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# Prenatal Dexamethasone, as Used in Preterm Labor, Worsens the Impact of Postnatal Chlorpyrifos Exposure on Serotonergic Pathways

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## Abstract

This study explores how glucocorticoids sensitize the developing brain to the organophosphate pesticide, chlorpyrifos. Pregnant rats received a standard therapeutic dose (0.2 mg/kg) of dexamethasone on gestational days 17–19; pups were given subtoxic doses of chlorpyrifos on postnatal days 1–4, (1 mg/kg, <10% cholinesterase inhibition). We evaluated serotonin (5HT) synaptic function from postnatal day 30 to day 150, assessing the expression of 5HT receptors and the 5HT transporter, along with 5HT turnover (index of presynaptic impulse activity) in brain regions encompassing all the 5HT projections and cell bodies. These parameters are known targets for neurodevelopmental effects of dexamethasone and chlorpyrifos individually. In males, chlorpyrifos evoked overall elevations in the expression of 5HT synaptic proteins, with a progressive increase from adolescence to adulthood; this effect was attenuated by prenatal dexamethasone treatment. The chlorpyrifos-induced upregulation was preceded by deficits in 5HT turnover, indicating that the receptor upregulation was an adaptive response to deficient presynaptic activity. Turnover deficiencies were magnified by dexamethasone pretreatment, worsening the functional impairment caused by chlorpyrifos. In females, chlorpyrifos-induced receptor changes reflected relative sparing of adverse effects compared to males. Nevertheless, prenatal dexamethasone still worsened the 5HT turnover deficits and reduced 5HT receptor expression in females, demonstrating the same adverse interaction. Glucocorticoids are used in 10% of U.S. pregnancies, and are also elevated in maternal stress; accordingly, our results indicate that this group represents a large subpopulation that may have heightened vulnerability to developmental neurotoxicants such as the organophosphates.

**Keywords**

Chlorpyrifos; Dexamethasone; Glucocorticoids; Organophosphate pesticides; Preterm delivery; Serotonin

## 1. INTRODUCTION

One of the most important issues in developmental neurotoxicity is the existence of subpopulations that are especially vulnerable to environmental agents. Given the widespread exposures of the human population to thousands of neurotoxic chemicals and their likely contribution to the explosive increase in neurodevelopmental disorders (Grandjean and Landrigan, 2006), the identification of factors that render individuals more sensitive is particularly important in setting safety limits as well as in informing targeted populations to avoid exposure. The organophosphate pesticides provide a prime example: polymorphisms of the enzyme that breaks down these neurotoxicants clearly delineate a sensitive subpopulation (Povey, 2010). In contrast to the intense focus of many researchers on genetic factors that determine vulnerability, relatively little attention has been paid to the issue of how an individual's "chemical history" might create differential susceptibility, namely, whether prenatal exposures to neuroactive drugs or chemicals sensitize an individual to neurotoxicants encountered later in life. We recently conducted studies both in vitro (Slotkin et al., 2012) and in vivo (Slotkin et al., 2013), exploring the possibility of such an interaction between prenatal dexamethasone treatment, as used in the management of preterm labor (Gilstrap et al., 1995) and postnatal exposure to chlorpyrifos, one of the most commonly used organophosphates. This particular combination encompasses a relatively large subpopulation. Because glucocorticoids are the consensus treatment to prevent neonatal respiratory distress syndrome in preterm labor (Gilstrap et al., 1995), approximately 10% of all live births in the U.S. involve this treatment (Matthews et al., 2002); an even larger population is exposed to prenatal glucocorticoids endogenously from maternal stress. Superimposed on this subgroup, organophosphate exposures are virtually ubiquitous (Casida and Quistad, 2004).

In our earlier study, we showed that prenatal dexamethasone treatment sensitized developing rats to the subsequent effects of postnatal chlorpyrifos exposure on development of cholinergic neurotransmitter systems (Slotkin et al., 2013), including effects that were unique to the dual exposure (i.e. not seen with either agent alone); this did not reflect simply a change in chlorpyrifos pharmacokinetics or pharmacodynamics. Indeed, modeling the same treatments in neuronotypic cell cultures (Slotkin et al., 2012), we found many of the same interactions, indicating that the adverse effects of the combined exposure largely reflected their convergence on common pathways that interfere with neuronal cell replication and differentiation. In the current work, we extended our observations to serotonin (5HT) systems; 5HT pathways are targeted both by chlorpyrifos and dexamethasone individually (Aldridge et al., 2003, 2004, 2005a, b; Slotkin et al., 1996, 2006a; Slotkin and Seidler, 2005, 2007b, 2008b, 2010) and their effects on this neurotransmitter likely contributes to an adverse impact on emotional behaviors (Aldridge et al., 2005a; Mauro and Zhang, 2007; Nagano et al., 2008, 2012; Ricceri et al., 2003, 2006).

Our treatment models have been described earlier (Slotkin et al., 2013). First, we gave 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, 2006; Slotkin et al., 1996, 2006a). Chlorpyrifos was given daily on postnatal days (PN) 1–4 at a dose of 1 mg/kg, a regimen that is not systemically toxic, producing only barely-detectable inhibition of brain cholinesterase, but that nevertheless disrupts brain development (Slotkin, 1999, 2004, 2005; Song et al., 1997). This exposure model successfully predicts both the neurobehavioral deficits and abnormalities of brain structure seen in children exposed prenatally to supposedly "safe" levels of chlorpyrifos (Bouchard et al., 2011; Engel et al., 2011; Rauh et al., 2006, 2011, 2012). Our study thus encompassed four treatment paradigms: control, dexamethasone alone, chlorpyrifos alone, and dexamethasone followed by chlorpyrifos.

