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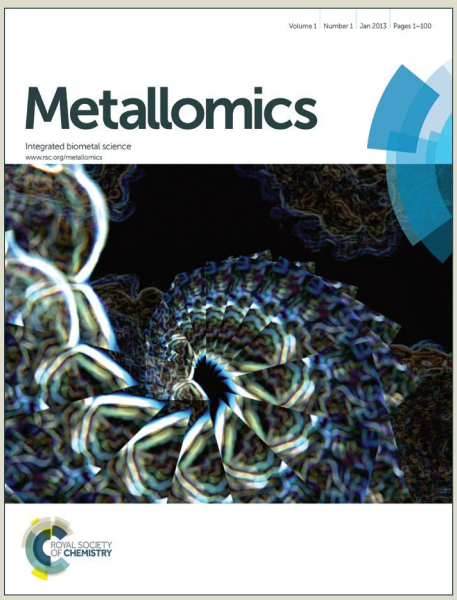

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ARTICLE

Embryonic exposure to 10 µg/L lead results in female-specific expression changes in genes associated with nervous system development and function and Alzheimer's disease in aged adult zebrafish brain

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A developmental lead (Pb) exposure has been proposed as an environmental risk factor for adult neurodegenerative diseases including Alzheimer's disease (AD). Recent animal studies showed pathological characteristics of AD in adults with a developmental Pb exposure, but additional studies are needed to investigate this phenomenon. To further assess the relationship between an embryonic Pb exposure and latent neurological alterations, the brain of adult female and male zebrafish aged 12 months that were exposed to a control treatment or 10 µg/L Pb only during embryogenesis (1- 72 hours after fertilization) were analyzed on a zebrafish-specific microarray platform. Gene ontology and pathway analysis revealed similarities in the top disease and functional categories in both sexes, but females had 4.3 times more genes altered than males. In addition, alterations in genes associated with nervous system development and function were more pronounced with a set of 89 genes associated with AD including amyloid precursor protein (*APP*), apolipoprotein (*APOE*), and sortlin-related receptor precursor (*SORL1*) observed to be changed in adult females. Our observations suggest that an embryonic exposure to Pb at levels as low as 10 µg/L disturb global gene expression patterns in a sex-specific manner that could lead to neurological alterations in later life. With these findings, future studies investigating the adverse neurological outcomes of these changes in gene expression will facilitate our understanding of the impact of an embryonic 10 µg/L Pb exposure on neurological disease pathogenesis and the inclusion of additional concentrations will broaden our knowledge of dose-dependent changes.

Introduction

Environmental contamination by lead (Pb) is a serious ongoing environmental health problem. In recent history Pb was used as an additive for gasoline and paint products which resulted in widespread human exposure. In many countries Pb is now removed from these products (e.g., the United States, Canada, European countries and others); however, remnants of Pb containing products still exist across the globe, contributing to environmental contamination and continual exposure to lower doses of Pb in these countries and higher doses in others.[1-3]

Adverse health consequences of Pb exposure are dose-dependent with detrimental effects of lower doses most severe during development. It is well-known that blood Pb levels lower than 10 µg/dL are related to a decreased intelligence quotient, poor academic performance, and an increased risk for attention deficit hyperactivity disorder in children.[4-6] These studies contributed to and support a recent revision by the Centers for Disease Control and Prevention to lower the reference blood level in children to 5 µg/dL.[7,8] Moreover, developmental Pb exposure can have long term adverse health outcomes which may not be immediately detectable at the time of exposure. For example, a developmental Pb exposure is suggested as an environmental trigger for adult neurodegenerative diseases such as Alzheimer's disease (AD).[9] Laboratory studies using rodent and non-human primate models have provided evidence that early life Pb exposure results in transcriptional and histopathological changes related with AD in aged adult animal brains (e.g., Basha et al., 2005; Wu et al., 2008).[10,11] Organisms undergoing critical steps of development are sensitive to subtle changes in their surroundings[12] and there is still an open question whether a developmental exposure to lower doses of Pb are responsible for a range of negative effects on one's long term neurological health such as an environmental risk factor for neurodegenerative diseases such as AD.

Given our current understanding of the long term health effects of early life Pb exposure, we hypothesized that an exposure to 10 µg/L (ppb) Pb during a critical window of development would result in expression changes in genes associated with neurodegenerative diseases in brains of aged adult animals. To test this hypothesis, we applied the zebrafish model which has several strengths for *in vivo* experimentation, including small size, rapid growth, a shorter life

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span, a completed genome sequence, and genetic similarity to humans.[13] The zebrafish has been proposed as a promising model for neurodegenerative diseases and studies using zebrafish are successfully demonstrating neurological impacts of developmental Pb exposure.[14,15] In our previous studies, we showed that an embryonic exposure to 100 µg/L Pb resulted in significant expression alterations in a number of genes related with neurological diseases, altered protein expression, and decreased axonal density in exposed zebrafish, but did not result in an increase in apoptotic cells in the brain or alter brain size.[16-18] In an additional study that also included treatments of 10 and 50 µg/L Pb during embryogenesis, no significant changes in head length, head width, or total larval length was observed, but alterations in genes associated with the gamma-aminobutyric acid (GABA)-ergic system and a decrease in GABA occurred at all three treatment concentrations.[19] Moreover, other studies show neurobehavioral changes appearing in adult zebrafish developmentally exposed to Pb, including altered color preference, social behavior, visual response, and cognitive abilities at concentrations ranging from 3 nM to 10 µM.[14,15,20,21] To determine later in life implications of an embryonic exposure from 1-72 hours post fertilization (hpf) to a lower Pb concentration, transcriptomic analysis was completed in the aged adult brain with a comparison between adults exposed to 10 µg/L (0.048 µM) Pb only during embryogenesis or to control conditions. This exposure is previously reported to result in a whole body dose of 295 ng/g Pb at the conclusion of exposure (at 72 hpf).[19] Analysis was performed by sex in order to determine if sex-specific differences on gene expression profiles was observed.

