TOXICOLOGICAL SCIENCES **92(2)**, 500–506 (2006)
doi:10.1093/toxsci/kfl004
Advance Access publication May 4, 2006

Effect of Developmental Exposure to Chlorpyrifos on the Expression of Neurotrophin Growth Factors and Cell-Specific Markers in Neonatal Rat Brain

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Received January 19, 2006; accepted April 20, 2006

Chlorpyrifos (CPS), a known neurotoxicant, is a widely used agricultural organophosphorus insecticide. The effects of postnatal exposure to CPS on the expression of mRNA for two factors critical to brain development, nerve growth factor (NGF) and reelin, were investigated in the forebrain of rats. In addition, the expression of mRNA for the muscarinic acetylcholine receptor (mAChR) M_1 subtype and cell-specific markers for developing neurons (β-III tubulin), astrocytes (glial fibrillary acidic protein, GFAP), and oligodendrocytes (myelin-associated glycoprotein, MAG) was also investigated. Oral administration of CPS (1.5 or 3.0 mg/kg) or the corn oil vehicle was performed daily from postnatal days (PNDs) 1 through 6. No signs of overt toxicity or of cholinergic hyperstimulation were observed after CPS administration. Body weight was significantly different from controls on PND7 in both males and females exposed to 3.0 mg/kg CPS. Quantitative PCR was performed on the forebrain. The expression of NGF, reelin, and M_1 mAChR mRNA was significantly reduced with both dosages of CPS in both sexes. β-III Tubulin mRNA expression remained unchanged after exposure, whereas MAG mRNA expression was significantly decreased with both dosages of CPS in both sexes, suggesting effects on the developing oligodendrocytes. In contrast, GFAP mRNA levels were significantly increased with both dosages of CPS in both sexes, suggesting increased astrocyte reactivity. Our findings indicate that dosages of CPS which cause significant cholinesterase inhibition but do not exert overt toxicity can adversely affect the expression levels of critical genes involved in brain development during the early postnatal period in the rat.

Key Words: **chlorpyrifos; nerve growth factor; reelin; β-III tubulin; brain development; astrocytes; oligodendrocytes; muscarinic receptor.**

INTRODUCTION

In recent years, great concern has been raised about the negative impact of organophosphorus (OP) insecticide exposure on the nervous system in children. These concerns have led to the elimination of the household uses of several insecticides, such as chlorpyrifos (CPS) and diazinon. However, even though this restriction has proven beneficial in urban areas (Whyatt *et al.*, 2004), the continued use of the OP insecticides in agriculture still provides the opportunity for childhood exposure. In fact, children in agricultural areas encounter higher exposures of insecticides than do children in nonagricultural areas (Bradman *et al.*, 1997; Simcox *et al.*, 1995). It is also generally acknowledged that children living in agricultural worker households or living in close proximity to insecticide-treated farmland would have increased exposures compared to other children living in the same community. Household residue analysis of OP insecticides have supported this idea (Lu *et al.*, 2002). However, the presence of similar levels of diethyl OP residues in the urine of children regardless of age, parental occupation, or residential proximity to fields suggests that insecticide spraying in an agricultural region can increase children's exposure in the absence of parental work contact with insecticides or residential proximity to OP-treated farmland (Koch *et al.*, 2002). Thus, all children living in agricultural communities may be at increased risk to OP insecticide exposure.

CPS was the most widely used OP insecticide prior to its restriction from household uses, but it is still heavily used in the agricultural industry. Postnatal exposure to CPS has been suggested to affect a plethora of neurological targets, and many of these studies, including our own, observe these effects at levels of CPS which do not produce the characteristic signs of OP toxicity. However, alteration of neurological targets by CPS is either accompanied by cholinesterase (ChE) inhibition or occurs following periods of ChE inhibition, indicating perturbation of the normal patterns of cholinergic neuronal activity. It is possible that a great proportion of the CPS-induced neurotoxic effects may be due to either direct or indirect consequences of the disruption of the cholinergic activity.

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Cholinergic neuronal activity is known to be involved in the regulation of critical genes involved in development such as the neurotrophin nerve growth factor (NGF) and other transcription factors in the developing brain (da Penha Berzaghi *et al.*, 1993). Normal development of the nervous system requires the concomitant and coordinated ontogeny of proliferation, migration, differentiation, synaptogenesis, gliogenesis, myelination, and apoptosis to occur in a temporally and regionally dependent manner. Disturbance of neuronal activity could cause perturbations in the normal profiles of gene expression during these critical developmental processes and could result in long-term irreversible consequences that affect the structure and function of the nervous system.

Our previous study demonstrated that CPS can alter protein levels of NGF (Betancourt and Carr, 2004) in the postnatal period, but the effects of CPS on NGF at the gene expression level are unknown. The purpose of this study was to investigate the effects of developmental exposure to CPS on gene expression during the first postnatal week using similar dosages. In line with our previous focus on NGF, we measured NGF mRNA levels in the forebrain of neonatally exposed male and female rats. Since previous studies have suggested that cholinergic neuronal activity is involved in the regulation of neurotrophin expression in the developing brain and involves the muscarinic acetylcholine receptors (mAChR) of the subtype M_1/M_3 (da Penha Berzaghi *et al.*, 1993), we also measured the expression of the M_1 mAChR.

