post hoc* analysis indicated significant values in the group receiving 0.6 mg/kg/day group as compared to controls ($p < 0.04$) or to 0.2 mg/kg/day ($p < 0.006$). Upregulation thus occurred after as few as four injections (2 days of treatment), with a threshold of between 0.2 and 0.6 mg/kg/day.

Figure 1 Effects of adolescent nicotine administration on body and brain region weights, presented as the percent change from control values (see Table 1). Nicotine was administered for 1 week beginning on PN30, so that the PN37 age point in each graph represents values obtained during the nicotine exposure, whereas PN45 and PN65 represent 1 week and 1 month after the end of nicotine treatment. For body weights, ANOVA indicated an interaction of nicotine treatment × route of administration, necessitating separate, lower-order tests for each route (see text). There were no significant differences in body weight for the nicotine infusion groups, but those receiving the highest dose of nicotine via injections showed significant deficits (see ANOVA in the upper right panel); separate testing for each age was not carried out because of the absence of a treatment × age interaction. For brain region weights, separate testing for each route of administration was not carried out because of the absence of an interaction of treatment × route. The main effects of nicotine across both routes were found only on PN65, representing a small overall increase in the group receiving the lowest dose of nicotine (see text); significance was not maintained when values were separated by route of administration and brain region.

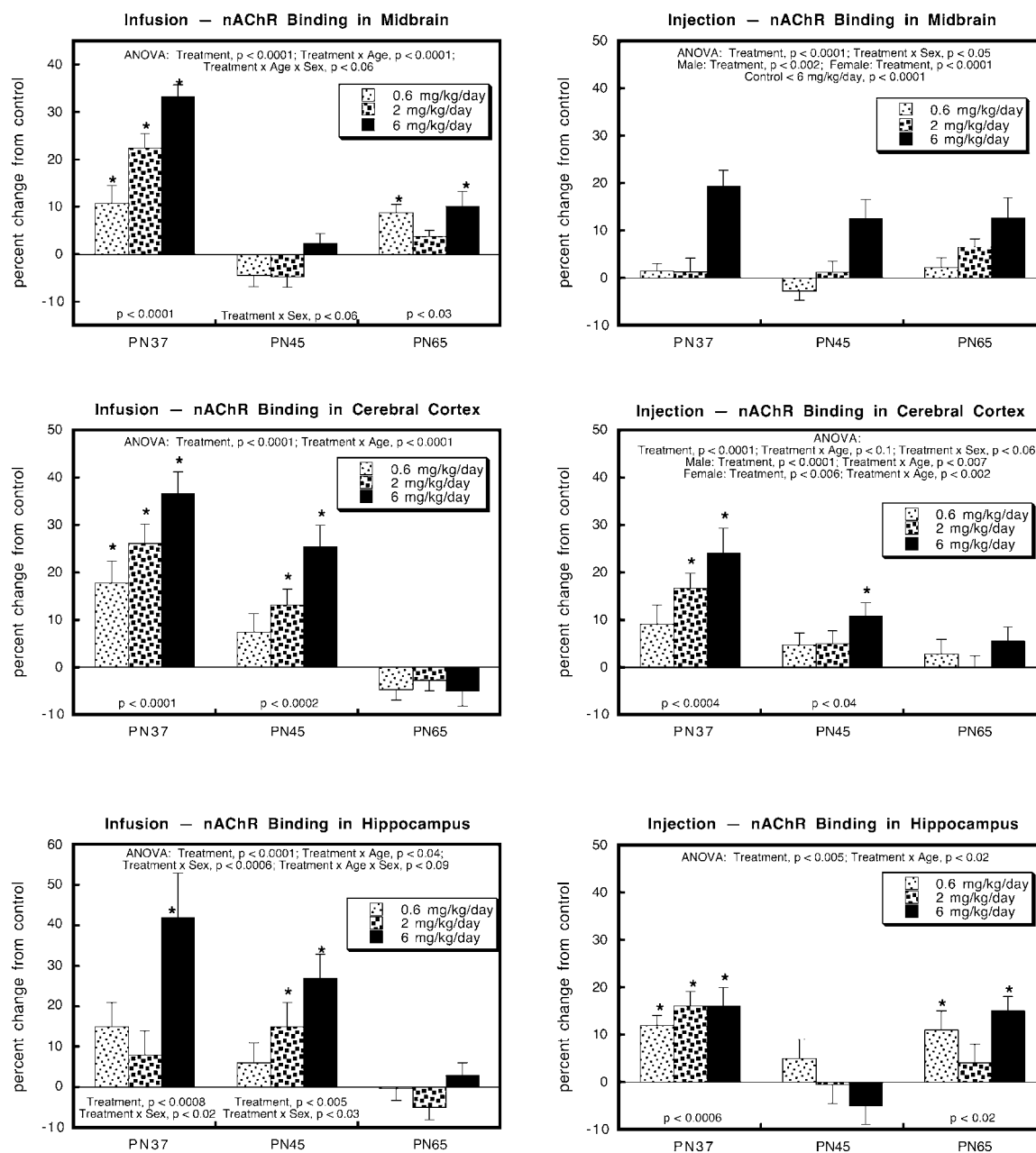


Figure 2 Effects of adolescent nicotine administration on nAChR binding, presented as the percent change from control values (see Table 1). Nicotine was administered for 1 week beginning on PN30, so that the PN37 age point in each graph represents values obtained during the nicotine exposure, whereas PN45 and PN65 represent 1 week and 1 month after the end of nicotine treatment. ANOVA for each route and region appears at the top of each panel. Where the initial test identified an interaction of treatment \times age, lower-order tests were conducted at each age, as shown at the bottom of each panel, and individual differences for each group were then assessed by Fisher's Protected Least Significant Difference (asterisks). In the absence of a treatment \times age interaction, only the main effect of nicotine was reported. Sex differences in the response to nicotine are described in the text.

Effects on ChAT Activity

Across all doses, ages, regions, both routes, and both sexes, adolescent nicotine treatment elicited a significant main effect ($p < 0.0002$; control vs nicotine 6 mg/kg/day, $p < 0.02$) as well as interactions of treatment \times region ($p < 0.0001$), treatment \times route \times age ($p < 0.08$), treatment \times age \times sex ($p < 0.02$), and treatment \times sex \times region ($p < 0.09$). Again, the data were subdivided into separate regions and routes of administration for further analysis.

Unlike the uniform increases seen for effects of adolescent nicotine on nAChRs, the effects on ChAT differed substantially among the three regions (Figure 3). In the midbrain, nicotine evoked a significant decrease in ChAT (main treatment effect, $p < 0.0001$) that was greater for the injection route than for the infusion route (treatment \times route interaction, $p < 0.04$). Considered separately, the infusion route had only a marginal effect overall, achieving significance only at the highest dose. In contrast, midbrain ChAT significantly decreased at all doses when

