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Gene Responses in the Central Nervous System of Zebra sh Embryos Exposed to the Neurotoxicant Methyl Mercury

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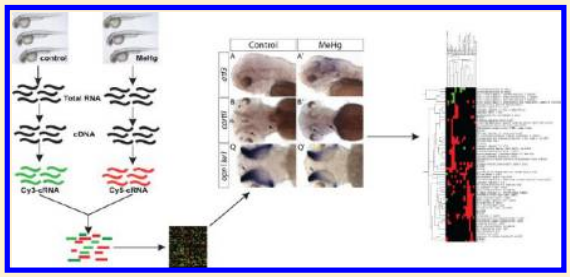
* *Supporting Information*

ABSTRACT: Methyl mercury (MeHg) is a neurotoxicant with adverse e ects on the development of the nervous system from sh to man. Despite a detailed understanding of the molecular mechanisms by which MeHg a ects cellular homeostasis, it is still not clear how MeHg causes developmental neurotoxicity. We performed here a genome-wide transcriptional analysis of MeHg-exposed zebra sh embryos and combined this with a whole-mount in situ expression analysis of 88 MeHg-a ected genes. The majority of the analyzed genes showed tissue- and region-restricted responses in various organs and tissues. The genes were linked to gene ontology terms like oxidative stress, transport and cell protection. Areas even within the central nervous system (CNS) are a ected di erently resulting in distinct cellular stress responses. Our study revealed an unexpected heterogeneity in gene responses to MeHg exposure in di erent tissues and neuronal subregions, even though the known molecular action of MeHg would predict a similar burden of exposed cells. The overall structure of the developing brain of MeHg-exposed embryos appeared normal, suggesting that the mechanism leading to di erentiation of the CNS is not overtly a ected by exposure to MeHg. We propose that MeHg disturbs the function of the CNS by disturbing the cellular homeostasis. As these cellular stress responses comprise genes that are also involved in normal neuronal activity and learning, MeHg may a ect the developing CNS in a subtle manner that manifests itself in behavioral de cits.



INTRODUCTION

Mercury is a well-known teratogenic and neurotoxin in humans.[1−3] Both natural geological sources, industrial activities and enrichment of mercury and its organic derivatives like methylmercury (MeHg) through the food chain can contribute to human exposure.[4−6] While Hg levels in surface waters, groundwater, and oceans are usually low in the range of 20 ng/L,[7] areas heavily polluted by anthropogenic mercury sources can have MeHg levels of more than 2000 ng/L.[8] The concentration of MeHg can increase by accumulation in the food chain million-fold.[9−11] About 95% of MeHg in the diet is absorbed by the gastrointestinal tract[12] and passes to all main tissues even penetrating the blood-brain barrier.[13,14] MeHg poisoning can result in sensory disturbances, de cits in motor coordination, and somatosensory and psychiatric disorders. The developing human brain is more susceptible to MeHg than the adult brain. Children exposed during pregnancy to subacute doses of MeHg showed impairments in motor coordination, speech and involuntary movement due to damage of the brain, particularly the cerebral and cerebellar cortices.[1−3] After the Minamata poisoning, median hair content of mercury was 30 ppm and the maximum was 920 ppm.[1] The EPA regards 5.8 μg Hg/L blood as safe (http://www.epa.gov/hg/exposure.htm). A study of children on the Faroe island suggest that MeHg concentrations below 10 ppm in the hair of the mother correlate with de cient performance in attention, memory and language of her o -spring.[15]

MeHg exposure has been shown to result in various cellular changes such as lipid peroxidation, DNA damage, membrane structure alteration, mitochondrial dysfunction, cell cycle alteration, apoptosis, and necrosis.[16] The interactions with sulfhydryl groups, the induction of oxidative stress and the disruption of calcium ion homeostasis have been reported to be the three major and critical mechanisms.[17−24] It is, however, not clear how MeHg acts as a neurotoxicant. Hence vertebrate models are needed to investigate the neurotoxic e ects in detail.

The zebra sh embryo is highly sensitive to MeHg. Exposure of zebra sh embryos to 20 and 30 μg/L MeHg led to impaired development of the caudal n and caused an abnormal tail exure.[25−27] Doses between 10 and 20 μg/L MeHg induced faint heartbeats, severe edema, upward exures of the body axis, reduced swimming activity, impaired prey capture performance, a delayed mortality syndrome, behavioral de cits[28] and impaired tail formation.[27] Global transcriptome study on zebra sh embryos showed that acute exposure to 60 μg/L

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MeHg induced genes involved in the acute in ammatory response, amino acid metabolism, transmembrane receptor protein tyrosine kinase signaling pathways, and the insulin receptor signaling pathway.[26] Similar transcriptome studies in adult zebra sh indicated that sublethal acute or chronic exposure to MeHg or Hg caused gene expression changes linked to a variety of biological and molecular processes, such as oxidative stress, immune defense, DNA damage, apoptosis, lipid peroxidation, and glycolysis/gluconeogenesis.[29−32] Investigation of the localization of MeHg in adult zebra sh after trophic and chronic MeHg exposure revealed that the brain accumulated high levels of MeHg among the three organs examined.[33] Another study concerning the visual system showed that MeHg could pass through the blood-retina barrier and could accumulate in the retina.[34]

To investigate the mode of action of the neurotoxicant MeHg, we carried out systematic gene expression analysis using zebra sh embryos as models. By a combination of microarray and in situ expression studies, we show that sublethal concentrations of MeHg induce tissue speci c gene expression changes in the CNS and in many other organs of the 3-day-old zebra sh embryo. By gene ontology analysis with functions inferred from those of mammalian orthologous genes, most of these genes were linked to cellular stress responses. Our data suggest that MeHg a ects neuronal cell homeostasis, thereby a ecting the developing brain.

