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Embryonic atrazine exposure and later in life behavioral and brain transcriptomic, epigenetic, and pathological alterations in adult male zebrafish

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Abstract

Atrazine (ATZ), a commonly used pesticide linked to endocrine disruption, cancer, and altered neurochemistry, frequently contaminates water sources at levels above the US Environmental Protection Agency's 3 parts per billion (ppb; μg/L) maximum contaminant level. Adult male zebrafish behavior, brain transcriptome, brain methylation status, and neuropathology were examined to test the hypothesis that embryonic ATZ exposure causes delayed neurotoxicity, according to the developmental origins of health and disease paradigm. Zebrafish (*Danio rerio*) embryos were exposed to 0, 0.3, 3, or 30 ppb ATZ during embryogenesis (1–72 hours post fertilization (hpf)), then rinsed, and raised to maturity. At 9 months post fertilization (mpf) males had decreased locomotor parameters during a battery of behavioral tests. Transcriptomic analysis identified altered gene expression in organismal development, cancer, and nervous and

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Authors' contributions

KH acquired and analyzed behavioral and transcriptomic data, performed histological examination of brains and morphometric analysis of scanned images, collected body size and brain weight data, and was a major contributor in writing the manuscript. LL acquired and analyzed methylation data. BT analyzed behavioral videos. CY designed the methylation experiment and analyzed methylation data. JF supervised experimental design and data analysis and assisted in manuscript preparation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All fish were treated humanely with regard to prevention and alleviation of suffering, in compliance with United States law. All protocols were approved by the Purdue University Animal Care and Use Committee (protocol #1110000088).

Consent for publication

Not applicable.

Availability of data and materials

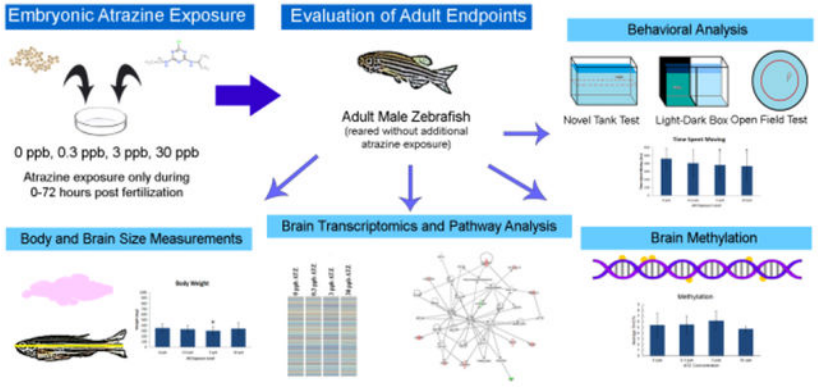
The transcriptomic datasets generated during the current study are available in the NCBI Gene Expression Onmibus (GEO) repository, [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112504]. Other data from the manuscript is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

reproductive system development and function pathways and networks. The brain was evaluated histopathologically for morphometric differences and decreased numbers of cells were identified in raphe populations. Global methylation levels were evaluated at 12 mpf, and the body length, body weight, and brain weight were measured at 14 mpf to evaluate effects of ATZ on mature brain size. No significant difference in genome methylation or brain size was observed. The results demonstrate that developmental exposure to ATZ does affect neurodevelopment and neural function in adult male zebrafish, and raises concern for possible health effects in humans due to ATZ's environmental presence and persistence.

Graphical Abstract



Keywords

zebrafish; atrazine; developmental origins of health and disease; neurotoxicity; transcriptomics

Introduction

The Developmental Origins of Health and Disease (DOHaD) paradigm advances that developmental exposure to environmental stressors can cause genetic, epigenetic, or functional changes in tissues that are associated with later life disease [1]. Although, this hypothesis was first proposed by Barker and Osmond in 1986 after observing increased risk of heart disease in adults who had poor nutrition during development [2, 3], the concept has been expanded to include the later life effects of developmental exposure to environmental toxicants [1, 4]. Immune system dysfunction, obesity and metabolic syndrome, altered neurodevelopment and neurological deficits, and cancer have been linked to developmental exposure to environmental toxicants [5]. One class of chemicals frequently implicated in causing later life health effects within the DOHaD framework are endocrine disrupting chemicals (EDCs).

An EDC is an exogenous chemical that can disrupt the normal action of hormones. Alterations in normal hormone action can cause irreversible changes in tissue and organ structure or function that can cause adverse health outcomes throughout the entire life course of an organism [6–8]. The developmental period is thought to be the most sensitive to the effects of EDCs [9], and while no altered phenotype might be detectible early on, the

functional implications may be observed later in life [10]. Exposure to low-doses of EDCs can have significant health effects; however, and because EDCs often cause non-monotonic dose response curves [11], the effects of EDCs cannot be predicted by high dose tests [10]. EDCs include industrial compounds, pharmaceuticals, and pesticides [7, 12].

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine; ATZ), is common herbicide and suspected EDC. ATZ is one of the two most commonly used agricultural herbicides in the United States based on weight of active chemical applied [13]. ATZ is water soluble and can leach from fields into surface and ground water sources [14] where it can persist in the environment [14–17]. ATZ is the most common pesticide detected in agricultural stream water, agricultural groundwater, and urban groundwater sources [18] with concentrations up to 224 parts per billion (ppb; μg/L) recorded in Midwestern streams [19]. ATZ is regulated in drinking water by the US Environmental Protection Agency (EPA) with a maximum contaminant level (MCL) of 3 ppb [13]. However, the European Union banned ATZ in 2003 because of environmental persistence and groundwater contamination concerns [20]. Drinking water contamination is considered the most hazardous exposure to the public, as ATZ levels in food are well below the EPA's level of concern [13].

As an EDC, ATZ is reported to result in abnormal metamorphosis and feminization in amphibians [21–23], disruption of the hypothalamic–pituitary–gonadal axis in rodents [24–29], altered neurotransmission in rodents and zebrafish [30–35], and reproductive abnormalities including decreased semen quality in men [36] and increased risk of breast cancer in women [37]. The mechanism of ATZ-related endocrine disruption is still under investigation, but appears to be related to disrupted intracellular signaling through the inhibition of type 4 cyclic nucleotide phosphodiesterases (PDE4), increased cyclic adenosine monophosphate (cAMP) levels, and decreased expression of steroidogenic proteins [38–41]. ATZ exposure is also suspected to cause epigenetic changes in methylation and microRNA activity [42–44], which might provide a mechanism for a DOHaD as the delayed health effects of many EDCs are caused by chemical induced epigenetic alterations [10, 45].

The zebrafish (*Danio rerio*) model has easy husbandry and fecund reproduction, a sequenced genome, and conserved metabolic pathways, which are utilized in toxicological research [46–50]. Previous studies from our laboratory using the zebrafish model suggest embryonic exposure to ATZ may alter brain development and function. Expression of genes related to neuroendocrine system development were altered in 72 hours post fertilization (hpf) larval zebrafish with exposure to low levels (0.3, 3, or 30 ppb) of ATZ [51] and adult, 6 months post fertilization (mpf) zebrafish with embryonic ATZ exposure had sex-specific gene expression alterations in pathways related to nervous system development and function, while 9 mpf female zebrafish had decreased 5-Hydroxyindoleacetic acid (5-HIAA; a serotonin metabolite) and decreased serotonin turnover [35, 52].

In this study, we further investigated the developmental origins of ATZ-related neurotoxicity by testing the hypothesis that embryonic ATZ exposure results in later-life changes in behavior, the brain transcriptome, brain methylation, brain histopathology, and body and brain size. We evaluated the performance of 9 mpf male zebrafish in a battery of three neurobehavioral tests of anxiety, the novel tank test (NTT), the light dark box (LDB), and an

open field test (OFT) to determine if embryonic ATZ exposure causes functional changes in neurobehavior. We also performed transcriptomic analysis of male zebrafish brain at 9 mpf to evaluate the persistence of gene expression changes through the zebrafish life course and to provide a transcriptomic basis of any behavioral phenotype observed. To determine if embryonic exposure to ATZ causes physical disruptions of the nervous system, we evaluated male zebrafish brain sections for histopathological changes and performed morphometric analysis of cellular density in three regions of the zebrafish brain that have been associated with neurological conditions [53]: the dorsal telencephalon (similar to the mammalian hippocampus and amygdala), the posterior tuberculum (similar to basal nuclei), and the raphe populations. We evaluated the global methylation status of male zebrafish brain to determine if previously observed decreased global methylation levels [43] were persistent throughout the life course. Finally, we evaluated body size and brain size to determine if embryonic ATZ exposure affected overall growth and development over the zebrafish life course.

Results

Behavior

The 9 mpf fish behavior was assessed to evaluate for neurological function. For the NTT, male zebrafish with embryonic ATZ exposure had a general trend for decreased movement related parameters with increasing embryonic ATZ exposure, although the difference between treatments was not significant for distance moved ($p = 0.1121$) or velocity ($p = 0.1109$) (Figure 1 A–B). The trending decrease in the time male zebrafish spent moving reached significance in the 3 and 30 ppb exposures ($p = 0.0279$; Figure 1C). Although there were no differences between exposures in the frequency of upper zone entry ($p = 0.2622$), time spent in the upper zone ($p = 0.1700$), frequency of bottom zone entry ($p = 0.2600$), or time spent in bottom zone ($p = 0.1652$; Figure S1), all male zebrafish with embryonic ATZ exposure (0.3, 3, and 30 ppb) had a significantly increased latency to the first upper zone entry compared to controls ($p = 0.0383$; Figure 1D).

For the LDB test, the males with embryonic exposure to 30 ppb ATZ had significantly decreased distance moved ($p = 0.0086$), and decreased velocity ($p = 0.0085$) compared to controls, but both the 0.3 ppb and the 30 ppb exposures had a significant decrease in time spent moving compared to controls ($p = 0.0257$) and a significant increased latency to the first light zone entry ($p = 0.0179$; Figure 2). There was no difference between treatments in the frequency of male light zone entry ($p = 0.0701$), time males spent in the light zone ($p=0.4507$), frequency of dark zone entry ($p = 0.0653$), or time spent in the dark zone ($p = 0.4468$; Figure S2).

For the OFT, male zebrafish with embryonic ATZ exposure had a trend towards decreasing activity with increasing embryonic ATZ exposure, however, it did not reach significance for distance moved ($p = 0.1929$), velocity ($p = 0.1948$), or time spent moving ($p = 0.0798$) (Figure S3). Additionally, there was no significant differences in latency to the first inner zone entry ($p = 0.4264$; Figure S3), frequency of inner zone entry ($p=0.0567$), time spent in the inner zone ($p = 0.1547$), frequency of outer zone entry ($p = 0.0545$) or time spent in the outer zone ($p = 0.1498$; Figure S4) between treatments.

Brain Transcriptome

Transcriptomic analysis of brain from 9 mpf male zebrafish with embryonic ATZ exposure identified 123 mapped genes with altered expression in the 0.3 ppb exposure, 95 genes with altered expression in the 3 ppb exposure, and 121 genes with altered expression in the 30 ppb exposure group (Figure S5). In the male brain, 25 genes were altered in all three treatment groups (GSE112504; Figure 3; Table 1).

