

Repeated Developmental Exposure of Mice to Chlorpyrifos Oxon Is Associated with Paraoxonase 1 (PON1)-Modulated Effects on Cerebellar Gene Expression

Toby B. Cole,^{*,†,‡} Richard P. Beyer,^{*} Theo K. Bammler,^{*} Sarah S. Park,^{†,‡} Federico M. Farin,^{*} Lucio G. Costa,^{*} and Clement E. Furlong^{†,‡,1}

^{*}Department of Environmental and Occupational Health Sciences; [†]Division of Medical Genetics, Department of Medicine; and [‡]Department of Genome Sciences, University of Washington, Seattle, Washington 98195

¹To whom correspondence should be addressed at Division of Medical Genetics, University of Washington, 1959 NE Pacific Street, Box 357720, Seattle, WA 98195-7720. Fax: (206) 543-3050. E-mail: clem@uw.edu.

Received April 6, 2011; accepted June 8, 2011

Microarray analysis was used to examine effects of repeated postnatal exposure to chlorpyrifos oxon (CPO) on gene expression in the cerebellum of genetically modified mice. The high-density lipoprotein-associated enzyme paraoxonase 1 (PON1) plays a significant role in the detoxication of CPO, which is present in exposures and generated from chlorpyrifos (CPF) *in vivo* following exposure. Two factors are important in modulating toxicity of CPO, the Q192R *PON1* polymorphism and PON1 plasma level, which is low at birth and increases throughout postnatal development. Mice used in these studies included wild type (*PON1*^{+/+}), *PON1* knockout (*PON1*^{-/-}), and two transgenic lines (*tgHuPON1*_{Q192}, *tgHuPON1*_{R192}) expressing either human *PON1*_{Q192} or *PON1*_{R192} on the *PON1*^{-/-} background. *PON1*_{R192} hydrolyzes CPO more efficiently than *PON1*_{Q192}. All four genotypes exposed to CPO (0.35 or 0.50 mg/kg/day) daily from postnatal day (PND) 4 to PND 21 showed significant differences in gene expression on PND 22 compared with controls. Pathway analysis and Gene Set Analysis revealed multiple pathways and gene sets significantly affected by CPO exposure, including genes involved in mitochondrial dysfunction, oxidative stress, neurotransmission, and nervous system development. Comparison between genotypes revealed specific genes, gene sets, and pathways differentially affected between *tgHuPON1*_{Q192} and *tgHuPON1*_{R192} mice and between *PON1*^{-/-} and *PON1*^{+/+} mice following CPO exposure. Repeated CPO exposure also resulted in a dose-related decrease in brain acetylcholinesterase activity during postnatal development in *PON1*^{-/-} and *tgHuPON1*_{Q192} mice but not in *PON1*^{+/+} or *tgHuPON1*_{R192} mice. These findings indicate that *PON1* status plays a critical role in modulating the effects of neonatal CPO exposure in the developing brain.

Key Words: chlorpyrifos; chlorpyrifos oxon; paraoxonase; organophosphorus insecticides; microarrays; neurotoxicity.

Despite restrictions imposed on residential use, the organophosphorus (OP) insecticide chlorpyrifos (CPF) is still widely used (Heudorf *et al.*, 2006). CPF undergoes oxidative desulfur-

ation in the liver to form CPF oxon (CPO), which inhibits serine active-site enzymes, including acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and carboxylesterase (CES) (Chambers *et al.*, 1990; Shenouda *et al.*, 2009). The oxon form itself is present in most, if not all exposures, representing 2% to more than 17% of OP residues (Cal-EPA, 1998; Vidal *et al.*, 1998; Yuknavage *et al.*, 1997), with CPO residues in green beans and tomatoes reported to be as high as 0.14–0.21 mg/kg (Vidal *et al.*, 1998). Despite the presence of CPO in exposures, safety studies for CPF have been carried out only with highly pure parent compound (Nolan *et al.*, 1984). CPO is bound stoichiometrically by B-esterases, including CES (Chambers *et al.*, 1990); however, it is efficiently hydrolyzed catalytically by the plasma enzyme, paraoxonase 1 (*PON1*) (Furlong, 2008).

The *PON1*_{Q192R} polymorphism affects the catalytic efficiency of hydrolysis of some substrates, including CPO (Furlong, 2008), with significant consequences *in vivo*. *PON1* knockout (*PON1*^{-/-}) mice are dramatically more sensitive than wild-type (*PON1*^{+/+}) mice to the toxicity of CPO and diazoxon (DZO) (Furlong, 2008; Li *et al.*, 2000; Shih *et al.*, 1998). Injection of rodents with purified human *PON1* (Furlong, 2008) or engineered recombinant *PON1* (Stevens *et al.*, 2008) provided protection against OP toxicity. Of the purified human *PON1* alloforms, *PON1*_{R192} provided greater protection than human *PON1*_{Q192} against exposures to CPF and CPO (Li *et al.*, 2000). *PON1*_{Q192} and *PON1*_{R192} provided equivalent protection against DZO exposure (Furlong, 2008; Li *et al.*, 2000). Humanized transgenic mice expressing *tgHuPON1*_{Q192} in the absence of mouse *PON1* were more sensitive to CPF and CPO than mice expressing *tgHuPON1*_{R192} (Cole *et al.*, 2003, 2005) and were also more sensitive to the interactive toxicity of mixtures of OP compounds that included CPO (Jansen *et al.*, 2009).

PON1 levels vary by at least 14-fold among adults and 26-fold among infants (Furlong *et al.*, 2006). An individual's "PON1 status," which encompasses both their *PON1*_{Q192R}

functional genotype and plasma PON1 level, can be determined with high-throughput microtiter-plate assays (Richter and Furlong, 1999; Richter *et al.*, 2008). PON1 levels are 3- to 4-fold lower in children than in adults (Furlong *et al.*, 2006) and do not reach adult levels until 6 months to 7 years of age (Cole *et al.*, 2003; Huen *et al.*, 2009). Children face additional risk due to their closer proximity to the multiple pathways of aggregate exposure, including oral ingestion, inhalation, and dermal exposure to OPs in food, air, and dust (Fenske *et al.*, 2005).

In a study of Washington State pesticide handlers using CPF within the previous 30 days, both the presence of PON1_{Q192} and low plasma PON1 level were significantly associated with inhibition of BChE, a sensitive biomarker of OP exposure (Hofmann *et al.*, 2009). South African fruit-farm workers carrying the PON1_{Q192} allele were nearly three times as likely as PON1_{R192} homozygotes to report multiple symptoms of OP toxicity (Lee *et al.*, 2003).

Whereas OP compounds originally were thought to act via the common mechanism of AChE inhibition, studies over the past decade have identified additional mechanisms of toxicity (Jameson *et al.*, 2007; Pope, 1999; Schuh *et al.*, 2002). Exposure of neonatal rats to subtoxic doses of CPF caused decreased cell density in the cerebellum and other brain regions (Whitney *et al.*, 1995). Gestational exposure of mice to CPF by Moreira *et al.* (2010) identified multiple dose-dependent effects on gene expression, at least some of which were unrelated to AChE inhibition. Chronic CPF exposure also can cause OP-induced delayed neuropathy (Lotti *et al.*, 1986). In primary human astrocytes (Mense *et al.*, 2006), PC12 cells (Slotkin and Seidler, 2009), adult rats (Stapleton and Chan, 2009), and neonatal rats (Slotkin and Seidler, 2007), CPF altered the expression of multiple genes involved in nervous system development and function. Exposure to CPF doses as low as 1.5 mg/kg caused reduced expression of nerve growth factor, reelin, and myelin-associated glycoprotein (Betancourt *et al.*, 2006). CPF exposure affected neuronal replication and differentiation, synapse function, and behavior, with toxicity extending fairly late into the period of brain development (Aldridge *et al.*, 2005).

In contrast to CPF, studies on CPO effects have been sparse, despite its prevalence in the environment (Cal-EPA, 1998; Vidal *et al.*, 1998; Yuknavage *et al.*, 1997) and its dramatically higher toxicity (Huff *et al.*, 1994; Cole *et al.*, 2005). CPO binds to muscarinic receptors (Huff *et al.*, 1994) and inhibits phosphorylation and internalization of muscarinic M2 receptors in transfected CHO cells (Udarbe Zamora *et al.*, 2008). In human sperm, CPO and other oxons produced more extensive chromatin alteration and DNA damage than did the parent phosphorothioates (Salazar-Arredondo *et al.*, 2008). Pesticide effects on semen quality and DNA integrity are modulated by the PON1_{Q192R} polymorphism (Perez-Herrera *et al.*, 2008).

Previously, Whitney *et al.* (1995) demonstrated effects of subtoxic doses of CPF on cell density in the cerebellum. To gain insight into the mechanisms of neurotoxicity of CPO in

this region and to ascertain the importance of the PON1_{Q192R} polymorphism for protecting against developmental toxicity of CPO, we used whole-genome microarrays to measure gene expression changes associated with repeated CPO exposure of developing (PND 4–21) PON1^{-/-}, tgHuPON1_{Q192} transgenic, tgHuPON1_{R192} transgenic, and PON1^{+/+} mice.

MATERIALS AND METHODS

Animals. PON1 knockout (PON1^{-/-}) mice (Shih *et al.*, 1998) and mice expressing either the human PON1_{R192} or PON1_{Q192} transgene (tgHuPON1_{R192} or tgHuPON1_{Q192}) in place of endogenous mouse PON1 (Cole *et al.*, 2003, 2005) were provided by Drs Diana M. Shih, Aaron Tward, and Aldons J. Lusis (University of California, Los Angeles (UCLA), Los Angeles, CA). Mice with at least one copy of the transgene were crossed with same genotype animals to produce both PON1^{-/-} mice and transgenic mice (tgHuPON1_{R192} or tgHuPON1_{Q192}) in the same litter. Wild-type (PON1^{+/+}) mice were bred from the same congenic B6.129 strain background. Presence of PON1_{Q192} or PON1_{R192} enzyme activity in heparinized saphenous-vein plasma was detected by measuring the rate of hydrolysis of either the alloform-neutral substrate, phenyl acetate (Cole *et al.*, 2003, 2005), or diazoxon, which is alloform-neutral at 0.5M NaCl (Richter and Furlong, 1999) and has no background activity in PON1^{-/-} mice (Cole *et al.*, 2003; Stevens *et al.*, 2008). PCR-based genotyping was used to determine the presence of the transgenes. Arylesterase (AREase) and diazoxonase (DZOase) assays were carried out in a microtiter plate reader (SpectraMax Plus, Molecular Devices), using 0.5 μ l (AREase) and 1.25 μ l (DZOase) of plasma per well. Only the initial linear rates of hydrolysis were used for calculations.

Mice were housed in specific pathogen free (SPF) or modified SPF facilities with a 12-h dark/light cycle and unlimited access to food and water. The animal use protocols used were approved by the Institutional Animal Care and Use Committee at the University of Washington. All animal experiments were carried out in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals, as adopted by the National Institutes of Health.

Chemicals. CPO (CAS 5598-15-2; 98% purity) was purchased from Chem Service (West Chester, PA). Acetylthiocholine, 5,5'-dithio-bis-nitrobenzoic acid (DTNB), *p*-nitrophenyl valerate, and phenyl acetate were from Sigma-Aldrich (St Louis, MO). All other analytical grade chemicals were obtained from commercially available sources.

