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Atrazine Exposure Elicits Copy Number Alterations in the Zebrafish Genome

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Abstract

Atrazine is an agricultural herbicide used throughout the Midwestern United States that frequently contaminates potable water supplies resulting in human exposure. Using the zebrafish model system, an embryonic atrazine exposure was previously reported to decrease spawning rates with an increase in progesterone and ovarian follicular atresia in adult females. In addition, alterations in genes associated with distinct molecular pathways of the endocrine system were observed in brain and gonad tissue of the adult females and males. Current hypotheses for mechanistic changes in the developmental origins of health and disease include genetic (e.g., copy number alterations) or epigenetic (e.g., DNA methylation) mechanisms. As such, in the current study we investigated whether an atrazine exposure would generate copy number alterations (CNAs) in the zebrafish genome. A zebrafish fibroblast cell line was used to limit detection to CNAs caused by the chemical exposure. First, cells were exposed to a range of atrazine concentrations and a crystal violet assay was completed, showing confluency decreased by ~60% at 46.3 μ M. Cells were then exposed to 0, 0.463, 4.63, or 46.3 μ M atrazine and array comparative genomic hybridization completed. Results showed 34, 21, and 44 CNAs in the 0.463, 4.63, and 46.3 μ M treatments, respectively. Furthermore, CNAs were associated with previously reported gene expression alterations in adult male and female zebrafish. This study demonstrates that atrazine exposure can generate CNAs that are linked to gene expression alterations observed in adult zebrafish exposed to atrazine during embryogenesis providing a mechanism of the developmental origins of atrazine endocrine disruption.

Keywords

array comparative genomic hybridization; atrazine; copy number alterations; transcriptomics; zebrafish

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1. Introduction

Structural genomic variation is common within the human genome and consists of multiple components such as single nucleotide polymorphisms (SNPs), tandem repeats, transposable elements, and structural alterations (deletions, duplications, and inversions) (Freeman et al., 2006; Stankiewicz and Lupski, 2010). Of these, differences in genomic copy number are widely present and are the largest known genomic variation. Copy number changes can be either amplifications or deletions that can range in size from 50 base pairs (bp) to greater than a megabase (one million bp) (Freeman et al., 2006; Russo et al., 2015). Genomic copy number differences occur throughout the genome of healthy individuals and are generally referred to as copy number variants or CNVs as they are thought to be non-pathogenic. Currently, over 25,000 CNVs including 1,000 large CNVs (greater than 50 kb) are now identified. However, non-recurrent CNVs can also contribute to various disorders and diseases including obesity, cancer, and neurological disorders including autism spectrum disorder (ASD), attention-deficit hyperactivity disorder (ADHD), and schizophrenia, Alzheimer's disease and Parkinson's disease (Sebat et al., 2007; Stankiewicz and Lupski, 2010; Valbonesi et al., 2015; Walsh et al., 2008; Zhang et al., 2013). Despite recent advances in genomic technologies, limited knowledge surrounds the generation of the disease associated CNVs, which are then referred to as copy number alterations or CNAs, and the risk factors involved. Furthermore, it is hypothesized that in addition to genetic factors, exposure to environmental contaminants can lead to CNA development (Adewoye, et al., 2015; Arlt et al., 2014; Peterson and Freeman, 2014).

Endocrine disrupting chemicals (EDCs) are exogenous agents that disrupt endogenous hormone signaling (Swedenborg et al., 2009). EDCs are diverse in structure and are found in numerous products such as plasticizers, pharmaceuticals, and pesticides, making human exposure to these chemicals a likely event (Birnbaum and Fenton, 2003; Ma et al., 2010; Prins et al., 2007). Numerous challenges are identified and need to be overcome when aiming to understand the mechanisms of action of EDCs. Two primary challenges of EDCs are their characteristic non-monotonic dose-response and latency period between exposure and observable effects (Vandenberg 2012). Furthermore, studies implicate that a developmental exposure to EDCs can alter genetic and epigenetic processes which can result in adverse health consequences later in life and in multigenerational and/or transgenerational effects (Anway et al., 2006; Casati et al., 2012, 2013, 2015; Dolinoy et al., 2007; Martinez-Arguelles et al., 2015).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a pre-emergent herbicide that is applied throughout the Midwestern United States and other parts of the globe on a variety of agricultural crops including corn, sorghum grass, sugar cane, and wheat (Barr et al., 2007; Eldridge et al., 2008; Solomon et al., 2008). Due to atrazine's water solubility, mobility in soil, and long half-life, atrazine frequently contaminates potable water supplies and reaches levels above the maximum contaminant level (MCL) as set by the U. S. Environmental Protection Agency (EPA) of 3 parts per billion (ppb; µg/L) (U.S. EPA 2002; Fraites et al., 2009; Rohr and McCoy, 2010). As such the European Union banned the use of atrazine in 2003 (European Commission 2003; Sass and Colangelo, 2006). Numerous laboratory studies have shown that atrazine alters the neuroendocrine system primarily