Another important component in brain development is reelin, a large protein of the extracellular matrix that plays a key role in layer formation during early stages of development of the cerebral and cerebellar cortices (D'Arcangelo *et al.*, 1995). Reelin function has been associated with normal patterns of neuronal migration (Rakic and Caviness, 1995), and an abnormal migration of neurons has been linked to cognitive deficits, mental retardation, and motor disorders (Eksloglu *et al.*, 1996; Gleeson *et al.*, 1998). It has been suggested that reelin could represent an important target for OP neurotoxicity during development (Keller and Persico, 2003). Thus, we measured reelin mRNA expression.

It has been suggested that prenatal and postnatal CPS exposure targets both neuronal and glial development (Garcia *et al.*, 2003). Therefore, we also measured mRNA expression for cell-specific markers for developing neurons (β-III tubulin), astrocytes (glial fibrillary acidic protein, GFAP), and oligodendrocytes (myelin-associated glycoprotein, MAG) in the forebrain after developmental exposure to CPS.

MATERIALS AND METHODS

Chemicals. CPS was a generous gift from DowElanco Chemical Company (Indianapolis, IN). All other chemicals were purchased from Sigma Chemical Co (St Louis, MO).

Animals. Adult male and female Sprague Dawley rats (CD IGS) were obtained from Charles River Laboratories (Wilmington, MA) and were used for breeding. Animals were housed in an AAALAC-accredited facility in a temperature-controlled environment (22 ± 2°C) with a 12-h dark-light cycle with lights on between 0700 and 1900 h. LabDiet rodent chow and tap water were freely available during the experimentation. The day of birth was designated as postnatal day (PND) 0. Individual pups of each sex were assigned to each treatment within each litter. All procedures were approved by the Mississippi State University Institutional Animal Care and Use Committee.

Animal treatments. CPS was dissolved in safflower oil and administered orally at a volume of 0.5 ml/kg body weight. Rats were treated daily from PND1 through 6. The size of the litter was adjusted as much as possible in order to obtain litters of the same size (8–10 pups) and even distribution of male and female pups within each litter. The treatment groups were (1) safflower oil (control), (2) 1.5 mg/kg CPS (low dosage), and (3) 3 mg/kg CPS (high dosage). Pups for each treatment group were obtained from a minimum of four litters.

The dosages used in this study were designed to induce inhibition of ChE to further understand the potential risk of exposure in children. These dosages are above the oral repeated No Observed Effect Level (NOEL) for brain ChE inhibition (0.75 mg/kg) but below the oral repeated NOEL for signs of toxicity (4.5 mg/kg) for postnatal rats as reported by Zheng *et al.* (2000). However, it is unclear if these dosages are within the range of exposure levels in children. Previous studies have used dosages as high as 5 mg/kg and claimed that this dosage falls within the range of estimated fetal and neonatal exposures from environmental exposure or home use of CPS (Aldridge *et al.*, 2005). Most likely, CPS exposure in children will occur at levels lower than those used in this study with the exception of situations of high levels of contamination.

Daily body weights for pups were recorded. Rats were euthanized by decapitation on PND7 (24 h after the last exposure to CPS), and the brain was rapidly removed and dissected on ice to obtain the forebrain (anterior to the optic chiasma). Tissues were frozen immediately on dry ice and maintained at − 80°C until processed as described below. All dissection instruments and surfaces used for tissue dissection were previously washed and rinsed with RNaseZap solution (Ambion, Austin, TX) and distilled water.

Total RNA isolation. Total RNA from the forebrain was extracted with the Trizol reagent (Invitrogen, Carlsbad, CA). Tissues were transferred into a 2-ml glass tube without being thawed followed immediately by the addition of 600 μl of Trizol reagent. Tissues were disrupted and homogenized using a Wheaton motorized tissue grinder and a Teflon pestle. Chloroform (10% of the total volume) was added, and the sample was covered, shaken vigorously, and placed on ice for 10 min before being subjected to centrifugation for 15 min (13,000 × g). The aqueous phase was removed and isopropanol (60% of the Trizol volume) used to precipitate the RNA. The RNA pellet was washed by resuspending it in 75% ethanol. The suspension was centrifuged for 5 min (10,000 × g), dried, and redissolved in diethylpyrocarbonate-treated water (Ambion). Total RNA was treated with DNase (Invitrogen) to eliminate any contaminating genomic DNA.

RNA quantification and quality assessment. The RiboGreen RNA–specific quantification kit with DNase I (Invitrogen-Molecular Probes, Eugene, OR) was used in order to accurately determine total RNA concentration. RNA integrity was confirmed by direct visualization of 18S and 28S rRNA bands after agarose gel electrophoresis. After quantification, all RNA sample volumes were adjusted using in diethylpyrocarbonate-treated water so that the concentrations of the samples were within a 10-fold range.

Real-time PCR. The 18S rRNA was used as the internal standard for the duplex quantitative reverse transcriptase PCR (Q-PCR). Primers and probes for the reference and target genes (Table 1) were designed to span intron-exon boundaries (Molecular Beacon 2.0 software; Biosoft International, Palo Alto, CA). All primers were synthesized by MWG Biotech (High Point, NC), and all probes were synthesized by Sigma Genosys (The Woodlands, TX). Primer, probe, and template concentrations were optimized for 18S, NGF, MAG, GFAP, β-III tubulin, reelin, and M_1 individually followed by optimization in duplex

