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Gene Expression Profiles in Zebrafish Brain after Acute Exposure to Domoic Acid at Symptomatic and Asymptomatic Doses

Kathi A. Lefebvre,* Susan C. Tilton,† Theo K. Bammler,† Richard P. Beyer,† Sengkeo Srinouanprachan,† Patricia L. Stapleton,† Federico M. Farin,† and Evan P. Gallagher†,[1]

*Marine Biotoxins Program, National Oceanic and Atmospheric Administration (NOAA) Fisheries/Northwest Fisheries Science Center, 2725 Montlake Boulevard East, Seattle, Washington 98125; and †Department of Environmental and Occupational Health Sciences, School of Public Health and Community Medicine, University of Washington, Seattle, Washington 98105

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Domoic acid (DA) is a neuroexcitatory amino acid that is naturally produced by some marine diatom species of the genus *Pseudo-nitzschia*. Ingestion of DA-contaminated seafood by humans results in a severe neurotoxic disease known as amnesic shellfish poisoning (ASP). Clinical signs of ASP include seizures and neuronal damage from activation of ionotropic glutamate receptors. However, the impacts of DA exposure at levels below those known to induce outward signs of neurobehavioral exicitotoxicity have not been well characterized. To further understand the mechanisms of neurotoxic injury associated with DA exposure, we examined the transcriptome of whole brains from zebrafish (*Danio rerio*) receiving intracoelomic (IC) injection of DA at both symptomatic and asymptomatic doses. A majority of zebrafish exposed to high-dose DA (1.2 μg DA/g) exhibited clinical signs of neuroexcitotoxicity (EC_{50} of 0.86 μg DA/g) within 5–20 min of IC injection. All zebrafish receiving low-dose DA (0.47 μg DA/g) or vehicle only maintained normal behavior. Microarray analysis of symptomatic and asymptomatic exposures collectively yielded 306 differentially expressed genes (1.5-fold, $p \leq 0.05$) predominately represented by signal transduction, ion transport, and transcription factor functional categories. Transcriptional profiles were suggestive of neuronal apoptosis following an overwhelming of protective adaptive pathways. Further, potential molecular biomarkers of neuropathic injury, including the zebrafish homolog of human *NDRG4*, were identified and may be relevant to DA exposure levels below that causing neurobehavioral injury. In general, DA-modulated gene expression was consistent with other model species thereby validating zebrafish as an appropriate vertebrate model to study mechanisms of DA neurotoxicity. These data provide a basis for identifying pathways of DA-induced injury as well as biomarkers of asymptomatic and symptomatic DA exposure levels.

Key Words: **domoic acid; microarray analysis; zebrafish; excitotoxicity.**

Domoic acid (DA) is a neuroexcitatory amino acid that is naturally produced by some diatom species of the genus *Pseudo-nitzschia* (Bates *et al*., 1998). The toxin accumulates in filter-feeding marine organisms (Lefebvre *et al*., 2002; Wekell *et al*., 1994) and is transferred through the food web resulting in a severe neurotoxic illness known as amnesic shellfish poisoning (ASP). Acute signs of ASP include vomiting, diarrhea, confusion, disorientation, seizures, memory loss, coma, and death and were first documented in 1987 when approximately 100 people became ill and 3 people died after consuming DA-contaminated mussels in Prince Edward Island, Canada (Perl *et al*., 1990; Wright *et al*., 1989). Massive mortality events have subsequently been observed in marine mammal and sea bird populations due to the consumption of DA-contaminated fish (Lefebvre *et al*., 1999; Scholin *et al*., 2000; Sierra-Beltran *et al*., 1997; Work *et al*., 1993). In an effort to fully characterize behavioral excitotoxicity and neurologic sensitivity in the vertebrate system, several acute DA exposure studies have been performed in the laboratory. Acute signs of DA-induced neurobehavioral excitotoxicity appear to be relatively consistent across vertebrate species and include gagging, loss of balance and tremors in *Cynomolgus* monkeys, seizures and scratching in rats and mice, and corkscrew and circle swimming in fish (Grimmelt *et al*., 1990; Lefebvre *et al*., 2001, 2007; Tryphonas *et al*., 1990a,b). Intraperitoneal injection doses reported to induce these symptoms are also similar between vertebrate species in primates, rodents, and fish (Grimmelt *et al*., 1990; Lefebvre *et al*., 2001, 2007; Tryphonas *et al*., 1990a,b).

Several studies have shed light on the mechanism of DA toxicity. DA is a rigid structural analog of glutamate, the putative excitatory neurotransmitter in the vertebrate central nervous system (Hampson and Manalo, 1998; Levitan, 1991). The toxin binds with higher affinity than glutamate to kainate and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subclasses of ionotropic glutamate receptors (Hampson and Manalo, 1998). Excitotoxicity is initiated via activation of these receptors and is mediated by secondary activation of

[1] To whom correspondence should be addressed at Department of Environmental and Occupational Health Sciences, School of Public Health and Community Medicine, University of Washington, 4225 Roosevelt Way NE Suite 100, Seattle, WA 98105-6099. Fax: (206) 685-4696. E-mail: evang3@u.washington.edu.

N-methyl-D-aspartic acid (NMDA) receptors and subsequent Ca^{2+} influx (Berman and Murray, 1997; Berman *et al*., 2002). Previous studies have confirmed the presence of AMPA/kainic acid (KA) receptors and their roles in the activation of NMDA receptors in the zebrafish olfactory bulb (Edwards and Michel, 2002). Additionally, a recent study has confirmed the presence of AMPA receptor subunit expression in the zebrafish central nervous system (Hoppmann *et al*., 2008), further suggesting that zebrafish can serve as a useful model system for investigating the role of AMPA receptors in the vertebrate nervous system. Changes in intracellular Ca^{2+} homeostasis are a direct trigger for apoptotic and necrotic cell death (Carafoli, 2004; Green and Reed, 1998). Changes in intracellular Ca^{2+} homeostasis, such as those induced by NMDA receptor activation, are a direct trigger for apoptotic and necrotic cell death (Carafoli, 2004; Green and Reed, 1998). Consequently, in addition to neurobehavioral effects, acute exposure to DA causes neuronal cell death and obvious histopathological lesions in the brain, particularly in regions involved in memory processing such as the CA1 and CA3 regions of the hippocampus (Schimanski and Nguyen, 2005; Scholin *et al*., 2000; Strain and Tasker, 1991; Sutherland *et al*., 1990). In addition to cell death, elevated Ca^{2+} influx can also cause changes in gene expression via the induction of intracellular signaling cascades (Duchen, 1999). Previous studies have documented gene or protein expression changes in the central nervous system of rodents and fish due to acute exposure to DA (Ananth *et al*., 2003; Ryan *et al*., 2005; Salierno *et al*., 2006). Pathways altered by DA appear to involve altered regulation of transcriptional control genes and those involved with mediating synaptic signaling and inflammatory responses.

Neurobehavioral signs of toxicity and dose-response relationships for DA have been reasonably well defined for primate, rodent, and fish model species (Dakshinamurti *et al*., 1991; Grimmelt *et al*., 1990; Iverson *et al*., 1990; Lefebvre *et al*., 2007; Peng and Ramsdell, 1996; Tasker *et al*., 1991; Tiedeken *et al*., 2005). At DA exposures that result in neurobehavioral excitoxicity, neuronal necrosis and the spatial distribution of resulting brain lesions have also been well-characterized (Ananth *et al*., 2001; Berman *et al*., 2002; Peng *et al*., 1994; Scholin *et al*., 2000; Strain and Tasker, 1991; Tryphonas *et al*., 1990b). However, relatively little is known about the effects DA exposure at asymptomatic doses, and there is growing concern regarding the potential human health impacts of long-term low-level exposure to DA. Accordingly, a major goal of this study was to determine if low-level asymptomatic DA exposures (below levels that induce outwards signs of excitotoxicity) modulate gene expression in the vertebrate central nervous system. To this end, we examined whole-brain gene expression in a vertebrate model species (zebrafish) after intracoelomic (IC) exposure to DA at symptomatic and asymptomatic doses. The zebrafish model is increasingly being used to study neurotoxicity and mechanisms of human disease. Our approach in the current study was to establish a dose-response relationship for DA-induced neurobehavioral injury and acute toxicity in zebrafish to identify appropriate symptomatic and asymptomatic exposure doses to link to global brain gene expression. Our results provide a basis for identifying potential pathways of DA-induced injury as well as molecular markers for the effects of asymptomatic and symptomatic DA exposure levels.