Gene ontology analyses via Ingenuity Pathway Analysis (IPA) indicated the most enriched pathways associated with disease and biological functions for each treatment. IPA identifies human orthologs of zebrafish genes and the results use human gene notation. Male zebrafish with 0.3 ppb embryonic ATZ exposure had enrichment in pathways associated with cancer, endocrine system disease, cell morphology, and reproductive system development and function (Table S2). Within the endocrine pathway, 23 genes were associated with gonadal tumors and 45 genes were associated with tumorigenesis of the reproductive tract within that pathway. Males with 3 ppb embryonic ATZ exposure had enrichment in pathways associated with organismal injury and abnormalities, reproductive and endocrine system disease, cellular development, and nervous system development and function (Table S3). Within the nervous system pathway, 17 genes were associated with central nervous system solid tumor. Males with embryonic exposure to 30 ppb ATZ had enrichment in pathways associated with cancer, amino acid metabolism, and tissue development (Table S4), with 113 genes having an association with solid tumors. Gene ontology analyses of the 25 genes common to all three exposures identified the most enriched pathways associated with disease and disorders, molecular and cellular functions, and physiological system development and function (Table 2). Within these categories, there was enrichment of developmental disorder, cellular function and maintenance, organismal function, and reproductive system development and function pathways. Of the common genes in males, all 25 genes are associated with solid tumors and 5 of the 25 are also associated with abnormal morphology of the reproductive system. The top network associated with the common gene pathways is a developmental disorder, neurological disease, and organismal injury and abnormalities network (Figure 4).

Our laboratory had previously evaluated the alterations in the brain transcriptome of 6 mpf female and male zebrafish with embryonic ATZ exposure [35, 52]. The results of the gene expression changes in the 9 mpf adult fish from this study were compared to the results of the previous male study performed at 6 mpf to evaluate the persistence of gene expression changes over time. We found 27 genes that were altered in the 0.3 ppb exposure at 6 mpf and 9 mpf, 23 genes that were altered at both 6 mpf and 9 mpf after embryonic exposure to 3 ppb ATZ, and 29 genes that were altered in the 30 ppb exposure groups at 6 and 9 mpf (Table S5). A total of 7 genes (*CAVIN4, CDK5, HTRA2, IGFBP7, ITM2C, SULT2B1, TNNI2*; Table 3) had altered expression in all three embryonic exposure groups at both 6 mpf and 9 mpf.

qPCR Confirmation

The results of the microarray were independently confirmed by determining the relative expression of a subset of genes altered in the three ATZ treatments. The relative expression of six genes (*aqp1a, cdk5, cyp26b1, ifgbp7, itm2cb*, and *sult2b1*) was evaluated in all ATZ

exposures and correlated to the $\log_2$ fold change determined on microarray analysis. There was a significant strong positive correlation for all three male exposure groups (0.3 ppb: r = 0.98254, p = 0.0005; 3 ppb r = 0.98623, p = 0.0003; 30 ppb: r = 0.97087, p = 0.0013; Figure S6).

Histopathology

Histopathology was performed on 9 mpf zebrafish brain to evaluate for physical brain changes resulting from embryonic ATZ exposure. No obvious histopathological changes such as neoplasia, necrosis, or inflammation were observed in any section of any treatment (Figure S7 is a representative image). Morphometric evaluation found no significant difference between male zebrafish with embryonic ATZ exposure and control zebrafish in the number of cells per μm^2 in the dorsal telencephalon (p=0.0793) or the posterior tuberculum (p=0.1525), but there was a significant decrease in the cells per μm^2 in the raphe of males exposed to 30 ppb ATZ (p = 0.0215; Figure 5).

Methylation

Global methylation status was evaluated in 12 mpf zebrafish. The percent 5mC was not significantly different among the treatments (p = 0.5840; Figure S8).

Body and Brain Size

The body length, body weight, and brain weight to body weight ratio was assessed for each exposure at 14 mpf. Male zebrafish with embryonic ATZ exposure did not have any differences in body length between treatments (p=0.8887; Figure 6A), but the 3 ppb exposure group did have a significantly decreased body weight (p=0.0456) compared to controls (Figure 6B). However, there was no significant difference between treatments in brain weight (p=0.3562) or brain weight to body weight ratio (p=0.1028) (Figure 6C–D).

Discussion

The studies presented here investigated the developmental origins of ATZ-related adult neurotoxicity in male zebrafish. Exposure to ATZ has been implicated in altered neurodevelopment and function by our laboratory [35, 51, 52] and others [30, 33, 54–56]. We assessed the behavioral, transcriptomic, and pathologic changes in adult male zebrafish brain after embryonic exposure to low, environmentally relevant concentrations of ATZ with the aim to link embryonic ATZ exposure to both physical and functional outcomes later in life. We hypothesized changes in anxiety-related behaviors and serotonergic pathways would be observed based on our previous study of transcriptomic changes in 6 mpf, male zebrafish [52].

Zebrafish have well-characterized behavioral outcomes [57] and the NTT, LDB, and OFT are common models used to assess zebrafish behavior and anxiety [58–60]. After subjecting 9 mpf, male zebrafish with embryonic ATZ exposure to the NTT, LDB, and OFT, we determined that developmental ATZ exposure affects neurobehavioral endpoints. Male zebrafish with developmental ATZ exposure had hypomotile phenotypes, with decreased distance moved, velocity and time spent moving. Although the latency to zonal entry was

increased in all male exposures in the NTT and the 0.3 and 30 ppb groups on the LDB, this was attributed to the overall decrease in movement.

It should be noted that although there can be significant variation in zebrafish behavior from clutch to clutch and between testing days [59], our results represent the aggregate results of four separate clutches over multiple testing days. Even though time of day, and testing order account for less variability [59], our treatments were balanced in testing order and the behavioral tests were performed in the same order at roughly the same time each day. The non-monotonic nature of some of the results is consistent with the effects of low dose exposure to other EDCs [11].

Rodent studies investigating ATZ also support a developmental basis of altered behavior later in life. Belloni et al found that CD-1 mice with gestational exposure to 1 μg/kg/day ATZ had altered behavior profiles, with juvenile males having feminization of behavior and altered learning performance in adulthood [54]. Lin et al found changes in activity, time swimming on a forced swim test in males, and marble burying in juvenile C57BL/6 mice that had developmental exposure to 3000 ppb ATZ in maternal drinking water from gestational day 6 to postnatal day 23 [61]. In zebrafish, ATZ exposure has been reported to affect behavior in adults after chronic or subchronic exposure. Adult zebrafish exposed to ATZ at concentrations ranging from 5 to 3125 ppb for four weeks had altered light dark preference at all treatment concentrations [62] and adult zebrafish with 14-day-exposure to 1000 ppb ATZ had decreased shoaling behavior [63]. The effects of developmental ATZ exposure have also been studied in larval zebrafish. Liu et al found that 120 hpf larvae with exposure to 100 and 300 ppb ATZ up to 120 hpf had decreased free swimming speeds and decreased locomotor activity after exposure to light and dark stimuli [64]. In our laboratory, we found that larvae exposed to 30 ppb ATZ during embryogenesis had decreased distance moved, velocity, and time spent moving in a larval visual motor response test performed at 120 hpf [65]. The current study is the first to use zebrafish to test the developmental origins of adult behavioral changes after embryonic ATZ exposure. The altered behavioral outcomes in the adult male zebrafish with embryonic 0.3 and 3 ppb ATZ exposure suggests that exposure to ATZ during development alters behavior at lower doses than what is observed in either larval developmental or adult exposure studies. Larval behavioral tests are often used in high-throughput toxicity screening of chemicals [66, 67], but our results suggest that larval screening tests might not fully characterize the late life effects of embryonic toxicant exposure.

We evaluated the brain transcriptome of 9 mpf zebrafish with embryonic ATZ exposure to identify possible genes or pathways that could explain the observed changes in neurobehavior and to determine if embryonic exposure to ATZ causes lasting alterations in gene expression later in life. Please note IPA identifies the human ortholog of zebrafish genes altered on a zebrafish specific microarray; therefore, this discussion uses human gene notation for consistency with the pathway analysis. In 9 mpf males, the genes common to all embryonic exposures were associated with developmental disorders, cellular functions, and reproductive system development and function; however, the genes are part of a larger network that influence neurological disease. Looking closer at the genes commonly altered in male zebrafish, 5 of the genes (*AQP1*, *CDK5*, *CYP26B1*, *HTRA2*, and *TNNI2*) are

associated with disease of the basal nuclei and movement disorders, which could suggest disruption of the dopaminergic system in male zebrafish with embryonic ATZ exposure. For instance, *CDK5*, a member of the cyclin-dependent kinases family, has roles in neuronal survival, synaptic plasticity, learning and memory, and behavior. As reviewed by Shah and Lahiri, dysreguation of *CDK5* is associated with Alzheimer's disease, Parkinson's disease, and Huntington's disease [68]. Also of note, cell-to-cell signaling and interaction pathways were altered in the 3ppb and 30 ppb exposures. Disrupted cell-to-cell signaling has also been reported as a mechanism of endocrine disruption, later-life toxicity, and epigenetic toxicity [69–71].

We evaluated the persistence of brain gene expression alterations caused by embryonic ATZ exposure throughout adult life stages by comparing genes altered in 6 mpf male zebrafish with embryonic ATZ exposure [52] to the results of our analysis. We identified 7 genes (*CAVIN4*, *CDK5*, *HTRA2*, *IGFBP7*, *ITM2C*, *SULT2B1*, and *TNNI2*) that were altered in all three male exposures at 6 and 9 mpf. Interestingly, *CDK5, HTRA2, ITM2C*, and *SULT2B1* are all related to the nervous system, neurodevelopment, or neurotransmission while *IFGBP7, SULT2B1*, and *TNNI2* have functions related to hormone biosynthesis, modification, receptor action, or cellular response. Among the individual exposures, the 3 ppb and 30 ppb embryonic exposures had a greater number of genes with persistent changes than the 0.3 ppb exposure. Additionally, the number of altered genes with functions related to the nervous system also increased with increasing embryonic ATZ exposures. The 0.3 ppb embryonic exposure did not have any additional changes in the expression of genes related to neurodevelopment or function, but the 3 ppb exposure had altered expression of 3 additional genes, *DLX2*, *ERBB4*, and *GSX2*, with functions related to brain development or synaptic function. Exposure to 30 ppb ATZ during embryogenesis altered the expression of 6 unique genes: *AK7*, *EN1, PRDX3, PRKCD, PRKCE*, and *SSH1*. Although there was no additional overlap in genes between the 3 and 30 ppb exposures, the increasing number of genes with persistent altered expression could suggest increased nervous system dysregulation, which would tie into the greater behavioral effects observed in the 30 ppb embryonic exposure group.

Previous transcriptomic analysis of 72 hpf larval zebrafish with embryonic ATZ exposure identified changes in the expression of genes associated with neuroendocrine system and function, reproductive system function, cell cycle, and carcinogenesis [51]. In addition to the genes related to nervous system development and function, the number of genes linked to different types of cancer in each exposure in our study is noteworthy, and holds with the enrichment of carcinogenesis pathways in the 72 hpf transcriptomic evaluation [51]. Moreover, recent proteomic analysis of 120 hpf larval zebrafish with embryonic (1–72 hpf) ATZ exposure identified 15 proteins with altered levels that were associated with urogenital and genital tract cancers [65]. Previous brain microarray analysis of 6 mpf male zebrafish with embryonic ATZ exposure also found alterations in pathways that relate to reproductive function and the nervous system [52]. Expression of genes with functions related to neurite growth, synapse formation and plasticity, and genes in the serotonin pathway were changed in 6 mpf adult male zebrafish with embryonic ATZ exposure [52]. Other studies have also linked ATZ to changes in neurotransmission. Male C57BL/6 mice with short-term (10 day) oral exposure to 5–250 mg/kg/day ATZ had changes in the plasma metabolome in pathways

that suggest disruption of α-linolenate, tryptophan, and tyrosine metabolism [72]. Tyrosine is the precursor of dopamine and tryptophan the precursor of serotonin; disruption of these pathways could provide one basis for altered neurotransmission after ATZ exposure. Multiple studies have identified changes in dopamine levels or pathway functions in rodents with either acute or developmental ATZ exposure [30–34, 55, 56, 73–75].

