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Holder, K, Veichweg, S, **Burke, RD**, Mong, J. Medial Amygdala (MePD) Catecholamines Mediate Methamphetamine-enhanced Proceptive Behaviors. 2011 Society for Neuroscience Meeting Planner, Program No. 714.13. Washington, DC: Society for Neuroscience, 2011. Online.

---ABSTRACT---

Title of Dissertation:

A Structural, Functional, and Behavioral Evaluation of the Developmental Neurotoxicity of the Organophosphorus Insecticide Chlorpyrifos in Guinea Pigs: Mechanistic Implications

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Abstract:

Organophosphorus pesticides are among the most heavily used agricultural insecticides in the United States and throughout the world. Exposure of pregnant women to doses of chlorpyrifos, a prominent organophosphorus insecticide, that do not result in overt maternal intoxication has been associated with increased risk of attention deficit hyperactivity disorder, learning and memory impairment, and tremors in children. Learning and memory impairments are especially pronounced in boys and are readily reproduced in developmental rodent models of chlorpyrifos exposure. The cognitive deficits observed in these models are spatial in nature and can be mimicked by administration of GABAergic potentiating ligands. As such, the present study was designed to test the hypothesis that *in-utero* exposure to toxicologically relevant doses of CPF that do not elicit overt signs of maternal toxicity results in cognitive deficits, particularly in male offspring, that correlate with an increase of GABAergic transmission

or reduction of glutamatergic transmission in hippocampal CA1 pyramidal neurons. To address this hypothesis we used a complementary multidisciplinary approach, including biochemical, behavioral, electrophysiological, and immunohistochemical assays, and a translationally relevant model – the guinea pig. Results here demonstrate that guinea pigs exposed prenatally to chlorpyrifos (25 mg/kg/day, GDs 53 to 62) presented in the Morris water maze (MWM) significant learning deficits, which were more pronounced among males. Electrophysiological experiments revealed that the frequency of spontaneous inhibitory spontaneous synaptic currents was significantly higher in the CA1 pyramidal neurons of chlorpyrifos-exposed animals. This increase was positively correlated with learning deficits in the MWM. Immunohistochemistry provided evidence of a selective increase in the microglia population in the CA1 field of chlorpyrifos-exposed animals. Taken together with the finding that expression of the pro-inflammatory cytokine TNF- α was also higher in the hippocampus of CPF-exposed animals, these results strongly indicated that prenatal exposure to chlorpyrifos was associated with a chronic neuroinflammatory process. Based on the results presented here a novel mechanism is proposed in which prenatal exposure to chlorpyrifos triggers a chronic neuroinflammatory process that contributes to enhancement of hippocampal GABAergic neurotransmission, which in turn increases the inhibitory tone to CA1 pyramidal neurons and this in turn contributes to the associated cognitive deficits.

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of the Organophosphorus Insecticide Chlorpyrifos in Guinea Pigs:
Mechanistic Implications

by
Richard D. Burke

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---LIST OF ABBREVIATIONS---

Abbreviation	Definition
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACSF	Artificial cerebral spinal fluid
ADHD	Attention deficit hyperactivity disorder
ADP	Adenosine diphosphate
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
APV	(2R)-amino-5-phosphonovaleric acid
ASChI	Acetylthiocholine iodide
ATP	Adenosine triphosphate
AU	Arbitrary units
BuChE	Butyrylcholinesterase
BuChI	Butylthiocholine iodide
BW284c51	1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide
C	Celsius
CA	Cornu ammonis
CB	Calbindin
ChAT	Choline acetyl transferase
ChE	Cholinesterase
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPF	Chlorpyrifos
CPO	Chlorpyrifos oxon
CR	Calretinin
DGEC	Ectal arm of the dentate gyrus
DH β E	dihydro- β -erythroidine hydrobromide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTNB	5-5'-dithiobis(2-nitrobenzoic) acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
EPSCs	Excitatory postsynaptic currents
g	Gram
GABA	gamma-Aminobutyric acid
GD	Gestation day
GFAP	Glial fibrillary acidic protein
GL	Granular layer
h	Hour

Abbreviation	Definition
HACT	High affinity choline transporter
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IACUC	Institutional Animal Care and Use Committee
Iba1	Ionized calcium-binding adapter molecule 1
IHC	Immunohistochemistry
IPSCs	Inhibitory postsynaptic currents
Iso-OMPA	Tetraisopropylpyro-phosphoramidate
ITI	Intertrial interval
KCC2	K^+ - Cl^- cotransporter
kg	Kilogram
kHz	Kilohertz
LD ₅₀	Lethal dose 50 percent
mAChRs	Muscarinic acetylcholine receptors
MBP	Myelin basic protein
mg	Milligram
mGluR	Metabotropic glutamate receptor
min	Minute
mIPSCs	Miniature inhibitory postsynaptic currents
ml	Milliliter
MLA	Methyllycaconitine
mM	Millimolar
mm	Millimeter
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mV	Millivolt
MWM	Morris water maze
MΩ	Megaohm
nAChRs	Nicotinic acetylcholine receptors
NeuN	Neuronal nuclei protein
NKCC1	Na^+ - K^+ - Cl^- cotransporter
nm	Nanomolar
NMDA	N-methyl-D-aspartate
OP	Organophosphate
P2Y	Purinergic g-protein coupled receptor two
PBS	Phosphate buffered saline
pg	Picogram
PND	Postnatal day
PO	Peanut oil
PV	Parvalbumin
QX-314	Lidocaine N-ethyl bromide
RBC	Red blood cell
RCF	Relative centrifugal force

Abbreviation	Definition
s	second
sc	Subcutaneous
SEM	Standard error of the mean
SLM	Stratum lacunosum
SO	Stratum oriens
SP	Stratum pyramidal
SR	Stratum radiatum
TBS-T	Tris-buffered saline containing 0.1% Tween 20
TCPY	3,5,6-trichloro-2-pyridinol
TNF- α	Tumor necrosis factor alpha
TTX	Tetrodotoxin
U.S.	United States of America
VAChT	Vesicular acetylcholine transporter
μm	Micrometers
μM	Micromolar

Chapter 1: Introduction

1.1. Public Health Relevance of Prenatal Sub-Acute Exposures to the Organophosphorous Pesticide Chlorpyrifos.

Organophosphorus (OP) pesticides are among the most heavily used agricultural insecticides. Due to the effectiveness, ease of application, and low cost of OP pesticides, their use is predicted to grow worldwide through 2022 (Grand View Research, 2014). Unfortunately, these pesticides are toxic not only to insects but to humans as well and their widespread use has become a major global public health issue.

The fatalities and poor health outcomes resulting from acute and/or chronic exposures of humans to OP pesticides are well documented. In 2001, the World Health Organization reported that acute OP pesticide poisoning amounted to more than three million cases per year, with more than one million cases being attributed to occupational exposure (WHO, 2004). Just as alarming are the conservative estimates that approximately a third of the yearly number of suicides in the world, approximately 250,000 deaths, are due to OP pesticide self-poisoning (Gunnell et al., 2007). Finally, epidemiological studies have provided compelling evidence that acute or continued exposure to sub-acute levels of OP pesticides are associated with increased risks of debilitating neurological disorders, especially in developing children (Dahlgren et al., 2004; Engel et al., 2011; Levin and Rodnitzky, 1976; Rosas and Eskenazi, 2008; Savage et al., 1988).

In 2001, the United States (U.S.) Environmental Protection Agency (EPA) restricted in the U.S. residential application of the most abundantly used OP pesticide, chlorpyrifos (CPF), out of concern for the neurological deficits associated with prenatal

and early age exposure of humans to CPF (Grube et al., 2011). As of 2006, however, CPF could still be detected in 78% of U.S. homes surveyed (Stoudt et al., 2009). Likewise, agricultural exposures were not addressed by the EPA restriction, and American agricultural workers as well as their families have been found to have the primary CPF metabolite, 3,5,6-trichloro-2-pyridinol (TCPY), and dialkylphosphate metabolites in their systems (Fenske et al. 2002; Eskenazi et al. 2004 and 2007). Thus, exposure to CPF will remain for years to come a serious public health concern, particularly for children in both agricultural and residential settings in the U.S. and throughout the world.

The global public health relevance of CPF toxicity, the need for a comprehensive understanding of the mechanisms that underlie the neurological deficits triggered by exposure to CPF, and the urgency to develop treatments to mitigate these impairments underscore the importance of preclinical studies in which the clinical manifestations of human OP toxicity can be replicated.

1.2. Neurobehavioral Phenotypes Associated with Developmental Exposure to Sub-acute Levels of CPF

1.2.1. Clinical Findings

In utero exposure to OP pesticide levels that do not result in overt maternal intoxication have been associated with increased risk of attention deficit hyperactivity disorder (ADHD) (Rauh et al., 2006; Eskenazi et al., 2007; Marks et al., 2010; Fortenberry et al., 2014), learning and memory impairment (Rauh et al., 2006 and 2011), and tremors (Rauh et al., 2015) among children. CPF has been specifically implicated, as these associations maintain significance when biomarkers for CPF exposure, including

maternal urine levels of the CPF metabolite TCYP and cord blood levels of TCPY and CPF, were evaluated. In fact, an inverse relationship between CPF cord blood concentration at birth and full-scale memory and intelligence quotient has been documented in seven-year-old children. Following normalization for maternal education and home environment, for every standard deviation (4.61 pg/g) increase in CPF cord blood, working memory decreased 2.8% and intelligence quotient declined by 1.4% (Rauh et al, 2011). Children from this cohort also had significant dose-dependent thinning of the frontal and parietal cortices as revealed by magnetic resonance imaging (MRI) (Rauh et al 2012). Generally, these associations are stronger and apparent at lower exposure levels in male than in female children, a finding that suggests that males appear to be especially susceptible to the neurotoxicity of sub-acute *in utero* CPF exposure.

1.2.2. Preclinical Studies

Learning and memory deficits and ADHD-related behaviors associated with prenatal exposure to CPF in humans have also been observed in animal models of sub-acute developmental CPF exposure. ADHD-like behaviors, including hyperactivity, motor agitation, and increased open field activity have been observed in rats subjected to sub-acute prenatal CPF exposure (Grabovska and Salyha, 2015; Samsam et al., 2005). Since it is difficult to assess “attentiveness” in rat and mouse models (Antunes and Biala, 2011; Clark and Martin, 2005; Clark and Squire, 2010; Fantz, 1964), most studies have focused on the evaluation of learning and memory in murine models of developmental exposure to sub-acute doses of OP pesticides. Specifically, hippocampal dependent learning and spatial, working, and reference memory have been evaluated in the Morris

Water Maze (MWM), T-maze, and 16-arm radial maze following developmental sub-acute exposure of rats and mice to CPF.

As illustrated in Table 1, performance deficits in learning and memory have been noted in adult rats and mice developmentally exposed to CPF doses as low as 1 mg/kg/day. These findings, taken together with reports that substantially larger doses of CPF are needed to induce cognitive deficits in adult mice and rats (Terry et al., 2003; Yan et al., 2012; Maurissen et al., 2000; Moser et al., 2005), support the notion that the developing mammalian brain is exquisitely sensitive to the toxicity of CPF.

Table 1: Hippocampal Dependent Behavioral Consequences of Sub-acute CPF Exposure in Murine Models.

Species	Dose Period	Dose (mg/kg/day)	Route	Examine	Test	Effect	Author
rat	30 days ^A	18	sc ^B (DMSO)	Post dose	MWM	↓Acquisition	Terry et al, 2007
	28 days ^A	5, 10	Gavage (oil)			↓ Probe ↓Relocations	Yan et al, 2012
	14 days ^A	18, 25	sc ^B (oil)			↓Acquisition ↓Probe ^C	Terry et al, 2003
	GD 9-12	5	sc ^B (DMSO)	PND weeks 8-13	16-arm Maze	↑Working, ↑Reference memory errors	Icenogle et al, 2004
	GD 17-20	1, 5					Levin et al, 2002
	PND 1-4	1		PND weeks 4-8, 8-13	T and 16- arm Mazes	↑Reference memory errors	Levin et al, 2001
	PND 11-14	5					
mice	GD 9-18	3		PND 80	MWM	↓Acquisition	Turgeman et al, 2011
	GD 13-17	5		PND 45	T-Maze	↑Lose-shift errors	Chen et al, 2012

A - Adult animals; **B** - Subcutaneous; **C** - Probe trial deficits with hindered task acquisition

1.3. The Role of the Hippocampus on Cognitive Deficits Associated with Exposure of the Developing Brain to CPF

Preclinical studies have examined the consequences of early life exposure to sub-acute levels of CPF on the structural integrity of the brain. These studies have provided evidence of decreased or unaffected neuronal cell counts and increased glial proliferation in brain regions that control mood, behavior, and cognition, including the hippocampus (Garcia et al., 2002; Roy et al., 2004, 2005). An MRI clinical study also revealed significant associations between prenatal CPF exposure and abnormalities in morphological measures of the cerebral cortical surface of children (Rauh et al., 2012). In particular, Rauh and collaborators (2012) reported increased cortical surfaces, which were primarily due to enlargements in the underlying white matter, in the superior and posterior middle temporal gyri, where the hippocampal formation is located.

The hippocampus and its neural networks govern many forms of learning and memory including visual spatial, episodic, declarative, and flexible relational categories (reviewed by Burgess et al, 2002). Therefore, it is tempting to speculate that disruption of the structural and/or functional integrity of the hippocampus is an important determinant of the neurological disorders that develop following early life exposure to sub-acute levels of CPF.

1.4. Effects of CPF on Synaptic Transmission

It is well accepted that synaptic plasticity, manifested as long-lasting, activity-dependent increases and decreases in the efficacy of synaptic transmission, is essential for the development of neural circuitries that encode learning and memory. It is,

therefore, not surprising that changes in synaptic transmission within different brain regions, including the hippocampus, trigger cognitive deficits. For example, intrahippocampal infusion of drugs that block the activity of nicotinic acetylcholine receptors, potentiate type A gamma-aminobutyric acid (GABA_A) receptor activity, or that block the N-methyl-D-aspartate (NMDA) type of glutamate receptors causes memory impairment (Kim et al., 2012; Levin, 2002; Watson and Stanton, 2009; Wiescholleck and Manahan-Vaughan, 2013).

Sub-acute developmental CPF exposure has been shown to alter the activity of the cholinergic system (Qiao et al., 2003 and 2004). Animals exposed early in life to sub-acute doses of CPF have lasting decreases in choline acetyl transferase (ChAT) expression and high affinity choline transporter (HACT) activity (see Table 2). ChAT, the enzyme that catalyzes the synthesis of acetylcholine (ACh) from acetyl-CoA and choline, has been long used as a marker of cholinergic neurons (Aubert et al., 1996; Happe and Murrin, 1992; Navarro et al., 1989). On the other hand, HACT activity is generally taken as a measure of presynaptic choline uptake. HACT is positively correlated with neuronal impulse activity, and is, thus, used as a surrogate measure for neurotransmission at the synapse (Klemm and Kuhar, 1979; Simon et al., 1976).

Previous studies have also reported that early life exposure of rats to sub-acute doses of CPF during a critical period of brain development results in persistent activation of serotonergic systems and serotonin-associated behavioral abnormalities (Aldridge et al., 2005). It also reduces dopaminergic activity in the hippocampus (Aldridge et al., 2005b; Slotkin et al., 2002).

To date, no study has assessed glutamatergic and GABAergic transmissions in the hippocampus following pre- and/or neonatal exposure to sub-acute doses CPF. Yet, evidence supports a role for glutamatergic and GABAergic activity in the hippocampus on cognitive processing (Martin et al., 2000; Malenka and Bear, 2004). Specifically, learning and memory deficits have been observed following intrahippocampal administration of the NMDA receptor antagonist MK-801 and the GABA_A receptor potentiating ligand diazepam to rats (Watson and Stanton, 2009; Kim et al., 2012).

Within the hippocampus, a brain region rich in cholinergic innervation, ACh interacts with muscarinic and nicotinic receptors to modulate gamma-aminobutyric acid (GABA) and glutamatergic transmission (reviewed in Albuquerque et al, 2009; Cobb and Davies, 2004). As such, a persistent decrease in cholinergic synapse number and/or function, as seen in the hippocampus of rats prenatally exposed to CPF (Qiao et al., 2003 and 2004), can have a significant and lasting effect on GABAergic and/or glutamatergic transmission within the hippocampus. In addition, as discussed in the next section, glutamatergic and GABAergic synaptic transmissions are affected in different ways by neuroinflammation, a response that has been seen in the brain of rats neonatally exposed to sub-acute doses of CPF (Zhang et al., 2015). Therefore, identifying the effects of developmental sub-acute CPF exposure on synaptic transmission, particularly in the hippocampus, and their correlations with neurological deficits will lay the groundwork for a better understanding of the mechanisms underlying the developmental neurotoxicity of CPF.

Table 2: Cholinergic Effects Subsequent to Sub-acute Developmental CPF Exposures.

Species	Dose Period	Dose (mg/kg/day)	Route	Examined (PND)	Effect	Area	Author
Rat	GD 9 to 12	1, 5	sc ^A , DMSO	30	↑ChAT	Hippocampus	Qiao et al, 2004
				60			
				30	↓HACT		
				60			
	GD 17 to 20	1, 5	sc ^A , DMSO	4	↑ChAT	Forebrain	Qiao et al, 2003
				10			
				15			
				4	↓HACT		
				10			
				21	NE ^B Either		
				30	↓HACT	Hippocampus	
				60			
	GD 6 to 20	7	gavage, oil	9	↓ChAT	Whole Brain	Richardson and Chambers, 2004
				12			
				30			
				6	↓HACT		
				12			
				30			
				3	↓VAch T		
				6			
				9			
				30			
	PND 1 to 4	1	sc ^A , DMSO	5	↓ChAT	Forebrain	Dam et al, 1999
				10			
5				NE ^B HACT			
10				↓ChAT			
30							
60							
PND 11 to 14	5	sc ^A , DMSO	30	↓HACT	Hippocampus	Slotkin et al, 2001	
			60				
			30	↓ChAT			
			60				
			15	NE ^B ChAT	Forebrain		Dam et al, 1999
			20				
			15	↓HACT			
			20				
30	↓ChAT						
60							
30	↓HACT						
60							

A - Subcutaneous; **B** - No effect

1.5. Neuroinflammation

As mentioned in the previous section, neonatal exposure of rats to a sub-acute dose of CPF triggered the activation of inflammatory pathways in the brain (Zhang et al., 2015). Neuroinflammation refers to the collective immunological response to various insults to the central nervous system (CNS) (Reviewed by DiSabato et al., 2016). This response is glial mediated, comprised of innate and acquired components, and is aimed at the removal of noxious stimuli. When the initiating factor is unable to be removed, neuroinflammation can become chronic. It is primarily mediated by microglial activation and production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) (reviewed by Franco and Fernandez-Suarez, 2015). Of interest, following exposure of neuronal cultures to CPF, TNF- α expression spikes above homeostatic control levels (Heydary et al., 2015; Li et al., 2012).

TNF- α can alter glutamatergic and GABAergic neurotransmission via a number of mechanisms that include but are not restricted to: (i) increased expression of the Na⁺-K⁺-Cl⁻ cotransporter NKCC1 (Topper et al., 1997; Ramia and Kreydiyyeh, 2009), (ii) increased gliotransmitter release (Bezzi et al., 2001), (iii) increased surface expression of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors (reviewed in Olmos and Lladó, 2014), and (iv) increased internalization of GABA_A receptors on neurons (reviewed in Olmos and Lladó, 2014).

The electrochemical gradients of chloride in the brain are controlled by cation-chloride cotransporters such as NKCC1 and the K⁺-Cl⁻ cotransporter KCC2 (reviewed by Blaesse et al., 2009). In the CNS, NKCC1 is expressed by neurons, endothelial cells, and glia and is the primary source of chloride uptake. On the other

hand, KCC2, the major chloride extruder, is expressed specifically by neurons. While NKCC1 expression prevails in the developing brain, KCC2 expression prevails in the mature brain (reviewed by Loscher et al., 2013). In practice, this developmental shift results in the transition of GABA from a depolarizing to a hyperpolarizing neurotransmitter through the developmental switch of the electrochemical gradient of chloride. Therefore, in the developing brain, TNF- α -induced increased expression of NKCC1 can potentially delay the shift of GABA signaling from excitatory to inhibitory.

TNF- α has been shown to increase astrocyte calcium uptake (Abdura et al., 2015) and facilitate release of glutamate, GABA, and adenosine triphosphate (ATP) from astrocytes (Bezzi et al., 2001). Astrocytes participate in the control of inhibitory networks (Benedetti et al., 2010; Kang et al., 1998; Liu et al., 2004) as ATP released from astrocytes facilitates hippocampal GABAergic interneuron firing through activation of P2Y receptors (Bowser and Khakh, 2004). An increase in GABAergic transmission has the potential to inhibit spatial learning and memory as seen in humans administered benzodiazepines (Nakamura-Polacios and Roelke, 1997). Therefore, one could hypothesize that, by triggering a lasting neuroinflammatory response in the brain, early life exposure to CPF leads to a sustained increase in hippocampal GABAergic synaptic transmission and this, in turn, contributes to the development of cognitive deficits.

1.6. Potential Mechanisms Underlying the Developmental Neurotoxicity of CPF

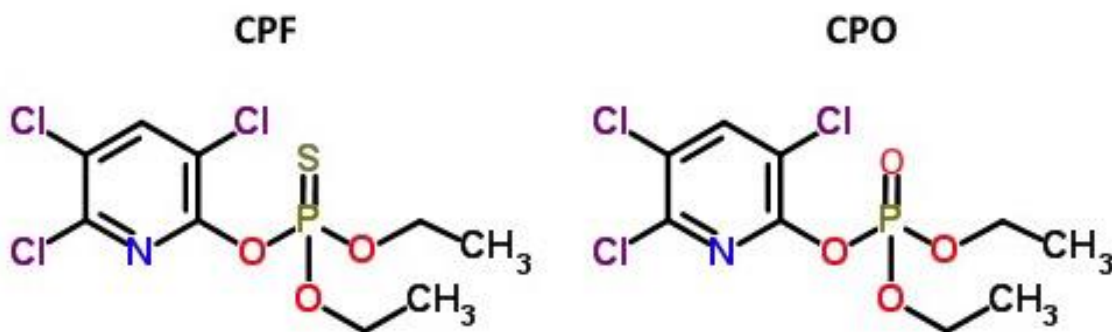
1.6.1. Inhibition of the Catalytic Activity of AChE

Acute OP toxicity is primarily the result of inhibition of acetylcholinesterase (AChE), the enzyme that metabolizes the neurotransmitter ACh. OPs that contain a P=O

moiety phosphorylate a hydroxyl group of the serine residue in the enzyme's active site, precluding substrate interaction, and, thereby, allowing for the accumulation of ACh. Overstimulation of muscarinic receptors (mAChRs) by accumulated ACh leads to a syndrome characterized by miosis, profuse secretions, bradycardia, bronchoconstriction, hypotension, and diarrhea. Overstimulation of nicotinic receptors (nAChRs) triggers tachycardia and skeletal muscle fasciculation, while their subsequent desensitization contributes to muscle weakness. The broad range of CNS-related acute effects includes anxiety, restlessness, confusion, ataxia, tremors, seizures, and central cardiorespiratory paralysis (Eaton et al., 2008).

CPF itself does not inhibit the enzymatic activity of AChE (Chambers et al., 1992). AChE inhibition is accomplished by its oxon metabolite (CPO), which is a potent AChE inhibitor and is generated *in vivo* by oxidative desulfurization of CPF (Figure 1) (Eaton et al., 2008).

Figure 1: CPF and CPO Chemical Structure



Chemical structures shown are representative of CPF and CPO. CPF is metabolized through oxidative desulfurization to produce CPO, which contains a serine hydroxylase inhibiting P=O moiety.

Source: www.chemspider.com (Royal Society of Chemistry)

The contribution of AChE inhibition to the developmental neurotoxicity induced by sub-acute exposure to CPF is poorly understood. Many studies have reported cognitive deficits in rats and mice exposed pre- or neonatally to doses of CPF that induce no significant inhibition of AChE activity. Others, however, have reported that these effects develop after exposure of mice or rats to CPF doses that induce significant inhibition of AChE in the CNS while producing no overt sign of toxicity (Terry et al., 2003 and 2007). Therefore, enzymatic AChE inhibition has not been dissociated entirely from sub-acute OP toxicity.

1.6.2. Inhibition of Non-canonical Functions of AChE in the Developing Brain

The function of AChE is not limited to the breakdown of ACh. During early development, AChE has a non-canonical neurotrophic function supporting neurite outgrowth and regional patterning (Layer and Willbold, 1995; Robertson and Yu, 1993). Periods of extensive neurite outgrowth prior to synaptogenesis have been correlated with transient increases in AChE mRNA and protein expression (Kostovic and Goldman-Rakic, 1983; Kristt, 1989; Robertson and Yu, 1993; Brimijoin and Hammond, 1996). This transient increase is vital for synaptogenesis, as pharmacological inhibition by physostigmine or antibody blocks neurite outgrowth (Bigbee et al., 1999). Of interest, at concentrations that did not reduce the cholinergic activity or synthesis of AChE, both CPF and CPO inhibited neurite outgrowth in primary cultures of neurons from wild type mice but not from AChE nullizygous mice (Yang et al, 2008). Neurite outgrowth inhibition was restored in the nullizygous mouse cultures upon transfection with AChE

(Yang et al., 2008). This strongly suggests that CPF and CPO impair neurite outgrowth through the inhibition of non-canonical functions of AChE. As such, inhibited growth and neural patterning in the developing brain emerges as a potential mechanism contributing to the neurotoxicity associated with sub-acute *in utero* CPF exposure.

1.6.3. AChE-Unrelated Mechanisms

Direct interactions of CPF with AChE-unrelated targets may play a central role in the development of neurological deficits following early life exposure to the pesticide. For example, CPF may induce neurodegeneration by disrupting the axonal transport of nutrients from the cell body to axon terminals of neurons (Grigoryan et al., 2008; Prendergast et al., 2007). CPO has been shown to bind directly to such structural proteins as tubulin and kinesin, which are essential for axonal transport (reviewed in Androustopoulos et al., 2013; Terry, 2012).

Additional mechanisms that have been proposed to contribute to the delayed neurotoxicity of CPF include exacerbated oxidative stress, imbalanced intracellular Ca^{2+} homeostasis, increased signaling mediated by inflammatory mediators such as interleukins and cytokines, changes in cellular signaling mediated by neurotrophin receptors and protein kinases, and mitochondrial disruption (reviewed in Androustopoulos et al., 2013; Banks and Lein, 2012; Terry, 2012). Identifying cellular and molecular mechanisms affected by developmental exposure to CPF and their relationship to the clinical manifestations resulting from early life exposure to this pesticide can have a significant impact on the discovery of effective therapeutic interventions.

1.7. Shortfall of Preclinical Studies: The Murine Animal Models

Researchers examining the developmental neurotoxicity of CPF and other OP pesticides have relied heavily on the use of altricial rodents, particularly rats and mice (Clancy et al., 2007). There are numerous reasons underlying the selection of these animal species. First, their short generation time and accelerated lifespan greatly shorten the time required to complete the studies. Second, most behavioral tests have been developed and validated for rats and mice. Third, antibodies against thousands of proteins are commercially available for both species, and, thereby, expedite assessment of the cellular and molecular markers.