through the hypothalamus-pituitary-gonadal (HPG) axis (Cooper et al., 2000; Foradori et al., 2009, 2013; Weber et al., 2013; Wirbisky et al., 2016a). In addition, genetic, epigenetic, and cellular mechanisms altered by atrazine exposure are under investigation (Karmaus and Zacharewski, 2015; Kucka et al., 2012; Pogrmic-Majkic et al., 2009, 2010, 2014; Wirbisky et al., 2016a, 2016b).

The genotoxicity of atrazine has been under investigation since the early 1990s and has provided contradictory evidence. The majority of *in vitro* studies have been conducted in Chinese hamster ovary (CHO) and human lymphocyte cells. Positive studies indicate that atrazine elicits genotoxicity through chromosomal aberrations (CA), sister chromatid exchanges (SCEs), and increases in the coefficient of variation (CV) at a variety of concentrations and exposure periods (Biradar and Rayburn, 1995; Lioi et al., 1998; Rayburn et al., 2001; Taets et al., 1998). While contrasting studies do not demonstrate any significant genotoxicity (Dunkelberg et al., 1994; Kligerman et al., 2000a, 2000b; Roloff et al., 1992; Surrallés et al., 1995; Zeljezic et al., 2006). *In vivo* studies conducted on a variety of animal models including fish, anuran, and rodent have also shown results indicating no evidence of genotoxicity (Adeyemi et al., 2015; Cavas 2011; Freeman and Rayburn 2004). While other studies reported increases in micronuclei, nuclear abnormalities, and DNA damage (Clements et al., 1997; Gebel et al., 1997; Tennant et al., 2001; de Campos Ventura et al., 2008).

While various *in vitro* and *in vivo* models have been utilized to investigate the genotoxicity of atrazine, this study utilized a zebrafish fibroblast cell line derived from AB wild-type embryos. This cell line is well characterized, routinely monitored for cytogenetic changes, and has been used in previous zebrafish cytogenetic studies (Freeman et al., 2007; Peterson and Freeman, 2014). In addition, the use of this zebrafish cell line will provide ease in moving into *in vivo* studies utilizing zebrafish embryos as the zebrafish is a strong complementary vertebrate model used throughout many biological disciplines. A finished genome sequence and a highly conserved genetic homology to humans permits translation of molecular mechanisms observed in the zebrafish to humans (de Esch et al., 2012; Howe et al., 2013). The development of array comparative genomic hybridization (aCGH) and next-generation sequencing has allowed for the detection of CNVs and CNAs throughout the genome (Russo et al., 2015). Due to an established CNV map for the zebrafish, studies have been completed investigating CNAs in cancer models and following chemical exposure (Brown et al., 2012; Chen et al., 2013; Freeman et al., 2009; Peterson and Freeman, 2014; Zhang et al., 2013).

In this study, zebrafish fibroblast cells were used to test the hypothesis that an atrazine exposure will cause genotoxicity through the generation of CNAs detectable with the use of aCGH technology. Although genotoxicity of atrazine has been investigated in past studies through the various endpoints previously mentioned, this is the first study to assess if atrazine exposure will result in genotoxicity by evaluating the generation of CNAs. Furthermore, in order to assess potential genetic mechanisms behind the developmental origins of health and disease hypothesis, which states that developmental chemical exposure can contribute to disease onset during adulthood, links between CNAs and previously

identified gene expression alterations in adult male and female zebrafish exposed to atrazine during embryogenesis was assessed (Wirbisky et al., 2015, 2016a, 2016b).

2. Materials and Methods

2.1 Zebrafish fibroblast cell line

A zebrafish fibroblast cell line established from approximately 100 embryos of the AB wildtype zebrafish strain was used in this study and is described in Freeman et al. (2007).

