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The Developmental Neurotoxicity of Tobacco Smoke Can Be Mimicked by a Combination of Nicotine and Benzo[a]Pyrene: Effects on Cholinergic and Serotonergic Systems

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ABSTRACT

Tobacco smoke contains polycyclic aromatic hydrocarbons (PAHs) in addition to nicotine. We compared the developmental neurotoxicity of nicotine to that of the PAH archetype, benzo[a]pyrene (BaP), and also evaluated the effects of combined exposure to assess whether PAHs might exacerbate the adverse effects of nicotine. Pregnant rats were treated preconception through the first postnatal week, modeling nicotine concentrations in smokers and a low BaP dose devoid of systemic effects. We conducted evaluations of acetylcholine (ACh) and serotonin (5-hydroxytryptamine, 5HT) systems in brain regions from adolescence through full adulthood. Nicotine or BaP alone impaired indices of ACh presynaptic activity, accompanied by upregulation of nicotinic ACh receptors and 5HT receptors. Combined treatment elicited a greater deficit in ACh presynaptic activity than that seen with either agent alone, and upregulation of nAChRs and 5HT receptors was impaired or absent. The individual effects of nicotine and BaP accounted for only 60% of the combination effects, which thus displayed unique properties. Importantly, the combined nicotine + BaP exposure recapitulated the effects of tobacco smoke, distinct from nicotine. Our results show that the effects of nicotine on development of ACh and 5HT systems are worsened by BaP coexposure, and that combination of the two agents contributes to the greater impact of tobacco smoke on the developing brain. These results have important implications for the relative safety in pregnancy of nicotine-containing products compared with combusted tobacco, both for active maternal smoking and secondhand exposure, and for the effects of such agents in "dirty" environments with high PAH coexposure.

Key words: acetylcholine; benzo[a]pyrene; developmental neurotoxicity; nicotine; serotonin; tobacco smoke.

It is now incontrovertible that maternal smoking during pregnancy or exposure to secondhand smoke, contribute to an increased risk of neurodevelopmental disorders (Pauly and Slotkin, 2008). Although nicotine clearly provides a major source for the adverse effects of tobacco smoke on brain development (Pauly and Slotkin, 2008; Slikker *et al.*, 2005), there are thousands of other smoke components that may contribute. In animal and cell culture models, we recently showed that perinatal tobacco smoke exposure has a greater impact on neurodifferentiation, synaptic function and behavioral performance than does an equivalent exposure to nicotine alone (Hall *et al.*, 2016; Slotkin *et al.*, 2014, 2015). The question remains as to which constituents of tobacco smoke besides nicotine are the most important contributors to neurodevelopmental effects. In this study, we specifically explored the role of polycyclic aromatic hydrocarbons (PAHs), which are produced when tobacco is combusted.

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Smoking is the main source of nonoccupational PAH exposure (Kim *et al.*, 2013) and secondhand tobacco smoke is the primary PAH source in nonsmokers (Suwan-ampai *et al.*, 2009). Furthermore, PAHs have been implicated in impaired neurobehavioral development in children (Perera *et al.*, 2003, 2006).

We focused on benzo[a]pyrene (BaP) as an exemplar of PAH actions for several reasons. First, each cigarette delivers approximately 25 ng of BaP (Kaiserman and Rickert, 1992), so that a pack-a-day smoker receives approximately 25% of average daily BaP exposure from smoking alone (Hattemer-Frey and Travis, 1991). Second, simultaneous exposure to nicotine and BaP are a hallmark of secondhand smoke (Aquilina *et al.*, 2010), and exposures are sufficient to detect BaP epoxide-DNA adducts in the fetus (Sanyal *et al.*, 2007). Third, because of its biological activity, BaP is the gold standard used as a surrogate for total PAH exposure, which is typically represented as "BaP equivalents" (World Health Organization, 2010). Fourth, prior studies have established that BaP is itself a neurobehavioral teratogen (Chen *et al.*, 2012; McCallister *et al.*, 2008, 2016). Finally, our prior studies in cell culture models indicated that BaP can synergize with nicotine to disrupt neurodifferentiation (Slotkin *et al.*, 2013), suggesting that the combined exposure to both agents in vivo could lead to effects not necessarily predicted just from the actions of each agent alone.

Our studies were modeled on our prior work with nicotine and tobacco smoke extract, which showed persistent neurochemical and behavioral effects that evolved over the course from adolescence to full adulthood (Hall *et al.*, 2016; Slotkin *et al.*, 2015). To avoid stress-related confounds, the agents were delivered by osmotic minipumps, implanted prior to mating, and set to deliver nicotine, BaP, or both agents (in separate pumps) throughout gestation and into the first postnatal week; because rats are altricial, the postnatal exposure period corresponds to stages of brain development in the late second to third trimester human fetus. We then evaluated the impact on synaptic development from adolescence through adulthood, focusing on acetylcholine (ACh) and serotonin (5-hydroxytryptamine, 5HT), two neurotransmitter systems known to be targets for nicotine and tobacco smoke (Slikker *et al.*, 2005; Slotkin *et al.*, 2015). We assessed the impact on brain regions comprising the major ACh and 5HT projections and their corresponding cell bodies. For ACh, we evaluated the activity of choline acetyltransferase (ChAT), the concentration of presynaptic high-affinity choline transporters (hemicholinium-3 [HC3] binding) and the concentration of α4β2 nicotinic ACh receptors (nAChRs). ChAT and high-affinity choline transporters are both constitutive components of ACh nerve terminals but they differ in their regulatory mechanisms and hence in their functional significance. ChAT is the enzyme that synthesizes ACh, but is not regulated by nerve impulse activity, so that its presence provides an index of the development of ACh projections (Slotkin, 2008). In contrast, HC3 binding to the choline transporter is directly responsive to neuronal activity (Klemm and Kuhar, 1979), so that comparative effects on HC3 binding and ChAT enable the characterization of both the development of cholinergic innervation and presynaptic impulse activity. For that determination, we calculated the HC3/ChAT ratio as an index of presynaptic activity relative to the number of cholinergic nerve terminals (Slotkin, 2008). The α4β2 nAChR is the most abundant nAChR subtype in the mammalian brain and regulates the ability of ACh systems to release other neurotransmitters involved in reward, cognition and mood (Dani and De Biasi, 2001). For 5HT systems, we focused on $5HT_{1A}$ and $5HT_2$ receptors, the subtypes that are known to be major targets for developmental neurotoxicity of nicotine (Slikker *et al.*, 2005; Slotkin *et al.*, 2007), and that play major roles in 5HT-related mental disorders, including depression (Maes and Meltzer, 1995).

MATERIALS AND METHODS

Animal treatments. All experiments were carried out humanely and with regard for alleviation of suffering, with protocols approved by the Duke University Animal Care and Use Committee and in accordance with all federal and state guidelines. Sprague-Dawley rats were shipped by climate-controlled truck (transportation time < 1 h) and were allowed to acclimate to the housing facility for 2 weeks prior to treatment. At 9 weeks of age, each animal received implants of two Type 2ML4 Alzet osmotic minipumps, inserted subcutaneously on opposite sides of the back. The pumps were implanted under anesthesia (60 mg/kg ketamine + 0.15–0.50 mg/kg dexmedetomidine given i.p; followed post-implant by 0.15 mg/kg atipamezole + 5 mg/kg ketoprofen given s.c. and topical bupivacaine) and the animals were allowed to recover for 3 days. Mating was then initiated by including a male rat in the cage for a period of 5 days. Although the pumps are marketed as a 4-week delivery device, it actually takes approximately 35 days for the reservoir to be exhausted completely (information supplied by the manufacturer) and thus the infusions terminated on postnatal day (PN) 8 ± 2 days (the insemination date varied among different mating pairs). In earlier work, we measured plasma nicotine levels to confirm the termination of nicotine absorption coinciding with the calculated values (Trauth *et al.*, 2000).

There were 4 treatment groups, each comprising 9–12 dams: control (dimethylsulfoxide vehicle in both pumps); nicotine bitartrate dissolved in water and titrated to a pH of 6.0, calibrated to deliver 2 mg/kg/day of nicotine free base at the start of the infusion period, with the second pump containing equivalent concentrations of sodium bitartrate at the same pH; BaP in dimethylsulfoxide vehicle, calibrated to deliver 30 μg/kg/day at the start of the infusion period, with the second pump containing dimethylsulfoxide; and a group that received 2 pumps, one with nicotine and 1 with BaP. Because body weights increased with gestation, the dose rate fell by approximately one-third by the end of gestation and then rose back toward the original values with the postpartum weight loss. Thus, the nicotine dose rate for the 2 mg/kg group remained well within the range that produces nicotine plasma levels similar to those in moderate smokers (Fewell *et al.*, 2001; Trauth *et al.*, 2000); likewise, the BaP dose rate was at the lower end of exposures known to have significant effects on the development of ion channels (Brown *et al.*, 2007) or on neurobehavioral endpoints (McCallister *et al.*, 2008, 2016). Further, we performed preliminary studies with BaP to ensure that the chosen dose rate did not have significant effects on fertility, maternal weight gain, litter size, or postnatal growth.

Parturition occurred during gestational day 22, which was also taken as PN0, and litters were culled to 8–10 pups to ensure standard nutrition. Weaning occurred on PN21. On PN30, 60, 100, and 150, animals were decapitated and brain regions were dissected for determination of ACh and 5HT synaptic markers: frontal/parietal cortex, temporal/occipital cortex, hippocampus, striatum, midbrain, and brainstem. The 2 cortical regions were sectioned at the midline and the right half used for the current determinations. The left halves of the cortical regions were reserved for future studies, along with the cerebellum, which is sparse in ACh and 5HT projections. Tissues were frozen in liquid nitrogen and stored at −80°C until assayed. Each treatment

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group comprised 12 animals at each age point, equally divided into males and females, with each litter contributing no more than one male and one female to any of the determinations.

Assays. Assays were conducted on each individual tissue, so that each determination represented a value from the corresponding brain region of one animal. The techniques were identical to those used in our earlier study of nicotine and tobacco smoke extract (Slotkin *et al.*, 2015), and accordingly, will be described only in brief. Tissues were thawed in 79 volumes of ice-cold 10 mM sodium-potassium phosphate buffer (pH 7.4) and homogenized with a Polytron (Brinkmann Instruments, Westbury, New York). Aliquots of the homogenate were assayed for ChAT using 50 μM [^{14}C]acetyl-coenzyme A as a substrate and activity was determined as the amount of labeled ACh produced relative to tissue protein. For binding measurements, the cell membrane fraction was prepared from the same tissue homogenate and aliquots were assayed for: (1) HC3 binding, using a ligand concentration of 2 nM [^{3}H]HC3 with or without 10 μM unlabeled HC3 to displace specific binding; (2) nAChR binding, using 1 nM [^{3}H]cytisine with or without 10 μM nicotine as a displacer; (3) $5HT_{1A}$ receptor binding with 1 nM [^{3}H]8-hydroxy-2-(di-n-propylamino)tetralin, displaced with 100 μM 5HT; and (4) $5HT_2$ receptor binding with 0.4 nM [^{3}H]ketanserin, displaced with 10 μM methylsergide. Ligand binding was calculated relative to the membrane protein concentration.

Some of the regions had insufficient amounts of tissues to permit all assays to be performed. Accordingly, we did not obtain values for nAChRs in the striatum, nor for the 5HT receptors in either the striatum or hippocampus.