We conducted longitudinal evaluations of the effects on 5HT systems spanning adolescence, young adulthood and full adulthood, so as to focus on persistent alterations that are most likely to influence behavioral performance, in keeping with earlier observations that the impacts of both dexamethasone and chlorpyrifos emerge over these stages (Aldridge et al., 2003, 2004, 2005a, b; Slotkin et al., 1996, 2006a; Slotkin and Seidler, 2005, 2007b, 2008b, 2010). We assessed multiple indices of 5HT synaptic function in all the brain regions comprising the major 5HT projections (frontal/parietal cortex, temporal/occipital cortex, hippocampus, striatum) as well as those containing 5HT cell bodies (midbrain, brainstem). We measured three 5HT synaptic proteins known to be highly affected by developmental exposure to both dexamethasone and chlorpyrifos (Aldridge et al., 2003, 2004, 2005a, b;; Slotkin et al., 1996, 2006a; Slotkin and Seidler, 2005, 2007b, 2008b, 2010), the $5HT_{1A}$ and $5HT_2$ receptors, and the presynaptic 5HT transporter. The two receptors play major roles in 5HT-related mental disorders, including depression (Arango et al., 2001; Fujita et al., 2000; Yatham et al., 1999, 2000), and the transporter, which regulates the synaptic concentration of 5HT, is the primary target for antidepressant drugs (Maes and Meltzer, 1995; Nemeroff, 1998; Nutt, 2002). Then, as an index of presynaptic impulse activity, we assessed the turnover of 5HT by measuring concentrations of 5HT and its principal metabolite, 5-hydroxyindoleacetic acid.

## 2. MATERIALS AND METHODS

### 2.1. Animal treatments

All experiments were carried out humanely and with regard for alleviation of suffering, 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 were shipped by climate-controlled truck (total transit time < 1 h), housed individually and allowed free access to food and water; shipping occurred on GD11, so that animals had nearly a week in-house before treatments commenced. 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 GD17, 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). 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 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, including robust effects on serotonergic systems,

without eliciting growth retardation or any other signs of systemic toxicity (Aldridge et al., 2003, 2004, 2005a, b; Slotkin and Seidler, 2005). 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.

On PN30, 60, 100 and 150, animals were decapitated and brain regions were dissected for determination of 5HT receptor and transporter binding, involving regions containing 5HT neuronal cell bodies (midbrain, brainstem) as well as synaptic projections (frontal/parietal cortex, temporal/occipital cortex, hippocampus, striatum). The two cortical regions were sectioned at the midline and the left half used for the binding determinations, whereas the right half was used for measurement of 5HT concentrations and turnover. The cerebellum, which is sparse in 5HT projections, was reserved for future studies. Tissues were frozen in liquid nitrogen and 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. Assays were conducted on each individual tissue, so that each determination represented a value from the corresponding brain region of one animal.

### 2.2 5HT receptors and transporter

All of the ligand binding methodologies used in this study have appeared in previous papers (Aldridge et al., 2004; Slotkin et al., 2006b; Slotkin and Seidler, 2005, 2007a), so only brief descriptions will be provided here. Tissues were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in ice-cold 50 mM Tris (pH 7.4), and the homogenates were sedimented at 40,000 × *g* for 15 min. The pellets were washed by resuspension (Polytron) in homogenization buffer followed by resedimentation, and were then dispersed with a homogenizer (smooth glass fitted with Teflon pestle) in the same buffer. An aliquot was assayed for measurement of membrane protein (Smith et al., 1985).

Two radioligands were used to determine 5HT receptor binding: 1 nM [$^3$H]8-hydroxy-2-(di-n-propylamino)tetralin for the $5HT_{1A}$ receptor, and 0.4 nM [$^3$H]ketanserin for the $5HT_2$ receptor. Binding to the presynaptic 5HT transporter site was evaluated with 85 pM [$^3$H]paroxetine. For the $5HT_{1A}$ and 5HT transporter sites, specific binding was displaced by addition of 100 μM 5HT; for the $5HT_2$ receptor, we used 10 μM methylsergide for displacement.

### 2.3. 5HT concentration and turnover

Tissues were thawed and homogenized in ice-cold 0.1 M perchloric acid and sedimented for 20 min at 40,000 × *g*. The supernatant solution was collected and aliquots were used for analysis of 5HT and 5-hydroxyindoleacetic acid by high-performance liquid chromatography with electrochemical detection (Xu et al., 2001). Concurrently-run standards were used to calculate the regional concentrations. Transmitter turnover was calculated as the 5-hydroxyindoleacetic acid/5HT ratio.

### 2.4. Data analysis

Data were compiled as means and standard errors. The initial comparisons were conducted by a global ANOVA (data log-transformed because of heterogeneous variance among regions, measures and ages) incorporating all the variables and measurements so as to avoid an increased probability of type 1 errors that might otherwise result from multiple tests of

the same data set. The variables in the global test were prenatal treatment (saline, dexamethasone), postnatal treatment (DMSO vehicle, chlorpyrifos), brain region, age and sex, with multiple dependent measures ($5HT_{1A}$ receptor, $5HT_2$ receptor and 5HT transporter for the ligand binding determinations; 5HT concentration and turnover for the HPLC determinations); in both cases, the dependent measures were treated as repeated measures, since multiple determinations were derived from the same sample. Where we identified interactions of treatment with the other variables, data were then subdivided for lower-order ANOVAs to evaluate treatments that differed from the corresponding control. As permitted by the interaction terms, individual treatments that differed from control were identified with Fisher's Protected Least Significant Difference Test. Significance was assumed at the level of $p < 0.05$. 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.