2.2 Cytotoxicity Assay

Atrazine (CAS #1912-24-9, Chem Service, West Chester, PA, 98% purity) stock (500,000 ppb or 2315 μM) was prepared using dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO). A cytotoxicity assay was completed following similar protocols as previously described (Freeman and Rayburn, 2006; Peterson and Freeman, 2014; Plewa et al., 2002). Briefly, cells were harvested from cell culture flasks following a standard trypsin protocol and cell concentration was determined. The cytotoxicity assay was set up in 96 well plates with 14,000 cells per well with appropriate amount of media and chemical stock to make up 1 – 1,125 μM . Plate set-up included a first column blank and a second column negative control. Each plate contained four subsample wells per chemical concentration. Plates were placed in an incubator at 28°C and 5% carbon dioxide for 72 hours (the equivalent of 1.5 cell cycle lengths). After 72 hours, cells were fixed in 50% methanol and stained with 1% crystal violet in 50% methanol, and excess crystal violet solution was washed from the plate. Absorbance was read on a microplate reader at 595 nm (SpectraMax M2, Molecular Devices). Readings from the four subsample wells of each test concentration was averaged. Four replicate plates (containing four subsample wells per chemical concentration) were completed and the average percent negative control values for each test concentration was plotted and fit to a sigmoidal curve using SigmaPlot (SigmaPlot 10.0) to determine appropriate test concentrations for copy number alteration (CNA) analysis. Test concentrations for aCGH were at the 60% negative control value and at concentrations where no impacts on cell confluency were observed.

2.3 aCGH analysis of copy number alterations

Zebrafish cells were exposed to 0, 0.463, 4.63, or 46.3 μM atrazine (100, 1000, or 10,000 ppb, respectively) or a control treatment (media with no additional chemical treatment). 7.5×10^5 cells were initially seeded into each petri dish ($n=4$). After set-up, petri dishes were placed in an incubator at 28°C and 5% CO_2 for 72 hours (the equivalent of 1.5 cell cycle lengths). After 72 hours, cells were harvested and genomic DNA was isolated following a standard phenol:chloroform isolation method as previously described (Freeman et al., 2009; Peterson and Freeman, 2014). A zebrafish specific oligonucleotide platform was designed based upon the Zv9 zebrafish genome build. A $2 \times 400\text{K}$ array containing 418,551 unique probes approximately 60 nucleotides in length with a median spacing of 2.942 kbp was constructed using SureDesign by Agilent Technologies (Agilent Technologies, Santa Clara, CA) with avoidance of standard masked regions and restriction sites (Table 1). Array CGH analysis was performed following manufacturer's protocol (Version 7.3) using a two color hybridization strategy. Briefly, 1 μg of test DNA and 1 μg of reference DNA were

fluorescently labeled with Cy5 and Cy3, respectively. Dye incorporation was assessed using a NanoDrop ND 1000 spectrophotometer. Cy5 test DNA and Cy3 reference DNA were combined and hybridized to the array for 40 hours at 67°C. Following hybridization, arrays were washed according to manufacturer's recommendations (Agilent Technologies, Santa Clara, CA). Arrays were then scanned on a SureScan microarray scanner (Agilent Technologies, Santa Clara, CA) at 3 microns. Array image data was extracted using Agilent Feature Extraction Software 11.5 (Agilent Technologies, Santa Clara, CA). Microarray analysis was performed following MIAME guidelines (Brazma et al., 2001). One biological replicate was removed from the analysis as it did not meet quality control standards resulting in 3 biological replicates for this analysis (n=3). aCGH analysis was completed using Agilent Genomic Workbench 7.0.4 following the aberration detection method 2 (ADM2) algorithm. ADM2 identifies all aberrant intervals in a given sample with consistently high or low log ratios based on statistical score. The ADM2 algorithm searches for intervals in which a statistical score based on the average quality weighted log ratio of the sample and reference channels exceeds a user specific threshold and reports contiguous genomic regions as aberrant regions. In this study calls were determined which contained at least six consecutive probes to obtain a high degree of confidence (Agilent Technologies, Santa Clara, CA). Microarray data is deposited in the NCBI Gene Expression Omnibus (GEO).

2.4 Quantitative polymerase chain reaction (qPCR) microarray confirmation

qPCR was performed on a subset of selected CNAs altered in the aCGH analysis using the BioRad SSOAdvance SYBR Green Supermix kit according to the manufacturer's recommendations. Primers of specific CNA regions were designed using the Primer3 website (Supplementary Table 1). qPCR was performed following similar methods as previously described (Weber et al., 2013; Wirbisky et al., 2015) following the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al. 2009). Similar to as performed in previous studies in our laboratory (Wirbisky et al., 2014, 2015, 2016a, 2016b) several genome regions were assessed to determine the best reference sequence to be used for this data set (data not shown). A primer targeting sequence containing the gene β -ACTIN was found to be the most consistent and least variable for this analysis. qPCR was performed on the same samples as used in the microarray analysis (n=3). Experimental samples were run in triplicate (technical replicates) and gene expression was normalized to β -ACTIN. Efficiency and specificity were checked with melting and dilution curve analysis and no-template controls. In the determination of microarray confirmation by qPCR, the relative expression (2^{-Cq}) of the treatments was divided by the 2^{-Cq} of the control to confirm the addition or deletion of CNA with a value of greater than or less than one as performed previously (Brown et al., 2012).