MATERIALS AND METHODS

Chemicals and biochemicals. DA was purchased from Sigma Chemical Corp. (St Louis, MO). Trizol reagent, superscript first-strand synthesis kit, formamide, 5-sulfosalicylic acid, Denhardt's solution, CotI DNA, high-performance liquid chromatography (HPLC) grade water, *Taq*Man probe, *Taq* polymerase, sequence-specific PCR primers and probes, and other molecular biology reagents were purchased from Invitrogen Inc. (Carlsbad, CA). The RNAse inhibitor was purchased from Ambion, Inc. (Austin, TX). Superscript enzyme, dithiothreitol, 2′-deoxyadenosine 5′-triphosphate, 2′-deoxyguanosine 5′-triphosphate, 2′-deoxythymidine 5′-triphosphate, and 2′-deoxycytidine 5′-triphosphate were purchased from Millipore Corp. (Bedford, MA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO) or Fisher Scientific (Orlando, FL). The Affymetrix GeneChip Zebrafish Genome Arrays were purchased from Affymetrix Inc. (Santa Clara, CA).

Experimental animals. Wild-type zebrafish (*Danio rerio*, AB strain) were obtained from the University of Oregon, Zebrafish International Resource Center (Eugene, OR) and maintained at the Northwest Fisheries Science Center (NWFSC) in a self-contained recirculating aquaculture ZMOD system (Marine Biotech Inc., Beverly, MA). The facility is operated according to standard procedures for zebrafish husbandry and research (Westerfield, 1995). Fish were maintained at 26°C, kept on a 1400:1000 h light-dark cycle, and fed daily with purified ground salmon feed that was prepared at the NWFSC and brine shrimp. Fish were not fed on the morning of each exposure experiment.

DA exposures for quantifying neurobehavioral toxicity. To quantify DA-induced neurobehavioral excitotoxicity in zebrafish, adult fish were exposed to DA via IC injection at several doses. For injection, fish were netted individually, placed into Ziploc bags and injected through the bag at a point midway between the pectoral fins and the anus. Contents (10 μl) were slowly injected into the IC cavity. IC injections consisted of a vehicle control of phosphate-buffered saline (PBS) only or DA dissolved in PBS. Eight exposure doses (0.21 ± 0.01, 0.25 ± 0.03, 0.32 ± 0.04, 0.47 ± 0.07, 0.82 ± 0.09, 1.2 ± 0.15, 1.6 ± 0.1, and 3.2 ± 0.5 μg DA/g body weight; mean + SD; $n = 4$ fish per treatment) were used. After injection, four fish from each treatment were placed in 1-l clear plastic tanks and observed for 6 h. Behavioral toxicity was quantified by the presence or absence of circle or spiral swimming. Time to the onset of excitotoxicity (i.e., the time period between injection and the first visible clinical signs of toxicity) and the number of fish affected were recorded for each treatment. Percentages of fish affected at each dose were used to generate a dose-response curve and an EC_{50} for behavioral toxicity using Graphpad Prism software (San Diego, CA). All DA exposure doses were confirmed by high performance liquid chromatography-ultraviolet detection (HPLC-UV) analysis (see *HPLC verification of DA exposure doses* below).

Acute symptomatic and asymptomatic DA exposures for gene expression analysis. Acute symptomatic and asymptomatic exposure doses were chosen based on results from the previously described neurobehavioral toxicity study. Intraceolomic injection exposures consisted of three treatments. Control fish ($n = 15$) were injected with the vehicle (PBS only), low-dose fish ($n = 15$) were injected with an asymptomatic dose of 0.47 ± 0.07 μg DA/g total fish weight (mean ± SD), and high-dose fish ($n = 30$) were injected with a dose of 1.2 ± 0.2 μg DA/g total fish weight (mean ± SD) which is a dose shown to

induce excitotoxic symptoms in the present study. Twice as many zebrafish were injected with the high dose to insure the survival of 15 fish in the event of 50% mortality. All fish were adults and at 14 months of age at the time of exposure. The source population was approximately 50% male and 50% female. Individual sexes were not recorded.

Zebrafish were injected in three groups of 5 control, 5 low-dose, and 10 high-dose fish per group. Immediately following injection, five zebrafish from each treatment were placed in 1-l clear plastic tanks and observed for 6 h. A total of three control tanks, three low-dose tanks, and six high-dose tanks containing five fish per tank were observed. The number of fish exhibiting behavioral toxicity (i.e., circle or spiral swimming) was recorded for each tank. At 6 h, all fish were euthanized by decapitation followed by rapid removal of the brain for RNA extraction. During brain removal surgeries, the skullcap was opened, and the brain removed, rinsed in ice cold PBS, weighed, and immediately frozen in liquid nitrogen. The length of brain removal surgeries, from euthanasia to freezing in liquid nitrogen, averaged 1.6 ± 0.5 min (mean ± SD, $n = 43$). The entire brain was removed after cutting the brain stem and at the optic nerves as close to the eyes as possible. RNA was extracted from whole brain with no attempt to distinguish neural versus glial tissues. To insure a consistent exposure period from injection to brain removal, the injection times were staggered for each group. Exposure time, from injection to euthanasia, averaged 6 ± 0.2 h (mean ± SD, $n = 43$) for all fish. Survival rates and the percentages of fish affected were calculated for each treatment.

HPLC verification of DA exposure doses. Prior to each experiment, aliquots of each IC injection dose were taken for DA quantification via standard HPLC-UV detection methods (Quilliam *et al*., 1989). In brief, samples were quantified using an isocratic elution profile on a Hewlett-Packard 1090 HPLC equipped with a diode array detector set at 242 nm with a bandwidth of 10 nm. The reference signal was set at 450 nm with a bandwidth of 10 nm. A reverse phase Vydac C_{18} column (catalog #201TP52, 2.1 × 25 mm, Separations Group, Hesperia, CA) equipped with a Vydac guard column (particle size 5 μm) was used. The mobile phase (90/10/0.1, water/MeCN/TFA) was degassed with helium for 10 min prior to analysis. DACS-1D certified DA standard (National Research Council of Canada, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, NS, Canada) was used as a reference standard and for generating calibration curves for DA quantification.

RNA isolation, processing and array hybridization. Total RNA was extracted from individual snap frozen zebrafish brains using a standard TRIzol procedure with the inclusion of 1 μl RNAse inhibitor/sample. Following determination of RNA concentrations by UV absorbance, the integrity of each RNA sample was verified using an Agilent 2100 Bioanalyzer (Santa Clara, CA). Samples devoid of significant contamination and RNA degradation (as measured by the ratio of 28S–18S peaks) were used for microarray analysis. In total, nine microarrays were used, including three for the control group, three for the low DA dose group, and three for the high dose group. Separate pools of RNA from five individuals were hybridized to each array resulting in three biological replicates (a total of 15 individuals) per treatment group. The Affymetrix One Cycle Target Labeling and Control Reagents Kit were used according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). These methods included the synthesis of first- and second-strand cDNAs, the purification of double-stranded cDNA, the synthesis of cRNA by *in vitro* transcription, the recovery and quantitation of biotin-labeled cRNA, the fragmentation of this cRNA and subsequent hybridization to the Affymetrix GeneChip Zebrafish Genome Array, the posthybridization washings, and the detection of the hybridized cRNAs using streptavidin-coupled fluorescent dye. Hybridized arrays were scanned with an Affymetrix GeneChip 3000 scanner. Image generation and feature extraction were performed using Affymetrix GeneChip Operating Software (GCOS). Cell intensity files were further processed in Bioconductor (http://bioconductor.org) (Gentleman *et al*., 2004).

Microarray data analysis. Raw microarray data were preprocessed and analysed with Bioconductor (Gentleman *et al*., 2004). Several quality control steps were followed to insure that the data were of high quality: (1) visual inspection of the GCOS chip images, (2) visual inspection of the chip pseudoimages generated by the Bioconductor affyPLM package, (3) generation of percent present calls and average background signals with the Bioconductor simpleaffy package, (4) generation and inspection of histograms of raw signal intensities, and (5) generation and comparison of the Relative Log Expression and Normalized Unscaled Standard Errors using the Bioconductor affyPLM package in order to ensure the arrays passed the manufacturer's specifications (which all microarrays did pass). The data were normalized with the Bioconductor gcrma package. From the normalized data, genes with significant evidence for differential expression were identified using 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 the Bioconductor package qvalue (Tusher *et al*., 2001), which allows for selecting statistically significant genes while controlling the estimated false discovery rate. In order to investigate categories of genes where the constituent genes show coordinated changes in expression over the experimental conditions, we carried out biological category analysis via the cumulative hypergeometric distribution method of determining enhanced gene ontology (GO) categories (Camon *et al*., 2004) using the Bioconductor package GOstats (Gentleman, 2005). This approach uses filtered gene lists and identifies GO categories by evidence of overrepresentation of significant genes.