Exposure of mice to CPO. Timed matings of the humanized transgenic mice (tgHuPON1_{Q192} \times tgHuPON1_{Q192}; tgHuPON1_{R192} \times tgHuPON1_{R192}) were used to generate litters comprised of both PON1^{-/-} and tgHuPON1_{R192} or tgHuPON1_{Q192} mice. PON1^{+/+} litters, and in some cases PON1^{-/-} litters, were generated by timed mating of homozygous (PON1^{-/-} or PON1^{+/+}) males and females. On postnatal day (PND) 4, litters were culled to six mice (three males and three females, when possible). Mice were injected sc (1 μ l/g bodyweight) with CPO (0, 0.15, 0.18, 0.25, 0.35, or 0.50 mg/kg/day) daily from PND 4 to PND 21. A 1 mg/ml stock solution of CPO (Chem Service, 98% purity) dissolved in dimethyl sulfoxide (DMSO) was used to make serial dilutions for injections. Control mice received vehicle (DMSO) alone. All mice within a litter received the same dose of CPO, and the litter was used as the unit of dosage; i.e., only one mouse was used per litter for each endpoint.

The experiments reported here were part of a larger study that also examined other endpoints of toxicity (Cole, Fisher, Walter, Burbacher, Costa, and Furlong, in preparation). Overall, the experiments were conducted with eight separate cohorts consisting of a total of 75 PON1^{-/-} litters, 30 PON1^{+/+} litters, 18 tgHuPON1_{Q192} litters, and 18 tgHuPON1_{R192} litters. Mice used for measurement of cerebellar gene expression ($n = 6$ male mice per group; one mouse per litter) came from one of these cohorts, consisting of six litters of each genotype for each of three dose groups: 0.50 mg/kg/day CPO, 0.35 mg/kg/day CPO, and the vehicle control (DMSO) group. One male mouse was used

per litter for measurement of gene expression. One male and one female mice were used per litter for measurement of brain acetylcholinesterase (AChE) activity. The remaining mice were used for assessment of histopathology or neurobehavior, the results of which are not reported here (Cole, Fisher, Walter, Burbacher, Costa, Furlong, in preparation). Bodyweights of all mice were measured daily just prior to dosing, and the dates of appearance of developmental landmarks (eye opening, pinna detachment, hair growth) were recorded. At weaning (PND 22), mice to be used for gene expression assessment ($n = 6$ male mice for each genotype and treatment group; one mouse from each litter) were euthanized by CO₂ asphyxiation, 24 h after the last injection. Upon dissection, the brains were bisected sagittally, and cerebella were separated and immersed in 10 volumes of RNAlater solution (Ambion, Austin, TX) for stabilization of RNA prior to extraction. For AChE measurement, dissected brains were immediately frozen on dry ice and stored at -80°C until analysis. Statistical differences in bodyweight among treatment groups were determined by calculating the mean bodyweights of males and females for each litter and using Student's *t*-test to test for statistical significance of the differences between means.

Brain AChE assays. Brains frozen and stored at -80°C were thawed and homogenized for 30 s in 0.1M sodium phosphate, pH 8.0 using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Brain AChE activity was measured as described (Cole *et al.*, 2005; Jansen *et al.*, 2009), using a SpectraMax Plus microtiter plate reader (Molecular Devices). The initial rates of 5-thio-2-nitrobenzoate formed during the assay (mOD/min) were pathlength-corrected and converted to U/g of wet tissue using an extinction coefficient of $13.6\text{mM}^{-1}\text{cm}^{-1}$. AChE activity is expressed as U/g of wet tissue ($U = \mu\text{mol}$ of acetylthiocholine hydrolyzed per minute). Statistical differences in brain AChE activity between treatment groups and genotypes were determined using Student's *t*-test.

RNA extraction, labeling, and microarray hybridization. The bisected cerebella in RNAlater were incubated for at least 12 h at 4°C , then stored at -20°C . Total RNA was isolated using TRIZOL Reagent and the QIAamp Tissue Kit from QIAGEN Inc., according to the manufacturer's established protocols. The quality of the total RNA was evaluated using an Agilent 2100 Bioanalyzer. Only samples with high integrity (integrity number > 8), adequate RNA quantity, and an $A_{260}:A_{280}$ ratio of 1.8–2.1 were used for further analyses. Sixty RNA samples ($n = 5$ –6 from all but two of the experimental groups) met these requirements. The exceptions were $PON^{+/+}$ and $PON1^{-/-}$ mice exposed to 0.50 mg/kg/day CPO, for which there were three and four samples, respectively. A NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) was used to determine RNA concentrations based on A_{260} measurements.

Affymetrix GeneChip whole transcript sense target labeling and hybridization. RNA samples isolated from individual cerebella were labeled and hybridized individually to Affymetrix Mouse Genome ST 1.0 microarrays. Processing of the RNA samples was carried out according to the Affymetrix GeneChip Whole Transcript Sense Target labeling protocol (for details, see <http://www.affymetrix.com/index.affx>). Briefly, double-stranded cDNA was synthesized with random hexamers tagged with a T7 promoter sequence. The double-stranded cDNA was subsequently used as a template and amplified by T7 RNA polymerase producing many copies of antisense cRNA. In the second cycle of cDNA synthesis, random hexamers were used to prime reverse transcription of the cRNA from the first cycle to produce single-stranded DNA in the sense orientation. In order to reproducibly fragment the single-stranded DNA and improve the robustness of the assay, a novel approach was utilized where dUTP was incorporated in the DNA during the second-cycle first-strand reverse transcription reaction. This single-stranded DNA sample was then treated with a combination of uracil DNA glycosylase (UDG) and apurinic/aprimidinic endonuclease 1 (APE-1) that specifically recognizes the unnatural dUTP residues and cuts the DNA strand. DNA was labeled by terminal deoxynucleotidyl transferase (TdT) with the Affymetrix proprietary DNA Labeling Reagent covalently linked to biotin. The biotin-labeled DNA fragments were hybridized to the array, washed, and stained with streptavidin and a fluorescent anti-streptavidin antibody. Following an additional wash step, the arrays were scanned with an Affymetrix GeneChip 3000 scanner. Image generation and feature extraction was performed using Affymetrix AGCC

Software. For MIAME compliance (Brazma *et al.*, 2001) raw data, final processed data, sample and array annotation, and experimental design have been submitted to the NCBI Gene Expression Omnibus (GEO) and are available at <http://www.ncbi.nlm.nih.gov/geo/>, accession #GSE25250.

Microarray data quality control. Several microarray data QC metrics were applied to assure high quality of the data. The first QC step was a visual inspection of the scanned image to check for fluorescent signal artifacts. In addition, the background signals were assessed and required to be below the manufacturer's recommended threshold. The distribution of the fluorescent signals of arrays within a given experiment was assessed by box plots and outliers were identified. In addition, normalized un-scaled standard errors were calculated for each array and outliers were identified. Only data that passed all QC steps were included in the analyses.

Microarray data analysis: individual gene analysis. Raw microarray data were processed and analyzed with Bioconductor (Gentleman *et al.*, 2004) and normalized using the Robust Multi-Array method from the Bioconductor Affy package. Using the normalized data, genes with significant evidence for differential expression were identified with the limma package (Smyth, 2004) in Bioconductor. *p* Values were calculated with a modified *t*-test in conjunction with an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. *p* Values were adjusted for multiplicity with Bioconductor's *q*value package (Storey and Tibshirani, 2003), which allows for selecting genes with statistically different levels of expression while controlling the estimated false discovery rate.

Ingenuity Pathway Analysis. Functional and network analyses of statistically significant gene expression changes were performed using Ingenuity Pathways analysis (IPA) (Ingenuity Systems, Redwood City, CA). Analysis considered all genes from the data set that met the 1.5-fold ($p < 0.05$) change cutoff and that were associated with biological functions and canonical pathways in the Ingenuity Pathways Knowledge Base. For all analyses, Fisher's exact test was used to determine the probability that each biological function assigned to the genes within each data set was not due to chance alone.

Gene Set Analysis. Gene set analysis (GSA) was used to investigate categories of genes where the constituent genes showed coordinated changes in expression for the various experimental comparisons. GSA is a recently established category/pathway analytical method and GSA software is freely available as R code (Efron and Tibshiran, 2007). GSA considers all genes in the experiment and allows for the identification of gene sets/pathways with genes that show modest but concordant changes in gene expression. Five databases of gene sets were used for GSA; three from the Gene Ontology (Camon *et al.*, 2004) (biological process [BP], molecular function [MF], and cellular component [CC]) and five from the Broad Institute's Molecular Signature Database (http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html) (Subramanian *et al.*, 2005).

Quantitative real-time PCR (qRT-PCR) analysis. Reverse transcription (RT) was performed according to the Applied Biosystems Inc. (Foster City, CA) protocol using total RNA and the SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The PCR primers and the dual-labeled probes for the specific genes of interest were contained in the Applied Biosystems (AB) inventoried TaqMan Gene Expression Assays mix and processed according to AB's protocol. For these gene expression measurements, 2 μl of cDNA were included in a PCR reaction (20 μl final volume) that also contained the appropriate forward and reverse primers, probe, and TaqMan Gene Expression Master Mix (Applied Biosystems Inc.).

Amplification and detection of PCR amplicons were performed with the ABI PRISM 7900 system (Applied Biosystems Inc.) with the following PCR profile: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 30 s and 62°C for 1 min. The 18S rRNA amplification plots derived from serial dilutions of an established reference sample were used to create a linear regression formula and calculate expression levels. The expression level of the house keeping gene, *fibroblast growth factor intracellular-binding protein (Fibp)*, was utilized as an internal control for normalizing the data. *Fibp* expression was not different among genotypes and did not change following exposure to CPO.

RESULTS

There were no overt toxic effects associated with repeated postnatal exposure to CPO, and mice developed apparently normally, with no delay in the appearance of developmental landmarks. Repeated exposure of male (but not female) $PON1^{-/-}$ mice to 0.50 mg/kg/day CPO was associated with reduced bodyweight on PND 21 (8.13 ± 0.33 g vs 9.78 ± 0.50 g; $p = 0.027$, Student's *t*-test). Bodyweights of mice in the other treatment groups and genotypes were not significantly affected by exposure to 0.35 mg/kg/day CPO or 0.50 mg/kg/day CPO, although there was a nonsignificant tendency toward reduced bodyweight in male $PON1^{-/-}$ mice exposed to 0.35 mg/kg/day CPO (9.46 ± 0.64 g vs 9.78 ± 0.50 g; $p = 0.695$, Student's *t*-test). At higher doses (≥ 0.625 mg/kg/day), repeated exposure of $PON1^{-/-}$ or $tgHuPON1_{Q192}$ mice to CPO was associated with lethality after 3–5 days.