2.5 aCGH comparison to gene expression microarray data of adult male and female zebrafish exposed to atrazine during embryogenesis

The CNAs corresponding to a known gene (identified through the ADM2 algorithm) were compared to the lists of previously identified altered genes from adult male and female brain and gonad tissue identified through gene expression microarray analysis (Wirbisky et al., 2015, 2016a, 2016b).

3. Results

3.1 Cytotoxicity assay

Test concentrations showed an EC₅₀ value of 129 μ M. In addition, test concentrations showed a 60% change in confluency at 46.3 μ M with no alterations in confluency at 4.63 or 0.463 μ M, respectively. Therefore, these concentrations were chosen for aCGH analysis (Figure 1).

3.2 aCGH for CNA analysis

Atrazine exposure elicited 34, 21, and 44 CNAs in the 0.463, 4.63, and 46.3 μ M treatments, respectively (Supplementary Table 2). In total, 99 CNAs were called within 45 regions (Figure 2; Supplementary Table 2). 53 CNAs from 10 different genomic regions were present in all three treatment groups (Supplementary Table 2). CNA analysis showed 17 copy number alteration regions (CNARs) which consisted of 69 calls and ranged in size from 14.1-645.6 kb (Table 2). In addition, atrazine caused 30 singlet CNAs which ranged in size from 5.8-408.9 kb. Atrazine exposure elicited 4 deletions on chromosomes 7, 16, 22, and 23 in the 46.3 μ M treatment. These deletions ranged in size from 20,647 to 408,887 bp. The deletions on chromosomes 7 and 16 were genic, corresponding to the genes *irx1a* and *hsd11b2*, respectively. The deletions located on chromosomes 22 and 23 were non-genic. 95 out of the 99 calls were amplifications consisting of 52 genic and 43 non-genic CNAs. No CNAs were identified on chromosomes 6, 10, 11, or 24. In addition, chromosome 8 consisted of the most CNAs, representing 23.2% of identified calls.

3.3 qPCR confirmation of aCGH CNA analysis

76 CNAs were selected for independent qPCR confirmation (Supplementary Table 2). These consisted of 24 singlets and 12 CNARs. 52 CNAs were confirmed, generating a 68% confirmation rate.

3.4 aCGH comparison to previously identified mRNA alterations in adult male and female zebrafish exposed to atrazine during embryogenesis

Genic CNAs (Supplementary Table 2) were compared to previously identified mRNA alterations in adult zebrafish (Wirbisky et al., 2015, 2016a, 2016b). In previous studies, zebrafish embryos were exposed to 0, 0.3, 3, or 30 ppb atrazine (0.0014, 0.014, or 0.14 μ M, respectively) throughout embryogenesis (1-72 hours post fertilization). After the exposure period, larvae were rinsed and allowed to mature until adulthood. Brain and gonad tissue was dissected and microarray analysis was conducted to identify mRNA alterations (Wirbisky et al., 2015, 2016a, 2016b). Comparison of these previously shown mRNA alterations with the CNA results of this paper showed altered expression of 9 unique genes associated with 14 CNA amplifications and 1 deletion. Of the 15 referenced CNAs (listed below), 10 were confirmed by qPCR analysis (Supplementary Table 2). Analysis of CNAs corresponding to altered mRNA expression showed no similar targets in male gonad tissue. However, male brain tissue showed up regulation of *cdh4* (CNAs 7 and 64) and *arl6* (CNA 1) and down regulation of *hsd11b2* (CNA 65) in the 0.3 ppb atrazine treatment (0.0014 μ M). The 3 ppb atrazine treatment (0.014 μ M) showed up regulation of *arl6* (CNA 1), *thrb* (CNA

89), *hdc* (CNA 51), and *cdh4* (CNAs 7 and 64). In addition, the 30 ppb treatment (0.14 μ M) also showed up regulation of *cdh4* (CNAs 7 and 64) and *hdc* (CNA 51). The female gonadal tissue in the 0.3 ppb atrazine treatment (0.0014 μ M) had up regulation of mRNA, which corresponded to CNAs containing *cdh4* (CNAs 7 and 64), *ogdh* (CNA 15), *thrb* (CNA 89), and *hdc* (CNA 51). The 3 ppb atrazine treatment (0.014 μ M) also showed down regulation of *ogdh* (CNA 15). No corresponding mRNAs were altered in the 30 ppb atrazine treatment (0.14 μ M). The female brain tissue had down regulation of *pbx4* (CNAs 3, 37, 58 and 60) in the 0.3 ppb treatment group (0.0014 μ M) and *ogdh* (CNA 15) in the 0.3 (0.0014 μ M) and 30 ppb (0.14 μ M) treatment groups; while an up regulation was reported for *flad1* (CNA 83) and *isg2012* (CNAs 24, 25, and 85) in the 0.3 ppb (0.0014 μ M) treatment group.