The decrease in locomotion observed in male zebrafish with embryonic ATZ exposure might point to disruptions in dopaminergic neurotransmission, which is supported by other studies [31–33, 55, 56, 75]. In this study, we identified alterations in expression of genes related to movement disorders and basal nuclei disease; however, we did not find gene expression changes in direct dopaminergic pathways, and previously neurotransmitter analysis did not identify changes in dopamine or dopamine metabolites in zebrafish brain [35]. It is also possible that the decreased activity of male zebrafish is related to a change in overall brain arousal state, rather than in movement related pathways, suggesting alterations in serotonergic pathways as supported by the work of our laboratory [35, 52] and others [33, 72, 76]. Although we did not have changes in the expression of genes directly related to serotonin production or metabolism in this study, it is likely that both the serotonergic and dopaminergic systems have altered function.

We performed morphometric analysis on three regions from 9 mpf male zebrafish brain to semi-quantitatively evaluate for subtle differences in tissue morphology, namely the cellular density via the number of cells per μm^2 area [77]. The utility of morphometric histopathologic techniques in the evaluation of EDCs has previously been recognized [78] and the dorsal telencephalon, posterior tuberculum, and raphe populations were identified as regions of interest based on suspected functional homology of these areas and their potential link to neurological diseases and disorders in humans [53]. The dorsal telencephalon consists of the medial dorsal telencephalon (sometimes called medial dorsal pallium) and the lateral dorsal telencephalon (sometimes called lateral dorsal pallium). The medial dorsal telencephalon is suggested to have a function homologous to the amygdala while the lateral dorsal telencephalon is suggested to be similar to the hippocampus [79–81]. The posterior tuberculum in teleost fish is considered to have features suggestive of an ancient basal ganglion, mainly the presence of dopaminergic cells that project to the striatum [82]. Finally, the raphe populations are classically considered populations of serotonergic neurons with projections that extend to the telencephalon, cerebellum, and spinal cord [83, 84]. Due to planar limitations associated with paraffin embedding and sectioning, the two regions of the dorsal telencephalon and the superior (dorsal) and inferior (caudal) raphe populations were respectively considered together.

The sections from males with 30 ppb embryonic ATZ exposure had significantly fewer cells per μm^2 of raphe area, suggesting altered microanatomy in an area classically associated with serotonin. However, dopaminergic neurons can also be identified in raphe populations and these neurons have been linked to phenotypes of social isolation in mice [85]. If the decreased cellularity represents a reduction in serotonergic neuron numbers, then behavioral alterations observed in males with embryonic ATZ exposure might be related to an altered state of arousal. However, in the determination of cells per μm^2, the number of nuclei, including those from all neuronal populations, glial cells, and microglia, were counted in

each brain region. Consequently, we could not determine if the decrease in cellular density is attributed to decreased numbers of neurons, glia, or microglia in the H&E sections evaluated. A decrease in number of any of those subpopulations associated with embryonic toxicity could disrupt normal brain function later in life and further investigation is required.

Evaluation of the body and brain size of male 14 mpf zebrafish with embryonic ATZ exposure only indicated a slight decrease in body weight for the male zebrafish with embryonic exposure to 3 ppb ATZ. The significance of this result is unclear, as there is no significant difference in either body length or brain weight between embryonic exposures. The lack of gross differences in brain weight or the brain weight to body weight ratio suggests that the decreased cellular density in the raphe areas do not significantly contribute to a difference in brain size, although it might contribute to altered brain function.

Finally, although ATZ has previously been found to affect the epigenome of 72 hpf zebrafish larvae by decreasing global methylation levels [43], in this study, the percent 5mC in genomic DNA isolated from 12 mpf male zebrafish brains was not changed. It is possible that the epigenetic alterations observed at 72 hpf are corrected by maturity and are not transmitted to offspring. Alternatively, although the male zebrafish brain did not have significant differences in global methylation levels after embryonic ATZ exposure, methylation changes might be occurring in specific organs not evaluated in this study or at specific sites within the genome that would be missed in this analysis since results represent the sum change in methylation events (i.e., hyper- and hypomethylation are equalized). It is also conceivable that, since ATZ is an EDC with sex-specific effects, persistent epigenetic alterations might be present in and limited to females. As zebrafish do not sexually differentiate until 5–7 weeks of age, the decreased methylation observed at 72 hpf [43] might reflect alterations in the epigenome of zebrafish that will go on to develop into females. ATZ is implicated in causing multigenerational toxicity, as zebrafish exposed to 0.3, 3, and 3 ppb ATZ during embryogenesis had offspring with altered head morphology when mated to similarly exposed partners [86]. Transgenerational toxicity has been observed in F3 generation Hsd:Sprague Dawley rats after the F0 generation was exposure to ATZ [44]. In a study by McBirney et al, F3 rats from an ATZ lineage had increased frequency of reproductive abnormalities, early onset of puberty in females, and had a lean, hyperactive phenotype [44].

Conclusions

In this study, we investigated the developmental origins of ATZ-related neurotoxicity in male zebrafish. In summary, we identified an embryonic ATZ exposure-related decrease in locomotion on two behavioral tests of stress and anxiety. On brain transcriptomic evaluation, male zebrafish with embryonic ATZ exposure had expression changes in genes related to cell function, cancer, and the reproductive and nervous systems. Interestingly, genes with persistently altered expression have functions related to the nervous system and hormone action. Furthermore, we observed alterations in cellular density within the raphe brain populations. Finally, we found no significant difference in overall brain size or in the brain percent 5mC in male zebrafish with embryonic ATZ exposure. Although we have behavioral, transcriptomic, and pathological support for a developmental origin of ATZ-

related neurotoxicity, further characterization is needed to better understand the physiological implication of the morphometric changes and to identify how the altered genes interact to alter neurobehavior. The assessment of later life nervous system outcomes after developmental ATZ exposure is important because of the human health implications in heavy ATZ use areas.

Methods

Zebrafish husbandry and treatment

A stock solution of technical grade (98.1% purity) ATZ (CAS 1912-24-9) was prepared at 10 parts per million (ppm; mg/L) [35, 51]. Aliquots of the stock solution were diluted with filtered aquaria water to achieve 0.3, 3, and 30 ppb exposure solutions. The 0 ppb negative control was filtered aquaria water. AB wild-type adult zebrafish (*Danio rerio*) were used to obtain embryos [87, 88]. Adult fish were kept on a 14:10 light-dark cycle and system water was at 26–28°C, with pH 7.0–7.3, and 470–550 μS conductivity. Fish and aquaria were monitored twice daily and fed a mixture of brine shrimp (*Artemia franciscana*), Golden Pearls 500–800 μm (Artemia International), and Zeigler adult zebrafish food. Embryos (4–8 cell stage) were collected immediately following the breeding interval, rinsed, randomly sorted into treatment groups, exposed to 0, 0.3, 3, or 30 ppb ATZ, and incubated at 28.5°C. ATZ exposure lasted from immediately after collection to 72 hpf (the end of embryogenesis). At 72 hpf, the larvae were rinsed in filtered aquaria water to remove ATZ exposure and then reared under normal conditions until 9, 12, or 14 months post fertilization (mpf). During rearing, larval zebrafish were fed Zeigler Larval AP100 powdered diet, paramecia, and brine shrimp and had a stocking density of 50 larvae/L; juvenile fish were fed brine shrimp and Golden Pearls 300–500 μm (Artemia International) and had a stocking density of 5–10 fish/L; and adult zebrafish were kept at a 5–10 fish/L density and fed similarly to the breeding colony. The sex ratio in raised zebrafish was approximately 1:1, male zebrafish were chosen for this series of experiments due to the previous identification of sex specific effects of ATZ in 6 mpf male zebrafish [52]. All protocols were approved by the Purdue University Animal Care and Use Committee and all fish treated humanely with regard to prevention and alleviation of suffering.

Zebrafish Behavior

At 9 mpf, 10 male zebrafish (considered subsamples) were randomly chosen from each of 4 biological replicates (N=4, with a total of 40 zebrafish per treatment analyzed; biological replicate defined as zebrafish grown from separate clutches) to undergo a battery of three behavioral tests: the Novel Tank Test (NTT), the Light-Dark Box (LDB), and the Open Field Test (OFT). Before testing, the zebrafish were placed in approximately 8 × 9 × 6 cm holding tanks, which were kept in a water bath heated to 28°C and allowed to acclimate 10 minutes before starting the first test. All fish saw the tests in the same order, but treatments were varied in their testing order to reduce bias. After each test, fish were placed back in the holding tank and allowed to recover for 80–160 minutes before starting the next test. 1–2 subsamples per treatment were tested per day. All behavior was tracked at a rate of 5 frames per second with an Ikegami ICD-49 Super-Cube DSP Monochrome Camera connected to a PC equipped with Noldus EthoVision XT 12. Fish were identified based on dynamic

subtraction of background images with 1 pixel contour erosion followed by 1 pixel contour dilation and no track smoothing. Two observers (KH and BT) manually confirmed correct video tracking and recognition of zebrafish subjects.

For the NTT, a clear 1.5 L polycarbonate, 25 × 6 × 16 cm, tank (Marine Biotech/Aquatic Habitats, Apopka, FL) was filled to a depth of 12 cm. The tank was divided into a bottom zone and an upper zone at the midpoint of the water level. The camera was set up horizontally to the testing arena. Zebrafish were transferred by net to the center of the testing area and gently dropped into the arena. Video recording started 1 second after the fish were detected, but movement traces and evaluations of behavior did not begin until zebrafish dropped into the bottom zone. The movement of the zebrafish was recorded for a period of 10 minutes. Parameters evaluated included distance moved, velocity, time moving, time spent in bottom and upper zones, frequency of zone entries, and latency to upper zone entry.

A similar 25 × 6 × 16 cm tank filled do a depth of 6 cm was used for the LDB. Half of the tank was externally covered with black construction paper on three walls and the other half of the tank was externally covered with white construction paper, creating two approximately 12.5 × 6 × 6 cm zones (dark zone and light zone). The bottom of the tank was covered with white construction paper to aid in detection of the zebrafish subject. The camera was located above the testing arena and orientated downwards. Zebrafish were transferred by net to the center of the testing area and gently dropped into the arena. Video recording started 1 second after the fish were detected and the movement of the zebrafish was recorded for a period of 10 minutes. Parameters evaluated included distance moved, velocity, time moving, time spent in zones, frequency of zone entries, and latency to light zone entry.

The OFT used a 30 cm diameter, white ceramic, circular arena with a wall height of 6 cm filled to a depth of 2 cm. An inner zone with a diameter of 18 cm created a 6 cm wide peripheral outer zone. The camera was located above the testing arena and orientated downwards. Zebrafish were transferred by net to the center of the testing area and gently dropped into the arena. Video recording started 1 second after the fish were detected and the movement of the zebrafish was recorded for a period of 10 minutes. Parameters evaluated included distance moved, velocity, time moving, time spent in inner and outer zones, frequency of zone entries, and latency to inner zone entry.