Inhibition of Brain AChE

Daily exposure to CPO from PND 4 to 21 resulted in depression of AChE activity in the brains of $PON1^{-/-}$ mice and $tgHuPON1_{Q192}$ transgenic mice, but not $PON1^{+/+}$ mice or $tgHuPON1_{R192}$ transgenic mice, on PND 22 (Fig. 1). First, to address the importance of PON1 status for protecting against CPO exposures during early neonatal development, a dose response of CPO on brain AChE inhibition was measured in $PON1^{+/+}$ mice and $PON1^{-/-}$ mice on PND 4, 2 h after CPO exposure (Fig. 1A). The EC_{50} of CPO for acute inhibition of brain AChE on PND 4 was 0.63 mg/kg in $PON1^{+/+}$ mice and 0.23 mg/kg in $PON1^{-/-}$ mice (Fig. 1A), indicating a greater than 2.5-fold difference in sensitivity of $PON1^{-/-}$ neonates to CPO. In mice of all genotypes, brain AChE was low (~ 2 units/g wet weight) on PND 4 and increased throughout postnatal development, reaching levels of 6–8 units/g by PND 21 (Fig. 1B, data not shown for all genotypes). Daily exposure from PND 4 to 21 was associated with depression of brain AChE (Figs. 1B and 1C), and this effect was modulated by $PON1$ genotype (Fig. 1C). Exposure of $PON1^{-/-}$ mice to 0.25 mg/kg CPO on PND 4, and subsequently on PND 5, resulted in 44 and 36% inhibition of brain AChE, respectively, 2 h following CPO exposure (Fig. 1B). On PND 13, after daily exposure of $PON1^{-/-}$ mice to 0.25 mg/kg/day CPO from PND 4 to 12, brain AChE was inhibited by 17–20% compared with vehicle-treated mice (Fig. 1B) but there was no additional acute inhibition of brain AChE measured 2 h after administration of the CPO dose on PND 13 (Fig. 1B). Similarly, on PND 21, after daily exposure to 0.25 mg/kg/day CPO from PND 4 to 20, brain AChE was inhibited by 26–27% compared with vehicle-treated mice (Fig. 1B), but there was no additional acute inhibition of brain AChE measured 2 h (Fig. 1B) or 4 h (not shown) after administration of the CPO dose on PND 21. Brain AChE activity measured on PND 22, 24 h after the last administration of CPO, was lower in $PON1^{-/-}$ mice and $tgHuPON1_{Q192}$

transgenic mice (exposed to CPO daily from PND 4 to 21) than in control mice (Fig. 1C). Brain AChE activity decreased in a dose-dependent manner, with maximal inhibition of $41 \pm 6\%$ in $PON1^{-/-}$ mice and $22 \pm 5\%$ in $tgHuPON1_{Q192}$ transgenic mice at the 0.5 mg/kg/day dose of CPO (Fig. 1C). $PON1^{+/+}$ mice and $tgHuPON1_{R192}$ mice were resistant to the inhibition of brain AChE (Fig. 1C). None of these doses of CPO inhibited brain AChE acutely on PND 21 in any of the genotypes used in these experiments (Fig. 1B; data not shown for all genotypes and doses).

Gene Expression in the Cerebellum

Previously, Whitney *et al.* (1995) demonstrated effects of subtoxic doses of CPF on cell density in the cerebellum. To determine the effects of CPO on gene expression in this region and to assess differences in the protective capacities of the $PON1_{Q192}$ and $PON1_{R192}$ alloforms, RNA samples were isolated from individual cerebella, then labeled and hybridized individually to Affymetrix Mouse Genome ST 1.0 microarrays ($tgHuPON1_{Q192}$ and $tgHuPON1_{R192}$ mice: $n = 5$ [DMSO], $n = 5$ [0.35 g/kg/day CPO], $n = 5$ [0.50 mg/kg/day CPO]; $PON1^{+/+}$ mice: $n = 6$ [DMSO], $n = 6$ [0.35 mg/kg/day CPO], $n = 3$ [0.50 mg/kg/day CPO]; $PON1^{-/-}$ mice: $n = 5$ [DMSO], $n = 6$ [0.35 mg/kg/day CPO], $n = 4$ [0.50 mg/kg/day CPO]). Following data normalization, mean intensity values for each genotype and treatment group were used to make multiple comparisons among all genotypes and treatment groups (Supplementary Table 1).

Individual Gene Analysis

Initial analyses focused on changes in expression of individual genes for comparisons of interest, using a statistical cutoff of $p < 0.05$ and $|\text{fold change}| > 1.5$. More stringent cutoff values for fold change resulted in unacceptable filtering of the more subtle effects on gene expression and in difficulties with data interpretation due to the low numbers of affected genes. Subsequent GSA that incorporated lower level effects, discussed in more detail below, allowed a more powerful statistical analysis of the CPO effects among genotypes. Supplementary Table 2 lists the transcripts for which there were significant ($p < 0.05$) differences in expression between $PON1^{+/+}$ and $PON1^{-/-}$ mice, or between $tgHuPON1_{Q192}$ and $tgHuPON1_{R192}$ mice, following treatment with 0.50 or 0.35 mg/kg/day CPO, or between the respective control groups (DMSO alone). Supplementary Table 2 also lists, for each of the four genotypes, the individual transcripts for which there were significant ($p < 0.05$) differences in expression between mice treated with 0.5 mg/kg/day CPO and mice treated with DMSO or between mice treated with 0.35 mg/kg/day CPO and mice treated with DMSO.

Figure 2 shows heat maps for the 1261 transcripts that were differentially expressed ($p < 0.05$; $|\text{fold change}| > 1.5$) following treatment of $PON1^{+/+}$, $PON1^{-/-}$, $tgHuPON1_{Q192}$, and $tgHuPON1_{R192}$ mice with 0.35 and 0.50 mg/kg/day CPO.

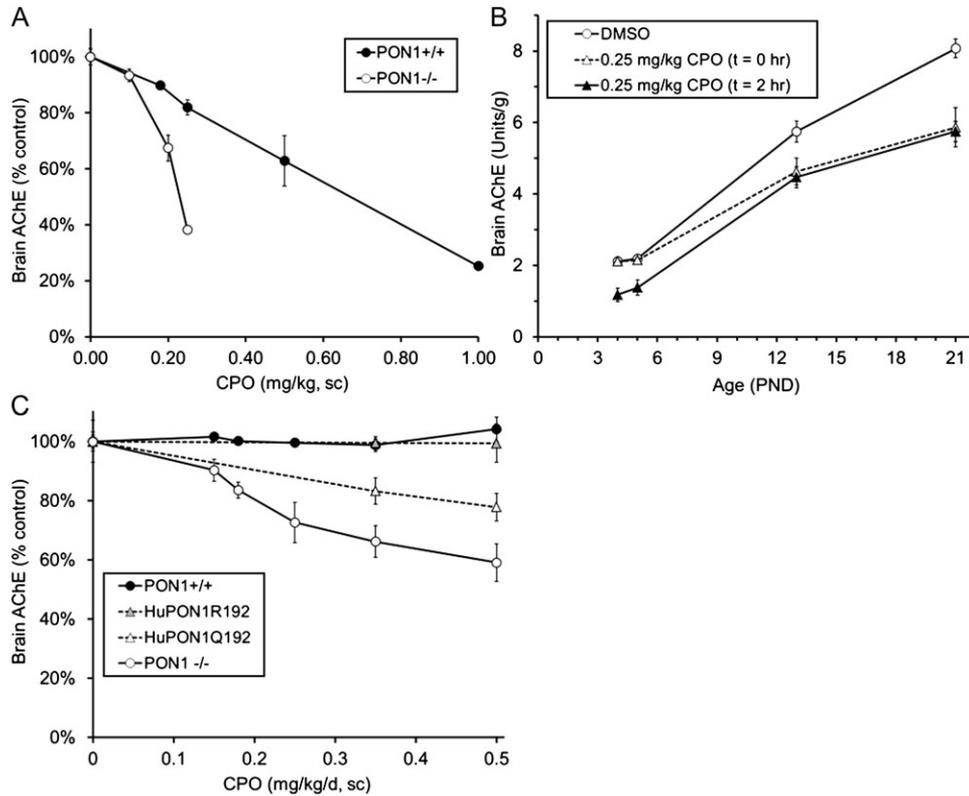


FIG. 1. AChE activity is lower in mice exposed repeatedly to CPO than in mice exposed to vehicle control. (A) Acute inhibition of brain AChE in *PON1*^{-/-} and *PON1*^{+/+} mice at PND 4, 2 h following exposure to different doses of CPO. Values are expressed as mean \pm SE ($n = 3-5$). EC₅₀ values for CPO inhibition of brain AChE in *PON1*^{+/+} and *PON1*^{-/-} mice were 0.63 and 0.23 mg/kg, respectively. (B) Brain AChE activity (Units/g wet weight \pm SE) in *PON1*^{-/-} mice on PND 4 ($n = 3-4$), PND 5 ($n = 3$), PND 13 ($n = 3-5$), and PND 21 ($n = 3-9$) during repeated exposure to 0.25 mg/kg/day CPO. Clear circles: AChE activity in mice exposed to DMSO. Clear triangles/dashed line: AChE activity measured just before administration of the CPO (i.e., at $t = 0$; 24 h after the previous dose). Solid triangles/solid line: AChE activity measured 2 h after administration of the CPO on that day. There was acute inhibition of brain AChE 2 h after CPO exposure on PND 4 and PND 5, but not on PND 13 or PND 21. However, brain AChE activity prior to dosing was lower in CPO-treated mice than in control mice on PND 13 and PND 21. (C) Brain AChE on PND 22 after repeated exposure of *PON1*^{+/+}, *tgHuPON1*_{R192}, *tgHuPON1*_{Q192}, and *PON1*^{-/-} mice to 0.15, 0.18, 0.25, 0.35, or 0.50 mg/kg/day CPO from PND 4 to 21. Data are expressed as % of control (\pm SE; $n = 5-11$).

Of the 1261 transcripts (representing 4.4% of the 28,818 transcripts on the array), 758 corresponded to known genes. Unsupervised clustering indicated distinct patterns of gene expression among mice of the different genotypes, with the 0.35 and 0.50 mg/kg/day treatment groups clustering together for each of the four genotypes. *PON1*^{-/-} mice had gene expression patterns that were most dissimilar from the other genotypes (Fig. 2). Four-way Venn diagrams of the 812 transcripts that were differentially expressed following treatment with 0.50 mg/kg/day CPO and 622 transcripts differentially expressed following treatment with 0.35 mg/kg/day CPO indicated that there was little overlap in affected transcripts among mice of the four genotypes (Fig. 3). Most of the significant ($p < 0.05$) effects on gene expression reflected less than a 1.5-fold change, probably due to the relatively low doses used in the study. Of the 732 transcripts differentially expressed between genotypes in control mice (exposed to DMSO), the highest number of differentially expressed genes were in the *PON1*^{-/-} versus *PON1*^{+/+} comparison (Supplementary Table 2). Surprisingly, *PON2* expres-

sion was reduced in the *PON1*^{-/-} mice compared with *PON1*^{+/+} mice (14–36% lower by microarray analysis, $p < 0.01$), regardless of treatment. This observation was confirmed using qRT-PCR which demonstrated decreased expression of *PON2* in the cerebella of *PON1*^{-/-}, *tgHuPON1*_{Q192}, and *tgHuPON1*_{R192} mice compared with *PON1*^{+/+} mice (Table 1). *PON1* gene disruption in these three lines of mice likely affected the regulatory sequences that control expression of the nearby *PON2* gene. *PON2* expression was not affected by CPO exposure (fold change [mean of all four genotypes] for 0.35 mg/kg/day CPO vs. DMSO, -1.017 ; fold change [mean of all four genotypes] for 0.50 mg/kg/day CPO vs. DMSO, $+1.073$), an observation that was confirmed by qRT-PCR (Table 1). As expected, *PON1* was expressed in the cerebellum of *PON1*^{+/+} mice but not in *PON1*^{-/-} mice.