Data analysis. The initial statistical comparisons were conducted by a global ANOVA (data log-transformed because of heterogeneous variance among regions, measures and ages) incorporating all the variables and measurements in a single test so as to avoid an increased probability of type 1 errors that might otherwise result from multiple tests of the same data set. The variables in the global test were treatment (vehicle, nicotine, BaP, and nicotine + BaP), brain region, age and sex, with multiple dependent measures (hereafter, designated simply as "measures"): ChAT, HC3 binding and nAChR binding for the ACh synaptic makers; $5HT_{1A}$ and $5HT_2$ receptor binding for the 5HT synaptic markers. For both transmitter systems, the dependent measures were treated as repeated measures, since all the determinations were derived from the same sample. Where we identified interactions of treatment with the other variables, data were then subdivided for lower-order ANOVAs to evaluate treatments that differed from the corresponding control or from each other. As permitted by the interaction terms, individual treatments that differed from control or from each other were identified with Fisher's Protected Least Significant Difference Test. However, where treatment effects were not interactive with other variables, we report only the main treatment effects without performing lower-order analyses of individual values. Significance was assumed at the level of $p < .05$, 2-tailed.

Data were compiled as means and SEs. To enable ready visualization of treatment effects across different regions, ages, and measures, the results are given as the percent change from control values but statistical procedures were always conducted on the original data, with log transforms because of heterogeneous variance as noted above. In addition, the log-transform evaluates the treatment differences as a proportion to control values, rather than as an arithmetic difference. This was important because of technical limitations: on any single day, we could conduct assays for all treatment groups and both sexes, but for only one region at one age point. Accordingly, representing the data as proportional differences (percent control) enables a full comparison of treatment effects and treatment interactions with all the other variables, even though absolute values for the controls cannot be compared across regions and ages (since assays for each region and age point were run on separate days). Graphs were scaled to encompass the different dynamic ranges of the changes in the various parameters. The original values for each set of determinations appear in the Supplementary Tables.

There were two separate issues addressed in the data analysis. First, we determined whether the effects of nicotine, BaP, or the combination, differed from control values and from each other. Second, we wanted to assess whether the effects of the combination were additive (sum of individual effects of nicotine and BaP), or nonadditive (synergistic, less than additive or antagonistic). The first issue required an ANOVA regarding the 4 treatment groups as one factor ("treatment"), followed by post-hoc comparisons for intergroup differences. The second issue required that the nicotine and BaP treatments be considered as 2 separate factors, with the interaction term (nicotine × BaP) thus testing for additivity: no significant nicotine × BaP interaction if the effects of the combination were indistinguishable from additive effects; significant interaction if effects were synergistic, less than additive or antagonistic.

Finally, we used linear regression and multiple regression analyses to determine the extent to which the combined exposure to nicotine + BaP could be accounted for by either agent alone, or by the summation of the individual effects of nicotine and BaP.

Materials. Animals were purchased from Charles River Laboratories (Raleigh, North Carolina) and osmotic minipumps (model 2ML4) were from Durect Corp. (Cupertino, California). PerkinElmer Life Sciences (Boston, Massachusetts) was the source for [^{3}H]HC3 (specific activity, 125 Ci/mmol), [^{3}H]cytisine (specific activity 35 Ci/mmol), [^{3}H]8-hydroxy-2-(di-n-propylamino)tetralin (specific activity, 135 Ci/mmol), [^{3}H]ketanserin (63 Ci/mmol), and [^{14}C]acetyl-coenzyme A (specific activity 6.7 mCi/mmol). Methylsergide was obtained from Sandoz Pharmaceuticals (E. Hanover, New Jersey) and all other chemicals came from Sigma Chemical Co. (St Louis, Missouri).

RESULTS

Maternal, Litter, and Growth Effects

None of the treatments had any significant effect on maternal weight gain during or after pregnancy or on the proportion of dams giving birth (data not shown). There was a small but statistically significant reduction in litter size in the nicotine group ($p < .02$ vs control or vs the group receiving the combination of nicotine + BaP): control, 13.4 ± 0.6 pups per litter ($n = 10$), nicotine 11.2 ± 0.6 ($n = 12$), BaP 12.1 ± 0.8 ($n = 11$), nicotine + BaP 13.4 ± 0.5 ($n = 9$). However, in an earlier study with a larger cohort, we did not observe a decrease in the nicotine group (Slotkin *et al.*, 2015). There were no detectable treatment effects on the sex ratio of the litters: control 47% ± 4% male, nicotine 43% ± 4%, BaP 51% ± 4%, and nicotine + BaP 52% ± 4%. The offspring displayed no significant treatment-related changes in body weight or brain region weights throughout adolescence and adulthood (Supplementary Table 1).

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Global Statistical Analyses of ACh Synaptic Markers

Because nAChRs were not determined in one of the brain regions (striatum), there were 2 different ways of performing global statistical analyses for ACh synaptic markers. First, we examined all 3 markers (ChAT activity, HC3 binding, and nAChR binding) across 5 of the regions, excluding the striatum. We identified a significant main effect of treatment ($p < .0001$) as well as interactions of treatment × sex ($p < .0002$), treatment × measure ($p < .0001$), and treatment × measure × age ($p < .01$). Second, we excluded the nAChR measurements and evaluated ChAT activity and HC3 binding across all 6 regions, and likewise found a significant treatment main effect ($p < .0001$), treatment × sex interaction ($p < .004$), treatment × measure interaction ($p < .0001$), and treatment × measure × age interaction ($p < .03$). Treating the treatments as 2 separate factors (nicotine, BaP), we further identified a nicotine × BaP interaction ($p < .0001$ for the 5 regions with all measures, $p < .0001$ for the 6 regions with ChAT and HC3 measures) and a nicotine × BaP × sex interaction ($p < .04$ for the 5 regions with all measures), connoting nonadditive effects of the 2 treatments. Accordingly, we evaluated each of the measures separately for main treatment effects and interactions of treatment with other variables, and for the interaction of nicotine × BaP in the 2-factor treatment analysis.

ChAT Activity

Comparing all 4 treatment groups, ANOVA identified a main effect of treatment on ChAT activity ($p < .0001$), as well as a treatment × sex interaction ($p < .0001$). With treatments considered as 2 separate factors, we further found a nicotine × BaP interaction ($p < .0001$) and a nicotine × BaP × region interaction ($p < .05$). Accordingly, we examined each treatment for differences from control and for interactions of treatment with sex and region, but not for an interaction with age; although age was still retained as a factor in the statistical analysis, any age-related interactions were ignored because of the absence of a significant interaction in the higher-order test (Figure 1).

Nicotine treatment evoked a significant overall elevation in ChAT activity that was restricted to males (Figure 1A). BaP also evoked a significant increase in ChAT that was selective for males (Figure 1B); this treatment also showed regional selectivity (treatment × region interaction, individually significant for midbrain and brainstem), but that relationship needs to be viewed cautiously, since the global ANOVA for ACh synaptic markers did not detect a significant interaction with region across all the markers. The overall effect of BaP on ChAT was significantly smaller than that of nicotine ($p < .008$). Combined treatment with nicotine + BaP likewise produced a significant elevation in ChAT that was specific to males (Figure 1C); the effect was smaller than that of nicotine ($p < .05$) but indistinguishable from that of BaP alone.

Because of the complexity of the results, it is difficult to visualize a comparison of the net effects of the 3 different treatments. Accordingly, we developed a simplified graphical representation of the data, calculating the mean values for main treatment effects, collapsed across region and age (Figure 1D). This streamlined picture dilutes the effects seen for specific regions or ages by averaging them with data points for which there was no effect or an opposite effect, so that the absolute magnitude becomes smaller; in addition, the variability term is no longer meaningful, since values are collapsed across factors that interact with treatment and that contribute to the overall variance. Despite these limitations, there were obvious overall patterns that correspond to the net outcomes presented in Figures 1A–C. All of the treatments produced elevations in ChAT selectively in males, with the greatest effect obtained with nicotine. Further, the combination of nicotine + BaP produced an outcome that was clearly less than additive, since the effects were smaller than those seen with nicotine alone; this is reflected in the significant nicotine × BaP interaction, as noted above.

HC3 Binding

ANOVA across all the treatment groups showed a significant main treatment effect on HC3 binding ($p < .0001$) as well as a treatment × age interaction ($p < .04$). Regarding the nicotine and BaP treatments as separate factors, we further identified interactions of nicotine × BaP ($p < .0009$) and nicotine × BaP × sex ($p < .05$), again indicating nonadditive effects of the 2 treatments. Accordingly, we examined each treatment for main effects and for interactions with age and sex, but not region; region was retained in the statistical model, but interactions with region were ignored because of the absence of interactions in the higher-order test (Figure 2).

As was true for ChAT activity, nicotine produced a net elevation of HC3 binding that was selective for males (Figure 2A). However, BaP elicited the opposite effect, an overall *decrease*, and the effect was specific to females, not males (Figure 2B); the effect of BaP was robustly significant from that of nicotine ($p < .0001$), reflecting the opposite directions of change. BaP also showed an age progression, with the deficits limited to younger ages (PN30 and PN60) and waning in adulthood (PN100 and PN150). Combined treatment with nicotine + BaP (Figure 2C) elicited a pattern that differed from that of either nicotine ($p < .0001$) or BaP ($p < .0001$) alone; values were reduced in both males and females and the deficit persisted into full adulthood.

Again, the simplified pattern of main effects places these differences in stark relief (Figure 2D). Nicotine showed an overall elevation in males, whereas BaP showed a decrease in females. The group receiving the combined treatment uniquely showed deficits in both sexes that were clearly nonadditive for males, but indistinguishable from additivity in females; this is reflected in the significant nicotine × BaP × sex interaction noted above. But the main point is that the reduction in HC3 binding evoked by the combination of nicotine + BaP was worse overall than for either of the individual treatments.

HC3/ChAT Ratio

The HC3/ChAT ratio gives a snapshot of ACh presynaptic activity (Slotkin, 2008). The overall ANOVA across all treatment groups identified a main treatment effect ($p < .0001$) and an interaction of treatment × age ($p < 0.01$). The 2-factor interaction of nicotine × BaP was not significant. Accordingly, we examined the main effect of treatment and its interaction with age separately for the 3 groups, retaining region and sex as ANOVA factors but ignoring any resultant interaction of treatment with the latter 2 variables (Figure 3).

Nicotine produced a small, but significant overall reduction in HC3/ChAT (Figure 3A). BaP evoked a more robust decrease ($p < .007$ vs the effect of nicotine) that showed an age progression, with lower values early in development, eventually resolving in full adulthood (Figure 3B). Combined treatment with nicotine + BaP produced the largest net deficit in the HC3/ChAT ratio, significantly greater than the effect of either nicotine ($p < .0001$) or BaP ($p < .0001$) alone (Figure 3C); for this group, the deficit did not resolve by PN150. Values collapsed across age and region again illustrate the overall effects of the different treatment (Figure 3D): a small decline for nicotine, a larger effect for BaP,