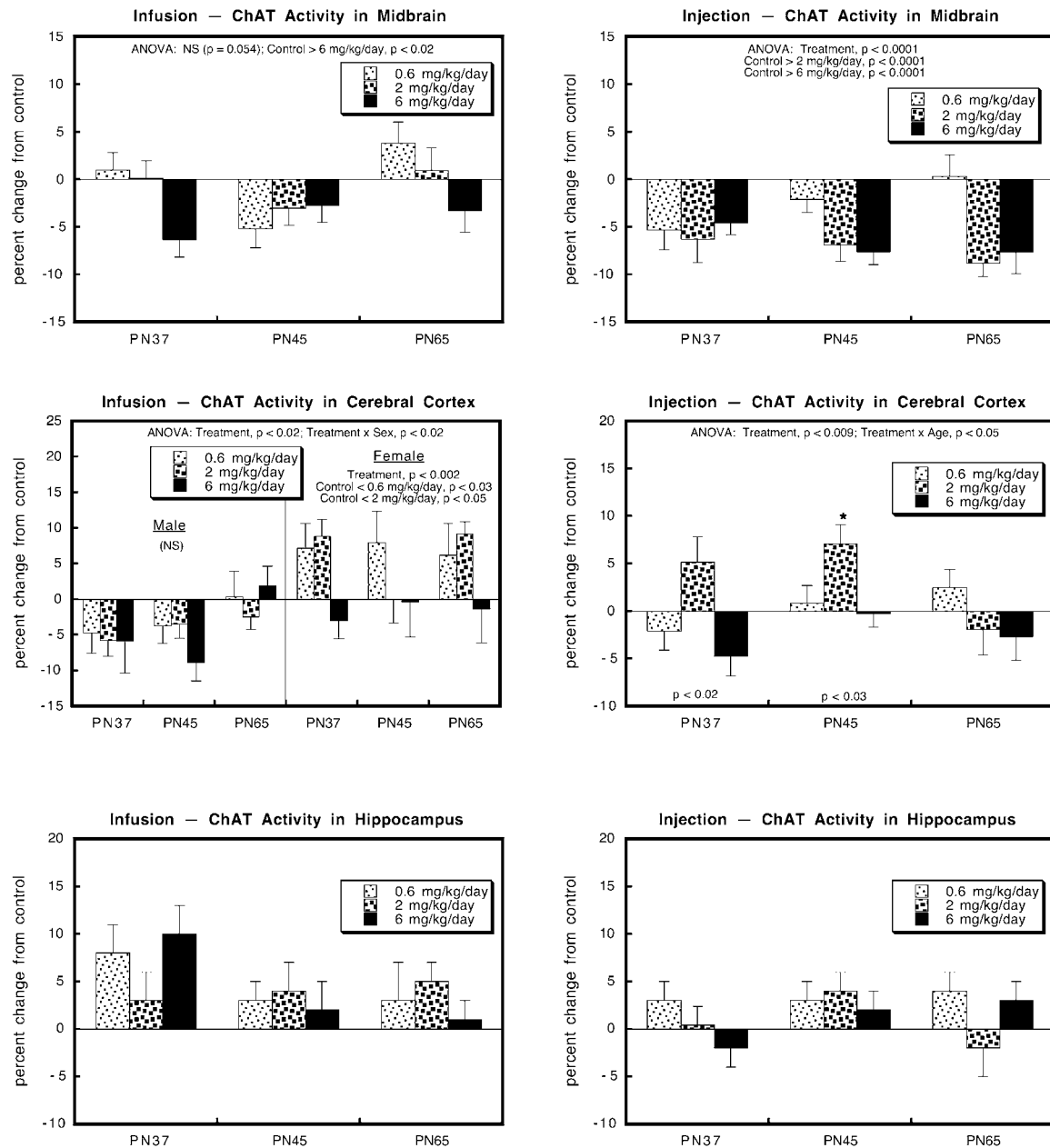


Figure 3 Effects of adolescent nicotine administration on ChAT, presented as the percent change from control values (see Table 1). Nicotine was administered for 1 week beginning on PN30, so that the PN37 age point in each graph represents values obtained during the nicotine exposure, whereas PN45 and PN65 represent 1 week and 1 month after the end of nicotine treatment. ANOVA for each route and region appears at the top of each panel. Where the initial test identified an interaction of treatment \times age, lower-order tests were conducted at each age, as shown at the bottom of each panel, and individual differences for each group were then assessed by Fisher's Protected Least Significant Difference (asterisks). In the absence of a treatment \times age interaction, only the main effect of nicotine was reported. Sex differences are shown only where there was a significant treatment \times sex interaction.

nicotine was given by injection, with the effect remaining prominent for the two higher doses even 1 month after discontinuing nicotine exposure. Although the cerebral cortex likewise showed a significant main effect of nicotine on ChAT ($p < 0.0003$), the response pattern was quite different, displaying distinct sex dependence (treatment \times sex, $p < 0.05$). For the infusion model, female rats showed hormesis, with promotion at the lower doses of nicotine, but a loss of the effect at 6 mg/kg/day. Males did not show any increase, and if anything, displayed lower ChAT activities. A biphasic effect on cerebrocortical ChAT was also apparent for the nicotine injection paradigm, with

significant increases at 2 mg/kg/day, but not 6 mg/kg/day. In the hippocampus, analysis across both routes of administration showed a significant overall increase in ChAT: main treatment effect, $p < 0.04$; $p < 0.004$ for 0.6 mg/kg/day; and marginally significant at 2 or 6 mg/kg/day ($0.1 > p > 0.05$) but the effect was too variable to maintain significance when either route was analyzed separately.

