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Neuroendocrine regulation of the stress response in adult zebrafish, *Danio rerio*

Michail Pavlidis *, Antonia Theodoridi, Aleka Tsalafouta

University of Crete, Department of Biology, P.O. Box 2208, GR-70013 Heraklion, Crete, Greece

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ABSTRACT

The main objectives of this study were to investigate the dynamics of the cortisol stress response and the underlying molecular regulation in adult zebrafish exposed to acute and long-term stressors that differed in nature, duration and relative intensity. Fish showed a very rapid and prolonged increase in trunk cortisol concentrations, starting at around 15 min and returning to basal levels at around 2 h following exposure to acute stressors. In addition, acute stress affected significantly brain mRNA expression levels of several genes (corticotropin-releasing factor, *crf*; pro-opiomelanocortin, *pomc*; glucocorticoid receptor, *gr*; MR/GR ratio; prolactin, *prl*; hypocretin/orexin, *hcrt*; brain-derived neurotrophic factor, *bdnf*; *c-fos*). Exposure of fish to unpredictable relatively low-grade environmental and husbandry stressors (SP-1) did not affect the overall behaviour of fish, as well as trunk cortisol concentrations. Fish exposed to relatively higher-grade long-term stressors (SP-2) showed elevated cortisol levels as well as significant changes in most of gene transcripts. In particular, fish exposed to SP-2 showed statistically significant upregulation in brain *gr*, *mr*, *prl* and *hcrt* compared to SP-1 and control individuals. The highest mean values of *bdnf* transcripts were found in SP-2 exposed zebrafish and the lowest in control fish, while an approximately 5 to 6-fold upregulation was observed in *c-fos* mean relative mRNA levels of long-term stress-exposed fish, regardless of stressor intensity, compared to control zebrafish. In conclusion, we developed realistic acute and unpredictable long-term stress protocols, based on husbandry and environmental stressors and physical, chemical, mechanical and social stimuli that fish may experience either in nature or under intensive rearing conditions.

1. Introduction

The concept of stressors, the notion of coping and the distinction between eustress and distress were introduced several decades ago by Selye (Selye, 1936, 1946; Tache and Selye, 1985). With the conceptualisation of *perception*, *cognition* and *appraisal*, Sterling and Eyer (1988), McEwen (1998, 2000) and McEwen and Wingfield (2003) developed the concepts of allostasis, allostatic load, mediators of allostasis and coping strategies (Koolhaas et al., 1999). Romero et al. (2009), in a recent attempt to integrate homeostasis, allostasis and stress, proposed the reactive scope model, taking into consideration both the circadian and seasonal fluctuations of the concentration of a physiological mediator (e.g., blood cortisol) as well as a low and a high stable threshold below and above which homeostatic failure and homeostatic overload occur, respectively. The range between these two thresholds is termed reactive scope, which is the physiological range of the mediator. The acute stress response, described as the cascade of endocrine and metabolic changes following exposure of an individual to stimuli of high intensity and short duration, has been well investigated in various fish species. In general, there is a primary response involving the release in the circulation of catecholamines and cortisol, a secondary response comprising changes in several metabolic and physiological parameters (e.g., blood glucose, lactate, osmotic pressure, liver glycogen), and a tertiary one involving whole animal changes in the case where the individual is unable to acclimate or adapt (Barton, 2002; Barton and Iwama, 1991; Fanouraki et al., 2011; Tort et al., 2011; Wendelaar Bonga, 1997). However, the concept of chronic stress still causes serious constraints due to the absence of concrete operational definitions, validated protocols and reliable indicators for diagnosis. An appropriate methodology for investigating the effects of animal exposure to long-term stressors is lacking. Nevertheless, despite the important differences in form and context of the chronic-stress concept, there is a general agreement that the interactions between the intensity, duration, frequency,