Microarray Evaluation of Adult Zebrafish Brain Transcriptome

At 9 mpf, 1 fish randomly identified from each treatment group (0, 0.3, 3, or 30 ppb ATZ) in each of four biological replicate (N = 4, with a total of 4 zebrafish per treatment analyzed) and was euthanized in 0.4 mg/ml buffered tricaine-S. The brains were dissected, homogenized in Trizol (Life Technologies), and then flash frozen in liquid nitrogen and stored at −80°C until further analysis. Total RNA was extracted using the RNeasy MinElute Cleanup Kit (Qiagen). For transcriptomic microarray analysis, a custom one-color multiplex zebrafish 4×180K expression platform (Agilent Technologies) was used to compare differences in transcript expression between treatments as previously described [86, 89]. The platform has 4 arrays each with 180K probes interrogating 36K known and predicted targets (3–5 probes per target) based on the Ensembl and UCSC Genome Databases. Samples were

hybridized to the array, arrays washed in buffer solutions, and arrays scanned (Agilent Technologies SureScan Microarray Scanner). Data was extracted using Agilent Feature Extraction Software 12.0. Microarray analysis was performed according to MIAME guidelines [90]. GeneSpring 14.9 (Agilent Technologies) used for statistical analysis, with normalization was via percentile shift with a percentile target of 75. Gene lists were uploaded into IPA for gene ontology and molecular pathway analysis. IPA identifies human orthologs of zebrafish genes, which are subsequently used for pathway analysis.

Quantitative Polymerase Chain Reaction (qPCR) Confirmation of Microarray

qPCR was performed was performed on a subset of genes that were significantly altered in the male microarray (*aqp1a, cdk5, cyp26b1, ifgbp7, itm2cb*, and *sult2b1*) to confirm and technically validate the microarray results. The genes were chosen as representative genes, as all six were differentially expressed in all exposures, and three genes had negative fold-changes (*aqp1a, cdk5, cyp26b1*) while the other three (*ifgbp7, itm2cb*, and *sult2b1*) had positive fold-changes. cDNA was synthesized from the same RNA samples used for the microarray (N = 4, with a total of 4 zebrafish per treatment analyzed) using the SuperScript First-strand synthesis system (Invitrogen) as previously described [91]. Primers specific to the target genes (Table S1) were designed using Primer3 and checked using NCBI Primer-BLAST [92]. qPCR was performed on a Bio-Rad CFX Connect Real-Time PCR System with the Bio-Rad SsoAdvanced SYBR Green Supermix. qPCR was completed following MIQE guidelines [93] and as previously described by our laboratory [51, 87, 94, 95]. As in previous studies [35, 51], *β-actin* was chosen as a reference gene due to consistent expression that did not vary across atrazine exposures (Figure S9). Experimental samples were run in triplicate to provide technical replicates and gene expression was normalized to *β-actin* (gene of interest/β-actin). Melting and standard dilution curves and no template controls were evaluated to ensure appropriate efficiency (100±10%) and specificity.

Zebrafish Brain Collection and Histopathology

At 9 mpf, 6 fish were randomly identified from each treatment group (0, 0.3, 3, or 30 ppb ATZ) in each of three biological replicates (N = 3, with a total of 18 zebrafish per treatment analyzed) and euthanized via anesthetic overdose with buffered tricaine. An incision was made in the ventral abdomen and the zebrafish were fixed *in toto* with 4% paraformaldehyde at 4°C for 7 days at a 1:10 volume ratio, rinsed with phosphate buffered saline, and decalcified at room temperature in 20% EDTA (ethylenedinitrilotetraacetic acid, disodium salt dihydrate; pH 8.0) for 10 days with rocking agitation. Once fixed and decalcified, the heads were removed, routinely processed, embedded in paraffin (3 heads per treatment per block), sectioned twice (bilateral midsagittal sections, approximately 750 μm apart), and stained with hematoxylin and eosin (H&E). Slides were scanned into Aperio Digital Pathology (Leica Biosystems Inc.). The slides were evaluated by a board certified veterinary pathologist (KH) for pathologic changes. Regions of interest (dorsal telencephalon, posterior tuberculum, and raphe) were identified and cells counted to determine the number of cells per μm^2 area with ImageScope (Leica Biosystems Inc.) software.

Evaluation of Global Brain Methylation

At 12 mpf, 6–7 zebrafish per treatment were euthanized via overdose with buffered tricaine and the brains were removed. Genomic DNA was extracted following a standard protocol [43, 96] slightly modified for smaller brain samples (see supplemental material for detailed methods). Global 5-methylcytosine (5mC) was quantified using the 5-mC DNA ELISA Kit (Zymo Research). Each sample was repeated four times and the average value was used for analysis.

Body and brain size analysis

At 14 mpf, 2–34 zebrafish (considered subsamples) per treatment were taken from each of 3 replicates (N = 3, with a total of 14–56 zebrafish per treatment total). After euthanasia via anesthetic overdose with buffered tricaine, the body length (snout to tip of caudal fin) and body mass was measured. The brain was then dissected and weighed to determine brain mass. Due to the focus on neurotoxicity, no other tissues or organs were examined in this study.

Statistical Analysis

For the behavior, body metrics, histopathology, and methylation experiments, statistical significance was determined by ANOVA on SAS 94 software. A Fisher's Least Significant Difference (LSD) post hoc test was used when the ANOVA was significant ($\alpha = 0.05$) to identify differences between treatment groups. Significance of microarray results was determined using GeneSpring 14.9 (Agilent Technologies) using an ANOVA ($\alpha = 0.05$) with Tukey HSD post-hoc test ($\alpha = 0.05$) and a 1.5 fold change criteria for significance. Microarray confirmation by qPCR was assessed by a Pearson's correlation using $\log_2$ fold change and $\log_2$ relative expression ($\alpha = 0.05$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Heindel JJ, Balbus J, Birnbaum L, Brune-Drisse MN, Grandjean P, Gray K, Landrigan PJ, Sly PD, Suk W, Cory Slechta D, et al.: Developmental Origins of Health and Disease: Integrating Environmental Influences. Endocrinology 2015, 156:3416–3421. [PubMed: 26241070]
2. Barker DJP, Osmond C: Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1986, i:1077–1081.
3. Barker DJP, Godfrey KM, Gluckman PD, Harding JE, Owens JA, Robinson JS: Fetal nutrition and cardiovascular disease in adult life. The Lancet 1993, 341:938–941.

4. Haugen AC, Schug TT, Collman G, Heindel JJ: Evolution of DOHaD: the impact of environmental health sciences. Journal of developmental origins of health and disease 2015, 6:55–64. [PubMed: 25471238]
5. Schug TT, Barouki R, Gluckman PD, Grandjean P, Hanson M, Heindel JJ: PPTOX III: environmental stressors in the developmental origins of disease--evidence and mechanisms. Toxicol Sci 2013, 131:343–350. [PubMed: 22956631]
6. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC: Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. Endocrine Reviews 2009, 30:293–342. [PubMed: 19502515]
7. Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, Toppari J, Zoeller RT: EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocrine Reviews 2015, 36:E1–E150. [PubMed: 26544531]
8. Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS: Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environmental Health Perspectives 2003, 111:994–1006. [PubMed: 12826473]
9. Bern H: The Fragile Fetus. In Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection. Edited by Colborn T, Clement C. Princeton, N.J.: Princeton Scientific Publishing Co., Inc.; 1992: 9–15. [Mehlman M (Series Editor): Advances in Modern Environmental Toxicology].
10. Barouki R, Gluckman PD, Grandjean P, Hanson M, Heindel JJ: Developmental origins of non-communicable disease: implications for research and public health. Environ Health 2012, 11:42. [PubMed: 22715989]
11. Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee DH, Shioda T, Soto AM, vom Saal FS, Welshons WV, et al.: Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses. Endocrine Reviews 2012, 33:378–455. [PubMed: 22419778]
12. Wirbisky SE, Freeman JL: Atrazine Exposure and Reproductive Dysfunction through the Hypothalamus-Pituitary-Gonadal (HPG) Axis. Toxics 2015, 3:414–450. [PubMed: 28713818]
13. U.S. Environmental Protection Agency: Decision documents for atrazine. 2006.
14. Thurman EM, Goolsby DA, Meyer MT, Kolpin DW: Herbicides in Surface Waters of the Midwestern United-States - the Effect of Spring Flush. Environmental Science & Technology 1991, 25:1794–1796.
15. Dao T, Lavy T, Sorensen R: Atrazine degradation and residue distribution in soil. Soil Science Society of America Journal 1979, 43:1129–1134.
16. Schwab AP, Splichal PA, Banks MK: Persistence of Atrazine and Alachlor in Ground Water Aquifers and Soil. Water, Air, & Soil Pollution 2006, 171:203–235.
17. Vonberg D, Hofmann D, Vanderborght J, Lelickens A, Köppchen S, Pütz T, Burauel P, Vereecken H: Atrazine Soil Core Residue Analysis from an Agricultural Field 21 Years after Its Ban. Journal of Environmental Quality 2014, 43:1450–1459. [PubMed: 25603092]
18. Gilliom R, Barbash J, Crawford C, Hamilton P, Martin J, Nakagaki N, Nowell L, Scott J, Stackelberg P, Thelin G, Wolock D: The Quality of Our Nation's Waters—Pesticides in the Nation's Streams and Ground Water, 1992–2001: U.S. Geological Survey Circular 1291,172. 2006.
19. Battaglin WA, Furlong ET, Burkhardt MR, Peter CJ: Occurrence of sulfonylurea, sulfonamide, imidazolinone, and other herbicides in rivers, reservoirs and ground water in the Midwestern United States, 1998. Science of the Total Environment 2000, 248:123–133.
20. European Commission: Review report for the active substance atrazine; European Commission Health and Consumer Protection Directorate-General. SANCO/10496/2003-final; 2003.
21. Brodeur JC, Sassone A, Hermida GN, Codugnello N: Environmentally-relevant concentrations of atrazine induce non-monotonic acceleration of developmental rate and increased size at metamorphosis in Rhinella arenarum tadpoles. Ecotoxicol Environ Saf 2013, 92:10–17. [PubMed: 23499184]
22. Freeman JL, Beccue N, Rayburn AL: Differential metamorphosis alters the endocrine response in anuran larvae exposed to T-3 and atrazine. Aquatic Toxicology 2005, 75:263–276. [PubMed: 16213604]

23. Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A: Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences of the United States of America 2002, 99:5476–5480. [PubMed: 11960004]
24. Cooper RL, Stoker TE, Goldman JM, Parrish MB, Tyrey L: Effect of atrazine on ovarian function in the rat. Reproductive Toxicology 1996, 10:257–264. [PubMed: 8829248]
25. Foradori CD, Hinds LR, Quihuis AM, Lacagnina AF, Breckenridge CB, Handa RJ: The Differential Effect of Atrazine on Luteinizing Hormone Release in Adrenalectomized Adult Female Wistar Rats. Biology of Reproduction 2011, 85:684–689. [PubMed: 21677308]
26. Foradori CD, Zimmerman AD, Hinds LR, Zuloaga KL, Breckenridge CB, Handa RJ: Atrazine Inhibits Pulsatile Gonadotropin-Releasing Hormone (GnRH) Release Without Altering GnRH Messenger RNA or Protein Levels in the Female Rat. Biology of Reproduction 2013, 88:7. [PubMed: 23175773]
27. Friedmann AS: Atrazine inhibition of testosterone production in rat males following peripubertal exposure. Reproductive Toxicology 2002, 16:275–279. [PubMed: 12128101]
28. Song Y, Jia ZC, Chen JY, Hu JX, Zhang LS: Toxic Effects of Atrazine on Reproductive System of Male Rats. Biomedical and Environmental Sciences 2014, 27:281–288. [PubMed: 24758756]
29. Victor-Costa AB, Bandeira SMC, Oliveira AG, Mahecha GAB, Oliveira CA: Changes in testicular morphology and steroidogenesis in adult rats exposed to Atrazine. Reproductive Toxicology 2010, 29:323–331. [PubMed: 20045047]
30. Bardullas U, Giordano M, Rodriguez VM: Chronic atrazine exposure causes disruption of the spontaneous locomotor activity and alters the striatal dopaminergic system of the male Sprague-Dawley rat. Neurotoxicol Teratol 2011, 33:263–272. [PubMed: 20850525]
31. Coban A, Filipov NM: Dopaminergic toxicity associated with oral exposure to the herbicide atrazine in juvenile male C57BL/6 mice. J Neurochem 2007, 100:1177–1187. [PubMed: 17217422]
32. Li Y, Sun Y, Yang J, Wu Y, Yu J, Li B: Age-dependent dopaminergic dysfunction following fetal exposure to atrazine in SD rats. Environ Toxicol Pharmacol 2014, 37:1275–1282. [PubMed: 24863964]
33. Lin Z, Dodd CA, Xiao S, Krishna S, Ye X, Filipov NM: Gestational and lactational exposure to atrazine via the drinking water causes specific behavioral deficits and selectively alters monoaminergic systems in C57BL/6 mouse dams, juvenile and adult offspring. Toxicol Sci 2014, 141:90–102. [PubMed: 24913803]
34. Rodriguez VM, Limon-Pacheco JH, Mendoza-Trejo MS, Gonzalez-Gallardo A, Hernandez-Plata I, Giordano M: Repeated exposure to the herbicide atrazine alters locomotor activity and the nigrostriatal dopaminergic system of the albino rat. Neurotoxicology 2013, 34:82–94. [PubMed: 23123945]
35. Wirbisky SE, Weber GJ, Sepulveda MS, Xiao C, Cannon JR, Freeman JL: Developmental origins of neurotransmitter and transcriptome alterations in adult female zebrafish exposed to atrazine during embryogenesis. Toxicology 2015, 333:156–167. [PubMed: 25929836]
36. Swan SH, Kruse RL, Liu F, Barr DB, Drobnis EZ, Redmon JB, Wang C, Brazil C, Overstreet JW, Study for Future Families Research G: Semen quality in relation to biomarkers of pesticide exposure. Environmental Health Perspectives 2003, 111:1478–1484. [PubMed: 12948887]
37. Kettles MA, Browning SR, Prince TS, Horstman SW: Triazine herbicide exposure and breast cancer incidence: An ecologic study of Kentucky counties. Environmental Health Perspectives 1997, 105:1222–1227. [PubMed: 9370519]
38. Sanderson JT, Boerma J, Lansbergen GWA, van den Berg M: Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. Toxicology and Applied Pharmacology 2002, 182:44–54. [PubMed: 12127262]
39. Abarikwu SO, Farombi EO, Kashyap MP, Pant AB: Atrazine induces transcriptional changes in marker genes associated with steroidogenesis in primary cultures of rat Leydig cells. Toxicology in Vitro 2011, 25:1588–1595. [PubMed: 21693180]

40. Kucka M, Pogrmic-Majkic K, Fa S, Stojilkovic SS, Kovacevic R: Atrazine acts as an endocrine disrupter by inhibiting cAMP-specific phosphodiesterase-4. Toxicology and Applied Pharmacology 2012, 265:19–26. [PubMed: 23022511]
41. Roberge M, Hakk H, Larsen G: Atrazine is a competitive inhibitor of phosphodiesterase but does not affect the estrogen receptor. Toxicology Letters 2004, 154:61–68. [PubMed: 15475179]
42. Wirbisky SE, Weber GJ, Schlotman KE, Sepulveda MS, Freeman JL: Embryonic atrazine exposure alters zebrafish and human miRNAs associated with angiogenesis, cancer, and neurodevelopment. Food and Chemical Toxicology 2016, 98:25–33. [PubMed: 27046698]
43. Wirbisky-Hershberger SE, Sanchez OF, Horzmann KA, Thanki D, Yuan C, Freeman JL: Atrazine exposure decreases the activity of DNMTs, global DNA methylation levels, and dnmt expression. Food Chem Toxicol 2017, 109:727–734. [PubMed: 28859886]
44. McBirney M, King SE, Pappalardo M, Houser E, Unkefer M, Nilsson E, Sadler-Riggleman I, Beck D, Winchester P, Skinner MK: Atrazine induced epigenetic transgenerational inheritance of disease, lean phenotype and sperm epimutation pathology biomarkers. PLoS One 2017, 12:e0184306. [PubMed: 28931070]
45. Dolinoy DC, Jirtle RL: Environmental epigenomics in human health and disease. Environmental and Molecular Mutagenesis 2008, 49:4–8. [PubMed: 18172876]
46. Bailey J, Oliveri A, Levin ED: Zebrafish model systems for developmental neurobehavioral toxicology. Birth Defects Res C Embryo Today 2013, 99:14–23. [PubMed: 23723169]
47. Hill AJ, Teraoka H, Heideman W, Peterson RE: Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci 2005, 86:6–19. [PubMed: 15703261]
48. de Esch C, Slieker R, Wolterbeek A, Woutersen R, de Groot D: Zebrafish as potential model for developmental neurotoxicity testing: A mini review. Neurotoxicology and Teratology 2012, 34:545–553. [PubMed: 22971930]
49. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, et al.: The zebrafish reference genome sequence and its relationship to the human genome. Nature 2013, 496:498–503. [PubMed: 23594743]
50. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF: Stages of embryonic development of the zebrafish. Dev Dyn 1995, 203:253–310. [PubMed: 8589427]
51. Weber GJ, Sepulveda MS, Peterson SM, Lewis SS, Freeman JL: Transcriptome Alterations Following Developmental Atrazine Exposure in Zebrafish Are Associated with Disruption of Neuroendocrine and Reproductive System Function, Cell Cycle, and Carcinogenesis. Toxicological Sciences 2013, 132:458–466. [PubMed: 23358194]
52. Wirbisky SE, Sepulveda MS, Weber GJ, Jannasch AS, Horzmann KA, Freeman JL: Embryonic Atrazine Exposure Elicits Alterations in Genes Associated with Neuroendocrine Function in Adult Male Zebrafish. Toxicol Sci 2016, 153:149–164. [PubMed: 27413107]
53. Kozol RA, Abrams AJ, James DM, Buglo E, Yan Q, Dallman JE: Function Over Form: Modeling Groups of Inherited Neurological Conditions in Zebrafish. Frontiers in Molecular Neuroscience 2016, 9:55. [PubMed: 27458342]
54. Belloni V, Dessi-Fulgheri F, Zaccaroni M, Di Consiglio E, De Angelis G, Testai E, Santochirico M, Alleva E, Santucci D: Early exposure to low doses of atrazine affects behavior in juvenile and adult CD1 mice. Toxicology 2011, 279:19–26. [PubMed: 20624442]
55. Filipov NM, Stewart MA, Carr RL, Sistrunk SC: Dopaminergic toxicity of the herbicide atrazine in rat striatal slices. Toxicology 2007, 232:68–78. [PubMed: 17218051]
56. Sun Y, Li YS, Yang JW, Yu J, Wu YP, Li BX: Exposure to atrazine during gestation and lactation periods: toxicity effects on dopaminergic neurons in offspring by downregulation of Nurr1 and VMAT2. Int J Mol Sci 2014, 15:2811–2825. [PubMed: 24552878]
57. Kalueff AV, Gebhardt M, Stewart AM, Cachat JM, Brimmer M, Chawla JS, Craddock C, Kyzar EJ, Roth A, Landsman S, et al.: Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. Zebrafish 2013, 10:70–86. [PubMed: 23590400]
58. Kalueff AV, Stewart AM, Gerlai R: Zebrafish as an emerging model for studying complex brain disorders. Trends in Pharmacological Sciences 2014, 35:63–75. [PubMed: 24412421]

59. Kalueff AV, Echevarria DJ, Homechaudhuri S, Stewart AM, Collier AD, Kaluyeva AA, Li S, Liu Y, Chen P, Wang J, et al.: Zebrafish neurobehavioral phenomics for aquatic neuropharmacology and toxicology research. Aquatic Toxicology 2016, 170:297–309. [PubMed: 26372090]
60. Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, et al.: Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res 2009, 205:38–44. [PubMed: 19540270]
61. Lin Z, Dodd CA, Filipov NM: Differentiation state-dependent effects of in vitro exposure to atrazine or its metabolite diaminochlorotriazine in a dopaminergic cell line. Life Sci 2013, 92:81–90. [PubMed: 23142650]
62. Steinberg CEW, Lorenz R, Spieser OH: EFFECTS OF ATRAZINE ON SWIMMING BEHAVIOR OF ZEBRAFISH, BRACHYDANIO-RERIO. Water Research 1995, 29:981–985.
63. Schmidel AJ, Assmann KL, Werlang CC, Bertoncello KT, Francescon F, Rambo CL, Beltrame GM, Calegari D, Batista CB, Blaser RE, et al.: Subchronic atrazine exposure changes defensive behaviour profile and disrupts brain acetylcholinesterase activity of zebrafish. Neurotoxicology and Teratology 2014, 44:62–69. [PubMed: 24893294]
64. Liu Z, Wang Y, Zhu Z, Yang E, Feng X, Fu Z, Jin Y: Atrazine and its main metabolites alter the locomotor activity of larval zebrafish (Danio rerio). Chemosphere 2016, 148:163–170. [PubMed: 26803580]
65. Horzmann KA, Reidenbach LS, Thanki DH, Winchester AE, Qualizza BA, Ryan GA, Egan KE, Hedrick VE, Sobreira TJP, Peterson SM, et al.: Embryonic atrazine exposure elicits proteomic, behavioral, and brain abnormalities with developmental time specific gene expression signatures. J Proteomics 2018, Submitted..
66. Truong L, Reif DM, St Mary L, Geier MC, Truong HD, Tanguay RL: Multidimensional in vivo hazard assessment using zebrafish. Toxicol Sci 2014, 137:212–233. [PubMed: 24136191]
67. Reif DM, Truong L, Mandrell D, Marvel S, Zhang G, Tanguay RL: High-throughput characterization of chemical-associated embryonic behavioral changes predicts teratogenic outcomes. Arch Toxicol 2016, 90:1459–1470. [PubMed: 26126630]
68. Shah K, Lahiri DK: Cdk5 activity in the brain - multiple paths of regulation. J Cell Sci 2014, 127:2391–2400. [PubMed: 24879856]
69. Trosko JE: The gap junction as a “Biological Rosetta Stone”: implications of evolution, stem cells to homeostatic regulation of health and disease in the Barker hypothesis. Journal of cell communication and signaling 2011, 5:53–66. [PubMed: 21484590]
70. Kubincová P, Sychrová E, Raška J, Basu A, Yawer A, Dydowiczová A, Babica P, Sovadinová I: Polycyclic Aromatic Hydrocarbons and Endocrine Disruption: Role of Testicular Gap Junctional Intercellular Communication and Connexins. Toxicol Sci 2019, 169:70–83. [PubMed: 30668803]
71. Chipman JK, Mally A, Edwards GO: Disruption of Gap Junctions in Toxicity and Carcinogenicity. Toxicological Sciences 2003, 71:146–153. [PubMed: 12563100]
72. Lin Z, Roede JR, He C, Jones DP, Filipov NM: Short-term oral atrazine exposure alters the plasma metabolome of male C57BL/6 mice and disrupts alpha-linolenate, tryptophan, tyrosine and other major metabolic pathways. Toxicology 2014, 326:130–141. [PubMed: 25445803]
73. Hossain MM, Filipov NM: Alteration of dopamine uptake into rat striatal vesicles and synaptosomes caused by an in vitro exposure to atrazine and some of its metabolites. Toxicology 2008, 248:52–58. [PubMed: 18423833]
74. Li Y, Sun Y, Yang J, Wu Y, Yu J, Li B: The long-term effects of the herbicide atrazine on the dopaminergic system following exposure during pubertal development. Mutat Res Genet Toxicol Environ Mutagen 2014, 763:23–29. [PubMed: 24561379]
75. Lin Z, Dodd CA, Filipov NM: Short-term atrazine exposure causes behavioral deficits and disrupts monoaminergic systems in male C57BL/6 mice. Neurotoxicol Teratol 2013, 39:26–35. [PubMed: 23770127]
76. Rajkovic V, Djolai M, Matavulj M: Alterations in jejunal morphology and serotonin-containing enteroendocrine cells in peripubertal male rats associated with subchronic atrazine exposure. Ecotoxicol Environ Saf 2011, 74:2304–2309. [PubMed: 21839517]
77. Shackelford C, Long G, Wolf J, Okerberg C, Herbert R: Qualitative and quantitative analysis of nonneoplastic lesions in toxicology studies. Toxicol Pathol 2002, 30:93–96. [PubMed: 11890482]

78. van der Ven LTM, Wester PW, Vos JG: Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (Danio rerio). Environmental Toxicology and Chemistry 2003, 22:908–913. [PubMed: 12685728]

79. Ganz J, Kroehne V, Freudenreich D, Machate A, Geffarth M, Braasch I, Kaslin J, Brand M: Subdivisions of the adult zebrafish pallium based on molecular marker analysis. F1000Research 2014, 3:308. [PubMed: 25713698]

80. Northcutt RG: Connections of the lateral and medial divisions of the goldfish telencephalic pallium. J Comp Neurol 2006, 494:903–943. [PubMed: 16385483]

81. Maximino C, Lima MG, Oliveira KR, Batista Ede J, Herculano AM: "Limbic associative" and "autonomic" amygdala in teleosts: a review of the evidence. J Chem Neuroanat 2013, 48–49:1–13.