Effects on [^3H]HC-3 Binding

Multivariate ANOVA incorporating all the variables indicated a highly significant main treatment effect

($p < 0.0001$), but no interactions of treatment with other variables. Accordingly, lower-order tests of different routes, regions, ages, and sexes were not justified. Nicotine administration elicited a decrease in HC-3 binding that was significant at all doses (Figure 4): $p < 0.03$ for the groups receiving 0.6 mg/kg/day, $p < 0.0001$ for those receiving 2 mg/kg/day, and $p < 0.0001$ for the groups receiving 6 mg/kg/day. The effect also displayed a clearcut dose-effect relation, with significant differences among all the nicotine treatment groups ($p < 0.02$ comparing 0.6 to 2 mg/kg/day, $p < 0.0001$ comparing 0.6 to 6 mg/kg/day, $p < 0.002$ comparing 2 to 6 mg/kg/day); collapsed across all the regions, ages, and

both sexes, the effects at 2 mg/kg/day were twice those obtained at 0.6 mg/kg/day, and those seen at 6 mg/kg/day were three times those of the lowest dose. Importantly, deficits in HC-3 binding remained robustly detectable on PN65 ($p < 0.002$ for main treatment effect), 1 month after the termination of nicotine exposure.

DISCUSSION

Our earlier work with adolescent nicotine exposure indicated significantly greater and more persistent effects on nAChR upregulation and on indices of cholinergic