Microarray confirmation by real-time quantitative reverse transcriptase-PCR. Differentially expressed genes of interest were selected for confirmation of the microarray results by real-time quantitative reverse transcriptase (qRT)-PCR. Genes for confirmation were chosen because they were differentially up- or downregulated or resulted in no change by microarray analysis. Total RNA isolated from individual zebrafish brains ($n = 15$ control, $n = 13$ low dose, $n = 18$ high dose) was treated with DNase (Invitrogen) according to manufacturer's protocol. Final cDNAs were diluted to a volume of 200 μl with 2 or 4 μl of each used in subsequent PCR reactions. PCR primers were designed using Oligo Primer Analysis Software, v. 6.71 (Cascade, CO) and verified for specificity using BLAST software (Supplementary Table 1). The 25 μl PCR mixture consisted of the appropriate forward and reverse primers (0.16 μM each), 4 μl cDNA, buffers, salts, and SYBR Green PCR master mix. Amplification and detection of the fluorescence were measured using 7900HT FAST Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following PCR reaction profile: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 30 s, and 60°C for 60 s, followed by a dissociation curve. SYBR green technology was utilized to measure expression of all genes, except *NDRG4* which utilized *Taq*Man. The primers and probe used to measure *NDRG4* expression were designed and verified by Applied Biosystems. For *NDRG4*, the PCR mixture (20 μl final volume) consisted of the appropriate forward and reverse primers (0.35 μM each), 2 μl cDNA, 150nM *Taq*Man probe, and 1× FAST Universal PCR Master Mix. Amplification and detection of the fluorescence were measured using 7900HT FAST Real-Time PCR System with the following PCR reaction profile: 1 cycle of 95°C for 15 s, 40 cycles of 95°C for 1 s, and 60°C for 20 s. DNA amplification was quantified (pg) from the *C* (*T*) value based on standard curves to ensure quantification was within a linear range. All signals were normalized against β-actin, and fold-change ratios were calculated for treated samples compared to controls. Expression of β-actin was not altered by treatment based on either microarray analysis or real-time qRT-PCR and so was found to be an appropriate housekeeping gene for normalization in this study.

RESULTS

Neurobehavioral Toxicity

Zebrafish exposed to DA exhibited clinical signs consistent with excitotoxicity in the form of rapid erratic darting behavior, circle swimming, and spiral swimming within 5–20 min of IC injection with DA dissolved in PBS. We used circle and spiral swimming as biomarkers of excitotoxicity resulting from DA exposure. After combining behavioral data from all doses, an

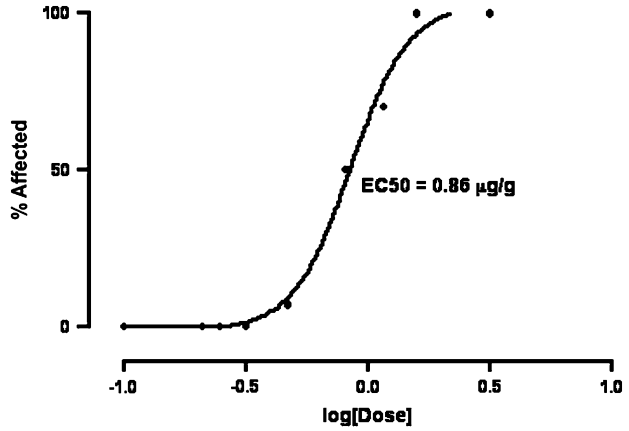

FIG. 1. Dose-response relationship of IC injection doses of DA and the percentage of zebrafish (*Danio rerio*) affected at each dose ($n \geq 4$ at each dose). Circular and/or spiral-swimming behavior were used to quantify excitoxicity in fish. Effective concentration (EC_{50}) = the dose at which 50% of the fish tested were affected.

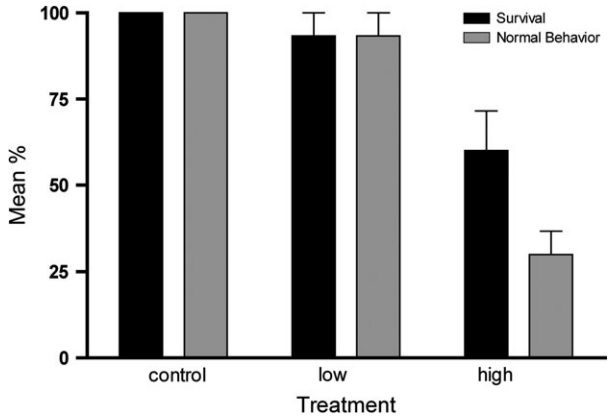

FIG. 2. Percent survival and the percentage of zebrafish exhibiting normal behavior at 6 h after IC injection exposure to 0.47 µg/g total body weight (low asymptomatic dose) and 1.2 µg/g total body weight (high symptomatic dose) DA as well as controls (mean ± SD). Brain tissues from surviving fish were used for gene expression analyses. Excitotoxic behavior was not observed in any of the control or low-dosed fish during the 6-h observation period. A majority of zebrafish injected with the high dose exhibited excitotoxic behavior.

EC_{50} of 0.86 µg DA/g total body weight was calculated (Fig. 1). At doses greater than or equal to the EC_{50} for excitotoxic behavior, death was often observed within 20 min after exposure (data not shown). At doses lesser than or equal to the EC_{50}, several fish regained normal swimming behavior after exhibiting neuroexcitotoxic behavior. Control zebrafish IC injected with vehicle (PBS) maintained normal swimming behavior and had no signs of neurobehavioral impairment throughout the observation period.

Symptomatic and Asymptomatic DA Exposures and Microarray Analysis

In order to characterize the response of the brain to asymptomatic and symptomatic DA exposure, gene expression profiles from whole zebrafish brain were compared between animals that were IC injected with 0.47 ± 0.07 and 1.2 ± 0.2 µg DA/g total fish weight (mean ± SD), respectively, and control animals IC injected with PBS. Each microarray ($n = 3$ per treatment) consisted of four to five pooled individual brains. Percent survivals were 100 ($n = 3$ tanks with five fish per tank), 93 ± 12 ($n = 3$ tanks with five fish per tank), and 60 ± 28 ($n = 6$ tanks with five fish per tank) (mean ± SD) for control, asymptomatic (low), and symptomatic (high) treatments, respectively (Fig. 2). The percentages of fish exhibiting normal behavior were 100 ($n = 3$ tanks with five fish per tank), 93 ± 12 ($n = 3$ tanks with five fish per tank), and 30 ± 17 ($n = 6$ tanks with five fish per tank) (mean ± SD) for control, asymptomatic, and symptomatic treatments, respectively (Fig. 2). None of the low-dose treatment fish used in microarray analyses exhibited neurobehavioral signs of excitotoxicity. In contrast, at least 50% of the high-dose treatment fish analysed by microarray exhibited neurobehavioral signs of excitoxicity (Fig. 2).

