

# Subtoxic chlorpyrifos treatment resulted in differential expression of genes implicated in neurological functions and development

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**Abstract** Chlorpyrifos (CPF), a commonly used organophosphorus insecticide, induces acetylcholinesterase inhibition and cholinergic toxicity. Subtoxic exposure to CPF has long-term adverse effects on synaptic function/development and behavioral performance. To gain insight into the possible mechanism(s) of these observations, this study aims to investigate gene expression changes in the forebrain of rats treated with subtoxic CPF doses using DNA microarrays. Statistical analysis revealed that CPF treatment resulted in differential expression of 277 genes. Gene ontology and pathway analyses revealed that these genes have important roles in nervous system development and functions including axon guidance, dorso-ventral axis formation, long-term potentiation, synaptic transmission, and insulin signaling. The results of biological associated network analysis showed that Gsk3b is highly connected in several of these networks suggesting its potential role in cellular response to CPF exposure/neurotoxicity. These findings might serve as

the basis for future mechanistic analysis of the long-term adverse effects of subtoxic CPF exposure.

**Keywords** Chlorpyrifos · Neurotoxicity · Transcriptomic profiling · Pathway analysis · Biological association networks

## Introduction

Chlorpyrifos (diethyl 3,5,6-trichloro-2-pyridyl phosphorothionate) is a broad-spectrum organophosphorus (OP) insecticide with numerous agricultural crop and urban pest control uses. Recent concerns about its health effects have led to its ban in residential applications. Despite the restrictions imposed on its use, CPF continues to be one of the most commonly used OP insecticides. CPF is designed to produce acute cholinergic effects through the inhibition of acetylcholinesterase (AChE) (Eto 1979; Echobichon 1982; Murphy 1986). Although CPF itself is a weak anticholinesterase compound, it undergoes cytochrome P-450-mediated bioactivation to CPF-oxon, which can bind to and inhibit AChE to elicit cholinergic toxicity (Forsyth and Chambers 1989; Chambers and Chambers 1989). The resultant accumulation of the neurotransmitter acetylcholine (ACh) in synaptic junctions leads to excessive stimulation of post-synaptic cells and results in cholinergic toxicity. Signs of toxicity include autonomic dysfunction, muscle fasciculations, seizures and convulsions, and respiratory distress (Dow Agro Sciences 2003). CPF may also cause delayed neurotoxicity known as organophosphate-induced delayed neuropathy (OPIDN) (Pope et al. 1993). CPF exhibits only moderate acute toxicity in many mammalian species (e.g. acute oral LD<sub>50</sub> of 97–276 mg/kg in rats), largely due to effective detoxification of the active metabolite, CPF-oxon,

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by A-esterases and carboxylesterases (Karanth and Pope 2000).

Acetylcholine also plays a trophic role in brain development. Accordingly, chemicals that promote or interfere with the functions of this neurotransmitter evoke neurodevelopmental abnormalities by disrupting the timing or intensity of neurotrophic actions. Exposure to CPF has long-term, adverse effects on specific processes involved in brain cell replication and differentiation, synaptic function/development, and ultimately behavioral performance (Slotkin 2004). CPF may elicit CNS cell damage, in part, through noncholinergic mechanisms that involve alterations in the expression and/or activity of transcription factors that regulate cell replication, differentiation, and apoptosis. Early neonatal exposure to CPF resulted in a significant elevation of c-fos in the forebrain, whereas its expression was suppressed after late postnatal exposure (Dam et al. 2003). Recent findings indicate that developmental neurotoxicity of CPF extends to late phases of brain maturation including adolescence (Slotkin 2004).

Subtoxic exposure to CPF, which does not cause cholinergic hyperstimulation or exert overt toxicity, can adversely affect the expression levels of critical genes involved in brain development during the early postnatal period in the rat. For instance, exposure to 1.5 or 3.0 mg/kg CPF resulted in significant reduction in the mRNA levels of nerve growth factor and reelin, two factors critical to brain development in the forebrain region (Betancourt et al. 2006). CPF also has significant inhibitory effect on the developing oligodendrocytes, as evidenced by decreased myelin-associated glycoprotein mRNA expression (Betancourt et al. 2006). In contrast, glial fibrillary acidic protein mRNA levels were significantly increased indicating enhanced astrocyte reactivity. Treatment with subtoxic doses of CPF also resulted in significant inhibition of DNA and protein synthesis followed by subsequent deficits in cell numbers in the cerebellum, forebrain, and brainstem of neonatal rats (Whitney et al. 1995). Although the molecular mechanism of DNA and protein synthesis inhibition by CPF is not clear, it is not secondary to generalized cell damage or suppression of cell metabolism as indicated by the maintenance of normal ornithine decarboxylase activities (Whitney et al. 1995). CPF has been reported to induce oxidative damage of cellular constituents including lipid peroxidation and nuclear DNA single-strand breaks in hepatic and brain tissues, as well as in cultured PC-12 cells by a poorly understood mechanism (Bagchi et al. 1995). Besides the toxic effects described above, CPF has also been shown to have direct effects on gonadotropin-releasing hormone (GnRH) gene expression, cell survival, and neurite outgrowth in a neuronal cell line (Gore 2002).

In a recent transcriptomic study of primary human astrocytes, CPF significantly changed the expression of a large

number of genes including molecular chaperones, signal transducers, transcription regulators, and transporters, as well as genes involved in behavior and development (Mense et al. 2006). Up-regulation of the interferon-gamma and insulin signaling pathways resulting from up-regulation of extracellular signal-regulated kinase 1/2, interleukin 6, and glial fibrillary acidic protein was observed. Based on this observation, the authors suggested that activation of the inflammatory response in astrocytes might be an important mechanism of CPF neurotoxicity.

Due to its widespread use, exposure to subtoxic levels of CPF might be a commonly encountered health hazard. Rats exposed to subtoxic doses of CPF show morphological changes in the brain, as well as long-term adverse effects on synaptic function/development and behavioral performance. Despite these observations, the effect of subtoxic CPF exposure on global gene expression *in vivo* has not been reported. In an attempt to gain new insights into the consequences of subtoxic CPF exposure at the molecular level, this study was undertaken to investigate gene expression changes resulting from CPF exposure using DNA microarrays. Data analyses using gene ontology search, pathway analysis, and biological association network analysis revealed that the differentially expressed genes are involved in several pathways with essential roles for neurological functions. These findings might help in defining future directions for investigating the mechanism of long-term adverse effects of subtoxic CPF exposure.

## Materials and methods

### Animals, CPF treatment and histopathology

Animal use in this study was conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996, and the Animal Welfare Act of 1966, as amended. Male Fischer 344 rats weighing 190–263 g (11–12 weeks old) were obtained from Charles River Laboratories. Groups of five animals were dosed at approximately 8 am by oral gavage with one of six doses of CPF (0.5, 1.0, 5, 10, 30, and 50 mg/kg) in corn syrup. Control animals were dosed with corn syrup alone. CPF-treated and control animals were sacrificed by CO<sub>2</sub> inhalation 96 h after CPF treatment. A section of the forebrain (prosencephalon) was dissected into sections weighing ~30 mg each, immediately frozen in liquid nitrogen, and stored at –80°C until processing for total RNA extraction.