While these advantages are significant, there are major issues that hinder the translational capacity of developmental studies performed in rats and mice. For instance, humans are neurologically precocial at birth, displaying advanced neural and perceptual development (Clancy et al., 2000; Verley, 1977), while rats and mice are comparatively immature at birth. A common “rule of thumb,” based on brain growth spurt and development of the GABAergic system in the brain, equates rat postnatal days (PNDs) two through seven to the human fetal age during the third trimester of pregnancy (Clancy et al., 2007 and ref. therein). Consequently, *in utero* exposure of rats and mice to drugs and toxicants does not target the same developmental stages of the brain as those targeted by *in utero* exposure of humans. In addition, while rats and mice have high levels of circulating carboxylesterases, which metabolically inactivate OP compounds, humans have low levels of these enzymes (Fonnum et al., 1985; Kaliste-Korhonen et al., 1996; Li et al., 2005). Since age-related differences in sensitivity of rats to CPF have been correlated with fluctuating levels of these enzymes, it is difficult to extrapolate to

humans results obtained from developing rats exposed to CPF (Benke and Murphy, 1975; Pope et al., 1991; Moser et al., 1998). Finally, there are substantial differences between the placental structure of rats and mice and that of humans, making it difficult to compare placental transfer of chemicals in humans to that in rats and mice (Pentsuk and van der Laan, 2009).

1.8. Guinea Pig: A Translationally Relevant Animal Model to Assess the Developmental Neurotoxicity of CPF.

It is in this context that the guinea pig emerges as a potential unique animal model for assessment of the developmental neurotoxicity of OP pesticides. Like humans, whom undergo a developmental brain growth spurt characterized by glial proliferation, axonal spreading, dendritic arborization, and synaptogenesis from mid-gestation to parturition, the brain growth spurt of the guinea pig initiates around gestational day 50 (GD 50) and concludes at parturition (approximately GD 65) (Dobbing and Sands, 1970; Dobbing and Sands, 1973). A roughly parallel developmental timeline allows for a better approximation of human gestational exposure. Moreover, this parallelism extends to adult neuroanatomy. Once developed, the overall brain structure of guinea pigs is remarkably similar to that of humans, particularly in the Circle of Willis and limbic regions, including the hippocampus (Librizzi, 1999).

The sensitivity of guinea pigs to the acute toxicity of OP compounds is also more similar to that of non-human primates and humans than to that of other rodents (Inns and Leadbeater, 1983; Maxwell et al., 1987); in part because levels of circulating carboxylesterases are lower in guinea pigs than in rats or mice. Further, unlike murine

models, guinea pigs have circulating carboxylesterase levels comparable to that of an adult animal at birth (Chow and Ecobicho, 1974). As such, the guinea pig lacks the carboxylesterase dependent age-related differences in OP sensitivity found in these models.

Finally, the transfer of chemicals through and the morphological structure of the placenta of humans are more comparable to those of guinea pigs than to those of rats and mice (Enders, 1965; Pentsuk and van der Laan, 2009). For example, during the final third of gestation, placental immunoglobulin G transfer is two times higher in the guinea pig and roughly a 1000 fold lower in the rat than in the human (Pentsuk and van der Laan, 2009). For these reasons, the guinea pig emerges as a translationally relevant animal model to assess developmental toxicity of OP compounds.

Chapter 2: Objectives

The primary objective of the present study was to test the overarching hypothesis that *in-utero* exposure to doses of CPF that do not elicit overt signs of maternal toxicity results in cognitive deficits, particularly in male offspring, that correlate with an increase of GABAergic transmission or reduction of glutamatergic transmission in Cornu Ammonis one (CA1) pyramidal neurons. To address this hypothesis, pregnant guinea pigs were exposed once per day for approximately the last ten days of pregnancy to vehicle (peanut oil) or to a dose of CPF (25 mg/kg, sc) that did not induce maternal toxicity but was anticipated to cause a degree of blood AChE inhibition that is toxicologically relevant to humans. Behavior of prepubertal guinea pigs prenatally exposed to CPF or vehicle was examined in the open field and MWM. Following the conclusion of testing, the whole-cell mode of the patch-clamp technique was used to evaluate synaptic transmission in CA1 pyramidal neurons in hippocampal slices obtained from adult male offspring. Immunohistochemistry and Western blots were also used to determine whether CPF-induced changes in synaptic transmission were likely due to alterations in the number of neurons and/or glial cells in the hippocampus and to shed light into potential molecular mechanisms that are likely to contribute to the effects of prenatal CPF exposure on hippocampal function. The questions addressed in this proposal are outlined below.

2.1. Following daily sc injection of guinea pigs with CPF (25 mg/kg) between approximate gestation days 53 and 63, what degree of cholinesterase inhibition is achieved in the blood and brain of dams and their offspring?

One set of experiments was designed to evaluate the effect of sub-acute (0.05xLD₅₀; 25 mg/kg/day, sc) gestational (GDs 53 to 63) CPF exposure on gestational outcome and cholinesterase enzymatic function in both dams and offspring. These experiments had three objectives: **(i)** confirming that the exposure paradigm does not result in overt maternal toxicity and cholinergic crisis, **(ii)** establishing the level of blood cholinesterase inhibition associated with the chosen exposure paradigm to facilitate inter-species and human exposure comparison, and **(iii)** providing evidence that CPF administered to the pregnant guinea pigs crossed the placenta and the blood brain barrier of the fetuses. To this effect, a modified Ellman colorimetric assay was used to assess the activity of butyrylcholinesterase (BuChE) and AChE in plasma and red blood cell (RBC) blood compartments, respectively, and in four brain regions (hippocampus, thalamus, cerebral cortex, and cerebellum). Guinea pig dams administered peanut oil (vehicle) during the dosage period and their offspring were used as controls. The dependent variables were sample absorbance at 412 nm over ten minutes, while the independent variable is CPF exposure. Results of these experiments are presented in Chapter 4.

2.2. Does sub-acute CPF exposure during the period of the major brain growth spurt result in changes to hippocampal synaptic transmission that correlate with cognitive deficits in male guinea pigs?

Offspring from dams exposed to CPF dissolved in peanut oil or vehicle (peanut oil) as described in section 2.1 were evaluated behaviorally beginning on approximately PND 30. First, locomotor activity was evaluated on two consecutive days in an open field arena. Then, immediately following the completion of the second open field test, novel object exploration was assessed. The dependent variables analyzed in the open field were locomotor activity, center time and distance traveled, center entries, wall-zone time and distance traveled and rearing, while independent variables included treatment and novel objects. Finally, on approximately PND 35, cognitive behavior was evaluated in the MWM. Cognition was evaluated through: **(i)** acquisition of a spatial task involving both procedural and spatial learning, **(ii)** probe trials measuring memory retention, **(iii)** and platform relocation trials evaluating spatial learning more specifically. The dependent variables analyzed in the MWM were swim speed, escape latency, total distance traveled, time in quadrant, distance traveled in quadrant, and virtual platform and annulus 40 crossings. Independent variables across both behavioral evaluations included treatment and platform location. Results of these cognitive behavioral assessments are presented in Chapter 5.

Hippocampal synaptic activity was evaluated beginning on PND 63 in the male offspring that were tested in the MWM. By means of the whole-cell mode of the patch-clamp technique, spontaneous and miniature inhibitory postsynaptic currents (IPSCs) and excitatory postsynaptic currents (EPSCs) were recorded from CA1

pyramidal neurons. Dependent variables include spontaneous and miniature IPSC and EPSC amplitude, frequency, and inter-event intervals, while the independent variable is *in utero* CPF exposure. Results of these experiments are detailed in Chapter 5.

2.3. Does sub-acute CPF exposure during the period of the major brain growth spurt result in changes in neuronal and/or glial architecture within the hippocampus of male guinea pigs?

Immunohistochemistry (IHC) was used to assess changes in hippocampal neuronal and glial cell markers in male offspring of dams exposed to CPF or vehicle as described in section 2.1. IHC staining was conducted on brain tissue that was obtained from animals transcardially perfused with 4% paraformaldehyde, blocked, cryoprotected, frozen, and sectioned in a microtome. Molecular markers of neuronal cell bodies, white matter, astrocytes, microglia, and cholinergic and GABAergic neurons were evaluated within the CA1 field of the hippocampus. Dependent variables include cell counts, intensity labeling (in pixels), and measurements (μm), while the independent variable is maternal exposure status. Results of these experiments are detailed in Chapter 6.

Chapter 3: Methods

3.1. Animal Care and Treatment

Hartley albino guinea pig dams [CrI:(HA)Br] were ordered from Charles River Laboratories (Wilmington, MA) to arrive at the testing facility on presumed gestation days 33 to 38 (GDs 33 to 38). Upon arrival, dams were singly housed in a light and temperature controlled facility ($21 \pm 0.5^{\circ}\text{C}$; 12-h light/dark cycle), provided food and water ad libitum, and randomized to either exposure (CPF) or vehicle control groups (PO). Following an acclimation period, dams were injected (sc) between the shoulder blades with a $0.05 \times \text{LD}_{50}$ dose of CPF (25 mg/kg/day, 0.5 ml/kg) or peanut oil (0.5 ml/kg) for up to ten consecutive days beginning approximately on GD 53. Dosing continued until either parturition or the conclusion of the dosing period resulting in an average of 9.6 and 8.6 doses for CPF- and vehicle- assigned dams, respectively. The number of doses administered was not significantly different between treatment groups (one-way ANOVA). Offspring were born on GDs 66 to 71, were weaned on PNDs 18 to 23, and subsequently group housed by sex and treatment group. Guinea pig handling and maintenance conformed to appropriate federal standards and adhered to the principles of the 1996 Guide for the Care and Use of Laboratory Animals.

3.2. Cholinesterase Inhibition

A colorimetric assay (Ellman et al., 1961) was used to measure cholinesterase activity in blood compartments and brain tissue obtained from male and female offspring within 24 hours of birth. Guinea pigs were euthanized in a CO_2 chamber according to the

IACUC-approved protocol. Blood was collected from dams and offspring by cardiopuncture into heparinized tubes. To separate plasma and RBCs, whole blood (0.5 ml) was centrifuged for 5 min at room temperature at 10,000 RCF. The plasma layer was transferred to a new tube and centrifuged again. The RBC pellet was washed three times with three volumes of phosphate buffer solution (PBS, pH 7.4). After the third wash, the RBC pellet was re-suspended to its original volume with phosphate buffer (100 mM Na₂HPO₄/KH₂PO₄, pH 7.0). Whole blood, plasma, and RBC samples (0.1 ml aliquots) were stored at -80°C.

Brains were removed, washed with 0.9% saline, and dissected into four sets: hippocampus, striatum, thalamus, and cerebellum. The remaining tissue was labeled cerebral cortex. The dissected brain tissues were flash frozen in liquid nitrogen and pulverized. Brain samples (≤ 0.5 g) were suspended in 1.0 ml of a buffer consisting of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH, 7), 5 mM EDTA, 1 M NaCl, 1% Triton X-100, and anti-proteases (Complete Mini; Roche Molecular Biochemicals, Indianapolis, IN). Samples were then sonicated on ice twice (20 s each time) and centrifuged briefly at 10°C to remove insoluble material. The clarified supernatants (0.2 ml aliquots) were stored at -80°C.

On the day of the assays, sample aliquots were thawed at 37°C and diluted to 1 ml with PBS (100 mM, pH 8). The resulting solution was placed on ice. The Ellman assay, modified for 96-well microplates, was used to determine specific cholinesterase activities as follows. Diluted samples (0.10 ml) were transferred to wells of three master microplates. PBS (100 mM, pH 8, 0.15 ml) was added to the wells of the plate used for determination of total cholinesterase (ChE) and brain butyrylcholinesterase (BuChE)

activity. For the determination of AChE activity, PBS (100 mM, pH 8, 0.15 ml) containing the BuChE inhibitor tetraisopropylpyro-phosphoramidate (iso-OMPA; final concentration, 100 μ M) was added, while the AChE inhibitor 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51; final concentration, 5 μ M) was added to determine blood BuChE activity. The samples were then incubated at room temperature for 15 min.

For the enzymatic assays, 0.05 ml of sample from a master plate was transferred to a reaction plate and mixed with 0.10 ml of 5-5'-dithiobis(2-nitrobenzoic) acid (DTNB, 2 mM) in phosphate buffer (100 mM, pH 8). After 10 min incubation at room temperature, 0.05 ml of butylthiocholine iodide (BuChI, 4 mM) in phosphate buffer (100 mM, pH 7) was added to the reaction plate for the determination of brain BuChE activity, while 0.05 ml of acetylthiocholine iodide (ASChI, 4 mM) in phosphate buffer (100 mM, pH 8) was added to the remaining reaction plates. The change in absorbance (AU) at 412 nm (for brain and plasma samples) or 436 nm (for RBC samples) was measured at 15 s intervals for 10 min in a SpectraMax PC340 microplate reader (Molecular Devices, Sunnyvale, CA). Baseline absorbance changes were measured in samples free of substrate. Each assay was repeated in triplicate. Results were captured using the SoftMax® Pro 4.0 software (Molecular Devices, Sunnyvale, CA) and transferred to Excel spreadsheets (Microsoft, Redmond, WA) for analysis. Conversion factors, 26 nmoles/AU and 32 nmoles/AU, were used to convert the rates of change in absorbance (AU/min) to rates of change in substrate (nmoles/min) at 412 nm and 436 nm, respectively. These values were determined empirically using the reaction of L-cysteine standards with DTNB [Ref. Thermo Fisher Scientific Inc. (2011). Instructions, Ellman's

Reagent 22582. https://tools.thermofisher.com/content/sfs/manuals/MAN0011216_Ellmans_Reag_UG.pdf]. Protein concentrations, determined using the bicinchoninic acid assay (Pierce Chemical, Rockford, IL) were used to normalize the results for the tissue samples. Results are presented as mean \pm standard error of the mean (SEM). Statistical analysis was performed using a two-way random factor ANOVA with sex and prenatal exposure as fixed factors and mother as random factor.

3.3. Open Field

3.3.1. Testing

Locomotor activity and preference for novelty were evaluated in an open field on approximately PND 30. Briefly, the open field apparatus consisted of a black, non-glare Starboard plastic box (120 cm \times 120 cm \times 60 cm). Four incandescent lamps hung over the apparatus generated evenly distributed light with intensity of 20 Lux on the floor of the field. Conair sound machines located on both sides of the apparatus were set to generate “white noise” on one side and “running stream” sound on the opposite side. Each apparatus was surrounded with black polyester curtains. During testing, the floor of the open field arena was covered with the same gray paper bedding as used for housing to reduce anxiety. For the purpose of data analysis, a virtual center zone (40 cm \times 40 cm) was delineated 40 cm from the walls of the open fields. For the novel object exploration test, a cylindrical glass jar (height: 8.8 cm; diameter: 7.2 cm) with a black plastic lid was placed in the center of the arena.

Four days before testing begun, animals were taken to the testing room for 3-4 h of acclimation on each of two consecutive days. Three animals were concurrently tested

in three sets of open fields. Before testing started, animals were weighed and placed in waiting cages adjacent to the open field apparatus for around 1-3 min. Then, animals were placed in the center of the arena and allowed to explore the open field for 10 min. The test was repeated 24 h later. Immediately after the second test, all animals were placed in the waiting cage adjacent to the open field for 3-5 min while a novel object was positioned in the center of the open field. Animals were then placed in the corner of the apparatus and allowed to explore for an additional 10 min.

3.3.2. Data Analysis

Data were collected and videos were recorded using the Any-Maze software (Stoelting Co., Wood Dale, IL). Locomotor activity as well as time and distance traveled in different zones of the open field were analyzed using the Any-Maze software. Rearing was evaluated and manually counted by one observer who watched all available videos. The tracking system was unable to differentiate between the head and the tail base of a guinea pig and, thereby, did not allow for automated assessment of head contact with the novel object. As a result, distance, number of entries, and time traveled in the center zone were taken as measures of novel object exploration.

3.4. Morris Water Maze

3.4.1. Testing

Spatial learning and memory were analyzed in the MWM using procedures similar to those described in Mamczarz et al. (2011). Briefly, the MWM consisted of a large grey galvanized metal circular tank (198 cm in diameter, 60 cm tall), filled with

water to a depth of 40 cm. Water was made opaque by adding tempera paint. The maze was divided virtually into four quadrants (N, S, E, W) and a round black painted escape platform (20 cm diameter) was submerged in the center of one of the quadrants. MWM testing began on approximately PND 35 with a five day training (acquisition) period. For each day of training, 4 trials per animal were conducted with a maximum length of 90 s each. On the third and fourth days after conclusion of the acquisition phase, the guinea pigs were subjected to a probe test. During the test, the platform was removed from the MWM and the guinea pigs were allowed to swim freely for 120 s.

After conclusion of the probe tests, guinea pigs were subjected to two platform relocation tests. During the first test, the escape platform was relocated from its original position in southwest quadrant to the opposite (northwest) quadrant. During the second test, the platform was positioned in the adjacent right (northeast) quadrant. On each day, before the first trial, each animal was placed on the platform and allowed to stay on it for 20 s. Each animal was then removed, dried, and placed on a waiting cage adjacent to the pool for another 30 s, after which time the animal was placed pseudo-randomly in the pool facing the wall. Each animal was allowed to swim for 120 s to find the hidden platform. At the time an animal climbed and stayed on the platform for 2 s, the animal was rescued, dried, and placed in its home cage. If an animal did not find the platform in 120 s, it was gently guided by the experimenter to the platform and allowed to stay on it for 15 s before being rescued. On each day, each animal was given 4 trials interspaced by 1 h.

3.4.2. Data Analysis

Video data from the MWM was analyzed with Any-Maze software (Stoelting Co.; Wood Dale, IL). Escape latency (defined as the time it took for the animals to climb and stay on the hidden platform for 2 s) and total distance traveled were measured in each trial during the acquisition phase and the platform relocation tests. On each training day, escape latency for any given animal refers to the mean escape latency measured during the 4 trials on that day. The same applied to the distance. During the acquisition phase, learning curves are presented as escape latency or distance vs. training day. During the platform relocation tests, learning curves are presented as escape latency or distance vs. trial. A random effect repeated measure ANOVA using treatment as fixed factor and mother as a random factor was used to analyze the data. The Tukey-Kramer post-hoc test was used for comparison of results obtained from CPF-exposed offspring to those obtained from control (vehicle-exposed) offspring.

Time and distance in the quadrants, number of platform crossings, and time and distance in the annulus 40 (a virtual area of 40-cm diameter in the center of each quadrant) were measured during the probe tests.

3.5. Electrophysiology

3.5.1. Recordings

On PNDs 63 to 79, offspring were euthanized by CO₂ asphyxiation followed immediately by decapitation. Their brains were removed from the skull and the hippocampi were dissected out in cold artificial cerebral spinal fluid (ACSF) consisting of (in mM) NaCl, 125; KCl, 2.5; CaCl₂·2H₂O, 2; MgCl₂·6H₂O, 1; NaH₂PO₄·H₂O, 1.25;

NaHCO₃, 26; and dextrose, 25. Transverse slices (300 μm thickness) were obtained from the medial portion of one hippocampus of each animal using a vibratome (Leica VT1000S; Leica Microsystems Inc., Deerfield, IL). The other hippocampus from each animal was flash frozen in liquid nitrogen and stored at -80°C for subsequent Western blot analysis, as described in section 3.6.

Hippocampal slices were kept for at least 1 h in a beaker filled with oxygenated ACSF before being transferred to the recording chamber, where they were continuously superfused with oxygenated ACSF at 2 ml/min. In the recording chamber, slices were kept submerged in ACSF and held in place by four nylon fibers. Patch pipettes were pulled from borosilicate glass capillaries (i.d. 1.2 mm; World Precision Instruments Inc., Sarasota, FL) with a horizontal puller (Sutter Instrument Company, Novato, CA). When filled with the internal solution, pipettes had resistances ranging from 2.3 to 4.5 MΩ. The internal solution consisted of (in mM): Cs methane sulfonate, 130; CsCl, 10; ethylene glycol tetraacetic acid (EGTA), 10; HEPES, 10; MgCl₂, 3; and lidocaine N-ethyl bromide (QX-314), 5. The pH of the pipette solution was adjusted to 7.3 with a solution of 1 mM CsOH. Biocytin (0.5%, 5 mg/ml) was added to the pipette solution for post-hoc reconstruction of the images of the pyramidal cells from which recordings were obtained.

Spontaneous EPSCs and IPSCs were recorded by means of the whole-cell mode of the patch-clamp technique from the somata of CA1 pyramidal cells using an LM-EPC7 amplifier (List Electronics, Darmstadt, Germany). Signals were filtered at 2 kHz, digitized at 10 kHz, and recorded with the pClamp 9.2 software (Molecular Devices, Sunnyvale, CA). The series resistance was monitored continuously during the recordings, primarily ranged between 25 and 50 MΩ, and was not compensated for.

Recordings lasted 5 min and were terminated if the series resistance changed by $\geq 20\%$. To minimize bias, only one recording per type, per slice, was collected for analysis.

EPSCs were recorded at the reversal potential for GABAergic currents (-50 mV, under present conditions), while IPSCs were recorded at the reversal potential for glutamatergic currents (0 mV, under present conditions). To confirm the glutamatergic or GABAergic nature of the recorded currents, slices were superfused with ACSF containing the NMDA receptor antagonist APV (100 μM) plus the AMPA receptor antagonist CNQX (10 μM) or the GABA_A receptor antagonist bicuculline (10 μM). Following the recording of IPSCs, a subset of slices were superfused with ACSF containing tetrodotoxin (TTX, 0.2 μM) and miniature IPSCs were recorded at 0 mV.

3.5.2. Data Analysis

Frequency and amplitude of synaptic events were measured using the Mini Analysis 6.0.3 software (Synaptosoft Inc., Decatur, GA). The threshold amplitude for automated detection of spontaneous and miniature EPSCs and IPSCs was set at twice the baseline noise (root mean square). Events that did not show a typical synaptic waveform were rejected manually. Data are expressed as means \pm SEM of results obtained from CA1 pyramidal cells of various animals. The data were analyzed for normality (Shapiro-Wilk) and heteroscedasticity (Levene's test). Normally distributed data with equal variance were analyzed for statistical significance using one-way ANOVA, while data failing either the Shapiro-Wilk or Levene's tests was analyzed on ranks using the Mann-Whitney rank sum test. Inter-event intervals, cumulative inter-event interval

distribution, and amplitude distribution were analyzed statistically using the Mann-Whitney rank sum test.

3.6. Western Blots

Western blots were used to evaluate cross-species primary antibody activity and analyze protein expression in hippocampal extracts. To this end, frozen hippocampal tissue was pulverized and suspended in a buffer containing 10 mM Tris-HCl (pH, 7.5), 1% Triton X-100, 1 mM EGTA, 5mM dithiothreitol (DTT), and protease inhibitors (Roche, Indianapolis, IN). Cellular lysates were obtained with the aid of a Polytron homogenizer (Kinematica AG; Lucerne, Switzerland). The lysates were centrifuged at 14,000 g for 30 min at 4°C and supernatant protein concentration was determined with the Bradford assay. Proteins (5 µg/well) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 26 well 10% Bis-Tris Midi Gels (Life Technologies; Frederick, MD) and semi-dry transferred to nitrocellulose membranes using the iBlot system (ThermoFisher Scientific; Rockford, IL). The membranes were then washed twice (15 min each) with Tris-buffered saline containing 0.1% Tween 20 (TBS-T), blocked with TBS-T containing 5% non-fat dried milk to preclude non-specific binding for 1.5 h at room temperature, and incubated with the appropriate primary antibody (Table 3) for 24 h at 8°C in 5% milk TBS-T. Following the primary incubation, membranes were washed three times (15 min each) with TBS-T and incubated with the appropriate secondary antibody (Table 3) for 1.5 h at room temperature in 5% milk TBS-T. After washing three additional times (15 min each) with TBS-T, signal was visualized with a chemiluminescence detection kit (Thermo Scientific;

Rockford, IL) and developed onto film (GE Limited Healthcare; Buckinghamshire, UK). Where appropriate, after development, membranes were stripped and probed for a constitutive marker of total protein, beta actin, for use as a loading control. Membranes designated to be stripped were washed five times (10 min each) in TBS-T, covered in Restore Western Blot Stripping Buffer (Thermo Scientific; Rockford, IL) for 15 min, rinsed with DiH₂O, and washed an additional three times (10 min each) with TBS-T. The western protocol was then repeated from the block through development.

Bands corresponding to protein expression, visible on the developed film, were analyzed using ImageJ software (National Institute of Mental Health; Bethesda, MD). Band intensity was optimized to within linear range experimentally through the manipulation of protein loading and primary and secondary antibody concentrations. Specifically, protein was pooled, serially diluted (1:1), and each concentration was loaded into the gel. This gel was then processed and developed as indicated previously using the manufacturer's suggested antibody concentrations. In some cases, where a loading concentration was not in linear range, primary and/or secondary concentrations were altered and the experiment was repeated until acceptable conditions were ascertained (Table 3).

3.7. Immunohistochemistry (IHC)

3.7.1. Tissue Sectioning

On PNDs 71 to 87, male guinea pigs were anesthetized with 4% isoflurane and transcardially perfused with 1% then 4% paraformaldehyde (pH 7.4) formulated in phosphate buffered saline (PBS). The brains were carefully removed from the skulls and

cut grossly into three blocks with incisions through the preoptic area and cerebral transverse fissure. The blocks of tissue were placed in 4% paraformaldehyde for five days, then PBS for two days, and finally sunk in 30% sucrose for at least five days. Brains were frozen on dry ice and sectioned at 20 μ M using a Leica cryostat (Leica CM 1900; Leica Microsystems Inc., Deerfield, IL). The brain slices were placed on gelatin-subbed slides. Sections in the range of -3.92 mM to -4.52 mM Bregma (Paxinos and Watson, 1996) were used in the present study. Three sections spaced 120 μ M apart were used per animal, per stain. Care was taken to standardize the anatomical positioning across all guinea pigs.

Table 3: Western Blot Antibodies

Type	Marker	Company	Species	Antigen Source	Weight (Kd)	Dilution	Secondary Dilution
Primary	Beta Actin	Sigma ^A	Mouse	Synthetic	42	1:2000	1:5000
	Calbindin			Bovine	28	1:3000	1:4000
	GFAP			Synthetic	51	1:500	
	NeuN	EMD ^B	Mouse	65	1:6000		
	Calretinin		Goat	Rat	31.5	1:10,000	
	TNF α	Abcam ^C	Rabbit	Guinea pig	17	1:1000	
	Parvalbumin			Rat	11		
Secondary ^D	Goat	Life Tech ^E	Donkey	NA ^F			
	Mouse	Sigma ^A					
	Rabbit						

A - Sigma-Aldrich (St. Louis, MO); **B** - EMD Millipore (Temecula, CA); **C** - Abcam (Cambridge, MA); **D** - Cross adsorbed, horse radish peroxidase conjugated; **E** - Life Technologies (Frederick, MD); **F** - Not applicable

3.7.1. IHC labeling

Sections selected for staining were removed from storage (8 °C) and brought to room temperature on a slide warming plate (Barnstead-Lab line, Melrose Park, TX) for 45 min. Staining conditions including washes, penetration steps, antigen retrieval, antibody concentrations, and development method were determined experimentally with

superfluous tissue from vehicle treated guinea pigs. All experimental steps except barrier pen (PAP) incubations and heating were conducted while rocking and all conditions per development method were run in parallel on individual slides; the most promising combination was either selected for the full-scale experiment or marked for further refinement. Primary antibody concentrations and incubation times were optimized through the use of a dilution series and signal specificity was determined with two negative controls - one omitting primary antibodies, the other omitting secondary antibodies. The development method and appropriate antibody concentrations for each marker are detailed in Table 4.