For each set of assays, all four experimental groups were included (control, dexamethasone, chlorpyrifos, dexamethasone + chlorpyrifos), as well as both sexes and all three binding measures, but on a single day, we could assay only one region at one age. Accordingly, comparisons of absolute values can be made between treatment groups and sexes but not between regions and ages (which were run in separate assay batches). Accordingly, to enable ready visualization of treatment effects across regions, sex, ages and measures, the results were compared as the percent change from control values (to eliminate any batch-to-batch differences in the assays), although statistical procedures were always conducted on the original data. Graphs were scaled to encompass the different dynamic ranges of the changes in the various parameters. The absolute values for each set of determinations appear in the Supplemental Tables.

The design of the studies required two different ways of analysis 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 one-dimensional factor in the statistical design. Then, to determine whether the effects of dexamethasone and chlorpyrifos were interactive, the treatment factors were changed to a two-dimensional design. In this formulation, synergistic, less-than-additive or unique (i.e. opposite to the individual treatments) effects appear as significant interactions between the two treatment dimensions, whereas simple, additive effects do not show significant interactions.

### 2.5. Materials

Animals were purchased from Charles River Laboratories (Raleigh, NC) and chlorpyrifos was obtained from Chem Service (West Chester, PA). PerkinElmer Life Sciences (Boston, MA) was the source for radioligands: [$^3$H]8-hydroxy-2-(di-n-propylamino)tetralin (specific activity, 135 Ci/mmol), [$^3$H]ketanserin (63 Ci/mmol) and [$^3$H]paroxetine (19.4 Ci/mmol). Methylsergide was obtained from Sandoz Pharmaceuticals (E. Hanover, NJ) and all other chemicals came from Sigma Chemical Co. (St. Louis, MO).

## 3. RESULTS

### 3.1. Maternal, litter and growth effects

The animals used in this study were littermates of those used for our earlier study of acetylcholine systems and the effects on litter characteristics and growth were published previously (Slotkin et al., 2013). Dexamethasone exposure reduced weight gain in the pregnant dams by about 10%; at birth, pups also showed a 10–15% weight deficit that was reduced to 5–10% by weaning and into adulthood. Notably, the group receiving both

dexamethasone and chlorpyrifos did not show any neonatal morbidity or mortality, and actually had less growth retardation than the group given dexamethasone alone.

### 3.2. Global statistics for 5HT receptor and transporter binding

We first conducted a global ANOVA to evaluate all factors (prenatal treatment, postnatal treatment, sex, brain region, age) and all three dependent measures ($5HT_{1A}$ receptor binding, $5HT_2$ receptor binding, 5HT transporter binding; repeated measures) in a single test, and found significant main treatment effects ($p < 0.0001$) as well as interactions of treatment × sex ($p < 0.0005$), treatment × age ($p < 0.0009$), treatment × age × region ($p < 0.02$), treatment × measure ($p < 0.0001$), treatment × sex × measure ($p < 0.04$), treatment × age × measure ($p < 0.05$) and treatment × sex × age × measure ($p < 0.02$). In light of the sex-dependence of the treatment effects, we then subdivided the data for males and females and found that the treatment effects were sustained: males, $p < 0.0001$ for the main treatment effect, $p < 0.05$ for treatment × age, $p < 0.0001$ for treatment × measure, $p < 0.05$ for treatment × age × region, and $p < 0.02$ for treatment × age × measure; females, $p < 0.0001$ for treatment, $p < 0.05$ for treatment × age, $p < 0.0001$ for treatment × measure. When the dexamethasone and chlorpyrifos treatments were considered as two dimensions, there was a dexamethasone × chlorpyrifos interaction ($p < 0.0001$) that was similarly sex-dependent (dexamethasone × chlorpyrifos × sex, $p < 0.02$), as well as age- and measure-dependent ($p < 0.004$ for dexamethasone × chlorpyrifos × age, $p < 0.0001$ for dexamethasone × chlorpyrifos × measure, $p < 0.008$ for dexamethasone × chlorpyrifos × age × region). This indicated that the results for the group receiving the combined treatments did not reflect simple, additive effects of dexamethasone and chlorpyrifos. Based on these global results, we analyzed the data separated into the different measures and divided for males and females, and then looked for treatment effects and interactions of treatment with the remaining variables (age, region). However, for each set of measures, we again preceded the lower-order tests by ANOVA for all the factors (treatment, sex, region, age) in a single test to ensure that further subdivisions were justified.

### 3.3. $5HT_{1A}$ receptors

For $5HT_{1A}$ receptor binding, ANOVA across all factors identified a main treatment effect ($p < 0.0002$) that depended on sex ($p < 0.05$ for treatment × sex) and the treatment effects were sustained after subdivision of the data into males ($p < 0.05$) and females($p < 0.0003$). By itself, prenatal dexamethasone treatment evoked regionally-selective alterations in males, with significant reductions in the frontal/parietal cortex and elevations in the striatum (Fig. 1A); in contrast, females showed an overall reduction across regions (main treatment effect, $p < 0.02$). Chlorpyrifos exposure elicited opposite effects in males and females, with a significant overall elevation of $5HT_{1A}$ receptor binding in males but a reduction in females (Fig. 1B). In males, combined exposure to both dexamethasone and chlorpyrifos produced effects that were distinct from those seen with either treatment alone, characterized by an overall reduction in receptor binding (Fig. 1C); this represented a statistically significant outcome from either dexamethasone or chlorpyrifos alone ($p < 0.004$ and $p < 0.009$, respectively), and consequently, there was a significant dexamethasone × chlorpyrifos interaction ($p < 0.05$) when regarding the treatments as two dimensions in the ANOVA. In females, the combined exposure produced a change in the same direction (decrease) as that seen with either dexamethasone or chlorpyrifos, but the effect was larger; the dexamethasone × chlorpyrifos interaction term was not significant in females, indicating that the augmented effect was not distinguishable from simple additivity of the two individual agents.