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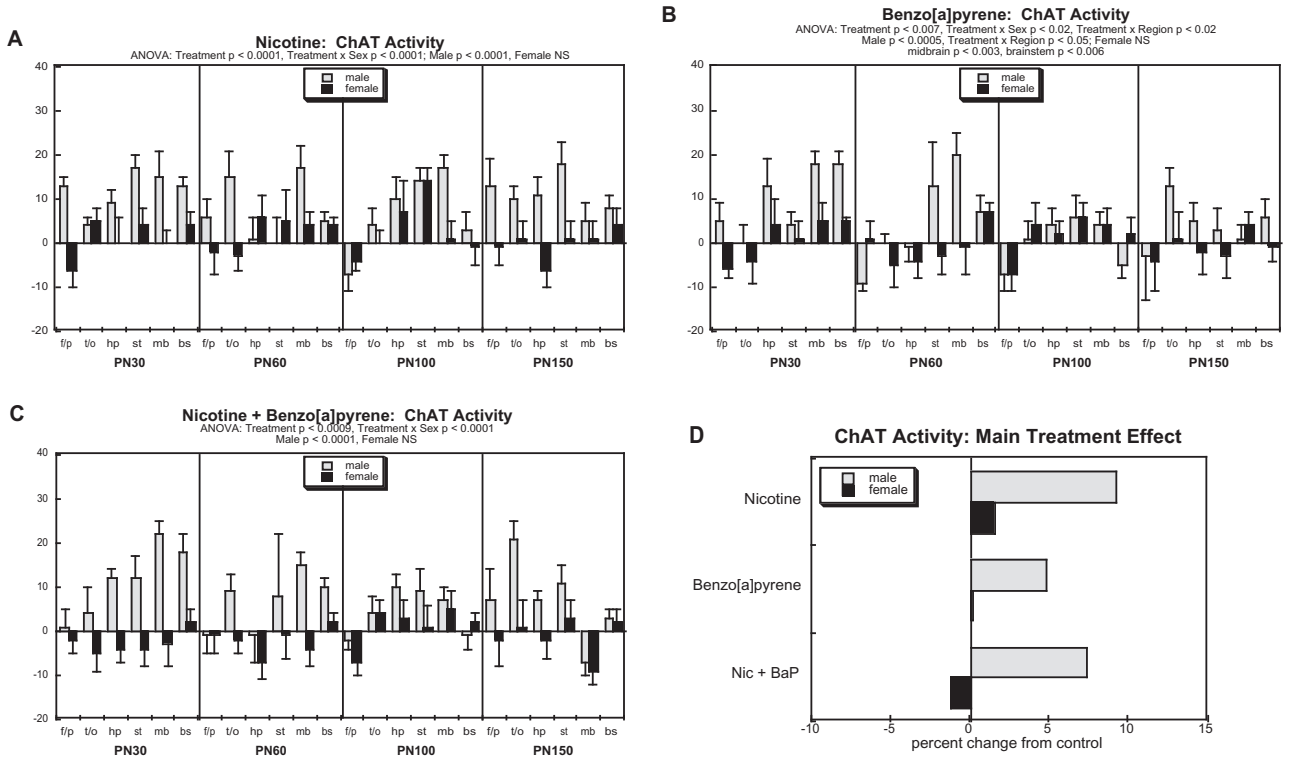

Figure 1. Effects of nicotine (A), BaP (B), and combined treatment (C) on ChAT activity. Data represent means and SEs, presented as the percent change from control values; complete original data are shown in Supplementary Table 2. Multivariate ANOVA for each treatment appears at the top of the panels. Lower-order tests were carried out in accordance with the interactions of treatment with other variables. Panel (D) shows the simple main treatment effects, collapsed across age and region. Abbreviations: f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; hp, hippocampus; st, striatum; mb, midbrain; bs, brainstem; NS, not significant; Nic, nicotine.

and the greatest deficit from nicotine + BaP, indistinguishable from the additive impact of the 2 individual treatments.

nAChR Binding

Across all treatment groups, ANOVA identified a main treatment effect ($p < .0001$) without any significant interactions with the other factors. With nicotine and BaP considered as separate treatment factors, we found a nicotine × BaP interaction, again indicating nonadditive effects, but without any significant impact of other factors on this outcome. Accordingly, examinations for each treatment were conducted with the other factors retained in the analysis, but without reporting any resultant interactions with treatment (Figure 4).

Nicotine produced a significant overall elevation in nAChR binding (Figure 4A), an effect that was smaller (nonsignificant) for BaP alone (Figure 4B) and absent for combined treatment with nicotine + BaP (Figure 4C). Indeed, the combination elicited changes that were entirely distinct from those of nicotine or BaP alone ($p < .0001$ and .003, respectively). The collapsed view of main effects likewise showed a robust increase for the nicotine group and a smaller increase with BaP (Figure 4D). The nicotine + BaP group showed an effect that was clearly less than additive for males and actually in the opposite direction (decrease) for females, reflecting the nicotine × BaP interaction noted earlier.

Global Statistical Analyses of 5HT Receptor Binding

Global statistical analysis across both 5HT receptor subtypes identified a main effect of treatment ($p < .0001$) as well as interactions of treatment × sex ($p < .03$), treatment × subtype ($p < .007$) and treatment × subtype × age ($p < .004$). Regarding nicotine and BaP as separate treatment factors, we also identified a significant interaction of nicotine × BaP ($p < .0001$), connoting nonadditive effects. Accordingly, we examined treatment effects, interactions of treatment with other factors, and the 2-factor interaction of nicotine × BaP, separately for the 2 receptor subtypes.

5HT1A Receptors

Across all groups, we found a main effect of treatment ($p < .0001$) on $5HT_{1A}$ receptor binding, without significant interactions with the other factors. For the 2-factor treatment arrangement, we identified a nicotine × BaP interaction ($p < .0008$), indicative of non-additive effects, but again, there were no interactions with other factors. Accordingly, we limited our comparisons to main treatment effects, retaining the other factors in the ANOVA but disregarding any resultant interactions with treatment (Figure 5).

Developmental nicotine exposure elicited a significant and robust upregulation of $5HT_{1A}$ receptor binding (Figure 5A) whereas the effects of BaP (Figure 5B) or nicotine + BaP (Figure 5C) were not statistically significant. These patterns were even more evident when results were collapsed across treatment and region (Figure 5D). Most notably, the group receiving combined treatment showed the smallest effect, clearly much less than the sum of the effects of nicotine and BaP alone and significantly lower than that of animals exposed just to nicotine ($p < .0001$).

5HT2 Receptors

As was true for the $5HT_{1A}$ receptor subtype, there was a main treatment effect for $5HT_2$ receptors ($p < .0001$) without any

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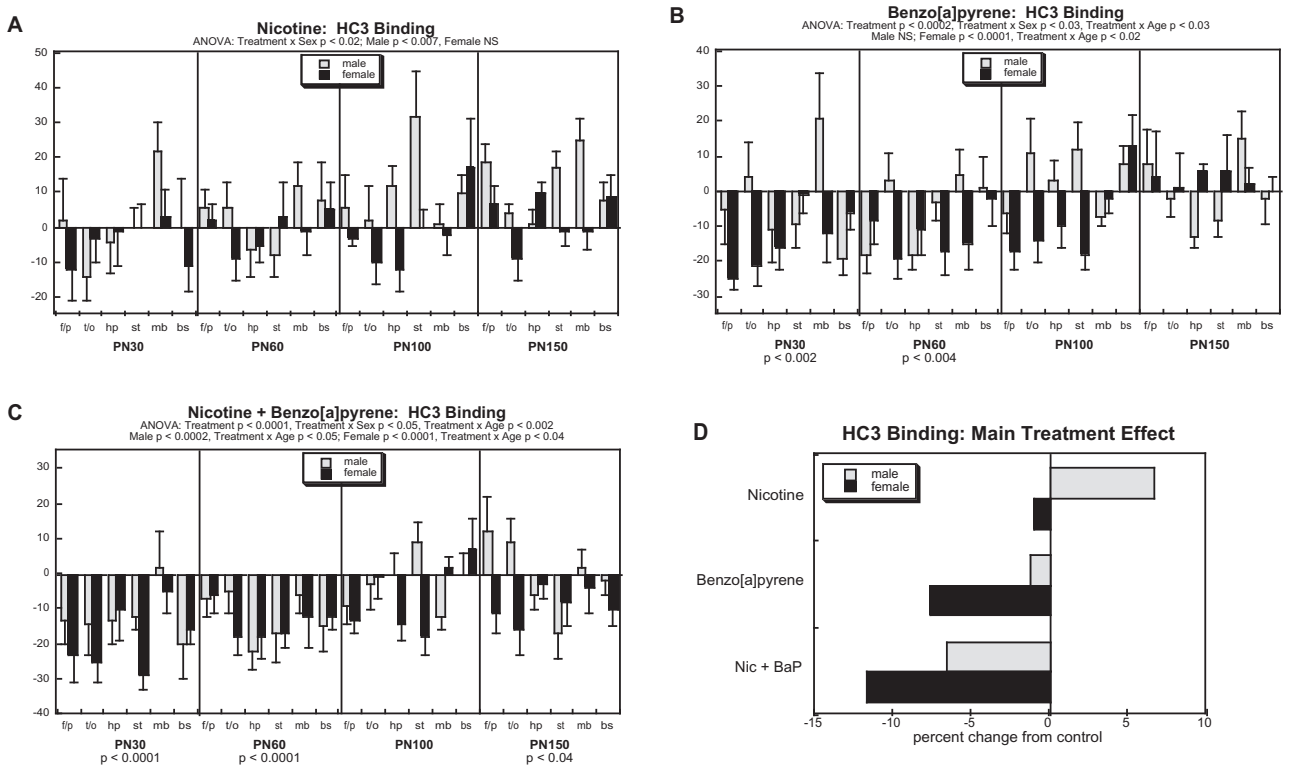

Figure 2. Effects of nicotine (A), BaP (B), and combined treatment (C) on HC3 binding. Data represent means and SEs, presented as the percent change from control values; complete original data are shown in Supplementary Table 3. Multivariate ANOVA for each treatment appears at the top of the panels. Lower-order tests were carried out in accordance with the interactions of treatment with other variables. Panel (D) shows the simple main treatment effects, collapsed across age and region. Abbreviations: f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; hp, hippocampus; st, striatum; mb, midbrain; bs, brainstem; NS, not significant; Nic, nicotine.

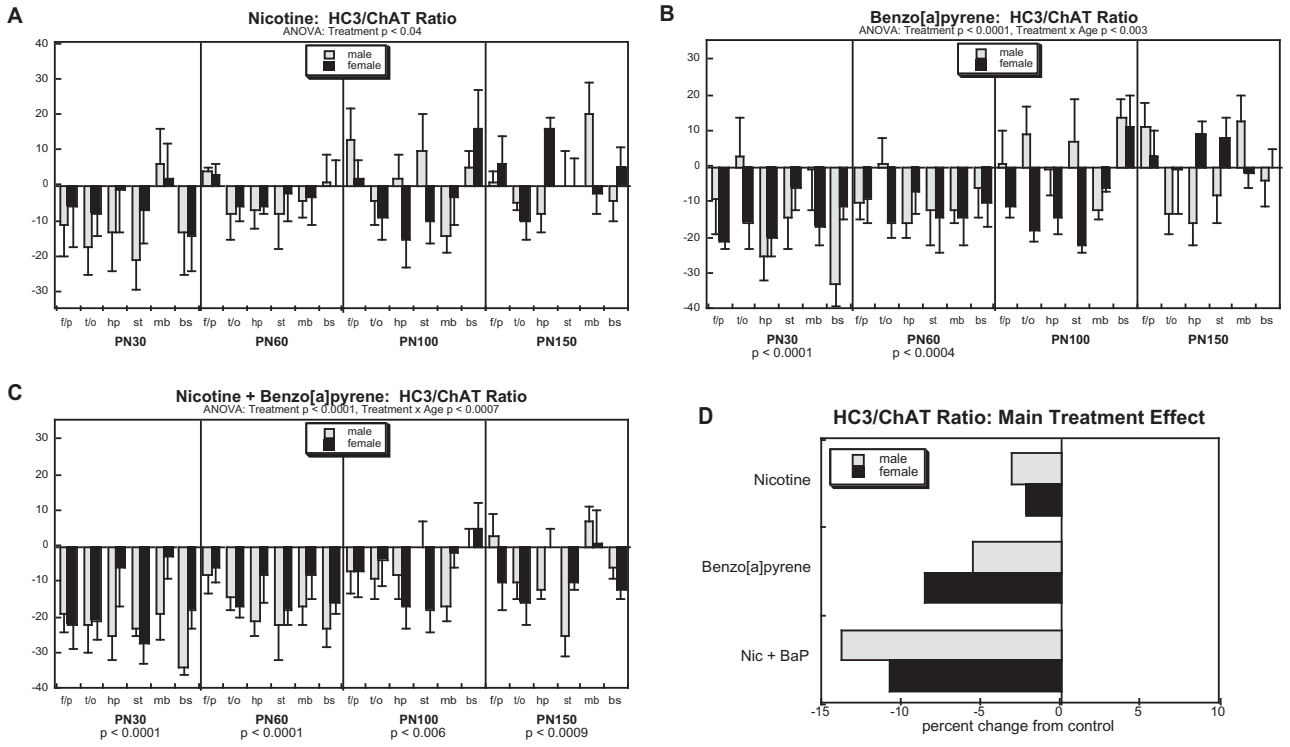

Figure 3. Effects of nicotine (A), BaP (B), and combined treatment (C) on the HC3/ChAT ratio. Data represent means and SEs, presented as the percent change from control values; complete original data are shown in Supplementary Table 4. Multivariate ANOVA for each treatment appears at the top of the panels. Lower-order tests were carried out in accordance with the interactions of treatment with other variables. Panel (D) shows the simple main treatment effects, collapsed across age and region. Abbreviations: f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; hp, hippocampus; st, striatum; mb, midbrain; bs, brainstem; Nic, nicotine.