Results & discussion

In this study global gene expression analysis using brains of aged adult zebrafish with or without an exposure to 10 µg/L Pb throughout embryogenesis was completed to determine the implications of this exposure and to investigate if the genetic expression profiles were similar in each sex. This study confirmed that an embryonic Pb exposure at 10 µg/L (0.048 µM, equivalent to 1 µg/dL) was sufficient to cause alterations in transcriptomic profiles in aged adult zebrafish brain (GSE74037 and GSE74038; Supplementary Information Fig 1 and Supplementary Information Tables 1 and 2). Data obtained from the microarray analysis were further validated by quantitative polymerase chain reaction (qPCR) on a subset of genes with altered expression in the microarray analysis (Fig 1; Supplementary Information Table 3). A significant positive correlation was found between data from the two different sets of experiments (i.e., qPCR data and microarray data) (Pearson's correlation coefficient, $R=0.778$; $p < 0.05$).

In aged adult female zebrafish, an embryonic exposure to 10 µg/L Pb resulted in significant expression alterations of 1836 unique genes matched to their annotated human gene homologs and then classified according to their roles in specific diseases and biological functions by IPA (GSE74037; Supplementary Information Table 1).[22] The top physiological system development and functions were associated with organismal survival, organismal development, nervous system development and function, tissue development, and behavior (Table 1). Genes were specifically associated with the development of neurons and proliferation of neuronal cells. The top molecular and cellular functions associated with the altered gene set were cellular growth and proliferation, cell morphology, cellular assembly and organization, cellular function and maintenance, and cell death and survival including genes involved in microtubule dynamics, organization of the cytoplasm and cytoskeleton, and formation of plasma membrane projections and cellular protrusions (Table 2). The top diseases and disorders were associated with cancer, organismal injury and abnormalities, gastrointestinal disease, reproductive system disease, and developmental disorder (Table 3). Genes in this set were associated with malignant solid tumor, tumorigenesis of tissue, neoplasia of epithelial tissue, and abdominal neoplasm.

In the aged brain of adult male zebrafish, an embryonic exposure to 10 µg/L Pb resulted in significant changes in expression of 427 unique genes with mapping information to their human homolog (GSE74038; Supplementary Information Table 2).[23] Those annotated genes were classified according to their roles in specific diseases and biological functions by IPA. The top physiological system and functions were associated with organismal survival, organismal development, cardiovascular system development and function, connective tissue development and function, and skeletal and muscular system development and function including genes involved in morphology and abnormal morphology of bone (Table 4). The top molecular and cellular functions were associated with cellular development, cellular growth and proliferation, gene expression, cellular movement, and cell morphology including genes involved in neuritogenesis and proliferation of tumor cell lines (Table 5). The top diseases and disorders were cancer, organismal injury and abnormalities, gastrointestinal disease, developmental disorder, and skeletal and muscular disorders (Table 6). A number of genes in these groups were associated with malignant solid tumor, tumorigenesis of tissue, neoplasia of epithelial tissue, epithelial cancer, congenital anomaly of musculoskeletal system, and congenital malformation of skeleton.

There were 85 genes identified to be present in both the female and male altered gene expression lists (Supplementary Information Table 4). In this set of 85 molecules, most were involved in various types of cancer (77 out of 85 molecules) including malignant solid tumor, epithelial cancer, and abdominal neoplasm. Although the number of common genes was low, in addition to the associations with cancer there were a number of similar functional categories identified in the IPA analysis with the sex-specific altered gene expression lists (Tables 1-6). Organismal survival and organismal development were both in the top physiological system development and function pathways in both sexes (Tables 1 and 4). Genes in organismal survival were associated with morbidity or mortality, organismal death, perinatal and neonatal death, while genes in organismal development were associated with morphology and abnormal morphology of the body cavity and development of body axis, body trunk, and head in both sexes. There were also similarities in the top molecular and cellular functions altered by the embryonic Pb exposure including cellular growth and proliferation and cell morphology (Tables 2 and 5). Genes in the cellular growth and proliferation pathways were involved in proliferation of cells, tumor cell lines, connective tissue cells, and neuronal cells and in the formation of cells. Genes in the cell morphology pathway were involved in morphology and abnormal

morphology of cells, formation of cellular protrusions, and neuritogenesis. Lastly, the top diseases and disorders in both sexes were almost identical with functions associated with cancer, organismal injury and abnormalities, gastrointestinal disease, and developmental disorder. The exceptions were reproductive system disease only in females and skeletal and muscular disorders in only males (Tables 3 and 6). All categories in cancer and organismal injury and abnormalities, and gastrointestinal disease were identical, excluding abdominal neoplasm in the females and epithelial cancer in the males. In addition in developmental disorder, growth failure and dysgenesis was included for the females, while males included multiple congenital anomalies and hypertrophy of the heart.