4. Discussion

Although numerous studies have investigated CNAs primarily in cancer and neurological disorders, the investigation into the generation of CNAs following exposure to chemicals or other environmental agents is limited (Adewoye et al., 2015; Arlt et al., 2014; Peterson and Freeman, 2014). As the identification of copy number variation within the zebrafish genome has been completed (Brown et al., 2012), this initial assessment utilized zebrafish fibroblast cells for the study of CNA formation following atrazine exposure to eliminate detection of copy number variants that would be present among different individual fish. The genotoxicity of atrazine has been under investigation and has produced conflicting results which have ranged from evidence of no genotoxicity (Kligerman et al., 2000a; Surrallés et al., 1995; Zeljezic et al., 2006), to the generation of chromosomal aberrations (CA), sister chromatid exchanges (SEC), and increases in coefficient of variation (CV) at various dose levels and exposure periods (Biradar and Rayburn, 1995; Lioi et al., 1998; Rayburn et al., 2001; Taets et al., 1998).

In this study, a range of atrazine concentrations was used to investigate whether atrazine exposure would generate CNAs. The highest test concentration (46.3 μ M) elicited a decrease in cell confluency to ~60%, while the other test concentrations (4.63 and 0.463 μ M) caused no alterations in cell confluency. The generation of CNAs following atrazine exposure exhibited a non-monotonic dose-response; a phenomenon typical of neuroendocrine disrupting chemicals (Vandenberg 2012). In addition, CNAs were detected in similar genomic regions among multiple test concentrations of atrazine indicating potential hotspots of genomic instability and a nonrandom genotoxic mechanism for atrazine. Results from the CNA analysis showed 17 CNARs which ranged in size from 14.1-645.6 kb. In addition, atrazine caused 30 singlet CNAs which ranged in size from 5.8-408.9 kb. To our knowledge this is the first study in which the genotoxicity of atrazine through the generation of CNAs has been assessed. Additional studies utilizing ethyl methanesulfonate, cytosine arabinoside, hydroxyurea, or ionizing radiation were recently completed (Adewoye et al., 2015; Arlt et al., 2011, 2014; Peterson and Freeman, 2014). Arlt et al. (2011) and Adewoye et al. (2015) both observed copy number changes following exposure to ionizing radiation, while Arlt et al. (2014) observed copy number changes following exposure to hydroxyurea in human cells. In an earlier study by our laboratory group we observed CNAs following exposure to the known genotoxicants ethyl methanesulfonate or cytosine arabinoside in zebrafish fibroblast cells (Peterson and Freeman, 2014). The findings of each of these studies support

the potential for exposure to various agents to generate CNAs and the need to investigate this mechanism of genotoxicity in future studies.

To confirm the CNAs observed by aCGH, qPCR was performed on a subset of randomly selected targets which encompassed both CNARs and singlets. Although the qPCR validation was performed on the same samples as used in the aCGH analysis, a confirmation rate of 68% was observed. During the analysis, two CNARs (Chr8: 32502115-32538091 and Chr14: 53430371-53444925) could not be confirmed since the regions were masked in N-repeats; therefore, qPCR primers could not be designed. Although qPCR is a reliable tool to confirm aCGH results (Wang et al., 2013), a few different factors may contribute to the observed confirmation rates. One contributing factor is the resolution of the microarray used in this experiment. The resolution of the microarray design is 2.94 kb (i.e. probes are present approximately every 3,000 bp). Therefore, it is possible to overestimate the size of a CNA and/or a single CNA may be called when multiple CNAs are present within the regions (i.e. multiple breakpoints). In addition, the primers designed to confirm CNAs are 100-200 bp in length. Therefore, due to their smaller size, the exact CNA may be missed. Future studies with a lower resolution aCGH platform and/or high resolution DNA sequencing to walk the genome is needed to provide more information on the break points of the CNAs to further refine size and structure.

The identification of the association between CNAs and gene expression is beginning to be assessed in order to identify key genetic events in disease progression, primarily in cancer models (e.g., Callagy et al., 2005; Cava et al., 2014; Chin et al., 2007; Kotliarov et al., 2012). Therefore, to further understand the mechanism of action of atrazine and its impacts on the genome, a comparative analysis was completed between CNAs generated *in vitro* by an atrazine exposure (CNAs which had a defined corresponding gene) and alterations in mRNA (gene expression changes) elicited by an embryonic atrazine exposure in adult male and female zebrafish. Overall, 15 CNAs were identified which corresponded to 9 unique genes previously identified in adult zebrafish (Wirbisky et al., 2015, 2016a, 2016b).