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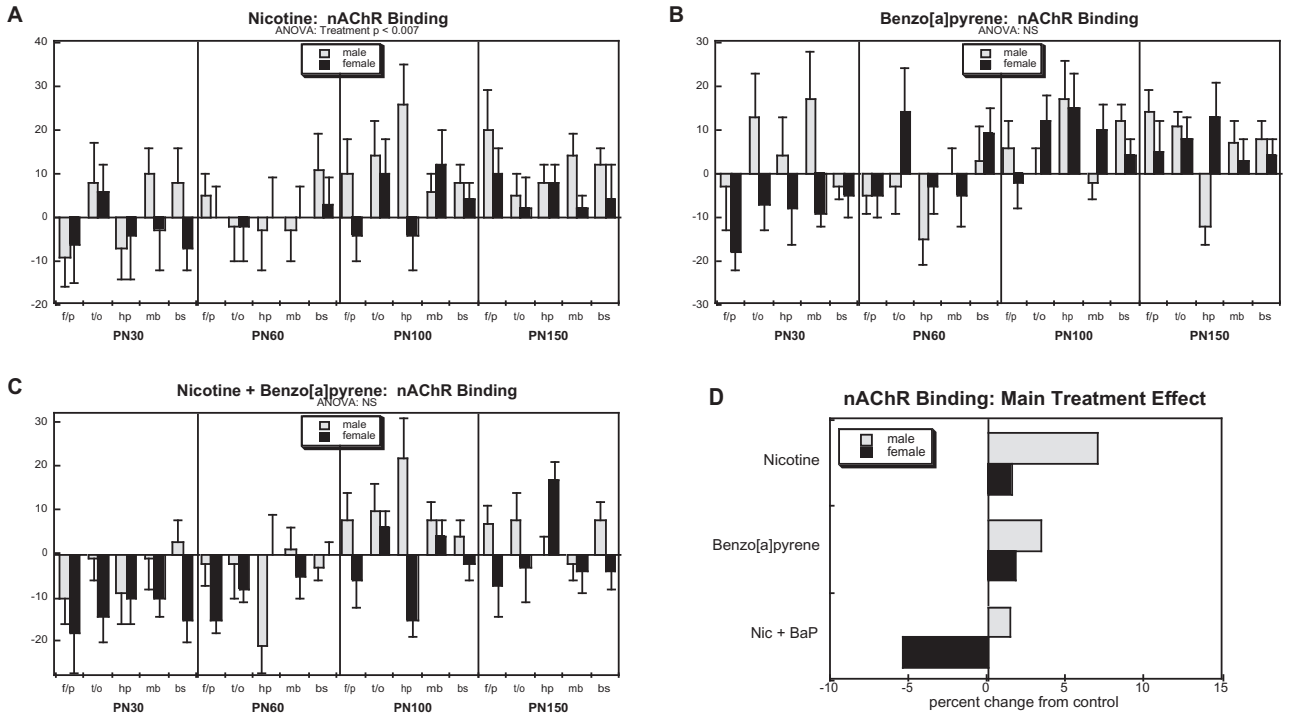

Figure 4. Effects of nicotine (A), BaP (B), and combined treatment (C) on nAChR binding. Data represent means and SEs, presented as the percent change from control values; complete original data are shown in Supplementary Table 5. Multivariate ANOVA for each treatment appears at the top of the panels. Lower-order tests were not carried out because of the absence of interactions of treatment with other variables. Panel (D) shows the simple main treatment effects, collapsed across age and region. Abbreviations: f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; hp, hippocampus; mb, midbrain; bs, brainstem; NS, not significant; Nic, nicotine.

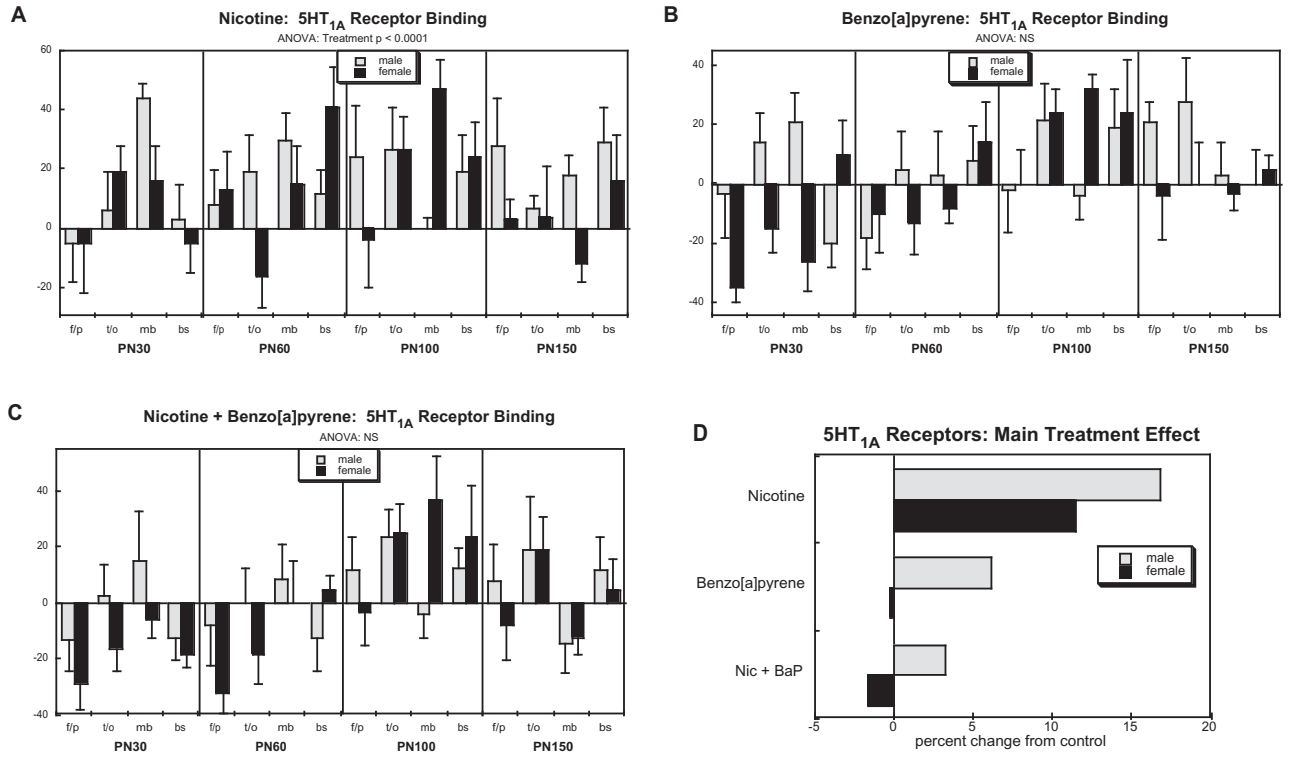

Figure 5. Effects of nicotine (A), BaP (B), and combined treatment (C) on $5HT_{1A}$ receptor binding. Data represent means and SEs, presented as the percent change from control values; complete original data are shown in Supplementary Table 6. Multivariate ANOVA for each treatment appears at the top of the panels. Lower-order tests were not carried out because of the absence of interactions of treatment with other variables. Panel (D) shows the simple main treatment effects, collapsed across age and region. Abbreviations: f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; mb, midbrain; bs, brainstem; NS, not significant; Nic, nicotine.

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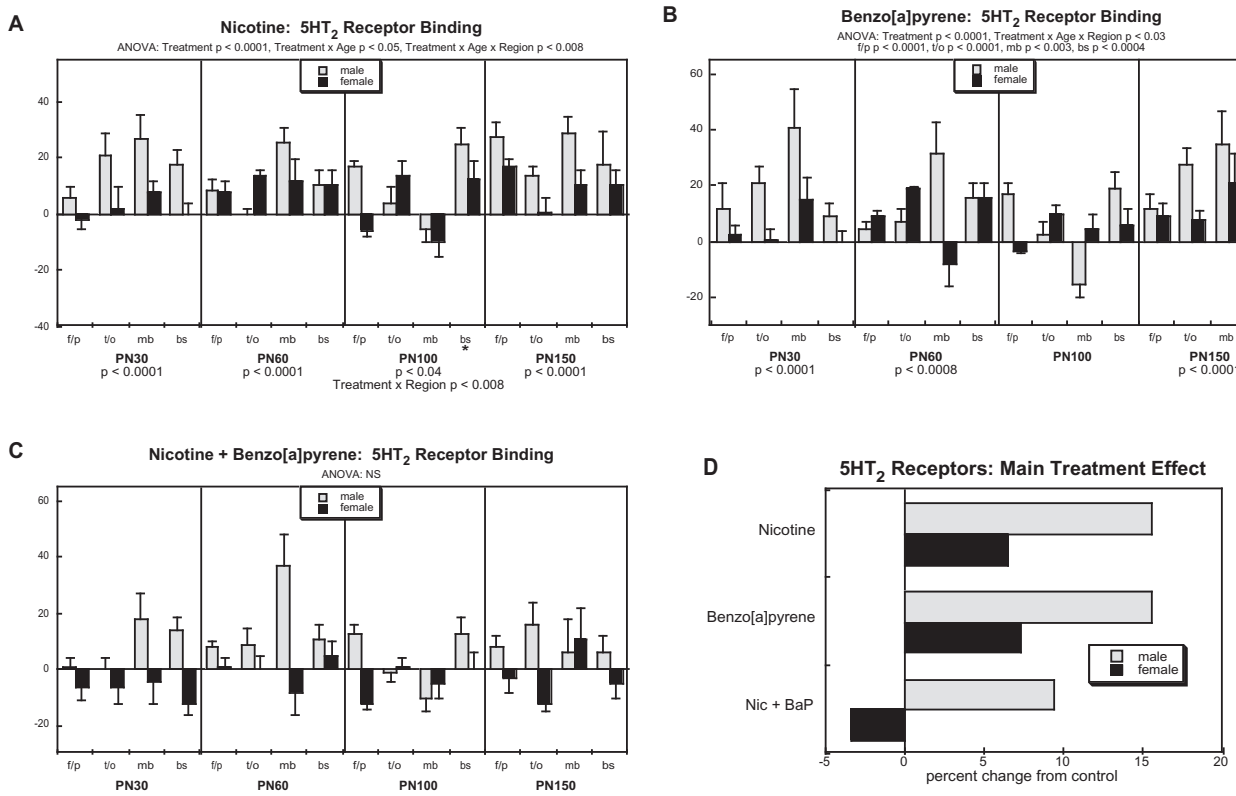

Figure 6. Effects of nicotine (A), BaP (B), and combined treatment (C) on $5HT_2$ receptor binding. Data represent means and SEs, presented as the percent change from control values; complete original data are shown in Supplementary Table 6. Multivariate ANOVA for each treatment appears at the top of the panels. Lower-order tests were carried out in accordance with the interactions of treatment with other variables. Asterisk in panel (A) shows the region displaying a significant difference on PN100, the only age point where there was a significant treatment × region interaction. Panel (D) shows the simple main treatment effects, collapsed across age and region. Abbreviations: f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; mb, midbrain; bs, brainstem; NS, not significant; Nic, nicotine.

interactions with the other factors. Considering nicotine and BaP as separate treatment factors, we again found a significant nicotine × BaP interaction ($p < .0001$) connoting non-additive effects, but for this subtype, there were also interactions of nicotine × BaP × age ($p < .02$) and nicotine × BaP × age × region ($p < .03$), so we examined each treatment for main effects and the interactions with those factors (Figure 6).