Although there were strong similarities among the two sexes in the pathways and functional categories for the genes altered in the brain of adult zebrafish exposed to 10 µg/L Pb during embryogenesis, about 4.3 times more genes were altered in the aged adult female zebrafish than in males. In particular, aged adult females exhibited expression profile changes of a substantial number of genes involved in nervous system development and function including the proliferation of neuronal cells, development of the central nervous system, and morphology and abnormal morphology of the nervous system that weren't as pronounced in the males. Moreover, female zebrafish exhibited a number of genes (89 genes) involved in AD including the known genetic risk factors *APP*, *APOE*, and *SORL1* (Supplementary Information Fig 2). *APP* codes for amyloid beta (Aβ) precursor protein of which proteolitically cleaved products can accumulate in the brain and constitute pathological hallmarks (i.e., Aβ plaques) of AD.[9] Apolipoprotein E (*APOE*) is a known AD risk gene, as an association has been found between the presence of a specific allele of APOE and increased risk for AD.[9] Moreover, APOE also plays a role in the transport of brain cholesterol, an important biological material required for cell membrane construction, synthesized in astrocytes to neurons.[24] Sortilin-related receptor, L (DLR class) A repeats-containing (*SORL1*) is a recently identified genetic risk factor of AD that functions in pathways for reducing Aβ production.[25] In addition, females also showed a significantly altered expression of beta-site APP-cleaving enzyme 1 (*BACE1*) that contributes to digestion of APP to produce Aβ.

In females, we also observed expression profile changes in specific sets of genes comprising a molecular network associated with cell-to-cell signalling and interaction, nervous system development and function, and cellular assembly and organization (Fig 2). The network included important molecules, such as neurexin 1 (*NRXN1*), neurexin 2 (*NRXN2*), and synaptotagmin 1 (*SYT1*), each of which plays a role in synaptic transmission. Neurexins are cell adhesion molecules on presynaptic neurons that interact with neuroligin on the postsynaptic side, mediating release of synaptic vesicles containing neurotransmitters.[26] *SYT1* is known to function as a calcium ion sensor that triggers calcium-dependent neurotransmitter release.[27] This network also included glutamate receptors (ionotropic glutamate receptor 3 [*GRIA3*] and 4 [*GRIA4*]) in the family of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. The AMPA receptors are involved in excitatory synaptic transmission and also synaptic plasticity.[28] These findings are consistent with our earlier microarray study in which significant expression profile changes of genes associated with the functional categories of long term depression of cells, synaptic transmission, and density and quantity of synaptic vesicles were observed in zebrafish at 72 hpf with an embryonic treatment of 100 µg/L Pb.[16] In addition, in a recent study, we observed time-point dependent alterations in expression of GABA transporters and GABA in zebrafish embryos developmentally treated with 10 µg/L Pb.[19] Taken together with the previous findings on the GABAergic system, results of this study suggest that an embryonic Pb exposure at 10 µg/L interrupts cell-to-cell communication across synapses, the impacts of which might be limited to the female sex.

Mechanisms underlying the gene expression alterations occurring in the aged animal brain have not been fully elucidated in relation to AD, but recent studies suggest an association between epigenetic modifications and latent transcriptional changes in developmentally Pb treated animals.[29-31] In Bihaqi et al. (2011), aged monkeys which had been Pb exposed during infancy showed significant changes in expression of brain genes and also proteins related to DNA methylation and histone modification pathways compared to aged matched control subjects.[29] Moreover, in studies comparing profiles of global gene expression and methylation from aged mice with a developmental Pb exposure to young male controls, aged animals exhibited significantly altered expression of brain genes of which suppression was related to DNA methylation profile patterns.[30, 31] Likewise, although epigenetic pathways in zebrafish are not clearly understood, it is speculated that similar epigenetic modifications observed in the aged non-human primate and rodent studies might be triggered in zebrafish by an early life Pb exposure. Our sex-specific microarray data show that an embryonic 10 µg/L Pb exposure resulted in female sex-specific changes in expression profiles of genes associated with nervous system development and function with a number of genes associated with AD. The male and female differences in gene expression can be attributed, in part, by sex hormonal differences which is suggested to be associated with epigenetic pathways leading to gene expression alterations.[32] However, it is still an open question at present whether the female sex is more genetically susceptible to a developmental Pb exposure since there are discrepancies among animal studies with different exposure concentrations, durations, and sexes of Pb treated animals.[33-35] For example, Schneider et al. (2012) reported differential patterns of global gene expression occurring in the hippocampus of female and male rats influenced by either lower (250 mg/L) or higher (750 mg/L) levels of Pb exposure during specific time-points (perinatal or postnatal Pb exposure).[35] In another study, a developmental Pb exposure through gestation and the early postnatal day period resulted in sex-specific changes in behavior and also in expression of a social behavior-related gene.[33] Overall our results indicate a greater genetic sensitivity of the aged adult female brain in response to a developmental 10 µg/L Pb exposure than that of males. Future studies with additional Pb concentrations and time-points of exposure in both sexes will further our understanding of the long term influences of a developmental Pb exposure on neurological disease pathogenesis.

Conclusions

This study demonstrates the impacts of an embryonic exposure to 10 µg/L Pb on global gene expression in brains of aged adult female and male zebrafish. While pathway analysis indicated similarities in the top diseases and functions that were altered in both sexes, the females had 4.3 times more genes changed from the embryonic Pb exposure. In addition, expression alterations in genes involved in nervous system development and function were more pronounced in adult females. Moreover, a number of genetic risk factors of AD had altered gene expression in females. Overall our observations support that a developmental exposure to Pb at concentrations as low as 10 µg/L can have long term neurological implications.

Experimental

Zebrafish husbandry

Zebrafish (AB stain) were housed in a Z-Mod System (Aquatic Habitats, Apopka, FL) on a 14:10 light:dark cycle at 28℃. Water quality was monitored by daily measurement of conductivity, pH, and temperature. Fish were properly maintained, fed, and bred following established guidelines in Westerfield (2007).[36] All animal protocols were approved and performed in accordance with Purdue University's Institutional Animal Care and Use Committee guidelines.

