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14. ABSTRACT

Chlorpyrifos (CPF), an organophosphorus pesticide (OP), is one of the most widely used pesticides in the world. Subchronic exposures to CPF that do not cause cholinergic crisis are associated with problems in cognitive function (i.e., learning and memory deficits), but the biological mechanism(s) underlying this association remain speculative. To identify potential mechanisms of subchronic CPF neurotoxicity, adult male Long Evans (LE) rats were administered CPF at 3 or 10 mg/kg/d (s.c.) for 21 days. We quantified mRNA and non-coding RNA (ncRNA) expression profiles by RNA-seq, microarray analysis and small ncRNA sequencing technology in the CA1 region of the hippocampus. Hippocampal slice immunohis- tochemistry was used to determine CPF-induced changes in protein expression and localization patterns. Neither dose of CPF caused overt clinical signs of cholinergic toxicity, although after 21 days of exposure, cholinesterase activity was decreased to 58% or 13% of control levels in the hippocampus of rats in the 3 or 10 mg/kg/d groups, respectively. Differential gene expression in the CA1 region of the hippocampus was observed only in the 10 mg/kg/d dose group relative to controls.

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Repeated exposure to neurotoxic levels of chlorpyrifos alters hippocampal expression of neurotrophins and neuropeptides

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ABSTRACT

Chlorpyrifos (CPF), an organophosphorus pesticide (OP), is one of the most widely used pesticides in the world. Subchronic exposures to CPF that do not cause cholinergic crisis are associated with problems in cognitive function (i.e., learning and memory deficits), but the biological mechanism(s) underlying this association remain speculative. To identify potential mechanisms of subchronic CPF neurotoxicity, adult male Long Evans (LE) rats were administered CPF at 3 or 10 mg/kg/d (*s.c.*) for 21 days. We quantified mRNA and non-coding RNA (ncRNA) expression profiles by RNA-seq, microarray analysis and small ncRNA sequencing technology in the CA1 region of the hippocampus. Hippocampal slice immunohistochemistry was used to determine CPF-induced changes in protein expression and localization patterns. Neither dose of CPF caused overt clinical signs of cholinergic toxicity, although after 21 days of exposure, cholinesterase activity was decreased to 58% or 13% of control levels in the hippocampus of rats in the 3 or 10 mg/kg/d groups, respectively. Differential gene expression in the CA1 region of the hippocampus was observed only in the 10 mg/kg/d dose group relative to controls. Of the 1382 differentially expressed genes identified by RNA-seq and microarray analysis, 67 were common to both approaches. Differential expression of six of these genes (*Bdnf*,*Cort*, *Crhbp*, *Nptx2*, *Npy* and *Pnoc*) was verified in an independent CPF exposure study; immunohistochemistry demonstrated that CRHBP and NPY were elevated in the CA1 region of the hippocampus at 10 mg/kg/d CPF. Gene ontology enrichment analysis suggested association of these genes with receptor-mediated cell survival signaling pathways. miR132/212 was also elevated in the CA1 hippocampal region, which may play a role in the disruption of neurotrophin-mediated cognitive processes after CPF administration. These findings identify potential mediators of CPF-induced neurobehavioral deficits following subchronic exposure to CPF at a level that inhibits hippocampal cholinesterase to less than 20% of control. An equally significant finding is that subchronic exposure to CPF at a level that produces more moderate inhibition of hippocampal cholinesterase (approximately 50% of control) does not produce a discernable change in gene expression.

Abbreviations: CPF, chlorpyrifos; OP, organophosphorus; LE, Long Evans; s.c., subcutaneous; ChE, cholinesterase; *b.w.*, body weight; aRNA, antisense RNA; K_2-EDTA, potassium ethylene diamine tetraacetic acid; seq, sequencing; GO, gene ontology; FDR, false discovery rate; FC, fold change of difference; DEG, differentially expressed gene; NPY, neuropeptide Y; CORT, cortistatin; BDNF, brain derived neuronal factor; NPTX2, neuronal pentraxin II; CRHBP, corticotropin-releasing factor-binding protein; PNOC, prepronociceptin; SRGN, serglycin; SLIT3, slit homolog 3; MDK, midkine; SYT1, synaptotagmin-1; SPTBN1, spectrin; SEC23A, SEC23-related protein A; PTEN, phosphatase and tensin homolog; MAP2K4, mitogen-activated protein kinase kinase 4; MAP1B, microtubule-associated protein 1B; GABRG2, gamma-aminobutyric acid receptor; DYRK1A, dual specificity tyrosine-phosphorylation-regulated kinase 1A; CDH13, cadherin 13; ADCYAP1R1, pituitary adenylate cyclase-activating polypeptide receptor; ACOX1, acyl-coA oxidase 1; SYT4, synaptotagmin 4; SEPT3, septin 3; ADORA1, adenosine a1 receptor; AKAP6, a-kinase anchor protein 6; MADD, multiple acyl-coA dehydrogenase deficiency; MAPK1, mitogen-activated protein kinase 1.

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1. Introduction

Organophosphorus (OP) pesticides are among the most widely used pesticides in the world. Every year, about 200,000 people die from OP pesticide self-poisoning in the developing world, whereas far fewer deaths occur from unintentional poisonings (Eddleston et al., 2008). High dose OP poisoning causes severe cholinergic crisis and type II respiratory failure (i.e., intermediate syndrome), resulting in fatality in 15–30% cases (Eddleston et al., 2008). OP (acute) poisoning is diagnosed by measuring butyrylcholinesterase activity in the plasma or acetylcholinesterase activity in whole blood, although the relative degree of inhibition varies with various pesticides (Eddleston et al., 2008). Only whole blood acetylcholinesterase activity inhibition correlates with clinical features of OP toxicity (Eddleston et al., 2008), which is primarily caused by hyperstimulation of cholinergic receptors (Fukuto, 1990).

In 2011, Drs. Rohlman, Anger and Lein conducted a review of PubMed and Medline peer-reviewed journals and found 24 articles (between the years of 1950 and 2010) that linked prolonged (chronic) exposures (i.e., occupational exposures) to adverse neurobehavioral effects in humans (Rohlman et al., 2011). Although many factors play a role in OP toxicity (e.g., dose, duration and frequency of exposure, host response and susceptibility factors), Rohlman et al. (2011) found that most studies anchored neurobehavioral test performance to a measure of duration of exposure, urinary metabolite (exposure) or ChE activity (effect). A range of adverse effects were noted in eight major domains of neurobehavioral functioning (Rohlman et al., 2011), including sustained attention (Rothlein et al., 2006; Rohlman et al., 2007), deficits in attention/short-term memory (Abdel Rasoul et al., 2008), memory (Farahat et al., 2003; Roldan-Tapia et al., 2005; Eckerman et al., 2007), and perception (Abdel Rasoul et al., 2008).