To illustrate the differences among the treatment groups, we calculated the mean effect on $5HT_{1A}$ receptor binding, collapsing the values across all the interactive variables so as to

show only the main treatment effects (Fig. 1D). This simplified picture dilutes the alterations seen in specific regions or at particular ages by averaging them with the regions or ages for which there was no effect or an opposite effect, so that the absolute magnitude becomes smaller. Despite these limitations, there was an obvious sex difference in the overall patterns, with males showing treatment-related increases in binding for dexamethasone or chlorpyrifos alone, whereas females showed net decreases, reflecting the overall treatment × sex interaction seen in the global ANOVA. For males, the combined treatment with dexamethasone and chlorpyrifos produced a decrease instead of the increases seen with the individual agents, clearly an effect that did not reflect additive properties (dexamethasone × chlorpyrifos interaction, $p < 0.05$). For females, the worsened outcome of the combined treatment was not significantly different from additive individual effects of dexamethasone and chlorpyrifos.

### 3.4. $5HT_2$ receptors

For $5HT_2$ receptor binding, ANOVA across all factors identified a main treatment effect ($p < 0.0001$) that depended on sex, age and brain region: $p < 0.05$ for treatment × sex, $p < 0.02$ for treatment × age, $p < 0.0001$ for treatment × age × region. Dexamethasone treatment evoked overall elevations in males (Fig. 2A). For females, the effects were more diverse, with small but consistent increases in receptor expression in the midbrain and decreases in the temporal/occipital cortex; at one age (PN100), the brainstem showed a large decrease in $5HT_2$ receptor binding but the effect was transient, disappearing by PN150. For chlorpyrifos treatment, both males and females showed significant overall increases in $5HT_2$ binding (Fig. 2B); superimposed on the global treatment effect, females exhibited regionally- and age-selective changes, albeit consistently in the upward direction. The group receiving combined treatment showed a different spectrum of effects from that seen with either dexamethasone or chlorpyrifos alone (Fig. 2C). In males, there was still a net increase in $5HT_2$ receptor binding but the effect declined with age, so that by PN150 there were no longer any significant increases. In females, there was a significant overall decrease in the group receiving both dexamethasone and chlorpyrifos, an effect opposite to that seen with chlorpyrifos alone. Accordingly, the effects of the combined treatment for males and females were statistically distinguishable from those of chlorpyrifos alone ($p < 0.0001$), and the two-factor interaction term was highly significant (dexamethasone × chlorpyrifos, $p < 0.0001$), indicating a net effect that could not be accounted for by simple additivity of the individual effects of dexamethasone and chlorpyrifos.

The main treatment effects collapsed across age and region again illustrate the key points of these results (Fig. 2D). In males, both dexamethasone and chlorpyrifos elicited overall increases in $5HT_2$ receptor binding, as did the group receiving combined treatment, but the latter effect was smaller than with either of the individual treatments, clearly less than expected from simple additivity (dexamethasone× chlorpyrifos, $p < 0.0001$). In females, dexamethasone had a relatively neutral effect whereas chlorpyrifos caused a clear-cut increase. However, the combined treatment produced a significant decrease, an effect distinct from with either agent alone; again, the highly significant interaction term (dexamethasone × chlorpyrifos, $p < 0.0001$) confirmed that this outcome was not predicated on additive effects of the two individual treatments.

### 3.5. 5HT transporter

Global statistical analysis of the effects on 5HT transporter binding showed a significant main treatment effect ($p < 0.0001$) and interactions of treatment × sex ($p < 0.0006$) and treatment × sex × age ($p < 0.005$). By itself, dexamethasone elicited changes in opposite directions in the two sexes, with net increases in males but decreases in females (Fig. 3A). The time course also differed, with effects generally increasing with age in males and

diminishing in females. Chlorpyrifos likewise evoked significant overall elevations in 5HT transporter binding in males, with increasing effect over time (Fig. 3B); in females, effects shifted from early decreases in adolescence, to net increases by PN150. Prior exposure to dexamethasone significantly diminished the effect of chlorpyrifos in males ($p < 0.0001$); the consistent increase seen with chlorpyrifos alone was converted to a more variable pattern, with significant decreases in young adulthood (PN60), changing to increases by PN100, and nonsignificant changes by PN150. Accordingly, there was a highly-significant ($p < 0.0001$) dexamethasone × chlorpyrifos interaction when the treatments were changed to two dimensions, and in addition, there was a shift in the time course of effect (dexamethasone × chlorpyrifos × age, $p < 0.02$). In contrast, for females, the combined treatment produced a small but significant overall decrement, without the age-dependence seen with either agent alone (dexamethasone × chlorpyrifos × age, $p < 0.0008$).

Collapsing the main treatment effects across the other variables shows that, in males, the net effect of combined treatment was smaller than that of either treatment alone, and certainly lower than would be expected from additive effects (Fig. 3D). For females, the net effect of combined treatment was small and similar in magnitude to the main effect of dexamethasone alone.

### 3.6. Temporal course of effects on binding parameters

The global, repeated-measures ANOVA showed a highly significant interaction of treatment × age ($p < 0.0009$). However, subdivision of the data into the three individual measures tended to obscure this relationship. Accordingly, we also evaluated the data collapsed across all three binding parameters and brain regions, focusing instead on the main treatment effects over time, separated by sex because of the significant treatment × sex interaction ($p < 0.0005$). For either dexamethasone or chlorpyrifos alone, males showed a progressive increase in binding with age, whereas the group receiving both agents did not (Fig. 4A); the lack of temporal progression in the dexamethasone + chlorpyrifos group was thus significantly distinct from that predicted from the individual agents (dexamethasone × chlorpyrifos × age, $p < 0.03$). In contrast, females generally did not show progressive changes, with the exception of chlorpyrifos treatment alone, where there was a jump between PN100 and PN150 (Fig. 4B), a change that was caused by an increase in only one of the three binding parameters ($5HT_2$ receptors); in this case, the interaction term was not significant.