Nicotine produced a net upregulation of $5HT_2$ receptors, with some variation among regions in young adulthood (Figure 6A). A similar pattern was observed for BaP (Figure 6B), but for the group receiving the nicotine + BaP combination (Figure 6C), effects were much less notable (nonsignificant vs control, $p < .0001$ vs nicotine, $p < .0001$ vs BaP). Collapsing the values across region and age, the robust upregulation with either nicotine or BaP was clearly evident (Figure 6D), whereas with the combination treatment, effects were smaller (male) or in the opposite direction (female). These differences were reflected in the highly significant nicotine × BaP interaction noted above.

Regression Relationships Between Treatments

We used regression analysis to provide an overall picture of the extent to which the individual effects of nicotine and BaP could account globally for the effects of combined treatment with nicotine + BaP, across all the various ACh and 5HT synaptic parameters. If an individual agent were entirely responsible for the effect of the combination treatment, then one would expect a high correlation (ideally, $r = 1$) that would account entirely for variance seen with the combination (r^2=1), and the regression line would go through origin and have a slope of unity. The regression of nicotine + BaP against nicotine alone (Figure 7A) showed a highly significant correlation, but could account for only about half of the effect of the combined treatment ($r^2 = 0.48$); furthermore, the regression line did not pass through the origin (significantly negative value) and the slope was significantly lower than unity, indicating that the effects of nicotine would have predicted a greater change than was actually obtained with the combination of nicotine + BaP. The same was true for the regression relationship to BaP (Figure 7B): roughly 50% accountability for the effect of nicotine + BaP, with a negative intercept and a slope < 1.

We then conducted a multiple regression with both nicotine and BaP as regressors. If the effect of the combined treatment with nicotine + BaP represented additive effects, then the r^2 value would approach unity, reflecting complete accountability for the response when both treatments were included in the regression. We found a correlation coefficient of $r = 0.79$, which was significantly better ($p < .02$) than the coefficient for nicotine alone, but not significantly better than that of BaP alone. Further, the r^2 value obtained (0.62) indicated that nearly 40% of the effect could still not be accounted for by considering the 2 individual treatments ($p < .0001$ vs unity). As shown by the primary results (Figs. 1–6), that statistical finding reflects a lower-than-expected set of values for the combination, resulting from greater suppression of ACh presynaptic activity, and a lowering of the compensatory upregulation of nAChRs and 5HT receptors relative to nicotine or BaP alone.

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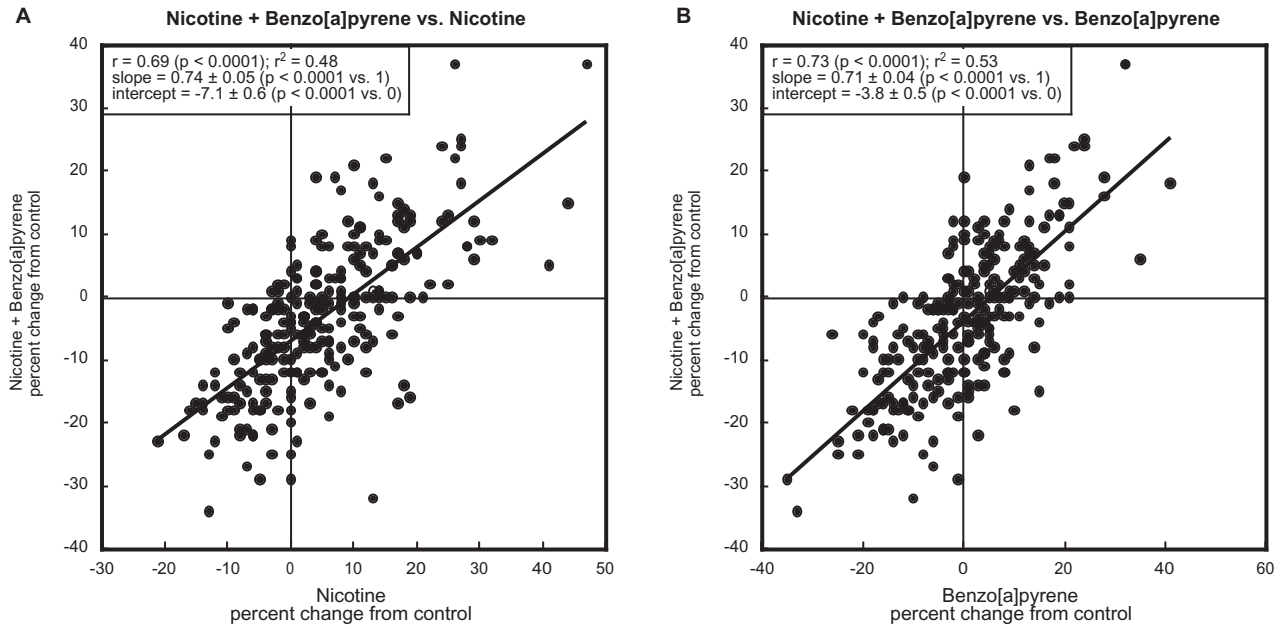

Figure 7. Correlations across all parameters for effects of nicotine + BaP versus nicotine alone (A) or BaP alone (B), combining data from Figures 1–6.

DISCUSSION

There are three principal findings: (1) nicotine and BaP converge on a common set of ACh and 5HT abnormalities, albeit via different mechanisms; (2) combining both treatments produces effects that are unique and distinguishable from those of the individual agents, constituting a greater loss of ACh presynaptic function and a suppression of compensatory changes in nAChRs and 5HT receptors; and (3) the combined effects recapitulate the outcomes seen from tobacco smoke exposure. By themselves, nicotine and BaP both led to reduced ACh presynaptic activity in adolescence and adulthood, indicated by a decrease in the HC3/ChAT ratio, with a greater net effect for BaP. However, for nicotine, the primary cause was an increase in ChAT, whereas for BaP there was a principal deficiency in HC3 binding, implying that the two agents target different events in ACh synaptic development that nevertheless lead to a net decrease in presynaptic activity. For nicotine, there is a component of ACh hyperinnervation (elevated ChAT) accompanying (or perhaps triggering?) a suppression of presynaptic activity (reduced HC3/ChAT), whereas for BaP, the reduction in neural activity is primary (deficit in HC3 binding). Both agents also evoked compensatory upregulation of receptors that can offset the functional loss of ACh activity, but again, there were differences between the effects of nicotine and BaP. Nicotine evoked robust upregulation of nAChRs and both 5HT receptor subtypes, whereas with BaP, increases were limited to $5HT_2$ receptors. Prior studies with other developmental cholinotoxicants show that neurobehavioral deficits resulting from ACh deficiencies are offset by increased dependence on 5HT receptors (Aldridge *et al.*, 2005). We have already shown that prenatal nicotine exposure leads to a rise in 5HT presynaptic activity (Slotkin and Seidler, 2010), so that the 5HT receptor upregulation observed here, would further serve to compensate for the ACh loss.

If developmental exposure to nicotine or BaP both suppress ACh presynaptic activity, then one would expect combined nicotine + BaP treatment to elicit even larger effects than with either agent alone. Indeed, this was the case for the impact on ACh presynaptic activity: the combination evoked a larger decrease in the HC3/ChAT ratio than was seen with just nicotine or BaP. Interestingly, that outcome was predicted by our prior study with cell culture models, that showed that nicotine augments the ability of BaP to impair neurodifferentiation into the ACh phenotype (Slotkin *et al.*, 2013). In contrast, though, the combination had a unique impact on compensatory receptor changes, reducing or eliminating the upregulation instead of augmenting it. The implication is clear: combined nicotine + BaP exposure worsens the ACh presynaptic deficit while at the same time interfering with compensatory mechanisms that would ordinarily offset the functional deficits. The net consequence is likely to be a profile of neurobehavioral impairment distinct from that of either agent alone, and studies are currently underway to assess this hypothesis.

Using regression analysis, we were able to quantify the discrete contributions of nicotine and BaP to the overall effects of the combined treatment, and to distinguish the unique effects of the combination from those of the individual agents. Across all of the measurements made in this study, nicotine could explain about half of the effect seen with the nicotine + BaP treatment, but the regression slope of <1 and negative y-intercept indicated that the combination had lower overall values than expected from those of nicotine; the lower overall values reflect the greater decrease in HC3/ChAT and the smaller increases (or outright decreases) in receptor binding. Roughly the same outcome was obtained for the regression of the combination against BaP. If the two agents did not interact, then multiple regression against both nicotine and BaP should have resulted in a near-complete accountability for the effects of the combination. In fact, there was little or no improvement over the relationship to either agent alone, indicating that the deviations seen with the combination were indeed unique effects, not predicted by those of either nicotine or BaP; the r^2 value indicates that these unexpected (ie, nonadditive) effects represent about 40% of the total pattern for nicotine + BaP.

The key point, then, is whether the unique effects of the nicotine + BaP combination can explain the differences between developmental exposure to nicotine alone versus tobacco

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Table 1. Effects of Nicotine + BaP Compared With Tobacco Smoke

	Nicotine + BaP	Tobacco Smoke (Slotkin *et al.*, 2015b)
ChAT	↑male Figure 1D	↑male
HC3	↓↓ Figure 2D	↓↓
HC3/ChAT	↓↓ male>female Figure 3D	↓↓ male>female
nAChR	↓female>male Figure 4D	↓female>male
$5HT_{1A}$	± Figure 5D	↓ female
$5HT_2$	↑male ↓female Figure 6D	↑male ↓female

smoke. Indeed, in our earlier study, we found that tobacco smoke elicited a more profound loss of ACh presynaptic activity and interfered with the compensatory upregulation of nAChRs and 5HT receptors (Slotkin *et al.*, 2015), precisely the difference seen here for nicotine vs the nicotine + BaP combination. The specific parallels between nicotine + BaP and tobacco smoke appear in Table 1 for each individual parameter. The concordance is striking, including direction and magnitude of change and sex selectivity for each of the markers, with the exception of $5HT_{1A}$ receptors. Our findings thus point strongly to the conclusion that it is specifically the combination of nicotine and PAHs that provides the major impetus for distinction between the developmental neurotoxicity of tobacco smoke and that of nicotine, at least as directed toward ACh and 5HT systems.

Our results also provide indirect evidence for the importance of the postnatal exposure period for nicotine's effects, and hence potentially for those of tobacco smoke as well. In our previous study with this nicotine regimen (Slotkin *et al.*, 2015), the pump had a different reservoir volume (ie, different production lot), so that the postnatal exposure period was about 50% longer than in this (ie, exposure terminated on PN12 ± 2 days, compared with PN8 ± 2 days here). Although most of the effects were same for the two studies, there were some differences: the longer postnatal exposure period elicited a decrease in HC3 binding and hence a larger decrease in the HC3/ChAT ratio, and also impaired the upregulation of $5HT_{1A}$ receptors. Likewise, if the exposure period were shortened to exclude postnatal exposure altogether (prenatal exposure only), the resultant effect was an increase in the HC3/ChAT ratio instead of a decrease, accompanied by upregulation of both 5HT receptor subtypes (Slotkin and Seidler, 2015). These comparisons emphasize the relative importance of the postnatal exposure period, which corresponds to the latter stages of gestation in human development, a period in which ACh systems are especially sensitive to nicotine (Nunes-Freitas *et al.*, 2011). The longer the postnatal exposure, the greater the loss of ACh presynaptic activity, and the less the compensatory upregulation of 5HT receptors. Indeed, the difference in duration of postnatal exposure is likely responsible for the one point of disagreement between the effects of nicotine + BaP versus tobacco smoke ($5HT_{1A}$ receptor upregulation), since the tobacco smoke exposure lasted into the second postnatal week (Slotkin *et al.*, 2015), as opposed to the first postnatal week in this study.

