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Ancestral Dvelopmental Exposure to Methylmercury Induces Transgenerational Inheritance of Visual and Neurobehavioral Deficits

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ANCESTRAL DVELOPMENTAL EXPOSURE TO METHYLMERCURY INDUCES TRANSGENERATIONAL INHERITANCE OF VISUAL AND NEUROBEHAVIORAL DEFICITS

by

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ABSTRACT
ANCESTRAL DVELOPMENTAL EXPOSURE TO METHYLMERCURY INDUCES TRANSGENERATIONAL INHERITANCE OF VISUAL AND NEUROBEHAVIORAL DEFICITS

by

Thomas A. Kalluvila

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Methylmercury (MeHg) is an environmental neurotoxicant of global concern. It is considered one of the top ten chemicals of public health concern by the World Health Organization. Prenatal exposure to MeHg has been associated with altered neurodevelopment, neurobehavioral and neurocognitive functions. The effects of low dose MeHg exposure are more subtle and can range from impaired motor function to sensory defects. Using quantitative neurobehavioral assays and zebrafish as a model organism, our laboratory has demonstrated that developmental MeHg exposure causes neurological dysfunction in adult zebrafish. Recently, a wide range of environmental insults (*e.g.*, pesticides, fungicides, plasticizers and endocrine disruptors) has been shown to induce disease phenotypes in individuals whose great grandparents were exposed to the toxicant. This phenomenon is known as transgenerational inheritance. To date, studies have shown that in transgenerational inheritance of diseases due to

developmental exposure, heritable changes occurred in the epigenome not the genome and were transmitted to subsequent generations without further exposure. Based on this evidence, we hypothesized that ancestral developmental exposure to MeHg induces transgenerational neurobehavioral deficits in F3 generation zebrafish. The F1-generation zebrafish embryos (less than 4 hour post fertilization) were exposed to either 0, 0.001, 0.003, 0.01, 0.03 or 0.1 µM MeHg for 24 hrs. These concentrations of MeHg are sublethal and environmentally relevant. Quantitative neurobehavioral assays for visual startle response and locomotor activity were used to assess MeHg-induced neurotoxicity in F3 generation. Our study demonstrated that developmental exposure to MeHg induces transgenerational visual deficits and locomotor dysfunctions in zebrafish. Altered retinal electrophysiology was also observed in the transgenerational population with visual deficits. To identify the genes and pathways involved with the phenotypes observed in the transgenerational population, we analyzed the whole transcriptome of the brain and retina of the transgenerational lineage animals, using RNAseq. Tissue specific altered gene expression was observed in both brain and retina. This is the first evidence of a transgenerational transcriptome induced by ancestral developmental exposure to MeHg. Gene set enrichment analysis revealed the correlation between dysregulated functional pathways and the observed phenotypic variation, including vision, phototransduction, motor activity, and retinal electrophysiology. Our studies also identify that the mode of germline transmission varies between the transgenerational phenotypes. This research has identified new mechanisms

associated with MeHg-induced phenotypes which may have significant impacts on public health, in terms of developing biomarkers to identify susceptible populations and developing preventative measures. The long term effects of MeHg observed in this study could be used to improve the awareness of reproductive age group women to monitor the type of fish that they consume. Since we observed the neurobehavioral deficits in a fish species, our findings have ecological impacts including the feeding behavior of fish, survival and reproduction. The findings made in this thesis also set the stage for future research into the identification of new transgenerational phenotypes associated with ancestral developmental exposure to MeHg.

Dedication

In memory of my grandfather,

Yohannan Oommen

(1914-2006)

I am dedicating my dissertation to my beloved grandfather. One of the best things that happened in my life was the opportunity to spend my first 20 years of life with him. It gave me the opportunity to learn life in India before and after independence. The struggle that my ancestors had to go through just to have a simple life.

He lost his wife in his late 30s and raised his six children as a single parent. He dedicated rest of his life to his children and grandchildren. He taught me the basics of agriculture, carpentry, mathematics and astronomy. I still remember the days that we both wake up 4’o clock in the morning and start milking cows. He taught me how to face life and encouraged to embrace learning to improve life.

I lost him on the very first day I started my Ph.D. program at ZSPH. The inspiration that he had given me helped me to navigate the ups and downs of my academic journey. As I am ending this chapter of my life, I hope he would be proud of me.

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One of the advantages of my academic progress was the support and guidance of brilliant committee members. I thank Dr. Niladri Basu for helping me with the analysis of my samples in his lab and for the creative support for helping me to understand my research topic at a broader level. Thank you Dr. Michael Laiosa for serving not only as a committee member but also as an academic guide throughout the program. Thank you Dr. Kurt Svoboda for helping me in designing my experiments, helping as graduate program coordinator and serving as a great mentor. Thank you Dr. Peter Tonetallo for allowing me to be a part of your lab and learn and apply complex bioinformatics tools and your guidance in understanding the meaning of molecular data. Thank you Dr. Todd Miller to be in my committee for the preliminary examination.

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CHAPTER 1

DISSERTATION INTRODUCTION

I. Atmospheric emission of mercury

Heavy metals such as cadmium (Cd), lead (Pb) and mercury (Hg) and the metalloid arsenic (As) are among the top ten chemicals or groups of chemicals of global public health concern identified by the World Health Organization (WHO 2013). There are 92 naturally occurring metals and about 30 of them have toxic effects on humans. The neurotoxic effects of Cd, Pb, As, and Hg have been well documented (Wang and Du 2013; Mason et al. 2014; Bartrem et al. 2014; Ishi and Tamaoka 2015; Rodríguez-Barranco et al. 2015). Humans have been using these metals for thousands of years. However, industrialization and globalization have caused the emission of atmospheric toxicants to reach an all time high in the history of this planet (Izatt et al. 2014).

Glacier ice core studies have revealed that the environmental release of metals have greatly accelerated during the past century (Schuster et al. 2002; Barbante et al. 2004). Hg is released into the atmosphere through both natural and anthropogenic activities. Anthropogenic activities contributed 52% of Hg identified in the Upper Fremont Glacier in the Wind River mountain range of Wyoming during the past 270 years. However, human activities account for 72% of Hg released during the past century, a 20-fold increase from the preindustrial era (Fig 1.1) (Schuster et al. 2002). Human activities that cause environmental emission of Hg include artisanal and small scale gold production, coal combustion, the chlor-alkali industry and the cement industry. Artisanal and small scale gold production is the leading source of anthropogenic Hg (37%) followed by coal burning (24%) (Fig 1.2)(UNEP 2013). It is estimated that up to 15 million

people are working in the poorly-regulated artisanal gold mining sector including 3 million women and children around the world (UNEP 2012).

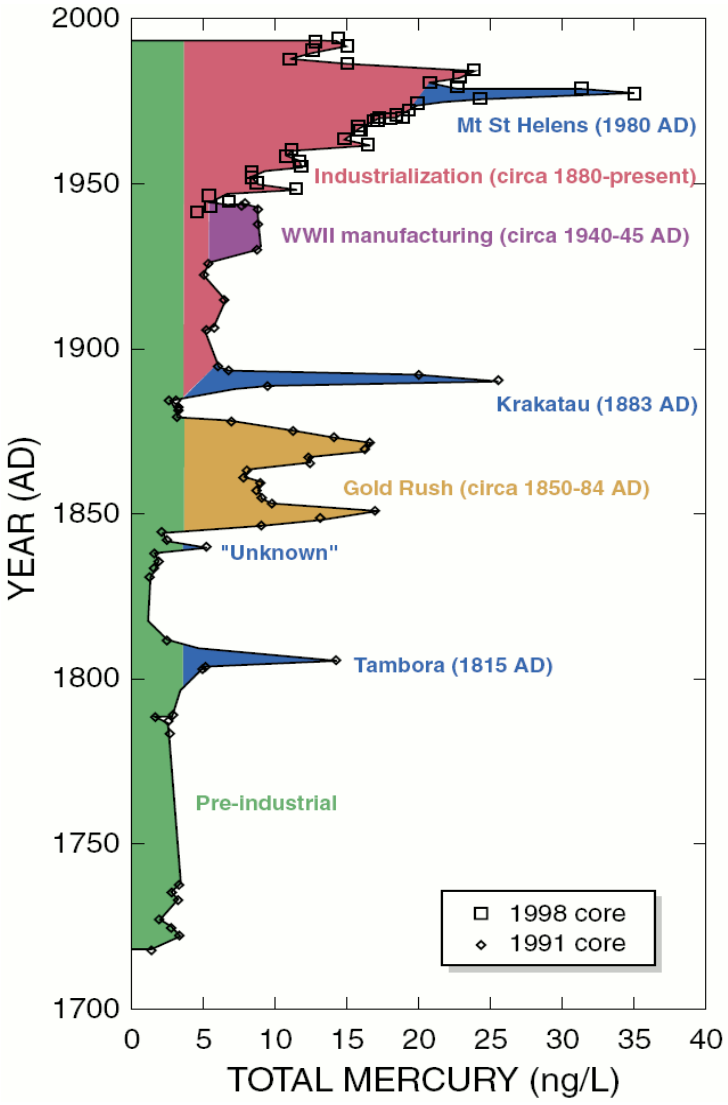

Figure 1.1. Atmospheric Hg deposition analysis. The patterns of mercury deposition in the ice core samples were tested. Both natural (volcanoes) and anthropogenic activities attribute the emission of Hg. Industrialization era significantly contributed the emission of Hg to the environment indicating the human activities as the primary source of environmental contamination of Hg (Image courtesy Schuster et al. 2002).

Anthropogenic activities along with natural emission of mercury have accelerated the burden of MeHg in the environment. Climate change has impacted the transport pathways, speciation and cycling of mercury within Arctic ecosystems and resulted in increased emission of Hg to the environment and bioaccumulation of MeHg in fish species (Booth and Zeller 2005; Stern and MacDonald 2005; Leitch et al. 2007). Projections generated using the global climate-chemistry model system have estimated that atmospheric Hg levels in the U.S. will significantly increase by 2050 due to the combined effect of human activities and climate change (Lei et al. 2014). The model also predicts that a domestic reduction of Hg emission could mitigate the effects of climate change in the U.S. However, regulations in other parts of the world are also required in global reduction of environmental Hg emission and related adverse health outcomes in different ecosystems and humans.

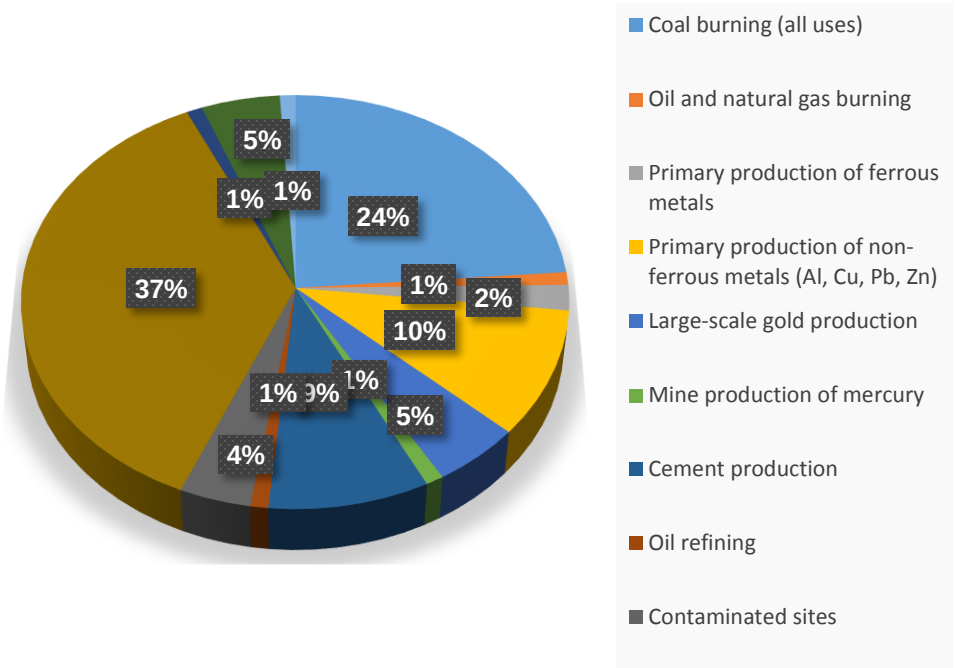

Figure 1.2. Sources of environmental mercury emission. Artisanal gold mining and coal combustion are the major anthropogenic sources of mercury in the environment. The mercury emission in 2010 was estimated at 1960 metric tons due to human activities (*Modified from* UNEP 2013).

II. Human exposure and health effects of Hg

Mercury exists in metallic, inorganic, and organic forms. Like other metals, mercury enters the food chain through the atmosphere, soil, and water. Once mercury enters water or soil, under anoxic conditions, bacteria convert inorganic mercury to the more highly neurotoxic form, methylmercury (MeHg) (Batten and Scow 2003). MeHg enters the food web through the consumption of the mercury converting bacteria by zooplankton (Kainz et al. 2006). The consumption of plankton results in the accumulation of MeHg in fish species (Rolfhus et al. 2011), with higher levels of MeHg accumulating in larger carnivorous species (A Kehrig et al. 2009) leading to bioaccumulation. Bioaccumulation, the accumulation of a chemical in an organism, of MeHg in predator fish has been associated with the amount of food consumed each year (Donald et al. 2015). MeHg also biomagnifies along the food chain as the concentration of this pollutant exceeds the background levels in higher-level predators (Lavoie et al. 2013; Cardoso et al. 2014). In humans, virtually everyone has accumulated some level of mercury in their tissues primarily through the ingestion of MeHg contaminated fish (Ström et al. 2011). Human populations whose diet contains a significant amount of fish tend to bioaccumulate this toxicant and suffer increased health risks (Buchanan et al. 2015).

Development is the life-stage most sensitive to MeHg toxicity. As the “developmental origins of health and diseases ” hypothesis suggests, the

environment during development plays a significant role in the health of a person later in life (Barker 1990). The adverse health effects of developmental exposure to MeHg were reported as early as 1865 with symptoms including ataxia, tunnel vision, altered facial sensation, loss of coordination and deafness (Grandjean et al. 2010). The two major outbreaks of MeHg poisoning in Minamata (1956) and Iraq (1971) revealed the public health impacts of this environmental toxicant in humans (Greenwood 1985; Harada 1995). The Minamata outbreak was the first evidence of the multigenerational effect of MeHg due to developmental exposure. Congenital Minamata disease. developed in children due to intrauterine exposure to MeHg (Harada 1978). There were no abnormalities observed in either mothers or fetuses during pregnancy. The clinical symptoms started to develop at the beginning of 6^{th} month after birth in those children prenatally exposed to MeHg. Children born after 1955 (Minamata outbreak) developed neurodevelopmental and neurobehavioral disorders such as instability of neck, convulsions, primitive reflex and intelligence disturbance (Harada 1978).

Minamata outbreak revealed the public health impacts of developmental toxicity of MeHg. Neurotoxic effects of environmental and occupational exposure to MeHg have been reported around the world during the past decades (Ohlander et al. 2013; Mostafazadeh et al. 2013; Peplow and Augustine 2014; Gibb and O'Leary 2014; De Miguel et al. 2014). It has been well established that prenatal exposure to high doses of MeHg can lead to widespread brain damage and impaired neurological development resulting in defects ranging from severe cerebral palsy and mental retardation to impaired motor and sensory functions.

The effects of low dose MeHg exposure are more subtle and can range from impaired motor function and sensory defects to slight deficiencies in learning and memory.

In 2001, the EPA set the reference blood level for maternal mercury at 5.8µg/L, the concentration below which the detrimental effects of Hg are expected to be minimal (including sensitive populations). However, Hg concentration in umbilical cord blood is 1.7 times higher than the maternal blood (Stern and Smith 2003; Rice 2004). Given the amplification of Hg in cord blood, the safe level for Hg in maternal blood is 3.5µg/L. Thus, maternal blood concentrations of 3.5µg/L actually result in embryos exposed to 5.95µg/L MeHg. Studies have shown that the fetus is exposed to nearly twice the mercury compared to maternal blood levels through placental transfer and accumulation in cord blood (Ou et al. 2014). In the U.S, it is estimated that 1 to 3 percent of women of child bearing age are at risk from MeHg exposure (EPA 2014). Studies have also shown that Hg levels in the fish species of Great Lakes are also on the rise resulting in wide-spread advisories on fish consumption (Bhavsar et al. 2011; Evers et al. 2011; Wiener et al. 2012). In support of these data, recent analysis of blood mercury concentrations of newborns from the U.S. Lake Superior Basin showed that 8% of newborns had blood mercury levels above the reference dose (5.8µg/L) and 1% of newborns had mercury levels above 58µg/L (Mercury Levels in Blood from Newborns in the Lake Superior Basin GLNPO ID 2007). The environmental burden of Hg is on the rise and the adverse health impacts of

developmental exposure to MeHg have been identified in different species including humans.

III. MeHg induced neurobehavioral deficits and zebrafish model

Prenatal exposure to MeHg results in significant neurodevelopmental deficits in neonates and these deficits can be observed as neurobehavioral dysfunctions later in life (Boucher et al. 2014; Obi et al. 2015; Jacobson et al. 2015). A "behavior" is defined as anything an organism does involving action and response to stimuli; it requires the ability to perceive the stimulus as well as the ability to respond appropriately (Anholt and Mackay 2010). A reaction to a stimulus involves complex biological processes including stimulus transduction, transmission, integration and response. Behavioral response to a stimulus depends on factors such as genetics, development and functional properties of organs involved in stimulus perception and processing, social, psychological and environmental conditions. Neurotoxicants alter the normal structure or function of the nervous system and these changes can often be observed as changes in behavior (Tierney 2011). Quantitative neurobehavioral assays are extremely useful and sensitive indicators of neurotoxicity as the chosen endpoints require functional integration of all nervous system components, while the analyses do not require an understanding of the underlying neurobiological mechanisms of toxicity (Tierney 2011)). High level exposure to neurotoxicants tend to cause devastating effects to motor and cognitive functions which are easily identified, but the effects of low level exposures are much more difficult to recognize as

they tend to result in subtle changes to normal cognition or behavior. A powerful, well-established animal model is necessary to orchestrate appropriate experimental design to identify subtle neurobehavioral changes and the mechanistic underpinnings.

Zebrafish is a powerful model organism for human developmental and disease research due to similarity in physiological, morphological, and genetic characteristics with humans (Bibliowicz et al. 2011). Zebrafish has also be used in neurodevelopmental and behavioral studies (Tierney 2011; Nishimura et al. 2015). Our laboratory has been using zebrafish as a model organism to elucidate the neurobehavioral effects of exposure to toxicants such as ethanol (Carvan et al. 2004) and MeHg (Smith et al. 2010; Xu et al. 2012); and gene expression changes due to dioxin (Liu et al. 2014) and MeHg (Liu et al. 2013) . Our laboratory also identified the neurotoxic effect of direct developmental exposure to MeHg by measuring visual startle response in adults (Weber et al. 2008), demonstrating the zebrafish equivalent of the “fetal origins of adult disease”. The study describes significant visual deficits due to developmental exposure to MeHg with potential linkage to altered retinal electrophysiology. Based on our previous knowledge and expertise with the visual startle response assay as a tool to measure neurobehavior, we further investigated the potential of developmental exposure to MeHg to induce neurobehavioral deficits in subsequent generations of zebrafish without additional exposure, a phenomenon known as transgenerational inheritance. Zebrafish have been identified as one of the best vertebrate model systems for studies involving environmental toxicant

induced transgenerational onset of diseases due to its short generation time (Baker et al. 2014) and well defined genome and epigenome sequences.

IV. Transgenerational inheritance of phenotypes

A phenotype is simply an observable characteristic (physical, biochemical, physiological or behavioral) exhibited by an individual. If direct exposure to a toxicant influences a phenotype in multiple generations (e.g. child and grandchild, or F1 and F2), it is defined as a multiple generation phenotype. In contrast, a transgenerational phenotype requires transmission of the phenotype in the absence of direct exposure (e.g. great grandchild, or F3) (Skinner 2014). Recently, a number of environmental toxicants such as vinclozolin (Guerrero-Bosagna et al. 2012) and DDT (Skinner et al. 2013) have been shown to cause heritable changes in DNA function without changing the DNA sequence, known as epigenetic changes. The altered epigenetic information is inherited through the germline in the absence of direct environmental exposure and leads to phenotypic variation. This type of phenotypic inheritance in subsequent generations without direct exposure is known as transgenerational inheritance (Fig 1.3).

Mutational genetic changes such as additions or deletions in the coding or transcriptional regions of the genome could lead to altered physiology and to disease phenotypes. However, less than 1% of human diseases are associated with changes in DNA sequence. The incidence of majority of the diseases are not

associated with random genetic mutations. We also know that social and environmental factors are associated with disease etiology without manipulating DNA sequence. Recently, an exponential growth in the incidence of health conditions such as diabetes and cardiovascular diseases without any specific triggering factors has been observed in different human subpopulations. The lack of genetic change and onset of disease phenotypes without any specific identifiable cause directs the recent research efforts toward epigenetic transgenerational inheritance (Skinner 2008). Life style factors such as stress, nutrition, alcohol and smoking are associated with transgenerational inheritance of diseases (Matthews and Phillips 2012; Nilsson and Skinner 2015). Developmental exposure to environmental pollutants such as pesticides, bisphenol A, dioxin and hydrocarbon compounds have also been associated with the onset of adult diseases and their transgenerational inheritance (Manikkam et al. 2012). Mounting evidences of transgenerational onset of different diseases are emerging including cryptorchidism (Chen et al. 2015), metabolic diseases (Stegemann and Buchner 2015) and respiratory diseases (Krauss-Etschmann et al. 2015).

The dynamic nature of development leads to critical periods of genetic and epigenetic susceptibility. Primordial germ cells undergo epigenetic reprogramming during fetal gonadal determination (Skinner et al. 2010). Alteration of the epigenome at this time can lead to their transmission to subsequent generations resulting in transcriptomic and physiologic alterations without direct exposure to the environmental factor—transgenerational

inheritance. Given the fact that direct developmental exposure to MeHg induces neurobehavioral deficits, this thesis investigates the potential of developmental exposure to MeHg to induce transgenerational inheritance of neurobehavioral deficits.

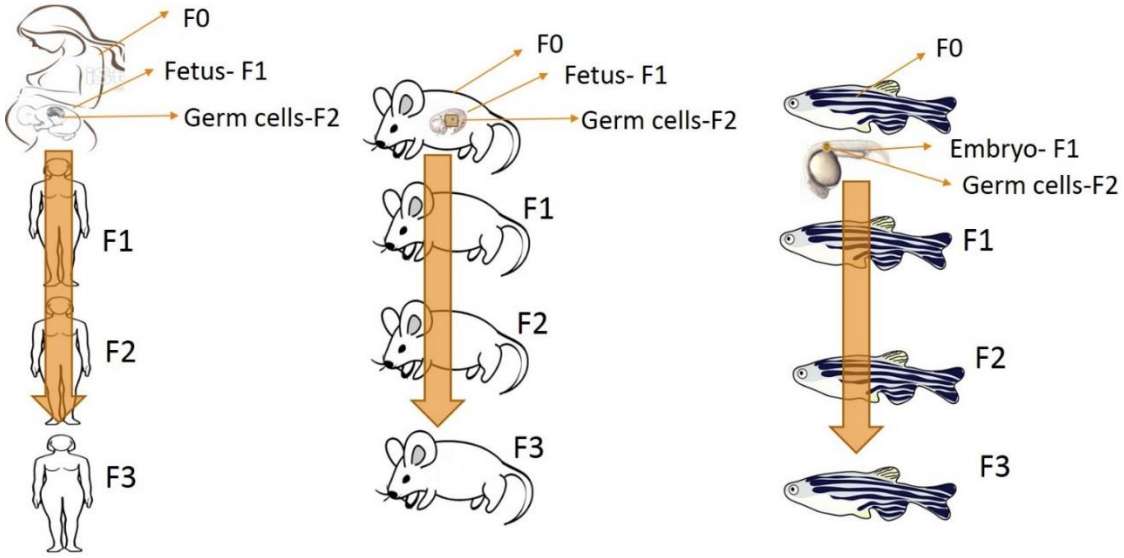

Figure 1.3. Schematic comparison of transgenerational inheritance between different species. A transgenerational phenotype requires the absence of a direct exposure and onset of a phenotype to minimally the F3 lineage. Both F1 organism and F2 primordial germ cells are directly exposed. In Mice and humans, exposure happens *in utero*. In zebrafish, developing embryos are exposed to MeHg externally.

In mammals, prenatal exposure results in direct effects on both F1 and F2 generations (Fig 1.3). In zebrafish, embryogenesis occurs outside of the body. This provides an excellent opportunity to closely monitor the developmental stages and physiological variations. Our early exposure paradigm with persistent chemicals in zebrafish mimics mammalian maternal transfer to the fetus in that lipophilic persistent chemicals first accumulate in the yolk and the embryo receives a measured dose throughout development as it utilizes the yolk as an

energy source. To keep nomenclature parallel to that of humans and laboratory mammals and to reflect the parallel with placental transfer, zebrafish embryos that are directly exposed to MeHg are considered as the first (F1) generation. The second generation (F2) fish develops from the germ cells of the embryo, which were directly exposed to MeHg. The third generation (F3) is the first generation that was not directly exposed to MeHg but inherited the altered physiology from the MeHg exposed first generation. Studies have shown that the inheritance of phenotypic variations occur in F3 generations and permanent alteration in DNA methylation of the germlines is one of the mechanisms identified in such inheritances (Guerrero-Bosagna et al. 2012; Skinner et al. 2013; Manikkam et al. 2014; Baker et al. 2014).

V. Role of DNA methylation in transgenerational inheritance

Genetic and epigenetic molecular mechanisms play key roles in the transmission of phenotypes to offspring. Epigenetic changes cause alterations in DNA function without changing the underlying DNA sequence. Three major molecular mechanisms of epigenetics have been identified as DNA modification, histone modification and RNA associated gene silencing through small and large non coding RNAs (Egger et al. 2004; Holoch and Moazed 2015). In eukaryotes, DNA methylation is the addition of a methyl group to the cytosine bases of DNA mediated by the enzyme DNA methyltransferase (Dnmt). The methyl group is methyl group is removed from the universal methyl donor S-Adenosylmethionine (SAM). Methylation of the cytosines in CpG islands in the promoter regions of

genes limit the access to transcription factors resulting in altered phenotypes (Fig 1.5). Hypermethylation, methylation above the normal level, prevents the transcription control proteins to access gene enhancers which will result in the dysregulation of gene expression (Suzuki and Bird 2008). DNA methylation usually occurs at specific CpG dinucleotides throughout the genome and is intimately involved in the control of gene expression. Methylation and demethylation at different sites along the whole genome occur to maintain normal cellular functions. The methylation pattern established in early embryogenesis is important in setting up the structural integrity of the genome and plays an important role in gene imprinting, gene transcription, normal cellular function, and the development of cells (Suzuki and Bird 2008b).

