Appendix C:
Effects on the Developing Brain

August 21, 2008

Prepared by:

Health Effects Division
Office of Pesticide Programs
US Environmental Protection Agency

Table of Contents

Table of Figures

Table of Tables

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
BMP	Bone morphogenic protein
Ch	Choline
ChAT	Choline acetyltransferase
cAMP	cyclic adenosine monophosphate
CREB/pCREB	cAMP response element binding protein/phosphroylated form
DMSO	dimethylsulfoxide
GD	Gestation day
HACU	High affinity Ch uptake transporter
mAChR	muscarinic ACh receptor
PC	Pheochromocytoma cells
PCR	Polymerase chain reaction
PND	Postnatal day
NGF	Nerve growth factor
TCP	3,5,6-trichloro-2-pyrindinol
USV	Ultrasonic vocalization

1. Introduction

Chlorpyrifos is an organophosphate insecticide that inhibits the catalytic function of acetylcholinesterase (AChE) that deactivates the neurotransmitter acetylcholine (ACh). The inhibition of the catalytic function of AChE in removing ACh from the synaptic region is generally regarded as the cause of its toxicity leading to observable clinical signs. In addition to the better-known synaptic catalytic function of AChE, there is also a morphogenic role (Brimijoin and Koenigsberger, 1999 and Bigbee et al, 1999) of this enzyme that may direct the architecture of the central and peripheral nervous systems to cause permanent damage. There is a growing body of literature on the effects of chlorpyrifos in the developing brain that indicate that gestational and early postnatal exposure can lead to both neurochemical and behavioral alterations into adulthood. As described below, several authors claim that there was no or only marginal inhibition of fetal or neonatal brain catalytic AChE activity in the pups that had effects persisting to adulthood. Slotkin (2006) has suggested that many mechanisms may be at work as shown in Figure 1 (Figure from Slotkin, 2006) for the effects leading to persistent consequences seen in adults following fetal or early postnatal exposure to chlorpyrifos. This figure also presents many of the references from Dr. Slotkin's laboratory and several of these will be briefly discussed below.

Chlorpyrifos oxon metabolically converted from chlorpyrifos is the active form that most effectively attacks the active site of AChE. Not shown in Figure 1 is that the active site of AChE contains a hydroxyl group from the amino acid serine that is phosphorylated by chlorpyrifos oxon to prevent the breakdown of ACh. There are other enzymes and proteins that also have serine amino acids in their active sites or allosterically situated near the active site and these serines may also be affected by organophosphate insecticides to cause inhibition or other adverse effect on the function of the protein. Alternatives to the inhibition of the catalytic function of AChE as the sole effect of organophosphates were proposed by others (Pope, 1999, Barone et al, 2000 and Casida and Quistad, 2004).

Figure 1: Multiple mechanisms underlying the developmental neurotoxicity of chlorpyrifos

(Please refer to Slotkin (2006) for references indicated in the caption but not listed in the references section of this review.)

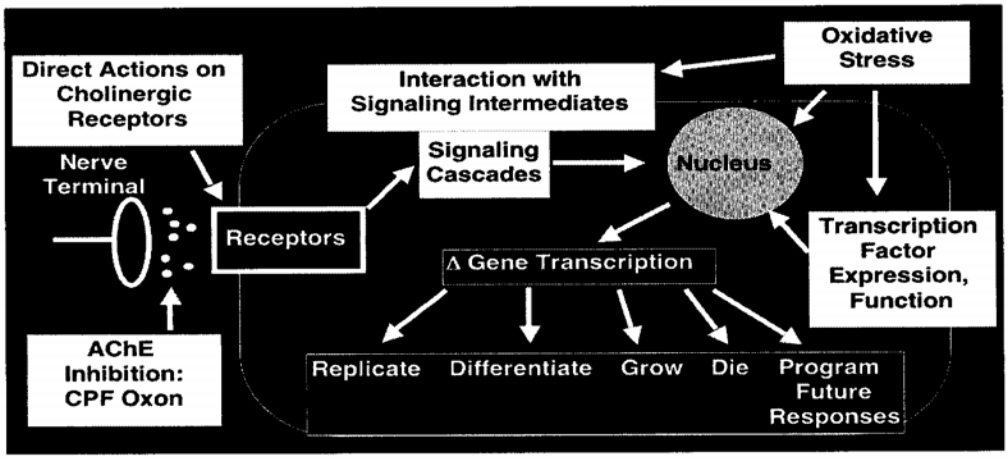

FIG. 1. Multiple mechanisms underlying the developmental neurotoxicity of chlorpyrifos. During development, neurotransmitter stimulation of target cell receptors and the resulting activation of signaling pathways controls the expression of genes that determine cell fate (Dreyfus, 1998; Hohmann, 2003; Lauder, 1985; Whitaker-Azmitia, 1991). At early stages of development, signals may promote cell replication, whereas later in development the same neurotransmitter, operating on the same receptors and signaling pathways, may be responsible for expression of genes controlling differentiation, growth, or apoptosis. Early input also programs the future responsiveness of the cell, priming signaling pathways and receptor expression to achieve the appropriate response to subsequent stimuli. Chlorpyrifos affects all these processes through a variety of mechanisms (Slotkin, 1999, 2004b; Yanai *et al.*, 2002, 2004). Inhibition of acetylcholinesterase (AChE) by chlorpyrifos (CPF)-oxon increases the concentration of acetylcholine, resulting in inappropriate and mistimed overstimulation of cholinergic receptors. Chlorpyrifos also interacts directly with nicotinic (Gupta, 2004; Katz *et al.*, 1997; Slotkin *et al.*, 2004; Smulders *et al.*, 2004; Wu *et al.*, 2003) and muscarinic (Betancourt and Carr, 2004; Bomser and Casida, 2001; Casida and Quistad, 2004; Chaudhuri *et al.*, 1993; Howard and Pope, 2002; Huff *et al.*, 1994, 2001; Huff and Abou-Donia, 1995; Song *et al.*, 1997; Ward and Mundy, 1996) cholinergic receptors and alters the expression and function of receptors for other neurotransmitters (Aldridge *et al.*, 2003, 2004, 2005) and of signaling intermediates such as adenylyl cyclase and G proteins (Casida and Quistad, 2004; Meyer *et al.*, 2003, 2004a,b, 2005; Olivier *et al.*, 2001; Song *et al.*, 1997). Through oxidative stress (Bagchi *et al.*, 1995; Crumpton *et al.*, 2000b; Garcia *et al.*, 2001; Gupta, 2004; Jett and Navoa, 2000; Karen *et al.*, 2001; Qiao *et al.*, 2005) and direct effects on nuclear transcription factor expression and function (Crumpton *et al.*, 2000a; Dam *et al.*, 2003; Garcia *et al.*, 2001; Schuh *et al.*, 2002), chlorpyrifos also alters the basic intracellular mediators of cell differentiation.

This current review is not meant to be an exhaustive review of this literature and it emphasizes some of the publications since the March 28, 2000 review by Agency staff. The March 28, 2000 review (Baetcke et al, 2000) of chlorpyrifos for potential hazard to children for increased sensitivity and susceptibility concluded that:

> "recent data have shown effects on the developing rat brain (*e.g.*, structural defects and changes in macromolecular synthesis, neurotransmitter levels and cell signaling). It is these neurodevelopmental effects that raised a high degree of concern for the potential unique susceptibility of the young to chlorpyrifos. The susceptibility parameters evaluated can not be directly linked, however, to cholinesterase inhibition or functional outcomes. Furthermore, whereas data are available to evaluate quantitative differences in the sensitivity of the young animal compared to the adult, there are insufficient data to establish the quantitative dose-response for the susceptibility indicators."

In this current (2008) review, the Agency attempted to evaluate two areas important for risk assessment and to provide context for the epidemiology studies as indicated by: 1) newer evidence demonstrating persistent effects in adult rodents following gestational and/or early postnatal exposure; and 2) relative sensitivity of observed effects with AChE inhibition.

The Agency's conclusion about the body of literature involving animal studies available up to August 2008 reinforces the concerns stated in 2000. Since the 2000 review, several studies elaborate on the alterations induced by chlorpyrifos exposure during gestation and early postnatal windows. These studies indicate changes in the serotonin and adenylyl cyclase systems, macromolecules including genes and mRNA. Independent laboratories report behavioral alterations in two mammalian species as well as in the zebrafish noted when assessed as adults. These effects are noted long after any initial inhibition of AChE has recovered and where little or no initial inhibition was detected. Although there are suggestions for direct targets for chlorpyrifos other than on the ACh system, there is no conclusive evidence to positively identifying such targets at this time. Specifically, it is still difficult to definitely discern the initiation of the neurochemical changes (i.e. serotonin, macromolecules etc) reported as a primary effect on these systems from either concurrent catalytic functional AChE inhibition or an effect on a morphogenic function of AChE or an interaction with ACh receptors or transporters. This inability to discern is usually due to study design issues and/or lack of time course information and/or pharmacokinetic data on tissue dosimetry. The studies do, however, provide qualitative descriptions of how the developing rodent brain has a susceptibility to chlorpyrifos exposure with consequences lasting into adulthood.

The first section below describes studies reporting the persistent behavioral effects in adults following gestational and early postnatal exposure to chlorpyrifos since detection of such behavioral effects are a significant concern for neurodevelopmental toxicity. In subsequent sections the interaction of chlorpyrifos with several neurochemical parameters based on *in vivo* and *in vitro* studies are described.

2. Behavioral Effects

Indications of gender-selective deficits in coordination skills and locomotor development following early postnatal exposure to chlorpyrifos at doses that did not indicate signs of AChE inhibition or overt toxicity were reported from the Slotkin laboratory as early as 2000 (Dam et al, 2000). In a later series of studies, the behavioral effects in adult rats, after subcutaneous exposure to chlorpyrifos (1 or 5 mg/kg in DMSO) during four different windows of development, were assessed: early gestation (GD 9-12; Icenogle et al 2004); late gestation (GD 17-20; Levin et al, 2002) or at PND 1-4 (Aldridge et al., 2005c; Levin et al., 2001) or PND 11-14 (Levin et al, 2001). As shown in Table 1, these studies report a variety of effects in the Figure 8 activity chamber, T-maze and radial arm maze cognitive tasks, or elevated plus maze, depending upon gender and/or window of administration. If exposure occurred during early gestation (days 9-12; Icenogle et al., 2004) the sexes were combined and learning and memory was adversely affected (more errors in the radial arm maze). Following chlorpyrifos dosing on GD 17-20, only females showed this effect on errors, whereas after dosing on PND 1-4, males showed more errors, and conversely, females showed fewer errors (Aldridge et al., 2005c; Levin et al., 2001). Early postnatal treatment at 1 mg/kg elicited more time spent in the open arms of an elevated plus maze in male rats only, and decreased chocolate milk preference in both sexes. Adult untreated rats show the expected demonstrated muscarinic dependence for reference memory as indicated by making more errors following scopolamine (a muscarinic antagonist) challenge in the radial arm maze. If, however, females were treated with chlorpyrifos (either gestationally or postnatally on PND 11-14 but not on PND 1-4) they did not show the characteristic increase in errors in the scopolamine challenge (Icenogle et al, 2004, Levin et al., 2001, 2002). Mecamylamine, a nicotinic antagonist, had no effect. In a study where the dosing was on PND 1-4, pretreatment with ketanserin, a 5HT2 receptor antagonist, increased radial arm maze errors in the chlorpyrifos groups only, indicating involvement of the serotonergic system in the cognitive outcomes. Thus, according to the authors, the effect of developmental exposure to chlorpyrifos appears to alter the neuronal circuitry to change the memory system away from muscarinic cholinergic dependence. The authors' interpretation was that there is a wide window of vulnerability to chlorpyrifos ranging from early gestation (neurulation) to late postnatal that can impair cholinergic circuits used in learning and memory.