As with any study in which maternal treatment extends into the neonatal period, it is impossible to rule out partial contributions of altered maternal physiology and/or behavior to adverse neurodevelopmental outcomes in the offspring, either directly or through interaction with direct drug effects. With prenatal treatments, this can be resolved by cross-fostering neonates to untreated dams, although this stressor too, might then contribute to any observed effects. Notably though, studies in which nicotine was given directly to the neonates produced adverse effects on ACh synaptic function, just as in this study (Liang *et al.*, 2006). Furthermore, for the combination of nicotine + BaP, we have likewise shown that these two agents directly disrupt neurodifferentiation in an in vitro model, and show interactions akin to those seen here (Slotkin *et al.*, 2013). It is thus unlikely that maternal effects alone can explain our findings.

Superimposed on the main effects of the nicotine and BaP treatments, we observed significant sex differences in the responses to treatment. For nicotine, these were similar to those noted in our earlier study (Slotkin *et al.*, 2015), characterized by greater effects in males than in females. Although both sexes showed decreases in the HC3/ChAT ratio, the index of presynaptic ACh activity, the individual parameters contributing to that index were quite different for the two sexes. In males, the index dropped primarily because of a profound elevation in ChAT, whereas in females the decrease reflected a drop in HC3 binding. This suggests that males experience a greater degree of cholinergic hyperinnervation accompanying the suppression of presynaptic activity, possibly representing a compensation for lost ACh tone. In support of that interpretation, males also showed greater upregulation of nAChR and $5HT_2$ receptor binding, which are also compensatory. It is notable that virtually the same pattern of sex differences was seen for the effects of BaP on 5 of the 7 measured parameters, suggesting that the differences in injury and compensatory response are common features of otherwise unrelated agents. However, the actual mechanisms underlying these sex differences remain to be elucidated.

Finally, our finding that the neurodevelopmental effects of tobacco smoke can be mimicked by the combination of nicotine and a PAH has important implications for public health. Given that tobacco smoke is more injurious to brain development than is nicotine (Slotkin *et al.*, 2015), we can now explicitly implicate PAHs, produced from combustion, as major contributors to that difference. In turn, this would lend support to the substitution of nicotine replacement products or e-cigarettes for tobacco smoking in pregnancy or for secondhand smoke exposure of pregnant women. However, nicotine is itself injurious to the fetal brain, so the relative improvement is predicated on keeping the nicotine consumption level at or below that achieved with cigarettes, and in no case is the use of nicotine a "safe" alternative. Second, PAHs are especially high in communities with polluted air (eg, inner cities) and epidemiological studies show a relationship between fetal exposure to PAHs, head circumference and cognitive performance (Perera *et al.*, 2003, 2006). Our findings imply that these populations will likewise experience a greater adverse impact from use of nicotine products in pregnancy and secondhand exposure because of the interaction of nicotine with PAHs.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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SUPPLEMENT

The Developmental Neurotoxicity of Tobacco Smoke Can Be Mimicked by a Combination of Nicotine and Benzo[a]pyrene: Effects on Cholinergic and Serotonergic Systems

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Edward D. Levin and Frederic J. Seidler

Contents:

TABLE S1: Body and Brain Region Weights (mean ± SE)

	Age	Male				Female			
		Control	Nic	BaP	Nic + BaP	Control	Nic	BaP	Nic + BaP
Body Weight (g)	30	115 ± 2	115 ± 4	112 ± 3	107 ± 2	104 ± 2	107 ± 3	105 ± 4	105 ± 2
	60	379 ± 9	394 ± 8	384 ± 11	392 ± 7	240 ± 7	246 ± 4	240 ± 1	236 ± 4
	100	538 ± 15	556 ± 12	579 ± 9	572 ± 10	295 ± 4	297 ± 11	295 ± 4	292 ± 5
	150	649 ± 24	717 ± 17	680 ± 19	677 ± 22	340 ± 9	331 ± 4	342 ± 13	322 ± 7
Region Weight (mg)									
frontal/parietal cortex	30	229 ± 6	244 ± 10	241 ± 14	243 ± 10	226 ± 11	232 ± 10	238 ± 8	206 ± 7
	60	279 ± 23	256 ± 10	245 ± 11	243 ± 12	221 ± 6	225 ± 8	239 ± 13	235 ± 13
	100	242 ± 10	249 ± 8	252 ± 9	243 ± 17	207 ± 12	225 ± 17	225 ± 10	226 ± 7
	150	249 ± 10	221 ± 11	263 ± 15	234 ± 14	216 ± 9	204 ± 9	234 ± 18	211 ± 14
temporal/occipital cortex	30	188 ± 9	176 ± 8	164 ± 8	158 ± 7	168 ± 10	159± 8	154 ± 10	175 ± 12
	60	174 ± 17	238 ± 24	215 ± 16	230 ± 20	202 ± 17	212 ± 15	215 ± 21	190 ± 16
	100	263 ± 22	247 ± 16	249 ± 13	249 ± 21	246 ± 18	233 ± 21	211 ± 19	248 ± 18
	150	257 ± 21	274 ± 16	230 ± 18	257 ± 18	252 ± 10	257 ± 5	229 ± 18	234 ± 15
hippocampus	30	119 ± 8	117 ± 6	112 ± 5	114 ± 2	115 ± 5	107 ± 5	107 ± 6	124 ± 6
	60	145 ± 5	144 ± 6	142 ± 10	140 ± 4	121 ± 9	135 ± 12	138 ± 3	130 ± 4
	100	144 ± 5	151 ± 5	147 ± 8	154 ± 6	130 ± 5	133 ± 6	136 ± 7	136 ± 5
	150	155 ± 7	154 ± 7	152 ± 7	153 ± 7	139 ± 9	145 ± 5	139 ± 6	138 ± 8
striatum	30	81 ± 6	77 ± 4	76 ± 3	73 ± 8	74 ± 3	74 ± 5	76 ± 4	82 ± 6
	60	89 ± 5	92 ± 4	102 ± 8	90 ± 7	73 ± 3	83 ± 6	74 ± 5	82 ± 6
	100	77 ± 8	86 ± 5	90 ± 7	106 ± 6	84 ± 9	97 ± 3	98 ± 7	80 ± 6
	150	98 ± 7	102 ± 7	98 ± 6	104 ± 6	92 ± 7	104 ± 4	101 ± 7	95 ± 5
midbrain	30	274 ± 11	284 ± 11	280 ± 11	287 ± 13	277 ± 6	283 ± 9	278 ± 14	270 ± 13
	60	330 ± 12	323 ± 27	340 ± 8	343 ± 14	321 ± 28	323 ± 12	314 ± 19	318 ± 12
	100	391 ± 5	368 ± 8	336 ± 18	330 ± 13	357 ± 20	325 ± 9	304 ± 12	330 ± 5
	150	376 ± 17	387 ± 11	387 ± 10	354 ± 17	316 ± 12	324 ± 3	334 ± 15	341 ± 13
brainstem	30	195 ± 8	169 ± 11	157 ± 6	163 ± 7	169 ± 9	161 ± 9	162 ± 8	154 ± 5
	60	248 ± 14	249 ± 9	245 ± 7	223 ± 14	219 ± 6	232 ± 13	216 ± 10	213 ± 5
	100	241 ± 6	253 ± 3	251 ± 13	258 ± 10	228 ± 13	227 ± 4	239 ± 7	236 ± 6
	150	278 ± 6	277 ± 10	248 ± 10	275 ± 10	247 ± 7	240 ± 7	252 ± 6	236 ± 6

Data represent mean ± SE obtained from six animals in each treatment group for each age and sex. For body weight, three-factor ANOVA (treatment, age, sex) indicates no significant treatment effects or interactions of treatment with the other factors.

For brain region weight, four-factor ANOVA (treatment, age, sex, region) indicates no significant treatment effects or interactions of treatment with the other factors. Note that weights for frontal/parietal cortex and temporal/occipital cortex are for the right hemisphere only, the portion used in this study.

Abbreviations: Nic = nicotine, BaP = benzo[a]pyrene

TABLE S2: ChAT Activity (mean ± SE)

Region	Postnatal Age	Male (pmol/min per mg protein)				Female (pmol/min per mg protein)			
		Control	Nic	BaP	Nic + BaP	Control	Nic	BaP	Nic + BaP
frontal/parietal cortex	30	0.82 ± 0.05	0.93 ± 0.02	0.86 ± 0.03	0.83 ± 0.03	0.89 ± 0.03	0.84 ± 0.04	0.84 ± 0.02	0.87 ± 0.03
	60	0.82 ± 0.02	0.87 ± 0.03	0.75 ± 0.02	0.81 ± 0.03	0.85 ± 0.05	0.83 ± 0.04	0.86 ± 0.03	0.84 ± 0.03
	100	0.89 ± 0.03	0.83 ± 0.04	0.83 ± 0.04	0.87 ± 0.02	0.90 ± 0.03	0.86 ± 0.02	0.84 ± 0.04	0.84 ± 0.03
	150	1.00 ± 0.07	1.13 ± 0.06	0.97 ± 0.10	1.07 ± 0.07	1.13 ± 0.06	1.12 ± 0.04	1.09 ± 0.08	1.11 ± 0.06
temporal/occipital cortex	30	0.53 ± 0.01	0.55 ± 0.01	0.53 ± 0.02	0.55 ± 0.03	0.57 ± 0.03	0.60 ± 0.02	0.55 ± 0.03	0.54 ± 0.02
	60	0.55 ± 0.03	0.63 ± 0.03	0.55 ± 0.01	0.60 ± 0.02	0.63 ± 0.02	0.61 ± 0.02	0.60 ± 0.03	0.62 ± 0.02
	100	0.67 ± 0.03	0.70 ± 0.03	0.68 ± 0.03	0.70 ± 0.03	0.70 ± 0.03	0.70 ± 0.02	0.73 ± 0.04	0.73 ± 0.02
	150	0.67 ± 0.01	0.74 ± 0.02	0.76 ± 0.03	0.81 ± 0.03	0.76 ± 0.03	0.77 ± 0.03	0.77 ± 0.05	0.77 ± 0.05
hippocampus	30	0.68 ± 0.03	0.74 ± 0.02	0.77 ± 0.04	0.76 ± 0.01	0.69 ± 0.03	0.69 ± 0.04	0.72 ± 0.04	0.66 ± 0.02
	60	0.77 ± 0.02	0.78 ± 0.04	0.76 ± 0.02	0.76 ± 0.05	0.82 ± 0.03	0.87 ± 0.04	0.79 ± 0.03	0.76 ± 0.03
	100	0.81 ± 0.02	0.89 ± 0.04	0.84 ± 0.03	0.89 ± 0.02	0.89 ± 0.03	0.95 ± 0.06	0.91 ± 0.03	0.92 ± 0.04
	150	0.96 ± 0.03	1.07 ± 0.04	1.01 ± 0.04	1.03 ± 0.02	1.08 ± 0.01	1.02 ± 0.04	1.06 ± 0.05	1.06 ± 0.04
striatum	30	1.96 ± 0.07	2.29 ± 0.06	2.04 ± 0.06	2.19 ± 0.09	2.17 ± 0.04	2.26 ± 0.09	2.20 ± 0.09	2.09 ± 0.10
	60	2.10 ± 0.12	2.10 ± 0.12	2.38 ± 0.21	2.27 ± 0.24	2.30 ± 0.14	2.42 ± 0.16	2.24 ± 0.09	2.29 ± 0.12
	100	2.32 ± 0.08	2.65 ± 0.07	2.46 ± 0.12	2.53 ± 0.12	2.31 ± 0.11	2.63 ± 0.07	2.45 ± 0.07	2.33 ± 0.11
	150	2.60 ± 0.06	3.07 ± 0.12	2.67 ± 0.13	2.89 ± 0.11	2.71 ± 0.07	2.73 ± 0.13	2.64 ± 0.14	2.79 ± 0.12
midbrain	30	0.67 ± 0.04	0.77 ± 0.04	0.79 ± 0.02	0.82 ± 0.02	0.77 ± 0.03	0.77 ± 0.02	0.81 ± 0.03	0.75 ± 0.04
	60	0.60 ± 0.04	0.70 ± 0.03	0.72 ± 0.03	0.69 ± 0.02	0.74 ± 0.03	0.77 ± 0.02	0.73 ± 0.05	0.71 ± 0.03
	100	0.70 ± 0.04	0.82 ± 0.02	0.73 ± 0.02	0.75 ± 0.02	0.77 ± 0.03	0.78 ± 0.03	0.80 ± 0.03	0.81 ± 0.03
	150	0.73 ± 0.03	0.77 ± 0.03	0.74 ± 0.02	0.68 ± 0.02	0.76 ± 0.03	0.77 ± 0.03	0.79 ± 0.02	0.69 ± 0.02
brainstem	30	1.20 ± 0.05	1.36 ± 0.02	1.41 ± 0.03	1.41 ± 0.05	1.29 ± 0.05	1.34 ± 0.04	1.36 ± 0.01	1.32 ± 0.04
	60	1.14 ± 0.03	1.20 ± 0.03	1.22 ± 0.05	1.25 ± 0.03	1.22 ± 0.03	1.27 ± 0.02	1.30 ± 0.03	1.25 ± 0.03
	100	1.17 ± 0.05	1.21 ± 0.05	1.11 ± 0.04	1.16 ± 0.03	1.24 ± 0.03	1.23 ± 0.05	1.27 ± 0.06	1.26 ± 0.03
	150	1.19 ± 0.04	1.29 ± 0.04	1.26 ± 0.05	1.22 ± 0.02	1.24 ± 0.02	1.29 ± 0.05	1.23 ± 0.03	1.26 ± 0.04