Table 4. IHC Antibodies

Type	Marker	Company	Host Species	Antigen Source	Developed	Dilution	Complex Dilutions
Primary	MBP	Novus ^A	Rat	Bovine	Fluor ^B	1:1000	1:3000
	Calbindin	Sigma ^C	Mouse		DAB	1:5000	
	GFAP			Synthetic	Fluor		
	NeuN	EMD ^D	Goat	Mouse	DAB	1:4000	1:2000
	Calretinin			Rat	Fluor	1:5000	1:3000
	ChAT			Human	DAB-PAP	1:100	
	Iba1	Novus	Fluor-PAP		1:500		
	Parvalbumin	Abcam ^E	Rabbit	Rat	DAB	1:4000	1:2000
	$\alpha 7$ nAChR	Alomone ^F			Fluor-PAP	1:200	
Secondary ^G	Rat	Life Tech ^H	Donkey	NA ^I			
	Rabbit						
	Mouse						
	Goat	Sigma					
Tertiary	Alexa Fluor	Life Tech	NA				
	Peroxidase	Jackson ^J					

A - Novus Biologicals (Littleton, CO); **B** - Fluorescent; **C** - Sigma-Aldrich (St. Louis, MO); **D** - EMD Millipore (Temecula, CA); **E** - Abcam (Cambridge, MA); **F** - Alomone Labs (Jerusalem, Israel); **G** - Highly cross adsorbed, biotin conjugated; **H** - Life Technologies (Frederick, MD); **I** - Not applicable; **J** - Jackson ImmunoResearch (West Grove, PA)

Once conditions were established, slides were washed twice (10 min each) in either PBS (pH 7.4) or penetrating PBS containing 0.2% Triton-X 100 (PBS-T). An

alcohol series was then used to further enable penetration for all slides except those stained for the $\alpha 7$ nAChR; these slides were not penetrated as cell-surface expression was desired. Slides were inserted sequentially in 50% (10 min), 75% (20 min), and then 50% (10 min) EtOH in DiH₂O and then washed twice in PBS-T (5 min each). All slides were then microwaved (5-6 min) in sodium citrate (10 mM, pH 6, ~ 99°C) to free epitopes occluded by paraformaldehyde crosslinking. Once removed from the microwave, they were allowed to cool in hot citrate for 20 min, placed in glycine (0.1 M, PBS, pH 7.4) for 30 min, washed three times in PBS (5 min each), and placed in 2% normal horse serum in PBS (pH 7.4) for 30 min to 1 h to block non-specific antibody interactions. Slides were then incubated in primary antibodies (1% NHS in PBS, pH 7.4) for 24 to 48 h at ~8 °C.

3.7.1.1. Fluorescent Development

Following incubation with the primary antibodies, slides were: washed three times in PBS (5 min each), incubated in appropriate biotinylated secondary antibodies (1% NHS in PBS, pH 7.4) at room temperature for 30 min and 8 °C for 2 h, washed three times in PBS (5 min each), incubated with streptavidin-conjugated alexa fluor 594 (1% NHS in PBS, pH 7.4) at room temperature for 30 min and 8 °C for 2 h, and washed three final times with PBS (5 min each). The slides were then cover-slipped with hard-set vectorshield (Vector; Burlingame, CA) and stored at 8 °C protected from light. Within 48 h, the slides were visualized with an Axioskop 2 Plus microscope (Zeiss United States; Thornwood, NY). The microscope contained an internal 10x magnification lens in addition to the objective lens. Signal (Nikon rhodamine filter cube) and background

images (Nikon FITC filter cube) were captured with a Nikon Digital Sight camera (Lewisville, TX). The camera was attached to a 0.64x coupled lens.

3.7.1.2. 3,3'-Diaminobenzidine Development

Following incubation with the primary antibodies, slides were: washed three times in PBS (5 min each), incubated in appropriate biotinylated secondary antibodies (1% NHS in PBS, pH 7.4) at room temperature for 1 h, washed three times in PBS (5 min each), incubated with streptavidin-conjugated horse radish peroxidase (1% NHS in PBS, pH 7.4) at room temperature for 1 h and washed three times with PBS (5 min each). The development process was then begun by bathing the slides in 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS, 0.05 M in DiH₂O) for 10 min followed by immersion in a filtered solution of 3,3'-diaminobenzidine [0.0015% H₂O₂, DAB (160 mg/400 ml) in 0.05 M TRIS]. Slides were allowed to develop in this solution for 7 to 15 min before being washed in PBS (10 min) and dehydrated with an alcohol series. The developed tissue was placed sequentially in 50%, 75%, 95%, 95% and 100% EtOH for 3 min per concentration and then placed in xylene for 24 h prior to cover-slipping with DPX (Sigma-Aldrich; St. Louis, MO). Slides were visualized with the Axioskop 2 Plus microscope and bright field images were captured with a Nikon Digital Sight camera (Shinagawa, Tokyo, Japan).

3.7.3. Data Analysis

Images were analyzed using Nikon Elements Advanced Research software (Nikon Instruments Inc.; Lewisville, TX). The investigator was blinded to the treatment

group, and intensity and/or cellular counts were measured in defined regions of interest within the image. To minimize bias, cell counts/intensities were averaged per guinea pig. Background intensity was defined and subtracted from each image. Data are expressed as mean cells or intensity \pm the SEM. Following analysis for normality (Shapiro-Wilk) and heteroscedasticity (Levene's test), statistical significance was determined with one- and two-way ANOVAs. Where a significant main effect was determined, post hoc analysis was conducted via the Holm-Sidak method.

3.8. Chemicals

CPF, peanut oil, (-)bicuculline methiodide, (2R)-amino-5-phosphonovaleric acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), N-(2,6-Dimethylphenyl-carbamoylmethyl) triethylammonium bromide (QX-314), and tetrodotoxin (TTX) were purchased from Sigma Chemical Co. (St. Louis, MO). Iso-ompa, butylthiocholine, and acetylthiocholine were purchased from Sigma Chemical Co. (St. Louis, MO). 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) was purchased from Life Technologies (Frederick, MD).

Chapter 4: Cholinesterase Inhibition

4.1. Introduction

The translational capacity of data generated in preclinical toxicological studies is contingent upon several factors, including the appropriateness of the animal model. To date, all developmental studies with sub-acute doses of CPF have been conducted on altricial species, specifically rats and mice. However, striking differences exist between their CNS development and that of humans making it difficult to extrapolate sensitive gestational periods from these rodents to humans (Byrnes et al., 2003; Dobbing and Sands, 1970, 1973). In addition, the structure of the human placenta is quite distinct from that of the rat placenta, further complicating the translatability of developmental studies in rats to humans (Pentsuk and van der Laan, 2009). The rodent that more closely resembles humans in terms of brain development and placental structure is the guinea pig (Dobbing and Sands, 1970; Pentsuk and van der Laan, 2009).

Here, guinea pigs were exposed *in utero* to sub-acute doses of CPF during the gestational period spanning from the time of brain growth spurt, which peaks around GD 50, to the time of rapid brain myelination, which peaks around GD 60 (Dobbing and Sands, 1970). Starting on approximate GD 53 to 55 and lasting ten consecutive days, pregnant guinea pigs were injected subcutaneously every 24 hours with 25 mg/kg CPF (dissolved in peanut oil) or with peanut oil. This CPF dose regimen was selected to reproduce a number of salient features associated with exposures of humans to pesticides in agricultural and industrial settings and in households. First, the daily injections were intended to mimic the repetitive nature of occupational human exposures to OP pesticides

(Farahat et al., 2011). Second, the sc route of exposure was selected because it allows for a slow sustained release of the pesticide in the systemic circulation and, thereby, approximates human dermal exposures to this pesticide (Ellison et al., 2011). The dermal route has been reported to be one of the most relevant routes of exposure of humans to CPF (Farahat et al., 2010; Fenske et al., 2012; Lebailly et al., 2009; van Hemmen and Brouwer, 1995; Zartarian et al., 2000). Third, the daily dose of CPF injected in the pregnant guinea pigs was chosen to be below doses that induce overt signs of acute toxicity. The oral LD₅₀ of CPF in guinea pigs is 504 mg/kg, and in general, oral and sc LD₅₀s of OP compounds are very similar (McCollister et al., 1974). As a result, taking into account metabolism, the cumulative dose of CPF used here would be well below 0.5xLD₅₀, which is lower than the threshold for OP-induced acute toxicity (Shih and McDonough, 1997). The intention was to model a scenario in which occupational human exposure may be presumed safe.

The primary objective of the study described in this chapter was to determine whether the dose regimen of CPF administered to the pregnant guinea pigs is translationally relevant. Epidemiologically, occupational exposure of humans to CPF and other OP pesticides is evaluated on the basis of RBC AChE activity measured in people before and after they begin working with the pesticides. Thus, to be translationally relevant, the CPF exposures used in the present study must at a minimum produce a degree of RBC AChE inhibition that is comparable to that observed in humans following occupational exposures to CPF.

The second objective of the study described in this chapter was to provide evidence that CPF administered to the pregnant dams crossed the placenta and

subsequently the blood brain barrier of the fetuses. Considering that BuChE is far more sensitive than AChE to inhibition by OP compounds, the activities of both enzymes were assessed in tissue and blood of offspring born to the dams exposed to CPF or vehicle.

4.2. Experimental Design

Three pregnant guinea pigs were dosed subcutaneously with CPF (25 mg/kg/day) and two were dosed with vehicle (peanut oil) beginning approximately on presumed GD 53 to 55 for ten consecutive days. The blood and brains of offspring were harvested at parturition. Upon centrifugation, blood was separated into RBCs and plasma. The activity of AChE was measured in brain tissue and RBCs, whereas the activity of BuChE was measured in brain tissue and plasma as detailed in chapter 3 section 3.2. Among the brain regions analyzed were those known to be involved in processing of cognitive and motor functions, including the hippocampus, cerebral cortex, thalamus, and cerebellum.

4.3. Results

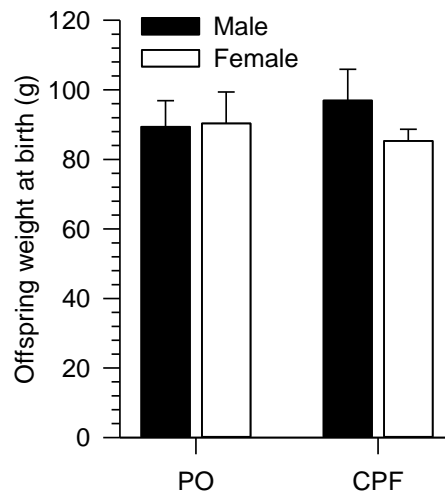
4.3.1. Clinical signs and offspring body weight

Acute intoxication induced by OP pesticides is characterized by clinical signs resulting from overactivation of muscarinic receptors and overstimulation followed by desensitization of nicotinic receptors due to accumulation of ACh in the peripheral and central nervous systems. Persistent stimulation of muscarinic receptors leads to a muscarinic syndrome characterized by miosis, profuse secretions, bradycardia, bronchoconstriction, hypotension, and diarrhea. Overstimulation of nicotinic receptors triggers tachycardia and skeletal muscle fasciculation, while their subsequent

desensitization contributes to muscle weakness and paralysis. The broad range of CNS-related acute effects includes anxiety, restlessness, confusion, ataxia, tremors, seizures, and central cardiorespiratory paralysis (reviewed in Hurst et al., 2012).

Repeated exposure to the dose of CPF used in the present study (i.e., 25 mg/kg/day for ten days starting on approximate GD 53 to 55) induced no clinical signs of acute toxicity in the dams or their offspring. In addition, at birth, the body weights of offspring from mothers exposed to CPF were comparable to those of offspring from mothers exposed to vehicle (Figure 2).

Figure 2: Body weight of offspring born to pregnant guinea pigs injected daily for 10 days with vehicle or CPF (25 mg/kg/day, sc) starting on GD 53 and 55.

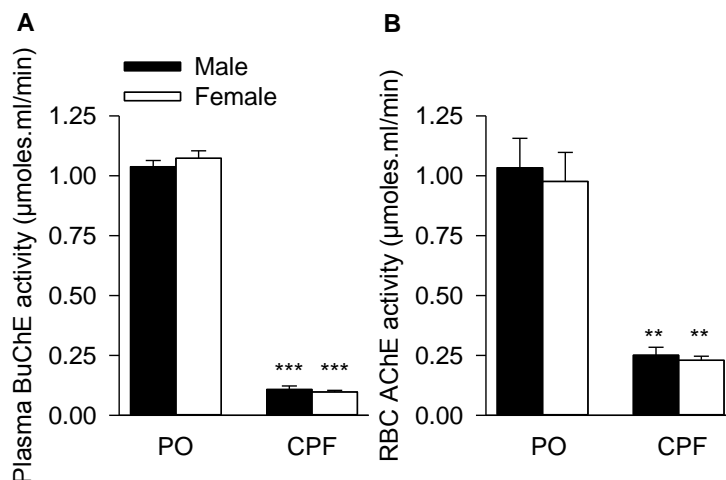


Body weights were measured within 24 h after animals were born. Graph and error bars represent mean and SEM of results obtained from offspring (n = 5 males and 4 females) born to pregnant guinea pigs that had been injected with peanut oil (PO, n = 2) and from offspring (n = 7 males and 4 females) born to pregnant guinea pigs that had been injected with CPF (n = 3).

4.3.2. BuChE and AChE activities in blood and brain

The activity of BuChE was approximately 80-90% lower in the plasma and in the various brain regions of male and female offspring born to dams injected with CPF than in the plasma and brain of offspring born to dams injected with peanut oil (Figures 3A and 4).

Figure 3: Effect of prenatal exposure to CPF on plasma BuChE and RBC AChE.

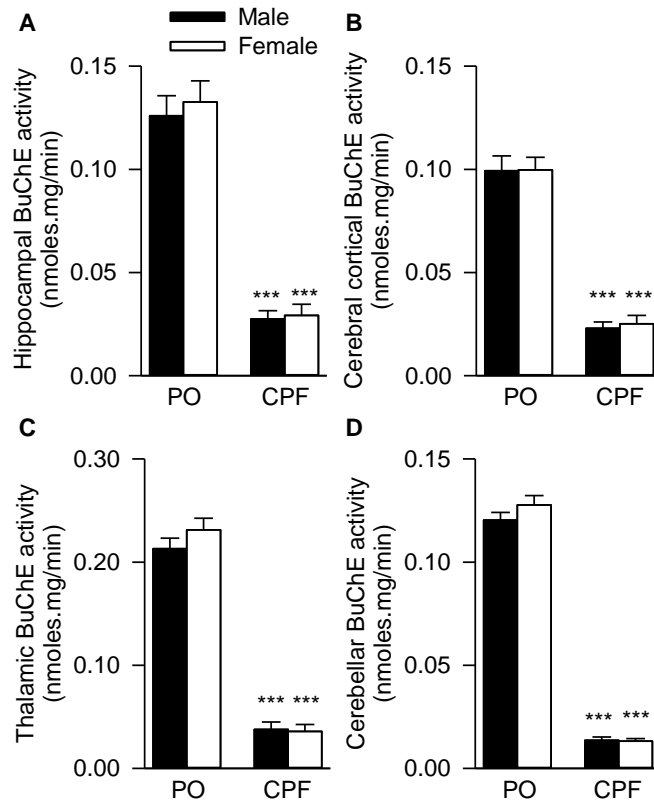


Measurements of plasma BuChE (A) and RBC AChE (B) are from the same animals as in Figure 1. A random effect ANOVA revealed a significant main effect of prenatal exposure on plasma BuChE and RBC AChE. There was no significant main effect of sex and no significant exposure x sex interaction on the activities of both enzymes. *Post-hoc* analysis using the Tukey-Kramer test revealed that plasma BuChE activity and RBC AChE activity were significantly lower among male and female offspring prenatally exposed to CPF than among those exposed to PO (**, $p < 0.01$; ***, $p < 0.001$).

A random factor ANOVA model revealed a significant main effect of prenatal exposure to CPF on plasma BuChE activity [$F(1,13) = 1575.77$; $p < 0.0001$]. There was no significant main effect of sex [$F(1,13) = 0.14$; $p = 0.710$] and no significant prenatal exposure x sex interaction [$F(1,13) = 0.16$; $p = 0.691$] on plasma BuChE activity. BuChE is generally taken as a sensitive biomarker of OP exposure because it is inhibited more effectively than AChE by most OP compounds, including CPF (Farahat et al., 2011). Thus, the present results support the notion that offspring were exposed to CPF *in utero*

following the daily exposure of the dams with the pesticide. The degree of BuChE inhibition observed in the plasma of CPF-exposed offspring was similar to that seen in the plasma of CPF-exposed dams (mean \pm SE: 90.2 \pm 1.97%, n = 3).

Figure 4: Effect of prenatal exposure to CPF on BuChE activity in different brain regions.

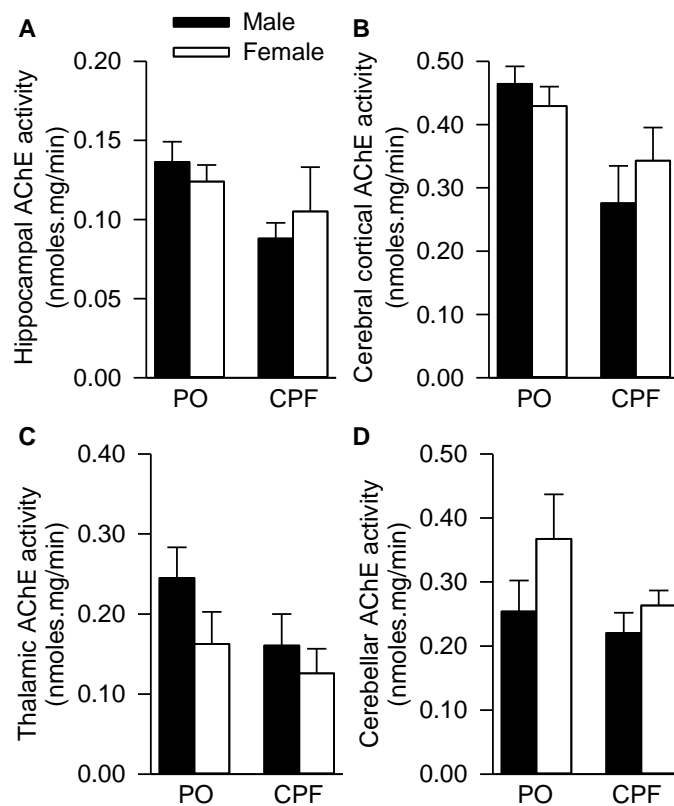


Measurements of BuChE activities are from the hippocampus (A), cerebral cortex (B), thalamus (C), and cerebellum (D) of the same animals as in Figure 1. The random effect ANOVA revealed a significant main effect of prenatal exposure on BuChE activity in the hippocampus [F(1,3) = 131.9, p = 0.0014], in the cerebral cortex [F(1,3) = 151.9, p = 0.0012], in the thalamus [F(1,3) = 123.7, p = 0.0016], and in the cerebellum [F(1,3) = 123.7, p = 0.0016]. There was no significant main effect of sex and no significant exposure x sex interaction on BuChE in any of the brain regions. The Tukey-Kramer *post-hoc* test revealed that in the different brain regions BuChE activity was significantly lower among male and female offspring prenatally exposed to CPF than among those exposed to peanut oil (PO; ***, p < 0.001).

The random factor ANOVA also revealed a significant main effect of prenatal exposure to CPF on RBC AChE [F(1,3) = 20.16; p = 0.02]. There was no significant main effect of sex [F(1,13) = 0.07; p = 0.798] and no prenatal exposure x sex interaction

[F(1,13) = 1.66; p = 0.220] on RBC AChE. Prenatal CPF exposure reduced by approximately 75% the activity of AChE in RBCs of both male and female offspring (Figure 3B). This degree of RBC AChE inhibition was comparable to that seen in RBCs from the CPF-exposed dams (mean ± SE: 81.6 ± 3.50%, n = 3). In contrast, the random factor ANOVA revealed that AChE activity in the hippocampus, cerebral cortex, thalamus, and cerebellum was not significantly different between CPF- and peanut oil-exposed offspring (Figure 5).

Figure 5: Effect of prenatal exposure to CPF on AChE activity in different brain regions.



Measurements of AChE activities are from the hippocampus (A), cerebral cortex (B), thalamus (C), and cerebellum (D) of the same animals as in Figure 1. The random effect ANOVA revealed no significant main effect of prenatal exposure or sex on AChE activity in the different brain regions. There was also no significant prenatal exposure x sex interaction.

4.4. Discussion

The acute toxicity of OP pesticides results primarily, though not exclusively, from their ability to block AChE in the peripheral and central nervous systems. Since RBC AChE is very sensitive to inhibition by most OP compounds, RBC AChE inhibition is commonly used as a surrogate endpoint for health risk assessment of OP intoxication (Chen et al., 1999; Strelitz et al., 2014).

On the day of birth, although neither CPF-exposed dams nor their offspring presented clinical signs of OP intoxication, they had approximately 75% lower RBC AChE activity than their control counterparts. Following repeated exposure to OP compounds, the degree of inhibition of RBC AChE does not reflect the degree of inhibition of tissue AChE, because recovery of AChE activity between exposures is due to combined reactivation of the inhibited enzyme and synthesis of new enzyme (Mason, 2000). While the half-life of AChE in skeletal muscles and brain ranges from two to three days (Rotundo and Fambrough, 1980; Wenthold et al., 1974), synthesis of new RBC AChE is only due to new RBC production and the half-life of RBCs is approximately 120 days (D'Alessandro et al., 2010). In fact, the degree of AChE inhibition in the brain of offspring prenatally exposed to CPF did not exceed 30%.

In the US, California and Washington have instituted programs in which RBC AChE activity can be longitudinally monitored in workers handling OP pesticides (Ames et al., 1989; Hofmann et al., 2010). At any time workers enrolled in these programs present RBC AChE activity at or below 70% of their baselines, they are removed from OP pesticide handling until their RBC AChE activity is within 20% of their personal baseline. However, this is not common practice within the US or

worldwide and evidence exists that RBC AChE activity can be reduced by as much as 40-80% from baseline in workers who otherwise present no overt clinical sign or symptom of OP intoxication (Ames et al., 1989; Farahat et al., 2011; Lakew and Mekonnen, 1998; Ohayo-Mitoko et al., 1999; Pathak et al., 2013; Singleton et al., 2015). Thus, based on measurements of RBC AChE, the CPF dose regimen the guinea pigs were subjected to in the present study translates in relevant levels of occupational exposure of people who handle OP pesticides throughout the world.

In conclusion, the results presented here demonstrate that the CPF dose regimen administered to guinea pig dams induced levels of RBC AChE inhibition that are relevant to humans occupationally exposed to this pesticide. They also provide evidence that CPF administered to the pregnant guinea pigs crossed the placenta and reached the brain of their offspring *in utero*. Therefore, this guinea pig model emerges as a translationally relevant preclinical model for assessment of the developmental neurotoxicity of CPF.

Chapter 5: Behavioral effects and changes in synaptic function associated with sub-acute developmental CPF exposure

5.1. Introduction

CPF is the OP pesticide most commonly used in agriculture worldwide and continues to be the top-selling OP pesticide for residential use in various countries. In part, because of the broad-spectrum pest control effectiveness and low cost of CPF, a global market analysis published recently reported that CPF sales will grow stably between 2015 and 2022 worldwide (Grand View Research, 2014).

In 2001, the U.S. Environmental Protection Agency banned the residential use of this pesticide in the U.S. The ban came upon the realization of the detrimental health effects of CPF, particularly in developing children (Israel, 2012). Epidemiology studies have provided evidence that *in-utero* exposure to CPF is associated with increased prevalence of attention deficit hyperactive disorder, pervasive developmental disorder, and reduced intelligence quotient among children (Rauh et al, 2006 and 2011). Nevertheless, CPF could still be detected in 78% of American households surveyed between 2005 and 2006 (Stout et al., 2009). Thus, exposure to CPF will remain for years to come a serious public health concern in both agricultural and residential settings in the US and throughout the world.

Although it is understood that the acute toxicity of OP compounds results mostly from the irreversible inhibition of AChE, the enzyme that metabolizes the neurotransmitter acetylcholine (Bajgar, 2004), the mechanisms underlying their developmental toxicity remain poorly understood. Studies performed in developing rats and mice have provided evidence that CPF doses well below the threshold to trigger overt

acute intoxication and large degrees of AChE inhibition (> 50%) (Bajgar, 2004) are sufficient to trigger neurological deficits, especially in learning and memory, that resemble those seen in children prenatally exposed to this CPF (Li et al, 2012). Several lines of evidence support the notion that a common mechanism of action is unlikely to explain the neurological deficits that develop in response to prenatal exposure to OP pesticides. For example, prenatal exposure to the OP pesticides CPF and diazinon results in increased expression of tryptophan hydroxylase, the rate-limiting enzyme for serotonin biosynthesis, and reduced expression of serotonin transporter genes in the brain. However, prenatal exposure to CPF enhances while prenatal exposure to diazinon suppresses the expression of most serotonin receptor subtypes in the brain (Slotkin and Seidler, 2008).

To date, developmental studies with sub-acute doses of CPF have been conducted on altricial species, specifically rats and mice. However, striking differences exist between their CNS development and that of humans making it difficult to extrapolate sensitive gestational periods from these rodents to humans (Byrnes et al., 2004; Dobbing and Sands, 1970 and 1979). In addition, the structure of the human placenta is quite distinct from that of the rat placenta, further complicating the translatability of developmental studies in rats to humans (Pentsuk and van der Laan, 2009). The rodent that more closely resembles humans in terms of brain development and placental structure is the guinea pig (Dobbing and Sands, 1979; Pentsuk and van der Laan, 2009). Therefore, the present study was designed to test the hypothesis that guinea pigs prenatally exposed to sub-acute doses of CPF develop cognitive deficits that bear resemblance to those observed in humans prenatally exposed to this pesticide and that

correlate with increased inhibitory synaptic transmission in CA1 pyramidal neurons, the major excitatory hippocampal output.

5.2. Experimental Design

To test this hypothesis, Dunkin Hartley guinea pig dams [CrI:(HA)Br] were injected sc once a day with CPF dissolved in peanut oil (25 mg/kg/day, 0.5 ml/kg) or peanut oil (0.5 ml/kg) for up to ten consecutive days beginning approximately on DG 53. Offspring were born on DGs 66 to 71, weaned on PNDs 18 to 23, and subsequently group housed by sex and treatment group.