Table 1: Effects following gestational or postnatal exposure to 1 or 5 mg/kg chlorpyrifos administered subcutaneously in DMSO

Reference	Icenogle (2004)	Levin (2002)	Levin (2001)	Aldridge (2005)	Levin (2001)
Window	GD 9-12	GD 17-20	PND 1-4	PND 1-4	PND 11-14
Dose	1 or 5 mg/kg[a]	1 or 5 mg/kg[b]	1 mg/kg	1 mg/kg	5 mg/kg
Assessment PND	~4-17 weeks	~4-17 weeks	~4-17 weeks	~7-17 weeks	~4-17 weeks
Chocolate Milk Preference	--[c]	--	--	♂/♀ ↓ preference	--
Elevated Plus Maze	↑center crosses	--	--	♂↑ time in open arms & ↑center crosses	--
Motor Activity	↑ habituation rate	↓ ♀ habituation rate	No effect	--	♂/♀ ↓ habituation rate
Radial arm maze (RAM) working & reference memory	↑ errors early in training	♀ ↑ errors early in training	♀ ↓ errors throughout, ♂ ↑ errors early in training	♀ ↓ errors throughout, ♂ ↑ errors early in training	No effect
T-maze activity & alternation	↓ activity early in session	↓ activity early in session	♂ ↓ activity middle of session	--	♂ ↓ activity middle of session
Ketanserin Challenge RAM	--	--	--	♂/♀ ↑ errors[d]	--
Scopolamine Challenge RAM	♂♀↓ scopolamine effect [d]	♀↓ scopolamine effect	No effect	--	♀↓ scopolamine effect
Mecamylamine Challenge RAM	No effect	No effect	No effect	--	No effect

[a] No statistical interaction of treatment and sex, therefore sexes were combined for analyses. Data are for the 5 mg/kg dose group which was most affected, 1 mg/kg dose group had little if any effect except in the T maze.
[b] Effects significant only at 1 mg/kg dose for RAM, both doses for T maze and motor activity
[c] -- not tested.
[d] Both sexes were combined.

Persistent effects of chlorpyrifos following gestational and early postnatal exposure were also demonstrated in the CD-1 strain mice in a series of three studies from the laboratory of Dr. Calamandrei. In the first study (Ricceri et al, 2003), postnatal exposure to chlorpyrifos during PND 1-4, or 11-14, to 1 or 3 mg/kg (subcutaneous administration in DMSO) was followed by several assessments (ultrasonic vocalization or USV measurements, homing response, locomotor activity, novelty seeking response, social interaction with an unfamiliar conspecific and passive avoidance learning and memory) from before weaning and up until about PND 60. This study included assessment of brain AChE activity and showed the G_4 tetramer form of the enzyme to be most sensitive. Total AChE was inhibited by about 20-23% (both dose groups) following the early postnatal exposure (assessed 1 hour after exposure), whereas inhibition was not noted four or twenty four hours after dosing. Notable persistent behavioral effects were apparent and included increased locomotor activity (on PND 25 in both of the dose groups of the PND 11-14 treatment groups only following post hoc ANOVA); more activity to environment novelty (on PND 35); and more agonistic responses (i.e. more marked aggressive grooming in males) in the social interaction test (on PND 45, all treatment groups). On PND 60, however, passive learning was reported as not affected in any of the treatment groups.

In a later study (Venerosi et al, 2006), exposure occurred during GDs15-18 at 3 or 6 mg/kg chlorpyrifos (oral administration in peanut oil), and the offspring were then also dosed on PND 11-14 at 1 or 3 mg/kg chlorpyrifos. Only female mice were assessed at four months after birth using a novel social recognition test with resident and novel female partners. The authors report that the highest dose prenatally followed by vehicle postnatally altered the patterns of ultrasonic vocalization, but in mice receiving the highest dose prenatally and either dose postnatally, these effects were not observed (Venerosi et al, 2006, Table 1). These results indicate that while prenatal chlorpyrifos alters social behavior, the outcomes are altered by subsequent postnatal exposure.

In a separate report (Ricceri et al, 2006), using the same gestational and postnatal exposure protocol and assessing the mice at PND 70, demonstrated that motor activity in the open field was increased in mice receiving the highest dose prenatally, and either vehicle or the low dose postnatally (Ricceri et al, 2006, Figure 2). Altered agonistic behaviors were noted in males (Figure 3)**.** Postnatal exposure increased maternal responsiveness toward pups (Figure 4). Females, rather than males, exposed to the high dose postnatally (Ricceri et al, 2006, Figure 5) showed more time in the open arms of the plus maze**,** a finding apparently contradicting that of Aldridge et al. (2005c, Figure 1) where the effect was only observed in males. Overall the authors' conclusions from these studies are that chlorpyrifos induces long-term alterations in sex-specific behavior patterns in the mouse with social responses the most affected.

Jett and coworkers (2001) exposed Long-Evans strain rats postnatally to chlorpyrifos at 0.3 or 7 mg/kg by subcutaneously administration in peanut oil. One set

(pre-weaning) was dosed every fourth day on PND 7, 11 and 15 and another set (post-weaning) was dosed on PND 22 and 26. Both males and females were tested but since no statistical differences in treatment effect were found, the authors combined both sexes for further analysis. They found that only the high pre-weaning dose group showed decreased spatial learning in the Morris water maze when the rats were assessed on PNDs 24 to 28. In contrast, both the post-weaning 0.3 and 7 mg/kg dose groups dosing were affected.

Table 2: Effects of chlorpyrifos in the Morris water maze following pre-weaning and early post-weaning exposure

Dosing [a]	Latency	Time in training	% of Rats Finding Platform ≤ 10 sec	AChE Inhibition
Pre-weaning				
Control (20)[b]	12.3±2.6	13.8±1.2	60%	--
0.3 mg/kg (19)	17.5±3.0	12.2±1.0	50%	None
7.0 mg/kg (17)	23.7±2.3*	7.7±1.0*	21%	None
Post-weaning				
Control (7)	9.0±2.3	17.0±1.4	71.4%	--
0.3 mg/kg (7)	22.7±4.7*	11.5±1.0*	28.5%	None
7.0 mg/kg (8)	22.3±4.4*	12.2±1.4*	25.0%	None

[a] Pre-weaning animals were dosed on PND 7, 11, and 15. Post-weaning rats were dosed on PND 22 and 26. * $p < 0.05$ by ANOVA

[b] The number in () is the number of combined males and females assessed for each group for the behavior study only.

The dose-response relationship in the post-weaning animals does not show a gradation in response, with similar quantitative effects in both dose groups. There was no effect on swimming speed. In this study, the dosing protocol leaves 4 day gaps between the successive doses, which is not comparable to the studies from Aldridge (2005) and Levin et al (2001, 2002) where dosing was daily over fixed four day intervals or to a guideline study where dosing is daily throughout most of gestation and lactation. The results also differ in that males and females were reported to have the same effect but the Levin group reports gender differences. The effects in the high dose pre-weaning group may reflect a prolonged effect, since assessment began a week after the last dose. The post-weaning dose study, however, is confounded since dosing occurred in the middle of testing, and may reflect the acute toxicity of chlorpyrifos. Whether the effect on learning and memory at 0.3 mg/kg is prolonged or not, the study demonstrates that there is an effect on spatial learning at this dose.

Neither dose (0.3 or 7 mg/kg) demonstrated inhibition of AChE in the cortex, hippocampus or cerebellum when assessed by these authors at PNDs 7 (3 hours after dosing) or on days 8 and 16 (possibly 24 hours post dosing) or on day 28 in all rats. A single dose of 7 mg/kg given orally to 17 day old rats would be expected to result in about 60% brain AChE inhibition (Moser and Padilla, 1998). Thus, there is an open question as to whether or not the method of administration (subcutaneous in peanut oil)

resulted in inhibition that was not detected during the times for enzyme assessment. Further discussion on the complexity of assessment for brain AChE inhibition in fetal and neonatal brain is presented in the accompanying chapters on cholinesterase inhibition and metabolism and pharmacokinetics.

One other paper assessed the effects of progressive doses (3 to 12 mg/kg, orally in corn oil) chlorpyrifos on PND 1-21 on locomotor activity and its correlation with AChE inhibition (Carr et al, 2001). These authors report that in early assessments (PNDs 10, 12, 14, 16, 18 and 20) when there was brain AChE inhibition, there were no differences in open field activity. After PND 25 and 30, when brain AChE was much reduced, locomotor activity was significantly decreased (Carr et al, 2001, Figure 2) in the middle and highest dose groups and apparently similarly in both sexes (i.e. males about 19 and 31% for the mid dose group and about 29 and 47% for the high dose group).

Chlorpyrifos was also demonstrated to affect response latency and spatial discrimination in a third species in the zebrafish assay (Levin et al, 2003) when exposed to concentration levels of 10 or 100 ng/ml on days 1-5 post fertilization with behavioral assessments starting at week 20. Some differences were that both 10 and 100 ng/ml reduced the escape/avoidance accuracy (Levin et al, Figures 2 and 3). The effect on spatial discrimination escape/avoidance response latency was increased at 10 ng/ml but was decreased at 100 ng/ml (Figures 4 and 5).

Yet another study (Lassiter and Brimijoin, 2008) using Long-Evans rats dosed with chlorpyrifos (by gavage in corn oil) during GD 7 to PND 21 resulted in the male rats dosed at 2.5 mg/kg, but not females, gained excess weight beginning at PND 45 and reaching a 10.5% increase by PND 72. Volumetric measurement of the males indicated they were 12% larger than the controls. The dose response curve was an inverted U shape indicating a smaller effect at 1 mg/kg, but no effect at 4 mg/kg (Lassiter and Brimijoin, 2008, Figure 1C).

In summary, the papers from the Slotkin/Levin and Calamandrei laboratories tested animals as adults and thus indicate that independent investigators have reported that adults have persistent behavioral effects following gestational and/or early postnatal exposure in two different mammalian species. The Levin laboratory shows that a third species (fish) may be affected. The studies from the Jett laboratory test much younger animals but still report behavioral differences after AChE inhibition has recovered or in the absence of inhibition in their assays. The Lassiter study, although not a behavioral study, also reports a persistent effect. Although these studies raise many questions, and each took a different approach with regard to dosing and behavioral assessments with different behavioral techniques, when taken together they provide a basis for concern for susceptibility for persistent effects of chlorpyrifos on neurodevelopment.