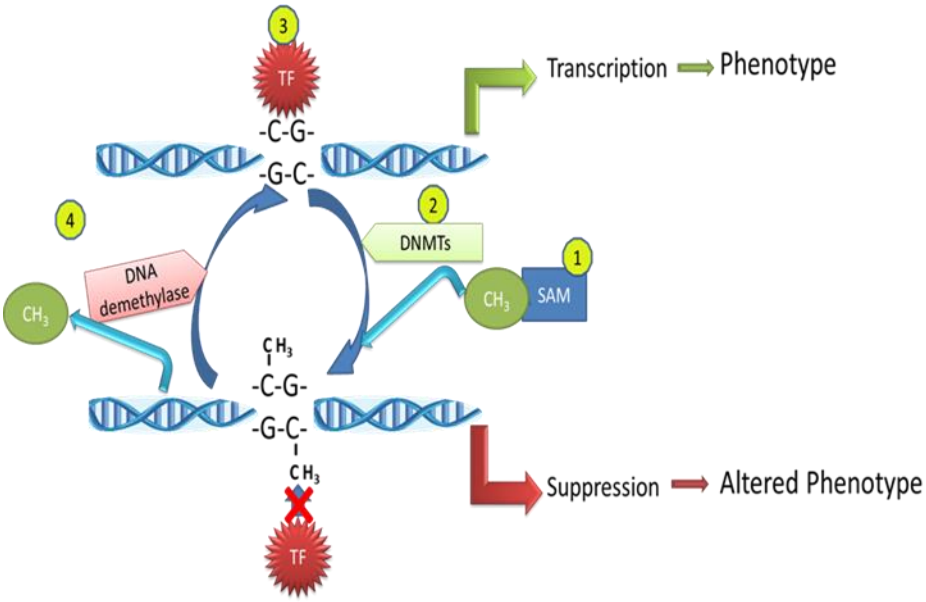

Figure 1.4. Role of DNA methylation in phenotypic variation. Cytosine nucleotides in the CpG islands of the promoter regions are normally unmethylated in the actively transcribed genes. DNA methyltransferases (DNMTs) transfer methyl group (-CH3) from SAM to cytosines to form 5-methylcytosines. DNA demthylase converts 5-methylcytoine to cytosines by removing the -CH3 group. Hypermethylation of the promoter region prevents access to transcription factors (TF) resulting in altered transcription and

phenotype. Numbers 1-4 indicate the major steps at which MeHg could influence the DNA methylation process.

Developmental exposure to toxicants such as Pb (Faulk et al. 2013; Li et al. 2015) and BPA (Goodrich et al. 2015; Nahar et al. 2015) are associated with alteration of DNA methylation patterns in humans. DNA methylation has been identified as one of the molecular mechanisms behind the transgenerational inheritance of disease phenotypes due to environmental exposure to toxicants (Skinner et al. 2013a; Skinner et al. 2013b; Manikkam et al. 2014). Exposure to environmental factors during fetal development could alter DNA methylation patterns and set the stage for adult disease susceptibility. Exposure to environmental factors during the critical window of primordial germ cell development could create permanent, heritable changes in the patterns of DNA methylation.

Environmental chemicals and their metabolites limit the availability of methyl donors such as S-adenosylmethionine (SAM) (Selhub 2002; Davis and Uthus 2004). The reduced availability of SAM is often associated with the depletion of glutathione (GSH) used in the detoxification pathway of chemicals and results in the alteration of DNA methylation pattern. Alteration of the methylation pattern in the promoter region of genes could lead to altered phenotypes through transcriptional modification and disruption of cellular and molecular pathways (Fig 1.4) (Irvine et al. 2002). Recently, DNA methylation changes in low density CpG regions known as “CpG deserts” located outside of the promoter regions of genes have also been associated with regulation of gene expression (Skinner

and Guerrero-Bosagna 2014). MeHg has been associated with alteration in DNA methylation in mammals including humans, rodents, and polar bears (Pilsner et al. 2010,Basu et al. 2014). Prenatal exposure to mercury in humans has also been associated with an altered epigenome and neurobehavioral outcomes (Maccani et al. 2015; Bakulski et al. 2015; Cardenas et al. 2015) Neurobehavioral deficits in rodents induced by perinatal exposure to MeHg are associated with hypermethylation of the promoter region of the brain-derived neurotrophic factor (BDNF) gene and the down regulation of this gene (Onishchenko et al. 2008). Given the potential of MeHg to induce transgenerational phenotypes, it is important to investigate the potential of MeHg to induce inheritable changes in DNA methylation.

VI. Linkage between MeHg exposure and gene expression

As previously stated, MeHg is associated with dysregulation of molecular pathways that are involved with neurodevelopment and neurobehavior. In zebrafish, developmental MeHg exposure results in the dysregulation of numerous genes involved in cellular homeostasis, oxidative stress responses, cell protection, and transport mechanisms (Ho et al. 2013). Brain development involves the expression of critical genes at specific embryonic stages that are associated with neural migration, neuronal growth and differentiation. *In utero* exposure to low levels of MeHg has been associated with the suppression of Rac1, Cdc42, and RhoA genes involved in the migration of cerebrocortical neurons during the early stage of brain development (Guo et al. 2013). The

evidence suggests that MeHg induces phenotypic variation by altering the expression of genes in critical pathways involved in neural development. Transgenerational inheritance of such phenotypic variations involve the transmission of altered epigenetic marks resulting in changes in the transcriptome (Fig 1.5) (Skinner et al. 2012; Skinner et al. 2013b). It is important to investigate whether MeHg can induce transgenerational neurobehavioral phenotypes and identify the differential expression of genes involved in the variation of phenotypes. The functional properties and the global roles of the differentially expressed genes are better elucidated by pathway analyses.

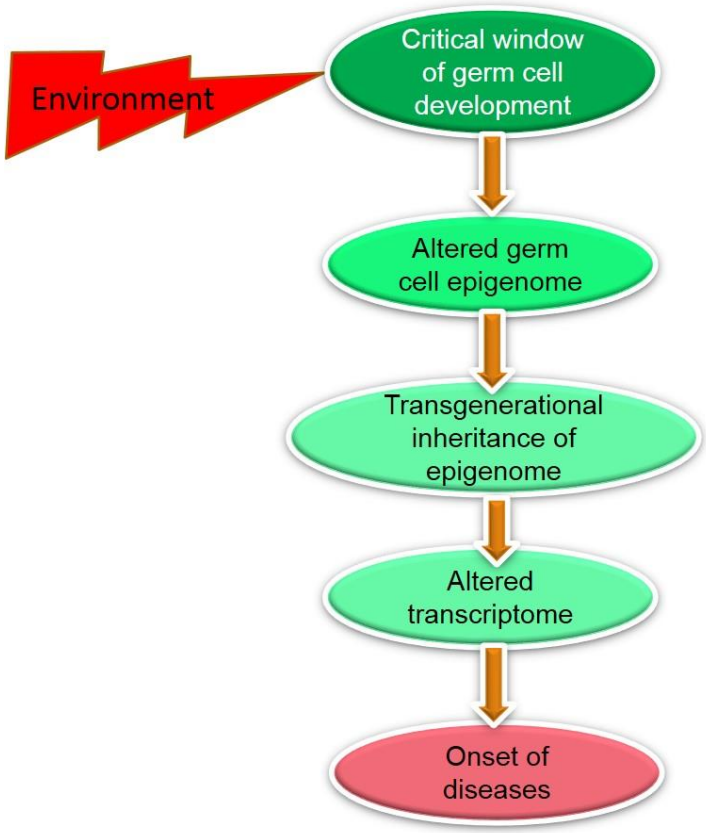

Figure 1.5. Transgenerational onset of disease etiology. DNA methylation pattern of germ cells is altered by direct exposure to environmental toxicants, these epigenetic mutations act like imprinted genes and are passed on to the offspring. Imprinted DNA methylation sites escape the genome-wide DNA methylation erasure events that occur during gonadal development and early embryogenesis. Therefore, these epimutations become programmed into the genome and can cause transgenerational onset of changes in gene expression leading to disease phenotypes in offsprings who inherit the epigenetically altered allele.

VII. Rationales of studies of the dissertation

The environmental burden of mercury has been steadily increasing due to human activities. Growth in the world population and the rise of a global middle class have accelerated the demand for gold and electronic items. Mercury-containing electronic waste (e-waste) is on the rise, and the recycling process for electric and electronic wastes that contain mercury has significantly increased

the re-emission of this toxicant into the environment (Ni et al. 2014; Lau et al. 2014; Julander et al. 2014). Mercury emission is directly linked to significant ecological and health impacts in geological areas close to gold mining and e-waste recycling sites. Mercury is transported by atmospheric processes around the globe, so a local contamination issue becomes a global health concern. For instance, species in arctic regions, far from any significant mercury source, have a high body burden of mercury (Pilsner et al. 2010).

The major source of mercury in the US is coal-fired power plants (US EPA). Even though, coal-fired power plants in the U.S. are showing a steady decrease in mercury emission, the environmental burden is predicted to rise due to increased global emission levels and changes in climate (Lei et al. 2014). Increases in atmospheric temperatures accelerate the mercury cycle and increase human exposure. In the U.S., humans are primarily exposed to mercury in its organic form, MeHg, through fish consumption or the use of fishmeal in the animal feed industry. Most women of reproductive age in the U.S. have low levels of mercury in their blood and, therefore, the risk to their offspring is minimal. However, there are subpopulations with an increased risk due to elevated blood mercury levels. In the U.S., high income people and Asian populations have the highest blood mercury levels due to an increased consumption of fish and seafood (Buchanan et al. 2015). Populations who consume contaminated fish from rivers and lakes are also at high risk regardless of whether they are recreational sport fishers or subsistence fishers feeding their families (Thomas et al. 2011; Loflen 2013; Burch et al. 2014). Prenatal exposure

to MeHg is the most toxic pathway that results in adverse health outcomes in humans. MeHg crosses the placental barrier and interferes with the development of the nervous system of the fetus. Cord blood mercury levels are often associated with physiological, behavioral and cognitive functions. Epidemiological studies have revealed the association between developmental exposures to MeHg with neurodevelopmental and neurobehavioral outcomes. The public health and economic impacts created by this condition is significant.

The transgenerational effect of MeHg has not been identified in any animal model prior to this study. Our studies were motivated by the potential of prenatal MeHg exposure to induce neurobehavioral deficits and the demonstrated ability to alter DNA methylation. The central hypothesis was that developmental exposure to MeHg would induce the transgenerational onset of neurobehavioral deficits. Previously published studies by our laboratory have shown the direct effect of MeHg exposure on visual reflex behaviors and retinal electrophysiology in zebrafish (Weber et al. 2008) . In the present study, zebrafish embryos were exposed to sublethal concentrations of MeHg relevant to environmental exposures and exposure-specific lineages were created to the F3, the first generation without direct exposure. As in the directly exposed lineages, the F3 generation demonstrated abnormalities in visual reflex behaviors and retinal electrophysiology. Locomotor behavior analysis was included for the F3 to identify the role of motor functions in explaining the visual startle response, presuming they had some locomotor defect that prevented a normal escape

response. Surprisingly, the exposed F3 lineages were actually hyperactivite relative to the unexposed control lineage.

After demonstrating that ancestral developmental exposure to MeHg resulted in transgenerational visual and locomotor deficits, we further investigated the mode of germline transmission and inheritance of the phenotypes to the F3 generation. Zebrafish from exposed F2 lineages were outcrossed with the unexposed F2 control lineage to create F3 populations in which only one parent came from an exposed F2 lineage. We observed that the two phenotypes were inherited independently, and that the visual reflex deficit and hyperactivity were inherited differently by the F3 generation. Inheritance of the visual deficits only required one parent from an exposed lineage; however, inheritance of hyperactivity required that both parents come from exposed lineages. These findings suggest that MeHg induced phenotypes are inherited transgenerationally through different modes of germline transmission, and that there are likely to be many more abnormal neurological phenotypes yet to be identified.

Ancestrally induced transgenerational phenotypes in the F3 lineages suggests that gene expression and molecular pathways associated with these phenotypes are dysregulated. The alteration of gene expressions involved in vision and the molecular mechanisms behind the transgenerational inheritance of visual defects due to developmental exposure to MeHg are not yet identified. We naturally hypothesized that genes and pathways associated with visual functions and cellular physiology would be dysregulated in the MeHg exposed F3 lineage. To test this hypothesis, we performed transcriptomic analysis of the retina and

brain of F3 lineage zebrafish by RNAseq. As expected, we found differential expression of genes associated with visual functions and retinal electrophysiology. However, we also identified dysregulation of genes and pathways associated with phenotypes that were not previously tested (e.g. circadian rhythm) which generates a whole new set of hypotheses for future studies.

Our findings have significant impact on the global fight against mercury pollution. They provide the basis for developing tools for identifying individuals and populations who are at high risk for MeHg-influenced neurobehavioral disorders even though they may have not been exposed directly. Public health interventions are only possible with the identification of high risk populations. This thesis has identified for the first time the potential for developmental exposure to MeHg to induce transgenerational neurobehavioral deficits. Our transcriptome findings have directed future studies that are likely to identify additional specific transgenerational phenotypes associated with MeHg toxicity. This thesis should provide a foundation for public health interventions to alleviate the neurotoxic effects of developmental MeHg exposure through preventative measures and the development for future therapeutic interventions.

CHAPTER 2

Ancestral developmental exposure to methylmercury induces transgenerational inheritance of visual and locomotor deficits

ABSTRACT

Exposure to the environmental toxicant methylmercury (MeHg) during embryonic development has been shown to induce a wide variety of neurobehavioral deficits in humans and animals. Direct exposure to MeHg during development induces altered retinal electrophysiology and neurobehavioral deficits including altered behavioral response to visual stimuli and learning deficits. However, the capacity of developmental MeHg exposure to induce transgenerational phenotypes is unknown. Given the potential of direct developmental exposure to MeHg to induce neurobehavioral deficits, we hypothesized that developmental exposure to MeHg induces transgenerational neurobehavioral phenotypic variations in zebrafish. In this study, we tested F3 zebrafish, whose grandparents (F1) were exposed to MeHg during early development, for visual startle response and locomotor activity. Exposure of the F1 grandparents to MeHg during development caused a significant reduction in visual startle responses, altered function of potassium ion channels of bipolar cells of the retina, and induced hyperactivity in the F3 generation zebrafish in the absence of additional exposure to MeHg. Furthermore, a dose dependent increase in the proportions of fish that exhibited a visual deficit or hyperactivity was observed. Finally, we found that direct exposure of zebrafish embryos to MeHg caused a decrease in global DNA methylation. Our data suggests that developmental exposure to MeHg induces transgenerational inheritance of neurobehavioral deficits and that altered DNA methylation may be the mechanism responsible for this transgenerational inheritance. This is the first

evidence of transgenerational neurotoxic effects from developmental exposure to MeHg in any species.

Keywords Methylmercury Zebrafish Transgenerational inheritance Neurobehavioral deficits DNA methylation

INTRODUCTION

It is well established that prenatal exposure to high doses of MeHg can lead to widespread brain damage and impaired neurological development resulting in defects ranging from severe cerebral palsy and cognitive impairment to impaired motor and sensory functions (Davidson et al. 2004; Ekino et al. 2007; Patel and Reynolds 2013). Exposure to MeHg at low levels during fetal development has been associated with neurodevelopmental defects, including visual deficits in humans (Shamlaye et al. 1995; Grandjean et al. 1997; Jedrychowski et al. 2006). The neurodevelopmental defects induced by developmental MeHg exposure can be permanent and have been linked to persistent changes in brain structure and activity in the exposed individual (Fox et al. 2012).

The 2009-2010 National Health and Nutrition Examination Survey reported that 2.14% of American women of childbearing age have blood MeHg concentrations above the reference dose level (5.8 µg/L), which is expected to cause developmental deficits in children due to prenatal exposure (Birch et al. 2014). Recent analysis of blood mercury concentrations of newborns from the Lake Superior Basin showed that 8% of newborns had blood mercury levels above the reference dose and 1% of newborns had mercury levels above 58 µg/L (McCann P et al. 2011). Global environmental emission of Hg has significantly increased during the past century and it is expected that levels will continue to increase, unless drastic measures are taken to reduce emissions.

The early developmental stages of an organism are susceptible to both genetic and epigenetic modifications resulting in permanent changes to the basic physiology of the organism (Skinner 2011). Exposure to certain environmental toxicants such as vinclozolin, DDT and methoxychlor during critical developmental windows has been shown to result in disease or dysfunction phenotypes that persist for multiple generations without further exposure. In order to be considered transgenerational, this phenotype must be observed in a generation that was never directly exposed to the toxicant. A directly exposed embryo (F1) will contain germ cells that contribute directly to the F2 generation; therefore, the F3 is the first generation without direct exposure. Ancestral exposure to the insecticide dichlorodiphenyltrichloroethane (DDT) in rats, for instance, results in obesity and related cardiovascular diseases in the F3 generation (Skinner et al. 2013). Ovarian disease, testis disease and obesity have been transgenerationally induced in rats by exposure to the endocrine disruptors bisphenol-A (BPA), bis (2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP) (Manikkam et al. 2013). Vinclozolin, a fungicide, has also been shown to induce diseases in male rat reproductive physiology in multiple generations including F3 without direct exposure (Anway et al. 2006). As demonstrated in these studies, exposure to environmental toxicants can induce permanent changes in the DNA methylation pattern of germ cells from developmentally exposed individuals and this altered epigenome was inherited by the following generations resulting in disease phenotypes without further exposure (Anway et al. 2006; Manikkam et al. 2013; Skinner et al. 2013).

Ancestral exposure to toxicants such as nicotine and BPA has been associated with transgenerational inheritance of neurobehavioral phenotypes including changes in social behavior and hyperactivity (Wolstenholme et al. 2012; Zhu et al. 2014). The transgenerational actions of prenatal MeHg exposure are still unclear. In order to close this knowledge gap, it is important to investigate the long-term adverse health outcomes of prenatal exposure to MeHg. Zebrafish are well characterized model organisms used to study visual defects and complex brain disorders due to the similarities in physiological, morphological, and genetic characteristics of zebrafish and humans (Bibliowicz et al. 2011; Kalueff et al. 2014). The short generation time (3 months) of zebrafish also makes it a great model organism for transgenerational studies. We have previously shown that direct developmental exposure to low doses of MeHg results in dysfunction in the electrophysiology of the retina, altered response to visual stimuli, as well as deficits in learning and memory in adult zebrafish (Smith et al. 2010; Weber et al. 2008). In our current study, we investigated the potential of ancestral developmental exposure to a range of environmentally relevant and higher and doses of MeHg to induce transgenerational visual and neurobehavioral deficits in the F3 generation.

MATERIALS AND METHODS

Animal studies

All animal care and experiments using animals or involving hazardous materials were pre-approved by the Institutional Animal Care and Use Committee, Environmental Protection, and Laboratory Safety offices of University Safety & Assurances of the University of Wisconsin-Milwaukee.

Zebrafish stock and egg collection

Adult EK strain zebrafish were originally obtained from Ekkwill Waterlife Resources, Gibsonton, FL. and have been maintained in the lab for over 10 years. Adult fish were housed at a maximum density of 10 adult fish/L in a flow-through dechlorinated water system maintained at 26 to 29 °C on a 14:10 h light: dark photoperiod at the Aquatic Animal Facility of the NIEHS-funded Children's Environmental Health Sciences Core Center at the University of Wisconsin-Milwaukee. For spawning, male and female fish (male:female ratio 1:2) were kept overnight in a 2 L plastic aquarium with a 3 mm nylon mesh bottom to separate newly fertilized eggs from the adults. The fish were moved to clean water 30min prior to the onset of light to ensure that only eggs fertilized within a narrow time period were used in experiments. Eggs were collected ≤ 1 h post fertilization (hpf) and placed into metal-free, plastic culture dishes (100 mm diameter × 50 mm depth) in E2 medium (Nüsslein-Volhard 2002; pH 7.2; in one liter -- 0.875 g NaCl, 0.038 g KCl, 0.120 g $MgSO_4$, 0.021 g $KH2PO_4$, and 0.006 g Na_2HPO_4; Hg-

free as determined by Inductively Coupled Plasma-Mass Spectrometer (ICP-MS).

Methylmercury exposure and breeding

The newly fertilized eggs were then transferred to 12-well non-treated culture plates (10 eggs/well) and rinsed twice with E2 medium. The eggs were exposed to 1.5 ml of E2 medium containing MeHg (0.0, 0.001, 0.003, 0.01, 0.03, 0.1 µM with ethanol as a carrier < 0.01% total volume). Following 24 h exposure, embryos were rinsed three times with E2 medium and transferred to 2L tanks (60 embryos per tank) containing static E2 medium for rearing. Starting at 5 dpf, eleutheroembryos were fed 5-100 micron Golden Pearl Reef & Larval Fish Diet (Brine Shrimp Direct, Ogden, UT). Platinum Grade *Artemia* nauplii (Argent Laboratories, Redmond, WA) were fed starting at 9 dpf. At 21 dpf, fish were transferred to 1L flow-through tanks and raised using standard husbandry techniques until 4 months of age, at which point they were used for behavior assays and breeding. Juvenile and adult fish were fed a combination of Platinum and Gold grade *Artemia* nauplii and Aquarian™ flake food (Aquarium Pharmaceuticals, Inc., Chalfont, PA).

In mammalian models of developmental toxicity, the pregnant animal is directly exposed to the toxicant and is designated as F0. The developing progeny which are exposed to the toxicant *in-utero* are considered the F1 generation. In our zebrafish model system, the directly exposed embryos are analogous to the F1 progeny in mammalian animal models. After the initial 24 h MeHg exposure of

the F1 embryos, there was no exposure to Hg during the entire life cycle beyond the background levels found in all fish food. Many different fish foods were screened, and those with the lowest levels of Hg (as determined by ICP-MS) were used as much as possible in this study. Adult F1 fish were inbred to create the F2 generation, which were inbred to create the F3 generation. F2 and F3 generations were never exposed to exogenous MeHg. However, the F2 generation is also considered to be directly exposed as they were primordial germ cells within the F1 embryos of the time of initial MeHg exposure. In our current study, we tested the F3 generational animals for transgenerational inheritance of neurobehavioral deficits.

Mercury determination

Mercury levels in embryos and food were determined by ICP-MS. Briefly, zebrafish embryos were exposed to MeHg as described above. Embryos were tested with (chorion-intact) and without (dechorionated) chorions. For dechorionation, embryos were first treated with 2mg/mL Proteinase-K for 2 min at 28^{o}C and then rinsed with E2 medium. The embryos were placed on a plate shaker at 75rpm to agitate and break the chorions. Embryos were rinsed again with E2 medium to remove the broken chorions. Chorion-intact and dechorionated embryos were analyzed separately. Embryos were transferred to pre-weighed 13x100 mm 8 mL Round Bottom Polystyrene Shelf Pack (RB PS SPK) tubes (SARSTEDT Inc, Newton, NC). Two hundred embryos were pooled for each of the 0.001µM, 0.003µM, 0.01µM and 0.03µM exposure groups, and

100 embryos were pooled for the 0.1µM and 0.3µM exposure groups. Pooling was identical for both chorion-intact and dechorionated embryos. All liquid was carefully removed and the embryos were weighed to determine tissue mass.

Digestion of the embryo or food samples was performed using 1.0mL of 20% Nitric Acid (Optima™ Fisher Scientific) with 2000 ppb gold standard (Gold Standard for ICP/MS - Claritas PPT Grade, SPEX CertiPrep™ Fisher Scientific). Each sample was transferred to a 3mL CEM MicroVessel and allowed to run in a CEM MARS 5 Microwave at 130^{o}C for 10 min at ≤ 50 psi. Samples were discarded if 1.0mL of each sample was not retrieved after the microwave digestion as this was evidence of improper MicroVessel sealing. After the cooling, the samples were diluted 1:4 with 5% nitric acid and 500 ppb gold and store at room temperature in RB PS SPK tubes.

Hg concentration was determined using a Micromass Platform ICP-MS (GV Instruments Ltd, Manchester, UK) controlled with MassLynx software. The concentration range was set to 10-100 ppb Hg for all isotopes. The response at each mass unit was acquired for 1 minute following a 1.5 minute solvent delay. Five concentrations of standards were prepared (0, 1, 10, 50, and 100 ppb) from commercially available ICP-MS standard solutions in 5% nitric acid with 500 ppb gold for calibration. Total Hg levels were statistically analyzed using one-way ANOVA with multiple comparisons versus control by Holm-Sidak method for post hoc test. The overall significance level was kept at 0.05.

Global DNA methylation

Zebrafish embryos were exposed to MeHg as described above. Fifty embryos were transferred to a 1.7 mL Eppendorf tube containing 250 µL RNAlater and quickly flash frozen in liquid nitrogen. Embryos were then stored at -80°C until DNA extraction. Samples were thawed to room temperature and all RNAlater was removed. DNA extraction was performed using the IBI Genomic DNA Mini-Kit-(Tissue; Midwest Scientific #IB47222) as described by the manufacturer with minor modifications for use with zebrafish embryos. Briefly, 200 µL of GT buffer and 10 µL of Proteinase K (10 mg/mL) were added to the embryos, vortexed, and incubated at 60°C for 15 min until fully dissociated. 200 µL of GBT buffer was added to the samples, vortexed, and incubated at 70° C for 5 min. RNA was removed using RNAse A (2 µL, 20 mg/mL; Invitrogen) with incubation at room temperature for 5 min. Washing and elution procedures were performed as directed by the manufacture's protocol. DNA was quantified in triplicate using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Pittsburgh, PA) and/or Qubit® 2.0 Fluorometer (Life Technologies, Grand Island, NY). The accuracy of this assay is dependent on precise quantification of DNA; therefore, multiple dilutions were quantified (initial concentration; triplicate measurements), after dilution to 25 ng/µL (quintuplicate measurements), and again after dilution to 12.5 ng/µL (triplicate measurements).

Methylated DNA quantification was performed using the MethylFlash Methylated DNA Quantification Kit – Colorimetric Assay (Epigentek,

Farmingdale, NY) according to the manufacturer's specifications with minor modifications. Samples, standard curves (0.25 ng, 0.5 ng, 0.75 ng, 1 ng, 2 ng, 5 ng ME4 positive control DNA), negative controls (5 ng ME3) and blanks (no DNA) were analyzed in duplicate. Spatial location on the plate was randomized to account for any variances in incubation time due to pipetting. To minimize well-to-well variation due to pipetting error or improper mixing of DNA and Binding Solution, stock solutions containing standards or sample DNA and ME2 Binding Solution were prepared for all samples prior to plate loading. The same stock solution was used for both of the technical duplicate wells. The methylated DNA capture (ME5 antibody), detection (ME6 antibody), and enhancement (ME7 solution) were carried out as directed by the manufacturer. Signal development (ME8) was monitored using a BioTek SynergyTM H4 Plate reader and stopped by the addition of ME9 when the absorbance at 370 nm reached 1.0 O.D. The stop reaction causes a spectral shift and absorbance of 450 nm was used to quantification methylated DNA. Reported data are normalized to the control group for comparison of data across multiple plates. One-way ANOVA (SigmaPlot 12.0) was used to analyze global methylation differences between treatment and control groups. Level of significance was set at $P < 0.05$.

