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Prenatal Drug Exposures Sensitize Noradrenergic Circuits to Subsequent Disruption by Chlorpyrifos

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Abstract

We examined whether nicotine or dexamethasone, common prenatal drug exposures, sensitize the developing brain to chlorpyrifos. We gave nicotine to pregnant rats throughout gestation at a dose (3 mg/kg/day) producing plasma levels typical of smokers; offspring were then given chlorpyrifos on postnatal days 1–4, at a dose (1 mg/kg) that produces minimally-detectable inhibition of brain cholinesterase activity. In a parallel study, we administered dexamethasone to pregnant rats on gestational days 17–19 at a standard therapeutic dose (0.2 mg/kg) used in the management of preterm labor, followed by postnatal chlorpyrifos. We evaluated cerebellar noradrenergic projections, a known target for each agent, and contrasted the effects with those in the cerebral cortex. Either drug augmented the effect of chlorpyrifos, evidenced by deficits in cerebellar β -adrenergic receptors; the receptor effects were not due to increased systemic toxicity or cholinesterase inhibition, nor to altered chlorpyrifos pharmacokinetics. Further, the deficits were not secondary adaptations to presynaptic hyperinnervation/hyperactivity, as there were significant deficits in presynaptic norepinephrine levels that would serve to augment the functional consequence of receptor deficits. The pretreatments also altered development of cerebrocortical noradrenergic circuits, but with a different overall pattern, reflecting the dissimilar developmental stages of the regions at the time of exposure. However, in each case the net effects represented a change in the developmental trajectory of noradrenergic circuits, rather than simply a continuation of an initial injury. Our results point to the ability of prenatal drug exposure to create a subpopulation with heightened vulnerability to environmental neurotoxicants.

Keywords

Cerebellum; Chlorpyrifos; Dexamethasone; Nicotine; Norepinephrine; Organophosphate pesticides

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1. INTRODUCTION

The continued, widespread use of organophosphate pesticides has resulted in virtually ubiquitous exposure of the entire population and ecosphere (Casida and Quistad 2004). Once considered as a safer alternative to organochlorine pesticides, the organophosphates are now known to be developmental neurotoxicants that target brain development at exposures well below the threshold for any discernible systemic signs of exposure (Eskenazi et al. 2008; Grandjean and Landrigan 2014; Slotkin 2004). Despite U.S. restrictions on household use of organophosphates such as chlorpyrifos and diazinon, both continue to be used in agriculture and in households in many other countries. It is now estimated that organophosphates are the number one environmental contributor worldwide to an adverse impact on IQ scores (Bellinger 2012), and are major contributors to the explosive increase in the incidence of neurodevelopmental disorders, termed a “silent pandemic” (Grandjean and Landrigan 2006, 2014). Indeed, organophosphates have been identified as one of the likely environmental causes of the rise in autism spectrum disorders (Grandjean and Landrigan 2014; Landrigan 2010; Shelton et al. 2012).

Given the ubiquity of organophosphate exposures, one of the critical issues is to establish whether there are subpopulations that are particularly susceptible, and much attention has been directed toward genetic polymorphisms that enhance their toxicity (Furlong et al. 2000, 2005). In recent work with developing rats, we pursued a different factor, namely whether prenatal exposure to other common chemicals alters the subsequent vulnerability of the developing brain to chlorpyrifos (Levin et al. 2014; Slotkin et al. 2013, 2014, 2015b; Slotkin and Seidler 2015). For prenatal exposures, we concentrated on nicotine and dexamethasone because of the large population (10–20% for each agent) exposed to these agents in utero: nicotine because of maternal smoking (Somm et al. 2009) and dexamethasone because of its use in the management of preterm labor (Gilstrap et al. 1995; Matthews et al. 2002). Additionally, prenatal tobacco smoke exposure and premature birth are highly linked: smoking during pregnancy accounts for 15–30% of all preterm deliveries (White et al. 1986; Wisborg and Henriksen 1995). Initially, we focused on cholinergic and serotonergic systems, known neurotransmitter targets for nicotine (Slikker et al. 2005; Slotkin 2004; Slotkin et al. 2015a; Xu et al. 2001), dexamethasone (Kreider et al. 2005a, 2005b, 2006; Slotkin et al. 2006) and chlorpyrifos (Aldridge et al. 2004, 2005a, 2005b; Slotkin 2004). In each case, we found that nicotine or dexamethasone sensitized these circuits to subsequent injury by chlorpyrifos, with adverse effects persisting into adulthood (Slotkin et al. 2013, 2014, 2015b; Slotkin and Seidler 2015).