Correlation of neurocognitive deficits to OP toxicity exposure (metabolites) or effect (AChE) markers, however, does not demonstrate a dose-dependent relationship (Rohlman et al., 2011). While there are several proposed mechanisms to explain OP induced neurotoxicity at prolonged (chronic) exposures, it is important to note that the neurotoxicity may not be mechanistically related to ChE inhibition at all (Rohlman et al., 2011). Oxidative stress, inflammation, and/or irreversible neuropathies could be mechanistically linked to chronic OP neurotoxicity and subsequent neurocognitive and neurobehavioral deficits (Rohlman et al., 2011).

In this study, we employed a toxicogenomic approach to investigate potential molecular mechanisms contributing to the OP neurotoxicity of chlorpyrifos (CPF) in the rat. The dose levels, route of administration and duration of exposure for this study were based on published exposure studies of human agricultural workers exposed to CPF for 15–30 days during the cotton growing season in Egypt (Farahat et al., 2010, 2011; Fenske et al., 2012). Using both a physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) model and experimental data, Ellison et al. (2011) reported that this dosing paradigm inhibits blood ChE activity in rats to an extent comparable to that reported in the Egyptian agricultural workers (Ellison et al., 2011).

Recent advances in gene expression profiling technology enable a highly sensitive evaluation of genome-wide transcriptomic responses to toxicants. cDNA microarray technology allows a simultaneous interrogation of many known transcripts in a high-throughput manner. RNA-seq offers several advantages over microarrays, such as the ability to quantify a broader dynamic range of expression levels, and to detect novel transcripts or splice variants. RNA-seq is also considered more sensitive in detecting low abundance transcripts, and allows more accurate determination of RNA expression levels than microarrays (Wang et al., 2009). Thus, toxicogenomics affords a global, unbiased approach to identifying potentially novel mechanisms of OP-induced neurotoxicity. In this study, we investigated the molecular effects of CPF exposure in the rat hippocampus using a toxicogenomics approach in the repeat-exposure rat model.

2. Materials and methods

2.1. Animals

All animals used in this study were housed and maintained in the animal facilities at WIL Research Laboratories, LLC (Ashland, OH) that are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Nine-week old Long Evans (LE) male rats were received from Charles River Laboratories, Inc. (Raleigh, NC) and acclimated for at least 10 days (d). The animals were housed individually in suspended stainless steel wire mesh cages in a humidity and temperature-controlled room (50% ± 20% and 25 °C) with a 12-hour (h) light/12-h dark cycle (lights on at 0600 h). Rats were provided rodent chow (Certified Rodent LabDiet 5002, PMI Nutrition International, LLC, St. Louis, MO) and reverse-osmosis treated water *ad libitum* throughout the study. Individual body weights were recorded twice weekly.

On the day prior to initiating test substance treatment, a random group assignment was performed using the WIL Toxicology Data Management System (WTDMS™) program based on body weight stratification in a block design (Stump et al., 2010). Ninety rats were randomly assigned to one of three treatment groups, each consisting of 30 rats (see Fig. 1 in Lee et al., 2015). An *a priori* power analysis indicated that 30 rats per treatment group were necessary for 80% power with an α of 0.05 based on the effect size presented in Lein et al. (2012).

2.2. Test substance administration

CPF (CAS no. 2921-88-2) was obtained from Chem Service, Inc. (Chester, PA) as a colorless crystalline solid (purity > 99.5%). The test substance formulations were prepared weekly in peanut oil, and the concentration and stability of CPF formulations were validated using gas chromatography. Two CPF formulations (3 or 10 mg/kg *b.w.*/d) or an equal volume of the vehicle (peanut oil) were administered by subcutaneous injection for 21 consecutive days (study days 1–21), approximately 24 h apart. Rats were 11 weeks old at the initiation of dosing, and individual body weights ranged from 350 g to 450 g. Each animal was monitored for any pain or discomfort throughout the duration of the study.

2.3. Sample collection, biochemical measurements and histological evaluation

Brain tissue and plasma samples for molecular and histological evaluation were collected on study day 21. Of the 30 rats per treatment group, 20 were randomly selected for biochemical and 'omics analyses, and 10 rats were euthanized for pathological evaluations (see Fig. 1 in Lee et al., 2015). The biochemical and histological assays were conducted at WIL Research Laboratories. Transcriptomics analyses were conducted at the authors' test facilities. Tissue specimens were flash frozen and shipped to the authors on dry ice.

2.3.1. Sample collection for ChE activity assay and RNA analysis

Rats selected for transcriptomics and biochemical analyses ($n = 60$; 20 rats per treatment group) were deeply anesthetized by an intraperitoneal (*i.p.*) injection of sodium pentobarbital (75 mg/kg *b.w.)* prior to the collection of blood and brain tissue. A total of approximately 6 mL of blood was drawn from each animal by cardiac puncture. One milliliter of blood was collected in a chilled collection tube containing sodium heparin as the anticoagulant, and the remaining 5 mL blood was collected in a pre-chilled K_2-EDTA collection tube with no gel barrier. Blood samples were centrifuged at $2000 \times g$ for 10 min at 4 °C. Whole blood samples were analyzed for ChE activity within 1 h of collection. Plasma isolated from samples collected in K_2-EDTA tubes after centrifugation was aliquoted in polypropylene tubes, flash frozen in liquid nitrogen and archived at −70 °C.

Following blood collection, brains were immediately removed, bisected and the CA1 region of the hippocampus and entorhinal cortex was removed from each hemisphere. Microdissection of the brains was performed on ice using sterile technique to prevent minimize RNAse activity. Representative sections of the hippocampus and cortex were placed in a sample collection tube containing 1% Triton X-100. The remaining tissue was transferred into individual sterile tubes, flash frozen in liquid nitrogen, and stored at −70 °C. ChE activity in whole blood, hippocampus and entorhinal cortex was determined using an assay based on a modification (Hunter et al., 1997) of the standard Ellman reaction (Ellman et al., 1961). For a detailed description of the ChE activity assay see File 1 in Lee et al. (2015).

2.3.2. Sample collection for biomarker evaluation and pathology

The rats not selected for 'omics analysis and the ChE inhibition assay ($n = 10$ rats/group, total $n = 30$) were evaluated by immunohistochemistry. Animals were deeply anesthetized by an *i.p.* injection of sodium pentobarbital (75 mg/kg *b.w.*) and perfused *in situ* with 4.0% paraformaldehyde in phosphate buffered solution. Following perfusion, the brain was removed and placed into perfusate for 24 h. After fixation, the brain was placed in 70% ethanol and stored at 4 °C until processed. Slide preparation and microscopic examination of hematoxylin–eosin stained paraffin sections was performed on the brain (focusing on the CA1 region of the hippocampus). Stained histologic sections were examined by light microscopy ($n = 3$ images per brain) by a pathologist blinded to treatment group. Brain sections were also stained for neuronal pentraxin II (NPTX2), neuropeptide Y (NPY) and corticotropin binding hormone releasing protein (CRHBP). Semi-quantitative analysis for each stain was performed on the CA1 region of the hippocampus using the following grading system: (1) Grade 1: minimal, <10% immunopositive cells; (2) Grade 2: mild, 10–<25% immunopositive cells; (3) Grade 3: moderate, 25–<40% immunopositive cells; (4) Grade 4, marked, >40% immunopositive cells. For detailed immunohistochemistry staining procedures see File 2 in Lee et al. (2015).

