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Amelioration Strategies Fail To Prevent Tobacco Smoke Effects On Neurodifferentiation: Nicotinic Receptor Blockade, Antioxidants, Methyl Donors

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Abstract

Tobacco smoke exposure is associated with neurodevelopmental disorders. We used neuronotypic PC12 cells to evaluate the mechanisms by which tobacco smoke extract (TSE) affects neurodifferentiation. In undifferentiated cells, TSE impaired DNA synthesis and cell numbers to a much greater extent than nicotine alone; TSE also impaired cell viability to a small extent. In differentiating cells, TSE enhanced cell growth at the expense of cell numbers and promoted emergence of the dopaminergic phenotype. Nicotinic receptor blockade with mecamylamine was ineffective in preventing the adverse effects of TSE and actually enhanced the effect of TSE on the dopamine phenotype. A mixture of antioxidants (Vitamin C, Vitamin E, N-acetyl-L-cysteine) provided partial protection against cell loss but also promoted loss of the cholinergic phenotype in response to TSE. Notably, the antioxidants themselves altered neurodifferentiation, reducing cell numbers and promoting the cholinergic phenotype at the expense of the dopaminergic phenotype, an effect that was most prominent for N-acetyl-L-cysteine. Treatment with methyl donors (Vitamin B12, folic acid, choline) had no protectant effect and actually enhanced the cell loss evoked by TSE; they did have a minor, synergistic interaction with antioxidants protecting against TSE effects on growth. Thus, components of tobacco smoke perturb neurodifferentiation through mechanisms that cannot be attributed to the individual effects of nicotine, oxidative stress or interference with one-carbon metabolism. Consequently, attempted amelioration strategies may be

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Conflict of interest statement

TAS has received consultant income in the past three years from the following firms: Acorda Therapeutics (Ardsley NY), The Calwell Practice (Charleston WV), Carter Law (Peoria IL), Gutglass Erickson Bonville & Larson (Madison WI), Alexander Hawes (San Jose, CA), Pardieck Law (Seymour, IN), Tummel & Casso (Edinburg, TX), and Chaperone Therapeutics (Research Triangle Park, NC).

partially effective at best, or, as seen here, can actually aggravate injury interfering with normal developmental signals and/or by sensitizing cells to TSE effects on neurodifferentiation.

Keywords

Antioxidants; Methyl donors; Neurodifferentiation; Nicotine; PC12 cells; Tobacco smoke extract

1. Introduction

In addition to its major contribution to perinatal morbidity and mortality (Abbott and Winzer-Serhan 2012; DiFranza and Lew 1995; Pauly and Slotkin 2008), prenatal tobacco smoke exposure substantially increases the risk of neurodevelopmental disorders, including learning disabilities, attention deficit/hyperactivity disorder and conduct disorders (Cornelius and Day 2009; DiFranza and Lew 1995; Gaysina et al. 2013; Pauly and Slotkin 2008; Wakschlag et al. 1997). These outcomes reflect, in large measure, the adverse impact of nicotine on brain development (Pauly and Slotkin 2008; Slikker et al. 2005; Slotkin 2004, 2008). Neurotransmitter signals provide key neurotrophic information for brain assembly (Dreyfus 1998; Hohmann 2003; Lauder 1985), so that the inappropriate timing and intensity of cholinergic receptor stimulation by nicotine preempts normal developmental processes, leading to defects in neuronal cell replication and differentiation, in axonogenesis and synaptogenesis, and in the formation of neural circuits (Pauly and Slotkin 2008; Slotkin 2008). However, given the advent of nicotine replacement products for smoking cessation, as well as alternative nicotine delivery devices, it becomes important to distinguish whether the thousands of other components of tobacco smoke also play a role in adverse neurodevelopmental outcomes. A number of animal studies have identified effects of cigarette smoke that, in general, resemble those of nicotine at the biochemical, structural and functional levels (Bruijnzeel et al. 2011; Fuller et al. 2012; Golub et al. 2007; Gospe et al. 2009; Lobo Torres et al. 2012; Sekizawa et al. 2008; Slotkin et al. 2006a, b). Nevertheless, these exposure models simply reinforce the resemblance between smoke exposure and the effects of nicotine, rather than distinguishing between them. Additionally, they add an uncontrolled variable, since repetitive, involuntary exposure in a smoke-filled chamber is likely to elicit stress.

To study the direct effects of tobacco smoke on neurodifferentiation without participation of these confounds, we recently compared nicotine to other tobacco smoke products in PC12 cells, a neuronotypic cell line widely used to study neurodifferentiation (Costa 1998; Teng and Greene 1994). This cell line is poorly responsive to nicotine despite the presence of nicotinic acetylcholine receptors, generally requiring concentrations as high as 100-200 μ M for a full effect (Abreu-Villaça et al. 2005; Avila et al. 2003; Gueorguiev et al. 2000). Using tobacco smoke extract (TSE) at concentrations where nicotine by itself had little or no effect, we found that TSE promotes the transition from cell replication to neurodifferentiation (Slotkin et al. 2014), resulting in deficits in cell numbers. Additionally, TSE alters neurodifferentiation outcomes, promoting emergence of the dopaminergic phenotype over the cholinergic phenotype. In the current study, we used these basic findings to address two issues. First, what are the mechanisms underlying the effect of TSE on