A summary of all differentially expressed genes resulting from low- and high-dose DA exposures are provided in Supplementary Table 2. Of the genes analysed on the Affymetrix GeneChip zebrafish array, 306 genes were significantly differentially regulated (up or down) above the 1.5-fold cut off ($p \leq 0.05$) in DA-exposed fish (Fig. 3). Interestingly, the majority of differentially expressed genes relative to control animals (223/306, 73%) were observed in animals exposed to the lower dose of DA (Fig. 3A). Of the 223 genes differentially expressed in the low-dose group, a majority (66%) were downregulated relative to controls. In contrast, a majority (85%) of the 106 genes differentially expressed in the high-dose group were upregulated. Of the 306 genes of interest, only 23 were shared by both treatments and in all 23 cases these genes were regulated in the same direction (either up or down) in each treatment. These trends in gene regulation are illustrated in Figures 3B and 3C. It is noteworthy that the most highly differentially expressed genes across the experiment were downregulated in the low dose and either upregulated or not changed in the high-dose treatments (Fig. 3B) relative to the controls ($n = 3$ for each group). Hierarchical clustering was used as a visualization tool to identify similarities among biological replicates within a treatment and differences in gene expression between treatments. Bidirectional hierarchical clustering of differentially expressed genes (1.5-fold, $p \leq 0.05$) indicated that there were distinct transcriptional profiles for each treatment group, which clustered under separate nodes (Fig. 3C). There was marked variability between biological replicates that may be due to sex ratio discrepancies and differences in uptake of DA into the brain of exposed fish. However, irregardless of the dose, gene profiling revealed that individual genes were generally regulated in the same direction (either up or down) by DA (Fig. 3C) and that the primary effect of dose was on

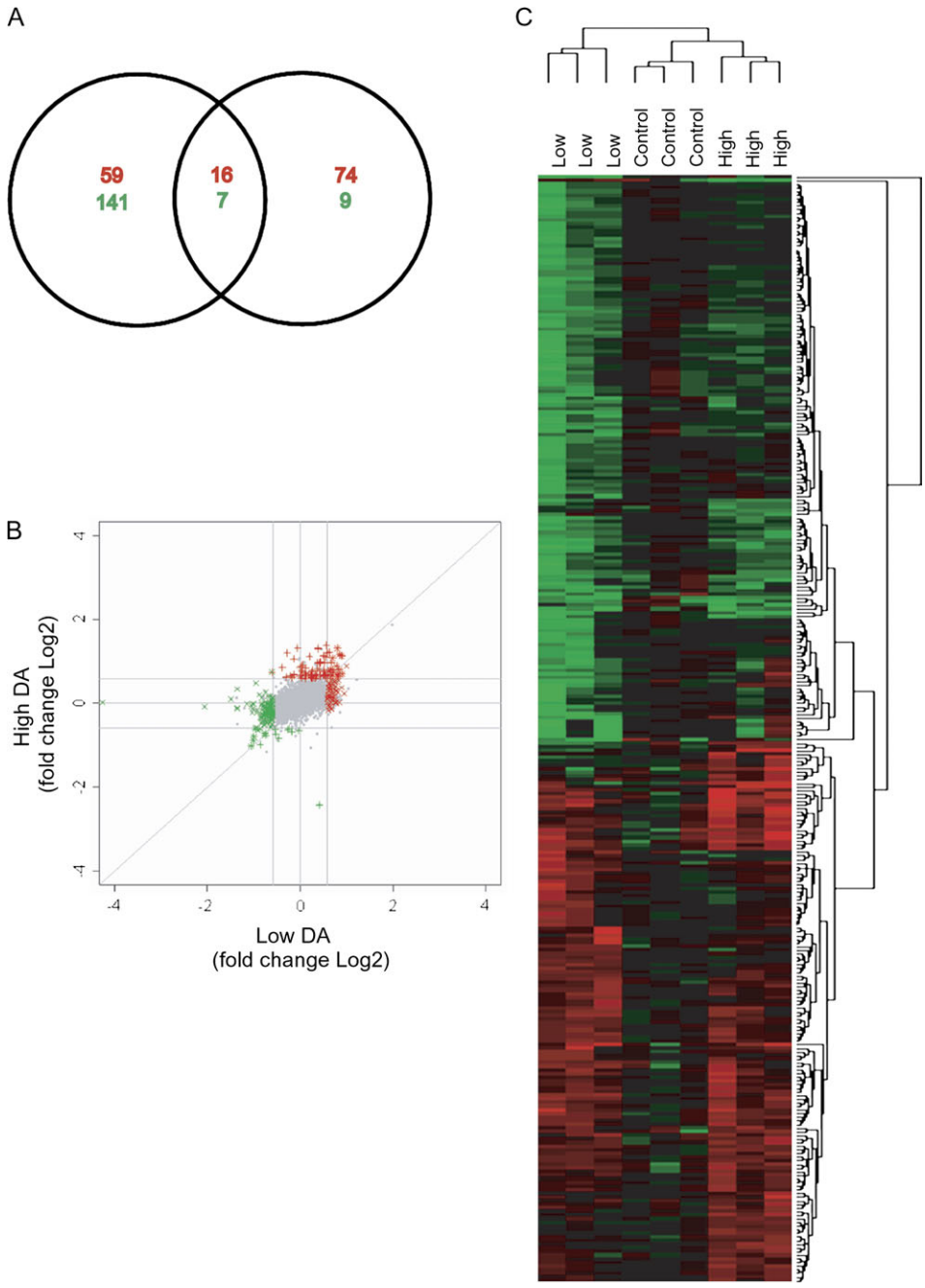

FIG. 3. Differential gene expression in zebrafish brain after IC injection exposure to 0.47 μg/g total body weight (low asymptomatic dose) and 1.2 μg/g total body weight (high symptomatic dose) DA. Each biological replicate ($n = 3$ per treatment) is a pool of four to five individuals. (A) Venn diagram showing number of genes significantly expressed ≥1.5-fold up or down ($p < 0.05$) in each treatment, with the numbers shown in red indicating upregulated and green downregulated. (B) Comparison of all the genes on the array between the high- and low-dose treatments (values are fold change [$\log_2$] compared to control animals). Genes significantly regulated up or down (≥1.5-fold; $p < 0.05$) are indicated in red or green, respectively, and correspond to the colored numbers in (A). (C) Bidirectional hierarchical clustering by Pearson correlation of genes significantly expressed ≥1.5-fold up or down ($p < 0.05$) in at least one treatment group. Red color, upregulation; green color, downregulation; and black, unchanged expression.

the extent of transcriptional change (measured by fold change from control).

Although only 7.5% of the differentially regulated genes were common to both treatments, the composition of functional groups was similar between low- and high-dose-treated fish (Fig. 4). Functional groups for the genes of interest (those 306 genes differentially regulated at least 1.5-fold, $p \leq 0.05$) were identified based on known zebrafish data or based on putative homology using the Gene Ontology (www.geneontology.org) and OMIM (www.ncbi.nlm.nih.gov/omim/) databases.

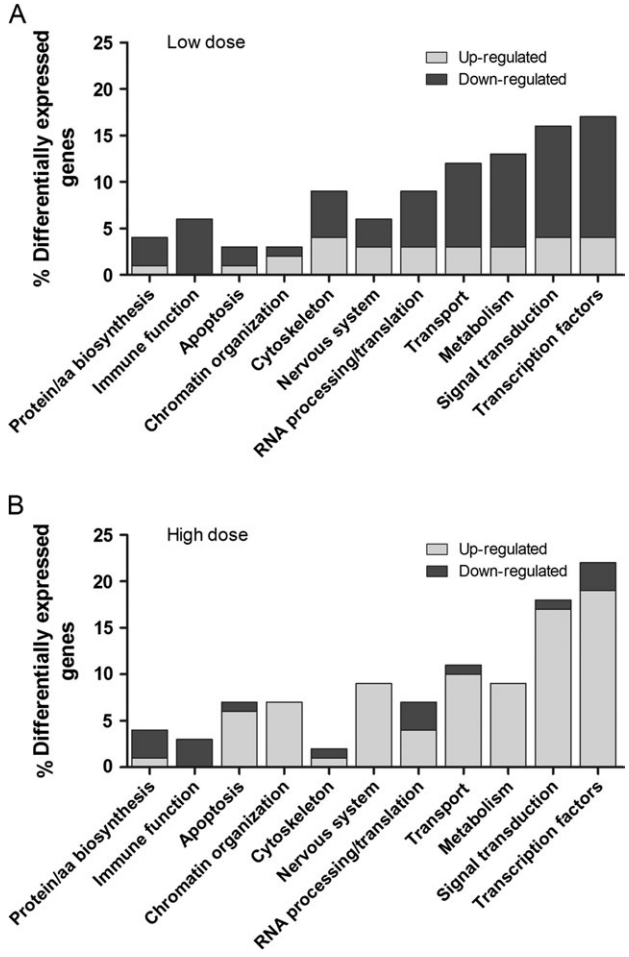

FIG. 4. Functional categorization of genes differentially expressed ≥1.5-fold up or down ($p < 0.05$) in zebrafish brain after exposure to (A) 0.47 μg/g total body weight (low asymptomatic dose) and (B) 1.2 μg/g total body weight (high symptomatic dose) IC injections of DA. Values are calculated as the percent of differentially expressed genes out of the total number of significant genes in the low or high dose (143 and 70 genes, respectively) with the exclusion of those having unknown function. In total, 37% and 35% of significantly differentially expressed genes were of unknown function in the low- and high-dose treatments, respectively.