Ingenuity Pathway Analysis (IPA)

IPA was used to determine the functional categories of differentially expressed genes. Genes that had a p value < 0.05 and a $|\text{fold change}| > 1.5$ were analyzed by IPA. Figure 4 shows

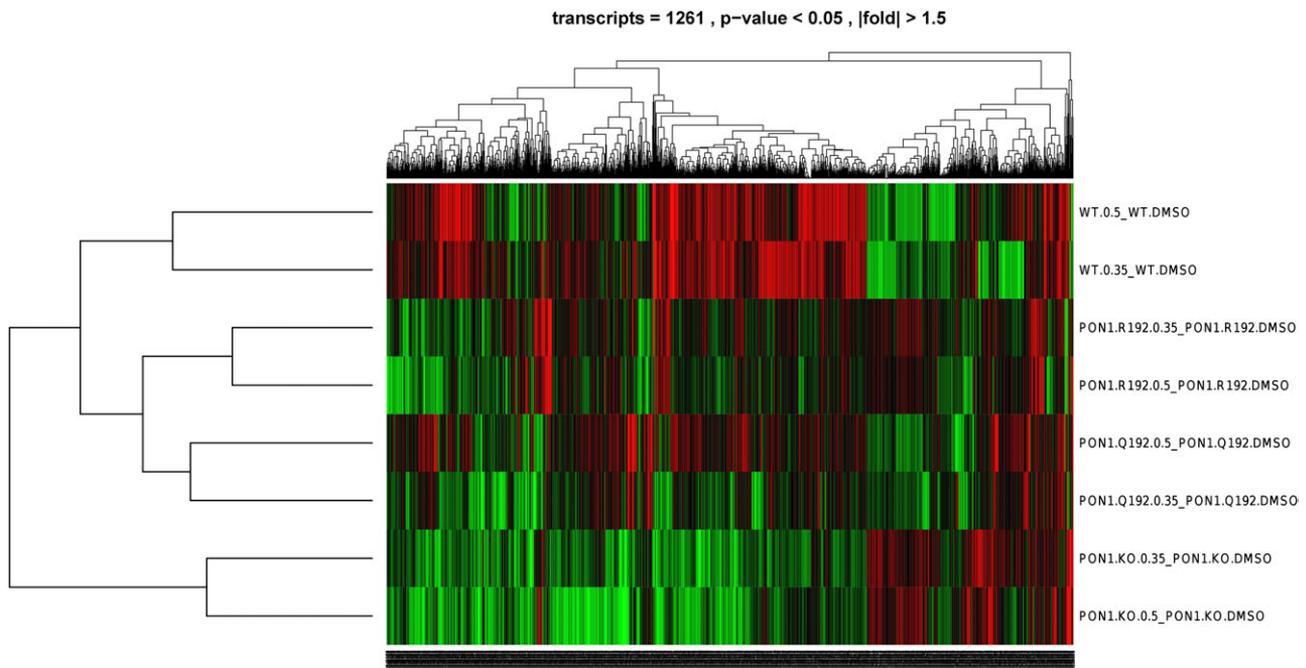


FIG. 2. Individual gene analyses. Heat map of 1261 transcripts differentially expressed ($p < 0.05$, $|\text{fold change}| > 1.5$) following treatment of $PON1^{+/+}$, $PON1^{-/-}$, $tgHuPON1_{Q192}$, or $tgHuPON1_{R192}$ mice with 0.35 or 0.50 mg/kg/day CPO. Red color indicates increased expression. Green color indicates decreased expression.

the top six canonical pathways identified by IPA based on these gene expression changes for the comparison of $PON1^{+/+}$ and $PON1^{-/-}$ mice exposed to 0.5 mg/kg/day CPO and for $tgHuPON1_{Q192}$ mice exposed to 0.5 mg/kg/day CPO compared with vehicle control. Mitochondrial dysfunction, oxidative phosphorylation, and protein ubiquitination were the top three canonical pathways that were elevated in $PON1^{-/-}$ mice exposed to 0.5 mg/kg/day CPO, as compared with $PON1^{+/+}$

mice exposed to 0.5 mg/kg/day CPO (Fig. 4A). Oxidative phosphorylation, mitochondrial dysfunction, and ubiquinone biosynthesis were also the top three pathways that were elevated in $tgHuPON1_{Q192}$ mice following exposure to 0.5 mg/kg/day CPO compared with the same genotype mice exposed to vehicle control (Fig. 4B). These results are consistent with the GSA described below, which also showed increased sensitivity of $PON1^{-/-}$ and $tgHuPON1_{Q192}$ mice to the effects of CPO.

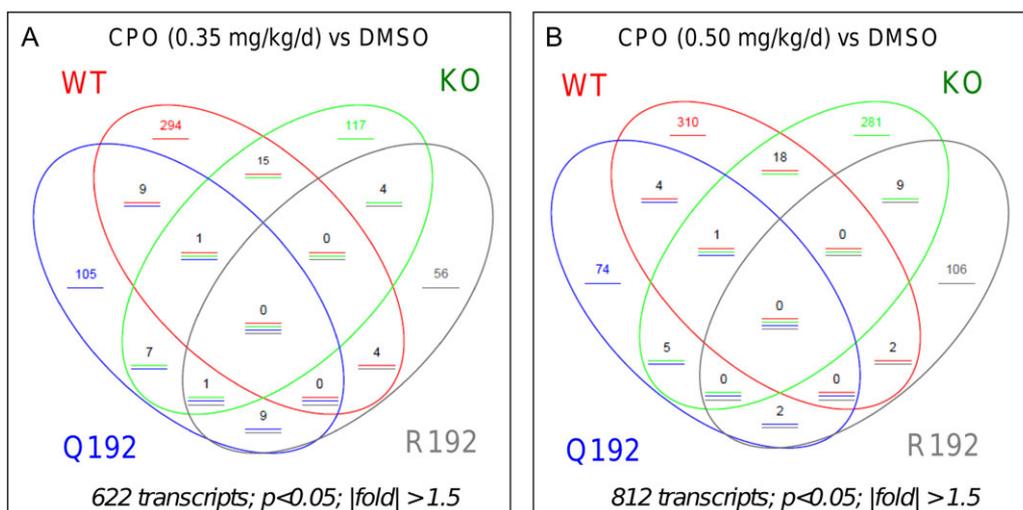


FIG. 3. Individual gene analyses. Four-way Venn diagrams of transcripts differentially expressed ($p < 0.05$; $|\text{fold change}| > 1.5$) following exposure of mice to (A) 0.35 mg/kg/day CPO or (B) 0.5 mg/kg/day CPO. The four compartments in each Venn diagram represent the number of transcripts with significantly affected expression in mice of each of the four indicated genotypes.

TABLE 1
PON2 Expression in $PON1^{+/+}$, $PON1^{-/-}$, $tgHuPON1_{Q192}$, and $tgHuPON1_{R192}$ Mice

	DMSO	0.35 mg/kg/day CPO	0.50 mg/kg/day CPO	All mice, regardless of treatment	% Decrease compared with $PON1^{+/+}$	N
$PON1^{+/+}$	3.72 ± 0.22	3.27 ± 0.18	3.20 ± 0.34	3.44 ± 0.21	—	15
$PON1^{-/-}$	2.15 ± 0.48	2.46 ± 0.40	2.24 ± 0.17	2.30 ± 0.22	33	15
$tgHuPON1_{Q192}$	2.51 ± 0.55	3.17 ± 0.61	2.82 ± 0.21	2.83 ± 0.27	17	15
$tgHuPON1_{R192}$	2.75 ± 0.46	2.71 ± 0.16	2.60 ± 0.16	2.69 ± 0.16	22	15

Note. *PON2* expression levels (adjusted quantity mean ± SE) in cerebellum, determined by qRT-PCR.

Gene Set Analysis (GSA)

The relatively low number of genes whose gene expression was altered greater than 1.5-fold was not surprising given the relatively low doses of CPO used for the study and signal dilution due to the use of the entire cerebellum. Because low-level exposures are likely to produce smaller effect sizes, GSA was used to further investigate the functional categories of differentially expressed genes. GSA provides the advantage of being able to test entire gene sets, rather than individual genes, for statistically significant enrichment among treatment groups. This is a particular advantage for data sets where effects on expression of individual genes may be minor, but when analyzed together as gene sets can become highly significant. For the category analysis, GSA was performed based on the three Gene Ontology hierarchies: BP, MF, and CC. In addition, five gene set collections were used from the Broad Institute (<http://www.broad.mit.edu/gsea/msigdb/index.jsp>). For each of the eight gene set collections, two summary files were generated for each of the 30 contrasts, for a total of 480 summary files.

Summary files contained the Gene Set name, GSA Enrichment Score, Raw *p* value, False Discovery Rate, Gene Set length, and a description of the gene set. Gene set enrichment was categorized as “positive” or “negative” based on the methods of Efron and Tibshirani (2007), which take into account the number of upregulated and downregulated genes, the degree of up- or downregulation, and the values of the individual *t*-statistics. While the interpretation is somewhat more complicated, the general idea is that in gene sets with positive enrichment, upregulation predominates in the comparison group (i.e., mice treated with CPO or mice of a specific genotype) compared with the control group (i.e., mice receiving vehicle control or mice of a different genotype), whereas downregulation predominates in gene sets with negative enrichment. For a more detailed explanation, see Efron and Tibshirani (2007).

Individual reports were generated for each gene set that was significantly enriched in any comparison. These individual reports contained GSA enrichment scores for the genes in the gene set versus GSA enrichment scores for all genes, GSA enrichment

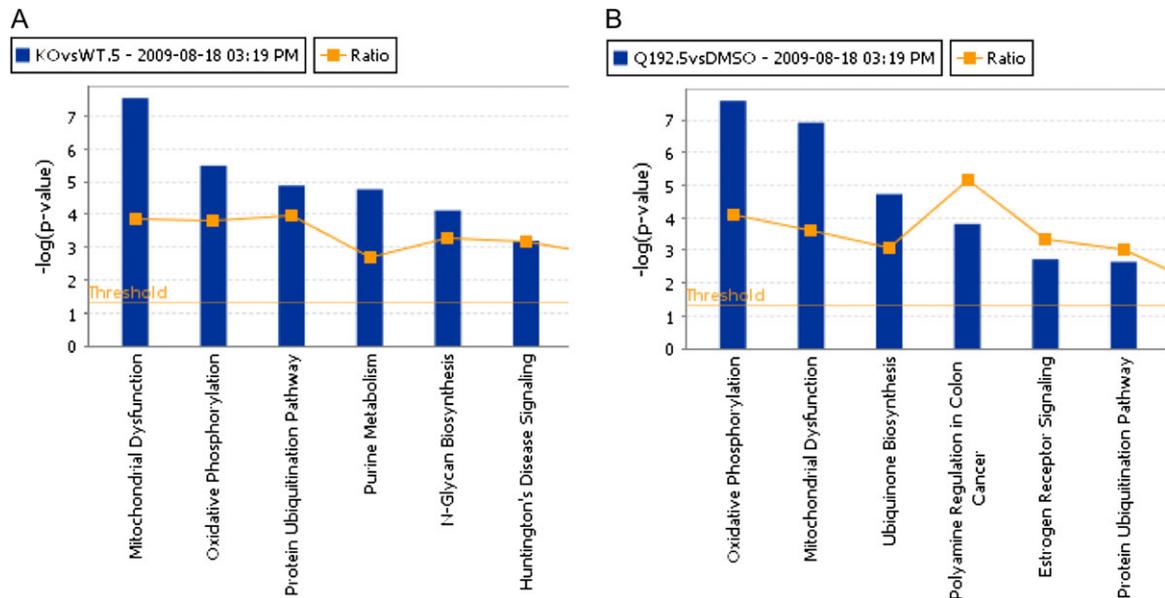


FIG. 4. IPA: Top six canonical pathways identified by IPA based on gene expression changes ($p < 0.05$; $|\text{fold change}| > 1.5$) between A) $PON1^{+/+}$ and $PON1^{-/-}$ mice exposed to 0.5 mg/kg/day CPO ($n = 3-4$), and B) $tgHuPON1_{Q192}$ mice exposed to 0.5 mg/kg/day CPO ($n = 5$) and $tgHuPON1_{Q192}$ mice exposed to vehicle control ($n = 5$).

scores for genes with hyperlinks to additional information, a heat map of the signal intensities with the average of the control values used as the baseline, and a heat map of the correlation of the above signal intensities to show how well the gene set genes were correlated across the samples. Supplementary Table 3 lists the CC, BP, and MF gene sets significantly ($p < 0.05$) enriched for the comparisons of 0.5 mg/kg/day CPO versus DMSO and 0.35 mg/kg/day CPO versus DMSO for all four genotypes. Supplementary Table 4 lists the CC, BP, and MF gene sets for the $PON1^{+/+}$ versus $PON1^{-/-}$ and $tgHuPON1_{Q192}$ versus $tgHuPON1_{R192}$ comparisons at 0.5 mg/kg/day CPO.