neurodifferentiation, and second, can we use that information to ameliorate the effects? We focused on three likely mechanisms: actions on nicotinic receptors (the target for nicotine), oxidative stress and interference with one-carbon metabolic pathways. Smoking causes fetal oxidative stress (Aycicek and Ipek 2008; Fayol et al. 2005; Gitto et al. 2002; Orhon et al. 2009) and consequently, attempts have been made to ameliorate the adverse effects through vitamin C supplementation. In vivo, Vitamin C prevents tobacco smoke-induced lung damage in the fetus and improves neonatal respiratory outcome (McEvoy et al. 2014; Proskocil et al. 2005). While Vitamin C also prevents oxidative damage to the developing brain (Slotkin et al. 2011), it increases fetal nicotine levels, enhancing damage attributable to inappropriate nicotinic receptor stimulation (Slotkin et al. 2005). Here, using an in vitro system that eliminates pharmacokinetic factors, we explored the ability of three antioxidants in combination or separately to ameliorate the effects of TSE: Vitamin C, Vitamin E and N-acetyl-L-cysteine (NAC). Likewise, smokers are often advised to supplement their diets with methyl donors, including Vitamin B12, folic acid, and choline (Boeke et al. 2013; Steegers-Theunissen et al. 2013). Accordingly we also evaluated amelioration strategies with these agents.

2. METHODS

TSE (Arista Laboratories, Richmond, VA) was prepared from Kentucky Reference cigarettes (KY3R4F) on a Rotary Smoke Machine under ISO smoke conditions. The smoke condensate was collected on 92 mm filter pads, which were then extracted by shaking for 20 min with dimethylsulfoxide, to obtain a solution of approximately 20 mg of condensate per ml. Condensate aliquots were stored in amber vials at -80°C until used. Two cigarettes were smoked to produce each ml of extract and the final product contained 0.8 mg/ml (5 mM) nicotine (determined by the manufacturer).

2.1 Cell cultures and TSE treatments

Because of the clonal instability of the PC12 cell line (Fujita et al. 1989), the experiments were performed on cells that had undergone fewer than five passages. As described previously (Qiao et al. 2003; Song et al. 1998), PC12 cells (American Type Culture Collection CRL-1721, obtained from the Duke Comprehensive Cancer Center, Durham, NC) were seeded onto poly-D-lysine-coated plates in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% horse serum (Sigma), 5% fetal bovine serum (Sigma), and 50 $\mu\text{g}/\text{ml}$ penicillin streptomycin (Invitrogen, Carlsbad, CA). Incubations were carried out with 5% CO_2 at 37°C , standard conditions for PC12 cells. For studies in the undifferentiated state, the medium was changed 24h after seeding to include test agents. TSE was evaluated at low and high levels ("TSE low" and "TSE high"), calculated to produce final concentrations of 1 μM or 10 μM nicotine in the culture medium, corresponding to a 1/5000 and 1/500 dilution of the condensate. As a positive control for comparison with TSE, we used nicotine bitartrate (Sigma) at a final concentration of 10 μM , a concentration previously verified in the PC12 model (Abreu-Villaça et al. 2005; Qiao et al. 2003; Slotkin et al. 2014). To control for the dimethylsulfoxide vehicle in the TSE samples, all cultures contained final concentrations of 0.2% dimethylsulfoxide (Sigma), which has no effect on

PC12 cell growth or differentiation (Qiao et al. 2001; Song et al. 1998). Studies were then conducted after 24h of exposure.

To initiate neurodifferentiation (Jameson et al. 2006b; Slotkin et al. 2007b; Teng and Greene 1994), the medium was changed to include 50 ng/ml of 2.5 S murine nerve growth factor (Promega Corporation, Madison, WI). TSE exposure commenced simultaneously with the addition of nerve growth factor, so as to be present throughout neurodifferentiation. The medium was changed every 48 hr with the continued inclusion of nerve growth factor and continued for either 4 days or 6 days, depending on the measured endpoint. At the end of each study, the cultures were examined under a microscope to verify the outgrowth of neurites.

2.2 Amelioration treatments

Each amelioration treatment was begun simultaneously with the exposure to TSE, whether in undifferentiated or differentiating cells. Control samples were always included that contained the ameliorant alone, so that each experiment consisted of four treatment groups: control, TSE alone, ameliorant alone, ameliorant + TSE. The final concentrations of the ameliorants were chosen from earlier studies that demonstrated the requisite effect of each with this model: nicotinic acetylcholine receptor blocker, 10 μ M mecamylamine (Slotkin et al. 2007a); antioxidants, 10 μ M Vitamin C (sodium ascorbate) (Slotkin and Seidler 2010), 10 μ M Vitamin E (α -tocopherol) (Slotkin and Seidler 2010), 5 mM NAC (Lee et al. 2012); methyl donors, 0.2 μ M Vitamin B12 (Savelkoul et al. 2012), 15 μ M folic acid (Savelkoul et al. 2012), 100 μ M choline (Knapp and Wurtman 1999) (all agents from Sigma). Vitamin E was dissolved in ethanol, yielding a final ethanol concentration of 0.05% in the medium; for experiments with this agent, all samples contained this ethanol concentration, which by itself has no effect on PC12 cell neurodifferentiation (Slotkin et al. 2007a). The final choline concentration is approximately five times the normal concentration already contained in the culture medium.