In the current study, we shifted our focus to a transmitter system and brain region that have been much less studied with these treatment combinations: noradrenergic systems in the cerebellum. The cerebellum may be a particularly sensitive target for chlorpyrifos, and prenatal exposure is associated with neurochemical and structural changes that are likely to contribute to sensorimotor deficits (Abdel-Rahman et al. 2004; Abou-Donia et al. 2006; Crumpton et al. 2000; Dam et al. 1999; Garcia et al. 2002; Krishnan 1993). Because the cerebellum is sparse in cholinergic projections, this region provides a distinctive model for exploring contributory neurotoxic mechanisms other than cholinergic hyperstimulation resulting from cholinesterase inhibition (Slotkin 2004, 2005); these alternative mechanisms

are the ones that most likely contribute to the structural and behavioral deficits seen in children with low environmental exposures (Rauh et al. 2011, 2012). Furthermore, the cerebellum is an important focus because of its structural and functional involvement in autism (Allely et al. 2014; Radell and Mercado 2014; Shevelkin et al. 2014; Skefos et al. 2014), especially in light of the association of organophosphate exposures with this disorder (Grandjean and Landrigan 2014; Herbert 2010; Landrigan 2010; Shelton et al. 2012). Developmental exposure to nicotine in combination with chlorpyrifos causes reactive gliosis and Purkinje cell loss in the cerebellum (Abou-Donia et al. 2006), but the physical damage from the combination does not seem to exceed that of either agent alone. In contrast, some of the behavioral effects show synergism (Abou-Donia et al. 2006), implying that there are interactions at the level of synaptic function that are distinct from Purkinje cell loss. To resolve this issue, we focused on noradrenergic pathways because the cerebellum is only sparsely innervated with cholinergic and serotonergic projections, the two neurotransmitter pathways that have been most studied to date. Norepinephrine systems are targeted for developmental disruption by nicotine (Navarro et al. 1988; Seidler et al. 1992; Slotkin and Seidler 2011), dexamethasone (Slotkin et al. 1991, 1992; Slotkin and Seidler 2011) and chlorpyrifos (Dam et al. 1999; Slotkin et al. 2002) individually.

We utilized prenatal nicotine and dexamethasone treatments that were designed to mimic typical human exposures. Nicotine was delivered throughout gestation via minipump infusion, at 3 mg/kg/day, which produces plasma levels associated with moderate smoking (Lichtensteiger et al. 1988; Murrin et al. 1987; Trauth et al. 2000). Dexamethasone was administered over a more restricted range, on gestational days (GD) 17–19, the stage in rat brain development that corresponds to the period in which glucocorticoids are used in preterm labor; we chose a dose (0.2 mg/kg) in the low therapeutic range (Gilstrap et al. 1995), and the three-dose regimen parallels the multiple glucocorticoid courses that are used in the vast majority of cases (Dammann and Matthews 2001). Chlorpyrifos was administered on postnatal days (PN) 1–4, at a dose (1 mg/kg) just at the threshold for barely-detectable inhibition of acetylcholinesterase (Song et al. 1997). This regimen produces developmental neurotoxicity without eliciting growth retardation or any other signs of systemic toxicity (Slotkin 1999, 2004), and more importantly, recapitulates both the neurobehavioral deficits and abnormalities of brain structure seen in children exposed prenatally to chlorpyrifos (Bouchard et al. 2011; Engel et al. 2011; Rauh et al. 2006, 2011, 2012). To monitor the effects on noradrenergic synaptic development, we measured the concentration of β -adrenergic receptors (β ARs) as well as norepinephrine levels. The β AR plays a critical trophic role in neurodevelopment (Barochovsky and Patel 1982; Kwon et al. 1996; Popovik and Haynes 2000) and abnormalities involving this subtype are associated with elevated autism risk (Cheslack-Postava et al. 2007; Connors 2008; Connors et al. 2005; Witter et al. 2009). Finally, to determine whether treatment effects were specific to the cerebellum, we made comparative measurements in subregions of the cerebral cortex.