### 3.7. 5HT levels & turnover

Assessments of 5HT levels and turnover were carried out in the cortical regions that are enriched in 5HT nerve terminals (frontal/parietal cortex, temporal/occipital cortex). The global, repeated-measures ANOVA incorporating measurements of both 5HT concentration and turnover, indicated a significant main treatment effect ($p < 0.05$) that depended on sex (treatment × sex, $p < 0.03$) and that differed in magnitude and time course between the concentration measure and the turnover measure (treatment × measure, $p < 0.0005$; treatment × measure × age, $p < 0.02$). Accordingly, we performed separate evaluations of 5HT concentration and turnover, divided into males and females. For 5HT concentrations, we did not detect any significant treatment effects or interactions of treatment with other variables (Supplemental Table 4). However, there were differences in turnover. By itself, dexamethasone did not evoke any significant overall effect (Fig. 5A), although values tended to be decreased in males and increased in females. In contrast, chlorpyrifos (Fig. 5B) produced a net decrease in turnover (main treatment effect, $p < 0.03$) that was most prominent in adolescence and diminished with age (treatment × age, $p < 0.02$; individually significant on PN30). The reduction in turnover caused by chlorpyrifos was statistically significant in males but not females. The group receiving dexamethasone + chlorpyrifos

(Fig. 5C) displayed more robust effects, with greater decrements than those seen with either agent alone ($p < 0.03$ vs. dexamethasone, $p < 0.05$ vs. chlorpyrifos). In addition, this group showed regional selectivity, with a significant overall effect limited to the frontal/parietal cortex. However, the time course still showed the progression from larger effects in adolescence to lessened effects in adulthood (treatment × age interaction, $p < 0.05$; individually significant decrements on PN30 and PN60). For the combination group, significant decreases were seen for both males and females. It should be noted that the treatment effects on 5HT turnover, which reflect neurotransmitter utilization, are more important than the lack of change in the static measure, 5HT concentration. Levels of 5HT are maintained by the rate-limiting enzyme, tryptophan hydroxylase, which is controlled by impulse activity, so that neurotransmitter levels are kept in balance despite increases or decreases in synaptic stimulation (Cooper et al., 1996).

When the dexamethasone and chlorpyrifos treatments were treated as two ANOVA dimensions, we found a highly-significant interaction that was sex-dependent (dexamethasone × chlorpyrifos × sex, $p < 0.003$). Collapsing the main treatment effects across region and age illustrated the interaction (Fig. 5D) The net effect of combined treatment in males was not statistically distinguishable from simple additivity of the decrements caused by each agent alone. In contrast, there was a significant interaction of dexamethasone × chlorpyrifos in females ($p < 0.05$); this reflected the fact that, whereas dexamethasone alone evoked a nonsignificant increase in turnover, and chlorpyrifos alone had relatively neutral effects, the group receiving both dexamethasone and chlorpyrifos showed a robust decrease in turnover.

## 4. DISCUSSION

In our earlier work on acetylcholine systems, we found that prenatal exposure to dexamethasone sensitized the developing brain to subsequent injury by chlorpyrifos in a sex-dependent manner, enhancing the long-term deficits of cholinergic function primarily in females, so that they lost their “protection” relative to males that was seen with just the organophosphate (Slotkin et al., 2013). In contrast, the results obtained here for 5HT systems show that prenatal exposure to dexamethasone affects the response to chlorpyrifos in both males and females in a similar direction, namely reducing the expression of 5HT synaptic proteins and 5HT turnover relative to the levels seen with chlorpyrifos alone. The net effect then, is a suppression of 5HT synaptic function. Superimposed on this pattern, there were important dissimilarities between males and females because the effects of chlorpyrifos itself differed between the sexes; that is, the starting point for comparing the combined treatment, namely the effects of chlorpyrifos alone, were not the same. Previous studies have shown that the adverse effects of chlorpyrifos on 5HT systems and associated behaviors are sex-dependent (Aldridge et al., 2004, 2005a; Slotkin and Seidler, 2005), likely reflecting the greater recuperative capacity of the female brain after neurotoxic injury (Arevalo et al., 2012; Arnold and Beyer, 2009; Hilton et al., 2004; Suzuki et al., 2006; Tanapat et al., 1999). Here, that difference was shown by the progressive increase in the expression of 5HT synaptic proteins in all the exposure models in males, but not in females. Further, whereas males showed increases across all the proteins, females showed differential regulation of individual binding parameters and a net overall decrease, thus demonstrating a completely different set of synaptic effects.

In males, individual treatments with either dexamethasone or chlorpyrifos evoked overall increases in the expression of 5HT synaptic proteins, with the effect increasing with age. Perhaps surprisingly, the group that received the combined treatment actually showed less of an increase, without a progression from adolescence to adulthood. Essentially, prenatal dexamethasone treatment attenuated the effects of chlorpyrifos on 5HT receptor and

transporter expression. There are two ways of interpreting this finding. First, dexamethasone might protect the developing brain from the adverse effects of chlorpyrifos. This seems highly unlikely, given the sensitization to damage in acetylcholine systems noted for the same combination treatment (Slotkin et al., 2013), or for their direct effects on neuronal cell replication and differentiation (Slotkin et al., 2012). Furthermore, a protective effect would not explain why the upregulation eventually becomes lower in the combined treatment group than in the group receiving just dexamethasone (Fig. 4, PN150). The second possibility is that the increases in 5HT receptors are compensatory for underlying deficits in 5HT neurotransmission, in which case, impairment of upregulation would be maladaptive. To test this hypothesis, we measured 5HT turnover as an index of presynaptic impulse activity. Consistent with this interpretation, 5HT turnover was impaired and the reduction was evident in adolescence, thus preceding the temporal increases in the expression of 5HT synaptic proteins. Dexamethasone pretreatment did not reduce the impact of chlorpyrifos on 5HT turnover, and in fact, the effects showed additive impairment. Consequently, attenuation of 5HT receptor upregulation in the group with combined exposure, in the face of greater deficits in 5HT synaptic activity, represents a worsened functional outcome. This conclusion is currently undergoing examination in tests of 5HT-related behaviors.