2.3 Assays

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

To assess 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 agents along with 1 μ Ci/ml of [3 H]thymidine (specific activity, 2 Ci/mmol; PerkinElmer Life Sciences, Waltham, MA). After 1 hr, the medium was aspirated and cells were washed repeatedly to allow unincorporated label to diffuse out, leaving only [3 H]thymidine that was incorporated into DNA remaining within the cell. Cells were then homogenized and an aliquot counted for radiolabel. This technique is a simplification of an established procedure (Bell et al. 1986; Slotkin et al. 1984), and we conducted preliminary experiments to show that it gave equivalent results. We corrected incorporation values to the amount of DNA present in each culture to provide an index of DNA synthesis per cell (Winick and Noble 1965) and the total DNA content was also recorded. The 24h time point was chosen to ensure that the cells were still in the log-phase of replication and well short of confluence.

For measurements of cell number and cell size, we relied on the fact that neural cells contain a single nucleus, so that the DNA content provides a measure of cell number (Winick and Noble 1965). Cells were harvested, washed, and the DNA and protein fractions were isolated and analyzed as described previously (Slotkin et al. 2007b). Since the DNA per cell is constant, cell growth entails an obligatory increase in the protein/DNA ratio (Qiao et al. 2003; Song et al. 1998).

To assess neurodifferentiation into dopamine and acetylcholine phenotypes, we assayed the activities of tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), respectively, using established techniques (Jameson et al. 2006a, b). Assessments were made 6 days after initiating neurodifferentiation with NGF, with continuous coexposure to test agents.

Effects on viability were assessed after 24h of exposure of undifferentiated cells, or 4 days of exposure of differentiating cells. The cell culture medium was changed to include trypan blue (1 volume per 2.5 volumes of medium; Sigma) and cells were examined for staining under 400× magnification, counting an average of 100 cells per field in four different fields per culture.

For evaluation of oxidative stress, we assessed the degree of lipid peroxidation in undifferentiated cells after 24h of exposure to test agents, and in differentiating cells after 4 days of exposure. The concentration of malondialdehyde (MDA) was measured with thiobarbituric acid (Guan et al. 2003). To give the MDA concentration per cell, values were calculated relative to the amount of DNA.

The 4 day time point for differentiating cells was chosen because effects on viability or oxidative stress would provide a presumptive, intervening mechanism for effects seen at the 6 day endpoint of neurodifferentiation.

2.4 Data analysis

Each study was performed using 2-4 separate batches of cells, with 3-4 independent cultures for each treatment in each batch; each batch of cells comprised a separately prepared, frozen and thawed passage. Results are presented as mean $\pm$ 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. The initial comparisons included factors of treatment and cell batch, and in each case, we found that the treatment effects for each type of experiment 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. Studies of amelioration of TSE effects were analyzed by two-factor ANOVA: factor 1= with or without TSE; factor 2 = with or without ameliorant. For these, the initial ANOVA assessed the main effects of TSE and ameliorants and the ameliorant $\times$ TSE interaction. Where the ameliorant $\times$ TSE interaction was significant, we evaluated individual differences between treatments, but in the absence of an interaction, only the main effects are reported without lower-order tests.

In addition to evaluating each individual set of measurements, we performed a global MANOVA to evaluate treatment effects across all measurements that were done on the same

samples, so as to avoid an increased probability of type I errors that could arise from multiple tests of the same cultures. At the same time, this test also increases statistical power by combining effects across multiple measurement types. Data were log-transformed because of heterogeneous variance across the different measures, and significance was assessed by Wilks' λ .

Significance for all tests was assumed at $p < 0.05$ (two-tailed).

3. RESULTS

3.1 Comparison of TSE with nicotine

In undifferentiated cells, 24h of TSE exposure elicited concentration-dependent inhibition of DNA synthesis, with the high TSE concentration producing a 22% decrement (Figure 1A). In contrast, 10 μ M nicotine by itself, equivalent to the nicotine concentration at high TSE, produced only a slight, nonsignificant reduction. The TSE effect on DNA synthesis was accompanied by a reduction in the total number of cells, assessed by measuring DNA content (Figure 1B). Again, an equivalent concentration of nicotine elicited a smaller, nonsignificant effect. Although the high concentration of TSE caused a significant increase in nonviable cells, this represented a small net change from about 3% of the total in controls to about 6% in the TSE group, so viability remained at 94%, compared to the control value of 97% (Figure 1C). By itself, 10 μ M nicotine was without significant effect on viability.

In differentiating cells, the high concentration of TSE reduced the number of cells after 4 days of exposure, an effect that was not shared by nicotine (Figure 1D). Unlike the case for undifferentiated cells, there were no significant changes in viability (Figure 1E). In our previous work in differentiating cells, we also found that TSE, but not nicotine, induced TH activity, connoting a shift in neurodifferentiation toward the dopaminergic phenotype (Slotkin et al. 2014). Based on these results, we evaluated the effects of ameliorant treatments against the high TSE concentration in the remaining studies.