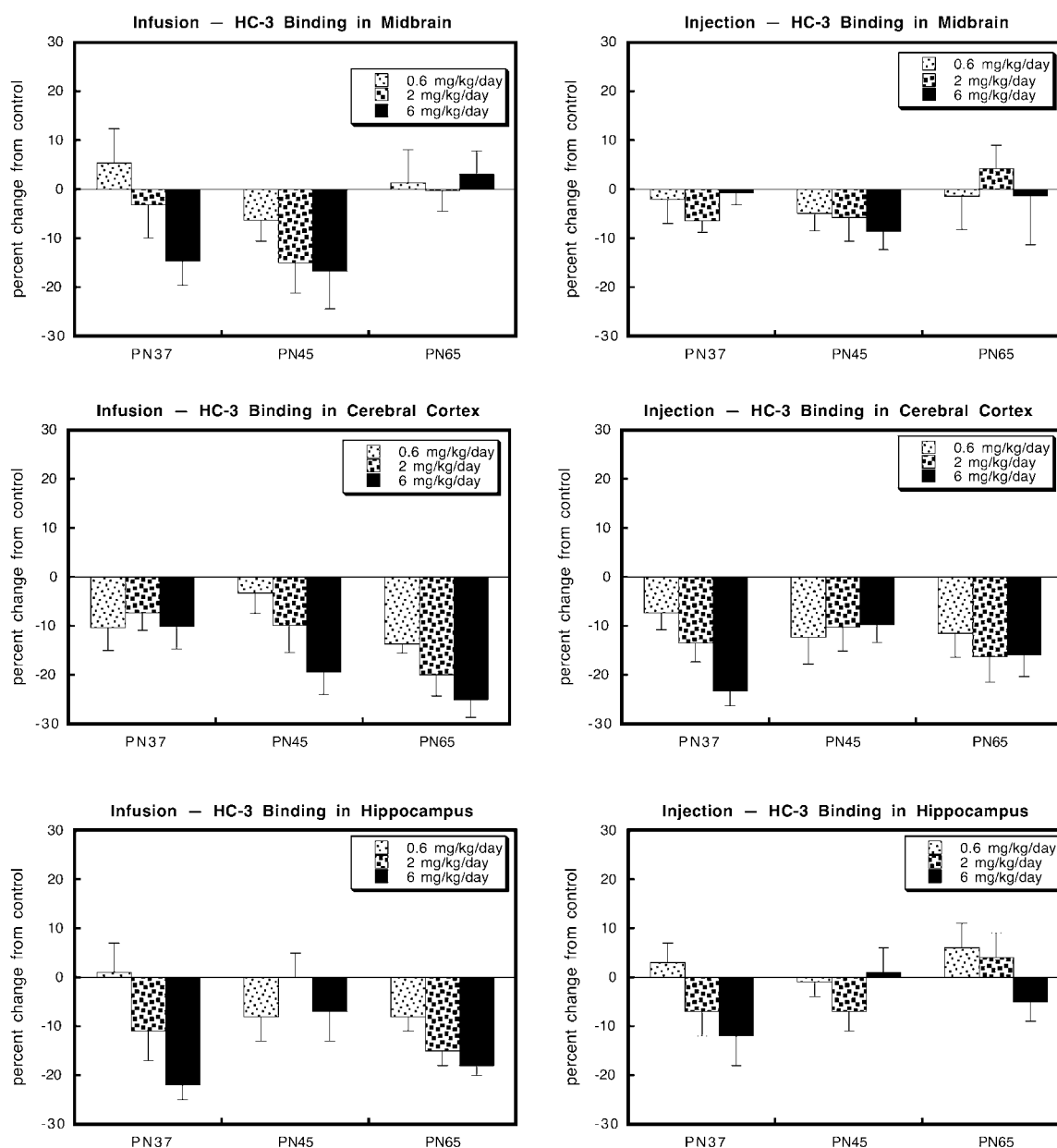


Figure 4 Effects of adolescent nicotine administration on [^3H]HC-3 binding, presented as the percent change from control values (see Table 1). Nicotine was administered for 1 week beginning on PN30, so that the PN37 age point in each graph represents values obtained during the nicotine exposure, whereas PN45 and PN65 represent 1 week and 1 month after the end of nicotine treatment. ANOVA was not determined for each route and region because the initial test indicated only a main treatment effect of nicotine, without interactions with other variables (see text). Effects of nicotine were significant overall ($p < 0.0001$) and were distinguishable at all doses: $p < 0.03$ at 0.6 mg/kg/day, and $p < 0.0001$ at the two higher doses.

activity than those found in adults (Happe and Murrin, 1992; Trauth *et al*, 1999, 2000a, 2001). Specifically, nAChR upregulation in the adolescent midbrain was twice that seen in adults and cerebrocortical values were equivalent, even when adults were exposed to nicotine at three times the plasma level; whereas persistent effects were seen 1 month post-treatment in adolescents, it was absent in adults. In the current study, we found that these changes can be elicited even with a short period of adolescent nicotine exposure (as little as 2 days to 2 week), and at a dose (0.6 mg/kg/day) that produces plasma nicotine levels only one-tenth that of typical smokers (Trauth *et al*, 2000b). Furthermore, we obtained evidence for persistent disruption of cholinergic function, well after the termination of nicotine treatment. Finally, continuous exposure to nicotine was not required for many of the changes: intermittent dosing via twice-daily nicotine injections was sufficient to produce nAChR upregulation and long-term alterations in biomarkers of cholinergic activity. In light of recent findings that adolescent smokers may develop signs of nicotine dependence within just a few days and with only a few cigarettes (DiFranza *et al*, 2000, 2002a,b), our findings provide evidence that these differences from the adult represent underlying biological differences in the adolescent brain. The ensuing paragraphs elaborate these essential conclusions in light of the specific questions fundamental to our experimental design.

