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Prenatal Dexamethasone Augments the Neurobehavioral Teratology of Chlorpyrifos: Significance for Maternal Stress and Preterm Labor

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Abstract

Glucocorticoids are the consensus treatment given in preterm labor and are also elevated by maternal stress; organophosphate exposures are virtually ubiquitous, so human developmental coexposures to these two agents are common. This study explores how prenatal dexamethasone exposure modifies the neurobehavioral teratology of chlorpyrifos, one of the most widely used organophosphates. We administered dexamethasone to pregnant rats on gestational days 17-19 at a standard therapeutic dose (0.2 mg/kg); offspring were then given chlorpyrifos on postnatal days 1-4, at a dose (1 mg/kg) that produces barely-detectable (<10%) inhibition of brain cholinesterase activity. Dexamethasone did not alter brain chlorpyrifos concentrations, nor did either agent alone or in combination affect brain thyroxine levels. Assessments were carried out from adolescence through adulthood encompassing T-maze alternation, Figure-8 maze (locomotor activity, habituation), novelty-suppressed feeding and novel object recognition tests. For behaviors where chlorpyrifos or dexamethasone individually had small effects, the dual exposure produced larger, significant effects that reflected additivity (locomotor activity, novelty-suppressed feeding, novel object recognition). Where the individual effects were in opposite directions or were restricted to only one agent, we found enhancement of chlorpyrifos' effects by prenatal dexamethasone (habituation). Finally, for behaviors where controls displayed a normal sex difference in performance, the combined treatment either eliminated or reversed the difference (locomotor activity, novel object recognition). Combined exposure to dexamethasone and chlorpyrifos results in a worsened neurobehavioral outcome, providing a proof-of-principle that prenatal

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glucocorticoids can create a subpopulation with enhanced vulnerability to environmental toxicants.

Keywords

Chlorpyrifos; Dexamethasone; Glucocorticoids; Organophosphate pesticides; Preterm labor; Thyroid hormone

1. Introduction

The existence of subpopulations that are especially vulnerable to toxicant effects is one of the most important issues in toxicology. Although a great deal of attention has been paid to genetic differences that underlie differential susceptibility, it is also likely that prior exposure to a drug or chemical could sensitize an individual to subsequent exposure to an unrelated toxicant. In the current study, we examined how prenatal exposure to dexamethasone, a glucocorticoid used to treat preterm labor, augments the neurobehavioral teratology of the widely-used organophosphate pesticide, chlorpyrifos.

Glucocorticoids have a profound effect on brain development and excess levels, whether from maternal stress or from pharmacologic administration, reduce the numbers of neurons, impair structural assembly and synaptic connectivity, and lead ultimately to neuroendocrine, behavioral and cardiovascular disorders (Cavalieri and Cohen, 2006; Drake et al., 2007; Meyer, 1985; Moritz et al., 2005; Pryce et al., 2011; Rokytka et al., 2008; Tegethoff et al., 2009). Nearly twenty years ago, the National Institutes of Health produced a consensus report endorsing the use of glucocorticoids in preterm labor so as to prevent neonatal respiratory distress syndrome (Gilstrap et al., 1995). Consequently, approximately 10% of all U.S. newborns, about 400,000 per year, are now exposed to glucocorticoids (Matthews et al., 2002), although only about 1% of these infants would actually have benefited from the treatment (Gilstrap et al., 1995). It is now evident that prenatal glucocorticoid use produces neurodevelopmental disorders in children, as expected from the findings in animal studies (Crowther et al., 2007; Hirvikoski et al., 2007; Needelman et al., 2008; Newnham, 2001; Peltoniemi et al., 2011). In addition to therapeutic administration, glucocorticoids are also increased by maternal stress. Stress is a potential factor in the net response to toxicant exposures, especially in light of the standard testing practice of extending the dose-response curve to the point of outright maternal toxicity.