### Gene expression profiling

Total RNA was isolated from one 30 mg-section of brain tissue from treated or control animals using Qiagen RNeasy

Mini kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. The quality of the isolated RNA was confirmed using the RNA 6000 Nano LabChip Kit with the Agilent 2100 Bioanalyzer System (Agilent Technologies, Palo Alto, CA). Fifteen micrograms of total RNA was used for first strand cDNA synthesis followed by second strand synthesis using the SuperScript Choice system (Invitrogen Corporation, Carlsbad, CA) in the presence of an oligo-(dT)24 anchored T7 primer (Prologo, Boulder, CO). The double-stranded cDNA was used as the template for in vitro transcription using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Inc., Farmingdale, NY). The resulting cRNA was purified and fragmented using the GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA). Fifteen micrograms of fragmented biotin-labeled cRNA was hybridized for 16 h at 45°C to an Affymetrix GeneChip array RAE230A. The hybridized arrays were then washed in the Affymetrix GeneChip Fluidics Station 400 with non-stringent washes at 25°C followed by stringent washes at 50°C as recommended by the manufacturer. Hybridized and stained GeneChips were scanned with an Affymetrix GeneArray Scanner and the fluorescence intensity captured using the Affymetrix GeneChip Operating Software (GCOS), according to standard Affymetrix procedures. The result generated by this procedure was stored as an image (.DAT) file.

#### Data processing and analysis

Absolute gene expression analysis (i.e., conversion of the scanned images to numerical gene expression values) was performed using the Affymetrix GCOS statistical algorithm. This algorithm also provides a qualitative measure for the signal referred to as “detection *P*-value”, which can be used to assign the genes into one of three groups, “Present” (P), “Marginal” (M), or “Absent” (A). To facilitate inter-experimental comparison, the gene expression signal was scaled using “All Probe Sets Scaling” with a target intensity of 500. The processed GeneChip data (.CHP file) was then published into a GCOS publish database for data analysis and data export.

The identification of differential gene expression was performed using the *t*-test analysis module of the Affymetrix Data Mining Tool (DMT). To increase the stringency of the analysis, the statistically significant genes identified in each dose of CPF were compared and only the genes with  $P < 0.05$  in two consecutive doses were considered as differentially expressed. The gene expression dataset was exported from DMT to Excel and text files for pathway analysis and other biological interpretation. GenMAPP, version 2.1 (Gene MicroArray Pathway Profiler, Gladstone Institute, University of California at San Francisco, San Francisco, CA) analysis and gene ontology analysis (using

the database for annotation, visualization, and integrated discovery, DAVID, <http://david.abcc.ncifcrf.gov>) of the differentially expressed genes was used to identify gene ontology categories and cellular/physiological processes and pathways that were significantly altered after CPF exposure. Biological association networks of the differentially expressed genes were constructed using PathwayArchitect, version 2.0.1 (Stratagene, La Jolla, CA), a tool that is based on the mining of the open literature, network modeling, and relevance statistics to map gene/protein associations and infer biological and functional interactions. The resulting association network maps were used without pruning for empirical exploration of their biological implications.

## Results

### Identification of gene expression changes

Gene expression changes in the forebrain isolated from rats 4 days after CPF exposure were identified using the *t*-test module of the Affymetrix DMT software. Initially, gene expression changes at each dose compared to the control group were identified. To increase the stringency of the analysis (i.e., to reduce false discovery due to multiple testing), only the probe sets with  $P < 0.05$  over at least two consecutive doses were considered as differentially expressed. The number of probe sets meeting this criterion increases gradually as the dose of CPF escalates and reaches a peak of 139 at 5 and 10 mg/kg then decreases to less than twenty at 30 and 50 mg/kg (Table 1) for up-regulated genes. The probe sets with significant expression changes across the entire dose range were then pooled to create the final list of a total of 287 probe sets. A small fraction of these probe sets were selected, and differential expression of the selected probe sets after CPF treatment was confirmed using qRT-PCR (data not shown).

A search of the DAVID database using this list revealed that these probes sets represent 277 unique genes and expressed sequences. Of these differentially expressed genes, 65 genes showed up-regulation, while 213 genes were down-regulated. The discrepancy between the total number of genes, 277, and the sum of the up-regulated and down-regulated genes, 278, is due to the presence of the gene *Ches1\_predicted* (checkpoint suppressor 1 (predicted)) in both the up- and down-regulated gene lists. The reason for this is that the two probe sets for the detection of this gene show differential expression at different doses. While the signal of one probe set showed up-regulation at the doses of 30 and 50 mg/kg, the signal of the other showed down-regulation at doses of 5 and 10 mg/kg. Although the exact reason for this observation is not clear,

**Table 1** Differential gene expression resulting from CPF treatments in the forebrain of rats identified using *t*-test

Dose	0.5 mg/kg	1.0 mg/kg	5 mg/kg	10 mg/kg	30 mg/kg	50 mg/kg
Number of probe sets down-regulated in two consecutive doses	12					
		58				
			139			
				37		
					19	
Total number of down-regulated probe sets	221					
Number of probe sets up-regulated in two consecutive doses	5					
		0				
			0			
				34		
					32	
Total number of up-regulated probe sets	66					
<b>Total number of probes sets showing significant change in signal intensity</b>	<b>287</b>					

$P < 0.05$  over two consecutive doses

it might be related to differential response of alternative processing/splicing of this gene to different doses of CPF.

Since a 2-log dose range (0.5–50 mg/kg) was used in this study, differential gene expression in the low-dose and high-dose ranges of 0.5–5 and 5–50 mg/kg, respectively, was also determined. This results in the identification of 5 up-regulated and 63 down-regulated genes after the low-dose treatments. On the other hand, 61 and 176 genes were up-regulated and down-regulated, respectively, after the high-dose treatments. Of these genes, 10 show differential expression in both dose ranges. The Affymetrix ID, the name/description of the genes, and the direction of expression changes of these genes are presented in supplemental data Table S1.

#### Gene ontology analysis of differentially expressed genes

To gain insight into the biological implications of gene expression changes resulting from CPF exposure, the differentially expressed genes were used to search the biological annotation databases of DAVID. This analysis revealed that a large number of biological processes and pathways were enriched (i.e. over-represented) in the final gene list. As shown in Table 2, among the enriched biological processes are nervous system development and function (e.g. axon guidance, dorso-ventral axis formation, long-term potentiation, and synaptic transmission), cell–cell interaction (e.g. cell adhesion and cell–cell signaling), and intracellular organization and biogenesis (e.g. intracellular protein metabolism, targeting and transport, as well as mRNA splicing). Performing this analysis using the lists of differentially expressed genes of low- and high-dose CPF treatments separately revealed that different sets of biological processes were selectively affected by these treatments (Table 3). Low-dose CPF treatment resulted in expression changes in the genes involved in cell organization and biogenesis (e.g.

regulation of protein metabolism and DNA-dependent transcription), MAPK signaling pathway, induction of apoptosis, neuroactive ligand–receptor interaction, and synaptic transmission. In contrast, the pattern of biological processes and pathway perturbations after high-dose treatments is similar to the result of the analysis using the entire list of differentially expressed genes as input data (Table 3). The lists of gene ontology categories and KEGG pathways affected by CPF treatments (i.e. low-dose range, high-dose range and the entire dose range) and the statistical significance as determined by the DAVID analysis is shown in supplemental data Table S2.