TABLE 1
Primer and Probes for the Reference and Target Genes

Gene	GenBank	Primer	Sequence 5′–3′
18S	X01117	S	CGTTGATTAAGTCCCTGCCCTT
		A	TCAAGTTCGACCGTCTTCTCAG
		P	ACACACCGCCCGTCGCTACTACCG
NGF	XM227525	S	GGACGCAGCTTTCTATCCTGG
		A	CCCTCTGGGACATTGCTATCTG
		P	ACGGTTCTGCCTGTACGCCGATCA
MAG	NM017190	S	GCGTCATGTATGCACCTTGGA
		A	GTGGAACACAGGATGGAGACTG
		P	CCTCTACCGCCACCACCGTCCCAT
GFAP	NM017009	S	CTAATGACTATCGCCGCCACCT
		A	CTCCTCCTGTTCGCGCATTT
		P	TCTCCAAGGACTCGTTCGTGCCGC
β-III Tubulin	NM139254	S	GCCAAGTTCTGGGAGGTCATC
		A	GTAGTAGACACTGATGCGTTCCA
		P	AGCATGGCATAGACCCCAGCGGCA
M_1	NM080773	S	ATGGTGATGCCCTTTCTCTGAG
		A	GAGGTGTTCATGGCAGCCG
		P	AGGCGCAGCCCCTTCCTCCAGTC
Reelin	NM080394	S	AAACTACAGCGGGTGGAACC
		A	ATTTGAGGCATGACGGACCTATAT
		P	TCCCCTCCCTAATGCGGCACTCAC

Note. S: Sense primer; A: antisense primer; and P: dual-labeled probe.

PCRs (iCycler; BioRad, Hercules, CA; Platinum Quantitative RT-PCR ThermoScript One-Step System; Invitrogen).

Q-PCR for rat NGF, M_1, MAG, GFAP, β-III tubulin, and reelin mRNA levels was done. Each PCR was done in triplicate in a final reaction volume of 50 μl. The thermal cycler conditions for the RT reaction were 65°C, 30 min; 95°C, 5 min; and 40 cycles of 95°C, 15 s and 65°C, 1 min. In all experiments, samples containing no template were included as negative controls. Standard curves of both the reference gene (18S) and each target gene were used to calculate the PCR efficiency for each gene (X-fold dilutions from 1:10 to 1:1000). The threshold cycle values (C_t values) of the target gene were normalized to the C_t values of 18S to correct for template variation. From the normalized mean C_t values for each target gene, the fold change was calculated using the method of $2^{(C_{t(\text{control mean})} - C_{t(\text{sample})} * \text{PCR efficiency})}$ using the PCR efficiency specific for each target gene. Percent change was calculated.

For comparison between treatment groups, the fold change of each individual sample (including both CPS and control samples) from the respective control C_t mean was calculated using the method of $2^{(C_{t(\text{control mean})} - C_{t(\text{sample})} * \text{PCR efficiency})}$. Within each gene and sex, statistical analysis was performed by ANOVA using the general linear model procedure followed by separation of means using Least Significant Difference and 95% CIs were determined. The criterion for significance was set at $p \leq 0.05$.

RESULTS

CPS treatment with either the low or the high dosage induced no signs of overt toxicity and no signs of cholinergic hyperstimulation such as lacrimation, diarrhea, or tremors even though based on our previous study, these dosages resulted in significant ChE inhibition (Betancourt and Carr, 2004). Body weights on PND7 were only significantly decreased from those of controls for the high dosage of CPS in males (16%) and females (21%) (data not shown). In the low dosage group, body weights were identical to control values.

With respect to the expression of components that have important roles in neuronal development, CPS treatment exerted negative effects in both sexes. NGF mRNA expression was significantly reduced in both females and males treated with both the low (16.8 and 15.7%, respectively) and high (23.3 and 22.8%, respectively) dosages of CPS (Fig. 1). Reelin mRNA expression was also significantly reduced in both females and males treated with both the low (6.5 and 10.3.%, respectively) and high (14.6 and 19.6%, respectively) dosages of CPS (Fig. 2). There were no significant differences between the low and high dosages with either NGF or reelin. With respect to the effects on M_1 mAChR expression, CPS treatment significantly reduced expression in both females and males treated with both the low (16.1 and 11.9%, respectively) and high (33.3 and 26.0%, respectively) dosages (Fig. 3). This pattern of decrease exhibited a clear dose-response effect.

With respect to the expression of the cellular markers, the effects varied. The expression of mRNA for the neuronal marker β-III tubulin was not affected by CPS treatment in either sex (Fig. 4). The expression of mRNA for the oligodendrocyte marker MAG was significantly reduced in both females and males with both the low (24.5 and 27.8%, respectively) and high (36.8 and 51.4%, respectively) dosages (Fig. 5). In contrast, the expression of mRNA for the astrocyte marker GFAP was significantly increased in both females and males treated with both the low (15.8 and 18.1%, respectively) and high (29.5 and 25.6%, respectively) dosages of CPS (Fig. 6).

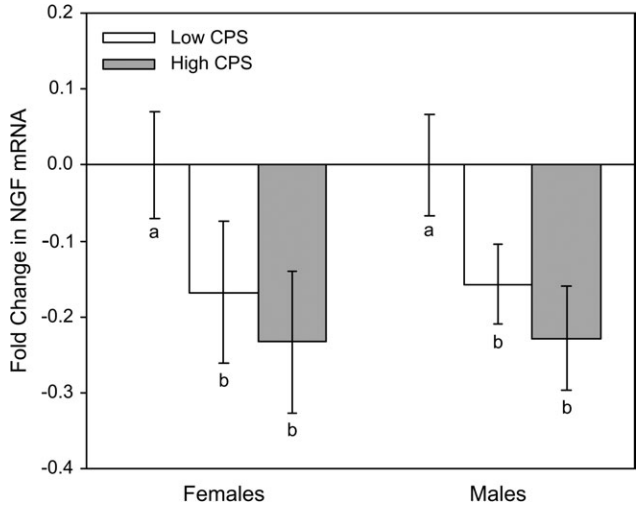

FIG. 1. Fold change in NGF mRNA in the forebrain of female and male rat pups exposed daily to either 1.5 or 3.0 mg/kg CPS from PND1 through 6 and sacrificed on day 7 (24 h after the last treatment). Values are expressed as fold change, and error bars indicate 95% CIs determined as described in the "Materials and Methods" section ($n = 5$). Bars within each sex with different lowercase letters are significantly different ($p \leq 0.05$) from one another.