2. METHODS

2.1 Animal treatments and tissue procurement

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 accord with all federal and state guidelines. The tissues used in this report were archived from earlier studies and maintained frozen at -45°C , so that no additional animals were actually used for this study. Details of animal husbandry, maternal and litter characteristics, and growth curves, have all been presented in earlier work from the original animal cohorts (Slotkin et al. 2013, 2014, 2015b; Slotkin and Seidler 2015). Separate animal cohorts were used for the two sets of studies, one cohort for the interaction of dexamethasone with chlorpyrifos, and the other cohort for the interaction of nicotine with chlorpyrifos. The use of archival tissue samples imposed some limitations, in that for some determinations, we did not have all brain regions available for all treatments at all the age points.

Timed-pregnant Sprague-Dawley rats were shipped by climate-controlled truck (total transit time $< 1\text{ h}$), housed individually and allowed free access to food and water. For the nicotine studies, there were four treatment groups, each comprising 10–12 litters: controls (prenatal vehicle infusion + postnatal vehicle injections), nicotine treatment alone (prenatal nicotine infusion + postnatal vehicle injections), chlorpyrifos treatment alone (prenatal vehicle infusion + postnatal chlorpyrifos injections), and those receiving the combined treatment (prenatal nicotine infusion + postnatal chlorpyrifos injections). On GD4, before implantation of the embryo in the uterine wall, each animal was quickly anesthetized with ether, a small area on the back was shaved, and an incision made to permit s.c. insertion of a Model 2ML2 Alzet minipump, after which the incision was closed with wound clips. The pumps contained nicotine bitartrate dissolved in bacteriostatic water so as to deliver 3 mg/kg/day of nicotine free base, with the dosage determined by the initial body weights of the dams ($215 \pm 2\text{ g}$); control pumps contained bacteriostatic water and equivalent concentrations of sodium bitartrate. Because weights increased with gestation, the dose rate fell accordingly to 2 mg/kg/day , but the dose rates remained well within the range that produces nicotine plasma levels similar to those in moderate smokers (Fewell et al. 2001; Trauth et al. 2000). It should be noted that the pump, marketed as a two week infusion device, actually takes approximately 17 days to be exhausted completely (information supplied by the manufacturer) and thus the nicotine infusion terminates on GD21; in earlier work, we confirmed the termination of nicotine delivery coinciding with the calculated values (Trauth et al. 2000). Parturition occurred during GD22, which was also taken as PN0. After birth, pups were randomized within treatment groups and litter sizes were culled to 10 (5 males and 5 females) to ensure standard nutrition. Control and dexamethasone-treated litters were then assigned to either the vehicle or chlorpyrifos postnatal treatment groups. Chlorpyrifos was dissolved in dimethylsulfoxide to provide consistent absorption (Whitney et al. 1995) and was injected subcutaneously at a dose of 1 mg/kg in a volume of 1 ml/kg once daily on postnatal days 1–4; control animals received equivalent injections of the dimethylsulfoxide vehicle. Pups were weighed, litters were re-randomized within treatment groups and dams were rotated among litters every few days to distribute differential effects of maternal caretaking equally among all litters, making sure that all the pups in a given litter were from

the same treatment group to avoid the possibility that the dams might distinguish among pups with different treatments; cross-fostering, by itself, has no impact on neurochemical or behavioral effects of these treatments (Nyirenda et al. 2001). Animals were weaned on PN21. At various ages, brain regions were dissected and frozen in liquid nitrogen, and then stored at -45°C until assayed. Each treatment group comprised 12 animals at each age point, equally divided into males and females, with each final litter assignment contributing no more than one male and one female to any of the treatment groups.

