

# Prenatal Nicotine Exposure Alters the Responses to Subsequent Nicotine Administration and Withdrawal in Adolescence: Serotonin Receptors and Cell Signaling

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Offspring of women who smoke during pregnancy are themselves more likely to take up smoking in adolescence, effects that are associated with a high rate of depression and increased sensitivity to withdrawal symptoms. To evaluate the biological basis for this relationship, we assessed effects on serotonin (5-hydroxytryptamine, 5HT) receptors and 5HT-mediated cellular responses in rats exposed to nicotine throughout prenatal development and then given nicotine in adolescence (postnatal days PN30-47.5), using regimens that reproduce plasma nicotine levels found in smokers. Evaluations were then made during the period of adolescent nicotine treatment and for up to one month after the end of treatment. Prenatal nicotine exposure, which elicits damage to 5HT projections in the cerebral cortex and striatum, produced sex-selective changes in the expression of 5HT<sub>1A</sub> and 5HT<sub>2</sub> receptors, along with induction of adenylyl cyclase (AC), leading to sensitization of heterologous inputs operating through this signaling pathway. Superimposed on these effects, the AC response to 5HT was shifted toward inhibition. By itself, adolescent nicotine administration, which damages the same pathways, produced similar effects on receptors and the 5HT-mediated response, but a smaller overall induction of AC. Animals exposed to prenatal nicotine showed a reduced response to nicotine administered in adolescence, results in keeping with earlier findings of persistent desensitization. Our results indicate that prenatal nicotine exposure alters parameters of 5HT synaptic communication lasting into adolescence and changes the response to nicotine administration and withdrawal in adolescence, actions which may contribute to a subpopulation especially vulnerable to nicotine dependence.

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## INTRODUCTION

Cigarette smoking typically begins in adolescence (Nelson *et al*, 1995; Pierce and Gilpin, 1996), and the earlier the onset, the greater the eventual daily consumption and the lower the probability of quitting (Chassin *et al*, 1996; Chen and Millar, 1998). Animal models of nicotine administration confirm that there is a biological basis for the greater susceptibility of adolescents to nicotine dependence and addiction, including greater and more persistent nicotinic acetylcholine receptor (nAChR) upregulation than in adults, outright neuronal loss in brain regions responsible for learning, memory and mood, and more pronounced alterations in synaptic activity upon withdrawal of nicotine (Abreu-Villaça *et al*, 2003a–c; Slotkin, 2002; Trauth *et al*,

1999, 2000a, 2001). These differences from nicotine effects in adults also carry over to behavioral outcomes (Adriani *et al*, 2002, 2003; Faraday *et al*, 2001, 2003; Levin, 1999; Trauth *et al*, 2000c).

Superimposed on the greater susceptibility of adolescents to nicotine dependence and addiction, there are subpopulations that are especially vulnerable, most notably those whose mothers smoked during pregnancy (Cornelius *et al*, 2000; Kandel *et al*, 1994; Niaura *et al*, 2001). Fetal nicotine exposure elicits neural cell death, alters cell proliferation and differentiation, and evokes lasting deficits in synaptic function and behavioral performance (reviews, Levin and Slotkin, 1998; Slotkin, 1992, 1998, 1999, 2004). Indeed, recent work has pointed toward persistent changes in the subsequent response to nicotine after prenatal nicotine exposure, findings that again point to biological mechanisms underlying differential susceptibilities in the adolescent. Rats given fetal nicotine exposures mimicking those in heavy maternal smoking display cholinergic hypoactivity that emerges at adolescence (Zahalka *et al*, 1992), accompanied by deficiencies of cholinergic responses mediated through nAChRs (Abreu-Villaça *et al*, 2004b; Seidler *et al*,

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1992) and further neuronal loss (Abreu-Villaça *et al*, 2004a); reinforcing the synaptic and neurochemical alterations, prenatal nicotine exposure also influences nicotine self-administration in adolescence (Klein *et al*, 2003).

The current study pursues the specific role of serotonin (5-hydroxytryptamine, 5HT) and some of its related cell signaling mechanisms in the interaction between prenatal nicotine exposure and the response to nicotine administration and withdrawal in the adolescent. Adolescent smokers have a high rate of depression (Goodman and Capitman, 2000; Martini *et al*, 2002; Patten *et al*, 2000; Wu and Anthony, 1999), and abnormalities of 5HT function are generally accepted to underlie the etiology and therapeutics of major depression (Maes and Meltzer, 1995). Depression is worsened by nicotine withdrawal, a major factor in failed attempts at smoking cessation (Colby *et al*, 2000; Salin-Pascual *et al*, 1995; Tsoh *et al*, 2000); again, this is a greater problem in adolescent smokers (Colby *et al*, 2000; Hurt *et al*, 2000). It is thus notable that nicotine administration to adolescent rats produces profound suppression of 5HT presynaptic activity upon cessation of nicotine administration, in conjunction with alterations in the ability of 5HT receptors to mediate cell signals in their target regions (Xu *et al*, 2001, 2002). Furthermore, prenatal nicotine exposure damages the same 5HT projections (Xu *et al*, 2001). Accordingly, in the present work, we carried out longitudinal evaluations of nicotine administration in adolescence and for up to 1 month after withdrawal of nicotine, comparing the effects in animals with or without prior exposure to prenatal nicotine. We assessed two 5HT receptor subtypes, 5HT<sub>1A</sub> and 5HT<sub>2</sub>, that are known to be affected in human depression (Arango *et al*, 2001; Fujita *et al*, 2000; Yatham *et al*, 1999, 2000). In addition, we evaluated cellular responses mediated through adenylyl cyclase (AC), a signaling pathway that plays critical roles in both dependence and withdrawal (Abreu-Villaça *et al*, 2003b; Nestler, 1992; Tzavara *et al*, 2002), and that we previously found to be affected by adolescent nicotine administration and abstinence (Abreu-Villaça *et al*, 2003b; Xu *et al*, 2002). Effects on AC responses can be mediated either through 'heterologous' mechanisms, where changes occur in the level or activity of AC itself, influencing the responses to all receptors that converge on cyclic AMP, or through 'homologous' mechanisms involving changes in response to a specific receptor. We assessed both alternatives. First, we measured basal AC and the response to two direct stimulants (forskolin, Mn<sup>2+</sup>) that bypass neurotransmitter receptors and that distinguish between AC isoforms (Slotkin *et al*, 2003; Zeiders *et al*, 1999). Second, we determined the AC response to 5HT input. The two 5HT receptor subtypes converge on AC through both stimulatory and inhibitory mechanisms (Barnes and Sharp, 1999; Duncan *et al*, 1999; Morin *et al*, 1992; Raymond *et al*, 1999; Rovescalli *et al*, 1993), so we evaluated the net balance of the AC response to 5HT itself, focusing on the shift from stimulatory to inhibitory responses noted previously for adolescent nicotine withdrawal (Xu *et al*, 2002). Determinations were made in brain regions comprising major 5HT terminal fields (cerebral cortex, striatum) as well as the brainstem, which contains the majority of 5HT cell bodies, and were contrasted with the cerebellum, a region sparse in cholinergic projections

and that should therefore respond to nicotine only indirectly, through multisynaptic pathways.

