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An embryonic 100 μg/L lead exposure results in sex-specific expression changes in genes associated with the neurological system in female or cancer in male adult zebrafish brains

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ABSTRACT

Developmental lead (Pb) exposure is linked to neurological health issues. Results from non-human primate and rodent studies suggest detrimental effects of an early life Pb exposure, showing transcriptional disturbances and pathological evidence of Alzheimer's disease in the adult animal brain. To elucidate the impacts of an embryonic Pb exposure on the adult brain, transcriptomic analysis was completed on the brain of zebrafish aged 12 months exposed to a control treatment or to an embryonic 100 μg/L Pb exposure by sex. In the adult female zebrafish brain, significant changes in expression profiles occurred in a number of genes involved in neurological disease and nervous system development and function. On the other hand, in adult males, a number of genes with significant expression alterations were found to be associated with cancer and tumors. p38 mitogen-activated protein kinase (p38 MAPK) was also indicated as an upstream regulator of observed gene expression changes. Western blot analysis confirmed activation of p38 MAPK in the form of phosphorylated p38 MAPK in the male zebrafish brain. In addition, we compared transcriptomic changes observed in this study to a previous study with an embryonic exposure of 10 μg/L Pb by sex, showing unique sets of genes dependent on Pb concentration. Overall, these results show sex-specific and concentration-dependent disturbances of global gene expression patterns in the brain of adult zebrafish exposed to Pb during embryogenesis.

1. Introduction

Lead (Pb) containing paint chips, water pipes, and automotive fuel find their way into the environment, contaminating water, soil, and air (reviewed in Tong et al., 2000). Although the application of Pb has been strictly regulated in industries in many countries, environmental contamination still occurs across the globe (Han et al., 2006; Nabulo et al., 2006; Rankin et al., 2005; Takaoka et al., 2006; Turner and Sogo, 2012; Wright et al., 2005). Moreover, as we saw in the most recent event in the city of Flint, Michigan, United States, where drinking water Pb contamination resulted in elevated blood Pb levels in young children, environmental Pb contamination is an ongoing threat to public health (Hanna-Attisha et al., 2016).

Environmental Pb exposure can lead to serious health issues including neurological problems even with exposures at very low concentrations. Health impacts of low levels of Pb exposure have been reported by several studies, especially in children of which the intelligence quotient (IQ), academic capability, and susceptibility to attention deficit hyperactivity disorder (ADHD) were shown to be associated with blood Pb levels of ≤ 10 μg/dL (Braun et al., 2006; Canfield et al., 2003; Surkan et al., 2007). Moreover, an early life exposure to low levels of Pb has been observed to manifest in pathological and molecular characteristics of neurodegenerative disease in later life in non-human primate and rodent studies. In those studies, organisms developmentally treated with low levels of Pb exhibited pathological features related to Alzheimer's disease (AD), a neurodegenerative disease frequently found in older adults, including increased levels of amyloid precursor protein (*APP*), β-amyloid, and pathological deposits that resembles senile plaques formed in their brains (Basha et al., 2005; Bihaqi et al., 2013; Wu et al., 2008). Recent studies have started incorporating the zebrafish model to assess Pb developmental neurotoxicity and the later in life adverse health outcomes from the developmental Pb exposure, reporting behavioral and molecular changes implying neurological interferences (e.g., Bault et al., 2015; Chen et al., 2012; Peterson et al., 2011; Rice et al., 2011; Weber and Ghorai, 2013; Wirbisky et al., 2014; Zhang et al., 2011). Studies from our laboratory have initially focused on providing an understanding of adverse impacts of an embryonic exposure to 100 μg/L Pb on zebrafish at early life stages, observing altered axon density, the gamma-aminobutyric acidergic (GABAergic) system, and gene expression profiles related to

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neurological development, function, and disease (Peterson et al., 2011; Wirbisky et al., 2014; Zhang et al., 2011). Our previous studies also investigated later in life effects from the 100 μg/L embryonic Pb exposure, reporting alterations in color preference but not in expression of known genetic risk factors of AD including *APP* (*appa* and *appb*), apolipoprotein E (*APOE*; *apoea* and *apoeb*), sortilin-related receptor precursor (*SORL1*; *sorl1*), or the presenilins (*psen1* and *psen2*) in adult zebrafish (Bault et al., 2015; Lee et al., 2016b).

Based on the previous findings regarding the Pb-induced adverse health effects, this study aimed to further assess global transcriptional changes associated with an embryonic Pb exposure at different concentrations. We first analyzed zebrafish brains aged 12 months to better understand the influences of sex on latent global gene expression profile changes associated with an embryonic 100 μg/L Pb exposure. We then assessed an upstream regulator of observed expression changes resulting from the embryonic 100 μg/L Pb exposure. In addition, we compared the findings of the present study with the embryonic 100 μg/L Pb exposure to those associated with a 10 μg/L embryonic Pb exposure (Lee and Freeman, 2016) to assess concentration-dependent differences.

2. Materials and methods

2.1. Zebrafish husbandry

The AB zebrafish strain was raised in a *Z*-Mod System (Aquatic Habitats, Apopka, FL) under appropriate laboratory conditions (pH, 7.2; conductivity, 470–520 μS; temperature, 28 °C; light/dark cycle, 14/10). Fish maintenance, feeding, and breeding were conducted following protocols in Westerfield (2007). Maintenance and experimental use of laboratory animals were carried out following established protocols, policies, and guidelines of Purdue Animal Care and Use Committee.

