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Benzo[a]pyrene Impairs Neurodifferentiation in PC12 Cells

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Abstract

Animal studies indicate neurobehavioral anomalies after prenatal exposure to benzo[a]pyrene (BaP). In order to determine if BaP directly affects neurodevelopment, we compared its effects to those of the organophosphate insecticide, chlorpyrifos (CPF), in undifferentiated and differentiating neuronotypic PC12 cells, evaluating indices of cell replication, cell number, neurite outgrowth and phenotypic differentiation. Unlike CPF, BaP did not inhibit DNA synthesis in undifferentiated cells. In cells undergoing nerve growth factor-induced differentiation, CPF reduced cell numbers (assessed by DNA content) whereas BaP increased them, suggesting a delay in the transition between cell replication and differentiation. Indices of cell enlargement (total protein/DNA) and neurite outgrowth (membrane protein/DNA) also showed opposite effects of CPF (increases) and BaP (decreases). We directly confirmed BaP impairment of neurodifferentiation by measuring markers for the two neurotransmitter phenotypes expressed by PC12 cells: tyrosine hydroxylase (dopamine phenotype) and choline acetyltransferase (acetylcholine phenotype). BaP significantly reduced both markers in differentiating cells, with a preferentially greater effect on the acetylcholine phenotype. Our results indicate that low, nontoxic levels of BaP can impair neurodifferentiation, resulting in excess cell numbers at the expense of the emergence of neurotransmitter phenotypes. BaP thus has direct actions on developing neuronal cells that could contribute to the adverse neurodevelopmental effects seen with in vivo exposures.

Keywords

Benzo[a]pyrene; Chlorpyrifos; In vitro; Neurodevelopment; Organophosphate insecticides; PC12 cells; Pesticides; Polycyclic aromatic hydrocarbons

INTRODUCTION

Human exposure to polycyclic aromatic hydrocarbons is virtually ubiquitous due to their generation as combustion products and inclusion in food. Although a great deal of attention has been focused on their carcinogenic properties, it is increasingly evident that these agents are also neuroactive, raising the specter that they may contribute to the emerging, “silent pandemic” of neurodevelopmental disorders [13]. Recent studies indicate a positive correlation between fetal exposure to these agents and adverse consequences such as reduced childhood head circumference and impaired neurobehavioral development [23,24]. However, surprisingly little has been done to establish a mechanistic link between exposure to polycyclic

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aromatic hydrocarbons and disruption of brain development. For benzo[a]pyrene (BaP), the prototype of this chemical class, neural and behavioral effects have been noted after exposure in adulthood [28]. With prenatal exposures, BaP alters later behavioral performance [45] and affects the expression of neurotransmitter receptors [6]; the latter effect can also be recapitulated by exposing postmitotic cultured neurons in vitro [6]. However, fetal BaP exposure also influences the expression of nuclear transcription factors that mediate the onset of neuronal cell differentiation [14], suggesting that there may be far more widespread effects of this agent in the developing brain, ultimately contributing to neurobehavioral impairment [46].

One of the major limitations of in vivo studies in animal models is the potential for confounding effects mediated by actions of the toxicant on maternal nutrition, endocrine status, neonatal caretaking, or other factors that impact neurobehavioral development of the offspring. Accordingly, the current study was undertaken to explore the possibility that BaP acts directly on developing neuronal cells to disrupt neurodifferentiation. We utilized PC12 cells, a neurotypic cell line derived from rat pheochromocytoma that has been widely exploited as a model of neuronal development [39]. Because they are transformed cells, the PC12 line enables the delineation of adverse effects aimed at mitotic activity, whereas cultured primary neurons do not maintain cell division and thus cannot detect adverse effects on this likely neurotoxic target. When PC12 cells are treated with nerve growth factor (NGF), they gradually cease dividing and instead undergo neurodifferentiation, with development of neuritic projections, electrical excitability, and neurotransmitter phenotypes for dopamine and acetylcholine [12,38,39]. In contrast, primary neurons provide a heterogeneous population of cell types, discoordinated staging of differentiation, and a large number of phenotypes. For those reasons, the PC12 model has been used to characterize essential features of the developmental neurotoxicity of a wide variety of chemicals, including pesticides, neurotoxic drugs, metals and organometals [1–3,5,7–11,16,17,19–22,25,26,29,34,38,40,42]. Our objective here was to evaluate the propensity of BaP to elicit developmental neurotoxicity, specifically in comparison to the organophosphate pesticide, chlorpyrifos (CPF), a known developmental neurotoxicant that has effects on PC12 cells that mirror its impact on brain development in vivo [30,31].