#### Pathway analysis of significant gene expression changes

To visualize the effect of CPF treatment on gene expression changes in specific cellular pathways, GenMAPP analysis was performed using the expression profiles of the differentially expressed genes as input data. Representative pathways, as well as the number of genes involved in these pathways and the direction of these gene expression changes are shown in Table 4. Consistent with the results of the gene ontology search described above, gene expression changes after CPF treatment were observed in the pathways related to neurological function, cell adhesion, and mRNA processing. Additionally, several signaling pathways including epidermal growth factor receptor 1 (Egfr1), interleukin 3 (IL-3), interleukin 6 (IL-6), insulin, and Wnt signaling pathways might also be affected by CPF treatment, due to differential expression of Kras (Kirsten rat sarcoma viral oncogene homolog), Prkca (protein kinase C, alpha), Map2k1 (mitogen activated protein kinase kinase 1), and Gsk3b (glycogen synthase kinase 3 beta) that are common players in these intracellular signaling cascades. Gene expression changes in two representative pathways, insulin signaling and long-term potentiation (LTP) are shown in Figs. 1 and 2, respectively.

**Table 2** Biological processes and pathways over-represented among the differentially expressed genes, the number of genes in each of these processes and pathways, and the statistical significance of enrichment based on the search of the NIAID DAVID biological annotation databases

Category	Term	Count	P-value
GOTERM_BP_ALL	Axon guidance	5	3.40E-02
KEGG_PATHWAY	Axon guidance	6	2.20E-02
GOTERM_BP_ALL	Cell adhesion	15	3.20E-02
SP_PIR_KEYWORDS	Celladhesion	9	8.80E-03
KEGG_PATHWAY	Cell adhesion molecules (CAMS)	7	1.90E-02
GOTERM_BP_ALL	Cell communication	60	1.20E-02
GOTERM_BP_ALL	Cell organization and biogenesis	42	2.50E-03
GOTERM_BP_ALL	Cell–cell signaling	23	4.70E-03
GOTERM_BP_ALL	Cellular localization	18	3.90E-02
KEGG_PATHWAY	Dorso-ventral axis formation	4	1.40E-02
GOTERM_BP_ALL	Establishment of cellular localization	18	3.60E-02
GOTERM_BP_ALL	Establishment of localization	61	5.60E-03
GOTERM_BP_ALL	Intracellular protein transport	13	2.90E-02
GOTERM_BP_ALL	Intracellular transport	18	3.10E-02
GOTERM_BP_ALL	Localization	61	7.00E-03
KEGG_PATHWAY	Long-term potentiation	6	1.40E-02
GOTERM_BP_ALL	Negative regulation of cell organization and biogenesis	4	2.50E-02
GOTERM_BP_ALL	Negative regulation of development	5	4.50E-02
GOTERM_BP_ALL	Negative regulation of protein metabolism	5	3.80E-02
GOTERM_BP_ALL	Nervous system development	22	7.00E-03
GOTERM_BP_ALL	Neurophysiological process	24	6.20E-03
GOTERM_BP_ALL	Nuclear mRNA splicing, via spliceosome	5	4.30E-02
GOTERM_BP_ALL	Protein depolymerization	4	3.30E-02
GOTERM_BP_ALL	Protein targeting	9	3.10E-02
GOTERM_BP_ALL	Protein transport	15	4.30E-02
GOTERM_BP_ALL	Regulation of cell organization and biogenesis	5	2.50E-02
GOTERM_BP_ALL	Regulation of protein metabolism	11	1.10E-02
GOTERM_BP_ALL	RNA splicing, via transesterification reactions	5	4.30E-02
GOTERM_BP_ALL	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	5	4.30E-02
GOTERM_BP_ALL	Spliceosome assembly	3	5.00E-02
GOTERM_BP_ALL	Synaptic transmission	19	3.90E-04
GOTERM_BP_ALL	System development	23	6.40E-03
GOTERM_BP_ALL	Transmission of nerve impulse	20	3.30E-04
GOTERM_BP_ALL	Transport	52	1.70E-02

In the insulin signaling pathway, five genes, Grb14 (growth factor receptor bound protein 14), Map2k1, Gsk3b, Prkca, and Ptpf (protein tyrosine phosphatase receptor type F) were down-regulated, while only one gene, Xbp1 (X-box binding protein 1, labeled as RGD 1303073 in Fig. 1), was up-regulated. Conversely, all six genes, including Grial (glutamate receptor, ionotropic, AMPA 1), Grin1 (glutamate receptor, ionotropic, N-methyl D-aspartate 1), Cacna1c (calcium channel, voltage-dependent, L type, alpha 1C subunit), Prkaca (protein kinase, cAMP-dependent, catalytic, alpha), Kras, and Map2k1, were down-regulated in LTP of synaptic transmission. The entire list of pathways and the direction of the gene expression changes

involved in these pathways are provided in supplemental data Table S3.

#### Biological association network analysis of differentially expressed genes

The biological association of the differentially expressed genes and their potential partners was explored using PathwayArchitect (version 2.0.1). A representative network map (binding complex network) is shown in Fig. 3. This network encompasses a variety of nodes with functions from voltage-gated ion channels to muscular dystrophy, Alzheimer's disease, and cancer. There are 40 gene/protein

**Table 3** Biological processes and pathways over-represented among the differentially expressed genes resulting from low- and high-dose CPF treatments, the number of genes in each of these processes and pathways, and the statistical significance of enrichment based on the search of the NIAID DAVID biological annotation databases