As described in Chapter 3, starting approximately on PND 30, locomotor activity of offspring was evaluated in two consecutive days in an open field arena. Novel object exploration was assessed immediately after completion of the second open field test. Training in the MWM started when animals were approximately 38 days old. First, cognitive performance was evaluated in a reference memory paradigm in which animals were trained to escape onto a hidden platform for five consecutive days. Memory retention was evaluated during a probe test conducted 72 h after completion of the MWM acquisition phase. Starting 24 h after completion of the probe test, the animals were subjected to two platform relocation tests.

On PNDs 63 to 79, male offspring were euthanized by CO₂ asphyxiation followed immediately by decapitation. Their hippocampi were harvested and cut in transverse slices (300- μ M thick). Spontaneous EPSCs and IPSCs were recorded from CA1 pyramidal cells voltage clamped at -50 mV and 0 mV, respectively.

Following the recording of spontaneous IPSCs, some slices was superfused with ACSF containing tetrodotoxin (TTX, 0.2 μ m) and recordings of miniature IPSCs were obtained at 0 mV.

5.3. Results

5.3.1. Pregnancy outcome

Pregnancy outcomes were analyzed for pregnant guinea pigs subjected to daily injections of peanut oil or CPF (25 mg/kg, sc) for ten consecutive days (Table 5). The number of pregnant guinea pigs that died during the injection period, number of miscarriages, number of perinatal deaths (i.e., number of offspring that died within the first 24 h after birth), number of litters with perinatal deaths, and mean litter size were comparable between both groups (Table 5). CPF-injected pregnant guinea pigs gained body weight at a rate similar to that of peanut oil-injected dams (Figure 6A) and presented no overt signs of acute toxicity during or after the period of injections.

Table 5: Number of maternal deaths, miscarriages, litters with perinatal deaths, offspring that died perinatally, and litter size per experimental group.

	Peanut Oil	Chlorpyrifos
Maternal deaths ^a	1/15	1/16
Miscarriages ^b	0/14	1/15
Litters with perinatal deaths ^c	7/14	5/14
Perinatal deaths ^d	14/70	10/73
Litter size ^e	5.10 \pm 0.55	4.41 \pm 0.42

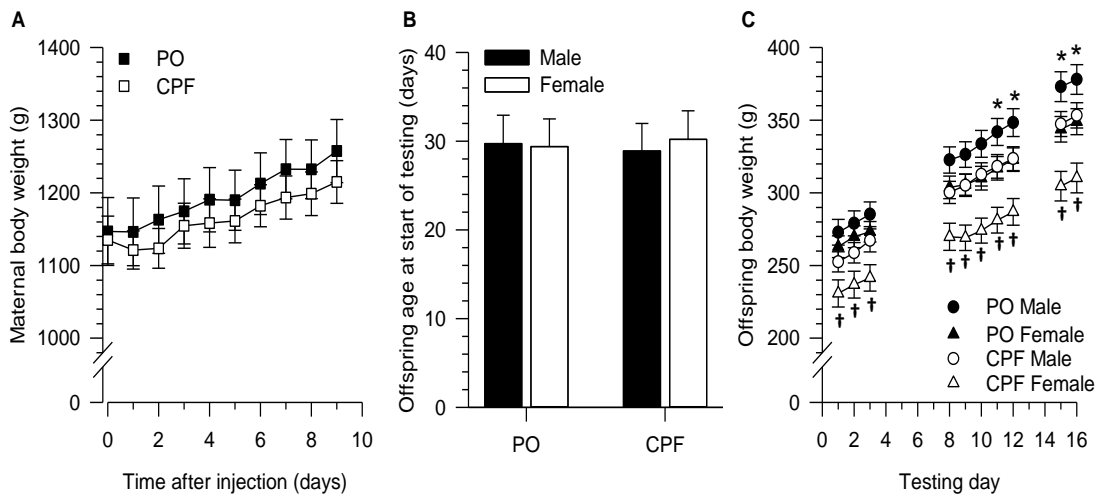
A - Number of pregnant guinea pigs that died during injections/total number of injected guinea pigs.

B - Number of pregnant guinea pigs that miscarried after beginning of injections/total number of pregnant guinea pigs that survived the injections. **C** - Number of litters with offspring that died within 24 h after birth/total number of viable litters. **D** - Number of offspring that died within 24 h after birth/total number of offspring. **E** - Litter size is presented as mean \pm SEM of total number of offspring per litter per group.

5.3.2. Body weight of offspring during testing

At the start of the behavioral tests, there were no significant differences in the ages of animals that had been prenatally exposed to peanut oil or CPF (Figure 6B). The body weights of these animals were recorded daily over the course of open field testing (days 1 to 3), water maze training (days 8 to 13), and water maze probe tests (days 15 to 16). Seven animals that were not able to swim in the water maze were not included in this analysis.

Figure 6: Effect of gestational exposure to CPF on maternal and offspring body weight gain.



A. Body weight of pregnant guinea pigs that were injected subcutaneously with peanut oil (PO) or CPF (25 mg/kg/day) for ten days starting on approximate GD 53 to 55. Body weights of the dams were measured daily immediately before each injection. Points and error bars represent mean and SEM, respectively, measured from 14 and 15 pregnant guinea pigs injected with peanut oil and CPF, respectively. **B.** Age of offspring at the beginning of behavioral testing. Graph and error bars represent mean and SEM, respectively, from 24 to 33 offspring/group. **C.** Body weight of offspring during open field testing (days 1 to 3), water maze training (days 8 to 13), and probe tests (days 15 to 16). As described in the text, a random effect ANOVA indicated significant main effect of sex ($p < 0.0001$), prenatal exposure ($p < 0.05$), and prenatal exposure \times sex interaction ($p < 0.001$). Analysis of the simple main effect of each factor in the significant interaction revealed that prenatal exposure to CPF significantly lowered body weight of females ($p < 0.05$), but not males. Data points and error bars represent mean and SEM, respectively, of results from 24 to 33 animals/group. According to the post-hoc Tukey-Kramer test: * $p < 0.05$ PO males vs. PO females; † $p < 0.05$ CPF males vs. CPF females. Data in A to C are only from offspring that were subsequently tested behaviorally and their mothers.

Although testing day, animal sex, and prenatal exposure had significant main effects on body weight [F(9, 1033) = 145.48, $p < 0.0001$; F(1,1033) = 180.83, $p < 0.0001$; and F(1,27) = 4.65, $p = 0.0401$, respectively], interpretation of the results was complicated by the significant testing day x sex and prenatal exposure x sex interactions [F(9, 1033) = 2.10, $p = 0.027$ and F(9, 1033) = 6.67, $p = 0.010$, respectively]. Analysis of the simple main effect of each factor in the significant interactions revealed that body weight of male and female offspring increased significantly with time [males: F(9, 1033) = 97.51, $p < 0.0001$; females: F(9, 1033) = 53.07, $p < 0.0001$]. It also revealed that prenatal exposure to CPF significantly reduced the body weight gain of female offspring [F(9, 1033) = 6.16, $p = 0.0132$], while having no significant effect on the body weight of males [F(9, 1033) = 3.20, $p = 0.0738$].

At the beginning of behavioral testing, animals that had been prenatally exposed to PO or CPF were approximately 30 days old (Figure 6B). Pairwise comparisons revealed that during the first ten days of testing, i.e. between approximately 30 and 40 days of age, control male and female offspring had comparable body weights (Figure 6C). Between the 11th and the 16th day of testing, when animals were approximately 41 to 46 days old, control male offspring became significantly heavier than their female counterparts (Figure 6C). These findings are in agreement with a previous report that male Dunkin-Hartley guinea pigs gain weight at faster rate than their female counterparts, with the body weight of males becoming considerably larger than that of females after approximately 40 days of age (Slob et al., 1973). In contrast, since the first testing day, male offspring that had been prenatally exposed to CPF were significantly heavier than their female counterparts (Figure 6C).

5.3.3. Open field and novel object exploration

There was a significant main effect of testing day on the total distance the animals traveled in the open field, with the locomotor activity decreasing significantly between the first and second days of testing [$F(1,198) = 137.81, p < 0.0001$] (Figure 7A). There was no significant main effect of sex or exposure and no significant interaction between these two factors on the total distance or on the inter-session decline of the total distance traveled by the animals in the open fields (Figure 7A). Therefore, prenatal exposure of guinea pigs to sub-acute doses of CPF had no significant effect on locomotor activity or on affect habituation, which is a form of non-associative memory.

There was a significant main effect of testing day on the distance traveled [$F(1,198) = 22.059, p < 0.0001$] and number of entries in the center zone [$F(1,198) = 23.35, p < 0.0001$, respectively], with exploration of the center of the field decreasing significantly between the first and second day of testing (Figures 7B and 7C). These results are in agreement with the notion that the animals habituated when successively exposed to the open fields. However, prenatal exposure and sex had no significant effect on distance, number of entries, and time in the center zone (Figure 7B through D).

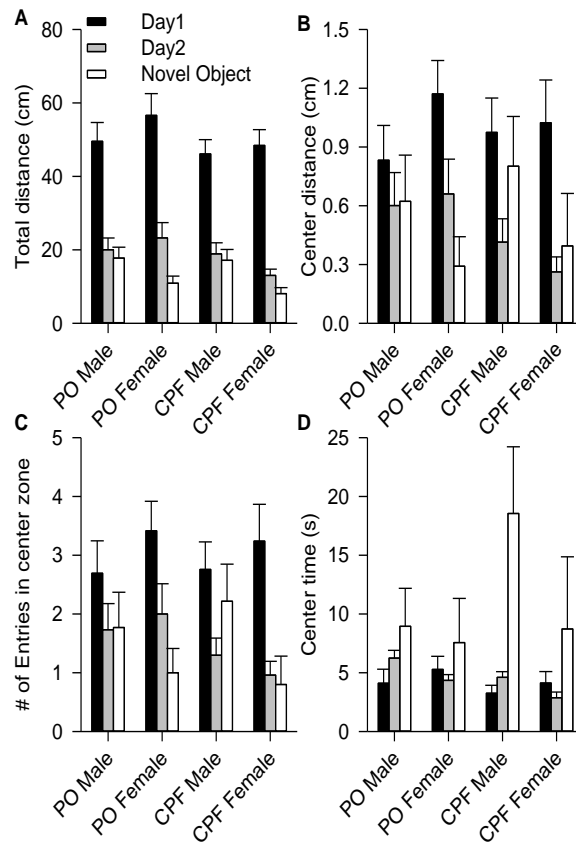
At the end of each open field session on the second day of testing, the animals were removed from the field and a novel object was placed in the center of the floor of the apparatus, as described in the Methods section. Behavior of the animals in the presence of the novel object was compared to that recorded during the preceding open field session. A three-way random effect ANOVA using test (i.e., novel object and 2nd day of open field), sex, and exposure as factors revealed a significant main effect of test

[F(1,198) = 7.44, p = 0.0069] and sex [F(1, 198) = 4.22, p = 0.0412] on the total distance traveled by the animals (Figure 7A). There was no significant interaction between sex and test [F(1,198) = 2.87, p = 0.0917] and no significant main effect of prenatal exposure on total distance (Figure 7A).

Distance and number of entries in the center zone during the novel object session were comparable to those measured in the preceding open field test without the object (Figure 7B,C). However, there was a significant main effect of test on time in the center zone [F(1,198) = 6.84, p = 0.0096], with the presence of the novel object triggering a significant increase of time the animals spent in the center of the fields (Figure 7C). Sex and prenatal exposure had no significant effect on the parameters measured during the novel object exploration test.

Exploration of the center of open fields is normally taken as a measure of anxiety-related behavior in rodents (Prut and Belzung, 2003). However, even control animals rarely entered and explored the center of the open fields (note in Figure 7B that distance in the center of the fields was < 2% of the total distance traveled in each test day). Therefore, results should not be interpreted as an indication of lack of effect of CPF on anxiety-related behavior.

Figure 7: Locomotor activity, habituation, and novel object exploration of prepubertal offspring prenatally exposed to CPF or PO.



Guinea pigs were allowed to explore the open field for 10 min on two consecutive days. Immediately after the second open field session, a 10-min novel object exploration test was performed in the same apparatus. Data points and error bars represent mean and SEM, respectively, of results obtained from 24-36 animals/group. As described in the text, a random effect ANOVA revealed no significant main effect of prenatal exposure or sex on the total distance the animals traveled in the open field on each of the two testing days (A). Likewise, prenatal exposure and sex had no significant effect on the distance (B), number of entries (C), and time (D) in the center zone of the field. Finally, as described in text, the total distance traveled in the field as well as the distance traveled and number of entries in the center zone declined significantly between the first and the second days of testing in the open field. There was no significant main effect of prenatal exposure or sex and no significant prenatal exposure x sex interaction in the inter-session decline of the exploration of open fields.

5.3.4. Morris Water Maze

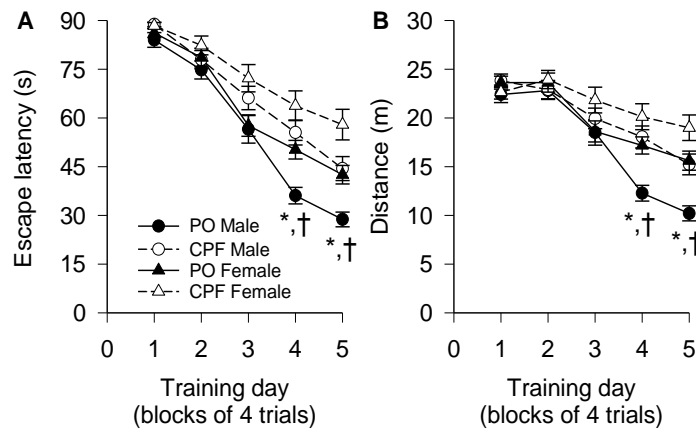
5.3.4.1. Reference Memory Training

During the first two days of the reference memory training in the MWM, seven animals were retired due to their inability to swim. The group of seven animals included four males and one female that had been prenatally exposed to CPF and two females that had been prenatally exposed to peanut oil.

Over the course of five days of training in the MWM animals progressively learned the task as suggested by a significant main effect of training day on the escape latency [$F(4,503) = 158.71, p < 0.0001$] and distance [$F(4,503) = 64.31, p < 0.0001$] (Figure 8). There was also a significant main effect of sex on both escape latency [$F(1,503) = 12.53, p = 0.0004$] and distance [$F(1,503) = 15.77, p < 0.0001$], with males outperforming females. Finally, prenatal exposure to CPF impaired the water maze performance of the offspring as suggested by a significant main effect of prenatal exposure on escape latency [$F(1,27) = 9.36, p = 0.0050$] and distance traveled to reach the hidden platform [$F(1,27) = 6.48, p = 0.0169$].

The training day x sex interaction was found to be significant [escape latency: $F(4,503) = 3.59, p = 0.0067$; distance: $F(4,503) = 4.40, p = 0.0017$] and analysis of simple main effects of each factor in this interaction revealed that the performance of male and female guinea pigs became significantly different on the 4th and 5th days of training [escape latency on 4th day: $F(1,503) = 10.66, p = 0.0012$; escape latency on 5th day: $F(1,503) = 16.86, p < 0.0001$; distance of 4th day: $F(1,503) = 12.36, p = 0.0005$; distance on 5th day: $F(1,503) = 21.93, p < 0.0001$].

Figure 8: Effect of prenatal exposure to CPF on the acquisition phase of the MWM task.



Graphs show (A) average escape latency and (B) average distance traveled per each training day. As described in the text, a random effect ANOVA indicated a significant main effect of sex ($p < 0.0005$ and $p < 0.0001$ for escape latency and distance, respectively), significant main effect of prenatal exposure to CPF ($p < 0.005$ and $p < 0.05$ for escape latency and distance, respectively), and significant prenatal exposure x sex interaction ($p < 0.05$ for escape latency). Analysis of the simple main effect of each factor in the significant interaction revealed that prenatal exposure to CPF significantly increased escape latency in both sexes ($p < 0.0005$ and $p < 0.05$ for males and females, respectively) and significantly increased the distance traveled in males ($p < 0.05$). Data points and error bars represent mean and SEM, respectively, of results obtained from 24-33 animals/group. According to Tukey-Kramer post-hoc test for pairwise comparisons: *, $p < 0.05$ PO males vs. CPF males; †, $p < 0.05$ PO males vs. PO females.

The training day x prenatal exposure interaction was also significant [escape latency: $F(4, 503) = 4.90$, $p = 0.0007$; distance: $F(4, 503) = 4.88$, $p = 0.0007$]. Analysis of the simple main effect of each factor in this interaction revealed that prenatal exposure to CPF significantly increased both the escape latencies and distances measured on the 3rd, 4th, and 5th training days [escape latency: 3rd day: $F(1, 503) = 9.97$, $p = 0.0049$; 4th day: $F(1, 503) = 15.27$, $p = 0.0001$; 5th day: $F(1, 503) = 13.55$, $p = 0.0003$. Distance: 3rd day: $F(1, 503) = 3.93$, $p = 0.048$; 4th day: $F(1, 503) = 12.85$, $p = 0.0004$; 5th day: $F(1, 503) = 12.02$, $p = 0.0006$]. The prenatal exposure x sex interaction was highly significant when escape latency was the dependent variable [$F(1, 503) = 4.58$, $p = 0.0329$]. Examination of this interaction revealed that the detrimental effect of prenatal CPF exposure was stronger

among male than female offspring Male: [F(1,503) = 10.72, p = 0.0011;
Female: F(1,503) = 1.78, p = 0.1827].

Post-hoc pairwise comparisons confirmed that the performance of control male guinea pigs was significantly better than that of their female counterparts and that of CPF-exposed male offspring on the 4th and 5th training days (Figure 8). Because the effect of CPF was weak among female offspring, *post-hoc* pairwise comparisons revealed no significant differences between control and CPF-exposed female offspring on each day of training (Figure 8).

Results obtained during the acquisition phase of the MWM indicate that spatial learning in the MWM is sexually dimorphic among guinea pigs, as is among rats and mice, with males outperforming females. They also revealed that prenatal CPF exposure impaired spatial learning more significantly among male than female guinea pigs and, consequently, reduced the sexual dimorphism of the task.

5.3.4.2. Memory retention

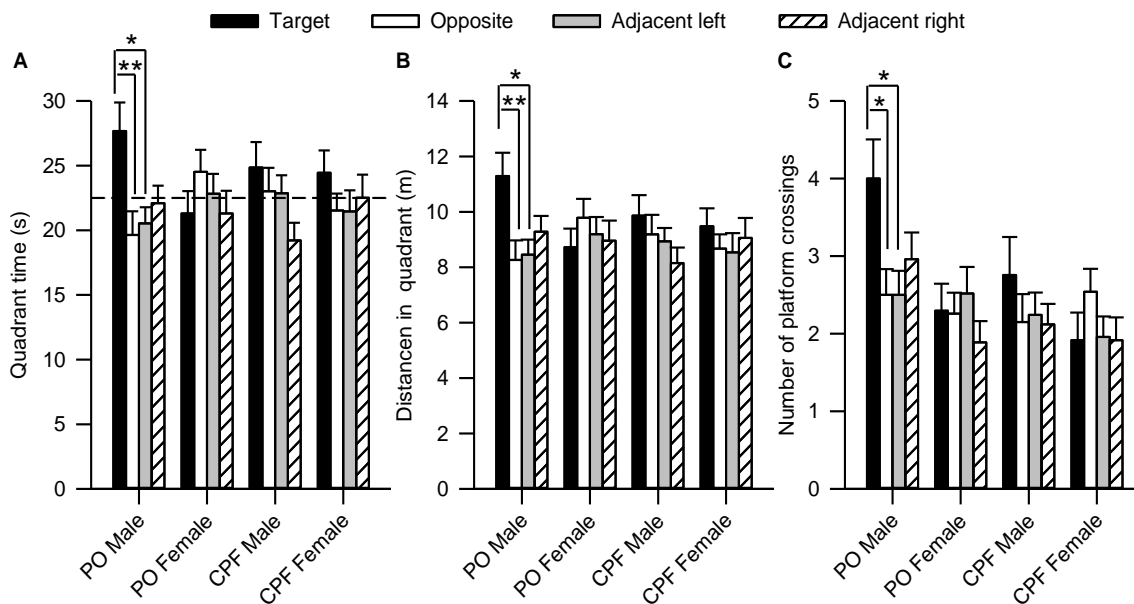
Spatial memory retention was assessed during a probe test performed 72 h after the last training session. In this test, the escape platform was removed from the pool and animals were allowed to swim freely for 90 s. Number of crossings of the platform area in the training quadrant as well as time and distance that was swum in that quadrant were taken as measures of memory retention.

Analysis of the total distance traveled during the first probe test showed that locomotor performance was not significantly affected by sex or prenatal exposure to CPF (Table 6). Analysis of time spent in the water maze quadrants by a three-way random

effect ANOVA revealed a significant main effect of quadrant [$F(3,397) = 2.89$, $p = 0.0352$] and a significant quadrant x prenatal exposure x sex interaction [$F(3,397) = 3.10$, $p = 0.0267$]. Analysis of the simple main effect of each factor in this interaction revealed that only male offspring that had been prenatally exposed to peanut oil spent more time in one of the quadrants [peanut oil males: $F(3,397) = 4.35$, $p = 0.0049$; CPF males: $F(3,397) = 2.37$, $p = 0.0701$; peanut oil females: $F(3,397) = 0.81$, $p = 0.4860$; CPF females: $F(3,397) = 0.60$, $p = 0.6171$]. The quadrant x prenatal exposure x sex interaction approached significance when distance swam in the quadrants was the dependent variable [$F(3,397) = 2.20$, $p = 0.087$]. Based on a within-subject comparison using a one-way random effect ANOVA followed by the Dunnett's *post-hoc* test, only offspring male prenatally exposed to peanut oil spent significantly more time and swam longer distances in the quadrant where the platform used to be located than in the other quadrants (Figure 9A,B).

To assess search accuracy during the probe tests, number of crossings of the platform area in the training quadrant was compared to number of crossings of equivalent areas positioned in the center of the non-training quadrants. The three-way random effect ANOVA revealed a significant main effect of sex [$F(1,397) = 5.36$, $p = 0.0211$] and a significant sex x quadrant interaction [$F(3,397) = 3.15$, $p = 0.0249$]. The one-way random effect ANOVA followed by the Dunnett's *post-hoc* test indicated that only male offspring that had been prenatally exposed to peanut oil crossed the platform position in the training quadrant more often than the same area in the other quadrants (Figure 9C).

Figure 9: Performance of prepubertal guinea pigs in a probe test performed 72 hours after completion of the acquisition phase of the Morris water maze training.



A. Time animals spent in each of the four quadrants of the pool. **B.** Distance traveled in each quadrant of the pool. **C.** Number of crossings of areas corresponding to the platform area virtually positioned in the center of each quadrant of the pool. As described in the text, a three-way random effect ANOVA revealed that prenatal exposure and sex had no significant main effect on any of the parameters measured during the probe test. There was also no significant prenatal exposure x sex interaction. Based on a within-subject comparison, a one-way random effect ANOVA followed by the Dunnett's post-hoc test revealed that only male offspring prenatally exposed to peanut oil (PO) showed significant preference towards the target quadrant and the platform area in the center of the target quadrant (*, $p < 0.05$; **, $p < 0.01$). Data points and error bars represent mean and SEM, respectively, of results from the same animals as in Figure 3.

According to the three-way random effect ANOVA, prenatal exposure to CPF and sex had no significant main effect on the % of time or distance the animals swam in the wall zone during the first probe test. However, there was a borderline significant prenatal exposure x sex interaction for the % of time [$F(1,79) = 4.02$, $p = 0.0484$]. Analysis of the simple main effect of each factor in this interaction indicated that offspring males prenatally exposed to peanut oil spent less time and swam shorter distances in the wall zone than did the animals in the other groups [prenatal exposure to peanut oil: $F(1,79) = 7.01$, $p = 0.0098$ for % time and $F(1,79) = 5.61$, $p = 0.0203$ for % distance; sex:

$F(1,79) = 6.25$, $p = 0.0145$ for % time and $F(1,79) = 5.84$, $p = 0.018$ for % distance].

Post-hoc multiple comparisons revealed that during the first probe test the thigmotactic behavior of male offspring that had been prenatally exposed to peanut oil was less pronounced than that of their female counterparts (Table 6). They also indicated that thigmotaxis was less pronounced among male offspring prenatally exposed to peanut oil than those exposed to CPF (Table 6).

Table 6: Overall swimming performance and thigmotactic behavior of male and female offspring prenatally exposed to PO or CPF.

	Peanut Oil		CPF	
	Male	Female	Male	Female
Total distance (m)	37.30 ± 0.49	36.67 ± 0.66	36.15 ± 0.67	35.8 ± 0.68
% Time in wall zone	23.3% ± 2.8 ^{*,†}	33.5% ± 2.2	35.1% ± 3.3	35.7 % ± 3.5
% Distance in wall zone	21.3% ± 2.8 [*]	29.6% ± 2.0	31.4% ± 3.1	32.6% ± 3.2

Thigmotactic behavior was defined as the percentage of time and the percentage of distance animals swam in a 20 cm-wide zone from the wall of the pool. Results are presented as mean ± SEM of measures obtained during the first probe test from the same animals as in Figure 5. According to the random effect ANOVA model followed by Tukey-Kramer *post-hoc* test for pairwise comparisons: *, $p < 0.05$ (peanut oil-exposed males compared to peanut oil-exposed females); †, $p < 0.05$ (peanut oil-exposed males compared to CPF-exposed males).

Results from the probe tests were not conclusive regarding the potential detrimental effects of prenatal exposure to CPF on memory retention. During the test, performed 72 h after the completion of training, only control male offspring presented signs of memory retention; they preferentially swam in the area where the platform used to be located. The lack of preference toward the target quadrant seen among CPF-exposed male offspring could be interpreted as evidence of significant memory deficits. Alternatively, it could have resulted from the fact that these animals did not reach the same near-asymptotic level of learning at the end of the acquisition phase as their control counterparts.

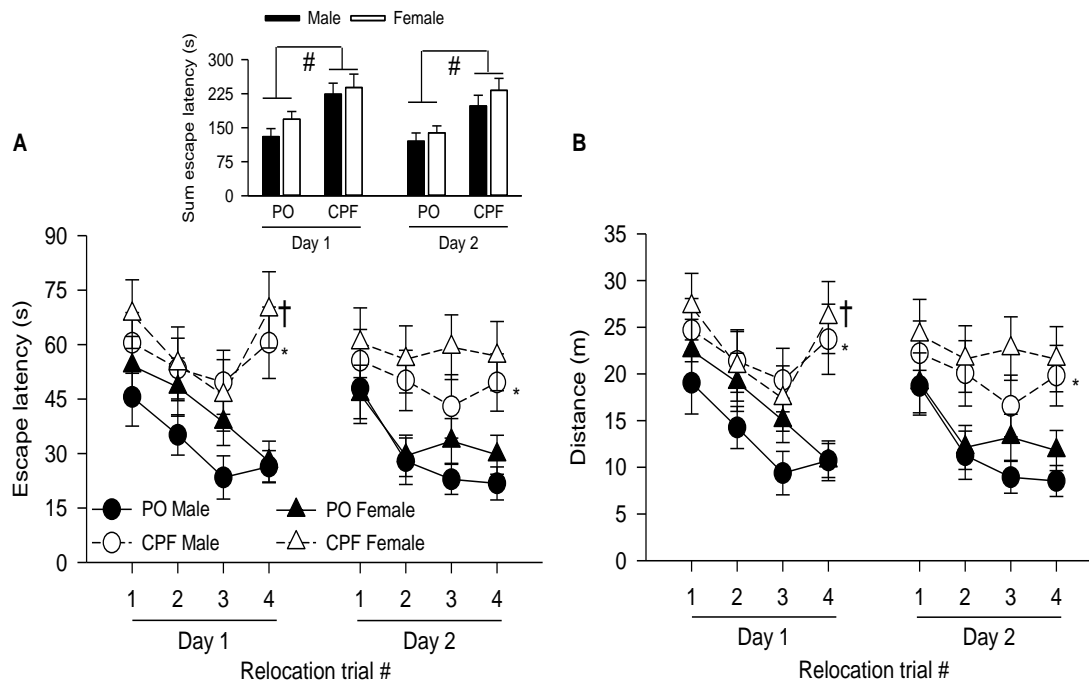
5.3.4.3. Platform relocations

The primary purpose of the relocation tests conducted here was to evaluate spatial learning of the animals after they had the opportunity to master the contextual and the procedural requirements of the task during the acquisition phase. The platform relocation tests were conducted in two consecutive days using the same protocol as that described for the acquisition phase with the exception that the inter-trial interval (ITI) lasted 1 h instead of 15 s.

