Chapter 11

Measuring Endocrine (Cortisol) Responses of Zebrafish to Stress

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Abstract

The zebrafish (*Danio rerio*) is rapidly becoming a popular model species in stress and neuroscience research. Their behavior, robustly affected by environmental and pharmacological manipulations, can be paralleled by physiological (endocrine) analysis. Zebrafish have a hypothalamic-pituitary-interrenal (HPI) axis, which is homologous to the human hypothalamic-pituitary-adrenal (HPA) axis. While mice and rats use corticosterone as their main stress hormone, both humans and zebrafish utilize cortisol. This protocol explains the whole-body cortisol extraction procedure and the use of the human salivary cortisol ELISA kit to measure the amount of cortisol in each zebrafish sample. The ability to correlate physiological data from individual fish with behavioral data provides researchers with a valuable tool for investigating stress and anxiety, and contributes to the utility of zebrafish neurobehavioral models of stress.

Key words: Zebrafish, physiological endpoint, HPA axis, HPI axis, stress, ELISA, cortisol.

1. Introduction

The hypothalamic-pituitary-adrenal (HPA) axis mediates the endocrine response to stress in humans and animals (1). Under stress, the paraventricular nucleus of the hypothalamus produces corticotropin-releasing factor (CRF), which is delivered to the anterior pituitary gland via the hypothalamic-hypophysial portal blood vessel system (2). CRF stimulates the anterior

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pituitary gland, causing the release of andrenocorticotropic hormone (ACTH) into the blood stream (3). When stimulated by ACTH, the adrenal cortex synthesizes glucocorticoid hormones from a cholesterol precursor (4, 5). Increased levels of glucocorticoids initiate metabolic effects that modulate the stress reaction (4, 6). These effects include the stimulation of gluconeogenesis, anti-inflammatory effects, and immune system suppression (7). The effects of the stress reaction are harmful in excess and are alleviated through a negative feedback to the hypothalamus and pituitary, which suppresses CRF and ACTH release (8, 3).

Mice and rats have traditionally been used for stress neuroendocrine research (9). Although they are phylogenetically closer to humans, rodent endocrine HPA systems utilize corticosterone as the main stress hormone (10). A similar mechanism has been found in teleosts, specifically zebrafish (*Danio rerio*) (11), whose hypothalamus-pituitary-interrenal (HPI) axis is homologous to HPA (**Fig. 11.1**). With cortisol being the main mediator of physiological response to stress, this makes zebrafish an excellent model for endocrine research (12–16). Here we report a simple protocol for analysis of whole-body zebrafish cortisol concentration as a physiological (endocrine) marker of stress and anxiety.

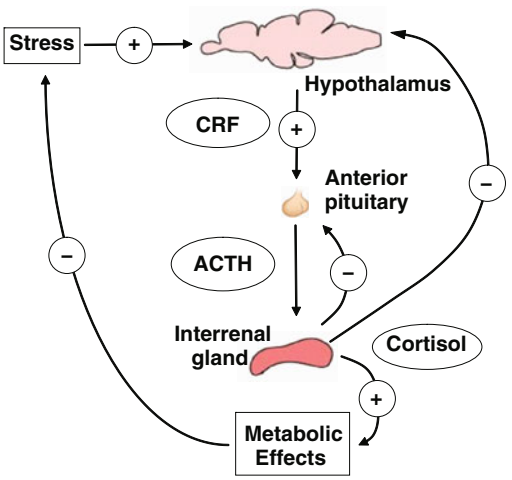

Fig. 11.1. The hypothalamic-pituitary-interrenal (HPI) axis in zebrafish. The hypothalamus secretes corticotropin-releasing factor (CRF), which stimulates the pituitary to release adrenocorticotropic hormone (ACTH). The interrenal gland, when stimulated by ACTH, secretes cortisol, the primary stress hormone in zebrafish. A negative feedback system acts on the hypothalamus to ensure homeostatic regulation. The human HPA axis functions similarly; however, it contains an adrenal gland in place of the interrenal gland.

2. Protocol

2.1. Animals and Housing

Adult (3–5 month old) zebrafish (male and female) can be purchased from commercial vendors. An acclimation period of at least 10 days must be given upon arrival into the laboratory environment. The fish are separated into groups of 20–30 and housed in 40-L home tanks filled with facility filtered water. The room and tank water temperature are maintained at 25–27°C with 12-h cycles of illumination with ceiling mounted artificial fluorescent lighting. Food is provided in the form of Tetramin Tropical Flakes (Tetra, USA). After behavioral study, fish are euthanized using 500 mg/L Tricane (Sigma-Aldrich, USA), and whole-body samples are used for cortisol assay (see further).

Cortisol extraction: Procedure for performing cortisol extraction on the whole body samples was adapted from Alderman and Bernier (13) and modified as described in (14, 17).