For the dexamethasone studies, there were four treatment groups, each comprising 12–14 dams: controls (prenatal saline + postnatal dimethylsulfoxide vehicle), dexamethasone treatment alone (prenatal dexamethasone + postnatal vehicle), chlorpyrifos treatment alone (prenatal saline + postnatal chlorpyrifos), and those receiving the combined treatment (prenatal dexamethasone + postnatal chlorpyrifos). On GD 17, 18 and 19, dams received subcutaneous injections of either saline vehicle or 0.2 mg/kg dexamethasone sodium phosphate, a dose at the lower range recommended for therapeutic use in preterm labor (Gilstrap et al. 1995). After birth, the animals received injections of vehicle or chlorpyrifos on PN1–4 as described above.

2.2 Assays

For βAR binding determinations, there were technical limitations imposed by the large number of membrane preparations that had to be examined. The overall strategy was to determine binding at a single, subsaturating ligand concentration to enable the detection of changes that originate either in altered K_d or B_{max} . Tissues were thawed and homogenized (Polytron; Brinkmann Instruments, Westbury, NY) in buffer containing 145 mM sodium chloride, 2 mM magnesium chloride, and 20 mM Tris (pH 7.5), and were then sedimented at $40,000 \times g$ for 15 min. The pellets were washed twice and then resuspended in the homogenization buffer. Aliquots of the suspension were incubated with 67 pM [^{125}I]iodopindolol in 145 mM NaCl, 2 mM MgCl_2 , 1 mM sodium ascorbate, 20 mM Tris (pH 7.5), for 20 min at room temperature; samples were evaluated with and without 100 μM isoproterenol to displace specific binding. Incubations were stopped by addition of 3 ml ice-cold buffer, and the labeled membranes were trapped by rapid vacuum filtration onto glass fiber filters, which were washed with additional buffer and counted by liquid scintillation spectrometry. Binding was then assessed relative to membrane protein (Smith et al. 1985).

For norepinephrine determinations, tissues were thawed on ice and deproteinized by homogenization in 0.1 N perchloric acid containing 3,4-dihydroxybenzylamine as an internal standard. Homogenates were sedimented at $26,000 \times g$ for 20 minutes, the supernatant solutions were decanted, and norepinephrine was then trace-enriched by alumina adsorption, separated by reverse-phase high performance liquid chromatography and quantitated by electrochemical detection (Seidler and Slotkin 1981); values were corrected for recovery of the internal standard.

2.3 Data analysis

To avoid the increase in type 1 errors that could occur from multiple statistical tests on the same data, each set of determinations was first evaluated using multivariate ANOVA

considering all relevant variables (treatment, sex, age, brain region) in a single test; data were log-transformed because of heterogeneous variance among the different ages and regions. Interactions of treatment with the other factors triggered subdivisions into the individual treatments, each of which was then tested with lower-order multivariate ANOVAs. As permitted by the interaction terms, individual differences between treatment groups and controls, or among the different treatments, were identified post-hoc 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 $p < 0.05$.

Data were compiled as means and standard errors. To facilitate comparison of the effects of the two prenatal treatments, results for the two studies were normalized against each other and combined in a common graph. For ready visualization of treatment effects across the different measures, ages and regions, the results are given as the percent change from control values; the original control values appear in Table 1. Statistical procedures were always conducted on the original data, with log transforms because of heterogeneous variance as noted above, and with treatment comparisons made against the contemporaneous control group.

2.4 Materials

Animals were purchased from Charles River Laboratories (Raleigh, NC) and osmotic minipumps from Durect Corp. (Cupertino, CA). Bacteriostatic water was obtained from Abbott Laboratories (N. Chicago, IL) and PerkinElmer Life Sciences (Boston, MA) was the source for [125 I]Iodopindolol (specific activity, 2200 Ci/mmol). Chlorpyrifos was obtained from Chem Service (West Chester, PA) and Sigma Chemical Co. (St. Louis, MO) was the source for all other reagents.