MATERIALS AND METHODS

Fish Maintenance and MeHg Treatment. Zebra sh were maintained and treated with MeHg as described.[26,27] For details see Suporting Information (SI) Material and Methods

DNA Microarray. Two-color DNA microarrays (zebra sh gene expression microarray chip 4 × 44K, Agilent Technologies) were hybridized according to the Agilent Low RNA Input Linear Ampli cation Kit protocol (Agilent Technologies). Three independent repeats were performed for both control and MeHg-treated samples. Two μg total RNA from each sample was reverse transcribed into cDNA (see below). cRNA was transcribed from the cDNA and labeled with Cyanine3 (Cy3) −CTP or Cy5-CTP uorescent dyes (PerkinElmer/NEN Life Science). The cRNA samples were puri ed with the RNeasy Mini Kit (Qiagen, Netherlands). Microarray chips were handled according to the user guide of the Agilent Microarray Hybridization Chamber Kit (Agilent Technologies). A dye swap was performed for each biological repeat.

Data Analysis. The software MATLAB (version R2010a; Math Works) or the MATLAB toolbox Gait-CAD[35] was modi ed by adding ltering functions, normalization methods and statistical tests. M-value (Log_2 of fold-change) transformations were performed to obtain a more symmetric distribution of the data. The raw microarray data were preprocessed by background subtraction and normalized by the LOWESS method.[36] Plots of the data distribution of each microarray were examined for control of the quality of hybridizations. The spike controls contained ten in vitro synthesized, polyadenylated transcripts in predetermined ratios for monitoring linearity, sensitivity, and accuracy. The data of the dye swaps were averaged using median. A one-sample t test was performed over the three biological replicates to identify statistically signi cant di erences ($p < 0.05$). The M-values of the replicates were averaged (using median). To reduce the false positives, we performed besides the t test a M-value cuto based on the variation of the M values. The false positives had rather small M-values. The variance of M-values over the replicate was expected to be smaller when arising from a true signal as compared to being caused by noise. To test this, the mean M-values were plotted against the coe cient of variation (CV) over the replicates. A CV value of one was generally used as cuto between small (CV < 1) and high variance (CV > 1). The M value cuto was determined by the point where the M-values scattered over the CV of one. Di erentially expressed spots (p-value <0.05) with M values greater than 1.4 had a small variance and therefore represented true biological signal. This double ltering method gave a better validation rate.[37] The microarray data were submitted to Gene Expression Omnibus (GEO) with the accession number GSE37970.

Gene Ontology Analysis. The regulated genes were assigned to the closest human homologue using BioMart (http://www.biomart.org/).[38] The gene ontology (GO) analyses were then carried out with the Gene Set Analysis Toolkit V2 (http://bioinfo.vanderbilt.edu/wg_gsat/).[39,40] Lists of the human homologues were blasted against the human genome. Hypergeometric test, Benjamini & Hochberg (1995) multiple test adjustment (MTC), and a signi cance level of 0.01 were used for the analysis. Three genes was the minimum number for a category to be determined as enriched.

Expression Analysis. In situ hybridization and immunohistochemistry was performed using standard protocols.[41,42] (For details see SI Material and Methods). Tissue annotations of in situ expression data were transformed into a matrix of gene expression (0 no change, 1 upregulated, −1 downregulated) and subjected to hierarchical clustering using the uncentered correlation metric and pairwise average linkage method.[43]

Real-time polymerase chain reaction (RT-qPCR) was carried out following suppliers instructions (For details see SI Material and Methods).

RESULTS

Analysis of the Transcriptome of MeHg-Exposed Embryos. We wished to identify genes whose expression was altered by exposure to MeHg in the developing zebra sh embryo and which might thus serve as reporters of developmental toxicity. Our previous transcriptome analysis[26] of MeHg-treated embryos covered only 10656 probes. We therefore repeated the analysis with the more comprehensive 4 × 44K chip design (Agilent Technologies), which contains 43 803 probes for 13 555 genes (Zebra sh genome assembly, Zv9, v66).

Embryos were exposed from 48 to 72 hpf to 60 μg/L MeHg and changes in gene expression were monitored by comparison with the water control using a two color protocol. Statistical analysis indicated that a total of 464 genes were up-regulated with M-values (log_2 of fold-change) greater than or equal to 1.3 ($p < 0.05$, SI Table S3) and 379 genes were down-regulated with M-values smaller than or equal to −1.3 ($p < 0.05$, SI Table S4).

We analyzed the gene ontology groups to which the MeHg-regulated genes belong with the Gene Set Analysis Toolkit V2 (http://bioinfo.vanderbilt.edu/wg_gsat/).[39,40] One large group of up-regulated genes is involved in apoptosis, and other genes were previously implicated in cell redox homeostasis, oxidative stress, and acute in ammatory responses, suggesting that MeHg caused cell stress and tissue damage. (Table 1, SI Figure 1A, A). Genes encoding phosphatases and endopeptidases were also up-regulated. Several genes involved in other cellular

Table 1. Ontology Groups of Genes Up-Regulated by MeHg

gene ontology	number of genes	adjusted *p*-value
apoptosis	36	3.00×10^{-4}
prostaglandin metabolic process	4	5.50×10^{-3}
calcium independent cell−cell adhesion	5	9.00×10^{-4}
cell redox homeostasis	6	5.50×10^{-3}
response to oxidative stress	12	3.00×10^{-4}
acute in ammatory response	8	2.60×10^{-3}
blood circulation	12	2.00×10^{-3}
MAPKtyrosine/serine/threonin phosphatase activity	4	4.00×10^{-4}
endopeptidase activty	19	1.00×10^{-4}
serine-type endopeptidase activity	11	5.00×10^{-4}
transcription factor activity	26	6.10×10^{-3}
aryldialkyphosphatase activity	3	2.00×10^{-4}

functions were down-regulated by MeHg exposure, such as transcriptional elongation, fatty acid metabolism, DNA repair, and particularly dehydrogenase and isomerase activities (Table 2, SI Figure 1B, B).