(a) Following behavioral study, whole body samples are collected and frozen at –20°C (or lower) for biological study to assess cortisol levels.

(b) Body samples are partially thawed, weighed, and then homogenized in 500 μL of ice-cold 1X phosphate-buffered saline (PBS) buffer. After recording weight (g), the whole body samples are dissected on ice into smaller parts for efficient homogenization. Homogenization can be performed using a Tissuemiser® from Fisher Scientific (USA). Note: Measuring the weight of the whole body sample prior to homogenization is absolutely necessary for determination of cortisol concentration following extraction and quantification (see further).

(c) The homogenizing rotor blade is washed with an additional 500 μL of ice cold 1X PBS and collected in a 2 mL tube containing the homogenate.

(d) The homogenizing rotor blade and probe must be washed with ethanol (100%) and deionized H_2O in-between each sample. This is an important step to minimize cross-contamination of samples.

(e) Samples are kept on ice throughout this process and then transferred to labeled glass extract-O tubes.

(f) 5 mL of diethyl ether (Fisher Scientific, USA) is added to each sample.

(g) The samples are vortexed for 1 min and then centrifuged at 5,000 rpm for 15 min.

(h) Following centrifugation, the organic layer containing cortisol was removed from each sample and placed in a

separate test tube. The process was repeated two (or three) times consistently throughout the experiment to ensure maximal cortisol extraction. The cortisol-containing layer (organic phase) is usually yellowish in color.

(i) Samples are kept overnight in the fume hood to allow for evaporation of ether. Other methods of drying the organic solvent could be used, such as a speed vacuum centrifuge equipped with a cryotrap, or the evaporation to dryness under nitrogen sparge.

(j) Ninety percent recovery was confirmed for this protocol using (8) [H]-testosterone as a tracer for evaluation.

Cortisol ELISA Assay:

(a) Cortisol is reconstituted in 1 mL of 1X PBS after ether evaporation and incubated overnight at 4°C.

(b) ELISA is performed per manufacturer's instructions to quantify cortisol concentrations using human salivary cortisol assay kit (Salimetrics LLC, USA).

(c) Absorbance from the reaction is measured in a VICTOR-WALLAC (Perkin Elmer, USA) or similar plate reader with the manufacturer's software package.

(d) Absorbance is adjusted by subtracting the measurements of non-specific binding wells to account for noise. Whole-body cortisol levels are then quantified using a 4-parameter sigmoid curve minus curve fit based on absorbance of standardized concentrations versus those observed in the samples. Cortisol levels are normalized based on the weight of the whole body sample and reported as relative circulating cortisol concentrations (ng/g body weight).

Troubleshooting (also see (17) *for details):*

(a) As circulating levels of cortisol fluctuate throughout the sleep/wake cycle, it is critical to perform behavioral experiments and sacrifice all subjects at the same time of day.

(b) Due to the volume of the tissue being homogenized, sectioning the whole body into smaller pieces prior to homogenization reduces the chance of losing material or jamming the equipment.

(c) The homogenizer must be carefully washed in ethanol and deionized water after each sample. Failure to fully wash and rinse the homogenizing blade will result in cross-contamination of samples that will confound results.

(d) Using glassware instead of plastic Eppendorf tubes helps reduce the loss of cortisol from samples. Cortisol tends to stick to the sides of plastic containers, and thus a percentage is lost upon each transfer. Additionally, ether degrades the plastic of Eppendorf tubes.

(e) Body size may affect the accuracy of cortisol detection. Use zebrafish of similar sizes. Embryonic or abnormally small zebrafish may result in a cortisol concentration below the ELISA assay's sensitivity threshold, and thus should be avoided if possible. Since the minimum threshold concentration has not been determined, it may be required to combine multiple samples when using embryonic subjects.

(f) Using a radioactive tracer (e.g., tritium) can be useful in determining the amount of cortisol lost during the extraction procedure; this proportion can be used to correct the concentration of cortisol per gram of fish for a more accurate analysis.

(g) Because equipment in different laboratories vary, it is possible to adapt the amount of ether used and the number of extractions done. Usually, a 1:3 to 1:5 solute:solvent ratio is used. To obtain the highest yield, repeat the extraction procedure several times. However, the amount of ether used and the number of extractions performed must be standardized for all samples used in the study.

(h) If the homogenate becomes an emulsion after adding ether, more ether before centrifugation may help separate the homogenate. However, if additional ether is used for separation, the remaining samples must similarly be treated for standardization and consistency of data. Additionally, increasing the speed of centrifugation can assist in phase separation.

(i) Before performing the salivary cortisol ELISA, make sure to graph the plate layout and the position of each sample (to assist in locating the samples for future quantification). Note that duplicating standards and samples is recommended to account for pipetting and human error.

(j) Always handle hazardous materials with care and according to Institutional and laboratory guidelines. Ether emits toxic fumes and thus must be handled and evaporated in a fume hood. Radioactive materials require proper attire and conduct.