Abbreviations: ACTH, adrenocorticotropic hormone; *avt*, arginine vasotocin; *bdnf*, brain-derived neurotrophic factor; *crf*, corticotropin-releasing factor; CRH, corticotropin-releasing hormone; *gr*, glucocorticoid receptor; *hcrt*, hypocretin/orexin; HPA axis, hypothalamic-pituitary-adrenal axis; HPI axis, hypothalamic-pituitary-interrenal axis; HR, high responding; LR, low responding; *mc2r*, melanocortin 2 receptor; *mr*, mineralocorticoid receptor; MSH, melanocyte-stimulating hormone; *pomc*, pro-opiomelanocortin; *prl*, prolactin; SP-1, Long-term stress protocol-1; SP-2, Long-term stress protocol-2; UCS, unpredictable chronic stress

* Corresponding author at: University of Crete, Department of Biology, GR-70013 Heraklion, Crete, Greece.
E-mail address: pavlidis@biology.uoc.gr (M. Pavlidis).

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(un)predictability and (un)controllability of environmental stimuli, the genetic background and life history of a given vertebrate individual are essential for its welfare (Korte et al., 2007).

Zebrafish, *Danio rerio*, is a vertebrate model in biomedical research and it has recently been proposed as a prominent model in the study of stress physiology and anxiety (Alsop and Vijayan, 2009; Egan et al., 2009; McGonnell and Fowke, 2006; Pavlidis et al., 2011, 2013). Following exposure to acute stressors like net handling, air exposure and increase in water current (vortex speed), zebrafish show a rapid increase (15 to 30 min post-stress) in whole-body cortisol levels and water-born cortisol, as well as differences in the expression of several genes involved in the regulation of the Hypothalamic–Pituitary–Interrenal (HPI) axis (Barcellos et al., 2007; Fuzzen et al., 2010; Pavlidis et al., 2013; Ramsay et al., 2009). Concerning chronic stress, stocking density and water quality are the main factors studied so far (Pavlidis et al., 2013; Ramsay et al., 2006). However, there is a lack of validated, reliable and repeatable protocols for evaluating the effects of unpredictable long-term stressors on zebrafish, and fish in general. In rats, chronic mild stress protocols have been developed to study depression (Schweizer et al., 2009; Willner, 2005), but in fish species there are only three recent studies in adult zebrafish (Chakravarty et al., 2013; Piato et al., 2011) and in European sea bass, *Dicentrarhus labrax* (Tsalafouta et al., 2014).

The objectives of this study were (a) to propose an unpredictable long-term stress protocol that can be used to elucidate the chronic-stress response in fish, and (b) to investigate the dynamics of the cortisol stress response and the underlying molecular regulation in adult zebrafish exposed to acute and long-term husbandry stressors.

2. Materials and methods

2.1. Experimental fish

Wild-type zebrafish (*D. rerio*) of Malaysian origin (Singapore import) were purchased through a Greek wholesaler. Fish were transferred to the installations of the Fish Physiology Laboratory at the Department of Biology, University of Crete, and placed in 250 L holding aquaria, equipped with biological filters (EHEIM external canister filter, EHEIM GmbH & Co. KG), facilities for temperature and photoperiod control, and air stones. Fish were maintained under a 12L:12D photoperiod regime and a water temperature of 25 to 26 °C. Oxygen and pH measurements were performed daily, and ammonia, nitrite and nitrate weekly. Fish were fed twice daily with commercial aliment (Tropical Fish Flakes, Prodac International S.r.l., Italy).

2.2. Experiment 1: acute stress response

To study the molecular and endocrine (cortisol) regulation of the acute stress response in zebrafish, 58 adult 1+ year-olds (mean body weight [b.w.] ± standard error of the mean [SEM]: 0.43 ± 0.02 g) were caught by net from the holding aquaria following a decrease of the water level to 25% of the initial volume, and placed in a 10 L bucket. Fish were chased for 5 min, exposed to air for 1 min and then transferred to 8 × 2.0 L beakers (6 fish per beaker, two beakers per sampling point). The fish were sampled at regular intervals (15, 30, 60 and 120 min post-stress) according to previous published data (Pavlidis et al., 2013; Ramsay et al., 2009). At each respective sampling point, fish were caught and placed in anaesthetic less than 1 min. Before the application of the stressor, 10 fish were caught and served as controls (0 h).

