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Regulation of zebrafish (*Danio rerio*) locomotor behavior and circadian rhythm network by environmental steroid hormones☆

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ABSTRACT

Environmental exposure of fish to steroid hormones through wastewater and agricultural runoff may pose a health risk. Thus far, ecotoxicological studies have largely been focused on the disruption of the sex hormone system, but additional effects have been poorly investigated. Here we report on the effects of a series of different natural and synthetic steroid hormones on the locomotor behavior and the transcriptional levels of core clock genes in zebrafish eleuthero-embryos (*Danio rerio*). Of the 20 steroids analyzed, progestins and corticosteroids, including progesterone and cortisol, significantly decreased the locomotor activities of eleuthero-embryos at concentrations as low as 16 ng/L, while estrogens such as 17β-estradiol led to an increase. Consistently, progestins and corticosteroids displayed similar transcriptional effects on core clock genes, which were remarkably different from those of estrogens. Of these genes, *per1a* and *nr1d2a* displayed the most pronounced alterations. They were induced upon exposure to various progestins and corticosteroids and could be recovered using the progesterone receptor/glucocorticoid receptor antagonist mifepristone; this, however, was not the case for estrogens and the estrogen receptor antagonist 4-hydroxy-tamoxifen. Our results suggest that steroid hormones can modulate the circadian molecular network in zebrafish and provide novel insights into their mode of actions and potential environmental risks.

1. Introduction

The presence of natural and synthetic steroid hormones in the environment and their adverse effects in wild animals, especially the disruption of sex hormone signaling pathways and reproduction in fish and amphibians, are of concern (Christen et al., 2010; Fent, 2015; Hutchinson et al., 2006; Kumar et al., 2015; Sumpter and Johnson, 2005). In addition, there is growing concern that steroid hormones and compounds with similar activities, especially estrogens, progestins, and glucocorticoids, could also pose a risk to mammals, including humans, through contaminated drinking water and seafood, in addition to their use in human and veterinary medicine and as growth promoters in livestock (Sumpter and Johnson, 2005; Benotti et al., 2009; Liu et al., 2015).

Effect assessment of environmental steroid hormones in vertebrates is limited to the hypothalamic−pituitary−gonadal (HPG) axis and related to specific endocrine endpoints, such as fecundity and hormone levels. In aquatic organisms, the decrease of fecundity and adverse reproductive effects, steroid hormone imbalances, feminization or masculinization, altered sexual behavior, changes in gonadal histology, and alteration of sex development have been reported upon various environmental steroid hormone exposure (Jobling et al., 2006; Nash et al., 2004; Overturf et al., 2014; Runnalls et al., 2013). Transcriptional analysis revealed that hormone signaling pathways including gonadal steroidogenesis were commonly the targets of steroids at environmentally relevant concentrations (Chen et al., 2016; Filby et al., 2007; Overturf et al., 2014; Siegenthaler et al., 2017). Despite the increasing evidence of their adverse endocrine disrupting effects, additional physiological outputs resulting in health impacts are rarely investigated.

In previous transcriptome studies, we observed that several progestins altered the prominent transcriptional responses of core clock genes in zebrafish. Compared to other pathways affected,

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including the HPG-axis, alteration of the circadian network was most prominent, both in adult zebrafish and eleuthero-embryos (Zhao et al., 2015a; Zucchi et al., 2013). Subsequent investigations confirmed this phenomenon and revealed similar regulation on central and peripheral circadian rhythm by several other progestins such as progesterone (P4) and corticosteroids such as fludrocortisone acetate (FLU) (Zhao et al., 2015b, 2016). We repeatedly observed remarkable transcriptional alterations of several key clock genes, such as *arntl1a*, *per1a,* and *nr1d1*, in response to these steroids (Zhao et al., 2015b, 2016). However, interactions between environmental steroids and the circadian rhythm network in wild organisms are poorly investigated. Considering that dozens of highly prescribed steroids enter the environment, the question arises whether deregulation of the circadian rhythm and associated physiological pathways are significant but unexplored effects of these steroid hormones.

The circadian rhythm is an endogenous, entrainable oscillation of about 24 h, although it can also be adjusted according to the environment by external stimuli, such as temperature, sunlight, and redox cycles (Bass, 2012). Recently, it has been demonstrated that steroids, such as dexamethasone (DEX) and 17β-estradiol (E2), can also reset the circadian time in rodents. The associated alterations in the regulation of the circadian rhythm were independent of the endogenous clock and indicated changes in the transcriptional levels of core clock genes as a possible mechanism. DEX, a synthetic glucocorticoid, induced circadian gene expression in cultured rat-1 fibroblasts and can transiently alter the phase of circadian gene expression, especially *Per1*, in the liver, kidneys, and heart (Balsalobre et al., 2000). The depletion of endogenous glucocorticoids through adrenalectomy or selective genetic deletion of brain glucocorticoid receptors blunts the rhythm of expression of the clock proteins (Amir et al., 2004; Lamont et al., 2005). Similarly, exogenously applied E2 shortens the period of the locomotor activity rhythm in rodents; moreover, significant transcriptional responses of the *Period* gene in the suprachiasmatic nucleus and uterus of rats were observed (He et al., 2007; Nakamura et al., 2008). In mammals, *Per1* was proposed to be the primary target of the circadian rhythm system responses to steroid hormone exposure. Although the detailed molecular mechanisms are not yet recognized, studies revealed that progesterone/glucocorticoid receptor (PR/GR) is required for the regulation of circadian rhythm activities in rodents exposed to progestins/corticosteroids (Rubel et al., 2012).