3.2 Mecamylamine (nicotinic receptor antagonist)

In undifferentiated cells, MANOVA across the two measurements (DNA synthesis, DNA content) showed a significant effect of TSE but not mecamylamine, and there was no interaction of mecamylamine $\times$ TSE, connoting a lack of significant antagonism (Figure 2). This conclusion was borne out with the individual measures. TSE elicited a significant decrease in DNA synthesis that was unaffected by mecamylamine treatment (Figure 2A). The same result was seen for DNA content in undifferentiated cells (Figure 2B).

This was not the case for the four measures made in differentiating cells, where the MANOVA revealed not only a main effect of TSE, but also a significant mecamylamine $\times$ TSE interaction. For DNA content, TSE produced the expected reduction but this was not blocked by mecamylamine (Figure 2C). Likewise, TSE increased cell size (protein/DNA ratio) but again, mecamylamine did not alter the effect (Figure 2D). However, for TH activity, mecamylamine significantly enhanced the induction caused by TSE, more than doubling the increase (Figure 2E). In contrast, there were no significant changes in ChAT with TSE or mecamylamine alone or in combination (Figure 2F).

3.3 Antioxidants (Vitamin C, Vitamin E, NAC)

Unlike the situation for mecamylamine, MANOVA for DNA synthesis and content in undifferentiated cells showed significant overall main effects of TSE and the antioxidant cocktail alone, as well as a significant interaction of antioxidants $\times$ TSE (Figure 3). However, because of the small magnitude of TSE's effects on undifferentiated cells, there was insufficient power to show a significant interaction for the two measures taken individually. For DNA synthesis, TSE caused a significant overall reduction, with antioxidant treatment reducing the effect from 18% inhibition to 13% inhibition (Figure 3A). For DNA content, the TSE effect was reduced from 9% to 3% (Figure 3B). Notably, though the antioxidants by themselves significantly increased DNA content, pointing to unexpected effects of the rescue agents, a finding that was reinforced when they were given to differentiating cells, described below.

In differentiating cells, MANOVA combining the four different types of measurements revealed significant effects of TSE and antioxidants individually, as well as a significant interaction of antioxidants $\times$ TSE (Figure 3). For DNA content, antioxidants by themselves evoked a deficit in cell number but at the same time, they blocked the decrement caused by TSE (Figure 3C). Antioxidants also partially protected the cells from the increase in protein/DNA ratio evoked by TSE (Figure 3D). In contrast, antioxidants provided no discernible protection from the effects of TSE on neurotransmitter phenotypes. By itself, TSE elicited about a 25% increase in TH (Figure 3E). In the presence of antioxidants, TSE produced the same increase over the antioxidant-exposed control group. Importantly, the antioxidants alone suppressed TH activity relative to the vehicle controls. The effect of antioxidants was even more notable for ChAT (Figure 3F). Whereas TSE by itself had little or no effect on ChAT, antioxidants alone produced an enormous increase and sensitized the cells to TSE: in the presence of antioxidants, TSE elicited a robust decrease in activity, an effect not seen with TSE in the absence of the antioxidants.

In light of the limited ability of antioxidants to protect against the effects of TSE, we next evaluated lipid peroxidation as an endpoint for oxidative stress. In undifferentiated cells, neither TSE concentration caused an increase in MDA levels after 24h of exposure, although a positive control, monovalent silver (Powers et al. 2010b), elicited a robust elevation (Figure 4A). In fact, the high concentration of TSE actually reduced MDA levels. Similarly, in differentiating cells, the high TSE concentration reduced MDA levels after 4 days of exposure, whereas monovalent silver elicited a clear-cut elevation (Figure 4B).

The unexpected, large effects of the antioxidant cocktail on neurotransmitter phenotypes led to an additional study to delineate whether any individual component was responsible. Vitamin C and NAC reduced TH but Vitamin E was without significant effect (Figure 5A). The combination of all three antioxidants was indistinguishable from additive effects of Vitamin C and NAC. For ChAT, only NAC evoked a significant increase by itself but addition of the other two antioxidants augmented the net effect above that achieved with NAC (Figure 5B).

3.4 Methyl donors (Vitamin B12, folic acid, choline)

In undifferentiated cells, MANOVA identified a significant main effect of TSE but failed to find a significant interaction of methyl donors $\times$ TSE (Figure 5). TSE inhibited DNA synthesis (Figure 6A) and reduced cell numbers (Figure 6B). The methyl donors alone had no significant effect, nor did they prevent the effects of TSE on either measure.

In differentiating cells, MANOVA identified main effects of TSE and methyl donors individually, but no significant interaction when the four measures were combined (Figure 6). TSE, as expected, reduced DNA content, but methyl donors increased DNA significantly (Figure 6C). The methyl donors did not provide any protection from the effects of TSE, and in fact, the TSE-induced decrement was actually larger because of the fact that the methyl donors alone increased DNA. As a result, we found a significant methyl donors $\times$ TSE interaction for DNA content but this should be interpreted with caution, since there was no significant interaction in the overall MANOVA. Likewise, we found that methyl donors did not protect differentiating cells from the effects of TSE directed toward cell size (Figure 6D), TH activity (Figure 6E) or ChAT activity (Figure 6F).