2.3. Experiment 2: long-term stress response

To develop useful long-term stress protocol based on ordinary husbandry parameters and to study its effect on molecular and endocrine factors involved in the regulation of the stress response in zebrafish, 128 adult 1+ year-olds (mean b.w. ± SEM: 0.55 ± 0.03 g) were used, at a female to male ratio of 1:2. Fish were transported from the holding 250 L glass aquaria to the experimental aquaria and the experiments started after a habituation period of one week. Each experimental aquarium was equipped with an internal aquarium filter (RESUN Magi — 200) and tank heater (RESUN THERM 25/300 — RH 9000) for temperature control. Water temperature was set at 25 to 26 °C and the photoperiod at 12L:12D. Water chemical parameters were monitored daily. Two protocols were designed and evaluated; both based on unpredictability and long-term duration, and differing in the nature and magnitude of the stressors applied. In both protocols three or four different types of low- or high-grade environmental and husbandry stressors were applied randomly on a daily basis for a total period of 12 days (Tables 1 & 2).

Long-term stress protocol 1 (SP-1, Table 1) consisted of relatively low-grade environmental and husbandry stressors. SP-1 included *optical* (increase in light intensity from 2.57 to 5.14 μmol m^{-2} s^{-1} for 30 min; lights on for 15 min twice during the night; lights off for 15 min twice during the day; exposure to blue or red spectrum for 30 min; introduction of plastic plant for 30 min); *mechanical* (high water current 600 L h^{-1} for 30 min); *chemical* (increase of water pH by 0.3 with the addition of 15 mL NaOH for 30 min; decrease of pH by 0.3 with the addition of 2.5 mL HCl for 30 min; administration of food extract by pulverizing food and dissolving it in 3 mL water); and *social* (crowding through the reduction of the water level to 1/3 of the initial water level, resulting in 6 fish per L compared to the initial 2 fish per L; introduction of novel object, i.e., plastic fish) stimuli. Full spectrum lights (Phillips, TLD 36W) were used to approximate natural sunlight and transparent filters to produce the blue (maximum absorption spectrum 450–475 nm) and red (maximum absorption spectrum 620–750 nm) spectra. Two 10 L aquaria with 20 fish per aquarium were used. An undisturbed group of fish held under identical conditions of water quality and stocking density served as controls. To minimize the number of sacrificed animals, forty-eight fish were euthanized to obtain brain and trunk samples.

Long-term stress protocol 2 (SP-2, Table 2) consisted of higher-grade, compared to SP-1, environmental and husbandry stressors. SP-2 included *optical* (lights off at day or lights on at night for 15 min three times), *husbandry* (chasing with a net for 5 min; restrained in the net for 5 min and exposed to air for 1.5 min) and *social* (crowding for 15 min through the decrease of the water level to 1/4 of the initial water volume, resulting in 8 fish per L compared to the initial 2 fish per L; isolation for 5 min in 80 mL beakers). Two 6 L aquaria with 12 fish in each were used. An undisturbed group of fish held under identical conditions of water quality and stocking density served as controls. To minimize the number of sacrificed animals, forty-eight fish were euthanized to obtain brain and trunk samples.

Crowding as a stressor applied in both protocols was based on the notion of keeping the same number of fish in a smaller water volume. Before, during and after the application of the stressor, oxygen levels were monitored, and remained at adequate levels (5.1–5.8 mg L^{-1}). Therefore, any stress response from the zebrafish was not due to poor water quality.

In all experiments, fish were caught by net and immediately sacrificed by immersion in ice-cold water to avoid any possible increase in cortisol concentrations due to the use of anaesthetic as well as degradation of brain RNA. The head and caudal fin were then cut and trunks were weighed, frozen in 1.5-mL eppendorfs on dry ice, and stored at −80 °C for cortisol determination. Brains were dissected and samples from two fish of the same sex were pooled and placed in liquid nitrogen for mRNA expression studies. In chronic-stress experiments, half of the fish per tank were sampled one day following the end of the experiment and the rest were exposed to acute stressors (chasing for 5 min with a net and exposure on air for 1 min) and sampled at 15 min post-stress. Brain samples were collected only from non-acutely-stressed fish.