3. Acetylcholine Receptor and Transporter Binding Studies

Chlorpyrifos dosing inhibits brain AChE following the metabolism of chlorpyrifos to its oxon analog and intact chlorpyrifos is much less potent as an inhibitor of this function. The resulting excess buildup of ACh causes a cholinergic crisis at critical doses, but may also have other modulation functions in the developing brain. In addition to postsynaptic events innervating downstream effects, there are also presynaptic events critical to maintaining the status of ACh and Ch in the synaptic region and synthesis of new ACh from Ch that are associated with increases and decreases in nerve activity. The density of pre- and postsynaptic receptors and high affinity transporters can be assessed to determine whether their function is affected by drug or toxicant treatment. The several studies below from independent laboratories demonstrate that chlorpyrifos alters several parameters that are associated with the ACh system.

The Slotkin protocol for dosing dams during on GD 9-12 and GD 17-20 as well as on PND 1-4 and 11-14 with chlorpyrifos in DMSO via subcutaneous injection was used with assessment of the fetuses and pups as well as adults for the biomarkers choline acetyl transferase (ChAT) and high affinity presynaptic choline transporter (HACU) and muscarinic ACh receptors (mAChR). Only some of the many responses can be mentioned here since the response varied with age, gender, brain region and dose. Following early gestational exposure on GD 9-12, ChAT was more obviously increased in the hippocampus and striatum in both adolescence and adulthood (Qiao et al, 2004, Figure 1). The HACU transporter was subnormal for both doses (1 and 5 mg/kg) in the hippocampus but in the striatum at 1 mg/kg on PND 30 it appeared elevated in contrast to reductions at other times and doses (Qiao et al, 2004, Figure 2). The density of the mAChR was also reduced especially on PND 60 in the hippocampus and striatum (Qiao et al, 2004, Figure 3).

Following later gestational exposure on GD 17-20 (Qiao, et al, 2003), there was little consistent change in the ChAT marker but there were changes of greater magnitude and in different brain regions in HACU marker, a marker that is responsive to nerve impulse activity, as determined by ^{3}H-hemicholonium-3 binding. Although the HACU system showed marked depression (Qiao et al, 2003, Figure 1B) up to nearly about[1] 20 to 30% on PNDs 4 and 10 in the forebrain, it had recovered to near control levels by weaning When the animals were assessed at PND 60 there were again reported decreases in the cerebral cortex (near about 20%), hippocampus (about 15%) and striatum (about 10% at 1 mg/kg to about18% at 5 mg/kg, Figure 1B). Thus, gestational exposure to chlorpyrifos led to changes in the HACU transporter when assessed as adults.

Following dosing at PNDs 1-4 at 1 mg/kg or PNDs 11-14 at 5 mg/kg the hippocampus, midbrain, striatum, brainstem and cerebral cortex were assessed on PNDs 30 and 60 (Slotkin et al, 2001) and variable effects were noted. Most brain

[1] In many cases the quantitative percentage value was read by eye from the Figures in the citations and when this was done the term “about” precedes the percent number.

regions generally (but not exclusively) demonstrated reductions in the biomarkers for ChAT and HACU but the effect on the HACU system appeared to be more pronounced in some of these structures. Because two dosing regimens and five brain regions were assessed at different times for both sexes, not all effects can be described here. One example is that of the effects in the hippocampus the structure that showed some of the larger changes in HACU density. Following early postnatal exposure to 1 mg/kg chlorpyrifos, at PND 30, the decrease in females was nearly 30% and in males it was about 18% (Slotkin et al, 2001, Figure 2). When assessed on PND 60, however, the male/female differences changed since the males had a 23% decrement but the females had only an 8% decrement in HACU density. If the animals were exposed to chlorpyrifos on PND 11-14 at the higher dose of 5 mg/kg, it was found that the effects on HACU density displayed a decrease of only about 3-5% at either PND 30 or 60 essentially reversing the effect seen following the lower dose given at the earlier window (Slotkin et al, 2001, Figure 2). Females also demonstrated a clear reversal at PND 30 when dosed at 5 mg/kg being only about 3% lower in contrast to the 30% decrease following the lower dose. However, after 60 days females were about equally reduced (compare 8% and 12% in Figure 2) for the 1 and 5 mg/kg doses in HACU density reduction. Mixed patterns of dose, gender and time of administration were also noted for the other 4 brain regions following early postnatal dosing. .

Data from the laboratory of Dr. Chambers reported on the effects of chlorpyrifos on the ACh receptor and transporter parameters in fetuses and neonatal pups following either gestational or oral post natal exposure (Richardson and Chambers, 2004). AChE activity was also assessed in order to correlate the inhibition with effects on the receptors and transporters. Following gavage administration of 3 or 7 mg/kg/day to pregnant dams on GDs 6-20 and assessing the pups on PNDs 1, 3, 6, 9, 12 and 30, the mAChR, ChAT activity, HACU system and the vesicular ACh transporter (VAChT) were found to be altered in brain. This dosing inhibited pup brain AChE activity by 15 and 30%, for the 3 and 7 mg/kg/day dose levels at PND 1, but no inhibition remained by PND 6 (Richardson and Chambers, 2004, Figure 1). The mAChR density was reduced in the high dose group similarly (17-21%) as demonstrated by three different ligands ([^{3}H]-*N*-methylscolpolamine, [^{3}H] quinuclidinyl benzilate and [^{3}H]-4-DAMP) but similar to AChE inhibition, the mAChR receptor density was equivalent to the controls by PND 6 (Richardson and Chambers, 2004, Figure 2). The 7-mg/kg dose caused up to about 12% inhibition of ChAT activity on PNDs 9, 12, and 30 (Richardson and Chambers, 2004, Figure 4). HACU density was decreased by about 25% on PND 6 in both the low and high dose groups and reduction persisted in the high dose group with a 14 to 21 % decrement on PNDs 12 and 30 Richardson and Chambers, 2004, Figure 5). The VAChT density was reduced 13-31% on PNDs 3 to 30 with the largest decrease appearing to be occurring on PND 12 in the high dose group (Richardson and Chambers, 2004, Figure 6). Since the ChAT, HACU and VAChT are all markers of presynaptic function, the authors (Richardson and Chambers, 2004) interpreted the findings that gestational exposure to 7 mg/kg/day chlorpyrifos causes long term alterations in presynaptic cholinergic neurochemistry.

The same group investigated the effects of repeated postnatal dosing of chlorpyrifos at a low dose of 1.5 mg/kg/day for PNDs 1-21 and a high dose group of 1.5 on PNDs 1-5, 3 mg/kg/day on PNDs 6-13 and 6 mg/kg/day on PNDs 14-21 (Richardson and Chambers, 2005). These dosing protocols caused inhibition of brain AChE in both the low (49%) and high (59%) dose groups on PND 6 with partial recovery by PND 30 with 20% inhibition remaining in the low dose group and 40% remaining in the high dose group (Richardson and Chambers, 2005, Figure 1). Similar to the gestational exposure to chlorpyrifos, total mAChR density was decreased on PNDs 12 and 22 when there was inhibition with some recovery by PND 30 (Richardson and Chambers, 2004, Figure 2). There were some differences in the densities of the M1. M2, M3 and M4 subtypes of mAChR with only the M1 and M3 being affected in the low dose group. ChAT activity (decreased 12%, Figure 5) and the VAChT (decreased 22%, Figure 7) were affected in the high dose group only on PND 30. The HACU was decreased at PND days 6 (about 20%/40%, 12 (about 8%/21%), 22 (about 20%/26%) and 30 (about 9%/24%) for the low/high dose groups (Figure 6). Overall, this study established that at doses that inhibit brain AChE there are transient decreases in mAChRs and more persistent deceases in presynaptic cholinergic neuron markers.

The differential effects of chlorpyrifos on neonatal (< 7 days old), juvenile (21 days) and adults (90 days) on ACh release were also assessed following relatively high doses of one half or 1 x of the LD_{10} (15, 47 or 136 mg/kg (subcutaneously in peanut oil) for the neonates, juveniles and adults, respectively) or doses that resulted in substantial inhibition of brain AChE (Won et al, 2001) that mostly reversed by 96 hours. These authors demonstrated that in tissue slices (Won et al, 2001, Table 1) prepared from the dosed animals and sacrificed at 4, 12, and 24 and 96 hours after dosing there were both inhibition (up to 20%, juveniles at 96 hours in the low dose) and stimulation (up to 37%, juveniles at 24 hours in the high dose) of ACh release. In contrast, neonates showed only up to 13% inhibition at 96 hours and adults showed an early inhibition followed by an increase with the effect most pronounced in the low dose. This study also showed that depolarization-stimulated ACh release from slices prepared from untreated rats was age dependent with the neonates having very little and the adults were maximal implying an inherent age difference in the function of ACh release. This study supports the above studies showing that chlorpyrifos can affect the synaptic events involved with ACh release with effects persisting after inhibition has reversed and differs with age.

The Slotkin, Chambers and Pope laboratories report effects on the cholinergic system that imply persistent effects, especially on presynaptic events. Direct comparison of the three laboratories is confounded because they used different routes of administration. Although the above effects on the ACh system were demonstrated, the relationship between chlorpyrifos and a morphogenic role of the ACh system was not specifically elucidated.

4. Serotonin

Serotonin (5HT) is one of the major neurotransmitters in the brain and, because of "the extraordinarily widespread projections within the brain and highly regulated pacemaker pattern of activity that is characteristic of serotonin neurons, a broad homeostatic function has been suggested for serotonergic systems" (Copper, Bloom and Roth, 1991). Historically, serotonin has been implicated in psychological models and more recently a class of selective serotonin uptake inhibitors has been extensively used for treatment of psychiatric disorders. Thus, studies on the possible interaction of chlorpyrifos with the 5HT system might suggest a possible pathway to perturbation in the developing brain for the developmental toxicity of chlorpyrifos. In a series of at least eight studies (Raines et al, 2001, Aldridge et al, 2003, Aldridge et al, 2004, Aldridge et al, 2005a,b and c, Slotkin and Seidler, 2005 and 2007a) from the laboratory of Dr. Theodore Slotkin and his co-workers, gestational or neonatal exposure to usually either 1 or 5 mg/kg/day applied subcutaneously in DMSO caused a variety of effects on the serotonin transporter and either the 5-HT_{1A} or 5-HT_2 receptors depending upon the brain region, gender, dose and window of administration and on when the pup was assessed including into adulthood. The variety of effects for all of these conditions is far too numerous to list here. Selected examples to show the complexity of the chlorpyrifos interaction with the serotonin system are as follows. Exposure of 1,2 or 5 mg/kg during GD 9-12 and assessment of the fetuses on GD 17 decreased the three serotonin parameters up to about 50% (for the $5HT_{1A}$ receptor) in whole brain (Aldridge et al, 2003, Figure 2). When the fetuses were assessed on GD 21 (same Figure), the decreases noted previously were reversed to increases in both the brainstem (largest effect up to almost 50%) and forebrain (maximum increase about 20%).