How Long does Nicotine Exposure Need to be in Order to Elicit Alterations in Cholinergic Activity?

Previous studies from our group demonstrated that adolescent rats exposed to nicotine for 2 weeks showed robust, persistent nAChR upregulation, with a regional pattern distinct from that of the adult (Trauth *et al*, 1999). In addition, there were lasting reductions in HC-3 binding that were larger than corresponding decrements in ChAT, thus indicative of reduced cholinergic synaptic activity (Trauth *et al*, 2000a). In the current study, we obtained a similar pattern and magnitude of nAChR upregulation with only 1 week of nicotine exposure, and in an additional experiment, with as little as 2 days' treatment. As before, in addition to these immediate effects, there were persistent effects on nAChRs, as well as long-lasting suppression of HC-3 binding, for up to 1 month post-treatment. Reductions in HC-3 binding could conceivably involve either loss of cholinergic synaptic terminals, or reductions in neuronal impulse activity. However, if the terminals were reduced, then ChAT activity would have shown the same reduction as did HC-3 binding. The fact that there was preferential loss of the activity-related marker, HC-3 binding, indicates that the effects represent a long-term reduction in cholinergic tone. Our results thus indicate that even a brief period of nicotine exposure in early adolescence is sufficient to cause global changes to both nAChRs and cholinergic activity. Notably, the nAChR ligand we used, [³H]cytisine, binds to the $\alpha 4\beta 2$ receptor, the most widely distributed subtype in the brain (Buisson and Bertrand, 2002). Upregulation of $\alpha 4\beta 2$ nAChRs is thought to be initiated by receptor desensitization (Fenster *et al*, 1999) and both desensitization and upregulation are hypothesized to play a role in

addiction mechanisms (Buisson and Bertrand, 2001, 2002; Dani and De Biasi, 2001; Quick and Lester, 2002).