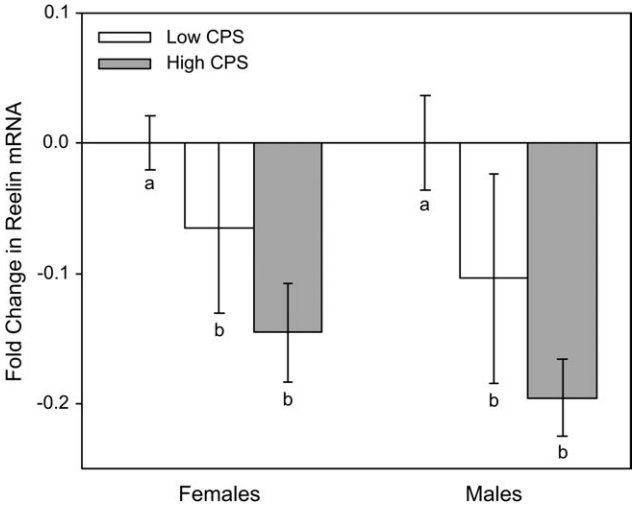

FIG. 2. Fold change in reelin mRNA in the forebrain of female and male rat pups exposed daily to either 1.5 or 3.0 mg/kg CPS from PND1 through 6 and sacrificed on day 7 (24 h after the last treatment). Values are expressed as fold change, and error bars indicate 95% CIs determined as described in the ''Materials and Methods'' section ($n = 5$–6). Bars within each sex with different lowercase letters are significantly different ($p \leq 0.05$) from one another.

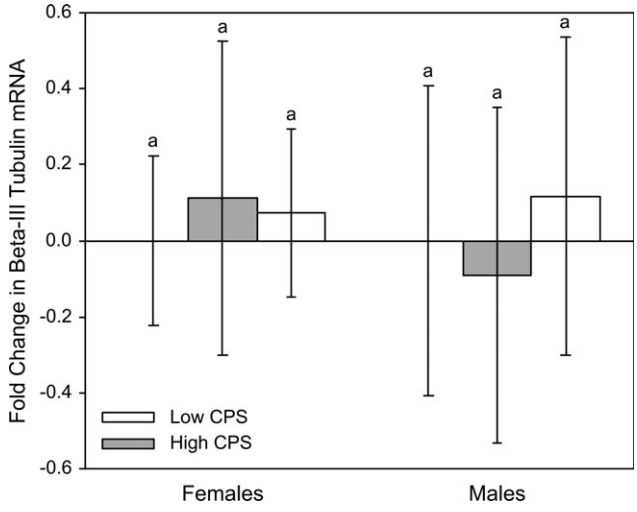

FIG. 4. Fold change in β-III tubulin mRNA in the forebrain of female and male rat pups exposed daily to either 1.5 or 3.0 mg/kg CPS from PND1 through 6 and sacrificed on day 7 (24 h after the last treatment). Values are expressed as fold change, and error bars indicate 95% CIs determined as described in the ''Materials and Methods'' section ($n = 5$–7). Bars within each sex with different lowercase letters are significantly different ($p \leq 0.05$) from one another.

DISCUSSION

In mammals, gene transcription is a step subjected to tight regulation especially during development. It has been shown that changes in mRNA levels in the CNS can account for numerous phenotypic differences in brain function (Alfonso *et al.*, 2002). Our previous results demonstrated that at the time period sampled in this study, forebrain ChE activity is significantly inhibited in a dose-dependent fashion, suggesting that CPS alters normal cholinergic activity (Betancourt and Carr, 2004). Those previous results taken with the results of this study demonstrate that the increased cholinergic stimulation induced by CPS exposure alters the normal pattern of gene expression which could potentially modify the highly

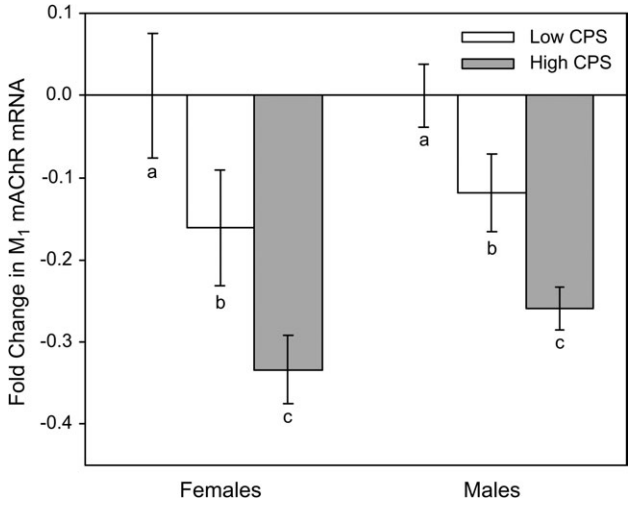

FIG. 3. Fold change in mAChR subtype M_1 mRNA in the forebrain of female and male rat pups exposed daily to either 1.5 or 3.0 mg/kg CPS from PND1 through 6 and sacrificed on day 7 (24 h after the last treatment). Values are expressed as fold change, and error bars indicate 95% CIs determined as described in the ''Materials and Methods'' section ($n = 5$–6). Bars within each sex with different lowercase letters are significantly different ($p \leq 0.05$) from one another.