During the first platform relocation test, there was a significant main effect of trial on escape latency [$F(3,340) = 4.49, p = 0.0042$] and distance traveled to reach the platform [$F(3,340) = 6.51, p = 0.0003$]. Sex had no significant main effect on escape latency or distance. However, there was a near-significant main effect of prenatal exposure on distance [$F(1,24) = 5.66, p = 0.056$] and a significant main effect of prenatal exposure to CPF on escape latency [$F(1,24) = 6.04, p = 0.0216$] (Figure 10A inset). There was also a significant trial x prenatal exposure interaction [escape latency: $F(3,340) = 2.94, p = 0.0334$; distance: $F(3,340) = 2.91, p = 0.0348$]. Examination of the simple main effect of each factor in this interaction revealed a significant effect of exposure on the 4th trial [escape latency: $F(1,340) = 13.47, p = 0.0003$; distance: $F(1,340) = 12.87, p = 0.004$]. *Post-hoc* analysis indicated that on the 4th trial male and female offspring prenatally exposed to CPF swam longer distances and took longer to find the hidden platform than did male and female offspring prenatally exposed to peanut oil (Figure 10A,B). During the second platform relocation test, trial also had a significant main effect on escape latency [$F(3,336) = 3.81, p = 0.0104$] and distance [$F(3,336) = 4.73, p = 0.0030$]. There was no significant main effect of sex on the

performance of the animal. However, prenatal exposure to CPF had a near-significant main effect on the distance traveled to reach the hidden platform [$F(1,24) = 4.13$, $p = 0.0533$] and a significant main effect on the escape latency [$F(1,24) = 4.90$, $p = 0.0366$] (Figure 10A inset). There was no significant sex x prenatal exposure interaction. *Post-hoc* analysis of the data revealed that escape latency and distance to reach the hidden platform during the 4th trial were longer among male offspring exposed

Figure 10: Effect of prenatal exposure to CPF on performance of guinea pigs trained in two platform relocation tests.



After the acquisition phase of the MWM and two probe tests, animals were given four trials interspaced by 1 h to find the relocated platform in each of two consecutive days. As described in the text, a random effect ANOVA revealed a significant main effect of prenatal exposure on the escape latency (A) measured during the 1st and 2nd relocation tests and a significant interaction between prenatal exposure and trial. Analysis of the simple main effect in the significant interaction revealed that escape latency was significantly longer among offspring prenatally exposed to CPF than among those prenatally exposed to peanut oil (PO. Inset: #, $p < 0.05$). The random effect ANOVA also revealed a near-significant main effect of the prenatal exposure to CPF on distance traveled to find the platform in the 1st and 2nd relocation tests ($p = 0.056$ and $p = 0.053$, respectively). (B). Data points and error bars represent mean and SEM, respectively, of results obtained from 22-26 animals/group. According to the Tukey-Kramer post-hoc test for multi-group comparisons: *, $p < 0.05$ PO males vs. CPF males; † $p = 0.05$ PO females vs. CPF females.

to CPF than among those exposed peanut oil (Figure 10A,B). The finding that CPF-exposed offspring also presented significant deficits in the platform relocation tests during which the animals had to learn to find the hidden platform in different locations using the same contextual information as that used during the acquisition phase supports the notion that spatial learning is impaired in animals prenatally exposed to CPF.

5.3.5. Electrophysiological analysis of synaptic transmission in male guinea pigs prenatally exposed to CPF

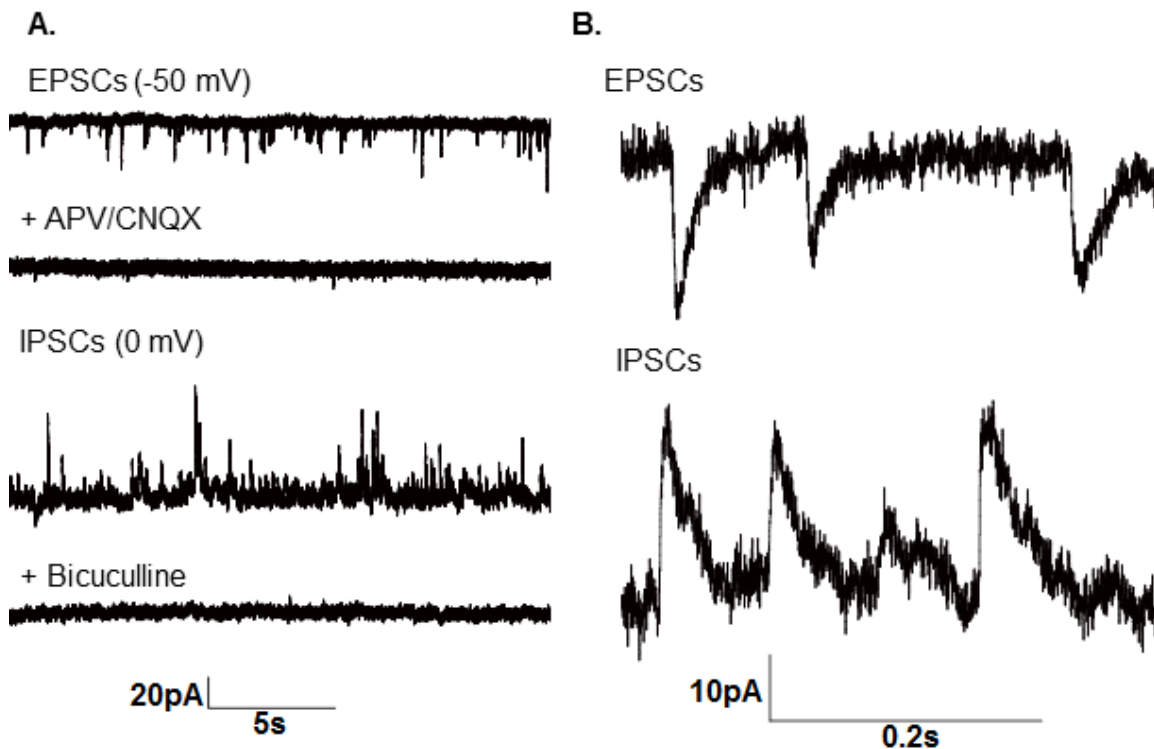
The patch-clamp technique is the gold-standard electrophysiological assay for evaluation of the activity of voltage- and ligand-gated channels and of synaptic transmission at the single-cell level in brain slices (Dunlop et al., 2008). Therefore, it was used in the present study to determine whether cognitive deficits in guinea pigs prenatally exposed to CPF were associated with deficits of glutamatergic transmission and/or increases in GABAergic transmission in CA1 pyramidal neurons.

The present electrophysiological study was limited to male offspring, because the detrimental effect of prenatal exposure of CPF on spatial learning was found to be more pronounced among males than females (see Figure 8). In addition, spatial learning in the MWM has been associated with evidence of a transient increase in synaptogenesis within the hippocampus (Eyre et al., 2003; Ramirez-Amaya et al., 1999). Spatial learning-induced increase in synaptogenesis appears to last for at least seven days after training in the MWM (Eyre et al., 2003; O'Malley et al., 2000) and is no longer detected 30 days after training in the MWM (Ramirez-Amaya et al., 2001). Therefore, to minimize the influence of the MWM training on synaptic transmission, EPSCs and IPSCs were recorded from patch-clamped neurons in hippocampal slices obtained from male guinea

pigs between two and four weeks after completion of the MWM tests. Due to the technically demanding and low-throughput nature of the patch-clamp technique, analysis of synaptic transmission was limited to 16 control and 19 CPF-exposed offspring.

The whole-cell configuration of the patch-clamp technique was used to record spontaneous EPSCs from CA1 pyramidal neurons voltage clamped at -50 mV, the reversal potential for Cl⁻-permeable channels, while spontaneous IPSCs were recorded at 0 mV, the reversal potential for glutamate-gated receptor channels. Thus, through manipulation of the membrane potential, spontaneous excitatory and inhibitory transmission could be recorded separately from the same neuron. In the present experiment, inward synaptic currents recorded at -50 mV were glutamatergic in nature, because they were fully inhibited after superfusion of the hippocampal slices with ACSF containing the NMDA receptor antagonist APV (100 μM) plus the AMPA receptor antagonist CNQX (10 μM) (Figure 11A). While both antagonists were required for a complete block, these currents were primarily mediated by AMPA receptors because the mean decay-time constant (τ_d) was relatively short (~ 13 ms) and the presence of Mg²⁺ in the ACSF blocks the slower NMDA receptor-mediated events (Ascher and Nowak, 1988). Outward synaptic currents recorded from CA1 pyramidal neurons voltage clamped at 0 mV were GABAergic in nature because they were inhibited by superfusion of the hippocampal slices with ACSF containing the GABA_A antagonist bicuculline (10 μM) (Figure 11A).

Figure 11: Pharmacological characterization of spontaneous postsynaptic currents recorded from CA1 pyramidal neurons.

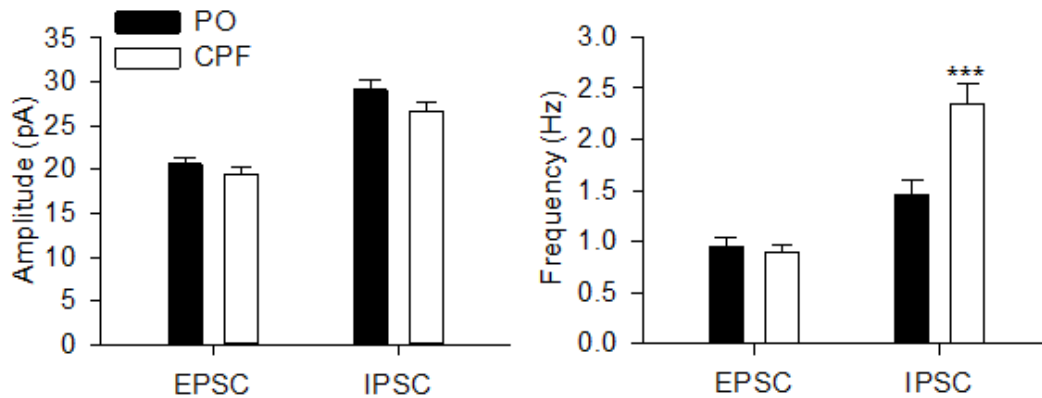


Representative recording samples of spontaneous synaptic currents obtained from CA1 pyramidal neurons in hippocampal slices from control male guinea pigs. (A.) EPSC and IPSC recordings before and during application of the receptor antagonists. Synaptic currents recorded at -50 mV were inhibited by superfusion of the hippocampal slice with ACSF containing APV (100 μ M) and CNQX (10 μ M). Synaptic currents recorded at 0 mV were blocked by bicuculline (10 μ M). (B.) Magnification of individual EPSC and IPSC events from condensed traces displayed in panel A.

Analysis of spontaneous synaptic activity recorded from CA1 pyramidal neurons in hippocampal slices from offspring that had been prenatally exposed to peanut oil (control) or CPF revealed no significant effect of CPF exposure on mean EPSC amplitude or frequency (Figure 12A,B). Likewise, prenatal CPF exposure did not significantly affect mean IPSC amplitudes (Figure 12A) or the distribution of IPSC amplitudes (Figure 13A). However, the mean IPSC frequency recorded from CA1 pyramidal neurons was significantly higher among CPF-exposed offspring than control

offspring [(U = 178) medians = 1.997, 1.277; p < 0.001] (Figure 12 B). The cumulative distribution of IPSC inter-event intervals recorded from neurons of CPF-exposed offspring was also significantly shifted to the left of the cumulative distribution of inter-event intervals recorded from neurons of control animals [(U = 707.5) medians = 0.995, 0.999, p = 0.006] (Figure 13B). Neither the mean τ_d (PO = 21.12 \pm 0.9; CPF = 20.62 \pm 0.85) nor the distribution of τ_d (Figure 13C) was significantly affected by CPF exposure.

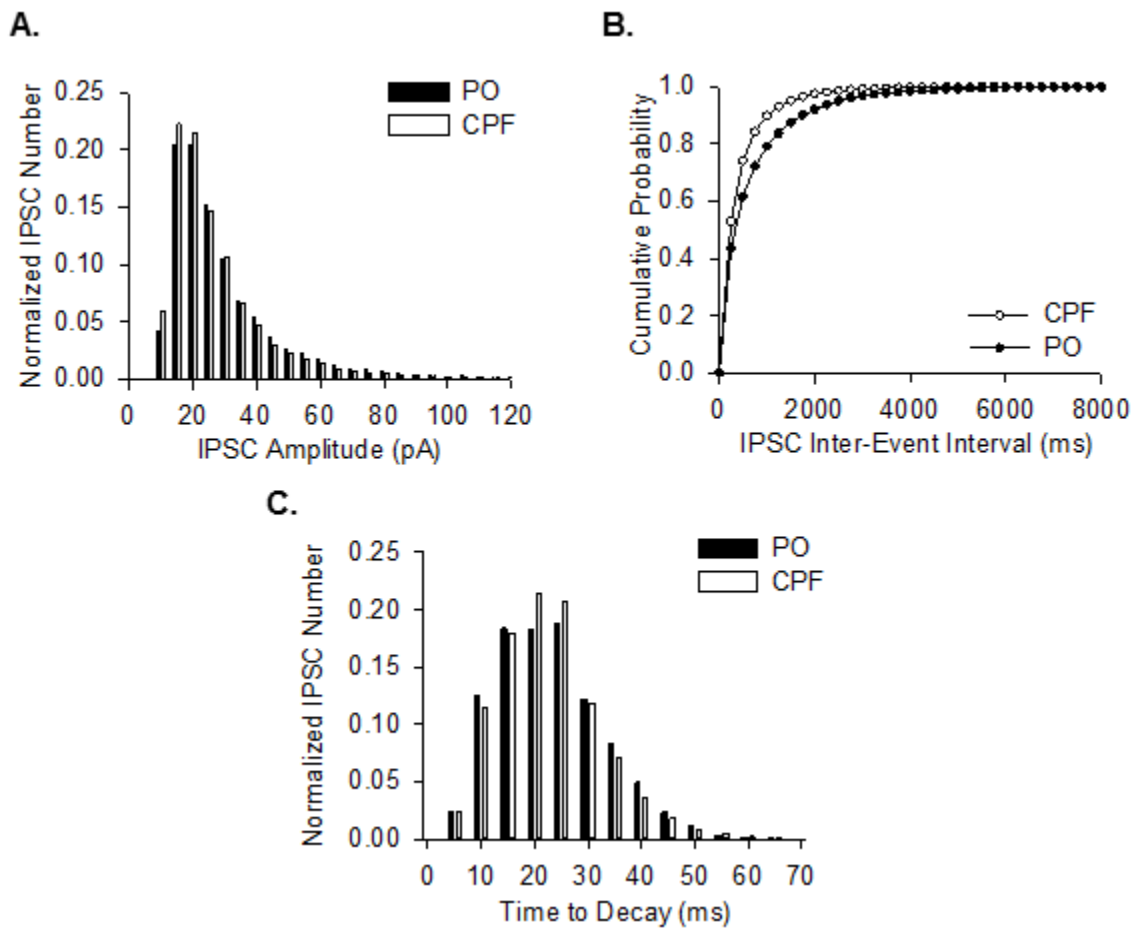
Figure 12: Mean EPSC and IPSC frequency and amplitude.



Graphs show electrophysiology data obtained from 19 CPF-exposed and 16 control male guinea pigs that were previously assessed in the MWM. EPSCs were recorded from pyramidal neurons at -50 mv from 17 CPF-exposed (29 recordings, one to three per animal) and 12 control (23 recordings, one to four per animal) guinea pigs. IPSCs were recorded at 0 mv from 13 CPF-exposed (30 recordings, one to four per animal) and 11 control (25 recordings, one to five per animal) guinea pigs. No significant differences were noted in mean EPSC or IPSC amplitude (A.). Likewise, no significant difference in mean EPSC frequency was observed (B.). Mean IPSC frequency however were significantly elevated in CPF-exposed offspring [(U = 178) medians = 1.997, 1.277; p ***<0.001]. Data are expressed as means \pm SEM.

To determine whether the higher IPSC frequency recorded from pyramidal neurons of CPF-exposed guinea pigs compared to control animals was presynaptic in origin; mIPSCs were recorded from neurons of CPF-exposed and control animals. mIPSCs were recorded from CA1 pyramidal neurons in hippocampal slices continuously

Figure 13: IPSC recordings obtained from the CA1 region of hippocampal slices from CPF- and PO-exposed male offspring.

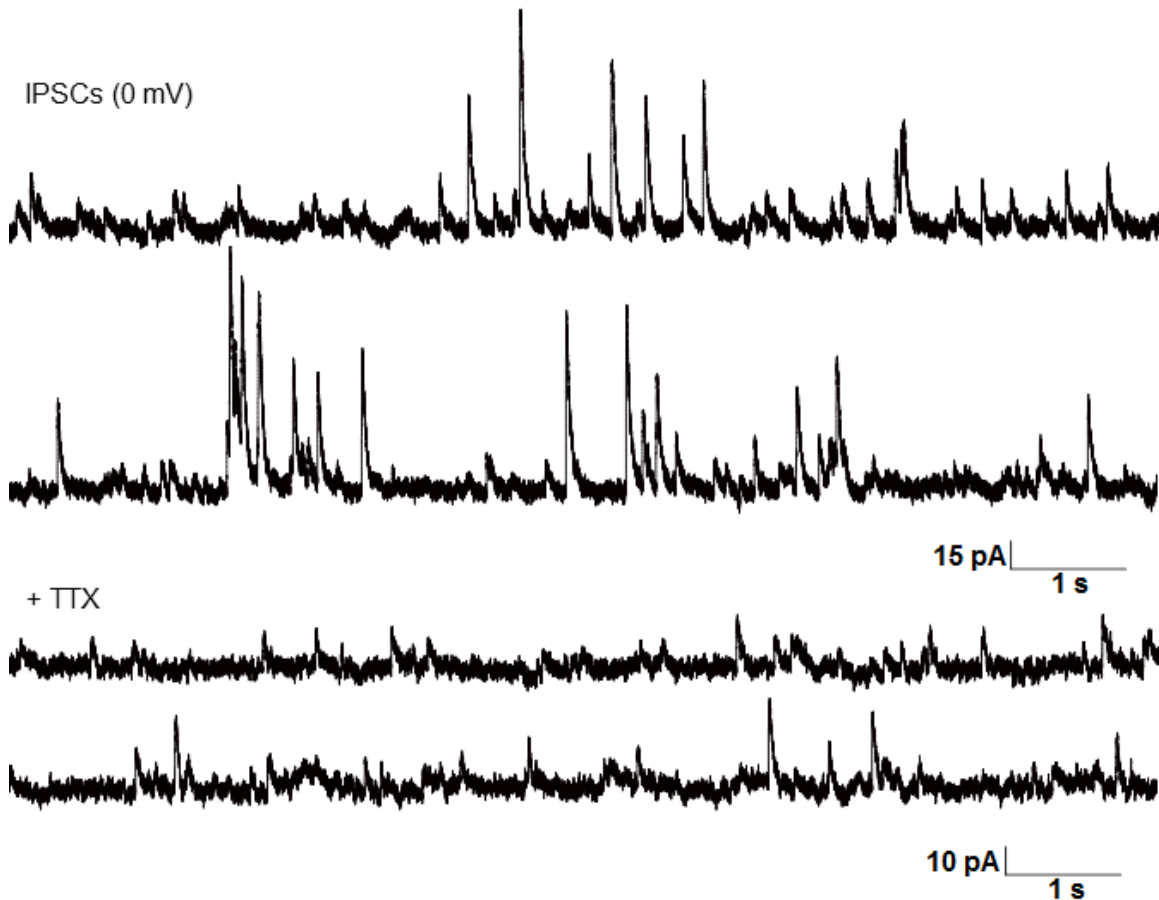


Graphs show electrophysiology data from 19 CPF-exposed and 16 control male guinea pigs that were previously assessed in the MWM. IPSCs were recorded at 0 mv from pyramidal neurons of 13 CPF-exposed (30 recordings, one to four per animal) and 11 control (25 recordings, one to five per animal) guinea pigs. There was no significant effect of CPF exposure on the distribution of IPSC amplitudes (A.) or the time to decay (C.). The cumulative distribution of IPSC inter-event intervals however was significantly shifted towards shorter intervals in CPF-exposed offspring [(U = 707.5) medians = 0.995, 0.999, p = 0.006].

superfused with ACSF containing the Na⁺-channel blocker tetrodotoxin (TTX, 0.2 μM).

Superfusion of the slices with TTX-containing ACSF suppressed the large-amplitude, action potential-dependent IPSCs (Figure 14). The mean amplitude, mean τ_d , and τ_d distribution of mIPSCs were not remarkably different between CPF-exposed and control

Figure 14: TTX application suppresses action potential dependent synaptic transmission.

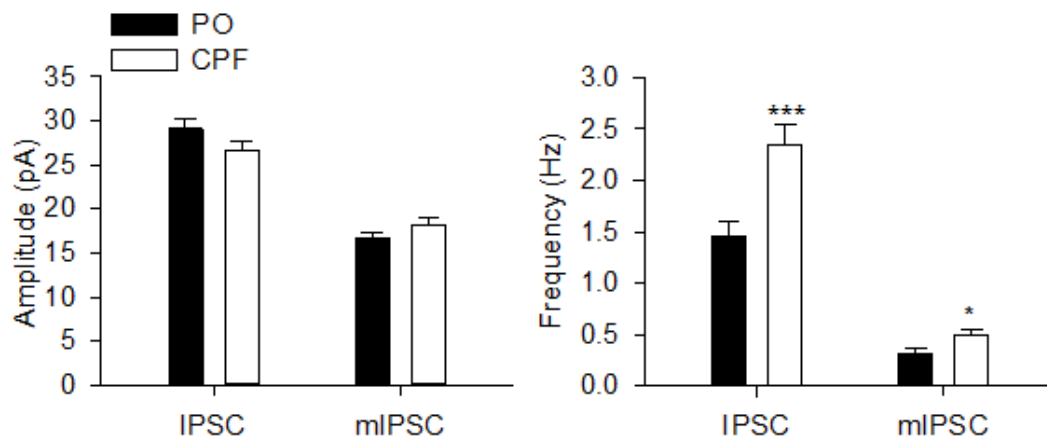


Representative recording samples of synaptic currents obtained from a CA1 pyramidal cell in a hippocampal slice of a control male guinea pig. Currents were recorded at 0 mV (IPSCs) for five minutes. Following this recording, the slice was superfused with ACSF containing TTX (0.2 μ M) for five minutes, after which time recordings continued for an additional 5 min in the presence of TTX. TTX abolished the high-amplitude, action potential-dependent IPSCs.

guinea pigs (Figures 15A. and 16C.). Likewise, there was no significant difference between the distribution of miniature event amplitudes recorded from neurons of control and CFP-exposed animals (Figure 16A). In contrast, the mean frequency of mIPSCs recorded from neurons of CPF-exposed guinea pigs was significantly higher than that recorded from neurons of control animals [$F(1,28) = 6.54$, $p = 0.016$] (Figure 15B). There was also a significant shift towards a shorter duration in the cumulative distribution

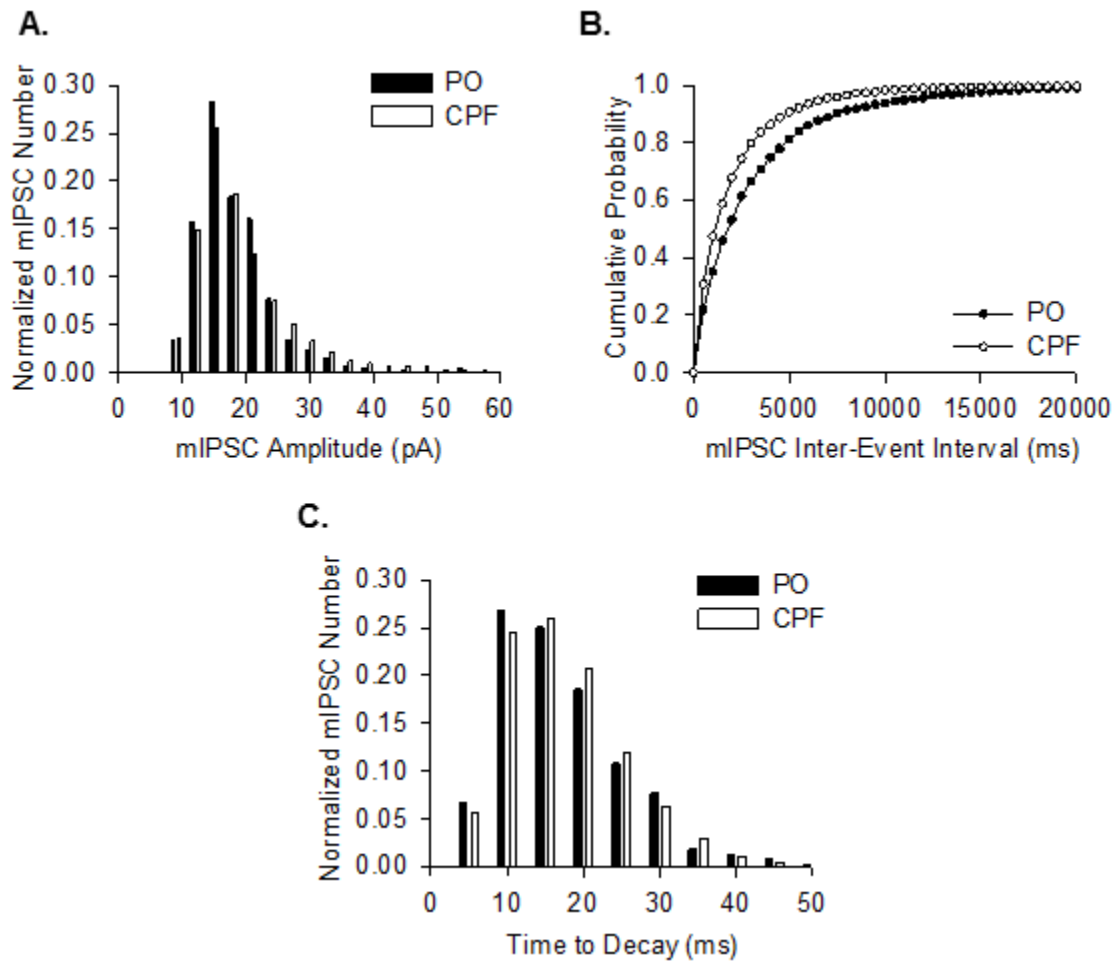
of mIPSC inter-event intervals recorded from neurons of CPF-exposed animals [(U = 729.5) medians = 0.989, 0.952; p = 0.005] (Figure 16B). Taken together, these results reveal that prenatal exposure to CPF results in a significant increase of GABAergic transmission onto CA1 pyramidal neurons and that this effect is unlikely related to increased postsynaptic GABA_A receptor expression or facilitation of GABA-induced activation of postsynaptic GABA_A receptors.