82. Wullimann MF: Ancestry of basal ganglia circuits: New evidence in teleosts. Journal of Comparative Neurology 2014, 522:2013–2018.

83. Herculano AM, Maximino C: Serotonergic modulation of zebrafish behavior: Towards a paradox. Progress in Neuro-Psychopharmacology and Biological Psychiatry 2014, 55:50–66. [PubMed: 24681196]

84. Lillesaar C: The serotonergic system in fish. Journal of Chemical Neuroanatomy 2011, 41:294–308. [PubMed: 21635948]

85. Matthews GA, Nieh EH, Vander Weele CM, Halbert SA, Pradhan RV, Yosafat AS, Glober GF, Izadmehr EM, Thomas RE, Lacy GD, et al.: Dorsal Raphe Dopamine Neurons Represent the Experience of Social Isolation. Cell 2016, 164:617–631. [PubMed: 26871628]

86. Wirbisky SE, Weber GJ, Sepúlveda MS, Lin T-L, Jannasch AS, Freeman JL: An embryonic atrazine exposure results in reproductive dysfunction in adult zebrafish and morphological alterations in their offspring. Scientific Reports 2016, 6:21337. [PubMed: 26891955]

87. Peterson SM, Zhang J, Weber G, Freeman JL: Global Gene Expression Analysis Reveals Dynamic and Developmental Stage-Dependent Enrichment of Lead-Induced Neurological Gene Alterations. Environmental Health Perspectives 2011, 119:615–621. [PubMed: 21147602]

88. Westerfield M: The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio). 5th edn. Eugene, Oregon: University of Oregon Press; 2007.

89. Wirbisky SE, Damayanti NP, Mahapatra CT, Sepulveda MS, Irudayaraj J, Freeman JL: Mitochondrial Dysfunction, Disruption of F-Actin Polymerization, and Transcriptomic Alterations in Zebrafish Larvae Exposed to Trichloroethylene. Chem Res Toxicol 2016, 29:169–179. [PubMed: 26745549]

90. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, et al.: Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet 2001, 29:365–371. [PubMed: 11726920]

91. Peterson SM, Freeman JL: RNA isolation from embryonic zebrafish and cDNA synthesis for gene expression analysis. J Vis Exp 2009:1470. [PubMed: 19684565]

92. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG: Primer3--new capabilities and interfaces. Nucleic Acids Res 2012, 40:e115. [PubMed: 22730293]

93. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, et al.: The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry 2009, 55:611–622. [PubMed: 19246619]

94. Freeman JL, Weber GJ, Peterson SM, Nie LH: Embryonic ionizing radiation exposure results in expression alterations of genes associated with cardiovascular and neurological development, function, and disease and modified cardiovascular function in zebrafish. Frontiers in Genetics 2014, 5:268. [PubMed: 25147559]

95. Zhang J, Peterson SM, Weber GJ, Zhu XQ, Zheng W, Freeman JL: Decreased axonal density and altered expression profiles of axonal guidance genes underlying lead (Pb) neurodevelopmental toxicity at early embryonic stages in the zebrafish. Neurotoxicology and Teratology 2011, 33:715–720. [PubMed: 21839828]

96. Dobrinski KP, Brown KH, Freeman JL, Lee C: Chapter 14 - Molecular Cytogenetic Methodologies and a BAC Probe Panel Resource for Genomic Analyses in the Zebrafish. In Methods in Cell

Biology. *Volume* 104. Edited by Detrich HW, Westerfield M, Zon LI: Academic Press; 2011: 237–257 [PubMed: 21924167]

Highlights

- Embryonic atrazine exposure affects neurodevelopment and neural function in adult male zebrafish
- Adult male zebrafish had decreased locomotion in behavioral tests
- Adult male zebrafish brain had altered gene expression in pathways associated with organismal development, cancer, and nervous and reproductive system development and function
- Adult male zebrafish brain had decreased cell numbers in raphe populations

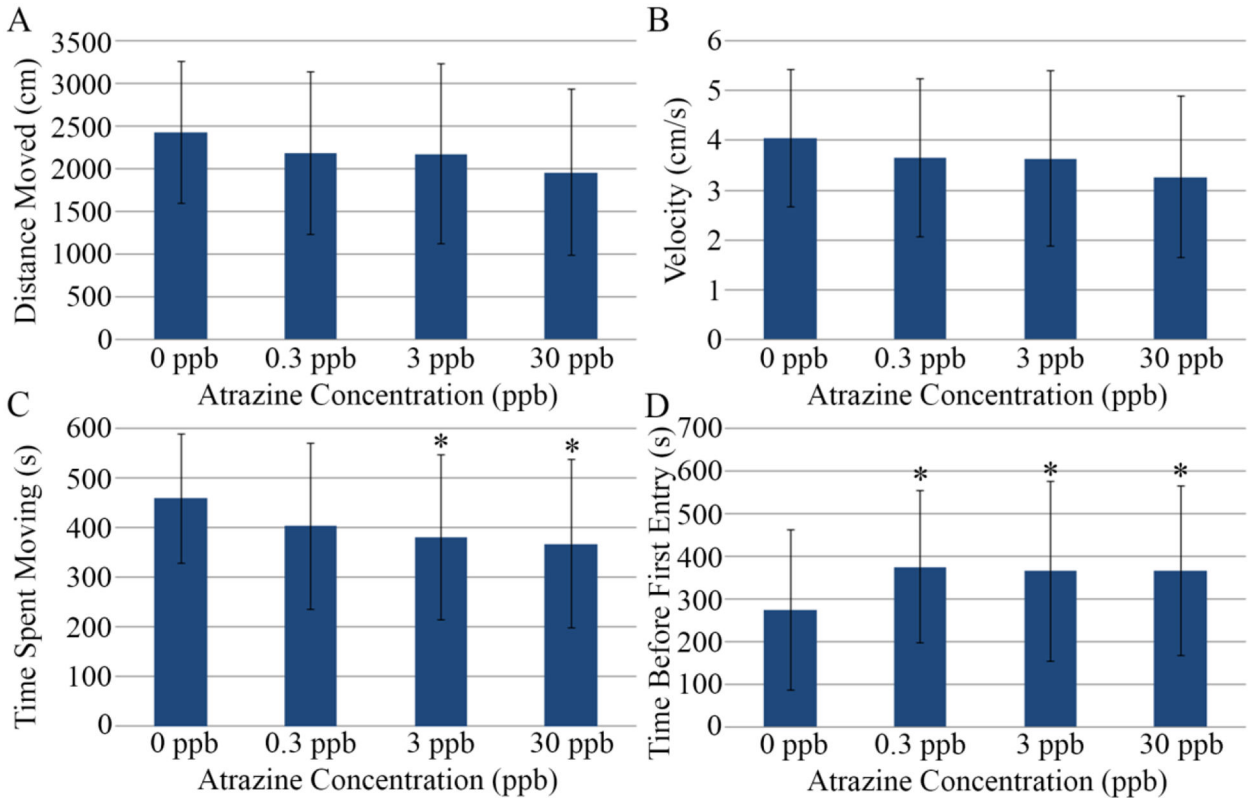

Figure 1.

NTT performance of male zebrafish with embryonic ATZ exposure. There was a general trend for decreased movement related parameters compared to controls for male zebrafish with increasing embryonic ATZ exposure in the distance moved (A), velocity (B), and the trend reached significance in the 3 and 30 ppb groups for the time spent moving (C). The latency to first upper zone entry was increased for all ATZ treatments compared to the controls (D). N = 4, 10 subsamples per treatment per replicate to total 40 fish per treatment, error bars represent standard deviation, *p<0.05.

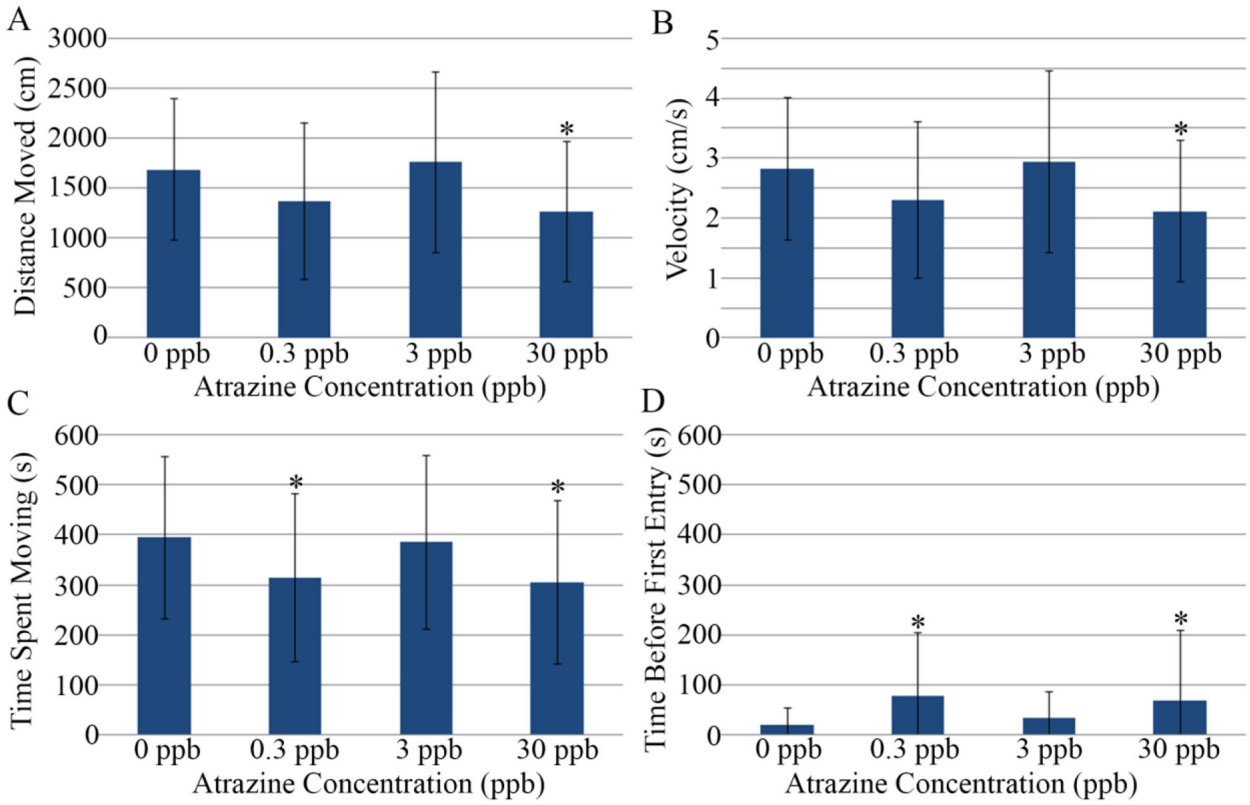

Figure 2.