Our evaluations were based on established protocols for screening of developmental neurotoxicants with PC12 cells [25–27,32–34,38]. In undifferentiated cells, we assessed antimitotic activity through measurement of [³H]thymidine incorporation into DNA, along with measuring DNA content to evaluate potential effects on cell number, since each neurotypic cell contains only a single nucleus [44]. In differentiating cells, we also measured DNA content, but then also evaluated effects on cell growth by monitoring the increase in cell size accompanying differentiation (total protein/DNA) as well as membrane outgrowth associated with the formation of neurites (membrane protein/DNA, membrane/total protein). Finally, we assayed the two enzymes delineating differentiation into the dopamine and acetylcholine phenotypes, tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), respectively.

MATERIALS AND METHODS

All of the techniques used in this study have appeared in previous papers and accordingly, only brief descriptions of procedures will be given.

Cell cultures

Because of the clonal instability of the PC12 cell line [12], the experiments were performed on cells that had undergone fewer than five passages. PC12 cells were grown as described earlier [8,27,38], in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10%

inactivated horse serum (Sigma Chemical Co., St. Louis, MO), 5% fetal bovine serum (Sigma), and 50 µg/ml penicillin streptomycin (Invitrogen); cells were incubated with 5% CO₂ at 37° C. For studies in the undifferentiated state, the medium was changed 24h after seeding to include varying concentrations of BaP (Sigma) or, for comparison, 50 µM CPF (Chem Service, West Chester, PA). Because of the limited water solubility of BaP and CPF, all test agents were dissolved in dimethylsulfoxide to achieve a final concentration in the culture medium of 0.1%, which has no effect on replication or differentiation of PC12 cells [25,27,38]; control cultures contained the same concentration of dimethylsulfoxide. For studies in differentiating cells, 24h after seeding, the medium was changed to include 50 ng/mL of 2.5 S murine NGF (Invitrogen) and dimethylsulfoxide with or without the test agents, and were examined after 6 days of exposure, with medium changes (including test agents) every 48h. We chose the CPF concentration to elicit a robust response for each of the effects to be compared to the actions of BaP, but below the threshold for outright cytotoxicity or loss of viability [2,9,10,16,25–27,34,38]. For BaP, we evaluated a concentration range spanning nearly two orders of magnitude (0.3 to 10 µM) after preliminary experiments determined the appropriate range; although we used only a single CPF concentration for reference in the present studies, we have previously published full concentration-response information for CPF [16,25,33,34,38].

DNA synthesis

Undifferentiated cells were exposed to the test agents for 24h and then, for the final hour of exposure, the medium was changed to include the test agents along with 1 µCi/ml of [³H] thymidine (specific activity, 2 Ci/mmol; PerkinElmer Life Sciences, Waltham, MA). After 1h, the medium was aspirated and cells were harvested. DNA was precipitated and separated by established procedures [4,35]. We corrected incorporation values to the amount of DNA present in each culture to provide an index of DNA synthesis per cell [44].

DNA and protein ratios

Differentiating cells were harvested after 6 days of exposure, washed, and the DNA and protein fractions isolated and analyzed as described previously [25,27,38,41]. To prepare the cell membrane fraction, the homogenates were sedimented at 40,000 × g for 10 min and the pellet was washed and resedimented. Aliquots of the final resuspension were then assayed for membrane protein.

Enzyme activities

Differentiating cells were harvested after 6 days of exposure and were disrupted by homogenization in a ground-glass homogenizer fitted with a ground-glass pestle, using a buffer consisting of 154 mM NaCl and 10 mM sodium-potassium phosphate (pH 7.4). Aliquots were withdrawn for measurement of DNA [41]. ChAT assays were conducted by published techniques [18] using a substrate of 50 µM [¹⁴C]acetyl-coenzyme A (specific activity 60 mCi/mmol; PerkinElmer). Labeled acetylcholine was counted in a liquid scintillation counter and activity calculated as pmol synthesized per hour per µg DNA. TH activity was measured using [¹⁴C]tyrosine as a substrate and trapping the evolved ¹⁴CO₂ after coupled decarboxylation [18,43]. Each assay contained 55 µM [1-¹⁴C]L-tyrosine (Moravek Biochemicals, Inc., Brea, CA; specific activity, 51 mCi/mmol, diluted to 3.3 mCi/mmol with unlabeled tyrosine) as substrate and activity was calculated on the same basis as for ChAT.