It is also notable that the pattern of persistent changes in biomarkers of cholinergic synaptic function seen here resemble those reported previously for prenatal nicotine exposure (Zahalka *et al*, 1992), which similarly evoked deficits in HC-3 binding independent of any comparable changes in ChAT. Our results indicate that the critical period in which nicotine can disrupt the programming of cholinergic function thus extends from fetal through adolescent stages. Furthermore, with the adolescent exposure paradigm, we found emergence of the deficits in HC-3 binding, hence reduced cholinergic synaptic activity, during the nicotine treatment period. Accordingly, the persistence of this effect 1 month post-treatment is not likely to reflect nicotine withdrawal, but rather a persistent effect of nicotine on the programming of cholinergic tone.

What is the Dose Threshold for these Effects?

Adolescent rats given infusions of 6 mg/kg/day of nicotine, display plasma nicotine levels comparable to those in typical smokers (25 ng/ml) (Trauth *et al*, 2000b). In order to determine whether similar effects could be elicited at much lower plasma nicotine concentrations, we reduced the dose to 2 or 0.6 mg/kg/day. Since the relation between dose and plasma levels is linear (Lichtensteiger *et al*, 1988), these regimens correspond to approximately 8 and 2.5 ng/ml, respectively; the lowest concentration produces a plasma level projected to be 10 times lower than that found in regular smokers. For nAChR upregulation, we found significant increases and the same persistence at all dose levels. Similarly, the long-lasting decrements in HC-3 binding remained detectable at all doses. Thus, the receptor changes that are thought to underlie nicotine dependence, as well as the persistent reductions in cholinergic synaptic activity, are both prominent features at exposure levels well below those of typical smokers. Indeed, the levels studied here encompass the so-called 'chippers,' who smoke up to five cigarettes per day and have nicotine plasma levels 20% those of regular smokers (Shiffman *et al*, 1990), as well as those with regular exposure to ETS, typically one-tenth those of active smokers (Eliopoulos *et al*, 1996; Eskenazi and Bergmann, 1995; Fried *et al*, 1995; Jauniaux *et al*, 1999; Kohler *et al*, 1999; Ostrea *et al*, 1994), but ranging up to levels indistinguishable from those in active, light smokers (Koren *et al*, 1998). Our findings thus raise the possibility that, in the adolescent brain, the biological mechanisms underlying nicotine dependence can be activated by nicotine exposures comparable to occasional smoking or even ETS exposure.

Does the Pattern of Exposure Matter?

Whereas our earlier work with the adolescent nicotine model concentrated on the effects of continuous, steady-state exposure to nicotine via continuous infusions (for a review see Slotkin, 2002), in the present work we compared nicotine infusions to the same daily doses delivered intermittently by twice-daily injections. This issue is particularly important in trying to understand the mechanisms that might underlie the onset of dependence in

adolescent humans who smoke only a few cigarettes (DiFranza *et al*, 2000, 2002a,b). Both treatment paradigms elicited nAChR upregulation across all three dose levels, although in general, the effect was greater with nicotine infusions. This difference was not unexpected, given the greater likelihood of nAChR desensitization with continuous exposure (Fenster *et al*, 1999), as intermittent exposure allows for some recovery in between doses. Nevertheless, significant nAChR upregulation was indeed found even with the lowest dose of injected nicotine, corresponding (after correction for interspecies differences) to a net consumption of as little as two cigarettes per day (Lichtensteiger *et al*, 1988; Trauth *et al*, 2000b); this fact, combined with the observation that the effects appear after as few as four injections (two days' exposure) reinforces the exquisite sensitivity of the adolescent brain to nicotine. Similarly, the long-term suppression of cholinergic synaptic activity, as indicated by reduced HC-3 binding, was fully evident with the injection paradigm. Again, our findings in adolescent rat brain thus provide evidence for biological mechanisms that are likely to underlie the emergence of nicotine dependence in adolescents within days after initiation of occasional tobacco use (DiFranza *et al*, 2000, 2002a,b).

What is the Role of Secondary Effects of Nicotine?

As detailed in the methods section, in contrast to rats that received nicotine infusions, rats that received the highest dose of injected nicotine exhibited clearcut signs of systemic toxicity, such as blanching of the skin and cyanosis (McFarland *et al*, 1991; Slotkin, 1992), as well as body weight deficits. Accordingly, the fact that nAChR upregulation and decrements in HC-3 binding were shared by both administration paradigms, involved doses below the threshold for systemic toxicity, and tended to be larger in animals receiving the infusions, all suggest that secondary toxic effects are not major contributors to these components of the effects of adolescent nicotine.