Exposure to 1 mg/kg chlorpyrifos during GD 9-12 resulted in elevation of $5HT_{1A}$ and $5HT_2$ receptors and the 5HT transporter in the cerebral cortex, midbrain and brainstem when the rats were assessed as adults (Aldridge et al, 2004, Figure 1A and B), meaning that the effect of chlorpyrifos during neurulation is permanent. In the cerebral cortex the elevation for the $5HT_{1A}$ receptor was increased about 20% following 1 mg/kg (Figure 1A) and about 27% following 5 mg/kg (Figure 1B) whereas females were only 2-3% higher than controls. Females were affected to about the same degree (10-15%) in midbrain for the $5HT_{1A}$ receptor at both doses but females had a higher elevation than males in the brainstem at 1 mg/kg but a lower value than males when dosed at 5 mg/kg. Persistent effects were also reported following exposure to 1 mg/kg chlorpyrifos on GD 17-20. An example is that in the striatum the $5HT_{1A}$ receptor elevations were as much as nearly 80% in males and 30% in females (Aldridge et al, 2004, Figure 2A). Following 5 mg/kg the effect in the striatum was only about 40% in males and almost nothing (about 5%) in females Figure 2B).

Following exposure to 1 mg/kg chlorpyrifos on PND 1-4 (Aldridge et al, 2004, Figure 3), serotonin parameters were generally increased in males and decreased in females in the cerebral cortex, hippocampus, striatum and midbrain, whereas in the brainstem both males and females showed elevations (to up to about 35%) with the males appearing to be slightly higher than the females. Following exposure to 5 mg/kg

chlorpyrifos on PND 11-14 (Figure 4), $5HT_{1a}$ receptors in males were again elevated to about 23% and with a few exceptions, the males were generally higher than the controls for the other parameters or not affected, but there were some cases where a parameter was decreased. Females were generally lower (i.e. the 5HT transporter was decreased about 15% in the striatum) or not affected.

The alterations in 5HT transporter and receptors when assessed as adults were said by the authors to likely contribute to the behavioral changes also seen in adults following gestational and/or neonatal exposure. These included males spending more time in the open area of the elevated plus maze; an anhedonia test with treated rats showing decreased a preference for chocolate milk over water and a reversal of the females to make more errors in the radial arm maze (Aldridge et al, 2005). Ketanserin (a 5HT receptor antagonist) elicited dose-dependent increases in working and reference memory errors in the adult chlorpyrifos rats. The authors interpreted the latter result to indicate an abnormal dependence on a serotonergic mechanism for memory (Aldridge et al, 2005c)

The above examples are only a very limited sampling from several papers from the Slotkin laboratory that were selected to show the variety of effects that can depend on the window of exposure, the variability of the magnitude of the differences relative to the control for some parameters and the persistence of some effects into adulthood. Since there is uncertainty associated with the true extent of brain AChE inhibition or its effects on a morphogenic role of ACh/AChE following the subcutaneous route of administration of chlorpyrifos especially in fetuses, it cannot be ascertained at this time if the effects of chlorpyrifos on the serotonin parameters are a secondary effect on the ACh system or a direct interaction with the serotonin transporter or its receptors. No *in vitro* data that show actual affinity of chlorpyrifos or chlorpyrifos oxon with the serotonin receptors or transporter to help characterize an association with these proposed targets were found in the literature. Many of the Figures in the supporting papers have large standard deviations. Thus, it is difficult to distinguish spontaneous variability from an effect of chlorpyrifos treatment especially when there are small differences from the controls. Furthermore, unlike some of the other neurochemical effects, attempts to replicate the serotonin effects have not yet been reported by other laboratories.

5. Adenylyl Cyclase

The role of the adenylyl cyclase system as the second messenger and the cascade of subsequent events in the nervous system are well known. Since many physiological processes such as transmitter functions affect it, it might be expected that alterations in this system would be affected by chlorpyrifos through its actions on the ACh and serotonin transmitter systems or on its interaction with other targets. Chlorpyrifos or its active oxon form may also directly affect the adenylyl cyclase system. The effects of chlorpyrifos resulting from the dosing protocol used by the Slotkin laboratory for early postnatal (PND 1-4 and 11-14) exposure demonstrated effects in the brain regions enriched (forebrain) or sparse (cerebellum) in cholinergic stimulation and in non-neuronal tissue (heart). In all three tissues, chlorpyrifos caused deficits in the

adenylyl cyclase cascade including on adenylyl cyclase itself, functioning of G-proteins that link neurotransmitter and hormone responses to cyclase activity, and expression of neurotransmitter receptors that act through this cascade (Song et al, 1997 and 1998). Some examples in the forebrain following the early dosing on PND 1-4 at 1 mg/kg were that adenylyl cyclase activity assessed on day 5 was slightly reduced (about 3%) but on PND10 was it reduced to about 20% (Song et el, 1997, Figure 2). Under these conditions, fluoride stimulated adenylyl cyclase activity was first elevated to about 10% at day 5 but was reduced to about 35% when assessed at day 10 (Song et al, 1997, Figure 2). Yet another example (Song et al, 1997, Figure 5) is that following dosing on PND 11-14 adenylyl cyclase on PND 15 was increased about 35% but on PND 20, it was reduced about 20% in the cerebellum. Only slight decreases in activity were noted in the fluoride-stimulated model following late postnatal exposure. Other examples of both decreases and increases in adenylyl cyclase were also apparent in this paper. Thus, a variety of effects on adenylyl cyclase can result following chlorpyrifos exposure depending upon window, gender and region investigated as well as stimulation conditions.

The Slotkin protocol for subcutaneous administration on GD 9-12 and 17-20 and PND 1-4 and 11-14 was also used to assess for persistent effects at PND 60 adults (Meyer et al 2003 and 2004). This work utilized several methods to activate the adenylyl cyclase system to help to understand what specific aspects of the adenylyl cyclase cascade may be affected. These included forskolin and Mn, which are direct adenylyl cyclase stimulants, and isoproterenol, which activates signaling through beta adrenoreceptors, coupled to stimulatory G proteins. Several brain regions were also investigated. Again a variety of effects were noted and only a few selected examples can be mentioned here since multiple doses and brain regions were assessed for both sexes. . Some examples of changes were that following early gestation exposure, only doses that elicited maternal toxicity (5 mg/kg) resulted in alterations in the adenylyl cyclase system. Dosing of 1 or 5 mg/kg on GD 17-20 resulted in gender and regional specific effects on adenylyl cyclase with males generally showing increases (up to about 20% in the striatum at 5 mg/kg following forskolin or Mn stimulation, Meyer et al, 2003, Figure 3B) and females decreases (down to about 15% in the striatum also at 5 mg/kg). Effects at 1 mg/kg were generally in the same direction but appear to be of lower magnitude in the striatum. There were decreases for males in the cerebellum (down to about 10% at 5 mg/kg) whereas there were increases in females (up to about 20%) demonstrating contrasting effects between the striatum and cerebellum (compare Meyer et al, 2003, Figures 3B and 3D). Following PND 1-4 exposure, in the cerebellum, males were decreased (to about 20%, for the basal and isoproterenol stimulation models (Meter et al, Figure 4B) but were increased about 18% following Mn stimulation, (Meyer et al, 2003, Figure 4B). Females were increased (to about 15-20%) for all conditions. Following dosing at PND 11-15 at 5 mg/kg and assessing at PND 60, the general pattern for most conditions was that there was a decrease in adenylyl cyclase activity (to about 18% in males and to almost about 27% in females, Meyer et al, 2003, et al, Figure 6) but with occasional increases. This paper also demonstrated that the specific binding of beta adrenoreceptor was reduced mostly in females but in males there were occasions of increases (Meyer et al, 2003, Figures 2 and 5). Overall, the

authors claim that exposures conducted on GD 17-20 and later all produced sex-selective alterations in cell-signaling cascades lasting into adulthood and these are shared by multiple neurotransmitters and hormonal inputs.

Another paper assessed the age related effects of chlorpyrifos on cAMP formation in the cortex of rats following oral dose levels that were one third and 1 x the LD_{10} for chlorpyrifos in neonatal, juvenile and adults in brain tissues removed 4, 24, and 96 hours after dosing. It was found that the low dose stimulated basal cAMP formation (Zhang et al, 2002, Table 4) in juveniles only (78%, 33% and 30% at 4, 24 and 96 hours) and all age groups were stimulated in the high dose group but the juveniles were probably most affected. . The high dose resulted in enhancement of the forskolin/Mn-stimulated formation of cAMP (Zhang et al, Table 5) in neonates (82% at 96 hours) and juveniles (77% at 24 hours and 32% at 92 hours) but inhibition in adults (17% at 96 hours). Since the study also demonstrated that oxotremorine (a mAChR agonist) inhibition of cAMP formation was further reduced by the high dose chlorpyrifos for all ages (about 19-26% at 96 hours, Figure 3), the authors concluded chlorpyrifos was possibly acting through a "direct interaction between CPF (or its oxon) and mAChRs or other components of the adenylyl cyclase cascade".

6. Macromolecular (proteins, genes, mRNA)

Macromolecular targets can be considered to include effects on genes, DNA synthesis and proteins. The 2000 chlorpyrifos risk assessment indicated that earlier studies (Whitney et al, 1995, Dam et al, 1998 and Song et al, 1998) utilized [^{3}H] thymidine to demonstrate inhibition of DNA synthesis. Since then, at least two independent laboratories have presented separate series of papers that demonstrate that gestational and/or early postnatal exposure can affect a variety of genes, proteins, as well as DNA synthesis or mRNA content.

The observations on macromolecular changes from the laboratory of Dr. Slotkin were reported in a series of papers as follows. The effects of chlorpyrifos *in vitro* in the C6 glioma cell line (Garcia et al, 2001) considered an established glial model showed that in undifferentiated cells, DNA synthesis was inhibited by concentrations starting at about > 1 micromolar. This effect of chlorpyrifos was not blocked by a combination of atropine and mecamylamine implying that neither muscarinic nor nicotinic receptors mediated the effect of chlorpyrifos on DNA synthesis (Garcia, et al, 2001, Figure 1). Chlorpyrifos also affected the adenylyl cyclase system at the level of G-proteins with undifferentiated cells being more affected than differentiated cells. In differentiated cells, however, the ability of chlorpyrifos to elicit formation of reactive oxygen species and evoke deficits in Sp1 nuclear transcription factor essential for differentiation was demonstrated (Garcia et al, 2001, Figure 6). The overall significance of these results are that *intact* chlorpyrifos applied *in vitro* may affect glial type cells and since glial development *in vivo* continues after neurogenesis, the window for vulnerability toward glial cells may continue for extended periods.