Embryonic Pb treatment

Zebrafish embryos were collected within 1-2 hpf and developmentally exposed to Pb acetate (10 µg/L Pb [0.048 µmol/L], Sigma Aldrich, St. Louis, MO) dissolved in aquaria water or aquaria water with no chemical treatment until 72 hpf (i.e., end stage of embryogenesis). This exposure was previously reported to result in a dose of 295 ng/g in the zebrafish.[19] At 72 hpf, larvae were transferred to clean aquaria water and maintained under control conditions with no additional Pb exposure until 12 months of age. This exposure procedure was repeated on multiple days to attain fish from different clutches.

Sample preparation

Adult zebrafish at 12 months of age were anesthetized using MS-222 and brains were obtained for female and male fish. Collected brain samples were thoroughly homogenized in Trizol Reagent (Life Technologies, Carlsbad, CA) and kept at -80°C until RNA isolation. RNA extraction was performed similarly as described in Peterson and Freeman (2009).[37] Extracted total RNA samples were purified on the RNeay Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol and then subjected to RNA quality and quantity measurements on the NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Sex-specific microarray analysis was carried out using the purified RNA samples. For sex-specific qPCR, the purified RNA was further converted into cDNA with the SuperScript® First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA) following the methods in Peterson and Freeman (2009).[37]

Sex-specific microarray transcriptome analysis

Sex-specific microarray analysis was performed to compare global brain gene expression profiles between aged fish with and without an embryonic exposure to 10 µg/L Pb exposure on a zebrafish custom 4x180K expression platform (Agilent Technologies, Santa Clara, CA) following MIAME guidelines.[38] This microarray is a multiplex format of 4 arrays each consisting of 180,000 60-mer probes interrogating ~36,000 known and predicted targets with approximately 3-5 probes per target. Arrays were hybridized using the one color hybridization strategy following the manufacturer's instructions and then washed and scanned on a SureScan Agilent microarray scanner (Agilent Technologies, Santa Clara, CA). Scanned images were extracted and then subjected to image data analysis using Agilent Feature Extraction Software version 11.5.1.1 (Agilent Technologies, Santa Clara, CA) and Agilent GeneSpring GX Version 12.5 (Agilent Technologies, Santa Clara, CA), respectively. A list of consistently expressed genes (Student's t-test, $p<0.05$) with significant alterations in gene expression profiles (mean absolute $\log_2$ expression ratio of at least 0.585 or over) was generated by sex as described in Peterson et al. (2011) using recommendations from the Microarray Quality Consortium.[16] Each gene list was imported into Ingenuity Pathway Analysis system (IPA, Qiagen, Redwood city, CA) to identify enrichment of genes related with different molecular pathways, networks, diseases, and function categories. Genes referred to in the results and discussion is reported as the human homologs of the genes identified to be altered by the microarrays. All microarray data is deposited in the GEO database (GSE74037 and GSE74038) .[22,23]

Sex-specific qPCR analysis for comparison to microarray observations

qPCR analysis was carried out to confirm results of microarray experiments. Primers for qPCR were designed with Primer3 to target randomly selected genes for female (i.e., *APP*, *ARID3B*, *BACE1*, *EMX1*, *GGCT*, *PRDX4*, *SLC25A14*) and male zebrafish (i.e, *GTF2F1, ROMO1, RPS15A, TRMT10C, TTLL2*) (Supplementary Information Table 3).[39] For qPCR analysis on both female (N = 4-6) and male zebrafish (N = 6), *RPL13A* was used as a reference gene following our preliminary tests to choose the most appropriate reference target with consistent and least variable gene expression (data not shown). qPCR experiments were performed using the SSOAdvanced SYBR Green SuperMix following the manufacturer's recommendations and the MIQE guidelines[40] on a BioRad CFX Connect™ Real Time PCR Detection System (Bio-Rad, Hercules, CA). In each experiment, expression of the gene of interest was first normalized to that of *RPL13A* and then compared between groups with and without an embryonic 10 µg/L Pb exposure. Experimental samples were run in triplicate (technical replicates) and gene expression was normalized to *RPL13A*. Efficiency and specificity were checked with melting and dilution curve analysis and no-template controls. Microarray confirmation by qPCR was assessed by a Pearson's correlation plotting microarray ($\log_2$ transformed gene expression fold change) and qPCR analysis ($\log_2$ transformed average gene expression in zebrafish with Pb exposure versus controls) ($p < 0.05$) using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp., Armonk, NY).

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Figure legends

Fig 1. Comparison of microarray data with qPCR results. Expression profiles of a set of genes generated in the microarray analysis ($\log_2$ expression) were compared with qPCR data ($\log_2$ expression ratio relative to *RPL13A*) in aged male and female zebrafish brain. Pearson's correlation analysis confirmed a significant very strong positive correlation between microarray data and qPCR observations ($p < 0.05$, $R=0.778$).

Fig 2. Female-specific network of genes involved in cell-to-cell signalling and interaction, nervous system development and function, and cellular assembly and organization. All molecules altered in the adult female brain are in bold. Figure was made in IPA with molecules listed as human protein annotation. (Abbreviations: APBA2, amyloid beta (Aβ) precursor protein-binding, family A, member 2; BAHCC1, BAH domain and coiled-coil containing 1; CBLN1, cerebellin 1 precursor; CLSTN1, calsyntenin 1; GRIA3, glutamate receptor, ionotropic, AMPA 3; GRIA4, glutamate receptor, ionotropic, AMPA 4; GRIK1, glutamate receptor, ionotropic, kainate 1; NAPA, N-ethylmaleimide-sensitive factor attachment protein, alpha; NAPB, N-ethylmaleimide-sensitive factor attachment protein, beta; NAPG, N-ethylmaleimide-sensitive factor attachment protein, gamma; NLGN1, neuroligin 1; NLGN3, neuroligin 3; NRXN1, neurexin 1; NRXN2, neurexin 2; PCDH10, protocadherin 10; PCDH17, protocadherin 17; PCDH19, protocadherin 19; PDZD2, PDZ domain containing 2; SLC6A9, solute carrier family 6 (neurotransmitter transporter, glycine), member 9; SRR, serine racemase; STX2, syntaxin 2; STX5, syntaxin 5; STX16, syntaxin 16; SYNGAP1, synaptic Ras GTPase activating protein 1; SYT1, synaptotagmin I; UNC5A, unc-5 netrin receptor A; VTI1B, vesicle transport through interaction with t-SNAREs 1B)