2.4. Total RNA isolation and transcriptomic analysis

Total RNA including small non-coding RNA (ncRNA) was isolated from the CA1 region of the rat hippocampus using miRNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The quantity and quality of RNA was determined using Nanodrop-1000 Spectrophotometer (Thermo Fisher Scientific, Rockford, IL) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA samples were stored at −80 °C until use. All RNA data generated from this study were submitted to the National Center for Biotechnology Information Gene Expression Omnibus (GSE74676, NCBI GEO; www.ncbi.nlm.nih.gov/geo/). For a detailed description of the transcriptomic methods see Lee et al. (2015).

2.4.1. Microarray analysis

Antisense RNA (aRNA) libraries were prepared from 100 ng of total RNA obtained from the CA1 region of rat hippocampus as described by the manufacturer (Affymetrix, Santa Clara, CA). Biotin-labeled aRNAs were fragmented and hybridized onto GeneChip Rat Genome 230 2.0 Arrays. Probe intensity data were processed and analyzed using the Partek Genomics Suites (PGS) v6.6 (Partek Inc., St. Louis, MO). For low and high abundance differentially expressed genes see File 4 in Lee et al. (2015).

2.4.2. RNA-sequencing

RNA-seq libraries were prepared using the TruSeq RNA sample prep kit V2 according to the manufacturer's recommendation (Illumina, San Diego, CA). The rat hippocampus RNA-seq libraries were clustered onto a TruSeq V3 flowcell, and subjected to a 100-cycle single read run (1×100 cycles) on an Illumina HiScanSQ sequencing instrument according to the protocols described by the manufacturer (HiScanSQ System User Guide (15015392J), Illumina, San Diego, CA).

Sequence reads were aligned to the Rat Genome Sequencing Consortium (RGSC) Rnor 5.0/rn5 build (March 2012) (https://www.hgsc.bcm.edu/other-mammals/rat-genome-project) using the TopHat short read alignment program v2.0 (Trapnell et al., 2009). Read counts were normalized to the total number of reads per kilo-base of transcript per million mapped reads (rpkm) and summarized to the gene level using a modified expectation maximization algorithm in PGS v6.6 (Xing et al., 2006). For detailed protocols and sequence mapping statistics see Lee et al. (2015).

2.4.3. Small RNA-sequencing

Small RNA libraries were generated from 1 μg of hippocampus total RNA using the TruSeq small RNA sample prep kit according to the manufacturer's protocol (Illumina, San Diego, CA). Multiplexed libraries were sequenced using the TruSeq V3 reagent kits on HiScanSQ system (Illumina, San Diego, CA). Sequence alignment was performed with the Bowtie short read aligner (Langmead et al., 2009) against the reference rat genome build UCSC Baylor 3.4/rn4 (November 2004). Aligned sequences were mapped and quantified against the miRBase (release 19) mature miRNA sequences (www.mirbase.org) (Kozomara and Griffith-Jones, 2011). The read count normalization and downstream statistical analyses were performed with PGS v6.6 (Partek Inc., St. Louis, MO). For detailed protocols and differentially expressed genes from RNA-seq analysis see File 4 in Lee et al. (2015).

2.5. Functional and pathway enrichment analysis

Based on the RNA-seq results, a Gene Ontology (GO) enrichment analysis was performed using the PGS v6.6 GO enrichment statistical analysis tool (Partek Inc., St. Louis, MO) and compared against functional gene groups obtained from the Gene Ontology Consortium (http://geneontology.org/) (Ashburner et al., 2000) (see File X in Lee et al., 2015). An upstream regulator prediction analysis was performed using the Upstream Regulator Analysis tool within the Ingenuity Pathway Analysis (IPA) package (Ingenuity® Systems, Redwood City, CA). An activation *z*-score was calculated based on the consistency between expected effects of an upstream regulator on gene expression and observed expression effects. A Fisher's exact test was used to determine the association between a set of molecules and a function or pathway. The expected expression effects were derived from the literature as well as Ingenuity Knowledge Base®. Molecules with an activation *z*-score ≥ 2.0 and $p < 0.05$ were considered to be

'activated', while molecules with an activation *z*-score ≤ -2.0 and $p < 0.05$ were predicted to be 'inhibited'.

A downstream effects prediction was performed using the Downstream Effects Analysis tool within IPA to identify biological functions associated with the observed gene expression profiles, and to predict the direction of the functional perturbation. An activation *z*-score for the downstream analysis was obtained based on the prior knowledge of expected causal effects between biological functions and genes. An activation *z*-score ≥ 2.0 with $p < 0.05$ identified significantly increased biological functions; the functions with *z*-scores ≤ -2.0 and $p < 0.05$ were predicted to be significantly decreased.

3. Results

3.1. Assessment of the clinical effects of CPF exposure in rats

Repeated administration of CPF at 3 or 10 mg/kg/d for 21 days did not lead to the development of overt toxicity in CPF-exposed rats. All animals survived to necropsy at 21 days, and there were no clinical signs of acute cholinergic crisis observed among the animals in the study during the 21 day treatment period. The difference in the net body weight gain over the treatment period was negligible between the rats administered 3 mg/kg *b.w.*/d CPF and the untreated controls. Body weight in rats treated daily with 10 mg/kg *b.w.*/d CPF was not different from controls until days 18–20, when CPF-treated rats weighed significantly less than the control animals ($p = 0.04$ by repeated measures ANOVA; Fig. 1).

ChE activity ($p < 0.01$) was significantly lower in hippocampus, cortex, and blood of CPF-exposed animals relative to controls. Blood ChE activity decreased to 1 and 10% of vehicle controls following administration of 3 mg/kg *b.w.* and 10 mg/kg *b.w.* CPF per day, respectively (Fig. 2, also see Table 1A–C in Lee et al., 2015). ChE activity in the cortex of rats administered 3 or 10 mg/kg *b.w.* CPF was reduced to 32% and 10%, respectively. In the hippocampus of the rats treated with 3 or 10 mg/kg *b.w.* CPF, ChE activity was 43% and 13% of untreated control animals, respectively.