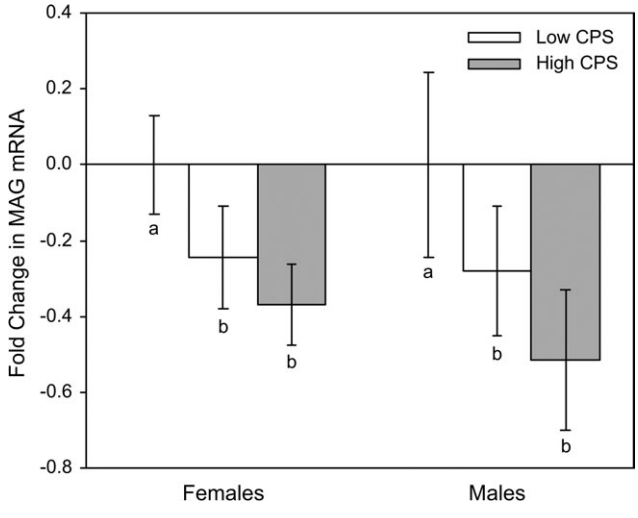

FIG. 5. Fold change in MAG mRNA in the forebrain of female and male rat pups exposed daily to either 1.5 or 3.0 mg/kg CPS from PND1 through 6 and sacrificed on day 7 (24 h after the last treatment). Values are expressed as expression ratios, and error bars indicate 95% CIs determined as described in the ''Materials and Methods'' section ($n = 5$). Bars within each sex with different lowercase letters are significantly different ($p \leq 0.05$) from one another.

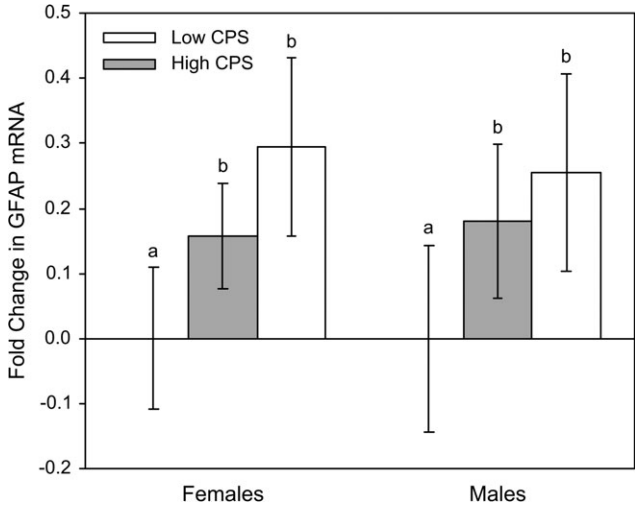

FIG. 6. Fold change in GFAP mRNA in the forebrain of female and male rat pups exposed daily to either 1.5 or 3.0 mg/kg CPS from PND1 through 6 and sacrificed on day 7 (24 h after the last treatment). Values are expressed as expression ratios, and error bars indicate 95% CIs determined as described in the "Materials and Methods" section ($n = 5$–6). Bars within each sex with different lowercase letters are significantly different ($p \leq 0.05$) from one another.

coordinated developmental processes, leading to perturbation of the normal patterns of neuronal development.

In this study, the dosages of CPS induced a statistically significant decrease in NGF mRNA levels which correlates well with our previous observations of decreased NGF protein levels following a similar treatment paradigm (Betancourt and Carr, 2004). It is known that during postnatal life, NGF is essential for the normal development of striatal and basal forebrain cholinergic neurons which project into the hippocampus and neocortex and that these neurons are important for attention, learning, and memory functions (Olton *et al.*, 1991). Thus, deficits on NGF mRNA followed by deficits on NGF protein levels may negatively alter the normal development of these cholinergic afferents. It has been shown that neonatal lesions to basal forebrain cholinergic afferents result in delayed cortical neuronal development and permanently altered cortical cytoarchitecture and cognitive behaviors (Hohmann and Berger-Sweeney, 1998).

Our previous results demonstrated significant reductions in the mAChR-binding sites following the low and high dosages of CPS in a dose-dependent fashion (Betancourt and Carr, 2004). In this study, M_1 mRNA levels were dose dependently decreased, suggesting that the downregulation of the M_1 mAChR subtype involves changes not only at the level of the synaptic membrane but also at the level of gene expression.

Our data indicate that developmental exposure to CPS elicits a significant statistical decrease in reelin mRNA expression, but it is not clear if the decreased expression is biologically relevant, especially in the low dosage group, where change in expression was not more than 10% in either sex. However, the variation in expression in the control samples was less than 3%, suggesting that the expression may be tightly regulated. It is known that reelin is synthesized in Cajal-Retzius cells, and some Cajal-Retzius cells contain mAChR (Chaudhuri *et al.*, 2005). While very little is known about the role of these receptors on Cajal-Retzius cells, it is possible that they could play a role in regulation of reelin expression. If true, it is possible that the decreased expression of reelin could be a by-product of the decreased levels of mAChR. In addition, epidemiological studies have found that reelin is located within a chromosomal region showing linkage with autism. While the precise etiology of autism has not been determined, it has been suggested that pesticide exposure could be an environmental factor involved in the pathology of this developmental disorder (D'Amelio *et al.*, 2005; Keller and Persico, 2003). In addition, children with autism have lower levels of an enzyme important in OP detoxication (Pasca *et al.*, 2006).