Gene Set Venn Diagrams

To facilitate analysis of the GSA data, Venn diagrams were used to determine overlapping gene sets among the treatment groups. The most useful of these comparisons were four-way Venn diagrams that compared significantly enriched gene sets (for 0.50 mg/kg/day CPO vs. vehicle control and 0.35 mg/kg/day CPO vs. vehicle control) among mice of each of the four genotypes. Two examples are shown in Figure 5, for gene sets in the CC and BP gene ontology categories. The results with these two categories of gene sets illustrate a finding that was also true for the other six GO and BROAD gene set categories (Supplementary Fig. 1) the only significant overlap in affected gene sets was between the $PON1^{-/-}$ mice and $tgHuPON1_{Q192}$ transgenic mice (Fig. 5). This is of particular interest because these two genotypes are known to be the most sensitive to the acute toxicity associated with exposure to CPO at higher doses.

Analysis of Specific Gene Sets

There were 98 gene sets that were significantly different at $p < 0.01$ between $PON1^{+/+}$ and $PON1^{-/-}$ mice exposed to 0.5 mg/kg/day CPO and an additional 60 gene sets that were significantly different at $p < 0.05$. There were 21 gene sets that were significantly different at $p < 0.01$ between $tgHuPON1_{Q192}$ and $tgHuPON1_{R192}$ mice exposed to 0.5 mg/kg/day CPO and an additional 74 gene sets that were significantly different at $p < 0.05$. Figure 6 shows heat maps of five selected gene sets that were significantly different ($p < 0.01$) between $PON1^{+/+}$ and $PON1^{-/-}$ mice exposed to 0.5 mg/kg/day CPO, and one of the gene sets that was significantly different between $tgHuPON1_{Q192}$ and $tgHuPON1_{R192}$ mice exposed to 0.5 mg/kg/day CPO. Figure 7 shows heat maps for one BP gene set (Synapse Organization; BP GO_0050808) that was significantly enriched for several different comparisons, including $tgHuPON1_{R192}$ mice versus $tgHuPON1_{Q192}$ mice exposed to 0.5 mg/kg/day CPO and $PON1^{+/+}$ versus $PON1^{-/-}$ mice exposed to 0.5 mg/kg/day CPO. Intriguingly, this gene set showed effects on the neurologins, transmembrane proteins that span the synapse, are involved in trans-synaptic signaling, and are members of the cholinesterase family. Virtually all of the heat maps showed stronger effects, as indicated by the larger magnitude of the changes in gene expression (both upregulation and downregulation), in the

$PON1^{-/-}$ mice and $tgHuPON1_{Q192}$ mice compared with the $PON1^{+/+}$ and $tgHuPON1_{R192}$ mice (Figs. 6 and 7).

Quantitative Real-Time PCR Confirmation

Microarray data for selected genes were confirmed independently by quantitative real-time PCR (qRT-PCR) (Fig. 8). Microarray data and qRT-PCR data indicated upregulation of ATPase, calcium transporting, plasma membrane 2 (Atp2b2), cholinergic receptor, nicotinic, beta polypeptide 4 (Chrb4), glutamate receptor, metabotropic 2 (Grm2), neuroligin 2 (Nlgn2), and synapsin II (Syn2), and downregulation of glutathione S-transferase, alpha 4 (Gsta4), and peptidylprolyl isomerase (cyclophilin)-like 6 (Ppil6), after repeated exposure to 0.5 mg/kg/day CPO. The upregulation or downregulation of these genes was reduced in mice exposed to 0.35 mg/kg/day CPO. $PON2$ expression was lower in the $PON1^{-/-}$ mice than in $PON1^{+/+}$ mice, regardless of exposure (Table 1). Figure 9 shows dose-dependent responses of repeated CPO exposure on the expression of specific genes, measured by qRT-PCR, in the cerebella of $PON1^{-/-}$ mice. There were dose-dependent increases in expression of $Nlgn2$, $Grm2$, and $Syn2$, and a dose-dependent decrease in expression of $Gsta4$ (Fig. 9).

DISCUSSION

This study showed that repeated developmental exposure to CPO is associated with alterations in gene expression and enzyme activity in the cerebellum and that $PON1$ status is important for modulating these effects. Whereas the levels of CPO used in these studies do not inhibit AChE when given acutely to adult animals, brain AChE activity was reduced after repeated exposure of mice from PND 4 to 21, and repeated developmental CPO exposure was associated with changes in the expression of many different genes in the cerebellum. To our knowledge, this is the first study examining the effects of repeated OP-oxon exposures on gene expression, and the first study to examine the modulation of gene expression effects by $PON1$ status. Previous studies have examined the effects of developmental exposure of mice, rats, or cultured cells to the parent organophosphorothioates (Mense *et al.*, 2006; Slotkin and Seidler, 2007, 2009; Stapleton and Chan, 2009; Moreira *et al.*, 2010).

By individual gene analysis, a total of 812 transcripts were differentially expressed ($p < 0.05$; |fold change| > 1.5) following treatment of mice with 0.50 mg/kg/day CPO, and 622 transcripts were differentially expressed following exposure of mice to 0.35 mg/kg/day CPO. IPA identified the top canonical pathways that contained these genes. However, many of the statistically significant effects on gene expression involved changes that were below 1.5-fold, which was not surprising given the relatively low-level exposures. GSA was particularly useful in determining classes of proteins whose expression was affected by CPO exposure. Specific categories of gene sets examined included the CC, BP, and MF Gene Ontology gene sets, and the five Broad Institute Gene Set collections.

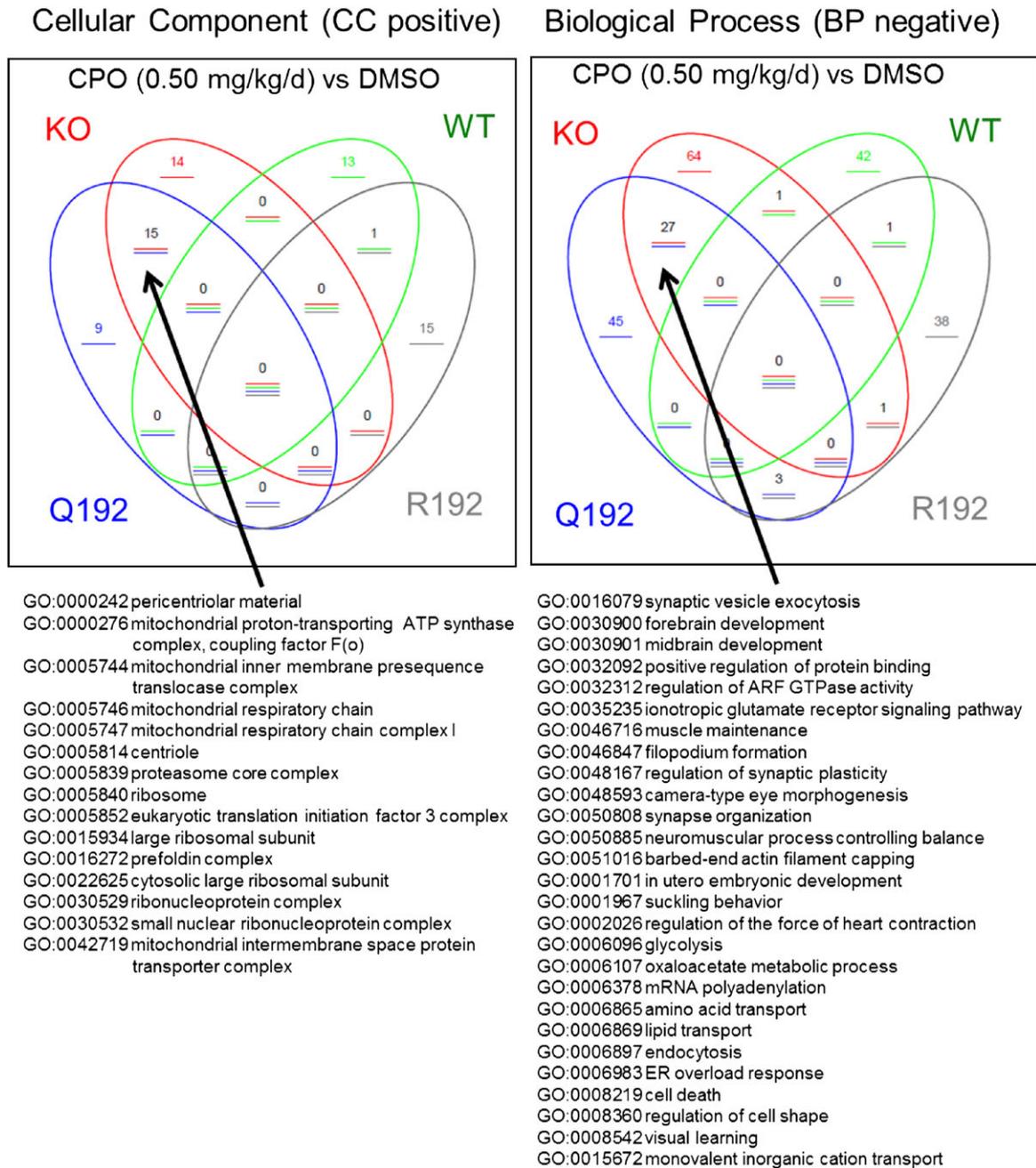


FIG. 5. Gene set analysis. Four-way Venn diagrams for significantly enriched gene sets in the CC and BP categories. The four compartments in each Venn diagram represent the numbers of gene sets with significant positive (CC) or negative (BP) enrichment for the 0.50 mg/kg/day CPO versus DMSO comparison, in mice of each of the four indicated genotypes. Gene sets overlapped between *PONI*^{-/-} and *tgHuPONI*_{Q192} mice (arrows) but not among other genotypes. Individual gene sets enriched in both *PONI*^{-/-} and *tgHuPONI*_{Q192} mice exposed to 0.5 mg/kg/day CPO are listed below the respective Venn diagrams.