Table 2. Ontology Groups of Genes down-Regulated by MeHg

gene ontology	number of genes	adjusted *p*-value
RNA elongation from RNA Pol II promoter	6	3.70×10^{-3}
fatty acid catabolic process	5	6.50×10^{-3}
double strand break repair	6	5.20×10^{-3}
transcription factor activity, nucleic acid binding	8	1.20×10^{-3}
acyl-CoA binding	4	1.30×10^{-3}
3-hydroxyacyl-CoA dehydrogenase activty	3	1.30×10^{-3}
peptidyl-prolyl cis−trans isomerase activity	5	1.50×10^{-3}

In Situ Analysis of Expression. Transcriptome analysis of whole zebra sh embryos does not allow examination of tissue-speci c changes in gene expression. In order to verify the microarray data and to derive at the same time information on tissue speci c expression of the induced genes, we carried out an in situ expression study with 88 signi cantly MeHg-regulated genes. Probes were subcloned (SI Table S1) and 72 hpf embryos were subjected to in situ hybridization. Embryos were treated with 60 μg/L MeHg from 4 to 72 hpf and expression patterns were compared to those of water controls. Among the 88 genes analyzed by in situ hybridization, the changes in expression levels of 60 genes were con rmed to be correlated with the microarray results (Figure 1 see also SI Figure 2). For example, *peroxiredoxin1* (*prdx1*) was up-regulated in the lateral line, the liver and the hypothalamus in response to MeHg (Figure 1A, A). Increased expression of *phosphoenolpyruvate carboxykinase 1* (*pck1*) was observed in the yolk syncytial layer and the liver (Figure 1B, B); *fibronectin1b* (*fn1b*) showed a strong up-regulation in the skin throughout the whole embryo (Figure 1C, C). *matrix metalloprotease* (*mmp13*) was up-regulated in cells scattered all over the body, the posterior blood island (PBI), where blood cells are produced, and along the edges of the pectoral and caudal ns (Figure 1D, D).

Clustering of Whole-Mount In Situ Hybridizations. The results derived from the whole-mount in situ hybridizations were classi ed using the following 17 anatomical terms:

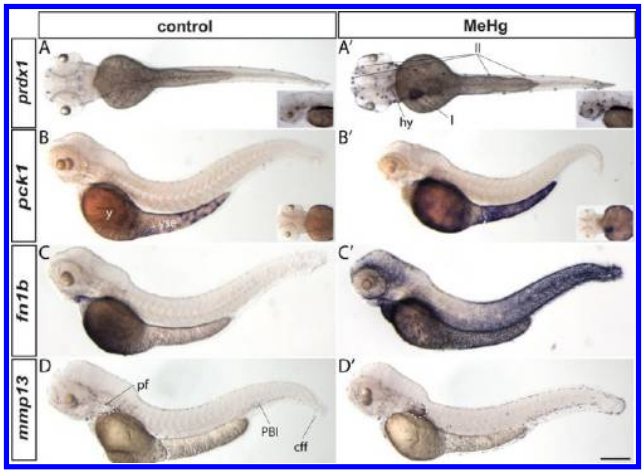

Figure 1. Changes in gene expression patterns after MeHg exposure. Control (A, B, C, D) and MeHg-treated (A , B , C , D) embryos (72 hpf) were hybridized with RNA probes complementary to *peroxiredoxin1* (*prdx1*, A, A), *phosphoenolpyruvate carboxykinase 1* (*pck1*, B, B), *fibronectin1b* (*fn1b*, C, C) and *matrix metalloprotease13* (*mmp13*, D, D) mRNA. Abbreviations, c , caudal n fold; hy, hypothalamus; l, liver; ll, lateral line; PBI, posterior blood island; pf, pectoral n; y, yolk; yse, yolk sac extension. Scale bar, A, A − D, D , 200 μm.

brain, eyes, olfactory bulb, branchial arches, heart, liver, gut, pronephros, somites, lateral line, pectoral ns, caudal n fold, blood vessels, skin, yolk syncytial layer (YSL), pigment cells, and notochord (Figure 2). Three major clusters of coexpressed genes were discernible: *complement factors b, 3a* and *4−2* are expressed in the YSL and the liver together with *pck1* (Figure 2). Other genes were coexpressed in the brain and the eyes. A third prominent cluster of coexpressed genes was found in the caudal ns and the skin. In 4 cases (*elf3*, *noxo1l*, *cbx7a*, and *slc16a9a),* expression was up-regulated in some tissues and was down-regulated in others (Figure 2). For example, the expression of *noxo1l* was increased in the lateral line, pectoral ns, caudal n fold and skin, but decreased in the branchial arches (SI Figure 2A, A). Taken together, our results demonstrate that most of the MeHg-regulated genes were expressed in a tissue restricted manner (Figures 1 and 2, SI Figure 2). Di erent tissues responded with distinct changes in gene expression.