LDB performance of male zebrafish with embryonic ATZ exposure. Male zebrafish with embryonic exposure to 30 ppb ATZ had significantly decreased distance moved (A) and velocity (B). Both the 0.3 and the 30 ppb exposures spent significantly less time moving (C) than controls and had an increased latency to the first light zone entry (D). N = 4, 10 subsamples per treatment per replicate to total 40 fish per treatment, error bars represent standard deviation, *p<0.05.

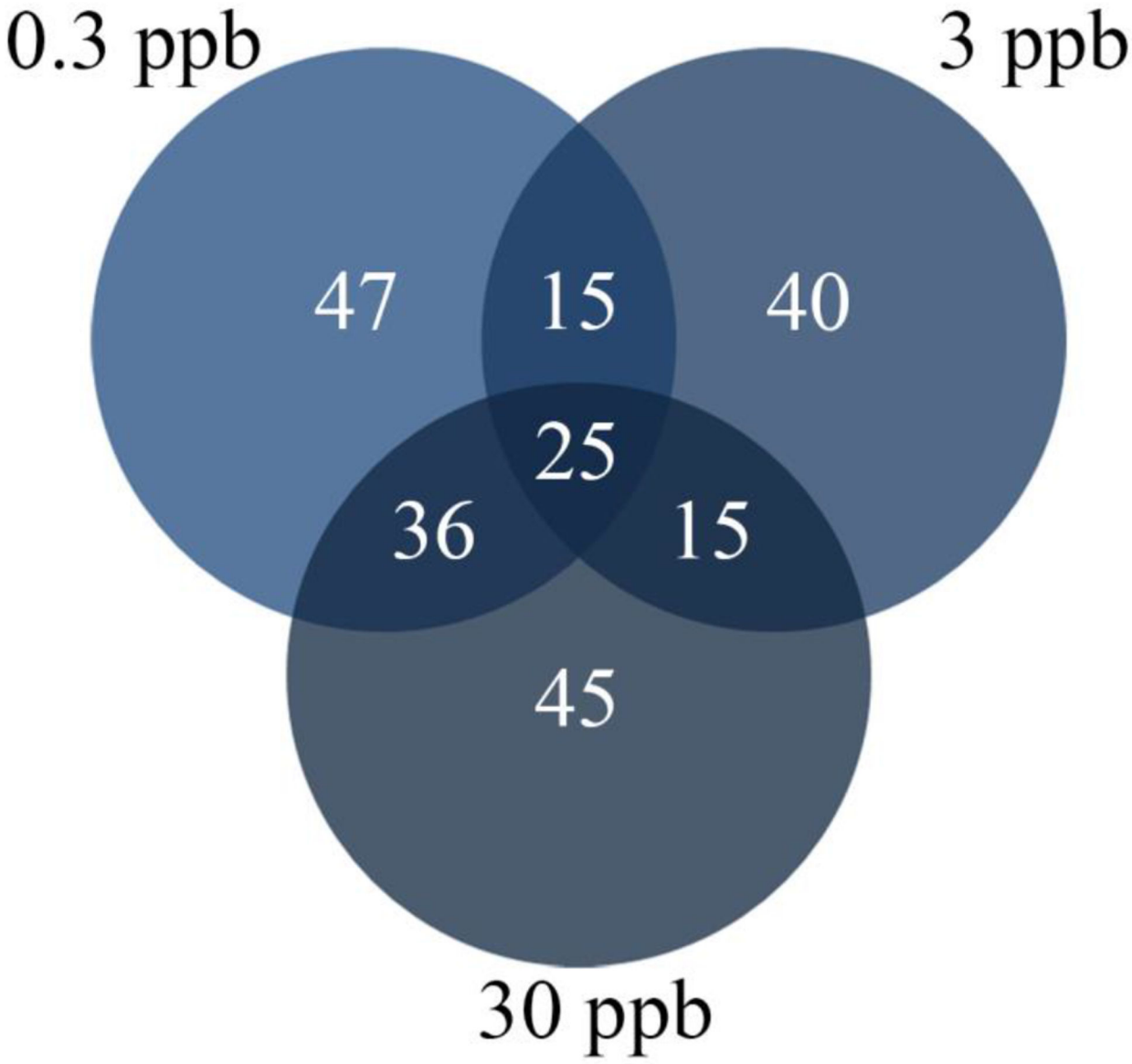

Figure 3.
Venn diagram of the number of mapped genes changed in each embryonic exposure group.

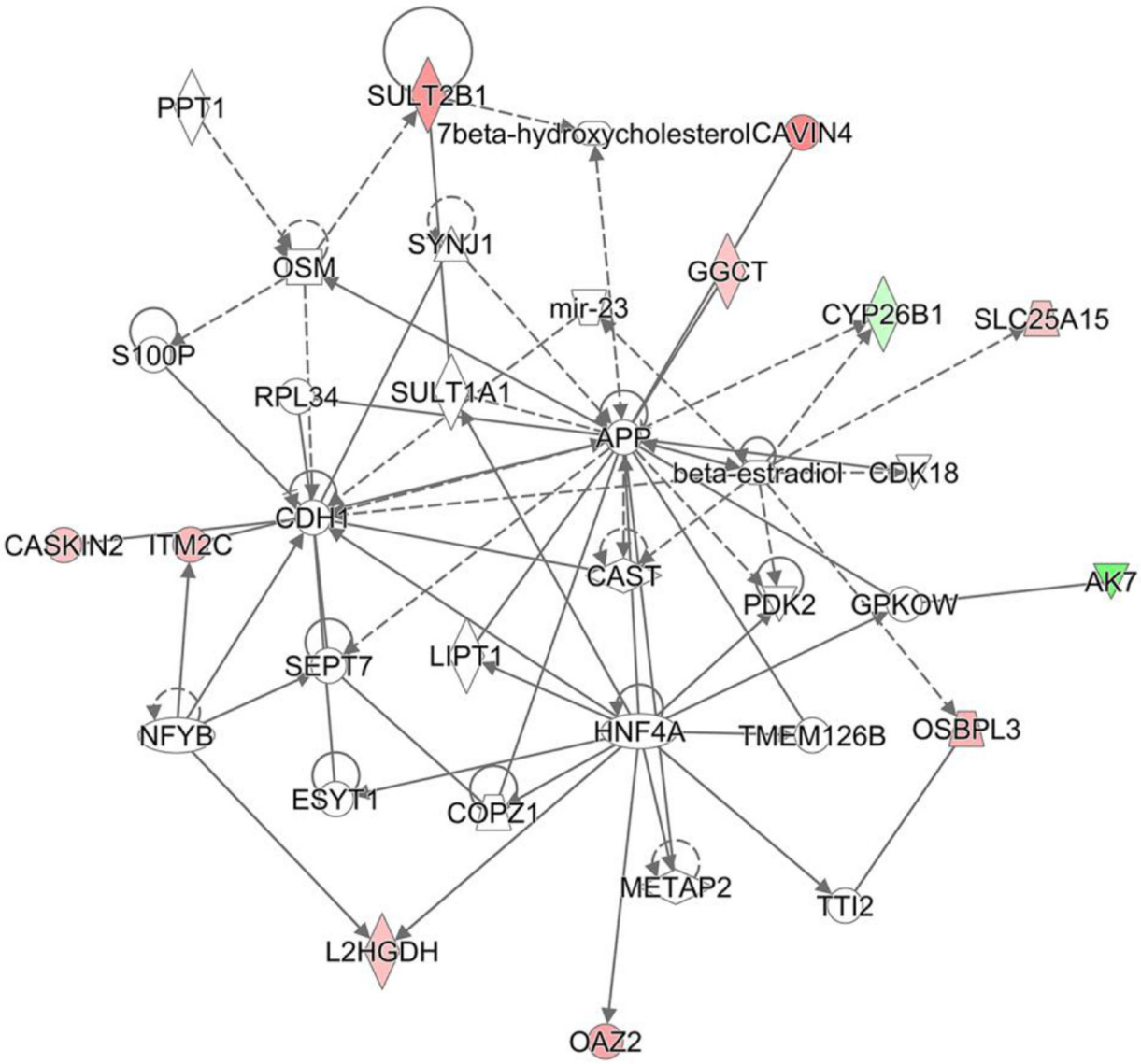

Figure 4.

Network analysis of genes changed in all three male ATZ exposure groups. Male zebrafish with embryonic ATZ exposure had in common altered expression of genes in a developmental disorder, neurological disease, organismal injury and abnormalities network. Red indicates upregulation, green indicates down regulation, with depth of color related to magnitude of change. Large ovals represent transcription regulators, small ovals represent hormones, squares represent cytokines, vertical diamonds represent enzymes, horizontal diamonds represent peptidases, inverted triangles represent kinases, trapezoids represent transporters, inverted trapezoids represent microRNA, and circles represent other molecules. Lines with arrows represent activation, lines without arrows represent interactions, solid lines represent direction interaction, and dashed lines represent indirect interaction. Gene symbols represent human orthologs of zebrafish genes.

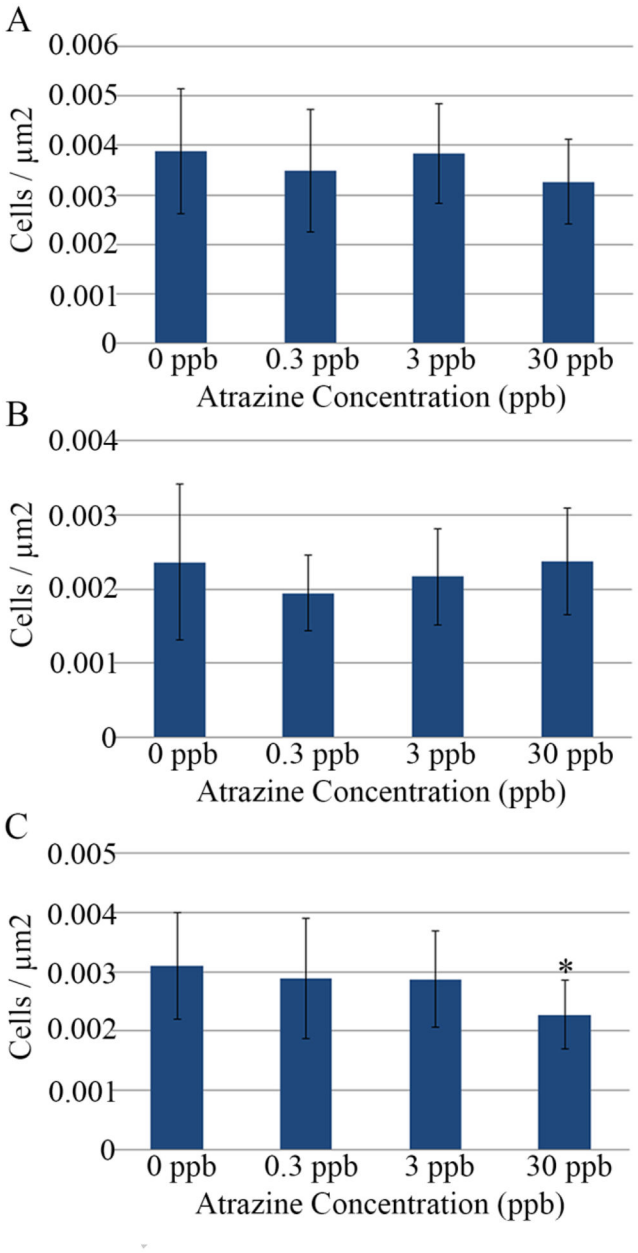

Figure 5.