Notwithstanding widespread prenatal exposure to glucocorticoids, relatively little attention has been paid to their potential role in modifying developmental toxicity. Our research group has been exploring how prenatal glucocorticoid treatment sensitizes the developing brain to subsequent exposure to organophosphates, the most widely used class of insecticides, to which nearly the entire human population is exposed (Casida and Quistad, 2004). In a pair of recent studies in rats, we found that dexamethasone given in late gestation augmented the adverse effects of chlorpyrifos, a commonly-used organophosphate pesticide, on development of cholinergic neurotransmitter systems (Slotkin et al., 2013a); notably, the effects of combined exposure showed both additive and synergistic deficits in indices of presynaptic and postsynaptic activity, with distinct sex-selective differences, and including effects that were unique to the dual exposure (i.e. not seen with either agent alone). When we modeled the same treatments in vitro in neuronotypic cell cultures (Slotkin et al., 2012), we were able to reproduce many of the same interactions, indicating that the adverse effects of the combined exposure reflected, in part, their convergence on convergent pathways that interfere with neuronal cell replication and differentiation.

In the current study, we examined how prenatal dexamethasone exposure modifies the neurobehavioral effects of a subsequent postnatal exposure to chlorpyrifos. We administered dexamethasone on gestational days (GD) 17-19, corresponding to the stage of brain development in which glucocorticoid therapy is typically given in preterm labor, using a dose (0.2 mg/kg) in the low therapeutic range (Gilstrap et al., 1995). The three-day regimen corresponds to multiple glucocorticoid courses, as used in approximately 85% of all cases (Dammann and Matthews, 2001), and the dose was chosen to produce submaximal effects to allow detection of interactions with chlorpyrifos (Kreider et al., 2005; Kreider et al., 2006; Slotkin et al., 1996; Slotkin et al., 2013a; Slotkin et al., 2006). Chlorpyrifos was given daily on postnatal days (PN) 1-4 at a dose of 1 mg/kg, a regimen that produces barely-detectable inhibition of brain cholinesterase that recovers quickly after the last exposure and that disrupts neurobehavioral development, but that is not systemically toxic (Slotkin, 1999; 2004; 2005; Song et al., 1997). This chlorpyrifos regimen causes long-term neural and behavioral dysfunction, including sex selective effects (Aldridge et al., 2004; Icenogle et al., 2004; Levin et al., 2002; Levin et al., 2001b; Ricceri et al., 2006; Slotkin, 2005; Slotkin et al., 2005), and the effects in animals successfully predict both the neurobehavioral deficits and abnormalities of brain structure seen in children exposed prenatally to chlorpyrifos (Bouchard et al., 2011; Engel et al., 2011; Horton et al., 2012; Rauh et al., 2011; Rauh et al., 2006; Rauh et al., 2012). Our study encompassed four treatment paradigms: control, dexamethasone alone, chlorpyrifos alone, and dexamethasone followed by chlorpyrifos. Tests of locomotor activity, investigatory behavior and emotional response to novelty, which have previously been found to be sensitive to either dexamethasone or chlorpyrifos, were selected for study of interactive effects. Rats of both sexes were tested because of the important sex-selective effects of developmental exposure to these compounds.

Finally, because early-life exposure to chlorpyrifos has been reported to alter thyroid structure and circulating thyroxine (T4) levels (De Angelis et al., 2009), we evaluated brain T4 concentrations during chlorpyrifos exposure and in adulthood to determine if prenatal dexamethasone could enhance thyroid toxicity; in addition, we measured brain chlorpyrifos concentrations so as to determine whether dexamethasone might alter the pharmacokinetics of the organophosphate.