## 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 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 (GD) 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 GD4, 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; ether is the preferred anesthetic for these types of studies because isoflurane and other halogenated anesthetics can damage the developing brain (Jevtovic-Todorovic *et al*, 2003; Levin *et al*, 1991), whereas ether does not (Slotkin *et al*, 1987). 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 GD21. 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 GD22, which was also taken as postnatal day (PN)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; crossfostering, by itself, has no impact on neurochemical or behavioral effects of nicotine exposure (Ribary and Lichtensteiger, 1989). Animals were weaned on PN21.

On PN30, 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 PN47 (Slotkin, 1998; Trauth *et al*, 1999, 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 conducted at four time points, one during adolescent nicotine administration (PN45) and three during the post-treatment period (PN50, PN60, and PN75). Animals were decapitated and the cerebral cortex, striatum, brainstem, and cerebellum were dissected (Trauth *et al*, 2000b), frozen in liquid nitrogen, and stored at  $-45^{\circ}\text{C}$  until assayed. For each treatment group, 12 animals were examined at each age point, equally divided into males and females, with each litter of origin contributing no more than one male and one female to any of the adolescent treatment groups.

In a supplemental experiment to delineate the immediate effects of prenatal nicotine exposure on early development of 5HT<sub>1A</sub> receptors, pregnant rats received vehicle or nicotine as already described and fetuses were removed on GD18. Additional animals were allowed to deliver normally and pups taken on PN4. A cut was made rostral to the thalamus to separate the forebrain from the brainstem and the tissues were then frozen as already described. For the GD18 determinations, only one fetus was selected from each of 10 dams per treatment group and sex was not determined; for the PN4 study, one male and one female were selected from each of four litters per treatment group.

### Tissue Preparation and Assays

All of the assay methodologies used in this study have appeared in previous papers (Aldridge *et al*, 2003; Meyer *et al*, 2003, 2004a, b), so only brief descriptions will be provided here. Tissues were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY, USA) in ice-cold 50 mM Tris (pH 7.4), and the homogenates were sedimented at 40 000 *g* for 15 min. The pellets were washed by resuspension (Polytron) in homogenization buffer followed by resedimentation, and were then dispersed with a homogenizer (smooth glass fitted with Teflon pestle) in the same buffer. An aliquot was assayed for measurement of membrane protein (Smith *et al*, 1985).

Two radioligands were used to determine 5HT receptor binding (Xu *et al*, 2002): 1 nM [<sup>3</sup>H]8-hydroxy-2-(di-*n*-propylamino)tetralin (specific activity, 135 Ci/mmol) for 5HT<sub>1A</sub> receptors (Park *et al*, 1999; Stockmeier *et al*, 1998), and 0.4 nM [<sup>3</sup>H]ketanserin (specific activity, 63 Ci/mmol) for 5HT<sub>2</sub> receptors (Leysen *et al*, 1982; Park *et al*, 1999). For 5HT<sub>1A</sub> receptors, incubations lasted for 30 min at 25°C in a buffer consisting of 50 mM Tris (pH 8), 0.5 mM MgCl<sub>2</sub>, and 0.5 mM sodium ascorbate; 100 μM 5HT was used to displace specific binding. For 5HT<sub>2</sub> receptors, incubations lasted 15 min at 37°C in 50 mM Tris (pH 7.4) and specific binding was displaced with 10 μM methylsergide. Incubations were stopped by the addition of excess of ice-cold incubation buffer (without radioligand or displacing agent) and the labeled membranes were trapped by rapid vacuum filtration

onto glass fiber filters that were presoaked in 0.15% polyethyleneimine. The filters were then washed twice with 3 ml of incubation buffer and radiolabel was determined. Since the cerebellum is sparse in 5HT terminals but enriched in noradrenergic projections, we assessed β-adrenoceptor (βAR) binding instead of 5HT receptors, using 67 pM [<sup>125</sup>I]iodopindolol (specific activity, 2200 Ci/mmol) as the radioligand, with nonspecific binding assessed by displacement with 100 μM isoproterenol.

AC activity was determined in aliquots of the same homogenate used for radioligand binding. 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 adenosine 5'-triphosphate, 10 μM guanosine 5'-triphosphate, 2 mM MgCl<sub>2</sub>, 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. 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 cyclic AMP by radioimmunoassay. AC activity was evaluated under different conditions. To define basal and maximal activity, assays were conducted in the absence of any additions (basal AC) and in the presence of two direct AC stimulants that bypass the participation of neurotransmitter receptors: 100 μM forskolin or 10 mM Mn<sup>2+</sup>. As the two stimulants act at different epitopes on the AC molecule, the preference for one over the other reflects shifts in molecular conformation, primarily influenced by the AC isoform (Zeiders *et al*, 1999). Third, we probed the AC response to 5HT receptor-mediated activation by determining the ratio of activity with 5HT to that without 5HT, evaluated in samples stimulated with forskolin, an approach that enables detection of potential inhibitory or excitatory actions (Aldridge *et al*, 2003, 2004; Chow *et al*, 2000; Slotkin *et al*, 1999; Xu *et al*, 2002). The concentrations of all the agents used here have been found previously to be optimal for effects on AC (Aldridge *et al*, 2003; Meyer *et al*, 2003, 2004a, b).

### Data Analysis

To avoid type I statistical errors that might result from repeated testing of the global data set, we first performed a global analysis of variance (ANOVA) (with log-transformed data because of heterogeneous variance) incorporating all the variables and measures: prenatal treatment, adolescent treatment, brain region, age, sex, and the multiple determinations (repeated measures) made on each membrane preparation for the different classes of measurements (receptor binding, AC activities). Lower-order ANOVAs were then run according to the interactions between treatment and the other variables. Where appropriate, this was followed by *post hoc* evaluations of each treatment group compared with the controls, using Fisher's Protected Least Significant Difference; however, where treatment effects did not interact with other variables only the main effect was recorded without testing of individual differences. Significance was assumed at the level of  $p < 0.05$  for main effects; however, for interactions at  $p < 0.1$ , we also examined whether lower order main effects were detectable

after subdivision of the interactive variables (Snedecor and Cochran, 1967).

The study design required two different ways of regarding 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.

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

## Materials

Animals were purchased from Charles River Laboratories (Raleigh, NC) and osmotic minipumps from Durect Corp. (Cupertino, CA). Bacteriostatic water was obtained from

Abbott Laboratories (N Chicago, IL) and methylsergide from Sandoz Pharmaceuticals (E Hanover, NJ). PerkinElmer Life Sciences (Boston, MA) was the source for radioligands and cyclic AMP radioimmunoassay kits were purchased from GE Healthcare (Piscataway, NJ). Sigma Chemical Co. (St Louis, MO) was the source for all other reagents.

## RESULTS

Across the age points from PN45 through PN75, prenatal nicotine treatment did not elicit significant reductions in body weight, but as reported earlier (Trauth *et al*, 1999, 2000b), adolescent nicotine reduced weights <10% during treatment, with a rapid return to normal after nicotine was discontinued (not shown). The combination of prenatal + adolescent nicotine exposure elicited the same pattern of slight weight deficits and recovery as seen with adolescent treatment alone. None of the treatments evoked significant deficits in brain region weights, in accord with previous results (Abreu-Villaça *et al*, 2004a,b; Trauth *et al*, 1999, 2000b). In control animals, there were only sporadic sex differences in any of the neurochemical measures; in fact, no more than would be expected at random from the overall number of comparisons (Table 1). Nevertheless, as described below, nicotine treatment elicited sex-selective effects on many of the parameters.