3.5 Antioxidants combined with methyl donors

In undifferentiated cells, the combination of antioxidants and methyl donors gave overall results resembling those of the antioxidants alone. MANOVA combining the measurements of DNA synthesis and DNA content showed main effects of TSE and the ameliorants separately as well as a significant interaction between them (Figure 7). The main effect of the antioxidants + methyl donor cocktail represented a significant increase for both DNA synthesis (Figure 7A) and DNA content (Figure 7B), in contrast to the decreases caused by TSE. The ameliorant treatments reduced the effect of TSE by about half, although the interaction was significant only for the effect on DNA content.

In differentiating cells, MANOVA across the four types of measurements identified significant main effects of TSE and the ameliorants separately, as well as a significant ameliorants $\times$ TSE interaction. The interaction connoted a shift in the response to TSE in the presence of the combination of antioxidants and methyl donors (Figure 7). For DNA content, we saw a pattern just like that obtained for antioxidants without the methyl donors (Figure 7C): TSE reduced DNA, as did the ameliorant cocktail, and the combination of antioxidants + methyl donors protected the cells from the effects of TSE to about the same extent as seen with just the antioxidants. For the protein/DNA ratio, the combined ameliorants by themselves produced a small but significant increase that had not been seen just antioxidants or just methyl donors (Figure 7D). Furthermore, the combination of antioxidants + methyl donors completely protected the cells from the increase evoked by TSE, whereas the protection was only partial with just the antioxidants (compare to Figure 3D). To verify this difference we compared the rescue caused by antioxidants + methyl donors to rescue attributable to just antioxidants or just methyl donors, using a three-factor ANOVA: factor 1 = with or without TSE; factor 2 = with or without ameliorant; factor 3 = antioxidants vs. antioxidants + methyl donors. We found a significant three-factor interaction ($p < 0.02$), indicating that the protection from the TSE effect provided by the ameliorants was greater for the antioxidants + methyl donors, connoting synergism.

For the neurotransmitter differentiation endpoints, the combination of antioxidants + methyl donors gave results similar to those seen with just the antioxidants. The ameliorant treatment lowered TH activity but did not block the induction caused by TSE (Figure 7E). The antioxidants + methyl donors elicited a large increase in ChAT activity (Figure 7F); under those conditions, addition of TSE elicited a substantial decrement in ChAT that was not seen when TSE was given without the ameliorants, resulting in a significant ameliorant $\times$ TSE interaction.

4. DISCUSSION

In our previous work, we found that the effects of TSE on differentiating PC12 cells could not be explained solely by the effects of nicotine (Slotkin et al. 2014). TSE accelerated neurodifferentiation, enhancing cell growth at the expense of cell numbers, augmenting neurite formation and promoting emergence of the dopaminergic phenotype. Whereas nicotine could produce some of these effects (reduced cell numbers, enhanced cell growth), much higher concentrations were required than were present in TSE, and nicotine was unable to switch neurotransmitter phenotypes or to promote neurite formation. In the present study, we extended these observations to cells in the undifferentiated state, showing that TSE had greater effects on DNA synthesis and formation of new cells than did nicotine. Additionally, TSE, but not nicotine, impaired cell viability, albeit that the effect involved only a small overall percentage of the cells. Interestingly, the reduced viability evoked by TSE was not seen in differentiating cells, reinforcing the earlier conclusion that reductions in cell numbers reflect promotion of the transition from cell replication to neurodifferentiation, rather than cytotoxicity (Slotkin et al. 2014). This inference was reinforced in the current study by our finding that TSE uniformly enhanced cell growth (Figures 2D, 3D, 6D, 7D), whereas cytotoxicity would be expected to impair growth. We also looked for lipid peroxidation consequent to oxidative stress as a likely mechanism independent of nicotinic receptor activation but failed to find any increase in MDA levels that would indicate such an effect. In fact, we found reductions in MDA at high TSE concentrations, regardless of differentiation state. Hence, a main conclusion of this study is that the effects of TSE comprise multiple mechanisms over and above any contribution of nicotine acting on cholinergic receptors, or of lipid peroxidation.

In addition to distinguishing between effects of TSE and nicotine on neural cell replication and differentiation, the central issue explored in this study was to investigate whether identifying the specific underlying mechanisms could be used to design amelioration strategies. Here, we specifically chose PC12 cells because their insensitivity to nicotine would enable us to pursue the actions of other TSE constituents operating through mechanisms over and above nicotinic receptor activation (Abreu-Villaça et al. 2005; Avila et al. 2003; Gueorguiev et al. 2000). Thus, it is not surprising that a nicotinic receptor antagonist (mecamylamine) was ineffective in preventing TSE effects on either undifferentiated or differentiating cells. Indeed, this can be taken as confirmation that the observed effects in this particular model reflect the contributions of TSE constituents other than nicotine. Unexpectedly, though, we found that mecamylamine augmented the ability of TSE to induce TH activity, indicating a synergistic promotion of differentiation into the dopaminergic phenotype. This likely reflects the fact that nicotinic receptor activation by

endogenous acetylcholine promotes differentiation into the opposite, cholinergic phenotype (Avila et al. 2003), and in that regard, receptor stimulation can act as a substitute for nerve growth factor (Yamashita and Nakamura 1996). The main point is that, rather than ameliorating the effect of TSE, mecamylamine actually worsened the imbalance of neurotransmitter phenotypes by preventing the emergence of the cholinergic phenotype, superimposed on the ability of TSE to promote the dopaminergic phenotype. As will be reinforced below, when amelioration strategies instead exacerbate the effects of TSE, or themselves cause abnormalities, they may be harmful instead of helpful.