Visual startle response

The behavioral response of the fish to visual stimuli was analyzed as previously described [Dowling et al. 1997, Weber et al. 2008]. Adult male and female fish (n = 20, 10M, 10F; 4-6 months old, 1.5-2.0 cm standard length) from all exposure groups were assigned random numbers to blind the observer to their exposure. Individual fish were placed in a stationary glass crystallizing dish (10 cm diameter, 5 cm depth, approximately 200ml of dechlorinated water) surrounded by a rotating white PVC drum with a black vertical stripe (1 cm width x 5 cm height). The drum speed was set to 10 rev/min and the black object elicited a startle response when it entered the fish's field of vision. When startled, zebrafish will elicit either a C-start escape response or an avoidance maneuver. After a 5-minute low light acclimation period, the PVC drum rotation was initiated and the response of fish to the rotating black bar was captured for 5 min using infrared digital video. All tests were conducted between the hours of 1300-1600 due to the zebrafish circadian rhythm which exhibit the most consistent light sensitivity and basal activity levels during the afternoon hours (Dowling et a. 1997). Blinded analysis was conducted on the videos of visual startle response and the number of C-start escape reactions exhibited by fish during the encounter with the rotating black bar was quantified. All data are expressed as responses relative to the control group. One-way ANOVA (SigmaPlot 12.0) was used to analyze visual startle response data with $P < 0.05$.

Retinal electrophysiology

Whole-cell voltage gated current responses were recorded from bipolar cells within intact zebrafish retinal slice preparations using methods described previously (Weber et al., 2008). All protocols and procedures were approved by the Animal Care and Use Committee at American University.

Adult zebrafish were maintained on a 14hr light: 10hr dark photoperiod at 26-28 ^{o}C, and fed Tetramin flakes daily until needed for experiments. Retinal slices were prepared following established protocols (Connaughton & Maguire, 1998; Connaughton and Nelson, 2000; Connaughton, 2003). Briefly, fish were removed from aquaria, dark adapted (at least 20 min), anesthetized in a 0.02% tricaine solution until gill ventilation stopped, and then decapitated. Retinas were removed from the eyes and mounted vitreal-side down on a piece of Millipore filter paper (0.45µm pore size). The filters along with the retina were then mounted onto Vaseline strips in the recording chamber, covered with the standard extracellular solution, and sectioned (100µm sections). Sections were rotated 90^{o} and viewed in cross-section using an Olympus BX50WI microscope fitted with a 40X water immersion lens and Hoffman modulation contrast optics.

Whole-cell voltage-gated currents were recorded in response to voltage steps from a holding potential of -60mV (-80 to +60mV, in 10mV increments). Recording chambers contained a standard extracellular solution composed of 120mM NaCl, 2mM KCl, 3mM $CaCl_2$, 1mM $MgCl_2$, 4mM HEPES, and 3mM D-glucose, brought to pH 7.4-7.5 with NaOH. Patch electrodes were made of thin walled, filamented borosilicate glass capillary tubes pulled to the desired tip

diameter (10-15MΩ) with a Flaming-Brown P-80 Micropipette Puller. Pipettes were filled with an intracellular solution composed of 12mM KCl, 104mM K-gluconate, 1mM EGTA, 4mM HEPES, and 100µM $CaCl_2$. Once mixed, the intracellular solution was brought to pH 7.4-7.5 using KOH. Lucifer Yellow (1% solution) was added to the intracellular solution to label recorded neurons. Labeled cells were visualized at the end of the experiment. Recordings were made using an Axopatch 1-D patch clamp amplifier and pCLAMP (ver. 8.0) software. Traces were analyzed in pCLAMP; graphs were made in SigmaPlot (ver. 12.5). Statistical analyses for the bipolar cell electrophysiology data were accomplished using SPSS 20 for Windows software and ANOVA (SigmaPlot 12.5, Systat software Inc, San Jose, CA).

Locomotor Activity

Locomotor activity was quantified using a Behavior Observation Box (BOB). Fish were placed in a clean glass crystallizing dish (10 cm diameter and 5 cm depth) in a light-tight chamber. The light intensity inside the chamber was produced by a standard computer monitor (76 Lux). The chamber was equipped with four Logitech c920 USB cameras (Logitech, Newark, CA) for capturing digital video of four arenas simultaneously. In our experiment, we used the two distant cameras at a time. The fish were acclimated for 5 minutes (min) after which a digital video was recorded for an additional 5 min. The files were obtained in M-MJPEG format using the Matlab Image Acquisition ToolboxTM (MathWorks, Natick, MA) at a 960 x 720 pixels resolution. Video was cropped to 600 x 600 pixels with ffmpeg

(https://www.ffmpeg.org/) prior to being imported into EthoVision XT (Noldus Information Technology Inc, Leesburg, VA) for automated analysis of distance traveled in 5 min. One-way ANOVA (SigmaPlot 12.0) was used to identify the difference in locomotor activity between the exposure and control groups. Level of statistical significance was set at $P < 0.05$.

RESULTS

Mercury accumulation in zebrafish F1 embryos

Zebrafish embryos were exposed to MeHg during the first 24hpf, then rinsed and placed in clean medium. The levels of Hg in the embryos were measured by ICP-MS at 24 hpf (chorion-intact or dechorionated), and 144 hpf (Fig 2.1). Developmental exposure to MeHg during the first 24hpf resulted in a dose-dependent accumulation of Hg at 144 hpf (Table 2.1). The chorion-intact 24hpf embryos had higher Hg concentrations (total ng/embryo) than both 24 hpf dechorionated and 144 hpf. Exposure to 0.01µM and 0.03 µM concentration resulted in approximately 47 times and 181 times more Hg than the control respectively in the chorion-intact embryos. Exposure to two highest doses (0.1µM and 0.3µM) resulted in significant increase in the accumulation of Hg at 144 hpf (one way ANOVA, $p < 0.05$). At 144 hpf, the 0.01µM exposure caused 1.6 times and 0.03 resulted in 18.1 times higher Hg accumulation than the control. The two lowest doses (0.001µM and 0.003µM) did not cause Hg accumulation above background levels observed in the control embryos. A regression analysis of the uptake of Hg due to exposure ranging from 0µM to 0.3µM at 144 hpf resulted with a R-square of 0.977. The *P* value for the significance of the interaction term is <0.001. The lowest three exposures resulted in significantly lower accumulation of Hg than the human reference dose for cord blood (5.8 µg/L), whereas, exposure to 0.03 µM MeHg resulted in a tissue dose (1.63 ng/embryo) comparable to the reference dose. The 0.1 µM MeHg exposure resulted in a tissue dose (1.76 ng/embryo) that is consistent with the higher range of in utero

exposures for fish consumers in the US reported by the latest NHANES and other epidemiological studies (Wolkin et al. 2012). The ICP-MS analysis included data from embryos exposed to 0.3µM but it was excluded from further analysis because of a low but significant incidence of abnormalities.

Table 2.1. MeHg treatment of zebrafish embryos and the resultant tissue dose in whole embryos

F1 exposure		Tissue Hg (ng/embryo)
(µM)	**(µg/L)**	
0	0	<0.1 (near detection limit)
0.001	0.216	<0.1
0.003	0.647	<0.1
0.01	2.16	0.18
0.03	6.47	1.63
0.1	21.6	1.76

Concentration of Hg presented in one embryo vs MeHg exposure in µM and equivalent µg/L. The amount of Hg accumulated in the embryo at 144 hpf after 24 hpf of developmental exposure to above mentioned concentrations of MeHg (One way ANOVA, $p = 0.001$).

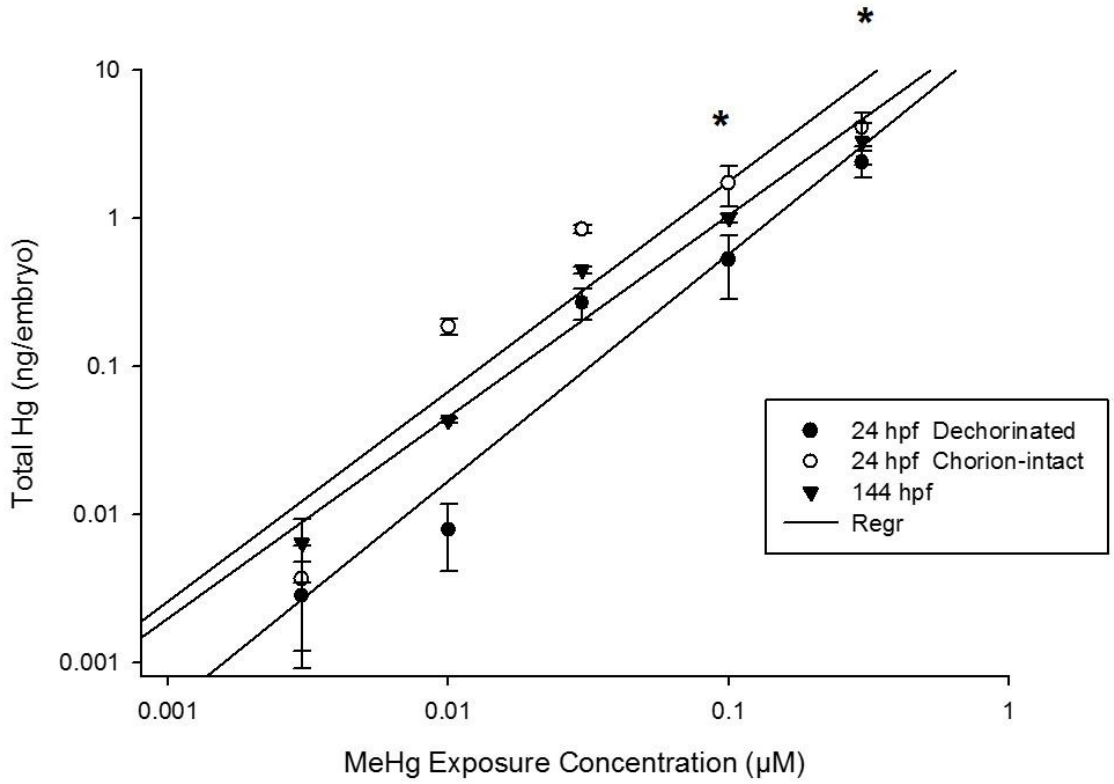

Figure 2.1. Hg uptake analysis. Concentration of Hg (ng per one embryo) present in zebrafish embryos at 24 hpf measured with chorion (○), 24 hpf without chorion (●) and 144 hpf (▼) at each exposure concentration (µM) of MeHg exposure. Exposure to the highest concentrations (0.1 and 0.3 µM MeHg) resulted in a significant increase in the accumulation of Hg at 144 hpf (one way ANOVA, * indicates $p < 0.05$).

Global DNA methylation analysis

Transgenerational onsets of disease phenotypes are often due to permanent heritable epigenetic modifications (Skinner et al. 2013). A number of studies have shown that the CpG islands associated with critical genes are differentially methylated in transgenerationally inherited diseases (Nilsson et al. 2012; Guerrero-Bosagna et al. 2012; Manikkam et al. 2012). Dietary exposure to MeHg in yellow perch did not cause alteration in global DNA methylation in the telencephalon brain (Basu et al. 2013). Global DNA methylation was not significantly changed in the F1 and F2 zebrafish embryos whose F0 female fish were fed with dietary MeHg (Olsvik et al. 2014). In our current study we quantified the changes in global DNA methylation during embryogenesis due to MeHg exposure during the 24 hpf (Fig 2.2). Analysis of DNA methylation revealed that MeHg exposure at 0.1µM resulted in global DNA hypomethylation relative to controls (ONEWAY ANOVA, Holm-Sidak method, p <0.001). This does not rule out changes in DNA methylation at lower concentrations. However, this method is not sensitive enough, nor does it have the necessary resolution, to detect the subtle differences that have the potential for dramatic influences on gene transcription.

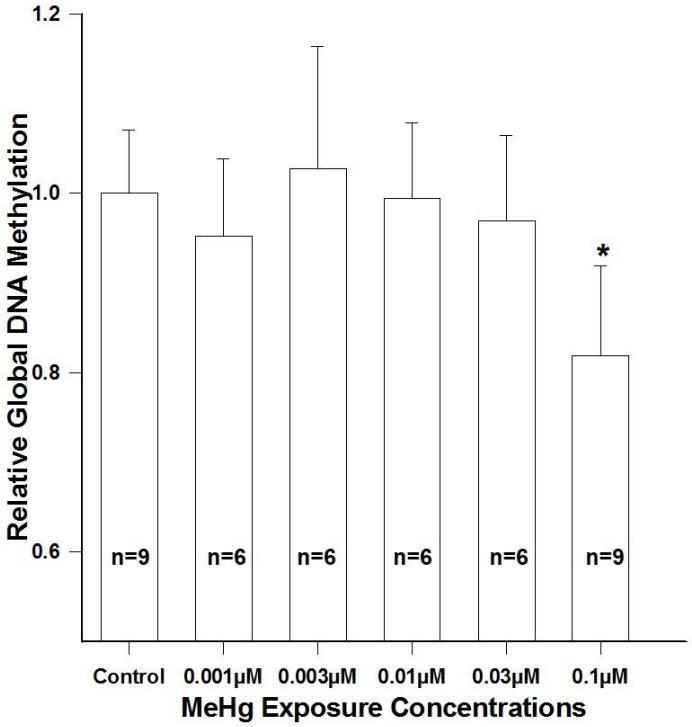

Figure 2.2. Alteration of DNA methylation by direct exposure to MeHg. Exposure of embryos for 12 hpf with different concentrations of MeHg resulted in altered DNA methylation. Error bars indicate standard deviation. Exposure to the highest concentration (0.1µM MeHg) resulted in a significant reduction in the magnitude of global DNA methylation (ONEWAY ANOVA, * indicates p <0.001).

Transgenerational visual deficits

We exposed zebrafish embryos to sublethal concentrations of MeHg at levels below or equivalent to human developmental exposure levels as described above. No overt morphological abnormalities were observed in any of the F1, F2 or F3 groups at concentrations up to 0.1 µM MeHg. We have previously reported dose-dependent visual startle deficits in adult F1 zebrafish following developmental exposure to MeHg (Weber et al. 2008). This phenotype persists into the F2 generation (data not shown). The visual startle response of F3 generation zebrafish (Fig 2.3) was significantly reduced in all exposure groups as compared to controls (one-way ANOVA, $p < 0.001$, $F = 10.842$, $df = 5$). Thus the fish, whose grandparents were exposed to MeHg during development, responded significantly less frequently to the rotating black bar than the controls. Unlike the F1 and F2 generations, the visual deficit observed in the F3 generation was not dose-dependent.

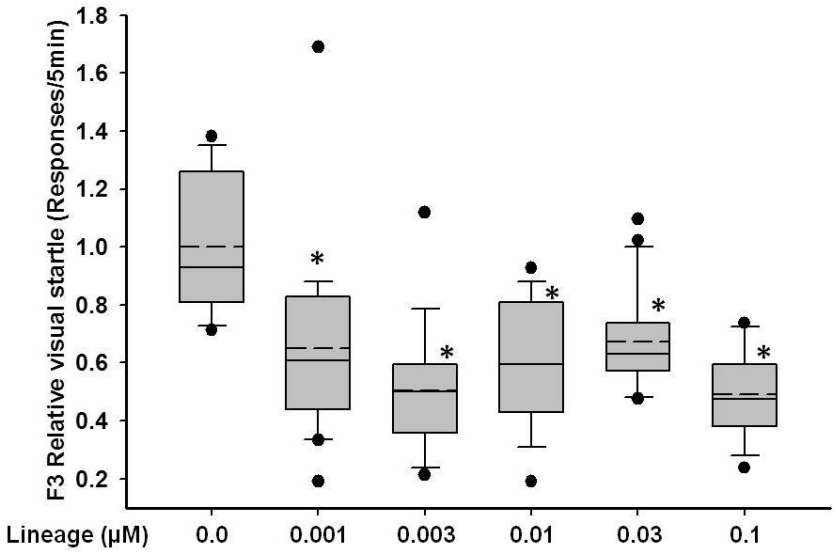

Figure 2.3. Transgenerational visual deficit in F3 zebrafish due to ancestral MeHg exposure. Twenty animals from each lineage were tested for their visual startle response. Relative response of all ancestrally exposed populations of F3 fish were significantly lower than the control group (ONEWAY ANOVA, ***** indicates p <0.001). Solid horizontal lines represent the median, dashed horizontal lines represent the mean, the box represents the 25th and 75th percentiles, the whiskers show the 5th and 95th percentiles, and outliers are represented by dots.

Transgenerational effects on retinal electrophysiology

In our previous study (Weber et al. 2008), the bipolar cells of the retina exhibit either a delayed rectifying I_K potassium current or a transiently activating and inactivating I_A potassium current in response to membrane depolarizations following developmental exposure to MeHg . In this study whole-cell voltage-gated currents were measured in retinal bipolar cells from the F3 zebrafish ancestrally exposed to MeHg. In most bipolar cells recorded from the F3 fish, the I_K current was present (N=47); while the remaining cells (N=27) expressed the I_A current. In both current groups, there was a clear trend of increasing current amplitude with increasing MeHg exposure concentration.

When examining changes in I_K current amplitude, mean peak values, elicited from a voltage step to +60mV in bipolar cells in retinas exposed to control, 0.01µM, or 0.03µM MeHg were significantly different (ANOVA, $p = 0.039$; Fig 2.4). A Tukey posthoc test revealed the difference to be between the 0.03µM and control groups; mean current amplitude between the 0.01µM and control groups were not significantly different. There was also a significant trend ($p = 0.012$) of increased current amplitude with increased MeHg concentration across exposure groups, with current amplitude increasing by 59% in the 0.01µM group and by 139% in the 0.03µM group (Table 2.2) compared to controls . Interestingly, peak current amplitudes recorded from cells within the highest MeHg exposure group (0.1µM) were reduced and comparable in value to peak current amplitudes recorded from cells in the 0.01µM exposure group (Table 2.1, Fig 2.4). If the 0.1µM exposure was included in the statistical analysis,

differences in current amplitude at +60mV became non-significant (ANOVA, p = 0.095). The range of peak current amplitudes recorded from individual bipolar cells was large, though the variability was smallest in the control animals (139-764pA). The greatest variability in peak current amplitudes was observed in bipolar cells from the 0.03μM exposure, which was also the exposure that showed the greatest increase in current amplitude.

There was also a significant trend of increased I_A current amplitude with increasing MeHg concentration (p=0.022) when control, 0.01μM, and 0.03μM exposure groups were compared (Table 2.3, Fig 2.5). However, mean peak current amplitudes recorded from bipolar cells in these three exposure groups, following a voltage step to +60mV, were not significantly different (ANOVA, p = 0.065). Current amplitude was increased by 32% in the 0.01μM exposure group and by 68% in the 0.03μM exposure group (Table 2.2). If the 0.1μM exposure group was included in the statistical analysis, changes in peak current amplitudes remained non-significant (ANOVA, p = 0.102). As noted for I_K, peak current amplitude varied among cells within a given exposure group. The smallest variability was seen in the 0.01μM exposure group (range of peak current amplitude = 628-878pA). Variability in peak current amplitudes in control 0.03μM and 0.1μM exposure groups were comparable.

Table 2.2. I_K current amplitude changes in response to ancestral MeHg exposure.

MeHg exposure group	I_K peak amplitude in response to +60mV step	Percent change in peak amplitude from control
Control	382 ±51.6pA (16)	--
0.01µM MeHg	584 ± 102.8pA (12)	53% increase
0.03µM MeHg	914 ± 313.9pA (7)	139% increase
0.1µM MeHg	561 ± 141.8pA (12)	47% increase

Mean peak amplitudes (+/- SE) and the corresponding percent change in amplitude of the delayed rectifying I_K current recorded from retinal bipolar cells in F3 generation zebrafish. Currents were elicited in response to a voltage step to +60mV. N's are given in parentheses. Control recordings include both water- and ethanol-treated fish.

Table 2.3. I_A current amplitude changes in response to ancestral MeHg exposure.

MeHg exposure group	I_A peak amplitude in response to +60mV step	Percent change in peak amplitude from control
Control	556 ± 83.1pA (12)	--
0.01µM MeHg	734 ± 53pA (4)	32% increase
0.03µM MeHg	936 ± 156.5pA (6)	68% increase
0.1µM MeHg	597 ± 123.5pA (5)	7% increase

Mean peak amplitudes (+/- SE) and the corresponding percent change in amplitude of the depolarization elicited A-type current recorded from retinal bipolar cells from F3 generation zebrafish. Currents were elicited in response to a voltage step to +60mV. N's are given in parentheses. Control recordings include both water- and ethanol-treated fish.

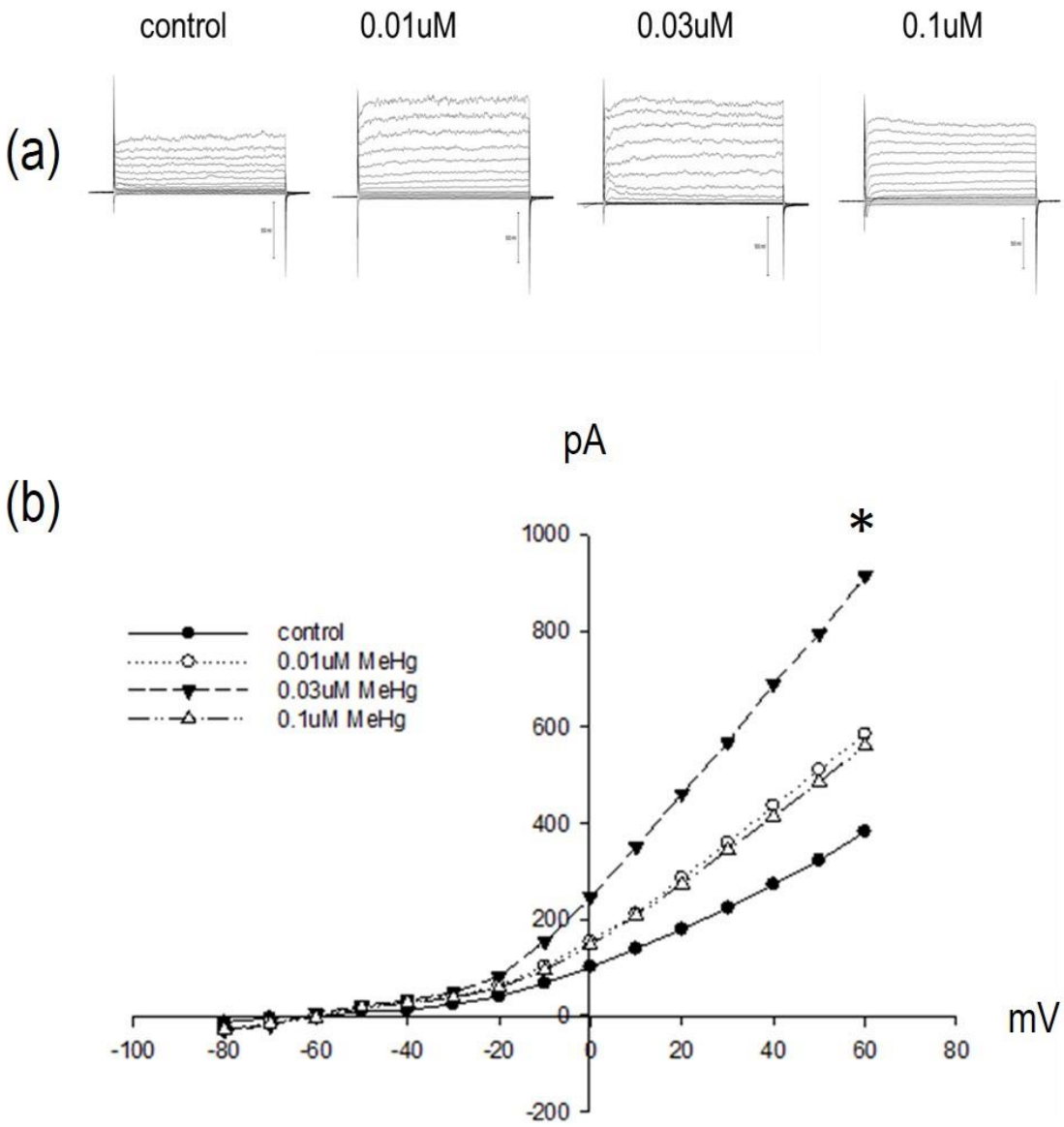

Figure 2.4. Change in mean peak amplitude of I_K current recorded from bipolar cells in retinas from F3 zebrafish ancestrally exposed to various concentrations of MeHg. (a) Representative whole-cell current traces recorded from bipolar cells in control (0μM), 0.01μM, 0.03μM, and 0.1μM MeHg exposure groups. (b) Current-voltage relationship showing the mean peak currents elicited at different voltage steps from a holding potential of -60mV. One-way ANOVA comparing peak currents at a voltage step to +60mV was not significant ($p = 0.095$), due to the reduction in current amplitude seen in the 0.1μM exposure (open triangles). If this high exposure is removed from the analysis, ANOVA results become significant ($p = 0.039$, *), with amplitude larger in the 0.03μM group (solid triangles) compared to control (solid circles) or the 0.01μM group (open circles). There was also a significant linear trend of increasing current amplitude with increasing MeHg exposure concentration for the control, 0.01μM, and 0.03μM exposure groups ($p = 0.012$).