Figure 15: Mean mIPSC and IPSC frequency and amplitude.



Graphs show mean IPSCs and mean mIPSCs from animals previously evaluated in the MWM. IPSCs were recorded at 0 mv from 13 CPF-exposed (30 recordings, one to four per animal) and 11 control (25 recordings, one to five per animal) guinea pigs, while mIPSCs were recorded from six CPF-exposed (16 recordings, two to three per animal) and seven control (14 recordings, one to three per animal) guinea pigs. No significant differences were noted in mean IPSC or mIPSC amplitude (A.). Mean IPSC and mIPSC frequency however were significantly elevated in CPF-exposed offspring {[U = 178) medians = 1.997, 1.277; p ***<0.001], [F(1,28) = 6.54, *p = 0.016], respectively} (B.). Data are expressed as means ± SEM.

Figure 16: Analysis of mIPSCs recorded from CA1 pyramidal neurons of hippocampal slices from CPF- and PO-exposed male offspring.



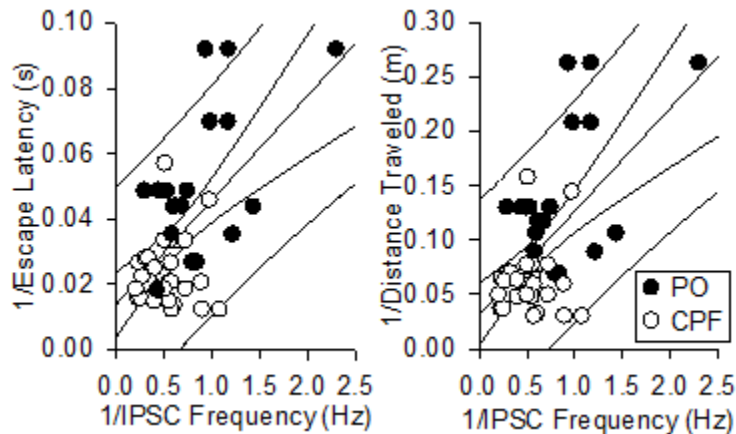
Electrophysiology data obtained from six CPF-exposed (16 recordings, two to three per animal) and seven control (14 recordings, one to three per animal) guinea pigs. There was no significant effect of CPF exposure on the distribution of IPSC amplitudes (A.) or τ_d (C.). The cumulative distribution of IPSC inter-event intervals however was significantly shifted towards shorter intervals in CPF-exposed offspring [(U = 729.5) medians = 0.989, 0.952, p = 0.005].

5.3.6. Correlation of MWM spatial learning deficits and IPSC frequency

Electrophysiological assessment of CPF-exposed guinea pigs revealed significantly increased GABAergic input to the CA1 region of the hippocampus as compared to control animals. With no observed concomitant increase in glutamatergic input, this increased inhibitory tone dampens the output of the excitatory CA1 circuit.

Considering that increases in GABAergic inhibition lead to deficits in spatial learning and memory (Timic et al., 2013) such as those observed in the MWM behavioral cohort (see sections 5.3.4.1. to 5.3.4.2.), the relationship between GABAergic IPSCs and MWM performance was examined. The platform relocation phase of the MWM testing was chosen for this assessment because it precludes the contextual and procedural requirements of the acquisition phase. This allows for a more refined isolation of hippocampal-dependent spatial learning.

Figure 17: MWM second relocation performance and IPSC frequency correlation in CPF- and PO-exposed male offspring.



Results of Pearson product moment analysis for IPSC frequency versus escape latency and distance traveled in the second relocation phase of MWM testing. While IPSC data was collected from 13 CPF exposed and 11 PO guinea pigs, three animals in the vehicle control group were excluded from the MWM due to a procedural error resulting in their testing at an earlier PND. As such, correlations represent data generated from 13 CPF exposed and 8 PO guinea pigs. There is a significant positive relationship between relocation two's mean escape latency (**A**) and distance traveled (**B**) with mean IPSC frequency $\{[r = 0.573, p < 0.0001], [r = 0.582, p < 0.0001], \text{ respectively}\}$. A double reciprocal plot was used to decrease the spread of the two data sets.

The relationship between mean escape latency, mean distance traveled, and mean CA1 IPSC frequency was examined (Figure 17). Pearson product moment correlation analysis revealed a significant positive relationship between relocation two's mean

escape latency ($r = 0.573$, $p < 0.0001$) and distance traveled ($r = 0.582$, $p < 0.0001$) with CA1 IPSC frequency. As such, increased GABAergic input to the CA1 pyramidal layer is associated with spatial learning deficits in the MWM.

5.4. Discussion

The present study is the first to provide direct evidence of a direct relationship between prenatal exposure to CPF and cognitive deficits in guinea pigs, which, like humans, are considered a precocial species. As shown here, prenatal exposure of guinea pigs to sub-acute doses of CPF had no significant effect on locomotor activity. In addition, it did not affect habituation, which is a form of non-associative memory. However, it significantly impaired spatial learning. This impairment was positively associated with increased inhibitory GABAergic input to the CA1 pyramidal region of the hippocampus. The implications of these findings are discussed in the sections that follow.

5.4.1. Effect of prenatal exposure of guinea pigs to CPF on locomotor activity and non-associative learning

Based on measurements of body weight, female guinea pigs prenatally exposed to CPF showed at prepubertal ages signs of slowed growth. However, neither their locomotor activity nor their locomotor habituation differed from those of control female guinea pigs. Body weight, locomotor activity, and locomotor habituation of prepubertal male guinea pigs were not significantly altered by the prenatal exposure to CPF. Locomotor habituation, a form of non-associative memory assessed here as a decrement in locomotor activity following repeated exposure of the animals to the same

environment, and locomotion are controlled in part by cholinergic activity in the hippocampus (Leussis and Bolivar, 2006). Specifically, when microinfused in the hippocampus muscarinic agonists or AChE inhibitors increase while muscarinic antagonists decrease locomotor habituation and activity in open fields (Izquierdo et al., 1992; Zheng et al., 1983). Thus, the findings that locomotor activity and habituation are comparable between control and CPF-exposed offspring suggest that the prenatal exposure to CPF did not result in significant changes in hippocampal cholinergic activity.

5.4.2. Spatial learning in the MWM is sexually dimorphic among control guinea pigs

Sexual dimorphism of spatial learning has been described in different animal species, with males outperforming female in most instances (Berger-Sweeney et al., 1995; Bimonte et al., 2000; Perrot-Sinal et al., 1996). However, the present study is the first to demonstrate that control pre-pubertal male guinea pigs performed significantly better than their female counterparts during the acquisition phase of the MWM. Since learning in the MWM is a hippocampus-dependent task (Chersi and Burgess, 2015), it is likely that the sexual dimorphism of the hippocampal morphology in guinea pigs contributes to the sex differences observed here in the MWM (Bartesaghi et al., 2003; Guidi et al., 2006).

Previous studies missed the sexual dimorphism of the learning performance of prepubertal guinea pigs in the MWM (Byrnes et al., 2004; Richardson et al., 2002). Numerous factors, including size of the tank, size and architecture of the platform, and duration of the training trials, influence the performance of rodents in this task

(Vorhees and Williams, 2006). The large tank, the two-step platform that facilitates climbing, and the long duration of the trials in the present study may have allowed for the animals to reach their best level of performance and, thereby, may have contributed to unmasking learning differences between male and female guinea pigs.

5.4.3. Learning and memory deficits in guinea pigs prenatally exposed to CPF

The sexual dimorphism observed during the acquisition phase of the MWM task in control guinea pigs was absent in guinea pigs prenatally exposed to CPF. This was due to the fact that during the acquisition phase of the task learning impairment was more severe among pre-pubertal male than female guinea pigs prenatally exposed to CPF. This finding is in line with reports that developmental exposure to CPF during the end of gestation and early postnatal ages abolishes the sex dimorphism of spatial learning in rats and mice (Aldridge et al., 2005; Johnson et al., 2009; Levin et al., 2001). In addition, this finding is of major interest because the correlation between prenatal CPF exposure and cognitive deficits in children is generally stronger among boys than girls (Horton et al., 2012; Marks et al., 2010; Rauh et al., 2012).

In the classical version of the MWM, animals first learn that they have to swim in order to escape onto a hidden platform from which they can be rescued (Izquierdo et al., 2006). Mechanisms regulating this procedural learning do not appear to be affected by the prenatal exposure to CPF because the performances of all animals were comparable in the three first days of training in the MWM. Following the procedural learning, animals learn to use the extra-maze cues to triangulate the position of the hidden platform

and guide themselves towards it (Izquierdo et al., 2006). The finding that learning differences between CPF-exposed and control offspring became significant on days four and five of training in the MWM suggests that the prenatal exposure to CPF had a significant detrimental effect on spatial learning. This notion is supported by the finding that CPF-exposed offspring also presented significant deficits in the platform relocation tests during which the animals had to learn to find the hidden platform in different locations using the same contextual information as that used during the acquisition phase. The platform relocation tests performed here differed from the more traditional delayed match-to-sample task used to evaluate episodic-like memory, because here the animals were placed on the platform 15 s prior to the first trial each day. To accurately evaluate episodic-like memory, animals would have been allowed to search randomly for the platform in the first trial and tested in a second trial for their ability to process and retain the spatial information needed to find the platform in the new position (Vorhees and Williams, 2006). Nevertheless, the results obtained from the platform relocation tests suggest that episodic-like memory may also be impaired in guinea pigs as it is in children prenatally exposed to CPF (Horton et al., 2012; Rauh et al., 2012).

Spatial learning deficits presented by male offspring prenatally exposed to CPF could be explained at least in part by increased thigmotaxis, which has been shown to inhibit acquisition of spatial relationships of contextual cues (Acheson et al., 2011). Increased thigmotactic behavior can be a result of increased anxiety (Treit and Fundytus, 1988) and/or damage to brain regions, such as the thalamus and the dorsal medial striatum, which are known to play a central role in the regulation of spatial search strategies (Cain and Boon, 2003). In the case of CPF-exposed female guinea pigs, spatial

learning deficits cannot be accounted for by increased thigmotactic behavior, because it was comparable to that of control female offspring. Spatial learning in the MWM is hippocampal-dependent but is also modulated by inputs from numerous brain regions, including the prefrontal cortex, the striatum, and the amygdala (Pooters et al., 2016). Of interest, a recent resonance magnetic imaging study of female guinea pigs prenatally exposed to CPF revealed that diffusion measures indicative of reduced white matter integrity within the striatum and amygdala correlated with their poor spatial learning performance in the MWM (Mullins et al., 2015).

Results from the probe tests were not conclusive regarding the potential detrimental effects of prenatal exposure to CPF on memory retention. During the first test, performed 72 h after the completion of training, only control male offspring presented signs of memory retention; they preferentially swam in the area where the platform used to be located. The lack of preference toward the target quadrant seen among CPF-exposed male offspring could be interpreted as evidence of significant memory deficits. Alternatively, it could have resulted from the fact that these animals did not reach the same near-asymptotic level of learning at the end of the acquisition phase as their control counterparts.

5.4.4. Increased GABAergic transmission in CA1 pyramidal neurons of guinea pigs prenatally exposed to CPF

The results of the present study indicate that *in utero* sub-acute CPF exposure has a lasting impact on the GABAergic system. A net increase in the ratio of inhibition to excitation was discovered in the CA1 pyramidal neurons of adult male offspring that had

been prenatally exposed to CPF. The mean frequency of spontaneous IPSCs and of mIPSCs recorded from CA1 pyramidal neurons was significantly higher than that recorded from neurons of control animals. Prenatal exposure to CPF had no significant effect on the amplitude or kinetics of the IPSCs and mIPSCs. The increase in GABAergic transmission was accompanied by no significant change in mean glutamatergic EPSC amplitude or frequency.

The increase in the frequency of mIPSCs suggested that the effect of the prenatal exposure to CPF on GABAergic transmission was presynaptic in nature. The fact that the amplitudes of the mIPSCs were comparable between CPF-exposed and control animals indicated that there was no change in the expression or activity of postsynaptic GABA_A receptors.

The observed increase in IPSC frequency recorded from CA1 pyramidal neurons of CPF-exposed animals could be accounted for by an increased interneuron count or increased interneuron excitability. Interneurons moderate the activity of many local hippocampal circuits (reviewed by Chamberland and Topolnik, 2012) and pyramidal cells are their primary postsynaptic target (Sik et al, 1995; Cobb et al, 1997). As such, an increase in interneuron number would account for the observed increase in IPSC frequency in pyramidal neurons. Alternatively, an increase in interneuron firing would lead to the same conclusion. Increased interneuron activity could stem from a variety of mechanisms that have been associated with developmental CPF exposure including: increased excitatory nAChR expression (Eells and Brown, 2009), increased theta rhythm frequency (Muller et al., 2014; Klausberger et al., 2003), and augmented glial

transmission (Benedetti et al., 2010; Kang et al., 1998; Liu et al., 2004; Bowser and Khakh, 2004).

Regardless of mechanism, an increase in the ratio of inhibition to excitation within the CA1 pyramidal layer, the primary excitatory output of the hippocampus proper, dampens hippocampal transmission to surrounding cortical structures. This reduction is of prime importance as cross-signaling between the hippocampus and the entorhinal cortex is required for spatial learning (Hafting et al., 2005; Bonnevie, et al., 2013). In fact, disruption of this connection through GABAergic potentiation has been shown in mice to manifest deficits in spatial learning in the MWM similar to those observed in this study (Timic et al., 2013). Considering CPF-exposed animals had both significant spatial learning impairments in the MWM and an increased inhibition to excitation ratio in hippocampus, the guinea pig's behavioral and electrophysiology results were analyzed for correlation. A significant positive relationship between IPSC frequency, escape latency, and distance traveled was revealed during the electrophysiology cohort's most impaired testing day, the second day of platform relocations. It is thus plausible that the observed decrease in excitatory hippocampal output underlies the deficits in spatial learning observed in the behavioral cohort.

The results presented here demonstrate that prepubertal guinea pigs prenatally exposed to sub-acute doses of CPF present cognitive deficits that are reminiscent of those observed in children exposed *in utero* to this pesticide (Horton et al., 2012; Marks et al., 2010; Rauh et al., 2012). These behavioral deficits occur concurrently to and are correlated with an increase in the ratio of inhibition to excitation within the primary excitatory output of the hippocampus.

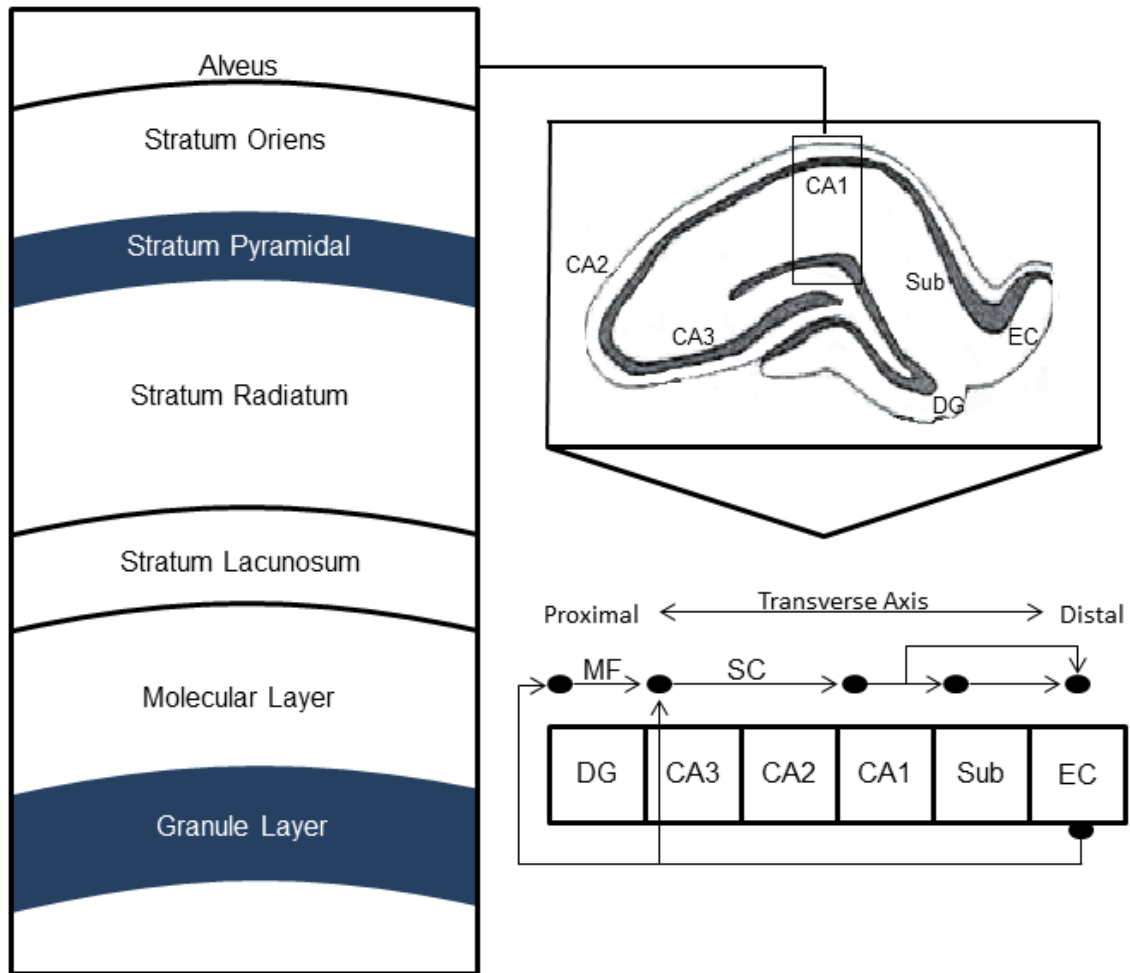
Chapter 6: Effects of Prenatal Exposure of Guinea Pigs to Chlorpyrifos on Neuronal and Glial Cells in the Hippocampus

6.1. Introduction

Cognitive deficits that have been observed in guinea pigs following prenatal exposure to the organophosphorus pesticide CPF resemble those observed in children prenatally exposed to this pesticide. In guinea pigs, the cognitive deficits correlated with an increase in GABAergic transmission on pyramidal neurons in the CA1 field of the hippocampus. However, mechanisms that can be important determinants of the effects of CPF on hippocampal functioning and cognition remain unknown.

The hippocampus is a heavily interconnected brain region involved in spatial learning and memory formation. The main excitatory neural circuit of the hippocampal formation is comprised of a mostly unidirectional loop, originating and terminating in the entorhinal cortex (Scharfman, 2007) (Figure 18). In brief, the entorhinal cortex projects to the dentate gyrus and CA3 pyramidal neurons via the perforant path. Together with mossy fiber input from the dentate gyrus, this signal is forwarded by the CA3 pyramidal neurons via Schaffer collaterals to CA1 pyramidal neurons. These neurons in turn project to the subiculum, which then forward the main hippocampal output back upon the entorhinal cortex, completing the loop. To assess if sub-acute developmental exposure to CPF induces morphological disruption of the hippocampal circuitry, markers of neuronal and glial populations were assessed immunohistochemically in the last region of this circuit within the hippocampus proper, the CA1 region, of guinea pigs prenatally exposed to CPF and control animals.

Figure 18: Diagrammatic Representation of Hippocampal Strata and Connectivity



A simplified diagrammatic representation of hippocampal lamination and connectivity. Legend: MF = Mossy fibers, SC = Schaeffer collaterals, DG = Dentate gyrus, Sub = Subiculum, EC = Entorhinal cortex.

The CA1 region of the hippocampus proper is a “laminated” structure organized into interconnected layers or strata. From the most proximal to the cortex, they consist of the stratum oriens (SO), stratum pyramidal (SP), stratum radiatum (SR), stratum lacunosum (SLM), molecular layer (ML), and granule layer (GL) (Figure 18).

Glutamatergic pyramidal neurons and granule cells of the dentate gyrus are located within the stratum pyramidale and granule layers, respectively, while GABAergic

interneurons are found throughout all strata. In combination with GABAergic projections from the septum and entorhinal cortex, these interneurons form the primary inhibitory network in the hippocampus (Chamberland and Topolnik, 2012). Tight regulation of this network is required for proper routing of hippocampal information (Buzsaki and Chrobak, 1995) and is accomplished by excitatory and inhibitory synaptic mechanisms.

Modulation of the excitability of the hippocampal neuronal network is accomplished through a number of mechanisms, including: (i) strata- and age-dependent differences in the expression of interneuron cholinergic receptors (Alkondon and Albuquerque, 2004; Alkondon et al., 2007), (ii) feed-forward inhibition, and (iii) feedback inhibition (Kullman, 2011). In the hippocampus, nAChR-dependent stimulation of GABAergic neurons is layer-specific, as interneurons of the various strata express dissimilar levels of nAChR subtypes (Alkondon and Albuquerque, 2004).

The two most predominant nAChR subtypes expressed by hippocampal GABAergic interneurons are the $\alpha 7$ nAChRs, which mediate fast-inactivating, methyllycaconitine (MLA)-sensitive currents, and the $\alpha 4\beta 2$ nAChRs, which mediate slow-inactivating, dihydro- β -erythroidine hydrobromide (DH β E)-sensitive currents (reviewed in Albuquerque et al. 2009). This difference in receptors leads to varying degrees of excitation of GABAergic interneurons in different layers, and, consequently, induces either inhibition or disinhibition of CA1 pyramidal neurons in response to ACh (Alkondon and Albuquerque, 2004). For example, in adult rats, $\alpha 7$ nAChRs are highly expressed in SLM interneurons, while they are expressed at low levels in SP interneurons. As a result of these low levels, SP interneurons are negligibly activated in response to cholinergic stimuli. They are, however, stimulated in response to glutamate

released from axon collaterals of pyramidal neurons they synapse with. Glutamatergic pyramidal neurons synapse on parvalbumin-expressing SP interneurons (Sik et al., 1995). Thus, pyramidal neuron firing excites the interneurons and provides GABAergic perisomatic inhibition.

Inhibitory modulation of the GABAergic network occurs concurrently to excitatory mechanisms. Primarily, this results from the high degree of interconnectivity between interneurons via GABAergic synapses (Vida et al. 1998; Cobb et al, 1997). In fact, one calretinin-expressing subset of interneurons synapses only with other GABAergic cells (Gulyas et al., 1996). This interaction can lead to disinhibition at the pyramidal level through GABAergic signaling. Moreover, parvalbumin-expressing SP interneurons have been shown to form functional autapses (Pawelzik et al., 2003), which constitute a negative feedback loop.

In addition to these synaptic methods of modulation, glial cells have also been implicated in the control of the activity of GABAergic networks (Benedetti et al., 2011; Kang et al., 1998; Liu et al., 2004). Interneuron firing results in a GABA_B-mediated increase in intracellular Ca²⁺ concentrations in astrocytes (Kang et al., 1998). This increase leads to glial release of glutamate, which, in turn, stimulates GABAergic neurons via kainate receptor activation on the interneurons (Liu et al, 2004). Likewise, Ca²⁺-dependent release of the purinergic neurotransmitter ATP has been shown to potentiate interneurons but not pyramidal cell firing within the hippocampus through P2Y receptor activation (Bowser and Khakh, 2004).

To assess the potential effects of developmental exposure to CPF on the structural integrity of the hippocampus, immunohistochemistry (IHC) was performed in hippocampal slices from control adult male guinea pigs and adult male guinea pigs prenatally exposed to CPF. Specifically assessed were markers of neuron cell bodies (NeuN), white matter tracts (MBP), SP/GL interneurons (parvalbumin), interneurons that synapse primarily onto other interneurons (calretinin), SO/SR interneurons (calbindin), and glia (GFAP). Also assessed by means of IHC were expression of the enzyme that catalyzes the synthesis of acetylcholine (choline acetyltransferase or ChAT) and expression of $\alpha 7$ nAChRs. Finally, expression of a marker of neuroinflammation (ionized calcium-binding adapter molecule one or Iba1), the effects of which are detailed in chapter 1.3.3, was also assessed immunohistochemically. The results of these stains indicate that sub-acute developmental CPF exposure does not lead to significant changes in the structure or cholinergic innervation of the CA1 hippocampal field, but does result in chronic neuroinflammation.

6.2. Experimental Design

Dunkin Hartley guinea pig dams [CrI:(HA)Br] were injected sc once a day with CPF dissolved in peanut oil (25 mg/kg/day, 0.5 ml/kg) or peanut oil (0.5 ml/kg) for up to ten consecutive days beginning approximately on DG 53. Offspring were born on DGs 66 to 71, weaned on PNDs 18 to 23, and subsequently group housed by sex and treatment group.

As described in Chapter 3, on PNDs 71 to 87, six male pups per treatment group were anesthetized with 4% isoflurane and transcardially perfused with 1% then 4% paraformaldehyde (pH 7.4) formulated in phosphate buffered saline (PBS). The brains were excised, blocked, placed in 4% paraformaldehyde for five days, then PBS for two days, and finally sunk in 30% sucrose for at least five days prior to cryostat sectioning (Leica CM 1900; Leica Microsystems Inc., Deerfield, IL). Brains were frozen on dry ice, sectioned at 20 μ M, and plated on gelatin subbed slides. Sections in the range of -3.92 mM to -4.52 mM bregma (Paxinos and Watson, 1996) were used on study. Three sections spaced by 120 μ M were used per animal, per stain. Care was taken to standardize the anatomical positioning across all guinea pigs. Sections were then stained for structural (NeuN, MBP, GFAP), interneuron (calbindin, calretinin, parvalbumin), cholinergic (ChAT, α 7 nAChR) and neuroinflammation (Iba1) protein markers and developed with either DAB or florescent methods. Staining within the strata of the CA1 hippocampal field was then evaluated. To provide additional insight, a western blot was also conducted for the pro-inflammatory cytokine, TNF- α , on hippocampal extracts from an additional six guinea pigs per treatment group of the same PND range.

6.3. Results

Sub-acute *in utero* CPF exposure results in dampened hippocampal output and associated spatial learning deficits in adult male guinea pigs (Chapter 5). To assess if these effects occur concurrent to structural changes within the CA1 hippocampal field, the present IHC study was conducted. The study was limited to male offspring because, as presented in the previous Chapter, the detrimental effect of prenatal exposure of CPF

on spatial learning was found to be more pronounced among males than females. Further, due to paucity of primary antibodies raised specifically against guinea pig derived antigens, this analysis was conducted with primary antibodies raised against other species. To account for this, primary antibodies were first tested with western blotting to assess promiscuity and detection of bands of the appropriate molecular weight (data not shown). Additionally, negative controls absent primary antibody were used to assure secondary/tertiary antibody specificity (data not shown). Only primary antibodies that produced clean westerns at approximately the predicted molecular weight and secondary antibodies that, in the absence of the primary antibody, generated minimal to no staining were used in these experiments.