3.2. Assessment of CPF effects on RNA profiles in the rat hippocampus

3.2.1. CPF-induced alteration of the mRNA profiles in the rat hippocampus

We evaluated perturbations of hippocampal gene expression in rats exposed to CPF by cDNA microarray and RNA-seq analysis. Despite ~50% ChE inhibition, microarray analysis indicated no significant changes in hippocampal mRNA expression relative to controls after repeated administration of CPF at the low dose (3 mg/kg *b.w.*/d for 21 days, raw microarray files are presented in Lee et al., 2015). CPF administration at the high dose (10 mg/kg *b.w.*/d), however, resulted in a significant alteration of the mRNA profile in the hippocampus. In the high dose group, among 19,502 probe sets detected in the rat hippocampus, 949 probe sets corresponding to 624 genes were differentially expressed relative to controls ($n = 20$ rats per dose group) with a False Discovery Rate (FDR) <0.05 (Table 1). Seventy probe sets corresponding to 50 unique Entrez Gene IDs met the fold-change (FC) threshold of 1.25 (see File 3 in Lee et al., 2015). A subset of hippocampal samples were randomly selected for RNA-seq analysis ($n = 7$ high dose CPF-treated and $n = 6$ controls). The RNA-seq study generated an average of 24 million reads mapping to the known rat transcript sequences ($n = 7$ high dose CPF-treated and $n = 6$ controls). The *ANOVA* analysis of the mapped reads yielded a list of 368 genes differentially expressed with a FC greater than 1.25 (see File 4 in Lee et al., 2015).

Of the 368 genes differentially expressed in high dose CPF-exposed rats, 20 genes (11 up-regulated and nine down-regulated

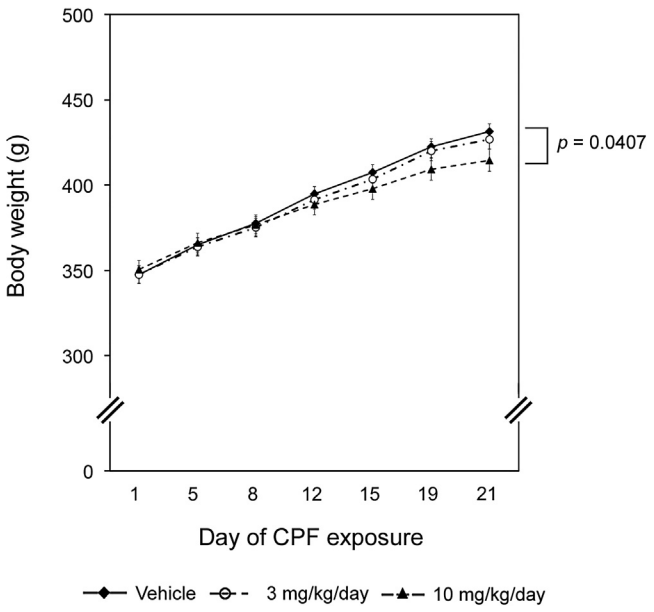

Fig. 1. Effects of CPF exposure on body weight.
Body weight measurements for Long Evans rats treated with vehicle or CPF. Data are presented as the mean ± SD; $N = 30$ per treatment group. Statistically significant differences between treatment groups were identified using repeated measures one-way ANOVA.

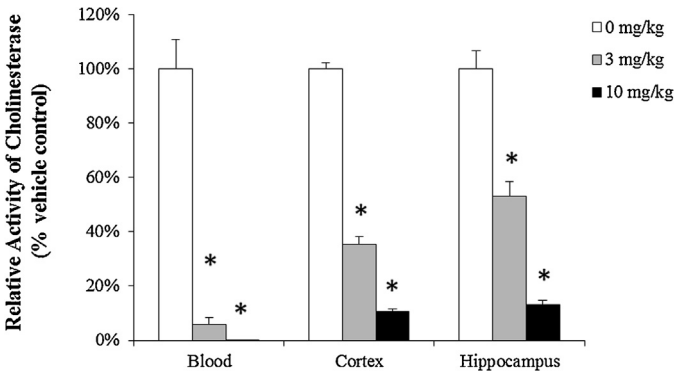

Fig. 2. Repeated daily exposure to CPF for 21 days significantly inhibits cholinesterase (ChE) activity.
ChE activity was measured in whole blood, the hippocampus and the entorhinal cortex of vehicle control and CPF-exposed animals after 21 days. Data are presented as the mean ± SE ($N = 10$). Statistically significant differences between treatment groups were identified using one-way ANOVA. White, 0 mg/kg/day CPF; gray, 3 mg/kg/day CPF; black, 10 mg/kg/day CPF; *$p < 0.05$; CPF, chlorpyrifos.

Table 1
Cross-platform comparison of CPF-induced differential gene expression.

	# Differentially expressed genes	
	Microarray	RNA-sequencing
FDR < 0.05	624	825
CPF > CTRL	276	218
CPF < CTRL	348	607
FDR < 0.05 and FC ≥ 1.25	50	368
CPF > CTRL	28	38
CPF < CTRL	22	330

CPF: 10 mg/kg/day CPF treatment for 21 days.
CTRL: vehicle control treatment for 21 days.
FDR: false discovery rate.
FC: fold change of difference.

after administration of CPF at 10 mg/kg/d) encode proteins that are secreted into the extracellular space, including the neuropeptides NPY, CORT, BDNF and CRHBP (Table 2). Biological function enrichment analysis (for the full GO enrichment analysis, see File 6 in Lee et al., 2015) identified 20 extracellular proteins involved in neuropeptide signaling pathways (CORT, NPY and PNOC), cell migration, axon guidance and synaptic transmission. A subset of these proteins was associated with physiological functions that are altered in neurological and psychological disorders (e.g., seizure disorders [BDNF, NPTX2, NPY, PNOC, SRGN], schizophrenia [BDNF, CRHBP, NPY, PNOC, SLIT3] and anxiety [BDNF, CRHBP, MDK, NPY, PNOC]).

Sixty-seven genes were differentially expressed (FDR < 0.05) in both the microarray and the RNA-seq analyses (Fig. 3a, also see File 5 in Lee et al., 2015) after high dose CPF exposure. Microarray analysis identified an additional 557 DEGs, most of which (>90%) displayed less than a ±1.25-fold change in mRNA abundance from control after CPF administration (10 mg/kg/*b.w*/d; Fig. 3b). RNA-seq identified an additional 758 DEGS (Fig. 3a). An independent experiment of the same design (male rats exposed to CPF at 10 mg/kg/d for 21 days) was conducted and DEGs were determined by microarray analysis (Hussainzada and Lein, *unpublished observations*). The results were compared with the 67 genes common to both the RNA-seq and microarray analyses. Six mRNAs (*Bdnf*, *Cort*, *Crhbp*, *Nptx2*, *Npy* and *Pnoc*) were common to the set of DEGs in all three transcriptomics analyses.