**Table 1** Control Values

Measure	Region	PN45		PN50		PN60		PN75	
		Male	Female	Male	Female	Male	Female	Male	Female
5HT <sub>1A</sub> receptors (fmol/mg protein)	Cerebral cortex	65±5	81±5 <sup>a</sup>	52±2	53±2	54±2	57±2	70±5	72±5
	Striatum	12±1	14±1	18±1	16±1	15±1	17±2	18±1	17±1
	Brainstem	19±1	20±1	22±1	21±1	31±1	33±2	40±2	41±1
5HT <sub>2</sub> receptors (fmol/mg protein)	Cerebral cortex	131±4	141±4	119±3	134±4 <sup>a</sup>	110±2	111±4	95±3	98±2
	Striatum	67±3	65±3	62±2	66±2	53±2	54±3	53±2	61±1 <sup>a</sup>
	Brainstem	18±1	18±1	16±1	17±1	14±1	15±1	14±1	14±1
Basal AC (pmol/min/mg protein)	Cerebral cortex	215±14	229±11	230±15	198±7	211±20	203±18	173±8	175±10
	Striatum	147±7	155±8	166±10	160±5	125±7	111±5	119±7	120±8
	Brainstem	188±5	172±9	123±8	110±4	134±5	123±5	94±3	97±3
	Cerebellum	179±9	164±18	182±16	179±18	177±11	172±11	182±7	183±7
Forskolin-stimulated AC (pmol/min/mg protein)	Cerebral cortex	1248±105	1256±103	1105±75	931±53	1097±137	1100±116	844±112	929±85
	Striatum	2895±321	3036±114	3408±221	3785±276	2716±178	2496±162	2326±254	2743±248
	Brainstem	390±24	360±12	318±18	301±14	428±20	370±26	303±5	301±17
	Cerebellum	685±60	657±76	591±57	584±44	708±44	681±49	679±38	668±44
Mn <sup>2+</sup> -stimulated AC (pmol/min/mg protein)	Cerebral cortex	2232±209	2258±199	1682±170	1567±166	1754±187	1662±155	1411±147	1597±151
	Striatum	2575±249	2831±192	3838±365	4195±346	2950±123	2777±185	2975±285	3036±204
	Brainstem	994±32	1009±54	798±33	808±12	711±52	648±55	758±36	818±31
	Cerebellum	2470±287	2232±249	2773±226	2639±225	2678±200	2539±224	2542±170	2522±186

<sup>a</sup>Values for which females differ significantly from males.

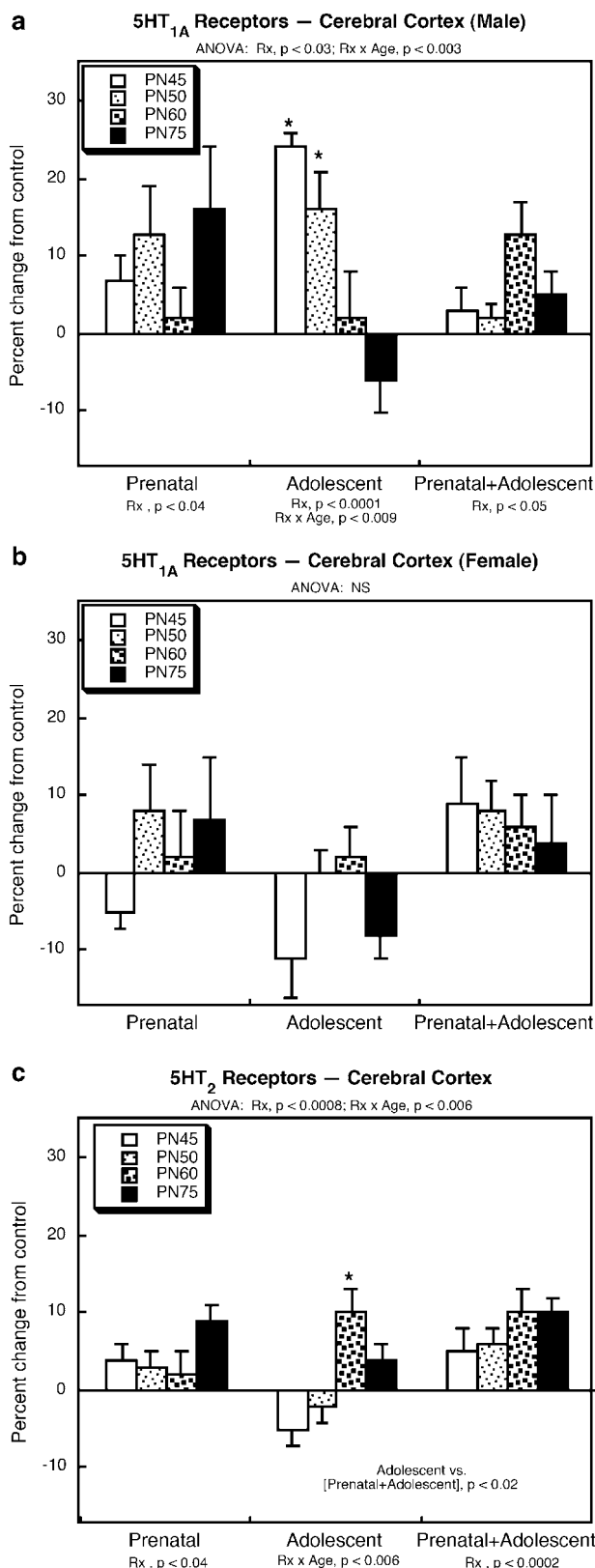
## Receptor Binding

For 5HT receptor binding, global (ANOVA) across all contributing variables indicated a main effect of treatment ( $p < 0.03$ ) and interactions of treatment  $\times$  sex ( $p < 0.02$ ), treatment  $\times$  sex  $\times$  region ( $p < 0.03$ ), treatment  $\times$  age  $\times$  region ( $p < 0.07$ ), treatment  $\times$  sex  $\times$  receptor subtype ( $p < 0.02$ ), treatment  $\times$  age  $\times$  subtype ( $p < 0.0001$ ), treatment  $\times$  region  $\times$  subtype ( $p < 0.02$ ), treatment  $\times$  sex  $\times$  age  $\times$  subtype ( $p < 0.0001$ ), treatment  $\times$  age  $\times$  region  $\times$  subtype ( $p < 0.004$ ), and treatment  $\times$  sex  $\times$  region  $\times$  subtype ( $p < 0.02$ ). Since the receptor subtype interacted with all the other variables, we then performed separate analyses for 5HT<sub>1A</sub> and 5HT<sub>2</sub> receptors. Both subtypes showed interactions of treatment with sex, age, and region: for 5HT<sub>1A</sub> receptors,  $p < 0.006$  for treatment  $\times$  sex,  $p < 0.05$  for treatment  $\times$  age,  $p < 0.02$  for treatment  $\times$  sex  $\times$  region, and  $p < 0.06$  for treatment  $\times$  age  $\times$  region; for 5HT<sub>2</sub> receptors,  $p < 0.004$  for the main effect of treatment,  $p < 0.0001$  for treatment  $\times$  age,  $p < 0.04$  for treatment  $\times$  sex  $\times$  age,  $p < 0.003$  for treatment  $\times$  sex  $\times$  region,  $p < 0.0007$  for treatment  $\times$  age  $\times$  region, and  $p < 0.07$  for treatment  $\times$  sex  $\times$  age  $\times$  region. Since the treatment effects differed among regions (interactions of other variables  $\times$  region), the results for each region were examined separately.