2. Methods

2.1. Animal subjects and treatments

Studies were conducted with protocols approved by the Institutional Animal Care and Use Committee and in accordance with all federal and state guidelines. Timed-pregnant Sprague-Dawley rats (Charles River, Raleigh, NC) were shipped by climate-controlled truck (total transit time < 1 h) on gestational day GD11, nearly a week before any treatment commenced. Animals were housed individually, allowed free access to food and water and, on GD17, were randomly assigned to 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); dexamethasone sodium phosphate was purchased from Sigma Chemical Co. (St. Louis, MO) and chlorpyrifos from Chem Service (West Chester, PA). On GD17, 18 and 19, dams received subcutaneous injections of either saline vehicle or 0.2 mg/kg dexamethasone, a dose at the lower range recommended for therapeutic use in preterm labor (Gilstrap et al., 1995). Parturition occurred during GD22, which was also taken as PN0. After birth, pups were randomized within treatment groups to dams with the same treatment as the birth dam 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 (Sigma) to provide consistent absorption (Whitney et al., 1995) and pups were 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. This regimen has been shown previously to produce developmental neurotoxicity without eliciting growth retardation or any other signs of systemic toxicity (Slotkin, 1999; 2004). 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). Randomization is important when small numbers of animals are to be used for determinations; otherwise, selection of one or two animals from an atypical litter could skew the results. Animals were weaned on PN21.

2.2. Analysis of brain chlorpyrifos and T4 levels

Two hr after the last injection of chlorpyrifos on PN4, one male and one female pup were selected from each of three litters in the two groups receiving chlorpyrifos treatment (chlorpyrifos alone, dexamethasone + chlorpyrifos); the animals were decapitated, the whole brain was dissected and frozen in liquid nitrogen, and stored at -45°C until assayed. For analysis of chlorpyrifos, tissues were first homogenized using glass beads in a Bullet Blender (Next Advance, Averill Park, NY, USA). Samples were then extracted into ethyl acetate, concentrated to dryness using nitrogen gas, washed three times with dichloromethane and then suspended in dichloromethane for analysis. Chlorpyrifos and its oxon metabolite were separated in the extract using an Agilent 6890N Gas Chromatograph fitted with a programmable temperature vaporization inlet and a DB-5ms capillary column (Agilent Technologies, Santa Clara, CA, USA). The analytes were detected using an Agilent 5975C single quadrupole mass spectrometer in negative chemical ionization mode. Laboratory blanks and matrix spikes were processed with the samples for quality assurance/quality control. Results were corrected for recovery ($\approx 90\%$) using internal standards (D_{10} -chlorpyrifos and D_{10} -diazinon).

For determination of brain T4 levels, animals were selected on PN4 (24 hr after the preceding day's chlorpyrifos injection) and in adulthood on PN150, with 8-12 animals per treatment group; for PN4, we utilized whole brain, whereas on PN150, we sampled the cerebellum. Tissue preparation, thyroid hormone extraction and analysis were all conducted using isotope-dilution liquid chromatography tandem mass spectrometry using a published method (Kunisue et al., 2011) with slight modifications. ^{13}C -labeled T4 and triiodothyronine (T3) were spiked into the tissues prior to extraction and used as internal standards, which averaged 75% and 85%, respectively. Cerebral T3 levels were too close to the assay detection limit ($\approx 0.3\text{ ng/g}$) to permit accurate assessment, so these are not reported.

2.3. Behavioral Test Battery

The tests selected covered a spectrum of behavioral functions including cognition (novel object recognition), emotional response (novel environment feeding) and spontaneous behavior (Figure-8 apparatus locomotor activity and T-maze exploration). All animals were tested on all of the following behavioral tests in the same sequence. None of the tests had high stress components that would have run the risk of carryover behavioral effects. At least a week interval was allowed between tests to further minimize any possible carryover effects. Behavioral assessment began with T-maze exploration at postnatal week 4. During week 5 locomotor activity and its habituation was tested in the Figure-8 apparatus. During week 7 fear was indexed with the Novel Environment Feeding Test. During week 9

investigatory behavior and memory was assessed with the Novel Object Recognition Test. Each apparatus and test procedure has been described in detail previously (Roegge et al., 2008; Swartzwelder et al., 2012; Timofeeva et al., 2008). For T-maze alternation, five trials were conducted during the test session and the endpoints were the percent of alternation between left and right arms, and average response latency. For the Figure-8 maze, animals were allowed to roam in the maze during a 1-hour session and photobeam breaks were recorded in twelve 5-min blocks to assess locomotor activity; to assess the speed of habituation, the blocks were analyzed for linear trend.