In the present study, we explored the circadian locomotor activities and transcriptional changes of zebrafish eleuthero-embryos in response to a series of steroid hormones that are present in the environment. In total, 20 steroid hormones were analyzed, which included 7 progestins, 6 corticosteroids, 4 estrogens, and 3 androgens. These steroids are naturally excreted and commonly prescribed in human and veterinary medicine. They were detected in environmental water bodies, seafood, and breast milk and therefore posed potential health risk to vertebrates, including humans, due to water exposure or contaminated seafood (Benotti et al., 2009; Christen et al., 2010; Fent, 2015; Hutchinson et al., 2006; Liu et al., 2015; Kumar et al., 2015; Sumpter and Johnson, 2005). Here we demonstrate the interference of steroids on the locomotor activity and transcription level of core clock genes involved in zebrafish circadian rhythms. These data provide novel insights into the understanding of steroid action and their potential environmental risks.

2. Materials and methods

Chemicals and zebrafish maintenance. Chemical information is provided in the Supporting Information. Abbreviations for steroids are: progesterone (P4), dexamethasone (DEX), 17a-ethinyl estradiol (EE2), testosterone (TTR), levonorgestrel (LNG), gestodene (GES), dydrogesterone (DDG), drospirenone (DRS), medroxyprogesterone acetate (MPA), norethindrone acetate (NET), cortisol (CRL), prednisone (PRE), prednisolone (PREL), betamethasone (BET), fludrocortisone acetate (FLU), estrone (E1), 17β-estradiol (E2), estriol (E3), androstenedione (ADD) and androsterone (ADR). Regular care and maintenance of adult zebrafish was performed as described previously (Zhao et al., 2015a, 2015b).

Experimental design. *Steroid hormone exposure.* Fertilized zebrafish eggs were incubated at 27 ± 1 °C, with a photoperiod of 14:10 h (light:dark). At 96 h post fertilization (hpf), 100 eleuthero-embryos from the overall eleuthero-embryo population were selected by simple random sampling and placed into a 150-mL covered glass beaker containing 100 mL of reconstituted fish water (reconstituted deionized water with salts: $CaCl_2 \times 2H_2O$ 147.0 g/L, KCl 2.9 g/L, $MgSO_4 \times 7H_2O$ 61.6 g/L, $NaHCO_3$ 32.4 g/L; the conductivity was at 470–480 μS/cm) as a replicate and incubated at 27 ± 1 °C under constant darkness. At 120 hpf (i.e. circadian time (CT) 0), steroid hormones at appropriate concentrations were added. In the case of progesterone (P4), DEX, 17α-ethinylestradiol (EE2) and testosterone (TTR) exposures, the experimental setup consisted of a solvent control (0.01% DMSO) and increasing concentrations of each steroid at nominal concentrations of 0.1 nM, 10 nM, and 1 μM. For further experiments with 16 other steroid hormones, the experimental setup consisted of a solvent control (0.01% DMSO) and 1 μM of each steroid. Low concentrations were selected to reflect environmentally realistic doses (Christen et al., 2010; Fent, 2015; Hutchinson et al., 2006; Kumar et al., 2015; Sumpter and Johnson, 2005), and high concentrations were chosen as pharmacologically relevant, depending on the transcriptional responses of zebrafish clock genes observed in our previous studies for progestins and glucocorticoids (Zhao et al., 2015a, 2015b, 2016). Each treatment consisted of three replicates (100 eleuthero-embryos *per* replicate). From each treatment, 12 eleuthero-embryos from the overall population (four eleuthero-embryos from each of the three replicates) were selected by simple random sampling and assigned gently into a 48-well cell culture plate for continuous 48-h swimming behavior analysis (between 120 hpf and 168 hpf). The water was completely changed every 24 h, with the reconstituted fish water containing appropriate steroid concentrations. At 126, 132, 138, and 144 hpf (e.g., CT 6, 12, 18, and 24, respectively), 15 eleuthero-embryos from each replicate were pooled and their RNA was stored for further molecular analysis. All experiments were conducted under constant darkness.

Antagonist exposure. RU486 was used to antagonize the transcriptional responses of eleuthero-embryos under P4 or DEX exposure. The experimental setup consisted of a solvent control (0.01% DMSO), positive control (1 μM P4 or DEX), RU486 (0.1 and 1 μM), and their mixtures (1 μM P4+0.1 μM RU486; 1 μM P4+1 μM RU486; 1 μM DEX+0.1 μM RU486, and 1 μM DEX+1 μM RU486). Similarly, 4-hydroxy-tamoxifen (4-OHT) was used to antagonize the transcriptional responses of EE2, and the experimental setup consisted of a solvent control (0.01% DMSO), positive control (1 μM EE2), 4-OHT (0.1 and 1 μM), and their mixtures (1 μM EE2+0.1 μM 4-OHT and 1 μM EE2+1 μM 4-OHT). Eleuthero-embryo exposure and sampling were processed as described above for steroid hormone exposure experiments.

Chemical analysis. Chemical analyses were performed by solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (HPLC-MS-MS). The analytical methods were described previously (Zhao et al., 2015b, 2016), and details about water sampling, analytical procedures, chemical recoveries, and the limits of quantification is provided in the Supporting Information (SI Text and Tables S1-S2).

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