Trophic factors play an important role in regulating many processes in vertebrate brain development including cell differentiation, neuronal proliferation and migration, axonal guidance, synapse formation, and glial maturation (Song *et al.*, 2002). Using gene expression for specific markers of neurons and glial cells including astrocytes and oligodendrocytes, we observed differential effects on the expression of markers for these various cell types.

β-III Tubulin is extensively used as a specific neuronal marker since it is initially expressed at the onset of neural differentiation and is the only β-tubulin present in the mammalian brain whose expression is neuron specific (Lee *et al.*, 1990). We observed no significant effects of CPS exposure on β-III tubulin mRNA levels in the forebrain. Our data agree with those of Garcia *et al.* (2003), who also reported no significant effects on the protein levels of the neuronal cell body marker neurofilament 68 following CPS exposure from PND1 to 4.

In most rat brain regions, gliogenesis and glial cell differentiation continues well into postnatal development (Aschner *et al.*, 1999). GFAP is the major intermediate filament of mature astrocytes and has been widely recognized as an astrocyte differentiation marker (Gomes *et al.*, 1999). In the rodent brain, GFAP mRNA levels increase between birth and PND15 with maximal transcripts present on PND3 and coincide with astrocyte proliferation (Riol *et al.*, 1992). Previously, Garcia *et al.* (2002) reported limited effects on GFAP protein levels following early sc postnatal exposure to CPS (PND1–4) with effects limited to decreased protein levels on PND5 in the male cerebellum only and a subsequent significant increase on PND10. In contrast, we observed increased GFAP mRNA levels in the forebrain on PND7 in both sexes following oral exposure to CPS. While it is possible that our observations on GFAP mRNA levels may not result in similar changes in protein levels, the increased expression does suggest treatment-induced changes in astrocyte reactivity. The basis for this increased reactivity is not clear but may be related to the CPS

exposure. It is known that CPS exposure can induce oxidative stress (Crumpton *et al.*, 2000; Qiao *et al.*, 2005), and the increased astrocyte reactivity may be a related response to the production of reactive oxygen species.

MAG is a minor transmembrane glycoprotein found in CNS myelin comprising ~ 1% of total myelin protein (Quarles *et al.*, 1973) that is essential for the maintenance of the mature myelinated unit. The mRNA for this marker of differentiated oligodendrocytes is present on PND7 and increases thereafter as oligodendrocytes mature (Rodriguez-Peña *et al.*, 1993). Our data suggest that the CPS exposure may have resulted in a decrease in those MAG-expressing cells. It is known that immature oligodendrocytes are more vulnerable to toxic insults than mature oligodendrocytes (Smith *et al.*, 2000), and this has been attributed in part to a developmentally related lack of antioxidant enzymes to control oxidative stress. It is possible that exposure has induced some cellular toxicity in the population of progenitor oligodendrocytes thereby decreasing the number of differentiated oligodendrocytes which would express MAG. However, another possibility is that the exposure may have resulted in a delay in the maturation of the progenitor oligodendrocytes. This is still significant since it is possible that even a transient delay in oligodendrocyte development may result in impaired myelin formation and maintenance (Deng and Poretz, 2003). However, our data are in contrast to those of Garcia *et al.* (2003), who reported no significant effects on the protein levels of the oligodendrocyte marker myelin basic protein (MBP) following CPS exposure from PND1 to 4. The mRNAs for MBP and MAG appear at the same time in developing oligodendrocytes (Dubois-Dalcq *et al.*, 1986). One would expect similar results between the two markers, but it may be that the reduced mRNA levels observed in this study may not result in decreased protein levels as observed by Garcia *et al.* (2003). In addition, as acknowledged by Garcia *et al.* (2003), the immunoblot methodology they used for detection of MBP levels may underestimate treatment effects (O'Callaghan *et al.*, 1999).

In conclusion, our findings indicate that neonatal exposure of rats to CPS can adversely affect the expression of genes involved in the regulation of neurological developmental processes. Even though reelin is a critical component in CNS development, the overall impact of the observed decreased expression of reelin in the developmental neurotoxicity of CPS is not clear and requires further investigation. The data presented here indicate that the decreased levels of NGF protein we have previously observed are due to CPS-induced effects at the level of gene transcription. This decrease in transcription may be due to altered cell signaling which has been reported to occur following CPS exposure (Auman *et al.*, 2000; Meyer *et al.*, 2003, 2004). However, it is known that activation of mAChR increases NGF expression (von der Kammer *et al.*, 2001), and we have previously reported that a decrease in mAChR levels occurs following CPS exposure as a physiological attempt to compensate for the cholinergic hyperactivity (Betancourt and Carr, 2004). It may be that the decrease in NGF expression is merely a by-product of the mAChR reduction. While this may be a simplistic explanation, a decrease in NGF levels may be detrimental to processes which NGF regulates, especially cholinergic neurons which depend on NGF for growth and maintenance.

Early neonatal exposure to CPS does not significantly affect the levels of neuronal markers, which is surprising but has been observed previously (Garcia *et al.*, 2003). However, the increased activation of astrocytes as suggested by increased expression of GFAP implies either increased oxidative stress or damage to other cellular populations. The simultaneous decreased expression of the marker for differentiated oligodendrocytes may be indicative of the fact that this population has taken the brunt of the damage. Several others have suggested that glial cells are much more susceptible to the negative effects of CPS than are neurons (Garcia *et al.*, 2002; Qiao *et al.*, 2001). Such effects on glial development by CPS exposure could compromise the future trophic and supportive functions of these cells.