To verify the observed changes in gene expression by an independent method, RT-qPCR was performed with selected genes including *arf4*, *atf3*, *c4−2*, *c6*, *cbx7a*, *homez*, *irf9*, *prdx1*, *ppp1r15a*, *txnl*, *zgc:101661*, and *opn1mw1*. Zebra sh embryos were exposed to 30 or 60 μg/L MeHg from 4 to 72 hpf and cDNA samples were subjected to real-time PCR analysis with gene-speci c primers. The expression levels of all the genes examined, except *homez*, were signi cantly regulated by treatment with 60 μg/L MeHg (Figure 3). In situ hybridization showed that *homez* mRNA was up-regulated in embryos with severe malformations (precardiac edema, reduced n folds, smaller brain, curved body), but not in embryos that were morphologically not a ected by MeHg treatment. This contributed to variation and thus reduced signi cance of the RT-qPCR results. *opn1mw1* showed a repression with an M-value of less than −2 after exposure to 60 μg/L MeHg. Down-regulation of other opsin genes was also noted (Figure 2, SI Table S4) suggesting that di erentiation of the retina is

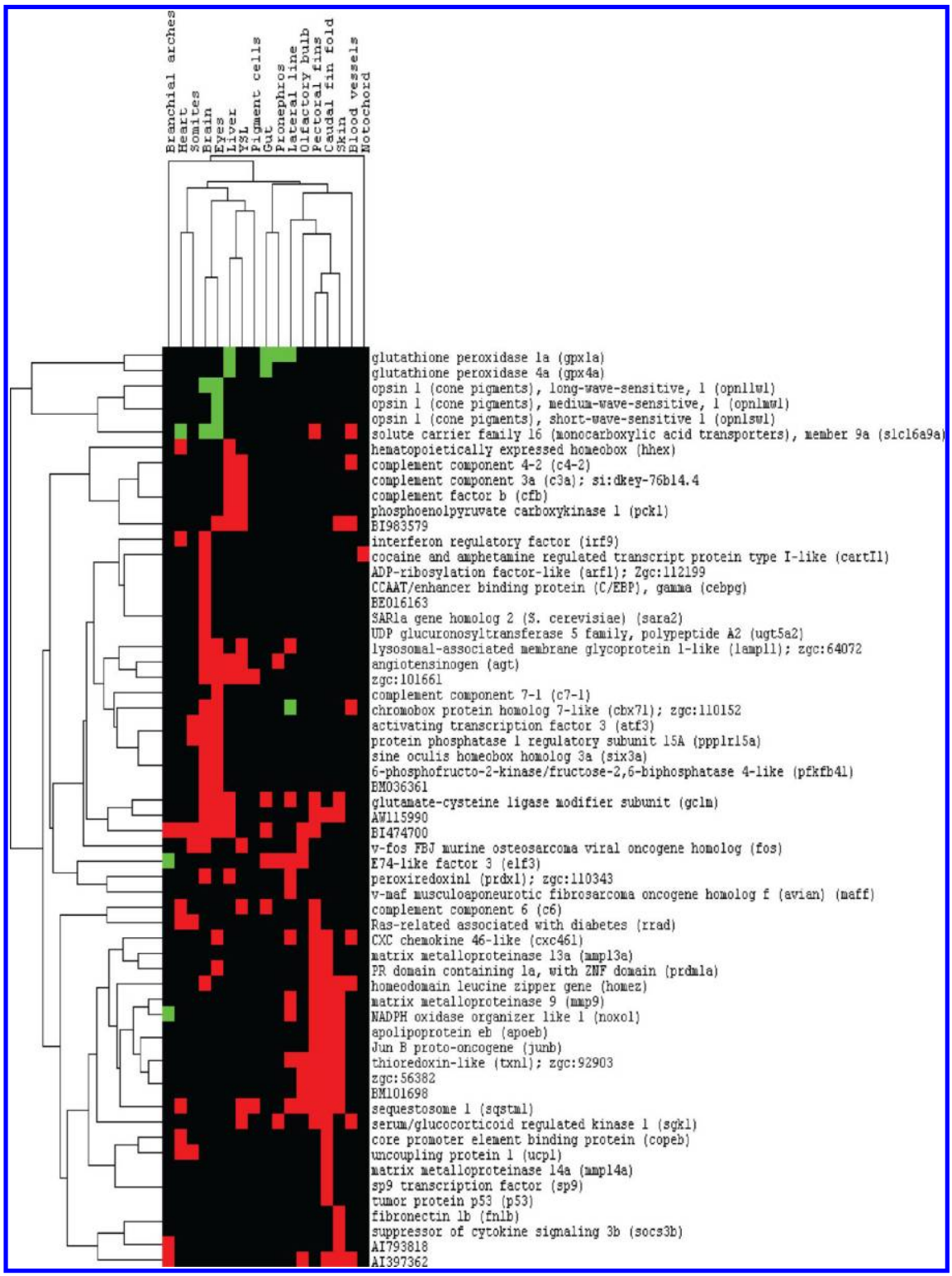

Figure 2. Hierarchical clustering of expression patterns of MeHg-regulated genes. The tissues scored are indicated at the top and the gene names are listed on the left. Red, up-regulation; green, down-regulation.

impaired by MeHg. *arf4*, *c4−2*, *c6*, *cbx7a*, *prdx1*, *txnl*, and *zgc:101661* showed signi cant changes also at 30 μg/L MeHg.

Genes Showing Ectopic Expression in the Brain of MeHg-Treated Embryos. MeHg is a known developmental neurotoxicant in humans.[1−3] We therefore focused our attention on MeHg-regulated genes that are expressed in the CNS of the zebra sh embryo. Among the 88 genes analyzed by in situ hybridization, 24 genes were speci cally regulated in the brain in response to MeHg exposure (Figure 2).