Category	Term	Count	<i>P</i> -value
Low-dose treatments (0.5–5 mg/kg)			
GOTERM_BP_ALL	Biopolymer metabolism	14	5.00E-02
GOTERM_BP_ALL	Biopolymer modification	10	5.00E-02
GOTERM_BP_ALL	Cell communication	20	1.10E-02
GOTERM_BP_ALL	Cell organization and biogenesis	13	3.20E-02
GOTERM_BP_ALL	Induction of apoptosis	4	4.60E-02
GOTERM_BP_ALL	Induction of programmed cell death	4	4.60E-02
KEGG_PATHWAY	MAPK signaling pathway	5	4.30E-02
GOTERM_BP_ALL	Negative regulation of caspase activity	2	2.90E-02
GOTERM_BP_ALL	Negative regulation of cell organization and biogenesis	3	1.10E-02
GOTERM_BP_ALL	Negative regulation of metabolism	5	4.50E-02
GOTERM_BP_ALL	Negative regulation of protein metabolism	3	4.20E-02
KEGG_PATHWAY	Neuroactive ligand-receptor interaction	2	3.20E-02
GOTERM_BP_ALL	Positive regulation of apoptosis	5	1.50E-02
GOTERM_BP_ALL	Positive regulation of biological process	9	4.20E-02
GOTERM_BP_ALL	Positive regulation of cellular process	8	4.50E-02
GOTERM_BP_ALL	Positive regulation of programmed cell death	5	1.50E-02
GOTERM_BP_ALL	Protein depolymerization	3	1.40E-02
GOTERM_BP_ALL	Regulation of apoptosis	6	4.70E-02
GOTERM_BP_ALL	Regulation of biological process	21	1.00E-02
GOTERM_BP_ALL	Regulation of cell organization and biogenesis	3	3.30E-02
GOTERM_BP_ALL	Regulation of cellular physiological process	18	1.10E-02
GOTERM_BP_ALL	Regulation of cellular process	19	1.20E-02
GOTERM_BP_ALL	Regulation of metabolism	14	5.40E-03
GOTERM_BP_ALL	Regulation of physiological process	19	1.10E-02
GOTERM_BP_ALL	Regulation of programmed cell death	6	5.00E-02
GOTERM_BP_ALL	Regulation of protein metabolism	5	2.40E-02
GOTERM_BP_ALL	Signal transduction	17	2.70E-02
GOTERM_BP_ALL	Synaptic transmission	6	3.90E-02
SP_PIR_KEYWORDS	Transcription	7	2.00E-02
SP_PIR_KEYWORDS	Transcription regulation	7	1.90E-02
GOTERM_BP_ALL	Transcription, DNA-dependent	10	4.10E-02
GOTERM_BP_ALL	Transmission of nerve impulse	6	5.00E-02
High-dose treatments (5–50 mg/kg)			
KEGG_PATHWAY	Axon guidance	5	4.70E-02
GOTERM_BP_ALL	Cell adhesion	14	1.90E-02
SP_PIR_KEYWORDS	Cell adhesion	9	3.10E-03
KEGG_PATHWAY	Cell adhesion molecules (CAM)	7	8.50E-03
GOTERM_BP_ALL	Cell communication	49	4.20E-02
GOTERM_BP_ALL	Cell organization and biogenesis	35	7.50E-03
GOTERM_BP_ALL	Cell–cell signaling	18	2.70E-02
GOTERM_BP_ALL	Cellular localization	17	1.80E-02
KEGG_PATHWAY	Dorso-ventral axis formation	4	8.70E-03
GOTERM_BP_ALL	Establishment of cellular localization	17	1.70E-02
GOTERM_BP_ALL	Establishment of localization	53	5.10E-03
GOTERM_BP_ALL	Establishment of protein localization	14	4.00E-02
GOTERM_BP_ALL	Intracellular protein transport	12	2.10E-02

**Table 3** continued

Category	Term	Count	<i>P</i> -value
GOTERM_BP_ALL	Intracellular transport	17	1.40E-02
SP_PIR_KEYWORDS	Ion channel	3	2.60E-02
GOTERM_BP_ALL	Localization	53	6.40E-03
GOTERM_BP_ALL	Negative regulation of cell differentiation	4	4.60E-02
GOTERM_BP_ALL	Negative regulation of development	5	2.60E-02
GOTERM_BP_ALL	Nervous system development	20	4.80E-03
GOTERM_BP_ALL	Neurophysiological process	19	2.90E-02
GOTERM_BP_ALL	Nuclear mRNA splicing, via spliceosome	5	2.50E-02
GOTERM_BP_ALL	Nucleocytoplasmic transport	6	2.90E-02
GOTERM_BP_ALL	Protein targeting	8	3.60E-02
GOTERM_BP_ALL	Protein transport	14	2.60E-02
GOTERM_BP_ALL	RNA splicing, via transesterification reactions	5	2.50E-02
GOTERM_BP_ALL	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	5	2.50E-02
GOTERM_BP_ALL	Spliceosome assembly	3	3.70E-02
GOTERM_BP_ALL	Synaptic transmission	15	3.60E-03
GOTERM_BP_ALL	System development	20	8.50E-03
GOTERM_BP_ALL	Transmission of nerve impulse	16	2.70E-03
GOTERM_BP_ALL	Transport	47	6.30E-03

nodes captured in this map. Of the 23 nodes showing differential expression, all but *Acs15* (acyl-CoA synthetase long-chain family member 5) were down-regulated. There were three highly connected nodes that function as the “central hubs” of this association network. These include *Taf1*, (TATA-binding protein-associated factor 1), *Noval* (neuron-oncological ventral antigen 1), and *Gsk3b*.

A list of the biological association networks identified using PathwayArchitect and the number of up-regulated and down-regulated genes in these networks are shown in Table 5. The entire list of genes and the direction of expression changes involved in the biological association networks of binding complexes, post-translational modification, transcription regulation, small molecule interaction, metabolism regulation, and transport regulation as determined using PathwayArchitect are provided in supplemental data Table S4. All of the differentially expressed genes captured in these network maps except *Acs15* (acyl-CoA synthetase long-chain family member 5), *Ggt13* (gamma-glutamyltransferase-like 3), *Scp2* (Sterol carrier protein 2), and *Slc10a2* (solute carrier family 10 (sodium/bile acid cotransporter family), member 2), were down-regulated after CPF exposure.

## Discussion

In this study, we examined gene expression changes following subtoxic CPF treatment in the forebrain using a rat model. Consistent with the reports in the literature, the doses of CPF used in this study do not result in overt toxic-

ity in terms of vascular congestion, cerebral hemorrhage, neuronal necrosis, and vacuolar degeneration as determined using hematoxylin and eosin staining (data available upon request). However, one animal treated with 50 mg/kg CPF had the appearance of periorbital hematoma, and two animals receiving 30 mg/kg CPF displayed a similar lesion and evidence of diarrhea. Despite this apparent lack of toxic response, differential expression of 277 genes was identified in this study. Instead of simply cataloging the gene expression changes resulting from CPF exposure, we seek to investigate the biological implications of these gene expression changes in the context of biological pathways/processes relevant to neurological functions. To achieve this goal, a search of the NIAID DAVID databases using the list of differentially expressed genes as input data was performed. Consistent with the potential impacts of CPF on neurological functions and development, genes involved in gene ontology terms and KEGG pathways such as nervous system development (GO:0007399) and neurophysiological process (GO:0050877), including dorso-ventral axis formation (KEGG PATH:ko04320) and axon guidance (KEGG PATH:ko04360), long-term potentiation (KEGG PATH:ko04720), transmission of nerve impulse (GO:0019226), and synaptic transmission (GO:0007268), were found to be significantly over-represented in the final gene list (see Table 2). Additionally, genes involved in biological processes such as cell signaling, mRNA splicing, regulation of protein metabolism, and intracellular protein transport/targeting were also enriched among the differentially expressed genes. Although these processes are not

**Table 4** Number of up- and down-regulated genes involved in different pathways under the categories of physiological processes, cellular processes, metabolic processes, and molecular functions identified in pathway analysis using GenMAPP