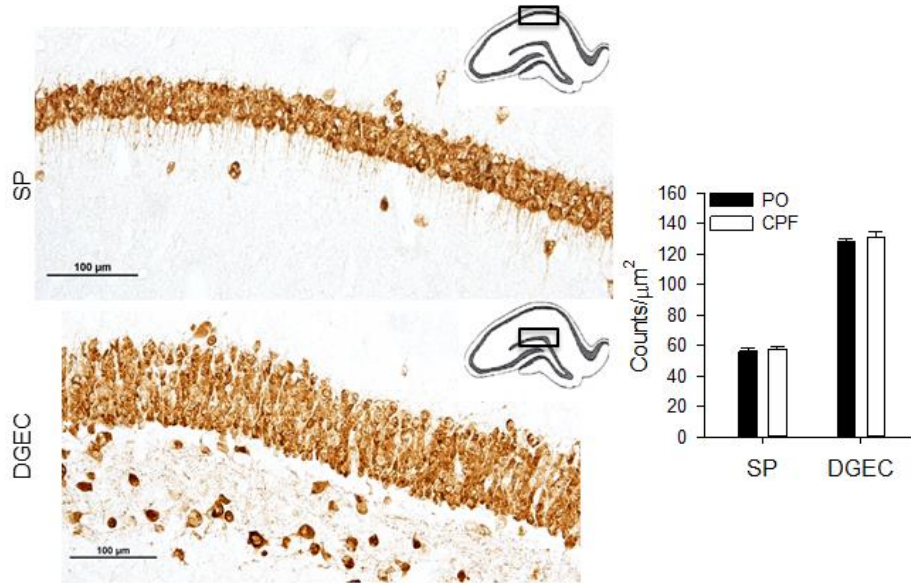
6.3.1. Assessment of neuronal and glial cells in the CA1 field of the hippocampus of control and CPF-exposed guinea pigs

NeuN-positive cells within the SP and the ectal limb of the dentate gyrus (DGEC) were quantified (Figure 19) and two-way ANOVAs with treatment and region as inter-animal factors showed no significant effect of prenatal CPF exposure on number of neurons. Moreover, the widths of the SP and DGEC did not vary significantly with prenatal CPF exposure.

To determine whether prenatal exposure to CPF affected myelination of axonal fibers, MBP immunoreactivity was analyzed in the SO and SR of the CA1 field of the hippocampus of control and CPF-exposed guinea pigs (Figure 20). Myelin basic protein (MBP) is a major constituent of the myelin sheath that surrounds axons in the nervous

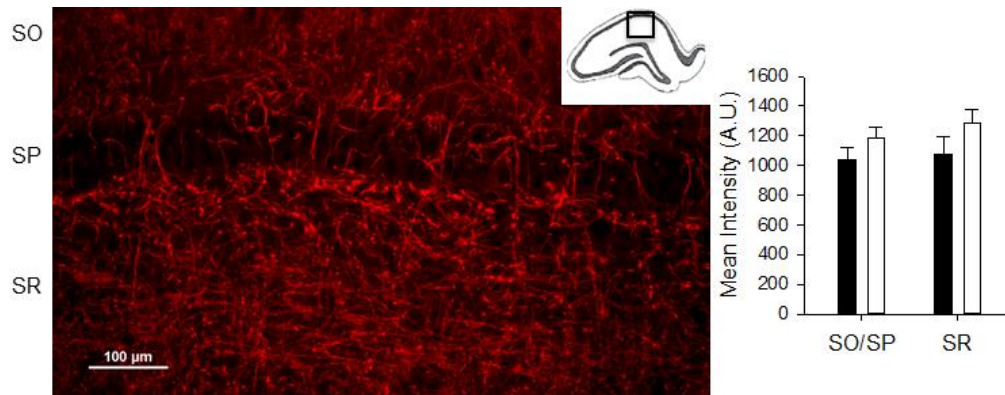
system. Two-way ANOVA with treatment and region as inter-animal factors revealed no significant effect of prenatal CPF exposure.

Figure 19: NeuN immunoreactivity in the CA1 hippocampal field of CPF-exposed and control male offspring.



Representative photomicrographs of NeuN immunoreactivity in the CA1 field of hippocampal slices from control guinea pigs. Magnification: 20x. CPF exposure did not significantly affect NeuN-positive cell counts. Data are displayed as mean \pm SEM.

Figure 20: MBP immunoreactivity in the CA1 hippocampal field of CPF-exposed and control male offspring.



Representative photomicrographs of MBP immunoreactivity in the CA1 field of hippocampal slices from control guinea pigs. Magnification: 20x. CPF exposure did not significantly affect MBP immunoreactivity. Data are displayed as mean \pm SEM.

Analysis of individual interneuron populations was also performed using antibodies that recognize the calcium binding proteins calbindin (CB), calretinin (CR), and parvalbumin (PV) (reviewed by Chamberland and Topolnik, 2012). Positively stained cells were quantified to assess if the observed presynaptic increase in CA1 GABAergic inhibition (see Chapter 5 section 5.3.5.) within the hippocampus of adult guinea pigs prenatally exposed to CPF is due to a relative increase in the number of GABAergic interneurons.

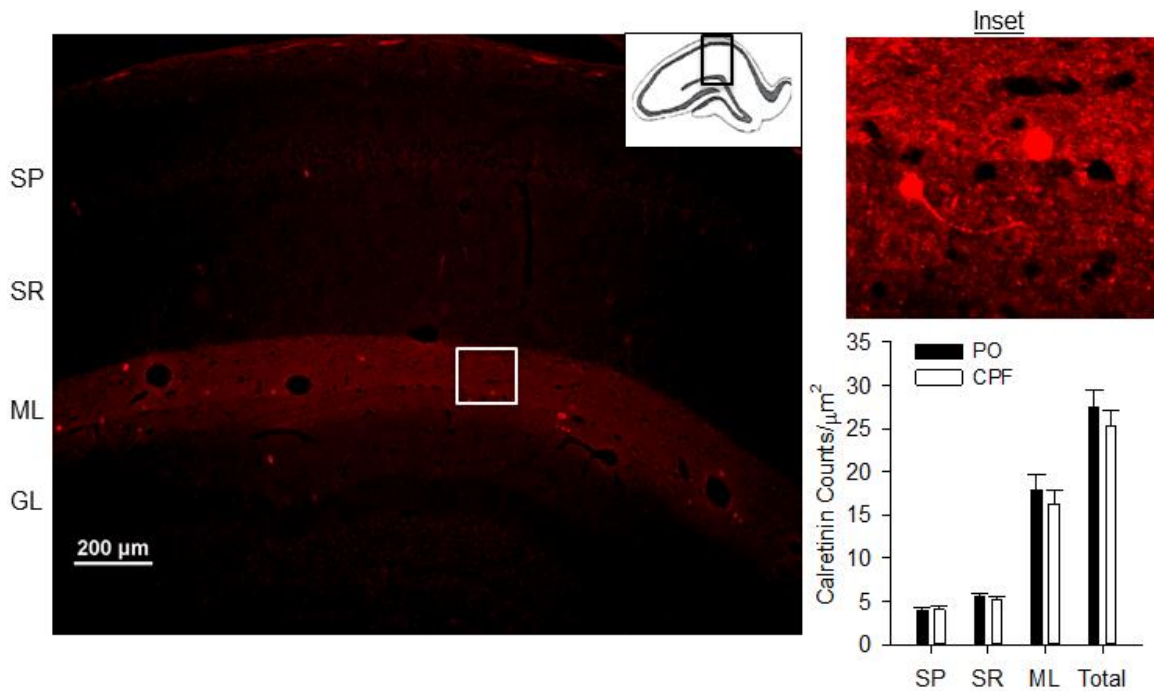
Previous studies have reported that in the guinea pig hippocampus, CB-expressing interneurons appear in the SO/alveus region (Rami et al., 1987), CR-expressing interneurons appear primarily in the ML (Murakawa and Kosaka, 1999), and PV-expressing interneurons appear primarily in the subgranular region of the DGEC (Nacher et al., 2000). The results presented here are in agreement with those reported by other laboratories. Thus, CB-positive neurons were most abundant in the SO/alveus, CR-positive neurons were found in the SP, SR, and ML, and PV-positive neurons were visualized in the SO, SP, and DGEC (Figures 21 through 23). Two-way ANOVA with hippocampal strata and treatment as inter-animal factors revealed no significant effect of prenatal CPF exposure on the number of CB-, CR-, and PV-positive neurons.

Figure 21: Calbindin immunoreactivity in the CA1 hippocampal field of CPF-exposed and control male offspring.



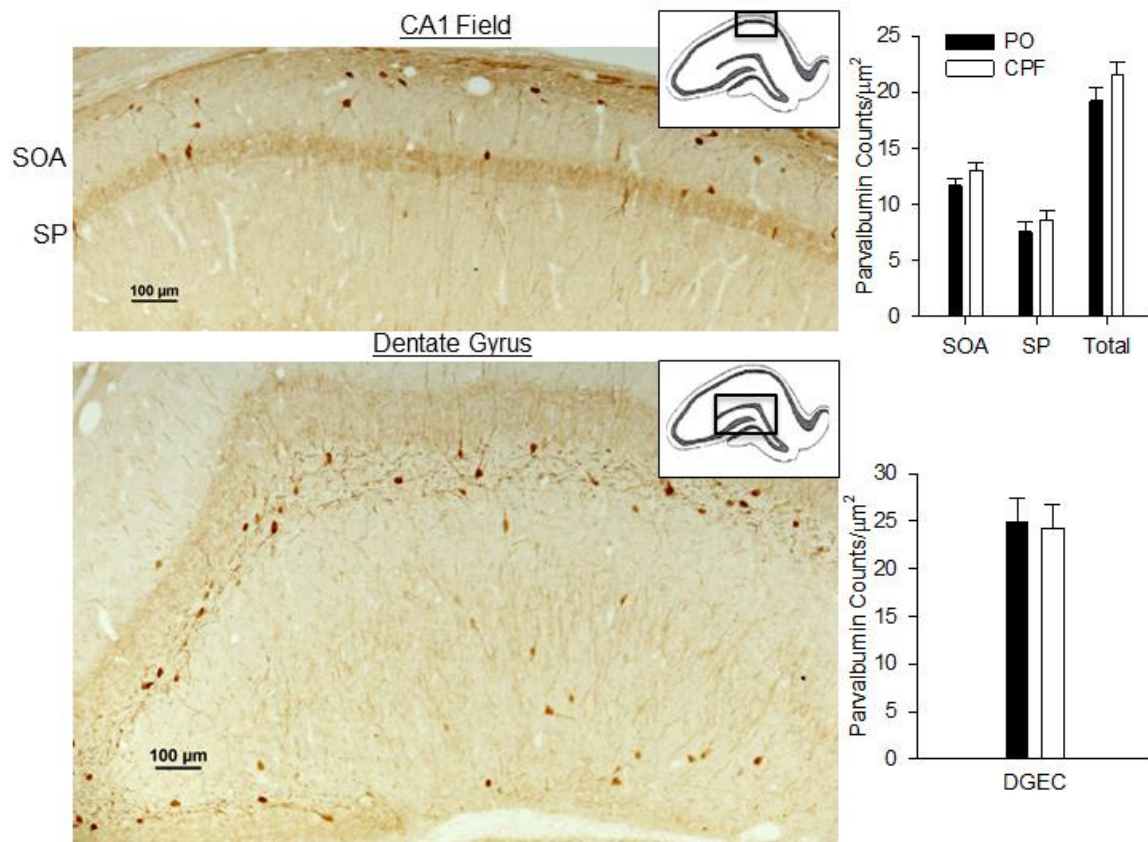
Representative photomicrograph of calbindin [20x (0.64x reduced)] immunoreactivity in the CA1 field of the hippocampus of control guinea pigs. The image was adjusted to highlight typical staining patterns. CPF exposure did not significantly affect cell calbindin positive cell counts or immunoreactivity. Data are displayed as mean \pm SEM.

Figure 22: Calretinin immunoreactivity in the CA1 hippocampal field of CPF-exposed and control male offspring.



Representative photomicrograph of calretinin [10x (0.64x reduced)] immunoreactivity in the CA1 field of the hippocampus of control guinea pigs. The image was adjusted to highlight typical staining patterns. CPF exposure did not significantly affect cell calretinin positive cell counts or immunoreactivity. Data are displayed as mean \pm SEM.

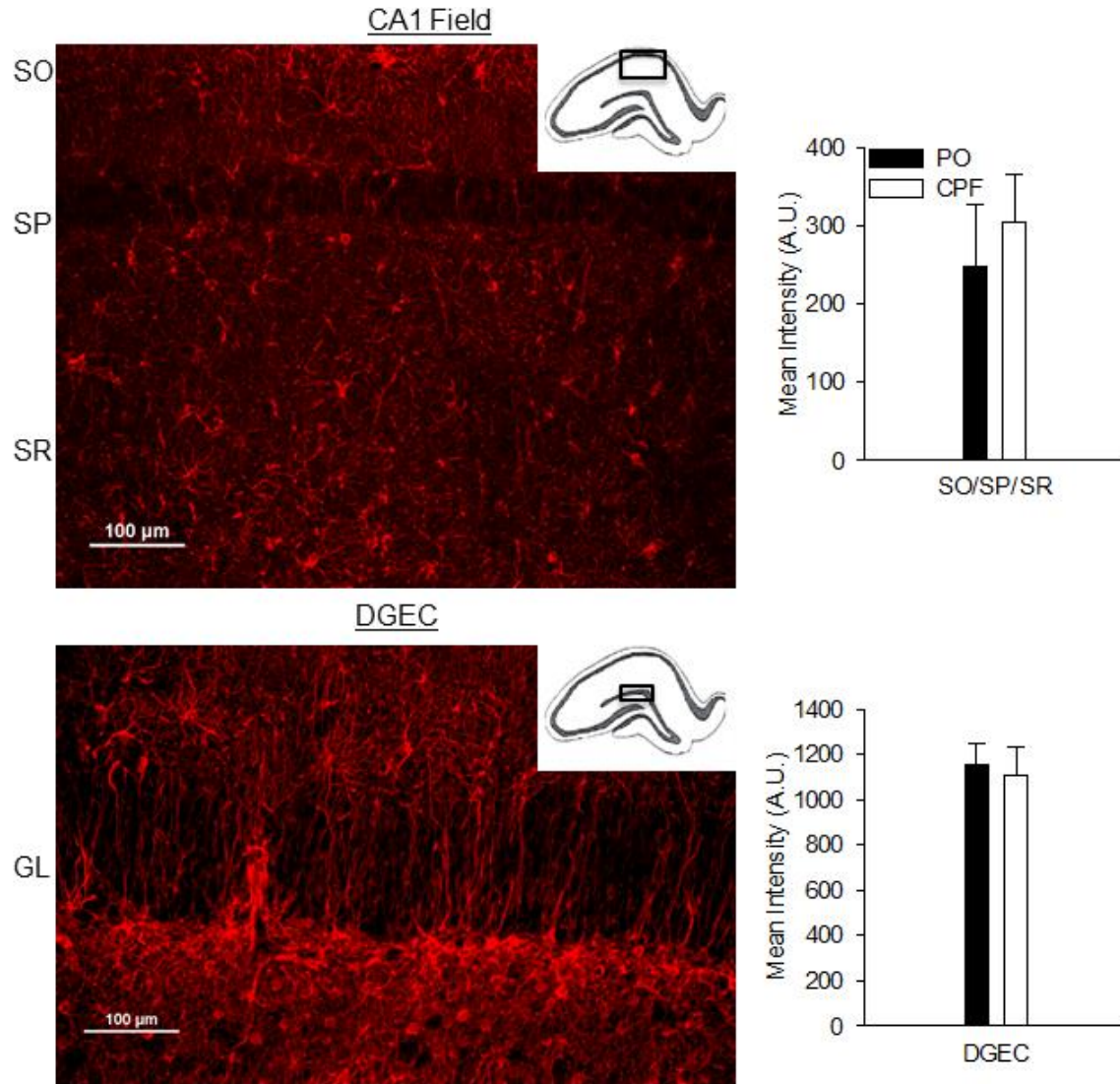
Figure 23: Parvalbumin immunoreactivity in the CA1 hippocampal field of CPF-exposed and control male offspring.



Representative photomicrographs (10x) of parvalbumin immunoreactivity in the SP and dentate gyrus of control guinea pigs. Fields shown in each image were adjusted to highlight typical staining patterns. CPF exposure did not significantly affect parvalbumin cell counts. Data are displayed as mean \pm SEM.

To examine the effects of prenatal exposure to CPF on the astrocytic population in the hippocampus, the pan-glial marker glial fibrillary acidic protein (GFAP) immunoreactivity was measured in the SO, SP, SR, and DGEC in the CA1 field of the hippocampus of guinea pigs prenatally exposed to CPF and control animals (Figure 24). A two-way ANOVA with treatment and location as inter-animal factors showed no significant effect of prenatal CPF-exposure.

Figure 24: GFAP immunoreactivity in the CA1 hippocampal field of CPF-exposed and control male offspring.



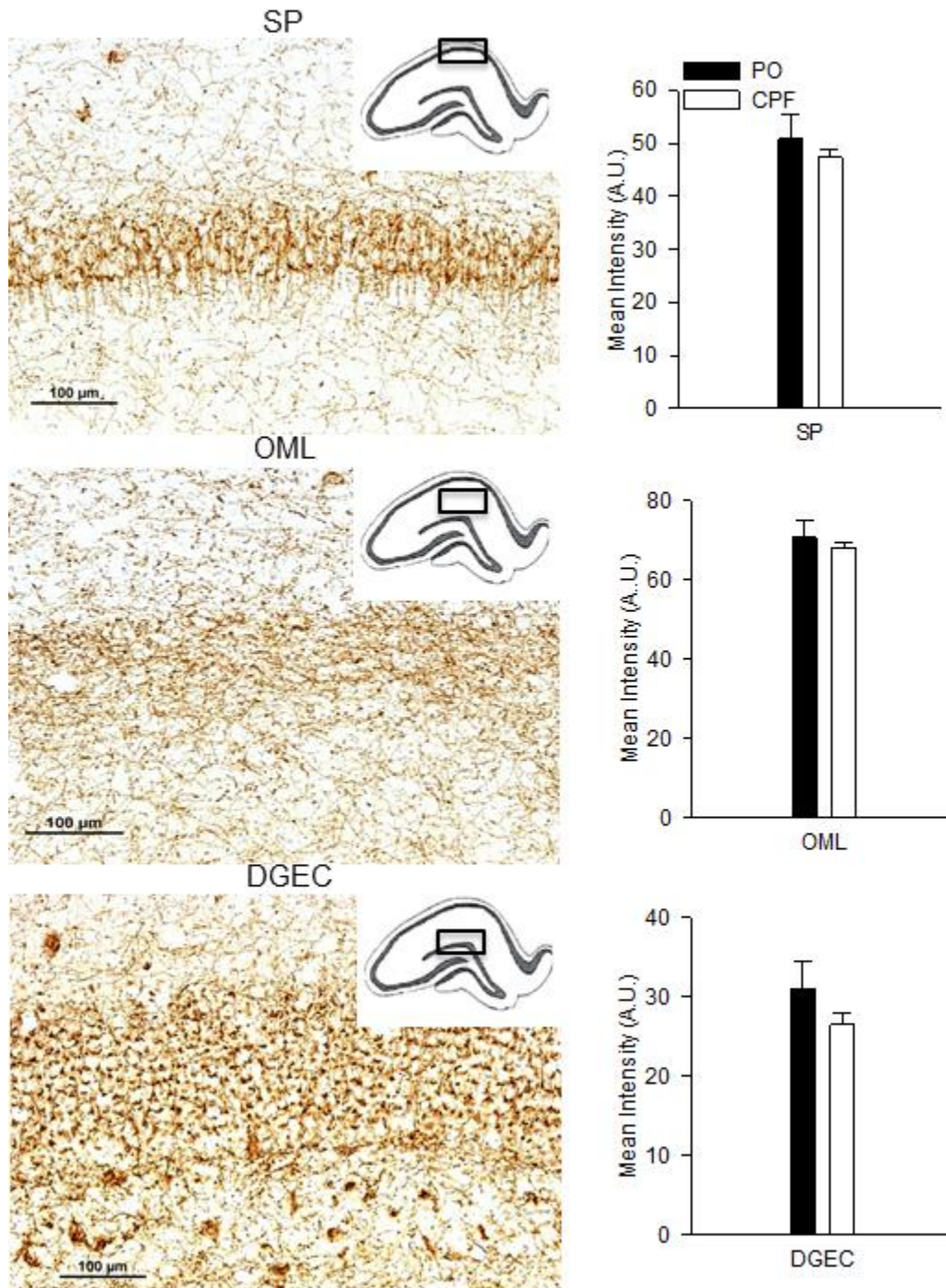
Representative photomicrographs (20x) of GFAP immunoreactivity in the CA1 field of the hippocampus of control guinea pigs. Fields shown in each image were adjusted to highlight typical staining patterns. Prenatal exposure to CPF had no significant effect on GFAP staining in the CA1 field of the guinea pig hippocampus. Data are displayed as mean \pm SEM.

6.3.2. Immunohistochemical assessment of the effects of prenatal exposure to on expression of ChAT and $\alpha 7$ nAChR subunits, which are involved in cholinergic signaling.

Previous studies reported that exposure of rats to CPF during critical periods of development significantly reduced expression of ChAT and of $\alpha 7$ nAChRs in the forebrain (Qiao et al., 2003 and 2004; Dam et al., 1999; Slotkin et al., 2001; Slotkin et al., 2004; Richardson and Chambers 2004). Therefore, CPF-induced alterations in cholinergic signaling could plausibly contribute to increased GABAergic transmission on CA1 pyramidal neurons. Here, ChAT and $\alpha 7$ nAChR immunoreactivities were analyzed in the CA1 field of the hippocampus of control and CPF-exposed guinea pigs.

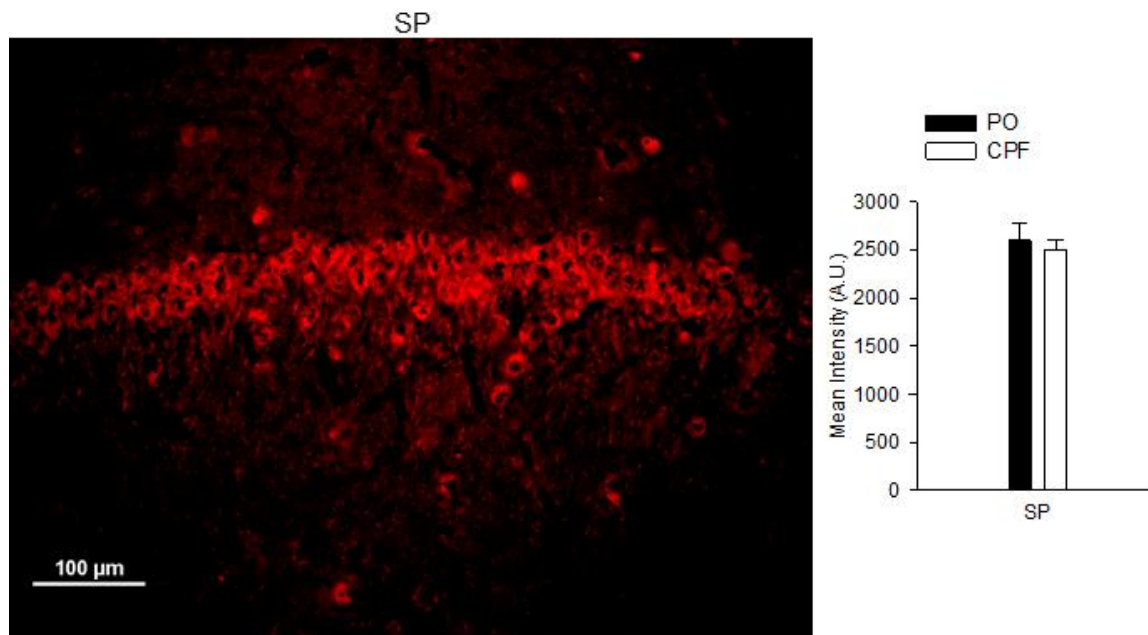
CPF exposure did not significantly affect ChAT immunoreactivity (Figure 25). Comparison of the intensity of ChAT immunoreactivity within the CA1 SP, ML, and DGEC between exposure groups by a two-way ANOVA with treatment and location as inter-animal factors revealed no significant main effect of CPF-exposure. Likewise, One-way ANOVA revealed no significant difference in $\alpha 7$ nAChR staining intensity between exposure groups in the CA1 pyramidal layer (Figure 26).

Figure 25: ChAT immunoreactivity in the CA1 hippocampal field of CPF-exposed and control male offspring.



Representative photomicrographs (20x) of ChAT immunoreactivity in the hippocampus of control guinea pigs. Fields shown in each image were adjusted to highlight typical staining patterns. CPF-exposure had no significant effect on staining intensity. Graph and error bars represent mean and SEM, respectively.

Figure 26: $\alpha 7$ nAChR immunoreactivity in the CA1 hippocampal field of CPF-exposed and control male offspring.



Representative photomicrograph (20x) of $\alpha 7$ nAChR immunoreactivity in the hippocampus of control guinea pigs. Fields shown in each image were adjusted to highlight typical staining patterns. CPF-exposure had no significant effect on staining intensity. Graph and error bars represent mean and SEM, respectively.