Data analysis

All studies were performed with multiple batches of cells, with several independent cultures for each treatment in each batch. Results are presented as mean ± SE, with treatment comparisons carried out by analysis of variance (ANOVA) followed by Fisher's protected least significant difference test for post hoc comparisons of individual treatments. In the initial test,

we evaluated two ANOVA factors (treatment, cell batch) and found that the treatment effects were the same across the different batches of cells, although the absolute values differed from batch to batch. Accordingly, we normalized the results across batches prior to combining them for presentation. Significance was assumed at $p < 0.05$.

RESULTS

In undifferentiated cells, exposure to BaP for 24h in concentrations up to 10 μM failed to evoke any inhibition of DNA synthesis (Figure 1); the lack of effect was clearly distinguishable from the significant inhibition evoked by 50 μM CPF. Similarly, there was no effect of BaP on cell number, as determined from DNA content (data not shown).

The results were quite different for cells undergoing NGF-induced differentiation. Exposure to BaP for six days evoked a concentration-dependent increase in DNA content, with significant effects evident even at 0.3 μM (Figure 2A); in contrast, CPF reduced the value. Indices of cell growth likewise showed significant effects of BaP in the opposite direction from those of CPF. BaP reduced the total protein/DNA ratio whereas CPF increased it (Figure 2B); again, there were significant effects of BaP even at 0.3 μM . The same pattern was seen for the membrane protein fraction, as evidenced by decreases in the membrane protein/DNA for BaP and increases for CPF (Figure 2C). The effects of BaP on the two protein fractions were equivalent, as there was no change in the membrane/total protein ratio (data not shown).

BaP exposure during differentiation suppressed the emergence of neuronal phenotypes, as evidenced by significant reductions in TH (Figure 3A) and ChAT (Figure 3B). The effect on the cholinergic phenotype was greater than that on the dopaminergic type, resulting in an increase in the TH/ChAT ratio (Figure 3C); again, even the lowest BaP concentration produced a significant increase in the ratio.

DISCUSSION

Our results provide some of the first evidence for a direct impairment of neurodifferentiation by BaP, with more potent effects than those of CPF, a known neuroteratogen. Unlike CPF, which affects both neuronal cell replication and differentiation, BaP was devoid of effects on the mitotic index or cell numbers in undifferentiated PC12 cells. With the onset of differentiation, CPF evoked a corresponding loss of cell numbers, as evidenced by a lowering of DNA content in the cultures, whereas BaP evoked substantial elevations, even at concentrations two orders of magnitude below that of CPF. An increase in cell number can come about in three ways: (a) enhancement of cell replication, (b) suppression of normally-occurring cell loss, or (c) interference with the transition from cell replication to neurodifferentiation. The first possibility is effectively ruled out by the fact that BaP did not evoke any increase in the mitotic index. Although a “beneficial” effect on cell loss seems unlikely, we nevertheless performed additional determinations to distinguish that possibility from the more likely outcome of impaired neurodifferentiation.

The total protein/DNA ratio, an index of net cell size, showed a clear-cut deficit in differentiating cells exposed to BaP, whereas CPF, which impairs cell replication without suppressing cell growth, evoked an increase. A reduction in cell size can come about either through general growth retardation (smaller cells) or through interference with the development of neuritic projections that accompany neurodifferentiation. Although the membrane protein/DNA ratio will fall for both cases, a cell that is simply smaller, will have an increased membrane/total protein ratio because of its higher surface-to-volume ratio. It is therefore important to note that BaP evoked equivalent changes in membrane and total protein (no change in the ratio), indicating that the reduction in overall cell size reflected a parallel loss of

membrane area, i.e. impairment of the neurite formation that is characteristic of neurodifferentiation. Finally, we performed a definitive test as to whether BaP suppresses neurodifferentiation, examining the increase in TH and ChAT activities that accompanies the emergence of the dopaminergic and cholinergic phenotypes. Here again, BaP evoked clear-cut deficits in both markers. Superimposed on this shared effect, we obtained evidence for a selective impairment of the cholinergic phenotype, since BaP evoked a net increase in the TH/ChAT ratio, reflecting a significantly greater impairment for ChAT than for TH. One likely target for impairment of neurodifferentiation is the pivotal role played by NGF, both in this model system and in the developing nervous system *in vivo*. Interference with the effect of NGF could readily contribute to a delay in the fall-off of DNA synthesis, deficits in cell growth, reduced neurite formation and impaired differentiation into neurotransmitter phenotypes. Nevertheless, this cannot provide a total explanation for the observed outcomes, since a straightforward effect on NGF sensitivity should not provide selective effects on ChAT vs. TH, and should actually cause reductions in cell numbers because of increased apoptosis. Again, future work should focus on whether NGF or other neurotrophic factors are a target for the effects of BaP on neurodevelopment.