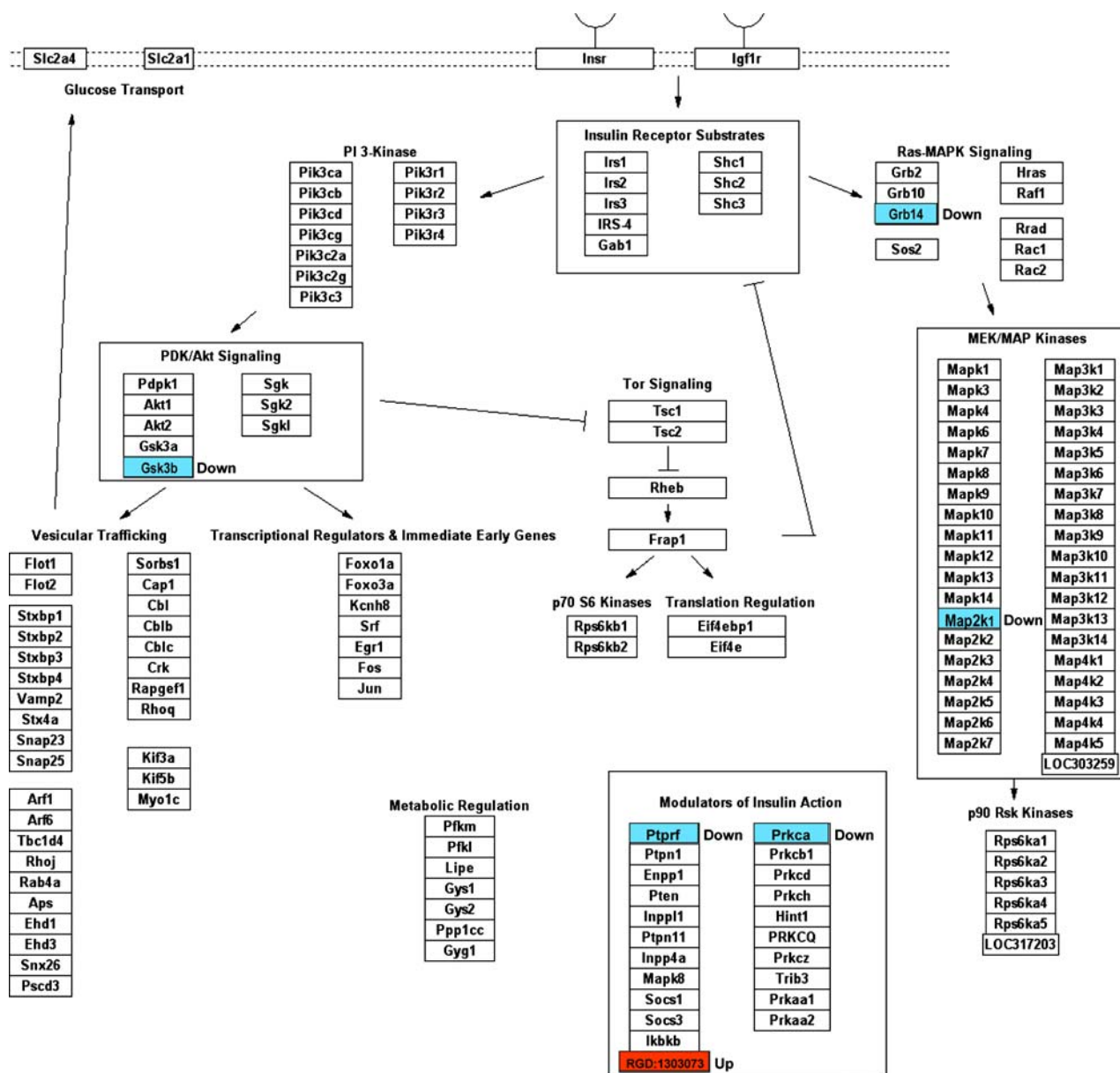
Category	Pathway	Down-regulated genes	Up-regulated genes
Physiological processes	Adipogenesis	2	0
	Calcium regulation in the cardiac cell	5	0
	Long-term potentiation	6	0
	Oxidative stress	3	0
	Smooth muscle contraction	5	0
Molecular functions	Small ligand GPCRs	1	1
Cellular processes	Androgen receptor signaling pathway	2	0
	B cell receptor signaling pathway	6	1
	Delta-Notch signaling pathway	2	0
	EGFR1 signaling pathway	8	1
	Focal adhesion	6	0
	G13 signaling pathway	2	0
	G protein signaling pathways	4	0
	IL-2 signaling pathway	3	0
	IL-3 signaling pathway	4	1
	IL-4 signaling pathway	0	2
	IL-5 signaling pathway	3	0
	IL-6 signaling pathway	4	1
	IL-7 signaling pathway	2	0
	Insulin signaling	5	1
	Integrin-mediated cell adhesion	2	0
	Kit receptor signaling pathway	3	1
	MAPK cascade	2	0
	MAPK signaling pathway	6	0
	mRNA processing	5	0
	Phosphatidylinositol signaling system	4	1
	Regulation of actin cytoskeleton	5	0
	Signaling of hepatocyte growth factor receptor	2	0
	T Cell receptor signaling pathway	4	0
TGF beta signaling pathway	2	0	
TGF beta receptor signaling pathway	4	0	
TNF-alpha/NF- $\kappa$ B signaling pathway	4	0	
Translation factors	2	0	
Wnt signaling pathway	6	0	
Metabolic processes	Inositol phosphate metabolism	2	0
	Purine metabolism	2	0

unique to neuronal tissues, perturbation of these essential cellular functions could also have significant impacts on neurological functions.

Since the doses used in this study cover a 2-log range, we were interested in investigating the effects of low-dose CPF treatments (i.e. 0.5–5 mg/kg) on biological processes and pathways that are relevant to neurological functions. As shown in Table 3, CPF exposure, even at such low doses, indeed results in perturbation of neuroactive ligand–receptor interaction (KEGG PATH:ko04080), transmission of nerve impulse (GO:0019226), and synaptic transmission

(GO:0007268). Low-dose CPF treatment also seems to affect cell survival by induction of programmed cell death (GO:0012502) and induction of apoptosis (GO:0006917). Perturbation of other important biological processes that might be relevant to the toxic effects of CPF include regulation of protein metabolism (GO:0051246), DNA-dependent transcription (GO:0006351), and MAPK signaling pathway (KEGG PATH:ko04010). These results are consistent with that of Whitney et al. (1995), who showed significant inhibition of DNA and protein synthesis following subtoxic CPF treatment.