Two CNAs containing the gene cadherin 4, type 1 (*cdh4*) underwent amplification at 0.463 and 46.3 μ M atrazine. *cdh4* was up regulated in the brain tissue of male zebrafish exposed to either 0.3, 3, or 30 ppb atrazine (0.0014, 0.014, or 0.14 μ M, respectively) during embryogenesis (Wirbisky et al., 2016b). In addition, the up regulation of *cdh4* was reported in female ovarian tissue in the 0.3 ppb atrazine treatment (0.0014 μ M). This gene encodes for the R-cadherin protein, which is expressed in various tissues with a wide range of functionality. In the brain, R-cadherin plays a role in establishing axonal guidance; while in other tissue, R-cadherin is involved in cell-cell cohesion, inhibition of apoptosis and cell signaling, and tumorigenesis (Du et al., 2011). Therefore, our results show that the generation of this CNA *in vitro* could be a potential genetic mechanism behind alterations in observed mRNA expression, although more studies are needed for further assessment.

An additional CNA amplified in the 0.463 μ M treatment contains ADP ribosylation factor like GTPase 6 (*arl6*). *arl6* was up regulated in the brain of male zebrafish exposed to either 0.3 or 3 ppb atrazine (0.0014 or 0.014 μ M, respectively) during embryogenesis. This gene plays a role in intracellular trafficking, protein transport, and cell signaling. It is primarily

associated with Bardet-biedl syndrome (BBS) leading to obesity and blindness. Research has characterized the expression of *arl6* interacting protein (*arl6ip*) throughout zebrafish development; however, its role during adulthood is still under investigation (Huang et al., 2009).

As atrazine is associated with neuroendocrine dysfunction primarily through the hypothalamus-pituitary-gonadal (HPG) axis as well as the hypothalamus-pituitary-adrenal (HPA) and hypothalamus-pituitary-thyroid (HPT) axes, it was of interest to identify CNAs containing genes involved in these neuroendocrine pathways. A deletion CNA contained the gene *hsd11b2* and this gene was down regulated in the brain of adult male zebrafish exposed to 0.3 ppb atrazine (0.0014 μ M) during embryogenesis. *hsd11b2* is a key gene in the metabolism of glucocorticoids by inactivating the conversion of cortisol to cortisone in aldosterone target tissues (Kosicka et al., 2013). Previous studies have shown that atrazine can impact the homeostasis of the HPA axis in adult female rodents (Fraites et al., 2009). Furthermore, results from this study also showed an amplification CNA associated with the thyroid hormone receptor beta (*thr β*) gene in the 46.3 μ M atrazine treatment. This amplification corresponds to an up regulation of *thr β* in the female gonad (0.3 ppb; 0.0014 μ M) and the male brain (3 ppb; 0.014 μ M). THR β is one of the two thyroid hormone receptors (in addition to *THR α*) which thyroid hormones bind. Thyroid hormones are vital for proper regulation of body metabolism, growth, development, and reproduction. Thyroid hormone receptors (THR α and THR β) have been identified in male and female zebrafish gonadal tissue (Morais et al., 2013; Wang et al., 2004). Although our previous studies (Wirbisky et al., 2016a, 2016b) did not investigate levels of thyroid hormone in the adult zebrafish, transcriptomic data and previous rodent and *X laevis* studies (Freeman et al., 2005; Ghinea et al., 1979; Kornilovskaya et al., 1996; Stoker et al., 2002) support the need for further investigation into the genetic alterations through CNA generation and gene expression alterations.

An additional CNA which was amplified corresponded to the gene histidine decarboxylase (*hdc*) which was upregulated in female gonad and male brain tissue. Histidine decarboxylase is the only enzyme that converts histidine to histamine, which is an important bioamine that contributes to inflammation, allergies, and neurotransmission (Ku et al., 2014). Although not investigated as predominately as the neuroendocrine dysfunction caused by atrazine, the inflammatory and immune responses are currently under investigation (Ma et al., 2015; Thompson et al., 2015; Zhao et al., 2013). As *hdc* expression is predominantly altered in adult male brain tissue, these data could provide support for further studies investigating the immune responses later-in-life following an embryonic atrazine exposure.

Although additional CNAs were identified which corresponded to gene expression alterations in *flad1*, *isg2012*, *ogdh*, and *pbx4*, further investigation into defining these gene targets as potential mechanisms of atrazine toxicity is required as these targets encompass a wide range of cellular processes (e.g., Couté et al., 2008; Kao et al., 2015; Sen et al., 2012; Torchetti et al., 2010). We observed limited commonality in CNAs related to transcriptional expression changes in specific tissue types among the two sexes (e.g., female and male brain or gonads). This observation was expected as there were limited similarities in the specific genes that were identified to be altered in our previous transcriptomic studies when

comparing the specific tissue types among the two sexes (Wirbisky et al., 2015, 2016a, 2016b). We also recognize the limitation presented based on the differences in exposure concentrations among the data sets being compared (i.e., 0.0014–0.14 μM (0.3–30 ppb) for the adult zebrafish transcriptomic analysis compared to 0.463–46.3 μM for the zebrafish fibroblast exposure). Even with these limitations, this comparison did indicate some connections between CNAs identified with the atrazine exposure in the zebrafish fibroblasts and the transcriptomic data sets from adult zebrafish that were exposed to atrazine during embryogenesis that warrant further research.