3.2.2. Immunohistochemical evaluation of neuropeptide proteins in the rat hippocampus

Protein expression of *Npy*, *Crhbp* and *Nptx2* were evaluated in the rat hippocampus by immunohistochemistry (Fig. 4 and Table 3). Administration of 10 mg/kg/d CPF caused an increase in the number of cells immunopositive for NPY and CRHBP within the CA1 region of the hippocampus. All animals showed NPTX2 immunoreactivity in the hippocampus, but there was no difference in the incidence or intensity of immunostaining for NPTX2 between treatment groups. However, upregulation of the *Nptx2* gene by CPF exposure at 10 mg/kg *b.w.*/d was validated at the level of mRNA transcription with the real-time, quantitative-PCR (qPCR) using independent samples (see Files 7 and 8 in Lee et al., 2015).

3.2.3. CPF-induced differential expression of microRNAs in the rat hippocampus

Small ncRNA profiles of CPF-exposed rat hippocampus (10 mg/kg *b.w.*/d CPF, $n = 11$) were compared to vehicle controls ($n = 11$) using Illumina sequencing technology. Mapping of the short read sequences against the database of known miRNAs (miRBase) revealed that repeated exposures to CPF at 10 mg/kg *b.w.*/d for 21 days resulted in the differential expression of two mature miRNAs (rno-miR-132-3p and rno-miR-212-5p) in the hippocampus (Table 4).

3.3. Functional network and pathway analysis

Upstream regulators and downstream effectors of the CPF-related gene expression changes were predicted using IPA analysis tools. Two potential upstream regulators were identified from the differential mRNA expression pattern observed in the high dose CPF-exposed rat hippocampus (Table 5), including miR-292-5p (*Adora1*, *Akap6*, *Madd*, *Mapk1*, *Syt4* and *Sept3*). The IPA downstream effects analysis identified enrichment in functions associated with seizure disorders and cognitive defects (i.e., long term depression, long-term potentiation of the synapse and long term depression of the synapse) (Table 5).

4. Discussion

Our study identifies novel molecular and cellular signatures of neurotoxicity associated with 21 days of subchronic exposure to CPF at 10 mg/kg/d (Fig. 5). We identified six “high confidence” genes encoding neuropeptides that are differentially regulated in

Table 2
Extracellular protein genes differentially expressed in the CPF-treated rat hippocampus (FDR < 0.05 and FC ≥ 1.25).

Gene symbol	Protein	Gene name	ANOVA *p*-value	FDR	Ratio [CPF/CTRL]
Cort	CORT	Cortistatin	5.62×10^{-5}	0.0397	2.86
Npy	NPY	Neuropeptide Y	2.73×10^{-6}	0.0146	1.98
Crhbp	CRHBP	Corticotropin releasing hormone binding protein	3.91×10^{-3}	0.0480	1.56
Bdnf	BDNF	Brain-derived neurotrophic factor	1.55×10^{-4}	0.0397	1.41
Mdk	MDK	Midkine (neurite growth-promoting factor 2)	1.03×10^{-3}	0.0455	1.37
Rbp4	RBP4	Retinol binding protein 4, plasma	2.96×10^{-3}	0.0479	1.35
Pnoc	PNOC	Prepronociceptin	1.40×10^{-3}	0.0455	1.34
Srgn	SRGN	Serglycin	1.27×10^{-3}	0.0455	1.33
Nptx2	NPTX2	Neuronal pentraxin II	1.39×10^{-3}	0.0455	1.32
Cgref1	CGREF1	Cell growth regulator with EF-hand domain 1	1.06×10^{-3}	0.0455	1.32
Mgp	MGP	Matrix Gla protein	5.50×10^{-3}	0.0492	1.25

Gene symbol	Protein	Gene name	ANOVA *p*-value	FDR	Ratio [CTRL/CPF]
Pdzd8	PDZD8	PDZ domain containing 8	5.42×10^{-3}	0.0491	1.25
Lrrn3	LRRN3	Leucine rich repeat neuronal 3	3.24×10^{-3}	0.0480	1.27
Fry	FRY	Furry homolog (*Drosophila*)	3.06×10^{-3}	0.0480	1.28
Specc1l	SPECC1L	Sperm antigen with calponin homology and coiled-coil domains 1-like	1.87×10^{-3}	0.0462	1.28
Wnt4	WNT4	Wingless-type MMTV integration site family, member 4	5.81×10^{-3}	0.0497	1.32
Slit3	SLIT3	Slit homolog 3 (*Drosophila*)	2.79×10^{-4}	0.0417	1.34
Slit1	SLIT1	Slit homolog 1 (*Drosophila*)	4.03×10^{-3}	0.0482	1.35
Spata2	SPATA2	Spermatogenesis associated 2	1.12×10^{-3}	0.0455	1.53
Negr1	NEGR1	Neuronal growth regulator 1	2.97×10^{-3}	0.0479	1.69

CPF: 10 mg/kg/day CPF treatment for 21 days.
CTRL: vehicle control treatment for 21 days.
FDR: false discovery rate.
FC: fold change of difference.

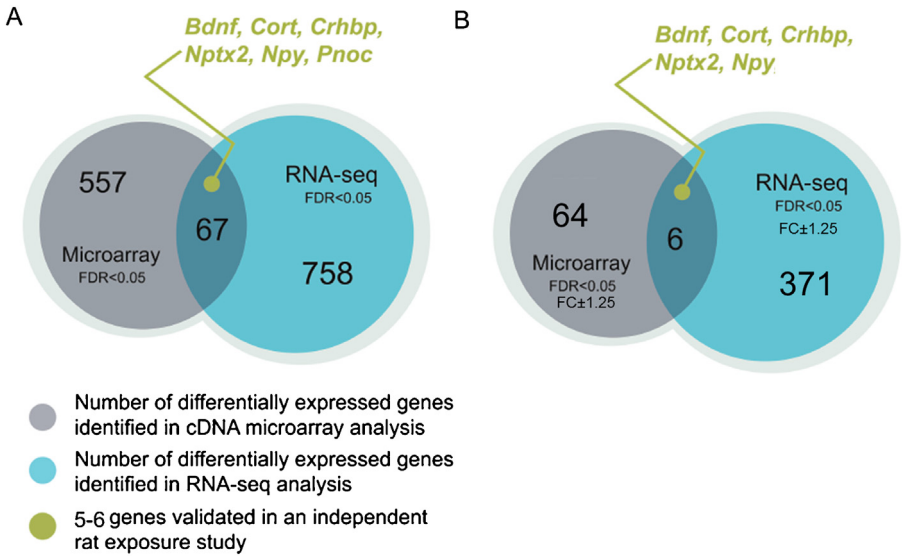

Fig. 3. Gene expression in the rat hippocampus is altered after 21 days exposure to CPF at 10 mg/kg/d.
Comparison of CPF-induced differential gene expression profiles determined by cDNA microarrays versus RNA-seq. (A) Venn diagrams comparing transcriptomics analyses with FDR < 0.05. (B) Venn diagrams comparing transcriptomics analyses with FDR < 0.05 and fold change (FC) ±1.25.