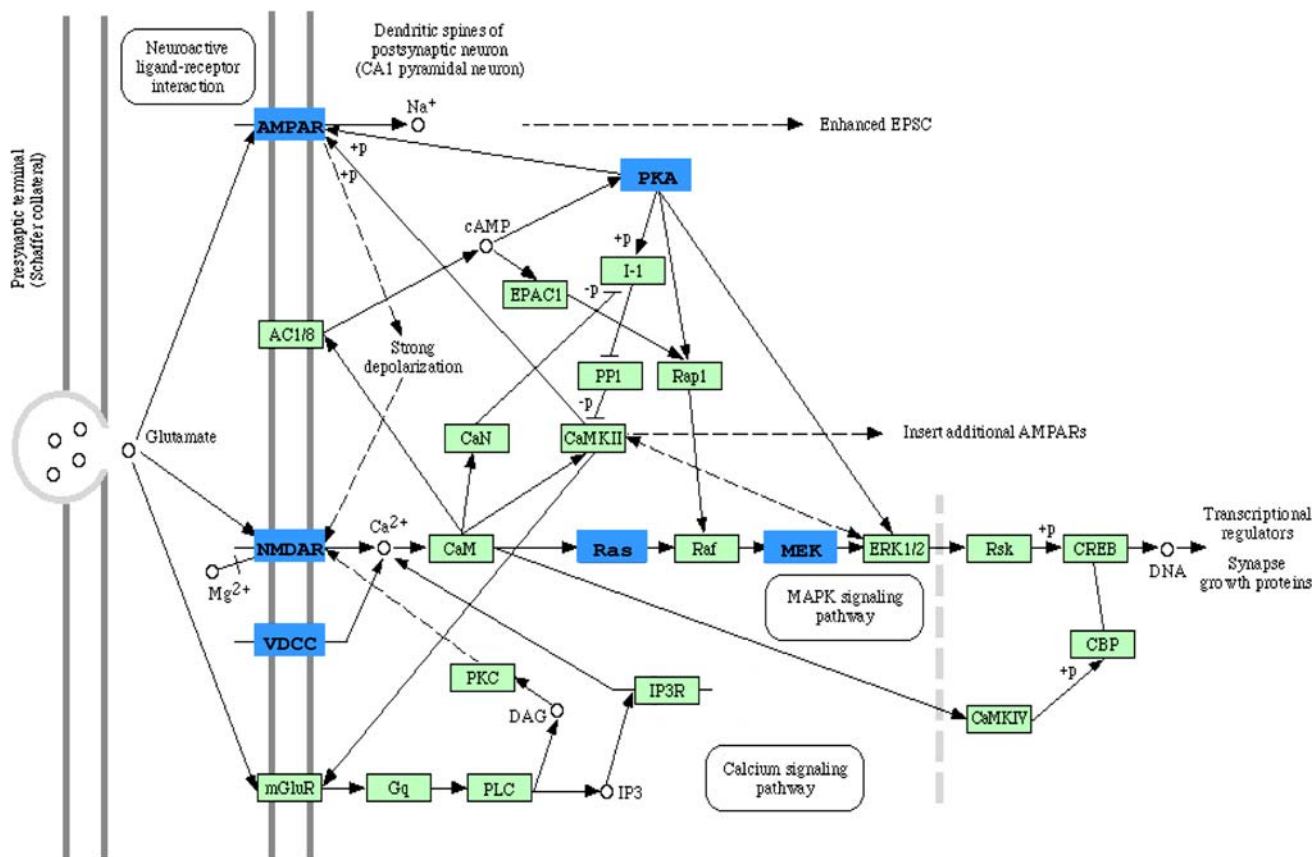
**Fig. 1** Gene expression changes involved in insulin signaling pathway. The expression profiles of the entire list of differentially expressed genes were used as input data in GenMAPP (version 2.1) analysis. A representative pathway illustrating the insulin signaling is shown.

Note that this pathway originated from the Diabetes Genome Anatomy Project. Five genes including Grb14, Map2k1, Gsk3b, Prkca, and Ptprf were downregulated (blue), while only one gene, Xbp1 (labeled as RGD 1303073), was up-regulated (red) in this pathway

As expected, the results of gene ontology analysis using the resultant gene list from high-dose CPF treatments (i.e. 5–50 mg/kg) is very similar to that of the analysis using the entire list of 277 differentially expressed genes. This observation is likely due to the fact that the relatively small number of differentially expressed genes identified in the low-dose treatments is overwhelmed by a significantly larger number of differentially expressed genes resulting from the high-dose treatments.

To further investigate the biological implication of gene expression changes resulting from CPF treatment, specific

cellular pathways in which the differentially expressed genes are involved were determined using GenMAPP. Besides the changes in the pathways as identified in the gene ontology analysis, several intracellular signaling pathways including EGFR, IL-3, IL-6, insulin, and Wnt signaling pathways were also down-regulated after CPF treatment, due to the down-regulation of several common players of these pathways such as Kras, Prkca, Map2k1, and Gsk3b (Table 4; Supplemental Data Table S3). IL-3 and IL-6 signaling pathways have been reported to be neuroprotective against ischemic brain injury (Akaneya et al.



**Fig. 2** Gene expression changes involved in long-term potentiation. The expression profiles of the entire list of the differentially expressed genes were used as input data in gene ontology analysis using DAVID, which revealed that six genes involved in long-term potentiation (LTP) in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway

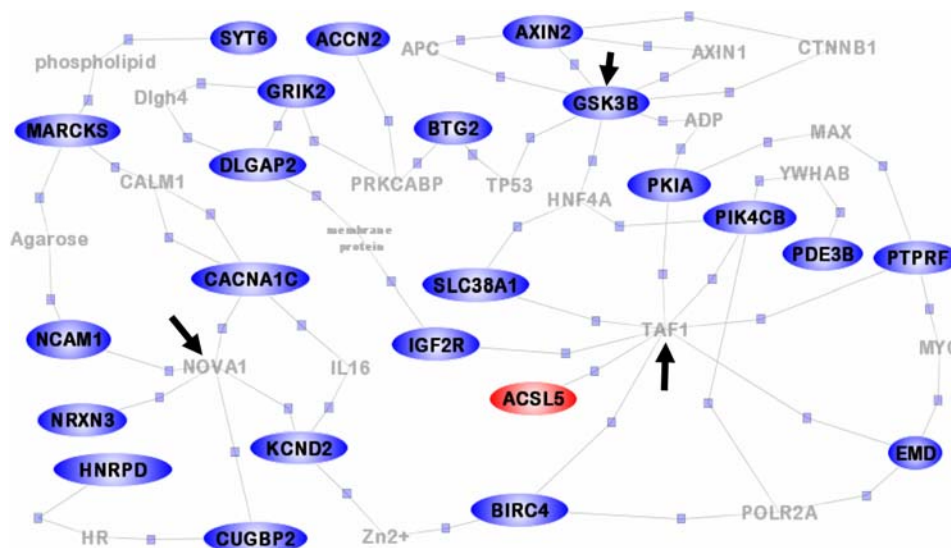
database were over-represented in the final gene list (Table 2). Illustrated is the KEGG pathway LTP of synaptic transmission. Note that all six genes including NMDAR (Grin1), AMPAR (Gria1), VDC (Cacna1c), PKA (Prkaca), Ras (Kras) and MEK (Map2k1) were down-regulated (blue) in this pathway

1995). Insulin and insulin-like growth factor (Igf) signaling pathways have also been shown to play an important role in neuroprotection against oxidative stress (Duarte et al. 2005), which has been suggested as one of the modes of action of CPF neurotoxicity (Banerjee et al. 2001; Kovacic 2003; Abdollahi et al. 2004; Abou-Donia 2003; Milatovic et al. 2006; Dettbarn et al. 2006). Consistent with this, oxidative stress response was also among the pathways identified in GenMAPP analysis (Table 4). It is therefore possible that down-regulation of these neuroprotective pathways might be relevant to the adverse effects of CPF exposure.

In addition to the neuroprotective activity against oxidative stress, insulin also exerts pleiotropic effects in neurons that include the regulation of neuronal proliferation/apoptosis, synaptic transmission, neuronal degeneration, and learning. Patients suffering from Alzheimer's and Parkinson's diseases exhibit reduced expression of insulin receptor (IR) in the brain (Moroo et al. 1994). Insulin-like growth factor 1 (Igf1), which also activates the insulin signaling pathway, is a neurotrophic factor that promotes neuronal growth, differentiation, and survival (Arsenijevic and

Weiss 1998). There are two signal transduction routes, the Pi3 k/Akt and the Mek/Erk routes in the insulin/IGF signaling pathway. Both of these routes play a role in the neuroprotective activity of glial-derived neurotrophic factor in the prevention of ethanol-induced B92 glial cell death (Villegas et al. 2006). Several genes involved in both the Pi3 k/Akt and the Mek/Erk signaling routes were found to be differentially expressed after CPF treatment (Fig. 1). Although not captured in this figure, down-regulation of insulin-like growth factor binding protein 5 (Igfbp5) and insulin-like growth factor 2 receptor (Igf2r) were also detected in this study (Supplemental Data Table S1).