5. Conclusions

Overall, results from this study indicate that atrazine elicits genotoxicity through the generation of CNAs. In addition, identified CNAs corresponded to gene expression alterations previously identified through microarray analysis in adult male and female zebrafish that were exposed to atrazine during embryogenesis. Although it is still under investigation of how CNAs correspond to alterations in gene expression, our data shows that CNA amplifications correspond to an up regulation in mRNA expression and CNA deletions tend to correspond to decreases in mRNA expression. It is acknowledged that these associations compare *in vitro* and *in vivo* data with various differences in dose levels and exposure periods. However, this preliminary study provides support of the genotoxicity of atrazine (*in vitro*) and begins to build a foundation in which future *in vivo* studies can be completed in order to identify the biological and functional significance of CNAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Cell cytotoxicity assay

The toxicity profile of atrazine was completed in the zebrafish fibroblast cell line to determine impacts on cell confluency in this specific cell line. From this analysis, atrazine concentrations were determined for array CGH experiments. Four replicate plates (containing four subsample wells per chemical concentration) were completed.

Figure 2. CNAs generated by atrazine exposure

This figure depicts the probe penetrance report showing the percentage of experimental arrays (samples) that have an aberration at each probe position for the 25 zebrafish chromosomes. Exposure to atrazine resulted in 99 CNAs among all atrazine treatments. These CNAs consisted of 45 CNARs (including singlets) at various locations among the 25 zebrafish chromosomes. Regions identified included both gains (41; red bars) and losses (4; green bars). Length of bar indicates percent of observation frequency with an aberration at each probe position among the experimental samples. For example, all 9 experimental samples had a gain detected within 35261578- 35907149 bp on chromosome 8 (i.e., 100% of the samples had a gain of varying sizes detected within this genomic region on chromosome 8; represented as a red bar to 100%).

Table 1

Genome coverage of the zebrafish array CGH platform.

Target ID	Total Probes	Median Probe Spacing (bp)	Coverage (%)
chr1:1-60348388	18796	2927	98.481606
chr2:1-60300536	18571	2949	98.67164
chr3:1-63268876	19619	2932	98.53819
chr4:1-62094675	17812	2930	94.466675
chr5:1-75682077	23270	2948	98.21061
chr6:1-59938731	18532	2943	98.4922
chr7:1-72726063	23999	2930	98.59621
chr8:1-56184765	17253	2947	98.31693
chr9:1-58232459	18012	2940	98.65244
chr10:1-46591166	14402	2949	98.64479
chr11:1-46661319	14508	2943	98.92596
chr12:1-50697278	15662	2948	98.328575
chr13:1-54093808	16757	2943	98.55145
chr14:1-53733891	16683	2943	98.935486
chr15:1-47442429	14606	2945	98.49317
chr16:1-58780683	18111	2952	98.54425
chr17:1-53984731	16780	2942	98.92435
chr18:1-49877488	15587	2938	99.21364
chr19:1-50254551	15595	2940	98.66511
chr20:1-55952140	17250	2954	98.70213
chr21:1-44544065	13798	2944	98.68743
chr22:1-42261000	13016	2937	98.18721
chr23:1-46386876	14417	2946	99.18174
chr24:1-43947580	13625	2947	98.65881
chr25:1-38499472	11890	2948	98.65577
Summary	418551	2.942 kb	98.433014

Table 2

Common copy number alteration regions among atrazine treatments.