3. RESULTS

3.1 Cerebellum

None of the treatments had any significant effect on cerebellum weights (data not shown). By itself, prenatal nicotine treatment evoked an overall elevation in cerebellar β AR binding (Figure 1A), whereas dexamethasone was without significant effect (Figure 1B). Postnatal exposure to chlorpyrifos produced initial elevations in adolescence that regressed to normal in adulthood (Figure 1C). When chlorpyrifos was preceded by prenatal nicotine treatment, this temporal pattern was exaggerated (Figure 1D): instead of returning to normal, the initial chlorpyrifos-induced elevation was replaced by significant deficits that emerged by PN60 and intensified in late adulthood. The combined exposure to nicotine + chlorpyrifos was statistically distinguishable from the effects of either treatment alone ($p < 0.0001$ vs. nicotine, $p < 0.0001$ vs. chlorpyrifos). Prenatal dexamethasone treatment likewise changed the response to postnatal chlorpyrifos exposure (Figure 1E), eliminating the initial β AR elevation in adolescence and, like nicotine, leading to deficits in adulthood.

Unlike its effects on β AR binding, prenatal nicotine treatment had no significant impact on cerebellar norepinephrine levels (Figure 2A). For dexamethasone, although there was a trend

toward the pattern of increases in adolescence followed by decreases in adulthood, the age-dependent effect did not achieve statistical significance (Figure 2B). In contrast, postnatal chlorpyrifos elicited that pattern more robustly, producing a significant age-dependent decrease in norepinephrine levels that was selective for males (Figure 2C). Surprisingly, nicotine pretreatment appeared to protect cerebellar norepinephrine levels from the effects of chlorpyrifos, reducing the magnitude of the effect to the point where values on PN150 were essentially normal (Figure 2D). The effect of the nicotine-chlorpyrifos combination was statistically distinguishable from that of chlorpyrifos alone ($p < 0.04$) but was indistinguishable from that of nicotine alone. In contrast to nicotine, dexamethasone did not provide protection against the chlorpyrifos-induced loss of cerebellar norepinephrine (Figure 2E).

3.2 Cerebral cortex

To compare to the cerebellum, we examined two cerebrocortical regions, the frontal/parietal cortex and the temporal/occipital cortex. None of the treatments had any significant effect on weights of either region (data not shown). For β AR measurements, we had tissue samples available only for the dexamethasone series. In general, effects on cerebrocortical β AR binding were distinct from those seen in the cerebellum. By itself, prenatal dexamethasone treatment evoked significant elevations in cerebrocortical β AR binding that emerged between adolescence and adulthood (Figure 3A), an effect that was absent in the cerebellum. Postnatal chlorpyrifos exposure produced a significant overall increase in β ARs that was restricted to males (Figure 3B), thus showing sex selectivity that was not seen in the cerebellum; further, whereas the cerebellum showed a temporal regression to normal, the cerebral cortex did not. When chlorpyrifos was preceded by prenatal dexamethasone treatment, cerebrocortical β AR binding displayed significant deficits in females, an effect not seen with either treatment alone (Figure 3C; $p < 0.0001$ vs. dexamethasone alone, $p < 0.004$ vs. chlorpyrifos alone); males given the combination showed the same overall chlorpyrifos-induced elevation as that seen with just chlorpyrifos.

For norepinephrine levels, we had samples available for all treatment groups in adulthood (PN100 and PN150 for the nicotine series, PN150 for the dexamethasone series). By itself, prenatal nicotine treatment caused a significant elevation in cerebrocortical norepinephrine levels in young adulthood that was no longer significant by full adulthood (Figure 4A). Dexamethasone had no effect at the full adult time point (Figure 4B). Although chlorpyrifos exposure caused significant reductions in cerebellar norepinephrine, it had no such effect in the cerebral cortex (Figure 4C). However, when chlorpyrifos was preceded by prenatal nicotine treatment, it did evoke significant changes in cerebrocortical norepinephrine levels, showing a persistent overall increase (Figure 4D); although effects were largest in the temporal/occipital cortex in females, variability was too high to achieve significant interactions of treatment $\times$ sex or treatment $\times$ region that would be required for interpretation of regional or sex differences. The enhanced effect of the combined treatment was statistically distinguishable from that of chlorpyrifos in the absence of nicotine pretreatment ($p < 0.05$); further, the positive effect of the combination on cerebrocortical norepinephrine levels was distinguishable from the absence of that effect in the cerebellum (treatment $\times$ region, $p < 0.04$). In contrast to the interaction of chlorpyrifos with nicotine,