In the cerebral cortex, 5HT<sub>1A</sub> receptors showed significant treatment effects that depended upon age (treatment  $\times$  age,  $p < 0.002$ ) and sex (treatment  $\times$  sex,  $p < 0.05$ ; treatment  $\times$  sex  $\times$  age,  $p < 0.08$ ), necessitating separate consideration of values in males and females. In males, prenatal nicotine exposure elicited a significant overall elevation in 5HT<sub>1A</sub> receptor binding (Figure 1a). When nicotine was given instead during adolescence, 5HT<sub>1A</sub> receptor binding was elevated during the treatment and returned to normal in the post-treatment period. Animals exposed to nicotine prenatally and then given nicotine in adolescence displayed a significant overall elevation in receptor binding akin to that evoked by the prenatal exposure alone. In fact, animals receiving nicotine prenatally showed a blunted 5HT<sub>1A</sub> upregulation in response to adolescent nicotine, as evidenced by a significant interaction of prenatal  $\times$  adolescent nicotine ( $p < 0.009$ ), regarding the treatments as two separate dimensions; the animals receiving the combined treatment did not show the augmented 5HT<sub>1A</sub> receptor binding found in the animals given adolescent treatment alone. In females, there were no overall effects of any of the nicotine treatments on cerebrocortical 5HT<sub>1A</sub> receptor binding (Figure 1b).

**Figure 1** Effects of prenatal and adolescent nicotine treatment on 5HT receptor binding in the cerebral cortex: (a) 5HT<sub>1A</sub> receptors in males; (b) 5HT<sub>1A</sub> receptors in females; (c) 5HT<sub>2</sub> receptors combined for males and females because of the absence of a treatment  $\times$  sex interaction (see text). Values are presented as the percent change from control (Table 1). ANOVA across all treatments and ages appears at the top of each panel and comparisons of each group to the control appears at the bottom; differences between nicotine administration groups are shown within the panel. Where there was a treatment  $\times$  age interaction, asterisks denote individual ages at which the nicotine-treated animals show significant differences from the corresponding control values but in the absence of the interaction, only main treatment effects are indicated. Rx=treatment; NS=not significant.

For cerebrocortical 5HT<sub>2</sub> receptors, there was only a main treatment effect and an interaction of treatment  $\times$  age, so values for males and females were combined for presentation (Figure 1c). Just as for 5HT<sub>1A</sub> receptors, prenatal nicotine exposure evoked a small but statistically significant

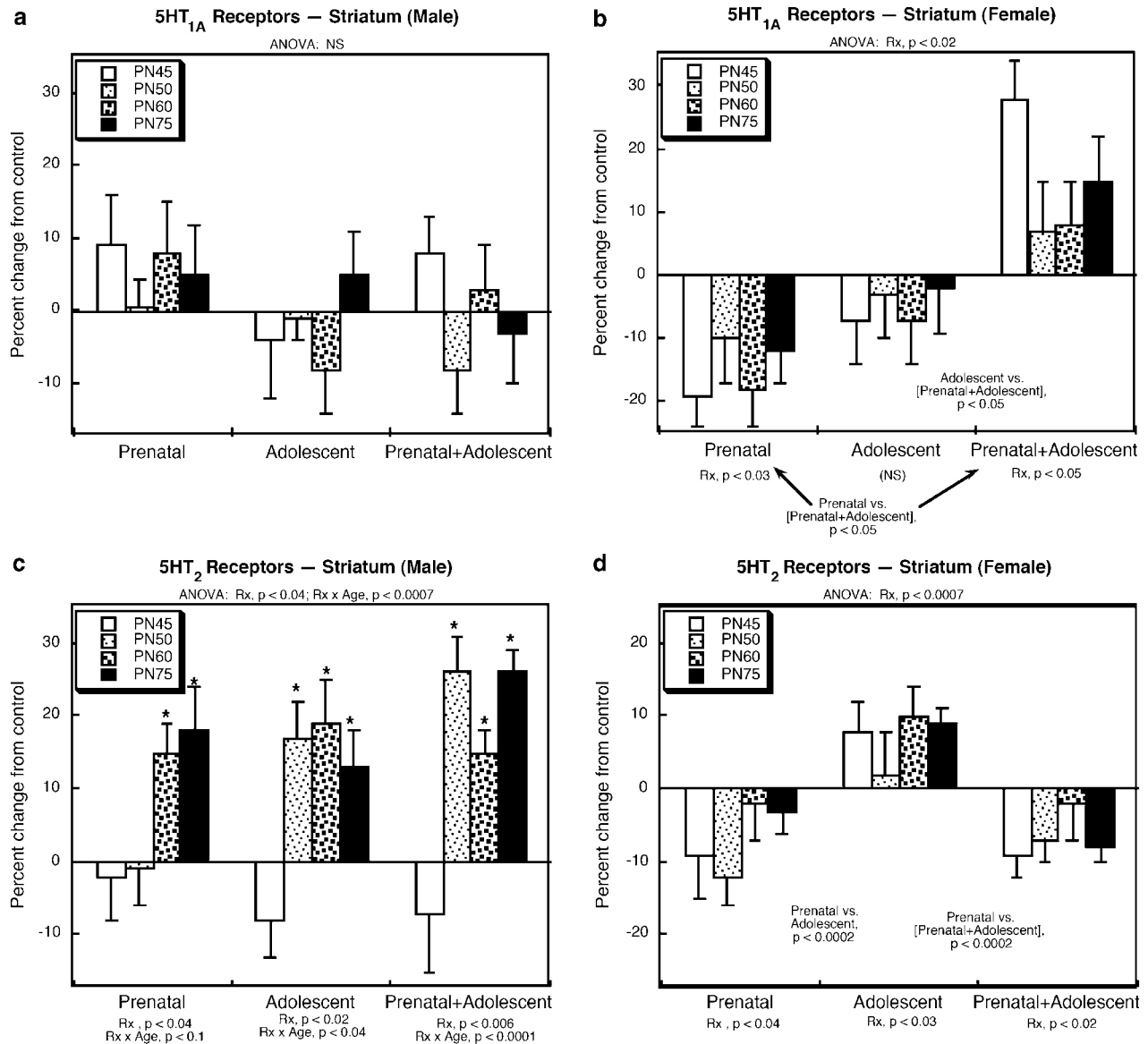


overall elevation of 5HT<sub>2</sub> receptor binding. The response to adolescent nicotine administration was far more modest than that seen for the other receptor subtype, with a significant elevation obtained only upon withdrawal of nicotine (PN60). Again, animals exposed to nicotine prenatally and then given nicotine in adolescence showed essentially the same receptor effects as those exposed just prenatally.

In the striatum, both 5HT<sub>1A</sub> and 5HT<sub>2</sub> receptors showed sex-dependent effects (treatment × sex interaction,  $p < 0.007$  and  $p < 0.004$  respectively), so values for males and females were separated for both subtypes. For 5HT<sub>1A</sub> receptors, males did not show any significant treatment-related changes (Figure 2a). Females exposed to nicotine prenatally showed significant overall reductions in 5HT<sub>1A</sub> receptor

binding but the effects of adolescent nicotine exposure were less notable and nonsignificant (Figure 2b). Females given fetal nicotine exposure showed a completely different response to adolescent nicotine administration, characterized by robust increases in 5HT<sub>1A</sub> receptors, an effect that was not produced by either prenatal or adolescent treatment alone (prenatal × adolescent nicotine,  $p < 0.007$ ). Regarding prenatal and adolescent nicotine treatments as separate dimensions in ANOVA, there was a significant interaction of the two treatments ( $p < 0.007$ ), confirming the fact that the group with combined exposure was statistically distinguishable from either of the two groups or from the summation of individual effects.