In females, the effects of chlorpyrifos alone were quite different, in agreement with earlier findings (Aldridge et al., 2004, 2005a; Slotkin and Seidler, 2005). Instead of an overall pattern of receptor upregulation, females showed differential effects on each protein: downregulation of $5HT_{1A}$ receptors, upregulation of $5HT_2$ receptors, and little net change in 5HT transporter expression. In earlier work with chlorpyrifos, we showed that, unlike males, females display changes in post-receptor coupling to cellular responses, characterized by a shift from excitatory to inhibitory responses (Aldridge et al., 2004). Thus, there is a similar impairment of 5HT neurotransmission, albeit by a different cellular mechanism. Here, we found a significantly larger impairment of 5HT turnover in females given the combined treatment when compared to either dexamethasone or chlorpyrifos alone, accompanied by reductions in 5HT receptor expression. Again, this points to a worsened outcome for the impact on synaptic transmission. Thus, as in our earlier work with cholinergic systems (Slotkin et al., 2013), prenatal dexamethasone treatment removes the relative protection of the female brain from the adverse effects of chlorpyrifos.

One concern about studies with multiple treatments is the potential for repeated handling or injection stress to influence the response to toxicant administration. It is therefore important to note that the results obtained in the present study for chlorpyrifos-induced upregulation of 5HT synaptic proteins, with preferential effects on males and progressive increases from young to full adulthood, are comparable to those obtained in earlier work where there were no additional vehicle injections of the pregnant dam (Aldridge et al., 2004; Slotkin and Seidler, 2005). Our current results for dexamethasone treatment alone (with the additional stressor of postnatal vehicle administration to the pups) are likewise similar to those in an earlier longitudinal study with a different, prenatal stressor involving maternal surgery in early gestation (Slotkin and Seidler, 2010). While it is not possible to rule out all potential interactions from the stress of multiple treatments, it is clear that a second prenatal or postnatal injection regimen is insufficient to change the overall patterns of the effects of dexamethasone or chlorpyrifos.

Twenty years ago, the National Institutes of Health issued a consensus report endorsing the use of glucocorticoids in the management of preterm labor occurring between 24 and 34 weeks of gestation (Gilstrap et al., 1995). Annually in the U.S., approximately 400,000 newborns receive this treatment (Matthews et al., 2002) in order to prevent about 6000 cases of neonatal respiratory distress syndrome and 2000 deaths (Gilstrap et al., 1995). It is increasingly clear that prenatal glucocorticoid treatment has long term liabilities that need to

be taken into account, especially given the much smaller population that benefits (Crowther et al., 2007; Hirvikoski et al., 2007; Needelman et al., 2008; Newnham, 2001; Peltoniemi et al., 2011). Our results indicate that one of those liabilities is likely to be susceptibility to the effects of environmental neurotoxicants encountered later in life.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Abbreviations

| | |
|---|---|
| **5HT** | 5-hydroxytryptamine, serotonin |
| **ANOVA** | analysis of variance |
| **GD** | gestational day |
| **PN** | postnatal day |

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## Highlights

- Coexposures to glucocorticoids (stress, preterm labor) and organophosphates are common
- Chlorpyrifos produced deficits in indices of 5HT synaptic function
- Dexamethasone exacerbated the effects of chlorpyrifos
- Glucocorticoids may create a subpopulation that is vulnerable to neurotoxicants

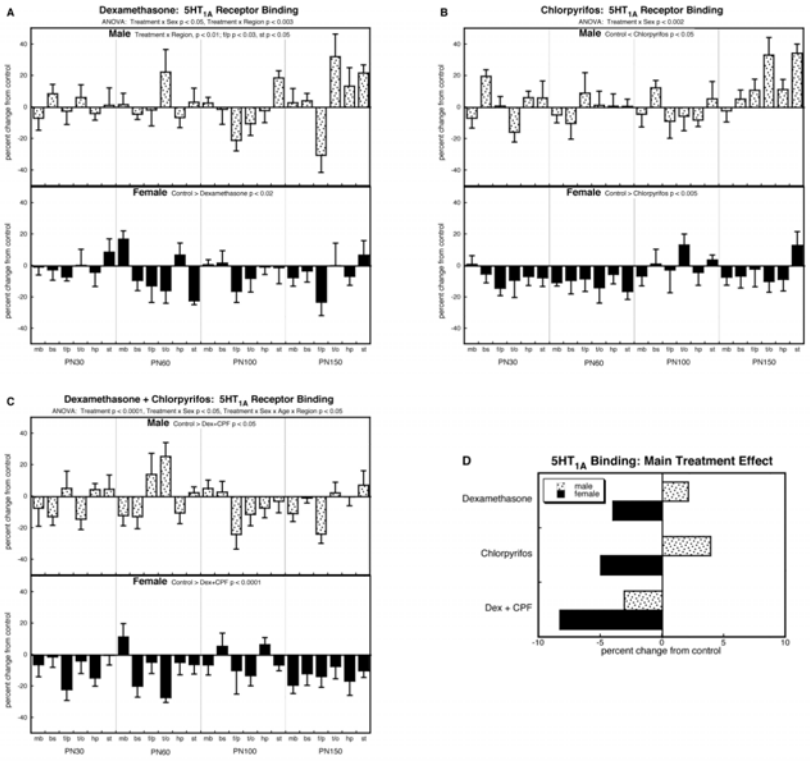


**Figure 1.**

Effects of dexamethasone (A), chlorpyrifos (B), and combined treatment (C) on $5HT_{1A}$ receptor binding (mean ± SE, n=6 for each sex in each treatment group), shown as the percent change from control values (see original values in Supplementary Table 1). Because the global ANOVA showed a significant treatment × sex interaction ($p < 0.05$), values were separated for males and females. Multivariate ANOVA for each treatment appears at the top of the panels. For dexamethasone (A), males showed an interaction of treatment × region and the regions for which differences were significant are indicated; females showed only a main treatment effect, so no lower-order tests were performed. For chlorpyrifos (B), and for combined treatment (C) both sexes showed only main treatment effects, so no lower-order tests were evaluated.. Panel (D) shows the simple main treatment effects for each sex, collapsed across all the other variables. Abbreviations: mb, midbrain; bs, brainstem; f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; hp, hippocampus; st, striatum; Dex, dexamethasone; CPF, chlorpyrifos.