The level of mRNA for *activating transcription factor 3 (atf3)*, a leucine zipper transcription factor of the CREB family, was induced in discrete patches in the brain in MeHg-treated

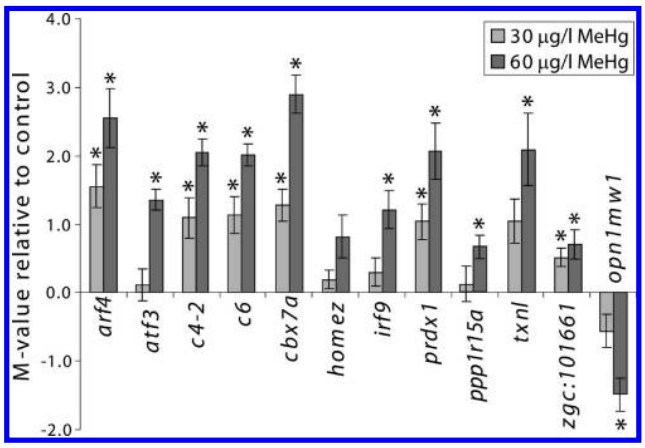

Figure 3. Changes in gene expression levels measured by quantitative real-time PCR. The changes in the expression levels of *arf4, atf3, c4–2, c6, cbx7a, homez, irf9, prdx1, ppp1r15a, txnl, zgc:101661,* and *opn1mwl* were determined by real-time PCR of cDNA from control and MeHg-treated embryos. Graph of M-value (log_2 of the fold change) relative to controls against MeHg concentrations is plotted. The mean ± SEM are presented. *t* test and Dunnett adjustment, $p < 0.05$ (*).

embryos (Figure 4A, A). Mammalian ATF3 responds to a wide variety of cellular stresses such as mechanically or toxicant-injured tissues, blood-deprived heart and postseizure brain,[44] and it protects hippocampal neurons from apoptosis.[45] The *cocaine and amphetamine regulated transcript protein type I-like (cartIl)* was weakly expressed in small groups or individual cells located bilaterally in the brain (Figure 4B). The level of *cart1l* transcripts was increased in these cells after MeHg exposure (Figure 4B, B , arrows). Mammalian Cart1 was ascribed a neuroprotective role.[46−48] The Polycomb related *chromobox protein homologue 7a (cbx7a)* was induced in an overlapping but di erent pattern in the brain in MeHg-treated embryos (Figure 4C, C , arrows). The mammalian homologue of *cbx7a* was previously shown to expand the life span of a wide variety of human cells.[49]

The expression of the transcription regulator *CCAAT/enhancer binding protein gamma (cebpg)* was elevated in the dorsal tectum by MeHg treatment (Figure 4D, D). *cebpg* expression was correlated with the expression of antioxidant gene expression in the human lung.[50] The transcription factor *FBJ murine osteosarcoma viral oncogene homologue (fos)* is an immediate early gene responding to cellular stress, growth factors and neuronal activity.[51,52] It was strongly induced in the brain of MeHg-exposed embryos (Figure 4E, E). Also the *glutamate-cysteine ligase modifier (gclm)* showed a general up-regulation in the brain in MeHg-treated embryos with stronger expression in the boundaries between forebrain/midbrain and midbrain/hindbrain and in the dorsal region of the tectum (Figure 4F, F). Gclm is a subunit of Glutamate-cysteine ligase, which together with glutathion synthetase maintains high glutathione levels in the cells as a major defense against oxidative stress.[53] Another protein involved in combating oxidative stress is *peroxiredoxin1 (prdx1)*.[54] The expression of *prdx1* was increased in the hypothalamus of the brain of MeHg-treated embryos (Figure 4G, G). The *protein phosphatase 1, regulatory subunit 15a (ppp1r15a)* was detected at higher levels in the tegmentum and rhombomeres of MeHg-treated embryos in comparison to controls (Figure 4H, H). The mammalian homologue of this gene responds to stressful conditions and its expression is correlated with apoptosis in mammals.[55]

Three developmental genes were also identi ed to be induced by MeHg treatment. The *homeodomain leucine zipper gene (homez)* was shown to act downstream of proneural genes in the control of neurogenesis in *Xenopus laevis*.[56] It was induced to varying levels in distinct regions throughout the brain by MeHg treatment (Figure 4I, I). However, its ectopic expression was correlated with major malformations of the embryo. While the mRNA of the transcription factor *interferon regulatory factor (irf9)* was barely detectable in control embryos, it showed strong expression in the brain of MeHg-treated embryos with stronger expression in the cells lining the ventricles (Figure 4J, J). In contrast to *irf9* and *homez* that showed ectopic expression in the brain, the expression of the homeobox transcription factor *sine oculis homeobox homologue 3a (six3a)* was increased in the normal domains of expression in the forebrain (Figure 4K, K). Together, the data from the three developmental genes suggests that MeHg directly or indirectly a ects the patterning of the brain and neurogenesis. In addition, we noted that in 60% MeHg-treated embryos (n = 3, 30 embryos examined in each experiment) the head was smaller than in controls (for example see Figure 4D, D , SI Figure 6). We thus examined the expression of genes involved in brain patterning including *sonic hedgehog* a (*shha*[57]), *paired box gene 6a* (*pax6a*[58]), *bone morphogenetic protein 2b* and *4* (*bmp2b, bmp4*[59]), *fibroblast growth factor 3 and 8* (*fgf3* and *fgf8*[60,61]), *wingless-type MMTV integration site family, member 1* (*wnt 1*[62]), *nestin*,[63] *wnt 5b*,[64] and *sox2* (Armant et al, in revision) and *crestin*,[65] and genes involved in neural transmission including *acetylcholinesterase* (*ache*[66]), *cannabinoid receptor 1* (*cb1*[67]) *serotonin transporter a* (*serta*[68]), *nuclear receptor-related factor 1* (*nurr1*[69]), and tyrosine hydroxylase (TH[70]). We did not detect signi cant deviations in the expression of these genes in MeHg-treated embryos relative to the controls (SI Figures 3 and 4). We also assessed whether cilia are a ected in MeHg treated embryos by staining with antiacetylated tubulin antibody. Cilia in the pronephros and the inner ear formed normally in MeHg-treated embryos (SI Figure 4I, I , J, J).