2.2. Embryonic exposure to 100 μg/L Pb

Adult zebrafish were bred to obtain embryos. Embryos were collected at 1 h post fertilization (hpf), pooled, and placed in a Petri dish (i.e., 50 embryos per group considered as subsamples). Embryos were then subjected to a 100 μg/L exposure of Pb-acetate (Sigma Aldrich, St. Louis, MO) or a control treatment (aquaria water only) until 72 hpf (the end of embryogenesis). Pb concentration of the treatment water solution was verified as previously described (Zhang et al., 2011). The experimental concentration and period of the developmental Pb exposure were determined according to our previous observations of global gene expression analysis on 72 hpf zebrafish (Peterson et al., 2011) and other previous studies (Wirbisky et al., 2014; Zhang et al., 2011), in which a developmental exposure to 100 μg/L Pb significantly changed expression of genes associated with nervous system development and function, and disease, decreased axonal density, and also disturbed the GABA-ergic system of zebrafish larvae. This exposure results in a whole body dose of 1139 ng/g Pb (Wirbisky et al., 2014). The embryonic 100 μg/L Pb exposure was stopped by transferring embryos to clean aquaria water at 72 hpf. Fish were then raised without chemical exposure until the age of 12 months for sex-specific experiments with brain tissue. This exposure regimen was repeated with embryos from multiple clutches over different weeks to attain adult fish from multiple biological replicates.

2.3. Brain RNA isolation and cDNA preparation

Adult zebrafish aged 12 months were anesthetized in MS-222. Brain tissue obtained from each subject was homogenized in Trizol Reagent (Life Technologies, Carlsbad, CA) to isolate RNA using the methods in Peterson and Freeman (2009). The RNA samples were purified with a RNeasy Mini Kit (Qiagen, Valencia, CA) as suggested by the manufacturer and then tested on a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) to check RNA quality and quantity. To convert RNA samples to cDNA, the protocols in Peterson and Freeman (2009) were applied using SuperScript® First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA). Synthesized cDNA samples were used for a sex-specific microarray analysis and quantitative polymerase chain reaction (qPCR).

2.4. Sex-specific transcriptomic analysis of the adult zebrafish brain exposed to 100 μg/L Pb during embryogenesis

Microarray analysis compared expression profiles of brain tissue of adult zebrafish at 12 months of age in the control treatment and those exposed to 100 μg/L Pb during embryogenesis by sex (N = 3) using a zebrafish-specific 12x135K expression platform (Roche NimbleGen, Madison, WI). This platform contains 135,000 60-mer oligonucleotide probes covering 37,157 targets (i.e., 3–5 probes per target). All experimental procedures were performed following MIAME guidelines with the one-color hybridization strategy (Brazma et al., 2001).

cDNA was labeled using the NimbleGen One-Color DNA Labeling Kit, following the manufacturer's instructions (Roche NimbleGen, Madison, WI). Quality (yield and specificity) of Cy3 labeled cDNA was measured on a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Cy3-labeled cDNA samples were mixed with hybridization mix containing 2× Hybridization Buffer, Hybridization Component A, and Alignment Oligo using NimbleGen Hybridization Kit, according to manufacturer recommendations (Roche NimbleGen, Madison, WI). The samples were incubated at 95 °C on a heat block for 5 min for denaturation. Samples were placed on arrays and hybridized at 42 °C overnight. Following the hybridization, array slides were washed using Wash Buffer I, II, and III in the NimbleGen Wash Buffer Kit (Roche NimbleGen, Madison, WI), and then dried briefly. Array slides were scanned on a SureScan Agilent microarray scanner (G2600D) with one-color scan setting (scan region, 61 × 21.6 mm; scan resolution, 2 μm; tiff file dynamic range, 20 bit; dye channel, green; green PMT gain, 100%). Array images were extracted with NimbleGen DEVA software (Roche NimbleGen, Madison, WI) in which fluorescence signal intensity values were normalized using quantile normalization and gene calls were generated using the Robust Multichip Average (RMA) algorithm. Then array data were analyzed on Array Star (DNASTAR, Inc., Madison, WI) to detect statistical differences between control and Pb exposed groups. A list of genes in each sex were generated by creating gene calls with significant expression alterations (i.e., ≥ 0.585 of a mean absolute $\log_2$ expression ratio) among consistently expressed genes by sex (Student *T*-test, $p < 0.05$) as shown in Peterson et al. (2011). The listed genes were matched to human gene symbols of their respective human homologs and further analyzed on QIAGEN's Ingenuity® Pathway Analysis (IPA, QIAGEN, Redwood City) to identify sets of genes involved in different diseases and functions and their associated molecular pathways and networks by sex. Functional relationships of molecules and gene-disease interactions were graphically visualized in IPA (QIAGEN, Redwood City). All data generated from the microarray are available in the Gene Expression Omnibus (GEO, GSE78879 and GSE78880). Genes are reported as the human homologs of the genes identified to be altered by microarrays in all subsequent sections.

2.5. Comparative analysis between sexes and Pb concentrations

To investigate impacts of sex differences on global gene expression patterns associated with a developmental 100 μg/L Pb exposure, the microarray data obtained in this study were subjected to data comparison analysis between sexes. The comparison of microarray data from adult female versus adult male zebrafish was performed with IPA (QIAGEN, Redwood City).

In addition, concentration-dependent effects of an embryonic Pb