For novelty-suppressed feeding rats were food-deprived for 24 hours prior to testing in the novel environment, and were then examined for 10 min, with measurements of the latency to begin eating, the number of eating sessions, amount of food consumed and the total time spent eating.

For the Novel Object Recognition Test, the animals were habituated to the apparatus in a ten-minute session conducted on two consecutive days so that the subjects would subsequently react to the novelty of the objects rather than the environment. Testing began on day three with a 5-minute information session (with identical objects, A/A session) followed by a 15 minute delay period. Then, in a subsequent 5-minute session, animals were presented with dissimilar objects (one novel object and one object that was used in the identical object session, A/B session) and the time spent with either object was recorded.

2.4. Data analysis

Data were compiled as means and standard errors. One male and one female from each litter was tested. Treatment effects were detected using multivariate ANOVA, incorporating all variables (prenatal treatment, postnatal treatment, sex) in a single test so as to avoid an increased probability of a type-1 error from repeated testing of the same data set; whenever variance was heterogeneous, values were log-transformed prior to statistical testing. Where appropriate, sequential test blocks were included in the analysis as a repeated measure. Further, the Novelty Suppressed Feeding Test involved four separate measurements related to the same behavior, so the measurements were combined for a single initial test in a multiple ANOVA (MANOVA) design, before subdividing the results into individual measures. When permitted by interactions of treatment with other variables, lower-order tests were conducted, followed by Fisher's Protected Least Significant Difference test to establish differences between individual groups. However, where there were no interactions, we report only the main treatment effects without further subdivision.

The design of the studies required two different ways of regarding treatment variables. To characterize the effects of dexamethasone alone, chlorpyrifos alone, or the combined treatment versus controls or versus each other, the four treatment groups were first considered as a single factor with four groups. To determine whether the effects of dexamethasone and chlorpyrifos were interactive, the treatments were changed to a two-factor (2×2) design. In this formulation, synergistic, antagonistic or less-than-additive effects appear as significant interactions between the two treatment factors, whereas simple, additive effects do not show significant interactions. Statistical significance for all tests was assumed at $p < 0.05$.

3. Results

3.1. Maternal, litter and growth effects

As previously published (Slotkin et al., 2013b) dexamethasone exposure caused a significantly reduced weight gain in the pregnant dams resulting in a 10% deficit compared to control dams and reduced 10-15% weight in the offspring during the postnatal period and

5-10% weight deficit into adulthood. Furthermore, the combined treatment with dexamethasone and chlorpyrifos did not result in any neonatal morbidity or mortality, and actually produced less initial growth retardation than dexamethasone alone. Prenatal dexamethasone exposure did not significantly alter brain chlorpyrifos levels measured 2 hr after injection on PN4, the time of peak effect on cholinesterase (Dam et al., 2000): chlorpyrifos alone, 54 ± 10 ng/g (n=6); dexamethasone + chlorpyrifos, 69 ± 6 (n=6); results for males and females are presented combined because of the absence of sex differences. We did not find any significant effects of chlorpyrifos alone on brain T4 levels, whether measured during neonatal chlorpyrifos administration or subsequently, in adulthood, nor did we find any impact of prenatal dexamethasone treatment alone or in combination with postnatal chlorpyrifos (Table 1).

3.2. T-Maze Exploration

Prenatal dexamethasone treatment reduced the average latency significantly as shown by a significant main treatment effect ($p < 0.03$; n=12 per treatment per sex): 27.1 ± 0.7 sec per trial for control, 24.8 ± 1.2 for dexamethasone alone, 27.3 ± 0.9 for chlorpyrifos alone, and 24.8 ± 1.0 for the group receiving combined treatment. There was no significant effect of chlorpyrifos, nor was there an interaction of the two treatment effects in the two-factor ANOVA design. Since there were no significant sex differences or interactions of sex $\times$ treatment, these are the values combined for males and females.

