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Developmental origins of neurotransmitter and transcriptome alterations in adult female zebrafish exposed to atrazine during embryogenesis

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ABSTRACT

Atrazine is an herbicide applied to agricultural crops and is indicated to be an endocrine disruptor. Atrazine is frequently found to contaminate potable water supplies above the maximum contaminant level of 3 μg/L as defined by the U.S. Environmental Protection Agency. The developmental origin of adult disease hypothesis suggests that toxicant exposure during development can increase the risk of certain diseases during adulthood. However, the molecular mechanisms underlying disease progression are still unknown. In this study, zebrafish embryos were exposed to 0, 0.3, 3, or 30 μg/L atrazine throughout embryogenesis. Larvae were then allowed to mature under normal laboratory conditions with no further chemical treatment until 7 days post fertilization (dpf) or adulthood and neurotransmitter analysis completed. No significant alterations in neurotransmitter levels was observed at 7 dpf or in adult males, but a significant decrease in 5-hydroxyindoleacetic acid (5-HIAA) and serotonin turnover was seen in adult female brain tissue. Transcriptomic analysis was completed on adult female brain tissue to identify molecular pathways underlying the observed neurological alterations. Altered expression of 1928, 89, and 435 genes in the females exposed to 0.3, 3, or 30 μg/L atrazine during embryogenesis were identified, respectively. There was a high level of overlap between the biological processes and molecular pathways in which the altered genes were associated. Moreover, a subset of genes was down regulated throughout the serotonergic pathway. These results provide support of the developmental origins of neurological alterations observed in adult female zebrafish exposed to atrazine during embryogenesis.

1. Introduction

During embryonic development, organisms exhibit a high level of developmental plasticity allowing for alterations of the genetic landscape in response to the surrounding environment which can ultimately result in a broad range of adult phenotypes (Feuer et al., 2014). It is within these genetic and epigenetic alterations that an increased risk of developing diseases in adulthood occurs. This concept is commonly referred to as the Developmental Origins of Health and Disease (DOHaD) hypothesis. The DOHaD hypothesis came into view during the late 1980's based upon a series of epidemiological studies which found an association between a reduction in fetal growth and the development of cardiovascular and metabolic disease in later life by Barker and colleges (Barker and Osmond, 1986; Barker et al., 1993). As research progressed, the number of diseases linked to a developmental origin has increased to include cardiovascular disease, type 2 diabetes, hypertension, and obesity (Dolinoy and Jirtle, 2008). The understanding of the genetic and epigenetic mechanisms behind the development of these diseases is still under investigation. Also, it is within this developmental plasticity that increases the vulnerability of developing organisms to toxicant exposure such as endocrine disrupting chemicals.

Endocrine disrupting chemicals (EDCs) are a group of chemicals that are diverse in structure. EDCs are found in many products such as plasticizers, pharmaceuticals, pesticides, and personal care products and can therefore result in exposures in diverse populations (Diamanti-Kandarakis et al., 2009). Since exposure to EDCs encompasses a broad spectrum of the population, public

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concern focused on investigating the harmful effects of EDCs and their mechanisms of toxicity has increased. EDCs pose significant harm to human health due to their ability to disrupt multiple processes including mimicry of endogenous hormones, alterations of hormone homeostasis, and disruption of hormone synthesis, transport, and metabolism (Diamanti-Kandarakis et al., 2009). Evidence suggests that EDCs do not adhere to classic dose-response toxicological principles; rather they are part of the 'low dose hypothesis' due to their ability to disrupt hormonal homeostasis at low levels and do not always follow a dose response (Diamanti-Kandarakis et al., 2009). Concern about the effects of EDCs during vulnerable developmental periods and childhood has been investigated in animal model systems (Belloni et al., 2011; Davis et al., 2011; Weber et al., 2013) and literature examining the lasting effects of a developmental exposure to EDCs and their contribution to the development of adult disease is under investigation (Birnbaum and Fenton, 2003; Ma et al., 2010).

Atrazine (ATZ) (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a common pre-emergent agricultural herbicide that is a reported endocrine disruptor and is indicated to be a potential carcinogen (Cooper et al., 2000; Freeman and Rayburn, 2005; Freeman et al., 2011; Wetzel et al., 1994). Atrazine has widespread use in the Midwest United States and is used to prevent broad leaf and grassy weeds in corn, sorghum grass, sugar cane, and wheat crops. Atrazine is commonly applied in the spring and summer months and has been shown to contaminate potable water supplies; often above the maximum contaminant level (MCL) set by the U. S. Environmental Protection Agency (EPA) of 3 μg/L (Ochoa-Acuña et al., 2009; Rohr and McCoy, 2010; U.S. EPA, 2002). Due to the persistence and mobility of atrazine in the environment its use was banned in European countries in 2004 (Fakhouri et al., 2010).