Functional data were available for approximately 63 and 65% of the genes that were differentially regulated in low and high treatments, respectively (Table 1 and Supplementary Table 2). Functionally identified genes fell into one of the following 11 categories: transcription factors, nervous system function, ion transport, signal transduction, immune function, apoptosis, RNA processing, metabolism, cytoskeleton, protein/amino acid biosynthesis, and chromatin organization. The highest percentages of differentially expressed genes in both low- and high-dose treatment fish belonged to the transcription factors and signal transduction functional groups (Fig. 4). Further analysis of gene expression patterns in each functional group revealed a distinct pattern of upregulation in high-dose treatment fish and downregulation in low-dose treatment fish (Fig. 4). In eight of the 11 functional groups, high-dose treatment fish had a higher percentage of upregulated genes, while the opposite trend was observed in low-dose treatment fish in which 10 of 11 functional groups had higher percentages of downregulated genes (Figs. 4A and 4B, respectively). These data suggest that there are differences in the impacts of symptomatic and asymptomatic DA toxicity. In addition, DA is a glutamate receptor agonist and, therefore, its effect on expression on glutamate related genes is of interest. While this study focused on genes whose expression changed with DA exposure, it is also informative to know which glutamate-related genes did not change. Supplementary Table 3 shows the expression of all genes on the array which had glutamate in the annotation regardless or whether they were differentially expressed or not. As observed in the supplementary table, approximately 8% of the glutamate pathway associated genes (2/26) were significantly altered by DA exposures, with several additional glutamate pathway associated genes approaching statistical significance.

Microarray Confirmation by Real-Time qRT-PCR

The expression of select genes differentially increased or decreased compared to controls in the microarray analysis, including *histH1*, *scn8a*, *dusp2*, *nolc1*, *stk3*, *cathB*, *nptx1*, and *ndrg4*, was confirmed by qRT-PCR (Fig. 5). The same RNA isolations were used for each technique; however, RNA was pooled and amplified for hybridization to the array ($n = 3$ per treatment, each a pool of four to five individuals), and individual brains were used for real-time qRT-PCR analysis ($n = 13$–15 per treatment). The mean expression ratios for each technique were compared for all replicates in a treatment. Overall, we were able to confirm gene expression profiles measured by microarray analysis using real-time qRT-PCR, which indicates that our strict criteria for determining differential gene regulation by array resulted in detection of meaningful changes that could be validated by other methods. The only discrepancy was expression of *scn8a*, which did not show a significant increase in expression by real-time qRT-PCR compared to the array data and may be due to differences in source RNA for the two techniques: individual samples for real-time qRT-PCR versus amplified and/or pooled samples for array analysis.

DISCUSSION

We have investigated the effects of DA exposure on global gene expression in the vertebrate central nervous system, with an emphasis on a phenotypic anchor of gene expression to neurobehavioral injury. In this regard, previous studies have characterized the neurobehavioral and histopathological effects of DA exposure in vertebrates (Dakshinamurti *et al.*, 1993; Gulland *et al.*, 2002; Lefebvre *et al.*, 2001; Silvagni *et al.*, 2005; Strain and Tasker, 1991; Sutherland *et al.*, 1990; Tryphonas *et al.*, 1990a), as well as documented gene expression changes associated with symptomatic exposure (Ananth *et al.*, 2001; Peng *et al.*, 1994;

Ryan *et al*., 2005). However, the impacts of DA exposure on gene expression at levels below those known to induce outward signs of neurobehavioral excitotoxicity have not been well characterized and the question remains as to whether or not low-level DA exposure impacts the vertebrate central nervous system. In the present study, we found that DA exposure resulted in brain gene expression that markedly differed with regards to up- or downregulation between high and low DA treatments. These data confirm that acute DA exposure does impact gene expression at both symptomatic and asymptomatic exposure concentrations and reveal a biphasic molecular response to DA. In addition, close examination of several DA regulated genes in zebrafish revealed that many were common to those previously reported for similarly exposed mammalian model species and provide evidence that mechanisms of DA-induced injury are similar across vertebrate species.

As reported, approximately 50% of the DA-regulated genes comprising both exposure groups and with known function fell into functional groups involved in signal transduction or transcriptional regulation. In particular, genes involved in transcriptional regulation made up the largest functional group of DA-regulated genes. This is not surprising as transcription factors are typically the earliest responders to chemical exposures. DA is a glutamate receptor agonist that executes excitotoxicity via cooperative interactions between NMDA, AMPA, and kainate glutamate receptor subtypes (Berman and Murray, 1997; Berman *et al*., 2002). This is consistent with previously reported findings in acute toxicity studies with KA, another excitotoxic glutamate agonist (Hampson and Manalo, 1998). As discussed, the Affymetrix zebrafish array platform used in the present study included 26 probe sets with the term "glutamate" occurring somewhere in the annotation. Of these 26 probe sets, however, only two (folylpolyglutamate synthase, glutamate receptor, ionotropic, N-methyl D-aspartate 1) were significantly altered at $p < 0.05$ (Supplementary Table 2). These data suggest that zebrafish glutamate pathways may not have been strongly impacted at the transcriptional level at the doses and time point used in the present study. However, it must be pointed out that little is known about transcriptional regulation of glutamate pathway associated genes by toxicants in zebrafish. In contrast, a large percentage of the genes regulated by KA in rat brain at 24-h postexposure were involved in overall transcriptional regulation (Hunsberger *et al*., 2005). Additionally, Ryan *et al*. (2005) reported that transcription factors were the first group of genes to respond to acute DA exposure in mice. We observed that transcription factors such as c-Fos and c-Jun were upregulated in zebrafish at 6-h postexposure, which is consistent with other studies using mammalian models (Peng and Ramsdell, 1996; Ryan *et al*., 2005). Additionally, increased c-Fos protein expression has also been observed with acute DA exposure in killifish at 1-h postexposure (Salierno *et al*., 2006) suggesting a critical role for these genes in regulating the expression of genes that protect against DA injury. In this regard, both c-Fos and c-Jun have been shown to be neuroprotective and to regulate neuronal excitability in the central nervous system of fish and mammals (Behrens *et al*., 1999; Jin *et al*., 2002; Salierno *et al*., 2006; Zhang *et al*., 2002).

In the present study, the CCAAT/enhancer binding protein (C/EBP) isoform d was upregulated in acutely exposed fish (Table 1). C/EBPs are a family of transcription factors that modulate many functions including cellular proliferation and differentiation, metabolism, and inflammation (Ramji and Foka, 2002). The gene product of another protein, COX-2, is a key enzyme involved in the synthesis of prostaglandins which are involved in inflammatory responses (Caivano *et al*., 2001). Previous studies have shown that COX-2 induction requires both C/EBP b and d in a biphasic manner with the C/EBP b isoform involved in the initial phase and the C/EBP d isoform essential to effect the second phase of COX-2 gene transcription (Caivano *et al*., 2001). In a time course study with acute symptomatic DA exposure conducted in mice, both b and d isoforms were similarly upregulated at 1 and 4 h (Ryan *et al*., 2005). However, in the mouse study, C/EBP b upregulation was highest at 1 h while C/EBP d upregulation was highest at 4 h (Ryan *et al*., 2005). Our observation of C/EBP d upregulation at 6 h is consistent with these findings and suggests that the second phase of COX-2 induction may have occurred. A gene profiling study with acute KA exposure also demonstrated upregulation of C/EBP isoforms at 24 h (Hunsberger *et al*., 2005). In addition to inflammatory response, both b and d isoforms of C/EBP have been shown to be involved in long-term memory formation in rats (Taubenfeld *et al*., 2001). Altered regulation of these transcription factors may impact memory formation. Impaired memory function is a hallmark of DA-induced neurobehavioral excitotoxicity (Clayton *et al*., 1999; Sutherland *et al*., 1990). It has also been shown that C/EBP b and d isoforms are induced via membrane depolarization, likely through Ca^{2+} regulation of CaM kinases (Taubenfeld *et al*., 2001), also a feature of DA excitotoxicity (Berman *et al*., 2002). Similarly, genes such as c-Fos, c-Jun, and C/EBP are highly conserved between vertebrate species (Kindy and Verma, 1990; Lyons *et al*., 2001) and the consistency of DA-induced regulation with acute exposure further demonstrates the usefulness of zebrafish as a suitable vertebrate model for addressing mechanisms of DA toxicity.