For striatal 5HT<sub>2</sub> receptors, the nicotine treatments elicited significant changes for both sexes, but again with



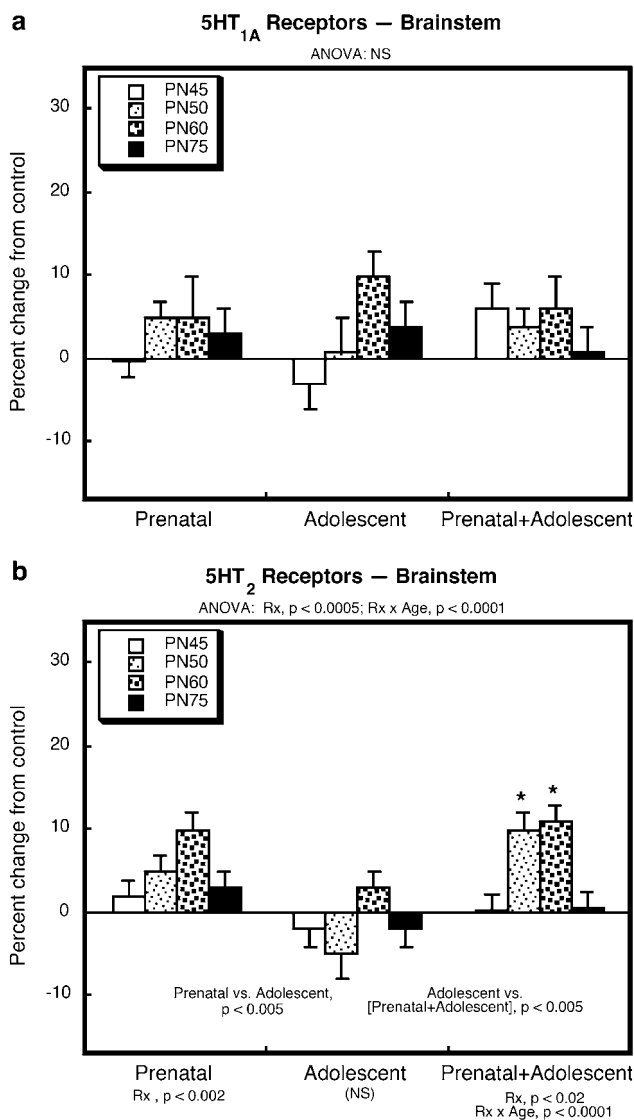
**Figure 2** Effects of prenatal and adolescent nicotine treatment on 5HT receptor binding in the striatum: (a) 5HT<sub>1A</sub> receptors in males; (b) 5HT<sub>1A</sub> receptors in females; (c) 5HT<sub>2</sub> receptors in males; (d) 5HT<sub>2</sub> receptors in females. Values are presented as the percent change from control (Table 1). ANOVA across all treatments and ages appears at the top of each panel and comparisons of each group to the control appears at the bottom; differences between nicotine administration groups are shown either within or below the panel as indicated. Where there was a treatment × age interaction, asterisks denote individual ages at which the nicotine-treated animals show significant differences from the corresponding control values but in the absence of the interaction, only main treatment effects are indicated. Rx = treatment; NS = not significant.

a different pattern in males vs females. In males, prenatal nicotine exposure evoked an increase in receptor binding with an onset corresponding to the eighth postnatal week (Figure 2c). Adolescent nicotine administration had a similar effect, with increases appearing only after the termination of nicotine treatment but persisting into adulthood. In this case, males exposed to nicotine prenatally and then given nicotine in adolescence showed an increase in 5HT<sub>2</sub> receptors that corresponded to the summation of the two individual effects, that is, no interaction of prenatal × adolescent nicotine, regarding the two treatments as separate dimensions in ANOVA. Females exposed to prenatal nicotine displayed an overall decrease in striatal 5HT<sub>2</sub> receptors, whereas adolescent nicotine treatment produced an increase, just as had been seen for males, albeit to a smaller degree (Figure 2d). When females were exposed to nicotine prenatally, the striatal 5HT<sub>2</sub> receptor response to adolescent nicotine was desensitized: the effects of the combined treatment were indistinguishable from that of prenatal nicotine alone, so that the adolescent nicotine treatment had essentially no effect in this group.

In the brainstem, 5HT<sub>1A</sub> receptors did not display significant effects of treatment (Figure 3a). For the 5HT<sub>2</sub> subtype, there were significant effects that did not depend on sex (no interaction of treatment × sex or of treatment × sex × age), so values for males and females were combined for presentation (Figure 3b). As was found in the cerebral cortex, 5HT<sub>2</sub> receptors in the brainstem showed overall upregulation in the animals exposed to prenatal nicotine. However, adolescent treatment had little or no effect and the combination of prenatal nicotine followed by adolescent treatment displayed essentially the same pattern as that achieved with fetal exposure alone.

We did not conduct measurements of 5HT receptors in the cerebellum because of the sparsity of 5HT innervation in that region. Instead, we assessed βAR binding, a corresponding measure for norepinephrine, the monoamine most prevalent in the cerebellum. Across all treatments, we found a main effect of nicotine ( $p < 0.02$ ) without interactions for age and sex, so for concision, the values are given collapsed across the four age points and combined for males and females: control,  $32.3 \pm 0.2$  fmol/mg protein; prenatal nicotine,  $31.1 \pm 0.2$  ( $p < 0.05$  vs control); adolescent nicotine,  $31.9 \pm 0.3$ ; prenatal + adolescent nicotine,  $30.3 \pm 0.2$  ( $p < 0.004$  vs control,  $p < 0.02$  vs adolescent nicotine). Thus, prenatal treatment decreased cerebellar βAR expression by a small but statistically significant extent, adolescent nicotine had little or no effect, and the combined treatment displayed effects akin to those of prenatal treatment alone.