A number of a ected genes are directly or indirectly involved in transport processes. The expression of *ADP-ribosylation factor 4 (arf4)* was up-regulated by MeHg in scattered cells in the brain, presumably microglia cells (Figure 4L, L). The GTP binding Arfs belong to the Ras superfamily and are involved in the regulation of membrane tra c and organization of the cytoskeleton.[71] The expression of the vesicular marker *lysosomal-associated membrane glycoprotein 1-like (lamp1l)* was elevated especially in the epithelial lining of the brain ventricles (Figure 4M, M). In contrast, the *solute carrier family 16 member 9a (slc16a9a)* involved in transport of monocarboxylic acids showed a general decrease in the expression in the brain after MeHg treatment (Figure 4N, N). *UDP glucuronosyltransferase 5, polypeptide A2 (ugt5a2)* belongs to the phase II detoxifying family of Ugt enzymes that modify compounds by attachment of a glucuronic acid residue to facilitate destruction or secretion.[72] *ugt5a2* was up-regulated in the dorsal region of the brain of MeHg-treated embryos (Figure 4O, O). *The SAR1a gene homologue (sara2),* a small GTPase, which is presumed to be involved in transport from the Golgi apparatus,[73] showed moderate up-regulation in the brain of embryos exposed to MeHg (Figure 4P, P).