prenatal dexamethasone treatment had no significant impact on the subsequent effects of chlorpyrifos on cerebrocortical norepinephrine levels (Figure 4E).

4. DISCUSSION

Our results indicate that either prenatal nicotine or dexamethasone treatment sensitizes developing noradrenergic circuits to subsequent damage by chlorpyrifos exposure, thus providing a proof-of-principle that fetal chemical exposures that are widespread in the human population can create a subpopulation with heightened subsequent vulnerability to commonly-encountered environmental toxicants. For the cerebellum, chlorpyrifos by itself produced initial, small elevations in β AR binding that regressed to normal by adulthood. But when chlorpyrifos exposure was preceded by fetal nicotine treatment, there were profound deficits that first emerged at the end of adolescence and intensified into adulthood. The effects of dexamethasone pretreatment were less intense but still showed the same increase in sensitivity to chlorpyrifos, with females displaying deficits in β AR binding throughout development, whereas in males, the decrements emerged in adulthood.

It was important to distinguish whether the reductions in β ARs represented a primary synaptic defect, or whether they were an adaptation to effects on presynaptic input; specifically, a deficit in β ARs could be an appropriate response to presynaptic noradrenergic hyperinnervation/hyperactivity, and would tend to normalize any impact on overall synaptic function. However, we did not find any elevations in cerebellar norepinephrine levels, and in fact, norepinephrine was reduced by each of the exposure paradigms. For chlorpyrifos alone, there was a preferential decrement in males, emerging after adolescence. With nicotine pretreatment, chlorpyrifos actually showed a smaller net effect that was still preferential for males, and after dexamethasone pretreatment we found essentially the same pattern as for chlorpyrifos alone. The presynaptic noradrenergic deficits would actually magnify the adverse functional impact of deficient β ARs, the opposite of an adaptive change. Our findings indicate further that the effects on norepinephrine and β ARs are entirely distinct; whereas nicotine enhanced the effect of chlorpyrifos on β ARs, it reduced the impact on norepinephrine. The latter observation is of particular interest because nicotine acts simultaneously as a neuroprotectant and a neurotoxicant, dependent on the differentiation stage of the affected cells (Slotkin et al. 2007). The present findings display both those effects of nicotine, directed in this case toward an interaction with chlorpyrifos.

If the impact of prenatal drug exposures on subsequent chlorpyrifos-induced neurotoxicity depends upon the developmental stage at which exposure occurs, then it would be expected that noradrenergic circuits in other brain regions would show a different spectrum of effects from the cerebellum, which is a late-developing region (Rodier 1988). Here, we compared the effects of the same treatment paradigms in the cerebral cortex, which develops earlier. By itself, chlorpyrifos caused an elevation in cerebrocortical β ARs, just as it had for the cerebellum, but in the cerebral cortex the effect was selective for males, and there was no regression of the effect in adulthood as had been seen for the cerebellum. More strikingly, dexamethasone alone produced a robust β AR elevation that progressed from adolescence to adulthood, an effect that was not seen in the cerebellum. Then, sequential exposure to dexamethasone followed by chlorpyrifos evoked yet another unique pattern, eliciting

significant β AR deficits in females, an effect that was not seen with either agent alone; regional differences in sex-dependence were also apparent in that males showed an increase in the cerebral cortex instead of the decrease seen in the cerebellum.