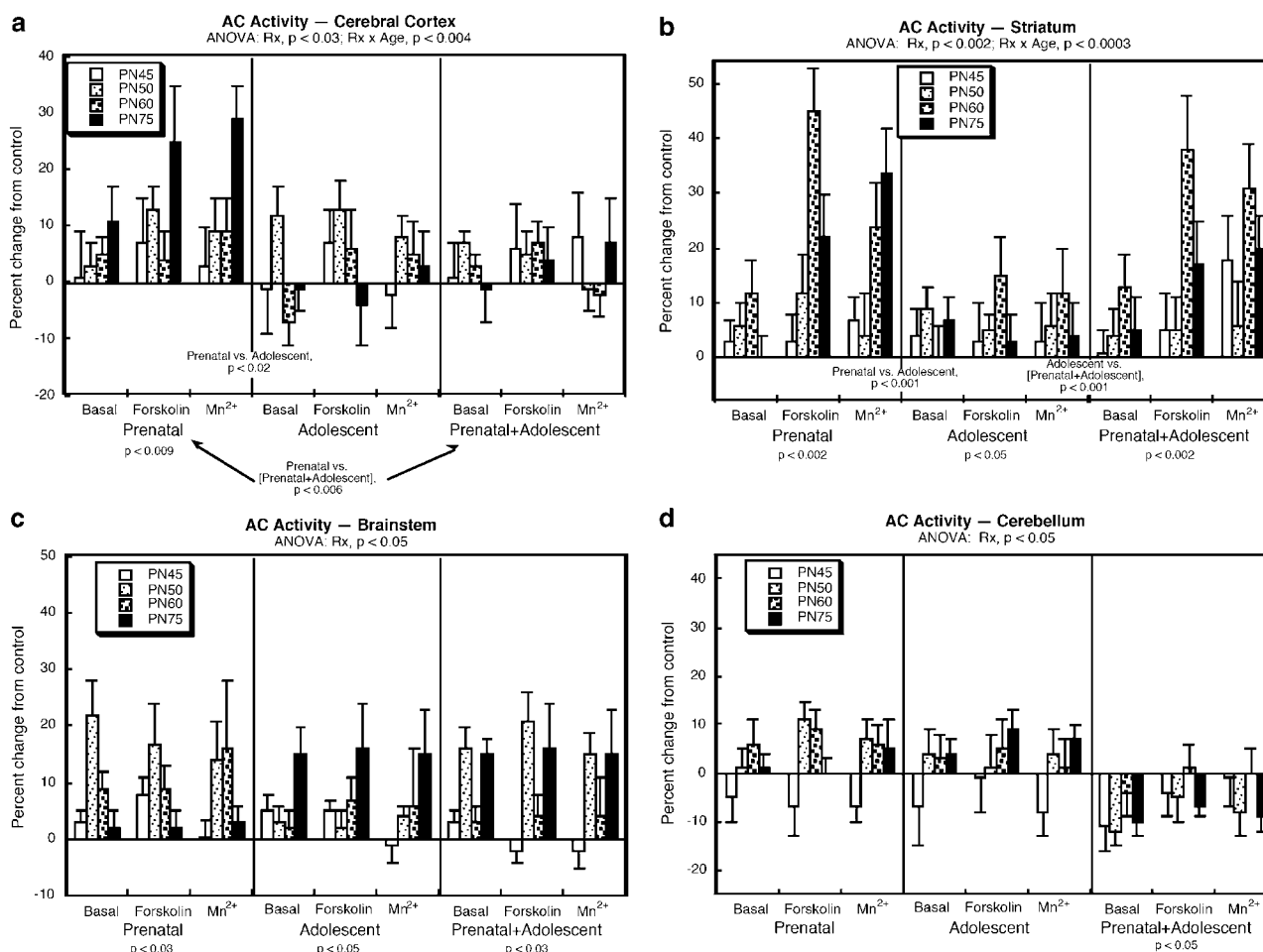
Effects on 5HT<sub>1A</sub> receptor expression early in life can influence the subsequent concentration of receptors in adulthood (Kusserow *et al*, 2004). Accordingly, we evaluated whether prenatal nicotine treatment affects 5HT<sub>1A</sub> receptors during the period in which the fetal brain is exposed to nicotine (GD18) or in the immediate postnatal period (PN4). We found an overall, significant increase in 5HT<sub>1A</sub> receptors in the forebrain ( $p < 0.04$  for the main treatment effect), without interactions with age or sex: GD18, control  $6.5 \pm 0.5$  fmol/mg protein, nicotine  $8.4 \pm 0.7$ ; PN4, control  $34 \pm 1$ , nicotine  $37 \pm 1$ .



**Figure 3** Effects of prenatal and adolescent nicotine treatment on 5HT receptor binding in the brainstem, shown combined for males and females because of the absence of a treatment × sex interaction (see text): (a) 5HT<sub>1A</sub> receptors; (b) 5HT<sub>2</sub> receptors. Values are presented as the percent change from control (Table 1). ANOVA across all treatments and ages appears at the top of each panel and comparisons of each group to the control appears at the bottom; differences between nicotine administration groups are shown within the panel. Where there was a treatment × age interaction, asterisks denote individual ages at which the nicotine-treated animals show significant differences from the corresponding control values but in the absence of the interaction, only main treatment effects are indicated. Rx = treatment; NS = not significant.

### Cell Signaling

Across all treatments, brain regions, AC measures (basal, forskolin-stimulated, and Mn<sup>2+</sup>-stimulated activity) and both sexes, ANOVA identified a main effect of treatment ( $p < 0.006$ ) that depended upon age, region, and measure, but not sex ( $p < 0.03$  for treatment × age,  $p < 0.02$  for treatment × region,  $p < 0.03$  for treatment × age × region,  $p < 0.0001$  for treatment × measure). Accordingly, results were examined separately for the various regions, but with values for males and females combined. In the cerebral cortex, prenatal nicotine exposure elicited a significant



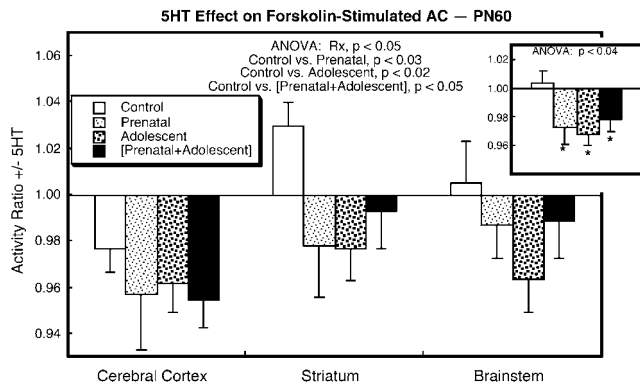
**Figure 4** Effects of prenatal and adolescent nicotine treatment on basal AC activity and the response to the direct AC stimulants, forskolin and  $Mn^{2+}$ , shown combined for males and females because of the absence of a treatment  $\times$  sex interaction (see text): (a) cerebral cortex; (b) striatum; (c) brainstem; (d) cerebellum. Values are presented as the percent change from control (Table 1). ANOVA across all treatments and ages, and all three AC measures, appears at the top of each panel and comparisons of each group to the control appears at the bottom; differences between nicotine administration groups are shown within or below the panel, as indicated. Separate statistical analyses for individual measures or ages was not undertaken because of the absence of treatment interactions with these two variables (see text), so that only main treatment effects are indicated. Rx = treatment.

overall increase in AC activities, whereas the effects of adolescent nicotine were smaller and did not achieve statistical significance (Figure 4a). However, when animals given prenatal nicotine were treated again with nicotine in adolescence, the net effect on AC activity was actually lower than the values achieved with the prenatal treatment alone; that is, in these animals, adolescent nicotine treatment actually reduced AC activity relative to the values achieved with just the prenatal treatment. Although none of the individual treatment paradigms showed a significant treatment  $\times$  age interaction, the overall data set did, reflecting a trend toward increased effect with advancing age.

For striatal AC, prenatal nicotine exposure produced a robust overall increase, again to a much greater extent than that evoked by adolescent treatment, both of which achieved statistical significance (Figure 4b). In this region, the combination of prenatal nicotine exposure followed by nicotine treatment in adolescence produced effects that were indistinguishable from those of prenatal nicotine alone. Again, there was an overall increase in effect with age (significant treatment  $\times$  age interaction). In the brainstem,

prenatal nicotine, adolescent nicotine, or the combination of both treatments all produced significant overall elevations of AC activity (Figure 4c). In contrast, the only effect seen in the cerebellum was a small, but statistically significant decrease in the animals receiving the combined prenatal and adolescent treatments (Figure 4d).