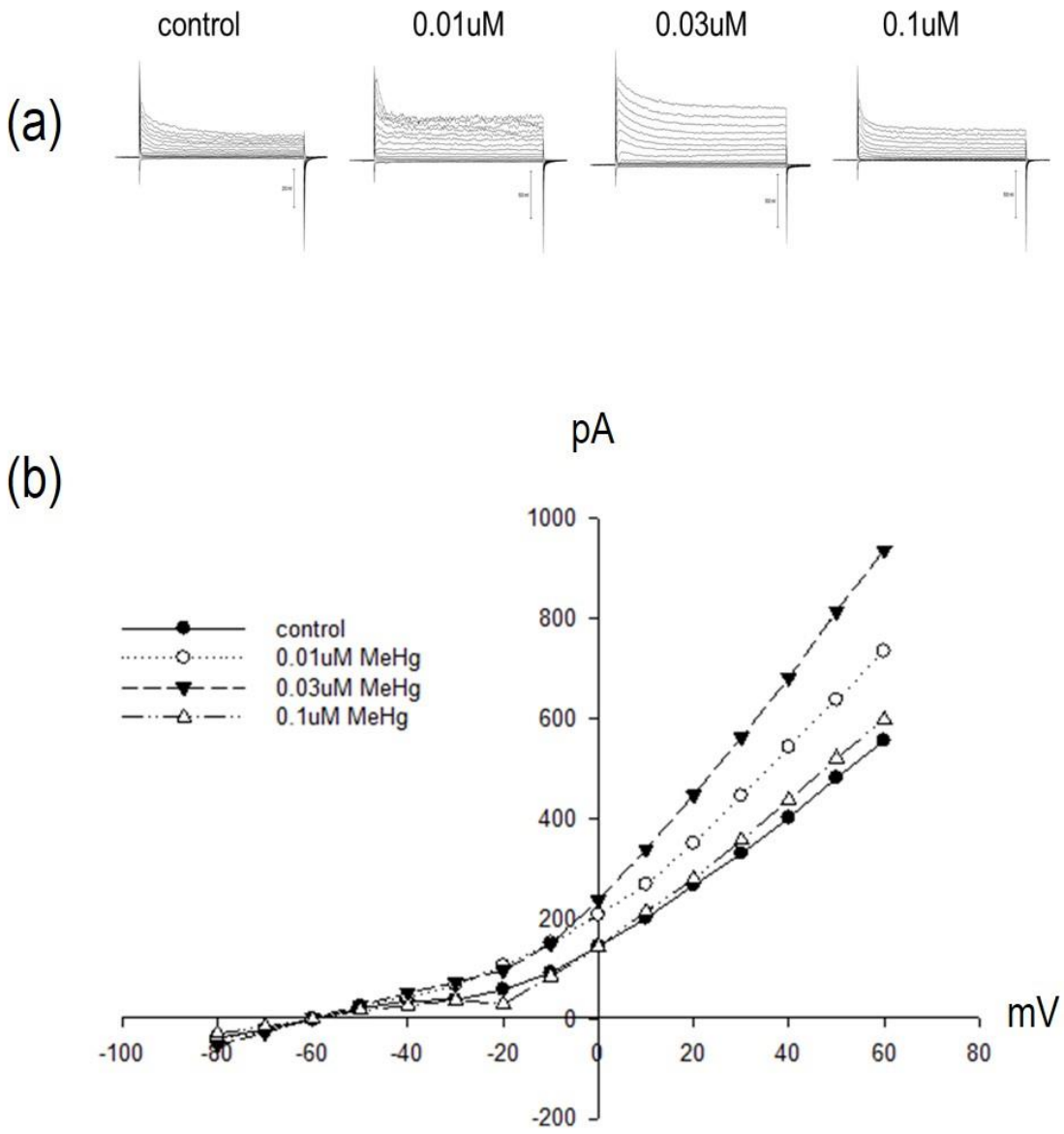

Figure 2.5. Change in mean peak amplitude of I_A current recorded from bipolar cells in retinas from F3 zebrafish ancestrally exposed to various concentrations of MeHg. (a) Representative whole-cell current traces recorded from bipolar cells in control (0μM), 0.01μM, 0.03μM, and 0.1μM exposure groups. (b) Current-voltage relationship showing the mean peak current amplitudes elicited at different voltage steps from a holding potential of -60mV. One-way ANOVA comparing peak currents at a voltage step to +60mV was not significant ($p = 0.102$). However, there was a significant linear trend in the data ($p = 0.022$) of increasing current amplitude with increasing MeHg exposure concentration (control, 0.01μM, and 0.03μM groups only).

Transgenerational effects on locomotor activity

Developmental exposure to MeHg has shown defects in locomotor activity in different species including decreased flight performance in songbirds (Carlson et al. 2014) decreased exploratory activity in mice (Onishchenko et al. 2007). Exposure to MeHg also results in neurobehavioral deficits in different fish species affecting their foraging ability, survival skills and swimming pattern (Alvarez et al. 2006; Jakka et al. 2007). In our study, developmental exposure to MeHg resulted in transgenerational inheritance of altered visual startle behavior in zebrafish. The altered response exhibited by the F3 population zebrafish to visual stimuli could have various causes including motor dysfunction, sensory damage, or damage in the signal relay and processing elements of the brain. In order to rule out motor dysfunction as a factor we quantified the spontaneous locomotor activity of adult F3 generation zebrafish (4-6 months old) ancestrally exposed to MeHg. Prenatal exposure to MeHg has been associated with motor dysfunctions and coordination (Montgomery et al. 2008). Locomotor analysis was conducted on the same F3 fish that were used for the visual startle response tests. The swimming distance traveled by the fish without any stimulus was recorded. Locomotor activity test revealed that all MeHg exposure groups of F3 fish were significantly hyperactive compared to the control group (one way ANOVA, $p < 0.006$, $F = 3.498$, $df = 5$). The fish of all exposure groups traveled more distance in 5 min than the control group (Fig 2.6).

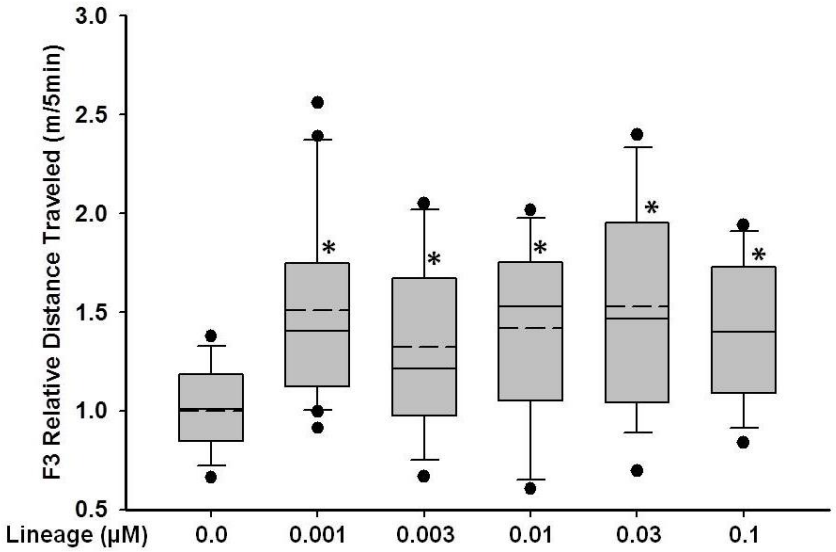

Figure 2.6. Transgenerational locomotor deficit in F3 zebrafish due to ancestral MeHg exposure. Twenty animals from each lineage were tested for their locomotor activity. The relative distance traveled in all ancestrally exposed populations of F3 fish were significantly higher than the control group (ONEWAY ANOVA, Holm-Sidak method, *indicates p <0.001). Solid horizontal lines represent the median, dashed horizontal lines represent the mean, the box represents the 25th and 75th percentiles, the whiskers show the 5th and 95th percentiles, and outliers are represented by dots.

Dose dependent expression of phenotypes

Although significant differences in the visual startle response and locomotor activity were observed in F3 zebrafish ancestrally exposed to MeHg as compared to controls, these differences were not dose-dependent when analyzed at the population level. As both neurobehavioral assays were performed on the same fish, we were able to determine the behavioral phenotypes exhibited by each individual. A fish was considered to have a defect in the behavioral response to visual startle if the number of responses fell below the 5th percentile as determined by the distribution of responses in the control group. A fish was considered to be hyperactive (as only increased locomotion was observed) if it traveled further in the locomotor assay than the 95th percentile as determined by the distribution of responses in the control group. Using these criteria, we were able to identify each individual fish as "normal" compared to controls or as having a defect (“outside the norm”) for each of the specific endpoints. As the ancestral MeHg dose increases, the proportion of fish that exhibit both phenotypes (visual defects and hyperactivity) increases (Fig 2.7). The proportion of fish exhibiting each individual neurobehavioral deficit compared to those expressing both phenotypes suggests that the phenotypes are inherited independently (Chi-square, p=0.224).

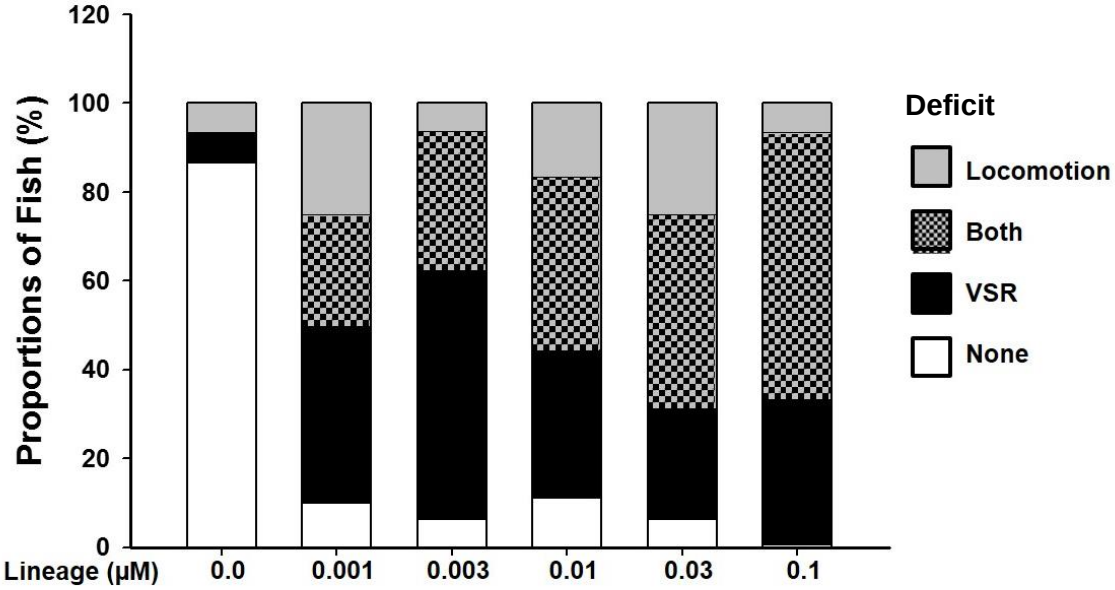

Figure 2.7. Proportion of the F3 populations showing transgenerational phenotypes. A fish is considered to have locomotor deficit, if the fish traveled further in the locomotor assay than the 95th percentile as determined by the distribution of responses in the control group. A fish is considered to have visual deficit, if the number of responses of the fish fell below the 5th percentile as determined by the distribution of responses in the control group. At lower concentrations, more fish exhibited a single deficit. The number of fish with both deficits had increased as the ancestral exposure concentration increased suggesting the independent inheritance of deficits.

DISCUSSION

Exposure to MeHg during prenatal nervous system development results in neurobehavioral disease phenotypes (Montgomery et al. 2008; Boucher et al. 2012; Ceccatelli et al. 2013). Developmental MeHg exposure has been shown to cause a wide range of defects in humans and animal models including teratogenic effects, defects in sensory functions, problems with learning and memory, Attention Deficit Hyperactivity Disorder (ADHD), and more subtle behavioral changes (Baraldi et al. 2002; Newland et al. 2006; Onishchenko et al. 2007; Smith et al. 2010; Mela et al. 2010; Boucher et al. 2012; Radonjic et al. 2013). Recently, a number of environmental toxicants including pesticides and fungicides have been shown to induce the transgenerational phenotypic variations. Thus far, the molecular mechanism behind transgenerational inheritance of phenotypes has been identified as permanent inheritable epigenetic modifications in the germline (Manikkam et al. 2012; Guerrero-Bosagna et al. 2012; Skinner et al. 2013). Given the neurotoxic effects of prenatal exposure to MeHg, we tested the potential of sublethal concentrations of MeHg to induce transgenerational neurobehavioral deficits in zebrafish. We have previously demonstrated that direct developmental exposure to low concentrations of MeHg results in altered visual functions and retinal electrophysiology of adult zebrafish (Weber et al. 2008). The endpoints tested in our current study include visual startle response, electrophysiology of bipolar cells of the retina and locomotor functions of the F3 generation zebrafish whose grandparents (F1) were developmentally exposed to MeHg. In the present study,

we demonstrate, for the first time, the potential of developmental MeHg exposure to induce transgenerational inheritance of neurobehavioral and electrophysiological deficits.

The concentrations of MeHg used in this study were sublethal and ecologically relevant. In 2001, the EPA set the reference dose for MeHg at 5.8 µg/L, the cord blood concentration below which the detrimental effects of MeHg are expected to be minimal (U.S. EPA 2014). According to 2009-2010 National Health and Nutrition Examination Survey (NHANES) data, total blood mercury levels range between 0.23 to 85.7 µg/L in the general U.S. population (Birch et al. 2014). The exposure regimen used in our study resulted in tissue doses ranging from <0.1 to 1.76 ng/embryo with the three lowest doses being significantly lower than the reference dose for humans. However, the tissue doses that we observed doesn't represent the concentration of Hg in the embryo because the majority is in the yolk at 24hpf. The threshold concentrations of dietary MeHg that causes adverse behavioral deficits in fish species has been identified as above 0.5 µg/g wet weight (Depew et al. 2012). Reproductive and subclinical effects were observed in fish at dietary concentrations below 0.2 µg/g wet weight. The exposure concentrations of MeHg that we used were below the ecological dietary threshold for fish. There were variation in behavioral effects reported in studies involved maternal dietary exposure to MeHg due to the amount of MeHg exposed to individual embryos (Alvarez et al. 2006). In an effort to avoid variation in exposure concentrations and behavioral outcomes, we

directly exposed zebrafish embryos to MeHg during the first 24 hpf and quantified the resultant embryo concentrations of Hg.

The embryos directly exposed to MeHg were raised as F1. Since the primordial germ cells present in F1 were also exposed, the F2 was also considered to be an exposed generation. The phenotypes observed in F3 were truly transgenerational phenotypes as the F3 generation was not directly exposed to MeHg neither as egg (F1) nor as germ cell (F2). There were no additional mercury exposure to F2 and F3 during their life cycles. We did not expect to have any mercury residues in F2 and F3 population fish due to both low concentrations of exposure and excretion of mercury from the F1 as they develop from larvae to adult. Finally, all zebrafish are exposed to background levels of Hg through their diet but our study populations are housed side-by-side and provided with the same routine care, thus the altered neurobehavioral and physiological differences observed are purely due to ancestral exposure.

Our current study revealed the potential of developing transgenerational neurobehavioral phenotypes upon developmental exposure to environmentally relevant levels of MeHg. The phenotypes tested include visual function, retinal electrophysiology and locomotion in both the F1 (directly exposed) and the F3 (ancestrally exposed) generations of zebrafish. Both F1 and F3 have shown visual deficits; however, the phenotypes were not dose-dependent. We have observed comparatively fewer effects on the visual startle response and retinal electrophysiology at the highest ancestral exposure concentration (0.1µM) than the second highest concentration (0.03 µM). This might be due to the subtle

negative impacts on health and fitness resulting in a differential distribution of the trait in the F3 populations.

In this study, the electrophysiology of the retinal bipolar cells in the F3 MeHg exposure lineage revealed dysfunctional potassium ion channels. Developmental exposure to MeHg has shown alteration of cellular and metabolic functions of the visual pathway leading to altered neurobehavioral outcomes (Glover et al. 2009; Ho et al. 2013). MeHg has also been associated with delayed latencies of visual evoked potentials in children of the Faroe Islands even without clinical conditions (Yorifuji et al. 2013). As previously reported, there was no significant difference in morphology of retina between the control and exposure groups in F1 zebrafish (Weber et al. 2008). However, the electrophysiology of the retinal bipolar cells was altered in F1 zebrafish. Direct MeHg exposure caused an enhanced outflow of K^+ currents due to an induced depolarization in the bipolar cells of the retina. We investigated the electrophysiology of three dosage groups of F3 fish (0.01, 0.03 and 0.1µM MeHg). The bipolar cells of F3 0.01 and 0.03 lineage showed similar electrophysiology as that of F1. A visual response to a stimulus requires coordinated cellular and molecular functions starting from the sensory cells of the retina to the skeletal muscular cells through the central and peripheral nervous systems. During phototransduction, bipolar cells of the retina are activated by photoreceptor cells (Leskov et al. 2000; Ebrey and Koutalos 2001). Thereafter, the bipolar cells relay signals to both ganglion cells and the optic tectum (*i.e.*, the final destination of the visual signals) of the brain with being. Proper function of potassium ion channels of the bipolar cells are required

for creating action potentials for signal transmission to ganglionic cells and to the brain. The normal function of Na+ and K+ voltage-gated cation channels is critical for the phototransduction process. Delayed potassium ion outward flow results in hyperpolarization of the bipolar cells and thus affect signal transmission to the ganglionic cells and beyond (Nelson and Connaughton 1995). We found that retinal electrophysiology was altered due to dysfunctional K+ ion channels in the bipolar cells of the retina due to ancestral developmental exposure to MeHg. Our findings revealed that delayed K^+ current in the bipolar cells of the retina likely to play role in the visual deficits exhibited by the F3 lineage fish. The fish with that exhibited visual deficits were also tested for their locomotor activity to better understand the mechanism behind the reduced response to visual stimuli.

The movement of the organism is dependent on the normal locomotor physiology. Prenatal exposure to MeHg has been linked to locomotor behavioral deficits in rodents (Bisen-Hersh et al. 2014). One of the mechanisms by which prenatal exposure to MeHg affects locomotor functions by reducing the dopaminergic transmission in the caudate putamen of the brain (Daré et al. 2003; Montgomery et al. 2008). As mentioned previously, we analyzed locomotion in order to eliminate problems in movement as a causative factor in the visual deficits. Our data demonstrates that these fish not only swim effectively, but they actually exhibit significant hyperactivity, compared to control fish. Thus, the F3-generation zebrafish have the physical ability to respond, but are not responding due to the visual stimuli provided in the visual startle response neurobehavioral assay. Studies have shown that motor functions of

male rats are impacted more than the female rats due to prenatal MeHg exposure (Rossi et al. 1997). Some other studies show that the locomotor functions are reduced in female progenies due to gestational MeHg exposure (Goulet et al. 2003; Gralewicz et al. 2009). In our study, we did not observe a gender difference in transgenerational locomotor hyperactivity due to ancestral MeHg exposure. Even though the exact mechanism by which MeHg induces transgenerational hyperactivity has not yet been fully characterized, our findings have wide-spread implications. Hyperactivity represents a newly identified transgenerational neurobehavioral defect induced by developmental MeHg exposure. There are mounting evidences of the association between developmental exposure to MeHg and attention-deficit hyperactivity disorder (ADHD) in children (Sagiv et al. 2012; Yoshimasu et al. 2014). Since zebrafish has been identified as an animal model for ADHD (Whalley 2015), future studies could be conducted in this model to elucidate the association between MeHg exposure, hyperactivity, and ADHD.

A number of recent studies have shown that environmental toxicants such as DDT, hydrocarbons, pesticides, fungicides, plastic, and dioxin induce a variety of transgenerational disease phenotypes (Manikkam et al. 2012; Manikkam et al. 2013; Skinner et al. 2013a; Skinner et al. 2013b; Tracey et al. 2013). These studies have identified the molecular mechanism responsible for transmission of the disease phenotypes as inherited epigenetic modification of DNA methylation in the germ cells. MeHg exposure causes oxidative stress, leading to DNA damage, at very high doses. However, random mutagenesis is not likely involved

in the inheritance of MeHg-induced phenotypes because of the relatively low doses used and the very high percentage of affected individuals in subsequent generations. Further studies are necessary to confirm that ancestral exposure to MeHg causes inheritable alteration of DNA methylation pattern in the germ cells and passed on to the offspring. The parental origin of the inheritance of transgenerational neurobehavioral phenotypes need to be further investigated. Imprinted DNA methylation sites escape the genome-wide DNA methylation erasure events which occur during gonadal development (Seisenberger et al. 2012) and early embryogenesis (Mhanni and McGowan 2004). Therefore, these epimutations become programmed into the genome and can cause changes in gene expression and function in offspring who inherit the epigenetically altered allele (Skinner et al. 2012). Transcriptome and methylome analysis of the F3 generation are required to identify the genes, pathways and the role of DNA methylation in the inheritance of phenotypes.

MeHg has been linked to decreases in global DNA methylation (Pilsner et al. 2010; Basu et al. 2013; Ceccatelli et al. 2013). However, significant variation in changes in global DNA methylation induced by MeHg exposure exists among different species (Basu et al. 2013; Basu et al. 2014). Dietary exposure to MeHg has not shown alteration in global DNA methylation either in yellow perch or zebrafish (Basu et al. 2013; Olsvik et al. 2014). The significant change in global DNA methylation that we observed in our study at 0.1µM exposure indicate the potential of direct exposure to MeHg during embryogenesis to alter DNA methylation. The direct exposure to MeHg resulted in genome-wide

hypomethylation of the embryo indicating the possibility of permanent alteration of epigenome and inheritance of disease phenotypes. Further investigations are needed to identify the gene specific changes in methylation and the differentially methylated regions (DMR).

It is almost six decades since the Minamata outbreak showed the adverse neurological impacts of developmental exposure to MeHg. The neurotoxic effects of MeHg due to prenatal exposure have been well established in a variety of species, but the potential to induce transgenerational effects was unknown. In the present study, we investigated the potential of developmental exposure to MeHg in inducing transgenerational neurobehavioral deficits in zebrafish. Our data suggests that developmental exposure to MeHg causes transgenerational inheritance of visual deficits, abnormal retinal electrophysiology and hyperactivity. We also report that MeHg has the ability to alter the DNA methylation pattern in directly exposed embryonic zebrafish. If the observations that we made in zebrafish are also seen in other species such as wild fish and humans, our findings will have considerable ecological and public health impacts in areas of risk assessment and interventions.

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CHAPTER 3

Ancestral Developmental Exposure to Methylmercury Induces Phenotype-Specific Germline Transmission of Transgenerational Neurobehavioral Deficits

ABSTRACT

Developmental exposure to the environmental neurotoxicant methylmercury (MeHg) is associated with neurobehavioral deficits. Our previous study has demonstrated the potential of MeHg to induce the transgenerational onset of visual and neurobehavioral deficits in the zebrafish of randomly-bred MeHg lineages. With the current study, we sought to identify the difference in the incidence of deficits between direct and transgenerational lineages as well as determine the parental origin of deficits transmission. We exposed zebrafish embryos to sublethal concentrations of MeHg and subsequently investigated visual response and locomotor behavior in the directly-exposed F1 individuals (grandparents) and in their descendant lineage (F3 generation or grandchildren). We used several breeding schemes to identify the parental origin of transgenerational inheritance and to explore the difference between random and selective breeding in F2 (parents) inheritance. To identify the parental origin of the deficits, we outcrossed F2 control and MeHg-exposure groups to generate F3 outcross populations. To identify the change in incidence of visual deficits due to selective breeding, we bred the F1 animals that exhibited severe visual deficits to create a F2 population. Our study demonstrated that the visual response was significantly reduced in both directly exposed groups and transgenerational lineages. Selective breeding resulted in higher incidence of visual deficits in the F2 population. The outcross study demonstrated that transgenerational inheritance of visual deficit to the F3 generation required either male or female germline transmission. No significant difference in locomotor behavior was observed in the F1 population; however, the phenotype was observed in the F3

generation. Outcross analysis revealed that the transgenerational inheritance of hyperactivity behavior required both maternal and paternal germline. The difference in the transgenerational inheritance of the visual startle deficit and hyperactivity phenotypes strongly suggests that MeHg-induced transgenerational phenotypes are the result of multiple independent genomic events with independent inheritance patterns.

Key words: MeHg Transgenerational Parental origin Selective breeding Outcross

INTRODUCTION

Developmental exposure to nutritional and environmental stressors has demonstrated the potential to induce epigenetic changes and transgenerational inheritance of phenotypic variation in several species (Skinner et al. 2010; Colombo et al. 2014). Transgenerationally-inherited stressor-induced phenotypes result from the germline (egg or sperm) transmission of a phenotype between generations in the absence of any additional environmental exposure (Skinner et al. 2013a; Skinner et al. 2013c; Manikkam et al. 2014). Transgenerational inheritance of environmentally-induced disease and dysfunction resulting from specific environmental toxicants (*e.g.*, vinclozolin, DDT, dioxin, and BPA) include cancers, obesity, reproductive defects, kidney diseases, and ovarian diseases (Manikkam et al. 2012; Guerrero-Bosagna et al. 2012; Manikkam et al. 2013; Skinner et al. 2013a; Tracey et al. 2013). Behavioral changes (*e.g.*, anxiety, mate preferences, and learning) are also suggested to be transgenerationally inherited and the result of ancestral exposure to environmental stressors (Crews et al. 2012).

The etiology of transgenerational inheritance of phenotypic variation or diseases involves, in many cases, the germline transmission of an altered epigenome between generations. Recent studies have demonstrated that exposure to environmental toxicants (*e.g.*, vinclozolin, DDT, dioxin, and BPA) results in transgenerational inheritance of adult onset dysfunction via permanent heritable changes in the DNA methylation pattern (epimutations) (Manikkam et al. 2012; Guerrero-Bosagna et al. 2012; Manikkam et al. 2013; Skinner et al.

2013a; Tracey et al. 2013). The window of susceptibility for altering the DNA methylation pattern leading to transgenerational inheritance is that of gonadal germline development during embryogenesis (Guerrero-Bosagna et al. 2014; Nilsson and Skinner 2015). Transcriptome analysis of transgenerationally affected populations has shown that the expression of specific genes in critical pathways is dysregulated by differentially methylated regions (DMRs) of the genome. The dysregulated genes associated with altered phenotypes are often clustered in regions within 2 to 5 megabases of the identified DMR (Skinner et al. 2012).

Most of the studies investigating the transgenerational inheritance of environmentally induced phenotypes use *in utero* exposure models with rodent. In this paradigm, exposure to an environmental stressor during gestation results in the direct exposure to three generations, the mother (F0), the embryo (F1) and the germline of the embryo (which will give rise to the F2). The phenotype observed in the F3 is considered a transgenerational phenotype because it is inherited in the absence of any direct exposure to a stressor/contaminant. Outcrossing studies (breeding with unexposed lineages) have shown that environmental toxicants may vary in the way that they transmit transgenerational environmental phenotypes from the F2 to the F3 and subsequent generations. For instance, gestational exposure to a high fat diet (Dunn and Bale 2011) or vinclozolin (Anway et al. 2005; Guerrero-Bosagna et al. 2012), an endocrine disruptor, result in the epigenetic transmission of transgenerational disease through the male germline. While, developmental exposure to methoxychlor, a

pesticide, is associated with the transgenerational inheritance of male obesity through the female germline. Conversely, transgenerational inheritance of female obesity from ancestral methoxychlor requires the exposure of both the male and female germlines (Manikkam et al. 2014b). The DDT-induced transgenerational inheritance of obesity, kidney disease, and gonadal disease are highly complex and suggest that the parental germline origins for transgenerational disease may be disease or organ specific (Skinner et al. 2013a). Finally, both male and female germline transmission of transgenerational phenotypic variations were observed due to environmental stressors such as dioxin, BPA, and JP-8 jet fuel (Manikkam et al. 2012, Manikkam et al. 2013, Tracey et al. 2013). The evidences suggest that there is no single mode of germline transmission of environmentally induced transgenerational phenotypes. The pattern varies between the environmental insult and the transgenerational phenotype. The germline mode of transmission of MeHg induced transgenerational neurobehavioral variation is previously unknown.