Regional distinctions were also apparent for the impact on norepinephrine levels. In the cerebral cortex, chlorpyrifos did not bring about the decrements seen in the cerebellum and the combined treatment paradigms produced completely different results in the two regions: for nicotine + chlorpyrifos, there were increases in the cerebral cortex but decreases in the cerebellum, and for dexamethasone + chlorpyrifos there were no significant effects in the cerebral cortex but profound decrements in the cerebellum. Our results thus indicate that the sensitization to chlorpyrifos evoked by prenatal drug exposures is not uniform for all noradrenergic circuits, but rather that the net impact depends on the developmental state of each brain region at the time of exposure.

Although our study was designed to test treatment outcomes rather than underlying mechanisms, the results nevertheless provide some important mechanistic information. First, the sensitization to chlorpyrifos caused by nicotine or dexamethasone does not represent simply an increase in systemic toxicity, heightened sensitivity to cholinesterase inhibition, or effects on chlorpyrifos pharmacokinetics; as detailed in our earlier studies, the pretreatments do not enhance the impact of chlorpyrifos on fetal or neonatal weight gain, on brain region weights, or on cholinesterase activity, nor do they affect chlorpyrifos levels in the neonatal brain (Levin et al. 2014; Slotkin et al. 2014, 2015b; Slotkin and Seidler 2015). Second, many aspects of the interaction of nicotine or dexamethasone with chlorpyrifos directed toward noradrenergic systems, have been recapitulated in vitro using PC12 cells, a neurodifferentiation model for the catecholaminergic phenotype (Qiao et al. 2003; Slotkin et al. 2007, 2012). Specifically, in the cell model, nicotine provided both protection from, and sensitization to chlorpyrifos dependent upon the developmental stage (Qiao et al. 2003; Slotkin et al. 2007), just as it did in vivo in the present work. Likewise, the cell model showed that dexamethasone rendered chlorpyrifos specifically injurious to the catecholamine phenotype (Slotkin et al. 2012). Thus, at least some of the sensitization seen here, represents an interaction at the cellular level. Nevertheless, a direct effect on neurodifferentiation cannot explain all the results. Many of our findings showed a temporal progression from adolescence to adulthood, rather than simply displaying persistence of an initial deficit, as would have been expected from a direct, immediate impact on cell development. Similarly, augmentation of an initial injury would not explain regional differences where effects are in opposite directions, nor would it necessarily account for sex differences where males are more affected for some parameters, but females are more sensitive for others. These factors of temporal progression, sex selectivity, and specificity for certain circuits, means that the net effect represents a change in the trajectory of development of noradrenergic function, not just immediate sensitization to damage.

4.1 Conclusions

Prenatal treatment with either nicotine or dexamethasone sensitizes cerebellar noradrenergic circuits to impairment caused by subsequent, neonatal chlorpyrifos exposure. Given the large number of individuals whose mothers smoked during pregnancy and/or who were

treated with glucocorticoids for preterm labor, our results point to the creation of a substantial subpopulation with heightened vulnerability to at least one member of a commonly-used class of pesticides with virtually ubiquitous human exposures (Casida and Quistad 2004). In the case of maternal smoking, substitution of alternative nicotine delivery devices, including transdermal patches or e-cigarettes, would not be expected to reduce the potential harm, since we found that nicotine produced sensitization to chlorpyrifos. The current results for noradrenergic systems are consonant with earlier studies of these specific interactions directed toward cholinergic or serotonergic circuits (Levin et al. 2014; Slotkin et al. 2014, 2015b; Slotkin and Seidler 2015), and therefore reinforce the idea that prenatal drug exposures can enhance the overall sensitivity of the developing brain to neurotoxicants. Our findings also provide the necessary, underlying defects at the level of synaptic function that verify the significance of morphological damage as reported earlier (Abdel-Rahman et al. 2004; Abou-Donia et al. 2006); specifically, because functional deficits provide more sensitive endpoints than gross anatomical effects, we were able to detect augmented synaptic changes that explain synergism at the behavioral level, even when enhanced toxicity cannot be detected at the morphological level. Most importantly, beyond the known liabilities associated with maternal smoking during pregnancy or with preterm delivery, our findings indicate the likelihood of heightened vulnerability to neurotoxic chemicals encountered later in life.