Data represent mean ± SE obtained from six animals in each treatment group for each age and sex. Results of multivariate ANOVA are provided in the main text. Note that the assays for each region and age were run in separate experiments, so absolute values cannot be compared strictly across ages or between regions. Accordingly, statistical comparisons in the main text were conducted on log-transformed data, which evaluates the treatment differences as a proportion to control values, rather than as an arithmetic difference. Representing the data as proportional differences (percent control) enables a full comparison of treatment effects and treatment interactions with all the other variables, even though absolute values for the controls cannot be compared across regions and ages.

Abbreviations: Nic = nicotine, BaP = benzo[a]pyrene

TABLE S3: HC3 Binding (mean ± SE)

Region	Postnatal Age	Male (fmol/mg protein)				Female (fmol/mg protein)			
		Control	Nic	BaP	Nic + BaP	Control	Nic	BaP	Nic + BaP
frontal/parietal cortex	30	16.9 ± 1.9	17.3 ± 2.1	16.0 ± 1.6	14.0 ± 1.2	17.3 ± 1.0	15.2 ± 1.6	13.0 ± 0.6	13.3 ± 1.3
	60	19.3 ± 1.0	20.5 ± 1.0	15.8 ± 1.1	17.9 ± 0.7	19.2 ± 1.4	19.5 ± 1.0	17.7 ± 1.3	18.0 ± 0.9
	100	21.6 ± 1.5	22.9 ± 2.0	20.2 ± 1.3	19.6 ± 1.0	23.4 ± 1.6	22.7 ± 0.5	19.5 ± 1.2	20.3 ± 1.0
	150	20.3 ± 0.8	24.2 ± 1.0	21.9 ± 2.1	22.7 ± 2.0	24.0 ± 0.4	25.6 ± 1.2	24.9 ± 3.1	21.4 ± 1.4
temporal/occipital cortex	30	11.1 ± 0.7	9.5 ± 0.8	11.5 ± 1.1	9.6 ± 1.0	12.5 ± 1.2	12.2 ± 0.9	9.9 ± 0.7	9.4 ± 0.8
	60	10.7 ± 0.5	11.3 ± 0.7	11.0 ± 0.8	10.2 ± 0.6	12.6 ± 1.0	11.5 ± 0.7	10.2 ± 0.7	10.3 ± 0.6
	100	12.4 ± 0.8	12.7 ± 1.2	13.8 ± 1.2	12.0 ± 0.9	14.3 ± 0.6	12.9 ± 0.9	12.3 ± 0.8	14.1 ± 0.9
	150	13.6 ± 0.8	14.1 ± 0.4	13.3 ± 0.7	14.8 ± 1.0	15.8 ± 1.5	14.3 ± 0.9	15.9 ± 1.5	13.3 ± 1.1
hippocampus	30	16.2 ± 0.6	15.6 ± 1.5	14.4 ± 1.5	14.1 ± 1.2	16.3 ± 0.4	16.1 ± 1.7	13.7 ± 1.0	14.6 ± 1.5
	60	14.5 ± 0.8	13.6 ± 1.2	11.9 ± 0.6	11.3 ± 0.7	14.3 ± 1.1	13.6 ± 0.7	12.7 ± 1.0	11.7 ± 0.9
	100	14.4 ± 0.8	16.1 ± 0.9	14.8 ± 0.9	14.4 ± 0.8	16.7 ± 0.3	14.7 ± 1.0	15.0 ± 1.1	14.4 ± 0.9
	150	17.8 ± 0.3	18.0 ± 0.7	15.4 ± 0.6	16.7 ± 0.7	16.1 ± 1.1	17.7 ± 0.5	17.1 ± 0.3	15.6 ± 0.6
striatum	30	82 ± 7	82 ± 5	75 ± 6	72 ± 3	95 ± 3	95 ± 7	94 ± 5	67 ± 4
	60	108 ± 3	99 ± 7	105 ± 6	90 ± 9	115 ± 13	118 ± 11	95 ± 8	96 ± 5
	100	96 ± 8	127 ± 12	108 ± 8	105 ± 6	117 ± 9	117 ± 6	96 ± 5	96 ± 6
	150	139 ± 10	162 ± 9	128 ± 7	116 ± 10	130 ± 5	128 ± 5	138 ± 13	119 ± 2
midbrain	30	12.9 ± 0.9	15.8 ± 1.0	15.6 ± 1.7	13.1 ± 1.3	15.7 ± 0.5	16.1 ± 1.3	13.8 ± 1.3	14.9 ± 1.0
	60	13.7 ± 1.2	15.4 ± 1.0	14.4 ± 0.9	12.9 ± 0.7	15.9 ± 1.5	15.8 ± 1.1	13.5 ± 1.1	14.0 ± 1.4
	100	12.0 ± 1.2	12.1 ± 0.7	11.1 ± 0.3	10.6 ± 0.5	12.1 ± 0.9	11.8 ± 0.7	11.8 ± 0.5	12.4 ± 0.4
	150	11.8 ± 0.7	14.8 ± 0.7	13.6 ± 0.9	12.0 ± 0.6	13.6 ± 0.8	13.5 ± 0.7	13.9 ± 0.7	13.0 ± 0.9
brainstem	30	9.8 ± 0.9	9.8 ± 1.4	7.9 ± 0.5	7.8 ± 0.1	9.3 ± 0.3	8.3 ± 0.7	8.7 ± 0.5	7.8 ± 0.4
	60	10.6 ± 1.1	11.5 ± 1.2	10.7 ± 1.0	9.0 ± 0.7	9.6 ± 0.3	10.1 ± 0.8	9.4 ± 0.8	8.4 ± 0.4
	100	7.3 ± 0.4	8.0 ± 0.4	7.9 ± 0.4	7.3 ± 0.5	7.0 ± 0.3	8.2 ± 1.0	7.9 ± 0.6	7.5 ± 0.6
	150	8.3 ± 0.5	9.0 ± 0.4	8.1 ± 0.6	8.1 ± 0.3	7.9 ± 0.3	8.6 ± 0.5	7.9 ± 0.3	7.1 ± 0.4

Data represent mean ± SE obtained from six animals in each treatment group for each age and sex. Results of multivariate ANOVA are provided in the main text. Note that the assays for each region and age were run in separate experiments, so absolute values cannot be compared strictly across ages or between regions. Accordingly, statistical comparisons in the main text were conducted on log-transformed data, which evaluates the treatment differences as a proportion to control values, rather than as an arithmetic difference. Representing the data as proportional differences (percent control) enables a full comparison of treatment effects and treatment interactions with all the other variables, even though absolute values for the controls cannot be compared across regions and ages.

Abbreviations: Nic = nicotine, BaP = benzo[a]pyrene

TABLE S4: HC3/ChAT ratio (mean ± SE)

Region	Postnatal Age	Male				Female			
		Control	Nic	BaP	Nic + BaP	Control	Nic	BaP	Nic + BaP
frontal/parietal cortex	30	20.8 ± 2.5	18.5 ± 1.9	19.0 ± 2.2	16.9 ± 1.0	19.5 ± 1.4	18.4 ± 2.2	15.5 ± 0.4	15.3 ± 1.3
	60	23.5 ± 1.2	24.5 ± 0.3	21.1 ± 1.1	21.6 ± 1.1	22.8 ± 1.1	23.4 ± 0.8	20.7 ± 1.6	21.4 ± 0.9
	100	24.3 ± 1.2	27.5 ± 2.1	24.6 ± 2.3	22.6 ± 1.4	25.9 ± 1.4	26.4 ± 1.2	23.1 ± 0.8	24.2 ± 1.8
	150	20.7 ± 1.2	20.8 ± 0.7	22.9 ± 1.5	21.3 ± 1.3	21.8 ± 1.3	23.2 ± 1.8	22.4 ± 1.6	19.7 ± 1.8
temporal/occipital cortex	30	21.1 ± 1.5	17.6 ± 1.7	21.7 ± 2.4	17.3 ± 1.7	21.9 ± 2.1	20.2 ± 1.4	18.5 ± 1.6	17.3 ± 1.2
	60	19.8 ± 1.7	18.2 ± 1.3	20.0 ± 1.4	17.1 ± 0.8	20.1 ± 1.3	18.9 ± 0.9	16.9 ± 0.8	16.7 ± 0.7
	100	18.7 ± 1.3	18.0 ± 1.4	20.4 ± 1.5	17.1 ± 1.2	20.5 ± 0.8	18.7 ± 1.3	16.8 ± 0.6	19.6 ± 1.5
	150	20.3 ± 1.2	19.2 ± 0.5	17.6 ± 1.2	18.2 ± 1.0	20.6 ± 1.4	18.6 ± 1.0	20.4 ± 2.5	17.4 ± 1.3
hippocampus	30	24.8 ± 1.0	21.5 ± 2.7	18.7 ± 1.7	18.6 ± 1.8	23.9 ± 1.9	23.7 ± 2.9	19.1 ± 1.3	22.5 ± 2.5
	60	18.8 ± 1.0	17.4 ± 0.9	15.8 ± 0.8	14.9 ± 0.7	17.3 ± 1.2	16.2 ± 0.4	16.1 ± 1.1	16.0 ± 1.3
	100	17.8 ± 1.2	18.2 ± 1.2	17.6 ± 1.2	16.3 ± 1.2	19.2 ± 0.4	16.4 ± 1.5	16.5 ± 1.0	15.9 ± 1.2
	150	18.4 ± 0.8	16.9 ± 0.9	15.4 ± 1.1	16.2 ± 0.6	14.9 ± 0.9	17.3 ± 0.5	16.3 ± 0.6	14.9 ± 0.8
striatum	30	43 ± 4	34 ± 3	37 ± 4	33 ± 1	45 ± 2	42 ± 4	43 ± 3	33 ± 3
	60	52 ± 4	48 ± 5	46 ± 5	41 ± 5	50 ± 5	49 ± 4	43 ± 5	41 ± 2
	100	42 ± 4	46 ± 4	45 ± 5	42 ± 3	50 ± 3	45 ± 3	39 ± 1	41 ± 3
	150	53 ± 3	53 ± 5	49 ± 4	40 ± 3	48 ± 2	48 ± 4	52 ± 3	43 ± 1
midbrain	30	19.8 ± 2.1	20.9 ± 2.0	19.7 ± 2.1	16.0 ± 1.4	20.6 ± 1.0	21.0 ± 2.1	17.0 ± 1.1	19.9 ± 1.2
	60	22.9 ± 1.6	21.9 ± 1.2	20.1 ± 1.0	19.0 ± 1.2	21.4 ± 1.6	20.7 ± 1.8	18.5 ± 1.6	19.7 ± 1.4
	100	17.2 ± 1.3	14.8 ± 0.9	15.2 ± 0.5	14.2 ± 0.8	15.7 ± 1.0	15.3 ± 1.2	14.8 ± 0.2	15.4 ± 0.6
	150	16.3 ± 0.8	19.5 ± 1.5	18.4 ± 1.2	17.5 ± 0.6	18.0 ± 1.0	17.7 ± 1.1	17.6 ± 0.7	18.1 ± 1.7
brainstem	30	8.3 ± 1.0	7.2 ± 1.0	5.6 ± 0.5	5.5 ± 0.2	7.2 ± 0.4	6.2 ± 0.7	6.4 ± 0.3	5.9 ± 0.4
	60	9.4 ± 1.0	9.5 ± 0.8	8.8 ± 0.8	7.2 ± 0.5	8.0 ± 0.3	8.0 ± 0.6	7.2 ± 0.6	6.7 ± 0.2
	100	6.3 ± 0.4	6.6 ± 0.3	7.2 ± 0.3	6.3 ± 0.3	5.7 ± 0.2	6.6 ± 0.6	6.3 ± 0.5	6.0 ± 0.4
	150	7.0 ± 0.4	6.7 ± 0.4	6.7 ± 0.5	6.6 ± 0.2	6.4 ± 0.3	6.7 ± 0.4	6.4 ± 0.3	5.6 ± 0.2