The adverse endocrine effects of atrazine caused by acute and chronic exposures during adulthood have been examined. Studies have implicated that atrazine disrupts the hypothalamus–pituitary–gonadal (HPG) axis by decreasing the pre-ovulatory surge of luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin (PRL) (Cooper et al., 2000; Foradori et al., 2009). Disruption of these hormones can lead to early reproductive senescence and dysfunction. Although these studies have shown the disruptive effects of atrazine on the endocrine system, they are representative of an exposure during adulthood. Understanding the effects of a developmental atrazine exposure and the later in life impact is of key importance. Literature noting the effects of a developmental atrazine exposure on the adult female and male reproductive system has shown a delay in mammary gland development and alterations in estrous cycles in female rodents (Davis et al., 2011; Rayner et al., 2005) and a delay in puberty and reduced testosterone levels (Fraites et al., 2011).

Although it is a well-regarded hypothesis that atrazine works by alterations throughout the HPG and hypothalamus–pituitary–adrenal (HPA) axes (Fraites et al., 2009), understanding its effects on the central nervous system (CNS) is necessary in elucidating its mechanism of action due to its ability to readily cross the blood brain barrier (BBB) (Ross et al., 2009). Multiple studies have started to examine the effects of atrazine on the dopaminergic system caused by developmental and adulthood exposures (Coban and Filipov, 2007; Lin et al., 2013a; Rodriguez et al., 2013). However, the effect of atrazine on other monoamine neurotransmitters such as serotonin (5-HT), as well as its effects on the GABAergic system is still under investigation (Das et al., 2000; Rajkovic et al., 2011).

There are considerable strengths in using the zebrafish model to define mechanisms associated with developmental toxicant exposure and the developmental origins of adult health and disease including *ex utero* fertilization and embryonic development, rapid embryogenesis, and a relatively short life span. Paired with these biological strengths are the structural and functional homology of the zebrafish CNS to humans and the conserved genetic, molecular, and endocrine pathways making the zebrafish a powerful model to assess the later life alterations caused by an embryonic atrazine exposure (de Esch et al., 2012; Howe et al., 2013). There are a few recent studies that are now utilizing the zebrafish to examine the contributions of toxicant exposure to the DOHaD hypothesis. These studies include the effects of embryonic methyl mercury (MeHg) (Xu et al., 2012), as well as TCDD (Barker et al., 2013). These studies provide valuable data and support of not only the DOHaD but also for utilizing the zebrafish as a primary model for investigation.

To begin to identify if an embryonic atrazine exposure may alter neurotransmitter profiles later in life, in this study zebrafish were exposed to 0, 0.3, 3 or 30 μg/L atrazine during embryogenesis. These concentrations of atrazine are likely found in the environment in which the general population is exposed including pregnant women, their fetuses, and young children, the primary demographic which the DOHaD hypothesis targets. Following the exposure period, zebrafish were rinsed and allowed to mature in clean water under normal laboratory conditions. Neurotransmitter levels were measured at 7 days post fertilization (dpf) and in mature adults (9 mpf) and then the transcriptomic profile of adult female brain tissue was defined to identify genetic mechanisms underlying observed neurological alterations.

2. Materials and methods

2.1. Zebrafish husbandry and atrazine exposure

Zebrafish (*Danio rerio*) wild-type AB strain were housed in Z-Hab systems (Aquatic Habitats, Apopka, FL) on a 14:10 hour light: dark cycle. Water quality was maintained at 28 °C, pH of 7.0–7.2, and conductivity range of 470–550 μS. Adult fish were bred in cages and embryos were collected and staged following established protocols (Peterson et al., 2011; Westerfield 2007). A stock solution of technical grade atrazine (98.1% purity) (CAS 1912-24-9; Chem Service, West Chester, PA) at a concentration of 10 mg/L was prepared near the solubility limit in water as previously described (Weber et al., 2013). Embryos were dosed with 0 (aquaria water only), 0.3, 3, or 30 μg/L atrazine from 1–3 dpf as previously described (Weber et al., 2013). After the exposure, larvae were rinsed with clean fish system water, housed in 4-liter tanks in the zebrafish systems and allowed to mature under normal growing conditions until 6–9 mpf. All protocols were approved by Purdue University's Institutional Animal Care and Use Committee (A3231-01) with all fish treated humanely and with regard for alleviation of suffering.

2.2. High performance liquid chromatography (HPLC) with electrochemical detection in larvae and adult zebrafish

Neurotransmitter analysis on larvae and adult male and female brain tissue was conducted similar to previously reported (Wirbisky et al., 2014). For the neurotransmitter analysis at 7 dpf, 30 larvae per treatment were pooled for analysis (considered as one biological replicate) (Fig. 1). For the adult neurotransmitter analysis, eight adult male and eight adult female zebrafish were collected from each treatment at 9 months post fertilization (mpf), anesthetized in MS-222 (4 mg/mL), and brain tissue dissected and submerged in 500 μL of 0.4 M perchloric acid ($HClO_4$). Samples were then sonicated (Power 40%, Pulse 2 s and stop for 1 s; Fisher Scientific, Model FB120, 120W) for 45 s per sample and centrifuged at 16,000 rcf for 35 min at 4 °C. The