In a subsequent paper (Garcia et al, 2002a), the effects of chlorpyrifos *in vivo*

(GD 17-21) on glial fibrillary acid protein (GFAP, an astrocyte marker) were investigated. GFAP was altered only at high dosages (20 or 40 mg/kg/day, subcutaneously in DMSO) that also elicited maternal and other fetal toxicity. If animals were, however, dosed postnatally, effects were seen at lower dosages. One example is that dosing on PND 1-4, caused decreases (about 24%) in GFAP in male pups on PND 5, an elevated response on PND 10 (about 18%) and a return to normal on PND 30 in the cerebellum following 1 mg/kg/day dose of chlorpyrifos (Garcia et al, 2002a, Figure 3). Exposure to chlorpyrifos on PND 11-14 at 5 mg/kg elicited decreased GFAP across all brain areas in both sexes on PND 15 (Garcia et al, 2002a, Figure 4). By PND 30, GFAP was increased in most regions in males, with the striatum being most affected (about 19%, (Garcia et al, 2002a, Figure 4). The conclusion was that chlorpyrifos dosing affects gliogenesis *in vivo* with the maximum effects corresponding to the peak period of gliogenesis and cell differentiation.

The concept of changing vulnerabilities following the exposure to chlorpyrifos during prenatal as compared to postnatal exposure was further established in another study (Garcia et al, 2002b) that assessed myelin basic protein (MBP, a marker for oligoendrocytes), neurofilaments 68 kD or NF68 (a marker for neuronal cell bodies) and neurofilament 200 (a marker of developing axons). Chlorpyrifos exposure during GD 17-21 (1 mg/kg/day and higher, Garcia et al, 2002b, Figure 2 A and B) produced an immediate enhancement by GD 21 for MBP and NF68: but by PND 30, there were deficits in the females but increases in males for some biomarkers in the striatum. Early postnatal exposure (PND 1-4) did not cause significant short or long-term changes in these biomarkers. Exposure on PND 11-14 reduced MBP levels in males on PNDs 15 and 20 throughout the brain and by PND 30, the biomarkers demonstrated decreases in all brain regions Garcia et al, 2002b, Figure 4A and B). In contrast, females showed both increases and decreases in MBP depending on the region on PND 15, 20 and 30 (Garcia et al, 2002b, Figure B and D). The authors claimed that the sex selective differences matched the behavioral differences that they reported in other publications.

The effects of exposure to chlorpyrifos on PND 1-4 at 1 mg/kg subcutaneously in DMSO using microarray techniques to assess for similarities and differences in transcriptional responses in both the brainstem and forebrain in male rat pups 24 hours after the last treatment (PND 5) were also investigated by this laboratory (Slotkin and Seidler, 2007b). In all, the expression of about 60% of 252 genes associated with pathways for general neural cell development, cell signaling, cytotoxicity and neurotransmitter systems were changed. There were far too many reported differences to list here. A representative change associated with chlorpyrifos is illustrated by there being a brain area specific 19% increase in gap43 (marker for neural growth) in the brainstem but no change in the forebrain (Slotkin and Seidler, 2007b, Figure 1). There was also about a 20% decrease in the *nefh* gene in the brainstem but no effect in the forebrain (also Figure 1). One other dramatic difference noted was the opposing effect on the ap1g2 gene since there was reported a nearly 30% increase in the brain stem but a nearly 35% decrease in the forebrain Slotkin and Seidler, 2007b, Figure 4). Thus there is much variability in these data confounding their interpretation. So far similar

data on female rats or on other windows such as gestational or late post-natal have not been reported.

In a separate series of studies reported from Dr. Carr and co-workers the effects of chlorpyrifos on neurotrophins and other proteins as well as on muscarininc ACh receptors (mAChR) were investigated with dosing on PND 1-6 by gavage in corn oil. The first paper (Betancourt and Carr, 2004), both chlorpyrifos and chlorpyrifos oxon described cholinesterase inhibition caused by chlorpyrifos (1.5 or 3 mg/kg/day) as persistent while that of chlorpyrifos oxon (0.25 or 0.35 mg/kg/day) as transient (Betancourt and Carr, 2004, Figure 1). The mAChR density (24% and 42% on day 4 and 24% and 40% on day 7, Figure 3) and nerve growth factor (NGF) protein (11% and 21% on day 4 and 19% and 22% on day 7 for the 1.5 and 3 mg/kg dose groups, respectively, Figure 5) were significantly decreased by chlorpyrifos. Treatment with the oxon analog did not affect either endpoint. No effects on the brain derived neurotrophic factor by either compound were noted. The authors interpreted these data to indicate that persistent AChE inhibition caused by chlorpyrifos administration but not the more transient inhibition caused by the oxon and deceased mAChR density may play a role in decreased NGF levels. In a succeeding paper (Betancourt et al, 2006), using the same dosing protocol but sacrificing on PND 7, quantitative PCR (polymerase chain reaction) showed reduced expression of NGF, reelin (a protein that plays a role in layer formation during early stages of development of the cerebral and cerebellar cortices) and M1 AChR m RNA. Myelin associated glycoprotein mRNA expression was also decreased but there was no effect on B-III tubulin mRNA. The authors interpreted these changes as a suggestion that chlorpyrifos exposure affects developing oligodendrocytes. There was also an increase on GFAP mRNA in both sexes (Figure 6) to indicate that astrocytes were affected.

In the third paper (Betancourt et al, 2007), chlorpyrifos was administered by gavage at dose levels of 4 or 6 mg/kg on PND 10-20 and assessments were made on PNDs 20 and 28. Some of the findings selected to show the variability in effects are as follows. At the higher dose on PND 20, NGF protein was increased (about 24%, Figure 3B) in the hippocampus but not in the frontal cortex (Figure 4B). NGF mRNA was decreased (about 38%, Figure 3A) in the hippocampus but increased (about 18%, Figure 4A) in the frontal cortex. On PND 28, NGF protein was decreased only slightly in the hippocampus but was about 23% decreased in the frontal cortex (compare Figures 3B and 4B). Also on day 28, however, NGF mRNA was increased about 23% in the hippocampus but there was no significant difference in the frontal cortex (compare Figures 3A and 4A). BDNF mRNA was increased in the frontal cortex as much as about 63 to 72% (Figure 6A) and in the hippocampus about 21 to 28% (Figure 5A) on PND 20 or 28, respectively. BDNF protein was increased on PND 20 (about 25% for unbound protein) but on PND 28 there was no statistical difference with a possible decrease in the hippocampus (Figure 5B); and in the frontal cortex there was a similar about 21% increase in unbound protein on PND 20 and no statistical difference on PND 28 (Figure 6B). Thus, although the mRNA remains elevated, the proteins (unbound) are not elevated. *c-fos* mRNA in both the hippocampus (about 46%) and cerebral cortex (about 42%) a marker of neuronal activation was increased (Figure 8).

In summary, two independent laboratories have recently presented data demonstrating alterations in macromolecules in developing brain following either gestational or postnatal exposure to chlorpyrifos. In addition, *in vitro* studies also demonstrate alterations in certain macromolecules.

7. Nerve Growth

There are several reports using a variety of techniques with exposure to chlorpyrifos, chlorpyrifos oxon and in some cases TCP showing effects on cell growth. In an *in vitro* study (Das and Barone, 1999) using PC12 cells, chlorpyrifos, its oxon analog or TCP all inhibited NGF-induced neurite outgrowth. These factors for cell growth were positively correlated with increased AChE in primed control cells suggesting a link between AChE and elaboration of neurite growth. Chlorpyrifos oxon inhibited both AChE activity and NGF induced cell growth, whereas the inhibition of cell growth in the presence of intact chlorpyrifos and TCP occurred in the absence of detectable AChE inhibition implying that intact chlorpyrifos and TCP may act via some other mechanism to affect cell differentiation.

In a later study (Schuh et al, 2002) primary cultures of hippocampal or cortical neurons were assessed for alterations in the Ca^{2+}/cAMP response element binding protein (cCREB) considered a critical molecule in brain development and cognitive function. Reported estimated EC_{50} values for effective concentrations in cortical neuron cultures were for chlorpyrifos (60 pM) or chlorpyrifos oxon (< 30 fM) following one hour of exposure and for TCP (<30 pM, following 7 days of exposure) with increases of the phosphorylated substrate being 300-400% (Schuh et al, Figures1, 2 and 3). At these low effective concentrations, AChE was not inhibited and cell viability was not affected. Inclusion of a cytochrome p-450 inhibitor to block the conversion of chlorpyrifos to the oxon did not prevent chlorpyrifos from affecting cCREB, an indication that intact chlorpyrifos affects the system although the oxon analog is more effective. Findings similar to the cortical neurons were noted in hippocampal neuronal but not in astrocyte cultures.

In a study (Howard et al, 2005) designed to determine if chlorpyrifos or its metabolites affect axonal or dendritic growth differentially, embryonic rat sympathetic neurons derived form the superior cervical ganglia were used. The authors reported that axonal outgrowth was significantly inhibited by chlorpyrifos (≥0.001 μM) and chlorpyrifos oxon (≥0.001 nM), but not by TCP. In contrast, all three chemicals enhanced BMP (bone morphogenic protein) and induced dendritic outgrowth. In these cultures, AChE activity was inhibited only at higher concentrations of chlorpyrifos (≥ 1 μM) relative to chlorpyrifos oxon (≥ 1 nM) and TCP did not inhibit activity. Thus, opposing effects of very low concentrations of intact chlorpyrifos on axonal and dendritic growth were reported to occur in the absence of significant inhibition of AChE.

A later report from this laboratory (Yang et al, 2008), further investigated the effects of chlorpyrifos or chlorpyrifos oxon on the possible mechanism for inhibiting

axonal growth and its relationship to different forms and functions of AChE using cultures of dorsal route ganglion from either wild type (AChE +/+) or nullizgous (AChE-/-) genotypes. In this study, the previous observation (Howard et al, 2005) was stated as being verified with both chlorpyrifos and its oxon inhibiting axonal growth at concentrations that did not affect the catalytic function of AChE or affect cell viability or increase protein synthesis in the AChE +/+ genotype. Samplings of the effects of chlorpyrifos and its oxon in axon length in the dorsal root ganglion are shown in Table 3 (below). A lack of dose response between the lowest concentrations raises a concern about whether or not there is an effect at these low concentrations (0.001 and 0.01 nmole for chlorpyrifos oxon and 1 and 10 nmole for chlorpyrifos.)