MeHg is a global environmental neurotoxicant. Environmental release of mercury from human activities has been rising since the industrial revolution. Current anthropogenic environmental releases of mercury are dominated by artisanal gold mining and coal combustion for the purpose of energy production. The World Health Organization has listed mercury as one of the top ten chemicals of global public health concern due to its toxic effects on humans (WHO). Humans are primarily exposed to MeHg through the consumption of contaminated fish and seafood (Davidson et al. 2006; Buchanan et al. 2015).

MeHg stays in the maternal blood and fetus is exposed to the toxicant during development through the umbilical cord blood. The developing embryo is much more sensitive to the neurodevelopmental and behavioral effects of MeHg than are other life stages, and these effects can persist throughout the entire lifespan (Castoldi et al. 2003; Bertossi et al. 2004; Huang et al. 2011).

In the U.S., people live in certain regions and some subpopulations are at risk from mercury exposure. In the U.S., studies based on the National Health and Nutrition Examination Survey (NHANES) found that total blood mercury levels are higher in the Northeastern region of the country (Mortensen et al. 2014). Asian ethnicity and higher income were also associated with higher blood mercury level due to greater seafood consumption (Buchanan et al. 2015). There is an estimated 1 to 3 percent of women of child bearing age are at risk from MeHg exposure in the U.S. (EPA 2014). Studies have also shown that Hg levels in the fish species of Great Lakes are also on the rise resulting in wide-spread advisories on fish consumption in this region (Bhavsar et al. 2011; Evers et al. 2011; Wiener et al. 2012). Total blood mercury at levels generally considered safe (below the US EPA reference dose) are also associated with a higher risk of developing disorders in reproductive-age females (Somers et al. 2015). Evidences suggest that developmental exposure to MeHg is a public health concern due to its adverse neurodevelopmental and neurobehavioral effects in offspring.

Our laboratory uses zebrafish as a model organism for human biomedicine and our previous studies have shown that ancestral developmental

exposure to MeHg induces transgenerational inheritance of visual and neurobehavioral deficits. Examination of the transcriptome in the brain and retina of the F3 generation revealed that numerous biological pathways related to neurological function are disrupted due to ancestral developmental MeHg exposure. In the study described herein, we used several breeding schemes to identify the parental origin of transgenerational inheritance of visual and neurobehavioral deficits by outcrossing males and females from MeHg-exposed zebrafish lineages, as well as to explore the difference between random and selective breeding in F2 inheritance of the same phenotypes. We have demonstrated that parental inheritance of transgenerational phenotypes are phenotype-specific. We also show that selective breeding greatly enhances the incidence of the visual deficit. The identification of phenotype-specific germline transmission involved in transgenerational inheritance of neurobehavioral deficits would serve critical roles in targeting susceptible subpopulations for interventions to reduce MeHg toxicity. Our findings also lead to future research to identify the mode of germline transmission of other phenotypes associated with MeHg exposure. Developmental and occupational exposure to MeHg is associated with both visual sensory and motor dysfunctions (Burbacher et al. 2005; Rodrigues et al. 2007; Barboni et al. 2009).

MATERIALS AND METHODS

Animal studies

All animal care and experiments using animals or involving hazardous materials were pre-approved by the Institutional Animal Care and Use Committee, Environmental Protection, and Laboratory Safety offices of University Safety & Assurances of the University of Wisconsin-Milwaukee.

Zebrafish stock and egg collection

Adult EK strain zebrafish were originally obtained from Ekkwill Waterlife Resources (Gibsonton, FL, USA) and have been maintained in our laboratory for over 10 years. Adult fish were housed at a maximum density of 10 adult fish/L in a flow-through dechlorinated water system maintained at 26 to 29 °C on a 14:10 h light: dark photoperiod at the Aquatic Animal Facility of the NIEHS-funded Children's Environmental Health Sciences Core Center at the University of Wisconsin-Milwaukee. For spawning, male and female fish (male: female ratio 1:2) were kept overnight in a 2 L plastic aquarium with a 3-mm nylon mesh bottom to separate newly fertilized eggs from the adults. The fish were moved to clean water 30 min prior to the onset of light to ensure that only eggs fertilized within a narrow time period were used in experiments. Eggs were collected ≤ 1 h post fertilization (hpf) and placed into metal-free, plastic culture dishes (100 mm diameter × 50 mm depth) in E2 medium (Nüsslein-Volhard 2002; pH 7.2; in one liter: 0.875 g NaCl, 0.038 g KCl, 0.120 g $MgSO_4$, 0.021 g $KH2PO_4$, and 0.006 g Na_2HPO_4; Hg-free as determined by Inductively Coupled Plasma-Mass Spectrometer (ICP-MS).

Methylmercury exposure

The newly fertilized eggs were then transferred to 12-well culture plates (10 eggs/well) and rinsed twice with E2 medium. The eggs were exposed to 1.5 ml of E2 medium containing MeHg (0.0, 0.001, 0.003, 0.01, 0.03, 0.1 μM with ethanol as a carrier [< 0.01% total volume]). Following a 24 h exposure, embryos were rinsed three times with E2 medium and transferred to 2 L tanks (60 embryos per tank) containing static E2 medium for rearing. Starting at 5 dpf, eleutheroembryos were fed 5-100 micron Golden Pearl Reef & Larval Fish Diet (Brine Shrimp Direct, Ogden, UT, USA). Platinum Grade *Artemia* nauplii (Argent Laboratories, Redmond, WA, USA) were fed starting at 9 dpf. At 21 dpf, fish were transferred to 1 L flow-through tanks and raised using standard husbandry techniques until 3 months of age, at which point they were used for behavior assays and breeding. Juvenile and adult fish were fed a combination of Platinum and Gold grade *Artemia* nauplii and Aquarian™ flake food (Aquarium Pharmaceuticals, Inc., Chalfont, PA, USA).

In mammalian models of developmental toxicity, the pregnant animal is directly exposed to the toxicant and is designated as F0. The developing progeny, which are exposed to the toxicant *in-utero*, are considered the F1 generation. In our zebrafish model system, the directly exposed embryos are analogous to the F1 progeny in mammalian animal models. After the initial 24-h MeHg exposure of the F1 embryos, there was no additional exposure to Hg during the entire life cycle, beyond the background levels inherent to all fish food.

Many different fish foods were screened, and those with the lowest levels of Hg (as determined by ICP-MS) were used as much as possible in this study.

Generation of F1, F2 and F3 population for selective breeding

Adult F1 fish that were exposed to MeHg as embryos were tested for visual deficits by visual startle reflex test as previously described (Weber et al. 2008). Ten male and ten female animals from each group (0.0, 0.001, 0.003, 0.01, 0.03, 0.1 μM MeHg) were tested. All fish were kept in individual tanks after testing to maintain individual identity during the analysis process.

Three male and three female F1 control animals were chosen randomly to create F2 control population. The three males and females that had the lowest number of responses in the visual startle reflex tests were chosen from each exposure group to generate the F2 exposure population. Two rounds of pair-wise breeding were conducted. After the breeding of a pair, the mates were switched for the second breeding, so that ultimately each of individual was spawned with two mates. Equal numbers of embryos from each pair for each spawning were combined to create the F2 population. The selection and spawning protocols were repeated on the F2 adult population to create the F3 generation. The F2 and F3 generations were never exposed to exogenous MeHg. However, the F2 generation is also considered to be directly exposed as they were the primordial germ cells within the F1 embryos at the time of initial MeHg exposure.

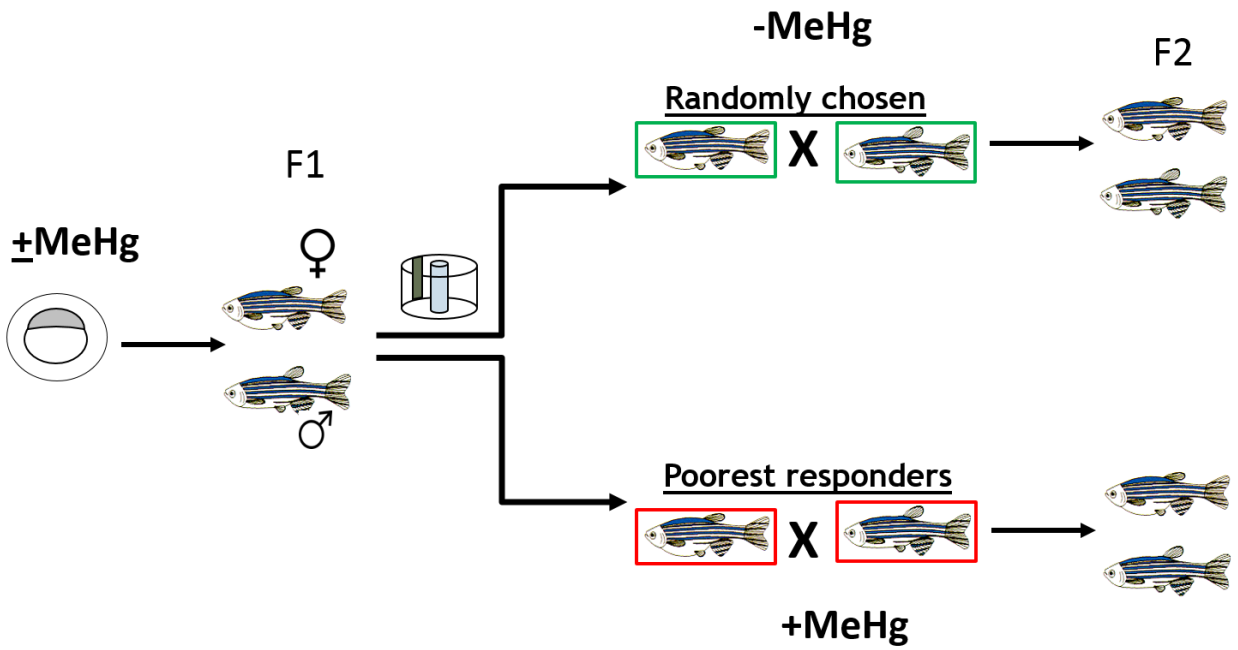


Figure 3.1. Experimental design for selective breeding. F1 animals were tested for visual startle response. Control animals were randomly chosen to generate F2 control while poorest responders of exposure groups were used to generated F2 exposure lineage. F2 population was then tested for their visual startle reflex.

Breeding scheme for outcross analysis

To identify the parental origin of transgenerational phenotypic transmission, we generated an outcross offspring population from the F2 animals. Briefly, zebrafish embryos were exposed to 0.0, 0.003 and 0.03 µM MeHg as described above. The selection of the exposure lineage was based on the population response from those lineages to visual response, retinal electrophysiology and locomotor activity. The F1 adults were inbred to generate F2 population. Outcrosses of the F3 generation were created by spawning the F2 control male with F2 MeHg-exposed females and F2 control female with F2 MeHg-exposed males (Fig 3.2). F3 outcross animals were, then, screened for their visual and locomotor behavioral functions.

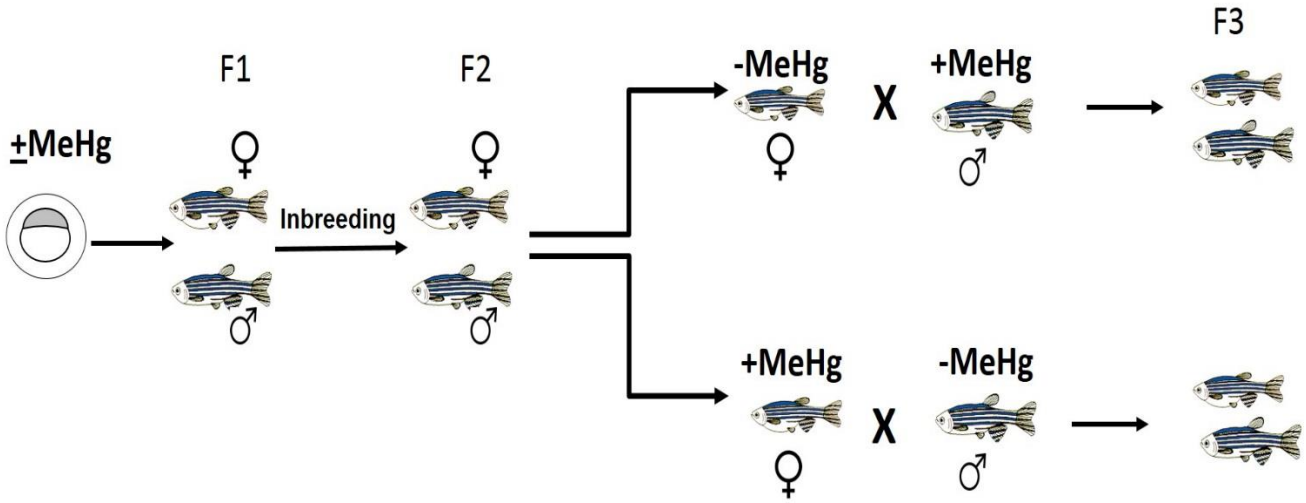


Figure 3.2. Experimental design for outcross. F1 animals were inbred to generate F2. F2 control females were spawned against F2 MeHg males. F2 MeHg females were spawned against F2 control males. F3 fish were tested for visual startle reflex and locomotion activity.

Visual startle response

The behavioral response of the fish to visual stimuli was analyzed as previously described [Dowling et al. 1997, Weber et al. 2008]. Adult male and female fish (n = 20, 10 of each gender; 3 months old from all exposure groups were randomly assigned numbers to blind the observer to their exposure. Individual fish were placed in a stationary glass crystallizing dish (10 cm diameter, 5 cm depth, approximately 200 ml of dechlorinated water) surrounded by a rotating white PVC drum with a black vertical stripe (1 cm width x 5 cm height). The drum speed was set to 10 rev/min and the black vertical stripe elicited a startle response when it entered the fish's field of vision. When startled, zebrafish will elicit either a C-start escape response or an avoidance maneuver. After a 5-minute l acclimation period in low light, the PVC drum rotation was initiated and the response of each fish to the rotating black bar was captured for 5 min using infrared digital video. All tests were conducted between the hours of 1300-1600 due to the zebrafish circadian rhythm, which exhibit the most consistent light sensitivity and basal activity levels during the afternoon hours (Dowling et a. 1997). Blinded analysis was conducted on the videos for the visual startle response and the number of C-start escape reactions exhibited per fish during the encounter with the rotating black bar was quantified. All data are expressed as responses relative to the control group. One-way ANOVA (SigmaPlot 12.0, Systat Software, San Jose, CA) was used to analyze visual startle response data with $P< 0.05$ as the threshold of statistical significance.

Locomotor Activity

Locomotor activity was quantified using a Behavior Observation Box (BOB) (Fig 3.3). Fish were placed in a clean glass crystallizing dish (10 cm diameter and 5 cm depth) in a light-tight chamber. The light intensity inside the chamber was produced by a standard computer monitor (76 Lux). The chamber was equipped with four Logitech c920 USB cameras (Logitech, Newark, CA) for capturing digital video of four arenas simultaneously. In our experiment, we used the two distant cameras at a time. The fish were acclimated for 5 minutes (min) after which a digital video was recorded for an additional 5 min. The files were obtained in M-MJPEG format using the Matlab Image Acquisition Toolbox™ (MathWorks, Natick, MA) at a 960 x 720 pixels resolution. Video was cropped to 600 x 600 pixels with ffmpeg (https://www.ffmpeg.org/) prior to being imported into EthoVision XT (Noldus Information Technology Inc, Leesburg, VA) for automated analysis of distance traveled in 5 min. One-way ANOVA (SigmaPlot 12.0) was used to identify the difference in locomotor activity between the exposure and control groups. Level of statistical significance was set at $P < 0.05$.

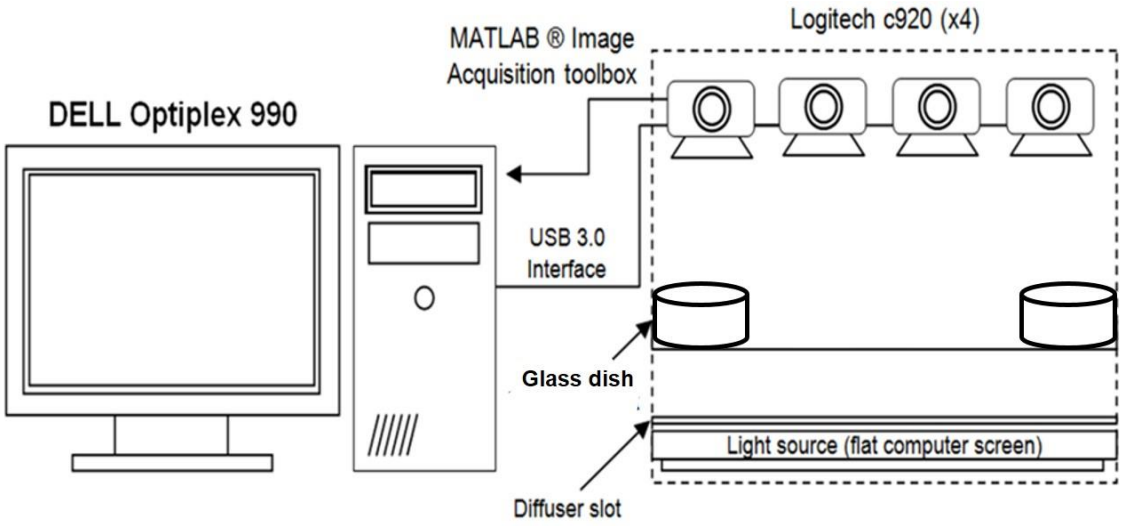


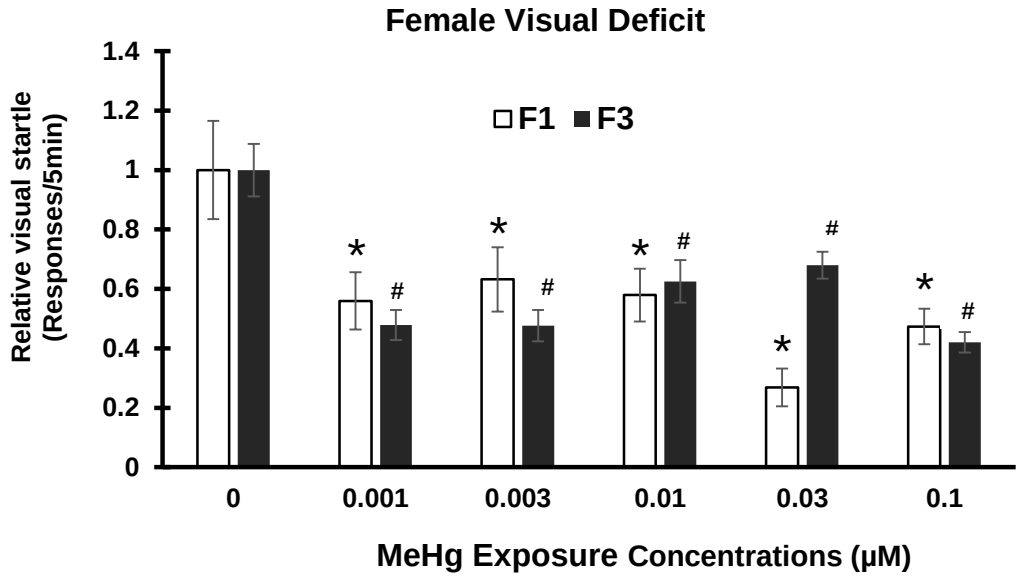
Figure 3.3. Schematic representation of locomotor activity recording. The recording chamber was equipped with Logitech c920 cameras. In our study, we placed fish in glass crystallizing dish and used the cameras at both ends to capture the movements of the fish. The cameras were operated from a remote computer running the MATLAB Image acquisition toolbox. Fish were acclimated for 5 min after which digital video was recorded for an additional 5 min.

RESULTS

Male and female transgenerational visual deficits

The difference in incidence of transgenerational visual deficits in male and female populations due to developmental exposure to MeHg zebrafish embryos was investigated. Zebrafish embryos were exposed to 0.0, 0.001, 0.003, 0.01, 0.03, 0.1 μM concentrations of MeHg during the first 24 hrs of development. The adult fish (F1) were directly exposed to MeHg as they developed from the MeHg exposed embryos. The F2 generation was exposed as primordial germ cells, making F3 generation the first generation without any direct exposure to MeHg. Adult F1 and F3 zebrafish were tested for visual deficits. Direct exposure to MeHg induced statistically significant visual deficits in both males and females relative to control (Fig. 3.4) (one-way ANOVA, $p < 0.05$). The incidence of visual deficits in F3 lineage males and females are shown in Figure 3.1. The visual response of females from all exposure concentrations was significantly reduced in the F3 lineage as compared to control (Fig. 3.4 A) (one-way ANOVA, $p < 0.05$, $F = 5.415$, $df = 5$). The visual response of the F3 lineage males in all exposure groups except the lowest were also significantly reduced (Fig. 3.4 B) (one-way ANOVA, $p < 0.05$, $F = 4.823$, $df = 5$). The F3 lineage males in the lowest concentration (0.001 μM) were also reduced relative to control males but the magnitude was not statistically significant ($p = 0.178$).

A



B

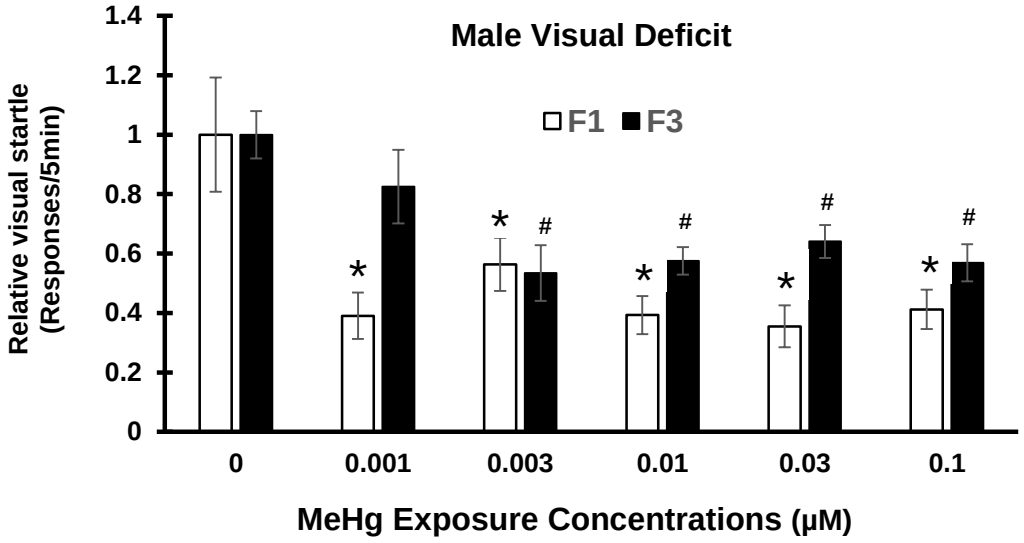


Figure 3.4. Direct and transgenerational visual deficits. Relative response of females (A) and males (B) compared to control males and females. Direct exposure (F1) groups are represented by white bars and transgenerational (F3) groups are shown as black bars. The significance difference of the exposure groups of each generation was tested against the control group of that generation. One-way ANOVA analysis was performed and * indicates F1 groups significantly different than F1 control group and # indicates F3 exposure groups significantly different than F3 control group (p <0.05).

Selective breeding of animals with visual deficits

The incidence of visual deficits between generations due to the selective breeding of the animals that exhibited visual deficits was investigated. There were significant difference in visual responses between the F1 control and all MeHg exposure groups (Figure 3.4). The incidences of visual deficits were calculated by determining the number of fish in each exposure group with a visual response below the 5^{th} percentile as determined by the distribution of responses in the control group. The lowest responding males and females of each F1 group were identified and spawned together to generate a F2 selective breeding (SB) population, as described above. The F1 control animals were randomly chosen for breeding to create a F2 lineage control population. The incidence of visual deficits in the F2 (SB) lineage was significantly higher than the corresponding F1 exposure group, with the exception of the 0.001 group (Table 3.1). These data suggest that at higher MeHg exposure concentrations, selective breeding of animals with visual deficits increases the incidence of the phenotype in the offspring.

Table 3.1. Incidence of visual deficits in F1 and F2 from selectively bred F1

MeHg exposure (µM)	F1 (%)	F2 (SB) (%)
0.0	5	5
0.001	52.6	45
0.003	40	70
0.01	42.1	75
0.03	66.6	90
0.1	60	70

Germline transmission of transgenerational visual deficit and hyperactivity

Transgenerational inheritance of disease phenotypes occurs through the transmission of an altered epigenome by the sperm, egg, or both. Our current study was designed to identify the parental origin of the transgenerational inheritance of visual deficits in zebrafish. F1 fish were exposed to MeHg as embryos and the F2 generation was created by inbreeding theseF1 fish. Since the F2 individuals were directly exposed to MeHg as primordial germ cells within the F1 embryo, a phenotype observed in F2 is considered multigenerational not transgenerational. The F2-lineage control males were outcrossed to the F2-lineage MeHg-exposed females and the F2-lineage control females were outcrossed to F2-lineage MeHg-exposed males to generate F3-lineage fish. The F3-lineage control and outcross fish were grown to adulthood and their visual function and locomotor behavior was evaluated (Figure 3.5). The visual response of all F3-outcross groups were significantly reduced compared to the control group (one way ANOVA, $p < 0.002$, $F = 8.281$, $df = 4$). This suggests that either

maternal or paternal germline is sufficient to transmit visual deficit to the F3 generation.

Locomotor behavior was tested by measuring the total distance traveled by fish in 5 min without any visual stimuli. No significant difference in the distance traveled by the F3-outcross lineage were observed (Fig 3.6) (one way ANOVA, $p < 0.05$). Our study demonstrates that the hyperactivity phenotype observed in inbred F3-lineage exposure groups was not exhibited by the F3-outcross lineage. This suggests that transgenerational transmission of hyperactivity phenotype requires both male and female germlines.