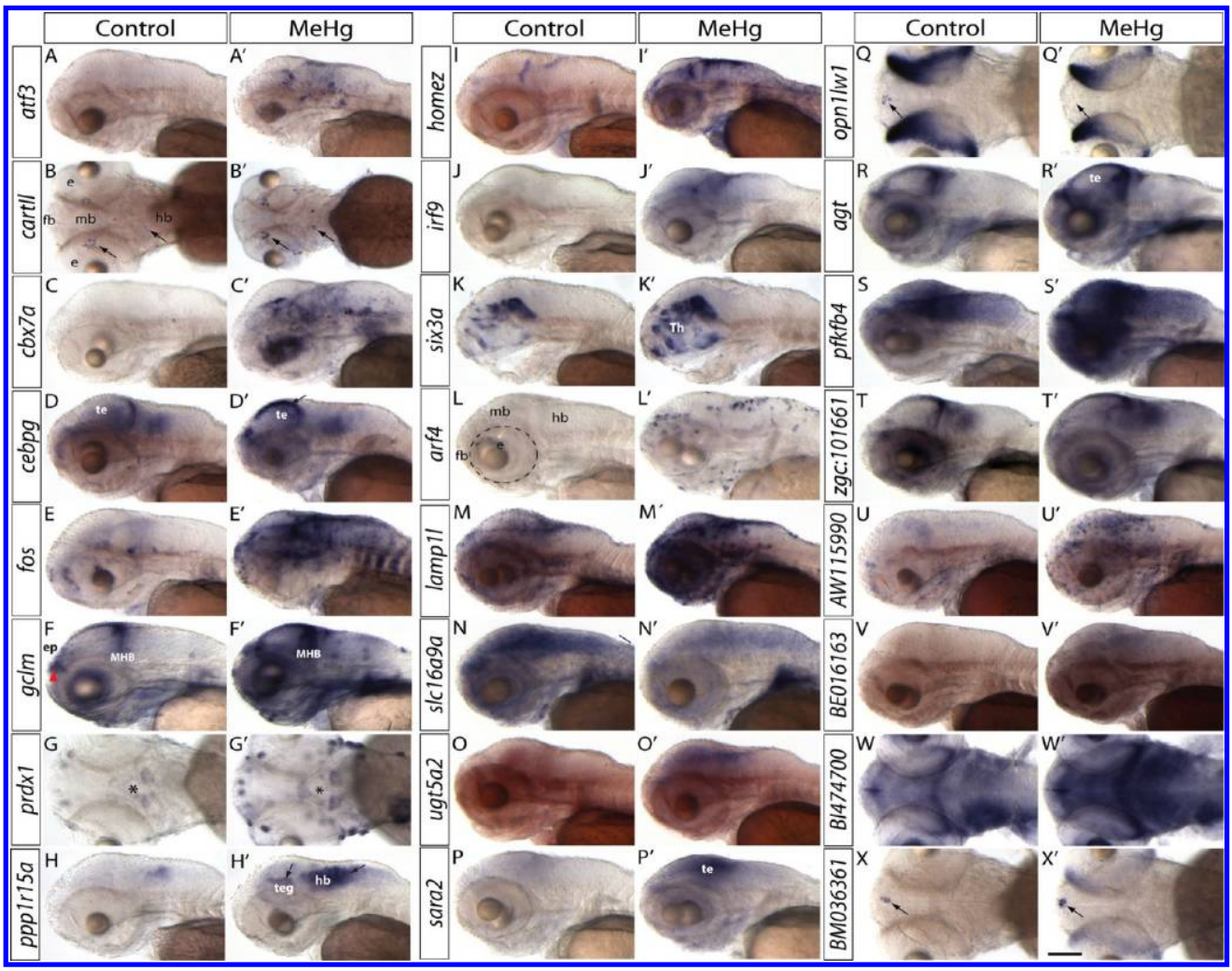

Figure 4. Changes in gene expression in the zebra sh brain after MeHg treatment. 72-hpf-old control (A to X) and MeHg-treated (A to X) embryos performed with in situ hybridization are shown. The dotted line in L indicates the position of the eye; the arrows in Q, Q , X, X mark the epiphysis. Up-regulations of *atf3* (A, A), *cartll* (B, B , the patches of cells with increased expression are indicated by arrows), *cbx7a* (C, C), *cebpg* (D, D), *fos* (E, E), *gclm* (F, F), *prdx1* (G, G , the asterisks indicate the hypothalamus), *ppp1r15a* (H, H), *homez* (I, I), *irf9* (J, J), *six3a* (K, K , eyes removed for better observation of the expression pattern in the brain), *arf4* (L, L), *lamp1l* (M, M), *ugt5a2* (O, O), *sara2* (P, P), *agt* (R, R), *pfkfb4l* (S, S), *zgc:101661* (T, T), *AW115990* (U, U), *BE016163* (V, V), *BI474700* (W, W), and *BM036361* (X, X), and down-regulations of *slc16a9a* (N, N) and *opn1lw1* (Q, Q) were observed in the brain of embryos exposed to MeHg. Abbreviations, e, eye; fb, forebrain; hb, hindbrain; mb, midbrain; ep, epiphysis; te, tectum; teg, tegmentum; Th, thalamus; MHB, midbrain-hindbrain boundary. Orientation of embryos: Lateral views with anterior to the left and dorsal side up (A, A , C, C - F, F , H, H - P, P , R, R - V, V), and dorsal views with anterior to the left and right side up (B, B , G, G , Q, Q , W, W , X, X). Scale bar, A, A - F, F , H, H - P, P , R, R - V, V , 100 μm, G, G , Q, Q , W, W , X, X , 80 μm.

The expression of *opsin 1 cone pigment long-wave-sensitive, 1 (opn1lw1)* was decreased in the epiphysis and in the retina after MeHg exposure (Figure 4Q, Q) suggesting that photoreceptor development is impaired in MeHg-treated embryos. The expression of *angiotensinogen (agt)* was slightly increased by MeHg treatment in areas of expression of control embryos such as the boundaries between forebrain/midbrain and midbrain/hindbrain and in the dorsal region of the tectum (Figure 4R, R). *6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4-like (pfkfb4l)* showed an increase in expression in the whole brain after MeHg treatment (Figure 4S, S). Five other genes with unknown identity (*zgc:101661*, *AW115990*, *BE016163*, *BI474700*, and *BM036361*) were also ectopically expressed in the brain after MeHg exposure (Figure 4T, T − X, X).

The genes up-regulated in the brain are involved in diverse biological processes and molecular functions, such as transcription regulation (*six3*, *atf3*, *homez*, *fos*, *cbx7a*, *cebpg*, *irf9*), developmental processes (*irf9*, *homez*, *six3a*) oxidative stress (*gclm*, *fos*, *atf3*, *prdx1*, *cebpg*), transport (*arf4*, *lamp1l*, *ugt5a2*, *sara2*, *slc16a9a*), immune response (*atf3*, *cebpg*, *irf9*, *prdx1*) and neuroprotection and cell survival (*cartIl*, *cbx7a*). Although a number of these genes are broadly up-regulated in the brain (*pfkfb4l*, *sara2*, *slc16a9a*, *W115990*, *BI474700*, *BE016163*), others show restricted patterns of expression, indicating that they are involved in region-speci c responses to MeHg exposure.

DISCUSSION

In this study, we combined transcriptional pro ling of MeHg-exposed embryos with a detailed in situ expression analysis. The aim was to examine tissue-speci c e ects of MeHg and in particular to identify changes in gene expression, which may

relate to the neurotoxic e ect of MeHg. The identi ed MeHg-regulated genes are involved in various biological processes and molecular functions, such as regulation of transcription, antioxidant defense, immune response, and transport. Altered expression of genes was noted in various organs including the brain, eyes, olfactory bulb, branchial arches, heart, liver, gut, pronephros, somites, lateral lines, pectoral ns, caudal n fold, blood vessels, skin, YSL pigment cells, and notochord. Many of the examined genes show tissue restricted expression. This indicates (1) that MeHg a ects many di erent tissues in the embryo and (2) that tissues respond with speci c gene responses. There were even di erences in the response in structures such as the caudal n and the pectoral n that are composed of similar cell types and are equally thin and exposed to the MeHg in the water. The expression of some genes was altered in either the pectoral n (*c6*, *gclm*, *rrad*, *slc16a9a*, *BI474700*) or the caudal n (*copeb*, *mmp14a*, *sp9*, *p53*, *ucp1*, *AI397362*) in response to MeHg. However, we found also similarities in the gene responses in some tissues. Prominent clustering of coexpression patterns was found in the liver and YSL as well as in the retina and brain. This suggests di erences, but also similarities, in the response to MeHg exposure in di erent tissues. In four cases, we found genes whose expression levels were decreased in some tissues and increased in others. In these cases, the microarray results of whole embryos scored the net change of expression. This underscores again that individual tissues can respond in a very speci c manner to MeHg exposure.