In contrast to the ineffectiveness of mecamylamine in ameliorating the effects of TSE, antioxidants produced clear-cut, but only partial protection, reducing the impact on DNA synthesis and content in undifferentiated cells, and on DNA content and protein/DNA ratio in differentiating cells. On the other hand, antioxidants provided no protection whatsoever against the shift in neurotransmitter phenotypes elicited by TSE and actually exacerbated the decrement in ChAT. This failure cannot be attributed just to an inadequate antioxidant dose, since this concentration of Vitamin C alone protects against oxidative damage caused by monovalent silver (Powers et al. 2010b), which we found here to have a much larger oxidative effect than TSE. Perhaps even more troubling, the antioxidants by themselves had unexpected effects, decreasing the number of cells in the differentiated state and grossly shifting neurodifferentiation toward the cholinergic phenotype at the expense of the dopaminergic phenotype. Analysis of the separate antioxidant components showed a hierarchy of effects on neurodifferentiation, with NAC producing the greatest shift away from the dopaminergic phenotype and toward the cholinergic phenotype. Vitamin C also affected TH but not ChAT, and the addition of Vitamins C and E augmented the effect of NAC. This probably reflects the fact that oxidative events play an obligatory role in neurodifferentiation (Katoh et al. 1997; Panchision 2009; Vieira et al. 2011), and thus, excessive antioxidant treatment can perturb normal developmental processes.

Even partial protection against TSE by antioxidants seemed puzzling, since we were unable to demonstrate significant lipid peroxidation in either undifferentiated or differentiating cells, and actually found a reduction in MDA levels at the higher TSE concentration. Nicotine alone causes oxidative stress and lipid peroxidation in PC12 cells (Qiao et al. 2005), so the current finding indicates that some additional components of TSE actually quench that activity. Interestingly, though, much higher concentrations of tobacco extract (not combusted) can produce oxidative stress (Yildiz et al. 1999), so the net response can be in either direction depending on dose, a not-unexpected consequence from a complex chemical mixture. It is also possible that the MDA endpoint is insufficiently sensitive, since oxidative damage can occur short of producing measurable accumulation of lipid peroxides. In particular, the high membrane lipid turnover in rapidly-growing cells could dilute effects on MDA (Araki and Wurtman 1997), although that would hardly explain the *decrease* seen at the high TSE concentration. Future studies focusing on more sensitive endpoints, such as generation of reactive oxygen species or mitochondrial dysfunction, should be able to resolve this issue. Certainly, the fact that antioxidants provide partial protection argues in favor of oxidative damage that is not detected with the MDA method. Nevertheless, our

results suggest that, at the concentrations used here, oxidative stress is at best, a minor contributor to the net effects on neurodifferentiation.

In any case, though, our results for MDA and for antioxidant amelioration are surprising in light of the clear-cut oxidative stress experienced *in vivo* with maternal smoking (Aycicek and Ipek 2008; Fayol et al. 2005; Orhon et al. 2009; Rossner et al. 2009), and the protection by antioxidants against fetal lung damage caused by maternal tobacco exposure (McEvoy et al. 2014; Proskocil et al. 2005). In that regard, TSE does not include volatile tobacco-smoke chemicals like HCN and CO that interfere with fetal oxygen delivery and utilization (Carmines and Rajendran 2008; Pettigrew et al. 1977; Robkin 1997), nor does an *in vitro* model incorporate hypoxic stress resulting from uteroplacental vasoconstriction and reduced placental oxygen transfer, which are major contributors to fetal hypoxia *in vivo* (Albuquerque et al. 2004; Bush et al. 2000; Clark and Irion 1992). Accordingly, the results seen here may underestimate the contribution played by oxidative stress for prenatal tobacco smoke exposure *in vivo*.

Notwithstanding these limitations, the inability of antioxidants to protect against the shift in neurotransmitter phenotype elicited by TSE indicates that this particular effect is unrelated to oxidative stress. A number of other oxidative stressors produce a similar shift toward the dopaminergic phenotype in the PC12 model: fipronil, chlorpyrifos, diazinon, parathion, dieldrin, monovalent silver and silver nanoparticles (Lassiter et al. 2009; Powers et al. 2010a, b; Slotkin et al. 2007b). However, divalent nickel, which is a reductant, also increases TH at the expense of ChAT (Slotkin et al. 2007b). It is thus obvious that multiple mechanisms can affect the balance of dopaminergic and cholinergic phenotypes, over and above contributions from oxidative stress or nicotinic receptor stimulation. As seen here, TSE elicited a profound shift despite its failure to increase lipid peroxidation, and the effects were not prevented by either antioxidants or mecamylamine. Alternative mechanisms are likely to include excitotoxicity triggered by changes in glutamate receptors (Slotkin et al. 2010; Slotkin and Seidler 2009) and stimulation of protein kinase signaling (Adigun et al. 2010). Indeed, we have already shown that combined exposure to two TSE products, benzo[a]pyrene and nicotine, alters neurotransmitter phenotypes in a manner unlike those of either compound given separately (Slotkin et al. 2013), pointing to complex interactions mediated by multiple mechanisms.