3.3. Locomotor Activity in the Figure-8 Maze

In the Figure-8 apparatus, the mean activity level over the test session showed a significant main effect of treatment ($p < 0.03$) and of sex ($p < 0.0005$), as well as a treatment $\times$ sex interaction ($p < 0.01$), necessitating separate comparisons of males and females in tests of the simple main effects of treatment within sex (Figure 1A). In males, either dexamethasone or chlorpyrifos treatment alone evoked significant ($p < 0.05$) locomotor hyperactivity. However, the group receiving both treatments showed even greater hyperactivity ($p < 0.0005$ vs. control), significantly more than chlorpyrifos alone ($p < 0.05$). In contrast, locomotor activity was not significantly affected by these treatments in females. It is also important to note that in controls, there was a sex difference in locomotor activity, with females significantly more active than males ($p < 0.0005$). Either treatment alone reduced this normal sex difference in locomotor activity and the combined treatment completely eliminated it. The two-way analysis of chlorpyrifos and dexamethasone as separate factors did not detect a significant interaction of these factors for locomotor activity in the figure-8 apparatus but confirmed the main treatment effects of chlorpyrifos ($p < 0.02$) and dexamethasone ($p < 0.004$) in males.

All treatment groups showed the reduction in locomotor activity over time ($p < 0.0001$ for main effect of block) that is characteristic of habituation (Figure 1B). The treatments significantly affected habituation, as shown by a treatment $\times$ block interaction ($p < 0.0005$). In this case, there was no treatment $\times$ sex interaction, so values for males and females were combined. To quantify the differences in habituation, the linear trend of habituation was calculated (Keppel, 1982). This showed a significant ($p < 0.0005$) main treatment effect (Figure 1C). By itself, dexamethasone treatment slowed the rate of habituation by 50% relative to controls. Chlorpyrifos alone evoked a small, nonsignificant increase in the slope. However, in animals pretreated with dexamethasone, chlorpyrifos raised the value by 50% relative to that of dexamethasone alone, so that the increase evoked by the combination became significant when compared to dexamethasone alone; accordingly, the two treatments were interactive ($p < 0.05$) in the two-factor ANOVA test. Because the augmented effect of chlorpyrifos was in the opposite direction (increase) from that of dexamethasone (decrease), the interaction with chlorpyrifos actually reversed the dexamethasone-induced deficit. The

two-factor test again confirmed the main treatment effects of both chlorpyrifos ($p < 0.002$) and dexamethasone ($p < 0.0001$).

3.4. Novelty-Suppressed Feeding

There were four measures taken for this test: latency to begin feeding, amount of food eaten, number of feeding bouts and time spent feeding. Accordingly, we first conducted a MANOVA across all the measurements in a single statistical test, and found a significant main treatment effect ($p < 0.03$, Wilks' Lambda). For latency to begin feeding, there were nonsignificant increases caused by chlorpyrifos or dexamethasone individually, but a larger, significant ($p < 0.005$) increase with the combined exposure (Figure 2A); the augmented effect was not distinguishable from the additive contributions of the individual treatments (no dexamethasone $\times$ chlorpyrifos interaction). Similarly, the amount of food eaten showed a decline that was significant only for the group receiving combined treatment ($p < 0.05$) (Figure 2B), as did the number of feeding bouts ($p < 0.05$) (Figure 2C). For feeding duration (Figure 2D), both dexamethasone ($p < 0.05$) and the combined treatment ($p < 0.005$) showed significant decrements, and again the latter effect was indistinguishable from additive actions of the two individual agents (no interaction of the two treatments in the two-factor ANOVA). The two-factor test again confirmed a significant main treatment effect of dexamethasone in the MANOVA ($p < 0.002$) and in each of the individual components ($p < 0.005$ for latency, $p < 0.05$ for amount of food eaten, $p < 0.02$ for feeding bouts, $p < 0.0005$ for feeding duration).