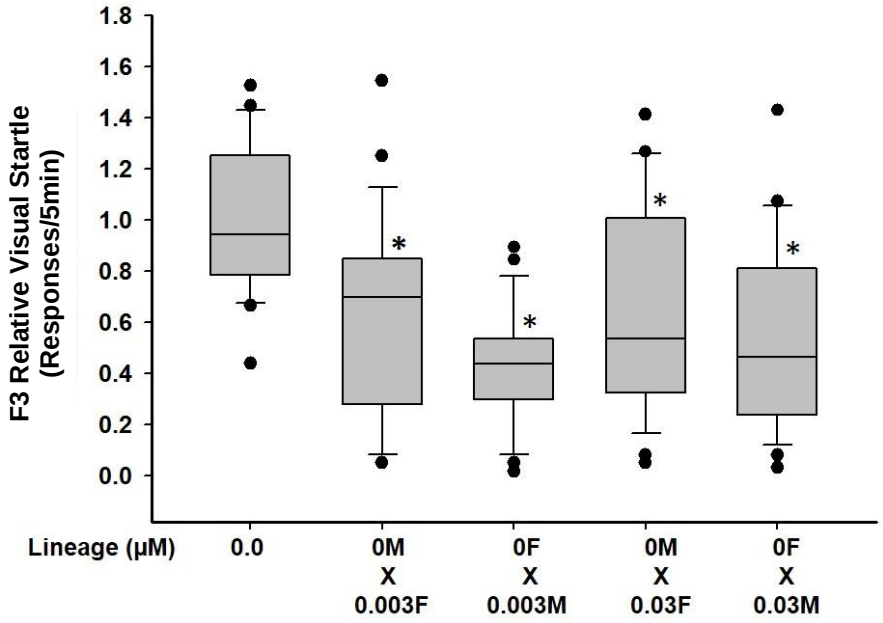


Figure 3.5. Germline transmission of transgenerational visual deficit. Visual startle response of fish to a rotating black bar was captured for 5 min. Relative visual response of F3-lineage control and outcross animals are shown. One way ANOVA analysis was performed and * indicates a statistically significant reduction in the visual response of outcross animals compared to the control group (p<0.002).

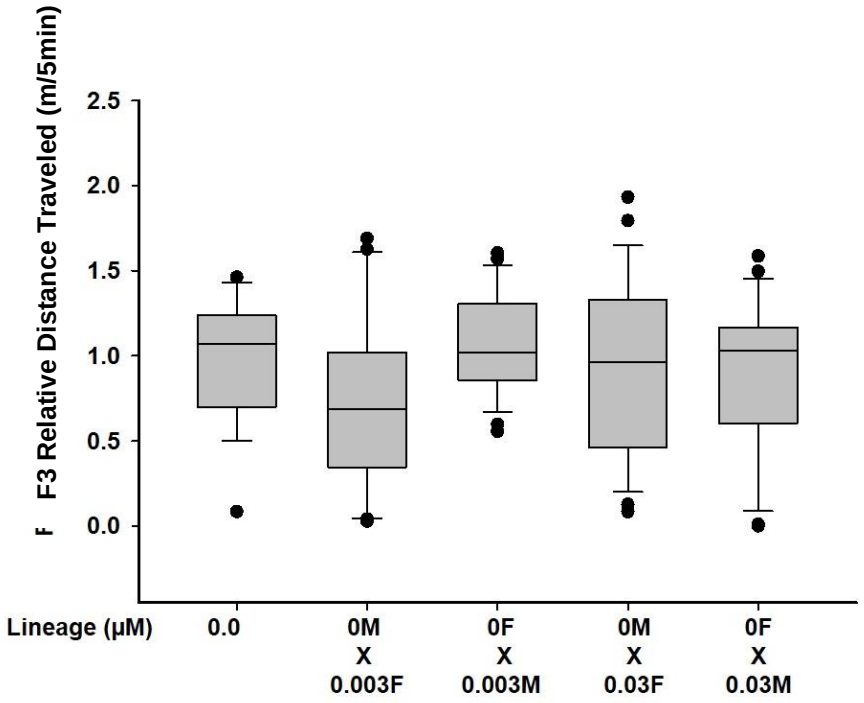


Figure 3.6. Germline transmission of locomotor behavior. Relative distance traveled in 5 min was recorded. One way ANOVA analysis revealed no statistically significant difference between that F3-outcross lineage compared to the control group (p<0.05).

Direct and transgenerational effect on locomotor behavior

A comparison of direct and transgenerational effect on locomotor behavior due to developmental exposure to MeHg was conducted. The swimming distance traveled by the fish without any stimulus was recorded. No statistically significant changes in locomotor function were detected in any F1 exposure population (Fig. 3.7) (one way ANOVA, $p < 0.05$). The F3 lineage of each exposure population traveled significantly farther than the F3 control lineage (Fig 3.6) (one way ANOVA, $p < 0.006$, $F = 3.498$, $df = 5$). Statistical analysis was also conducted to identify the difference in incidence in hyperactivity among males and females of both F1 and F3 generations, but no statistically significant differences were observed between the males and females of either direct or transgenerational populations (data not shown, $P>0.05$).

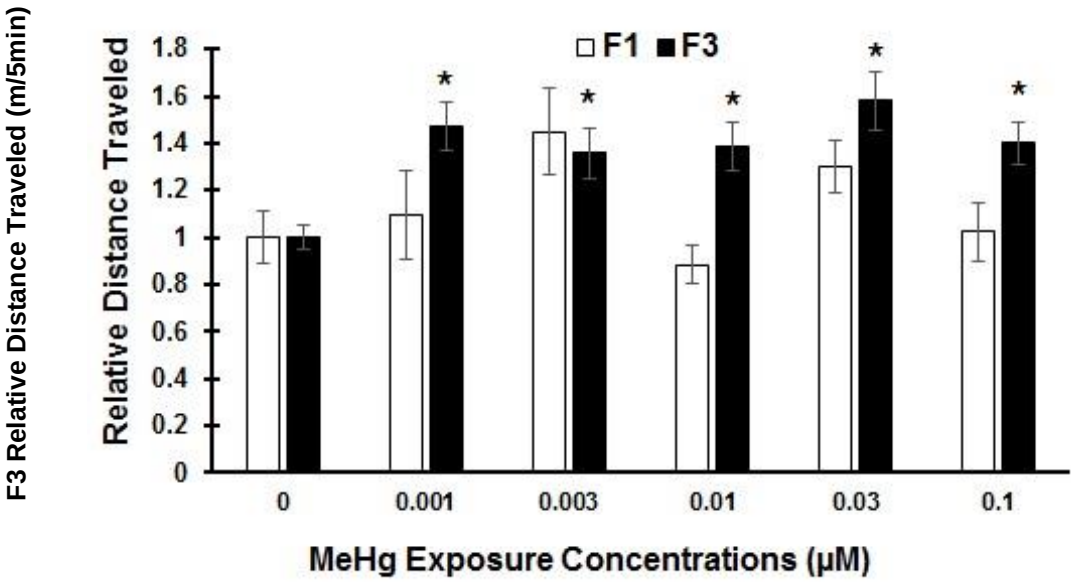


Figure 3.7. Direct and transgenerational locomotor activity. Relative distance traveled in 5 min was recorded. Direct exposure (F1) groups are represented by white bars and transgenerational (F3) groups are shown as black bars. One way ANOVA analysis was performed and * indicates that the F3 MeHg-exposed groups were significantly different than F3 control group (p <0.05).

DISCUSSION

The mechanism of environmentally induced transgenerational inheritance of phenotypic variation and diseases requires heritable epigenetic changes in the germline and transmission to subsequent generations without further exposure (Manikkam et al. 2012; Manikkam et al. 2013; Skinner et al. 2013a; Skinner et al. 2013b; Tracey et al. 2013). The germline mode of epigenetic transmission of disease phenotypes varies between environmental factors. The four possible ways of transmission of a phenotype through germlines are through the male only, through the female only, either male or female is sufficient and both male and female are required. Research has shown that ancestral vinclozolin exposure caused male infertility (?)up to four generations through male germline only (Anway et al. 2005). While female transmission of male obesity was observed in methoxychlor(a pesticide) -exposed transgenerational populations (Manikkam et al. 2014). Sex-specific inheritance of transgenerational diseases such as obesity were identified following ancestral DDT exposure (Skinner et al. 2013a). Female transgenerational obesity due to ancestral DDT exposure was transmitted through the male germline or combined effect of both male and female germlines. Male obesity was transmitted only though the female germline. Evidences suggest that there is no single mode of inheritance of transgenerational disease phenotypes. The germline through which a transgenerational phenotype is being inherited depends upon the triggering environmental factor and the phenotype.

The neurotoxic effects of MeHg due to prenatal exposure has been well documented (Farina et al. 2011; Bose et al. 2012; Grandjean et al. 2014). Our previous research has identified the potential of ancestral developmental exposure to MeHg to induce visual and locomotor behavioral deficits in zebrafish. Our current study indicates that the visual functions of both males and females in both direct (F1) and transgenerational (F3) populations were significantly affected by developmental exposure to MeHg. The effect of developmental exposure to MeHg on visual response in both F1 and F3 generations is similar to the actions of environmental toxicants such as vinclozolin (Anway et al. 2006) and methoxychlor (Manikkam et al. 2014b). Vinclozolin caused reduced spermatogenesis in F1 through F4 generations while methoxychlor yielded kidney disease in the females of both the F1 and F3 generations. Our findings, along with other studies on environmental toxicants, suggest that a phenotype can be induced by direct exposure during development of an organism and future generations could exhibit the same phenotype due to permanent inheritable changes made in the exposed generation. It is also noted that certain phenotype might not be necessarily observed in direct exposure lineage, however exhibited by the transgenerational lineage.

The incidence of visual deficits due to selective breeding of F1 animals with the phenotype was investigated. The F1 animals that exhibited significant visual deficits were inbred to generate F2 population. The percentage of F2 lineage fish with visual deficits in the lowest exposure concentration (0.001 µM) was not significantly different from the corresponding F1 group. The incidence of

visual deficits in F2 lineage fish in all other exposure groups were higher. Our current study demonstrated that incidence of visual deficits due to MeHg exposure had increased due to selective breeding of affected population. The incidence of transgenerational disease phenotypes varies with the environmental factor and the phenotype observed. There was a significant increase in female pubertal abnormalities and polycystic ovary diseases in F3 generation dioxin lineage rats compared to the F1 generation (Manikkam et al. 2012b). However, the incidences of the male pubertal abnormalities, primordial follicle loss and male tumor development were lower in F3 generation than the F1 generation dioxin lineage rats. The incidence of female kidney diseases, ovary diseases, male obesity and polycystic ovary conditions were higher in F3 generation methyoxychlor lineage rats than the F1 generation (Manikkam et al. 2014b). The F1 methoxychlor animals had higher incidences of male pubertal abnormalities, primordial follicle loss and uterine infection than the F3 group. The above mentioned studies did not do a selective breeding scheme, however they observed increase in incidence of some phenotypes but not all.

A transgenerational phenotype is not necessarily observed in all generations. In directly exposed organism, the whole system is being exposed to the toxicant resulting in system wide response to the exposure. In transgenerational inheritance, the phenotype inducing epigenetic change is being inherited through the germline targeting specific systems or phenotypes. Hence, the mechanistic pathway of disease induction could be different in direct and transgenerational populations. For example, obesity was developed in F3

generation DDT lineage rats but not in directly exposed F1 lineage (Skinner et al. 2013c). In this study we demonstrated no significant locomotor behavior in adult zebrafish due to direct exposure to MeHg. However, transgenerational onset of hyperactivity was observed due to ancestral developmental exposure to MeHg. There is mounting evidence of association between prenatal exposure to MeHg and hyperactivity disorder (Boucher et al. 2012; Sagiv et al. 2012; Hong et al. 2013; Yoshimasu et al. 2014). It is estimated that 9.5 % of children between the age of 4 to 17 years have attention deficit hyperactivity disorder as of 2011-2013 (Pastor et al. 2015). Further epidemiological studies are necessary to find out the association between prenatal exposure to MeHg and transgenerational hyperactivity behaviors. Our findings need to be further validated with ADHD animal model organisms such as the zebrafish mutant for the circadian rhythm-related gene *period1b* (Whalley 2015).

The parental origin of the transmission of transgenerational phenotypes due to developmental exposure to MeHg was investigated in this study. The germline through which the visual deficit and hyperactivity behaviors were passed on to the F3 generation was identified by an outcrossing study. The F2 lineage control males were bred against F2 lineage MeHg exposure females and vice versa to generate F3 outcross population. Visual deficit was observed in all F3-outcross offsprings, suggesting that either male or female germline was sufficient for the onset of visual deficit in F3 lineage fish. This is one of the first cases where only one parent was required to transmit a transgenerational phenotype due to ancestral exposure. Previous studies showed that

transgenerational disease phenotypes were inherited in different ways such as, only through female germline (Manikkam et al. 2014b), only through male germline (Anway et al. 2005; Dunn and Bale 2011) or requiring both germlines (Skinner et al. 2013c). We have shown that a very sensitive neurobehavioral phenotype, the visual response, could be transgenerationally inherited even if only one of the parents carry the dysregulated allele due to developmental exposure to MeHg.

Experiments were also conducted to identify the parental origin of transgenerational hyperactivity behavior. Locomotor behavior analysis of F3 outcross lineage indicated the requirement of germline transmission of both male and female combined. The inbred F3 lineage MeHg exposure groups were significantly hyperactive, however F3 outcross between exposure and control lineage did not induce hyperactivity, suggesting neither male nor female alone were sufficient to induce the phenotype. Similar modes of germline transmissions were observed in environmental exposures to toxicants such as dioxin and hydrocarbons (Manikkam et al. 2012a; Manikkam et al. 2012c). Ancestral DDT exposure resulted in obesity in F3 generation and it required the combination of both male and female germlines to induce the phenotype (Skinner et al. 2013c). Our study demonstrates the significance of equal role of male and female germlines to induce transgenerational neurobehavioral phenotypes.

Our study demonstrates the complexity of transgenerational disease transmission due to MeHg exposure. The two phenotypes that we evaluated were inherited differently. Our study suggests that MeHg-induced

transgenerational transmission of unique phenotypes, such as visual deficit and locomotor behavior, occur through different modes of parental origin. Further studies are necessary for the complete elucidation of the pattern of transgenerational inheritance and mechanisms. Our observations indicate that an environmental toxicant could potentially induce different transgenerational traits through different modes of transmission. Our findings have significant implications on different levels of addressing the environmental health impacts of mercury such as changing the previous belief of mercury toxicity only due to maternal exposure. Since male germline is sufficient for the onset of transgenerational visual deficit due to MeHg exposure, the target population to prevent such phenotypes should be widened to include the possibility of male germline transmission.

CHAPTER 4

Methylmercury Promotes Transgenerational Inheritance of Transcriptome Associated with Neurobehavioral Deficits

ABSTRACT

Methylmercury (MeHg) is a ubiquitous environmental toxicant that is often detected in the tissues of fish-eating species. It has been well established that prenatal exposure to MeHg can lead to widespread brain damage and impaired neurological development resulting in defects ranging from severe cerebral palsy and cognitive deficits to impaired motor and sensory function. A wide range of environmental toxicants have been shown to induce transgenerational inheritance of diseases via changes in DNA methylation—a well-known epigenetic modification. Our previous research has demonstrated that developmental MeHg exposure may yield transgenerational inheritance of neurological dysfunction in adult F3-lineage zebrafish via quantitative neurobehavioral assays that evaluated the visual startle response, retinal electrophysiology, and locomotor function. The objective of the current study was to examine the correlation between neurobehavioral phenotypes and the transcriptome activity in the brain and retina of F3 zebrafish by RNA sequencing (RNAseq). Transcriptomic analyses of F3 generation MeHg-treated zebrafish (compared to control) revealed significant gene dysregulation in both the brain and retina. There were 1648 and 138 differentially expressed genes in the retina and brain, respectively (FDR <0.05). Thirty-five genes were commonly dysregulated in both organs. Gene set enrichment analysis revealed significantly enriched pathways including: neurodevelopment, visual functions, phototransduction, and motor movement. Moreover, commonly dysregulated genes were associated with circadian rhythm and metabolic pathways, as well as

arginine and proline metabolism. To our knowledge, this is the first evidence of a transgenerational transcriptome induced by ancestral developmental exposure to MeHg in any species. If the transgenerational phenotypes, transcriptome, homologous biomarkers, or similar molecular pathways hold true for human populations, our findings have significant impact on global public health in terms of identifying the susceptible populations using biomarkers and preventing transgenerational inheritance of MeHg-induced neurobehavioral deficits.

Key words: Methylmercury Transgenerational inheritance Transcriptome Epigenome

INTRODUCTION

Developmental exposure to MeHg results in adverse neurodevelopmental and neurobehavioral outcomes. The precise expression of genes during embryogenesis is crucial in proper neurodevelopment (Kang et al. 2011). The expression of genes associated with neurodevelopment and behavior have shown to be dysregulated by MeHg exposure (Richter et al. 2011; Ho et al. 2013a; Engel and Rand 2014). Low level exposure to MeHg has been shown to disrupt the neuroendocrine pathways in the brain by altering the expression of genes associated with motor functions (Richter et al. 2014). In addition, Liu et al. (2013) have demonstrated that genes involved in iron ion homeostasis, glutathione transferase activity, regulation of muscle contraction, troponin I binding, and calcium-dependent protein binding were affected by dietary exposure to sublethal doses of MeHg. However, the role of altered gene expression in developmental MeHg-induced transgenerational phenotypic variation is unknown.

Our previous studies have shown the potential of developmental exposure to MeHg to induce transgenerational neurobehavioral deficits including a reduced visual startle response, altered electrophysiology of the bipolar cells of retina and hyperactivity in zebrafish. Little is known about the dysregulated genes or the associated biological, molecular, and functional pathways responsible for these MeHg-induced transgenerational neurobehavioral deficits. Studies have shown that direct exposure to MeHg alters the development and functions of the central nervous system in zebrafish by altering the expression of critical genes (Yang et

al. 2010; Cambier et al. 2012; Ho et al. 2013b). We also know that developmental exposure to MeHg induces transgenerational neurobehavioral phenotypic variation. The objective of this study was to identify the transgenerational transcriptome associated with MeHg-induced neurobehavioral deficits.

Our previous research demonstrated that ancestral developmental exposure to MeHg induces neurobehavioral deficits in F3-lineage zebrafish. Developmental exposure of zebrafish embryos (F1) and their progeny (F2) to MeHg resulted in visual deficits in both generations, but since the F2 lineage was exposed as germ cells, the F3 lineage was the first generation of zebrafish to not endure direct exposure to MeHg. The deficits such as a reduced visual response, altered functions of potassium ion channels of the retina and hyperactivity observed in the fish from F3 lineage represent the transgenerational inheritance of phenotypes. The current study focused on elucidating the genes dysregulated in the brain and retina of the affected population. The identification of the genes and pathways dysregulated in the F3 lineage brain and retina would help us to better understand the etiology of MeHg induced transgenerational neurotoxicity. To date, no other study has conducted genome-wide analysis of gene expression following ancestral exposure to MeHg.

Visual deficit was one of the transgenerational phenotypes induced by ancestral developmental exposure to MeHg. Proper gene function is critical for normal vision and response to a visual stimulus. Direct exposure during the prenatal and perinatal phases of pregnancy, as well as occupational exposure, to

mercury has been associated with visual dysfunctions (Lebel et al. 1998; Jedrejko and Skoczyńska 2011; Yorifuji et al. 2013). Direct exposure to MeHg has been shown to mediate the alteration of gene expression in visual pathway. MeHg affects the functions of the photoreceptor cells of the retina (Korbas et al. 2013) and the visual cortex of the brain (Berlin et al. 1975; O'Kusky 1985; Ethier et al. 2012). The development of photoreceptor cells in the retina has shown to be impaired due to the down regulation of *opn1lw1* associated with developmental exposure to MeHg (Ho et al. 2013c). Dietary exposure to MeHg has resulted in the cellular damage of the optic tectum in zebrafish brain suggesting impaired visual response leading to increase susceptibility to predation (Cambier et al. 2012). Since developmental exposure to MeHg induces transgenerational visual deficit, the objective of our current study focused on the identification of dysregulated genes and pathways involved in the transgenerational visual deficit.

Research has shown that MeHg can affect the retina leading to visual deficits in humans (Ekinci et al. 2014) and other species including fish (Tanan et al. 2006; Weber et al. 2008a; Mela et al. 2010), monkey (Warfvinge and Bruun 2000), and chicken (Papaconstantinou et al. 2003). Our previous studies have shown altered electrophysiology of the retinal bipolar cells in zebrafish due to both direct (F1) and transgenerational (F3) MeHg exposure lineages. In our current study we conducted a genome-wide transcriptome analysis of the retina of the F3-lineage zebrafish to identify the changes in gene expression and the related functional pathways leading to visual deficits. We also looked at the

dysregulation of genes associated with potassium ion channels in the retina of F3 zebrafish.

Recently, diverse environmental toxicants have been showen to alter the epigenome of an organism, specifically by changing the DNA methylation pattern (Guerrero-Bosagna et al. 2010; Manikkam et al. 2012; Skinner et al. 2013; Manikkam et al. 2014). Thereafter, the altered epigenome is passed on to subsequent generations, even in the absence of further toxicant exposure, and leads to adult diseases and deficits. DNA methylation patterns are established in the primordial germ cells during gonadal development for the male or female specific germline patterning (Burdge et al. 2009). During embryogenesis, environmental factors can interfere with the patterning of DNA methylation during gonadal development (Ceccatelli et al. 2013). Once a DNA methylation pattern has been altered during gonadal development, the epigenome becomes permanent and escapes the de-methylation which occurs after fertilization (erasure), in the same manner as imprinted genes (Hales et al. 2011; Crews et al. 2012).The inheritance of permanently altered DNA methylation regions (DMR) leads to cell-specific changes in gene expression which yields altered cell physiology (Manikkam et al. 2012; Guerrero-Bosagna et al. 2014; Li et al. 2014). Some tissues are resistant to the altered physiology and some are sensitive to such changes. The tissues that are sensitive to the dysregulated genes and pathways potentially lead to the onset of disease phenotypes.

MeHg-induced developmental neurotoxicity and the mounting evidence of environmentally driven epigenetic transgenerational inheritance of diseases

directed our focus towards the epigenetic effects of MeHg. Direct exposure to MeHg has been associated with alterations in DNA methylation in different species (Pilsner et al. 2010; Basu et al. 2013; Goodrich et al. 2013; Maccani et al. 2015; Bakulski et al. 2015). For example, hypomethylation was shown to be associated with the amount of mercury in the brain of polar bears (Pilsner et al. 2010). In addition, the exposure of embryonic stem cells to mercury has shown aberrant DNA methylation (Arai et al. 2011). Other evidence suggests that altered DNA methylation can result in altered phenotypes, including diseases. For example, neurobehavioral deficits have been observed due to perinatal exposure to MeHg and subsequent down regulation of *Bdnf* was associated with hypermethylation of the promoter region of the gene (Ceccatelli et al. 2013). The molecular mechanism by which MeHg alters DNA methylation is thought to be through altering the glutathione pathway.

MeHg is removed from the human body as a glutathione (GSH) conjugate. GSH is an important antioxidant found throughout the body and it is crucial for the metabolism of MeHg. Specifically, the mercury atom of MeHg binds directly to the thiol group of GSH (Farina et al. 2011).This complex, GS-$HgCH_3$, can then be transported out of the cell and excreted. It has been suggested that exposure to toxicants, including MeHg, has the capacity to deplete the cellular GSH stores, potentially to the degree that reactive oxygen species accumulate within the cell and cause oxidative damage (Lee et al. 2009; Farina et al. 2011). Subsequently, the depletion of GSH due to MeHg exposure causes homocysteine to be recruited into the GSH synthesis pathway. As homocysteine normally contributes

to both methionine and GSH synthesis pathways, increased cellular demand for GSH decreases the availability of homocysteine for methionine synthesis. This, in turn, results in the depletion of cellular S-adenosyl methionine (SAM), a critical methyl donor, and presumably reduce DNA methylation (Caudill et al. 2001). Current evidence suggests that if the DNA methylation pattern of germ cells is altered by direct exposure to environmental toxicants, these epigenetic mutations (*i.e.*, epimutations) act like imprinted genes and are passed on to the offspring. Imprinted DNA methylation sites escape the genome-wide DNA methylation erasure events which occur during gonadal development and early embryogenesis. Therefore, these epimutations become programmed into the genome and can cause changes in gene expression in offspring who inherit the epigenetically altered allele.

Ancestral developmental exposure to MeHg induces variation in neurobehavioral phenotypes. The alteration of phenotypes requires changes in gene expressions associated with the phenotypes. The current study identifies the genes and pathways dysregulated in the brain and retina of F3-lineage zebrafish. Genome wide transcriptome analysis was conducted to identify all dysregulated genes. Gene set enrichment analysis was performed to identify the functional pathways significantly enriched in the brain and retina and their association with the phenotypes observed. This approach also helped to identify potential variation in phenotypes that we previously has not screened. The current study revealed the involvement of different genes and pathways associated with MeHg induced transgenerational neurobehavior.

MATERIALS AND METHODS

Animal studies

All animal care and experiments using animals or involving hazardous materials were approved by the Institutional Animal Care and Use Committee, Environmental Protection, and Laboratory Safety offices of University Safety & Assurances of the University of Wisconsin-Milwaukee.

Zebrafish stock and egg collection

Adult EK strain zebrafish were originally obtained from Ekkwill Waterlife Resources (Gibsonton, FL, USA) and have been maintained in Children's Environmental Health Sciences Core Center for over 10 years. Adult fish were housed at a maximum density of 10 adult fish/L in a flow-through dechlorinated water system maintained at 26-29 °C on a 14:10 h light:dark photoperiod at the Aquatic Animal Facility of the NIEHS-funded Children's Environmental Health Sciences Core Center at the University of Wisconsin-Milwaukee. For spawning, male and female fish (1:2 ratio) were kept overnight in a 2 L plastic aquarium with a 3 mm nylon mesh bottom to separate newly fertilized eggs from the adults. The fish were moved to clean water 30 min prior to the onset of light to ensure that only eggs fertilized within a narrow time period were used in experiments. Eggs were collected ≤ 1 hours post fertilization (hpf) and placed into metal-free plastic culture dishes (100 mm diameter × 50 mm depth) in E2 medium (Nüsslein-Volhard 2002; pH 7.2; in 1 L: 0.875 g NaCl, 0.038 g KCl, 0.120 g $MgSO_4$, 0.021

g $KH2PO_4$, and 0.006 g Na_2HPO_4; Hg-free as determined by Inductively Coupled Plasma-Mass Spectrometer [ICP-MS]).

Methylmercury exposure and breeding

The newly fertilized eggs were transferred to 12-well non-treated culture plates (10 eggs/well) and rinsed twice with E2 medium. The eggs were exposed to 1.5 ml of E2 medium containing MeHg (0.0, 0.001, 0.003, 0.01, 0.03, 0.1 μM with ethanol as the vehicle; < 0.01% total volume). Following a 24-h exposure, embryos were rinsed three times with E2 medium and transferred to 2-L tanks (60 embryos per tank) containing static E2 medium for rearing. Starting at 5 dpf, eleutheroembryos were fed Golden Pearl Reef & Larval Fish Diet (5-100 micron; Brine Shrimp Direct, Ogden, UT, USA). Platinum Grade *Artemia* nauplii (Argent Laboratories, Redmond, WA, USA) were fed starting at 9 dpf. At 21 dpf, the fish were transferred to 1-L flow-through tanks and raised using standard husbandry techniques until 4 months of age. At this point the fish were used for behavior assays and breeding. Juvenile and adult fish were fed a combination of Platinum and Gold grade *Artemia* nauplii and Aquarian™ flake food (Aquarium Pharmaceuticals, Inc., Chalfont, PA, USA).