Is Nicotine a Neurotoxin in the Adolescent Brain?

The general conclusion that systemic toxicity of nicotine does not account for effects on the adolescent brain, leaves open the possibility of primary neurotoxicity of nicotine that may occur independent of systemic actions. Indeed, with fetal exposure, nicotine elicits specific, receptor-mediated effects on cell replication, differentiation, and synaptogenesis that contribute to its neurobehavioral teratogenicity (Levin and Slotkin, 1998; Slotkin, 1992, 1998, 1999), and at least some of these mechanisms persist into adolescence (Slotkin, 2002). Although direct evidence for adolescent brain cell injury caused by low-dose nicotine exposure is being pursued in a separate study (Abreu-Villaça *et al*, 2003), the current results for ChAT provide some evidence of neurotoxicant actions. ChAT is a constitutive cholinergic synaptic biomarker, so that its activity largely reflects the density of cholinergic innervation (Aubert *et al*, 1996; Happe and Murrin, 1992; Navarro *et al*, 1989; Slotkin *et al*, 1990; Zahalka *et al*, 1992, 1993). In our earlier work with a 2-week infusion of 6 mg/kg/day of

nicotine, we found small, but statistically significant decrements in ChAT (Trauth *et al*, 2000a) that corresponded to loss of neural cells (Trauth *et al*, 2000b). The current study again indicates that, in at least one region (midbrain), a 1-week regimen is sufficient to elicit the effect on ChAT. Notably, however, we obtained a more robust effect with nicotine injections, which produced deficits in ChAT even at the lowest dose. These differences between the two routes were not shared by the biomarker of cholinergic synaptic activity, HC-3 binding, which instead showed decrements across all brain regions and, if anything, greater midbrain effects for the infusion paradigm. Accordingly, the degree of synaptic damage may differ between the two administration paradigms; direct assessments of this possibility.

The pattern of nicotine-induced changes in ChAT was highly region-specific. Whereas decreases were seen in the midbrain, values in the cerebral cortex and hippocampus tended instead to be elevated. Even more intriguing, the promotional effect on ChAT in the latter two regions was not a monotonic function of dose, but rather displayed hormesis, with reversal of the effect at higher doses, a typical finding for other aspects of nicotine's biological effects (Furst, 1987). In fact, the biphasic effects on ChAT might be expected from the fact that the activation of nAChRs serves a trophic role in neurodevelopment (Coronas *et al*, 2000; Hohmann and Berger-Sweeney, 1998; Hohmann *et al*, 1988; Navarro *et al*, 1989; Pugh and Margiotta, 2000), while at the same time, excessive stimulation disrupts patterns of cell replication, differentiation, and synaptogenesis (Levin and Slotkin, 1998; Slotkin, 1992, 1998, 1999), progressing at high levels to outright cell damage (Abrous *et al*, 2002; Slotkin, 1992, 1998; Trauth *et al*, 2000b). The fact that neuronal cell replication continues into adolescence in late-developing regions may render these particular areas especially vulnerable to hormetic effects of nicotine (Altman and Bayer, 1990; McWilliams and Lynch, 1983; Zahalka *et al*, 1992, 1993). Alternatively, the elevations in ChAT may represent sprouting as a compensatory response to decrements in cholinergic synaptic function, although it would then be expected that the midbrain should show the same pattern. Obviously, the current results do not address the specific mechanism(s) for the hormetic response of ChAT to adolescent nicotine administration, and future studies will need to address the anatomical features that underlie the neurochemical changes, and perhaps more importantly, whether there is a parallel, biphasic effect on behavioral parameters.

Tobacco use by adolescents is a worldwide public health issue of widening severity, and the resultant rise in the number of life-long smokers will place an increasing burden on health care resources and on society in general. Nicotine dependence can be discerned in adolescents who smoke even occasionally (DiFranza *et al*, 2000, 2002a,b), so that this developmental period is a crucial component of the commencement of lifetime smoking. The present results indicate immediate and persistent alterations in nAChRs and cholinergic function in the adolescent rat brain even at nicotine doses one-tenth of those found in regular smokers, even with only intermittent dosing. Accordingly, our findings support the concept that the onset of nicotine dependence in the adolescent represents, in part, basic

differences in the biological responses to nicotine that are unique to this period of brain development.

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