The mitochondrion was conspicuous as a cellular component for which many genes showed differential expression with treatment and among genotypes, and the mitochondrial inner membrane presequence complex (GO:0005744), mitochondrial intermembrane space protein transporter complex (GO:0042719), mitochondrial respiratory chain (GO:0005746; GO:0005747), and mitochondrial proton-transporting ATP synthase complex coupling factor F(0) (GO:0000276) were among the 15 CC

positive gene sets that overlapped between the *PONI*^{-/-} and *tgHuPONI*_{Q192} groups. “Mitochondrial dysfunction” and “oxidative phosphorylation” also were identified by IPA as the top two canonical pathways affected by exposure based on analysis of individual genes with |fold change| > 1.5 and statistical significance of *p* < 0.05. The involvement of oxidative stress and mitochondrial dysfunction in CPO toxicity would be consistent with other studies suggesting oxidative stress as an important

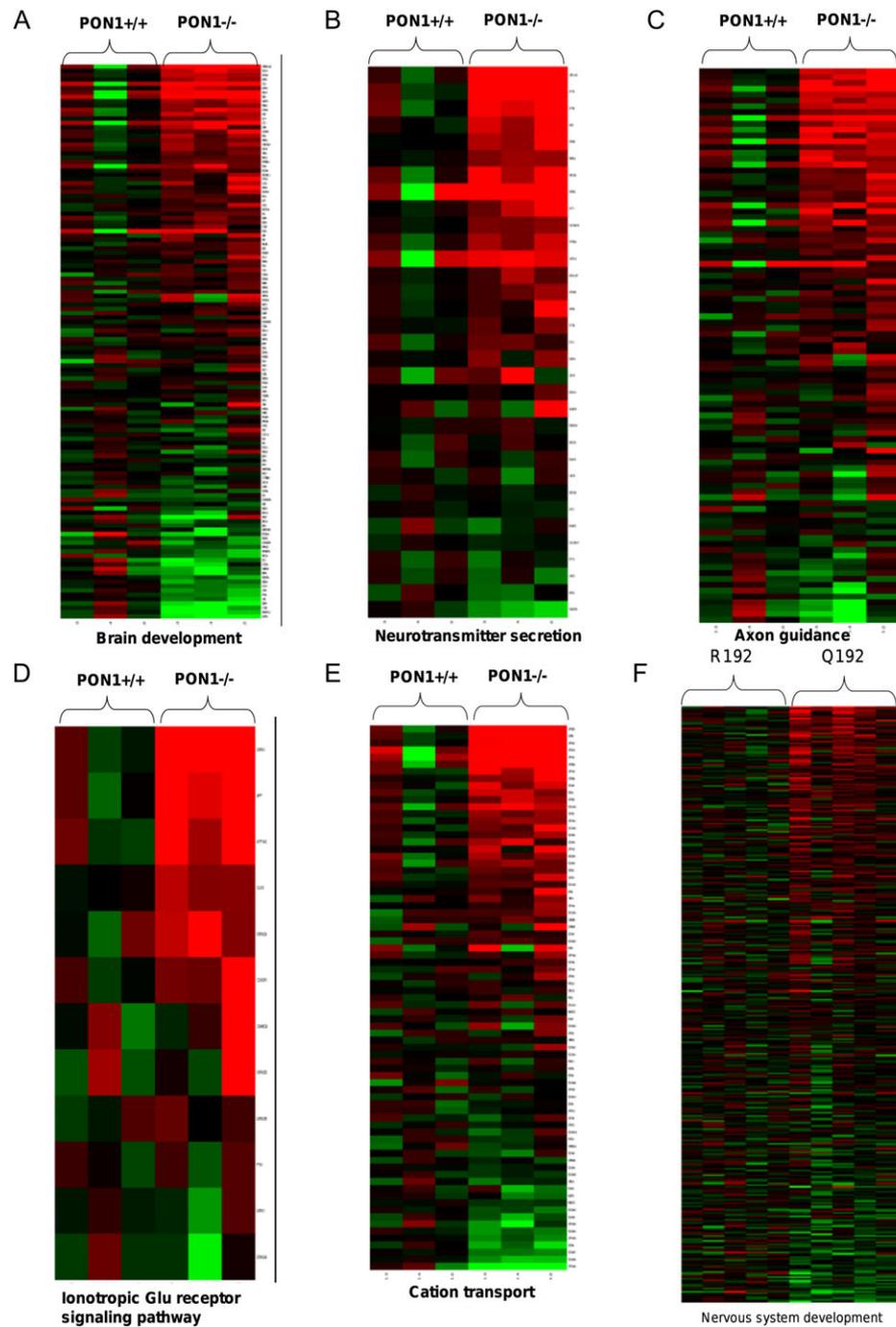


FIG. 6. GSA heat maps. Heat maps of some of the particularly interesting gene sets (BP; GSA positive) that were significantly enriched ($p < 0.01$) between *PON1*^{+/+} and *PON1*^{-/-} mice exposed to 0.5 mg/kg/day CPO (A–E) or between *tgHuPON1*_{Q192} and *tgHuPON1*_{R192} mice exposed to 0.5 mg/kg/day CPO (F). Red color indicates increased expression. Green color indicates decreased expression.

contributor to the toxicity associated with OP exposures (Moreira *et al.*, 2010; Slotkin and Seidler, 2009, 2007).

A total of 439 BP gene sets (217 with positive enrichment; 222 with negative enrichment) were affected by CPO exposure at the 0.5 mg/kg/day dose. The 27 BP negative-enrichment gene sets that overlapped between *PON1*^{-/-} and *tgHu-*

*PON1*_{Q192} mice exposed to 0.5 mg/kg/day CPO included Forebrain Development (GO:0030900), Midbrain Development (GO:0030901), and Cell Death (GO:0008219), as well as the Iontropic Glutamate Receptor Signaling Pathway (GO:0035235), Regulation of Synaptic Plasticity (GO:0048167), Synaptic Vesicle Exocytosis (GO:0016079),

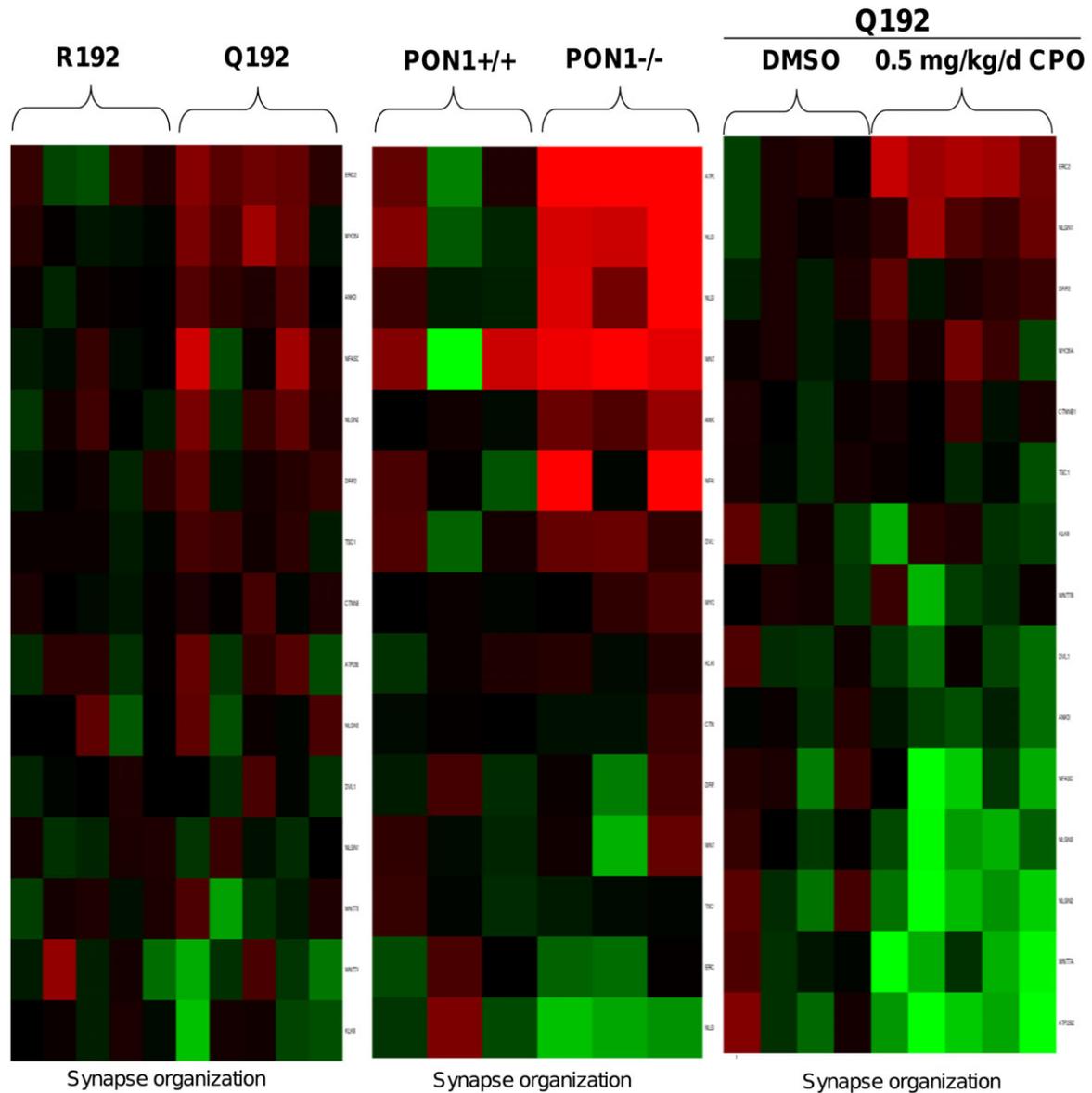


FIG. 7. Synapse Organization gene set. Heat maps of the Synapse Organization gene set (BP GO_0050808) for several different comparisons. This particular gene set was significantly enriched ($p < 0.01$) for a number of different comparisons: *tgHuPON1*_{R192} versus *tgHuPON1*_{Q192} mice exposed to 0.5 mg/kg/day CPO (left); *PON1*^{+/+} versus *PON1*^{-/-} mice exposed to 0.5 mg/kg/day CPO (middle); *tgHuPON1*_{Q192} mice exposed to vehicle control versus *tgHuPON1*_{Q192} mice exposed to 0.5 mg/kg/day CPO (right). Red color indicates increased expression. Green color indicates decreased expression.

and Synapse Organization (GO:0050808). The Synapse Organization gene set is comprised in part by the neuroligins, AChE family members whose active sites have been modified to form trans-synaptic signaling domains. This particular gene set was significantly enriched in comparisons both among genotypes (i.e., between *tgHuPON1*_{Q192} and *tgHuPON1*_{R192} mice that had been exposed to 0.50 mg/kg/day CPO and between *PON1*^{-/-} and *PON1*^{+/+} mice that had been exposed to 0.50 mg/kg/day CPO) and also for comparisons of CPO effects within individual genotypes (i.e., when comparing CPO-exposed mice of a given genotype to vehicle-treated mice of the same genotype). Similar pathways and GO categories were identified

in other studies examining the neurodevelopmental effects of CPF in neonatal rats (Slotkin and Seidler, 2007) and cultured cells (Slotkin and Seidler, 2009), raising the likelihood that many of the effects seen in these earlier studies were due to the bioconversion of CPF to CPO by liver CYP450 enzymes. The gene expression effects almost certainly represent a mixed pattern of toxic responses, repair processes, and adaptive responses; the use of a single time point following dosing did not allow for examination of temporal effects of CPO treatment on gene expression. Additionally, there is a possibility of masking of cell-specific effects due to the complexity of cell types in the cerebellum.