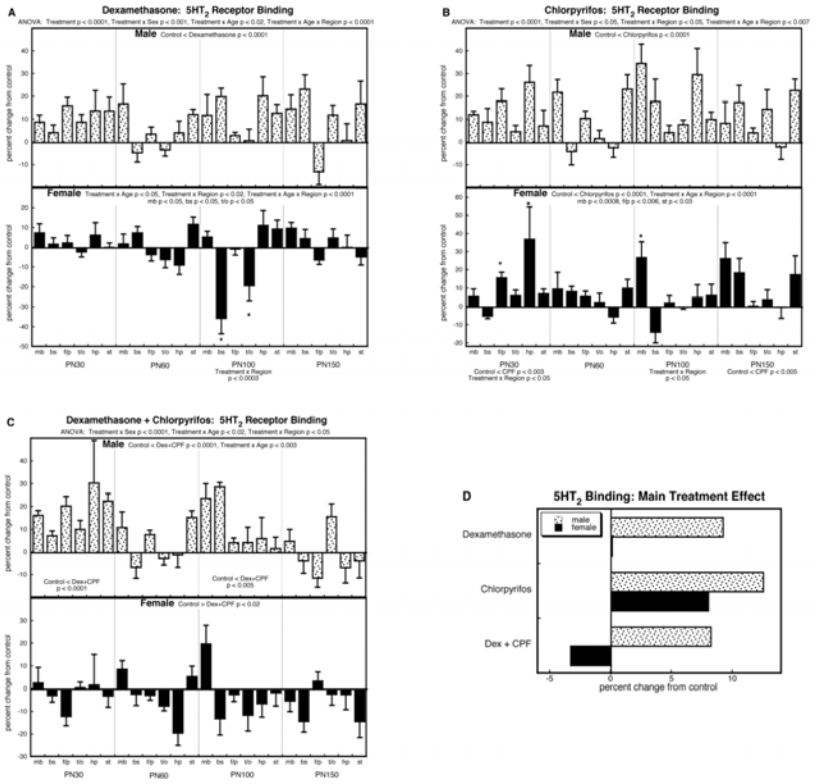


**Figure 2.**

Effects of dexamethasone (A), chlorpyrifos (B), and combined treatment (C) on $5HT_2$ receptor binding (mean ± SE, n=6 for each sex in each treatment group), shown as the percent change from control values (see original values in Supplementary Table 2). Because the global ANOVA showed a significant treatment × sex interaction ($p < 0.0001$), values were separated for males and females. Multivariate ANOVA for each treatment appears at the top of the panels. For males given dexamethasone (A) or chlorpyrifos (B), there was only a main treatment effect, so no lower-order tests were performed. However, females showed treatment interactions with region and age for both the dexamethasone (A) and chlorpyrifos (B) groups, so main treatment effects for each region are indicated at the top, and lower-order tests at each age are at the bottom; for ages that showed a treatment × region interaction, the individual regions that differ from control are indicated with asterisks. For the combined treatment group (C), males showed a main treatment effect as well as a treatment × age interaction, so lower-order tests were performed at each age and are shown at the bottom of the panel; females had only a main treatment effect, so no lower-order tests were performed. Panel (D) shows the simple main treatment effects for each sex, collapsed across all the other variables. Abbreviations: mb, midbrain; bs, brainstem; f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; hp, hippocampus; st, striatum; Dex, dexamethasone; CPF, chlorpyrifos.