In mammalian models of developmental toxicity, the pregnant animal is directly exposed to the toxicant and is designated as the F0 generation. The developing progeny are exposed to the toxicant *in-utero* and are considered the F1 generation. In our zebrafish model system, the directly exposed embryos resemble the F1 progeny in the mammalian models. After the initial 24-h MeHg exposure of the F1 embryos, there was no additional exposure to Hg during the

entire life cycle beyond the background levels inherent to all fish food. Many different fish foods were screened and those with the lowest levels of Hg (as determined by ICP-MS) were used as much as possible in this study. Adult F1 fish were inbred to create the F2 generation, which were subsequently inbred to create the F3 generation. The F2 and F3 generations were never exposed to exogenous MeHg. However, the F2 generation is also considered to be directly exposed as they were primordial germ cells within the F1 embryos at the time of the initial MeHg exposure. Ultimately, we tested the F3-generation zebrafish for transgenerational inheritance of neurobehavioral deficits.

Animal selection and sample collection

The screening of the F3-lineage zebrafish for the visual startle response, retinal electrophysiology, and locomotor activity revealed transgenerational inheritance of visual and motor deficits due to ancestral developmental exposure to MeHg (Chapter 2). The lowest responders of the F3-MeHg exposure lineage and the best responders of the F3-control lineage zebrafish in the visual startle assay were selected for the collection of brain, retina and sperm. The control and 0.03 µM MeHg ancestral exposure groups were used for the sample collection. For brain and retina collection, fish were euthanized via 0.1% tricaine (MS-222, Sigma-Aldrich, St. Louis, MO, USA) for at least 10 minutes after cessation of gill movement. Whole brain and retina tissues were removed and transferred to 250 µL of RNA*later*® (Life Technologies, Carlsbad, CA, USA) and then immediately frozen in liquid nitrogen. Thereafter, samples were stored at -80^{o}C until RNA extraction. Fish were anesthetized for sperm collection by using 0.004% tricaine.

Abdominal massage followed by microcapillary suction was used to collect sperm. Sperm samples were transferred to 250 µL of RNA*later*®, immediately frozen in liquid nitrogen, and then kept at -80ºC until DNA extraction.

RNA extraction and transcriptomic analysis by RNAseq

High quality total RNA was extracted from the brain and retina using RNeasy Micro Kit (QIAGEN, Valencia, CA, USA). The concentration of RNA was quantified using the NanoDrop-1000 (Thermo Fisher Scientific, Wilmington, Delaware, USA). RNA integrity was assessed via the Experion automated electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). Illumina TruSeq RNA Library Preparation and Sequencing was performed at the Biotechnology Center at the University of Wisconsin-Madison. Each library was generated using a paired-end approach following the Illumina “TruSeq RNA Sample Preparation Guide” and the Illumina TruSeq RNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA). Samples were run with 12 samples per lane, with 100 base pair, paired-end reads. Sequencing depth was 14-32 million reads per sample.

All bioinformatics procedures and analyses were performed by the UW-Milwaukee Laboratory for Public Health Informatics and Genomics (LPHIG). Adapters and low quality bases were removed from the initial 2x101bp Illumina TruSeq reads and trimmed using Cutadapt (Martin 2011). Illumina TruSeq adapters were removed as prescribed by the Cutadapt manual, using an error rate of 10% and a minimum overlap between the read and the adapter of five nucleotide bases. To alleviate sequencing-related GC biases at the 5’ end of

each read, the first seven bases were removed from all forward and reverse strand reads. FastQC was used to ensure that cleaned reads were of higher quality than initial raw reads supplied by the sequencer; per-base GC% and over-represented sequence statistics also confirmed adapter contamination was minimized. The cleaned reads for each sample were independently aligned to the reference zebrafish genome (Zv9, UCSC) using TopHat (v. 2.0.11) (Trapnell et al. 2009; Trapnell et al. 2010; Kim et al. 2013). The alignment output from TopHat was converted into a transcriptome using Cufflinks (v. 2.2.1), with the Zv9 Gene Transfer Format (GTF) as a guide; a mate-pair-distance of zero and a maximum of two mismatches bases per alignment was used. Alignment data was confirmed using RNAseQC (DeLuca et al. 2012) against the Zv9 reference transcriptome of zebrafish. Using these alignments, sample specific transcriptomes were assembled using Cufflinks (Trapnell et al. 2010), with the Zv9 transcriptome as a reference to correct fragment biases by better identifying the start/end point of each exon (Roberts et al. 2011). The transcriptome from each sample was then merged together into brain and retina specific transcriptome using Cuffmerge. Differential expression was conducted with Cuffdiff using pooled dispersion, geometric normalization, and the merged brain and retina transcriptome. TopHat alignments were grouped according to MeHg exposure levels. Differentially expressed genes were further used for gene-set enrichment analysis by WebGestalt (http://www.webgestalt.org or http://bioinfo.vanderbilt.edu/webgestalt/) and GSEA

(http://www.broadinstitute.org/gsea/index.jsp) (Broad Institute, Cambridge, MA) platforms.

RESULTS

In this study, we selectively harvested brain and retina from the F3-lineage fish from the exposure group that demonstrated the poorest performance in our panel of neurobehavioral assays. Total RNA was isolated from the brain and retina for the transcriptome analysis by RNAseq. Rigorous bioinformatics methods were exploited to ensure high quality transcriptomic analysis. Differential expression of genes in the brain and retina of F3-lineage zebrafish were identified. There were 1648 and 138 differentially expressed genes within the retina and brain (FDR <0.05), respectively. The majority of differential gene expression was tissue specific; however, 35 genes were commonly dysregulated (Fig 4.1).

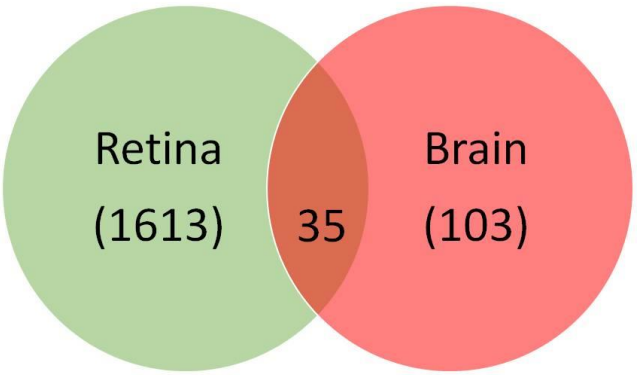


Figure 4.1. Number of genes differentially expressed in the brain and retina of F3-lineage zebrafish ancestrally exposed to MeHg during development. A distinct number of genes were dysregulated in both the brain and retina. There were also genes commonly dysregulated in both organs.

The molecular, cellular and biological functions of the differentially expressed gene (DEG) sets in the brain (Table 4.1) and retina (Table 4.2) were identified by Gene Ontology analysis. Differentially expressed genes involved in critical functional pathways included metabolic process, response to stimulus, ion binding, development, cellular components, as well as nucleotide and nucleic acid binding. In both organs, there were 7 genes differentially expressed that were associated with the *response to stimulus* biological function. Genes specifically associated with the transduction of light stimulus were differentially expressed both in the retina (20 genes) and the brain (19 genes). Genes involved in photoperiodism and circadian rhythm were dysregulated in the brain. Gene Ontology analysis of the commonly dysregulated genes in brain and retina demonstrated that genes were differentially expressed in response to abiotic stimulus (7 genes), response to light stimulus (6 genes) and photoperiodism (5 genes). The dysregulation of genes associated with the visual pathway both in the brain and retina supports our previous conclusion that MeHg may induce visual and neurobehavioral deficits in zebrafish (refer to Chapter 2). Genes associated with visual functions such *arntl1a, per3, clock, arntl1b, arntl2* were differentially expressed both in retina and brain. Ancestral developmental exposure to MeHg was found to induce transgenerational gene dysregulation within the brain and retina. Cluster analysis showed the variation among the individual animals between the control and MeHg-treatedF3-lineage zebrafish (Figures 4.2 and 4.3).

Table 4.1. Cellular, molecular and biological functions and number of genes dysregulated in the brain between control and MeHg lineage F3 generation.

Cellular component	# of genes	Biological process	# of genes	Molecular functions	# of genes
Membrane	24	Metabolic process	61	Ion binding	46
Nucleus	22	Response to stimulus	46	Molecular-transducer activity	23
Macromolecular-complex	13	Biological regulation	45	Nucleic acid binding	23
Cytoskeleton	8	Cell communication	19	Protein binding	21
Membrane enclosed-lumen	6	Multicellular-organismal process	19	Nucleotide binding	19
Cell protection	3	Localization	8	Hydrolase activity	10
Mitochondrion	2	Developmental-process	7	Transferase activity	10
Extracellular matrix	1	Cellular component-organization	4	Transporter activity	5
Cytosol	1	Death	4	Enzyme regulator-activity	4
Envelope	1	Reproduction	1	Lipid binding	1
Vesicle	1	Growth	1	Electron carrier- activity	1
Unclassified	81	Multi-organism process	1	Carbohydrate-binding	1
		Unclassified	49	Unclassified	49

Table 4.2. Cellular, molecular and biological functions and number of genes dysregulated in the retina between control and MeHg lineage F3 generation.

Cellular component	# of genes	Biological process	# of genes	Molecular functions	# of genes
Membrane	181	Metabolic process	386	Ion binding	291
Nucleus	133	Biological regulation	245	Nucleotide binding	163
Macromolecular complex	86	Response to stimulus	199	Nucleic acid binding	122
Cytoskeleton	50	Cell communication	142	Hydrolase activity	120
Membrane enclosed lumen	40	Multicellular organismal process	140	Transferase activity	102
Mitochondrion	20	Developmental process	126	Protein binding	101
Endomembrane system	14	Localization	98	Transporter activity	47
Envelope	14	Cellular component organization	66	Enzyme regulator activity	44
Chromosome	14	Death	15	Molecular transducer activity	43
Endoplasmic reticulum	12	Growth	15	Lipid binding	33

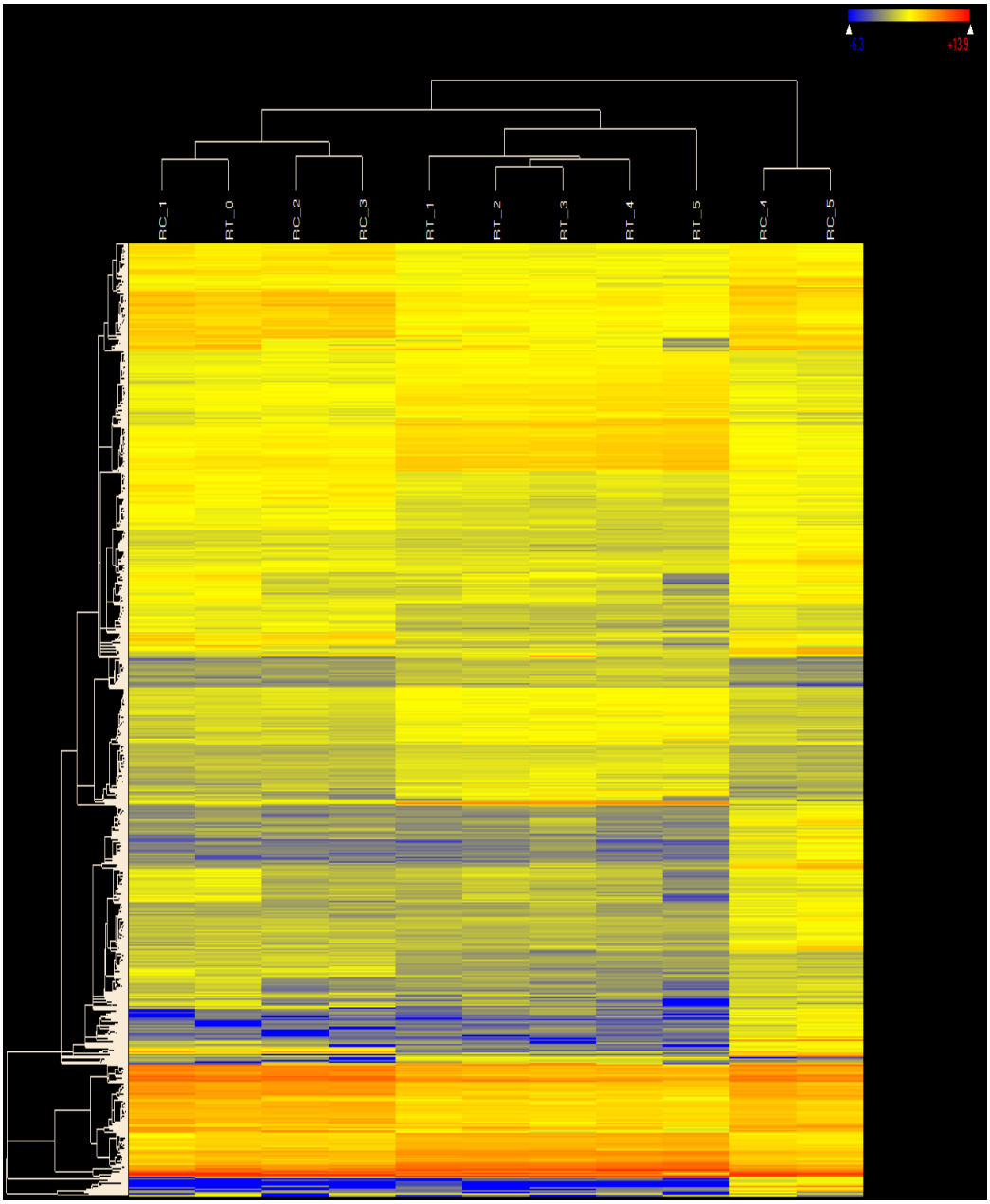


Figure 4.2. Heat map of DEGs in the retina of F3 lineage ancestrally exposed to MeHg during development. The DEGs in individual control (RC1-5) and MeHg exposure (RT0-5) animals were hierarchically clustered.

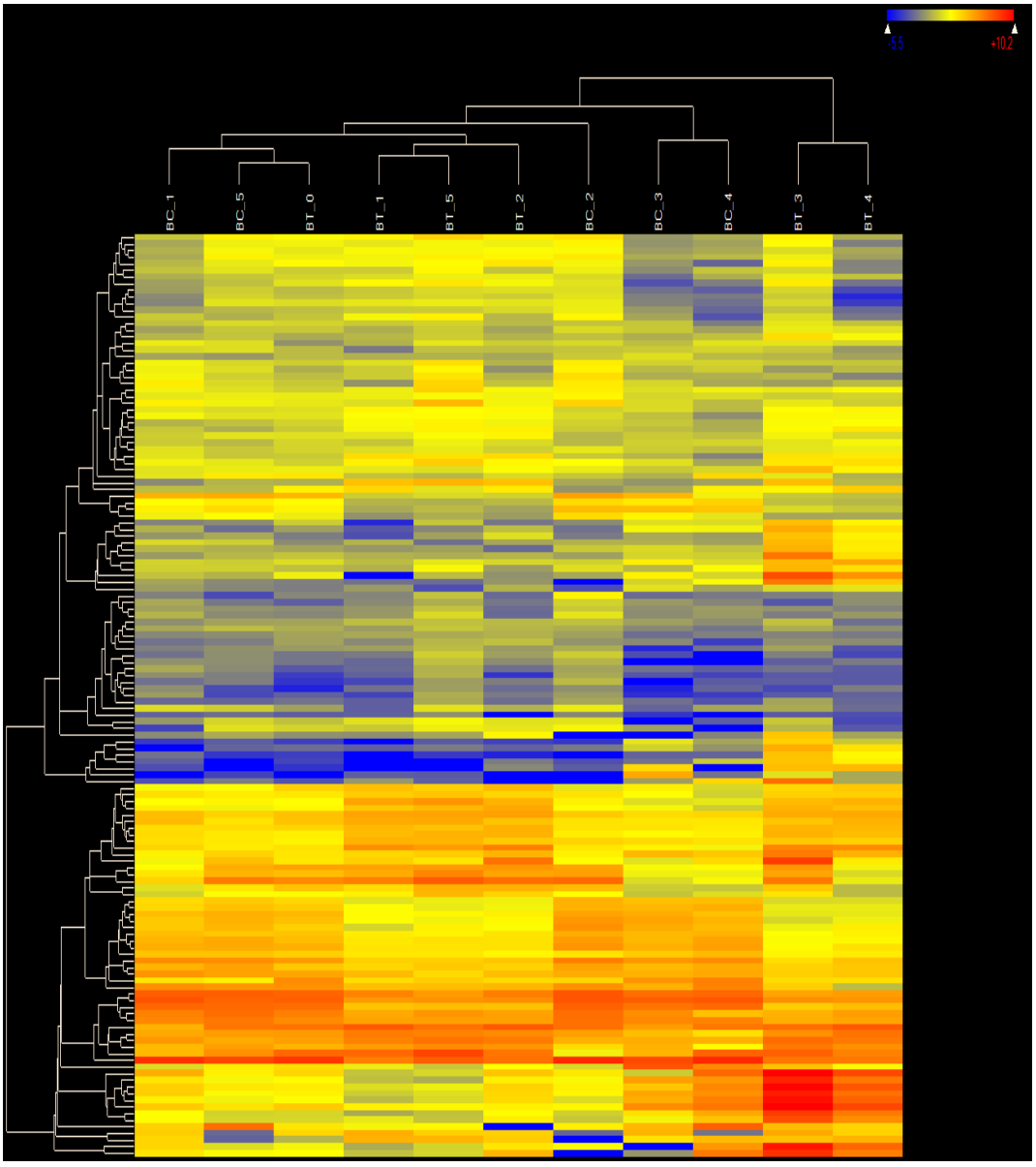


Figure 4.3. Heat map of DEGs in the brain of F3 lineage ancestrally exposed to MeHg during development. The DEGs in individual control (BC1-5) and MeHg exposure (BT0-5) animals were hierarchically clustered.

MeHg induced transgenerational transcriptome

Gene Set Enrichment Analysis (GSEA) of the DEGs of the brain and retina yielded significantly enriched pathways that included vision, electrophysiology, and neurodevelopment. In the retina, enrichment analysis of the Gene Ontology set revealed upregulation of 8-gene sets and downregulation of 3-gene sets out of 11 total sets. There were 8 and 11 gene sets that were upregulated and downregulated, respectively, out of 19 total sets tested in the pathway enrichment analysis. Human-phenotypic enrichment analysis resulted with the upregulation of 22-gene sets and downregulation of 18 gene set of 40 sets tested. The significant enrichment gene sets are shown in Table 4.3.

Table 4.3. GSEA of DEGs in the retina of F3 lineage.

Name	Size	ES	NOM p-val	FDR q-val
Nervous system development	36	0.3621	0	0
Axon guidance	28	0.3112	0.008016	0.012176
Extracellular region	32	0.2779	0.014286	0.036451
Generation of neurons	10	0.4494	0.02834	0.036722
Developmental biology	40	0.2435	0.024793	0.041788
Neurogenesis	10	0.4494	0.024194	0.045653
Development of central nervous system	13	0.3474	0.052738	0.081185
Abnormality of movement	55	0.2328	0.001976	0.095318
MAPK_Signaling pathway	22	0.259	0.081395	0.171972
Transmission of nerve impulse	18	0.2615	0.165306	0.17889
Circadian clock	14	0.2924	0.138067	0.230248
Autism	16	0.2563	0.219124	0.230417

Size refers to the number of genes from the dysregulated genes that are found in a particular gene set tested; NES is the normalized enrichment scores for the set; p-value is the nominal p-value associated with the NES; FDR q-value is the false discovery rate ratio. Gene sets that passed the FDR threshold 0.25 are shown.

Dysregulation of phototransduction pathway

WebGestalt analysis revealed that the phototransduction pathway was significantly dysregulated both in the brain and retina. As shown in Figure 4.5, critical genes involved in phototransduction pathway were dysregulated due to ancestral developmental exposure to MeHg. Genes associated with both light and dark vision were also dysregulated. Moreover, transcriptomic analysis revealed that 12 genes associated with potassium channels were dysregulated (Table 4.4). Among the other pathways affected, genes in the photoperiodism and circadian rhythm pathways were also differentially expressed (Fig 4.4).

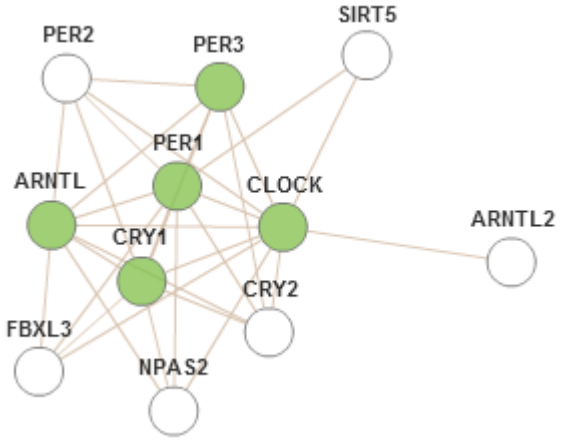


Figure 4.4. Protein network analysis of DEGS in the circadian rhythm pathway of F3 lineage brain. Green indicates dysregulated genes in the pathway.

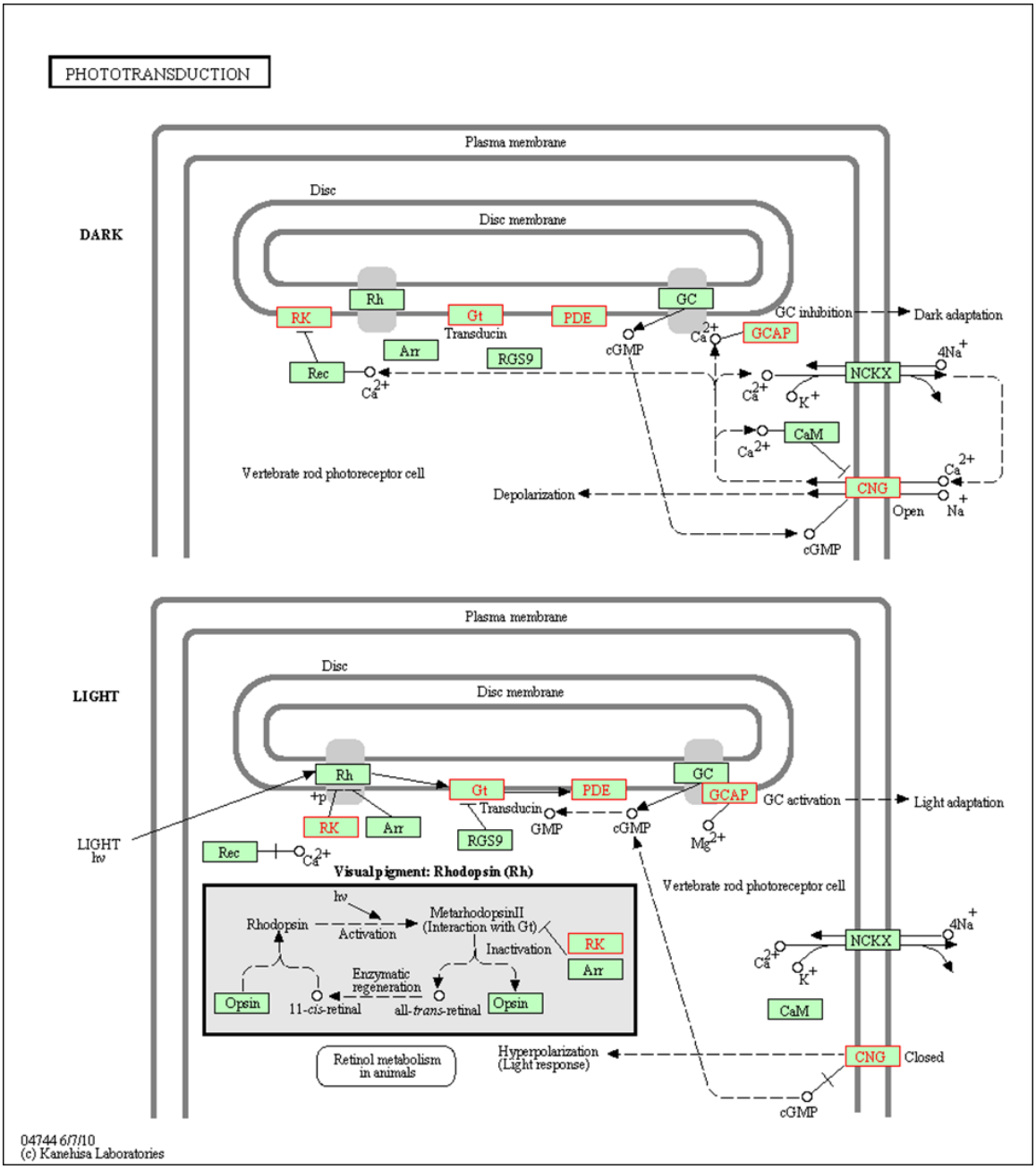


Figure 4.5. Phototransduction pathway showing the DEGs in the retina of F3 lineage ancestrally exposed to MeHg during development by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The genes in the red boxes are differentially expressed genes associated with visual transduction in the retina of F3 MeHg exposure lineage zebrafish.

Table 4.4. Genes associated with potassium ion channels dysregulated in the F3 lineage retina.

Gene Symbol	Gene Name
kcnj11l	potassium inwardly-rectifying channel, subfamily J, member 11, like
kcnk5a	potassium channel, subfamily K, member 5a
hcn4	hyperpolarization activated cyclic nucleotide-gated potassium channel 4
slc24a3	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3
kctd13	potassium channel tetramerization domain containing 13
LOC100334297	potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1-like
LOC571330	potassium channel subfamily K member 3-like
kcnk5b	potassium channel, subfamily K, member 5b
kcnd3	potassium voltage-gated channel, Shal-related subfamily, member 3
kcnk1a	potassium channel, subfamily K, member 1a
LOC563744	inward rectifier potassium channel 2-like
kcnd2	potassium voltage-gated channel, Shal-related subfamily, member 2

DISCUSSION

Developmental exposure to environmental toxicants has been associated with the induction of transgenerational phenotypes such as obesity, reproductive diseases, and kidney diseases (Manikkam et al. 2012). Alterated DNA methylation of the germline and the inheritance of the modified epigenome have been identified as the molecular mechanism behind the inheritance of phenotypes without direct exposure. In addition, research has shown that the dysregulation of the genes involved in critical cellular functions and pathways were caused by the inheritance of epimutations (Manikkam et al. 2012; Crews et al. 2012; Guerrero-Bosagna et al. 2014). Our previous study demonstrated that developmental exposure to MeHg may induce the transgenerational onset of visual and neurobehavioral phenotypes in F3-lineage zebrafish in the absence of further exposure. The identification of altered phenotypes in the F3-generation MeHg-treated descendants indicated that developmental exposure to MeHg resulted in inheritable changes in the epigenome. The objective of the current study was to survey the transgenerational transcriptome to identify whether the dysregulation of critical genes were associated with the transgenerational phenotypes observed.

