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Atrazine alters expression of reproductive and stress genes in the developing hypothalamus of the snapping turtle, *Chelydra serpentina*

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ABSTRACT

Atrazine is an herbicide used to control broadleaf grasses and a suspected endocrine disrupting chemical. Snapping turtles lay eggs between late May and early June, which could lead to atrazine exposure via field runoff. Our goal was to determine whether a single exposure to 2 ppb or 40 ppb atrazine during embryogenesis could induce short- and long-term changes in gene expression within the hypothalamus of snapping turtles. We treated eggs with atrazine following sex determination and measured gene expression within the hypothalamus. We selected genes *a priori* for their role in the hypothalamus-pituitary-gonad or the hypothalamus-pituitary-adrenal axes of the endocrine system. We did not identify any changes in gene expression 24-h after treatment. However, at hatching *AR, Kiss1R*, and *POMC* expression was upregulated in both sexes, while expression of *CYP19A1* and *PDYN* was increased in females. Six months after hatching, *CYP19A1* and *PRLH* expression was increased in animals treated with 2 ppb atrazine.

Our study shows persistent changes in hypothalamic gene expression due to low-dose embryonic exposure to the herbicide atrazine with significant effects in both the HPG and HPA axes. Effects reported here appear to be conserved among vertebrates.

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1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is one of the most widely used herbicides in the country, and is a suspected endocrine disrupting chemical (EDC). Atrazine (ATR) can be found in approximately 70% of surface streams and groundwater (Arlos et al., 2014) at concentrations averaging 20 ppb, however it has been found in excess of 100 ppb in some agricultural areas (Blanchard and Lerch, 2000). These levels far exceed the U.S. EPA's set maximum contaminant level of 3 ppb (USEPA, 2009).

The actions of many EDCs on hormone homeostasis often occur at extremely low dose exposures, and therefore do not always follow classic dose-response toxicological principles (Diamanti-Kandarakis et al., 2009). Some evidence shows physiological effects of ATR exposure at levels below 3 ppb (Neuman-Lee and Janzen, 2011). However, results vary from study to study, species to species, and with physiological outcome (reviewed in Cooper et al., 2007). For instance, serum levels of LH and PRL were significantly decreased by exposure to 50 mg/kg, 100 mg/kg, 200 mg/kg, and 300 mg/kg doses of ATR in Long-Evans rats, but only the 300 mg/kg dose had an effect in Sprague-Dawley rats (Cooper et al., 2000). Activity of several neurotransmitters was tested in zebrafish exposed to 0.3, 3, and 30 ppb ATR. Only 3 ppb altered activity of 5-hydroxyindoleacetic acid, while 0.3 and 3 ppb exposures altered serotonin turnover (Wirbisky et al., 2015). High doses of ATR decreased testosterone levels in male African clawed frogs, but estradiol levels and aromatase activity remained unchanged (Hecker et al., 2005). Therefore, the EPA maximum contaminant levels for ground and surface waters might be inappropriate for preventing endocrine disruption by ATR.

While ATR does not bioaccumulate (McMullin et al., 2003; Ross et al., 2009), persistence in soil and the environment can result in chronic exposure in animals and humans. The half-life of ATR in soil ranges from 49 to 119 days (Accinelli et al., 2001). The European Union banned the use of ATR in 2003 due to widespread environmental contamination (European Commission Health and Consumer Protection Directorate-General, 2003). The U.S. EPA

Abbreviations: AVPV, anteroventral periventricular nucleus; ATR, atrazine; HPG, hypothalamuspituitary-gonad axis; HPA, hypothalamus-pituitary-adrenal axis; Kiss1, kisspeptin; Kiss1R, kisspeptin receptor; GnRH, gonadotropin-releasing hormone; AR, androgen receptor; ESR, estrogen receptor; Cyp19a1, aromatase; PDYN, prodynorphin; POMC, proopiomelanocortin; SST, somatostatin; PRL, prolactin; PRLH, prolactin-releasing hormone; EDC, endocrine disrupting chemical.

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began reevaluating the safety of ATR in 2009, and according to a 2013 update, is considering whether new restrictions are necessary to protect environmental and public health (USEPA, 2013).

Acute and chronic exposure to ATR during development or adulthood can produce adverse effects. Exposure to ATR is linked to development of some cancers (Albanito et al., 2015; Schroeder et al., 2001), disruption of the dopaminergic system in the central nervous system (Coban and Filipov, 2007), impairment of fetal growth and development (Ochoa-Acuna et al., 2009), inhibition of the immune system (Thueson et al., 2015), altered sex ratios (Langlois et al., 2009), and various effects in the reproductive system (Cragin et al., 2011; Davis et al., 2011). ATR is shown to have endocrine-disrupting effects both by activating the Hypothalamus-Pituitary-Adrenal (HPA) axis (Fraites et al., 2009) and altering the Hypothalamus-Pituitary-Gonad (HPG) axis (Trentacoste et al., 2001). Exposure to ATR has also been linked to abnormal testis development and steroidogenesis across vertebrate taxa (Stoker et al., 2008; Victor-Costa et al., 2010).

Testosterone derivatives (dihydrotestosterone and estradiol) induce changes in gene expression within the hypothalamus during development, resulting in morphological and functional differences between sexes (Buedefeld et al., 2015; Phoenix et al., 1959). Hypothalamic cellular structure is established during pre- and postnatal development, is required for HPG regulation, and is imperative to fertility later in life. While atrazine's mechanism of action is not well-understood, one hypothesis is that ATR upregulates aromatase expression. ATR has been shown to upregulate aromatase activity and mRNA expression in human cell lines (Sanderson et al., 2000, 2001, 2002; Caron-Beaudoin et al., 2016). Aromatase upregulation leads to increased conversion of androgens into estrogens (Laville et al., 2006), which could have life-long effects on reproductive success. A second hypothesis is that ATR interferes with HPA regulation, indirectly impacting reproduction through stress pathways (Fraites et al., 2009).