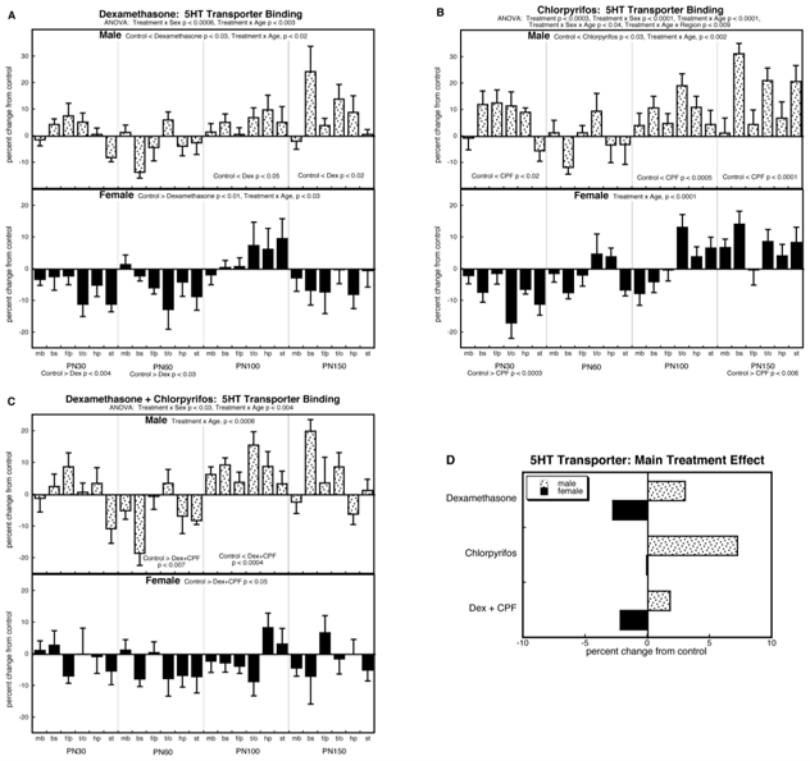


**Figure 3.**

Effects of dexamethasone (A), chlorpyrifos (B), and combined treatment (C) on 5HT transporter binding (mean ± SE, n=6 for each sex in each treatment group), shown as the percent change from control values (see original values in Supplementary Table 3). Because the global ANOVA showed a significant treatment × sex interaction ($p < 0.0006$), values were separated for males and females. Multivariate ANOVA for each treatment appears at the top of the panels. For males or females given dexamethasone (A), there was a main treatment effect as well as a treatment × age interaction, so lower-order tests were performed at each age and are shown at the bottom of the panel. Similarly, for chlorpyrifos (B), males showed a main treatment effect and a treatment × age interaction and females also displayed a treatment × age interaction; accordingly, lower-order tests were performed at each age and are shown at the bottom of the panels. For the combined treatment (C), males had a treatment × age interaction whereas females showed only a main treatment effect, so lower-order tests were performed only for males. Panel (D) shows the simple main treatment effects for each sex, collapsed across all the other variables. Abbreviations: mb, midbrain; bs, brainstem; f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; hp, hippocampus; st, striatum; Dex, dexamethasone; CPF, chlorpyrifos.

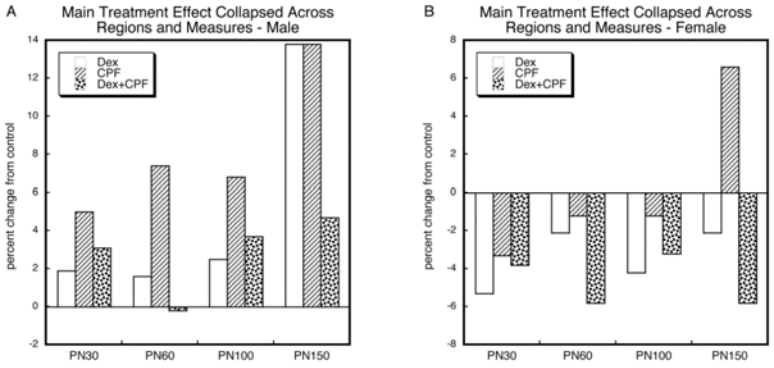


**Figure 4.**

Temporal progression of main treatment effects, collapsed across regions and measures. Values were separated for males (A) and females (B) because of the significant interaction of treatment × sex ($p < 0.0005$) in the repeated-measures ANOVA across all three binding parameters. Percent change was calculated using the geometric means of the original values in Tables 1, 2 and 3. For males, the temporal course of effects for the combined exposure group differs from what would be expected from the individual treatments (dexamethasone × chlorpyrifos × age, $p < 0.03$), whereas that interaction term is not significant in females. Abbreviations: Dex, dexamethasone; CPF, chlorpyrifos

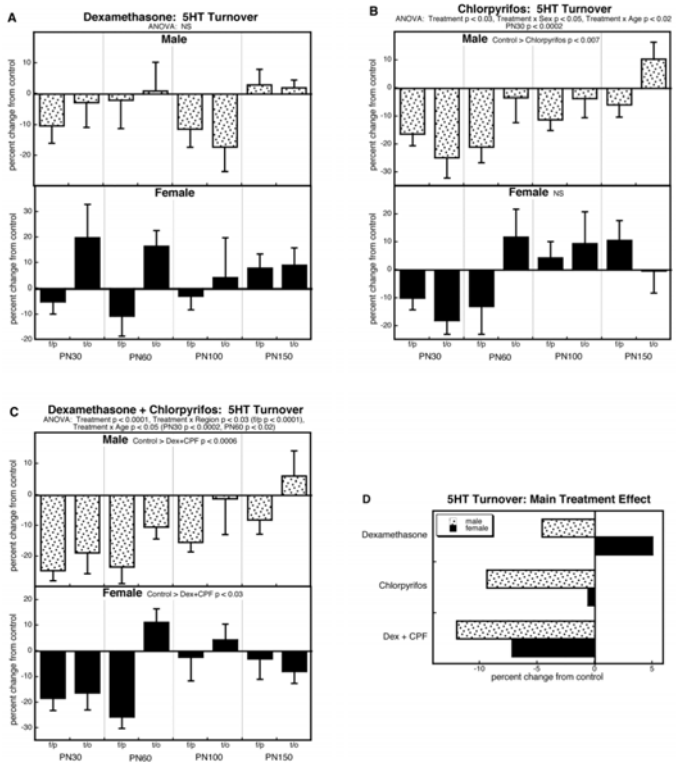


**Figure 5.**

Effects of dexamethasone (A), chlorpyrifos (B), and combined treatment (C) on 5HT turnover (mean ± SE, n=6 for each sex in each treatment group), shown as the percent change from control values (see original values in Supplementary Table 4). Because the global, repeated-measures ANOVA for 5HT concentration and turnover showed a significant treatment × sex interaction ($p < 0.03$), values were separated for males and females. Multivariate ANOVA for each treatment appears at the top of the panels. Dexamethasone alone had no significant effect (A). For chlorpyrifos (B), males showed a main treatment effect without interactions with other variables, so no lower-order tests were performed. Similarly, the group receiving combined treatment (C) showed main treatment effects for males and females, without the need for lower-order tests. Panel (D) shows the simple main treatment effects for each sex, collapsed across all the other variables. Abbreviations: f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; Dex, dexamethasone; CPF, chlorpyrifos; NS, not significant.