Given the excitatory nature of DA, it is also not surprising that genes involved in brain signal transduction processes were impacted by acute exposure. DA-regulated genes involved in signal transduction included a number of genes encoding for kinase and phosphatase enzymes. Others have shown that altered intracellular signal transduction mediated by glutamate receptors is likely an adaptive cellular protective mechanism against DA-induced neurotoxicity (Qiu and Curras-Collazo, 2006). The fact that DA stimulates a rapid and concentration-dependent increase in Ca^{2+}, which is mediated through the activation of NMDA receptors and correlated with neurotoxicity, underscores the role of signal transduction in these processes. Overexcitation of glutamate receptors results in

TABLE 1
Select Genes Differentially Regulated in Zebrafish Brain by DA in Four Representative Functional Groups (Apoptosis, Nervous System, Signal Transduction, and Transcription Factors)

Array ID[b]	Gene name[c]	Cluster	Average fold change (log_2)[a]			
			Low DA (0.46 μg/g)		High DA (1.16 μg/g)	
			Log_2	*p* value	Log_2	*p* value
Apoptosis						
Dr.7375.1.A1_at	EF-hand domain-containing protein 1 (*efhc1*)	1	**−0.764**	*0.001*	−0.194	*0.250*
Dr.2509.1.A1_a_at	Programmed cell death 6 interacting protein	1	**−0.713**	*0.036*	−0.299	*0.329*
Dr.4971.1.A1_at	Death-associated transcription factor 1-like	1	**−0.588**	*0.021*	−0.260	*0.249*
Dr.3374.1.S1_at	Cathepsin B	1	0.415	*0.703*	**−2.419**	*0.047*
Dr.22685.1.A1_at	Dual-specificity protein phosphatase (*dusp2*)	4	−0.133	*0.733*	**1.058**	*0.020*
Dr.1378.1.S1_at	Growth arrest and DNA-damage−inducible, beta (*gadd45b*)	6	0.026	*0.801*	**0.598**	*0.000*
Dr.11978.1.A1_at	Hypoxia-inducible factor 1, alpha subunit, like (*hif1a*)	6	0.269	*0.056*	**0.759**	*0.000*
Dr.12775.1.A1_at	BCL2/adenovirus E1b 19 kDa interacting protein 2 (*bnip2*)	6	**1.004**	*0.031*	**0.886**	*0.050*
Dr.12550.1.S1_at	Apoptotic chromatin condensation inducer 1b	5	**0.667**	*0.003*	0.450	*0.026*
Nervous system (synaptic transmission, neurotransmission, neurogenesis)						
Dr.11104.1.A1_at	NDRG family member 4 (*Homo sapiens*)	1	**−4.253**	*0.003*	0.018	*0.987*
Dr.24241.1.S1_at	Midkine-related growth factor b; neurite growth promoting factor 2	3	**−0.878**	*0.025*	0.209	*0.540*
Dr.8012.1.A1_at	Neuronal pentraxin I precursor	1	**−0.857**	*0.000*	−0.330	*0.053*
Dr.12845.1.S1_at	GABA(A) receptor-associated protein	3	**−0.733**	*0.023*	−0.270	*0.341*
Dr.16968.1.A1_at	Synaptotagmin X	1	**−0.618**	*0.022*	−0.204	*0.387*
Dr.9125.1.S1_at	Synaptotagmin XI (*syt11*)	6	0.224	*0.103*	**0.654**	*0.000*
Dr.19866.1.S1_at	Protocadherin 2 alpha (*pcdha2*)	6	0.328	*0.123*	**0.595**	*0.013*
Dr.9155.1.S1_at	N-ethylmaleimide-sensitive factor (*nsf*)	6	0.432	*0.041*	**0.961**	*0.000*
Dr.26373.1.S1_at	Chromogranin A (*chga*)	6	0.541	*0.075*	**0.660**	*0.036*
Dr.16770.1.A1_at	Similar to sodium- and chloride-dependent creatine transporter 1 (*ct1*)	6	0.558	*0.001*	**0.609**	*0.000*
Dr.9889.1.A1_at	Similar to immunoglobulin superfamily member 4	6	0.561	*0.017*	**0.656**	*0.008*
Dr.7509.1.A1_at	Similar to ectodermal-neural cortex 1 (*Danio rerio*)	5	**0.631**	*0.010*	0.333	*0.125*
Dr.37.1.S1_a_at	POU domain gene 12	5	**0.663**	*0.030*	−0.128	*0.633*
Dr.11290.1.A1_at	Midnolin	5	**0.668**	*0.005*	0.494	*0.025*
Dr.12616.1.S1_at	Ephrin B1	5	**0.766**	*0.032*	−0.009	*0.976*
Signal transduction (cell proliferation, cell cycle, signaling)						
Dr.24253.1.S1_at	Serine/threonine kinase 3 (STE20 homolog, yeast)	2	**−1.005**	*0.007*	**−0.883**	*0.013*
Dr.22971.1.A1_at	Probable serine/threonine protein kinase SNF1LK	3	**−1.001**	*0.007*	−0.010	*0.972*
Dr.5678.1.S1_at	Caveolin 1	3	**−0.828**	*0.046*	0.136	*0.715*
Dr.17560.1.S1_at	Transmembrane protein 9	1	**−0.824**	*0.001*	−0.348	*0.074*
Dr.2009.1.A1_at	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase, gamma	3	**−0.796**	0.007	−0.086	*0.719*
Dr.16985.1.A1_at	Similar to protein kinase C-like 2 (*prkcl2*) (*Gallus gallus*)	1	**−0.735**	*0.019*	−0.211	*0.435*
Dr.14419.1.S1_at	Guanine nucleotide-binding protein	1	**−0.722**	*0.003*	−0.431	*0.038*
Dr.3811.1.A1_at	Similar to pKU-beta protein kinase (*tlk1*)	1	**−0.715**	*0.038*	−0.195	*0.525*
Dr.3333.1.A1_at	Pleckstrin homology domain containing, family G4 (with RhoGef domain)	1	**−0.712**	*0.001*	−0.168	*0.279*
Dr.25734.1.A1_at	PTPL1-associated RhoGAP 1	1	**−0.706**	*0.002*	−0.137	*0.414*
Dr.26344.2.S1_a_at	Cell division cycle 42 (Rho GTPAse)	1	**−0.640**	*0.002*	−0.152	*0.326*
Dr.3827.1.A1_at	Similar to membrane protein mKirre	1	**−0.612**	*0.029*	−0.336	*0.190*
Dr.5298.1.A1_at	Ras-related protein Rab, GTP-binding protein	1	**−0.609**	*0.003*	−0.176	*0.268*
Dr.16778.1.A1_at	Regulator of G-protein signaling 9 (*Mus musculus*)	1	**−0.601**	*0.002*	−0.143	*0.327*
Dr.11202.1.A1_at	Calcium and integrin-binding family member 2	3	**−0.596**	*0.019*	−0.235	*0.293*
Dr.4260.1.A1_at	Similar to cytoplasmic protein NCK2 (NCK adaptor protein 2) (*G. gallus*)	3	**−0.586**	*0.015*	0.062	*0.760*
Dr.8587.1.A2_at	Insulin-like growth factor binding protein 1	4	−0.279	*0.437*	**1.198**	*0.007*
Dr.8283.1.S1_at	Muscle-specific beta 1 integrin binding protein similar to Mibp protein	4	−0.003	*0.990*	**0.857**	*0.007*
Dr.20083.1.A1_at	Cyclin G2 (*ccng2*)	6	0.037	*0.763*	**0.793**	*0.000*
Dr.17570.1.S2_at	MAP kinase–interacting serine/threonine kinase 2 (*mknk2*)	6	0.120	*0.407*	**0.828**	*0.000*