Besides the basic measurements of AC activity, we also evaluated whether any of the nicotine treatment paradigms altered the response of forskolin relative to  $Mn^{2+}$ ; there were no significant differences in the response ratio (not shown). Since earlier work demonstrated a shift in the AC response to 5HT receptor stimulation evoked by withdrawal from nicotine in adolescence (Xu *et al*, 2002), we next assessed this effect in animals exposed to nicotine prenatally, given nicotine in adolescence, or receiving the combination (Figure 5). In control animals, 5HT produced a net decrease in AC in the cerebral cortex and stimulation in the striatum, with a fairly neutral balance in the brainstem. As noted earlier, the magnitude of the 5HT-mediated AC responses reflects the small proportion of total AC activity specifically linked to 5HT receptors, as well as the fact that the different subtypes are linked both to stimulation and



**Figure 5** Effects of prenatal and adolescent nicotine treatment on the AC response to 5HT on PN60, assessed as the ratio of forskolin-stimulated activity with addition of 5HT to forskolin-stimulated activity without 5HT. ANOVA across all regions appears at the top of the panel and the inset shows the net effect collapsed across all three regions because of the absence of a treatment  $\times$  region interaction; in the inset, asterisks denote individual treatments that differ from the corresponding control.

inhibition (Aldridge *et al*, 2005; Xu *et al*, 2002). In each region, prenatal nicotine, adolescent nicotine, or the combined treatment altered the AC response to 5HT by increasing the inhibitory component. Collapsed across all three regions (because of the lack of treatment  $\times$  region interactions), the net effect of 5HT was relatively neutral in controls but inhibitory in each of the three different nicotine groups, all of which displayed approximately the same degree of effect (Figure 5 inset). Again, then, the prenatal treatment interfered with the ability of adolescent nicotine to cause a further effect.

## DISCUSSION

### Prenatal Nicotine Exposure

In earlier work, we found that prenatal nicotine exposure elicits persistent loss of 5HT projections and reactive sprouting at the cell bodies (Xu *et al*, 2001). Accordingly, the increase seen here for 5HT receptor binding in brain regions containing 5HT terminals likely represents compensatory upregulation consequent to the reduction in 5HT input. Indeed the magnitude of the changes in receptor expression approximates the degree of synaptic loss noted earlier (Xu *et al*, 2001). In keeping with this interpretation, the brainstem, which contains the majority of 5HT cell bodies, showed less effect, with no significant increases in 5HT<sub>1A</sub> receptors and only a small increase in the 5HT<sub>2</sub> subtype. Our findings further indicate that the changes in 5HT receptors originate very early in development, during and immediately after the period of prenatal nicotine exposure. Earlier work with animals genetically modified to over- or underproduce 5HT<sub>1A</sub> receptors showed that the perinatal period is a critical phase for programming future receptor expression and function (Kusserow *et al*, 2004), so that the ability of prenatal nicotine exposure to cause early receptor upregulation itself is likely to contribute additionally to the lasting effects on 5HT receptors in adolescence and adulthood. There were notable sex differences in the receptor effects, which will be discussed

later. Superimposed on the changes in 5HT receptor expression, prenatal nicotine exposure also altered 5HT-mediated AC signaling, enhancing the inhibitory component of the response. Again, this result is in keeping with conclusions reached from genetic models, where perinatal 5HT<sub>1A</sub> receptor overexpression was found to reduce 5HT-related anxiety in adulthood (Kusserow *et al*, 2004).

It is clear from our results that prenatal nicotine alters 5HT cellular responses not just through effects on homologous mechanisms but rather involves global, heterologous changes in AC itself. In fact, the largest changes in cell signaling involved the response to direct AC stimulants, forskolin and Mn<sup>2+</sup>, that bypass the requirement for receptor activation; there was no preferential shift in the response to forskolin vs Mn<sup>2+</sup>, so that it is most likely that the increase in AC catalytic activity represents overexpression of AC itself (Zeiders *et al*, 1999). Unlike the receptor effects, the increases in overall AC activity were similar in the cerebral cortex and brainstem, and only slightly larger in the striatum, and did not show the sex differences noted for receptor upregulation. Accordingly, these differences imply that the heterologous effects have a different originating mechanism from those targeted specifically toward 5HT systems. Indeed, the general effects of prenatal nicotine exposure on cellular development, differentiation, and synaptogenesis reflect actions mediated through nAChRs in the fetal brain, rather than affecting only 5HT projections (Slotkin, 1998, 2004). The smaller effects seen in the cerebellum are thus a likely consequence of the sparsity of cholinergic projections and receptors in that region. In any case, the heterologous effects on AC are of key importance in the neurobehavioral teratology of nicotine, given the convergence of multiple neurotransmitter and hormonal inputs on this signaling pathway. Future work is thus needed to examine AC responses and the corresponding behavioral changes to a variety of neurotransmitters in the prenatal nicotine model.

### Nicotine Administration in Adolescence

By itself, nicotine treatment in adolescence also elicited significant changes in 5HT receptor expression, 5HT-mediated cellular responses, and heterologous aspects of AC signaling, comparable to those reported earlier (Xu *et al*, 2002). In general, these were similar in direction to those noted for prenatal nicotine exposure, but frequently smaller in magnitude, in keeping with the lower vulnerability of the adolescent brain to nicotine-induced neurotoxicity and the closure of developmental events delineating synaptic organization and the programming of synaptic activity (Slotkin, 2002). As these have been discussed in detail in earlier reports (Abreu-Villaça *et al*, 2003b; Slotkin, 2002; Xu *et al*, 2002), only a few salient features will be noted here. With adolescent nicotine treatment, just as with prenatal nicotine exposure, we found upregulation of cerebrocortical 5HT<sub>1A</sub> and 5HT<sub>2</sub> receptors, except that there was a clear relationship to the periods of administration and discontinuation of nicotine in the adolescent model: the 5HT<sub>1A</sub> subtype was increased by nicotine treatment and returned to normal in the post-treatment period, whereas the 5HT<sub>2</sub> subtype was increased only after nicotine was discontinued. In the striatum, only the 5HT<sub>2</sub> subtype showed significant

adolescent nicotine-induced alterations, and again, this was primarily related to the period after discontinuing nicotine treatment. It is therefore highly likely that the receptor alterations with adolescent nicotine, unlike those after prenatal nicotine exposure, represent short-term responses to nicotine treatment and withdrawal, rather than effects on long-term programming of receptor expression. In accord with this interpretation, the primary outcome for 5HT-mediated cell signaling is also restricted to the post-treatment period and is transient (Xu *et al*, 2002). This was reproduced in the current study: as with prenatal nicotine, the net effect of adolescent nicotine on the AC response to 5HT was promotion of inhibitory responses during withdrawal on PN60. In additional studies, we found that the effect of discontinuing nicotine treatment was much more profound for the response to 5HT than for catecholaminergic receptors linked to AC, implying that there is a preferential effect of adolescent nicotine withdrawal on 5HT function (Abreu-Villaça *et al*, 2003b; Xu *et al*, 2002). Again, behavioral consequences of this alteration in 5HT signaling would be interesting to explore, especially given the specific relationship of 5HT to mood disorders (Maes and Meltzer, 1995) and the prevalence of depression in adolescent smokers (Goodman and Capitman, 2000; Patten *et al*, 2000; Wu and Anthony, 1999).