Acknowledgments

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Abbreviations

ANOVA	analysis of variance
βAR	β-adrenergic receptor
GD	gestational day
PN	postnatal day

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Figure 1.

β AR binding in cerebellum after exposure to: nicotine (A), dexamethasone (B), chlorpyrifos (C), nicotine + chlorpyrifos (D), or dexamethasone + chlorpyrifos (E). Data are mean $\pm$ SE, presented as the percent change from control values, which are shown in Table 1.

Multivariate ANOVA (factors of treatment, age, sex) appears at the top of each panel; where treatment interacted with age (C,D,E), asterisks denote individual ages where values differ significantly from the corresponding control. ANOVA across all treatments indicated a significant main treatment effect ($p < 0.0001$) and interactions of treatment $\times$ age ($p < 0.02$) and treatment $\times$ sex ($p < 0.05$); PN150 could not be included in this global test because the absence of values in B and E.

Figure 2.

Norepinephrine concentration in cerebellum after exposure to: nicotine (A), dexamethasone (B), chlorpyrifos (C), nicotine + chlorpyrifos (D), or dexamethasone + chlorpyrifos (E). Data are mean $\pm$ SE, presented as the percent change from control values, which are shown in Table 1. Multivariate ANOVA (factors of treatment, age, sex) appears at the top of each panel. ANOVA across all treatments indicated a significant main treatment effect ($p < 0.02$) and an interaction of treatment $\times$ sex ($p < 0.05$); PN30 and PN60 could not be included in this global test because the absence of values in A and D.

Figure 3.

β AR binding in cerebrocortical regions after exposure to: dexamethasone (A), chlorpyrifos (B) or dexamethasone + chlorpyrifos (C). Data are mean $\pm$ SE, presented as the percent change from control values, which are shown in Table 1. Multivariate ANOVA (factors of treatment, age, sex) appears at the top of each panel; where treatment interacted with age (A), asterisks denote individual ages where values differ significantly from the corresponding control. ANOVA across all treatments indicated a significant main treatment effect ($p < 0.0001$ and interactions of treatment $\times$ age ($p < 0.003$) and treatment $\times$ sex ($p < 0.0001$)).

Figure 4.

Norepinephrine concentration in cerebrocortical regions after exposure to: nicotine (A), dexamethasone (B), chlorpyrifos (C), nicotine + chlorpyrifos (D), or dexamethasone + chlorpyrifos (E). Data are mean $\pm$ SE, presented as the percent change from control values, which are shown in Table 1. Multivariate ANOVA (factors of treatment, age, sex in A,C,D; factors of treatment, sex in B,E) appears at the top of each panel. Multivariate ANOVA could not be run across all treatments simultaneously because of the absence of PN100 values for B and E.

TABLE 1Control Values (mean $\pm$ SE)

Region	Sex	β AR Binding (fmol/mg protein)				Norepinephrine (ng/g)			
		PN30	PN60	PN100	PN150	PN30	PN60	PN100	PN150
cerebellum	male	25 $\pm$ 1	38 $\pm$ 1	38 $\pm$ 1	47 $\pm$ 3	184 $\pm$ 11	229 $\pm$ 10	208 $\pm$ 10	201 $\pm$ 8
	female	26 $\pm$ 1	39 $\pm$ 1	38 $\pm$ 1	46 $\pm$ 2	187 $\pm$ 7	198 $\pm$ 10	197 $\pm$ 5	176 $\pm$ 9
frontal/parietal cortex	male	44 $\pm$ 2	41 $\pm$ 1	38 $\pm$ 1	—	—	—	451 $\pm$ 15	468 $\pm$ 15
	female	50 $\pm$ 3	44 $\pm$ 2	38 $\pm$ 2	—	—	—	415 $\pm$ 12	432 $\pm$ 22
temporal/occipital cortex	male	45 $\pm$ 2	37 $\pm$ 1	34 $\pm$ 1	—	—	—	276 $\pm$ 16	278 $\pm$ 12
	female	49 $\pm$ 1	42 $\pm$ 1	37 $\pm$ 1	—	—	—	259 $\pm$ 6	260 $\pm$ 10