Although PC12 cells provide an advantage for identifying direct mechanisms of toxicant exposure on neurodevelopment, they share the limitations common to all cell culture systems, namely difficulties in assessing neuronal-glia interactions or architectural aspects of brain region development, maternal-fetal or neonatal pharmacokinetics and related issues of bioavailability, dose and bioeffective concentrations. The translation of *in vitro* to *in vivo* exposure is especially problematic, given the fact that the PC12 model involves transformed cells that are inherently more resistant to toxicants than are primary neurons and that effects need to be observed over a much shorter period in cells as compared to long-term exposures for brain development *in vivo*. It is thus important to note that we observed effects at the same submicromolar concentrations as those achieved with BaP exposures in fetal rats that lead to adverse effects on brain development [6].

Our findings are thus indicative of a highly selective effect of BaP on neuronal development, namely impairment of neurodifferentiation, leading to excessive accumulation of cells at the expense of both neurite formation and the emergence of neurotransmitter phenotypes. If the same effects occur with *in vivo* BaP exposures, each of these effects has important ramifications for brain development. First, the proper modeling of brain architecture requires the elimination of “exuberant” neurons so as to permit the formation and reinforcement of proper synaptic connections. Impairment of neuronal cell loss, as seen here, is thus likely to produce widespread interference with the development of neural circuitry. Second, the guidance of axonal projections to their proper targets requires carefully-coordinated interactions of developing neurons, glia and trophic factors, and accordingly, the appropriate timing of neurodifferentiation is a critical event. Here, we found a substantial delay in the transition from cell replication to neurodifferentiation and a corresponding impairment of neurite formation and expression of neurotransmitter phenotypes. Again, this could potentially lead to widespread “miswiring” of neural circuits. Finally, we saw a selective effect on the cholinergic phenotype as distinct from the dopaminergic phenotype, an outcome that BaP shares with a number of other environmental toxicants that are known to disrupt brain development and to evoke corresponding behavioral deficits [15–17,32,34,36,37]. Future studies should thus focus on behaviors linked to these phenotypes as likely targets for the consequences of developmental exposure to BaP.

In conclusion, our studies indicate that BaP can disrupt neuronal cell differentiation, providing mechanistic links to the epidemiological findings for human fetal and childhood exposures to polycyclic aromatic hydrocarbons that show an impact on neurobehavioral development [24]. Accordingly, animal studies of BaP exposure that show corresponding synaptic and behavioral

deficits are likely to involve direct actions on brain development, beyond any indirectly-mediated effects on the maternal-fetal unit, endocrine status, or other contributing factors.

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Abbreviations

ANOVA	analysis of variance
BaP	benzo[a]pyrene
ChAT	choline acetyltransferase
CPF	chlorpyrifos
NGF	nerve growth factor
TH	tyrosine hydroxylase

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DNA Synthesis in Undifferentiated Cells