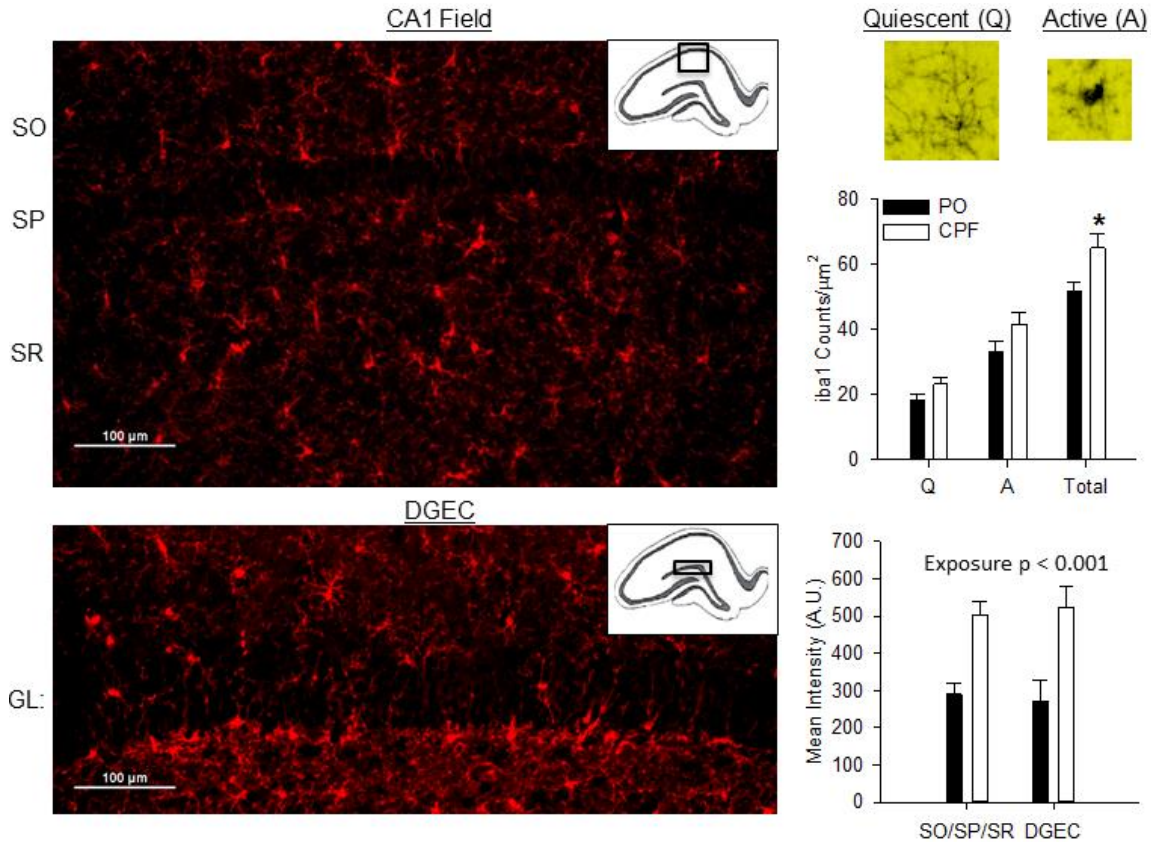
6.3.3. Immunohistochemical assessment of the effects of prenatal CPF exposure on microglia.

Microglia have been associated with neural pathology, scarring, and inflammation (Reviewed by: Casano and Petri 2015, Franco and Fernandez-Suarez, 2015) as well as changes in hippocampal synaptic transmission and plasticity (Gunderson et al., 2015). Here, an antiserum that recognizes the microglia specific Ca^{2+} -binding protein Iba1 was used as a pan-microglial marker. Microglia were counted in a defined region encompassing the SO, SP, and SR and categorized as active or inactive based upon their morphology (Kreutzberg, 1996; VanGuilder et al., 2011) (Figure 27). Total number of microglia was significantly higher [$F(1,9) = 5.794, p = 0.039$] in the CA1 field of the

hippocampus of CPF-exposed guinea pigs, and a two-way ANOVA with treatment and active state as inter-animal factors revealed a significant effect of CPF exposure [$F(1,18) = 6.278, p = 0.022$] on the number of active and quiescent microglia. Likewise, Iba1 intensity measurements from a similarly defined geographic region further supported CPF's effect on microglial number (Figure 27). Two-way ANOVA with treatment and region (SO/SP/SR, DGEC) as inter-animal factors shows a significant CPF-dependent elevation in Iba1 staining intensity [$F(1,18) = 23.744, p < 0.001$], no regional differences in the intensity of labeling [$F(1,18) = 0.00001, p = 0.997$], and no significant CPF exposure x hippocampal stratum [$F(1,18) = 0.172, p = 0.683$]. This increase in intensity could be accounted for by the increased Iba1-positive cell count in CPF-exposed guinea pigs compared to control guinea pigs.

To determine the onset of this increased Iba1 immunoreactivity, CPF-exposed ($n = 9$) and control ($n = 9$) guinea pigs were transcardially perfused on PNDs 7, 14, and 30 and processed as adult offspring for Iba1 IHC (Figure 27). Two-way ANOVA with CPF exposure and PND as inter-animal factors revealed a significant CPF-dependent elevation in Iba1 staining intensity [$F(1,11) = 11.76, p = 0.006$], no differences in the intensity of labeling with age [$F(2,11) = 0.749, p = 0.496$], and no significant CPF exposure x age interaction [$F(2,11) = 0.277, p = 0.763$]. These results indicate that Iba1 immunoreactivity is consistently elevated in CPF-exposed offspring from as early as PND 7.

Figure 27: Iba1 staining within the CA1 hippocampal field of CPF-exposed and control male offspring.

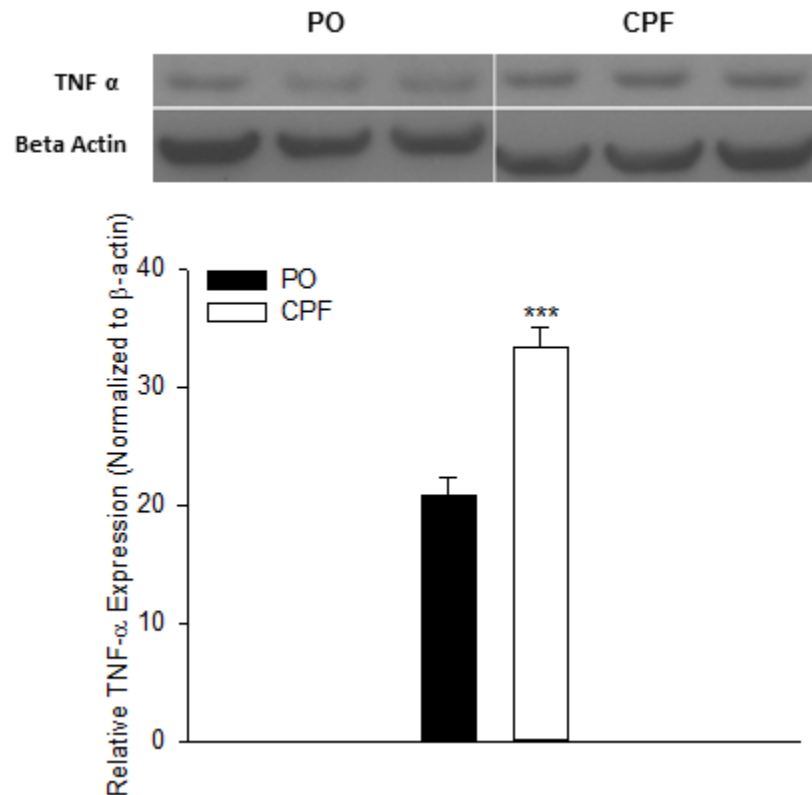


Representative photomicrographs (20x) of Iba1 immunoreactivity in control guinea pigs. Fields shown in each image were adjusted to highlight typical staining patterns. Total CA1 Iba1 positive cells were significantly elevated [$F(1,9) = 5.794$, $*p = 0.039$] in CPF-exposed guinea pigs. Two-way ANOVA with exposure and active state as inter-animal factors revealed a significant main effect of CPF-exposure on the number of quiescent (Q) and active (A) Iba1 positive cells [$F(1,18) = 6.278$, $p = 0.022$]. Likewise, two-way ANOVA with exposure and region as inter-animal factors showed a significant CPF-dependent increase in Iba1 staining intensity [$F(1,18) = 23.744$, $p < 0.001$]. Data are displayed as mean \pm SEM.

Increases in microglial number, such as those observed with prenatal CPF exposure, are associated with neuroinflammatory processes. Therefore, it is likely that this microglial increase occurred concurrent to neuro-inflammation and CPF-mediated hippocampal injury (Reviewed by Devinski et al, 2013). To test this, western blotting was conducted to evaluate whether prenatal exposure to CPF leads to increases in the expression of the pro-inflammatory cytokine, TNF- α , in the hippocampus. As a marker

for neuroinflammation (Olmos and Llado, 2014), TNF- α expression was assessed in hippocampal lysates from male guinea pigs of a similar age group that underwent electrophysiological evaluation. CPF-exposed guinea pigs had significantly higher hippocampal TNF- α expression [$F(1,10) = 31.27, p < 0.001$] than control guinea pigs (Figure 28). Combined with the observed increase in Iba1 staining, this result strongly indicates the presence of a chronic neuroinflammatory process in the hippocampus of guinea pigs prenatally exposed to CPF.

Figure 28: TNF- α expression in hippocampal lysates obtained from CPF-exposed and control guinea pigs.



Western blotting for TNF- α in hippocampal lysates obtained from six guinea pigs per experimental group. Blots were stripped and normalized for total loaded protein against beta actin. One-way ANOVA reveals a significant effect of CPF exposure on TNF- α expression [$F(1,10) = 31.27, ***p < 0.001$].

6.4. Discussion

The results presented here demonstrate that adult male guinea pigs prenatally exposed to a sub-acute dose of CPF present no significant alterations in the immunoreactivity of neuronal markers and in the astrocyte marker GFAP. Instead, compared to control animals, CPF-exposed guinea pigs presented a significant increase in the immunoreactivity of the microglia marker Iba1 in the CA1 field of the hippocampus and an increase in the expression of the inflammatory cytokine TNF- α in the hippocampus. The implications of these findings are discussed herein.

Prenatal exposure of adult male guinea pigs to a sub-acute dose of CPF had no significant effect on the number of NeuN-immunopositive cells and on number of CB-, PV-, and CR-immunopositive cells. These results support the notion that the cognitive deficits and the increase in GABAergic transmission in the CA1 field of the hippocampus seen in adult male guinea pigs prenatally exposed to CPF cannot be attributed to loss of excitatory neurons or increase in the number of interneurons within the hippocampus. They are also in agreement with previous reports that substantial neuronal death in the CA1 and CA3 fields of the hippocampus only develops following exposures to an acute dose (0.5xLD₅₀) that induces overt signs of toxicity (Mitra et al., 2008). Of interest, a recent magnetic resonance imaging study demonstrated that adult female guinea pigs developmentally exposed to CPF using the same paradigm as that used here present no signs of disruption of the overall structural integrity of the hippocampus (Mullins et al., 2013).

Immunoreactivity of the astrocytic marker GFAP and the marker of myelin sheaths MBP was comparable between adult male guinea pigs prenatally exposed to CPF and control animals. These findings did not recapitulate the decreases in GFAP and MBP immunoreactivity in the hippocampus of rats following sub-acute developmental CPF exposure (Roy et al 2004, Garcia et al, 2002). This discrepancy could stem from the lack of significant brain AChE inhibition in the brain of offspring guinea pigs prenatally exposed to CPF in the present study (Chapter 4). In both rat developmental studies, dosages of CPF that induced significant brain cholinesterase inhibition were used (Richardson and Chambers, 2005).

Decreased ChAT and $\alpha 7$ nAChR expression in the hippocampus have been associated with cognitive deficits in rodents and humans (see Orta-Salazar et al., 2014 and references therein) and previous studies have reported that developmental exposure of rats to CPF results in reduction of both ChAT and $\alpha 7$ nAChR expression in the brain (Table 2). The immunohistochemical analysis performed here, however, provided evidence supporting the notion that the developmental exposure of guinea pigs to CPF induced no significant alteration in the expression of ChAT and $\alpha 7$ nAChRs in the CA1 field of the hippocampus. This result runs counter to observations of a lasting cholinergic terminal deficit following sub-acute developmental CPF exposure in the rat (Table 2). The divergence of these results can likely be attributed to the lack of significant AChE inhibition in the brain offspring born to pregnant guinea pigs exposed to CPF in the present study as well as the developmental dissimilarities of the murine models.

While adult male guinea pigs that had been prenatally exposed to sub-acute doses of CPF presented no significant alteration in the number of neuronal or astrocytic cells,

they had a significant increase in Iba1 immunoreactivity in the CA1 field of the hippocampus. In addition, they had significantly higher hippocampal TNF- α levels than those measured in control guinea pigs. Considering that microglia mediate neuroinflammation in the CNS (reviewed by Franco and Fernandez-suarez, 2015) and are the primary source of pro-inflammatory cytokines, such as TNF- α , these results indicate the presence of chronic neuroinflammation in the hippocampus of CPF-exposed animals. The origin of this inflammation is likely multifactorial. Speculatively, CPF has been shown to disrupt the axonal transport of nutrients from the cell body to axon terminals in neurons (Grigoryan et al., 2008; Prendergast et al., 2007), exacerbate oxidative stress, increase cytokine and interleukin production, and disrupt mitochondrial function (reviewed in Androustopoulos et al., 2013). Any of these effects potentially could contribute to the chronic inflammation observed in adult animals following sub-acute developmental exposure to CPF. Regardless of underlying triggering mechanism, the increase of pro-inflammatory cytokines above homeostatic levels has been shown to profoundly affect neurotransmission. For example, TNF- α can alter glutamatergic and GABAergic neurotransmission via a number of mechanisms that include but are not restricted to: (i) increased expression of the Na⁺-K⁺-Cl⁻ cotransporter NKCC1 (Topper et al, 1997; Ramia and Kreydiyyeh, 2009), (ii) increased gliotransmitter release (Bezzi et al., 2001), (iii) increased surface expression of AMPA and NMDA receptors (reviewed in Olmos and Lladó, 2014), and (iv) increased internalization of GABA_A receptors on neurons (reviewed in Olmos and Lladó, 2014). In conclusion, adult male guinea pigs exposed prenatally to a dose regimen of CPF that can induce cognitive deficits and increase GABAergic transmission in the hippocampus present no overt sign

of neurodegeneration, no changes in number of GABAergic neurons, and no sign of astroglyosis in the hippocampus. Instead, they present with a chronic neuroinflammatory process, which has been associated with cognitive deficits in preclinical animal models and in humans (Habbas et al 2015; see also Ownby, 2010 and references therein). As such, through associated increases in gliotransmission and bimodal effects on glutamatergic and GABAergic synaptic transmission, it is possible that chronic neuroinflammation contributes to deficits in learning and memory observed in guinea pigs and humans developmentally exposed to CPF.

Chapter 7: Discussion

The results presented here support the hypothesis that *in-utero* exposure to doses of CPF that do not elicit overt signs of maternal toxicity results in cognitive deficits, particularly in male offspring, that correlate with an increase of GABAergic transmission in CA1 pyramidal neurons. The experiment conducted in Chapter 4 investigated the translational relevance of the chosen CPF exposure paradigm (25 mg/kg/day, sc) through the measurement of AChE activity in the brain and blood compartments of newborn pups. The results indicated that maternal CPF exposure during approximately the last ten days of gestation induced levels of RBC AChE inhibition (75%) that are relevant to humans occupationally exposed to this pesticide. They also provided evidence that CPF administered to the pregnant guinea pigs crossed the placenta and reached the brain of their offspring *in utero*. In Chapter 5, the effects of this sub-acute developmental CPF exposure on the motor activity and cognitive behavior of male and female offspring were evaluated. The results of these experiments are the first to demonstrate a direct relationship between prenatal CPF exposure and cognitive deficits in guinea pigs. Prenatal CPF exposure significantly impaired spatial learning in male and female offspring. The learning impairment was much stronger in male than female offspring prenatally exposed to CPF and, as a result, the normal sexual dimorphism of the animals in the MWM performance was abolished. This is of especial interest considering that the correlation between cognitive deficits and prenatal CPF exposure is stronger among boys than girls (Horton et al, 2012.; Marks et al., 2010; Rauh et al., 2012).

Given the stronger effect of prenatal CPF exposure on spatial learning of male guinea pigs and the integral role of the hippocampus in spatial learning, male guinea pigs were selected for evaluation of spontaneous hippocampal synaptic activity. As presented in Chapter 5, compared to control animals, CPF-exposed adult male guinea pigs presented a significant increase in GABAergic synaptic activity and no change in glutamatergic synaptic activity recorded from CA1 pyramidal neurons. This augmented inhibition serves to dampen hippocampal output to surrounding cortical structures and can be an important determinant of the deficits these guinea pigs exhibited in the MWM task. In support of this argument, a significant positive relationship was demonstrated between GABAergic frequency and escape latency and distance traveled within the MWM. In Chapter 6, experiments were conducted to compare the hippocampal architecture of CPF-exposed and naïve adult male guinea pigs. The results of these experiments revealed that while sub-acute developmental CPF exposure does not result in loss of excitatory neurons, gain of inhibitory neurons, or signs of astrogliosis, it does result in increased neuroinflammatory markers that are suggestive of a chronic inflammatory process in the adult hippocampus.

7.1. A possible mechanism underlying spatial learning deficits in CPF-exposed male guinea pigs.

The present study is the first to provide evidence that sub-acute prenatal exposure of precocial species to CPF results in an increase in markers of neuroinflammation in the hippocampus, an increase in GABAergic synaptic transmission on CA1 pyramidal neurons, and spatial learning deficits in the MWM. It is our conjecture that these findings generate a model of sub-acute CPF toxicity that can be further examined mechanistically

to unmask causative factors underlying cognitive behavioral deficits associated with prenatal CPF exposure. Once causation is established through further experimentation, it would become possible to target these processes remedially.

The initiating factor of this model appears to be chronic neuroinflammation, which develops quickly subsequent to developmental CPF exposure. In fact, elevated hippocampal Iba1 immunoreactivity in CPF-exposed guinea pigs was noted as early as PND 7. These findings support the idea of a rapid onset of an inflammatory process that is maintained chronically throughout adulthood. While the mechanisms by which CPF triggers neuroinflammation are undefined, several known actions of OP compounds have the potential to generate an inflammatory response. For example, OP compounds such as CPF are known to disrupt mitochondrial function, generate reactive oxygen species, alter cytosolic calcium levels, potentiate voltage-gated sodium channels, and interfere with tubulin polymerization (reviewed in Androutsopoulos et al. 2013). Likewise, compensatory upregulation of BuChE following CPF/CPO clearance could also play a role. Recently, it has been demonstrated that BuChE reduces circulating ghrelin levels in the blood and brain (Chen et al., 2015). Ghrelin is a gastric peptide with both central and peripheral effects indicated in the ontogeny of hunger. It has been shown centrally to decrease TNF- α (Guneli et al., 2010), protect against hippocampal cell death (Xu et al., 2009), affect GABAergic networks (Haam et al. 2014), and support spatial learning through increased hippocampal spine density (Diano et al., 2006). Thus, a compensatory increase in BuChE expression, similar to those observed in murine models following prolonged AChE inhibition (Zivin and Pregeli, 2008; Duysen and Lockridge 2011), would reduce ghrelin and contribute to neuroinflammation and spatial learning deficits.

Further support for this assertion is found in the offspring body weight data (Figure 6). Prenatal CPF exposure significantly reduced offspring bodyweight. This reduction could stem from decreased food intake as a result of ghrelin depletion.

Regardless of the initiating events, chronic neuroinflammation through pro-inflammatory cytokine signaling can lead to changes in synaptic transmission within the hippocampus, such as those documented in the present study. In adult male CPF-exposed offspring, hippocampal TNF- α expression was significantly elevated (Figure 28). Concurrently, increases in GABAergic transmission were observed at the level of CA1 pyramidal neurons. This enhancement of GABAergic transmission was noted without concomitant increases in glutamatergic synaptic transmission, indicating a selective activation of the GABAergic interneuron network (Figure 12). The selective activation of GABAergic transmission could be attributed to the elevated levels of TNF- α in the hippocampus of adult male guinea pigs prenatally exposed to CPF. TNF- α can activate the network of interneurons in the hippocampus through a number of distinct mechanisms that include: (i) increased NKCC1 expression and (ii) increased gliotransmitter release.

TNF- α has been shown to increase expression of NKCC1, a Na⁺, K⁺, Cl⁻ co-transporter that causes Cl⁻ influx into cells (Topper et al, 1997; Ramia and Kreydiyyeh, 2009). As a result, the ratio of NKCC1 to KCC2, the K⁺, Cl⁻ co-transporter that causes Cl⁻ efflux from neurons, increases. Elevation in this ratio increases intracellular concentrations of Cl⁻ in neurons (reviewed by Loscher et al., 2013). Under this condition, when ligand-gated ion channels permeable to Cl⁻, such as GABA_A receptors, are activated, Cl⁻ moves out of the neurons, in favor of its electrochemical

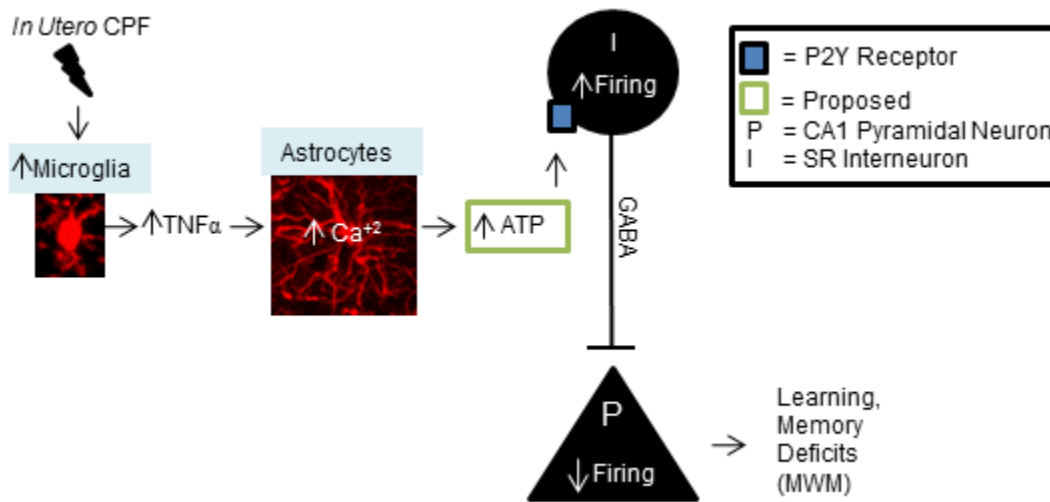
gradient. Consequently, GABAergic transmission becomes excitatory rather than inhibitory. Conceivably, the increased frequency of GABAergic synaptic events recorded from CA1 pyramidal neurons could be explained by an increased ratio in NKCC1 to KCC2 in GABAergic neurons causing these neurons to be depolarized in response to GABA release from synaptically interconnected interneurons. One could argue against this hypothesis based on previous reports that, in contrast to pyramidal neurons, interneurons appear to be impervious to changes in expression of NKCC1 and KCC2 (Banke and McBrain, 2006).

Alternatively, TNF- α has been shown to increase astrocyte Ca^{2+} uptake (Abdura et al., 2015) and facilitate gliotransmitter release (Bezzi et al., 2001). Of principle interest are the gliotransmitters ATP and glutamate. These transmitters have been shown to work in conjunction to both propagate astrocyte Ca^{2+} oscillations and increase the activity of inhibitory networks. Specifically, ATP and its degradation product adenosine diphosphate (ADP) activate metabotropic P2Y receptors with high affinity (Lazarowski et al., 2003) on astrocytes and interneurons (Bowser and Khakh 2004, Moore et al., 2000). These receptors increase cytosolic Ca^{2+} concentration and induce slow depolarization through the activation of non-specific cationic conductance and inhibition of potassium conductance (Bowser and Khakh 2004). On astrocytes, this excitation serves to propagate Ca^{2+} waves, and thus maintains gliotransmitter release, while on interneurons this excitation aids action potential firing. This P2Y-induced depolarization is limited to astrocytes and interneurons, as the receptor is expressed negligibly in pyramidal cells (Khakh et al. 2003; Bowser and Khakh 2004). Thus, astrocytic ATP release suppresses glutamatergic synapses through P2Y activation (Koizumi et al., 2003; Zhang et al.,

2003). Likewise, astrocytic glutamate release aids this suppression through interaction with excitatory type 1 metabotropic glutamate receptors (mGluR1) on interneurons (van Hooft et al., 2000; Krause et al. 2002).

The resultant increase in the ratio of inhibition to excitation within the CA1 pyramidal layer dampens the excitatory output of the hippocampus to surrounding cortical structures. This reduction is of prime importance as cross-signaling between the hippocampus and the entorhinal cortex is required for spatial navigation. The coordination of hippocampal place cells, which function in local spatial navigation, and entorhinal cortex grid cells, which coordinate a global spatial map, is integral to spatial learning (Hafting et al., 2005). Thus, it is plausible that the deficits in spatial learning seen in guinea pigs prenatally exposed to CPF are directly related to increased GABAergic inhibition of CA1 pyramidal neurons. This is supported by the manifestation of similar spatial learning deficits following temporary CA1 pyramidal inactivation by muscimol intrahippocampal application (Bonnievie, et al., 2013) or benzodiazepine ingestion (Timic et al., 2013). A diagrammatic representation of this proposed model of sub-acute developmental CPF toxicity is presented in Figure 29. While experimental work is required to establish a causal relationship, this model establishes a framework for the targeting of such experiments.

Figure 29: A proposed mechanism for the generation of spatial learning deficits subsequent to sub-acute developmental CPF exposure.



Schematic diagram of a potential mechanism underlying the spatial learning deficits that ensue following prenatal exposure to CPF. This mechanism is detailed in section 7.1. Briefly, *in utero* CPF exposure leads to the onset of chronic neuroinflammation and increased $TNF-\alpha$ expression. $TNF-\alpha$, in turn, increases gliotransmitter release, which facilitate increased presynaptic inhibition at the CA1 pyramidal layer. This increased ratio of inhibition to excitation in the pyramidal cells results in decreased excitatory output to the entorhinal cortex and, thus, contributes to spatial learning deficits.

Chapter 8: Conclusion and future directions

The results of the present study demonstrate for the first time that guinea pigs exposed *in utero* to the pesticide CPF develop chronic neuroinflammation, decreased hippocampal output, and cognitive deficits. These results are far reaching because: (i) the CPF dose regimen administered to the pregnant animals induced levels of RBC AChE inhibition that are relevant to humans occupationally exposed to this pesticide, (ii) the pattern of brain development of humans is closer to that of guinea pigs than of other rodents commonly used in preclinical studies, and (iii) the cognitive deficits observed in offspring are similar to those observed in male children. Thus, the guinea pig emerges as a unique model to advance our understanding of cellular and molecular mechanisms underlying the developmental neurotoxicity of OP pesticides.

Future work using this preclinical model of CPF neurotoxicity will focus on the assessment of the proposed mechanism for the generation of spatial learning deficits subsequent to sub-acute developmental CPF exposure. Specifically, adult brain and plasma BuChE activity and ghrelin levels will be assessed at different times following prenatal exposure to CPF to address their possible involvement in the initiation of the neuroinflammatory process. Next, the effect of the P2Y antagonists PPADs and MRS2179 on whole-cell CA1 pyramidal spontaneous GABAergic activity will be compared between CPF-exposed and control guinea pigs to assess the relative contribution of the P2Y receptor on presynaptic neurotransmission. An IHC study could also be performed to assess the NKCC1:KCC2 ratios in the hippocampus of CPF-exposed and control animals. Finally, a functional electrophysiological assay could

be carried out to determine whether the NKCC1 inhibitor bumetanide can decrease the high frequency of GABAergic synaptic events recorded from CA1 pyramidal neurons of CPF-exposed animals. Together, these studies could shed light into the mechanism(s) underlying the observed increase in presynaptic GABAergic signaling at the pyramidal layer. Subsequently, using drugs that specifically target those mechanisms, one could attempt to reverse and/or prevent the development spatial learning deficits in CPF-exposed guinea pigs. Remediation of the observed deficits through the antagonism of specific mechanisms would finally establish a causative relationship.

In conclusion, future experimentation is required to conclusively discern the mechanism underlying the cognitive deficits associated with prenatal CPF exposure. The present study, in addition to demonstrating that the guinea pig is a translationally relevant model for preclinical evaluation of the effects of prenatal exposure to CPF in the developing brain, provides a framework for identification of potential molecular mechanisms underlying the developmental neurotoxicity of this pesticide.

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