TABLE 1—*Continued*

Array ID[b]	Gene name[c]	Cluster	Average fold change (log_2)[a]			
			Low DA (0.46 μg/g)		High DA (1.16 μg/g)	
			Log_2	*p* value	Log_2	*p* value
Dr.58.1.A1_at	PI3K, regulatory subunit, polypeptide 3 (p55, gamma) isoform 4 (*D. rerio*)	6	0.333	*0.122*	**0.691**	*0.006*
Dr.7908.1.S1_at	Calmodulin 1a	6	0.348	*0.155*	**0.634**	*0.020*
Dr.8587.1.A1_at	Insulin-like growth factor binding protein 1	4	0.386	*0.307*	**1.294**	*0.005*
Dr.275.1.S1_at	Janus kinase 1 (*jak1*)	6	0.415	*0.008*	**0.783**	*0.000*
Dr.20346.1.S1_at	Similar to serine/threonine kinase 35 isoform 3 (*D. rerio*)	6	0.434	*0.057*	**0.672**	*0.008*
Dr.11348.1.S1_at	Similar to retinoblastoma-binding protein 6 (*rbbp6*) isoform 2	6	**0.688**	*0.012*	**0.670**	*0.014*
Dr.15883.1.S1_at	Protein phosphatase 2, regulatory subunit B (*ppp2r*)	6	**0.814**	*0.002*	**0.670**	*0.006*
Dr.24198.1.S1_at	Similar to protein phosphatase 1D magnesium-dependent, delta (*D. rerio*)	5	**0.602**	*0.000*	0.205	*0.041*
Dr.12854.1.A1_at	Similar to receptor protein tyrosine phosphatase LAR	5	**0.620**	*0.002*	0.473	*0.010*
Dr.11348.1.S1_x_at	Similar to retinoblastoma-binding protein 6 isoform 2 (*rbbp6*)	6	**0.664**	*0.013*	0.392	*0.102*
Transcription factors						
Dr.26166.1.A1_at	Similar to Sp3 transcription factor	3	**−1.489**	*0.001*	0.098	*0.749*
Dr.25185.2.S1_x_at	Similar to MAX dimerization protein 1	3	**−1.045**	*0.028*	−0.069	*0.867*
Dr.12836.2.A1_at	Inhibitor of DNA binding 2, dominant negative helix-loop-helix, b (*id2*)	3	**−1.024**	*0.029*	−0.018	*0.964*
Dr.12986.1.A1_a_at	*Danio rerio* c-fos mRNA	3	**−0.954**	*0.005*	0.476	*0.102*
Dr.15311.1.A1_at	Early growth response 1 (*egr1*) (*G. gallus*)	2	**−0.743**	*0.001*	0.269	*0.106*
Dr.13371.1.S1_at	Hypoxia-inducible factor 1, alpha subunit inhibitor	2	**−0.661**	*0.005*	0.020	*0.914*
Dr.17619.1.A1_at	cAMP-responsive element modulator	3	**−0.654**	*0.006*	0.169	*0.381*
Dr.9512.1.A1_at	Influenza virus NS1A binding protein a	1	**−0.611**	*0.001*	−0.234	*0.079*
Dr.15759.1.A1_a_at	Transcription initiation factor IID beta chain	1	**−0.609**	*0.003*	−0.277	*0.105*
Dr.93.1.A1_a_at	Zinc finger protein 207, a	1	**−0.608**	*0.002*	−0.515	*0.006*
Dr.25102.1.A1_at	Transducer of ERBB2, 1b	2	**−0.595**	*0.002*	0.121	*0.391*
Dr.431.1.S1_at	Hairy-related 6	1	**−0.592**	*0.002*	−0.288	*0.069*
Dr.7714.1.S1_at	Ets-related protein (*erm*)	1	**−0.751**	*0.004*	**−0.507**	*0.027*
Dr.5572.1.S1_at	Homeobox B5a (*hoxb5a*)	2	**−0.750**	*0.042*	**−0.761**	*0.040*
Dr.3920.1.S1_at	Oligodendrocyte lineage transcription factor 2 (*olig2*)	2	**−0.531**	*0.023*	**−0.613**	*0.012*
Dr.12986.1.A1_at	*Danio rerio* c-fos mRNA	4	−0.299	*0.203*	**0.621**	*0.019*
Dr.7710.1.A1_at	C-ETS-2 protein	4	−0.210	*0.429*	**0.660**	*0.028*
Dr.1280.1.A1_at	CCAAT/C/EBP, delta	4	−0.053	*0.883*	**1.317**	*0.004*
Dr.9438.1.A1_at	Similar to CBP/p300-interacting transactivator	6	0.010	*0.954*	**0.666**	*0.003*
Dr.14282.1.S1_at	Activating transcription factor 3 (*atf3*)	6	0.031	*0.781*	**0.893**	*0.000*
Dr.7608.1.A1_at	*Danio rerio* v-jun sarcoma virus 17 oncogene homolog (avian); C-Jun	6	0.067	*0.699*	**0.925**	*0.000*
Dr.19928.1.A1_at	Similar to PGC-1–related estrogen receptor alpha coactivator	6	0.103	*0.530*	**0.650**	*0.002*
Dr.8209.1.S2_at	Forkhead box O5	6	0.246	*0.121*	**0.606**	*0.002*
Dr.12410.1.S1_at	Sprouty (*Drosophila*) homolog 4 (*spry4*)	6	0.520	*0.149*	**0.744**	*0.050*
Dr.12671.1.S1_at	Ankyrin repeat domain 12	6	0.549	*0.025*	**0.634**	*0.012*
Dr.5175.1.A1_at	PHD finger protein 2	6	0.579	*0.021*	**0.604**	*0.017*
Dr.20685.1.A1_at	Similar to ankyrin repeat domain 12 (*G. gallus*)	6	**0.642**	*0.022*	**0.790**	*0.008*
Dr.12821.1.A1_at	Forkhead box protein G1B (*foxg1*)	4	**0.674**	*0.123*	**0.971**	*0.036*
Dr.4597.1.S1_at	Max-interacting protein	5	**0.607**	*0.014*	0.208	*0.327*
DrAffx.1.1.S1_at	CCAAT/C/EBP 1	5	**0.625**	*0.048*	0.089	*0.753*
Dr.20146.1.A1_at	Similar to bromodomain, testis specific	5	**0.822**	*0.001*	0.267	*0.145*
Dr.8302.3.S1_a_at	Aryl hydrocarbon receptor nuclear translocator 2	5	**0.841**	*0.016*	0.020	*0.947*

[a]Average fold change values represent background corrected and GCRMA normalized data calculated as signal ratios of treated compared to control animals ($n = 3$). Stringent criteria were used to filter for genes that were regulated at least **≥1.5-fold ($p \leq 0.05$).** Fold change values for genes that passed stringency criteria are in bold.

[b] Zebrafish Affymetrix array ID number.

[c] The most significant BLASTX is shown. If target probe has no significant (*E*-value < 10^{-6}) BLASTX hit, then the most significant BLASTN hit is shown. Genes have been categorized by function based on known zebrafish data or putative homolog using Gene Ontology and OMIM databases.