Table 3: Comparison of concentrations for inhibition of axon length and AChE inhibition in dorsal root ganglion in vitro by chlorpyrifos and chlorpyrifos oxon

Concentrations	Length inhibition(a)	AChE inhibition(b)	Length inhibition	AChE inhibition
	Chlorpyrifos Oxon		Chlorpyrifos	
0.001 nmole	23%(ns)/40%*	None	--	--
0.01 "	29%*/25%%	None	--	--
0.1 "	42%*/40%*	19%**	--	--
1 nmole	55%*/30%*	63%**	26%*/18ns[(c)]	None
10 "	61%*/35%*	84%**	29%*/42%*	None
100 "	--	--	48%*/52%*	48%**
1 µmole	--	--	42%*/56%*	63%**
10 "	--	--	45%*/54%*	88%**

(a) From Figure 1 Yang et.al. 2008). Values are approximates based on visual assessment of the bar lines in the Figure. A value of 155 was used as the control. Values of 120, 110, 90, 70 and 60 were used for the chlorpyrifos oxon calculations. Values of 115, 110, 80, 90 and 85 were used for the parent chlorpyrifos calculations.
(b) Similarly values for AChE inhibition were also obtained by visual analysis of the Figure 2 (Part C).
(c) Length inhibition has two entries. The first is from Figure 1. Refer to (a) above. The second is from Figure 3 (C) for chlorpyrifos and Figure 3 (D) for chlorpyrifos oxon to attempt to show reproducibility of the study.

An important aspect of this study is that these effects of chlorpyrifos oxon and chlorpyrifos on axon length were not seen in the AChE -/- genotype. Transcription of the DNA from the AChE+/+ line into the nullizgous genotype, however, resulted in restoration of the effects of chlorpyrifos and its oxon in inhibiting axonal growth (Yang et al, Figure 5). Overall, the effect on the morphogenic rather than on the catalytic function of AChE was discussed as being related to inhibition of axonal growth in these cultures *in vitro.*

The effects of chlorpyrifos in primary human fetal astrocytes (Mense et al, 2006) revealed a variety of effects as indicated by expression of gene subsets. The concentrations levels used were generally greater than 1 µM or concentrations levels

where effects on growth, survival and proper functioning of the cells were potentially affected. No comparison with chlorpyrifos oxon was made. Using microarray gene expression profiling, chlorpyrifos (25 μM) was reported to affect molecular chaperones, signal transduction, transcriptional regulators, transporters and factors involved in behavior and development.

The effects of chlorpyrifos oxon (range from 0.1 to 10 μM, with exposures up to 7 days) on microtubule-associated targets in hippocampal slices were assessed (Prendergast et al, 2007) at concentrations that inhibited AChE (15-60%). As early as 24 hours after application of chlorpyrifos oxon, microtubule associated protein was decreased with progressive decreases with time at the lowest test concentrations. Moreover, Injury to the *cornu ammonis (*CA) 1 pyramidal cell layer became evident at 3 days and injury progressed to day 7. Tubulin polymerization assays indicated inhibition (at ≥ 0.1 μM chlorpyrifos oxon) of the polymerization of purified tubulin and microtubule associated protein tubulin. Overall, the authors concluded that exposure to chlorpyrifos oxon produces a progressive decrease in neuronal viability that may be associated with impaired microtubule synthesis and/or function.

The effects of chlorpyrifos or chlorpyrifos oxon (at 30 μM) in differentiating PC12 line cells on two AChE isoforms splice variants were reported (Jameson et al, 2007). One form (AChE-R) is less well studied and is preferentially induced by cell injury and appears to promote repair and protect against neurodegeneration. The other form (AChE-S) is more abundant and is related to enhancement of neurotoxicity. Exposure to either chlorpyrifos or chlorpyrifos oxon resulted in enhanced gene expression for AChE-R by about 20% but only chlorpyrifos increased AChE-S expression by 20-40%. Thus, intact chlorpyrifos rather than the more potent AChE inhibitory oxon form induced the AChE-S isoform. The same paper, however, reported that there was no effect of dosing with 1 mg/kg chlorpyrifos (in DMSO on PNDs 1-4) on either AChE-S or AChE-R.

In another report from the same laboratory (Slotkin et al, 2007c), the effects of chlorpyrifos (30 μM) on DNA synthesis, cell number and size and cell signaling mediated by adenylyl cyclase in PC12 cell lines were reported. In undifferentiated cells, cholinergic antagonists (atropine and mecamylamine) had little or no protective effect against the antimitotic activity of chlorpyrifos. When however, nerve growth factor was used to stimulate differentiation, the ACh antagonists showed partial protection against cell loss and alteration in cell size, but were ineffective in preventing the deterioration of adenylyl cyclase signaling. Nicotine, an ACh agonist protected undifferentiated cells from the adverse effects of chlorpyrifos, but had mixed additive/protective effects on cell number in differentiating cells. Overall, the conclusions of the authors were that the data implied that cholinergic hyper-stimulation, oxidative stress and interference with the adenylyl cyclase signaling by chlorpyrifos may be factors in the developmental neurotoxicity of chlorpyrifos.

One other study (Caughlan et al, 2004) determined that chlorpyrifos oxon was only slightly more potent than intact chlorpyrifos in inducing apoptosis in rat cortical

cultures and suggested that apoptosis is a novel toxic endpoint of chlorpyrifos toxicity independent of AChE inhibition.

In summary, this limited sampling of *in vitro* studies provide a basis for stating that intact chlorpyrifos may affect targets in the nervous system independent of inhibition of AChE. The database, however, does not verify that such reactions occur *in vivo.*

8. Morphometric and Pathological Changes in the Brain

The preceding section presented the evidence for several alterations on the growth of nerve cells that may be related to intact chlorpyrifos, its oxon, and to some minor extent, TCP. There are some additional studies using brain morphometrics that indicate that there may also be some structural changes following *in vivo* exposure.

In the guideline developmental neurotoxicity study (DNT study, MRID No.: 44556901, copy of the Data Evaluations Record provided to the Panel and published in part, Maurissen et al, 2000), dams were dosed by gavage on GDs 6 through PND 11 at 0, 0.3, 1 or 5 mg/kg/day. According to the review of this study: The morphometric measurements in PND 12 pups differed only in the 5 mg/kg/day dose group and included decreased anterior to posterior length of the cerebellum, reduced height of the cerebellum, decreased thickness of the parietal cortex (13.8% in males and 5.5% in females), and decreased thickness of the hippocampal gyrus. These effects at the high dose occurred in the presence of maternal toxicity (*e.g*., maximum brain, RBC and plasma ChE inhibition) but in the absence of effects on dam body weights, food consumption, pregnancy parameters, or deaths among the dams. At PND 12, however, the pups in the 5 mg/kg dose group had lower body weight (17.4% for males and 18.6% for females) and brain weight (8.6% for males and 8.8% for females). In 1 and 5 mg/kg/day PND 66 offspring, effects on brain structure included statistically significant decreases in the thickness of the parietal cortex (4.2% at 1 and 5.1% at 5 mg/kg/day) and non-significant decreases in the thickness of the hippocampal gyrus (3.7% at 1 and 6.4% at 5 mg/kg/day). Brain weight at PND 66 for the 1 and 5 mg/kg dose groups was equivalent to the controls. The review pointed out that morphometric evaluation of the low-dose brains was not conducted meaning it is not known whether alterations are occurring at lower doses. Other findings in adults were that at the 5 mg/kg/day dose there was a suggestion of altered motor activity (decreased on PND13, increased thereafter to PND 60), alterations in auditory startle measurements (increased latency to peak response and decreased peak response amplitudes) at PND 22.

A subsequent publication from the Registrant (DOW AgroSciences) however, describing the developmental neurotoxicity study and in their discussion disagreed with the Agency's conclusion regarding brain morphometrics for the alleged morphometric effect at 1 mg/kg (Maurissen et al, 2000). It was argued that there is no dose response between the middle and the high dose groups for the decrease in thinner parietal cortex because the two values differ by only 0.9%. When the data are adjusted for body weight differences, the statistical difference is lost, with a concurrent loss of any dose

response. In the middle dose, there were no behavioral or functional effects and no inhibition of blood or brain AChE to support an effect on the morphometrics on this structure. Also, the rebuttal explained that from a neurogenesis perspective, the rat neocortex, parietal and frontal lobes develop mainly on GDs 14 to 20 and if treatment affected the cortical neurogenesis, it would be expected that parietal and frontal lobes would be similarly affected. If the effect on the parietal cortex occurred during gestation, it should be seen on PND 11 but was not. Overall, the DOW Company considers that "the statistically significant middle dose effect in the parietal cortex seen in females only at PND 66 was isolated, did not support a biologically plausible interpretation and was therefore, considered spurious."

9. Discussion of data on effects in the developing brain

Overview. The above sections mention only selected publications dealing with effects following gestational and neonatal exposure and from *in vitro* studies of the many publications reported from several different laboratories. The Agency is aware of other papers that could have been included in this survey. The selections discussed above were made to represent examples to show the reported potential for chlorpyrifos dosing to cause alterations in behavior in adults exposed gestationally and early prenatally. The papers dealing with neurochemical brain parameters were selected to show in some cases the magnitude of the effect and the variability in different brain structures as well as the differences in exposure windows and gender. The effects presumed to persist into adulthood in rats and mice at the lowest generally effective dose (1 to 5 mg/kg/day) are resummarized in Table 4. These studies show that when adults are tested for behavior, they show altered responses in certain tests (e.g. motor activity and learning and memory) in rats as well as social responses in mice. The behavioral alterations may represent the sum total of the expression of the neurochemical alterations discussed above or be caused by neurochemical alterations not yet discovered.

Table 4: Selected Effects in Adults Following Gestational and/or Early Postnatal Dosing of about 1 to 5 mg/kg Chlorpyrifos

Parameter	Effects Behavioral/Physical
Chocolate milk preference	Decreased preference
Body weight	Increased in males
Elevated plus maze	Increased time in open arms
Motor Activity	Both increases and decreases in multiple apparatuses, depending upon window of exposure and gender
Learning and memory Radial arm maze	Increased and decreased error rate during training depending on window of exposure and gender; challenges uncover altered function of cholinergic and serotonergic systems
Social interaction	Altered aggressive and maternal behaviors
	Neurochemical and Macromolecular
ACh (transporter and HACU)	Mostly decreased.
Adenylyl cyclase cascade	Various effects depending on gender, brain region, window of dosing and time of assay.
Brain morpho-metrics	Decreased thickness of the parietal cortex (HED DNT review conclusion).
Dopamine	Increased turnover at PND 30.
Myelin basic protein	Some differences persisting to PND 30.
Serotonin (transporter, $5HT_2$ and $5HT_{1A)}$	Various effects depending on gender, brain region, window of dosing and time of assay. Persisting to adulthood.

Consideration on AChE Inhibition and Brain Development.

Appendix B describes the brain, blood and peripheral AChE inhibition from the many studies in the literature during the several gestational and early postnatal windows where dosing was made. There are no reliable data that demonstrate fetal brain AChE inhibition following exposure during the GD 9-12 window. There is evidence of fetal brain AChE inhibition for exposure on GD 17-20 or late gestation but inhibition was not clearly evident around 1 mg/kg but doses of 2 mg/kg can be shown to result in 15-20% inhibition. Neonatal brain AChE inhibition was demonstrated at dose levels of 1 mg/kg in multiple studies.