ACKNOWLEDGMENTS

The authors wish to acknowledge the statistical expertise of Dr Carolyn Boyle. This study was funded in part by NIH 1P20-RR17661. This research was also partially supported by the Mississippi Agricultural and Forestry Experiment Station (MAFES) under MAFES project #MISV-339010 and the College of Veterinary Medicine, Mississippi State University. This paper is MAFES publication #J-10936 and Center for Environmental Health Sciences publication #110.

REFERENCES

Aldridge, J. E., Meyer, A., Seidler, F. J., and Slotkin, T. A. (2005). Developmental exposure to terbutaline and chlorpyrifos: Pharmacotherapy of preterm labor and an environmental neurotoxicant converge on serotonergic systems in neonatal rat brain regions. *Toxicol. Appl. Pharmacol.* **203,** 132–144.

Alfonso, J., Pollevick, G. D., Castensson, A., Jazin, E., and Frasch, A. C. (2002). Analysis of gene expression in the rat hippocampus using Real Time PCR reveals high inter-individual variation in mRNA expression levels. *J. Neurosci. Res.* **67,** 225–234.

Aschner, M., Allen, J. W., Kimelberg, H. K., LoPachin, R. M., and Streit, W. J. (1999). Glial cells in neurotoxicity development. *Annu. Rev. Pharmacol. Toxicol.* **39,** 151–173.

Auman, J. T., Seidler, F. J., and Slotkin, T. A. (2000). Neonatal chlorpyrifos exposure targets multiple proteins governing the hepatic adenylyl cyclase signaling cascade: Implications for neurotoxicity. *Dev. Brain Res.* **121,** 19–27.

Betancourt, A. M., and Carr, R. L. (2004). The effect of chlorpyrifos and chlorpyrifos-oxon on brain cholinesterase, muscarinic receptor binding, and neurotrophin levels in rats following early postnatal exposure. *Toxicol. Sci.* **77,** 63–71.

Bradman, M. A., Harnly, M. E., Draper, W., Seidel, S., Teran, S., Wakeham, D., and Neutra, R. (1997). Pesticide exposures to children from California' s Central Valley: Results of a pilot study. *J. Expo. Anal. Environ. Epidemiol.* **7,** 217–234.

Chaudhuri, J. D., Hiltunen, M., Nykanen, M., Yla-Herttuala, S., Soininen, H., and Miettinen, R. (2005). Localization of M2 muscarinic receptor protein in parvalbumin and calretinin containing cells of the adult rat entorhinal cortex using two complementary methods. *Neuroscience* **131,** 557–566.

Crumpton, T. L., Seidler, F. J., and Slotkin, T. A. (2000). Developmental neurotoxicity of chlorpyrifos *in vivo* and *in vitro*: Effects on nuclear transcription factors involved in cell replication and differentiation. *Brain Res.* **857,** 87–98.

D'Amelio, M., Ricci, I., Sacco, R., Liu, X., D'Agruma, L., Muscarella, L. A., Guarnieri, V., Militerni, R., Bravaccio, C., Elia, M., *et al.* (2005). Paraoxonase gene variants are associated with autism in North America, but not in Italy: Possible regional specificity in gene-environment interactions. *Mol. Psychiatry* **10,** 1006–1016.

da Penha Berzaghi, M., Cooper, J., Castren, E., Zafra, F., Sofroniew, M., Thoenen, H., and Lindhholm, D. (1993). Cholinergic regulation of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) but not neurotrophin-3 (NT-3) mRNA levels in the developing rat hippocampus. *J. Neurosci.* **13,** 3818–3826

D'Arcangelo, G., Miao, G. G., Chen, S. C., Soares, H. D., Morgan, J. I., and Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature* **374,** 719–723.

Deng, W., and Poretz, R. D. (2003). Oligodendroglia in developmental neurotoxicity. *Neurotoxicology* **24,** 161–178.

Dubois-Dalcq, M., Behar, T., Hudson, L., and Lazzarini, R. A. (1986). Emergence of three myelin proteins in oligodendrocytes cultured without neurons. *J. Cell Biol.* **102,** 384–392.

Eksloglu, Y. Z., Scheffer, I. E., Cardenas, P., Knoll, J., DiMario, F., Ramsby, G., Berg, M., Kamuro, K., Berkovic, S. F., Duyk, G. M., *et al.* (1996). Periventricular heterotopia: An X-linked dominant epilepsy locus causing aberrant cerebral cortical development. *Neuron* **16,** 77–87

Garcia, S. J., Seidler, F. J., and Slotkin, T. A. (2002). Chlorpyrifos targets developing glia: Effects on glial fibrillary acidic protein. *Dev. Brain Res.* **133,** 151–161.

Garcia, S. J., Seidler, F. J., and Slotkin, T. A. (2003). Developmental neurotoxicity elicited by prenatal or postnatal chlorpyrifos exposure: Effects on neurospecific proteins indicate changing vulnerabilities. *Environ. Health Perspect.* **111,** 297–303.

Gleeson, J. G., Allen, K. M., Fox, J. W., Lamperti, E. D., Berkovic, S., Scheffer, I., Cooper, E. C., Dobyns, W. B., Minnrath, S. R., Ross, M. E., *et al.* (1998). Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. *Cell* **92,** 63–72.

Gomes, F. C. A., Garcia-Abreu, J., Galou, M., Paulin, D., and Moura Neto, V. (1999). Neurons induce GFAP gene promoter of cultured astrocytes from trasgenic mice. *Glia* **26,** 97–108.