3.5. Novel Object Recognition

With the novel object recognition test, animals spent a greater amount of time investigating the novel object vs. the familiar object ($p < 0.0005$), validating the test. This differential investigation of the two objects was not found to be changed by treatment. We did find a significant main effect of treatment ($p < 0.05$) and a significant interaction of treatment $\times$ sex ($p < 0.05$), necessitating separate analysis of the values for males and females (Fig. 3). In males, there was a significant ($p < 0.01$) main effect of treatment on overall object exploration. Exposure to chlorpyrifos alone caused a significant ($p < 0.05$) increase in time spent in exploring the objects, whereas a larger, significant ($p < 0.001$) effect was seen for the group receiving the combined treatment. With the combined treatment the males had significantly ($p < 0.01$) greater total object exploration than those receiving dexamethasone alone. Again, the augmented effect was not distinguishable from simple additivity of the two individual treatments in the two-factor ANOVA, but confirmed the main effects of both chlorpyrifos ($p < 0.002$) and dexamethasone ($p < 0.05$). We did not observe a comparable treatment effect in females. Notably, there again was a normal sex difference in this behavior that was reversed in the group given combined dexamethasone + chlorpyrifos exposure. No treatment effects were seen in the initial pretest with the objects.

4. DISCUSSION

Results obtained in this study show that the behavioral abnormalities in the locomotor activity, novelty suppressed feeding and object exploration tests, caused by neonatal chlorpyrifos exposure were generally worsened in animals that received prenatal dexamethasone treatment. In turn, the endpoints evaluated here show that the underlying perturbations of neuronal cell differentiation identified for these treatments in cell cultures (Slotkin et al., 2012), or for indices of brain circuit development (Slotkin et al., 2013a), do indeed culminate in dysfunction. The implications are clear: either prenatal stress or pharmacotherapy of preterm labor have the potential to worsen the impact of subsequent toxicant exposure, thus creating a subpopulation with enhanced vulnerability to developmental neurotoxicity. Indeed, although our dexamethasone dose (0.2 mg/kg) is in the

therapeutic range for use in preterm labor, this is a much lower effective exposure in rats, given their higher surface area-to-weight ratio and metabolic rate, so that our results are likely to be equally valid for stress-induced increases in endogenous glucocorticoids.

The augmentation of chlorpyrifos' effects produced by prior exposure to dexamethasone reflected three distinct patterns. First, for behaviors where chlorpyrifos or dexamethasone individually had small, often nonsignificant effects that were in the same direction, the dual exposure produced larger, significant effects that reflected additivity (locomotor activity, novelty-suppressed feeding, novel object recognition). Second, where the effects were in opposite directions or were restricted to only one agent, we were able to demonstrate an enhancement of chlorpyrifos' effects caused by prior exposure to dexamethasone (habituation), essentially antagonizing the reduction evoked by dexamethasone. Third, for behaviors where controls displayed a normal sex difference in behavioral performance, the combined treatment enhanced the CPF effects eliminating or reversing normal sex-differences in behavior (locomotor activity, novel object recognition). Previous studies in both animals (Levin et al., 2001a) and children (Rauh et al., 2012) have shown that developmental exposure to chlorpyrifos characteristically interferes with sexual dimorphism of behavioral performance and brain structure; our findings indicate that this, too, is likely to be enhanced in subjects exposed to prenatal glucocorticoids.

Although the exact neural underpinnings of these persisting behavioral impairments remain to be fully elucidated, we did rule out two prominent possibilities. First dexamethasone did not alter the pharmacokinetics of chlorpyrifos, in keeping with our earlier finding of an equivalent, small degree of cholinesterase inhibition (<10%) in animals given chlorpyrifos alone as well as for dexamethasone + chlorpyrifos (Slotkin et al., 2013a). Likewise, dexamethasone did not enhance any chlorpyrifos effect on brain T4; chlorpyrifos had no effect in this study (two age points) but a more detailed time course shows, if anything, a transient *increase* in brain T4 from chlorpyrifos (Slotkin et al., 2013c). Instead, our results are compatible with previous conclusions that these treatments directly target developing neural pathways, with acetylcholine systems particularly vulnerable (Slotkin et al., 2013a). Indeed, since comparable effects were seen for dexamethasone-chlorpyrifos interactions in an in vitro model with neuronotypic cells (Slotkin et al., 2012), our results point to direct effects of the two agents converging on neuronal cell replication and differentiation. Future studies will be needed to address these mechanisms.