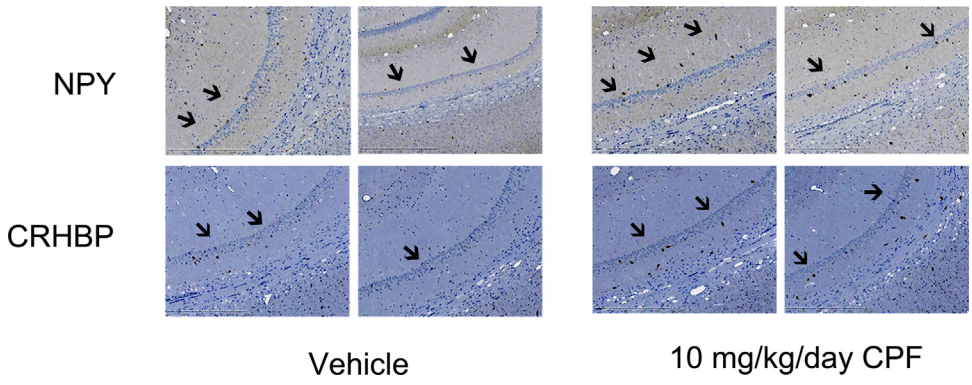

Fig. 4. CPF increases hippocampal expression of neuropeptides.
The expression of neuropeptides in the hippocampus of rats exposed for 21 days to vehicle or CPF at 10/mg/kg *b.w.*/d was determined immunohistochemically using antibodies specific for NPY and CRHBP.

Table 3
Quantitative analysis of NPY and CRHBP immunoreactivity in the rat hippocampus.

Protein	CPF dose	No. of animals analyzed	Grade 1 (minimal; <10% IR cells)	Grade 2 (mild; 10–25% IR cells)
NPY (neuronal peptide Y)	Vehicle	10	9	1
	3 mg/kg/day	10	6	4
	10 mg/kg/day	10	2	8
CRHBP (corticotropin-releasing factor-binding protein)	Vehicle	10	10	0
	3 mg/kg/day	10	9	1
	10 mg/kg/day	10	5	5

IR: immunoreactive.

the hippocampus after administration of CPF at a level that causes severe inhibition of brain cholinesterase. These neuropeptide-encoding genes have been associated with neurocognitive disorders previously (Table 6), supporting a role for these genes in the neurobehavioral deficits observed in animal studies of OP-induced neurotoxicity. We have determined in independent experiments that the CPF dosing paradigm used in this study causes deficits in performance in Pavlovian fear conditioning, which implicates amygdalar and hippocampal mechanisms in mediating CPF-induced neurotoxicity (manuscript in preparation).

Higher incidence rates of OP poisoning have been associated with insufficient regulation of the use of OP compounds, inadequate handling or storage techniques and lack or inadequate use of personal protective equipment (International Programme on Chemical Safety, 2004). Agricultural workers exposed to mild to moderate levels of OP pesticides displayed decreased memory and

Table 4
Differentially expressed miRNAs.

# miRNAs in the model[a]	# Mature miRNAs detected[b]	DE miRNA (FDR < 0.05)	CPF/CTRL ratio[c]
723	214	rno-miR-212-5p	1.34
		rno-miR-132-3p	1.85

DE: differentially expressed; FDR: false discovery rate; miRNA: micro RNA; rno-miR: *Rattus norvegicus* micro RNA; RGSC: rat genome sequencing consortium.
[a] miRBase, release 19 (RGSC 3.4/rn4).
[b] RPM (reads per million mapped miRNA) ≥ 5.
[c] CPF: 10 mg/kg/day CPF treatment for 21 days; CTRL: vehicle control treatment for 21 days.

Table 5
Upstream regulation and downstream effects of repeated exposures to 10 mg/kg/day CPF.

Functions	Activation *z*-score	*p*-value of overlap	# Molecules	Predicted state
Upstream regulation				
miR-292-5p	2.45	9.08×10^{-5}	6	Activated
Ethanol	−3.11	1.03×10^{-3}	14	Inhibited
Downstream effect				
Seizure disorder	2.50	1.38×10^{-8}	34	Increased
Seizures	2.30	4.45×10^{-8}	30	Increased
Outgrowth of neurites	−2.02	8.52×10^{-3}	23	Decreased
Aggregation of cells	−2.12	4.94×10^{-4}	15	Decreased
Long term depression	−2.15	1.26×10^{-5}	13	Decreased
Long-term potentiation of synapse	−2.17	1.93×10^{-6}	19	Decreased
Formation of plasma membrane projections	−2.20	2.7×10^{-5}	37	Decreased
Synaptic depression	−2.20	7.21×10^{-7}	17	Decreased
Long term depression of synapse	−2.20	2.52×10^{-4}	8	Decreased
Behavior	−2.21	1.9×10^{-13}	70	Decreased
Size of brain	−2.38	6.42×10^{-5}	12	Decreased
Formation of cellular protrusions	−2.43	7.73×10^{-4}	41	Decreased
Growth of neurites	−2.46	6.15×10^{-3}	26	Decreased
Microtubule dynamics	−2.72	9.33×10^{-5}	57	Decreased
Organization of cytoskeleton	−2.78	5.76×10^{-5}	63	Decreased
Organization of cytoplasm	−2.78	1.06×10^{-3}	64	Decreased
Long-term potentiation	−3.05	4.82×10^{-9}	30	Decreased
Coordination	−3.29	6.69×10^{-4}	17	Decreased
Fertility	−3.83	159×10^{-4}	16	Decreased
Size of body	−5.96	2.83×10^{-7}	53	Decreased

attention (Mackenzie Ross et al., 2010). Increased levels of anxiety and other mood disorders in commercial OP pesticide sprayers have also been reported (Roldan-Tapia et al., 2006). Similarities between human and rat OP neurotoxicity have previously been demonstrated (Bushnell and Moser, 2006; Costa, 2006). Sustained ChE inhibition results in systemic hypercholinergic activity, an effect hypothesized to lead to neurotoxicity even in the absence of clinical signs (Fukuto, 1990; Rohlman et al., 2014). Our study was designed to determine the hippocampal gene changes associated with repeated exposures to CPF at levels that did not cause signs of cholinergic crisis.