From the 88 genes analyzed by in situ hybridization, 24 genes showed speci c changes of expression in the brain of zebra sh embryos and are thus prime candidates for genes, which respond to or mediate the neurotoxic e ect of MeHg. These genes cover a wide range of biological and molecular functions, including transcription regulation such as *six3*,[74] *atf3*,[75,76] *homez*,[77] *fos*,[78,79] *cbx7a*,[49,80] *cebpg*[81] and *irf9*,[82] response to oxidative stress such as *gclm*,[83] *fos*[51,52] and *prdx1*,[84] apoptosis such as *agt*,[85] *atf3*[53] and *ppp1r15a*,[86] immune response such as *atf3*,[53] *cebpg*,[87] *irf9*[88] and *prdx1*,[54] cytokine production such as *cebpg*[89] and *irf9*[90] and blood vessel regulation such as *agt*[91,92] and *gclm*.[93] Some of the genes (*pfkfb4l*, *sara2*, *slc16a9a*, *AW115990*, *BI474700*, and *BE016163*) are expressed ubiquitously in the brain, others were restricted in their pattern of expression. This latter observation suggests regionally restricted responses in the brain to MeHg exposure. Given the known molecular interaction of MeHg with cellular processes and molecules such as sulfhydryl groups, the induction of oxidative stress and the disruption of calcium ion homeostasis,[17−24] many cell types should be a ected by MeHg. It is thus surprising that quite a number of the genes are induced in a regionally restricted manner in MeHg-exposed embryos. Thus, MeHg seems to a ect cells di erently leading to cell-speci c responses. In addition to regional di erences in the gene responses to MeHg exposure, unequal accumulation of MeHg[94,95] may contribute to these di erences.

Comparison with E ects Reported on Adult Zebra-sh. MeHg has been demonstrated to be hepatotoxic in adult zebra sh, rat and mummichog.[96,32,97] The increase in expression of antioxidant genes in the liver in MeHg-treated zebra sh embryos, such as *prx1*, *gclm*, and *uricase*, indicated that the liver su ered oxidative stress, which may be cause or consequence of hepatocellular damage.[98,85] Since the liver is an organ with a strong innate immunity,[99,100] reactive oxygen species may be also generated as an in ammatory response to tissue damage. In this context, it may be of relevance that we observed up-regulation of complement components *c3a*, *c4−2*, and *cfb* in the liver in response to MeHg exposure.

In a study by Gonzalez and co-workers,[33] the e ect of chronic exposure to MeHg was determined after dietary intake of MeHg for 7, 21, and 63 days. The changes in the expression levels of 13 genes known to be involved in antioxidative defenses, apoptosis, metal chelation, active e ux of organic compounds, mitochondrial metabolism were determined by real-time PCR. Although there were changes in the expression levels of some of the genes examined in the muscle and liver after MeHg exposure, no such changes were observed in the brain. In another study performed by Richter,[31] the acute e ect of MeHg exposure on the adult zebra sh brain was investigated by intraperitoneal injection of 0.5 μg MeHg/g body weight into female zebra sh. After 96 h, the sh were sacri ced for examination of gene expression levels using a 22K-probe microarray. Seventy-nine genes were shown to be up-regulated and 76 genes were down-regulated ($p > 0.01$) in response to MeHg. In contrast to the study performed by Gonzalez et al., but consistent with our measurements, they detected the up-regulation of oxidative stress and apoptosis genes in the brain after MeHg exposure.

A study of the visual system in adult zebra sh showed that MeHg could pass through the blood-retina barrier and accumulate in various regions of the retina, especially in the photoreceptor layer and in the inner and outer nuclear layer.[34] These observations are consistent with our data in the embryo that showed down-regulation of opsin expression in the developing retina. Taken together, our data derived from zebra sh embryos re ect the MeHg toxicity observed in adult zebra sh.

How Does MeHg Act As a Neurotoxicant? It was previously suggested that exposure of embryos to 10, 50, and 80 μg/L MeHg decreased cell proliferation in the neural tube.[101] However the authors of this previous study did not observe a dose-dependence, raising concerns whether the observed reduction in cell proliferation was indeed caused by MeHg. When exposing zebra sh embryos from 4 to 72 hpf to 60 μg/L MeHg, we did not observe changes in the overall pattering of the brain when probing with a number of developmental regulators (*shha*, *pax6a*, *crestin*, *nurr1*) and genes involved in neurotransmission (*cb1*, *serta*, TH). The induction of the developmental regulators *irf9*, *homez*, and *six3a* may thus be related to local repair processes rather than to a disturbance of the overall pattering of the brain. This suggests that the overall patterning of the brain is not signi cantly a ected. The e ects of MeHg on the developing nervous system may, however, be too subtle to be detected by overall morphological analysis. Clearly, the heads of MeHg-exposed embryos were smaller. We found increased or ectopic expression of a number of genes whose function is related to oxidative stress, transport and transcription regulation. It is intriguing that some of these genes (*c-fos*, *cebpg*, *atf*, *socs3*) are induced by cAMP in mammalian embryos.[102−106] In light of the view that the cAMP pathway and downstream genes like *c-fos* are involved in learning and memory in mammals,[107,108] it is tempting to speculate that MeHg-induced cellular stress may a ect the development and function of the nervous system by aberrantly inducing these regulators of brain function. This disturbance may account for the mental de cits seen in humans exposed during the prenatal period to low doses of MeHg.

ASSOCIATED CONTENT

* Supporting Information

Additional information including tables and gures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing nancial interest.

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