A genome-wide transcriptome approach was taken to identify not only the genes involved in the phenotypes of interest but also to discover dysregulation of genes in previously unidentified transgenerational phenotypes. The hypothesis tested in this study was that altered phenotypes due to ancestral developmental exposure to MeHg are the result of dysregulated genes in the F3 lineage

generation. Genome-wide transcriptomic analysis of the brain and retina of control and MeHg-exposed generations (F3-lineage zebrafish) was conducted to identify the transgenerational inheritance of altered gene functions. We chose the whole brain transcriptome analysis rather than just the optic tectum, region associated with vision. The function of the brain is a synchronous process (Birn 2012; Richiardi et al. 2015). Different anatomical regions of the brain functionally connected for neurobehavioral outcomes including vision and memory. Hence, the whole brain transcriptome was investigated to identify the common gene expression profiles among the different regions of the brain.

The analysis of transcriptome of brain demonstrated 1648 genes differentially expressed in the retina and 138 differentially expressed genes in the brain. There were 35 genes commonly dysregulated between brain and retina. This suggests that MeHg exposure during embryonic development alters the transgenerational transcriptome of the brain and retina differently. However, the overlap of commonly dysregulated genes indicates common pathways affected in both organs. A previous study has shown that maternal exposure to mercury resulted in the differential expression of 131 genes in the brain of mice pups (Glover et al. 2009). We have seen similar number of genes dysregulated in the brain tissue of the transgenerational offsprings of zebrafish developmentally exposed to MeHg. The differentially expressed genes are involved in wide variety of cellular, molecular, and biological functions including: neural development, neurogenesis, signaling pathways, and photoreceptor development.

Our analysis was focused on the differential expression of genes associated with visual functions, retinal electrophysiology, and locomotor activity. Pathway analysis demonstrated that 10 pathways were dysregulated in the brain, with at least two differentially expressed genes in each pathway. The top three significant (p<0.05) pathways with a maximum number of genes dysregulated were circadian rhythm (11 genes), phototransduction (10 genes), as well as arginine and proline metabolism (5 genes). Our findings of genes altered in circadian rhythm (*i.e., nr1d1, clock, cry1a, bhlhe40, arntl1b, per1b, per1a, arntl1a, per3, bhlhe41,* and *cry3),* and phototransduction (*i.e.*, *grk1b, grk1a, guca1b, pde6b, gngt1, pde6g, pde6a, gnat1,* and *guca1a*) were in conjunction with the visual deficits that we observed in the F3 descendants. Studies have shown the association of the activities of biological clock genes with visual and behavioral functions (Claridge-Chang et al. 2001; Owens et al. 2012; Friedrich 2013). Our current study reveals the MeHg induced transgenerational differential expression of genes associated with visual pathways in zebrafish.

One of the transgenerational phenotypes that we observed in F3-MeHg lineage was hyperactivity. Hyperactivity-type behavior is associated with abnormalities in circadian rhythm (Van Veen et al. 2010; Kooij and Bijlenga 2013; Huang et al. 2015). We have identified more than 10 genes in the circadian rhythm pathway that were dysregulated in the MeHg-exposed F3-lineage fish. One of these genes was *period 1b* (*per1b*). Huang et al. (2015) has identified hyperactivity behaviors in zebrafish that were mutant for *per1b*, suggesting that the *per1b*-mutant zebrafish as a promising model for attention deficit

hyperactivity disorders. Since we identified that F3-lineage zebrafish were hyperactive and that the critical gene associated with the hyperactivity behaviors (*i.e.*, *per1b*) was dysregulated, future studies using this mutant zebrafish model are necessary to further confirm the role of MeHg in inducing transgenerational ADHD.

Functional pathway analysis was conducted on the genes differentially expressed in the brain and retina. Visual gene networks in the retina were used for enrichment analysis for the identification of different pathways affected in the visual phototransduction process. Pathway analysis demonstrated that 25 pathways were dysregulated in the retina in which at least four genes were differentially expressed in each pathway. The top three significant ($p<0.05$) pathways with a maximum number of genes dysregulated were metabolic pathways (87 genes), cell cycle (26 genes) and purine metabolism (14 genes). The dysregulated pathways associated with visual functions were retinol metabolism (5 genes), phototransduction (5 genes) and circadian rhythm (12 genes). Human phenotypic pathway enrichment analysis of the dysregulated genes in the retina demonstrated that genes involved in retinal disease (27 genes), eye disease (29 genes), vision disorders (15 genes) and dim vision (12 genes) were differentially expressed.

Thirty-five genes associated with response to light stimulus, circadian rhythm, and photoperiodism were dysregulated in both brain and retina. Our findings indicate that developmental exposure to MeHg dysregulated genes involved with vision in both organs, thus suggesting a common molecular

mechanism prior to cellular differentiation. For example, *amd1 (adenosylmethionine decarboxylase 1),* which was dysregulated both in brain and retina*,* encodes for an essential enzyme which catalyzes the conversion of S-adenosyl methionine (SAM) to S-adenosylmethioninamine in the polyamine pathway. SAM is the methyl donor for the methylation of DNA. Therefore, the dysregulation of *amd1* could alter DNA methylation process. This finding suggest that ancestral MeHg exposure could alter the DNA methylation pathway via dysregulation of *amd1.* Other studies have reported that *amd1* also plays a role in embryonic development and self-renewal of embryonic stem cells (Nishimura et al. 2002; Zhang et al. 2012), suggesting that this gene may play a role in MeHg-mediated abnormalities in development.

Evidence has demonstrated that direct exposure to MeHg affects the functions of potassium ion (K^+) channels (Leonhardt et al. 1996; Yuan et al. 2005). For example, Weber et al. (2008) showed that direct MeHg exposure was associated with a delay in depolarization-elicited outward K^+ currents in the bipolar cells of zebrafish retina (Weber et al. 2008b). Our study on the transgenerational population of zebrafish demonstrated that K^+ channels were dysfunctional in F3 exposure lineage. Transcriptomic analysis of the retina from MeHg-exposed zebrafish (F3 lineage) revealed that 12 genes associated with potassium ion channels were dysregulated. These findings suggest that developmental exposure to MeHg induces transgenerational inheritance of altered gene expression related to potassium ion channels in the retina. However, since we used the whole retina for transcriptome analysis, it is not

clear that the dysregulated potassium ion channel genes were solely from the bipolar cells. Further studies are necessary to confirm that the dysregulated potassium ion channels genes are indeed located in the bipolar cells.

We have demonstrated that ancestral developmental exposure to MeHg alters the transcriptome of the brain and retina in zebrafish. Our results revealed that common genes were dysregulated in both organs, suggesting the presence of a common molecular mechanism of toxicity during development. There was an association observed between the neurobehavioral deficits and differentially expressed genes. Pathways involved with the phenotypes observed were significantly enriched by gene set enrichment analysis. To our knowledge, this is the first study to report an altered transgenerational transcriptome due to developmental exposure to MeHg.

The implications of these findings are significant in terms of public health—especially due to the rising global emission of mercury and the fact that a significant cohort of the human population depend on fish and seafood as their main source of protein. To this end, our findings suggest that the transgenerational effects of MeHg on neurobehavioral functions need to be addressed, coupled with a focus on preventing direct exposure to this neurotoxicant, when establishing public health policies. In the future, the identification of biomarkers associated with visual and neurodevelopmental deficits would aid in the eventual screening of susceptible human populations. Moreover, our study highlighted the dysregulation of novel pathways such as circadian rhythm and hyperactivity disorders. Subsequently, future studies are

necessary to explore the onset of transgenerational circadian rhythm and autistic spectrum disorder phenotypes due to ancestral developmental MeHg exposure.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

DISCUSSION AND FUTURE DIRECTIONS

MeHg is a global environmental neurotoxicant of both ecological and public health concern. Developmental exposure to MeHg has been associated with the onset of variations in neurobehavioral phenotypic variations later in life (Gilbert and Grant-Webster 1995; Ceccatelli et al. 2013; Maccani et al. 2015). Recently, the developmental exposure to environmental factors such as pesticides, fungicides, plasticizers, and endocrine disruptors have been shown to induce different phenotypic variations (e.g., metabolic, reproductive and cardiovascular diseases) in subsequent generations without further exposure (Skinner 2011; Skinner et al. 2013; Nilsson and Skinner 2015). DNA methylation, an epigenetic modification, has been identified as one of the molecular mechanisms that induces such phenotypic variations (Manikkam et al. 2014; Skinner et al. 2014; Skinner and Guerrero-Bosagna 2014). Hence, transgenerational epigenetic inheritance is “the germline (egg or sperm) transmission of epigenetic information between generations in the absence of any environmental exposure” (Skinner 2011). Developmental stage of an organism has been identified as the susceptible stage for environmental insults to create permanent inheritable changes in the germline.

The discovery that environmental insults have the potential to induce transgenerational phenotypes has further strengthen the theory of developmental origin of health and diseases (Barker et al. 1993). Developmental stages have been identified as susceptible to permanent epigenetic alteration that could potentially lead to the onset of diseases in generations that never had exposed to

the specific environmental factor. It has been well established that MeHg induces neurotoxicity due to developmental exposure (McKeown-Eyssen et al. 1983; Jedrychowski et al. 2006; Suzuki et al. 2010). Our previous study has shown that embryonic exposure to MeHg induces neurobehavioral deficits in zebrafish (Weber et al. 2008). In our current studies, we investigated the potential of developmental MeHg exposure to induce transgenerational neurobehavioral deficits.

The neurotoxic effects of mercury in humans and other species are well documented (Basu et al. 2005; Basu et al. 2007; Gandhi et al. 2014; Nakamura et al. 2014; Krey et al. 2015). Prenatal exposure to mercury adversely affects neurodevelopment and neurobehavioral functions during infancy, childhood, and later in life (Jedrychowski et al. 2006; Sagiv et al. 2012; Wu et al. 2014). We used neurobehavior as an assessment tool since it integrates the functions at various stages including molecular, cellular, organ system to organism levels. A problem in any one of the functional levels would affect the neural integration process leading to alteration in behavior. Visual response is one of the complex neurobehavioral functions dysregulated by developmental exposure to MeHg (Rice and Gilbert 1990; Weber et al. 2008). A response to a visual stimulus involves the coordinated functions of different components of nervous and muscular systems including retina, optic nerve, neurotransmitters, ion channels, tectum, cerebellum, motor neuron and skeletal muscles. Adverse effects of Hg exposure in visual nervous system physiology has been reported by different studies (da Costa et al. 2008; Fillion et al. 2013; Yorifuji et al. 2013).

Previous studies of our lab have shown that direct exposure to MeHg induces visual startle response, vibrational startle response, and learning and memory deficits in zebrafish (Carvan et al. 2004; Weber et al. 2008; Smith et al. 2010; Xu et al. 2012). We use zebrafish as a model organism due to its physiological, morphological and genetic similarities to humans, and in this study we wished to further explored the effects of MeHg and the potential for transgenerational actions. The ability of a direct exposure to influence multiple generations (i.e. F0, F1, and F2) is defined as a multiple generation phenotype. In contrast, a transgenerational phenotype requires transmission of the phenotype in the absence of direct exposure. Most of the transgenerational studies were conducted in the context of mammalian physiology and development, and we applied those principles to the zebrafish model system (Figure 1.4). Following dietary MeHg exposure of F0 adult females the F3 generation is the first not directly exposed to MeHg. The zebrafish model allows us to bypass F0 adult exposure and directly expose the F1 zebrafish embryos to waterborne MeHg. Again, the MeHg is rapidly absorbed by the yolk and distributed to the developing embryo (F1) and its' primordial germ cells (F2).In the current study, we screened the F3 generation for visual and locomotor behavioral deficits using the same approaches as previously published.

The findings of this thesis demonstrated that developmental exposure to MeHg induced both direct and transgenerational visual deficits in zebrafish. The present study also demonstrated that locomotor activity was not significantly affected in the F1 lineage (direct exposure). However, significant hyperactivity

was observed in the F3 exposure lineage (transgenerational). This study is the first study to show that developmental MeHg exposure can promote transgenerational neurobehavioral phenotypes. If these findings in zebrafish translate to human populations, the societal costs of mercury pollution are, at present, dramatically underestimated. Developmental disabilities are on the rise in the US among children between the ages of 3 and 17 (Boyle et al. 2011). There is an unexplained increase in neurobehavioral disorders such as autism and attention deficit hyperactivity disorder among the children in the US (Newschaffer et al. 2005; Safer 2015). Mercury is not the sole causative agent responsible for these changes, There is, as yet, no direct link between blood Hg levels and autism spectrum disorders (Yau et al. 2014). It is much more likely that developmental exposures to environmental chemical mixtures are influencing disease susceptibility. Future epidemiological studies are needed to elucidate the transgenerational potential of MeHg, alone and in combination with other common environmental chemicals, to induce such disorders. Our studies on the transgenerational inheritance of altered neurophysiology may provide guidance for the design of such studies.

In our previous studies, retinal electrophysiology was conducted to identify alteration in cellular physiology due to the absence of histopathological anomalies in the retina of the F1 generation. Zebrafish retina has three distinct neuronal cell layers, ganglion cell layer, inner and outer nuclear layers with seven major types of cell types as that of humans (Malicki 2000). There are 17 morphological types of bipolar cells identified in the zebrafish retina, transmitting

signals between the photoreceptor cells and ganglionic cells (Connaughton and Nelson 2000). Photons of the light cause hyperpolarization of the photoreceptor cone cells and a subsequent drop in the neurotransmitter glutamate release to bipolar cells (Leskov et al. 2000; Ebrey and Koutalos 2001). Bipolar cells depolarize due to decrease in inhibitory glutamate levels resulting in signal transduction to ganglionic cells from there to the optic tectum of the brain.

Our previous study has demonstrated developmental exposure to MeHg alters the functions of voltage gated K^+ ion channels of the bipolar cells of zebrafish retina (Weber et al. 2008). We found that retinal electrophysiology was also altered due to dysfunctional K^+ ion channels in the bipolar cells of the retina due to ancestral developmental exposure to MeHg. The normal functions of voltage gated cation channels, Na^+ and K^+ are critical for the phototransduction process. Delayed potassium ion outward flow results in hyperpolarization of the bipolar cells and thus affect signal transmission to the ganglionic cells and beyond. Our findings revealed one specific change in cellular physiology (there may be more) that is likely involved in visual deficits the F3 lineage fish. Bipolar cells are one of the structural components of visual nervous system. In addition to its role in phototransduction, K^+ ion channels are also directly involved with the locomotor activity (Manira et al. 1994; Hess and Manira 2001; Grillner et al. 2001). Voltage gate K^+ ion channels play crucial role in neural excitability (Pongs 1999) and signal propagation (Hoffman et al. 1997). The blockage of K^+ ion channels in the neurons of lamprey affected both single neuron firing and the generation of locomotor pattern, suggesting the role of K^+ ion channels in

locomotor activities (Grillner et al. 2001). It has also shown that the activity of the hippocampal interneurons can be altered by inhibiting K^+ ion channels leading to increased release of the neurotransmitter GABA resulting in anticonvulsant properties (Miller et al. 1997; Svoboda and Lupica 1998). In our current studies, we did not establish a correlation between MeHg induced locomotor dysfunctions and altered K^+ ion channels. Future studies are necessary to establish the association between developmental neurotoxicity of MeHg and locomotor dysfunctions due to altered functions of K^+ ion channels. In our current study, we looked at the functions of K^+ ion channels in only bipolar cells of the retina. Future studies are also necessary to identify the role of MeHg in other cell types in the retina (e.g. photoreceptors, ganglion cells and amacrine cells) brain (e.g., cells of medial septal glutamatergic circuit) and spinal cord (e.g., glutamatergic and glycinergic interneurons). The function of all the cell types in this pathway need to be elucidated for the better understanding of the influence of MeHg in visual startle response.

Previous studies have shown that transgenerational inheritance of phenotypic variations do not follow a specific parental germline transmission (Lawlor et al. 2003; Pembrey et al. 2006; Veenendaal et al. 2013; Bygren et al. 2014). Paternal grandfather's nutrition status was associated with mortality risk ratios of the grandsons not granddaughters (Pembrey et al. 2006b). DDT induced transgenerational phenotypes including obesity and testis diseases in males was identified to be transmitted through the female germline (Skinner et al. 2013). In our studies, we identified that both males and females of the F3 lineage fish were

affected by the ancestral MeHg exposure. Either male or female germline was sufficient to induce transgenerational visual deficit. However, both male and female germlines were required to induce locomotor deficit. The two phenotypes that we monitored were inherited differently, suggesting that MeHg induced phenotypes are inherited independently. The significance of the mode of germline transmission of a MeHg induced phenotype is that it provides the knowledge to target which parent for preventative measures for a specific phenotype. Future studies are necessary for understanding the sex specific transmission of environmentally-induced transgenerational phenotypes.

The visual deficit exhibited by the F3 lineage fish were followed up with genome wide transcriptome analysis to explore potential molecular mechanisms. Differential gene expression of brain and retina of F3 lineage zebrafish revealed a d transgenerational transcriptome with 1648 genes differentially expressed in the retina and138 genes dysregulated in the brain. Thirty-five genes were commonly dysregulated between brain and retina. Get set enrichment analysis of differentially expressed genes revealed significant enrichments of pathways such as neurodevelopment, neurogenesis, and phototransduction. The top three pathways significantly enriched in the brain were circadian rhythm, phototransduction and arginine and proline metabolism. Retinol metabolism, phototransduction and circadian rhythm pathways were shown to be significantly enriched in the retina. The commonly dysregulated genes in both brain and retina were involved with response to light stimulus, circadian rhythm, and photoperiodism. Disease pathway analysis of the differentially expressed genes

in the retina resulted in the enrichment of diseases including retinal diseases, eye disease, vision disorders and dim vision. Transcriptome analysis of brain and retina of F3 lineage has demonstrated that critical genes and pathways involved in visual functions were differentially expressed due to ancestral MeHg exposure. The dysregulated pathways revealed in our transcriptomic analysis are consistent with visual deficit, phenotype, observed in the F3 lineage animals

The altered potassium ion channel function of the bipolar cells of retina motivated us to screen differentially expressed genes in the retina associated with potassium ion channels. We identified 12 genes associated with potassium ion channels that were dysregulated in the retina of F3 lineage exposure groups. The retina contains several cell types, so our study did not confirm whether the dysregulated genes were located in the bipolar cells. Further analysis of the bipolar cell transcriptome is needed to confirm the role of the dysregulated potassium channels in bipolar cells.

One of the genes dysregulated both in retina and brain was *amd1*, which codes for an enzyme associated with the polyamine pathway. Polyamine pathway is involved with two major functions; embryonic development and DNA methylation. Previous studies have shown that MeHg exposure affects both development and DNA methylation. The discovery of differential expression of *amd1* gene identifies one of the possible pathways by which MeHg affects development and gene expression.

The RNAseq method that we used to identify the association between MeHg induced phenotype and the transcriptome turned out to be a powerful tool

in transgenerational inheritance research. The method provided us the opportunity to survey gene expression of the whole entire genome. We were also able to identify alterations in pathways such as circadian rhythm that were not included in our prior phenotype screening. Since we have identified the possible phenotypic pathways induced by MeHg exposure, future studies can be directed towards the identification of specific transgenerational phenotypes such as circadian rhythm and hyperactivity disorders.

The discoveries that we made in our current study have significant impact on both ecological settings and public health. Environmental impacts of Hg is not limited to human beings. Large carnivorous fish, fish eating birds, mammals and predators of fish eating animals are at risk for Hg toxicity (Varian-Ramos et al. 2014; Goutte et al. 2014; Lavoie et al. 2015). The reproductive success and the population of songbirds are adversely affected by mercury exposure (Varian-Ramos et al. 2014). The primary source of protein for the one third of the world's population is fish. Fish are vital components of every marine and aquatic ecosystems my. Exposure to MeHg has adverse impacts on cellular and physiological functions of fish species including higher oxidative stress (Claveau et al. 2015), reproduction (Crump and Trudeau 2009), feeding habit and habitat preference and survival skills (Alvarez et al. 2006). If the transgenerational neurobehavioral defects that we observed in zebrafish in the laboratory are true in the wild, it will have significant impact on the survival of different fish species leading to significant population reduction.

The significance of our findings is that in addition to other environmental toxicants, a heavy metal for the first time has been identified for its ability to induce phenotypic variations in multiple generations without further exposure. Our findings have opened the door to a new area of biomedical research in identifying transgenerational toxicity of metals such as Pb, Cd and As. The transgenerational potential of the metals have not yet elucidated. If other metals also show similar effects as that of Hg, our findings have monumental impact on global public health.

Our findings will have significant impacts on public health. Neurocognitive functions were significantly affected in the Minamata population exposed prenatally during Hg outbreak (Yorifuji et al. 2014). Transgenerational inheritance of altered phenotypes due to prenatal exposure to MeHg (or any environmental chemical) has yet to be identified in humans. To confirm our findings in human population, epidemiological studies need to be designed to assess effects over several generations with consideration of high level ancestral exposure to mercury and other persistent pollutants pose an especially difficult case since there are no unexposed human populations. Our study mimics the human condition. It is impossible to find fish food without trace levels of mercury—much like the human diet. We were able to identify the transgenerational phenotypes against the background noise induced by trace levels of Hg in an outbred animal model. This suggests that transgenerational phenotypes in humans will be discovered as part of well-designed longitudinal human studies.

Mercury pollution is a global environmental health concern. Countries around the world have come to a consensus around this significant threat which is outlined in the Minamata Convention treaty focused on reducing global emission of mercury from anthropogenic activities. All parties recognize this dire public health problem. Our discovery of transgenerational neurotoxic effects of mercury provides additional compelling evidence that reducing mercury emission is essential to protect public health.

Fish consumption provides important nutrients such as omega-3 fatty acid needed for fetal brain development. On the other hand, fish consumption is the major source of mercury in humans. The mercury levels in fish species vary and women of child-bearing age need to make informed intelligent choices about fish consumption. It is important to take necessary preventative interventions to reduce the bioaccumulation of mercury in reproductive age to prevent neurotoxic affects in our descendants, because we could transmit a silent ghost in our genome to our next generations causing diseases in them.

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THOMAS ACHANKUNJU KALLUVILA

EDUCATION

University of Wisconsin-Milwaukee

- **Doctor of Philosophy** – Environmental and Occupational Health,'09-Present **Dissertation:** "Methylmercury induced visual and neurodevelopmental deficits in zebrafish: The role of DNA methylation in the transgenerational inheritance of disease phenotypes."
  Advisor: Michael J. Carvan III, Ph.D.

- **Master of Science** – Clinical Laboratory Science, May 2005
  Thesis: "DNA methylation and the risk factors for the development of invasive breast cancer."

- **Bachelor of Science** – Medical Laboratory Technology, March 2001

RESEARCH EXPERIENCE

Project Assistant, Spring 2010-Present: Conducting research in the area of environmental toxicogenomics of methylmercury (MeHg). Identifying the role of DNA methylation in transgenerational disease phenotypes induced by developmental exposure to MeHg, using a zebrafish model. The study uses different approaches such as behavioral tests, electrophysiology analysis, and detection of DNA methylation by pyrosequencing and Next Generation Sequencing. Gene expression analysis is conducted by RNAseq technology.

Independent Study, Spring 2012: Learned and practiced bioinformatics tools to analyze next generation sequencing data in the Laboratory for Public Health Informatics and Genomics, ZSPH. The different platforms available for DNA and RNA sequencing were analyzed to identify cost effectiveness and efficiency. A raw sequencing data was obtained from the University of Utah to practice. The different tools of "Galaxy", a web based platform for biomedical research, was used to analyze the data.

Research Technologist, 2005-2006: Developed molecular diagnostic methods at the Medical College of Wisconsin. Involved in the development of a multiplex molecular test for the rapid identification of infectious diseases which could be used in bioterrorism events. Helped the department to write grants for funding from National Institute of Health.

Research Assistant, Summer 2003-Spring 2005: Began initial research in the Molecular Biology Lab of School of Health Sciences, UWM. Learned new techniques in molecular biology and conducted extensive research to identify alterations in DNA methylation of promoter regions of *GSTP1* and *RASSF1* genes in invasive breast tumor tissues. . Trained and supervised undergraduate students in conducting research projects in the lab.

Research Technologist, 2001-2002: Performed histopathological examination of biocompatibility of metal heart valves in pig models.

TEACHING EXPERIENCE

Instructor, Milwaukee Area Technical College, Fall 2006-Present
Teach different levels of Anatomy & Physiology classes. Responsible for teaching both lecture and lab classes and grading.

Graduate Teaching Assistant, UW-Milwaukee, Spring 2003-Fall 2004
Helped professors in conducting courses, lab practicals and examinations; evaluating lab reports and entering students grades in D2L and Blackboard. Helped undergraduate students in conducting experiments in the labs of Medical Microbiology, Hematology, Blood banking, Immunology, Clinical- Chemistry and Urinalysis courses. Prepared reagents, chemicals and media for the lab practical tests.

CONFERENCE PRESENTATIONS

Oral presentation, January 2014: "*Ancestral exposure to methylmercury induces transgenerational inheritance of neurobehavioral defects"*
• The First Annual Graduate Student Research Symposium, ZSPH.

Poster, June 2013: *"Transgenerational inheritance of visual deficits in zebrafish due to developmental exposure to methylmercury."*

• Aquatic Animal Models for Human Disease and Midwest Zebrafish Conference, Milwaukee, WI.

Poster, May 2012: *"Luminometric Assay to Detect Alteration in Global DNA Methylation Due to Methylmercury Exposure in Zebrafish."*
• The Contribution of Epigenetics in Pediatric Environmental Health, San Francisco, CA.

AWARDS

Stipend, April 2014. To attend the Wisconsin Environmental Health Association Spring Educational Conference, Madison, WI.

Travel Award, March 2014: Received financial support from the ZSPH and graduate school for presenting our research in the Society of Toxicology Annual meeting 2014 in Phoenix, AZ.

Crowd Pleaser Award, January 2014: For the oral presentation at the First Annual Graduate Student Research Symposium, ZSPH.
Scholarship, May 2012: Travel accommodations and conference fees for the The Contribution of Epigenetics in Pediatric Environmental Health conference in San Francisco, CA.

Graduate Project Assistantship, Fall 2009-Spring 2012: Awarded through the Zilber School of Public Health, UWM.

Chancellor's Graduate Award, Spring 2005. Awarded through the department of Clinical Laboratory Sciences, UWM. Based on outstanding academic achievements and progress in graduate research project.

Scientific Research Symposium Award, May 2004: Third place for the research poster competition by the College of Health Sciences, UWM.

Graduate Teaching Assistantship, Spring 2003-Fall 2004. Awarded through the department of Clinical Laboratory Sciences, UWM.