### Effects of Prenatal Nicotine Exposure on the Response to Nicotine in Adolescence

In earlier work, we found that prenatal nicotine exposure produces lasting deficiencies in cholinergic neurotransmission, including decreased presynaptic activity and lasting nAChR desensitization (Abreu-Villaça *et al*, 2004b; Seidler *et al*, 1992; Slotkin, 1998, 2002, 2004). As a result, nicotine administered in adolescence then produces a reduced net response and a smaller degree of nAChR upregulation (Abreu-Villaça *et al*, 2004b; Seidler *et al*, 1992). The current results indicate a similar loss of responsiveness for the effects of nicotine on 5HT systems. Thus, for many of the indices, animals exposed to prenatal nicotine did not show any further change upon nicotine administration in adolescence. The best examples are provided by the measurements of cerebrocortical 5HT<sub>1A</sub> and 5HT<sub>2</sub> receptor binding, striatal 5HT<sub>2</sub> receptors in females, AC activity in the striatum and brainstem, and the shift of the 5HT-mediated AC response toward greater inhibition; in each of these cases, animals given prenatal and adolescent nicotine displayed the same response as that achieved with prenatal nicotine alone, indicating essentially no response to adolescent nicotine. In contrast, the effects on striatal 5HT<sub>2</sub> receptor binding in males were additive, indicating that prenatal nicotine did not change the response to adolescent nicotine for this parameter. However, the most striking interactions were seen for cerebrocortical AC activity and for striatal 5HT<sub>1A</sub> receptors in females. Prenatal nicotine evoked a persistent elevation of AC in the cerebral cortex, and by itself, adolescent nicotine was only slightly (nonsignificantly) stimulatory. However, when animals with prenatal nicotine exposure were given nicotine in adolescence, the adolescent treatment actually reduced the effect of prenatal nicotine, lowering the net change to nearly zero; this implies that although adolescent nicotine

administration has a slight stimulatory effect by itself, when it is administered to animals exposed earlier to prenatal nicotine, it actually inhibits the AC response, neutralizing the long-term effect of the fetal treatment. For striatal 5HT<sub>1A</sub> receptors, prenatal or adolescent nicotine alone tended to reduce expression in females, but the combined treatment produced significant elevations, so that once again the response to adolescent nicotine was drastically altered by prenatal nicotine. For this latter unique effect, there may be a significant contribution from nicotine-induced neuronal damage. Nicotine selectively upregulates striatal  $\alpha 7$ nAChRs in adolescent rats (Slotkin *et al*, 2004), the subtype associated with plasticity and responses to damage (Broide and Leslie, 1999; Shimohama *et al*, 1998). Both smoking and nicotine release large concentrations of dopamine in the striatum (Brody *et al*, 2004), leading to oxidative damage (Qiao *et al*, 2005), as is characteristic of this neurotransmitter (Olanow and Arendash, 1994). Accordingly, there may be cumulative damage after prenatal and adolescent nicotine that contributes to atypical effects on striatal 5HT systems. If this is so, then antioxidant strategies may be able to prevent or offset the effects, a possible avenue for future work.

For virtually all the parameters, then, prenatal nicotine exposure elicits profound alterations in the response of 5HT systems and AC signaling to adolescent nicotine treatment, most of which involve lasting desensitization but some of which involve unique responses that are not typically seen with prenatal or adolescent nicotine alone. In fact, these patterns may not be specific to nicotine effects on 5HT systems, since we found similar desensitization for cholinergic systems (Abreu-Villaça *et al*, 2004b; Seidler *et al*, 1992). Indeed, given the prominent role of cholinergic input in regulating 5HT synaptic activity (Cucchiari and Commons, 2003; Rao *et al*, 2003; Seth *et al*, 2002), the parallel findings may reflect a primary effect of prenatal nicotine exposure on cholinergic synaptic activity and responsiveness, secondarily influencing 5HT systems (Abreu-Villaça *et al*, 2004b; Seidler *et al*, 1992; Slotkin, 1998, 2002, 2004). Superimposed on this overall effect, the cumulative neurotoxicity imposed by the combined prenatal and adolescent nicotine exposure may then account for the additional alterations in 5HT and AC responses that show directions of change opposite to those of either treatment alone (Abreu-Villaça *et al*, 2004a; Slotkin, 2002). Future work detailing the anatomical relationship of 5HT systems to nicotine-induced brain damage should elucidate these mechanisms. In any case, the relatively smaller effects of any of the treatments on the cerebellum, a region sparse in cholinergic projections and nAChRs, points to the importance of receptor-mediated events in producing the lasting effects on cell signaling.

Both prenatal and adolescent nicotine treatments, alone or in combination, displayed sex selectivity in the effects on 5HT receptor expression. These results are consistent with previous work with prenatal nicotine treatment, which showed male–female differences in neurochemical and behavioral outcomes and in the synthesis of sex steroids (Lichtensteiger and Schlumpf, 1985; Pauly *et al*, 2004; Sarasin *et al*, 2003; Segarra and Strand, 1989; Tizabi *et al*, 1997). These have distinct parallels in the effects of maternal smoking during pregnancy on behavior, which is similarly

gender-selective (Fergusson *et al*, 1998; Wakschlag and Hans, 2002; Wakschlag *et al*, 1997). Adolescent nicotine administration in animal models also shows distinct sex differences, both in the present study and in previous work from our and other laboratories (Faraday *et al*, 2001; Klein *et al*, 2004; Slotkin, 2002; Slotkin *et al*, 2002; Xu *et al*, 2002, 2003). Furthermore, gender selectivity also been reported for the initiation of smoking in individuals who were exposed *in utero* (Oncken *et al*, 2004).

The key remaining question is the extent to which prenatal nicotine exposure alters nicotine dependence or withdrawal in adolescence, and whether 5HT systems and AC signaling contribute to these outcomes. Although our studies do not address these issues directly, for the majority of biomarkers, the *in utero* treatment elicited a pattern of alterations in the same direction as those elicited by withdrawal of nicotine in the adolescent treatment model, only with a greater magnitude of effect. These animals are therefore in the same state of 5HT and AC function as would be expected in the adolescent during withdrawal after nicotine administration. Interestingly, this interpretation is in keeping with a recent hypothesis of the origin of nicotine dependence, the 'sensitization-homeostasis theory' (DiFranza and Wellman, 2005), which postulates that the stimulatory and inhibitory adjustments to nicotine administration produce reversible as well as permanent changes in activity of specific neuronal circuits. The fact that alterations in 5HT synaptic signaling emerge upon discontinuation of adolescent nicotine administration, and that prenatal nicotine exposure alters the pattern of such reactivity, are all in keeping with this model, and similarly suggest that persistent changes will leave prenatally exposed individuals vulnerable to relapse upon cessation of adolescent smoking (DiFranza and Wellman, 2005). Indeed, studies in adolescent smokers show emergence of both cognitive impairment and depression upon smoking cessation, contributing to the failure to quit (Colby *et al*, 2000; Goodman and Capitan, 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). Just as predicted by our findings in animals, these problems are worsened in adolescent smokers who were exposed *in utero* via maternal smoking (Oncken *et al*, 2004), with the differences from other adolescent smokers emerging prominently during withdrawal (Jacobsen *et al*, 2005, 2006). Accordingly, animal models of prenatal and adolescent nicotine exposure are likely to provide a biological basis for the creation of subpopulations that are especially vulnerable to nicotine dependence and withdrawal in adolescence.

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