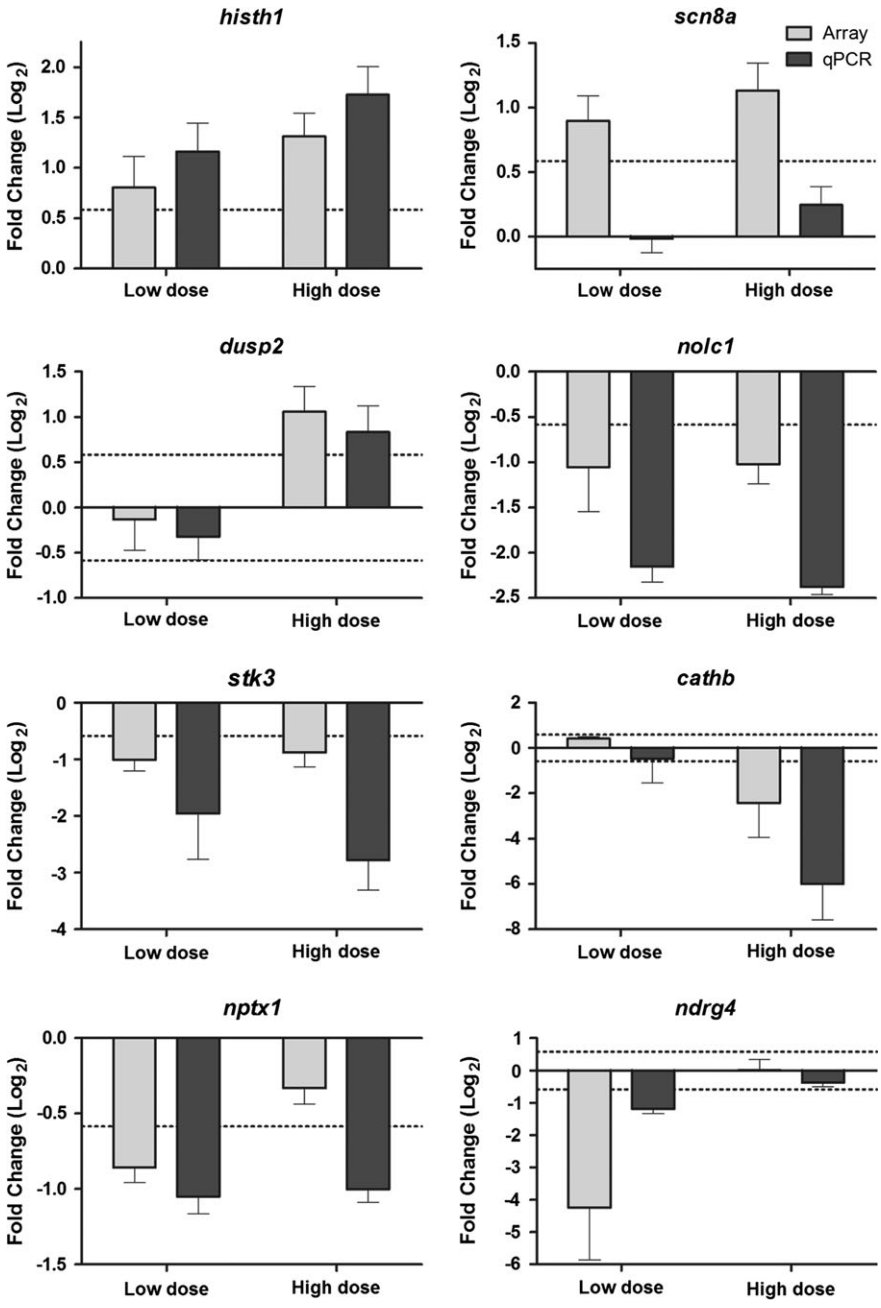

FIG. 5. Comparison of gene expression in zebrafish brain measured by microarray ($n = 3$ biological replicates, each a pool of four or five animals, gray bars) and real-time qRT-PCR ($n = 13$–15 individuals, black bars) after exposure to 0.46 μg/g total body weight (low asymptomatic dose) and 1.2 μg/g total body weight (high symptomatic dose) IC injections of DA. Values are expressed as average fold change ($\log_2$) with standard error compared to control animals as indicated for select genes.

a rapid increase in intracellular Ca^{2+}. Because mitochondria play a major role in cellular Ca^{2+} homeostasis, we hypothesized that expression of mitochondrial-related genes may be impacted by DA exposure. However, mitochondrial-related genes, which were identified by GO classification, were not differentially regulated in this data set by either treatment. In contrast, rapid changes in intracellular Ca^{2+} levels also act as a direct trigger for apoptosis (Green and Reed, 1998), and a number of apoptosis-related genes were differentially expressed in DA exposed fish. A closer examination of the apoptosis gene category revealed that genes with known function in the regulation of apoptosis were more markedly upregulated in the high dose of DA relative to the lower dose, suggesting an overwhelming of cellular adaptive responses and the onset of apoptosis associated with high-dose DA-induced neurobehavioral injury. The fact that only one apoptosis-related gene, *bnip2*, was shared between both low and high treatments further suggests differential effects of the low and high doses

on the modulation of apoptosis. *bnip2* encodes for a Bcl-2 binding protein involved in apoptosis, and both Bcl-2- and Binp2-coded proteins have been shown to protect cells from apoptosis induced by viral infection or other proapoptotic stimuli (Boyd *et al*., 1994). In the present study, expression of *bnip2* was shown to increase at 6-h postexposure in both high and low treatments. Bcl2 has also been shown to increase at 4 and 16 h after acute exposure in rats (Ananth *et al*., 2001). These similarities highlight the importance of monitoring apoptosis-regulating genes in DA-induced neurotoxicity.

In general, very few genes (7.5%) were shared between high- and low-dose treatments, and the general direction of altered gene regulation was different between the two doses relative to the control nonexposed animals. This is particularly interesting in light of the fact that the low- and high-dose DA exposures generally affected similar pathways (i.e., the differentially expressed genes in both doses spanned the same functional groups). In addition to a potential biphasic response on the modulation of apoptosis, another explanation for this phenomenon may be one associated with the relative degree of oxidative stress occurring at the different DA doses. It has been shown that oxidative stress is a mechanism of DA injury in rat cortex (Bondy and Lee, 1993) that is potentially related to glutamate-gated cation channel activation (Coyle and Puttfarcken, 1993). We have previously shown that oxidative stress by structurally dissimilar chemical pro-oxidants can elicit a general loss of hepatic steady-state mRNA expression in rats (Gallagher *et al*., 1995). Such observations are consistent with the overall downregulation of genes in the low DA–dosed fish. In contrast, higher levels of cellular oxidative stress may overwhelm protective pathways leading to the induction of apoptosis. Although an examination of two GO classifications, including ''oxygen and reactive oxygen species metabolism'' and ''response to oxidative stress'', did not reveal an overrepresentation in our DA zebrafish data set, little is known about the regulation of zebrafish genes that protect against oxidative stress relative to mammals. We were not able to rule out or demonstrate the effects of DA on brain oxidative injury in the present study, but future studies could shed light on the role of oxidative stress in the observed biphasic response to DA injury. Furthermore, the fact that 30% of the animals dosed at the EC_{50} exhibited normal behavior is intriguing and may potentially reflect pharmacological issues associated with dosing or interindividual differences in resistance to DA. Unfortunately, we could not correlate the observed behaviors to PCR values or microarray gene lists in individual animals. Future studies using larger numbers of arrays and individuals, as well as of DA uptake, may shed light on the nature of these observations.

One of the secondary goals of our study was to determine if we could identify molecular markers of asymptomatic DA exposure. One gene in particular, the nervous system related gene *ndrg4* was markedly downregulated in the low-dose DA-modulated gene set (and confirmed by real-time qRT-PCR), but not differentially expressed in the high-dose gene set. *ndrg4* is one of four members of the N-myc downstream-regulated gene (NDRG) family (Okuda *et al*., 2008). The specific functions of these genes remain obscure, but NDRG protein is involved in stress response, cell growth, and differentiation (Okuda *et al*., 2008), as well as aiding in neurite outgrowth (Ohki *et al*., 2002). In humans, NDRG1–3 are ubiquitously expressed, while *NDRG4* is specifically expressed in the heart and brain and therefore likely plays an important role in these organs (Zhou *et al*., 2001). This is intriguing because to date the heart and brain are the only organs that have been reported to have lesions in association with DA exposure (Silvagni *et al*., 2005; Strain and Tasker, 1991). Additionally, *NDRG4* expression has been shown to be dramatically decreased in Alzheimer's diseased brain compared with normal brain in human studies (Zhou *et al*., 2001). This is also interesting due to the similarities between Alzheimer's disease and the DA-induced neurotoxic illness ASP, as both conditions result in severe memory impairment and neuronal degeneration in the hippocampus (Ball, 1977; Dakshinamurti *et al*., 1993; Horn *et al*., 1996; Sutherland *et al*., 1990). Further studies are needed to determine whether the observed decrease in *NDRG4* expression is a cause or result of neuronal degeneration in Alzheimer's disease (Zhou *et al*., 2001) and whether it plays a similar role in low-level DA toxicity.

In conclusion, global gene expression profiles associated with acute DA exposures in zebrafish brain have provided several important findings with regards to human risk linked to DA-associated toxicity. For example, our data indicate that the regulation of genes involved in a number of biochemical pathways is impacted at DA doses below those that cause observable signs of behavioral injury. Of relevance is the downregulation of a host of genes involved in important subcellular processes, such as immune function, RNA processing, ion transport, metabolism, and signal transduction, after low-level DA exposures that did not elicit observable neurobehavioral injury, thus indicating potential neurological risk associated with asymptomatic exposures. One specific gene, the zebrafish homolog of human *NDRG4*, was markedly downregulated in the zebrafish model, has been associated with memory loss, and is under further investigation in other mammalian species as biomarkers of memory-related disease. Collectively, our data validate the zebrafish model for use in studies of DA neurotoxicity and indicate the relevance of these genes as potential biomarkers of neurodegeneration. Additional studies of DA-associated gene expression with environmentally relevant low-level chronic exposures and linked to cell injury end points will help elucidate the potential impacts of chronic asymptomatic DA exposure on the vertebrate central nervous system.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/. GSE12140 is the GEO series accession number. Supplementary Table 1 provides a list of PCR primer

sequences used for confirmation of microarray experiments. Supplementary Table 2 provides a list of all the differentially expressed genes on the array resulting from either low or high DA exposure. Supplementary Table 3 provides a list of all genes on the array with the term glutamate as a descriptor, whether they were differentially expressed by DA or not.

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