Steroid hormones produced by the gonads induce changes in gene expression within the hypothalamus during development via their receptors, resulting in morphological and functional differences between sexes (Phoenix et al., 1959; Aste et al., 2010; Buedefeld et al., 2015). The hypothalamus in turn regulates a wide variety of homoeostatic mechanisms, including water balance, growth, body temperature, stress response, and reproduction. In a simplistic description of the HPG axis, regulation occurs in a negative-feedback mechanism beginning with Kisspeptin (Kiss1) neurons, which signal to gonadotropin-releasing hormone (GnRH) neurons within the hypothalamus, which in turn release GnRH to the pituitary gland. GnRH stimulates the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from gonadotropes in the pituitary gland, which are then released into the bloodstream. LH and FSH receptors found in the gonads stimulate production of gonadal steroid hormones, including testosterone and estradiol. Testosterone and estradiol travel through the bloodstream, where they bind to receptors throughout the body. Sex steroids decrease activity of both GnRH neurons and gonadotropes. Testosterone derivatives (estradiol and/or dihydrotestosterone) are responsible for masculinization and defeminization of the brain in several vertebrate species (Aste et al., 2010; Rhen and Crews, 2002). The cellular structure and function required for this tightly controlled signaling mechanism is established during pre-and postnatal development, and is imperative to fertility later in life. Any disruption to this process could have life-long effects on reproductive success. Considering the evidence for ATR effects on gonads and the tight regulation of the HPG, ATR may impact the hypothalamus as well.

Atrazine disruption within the HP is supported by considerable evidence in mammals. Exposure to ATR in adulthood can disrupt the GnRH pulse generator, which regulates luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release (Foradori et al., 2009). Furthermore, embryos are often more vulnerable to environmental contaminants with exposures causing long-lasting defects. ATR exposure during development decreases fertility, delays puberty, reduces testosterone levels in males (Fraites et al., 2011; Swan, 2006), and delays mammary gland development and alters estrous cycles in females (Davis et al., 2011; Rayner et al., 2005).

The common snapping turtle (*Chelydra serpentina*) is a reptile found east of the Rocky Mountains, and is a species with temperature-dependent sex determination (Rhen and Lang, 1994). Eggs incubated at 26.5 °C will produce 100% males, while 31 °C produces 100% females in the best studied population in Minnesota. Snapping turtles lay large clutches of eggs (mean ~ 45 eggs) in late May or early June. Because ATR is applied to fields shortly before nesting season, nests are susceptible to exposure via run-off. Furthermore, snapping turtle eggs readily absorb atrazine in aqueous solutions and soil (de Solla et al., 2006; de Solla and Martin, 2011a; de Solla and Martin, 2011b). When eggs were exposed directly to 0.94 ug/g aqueous ATR, 83% of the chemical was recovered within the egg (de Solla and Martin, 2011a). When snapping turtle eggs were incubated in soil treated with ATR at application rates that mimic agricultural practices, ATR was recovered from the eggs, with higher levels occurring with higher levels and longer durations of exposure (de Solla and Martin, 2011a). Additionally, ATR has been detected in adult snapping turtle tissue (Douros et al., 2015), and therefore exposure could also occur through maternal transfer.

Based on evidence in other vertebrates, we tested the hypothesis that an acute, developmental exposure to ATR can induce sex-specific short- and/or long-term changes in gene expression within the hypothalamus of the snapping turtle. We do not know exposure patterns in the wild, but considering the depth of the nests and incidence of precipitation, a single exposure is plausible. Following embryonic treatment with 17β-estradiol or ATR, we used quantitative PCR to measure expression of genes selected *a priori* based on their critical involvement in the regulation of the HPG axis. Genes tested include kisspeptin (*Kiss1*), kisspeptin receptor (*Kiss1R*), gonadotropin-releasing hormone 1 and 2 (*GnRH1* and *GnRH2*), prolactin releasing hormone (*PRLH*), estrogen receptors 1 and 2 (*ESR1* and *ESR2*), androgen receptor (*AR*), and aromatase (*CYP19A1*). We also examined key genes in stress and growth pathways in the HPA by measuring prodynorphin (*PDYN*), proopiomelanocortin (*POMC*), and somatostatin (*SST*). We expected alterations in expression of genes involved in the reproductive axis, especially expression of CYP19A1, genes involved in the stress axis, including PDYN and POMC, and genes involved in growth inhibition, such as SST.

We also compared the effects of ATR on gene expression with effects of E2 exposure. We include E2 as a positive control based on two factors. One, the effects of ATR on gonads appear to be estrogenic, as seen in the demasculinizing effects in testes (de Solla et al., 2006; Hayes et al., 2010). Two, steroid hormones such as estradiol have been shown to influence HP development and sexual differentiation of the HP in various species (Morris et al., 2004; Lenz and McCarthy, 2010). Our results reveal that an acute, embryonic exposure to ATR can alter gene expression within the HPG and HPA.

2. Methods

2.1. Egg collection and treatment

Snapping turtle eggs, embryos, and hatchlings were treated according to protocols approved by the Institutional Animal Care and Use Committee at the University of North Dakota (UND). We collected eggs immediately after oviposition from north-central

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