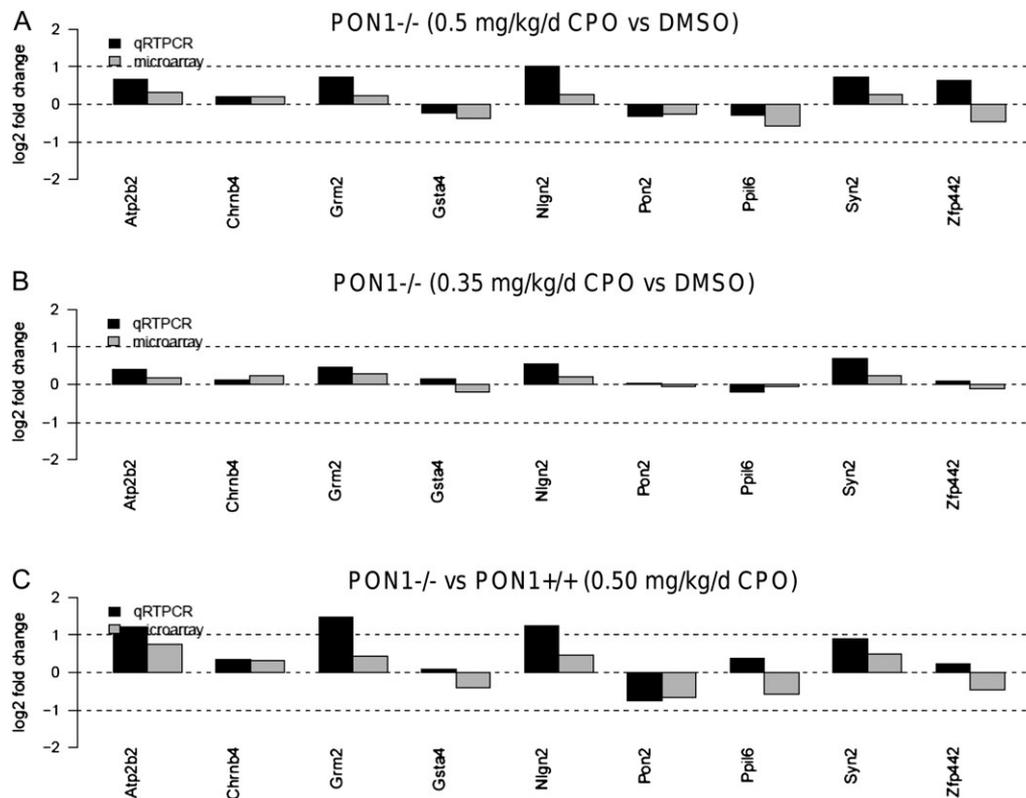


FIG. 8. qRT-PCR confirmation. The expression levels of selected genes were measured by qRT-PCR, standardized to the expression of the housekeeping gene, *Fibp*, and fold changes for the various comparisons compared with the results obtained by microarray analysis. (A) Fold changes in gene expression in *PON1*^{-/-} mice exposed to 0.50 mg/kg/day CPO ($n = 4$) versus DMSO-treated mice ($n = 5$), measured by microarray hybridization (gray bars) and qRT-PCR (black bars). (B) Fold changes in gene expression in *PON1*^{-/-} mice exposed to 0.35 mg/kg/day CPO ($n = 4$) versus DMSO-treated mice ($n = 5$). (C) Fold changes in gene expression for the *PON1*^{+/+} mice exposed to 0.5 mg/kg/day CPO ($n = 3$) versus *PON1*^{-/-} mice exposed to 0.5 mg/kg/day CPO ($n = 4$). The qRT-PCR and microarray results were similar, with the exception of *Zfp442*.

With only two dose groups used for the microarray studies (0.35 and 0.50 mg/kg/day), it was not possible to demonstrate conclusive dose-response relationships on gene expression. Nevertheless, a number of genes did show either increasing or decreasing gene expression with increasing dose that was confirmed by qRT-PCR. These included neuroligin 2 (*Nlgn2*), metabotropic glutamate receptor 2 (*Grm2*), glutathione S-transferase $\alpha 4$ (*Gsta4*), and synapsin II (*Syn2*).

Second, this study showed that *PON1* status was important in modulating the effects of CPO exposure, both on brain AChE inhibition and on gene expression effects in the cerebellum. *PON1*^{-/-} mice showed the most inhibition of brain AChE after exposure to CPO from PND 4 to 21, followed by *tgHuPON1*_{Q192} mice. This is consistent with lack of plasma CPO hydrolytic activity in the *PON1*^{-/-} mice and with the lower catalytic efficiency of CPO hydrolysis by the *PON1*_{Q192} alloform compared with the *PON1*_{R192} alloform. As with previous studies examining acute toxicity in adult mice (Cole *et al.*, 2005; Furlong, 2008; Jansen *et al.*, 2009; Li *et al.*, 2000), the *PON1*_{R192} alloform was more protective of CPO exposure than the *PON1*_{Q192} alloform. Furthermore, gene expression effects analyzed by GSA were the most similar between the

CPO metabolic-deficient *tgHuPON1*_{Q192} and *PON1*^{-/-} mice compared with the *tgHuPON1*_{R192} and *PON1*^{+/+} mice, which are more efficient at detoxifying CPO. Significantly enriched gene sets overlapped between the *tgHuPON1*_{Q192} and *PON1*^{-/-} mice, but not among mice of other genotypes. Direct comparison of *PON1*^{-/-} and *PON1*^{+/+} mice exposed to 0.5 mg/kg CPO identified 126 BP gene sets that were significantly differentially affected between the two genotypes, including Brain Development (GO:0007420), Neurotransmitter Secretion (GO:0007269), Axon Guidance (GO:0007411), Neurite Development (GO:0031175), Cell Death (GO:0008219), Ionotropic Glutamate Receptor Signaling Pathway (GO:0035235), Response to Reactive Oxygen Species (GO:0000302), and Cation Transport (GO:0006812). Similarly, direct comparison of *tgHuPON1*_{Q192} and *tgHuPON1*_{R192} mice exposed to 0.5 mg/kg CPO was informative for understanding genes and pathways that were affected differently between mice expressing these two *PON1* alloforms, which differ in efficiency of CPO detoxication. Figure 6 shows this difference between genotypes dramatically for the Nervous System Development (GO:0007399) BP gene set. In both the *PON1*^{-/-} versus *PON1*^{+/+} and *tgHuPON1*_{Q192} versus *tgHuPON1*_{R192} comparisons of mice treated with

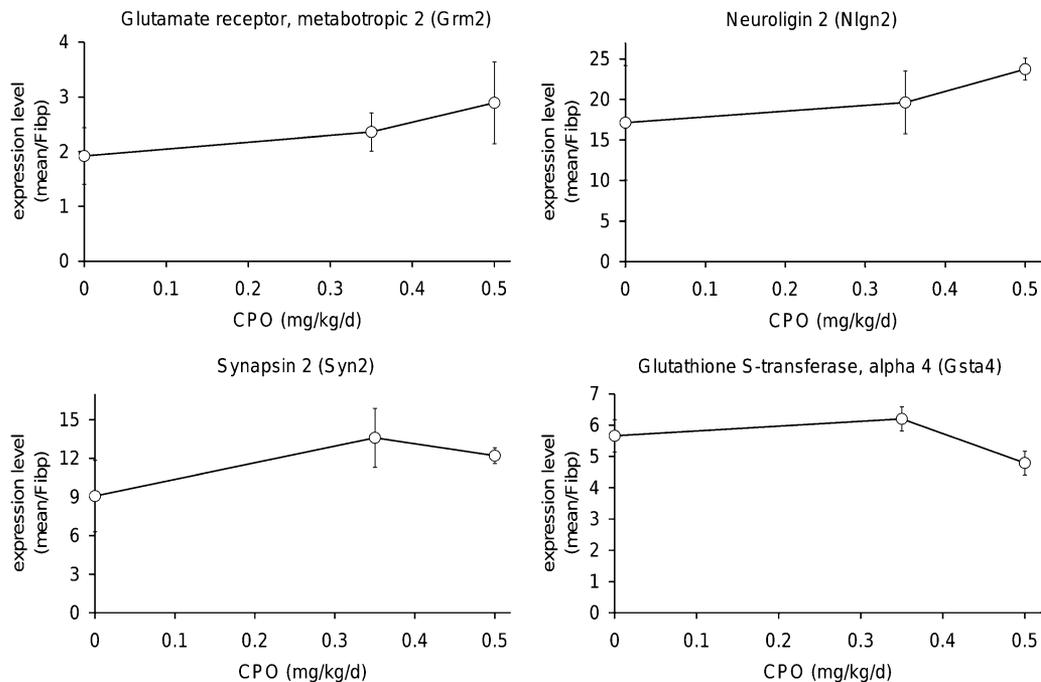


FIG. 9. Dose responses of repeated CPO exposure on expression of specific genes measured by qRT-PCR. Gene expression levels of *Nlgn2*, *Grm2*, *Syn2*, and *Gsta4* were measured from cerebella of *PON1*^{-/-} mice, standardized to the housekeeping gene, *Fibp*, and plotted (mean standardized expression level \pm SE; $n = 3-5$) as a function of dose.

0.5 mg/kg/day CPO, mitochondrial gene sets (e.g., mitochondrial respiratory chain GO:0005746 and mitochondrial intermembrane space GO:0005758) were prominent among the CC gene sets that were significantly differentially affected between genotypes. Analysis by both GSA and IPA showed that *PON1*^{-/-} mice and *tgHuPON1*_{Q192} mice were more sensitive than *PON1*^{+/+} and *tgHuPON1*_{R192} mice to the gene expression changes associated with CPO exposure.

As might be expected, there were differences in cerebellar gene expression among genotypes, even in the absence of CPO exposure. Confirming earlier studies showing *PON1* expression in the brain, in the current study, *PON1* was expressed in the cerebellum of *PON1*^{+/+}, *tgHuPON1*_{Q192}, and *tgHuPON1*_{R192} mice, but not *PON1*^{-/-} mice. Surprisingly, *PON2* expression was also reduced in the *PON1*^{-/-} cerebellum, regardless of treatment. *PON2* mRNA levels were 17–33% lower in the *PON1*^{-/-}, *tgHuPON1*_{Q192}, and *tgHuPON1*_{R192} mice than in the *PON1*^{+/+} mice. This was likely due to disruption of as-yet uncharacterized cis-regulatory elements when the nearby *PON1* gene was inactivated. It is possible that some of the differences in CPO-associated effects on gene expression between the *PON1*^{+/+} mice and mice of the other three genotypes were due to reduced expression of *PON2*, which is localized to mitochondria and could serve a protective role against oxidative damage.

These results indicate that both *PON1* activity and the *PON1*_{Q192R} alloform are important in modulating the develop-

mental effects of CPO exposure, even as early as PND 4, when *PON1* levels are low. At PND 4, the *PON1*^{-/-} mice were ~2.5-times more sensitive to brain AChE inhibition compared with *PON1*^{+/+} mice. It will be interesting to see if this modulation extends back into prenatal development and to determine the relative importance of maternal versus embryonic *PON1* status in protecting against OP exposures. Moreira *et al.* (2010) showed that gestational CPF exposure (at as low as 2 mg/kg/day maternal exposure) affected genes in the fetal brain that were involved in cell division, neurotransmission, and nervous system development. Determining the extent to which these effects are modulated by maternal and fetal *PON1* levels and *HuPON1*_{Q192R} genotype will provide valuable information for assessing the risks associated with OP exposures during pregnancy.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

FUNDING

National Institute of Environmental Health Sciences (P42 ES04696, U19 ES11387, P30 ES07033, R01 ES09883, and ES09601); United States Environmental Protection Agency (R826886).

ACKNOWLEDGMENTS

The authors thank the technical staff at the University of Washington Functional Genomics and Bioinformatics Laboratory for assistance with RNA isolation and quality control and members of the Furlong and Costa laboratories for helpful discussions and technical support. The transgenic and knockout mouse lines were kindly provided by Drs Aldons J. Lusis, Diana Shih, and Aaron Tward at the University of California, Los Angeles.