Of the six differentially expressed genes involved in the insulin signaling pathway (Fig. 1), Grb14 functions as an inhibitor of this pathway by controlling tyrosine dephosphorylation of the IR in a site-specific manner and thus inhibition of its catalytic activity (Béréziat et al. 2002). Overexpression of Grb14 was shown to have an inhibitory effect on both metabolic and mitogenic actions of insulin in rat brains (Kasus-Jacobi et al. 1997, 1998). However, down-regulation of Map2k1, a protein kinase down-stream



**Fig. 3** Gene expression changes involved in the binding complexes network. The expression profiles of the entire list of differentially expressed genes were used as input data to construct biological association networks using PathwayArchitect 2.0.1. The biological association network illustrating protein binding complexes is shown. The genes

up-regulated and down-regulated after CPF treatment are colored *red* and *blue*, respectively. Genes with no expression changes are shown by their names only. The *blue squares* represent binding interactions. The three highly connected nodes, TAF1, NOVA1, and GSK3B are indicated by *black arrows*

**Table 5** Number of up- and down-regulated genes involved in the binding complexes, metabolism regulation, post-translational modification, small molecule interaction, transport regulation, and transcription regulation networks identified in biological association network analysis using PathwayArchitect

Biological association network	Down-regulated genes	Up-regulated genes
Transport regulation	4	2
Metabolism regulation	5	2
Small molecules	20	1
Post-translational modification	9	0
Transcription regulation	10	1
Binding complexes	22	1

of Grb14, would suggest an overall down-regulation of the Mek/Erk signaling route of the insulin signaling pathway. The Pi3 k/Akt signaling route promotes cell survival by inhibiting Gsk3b activity, which is a pro-apoptotic enzyme capable of inhibiting the activation of several transcription factors regulating the expression of cell survival factors (Brunet et al. 2001). As inhibition of Gsk3b promotes cell survival after growth factor withdrawal (Bhat et al. 2002), down-regulation of this gene observed in this study might represent a cellular response to counter the toxic effect of CPF. Since Gsk3b activity can also be regulated by the phosphorylation of specific amino acid residues (i.e. Ser9 and Tyr216), future studies aimed at determining the phosphorylation state of these residues will be needed to address the effect of CPF treatment on Gsk3b activity.

Three other differentially expressed genes shown in this pathway map are Prkca, Ptpfr, and Xbp1 (labeled as RGD 1303073 in Fig. 1). Prkca, which functions as a physiological feedback inhibitor of the insulin signaling pathway, was down-regulated after CPF treatment. Prkca is constitutively associated with insulin receptor substrate 1 (Irs1) (Rosenzweig et al. 2002) and regulates insulin signaling through rapid dissociation from Irs1. Subsequent association with Akt occurs upon insulin stimulation (Cipok et al. 2006). Similar to the down-regulation of Gsk3b, down-regulation of Prkca might also represent a cellular defense in response to CPF exposure. Insulin signaling is also regulated by protein tyrosine phosphatases (PTPs) that act on the IR itself and/or its substrates. Increases in the abundance of Ptpfr protein expression down-regulate a number of insulin-stimulated cellular responses (Zhang et al. 1996). In contrast, inhibition of Ptpfr expression by antisense mRNA enhanced insulin receptor signal transduction. Therefore, down-regulation of Ptpfr after CPF treatment is consistent with the theme of down-regulation of Gsk3b and Prkca. Xbp1 functions as a modulator of the insulin signaling pathway. Transcription of Xbp1 is strongly enhanced by the endoplasmic reticulum stress response (i.e. unfolded protein response) and is up-regulated in brain trauma patients (Paschen et al. 2004). The up-regulation of Xbp1 observed in this study is consistent with the induction of programmed cell death as revealed by gene ontology analysis (see Table 3).

It is well recognized that CPF exposure, even at doses with no acute symptoms, is capable of producing long-term

defects. For instance, CPF exposure produced long-lasting neurobehavioral changes and altered response to challenges (Moser et al. 2005). Both acute and repeated CPF exposure resulted in selective deficits in the learning of response sequences (Cohn and MacPhail 1997) and in spatial learning in rats (Canadas et al. 2005). These defects however seem to be independent of the inhibition of AChE activity and muscarinic receptor binding by CPF, since recovery occurred prior to the detection of these behavioral defects (Canadas et al. 2005). In light of this, it is of particular interest that the results of gene ontology and pathway analyses of the differentially expressed genes indicate the down-regulation of several genes involved in LTP (see Table 2), a process essential for neuronal plasticity and long-lasting potentiation of synaptic efficacy in learning and memory formation. LTP involves the release of L-glutamate from presynaptic neurons and subsequent coactivation of the NMDA- and AMPA-receptors and a concurrent influx of  $\text{Ca}^{2+}$  into the postsynaptic cell via the activated receptor operated channels (Herron et al. 1986). As the genes for both NMDA- and AMPA-glutamate receptors (i.e. *Gria1* and *Grin1*) were down-regulated after CPF treatment (see Fig. 2), this pattern of gene expression changes would suggest defects in postsynaptic  $\text{Ca}^{2+}$  influx and subsequent activation of *Camk2*, an event essential for LTP. Another gene involved in postsynaptic  $\text{Ca}^{2+}$  influx, *Cacna1c* (L-type voltage-dependent calcium channel, labeled as VDCC in Fig. 2), was also down-regulated after CPF treatment. *Cacna1c* is activated by repetitive stimulation to generate sufficient  $\text{Ca}^{2+}$  influx required for transcription- and translation-dependent LTP, i.e. LTP3 (Impey et al. 1996; Moosmang et al. 2005). Since LTP3 comprises NMDA receptor-dependent and *Cacna1c*-dependent components (Morgan and Teyler 2001; Raymond and Redman 2006), and since both of the genes for NMDA receptor and *Cacna1c*, as well as those for their downstream effectors (*Kras* and *Map2k1*) were down-regulated after CPF treatment, CPF exposure might have a profound effect on LTP3. The observation that *Prkaca* (labeled as PKA in Fig. 2), a co-activator of CREB-mediated transcription and an important regulator of gene transcription essential for LTP3, was down-regulated also lends some support to this hypothesis. This is also consistent with the report by Nguyen and Woo (2003) that inhibition of *Prkaca* prevents the induction of transcription-dependent LTP3. Taken together, this differential gene expression pattern provides a possible mechanistic basis for the long-term behavioral defects associated with CPF exposure as reported in the literature (Cohn and MacPhail 1997; Canadas et al. 2005; Samsam et al. 2005; Bushnell et al. 2001).

To explore the potential interaction of the differentially expressed genes and the respective protein products with their potential partners in response to CPF exposure, bio-

logical association networks of binding complexes, post-translational modifications, transcription regulation, small molecule interactions, metabolism regulation, and transport regulation were constructed using PathwayArchitect (see Table 5; Supplemental Data Table S4).