Hohmann, C. F., and Berger-Sweeney, J. (1998). Cholinergic regulation of cortical development and plasticity. New twists to an old story. *Perspect. Dev. Neurobiol.* **5,** 401–425.

Keller, F., and Persico, A. M. (2003). The neurobiological context of autism. *Mol. Neurobiol.* **28,** 1–22.

Koch, D., Lu, C., Fisker-Andersen, J., Jolley, L., and Fenske, R. A. (2002). Temporal association of children's pesticide exposure and agricultural spraying: Report of a longitudinal biological monitoring study. *Environ. Health Perspect.* **110,** 829–833.

Lee, M. K., Rebhun, L. I., and Frankfurter, A. (1990). Posttranslational modification of class III beta-tubulin. *Proc. Natl. Acad. Sci.* **87,** 7195–7199.

Lu, C., Fenske, R. A., Simcox, N. J., and Kalman, D. (2002). Pesticide exposure of children in an agricultural community: Evidence of household proximity to farmland and take home exposure pathways. *Environ. Res.* **84,** 290–302.

Meyer, A., Seidler, F. J., Aldridge, J. E., Tate, C. A., Cousins, M. M., and Slotkin, T. A. (2004). Critical periods for chlorpyrifos-induced developmental neurotoxicity: Alterations in adenylyl cyclase signaling in adult rat brain regions after gestational or neonatal exposure. *Environ. Health Perspect.* **112,** 295–301.

Meyer, A., Seidler, F. J., Cousins, M. M., and Slotkin, T. A. (2003). Developmental neurotoxicity elicited by gestational exposure to chlorpyrifos: When is adenylyl cyclase a target? *Environ. Health Perspect.* **111,** 1871–1876.

O'Callaghan, J. P., Imai, H., Miller, D. B., and Minter, A. (1999). Quantitative immunoblots of proteins resolved from brain homogenates: Underestimation of specific protein concentration and of treatment effects. *Anal. Biochem.* **274,** 18–26.

Olton, D., Markowska, A., Voytko, M. L., Givens, B., Gorman, L., and Wenk, G. (1991). Basal forebrain cholinergic system: A functional analysis. *Adv. Exp. Med. Biol.* **295,** 353–372.

Pasca, S. P., Nemes, B., Vlase, L., Gagyi, C. E., Dronca, E., Miu, A. C., and Dronca, M. (2006). High levels of homocysteine and low serum paraoxonase 1 arylesterase activity in children with autism. *Life Sci.* **78,** 2244–2248.

Qiao, D., Seidler, F. J., and Slotkin, T. A. (2001). Developmental neurotoxicity of chlorpyrifos modeled in vitro: Comparative effects of metabolites and other cholinesterase inhibitors on DNA synthesis in PC12 and C6 cells. *Environ. Health Perspect.* **109,** 909–913.

Qiao, D., Seidler, F. J., and Slotkin, T. A. (2005). Oxidative mechanisms contributing to the developmental neurotoxicity of nicotine and chlorpyrifos. *Toxicol. Appl. Pharmacol.* **206,** 17–26.

Quarles, R. H., Everly, J. L., and Brady, R. O. (1973). Evidence for the close association of a glycoprotein with myelin in rat brain. *J. Neurochem.* **21,** 1177–1191.

Rakic, P., and Caviness, V. S., Jr. (1995). Cortical development: View from neurological mutants two decades later. *Neuron* **14,** 1101–1103.

Riol, H., Fages, C., and Tardy, M. (1992). Transcriptional regulation of glial fibrillary acidic protein (GFAP)-mRNA expression during postnatal development of mouse brain. *J. Neurosci. Res.* **32,** 79–85.

Rodriguez-Peña, A., Ibarrola, N., Iniguez, M. A., Munoz, A., and Bernal, J. (1993). Neonatal hypothyroidism affects the timely expression of myelin-associated glycoprotein in the rat brain. *J. Clin. Invest.* **91,** 812–818.

Simcox, N. I., Penske, R. A., Wolz, S. A., Lee, I. C., and Kalman, D. A. (1995). Pesticides in household dust and soil: Exposure pathways for children of agricultural families. *Environ. Health Perspect.* **103,** 1126–1134.

Smith, J., Ladi, E., Mayer-Proschel, M., and Noble, M. (2000). Redox state is a central modulator of the balance between self-renewal and differentiation in a dividing glial precursor cell. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 10032–10037.

Song, H., Stevens, C. F., and Gage, F. H. (2002). Astroglia induce neurogenesis from adult neural stem cells. *Nature* **417,** 39–42.

von der Kammer, H., Demiralay, C., Andersen, B., Albrecht, C., Mayhaus, M., and Nitsch, R. M. (2001). Regulation of gene expression by muscarinic acetylcholine receptors. *Biochem. Soc. Symp.* **67,** 131–140.

Whyatt, R. M., Rauh, V., Barr, D. B., Camann, D. E., Andrews, H. F., Garfinkel, R., Hoepner, L. A., Diaz, D., Dietrich, J., Reyes, A., *et al.* (2004). Prenatal insecticide exposures and birth weight and length among an urban minority cohort. *Environ. Health Perspect.* **112,** 1125–1132.

Zheng, Q., Olivier, K., Won, Y. K., and Pope, C. N. (2000). Comparative cholinergic neurotoxicity of oral chlorpyrifos exposures in preweanling and adult rats. *Toxicol. Sci.* **55,** 124–132.