Genome-wide profiling of protein coding RNAs and ncRNAs revealed increased expression of neurotrophins and secreted neuropeptides in the CPF-exposed rat hippocampus. Based on a knowledge-based pathway analysis and literature-based curation, we constructed a diagram illustrating putative molecular mechanisms that may be elicited in rat hippocampal cells following repeated exposure to CPF (Fig. 5). Neuropeptides such as NPY, or neurotrophins like BDNF, can bind to their cognate receptors to regulate downstream cell signaling pathways. The activation of neurotrophin-mediated signaling pathways results in phosphorylation of signaling proteins, including methyl CpG binding protein 2 (MeCP2) and cAMP response element-binding (CREB) protein. Phosphorylation of MeCP2 and CREB lead to the CREB-mediated transcriptional activation of numerous genes, including a subset of the genes and miRNAs identified in our study. CPF has been shown to trigger CREB phosphorylation (activation) in cultured hippocampal neurons at very low concentrations (Schuh et al., 2002).

Overall, the extracellular neuropeptides up-regulated by CPF promote neuronal survival (Table 6). They are involved in the regulation of various physiological processes and participate in modulating neurological functions in the brain (Borroni et al., 2009). Expression of these neuropeptides is critical in maintaining normal physiological balance and neurobehavioral functions, such as memory, learning and resilience to stress (Zhang et al., 2012). Conversely, dysregulation of these neuropeptide genes is associated with detrimental consequences, especially in neurobehavioral functions (Table 6). Cellular pathways involving these neuropeptides are tightly regulated by fast response factors such as phosphorylation, acetylation or ncRNAs. These epigenetic factors also play a central role in maintaining the balance between neuroprotection and neuronal dysfunction.

Our data suggest that CPF exposure significantly disrupts the mRNA expression of neuropeptide-encoding genes at levels that cause high (approximately 90%) but not moderate (approximately 50%) inhibition of brain cholinesterase. Five of the six “high confidence” DEGs encode neuropeptides that are secreted into the extracellular space and regulate communication between neuronal cells. *Crhbp* encodes a protein that binds to and regulates the activity of the neuropeptide molecule corticotropin-releasing hormone (CRH). Our results are concordant with other laboratories showing that repeated low-dose exposure to CPF alters the mRNA and protein expression of neurotrophins in the brain and the protein level (Betancourt et al., 2007). Perturbed neurotrophin signaling may result in deficits in hippocampal synaptic transmission. Neuropeptides may bind to receptors located on the cell membrane surface of the parent cell or neighboring cells, enabling

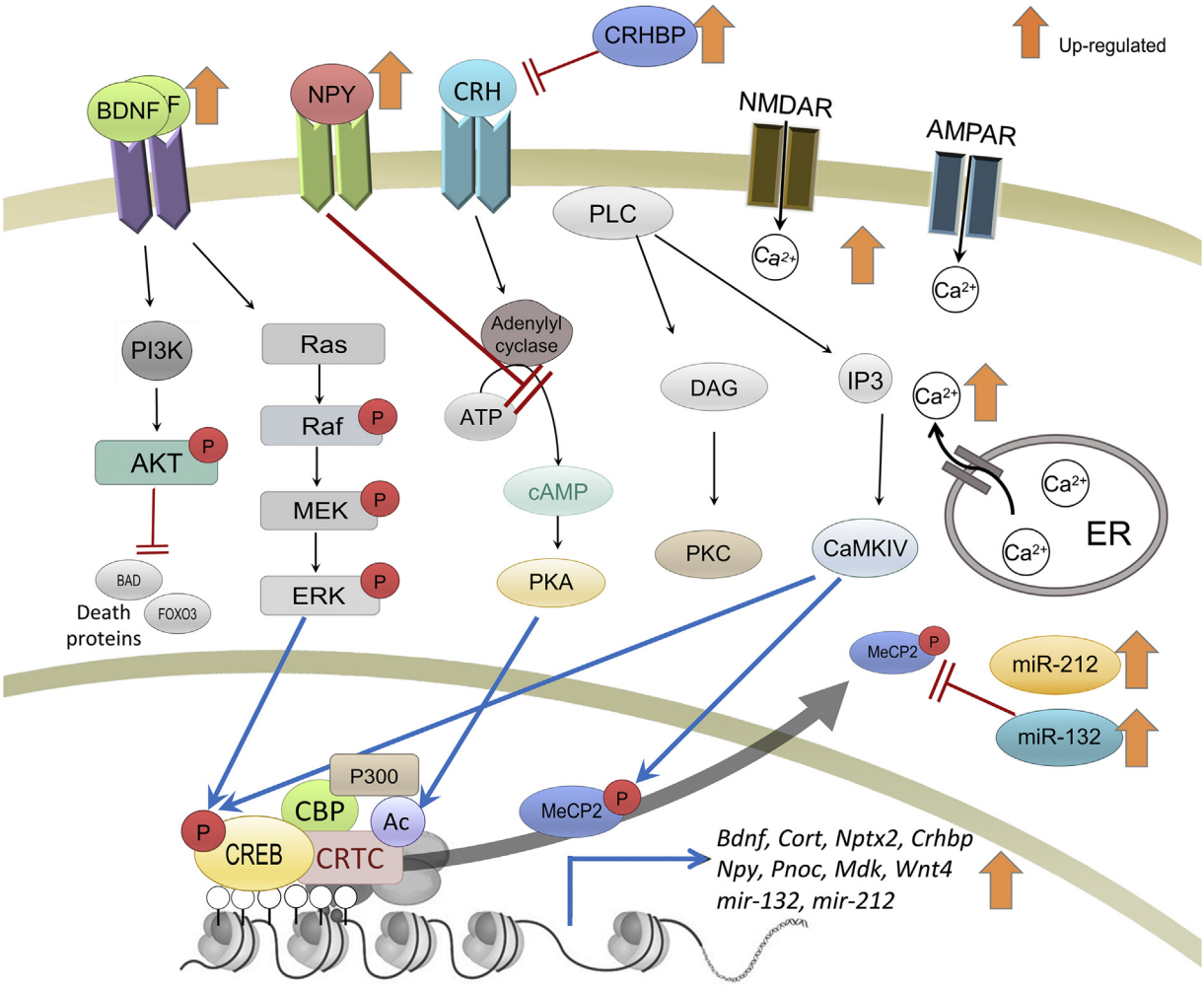

Fig. 5. Proposed mechanisms of CPF neurotoxicity in the hippocampus of rats exposed to CPF at 10 mg/kg/d for 21 days.

Table 6
Neuropeptide-encoding genes, their Functions and associated disorders.