24h Exposure, n=16
ANOVA: $p < 0.0002$

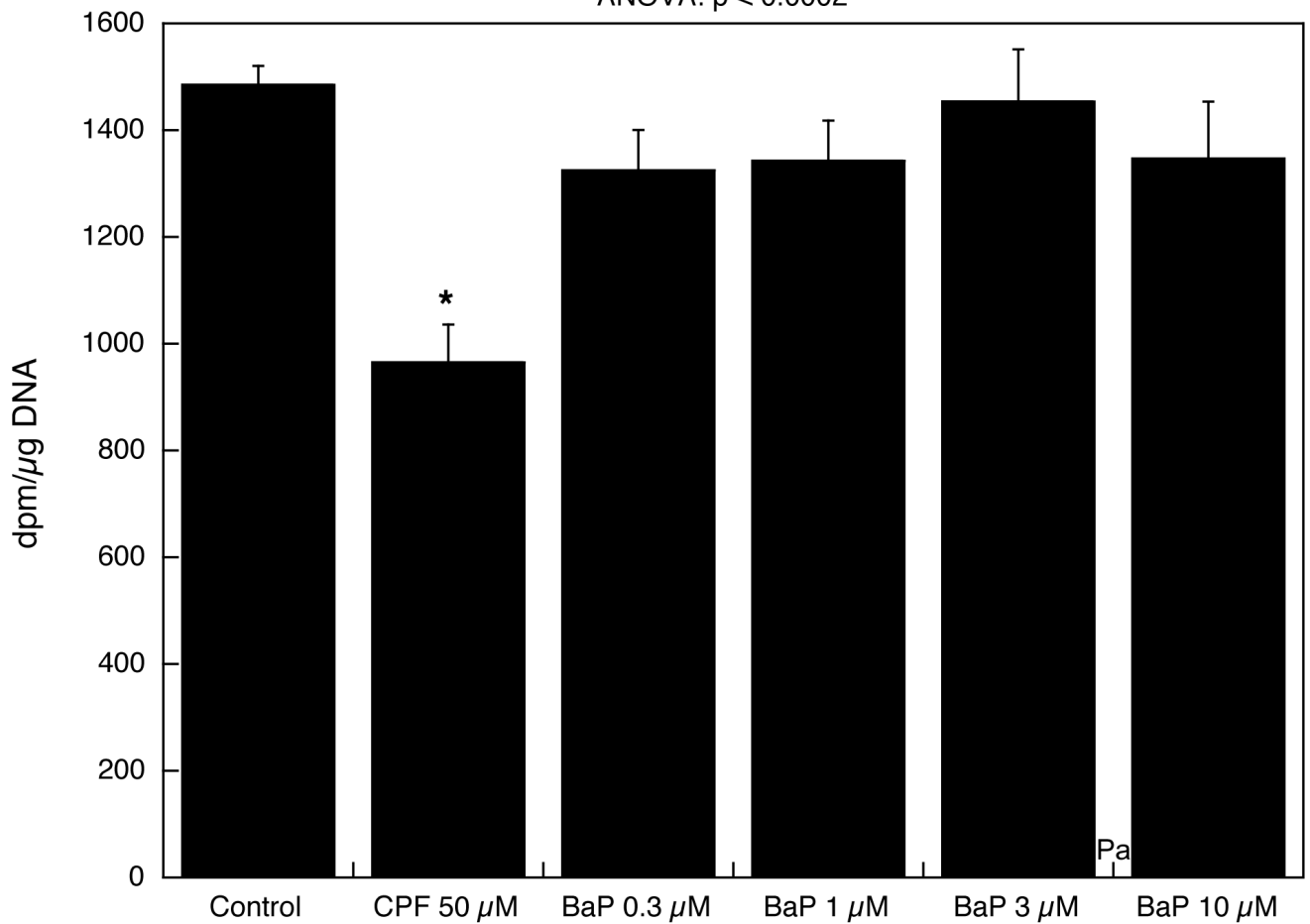


Figure 1.

Effects of benzo[a]pyrene (BaP) in comparison to chlorpyrifos (CPF) on DNA synthesis in undifferentiated PC12 cells. Data represent means and standard errors. ANOVA across all treatments appears at the top of the panel and asterisks denote values that differ significantly from the control.