Supplementary information figure legends

Supplementary Information Fig 1. Heat map of altered probe sets by a developmental Pb exposure in aged brain of adult female and male zebrafish. Heat map images show significantly altered probe sets (rows) by a developmental exposure to 0 µg/L Pb or 10 µg/L Pb (columns) in aged brains from adult female (left, n=3) and adult male zebrafish (right, n=4 for 0 µg/L Pb, n=3 for 10 µg/L Pb treated groups). Color range indicates different expression of each probe which is mapped to a color intensity value (blue, minimum -2 representing low expression; red, maximum 2 representing high expression).

Supplementary Information Fig 2. Altered genes in aged adult female zebrafish brain associated with Alzheimer's disease. The image shows indirect relationship (dashed line) of Alzheimer's disease with 89 molecules with altered expression profiles in adult female zebrafish exposed to 10 µg/L Pb during embryogenesis. Figure was made in IPA with molecules listed as human protein annotation. (Abbreviations: A2M, alpha-2-macroglobulin; ABAT, 4-aminobutyrate aminotransferase; ABCA1, ATP-binding cassette, sub-family A; ACLY, ATP citrate lyase; ACTB, actin, beta; ADAM10, ADAM metallopeptidase domain 10; AIFM1, apoptosis-inducing factor, mitochondrion-associated, 1; APLP2, amyloid beta (Aβ) precursor-like protein 2; APOE, apolipoprotein E; APP, amyloid beta (Aβ) precursor protein; AR, androgen receptor; ARNT2, aryl-hydrocarbon receptor nuclear translocator 2; BACE1; beta-site APP-cleaving enzyme 1; BLMH, bleomycin hydrolase; CALHM1, calcium homeostasis modulator 1; CAMK2B, calcium/calmodulin-dependent protein kinase II beta; CAPN1, calpain 1, (mu/I) large subunit; CASP3, caspase 3, apoptosis-related cysteine peptidase; CBS/CBSL, cystathionine-beta-synthase; CDK1, cyclin-dependent kinase 1; CFAP45, cilia and flagella associated protein 45; CHRM2, cholinergic receptor, muscarinic 2; CNR1, cannabinoid receptor 1 (brain); CST3, cystatin C; CTGF, connective tissue growth factor; CYP11A1, cytochrome P450, family 11, subfamily A, polypeptide 1; DDIT3, DNA-damage-inducible transcript 3; DHCR24, 24-dehydrocholesterol reductase; DICER1, dicer 1, ribonuclease type III; DLL1, delta-like 1 (Drosophila); DRD2, dopamine receptor D2; EEF2, eukaryotic translation elongation factor 2; EEF1G, eukaryotic translation elongation factor 1 gamma; FDPS, farnesyl diphosphate synthase; GABRA6, gamma-aminobutyric acid (GABA) A receptor, alpha 6; GABRB1, GABA A receptor, beta 1; GABRD, GABA A receptor, delta; GAD2, glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa); GAPDH, glyceraldehyde-3-phosphate; GFAP, glial fibrillary acidic protein; GRIA3, glutamate receptor, ionotropic, AMPA 3; GRIN3A, glutamate receptor, ionotropic, N-methyl-D-aspartate 3A; HDAC9, histone deacetylase 9; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMOX1, heme oxygenase 1; HNRNPU, heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A); HSPA9, heat shock 70kDa protein 9 (mortalin); HTT, huntingtin; IGSF8, immunoglobulin superfamily, member 8; IREB2, iron-responsive element binding protein 2; LARP4, La ribonucleoprotein domain family, member 4; LSS, lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase); MAPK8IP1, mitogen-activated protein kinase 8 interacting protein 1; MFN1, mitofusin 1; MTHFR, methylenetetrahydrofolate reductase (NAD(P)H); MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; MYH11, myosin, heavy chain 11, smooth muscle; NBAS, neuroblastoma amplified sequence; NR3C1, nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor); NRXN1, neurexin 1; OPA1, optic atrophy 1 (autosomal dominant); OSBPL10, oxysterol binding protein-like 10; PAK1, p21 protein (Cdc42/Rac)-activated kinase 1; PAXIP1, PAX interacting (with transcription-activation domain) protein 1; PCNA, proliferating cell nuclear antigen; PGR, progesterone receptor; POLB, polymerase (DNA directed), beta; PTGER4, prostaglandin E receptor 4 (subtype EP4); PTPRZ1, protein tyrosine phosphatase, receptor-type, Z polypeptide 1; PUM1, pumilio RNA-binding family member 1; RAB6A, RAB6A, member RAS oncogene family; ROCK2, Rho-associated, coiled-coil containing protein kinase 2; RTN3, reticulon 3; RTN4R, reticulon 4 receptor; S100B, S100 calcium binding protein B; SC5D, sterol-C5-desaturase; SCD, stearoyl-CoA desaturase (delta-9-desaturase); SLC6A3, solute carrier family 6