Morphometric analysis of zebrafish brain regions from males with embryonic ATZ exposure. Male zebrafish had no differences between treatments in the cells per µm2 of the dorsal telencephalon (A) or the posterior tuberculum (B), but the males with embryonic exposure to 30 ppb ATZ had significantly fewer cells per µm2 compared to controls (C). N = 3, 6 subsamples per treatment per replicate to total 18 fish per treatment, 2 brain sections evaluated per subsample, error bars represent standard deviation, *$p<0.05$.

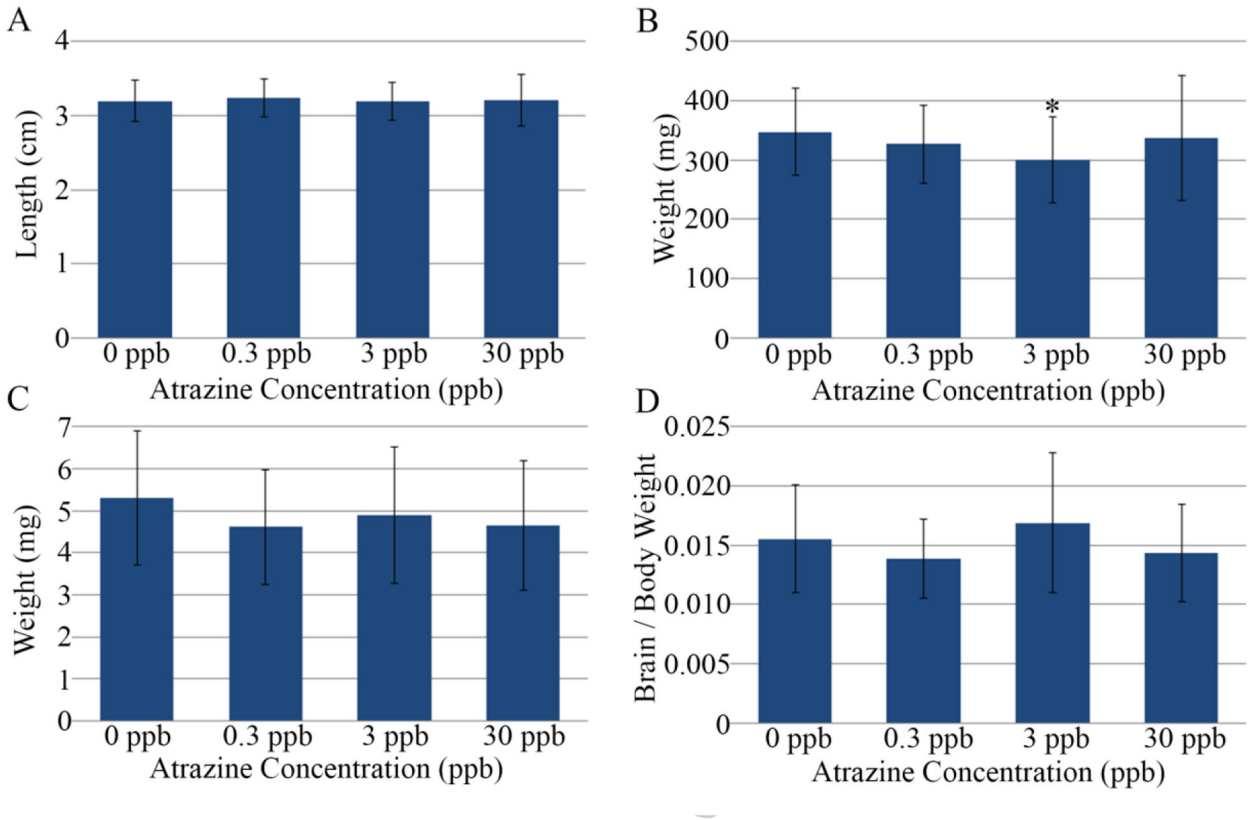

Figure 6.

Changes in body length and weight, and brain weight in males after embryonic ATZ exposure. There was no difference in body weight between male embryonic ATZ exposure groups (A), but the body weight was significantly decreased in the males with embryonic exposure to 3 ppb ATZ (B). There was no difference in brain weight (C) or brain weight to body weight ratio between the male exposures (D). N = 3, with 14–69 subsamples per treatment per replicate to total 42–207 fish per treatment. Error bars represent standard deviation. *$p < 0.05$.

Table 1.

List of genes changed in male brains common to all embryonic ATZ exposures

Name	Gene Symbol[a]	Biological Function[b]
Adenylate kinase 7	*AK7*	Axoneme assembly; brain development; cell projection organization; inflammatory response; phosphorylation; spermatogenesis
Aquaporin 1 (Colton blood group)	*AQP1*	Cellular homeostasis; cellular response to cAMP; cerebrospinal fluid secretion; lateral ventricle development; response to hormone stimulus
CASK interacting protein 2	*CASKIN2*	Protein-protein interactions
Caveolae associated protein 4	*CAVIN4*	Cardiac myofibril assembly; cell differentiation; muscle development; regulation of gene expression; Rho protein signal transduction
Coiled-coil domain containing 39	*CCDC39*	Axonemal dynein complex assembly; cilium movement; determination of left/right symmetry; heart development; sperm motility
Cyclin dependent kinase 5	*CDK5*	Associative learning; neuron and oligodendrocyte development and differentiation; dopaminergic and glutamatergic synaptic transmission
Cytochrome P450 family 26 subfamily B member 1	*CYP26B1*	Bone morphogenesis; cell fate; response to retinoic acid; embryonic limb morphogenesis; spermatogenesis; sterol metabolic process
Dihydrolipoamide branched chain transacylase E2	*DBT*	Branched chain family amino acid catabolic process; cellular nitrogen compound metabolic process; metabolic process
Dopey family member 2	*DOPEY2*	Cognition; endoplasmic reticulum organization; Golgi to endosome transport; protein transport
Family with sequence similarity 169 member A	*FAM169A*	--
Gamma-glutamylcyclotransferase	*GGCT*	Glutathione biosynthetic process; release of cytochrome c from mitochondria
HtrA serine peptidase 2	*HTRA2*	Adult locomotor behavior; forebrain development; negative regulation of cell cycle; neuron development; proteolysis; response to herbicide
Insulin like growth factor binding protein 7	*IGFBP7*	Cell adhesion; cellular response to hormone stimulus; post-translational protein modification; regulation of steroid biosynthesis
Integral membrane protein 2C	*ITM2C*	Negative regulation of amyloid precursor protein; nervous system development and differentiation; regulation of apoptosis
L-2-hydroxyglutarate dehydrogenase	*L2HGDH*	2-oxoglutarate metabolic process; cellular protein metabolic process; oxidation-reduction process
Macrophage stimulating 1 receptor	*MST1R*	Cellular component movement; defense response; hepatocyte growth factor receptor signaling pathway; immune system process
Ornithine decarboxylase antizyme 2	*OAZ2*	Regulation of catalytic activity; polyamine biosynthesis; regulation of intracellular protein transport; protein and amino acid catabolism
Oxysterol binding protein like 3	*OSBPL3*	Bile acid biosynthetic process; lipid transport; sterol transport; transport
Protein disulfide isomerase family A member 6	*PDIA6*	Apoptotic cell clearance; cell redox homeostasis; cellular protein metabolic process; post-translational protein modification
Periostin	*POSTN*	Cell adhesion; cellular response to fibroblast growth factor, estradiol, transforming growth factor beta, tumor necrosis factor, and vitamin K
Protein regulator of cytokinesis 1	*PRC1*	Cell cycle; cell division; cytokinesis; microtubule bundle formation; microtubule cytoskeleton organization
Peroxiredoxin 3	*PRDX3*	Apoptotic process; cell redox homeostasis; negative regulation of neuron apoptotic process; regulation of cell proliferation
Solute carrier family 25 member 15	*SLC25A15*	Mitochondrial ornithine transport; mitochondrial transport; transport; urea cycle
Sulfotransferase family 2B member 1	*SULT2B1*	Sulfate conjugation of hormones, neurotransmitters, and xenobiotic compounds; lipid metabolic process; steroid metabolic process
Troponin I2, fast skeletal type	*TNNI2*	Regulation of muscle contraction; co-activator of estrogen receptor-related receptor alpha; positive regulation of transcription

[a]Human ortholog of zebrafish gene

[b] Determined via Ingenuity Pathway Analysis

Table 2.

Top pathways altered in all three ATZ exposures

Disease and Disorders		
Name	**p-value**[a]	**# Molecules**[b]
Developmental Disorder	2.49E-02 – 4.74E-04	11
Hereditary Disease	2.32E-02 – 4.74E-04	15
Metabolic Disease	1.60E-02 – 4.74E-04	6
Organismal Injury and Abnormalities	4.96E-02 – 4.74E-04	25
Cardiovascular Disease	8.57E-03 – 5.06E-04	4
Molecular and Cellular Functions		
Name	**p-value**[a]	**# Molecules**[b]
Cellular Function and Maintenance	4.83E-02 – 7.22E-04	9
Cellular Movement	4.93E-02 – 7.22E-04	9
Cellular Development	4.73E-02 – 8.86E-04	8
Cellular Growth and Proliferation	4.73E-02 – 8.86E-04	5
Cell Cycle	4.17E-02 – 1.07E-03	6
Physiological System Development and Function		
Name	**p-value**[a]	**# Molecules**[b]
Organismal Functions	2.86E-02 – 3.09E-05	5
Renal and Urological System Development and Function	3.49E-02 – 8.86E-04	4
Reproductive System Development and Function	3.80E-02 – 9.29E-04	5
Embryonic Development	4.83E-02 – 9.67E-04	8
Organismal Development	4.88E-02 – 9.67E-04	8

[a]Derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.

[b]Classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

Table 3.

Genes altered in all embryonic exposures at 6 mpf* and 9 mpf

Name	Gene Symbol[a]	Biological Function[b]
Caveolae associated protein 4	*CAVIN4*	Cardiac myofibril assembly; cell differentiation; muscle development; regulation of gene expression; Rho protein signal transduction
Cyclin dependent kinase 5	*CDK5*	Associative learning; neuron and oligodendrocyte development; dopaminergic and glutamatergic synaptic transmission
HtrA serine peptidase 2	*HTRA2*	Adult locomotor behavior; forebrain development; regulation of cell cycle; neuron development; proteolysis; response to herbicide
Insulin like growth factor binding protein 7	*IGFBP7*	Cell adhesion; cellular response to hormone stimulus; post-translational protein modification; regulation of steroid biosynthesis
Integral membrane protein 2C	*ITM2C*	Negative regulation of amyloid precursor protein; nervous system development and differentiation; regulation of apoptosis
Sulfotransferase family 2B member 1	*SULT2B1*	Sulfate conjugation of hormones, neurotransmitters, and xenobiotic compounds; lipid metabolic process; steroid metabolic process
Troponin I2, fast skeletal type	*TNNI2*	Regulation of muscle contraction; co-activator of estrogen receptor-related receptor alpha; positive regulation of transcription

*Data from 6 mpf male zebrafish originally published in Wirbisky et al, 2016 [52].

[a]Human ortholog of zebrafish gene

[b]Determined via Ingenuity Pathway Analysis