There are inherent problems in assessing for brain AChE inhibition in fetuses and neonatal pups. The first being that unlike in the adult, new enzyme is rapidly being made such that not assessing for activity at the optimal time can potentially underestimate the true extent of inhibition. This is demonstrated rather clearly by the reference (Dam et al 2000, Figure 4) showing a marked difference between only 2 and

4 hours. As discussed in Appendix B, time course information is an important component of AChE studies. Since many studies do not assess for AChE inhibition until 24 or more hours after the last dose, inhibition may be underestimated in these studies.

Another inherent problem is that total brain AChE activity does not distinguish which isoform or function of AChE is being inhibited or otherwise affected. For example, 20% inhibition of total brain AChE could mean that a greater proportion and a biologically significant amount of a specific isoform or functional role was affected.

Lastly, the morphogenic role of AChE, if it is *affected* in some way by chlorpyrifos or its oxon, could still upset neurogenesis without actual detection of AChE inhibition. This was demonstrated by Yang et al (2008) where it was shown that the AChE +/+ genotype is needed for axonal growth inhibition by chlorpyrifos or its oxon at concentrations *in vitro* where there was no inhibition of AChE.

Problems with studies from the literature. There are several problems with the studies from the open literature that need to be considered. Many of the studies use subcutaneous administration, a route that is not related to human exposure. Many of the studies also use DMSO, a vehicle that is not generally used for regulatory toxicity studies because of its potential intrinsic toxicity and influence on absorption. Although HED is critical of both the use of subcutaneous dosing and DMSO, their use does not in itself eliminate concerns that the effects seen are biologically relevant to characterize the spectrum of possible adverse effects of chlorpyrifos.

There is much variation in the data as indicated by large standard deviations in many of the neurochemical studies. Many of the parameters were reported as showing gender and brain region as well dosing window and dose level differences and dependence often with directional changes in the response. The response at 5 mg/kg was often less than at 1 mg/kg. Since some of the differences from the control are small and even though they may achieve statistical significance but because there are many directional changes, it is often hard to accept that the differences are real without independent verification. One glaring example of the variance is in Figure 4 (Aldridge et al 2003, Figure 4) that attempts to show the dose response for the 5HTT in GD 21 forebrain over the range from 1 to 40 mg/kg. There is first what appears to be a reasonable increase with dose from 1 (increase about 15%) to 2 (increase about 60%) mg/kg but at 5 mg/kg there is only about a 25% increase. This is followed by a negative result at 10 mg/kg (about 60% decrease) and the next dose or 20 mg/kg has no effect while the succeeding dose of 45 mg/kg is again negative (about 90% decrease).
As noted in the Issue Paper, the Agency has placed more weight on effects which have been replicated in multiple laboratories. Some of the neurochemical findings have only been shown in a single laboratory. Similar finding from an independent laboratory would increase the overall confidence in these results. The overall confidence in the neurochemical studies would also benefit by using dosing methods more relevant to human exposure.

Many of the literature studies do not establish a dose response for *in vivo* studies. They were conducted at either a single dose or when dose response curves were attempted, there was no appearance of a monotonic dose response in some cases. Temporal responses are also lacking. A possible explanation for the lack of dose and temporal responses may be that as the dose level increases or dosing windows change, other targets become more significant or other responses to AChE inhibition overwhelm the response at the lower dose or earlier window. Since the brain is rapidly changing, the consequences of AChE inhibition or interaction with the morphometric role of AChE at different windows may well have different consequences. Thus, dose related and temporal relationships are difficult to establish. Since the literature studies do not show NOAELs in many cases, it leaves open questions regarding the relationship to the effect relative to the extent of brain AChE inhibition being the sole target.

Although independent laboratories report various effects that indicate alterations in certain neurochemical and behavioral parameters into adulthood, they do not use the same techniques for dosing, dosing windows and for assessment of the endpoints and thus do not actually verify each other. Independent verification of the key findings demonstrating effects into adulthood using the same and/or very similar techniques is important.

An aspect of some of the *in vivo* studies discussed above is that no AChE inhibition was detected during the windows of exposure at the dose level used. This is especially true for gestational exposures. This suggests that some other target was affected by chlorpyrifos or its oxon. Studies with muscarinic and nicotinic antagonists did not always show reversal of chlorpyrifos effects as might be expected. For example, mecamylamine, a nicotinic receptor antagonist did not block the DNA synthesis effect of chlorpyrifos (Whitney, et al, 1995) and neither mecamylamine nor atropine protected against the antimitotic effects of chlorpyrifos in P12 cells (Slotkin et al, 2007c). It may be possible that in the developing brain, ACh/AChE do not always act through the nicotinic or muscarinic receptors, opening up the possibility that alternative pathways may exist for the alterations following the interaction between chlorpyrifos and the cholinergic nervous system components. *In vitro* studies also raise concerns for possible non-cholinergic targets.

***Preliminary* Conclusions.** The Agency believes that, to date, there are insufficient data that support a series of key events in a mode of action analysis for pathways other than AChE inhibition. Moreover, the Agency believes that due to uncertainties surrounding dose response and time course information, there is insufficient information to determine primary targets of chlorpyrifos other than AChE. Additional data such as determining the affinity constants for chlorpyrifos for possible targets within the nervous system such as the serotonin receptors and transporter would be helpful in establishing what other systems may be a target for chlorpyrifos. The *in vitro* studies with chlorpyrifos raise many questions because in some cases very low levels were shown to be effective on some parameters. The same parameters, however, if they were

tested for were usually only tested for at the dose level of 1 mg/kg or higher *in vivo* and if effects were noted, no attempt was made to establish the NOAEL to correlate with the low concentration *in vitro*. Some of the *in vitro* data indicate that intact chlorpyrifos is effective in causing alterations in neurochemistry. There is, however, no conclusive data available at this time to determine the active form of chlorpyrifos in the fetal or neonatal brain that may be responsible for the alterations described.

The Agency has preliminarily concluded that gestational and/or early postnatal exposure to chlorpyrifos in laboratory animals may cause persistent behavioral effects into adulthood. There are also concurrent changes in brain neurochemistry based on both *in vivo* and *in vitro* studies that may underlie the persistent behavioral changes into adulthood. The doses *in vivo* known to cause the persistent effects, however, are above the current and proposed regulatory endpoints. The cholinergic nervous system including both AChE inhibition associated with ACh destruction at the synapse and the morphogenic role of this enzyme, as well as possible effects on transporters and receptors are considered the primary targets for chlorpyrifos at this time. The Agency, however, recognizes that other mechanisms may be involved, but further proof that they are *primary* targets and not secondary effects resulting from the interaction of chlorpyrifos or its oxon on the cholinergic nervous system is needed.

10. References

Aldridge, J.E., F.J. Seidler, A. Meyer, I. Thillai and T.A. Slotkin (2003). Serotonergic Systems targeted by developmental exposure to chlorpyrifos: Effects during different critical periods. EHP: 111:1736-1743.

Aldridge, J.E., F.J. Seidler and T.A. Slotkin (2004). Developmental exposure to chlorpyrifos elicits sex-selective alterations of serotonergic synaptic function in adulthood: Critical periods and regional selectivity for effects on serotonin transporter. EHP: 112:148-155.

Aldridge, J.E., A. Meyer, F.J. Seidler and T.A. Slotkin (2005a). Alterations in central nervous system serotonergic and dopaminergic synaptic activity in adulthood after prenatal or neonatal chlorpyrifos exposure. EHP. 113:1027-1031.

Aldridge, J.E., A. Meyer, F.J. Seidler and T.A. Slotkin (2005b) Developmental exposure to terbutaline and chlorpyrifos: Pharmacology of preterm labor and an environmental neurotoxicant converge on serotonergic systems in neonatal rat brain regions. TAP: 203 134-144.

Aldridge, J.E., E.D. Levin, F.J. Seidler and T.A. Slotkin (2005c) Developmental Exposure of rats to chlorpyrifos leads to behavioral alterations in adulthood, involving serotonergic mechanisms and resembling animal models of depression. Envionr. Health Perspect. 113:527-531.

Baetcke, K.,V. Dellarco, S. Makris and D. Smegal (2000) Chlorpyrifos Children's Hazard: Sensitivity and Susceptibility. Office of Pesticide Programs, USEPA, March 28, 2000.

Barone, S. Jr., K.P. Das, T.L. Lassiter and L.D. White (2000) Vulnerable Processes of Nervous System Development: A Review of Markers and Methods. NeuroToxicology 21(1-2): 15-36.

Betancourt, A.M. and R.L. Carr (2004) The Effect of Chlorpyrifos and Chlorpyrifos-Oxon on Brain Cholinesterase, Muscarinic Receptor Binding, and Neurotrophin Levels in Rats Following Early Postnatal Exposure. Toxicological Sciences 77:63-71.

Betancourt, A. M., S.C. Burgess and R.L. Carr (2006). Effect of Developmental Exposure to Chlorpyrifos on the Expression of Neurotrophin Growth Factors and Cell-Specific Markers in Neonatal Rat Brain. Toxicological Sciences 92(2):500-506.

Betancourt, A.M., N.M. Filipov and R.L. Carr (2007). Alteration of Neurotrophins in the Hippocampus of Young Rats Exposed to Chlorpyrifos and Methyl Parathion. Toxicological Sciences. 100(2):445-455.

Bigbee, J.W., K.V. Sharma, J.J. Gupta and J.L. Dupree (1999) Morphogenic Role of Acetylcholinesterase in Axonal Outgrowth during Neuroal Development. Environ. Health Perspect. 107(Suppl 1): 81-87.

Brimijoin, S. and C. Koenigsberger (1999) Cholinesterase in Neural Development: New Findings and Toxicologic Implications. Environ. Health Perspect. 107(Suppl 1):59-64.

Carr, R.L., H.W. Chambers, J.A. Guarisco, J.R. Richardson, J. Tang and J.E. Chambers (2001). Effects of Repeated Oral Postnatal Exposure to Chlorpyrifos on Open-Field Behavior in Juvenile Rats. Toxicological Sciences 59:260-267.

Casida, J.E. and G.B. Quistad (2004) Organophosphate Toxicology: Safety Aspects of Nonacetylcholinesterase Secondary Targets. Chemical Research in Toxicology 17(8): 983-998.

Caughlan, A., K. Newhouse, U. Namgung and Z. Xia (2004) Chlorpyrifos Induces Apoptosis in Rat Cortical Neurons that is Regulated by a Balance Between p38 and ERH/JNK MAP Kinases Toxicological Sciences78:125-134.

Cooper, J.R., F.E. Bloom and R.H. Roth (1991) *The Biochemical Basis of Neuropharmacology.* Oxforf University Press, p. 356.

Dam, K., F.J. Seidler and T.A. Slotkin (2000) Chlorpyrifos exposure during a critical neonatal period elicits gender-selective deficits in the development of coordination skills and locomotor activity. Developmental Brain Research 121:179-187.

Das, K.P. and S. Barone (1999) Neuronal Differentiation in PC12 Cells is Inhibited by Chlorpyrifos and Its Metabolites: Is Acetylcholinesterase Inhibition the Site of Action? Toxicology and Applied Pharmacology 160:217-230.