It is possible, but unlikely that treatment effects on maternal-pup interactions could have contributed to neurobehavioral impairment. Typically, impaired caretaking produces neonatal weight loss but instead, we observed a rapid postnatal recovery from the modest (10-15%) gestational growth impairment produced by this dexamethasone regimen (Slotkin et al., 2013a). It should be noted that, since we were modeling therapeutic use of dexamethasone, the dose was substantially lower and the duration of exposure shorter, than most animal studies whose primary goal is to demonstrate disruption of brain development by glucocorticoids. Similarly, although perturbation of pup behavior could impair maternal caretaking, the small magnitude of initial growth impairment and rapid postnatal recovery argue against this as a significant factor.

Our findings have important implications. The combined exposures not only cause long-term neurochemical changes as previously reported (Slotkin et al., 2013a), they cause long-term functional behavioral impairments. These results reinforce the documentation that an individual's "chemical history" may be just as important as genetic differences in determining the subsequent susceptibility to environmental toxicants. Specifically, our results point to heightened vulnerability after prenatal glucocorticoid exposure, a paradigm that is relevant to both maternal stress and to the use of these agents in preterm labor

(Gilstrap et al., 1995). Equally important, these animal studies can be readily translated into studies in human cohorts, since therapeutic use of prenatal glucocorticoids should be readily documented in medical histories. Finally, the impact of glucocorticoids will be important in considering toxicities identified by standard testing procedures, which typically mandate that exposures be extended past the threshold for maternal toxicity. The attendant stress is likely to influence the outcome of toxicant exposure, a factor that could contribute to, and explain nonmonotonic dose-effect relationships.

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Research Highlights

Combined exposure to dexamethasone and chlorpyrifos can result in a worsened neurobehavioral outcome.

For behaviors where controls displayed a normal sex difference in performance, the combined treatment either eliminated or reversed the difference.

Prenatal glucocorticoids can create a subpopulation with enhanced vulnerability to environmental toxicants.

Figure 1.

Performance in the Figure-8 Maze. Data are shown as mean $\pm$ SE compiled from 11-12 animals per sex per treatment group. (A) Mean activity level, (B) activity for each 5-minute period over the one-hour test session, showing habituation, (C) linear trend of habituation. For (A), values are shown separately for males and females because of a significant treatment $\times$ sex interaction ($p < 0.01$), whereas for (B) and (C) the values for males and females were combined because of the lack of a sex interaction. Abbreviations: Dex = dexamethasone, CPF = chlorpyrifos.

Novelty Suppressed Feeding

Figure 2. Novelty-Suppressed Feeding Test: (A) Latency to begin feeding, (B) Grams of food eaten), (C) Number of feeding bouts, (D) Time spent feeding. Data are shown as mean ± SE compiled from 12 animals per treatment group, with males and females combined because of the absence of a treatment × sex interaction.

Figure 3. Novel Object Recognition Test, showing the total time spent investigating the objects. Data represent mean $\pm$ SE compiled from 6 animals per treatment group for each sex.

TABLE 1**BRAIN THYROXINE LEVELS**

Age	Treatment	T4 (ng/g)
PN4	Control	0.66 ± 0.07
	Dexamethasone	0.70 ± 0.07
	Chlorpyrifos	0.81 ± 0.09
	Dexamethasone + Chlorpyrifos	0.78 ± 0.08
PN150	Control	6.9 ± 0.4
	Dexamethasone	8.9 ± 1.2
	Chlorpyrifos	7.0 ± 0.5
	Dexamethasone + Chlorpyrifos	7.3 ± 0.4

Data are mean ± SE from 8-12 animals in each group. Two factor ANOVA (treatment, age) indicates no significant main treatment effects, nor a treatment × age interaction. There was no significant main effect of sex, nor a treatment × sex interaction, so values are shown combined for males and females.