(neurotransmitter transporter), member 3; SOD1, superoxide dismutase 1, soluble; SORL1, sortilin-related receptor, L(DLR class) A repeats containing; SPTLC1, serine palmitoyltransferase, long chain base subunit 1; SREBF2, sterol regulatory element binding transcription factor 2; SRF, serum response factor (c-fos serum response element-binding transcription factor); STX2, syntaxin 2; TGM1, transglutaminase 1; TGM2, transglutaminase 2; TSHZ1, teashirt zinc finger homeobox 1; VEGFA, vascular endothelial growth factor A; XPO7, exportin 7)

Table 1. Top physiological system development and functions affected by an embryonic exposure to 10 µg/L Pb in aged adult female zebrafish brain

Diseases or Functions Annotation	p-Value[a]	# of Genes[b]
Organismal survival		
morbidity or mortality	3.05E-26	460
organismal death	4.72E-27	458
perinatal death	3.48E-07	108
neonatal death	6.09E-05	75
Organismal development		
morphology of body cavity	1.68E-06	194
development of body trunk	4.33E-08	191
abnormal morphology of body cavity	8.74E-07	188
development of body axis	2.82E-09	187
development of head	2.10E-10	181
Nervous system development and function		
morphology of nervous system	2.21E-12	157
development of neurons	3.75E-12	156
abnormal morphology of nervous system	3.23E-10	140
development of central nervous system	9.98E-14	137
proliferation of neuronal cells	3.20E-11	119
Tissue development		
development of neurons	3.75E-12	156
proliferation of neuronal cells	3.20E-11	119
neuritogenesis	6.45E-10	116
formation of brain	2.63E-10	104
growth of connective tissue	3.06E-04	102
Behavior		
behavior	4.12E-11	185
cognition	3.15E-07	86
learning	1.72E-07	81
locomotion	3.37E-06	65
feeding	6.75E-05	54

[a]p-Value: the probability of observing an enrichment of a particular set of genes with a given number of genes by chance alone.

[b]# of Genes: number of differentially expressed genes categorized into their related diseases or functions. A gene may be present in multiple diseases or functional categories.

Table 2. Top molecular and cellular functions affected by an embryonic exposure to 10 µg/L Pb in aged adult female zebrafish brain

Diseases or Functions Annotation	p-Value[a]	# of Genes[b]
Cellular growth and proliferation		
proliferation of cells	1.88E-21	622
proliferation of tumor cell lines	5.78E-11	268
formation of cells	1.73E-05	158
proliferation of neuronal cells	3.20E-11	119
proliferation of connective tissue cells	2.38E-05	100
Cell morphology		
morphology of cells	2.78E-16	350
abnormal morphology of cells	3.55E-09	218
formation of cellular protrusions	5.57E-09	163
formation of plasma membrane projections	1.82E-10	120
neuritogenesis	6.45E-10	116
Cellular assembly and organization		
organization of cytoplasm	3.14E-14	280
organization of cytoskeleton	1.16E-14	261
microtubule dynamics	3.25E-11	215
formation of cellular protrusions	5.57E-09	163
formation of plasma membrane projections	1.82E-10	120
Cellular function and maintenance		
organization of cytoplasm	3.14E-14	280
organization of cytoskeleton	1.16E-14	261
cellular homeostasis	5.37E-06	237
microtubule dynamics	3.25E-11	215
formation of cellular protrusions	5.57E-09	163
Cell death and survival		
cell death	2.52E-14	538
apoptosis	2.49E-12	431
necrosis	1.59E-10	411
cell death of tumor cell lines	2.19E-09	257
cell survival	3.60E-07	226

[a]p-Value: the probability of observing an enrichment of a particular set of genes with a given number of genes by chance alone.

[b]# of Genes: number of differentially expressed genes categorized into their related diseases or functions. A gene may be present in multiple diseases or functional categories.

Table 3. Top diseases and disorders affected by an embryonic exposure to 10 µg/L Pb in aged adult female zebrafish brain

Diseases or Functions Annotation	p-Value[a]	# of Genes[b]
Cancer		
cancer	1.90E-34	1576
malignant solid tumor	2.19E-35	1564
tumorigenesis of tissue	1.22E-25	1335
neoplasia of epithelial tissue	3.83E-27	1323
abdominal neoplasm	1.13E-25	1309
Organismal injury and abnormalities		
cancer	1.90E-34	1576
malignant solid tumor	2.19E-35	1564
tumorigenesis of tissue	1.22E-25	1335
neoplasia of epithelial tissue	3.83E-27	1323
abdominal neoplasm	1.13E-25	1309
Gastrointestinal disease		
digestive organ tumor	4.07E-21	1138
digestive system cancer	6.62E-21	1128
gastrointestinal tract cancer and tumors	6.68E-19	817
gastrointestinal tract cancer	3.76E-18	802
gastrointestinal carcinoma	5.52E-21	744
Reproductive system disease		
genital tumor	1.68E-14	643
tumorigenesis of genital organ	6.77E-14	622
genital tract cancer	1.16E-13	616
female genital neoplasm	5.55E-13	567
tumorigenesis of reproductive tract	3.43E-12	543
Developmental disorder		
congenital anomaly of musculoskeletal system	2.59E-12	149
growth failure	8.38E-16	136
congenital malformation of skeleton	1.54E-10	91
dysgenesis	1.04E-04	89
hypertrophy	6.30E-07	88

[a]p-Value: the probability of observing an enrichment of a particular set of genes with a given number of genes by chance alone.

[b]# of Genes: number of differentially expressed genes categorized into their related diseases or functions. A gene may be present in multiple diseases or functional categories.