Garcia, S.J., F.J. Seidler, T.L. Crompton and T.A. Slotkin (2001) Does the developmental neurotoxicity of chlorpyrifos involve glial targets? Macromolecular synthesis, adenylyl cyclase signaling, nuclear transcription factors and formation of reactive oxygen in C6 glioma cells. Brain Res. 891(1-2): 54-68.

Garcia, S.J., F.J. Seidler, D. Qiao and T.A. Slotkin (2002a) Chlorpyrifos targets developing glia: effects on glial fibrillary acidic protein. Brain Res. Dev. Brain Res. 133(2):151-61.

Garcia, S.J., F.J. Seidler and T.A. Slotkin (2002b) Developmental Neurotoxicity Elicited by Prenatal or Postnatal Chlorpyrifos Exposure: Effects on Neurospecific Proteins Indicate Changing Vulnerabilities. Environ. Health Perspect. 111:297-303.

Howard, A.S., R. Bucelli, D.A. Jett, D. Bruun, D. Yang and P.J. Lein (2005) Chlorpyrifos exerts opposing effects on axonal and dendritic growth in primary neuronal cultures. Tox. Appl. Pharmac. 207:112-124.

Icenogle, L.M., N.C. Christopher, W.P. Blackwelder, D.P. Caldwell, D. Qiao, F.J.

Seidler, T.A. Slotkin and E.D. Levin, (2004). Behavioral alterations in adolescent and adult rats caused by a brief subtoxic exposure to chlorpyrifos during neurulation. Neurotoxicology and Teratology 26:95-101.

Jameson, R.A., F.J. Seidler and T.A. Slotkin (2007) Nonenzymatic Functions of Acetylcholinesterase Splice Variants in the Developmental Neurotoxicity of Organophosphate: Chlorpyrifos, Chlorpyrifos Oxon and Diazinon. EHP 115:65-70.

Jett, D.A., R.V. Navoa, R.A. Beckles and G.L. McLemore (2001) Cognitive function and cholinergic neurochemistry in weanling rats exposed to chlorpyrifos. Toxicology and Applied Pharmacology 174:89-98.

Lassiter, T.L. and S. Brimijoin (2008) Rats gain excess weight after developmental exposure to the organophosphate pesticide, chlorpyrifos. Neurotoxicology and Teratology 30:125-130.

Levin, E.D., N. Addy, A. Nakajima, N.C. Christopher, F.J. Seidler and T.A. Slotkin (2001) Persistent behavioral consequences of neonatal chlorpyrifos exposure in rats. Developmental Brain Research 130:83-89.

Levin, E.D., N. Addy, A. Baruah, A. Elias, N. C. Christopher, F.J. Seidler and T.A. Slotkin (2002) Prenatal chlorpyrifos exposure in rats causes persistent behavioral alterations. Neurotoxicology and Teratology 24:733-741.

Levin, ED, E. Chrysanthis, K. Yacisin and E. Linney (2003) Chlorpyrifos exposure of developing zebrafish: effects on survival and long-term effects on response latency and spatial discrimination. Neurotoxicology and Teratology 25:51-57.

Maurissen, J.P.J., A.M. Hoberman, R.H. Garman and T.R. Hanley (2000) Lack of Selective Developmental Neurotoxicity in Rat Pups from Dams Treated by Gavage with Chlorpyrifos. Toxicological Sciences 57:250-263.

Mense, S.M., A.Sengupta, C. Lan, M. Zhou, G.Bentsman, D.J. Volsky, R.M. Whyatt, P.P. Perera and L.Zhang (2006) The Common Insecticides Cyfluthrin and Chlorpyrifos Alter the Expression of a Subset of Genes with Diverse Functions in Primary Human Astrocytes. Tox. Sciences 93(1): 125-135.

Meyer, A., F.J. Seidler, M.M. Cousins and T.A. Slotkin (2003). Developmental Neurotoxicity Elicited by Gestational Exposure to Chlorpyrifos: When is Adenylyl Cyclase a Target? Environ. Health Perspect. 111:1871-1876.

Meyer, A., F.J. Seidler, J.E. Aldridge, C.A. Tate, M.M. Cousins and T.A. Slotkin (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.

Moser, V.C. and S. Padilla (1998). Age- and Gender-Related Differences in the Time Course of Behavioral and Biochemical Effects Produced by Oral Chlorpyrifos in Rats. Toxicology and Applied Pharmacology 149:107-119.

Pope, C.N. (1999) Organophosphorus pesticides: do they all have the same mechanism of toxicity? J. Toxicol. Environ. Health B: Crit. Rev. 2:161-181.

Prendergast, M.A., R.L. Self, K.J. Smith, L. Ghayoumi, M.M. Mullins, T.R. Butler, J.J. Buccafusco, D.A. Gearhart and A.V. Terry, Jr. (2007) microtubule-associated targets in chlorpyrifos oxon hippocampal neurotoxicity. Neuroscience 146:330-339.

Qiao, D., F.J. Seidler, S. Padilla, and T.A. Slotkin (2002) Developmental Neurotoxicity of Chlorpyrifos: What is the Vulnerable Period? Environ. Health Perspect. 110:1097-1103.

Qiao, D., F.J. Seidler, C.A. Tate, M.M. Cousins and T.A. Slotkin (2003) Fetal Chlorpyrifos Exposure: Adverse Effects on Brain Cell Development and Cholinergic Biomarkers Emerge Postnatally and Continue into Adolescence and Adulthood. Environ. Health Perspect. 111:536-544.

Qiao, D., F.J. Seidler, Y.Abreu-Villaca, C.A. Tate, M.M. Cousins and T.A. Slotkin (2004) Chlorpyrifos Exposure During Neurolation: Cholinergic synaptic Dysfunction and Cellular Alterations in brain Regions of Adolescence and Adulthood. Developmental Brain Research 148:43-52.

Raines, K.M., F.J. Seidler and T.A. Slotkin (2001) Alterations in serotonin transporter expression in brain regions of rats exposed neonatally to chlorpyrifos. Dev. Brain Res. 130:65-72.

Ricceri, L., N. Markina, A. Valanzano, S. Fortuna, M. F. Cometa, A. Meneguz and G. Calamandrei. (2003). Developmental exposure to chlorpyrifos alters reactivity to environmental and social cues in adolescent mice. Toxicology and Applied Pharmacology. 181:189-201.

Ricceri, L., A. Venerosi, F. Capone, M.F. Cometa, P. Lorenzini, S. Fortuna and G. Calamandrei (2006) Developmental neurotoxicity of organophosphorous pesticides: Fetal and neonatal exposure to chlorpyrifos alters sex-specific behaviors at adulthood in mice. Toxicological Sciences 93(1):105-113.

Richardson, J.R. and J.A. Chambers (2004) Neurochemical Effects of Repeated Gestational Exposure to Chlorpyrifos in Developing Rats. Toxicological Sciences 77::83-90.

Richardson, J.R. and J.A. Chambers (2005) Effects of Repeated Oral Postnatal Exposure to Chlorpyrifos on CHolinergic Neurochemistry in Developing Rats. Toxicological Sciences 84:352-359.

Schuh, R.A., P.J. Lein, R.A. Beckles and D.A. Jett (2002) NonCholinesterase Mechanisms of Chlorpyrifos Neurotoxicity: Binding Protein in Cultured Neurons. To. Appl. Pharm. 182:176-185.

Slotkin, T.A., M.M. Cousins, C.A. Tate and F.J. Seidler (2001) Persistent Cholinergic Presynaptic deficits after neonatal chlorpyrifos exposure. Brain Research 902:229-243.

Slotkin, T.A. and F.J. Seidler (2005). The alterations in CNS serotonergic mechanisms caused by neonatal chlorpyrifos are permanent. Devel. Br. Res. 158:115-119.

Slotkin, T.A. (2006) Developmental Neurotoxicity of Organophosphates: A Case Study of Chlorpyrifos. In: *Toxicology of Organophosphates and Carbamate Compounds* Elsevier, Inc. pgs: 293-314.

Slotkin, T.A. and F.J. Seidler (2007a). Prenatal chlorpyrifos exposure elicits presynaptic serotonergic and dopaminergic hyperactivity at adolescence: Critical periods for regional and sex-selective effects. Reproductive Toxicology 23:421-427.

Slotkin, T.A., and F.J. Seidler (2007b) Comparative developmental neurotoxicity of organophosphates in vivo: Transcriptional responses of pathways for brain cell development, cell signaling, cytotoxicity and neurotransmitter systems. Brain Research Bull. 72:232-274.

Slotkin, T.A., E.A. MacKillop, I.T. Ryde, C.A. Tate, and F.J. Seidler (2007b) Screening for Developmental Neurotoxicity Using P12 Cells: Comparisons of Organophosphates with a Carbamate, an Organochlorine, and Divalent Nickel, EHP 115:93-101.

Slotkin, T.A., E.A. MacKillop, I.T.Ryde and F.J. Seidler (2007c) Ameliorating the Developmental Neurotoxicity of Chlorpyrifos: A Mechanisms-Based Approach in PC12 Cells EHP 115:1306-1313.

Song, X., F.J. Seidler, J.L. Saleh, J. Zhang, S. Padilla and T.A. Slotkin (1997) Cellular mechanisms for developmental Toxicity of chlorpyrifos: Targeting the adenylyl cyclase signaling cascade. Tox. Appl. Pharmacol. 145:158-174.

Song, X., J.D. Violin, F.J. Seidler and T.A. Slotkin (1998) Modeling the Developmental Neurotoxicity of Chlorpyrifos *In Vitro:* Macromolecular Synthesis in PC12 Cells. Toxicology and Applied Pharmacology 151:182-191.

Venerosi, A., G. Calamandrei and L. Ricceri (2006) A social recognition test for female mice reveals behavioral effects of developmental chlorpyrifos exposure. Neurotoxicology and Teratology 28:466-471.

Whitney, K.D., F.J. Seidler and T.A. Slotkin (1995) Developmental Neurotoxicity of Chlorpyrifos: Cellular Mechanisms. Toxicology and Applied Pharmacology 134: 53-62.

Won, Y.K., J. Liu, K. Olivier, Jr., Q. Zheng and C.N. Pope (2001) Age-related Effects of Chlorpyrifos on Acetylcholine Release in Rat Brain. NeuroToxicology 22:39-48.

Yang, D., A. Howard, D. Bruun, M. Ajua-Alemanj, C. Pickart and P.J. Lein (2007) Chlorpyrifos and Chlorpyrifos-oxon inhibit axonal growth by interfering with the morphometric activity of acetylcholinesterase . Tox. and Appl. Pharmacology 228(1):32-41.

Zhang, H., J.Liu and C.N. Pope (2002) Age-related effects of chlorpyrifos on muscarinic receptor-mediated signaling in rat cortex. Arch. Toxicol. 75:676-684.

Zheng, Q. K. Olivier, Y.K. Won and C.N. Pope (2000) Compartaive Cholinergic Neurotoxicity of Oral Chlorpyrifos Exposures in Pre-weaning and Adult Rats. Toxicological Sciences 55:124-132.