Data represent mean ± SE obtained from six animals in each treatment group for each age and sex. Results of multivariate ANOVA are provided in the main text. Note that the assays for each region and age were run in separate experiments, so absolute values cannot be compared strictly across ages or between regions. Accordingly, statistical comparisons in the main text were conducted on log-transformed data, which evaluates the treatment differences as a proportion to control values, rather than as an arithmetic difference. Representing the data as proportional differences (percent control) enables a full comparison of treatment effects and treatment interactions with all the other variables, even though absolute values for the controls cannot be compared across regions and ages.

Abbreviations: Nic = nicotine, BaP = benzo[a]pyrene

TABLE S5: nAChR Binding (mean ± SE)

Region	Postnatal Age	Male (fmol/mg protein)				Female (fmol/mg protein)			
		Control	Nic	BaP	Nic + BaP	Control	Nic	BaP	Nic + BaP
frontal/parietal cortex	30	67 ± 6	61 ± 5	65 ± 7	60 ± 4	68 ± 5	64 ± 6	56 ± 3	56 ± 4
	60	55 ± 2	58 ± 3	52 ± 2	54 ± 3	59 ± 4	59 ± 4	53 ± 3	50 ± 2
	100	48 ± 2	53 ± 4	51 ± 3	52 ± 3	53 ± 2	51 ± 3	52 ± 3	50 ± 3
	150	56 ± 4	67 ± 5	64 ± 3	60 ± 2	59 ± 2	65 ± 4	62 ± 4	55 ± 4
temporal/occipital cortex	30	78 ± 5	84 ± 7	88 ± 8	77 ± 4	87 ± 4	92 ± 5	81 ± 5	75 ± 5
	60	66 ± 4	65 ± 5	64 ± 4	65 ± 5	59 ± 4	58 ± 5	67 ± 6	54 ± 2
	100	51 ± 3	58 ± 4	51 ± 3	56 ± 3	50 ± 3	55 ± 4	56 ± 3	53 ± 2
	150	62 ± 4	65 ± 3	69 ± 2	67 ± 4	61 ± 4	62 ± 4	66 ± 3	59 ± 5
hippocampus	30	45 ± 3	42 ± 3	47 ± 4	41 ± 3	50 ± 2	48 ± 5	46 ± 4	45 ± 3
	60	33 ± 2	32 ± 3	28 ± 2	26 ± 2	32 ± 3	32 ± 3	31 ± 2	32 ± 3
	100	23 ± 2	29 ± 2	27 ± 2	28 ± 2	26 ± 1	25 ± 2	30 ± 2	22 ± 1
	150	26 ± 1	28 ± 1	23 ± 1	26 ± 1	24 ± 2	26 ± 1	27 ± 2	28 ± 1
midbrain	30	83 ± 7	91 ± 5	97 ± 9	82 ± 6	95 ± 7	92 ± 9	86 ± 3	85 ± 4
	60	73 ± 5	71 ± 5	73 ± 4	74 ± 4	74 ± 6	74 ± 5	70 ± 5	70 ± 4
	100	50 ± 3	53 ± 2	49 ± 2	54 ± 2	49 ± 2	55 ± 4	54 ± 3	51 ± 2
	150	56 ± 1	64 ± 3	60 ± 3	55 ± 2	58 ± 3	59 ± 2	60 ± 3	56 ± 3
brainstem	30	39 ± 3	42 ± 3	38 ± 1	40 ± 2	41 ± 2	38 ± 2	39 ± 2	35 ± 1
	60	36 ± 3	40 ± 3	37 ± 3	35 ± 1	33 ± 2	34 ± 2	36 ± 2	33 ± 1
	100	26 ± 1	28 ± 1	29 ± 1	27 ± 1	26 ± 1	28 ± 1	28 ± 1	25 ± 1
	150	25 = 1	28 ± 1	27 ± 1	27 ± 1	26 ± 1	27 ± 2	27 ± 1	25 ± 1

Data represent mean ± SE obtained from six animals in each treatment group for each age and sex. Results of multivariate ANOVA are provided in the main text. Note that the assays for each region and age were run in separate experiments, so absolute values cannot be compared strictly across ages or between regions. Accordingly, statistical comparisons in the main text were conducted on log-transformed data, which evaluates the treatment differences as a proportion to control values, rather than as an arithmetic difference. Representing the data as proportional differences (percent control) enables a full comparison of treatment effects and treatment interactions with all the other variables, even though absolute values for the controls cannot be compared across regions and ages.

Abbreviations: Nic = nicotine, BaP = benzo[a]pyrene

TABLE S6: 5HT Receptor Binding (mean ± SE)

Subtype and Region	Postnatal Age	Male (fmol/mg protein)				Female (fmol/mg protein)			
$5HT_{1A}$ Receptors		Control	Nic	BaP	Nic + BaP	Control	Nic	BaP	Nic + BaP
frontal/parietal cortex	30	96 ± 11	91 ± 13	93 ± 15	83 ± 11	107 ± 9	102 ± 17	70 ± 5	76 ± 9
	60	66 ± 9	71 ± 8	54 ± 7	61 ± 9	68 ± 11	77 ± 9	61 ± 9	46 ± 5
	100	49 ± 6	61 ± 9	48 ± 7	55 ± 6	67 ± 7	64 ± 11	67 ± 8	65 ± 8
	150	76 ± 10	97 ± 12	92 ± 5	82 ± 10	103 ± 3	106 ± 7	99 ± 15	95 ± 12
temporal/occipital cortex	30	116 ± 11	123 ± 15	132 ± 12	120 ± 13	129 ± 13	153 ± 11	110 ± 10	109 ± 11
	60	79 ± 6	94 ± 10	83 ± 10	79 ± 10	92 ± 12	77 ± 10	80 ± 10	75 ± 10
	100	59 ± 8	75 ± 8	72 ± 7	73 ± 6	63 ± 4	80 ± 7	78 ± 5	79 ± 7
	150	75 ± 2	80 ± 3	96 ± 11	89 ± 14	77 ± 5	80 ± 13	77 ± 11	92 ± 9
midbrain	30	39 ± 6	56 ± 2	47 ± 4	45 ± 7	50 ± 4	58 ± 6	37 ± 5	47 ± 3
	60	34 ± 3	43 ± 3	35 ± 5	37 ± 4	39 ± 5	45 ± 5	36 ± 2	39 ± 6
	100	23 ± 2	23 ± 1	22 ± 2	22 ± 2	19 ± 1	28 ± 2	25 ± 1	26 ± 3
	150	28 ± 1	33 ± 2	29 ± 3	24 ± 3	32 ± 2	28 ± 2	31 ± 2	28 ± 2
brainstem	30	40 ± 6	41 ± 5	32 ± 3	35 ± 3	39 ± 2	37 ± 4	43 ± 5	32 ± 2
	60	25 ± 3	28 ± 2	27 ± 3	22 ± 3	22 ± 3	31 ± 3	25 ± 3	23 ± 1
	100	16 ± 2	19 ± 2	19 ± 2	18 ± 1	17 ± 1	21 ± 2	21 ± 3	21 ± 3
	150	17 ± 1	22 ± 2	17 ± 2	19 ± 2	19 ± 1	22 ± 3	20 ± 1	20 ± 2
$5HT_2$ Receptors									
frontal/parietal cortex	30	169 ± 1	179 ± 7	189 ± 6	171 ± 5	181 ± 4	178 ± 6	186 ± 5	170 ± 9
	60	168 ± 3	183 ± 6	176 ± 4	181 ± 4	172 ± 5	186 ± 7	188 ± 3	173 ± 4
	100	138 ± 5	161 ± 3	162 ± 5	156 ± 4	168 ± 8	158 ± 4	163 ± 2	148 ± 3
	150	130 ± 3	167 ± 7	145 ± 7	141 ± 5	147 ± 3	172 ± 4	160 ± 8	143 ± 7
temporal/occipital cortex	30	80 ± 4	97 ± 6	97 ± 5	80 ± 3	100 ± 5	102 ± 8	101 ± 4	94 ± 6
	60	85 ± 5	85 ± 2	91 ± 4	93 ± 5	86 ± 3	98 ± 2	102 ± 1	86 ± 4
	100	74 ± 4	77 ± 5	76 ± 3	73 ± 2	73 ± 3	83 ± 4	80 ± 2	74 ± 2
	150	64 ± 2	73 ± 2	82 ± 4	74 ± 5	78 ± 2	79 ± 4	84 ± 4	69 ± 2
midbrain	30	22 ± 1	28 ± 2	31 ± 3	26 ± 2	26 ± 1	28 ± 1	30 ± 2	25 ± 2
	60	19 ± 2	24 ± 1	25 ± 2	26 ± 2	25 ± 3	28 ± 2	23 ± 2	23 ± 2
	100	20 ± 1	19 ± 1	17 ± 1	18 ± 1	20 ± 1	18 ± 1	21 ± 1	19 ± 1
	150	17 ± 1	22 ± 1	23 ± 2	18 ± 1	19 ± 1	21 ± 1	23 ± 2	21 ± 2
brainstem	30	22 ± 1	26 ± 1	24 ± 1	25 ± 1	25 ± 1	25 ± 1	25 ± 1	22 ± 1
	60	19 ± 1	21 ± 1	22 ± 1	21 ± 1	19 ± 1	21 ± 1	22 ± 1	20 ± 1
	100	16 ± 1	20 ± 1	19 ± 1	18 ± 1	16 ± 1	18 ± 1	17 ± 1	16 ± 1
	150	17 ± 1	20 ± 2	18 ± 2	18 ± 1	19 ± 1	21 ± 1	20 ± 1	18 ± 1

Data represent mean ± SE obtained from six animals in each treatment group for each age and sex. Results of multivariate ANOVA are provided in the main text. Note that the assays for each region and age were run in separate experiments, so absolute values cannot be compared strictly across ages or between regions. Accordingly, statistical comparisons in the main text were conducted on log-transformed data, which evaluates the treatment differences as a proportion to control values, rather than as an arithmetic difference. Representing the data as proportional differences (percent control) enables a full comparison of treatment effects and treatment interactions with all the other variables, even though absolute values for the controls cannot be compared across regions and ages.

Abbreviations: Nic = nicotine, BaP = benzo[a]pyrene