Table 4. Top physiological system development and functions affected by an embryonic exposure to 10 µg/L Pb in aged adult male zebrafish brain

Diseases or Functions Annotation	p-Value[a]	# of Genes[b]
Organismal survival		
morbidity or mortality	1.21E-10	120
organismal death	1.05E-10	119
perinatal death	3.03E-06	36
survival of organism	3.01E-04	35
neonatal death	6.44E-05	26
Organismal development		
morphology of body cavity	1.65E-08	67
abnormal morphology of body cavity	4.04E-07	61
development of body trunk	4.04E-07	60
development of body axis	2.81E-06	55
development of head	1.50E-06	53
Cardiovascular system development and function		
morphology of cardiovascular system	1.80E-07	40
angiogenesis	3.05E-03	39
abnormal morphology of cardiovascular system	1.21E-07	38
vasculogenesis	2.71E-03	33
morphology of heart	4.02E-05	25
Connective tissue development and function		
morphology of bone	1.96E-07	32
abnormal morphology of bone	2.92E-07	31
quantity of connective tissue	1.35E-03	29
abnormal morphology of skull	4.27E-06	18
differentiation of adipocytes	3.11E-03	14
Skeletal and muscular system development and function		
morphology of bone	1.96E-07	32
abnormal morphology of bone	2.92E-07	31
formation of muscle	7.43E-05	24
function of muscle	6.10E-05	22
growth of muscle tissue	1.90E-04	22

[a]p-Value: the probability of observing an enrichment of a particular set of genes with a given number of genes by chance alone.

[b]# of Genes: number of differentially expressed genes categorized into their related diseases or functions. A gene may be present in multiple diseases or functional categories.

Table 5. Top molecular and cellular functions affected by an embryonic exposure to 10 µg/L Pb in aged adult male zebrafish brain

Diseases or Functions Annotation	p-Value[a]	# of Genes[b]
Cellular development		
differentiation of cells	1.37E-05	96
proliferation of tumor cell lines	5.81E-10	85
development of neurons	7.51E-05	40
differentiation of nervous system	4.26E-05	29
neuritogenesis	7.71E-04	29
Cellular growth and proliferation		
proliferation of cells	5.80E-09	159
proliferation of tumor cell lines	5.81E-10	85
formation of cells	1.27E-04	47
proliferation of connective tissue cells	1.02E-03	29
proliferation of neuronal cells	2.64E-03	27
Gene expression		
expression of RNA	4.99E-05	91
transcription	8.26E-06	89
transcription of RNA	2.69E-06	86
transcription of DNA	9.99E-08	78
activation of DNA endogenous promoter	1.46E-06	62
Cellular movement		
cell movement	1.59E-03	82
migration of cells	3.08E-03	73
invasion of cells	9.47E-07	49
invasion of tumor cell lines	5.02E-07	41
cell movement of tumor cell lines	2.50E-04	41
Cell morphology		
morphology of cells	2.96E-05	84
abnormal morphology of cells	1.47E-03	53
formation of cellular protrusions	4.85E-03	38
neuritogenesis	7.71E-04	29
morphogenesis of neurons	3.50E-05	26

[a]p-Value: the probability of observing an enrichment of a particular set of genes with a given number of genes by chance alone.

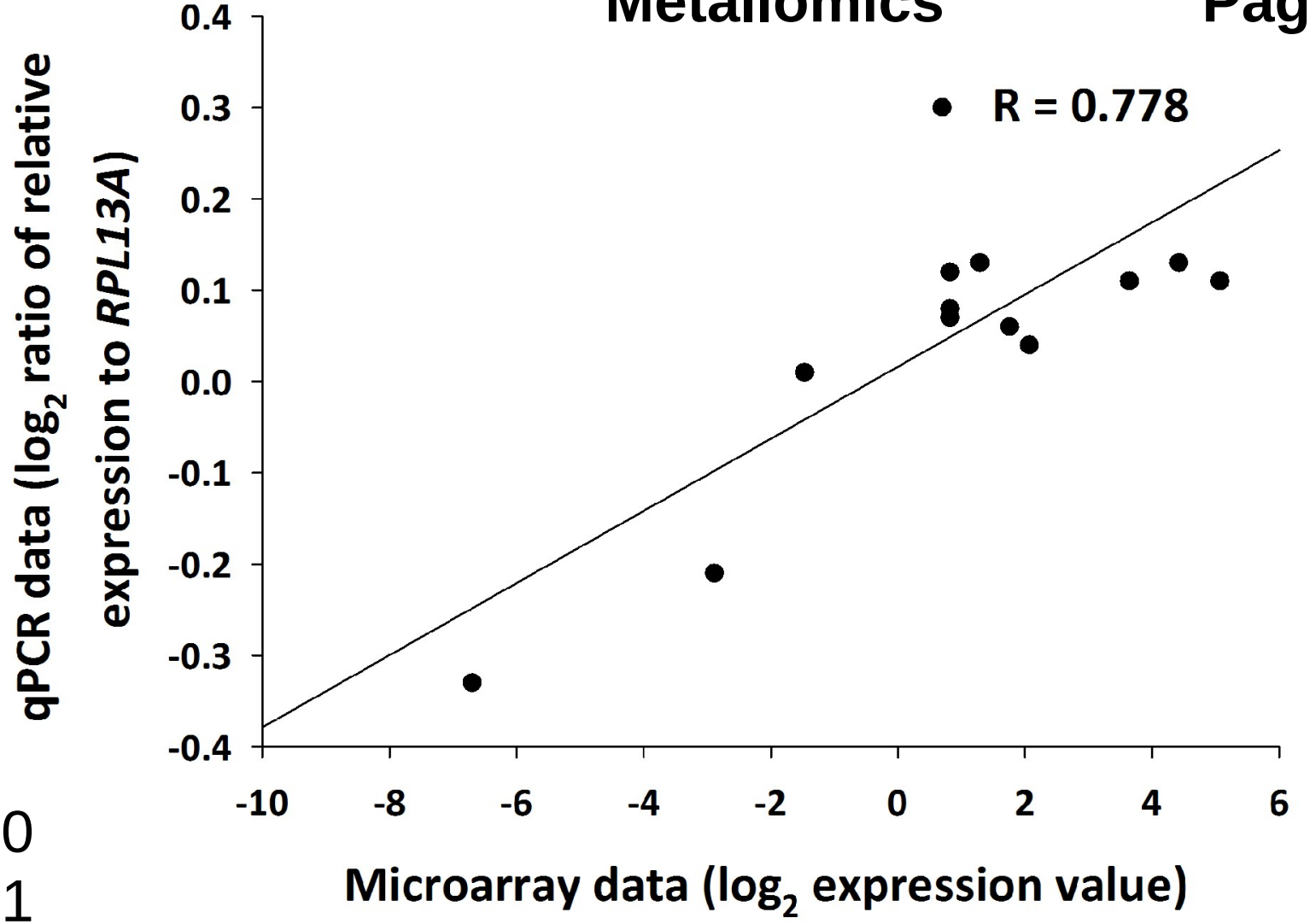
[b]# of Genes: number of differentially expressed genes categorized into their related diseases or functions. A gene may be present in multiple diseases or functional categories.