In contrast to the antioxidants, methyl donors provided no discernible protection against any of the effects of TSE, whether on undifferentiated or differentiating cells. The only significant effect was a methyl donor-induced increase in DNA in differentiating cells, which then led to a relative exacerbation of the loss caused by TSE. Combining the methyl donors with antioxidants produced results resembling those of the antioxidants alone, with one exception, namely a synergistic interaction in differentiating cells, promoting the protection against the increase in cell size evoked by TSE. Although we did not dissect which component of the methyl donor mixture was responsible for this interaction, the main finding for methyl donors is their relative ineffectiveness in offsetting the effects of TSE on neural cell replication or differentiation.

Any in vitro approach to developmental neurotoxicity has clear limitations (Coecke et al. 2007; Qiao et al. 2001; Song et al. 1998). In this case, we took advantage of the insensitivity of PC12 cells to nicotine (Abreu-Villaça et al. 2005; Avila et al. 2003; Gueorguiev et al. 2000), in order to evaluate additional mechanisms by which TSE affects neurodifferentiation, providing cause-and-effect relationships that would be much more difficult to establish in vivo. Notably, culture systems cannot model cell-to-cell interactions, architectural modeling of brain regions or more complex events in brain assembly, and consequently, sensitivity is likely to be greater in vivo than in vitro. The difference in sensitivity is exacerbated by the necessity to detect effects within a short span of hours to days in vitro, versus weeks of exposure in vivo (Coecke et al. 2007). This is especially true for transformed cell lines, such as PC12 cells, which are less sensitive to toxicants than are primary neurons. However, for toxicants that act on the cell cycle and that target specific neurodifferentiation endpoints, primary neurons are problematic, since they do not divide in culture and undergo heterogeneous neurodifferentiation, whereas PC12 cells undergo mitosis and differentiate uniformly (Coecke et al. 2007; Radio et al. 2008). Most importantly, though, culture models do not address maternal-fetal pharmacokinetics. Although we can use culture models to identify the direct effects of TSE and some of its underlying mechanisms, we do not yet know which TSE components pass through the placenta to enter the fetus, nor their relative concentrations in the fetal compartment. Accordingly, the current approach points to the potential neurodevelopmental effects that should be pursued with in vivo studies; these are underway in our laboratory.

4.1 Conclusions

There are several important conclusions from the present results. First, although nicotine is a major contributor to the neurodevelopmental damage associated with maternal smoking during pregnancy (Pauly and Slotkin 2008; Slikker et al. 2005; Slotkin 2004, 2008), the thousands of other components in tobacco smoke are likely to play a part. This is especially critical with regard to second-hand smoke exposure, which could then be more injurious than anticipated from measured levels of nicotine or its metabolites (DeLorenze et al. 2002; Koren et al. 1998; Luck et al. 1985). At the same time, the complex composition of tobacco smoke renders the identification of specific underlying mechanisms problematic. Even a combination of just two components, nicotine and benzo[a]pyrene, interact to produce effects on neurodifferentiation unlike either agent alone (Slotkin et al. 2013). In the current study, we were unable to reverse the effects of TSE on neurodifferentiation by using a nicotinic receptor antagonist, or through use of antioxidants and methyl donors separately or together. At best, we saw only a partial protection by antioxidants against cell loss, and no amelioration of adverse effects on emergence of neurotransmitter phenotypes. Even worse, we found that ameliorant strategies themselves had adverse effects on neurodifferentiation, likely because of their interference with the critical roles of endogenous cholinergic stimulation and redox status in normal neuronal cell development (Dreyfus 1998; Hohmann 2003; Katoh et al. 1997; Lauder 1985; Panchision 2009; Vieira et al. 2011). This becomes crucial in attempting to offset the adverse effects of maternal smoking through interventions targeting putative mechanisms individually and without regard for heterogeneity of responses in different tissues. For example, recent studies have recommended maternal Vitamin C supplementation to protect lung development and respiratory function in

offspring of smokers (McEvoy et al. 2014; Proskocil et al. 2005). However, this beneficial result is likely to be offset by adverse effects on brain development, both from augmentation of nicotine levels in the fetus (Slotkin et al. 2005) and from interference with natural oxidative signals involved in neurodifferentiation, as seen here. Ultimately, there is no better approach than to limit, or preferentially eliminate, prenatal tobacco smoke exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	analysis of variance
ChAT	choline acetyltransferase
MDA	malondialdehyde
NAC	N-acetyl-L-cysteine
TH	tyrosine hydroxylase
TSE	tobacco smoke extract

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Figure 1.