Gene symbol	Gene name	Encoded product	Functions	Associated diseases
Bdnf	Brain-derived neurotrophic factor	BDNF	Synaptic plasticity, Long-term potentiation (LTP), Long-term depression (LTD), Survival, Differentiation, Maintenance of nerve cells	Alzheimer's disease (Borroni et al., 2009), Huntington diseases (Canals et al., 2004), Epilepsy (Gall et al., 1991), Increase in cocaine self-administration behavior (Vargas-Perez et al., 2009)
Cort	Cortistatin	CORT	Inhibition of cAMP production, Depression of neuronal activity, Neuroprotection	Induction of slow-wave sleep, Reduction of locomotor activity
Crhbp	Corticotropin releasing hormone (CRH) binding protein	CRHBP	Inactivation of CRH (Cortright et al., 1995)	Exploratory behavior, Decreased anxiety, Bipolar disorder (Van Den Eede et al., 2007)
Npy	Neuropeptide Y	NPY	Seizure inhibition, Neuroprotection, Pain inhibition, Appetite stimulation	Anxiety effects (Heilig, 2004), Compulsive food consumption, Cocaine seeking behavior, Higher alcohol consumption (Hwang et al., 1999), Metabolic disease, Cardiovascular disease
Nptx2	Neuronal pentraxin II	NPTX2	Long-term plasticity, Individual difference in responding to a novel environment	Parkinson's disease (Moran et al., 2008), Pancreatic cancer
Pnoc	Prepronociceptin	PNOC	Modulation of nociceptive & locomotor behavior, Reduced neuronal excitability	Anxiety, Increased nociceptive sensitivity in PTSD (Zhang et al., 2012)

the amplification of CPF-exposure signals through activation of various signaling pathways (Rozengurt, 2002).

Neurotrophin-like signaling may contribute to the effects of CPF. Exposure to 3 mg/kg *b.w.*/d CPF did not induce statistically significant gene expression changes in the hippocampus, but treatment with 10 mg/kg *b.w.*/d elicited a strong up-regulation of secreted neuropeptide genes. Although the reason for this finding is unclear, it is conceivable that a threshold exists for induction of differential gene expression with subchronic exposures to CPF, and the 3 mg/kg *b.w.*/d CPF may not have been sufficient to trigger the activation of neurotrophin gene expression. Alterations in thresholds of neurotrophins may be the consequence or contribute to the onset of OP-induced neuropathies (Pope et al., 1995). Alternatively, molecular targets affected by 3 mg/kg *b.w.*/d CPF may be epigenetic mediators not detected by transcriptome analyses. It is also possible that we failed to discern alterations in gene expression that occurred only in a subset of the hippocampal cells because of a dilution effect. The results observed at the high dose level may also have resulted from the general toxicity implied by the slight decrease in body weight gain observed in the high dose group. However, the percent difference in weight between animals exposed to CPF at 10 mg/kg *b.w.*/d and vehicle controls animals is less than 3%, which is below the 20% decrease in body weight considered to be clinically significant in laboratory animals (National Research Council, 2011). Although this potential confounder cannot be ruled out completely, the downstream effects predicted by an unbiased gene set enrichment analysis are nearly all CNS-related, including endpoints coinciding with neurocognitive deficits and learning and memory endpoints (i.e., long term depression, long term potentiation of the synapse and long term depression of the synapse; see Table 5).

Epigenetic mechanisms may contribute to the molecular effects of CPF exposure. Recent evidence suggests that epigenetic processes are not limited to gene regulation in pluripotent/totipotent states, but also contribute to the modifications and maintenance of the transcriptional state of mature cells such as neurons (Day and Sweatt, 2011). External environmental stimuli can modify epigenetic states to trigger changes in gene expression in target cells, tissues or organisms (Lesiak et al., 2014). Small ncRNAs are present in various brain regions and exert epigenetic control of neurobehavioral functions, such as learning, memory and synaptic plasticity (Konopka et al., 2010). The small ncRNAs miR-132 and miR-212 are transcribed from the same miRNA gene cluster located on chromosome 10 in rats and chromosome 17 in humans. The expression of the miR-132/212 cluster is promoted by cAMP response element-binding protein (CREB) through direct binding to the CRE in their 5′ promoter region, and is also induced by neurotrophins such as BDNF (Remenyi et al., 2010). miR-132 and miR-212 are involved in synaptic function, long-term potentiation, cell survival and memorization processes (Wang et al., 2013). Dysregulation of miR-132 and miR-212 expression is implicated in numerous brain disorders, including Huntington's disease, Parkinson's disease, Rett syndrome, seizure, schizophrenia, cocaine addiction and neurodevelopmental disorders (reviewed in Wanet et al., 2012). Up-regulation of miR-132 and miR-212 in the CPF-exposed rat hippocampus suggests that these two miRNAs may regulate at least a subset of the transcriptome-level changes involved in neurotrophin signaling pathways, and that repeated CPF exposure may elicit initial fast adaptive transcriptional responses via a reversible epigenetic mechanism.

The protein products of miR-292 target genes (ADORA1, AKAP6, MADD and MAPK1) are involved in survival signaling pathway (Serchov et al., 2012). Studies indicate that miR-292-5p is one of the miRNAs that is down-regulated after ischemic preconditioning, and down-regulation of miR-292 target genes might be a neuroprotective mechanism following oxidative stress (Dharap and Vemuganti, 2010). Whether activated miR-292 induces a neuroprotective response in animals treated with CPF remains to be determined.

An important observation from these studies is that the lower CPF dose group (3 mg/kg/d) displayed 40–50% brain cholinesterase activity but did not show any statistically significant toxicogenomic changes. Only at high (~90%) levels of ChE inhibition caused by the higher CPF dose of 10 mg/kg/d are statistically significant toxicogenomic changes observed. The high dose CPF exposure also caused a slight (<3%) but statistically significant decrease in body weight, which may reflect generalized toxicity and metabolic changes that could also contribute to the toxicogenomic changes observed in these animals. Collectively, these observations indicate that phenotypic changes s associated with occupational CPF exposure level typical of developed countries are likely not the result of large scale transcriptomic changes throughout the hippocampus.

5. Conclusions

Our findings clearly demonstrate effects of 10 mg/kg/d CPF on gene expression with statistically significant transcriptomic changes in the brain observed only in animals exposed to CPF at levels that inhibit brain ChE by approximately 90%. Our results identify molecular processes that may contribute to neurobehavioral functions that others have observed after subchronic exposure to 10 mg/kg/d CPF (Lein et al. manuscript in preparation). While 3 mg/kg/d CPF led to some changes in gene expression, they failed to reach significance due to the strict criteria we have applied to minimize false positives caused by multiple testing. In animals exhibiting CPF-related changes in brain gene expression, our data support earlier studies that have implicated CREB-mediated transcriptional regulation in CPF neurotoxicity (Schuh et al., 2002). In addition, we present transcriptomic and epigenetic evidence implicating activation of neuropeptide- and neurotrophin-mediated pathways in the hippocampus of rats after repeated exposure to CPF. Further investigation, however, is necessary to determine whether perturbations of these homeostatic regulatory pathways are causally linked to neurotoxicity related to CPF exposure. Furthermore, it remains to be determined whether similar changes in gene expression accompany lower, more common exposures to CPF, which may constitute a more useful bioindicator of more typical CPF exposures.

Conflict of interest

The authors declare that there are no conflicts of interest.

Disclaimers

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Committee and conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (NRC, 2011) in facilities that are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

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