In the binding complex network, there are three highly connected nodes that function as the “central hubs” of this association network (see Fig. 3). These include *Taf1*, *Nova1*, and *Gsk3b*. *Taf1* is an essential component of the general transcription factor IID (TFIID), which nucleates assembly of the preinitiation complex for transcription by RNA polymerase II. Of the 14 TATA-binding protein-associated factors that compose TFIID, *Taf1* is one of the largest and most functionally diverse. Because of its important functions, it is perhaps not surprising that *Taf1* is the most connected node in this network. While the transcript level of *Taf1* was not changed after CPF treatment, all eight genes with connections to *Taf1*, except *Acs15*, were down-regulated after CPF treatment. Because of its important role in gene transcription, it is possible that a decreased *Taf1* activity might be responsible for the down-regulation of these eight genes. However, we would like to emphasize that this has yet to be formally demonstrated. Of the eight genes connected to *Taf1*, some have been previously reported to be related to neurological functions. For instance, *Slc38a1* functions as a transporter of glutamine, a precursor for the synaptic transmitter glutamate (Gu et al. 2001); *Pik4cb* is reported to have a role in neuronal differentiation and maturation (Nakagawa et al. 1996); *Birc4* is capable of inhibiting several caspases thereby functioning as an apoptotic suppressor (Takahashi et al. 1998); *Ptprf* is a regulator of insulin and Wnt signaling pathways that have essential roles in neuronal functions.

*Nova1*, which is the second most highly connected node in this network, is a neuron-specific antigen targeted in paraneoplastic opsoclonus myoclonus ataxia (POMA), an autoimmune neurologic disorder characterized by abnormal motor inhibition. *Nova1* regulates neuronal pre-mRNA metabolism and splicing in a specific subset of developing neurons. It is worth noting that genes involved in mRNA splicing (spliceosome assembly) were over-represented among the differentially expressed genes as revealed in the gene ontology analysis (see Table 2). Although *Nova1* was not significantly changed after CPF treatment, it is connected to five genes (including *Ncam*, *Nrxn3*, *Cacna1c*, *Cugbp2* and *Kcnd2*) that are down-regulated after CPF treatment. Ule et al. (2003) reported that *Nova1* has a role in regulating the processing and metabolism of the transcripts of these genes (Ule et al. 2003). Thus, it would be interesting to investigate if a decrease in the pre-mRNA processing/splicing accounts for the down-regulation of these genes. All five genes connected to *Nova1* have significant roles in neurological functions. Both *Ncam1* and

Nrxn3 are cell adhesion molecules/receptors involved in neuronal cell adhesion (Thiery et al. 1977; Ushkaryov et al. 1992; Ushkaryov and Südhof 1993; Geppert et al. 1992). Cacna1c has an essential role in LTP and inactivation of Cacna1c in the hippocampus and neocortex resulted in severe impairment of spatial learning and synaptic plasticity in mice (Moosmang et al. 2005). Cugbp2, which participates in many aspects of RNA metabolism including pre-mRNA alternative splicing (Choi et al. 1999), has a role in regulating specific splicing events of NMDA receptor (Grin1) transcript (Zhang et al. 2002). Cugbp2 is prominently induced during apoptosis of neuronal cells prior to caspase-3 activation (Nakagawa-Yagi et al. 2001) and antisense-mediated down-regulation of this gene protected hippocampal cultures against apoptosis induced by oxygen and glucose deprivations and prevented caspase-3 activation. Since Cugbp2 induction appears to be necessary for the execution of apoptosis, down-regulation of this gene after CPF exposure might suggest that the anti-apoptotic pathway is also activated after CPF treatment as a defense mechanism. Kcnd2 has numerous functions including regulating neurotransmitter release, insulin secretion, neuronal membrane excitability, cell volume, etc. (for review see Nelson and Quayle 1995).

Gsk3b is the third most highly connected node in this network. It is also among the most highly connected nodes in other biological association networks (data not shown). Gsk3b plays an important regulatory role in a variety of pathways including initiation of protein synthesis, cell proliferation, cell differentiation, apoptosis, and embryonic development (Doble and Woodgett 2003; Frame and Cohen 2001; Grimes and Jope 2001). It is a critical intermediate in pro-apoptotic signaling cascades that are associated with Alzheimer's disease (Chin et al. 2005). Nerve growth factor withdrawal or staurosporine treatment of neuronal cells led to increased Gsk3b activity with concomitant phosphorylation at Tyr216 and cell death (Bhat et al. 2000). Of the six genes connected to Gsk3b, only Axin2 showed differential expression after CPF treatment. Axin2 is a scaffold protein which binds  $\beta$ -catenin and Gsk3b (as well as several other proteins) and thus promotes the phosphorylation of  $\beta$ -catenin by Gsk3b at several residues, thereby facilitating the ubiquitination and proteasomal degradation of  $\beta$ -catenin (Ikeda et al. 1998). The connection between Gsk3b and Axin2 as observed in this network map, together with the down-regulation of these genes and another major regulator of Wnt signaling pathway, Ptpfrf, suggests a potential involvement of this pathway in the cellular response to CPF exposure.

Two other down-regulated genes captured in this network map may also be relevant to CPF neurotoxicity. For instance, Grik2 (Glutamate receptor, ionotropic, kainate 2)

may have a role in synaptic plasticity that is important for learning and memory. Dlgap2 (Discs, large Drosophila homolog-associated protein 2) may play a role in the molecular organization of synapses and neuronal cell signaling (Ranta et al. 2000). Grik2 and Dlgap2 have been shown to bind to each other and this interaction induces both JNK activation and neurotoxicity upon activation of kainate receptor and induction of mixed lineage kinase-mediated cellular signaling cascades via post-synaptic density protein 95 (Savinainen et al. 2001).

## Conclusion

In this study, differential expression of 277 genes was identified in the forebrain following CPF treatment in a rat model. We are aware that performing transcriptomic analysis at a single time point post-treatment is a major weakness of this study, which does not allow for the identification of the temporal effect of CPF treatment on gene expression. The gene expression profiles identified in this study may also represent a mixed pattern of adaptive responses, toxic responses, repair processes, and apoptotic events. As the kinetics of the cellular response after a toxic insult is highly dependent on the nature and magnitude of the insult, studies like this can only provide a snapshot of the transcriptomic response to CPF treatment. While this study does not provide a definitive picture concerning the final outcome of cellular responses to CPF exposure, detailed interpretation of the biological significance of gene expression changes as presented here does shed some light onto the potential impacts of CPF treatment on neurological functions. Specifically, the differential expression of genes involved in multiple pathways relevant to nervous system development and functions were successfully identified in this study. As demonstrated in this study, the use of a combination of several bioinformatics tools can significantly facilitate the interpretation of the biological significance of differential gene expression as detected in DNA microarray experiments. These findings might serve as the basis for future mechanistic analysis of the long-term adverse effects of subtoxic CPF exposure. For instance, analysis of temporal expression changes of genes involved in specific pathways (such as insulin signaling, long-term potentiation or other pathways relevant to neurological functions identified in this study) will undoubtedly provide further insights into the neurotoxicity resulting from CPF exposure.

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