Genomic Region of Overlap	Size (kb)	Concentration (μM)	CNA Genomic Region	Amplification	Length (kb)
Chr3: 44059724-44094120	34.4	0.463	Chr3: 44059724-44094120	0.534274	34.4
		4.63	Chr3: 44059724-44094120	0.523277	
		4.63	Chr3: 44059724-44094120	0.629812	
		46.3	Chr3: 44059724-44094120	0.529955	
Chr3: 50360510-50518188	157.7	0.463	Chr3: 50360510-50474748	0.413388	114.2
		4.63	Chr3: 50366222-50495424	0.253529	
		46.3	Chr3: 50360510-50518188	0.313299	
		46.3	Chr3: 50398748-50495424	0.54105	
Chr4: 28000808-28014937	14.1	0.463	Chr4: 28000808-28014937	0.690337	14.1
		0.463	Chr4: 28000808-28014937	0.760753	
		0.463	Chr4: 28000808-28014937	0.763522	
		4.63	Chr4: 28000808-28014937	0.695638	
		4.63	Chr4: 28000808-28014937	0.748757	
		46.3	Chr4: 28000808-28014937	0.712744	
Chr5: 44725633-44981001	255.4	0.463	Chr5: 44725633-44981001	0.40703	255.4
		46.3	Chr5: 44909510-44977511	0.572222	
Chr8: 27853872-27951513	97.6	0.463	Chr8: 27853872-27951513	0.355014	97.6
		0.463	Chr8: 27869151-27951513	0.286967	
		0.463	Chr8: 27869151-27951513	0.347453	
		4.63	Chr8: 27866351-27951513	0.31109	
		4.63	Chr8: 27869151-27951513	0.369476	
		4.63	Chr8: 27869151-27951513	0.295811	
Chr8: 32502115-32538091	36	46.3	Chr8: 27869151-27948666	0.577535	79.5
		46.3	Chr8: 27869151-27951513	0.407814	
					36

Genomic Region of Overlap	Size (kb)	Concentration (μM)	CNA Genomic Region	Amplification	Length (kb)
Chr8: 35261578-35907149	645.6	4.63	Chr8: 32502115-32538091	0.378012	36
		46.3	Chr8: 32502115-32538091	0.398497	36
Chr8: 35261578-35907149	645.6	0.463	Chr8: 35261578-35857774	0.266381	596.2
		0.463	Chr8: 35261578-35857774	0.305822	596.2
		0.463	Chr8: 35261578-35861320	0.327408	599.7
		4.63	Chr8: 35261578-35864120	0.310068	602.5
		4.63	Chr8: 35261578-35857774	0.285159	596.2
		4.63	Chr8: 35261578-35857774	0.265841	596.2
		46.3	Chr8: 35261578-35907149	0.35236	645.6
		46.3	Chr8: 35264378-35861320	0.277287	597
Chr9: 28799123-28892528	93.4	0.463	Chr8: 35264378-35864120	0.326299	600
		4.63	Chr9: 28799123-28889489	0.285325	90.4
Chr13: 28053587- 28100740	47.2	0.463	Chr9: 28799123-28892528	0.283453	93.4
		0.463	Chr13: 28053587- 28079850	0.408287	26.3
		4.63	Chr13: 28053587- 28100740	0.316946	47.2
Chr14: 17343313- 17407725	64.4	0.463	Chr13: 28053587- 28079850	0.401655	47.2
		4.63	Chr14: 17343313-17407725	0.436502	64.4
Chr14: 21073574-21134910	61.3	0.463	Chr14: 17343313-17389352	0.499064	46.0
		46.3	Chr14: 21073574-21134910	0.380523	61.3
Chr14: 53430371-53444925	14.6	0.463	Chr14: 21091293-21134910	0.432264	43.6
		0.463	Chr14: 53430371-53444925	0.878465	14.6
		4.63	Chr14: 53430371-53444925	0.97528	14.6
		46.3	Chr14: 53430371-53444925	0.744373	14.6
Chr16: 28132136-28249075	116.9	0.463	Chr14: 53430371-53444925	0.927222	14.6
		0.463	Chr16: 28132136-28232026	0.282434	99.9
		46.3	Chr16: 28132136-28249075	0.28236	116.9
Chr16: 28132136-28249075	116.9	0.463	Chr16: 28132136-28232026	0.296323	99.9
		46.3			

Genomic Region of Overlap	Size (kb)	Concentration (μM)	CNA Genomic Region	Amplification	Length (kb)
chr17: 23159085-23212834	53.7	0.463	Chr17: 23159085-23212834	0.459385	53.7
		46.3	Chr17: 23161940-23238852	0.281195	76.9
chr18: 15016943-15040400	23.5	0.463	Chr18: 15016943-15040400	0.468779	23.5
			Chr18: 15016943-15040400	0.494164	23.5
			Chr18: 15016943-15040400	0.429273	23.5
Chr22: 26943663-27091114	147.5	0.463	Chr22: 26943663-27050477	0.386284	106.8
			Chr22: 26943663-27054893	0.352951	111.2
			Chr22: 26943663-27054893	0.380817	111.2
			Chr22: 26943663-27050477	0.34241	106.8
			Chr22: 26943663-27050477	0.268398	106.8
			Chr22: 26943663-27091114	0.326314	147.5
Chr25: 25942877-26067318	124.4	0.463	Chr22: 26943663-27050477	0.394128	106.8
			Chr25: 25942877-26046450	0.336315	103.6
			Chr25: 25942877-26067318	0.301212	124.4
			Chr25: 25942877-26067318	0.303896	124.4
		46.3	Chr25: 25942877-26067318	0.351241	124.4