Table 6. Top diseases and disorders affected by an embryonic exposure to 10 µg/L Pb in aged adult male zebrafish brain

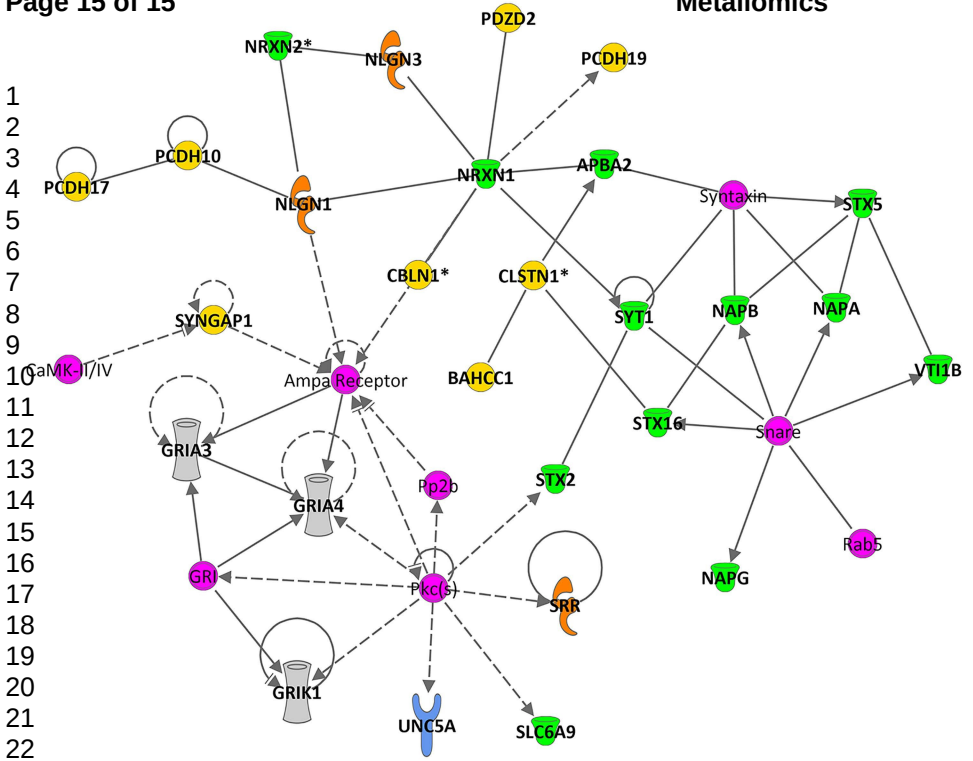
Diseases or Functions Annotation	p-Value[a]	# of Genes[b]
Cancer		
cancer	7.24E-10	371
malignant solid tumor	8.90E-11	370
tumorigenesis of tissue	8.08E-12	329
neoplasia of epithelial tissue	7.41E-13	328
epithelial cancer	5.98E-13	326
Organismal injury and abnormalities		
cancer	7.24E-10	371
malignant solid tumor	8.90E-11	370
tumorigenesis of tissue	8.08E-12	329
neoplasia of epithelial tissue	7.41E-13	328
epithelial cancer	5.98E-13	326
Gastrointestinal disease		
digestive organ tumor	3.84E-09	280
digestive system cancer	6.77E-09	277
gastrointestinal tract cancer and tumors	8.65E-11	214
gastrointestinal tract cancer	2.10E-10	210
gastrointestinal carcinoma	2.54E-08	185
Developmental disorder		
congenital anomaly of musculoskeletal system	5.41E-07	44
multiple congenital anomalies	3.87E-06	28
hypertrophy	1.26E-03	24
congenital malformation of skeleton	5.25E-04	23
hypertrophy of heart	4.50E-03	17
Skeletal and muscular disorders		
congenital anomaly of musculoskeletal system	5.41E-07	44
congenital malformation of skeleton	5.25E-04	23
congenital anomaly of limb	4.80E-04	12
osteoarthritis	1.22E-03	11
congenital anomaly of digit	1.31E-03	9

[a]p-Value: the probability of observing an enrichment of a particular set of genes with a given number of genes by chance alone.

[b]# of Genes: number of differentially expressed genes categorized into their related diseases or functions. A gene may be present in multiple diseases or functional categories.



Metallomics



Legends

- Group/complex
- Enzyme
- Ion channel
- Transmembrane receptor
- Transporter
- Other