REFERENCES

- Aldridge, J. E., Meyer, A., Seidler, F. J., and Slotkin, T. A. (2005). Alterations in central nervous system serotonergic and dopaminergic synaptic activity in adulthood after prenatal or neonatal chlorpyrifos exposure. *Environ. Health Perspect.* **113**, 1027–1031.
- Betancourt, A. M., Burgess, S. C., and Carr, R. L. (2006). Effect of developmental exposure to chlorpyrifos on the expression of neurotrophin growth factors and cell-specific markers in neonatal rat brain. *Toxicol. Sci.* **92**, 500–506.
- Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C. A., Causton, H. C., et al. (2001). Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat. Genet.* **29**, 365–371.
- California Environmental Protection Agency (Cal-EPA), Air Resources Board. (1998). Report for the application and ambient air monitoring of chlorpyrifos (and the oxon analogue) in Tulare County during spring/summer 1996. Projects No. C96-040 and C96-041:28 Cal-EPA, Sacramento, CA.
- Camon, E., Magrane, M., Barrell, D., Lee, V., Dimmer, E., Maslen, J., Binns, D., Harte, N., Lopez, R., and Apweiler, R. (2004). The Gene Ontology Annotation (GOA) database: sharing knowledge in Uniprot with Gene Ontology. *Nucleic Acids Res.* **32**, D262–D266.
- Chambers, H., Brown, B., and Chambers, J. E. (1990). Noncatalytic detoxication of 6 organophosphorus compounds by rat-liver homogenates. *Pestic. Biochem. Physiol.* **36**, 308–315.
- Cole, T. B., Jampsa, R. L., Walter, B. J., Arndt, T. L., Richter, R. J., Shih, D. M., Tward, A., Lusis, A. J., Jack, R. M., Costa, L. G., et al. (2003). Expression of human paraoxonase (PON1) during development. *Pharmacogenetics* **13**, 357–364.
- Cole, T. B., Walter, B. J., Shih, D. M., Tward, A. D., Lusis, A. J., Timchalk, C., Richter, R. J., Costa, L. G., and Furlong, C. E. (2005). Toxicity of chlorpyrifos and chlorpyrifos oxon in a transgenic mouse model of the human paraoxonase (PON1) Q192R polymorphism. *Pharmacogenet. Genomics* **15**, 589–598.
- Efron, B., and Tibshirani, R. (2007). On testing the significance of sets of genes. *Ann. Appl. Stat.* **1**, 107–129.
- Fenske, R. A., Lu, C., Curl, C. L., Shirai, J. H., and Kissel, J. C. (2005). Biologic monitoring to characterize organophosphorus pesticide exposure among children and workers: an analysis of recent studies in Washington State. *Environ. Health Perspect.* **113**, 1651–1657.
- Furlong, C. E. (2008). The Bert La Du Memorial Lecture: Paraoxonases: an historical perspective. In: *The Paraoxonases: Their Role in Disease, Development and Xenobiotic Metabolism* (M. M. B. Mackness, M. Aviram, and G. Paragh, Eds.), pp. 3–31. Springer, Dordrecht, The Netherlands.
- Furlong, C. E., Holland, N., Richter, R. J., Bradman, A., Ho, A., and Eskenazi, B. (2006). PON1 status of farmworker mothers and children as a predictor of organophosphate sensitivity. *Pharmacogenet. Genomics* **16**, 183–190.
- Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80.
- Heudorf, U., Butte, W., Schulz, C., and Angerer, J. (2006). Reference values for metabolites of pyrethroid and organophosphorous insecticides in urine for human biomonitoring in environmental medicine. *Int. J. Hyg. Environ. Health* **209**, 293–299.
- Hofmann, J. N., Keifer, M. C., Furlong, C. E., De Roos, A. J., Farin, F. M., Fenske, R. A., van Belle, G., and Checkoway, H. (2009). Serum cholinesterase inhibition in relation to paraoxonase-1 (PON1) status among organophosphate-exposed agricultural pesticide handlers. *Environ. Health Perspect.* **117**, 1402–1408.
- Huen, K., Harley, K., Brooks, J., Hubbard, A., Bradman, A., Eskenazi, B., and Holland, N. (2009). Developmental changes in PON1 enzyme activity in young children and effects of PON1 polymorphisms. *Environ. Health Perspect.* **117**, 1632–1638.
- Huff, R. A., Corcoran, J. J., Anderson, J. K., and Abou-Donia, M. B. (1994). Chlorpyrifos oxon binds directly to muscarinic receptors and inhibits cAMP accumulation in rat striatum. *J. Pharmacol. Exp. Ther.* **269**, 329–335.
- Jameson, R. R., Seidler, F. J., and Slotkin, T. A. (2007). Nonenzymatic functions of acetylcholinesterase splice variants in the developmental neurotoxicity of organophosphates: chlorpyrifos, chlorpyrifos oxon, and diazinon. *Environ. Health Perspect.* **115**, 65–70.
- Jansen, K. L., Cole, T. B., Park, S. S., Furlong, C. E., and Costa, L. G. (2009). Paraoxonase 1 (PON1) modulates the toxicity of mixed organophosphorus compounds. *Toxicol. Appl. Pharmacol.* **236**, 142–153.
- Lee, B. W., London, L., Paulauskis, J., Myers, J., and Christiani, D. C. (2003). Association between human paraoxonase gene polymorphism and chronic symptoms in pesticide-exposed workers. *J. Occup. Environ. Med.* **45**, 118–122.
- Li, W. F., Costa, L. G., Richter, R. J., Hagen, T., Shih, D. M., Tward, A., Lusis, A. J., and Furlong, C. E. (2000). Catalytic efficiency determines the in-vivo efficacy of PON1 for detoxifying organophosphorus compounds. *Pharmacogenetics* **10**, 767–779.
- Lotti, M., Moretto, A., Zoppellari, R., Dainese, R., Rizzuto, N., and Barusco, G. (1986). Inhibition of lymphocytic neuropathy target esterase predicts the development of organophosphate-induced delayed polyneuropathy. *Arch. Toxicol.* **59**, 176–179.
- Mense, S. M., Sengupta, A., Lan, C., Zhou, M., Bentsman, G., Volsky, D. J., Whyatt, R. M., Perera, F. P., and Zhang, L. (2006). The common insecticides cyfluthrin and chlorpyrifos alter the expression of a subset of genes with diverse functions in primary human astrocytes. *Toxicol. Sci.* **93**, 125–135.
- Moreira, E. G., Yu, X., Robinson, J. F., Griffith, W., Hong, S. W., Beyer, R. P., Bammler, T. K., and Faustman, E. M. (2010). Toxicogenomic profiling in maternal and fetal rodent brains following gestational exposure to chlorpyrifos. *Toxicol. Appl. Pharmacol.* **245**, 310–325.
- Nolan, R. J., Rick, D. L., Freshour, N. L., and Saunders, J. H. (1984). Chlorpyrifos: pharmacokinetics in human volunteers. *Toxicol. Appl. Pharmacol.* **73**, 8–15.
- Perez-Herrera, N., Polanco-Minaya, H., Salazar-Arredondo, E., Solis-Heredia, M. J., Hernandez-Ochoa, I., Rojas-Garcia, E., Alvarado-Mejia, J., Borja-Aburto, V. H., and Quintanilla-Vega, B. (2008). PON1Q192R genetic polymorphism modifies organophosphorus pesticide effects on semen quality and DNA integrity in agricultural workers from southern Mexico. *Toxicol. Appl. Pharmacol.* **230**, 261–268.
- Pope, C. N. (1999). Organophosphorus pesticides: do they all have the same mechanism of toxicity? *J. Toxicol. Environ. Health B Crit. Rev.* **2**, 161–181.

- Richter, R. J., and Furlong, C. E. (1999). Determination of paraoxonase (PON1) status requires more than genotyping. *Pharmacogenetics* **9**, 745–753.
- Richter, R. J., Jarvik, G. P., and Furlong, C. E. (2008). Determination of paraoxonase 1 status without the use of toxic organophosphate substrates. *Circ. Cardiovasc. Genet.* **1**, 147–152.
- Salazar-Arredondo, E., de Jesus Solis-Heredia, M., Rojas-Garcia, E., Hernandez-Ochoa, I., and Quintanilla-Vega, B. (2008). Sperm chromatin alteration and DNA damage by methyl-parathion, chlorpyrifos and diazinon and their oxon metabolites in human spermatozoa. *Reprod. Toxicol.* **25**, 455–460.
- Schuh, R. A., Lein, P. J., Beckles, R. A., and Jett, D. A. (2002). Noncholinesterase mechanisms of chlorpyrifos neurotoxicity: altered phosphorylation of Ca²⁺/cAMP response element binding protein in cultured neurons. *Toxicol. Appl. Pharmacol.* **182**, 176–185.
- Shenouda, J., Green, P., and Sultatos, L. (2009). An evaluation of the inhibition of human butyrylcholinesterase and acetylcholinesterase by the organophosphate chlorpyrifos oxon. *Toxicol. Appl. Pharmacol.* **241**, 135–142.
- Shih, D. M., Gu, L., Xia, Y. R., Navab, M., Li, W. F., Hama, S., Castellani, L. W., Furlong, C. E., Costa, L. G., Fogelman, A. M., *et al.* (1998). Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature* **394**, 284–287.
- Slotkin, T. A., and Seidler, F. J. (2007). Comparative developmental neurotoxicity of organophosphates in vivo: transcriptional responses of pathways for brain cell development, cell signaling, cytotoxicity and neurotransmitter systems. *Brain Res. Bull.* **72**, 232–274.
- Slotkin, T., and Seidler, F. (2009). Transcriptional profiles reveal similarities and differences in the effects of developmental neurotoxicants on differentiation into neurotransmitter phenotypes in PC12 cells. *Brain Res. Bull.* **78**, 211–225.
- Smyth, G. K. (2004). Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **3**, 1–25.
- Stapleton, A. R., and Chan, V. T. (2009). Subtoxic chlorpyrifos treatment resulted in differential expression of genes implicated in neurological functions and development. *Arch. Toxicol.* **83**, 319–333.
- Stevens, R. C., Suzuki, S. M., Cole, T. B., Park, S. S., Richter, R. J., and Furlong, C. E. (2008). Engineered recombinant human paraoxonase 1 (rHuPON1) purified from *Escherichia coli* protects against organophosphate poisoning. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 12780–12784.
- Storey, J. D., and Tibshirani, R. (2003). Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 9440–9445.
- Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S., *et al.* (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 15545–15550.
- Udarbe Zamora, E. M., Liu, J., and Pope, C. N. (2008). Effects of chlorpyrifos oxon on M2 muscarinic receptor internalization in different cell types. *J. Toxicol. Environ. Health A.* **71**, 1440–1447.
- Vidal, J. L. M., Gonzalez, F. J. E., Galera, M. M., and Cano, M. L. C. (1998). Diminution of chlorpyrifos and chlorpyrifos oxon in tomatoes and green beans grown in greenhouses. *J. Agric. Food Chem.* **46**, 1440–1444.
- Whitney, K. D., Seidler, F. J., and Slotkin, T. A. (1995). Developmental neurotoxicity of chlorpyrifos: cellular mechanisms. *Toxicol. Appl. Pharmacol.* **134**, 53–62.
- Yuknavage, K. L., Fenske, R. A., Kalman, D. A., Keifer, M. C., and Furlong, C. E. (1997). Simulated dermal contamination with capillary samples and field cholinesterase biomonitoring. *J. Toxicol. Environ. Health* **51**, 35–55.