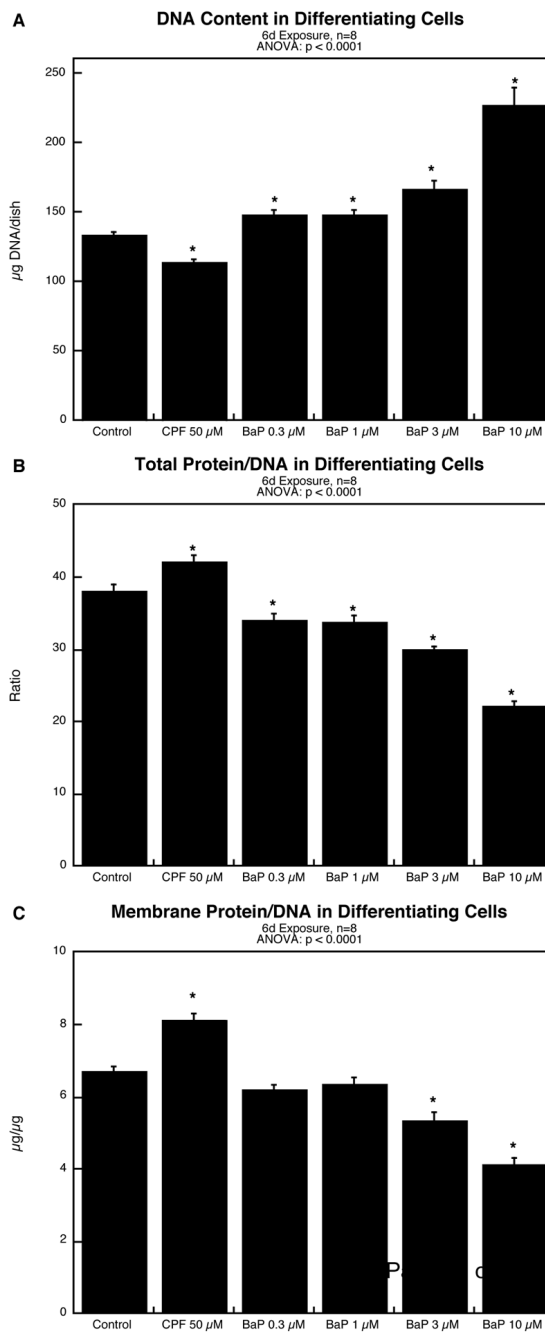


Figure 2. Effects of benzo[a]pyrene (BaP) in comparison to chlorpyrifos (CPF) in differentiating PC12 cells after 6 days of NGF-induced differentiation: (A) DNA content; (B) total protein/DNA ratio; (C) membrane protein/DNA ratio. Data represent means and standard errors. ANOVA across all treatments appears at the top of each panel and asterisks denote values that differ significantly from the control.

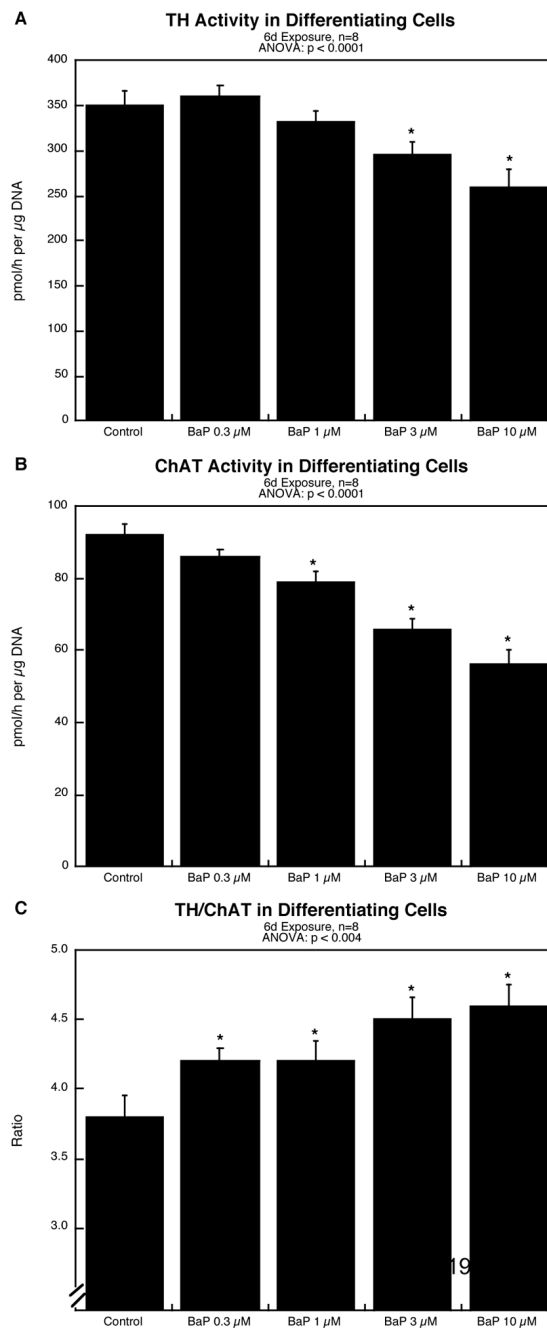


Figure 3.

Effects of BaP on differentiation into dopamine and acetylcholine phenotypes after 6 days of NGF-induced differentiation: (A) tyrosine hydroxylase activity, (B) choline acetyltransferase activity, (C) TH/ChAT ratio. Data represent means and standard errors. ANOVA across all treatments appears at the top of each panel and asterisks denote values that differ significantly from the control.