Comparison of TSE effects with nicotine in undifferentiated cells after 24h of exposure, and in differentiating cells after 4 days of exposure: (A) DNA synthesis in undifferentiated cells; (B) DNA content in undifferentiated cells; (C) trypan blue exclusion in undifferentiated cells; (D) DNA content in differentiating cells; (E) trypan blue exclusion in differentiating cells. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVA across all treatments is shown at the top of each panel and asterisks denote values that differ significantly from the control.

Figure 2.

Effects of mecamylamine on the response to TSE in the undifferentiated state (24h exposure) and during differentiation (6 day exposure): (A) DNA synthesis in undifferentiated cells, (B) DNA content in undifferentiated cells, (C) DNA content in differentiating cells, (D) protein/DNA ratio in differentiating cells, (E) tyrosine hydroxylase activity in differentiating cells, and (F) choline acetyltransferase activity in differentiating cells. Data represent means and standard errors of the number of determinations shown in parentheses. MANOVAs above each set of panels combine all the measurements for each

differentiation state, and two-factor ANOVAs are shown at the top of each panel for each individual measure. Where there was a mecamylamine $\times$ TSE interaction (E), asterisks denote whether the TSE group is significantly different from the corresponding control value. Abbreviation: NS, not significant.

Figure 3.

Effects of a mixture of antioxidants (10 μM Vitamin C + 10 μM Vitamin E + 5 mM NAC) on the response to TSE in the undifferentiated state (24h exposure) and during differentiation (6 day exposure): (A) DNA synthesis in undifferentiated cells, (B) DNA content in undifferentiated cells, (C) DNA content in differentiating cells, (D) protein/DNA ratio in differentiating cells, (E) tyrosine hydroxylase activity in differentiating cells, and (F) choline acetyltransferase activity in differentiating cells. Data represent means and standard errors of the number of determinations shown in parentheses. MANOVAs above each set of

panels combine all the measurements for each differentiation state, and two-factor ANOVAs are shown at the top of each panel for each individual measure. Where there was an antioxidants $\times$ TSE interaction, asterisks denote whether the TSE group is significantly different from the corresponding control value and daggers indicate whether the antioxidants alone are different from the vehicle control. Abbreviation: NS, not significant.

Figure 4.

Effects of TSE on MDA concentrations, compared to a positive control, 10 μM AgNO₃: (A) undifferentiated cells after 24h of exposure; (B) differentiating cells after 4 days of exposure. Data represent means and standard errors of the number of determinations shown in parentheses. ANOVA across all treatments is shown at the top of each panel and asterisks denote values that differ significantly from the control.

Figure 5. Effects of individual antioxidants and the combination of all three, on TH (A) and ChAT (B). Data represent means and standard errors of the number of determinations shown in parentheses. ANOVA across all treatments is shown at the top of each panel and asterisks denote values that differ significantly from the control.

Figure 6.

Effects of a mixture of methyl donors (0.2 μ M Vitamin B12 + 15 μ M folic acid + 100 μ M choline) on the response to TSE in the undifferentiated state (24h exposure) and during differentiation (6 day exposure): (A) DNA synthesis in undifferentiated cells, (B) DNA content in undifferentiated cells, (C) DNA content in differentiating cells, (D) protein/DNA ratio in differentiating cells, (E) tyrosine hydroxylase activity in differentiating cells, and (F) choline acetyltransferase activity in differentiating cells. Data represent means and standard errors of the number of determinations shown in parentheses. MANOVAs above each set of

panels combine all the measurements for each differentiation state, and two-factor ANOVAs are shown at the top of each panel for each individual measure. Where there was a methyl donors $\times$ TSE interaction (C), asterisks denote whether the TSE group is significantly different from the corresponding control value and the dagger indicates whether the methyl donors alone are different from the vehicle control. Abbreviation: NS, not significant.

Undifferentiated Cells

MANOVA: TSE $p < 0.0001$, Antioxidants + Methyl Donors $p < 0.0001$,
Antioxidants + Methyl Donors $\times$ TSE $p < 0.02$

Differentiating Cells

MANOVA: TSE $p < 0.0001$, Antioxidants + Methyl Donors $p < 0.0001$,
Antioxidants + Methyl Donors $\times$ TSE $p < 0.0001$

Figure 7.

Effects of a combined antioxidants and methyl donors on the response to TSE in the undifferentiated state (24h exposure) and during differentiation (6 day exposure): (A) DNA synthesis in undifferentiated cells, (B) DNA content in undifferentiated cells, (C) DNA content in differentiating cells, (D) protein/DNA ratio in differentiating cells, (E) tyrosine hydroxylase activity in differentiating cells, and (F) choline acetyltransferase activity in differentiating cells. Data represent means and standard errors of the number of determinations shown in parentheses. MANOVAs above each set of panels combine all the

measurements for each differentiation state, and two-factor ANOVAs are shown at the top of each panel for each individual measure. Where there was an [antioxidants + methyl donors] $\times$ TSE interaction, asterisks denote whether the TSE group is significantly different from the corresponding control value and daggers indicate whether the antioxidants alone are different from the vehicle control. Abbreviation: NS, not significant.