(k) Laboratory temperature may affect the outcome of the extractions. To prevent confounding the results, be sure to keep the lab temperature standardized throughout this procedure.

(l) Samples can be stored for a long period (several months) at –20°C or –80°C before cortisol extraction procedure.

(m) In order to prevent cross-contamination, pipette tips must be changed after use and equipment must be cleansed after contact per sample.

(n) After centrifuging, the organic (containing cortisol) phase layer is the top layer and hence is the layer to be collected and stored.

(o) To prevent confusion, label each test tube properly. Be aware that ether will wash away marker labels on glass tubes if spilled.

(p) Take caution when pipetting ether, as it tends to leak initially.

2.2. Typical Results

Figure 11.2 represents data collected after drug exposure and withdrawal experiments performed on zebrafish in our laboratory in 2009. The consistency of the results in respect to increased whole-body cortisol concentrations following introduction of stressful stimuli is in line with behavioral data gathered in these and previous studies (14, 17). Zebrafish behavioral research frequently uses the novel tank paradigm, a test that exploits the instinctive anxiety-like behavior induced by exposure to a novel environment. Numerous studies reported that new environment as well as additional stressors (e.g., presence of predators, alarm pheromone or drug withdrawal syndrome), lead to specific behavioral phenotypes (representative of anxiety) including decreased exploration, increased freezing, and increased erratic (darting) movements. In **Fig. 11.2**, paralleling this anxiety-like behavior (behavioral data not shown), whole-body cortisol analysis of anxious drug withdrawal zebrafish predictably reveals significantly increased cortisol concentrations.

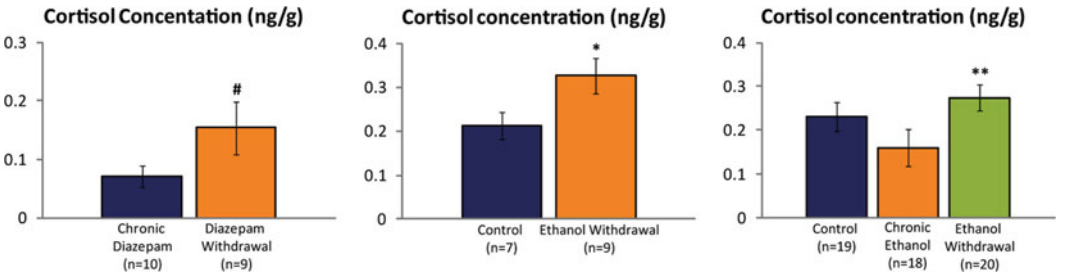

Fig. 11.2. Zebrafish endocrine responses (whole-body cortisol, ng/g fish) to withdrawal from diazepam and ethanol. *Left* to *right*: 72-h withdrawal from chronic diazepam (72 μg/ml, 2 weeks); 12-h withdrawal from chronic ethanol (0.3%, 1 week); chronic ethanol exposure (0.3%, 1 week) and 12-h withdrawal from chronic ethanol (0.3%, 1 week). Data are presented as mean ± SEM (*p<0.05, **p<0.01, #p=0.05–0.1, trend, U-test).

3. Discussion

The protocol described here (also see (14, 17)) represents a significant modification of previously employed cortisol assays for assessing physiological stress in zebrafish. In prior studies, human

serum cortisol kits necessitated combining multiple fish for a single cortisol sample. Here, we used human salivary cortisol kits that are sensitive to a full range of cortisol levels from 0.003 to 3.0 μg/dL. Such high sensitivity enables quantification of whole-body cortisol in individual fish samples. This methodological modification has significant consequences for the utility of zebrafish to study anxiety and stress disorders, because smaller sample groups can be used, and additional data becomes available to correlate behavioral and endocrine responses to stress in individual fish (14, 17).

Analysis of the physiological (neuroendocrine) responses to stress in zebrafish is a valuable tool complementing behavioral studies. The cortisol extraction procedure and human salivary ELISA assay are relatively simple, inexpensive, and can be easily adopted in a variety of laboratory settings. Additional modifications to the protocol may enhance the yield further, as discussed in the troubleshooting section. Likewise, statistical analysis of correlation between behavior and endocrine response may further assist in data interpretation. For example, the Spearman's rank correlation coefficient, used to assess the relationship between two variables, can determine the level of correlation between behavioral data and cortisol concentration values.

Overall, the ability to parallel physiological responses (i.e., cortisol production) with behavioral responses provides researchers with an important tool for investigating stress-related responses (17). The use of human salivary cortisol assays provides physiological evidence measuring the endocrine stress response in individual zebrafish that can be associated with anxious behavioral responses. The method is also cost-effective, as compared to measuring cortisol levels using more expensive tools, such as mass spectrometry or gas chromatography. This new protocol offers a simple, fast, reliable, and cost-effective method to measure the endocrine stress response in zebrafish.

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