Progress in Neuro-Psychopharmacology & Biological Psychiatry 35 (2011) 561–567

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Unpredictable chronic stress model in zebrafish (*Danio rerio*): Behavioral and physiological responses

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ARTICLE INFO

Article history:
Received 5 November 2010
Received in revised form 25 November 2010
Accepted 18 December 2010
Available online 25 December 2010

Keywords:
Anxiety
Cortisol
Depression
Unpredictable chronic stress protocol (UCS)
Zebrafish

ABSTRACT

Zebrafish (*Danio rerio*) have emerged as a promising model organism to study development, toxicology, pharmacology, and neuroscience, among other areas. Despite the increasing number of studies using zebrafish, behavioral studies with this species are still elementary when compared to rodents. The aim of this study was to develop a model of unpredictable chronic stress (UCS) in zebrafish. We evaluated the effects of UCS protocol during 7 or 14 days on behavioral and physiological parameters. The effects of stress were evaluated in relation to anxiety and exploratory behavior, memory, expression of corticotrophin-releasing factor (CRF) and glucocorticoid receptor (GR), and cortisol levels. As expected, UCS protocol increased the anxiety levels, impaired cognitive function, and increased CRF while decreased GR expression. Moreover, zebrafish submitted to 7 or 14 days of UCS protocol presented increased cortisol levels. The protocol developed here is a complementary model for studying the neurobiology and the effects of chronic stress in behavioral and physiological parameters. In addition, this protocol is less time consuming than standard rodent models commonly used to study chronic stress. These results confirm UCS in zebrafish as an adequate model to preclinical studies of stress, although further studies are warranted to determine its predictive validity.

1. Introduction

Zebrafish (*Danio rerio*), a fish native to India, is a promising player in disease research and drug screening. It has been used to study development (Fetcho and Liu, 1998), neuroscience (Becker and Becker, 2008), pharmacology (Bencan et al., 2009; Egan et al., 2009), toxicology (Komjarova and Blust, 2009), behavior (Wong et al., 2010; Mathur and Guo, 2010), and teratology (Yang et al., 2009). This species has relatively high genetic homology to humans and provides many advantages when compared to other vertebrates, such as low cost, easy handling and maintenance, and fast reproduction (Barbazuk et al., 2000; Guo, 2004; Egan et al., 2009).

Despite specific embryological distinctions, the zebrafish brain is neuroanatomically and functionally comparable to mammals (Guo, 2004). Several neurotransmitter systems have been documented in zebrafish, e.g. dopaminergic (Schweitzer and Driever, 2009; Kastenhuber et al., 2010; Yamamoto et al., 2010), serotoninergic (Lillesaar et al., 2007), noradrenergic (Kastenhuber et al., 2010), and purinergic (Rosemberg et al., 2007). In zebrafish the stress system is represented by the hypothalamus–pituitary–interrenal (HPI) axis, which has already been characterized in detail (Alsop and Vijayan, 2008; Alderman and Bernier, 2009; Alsop and Vijayan, 2009). Similarly to the mammalian hypothalamus–pituitary–adrenal (HPA) axis, the zebrafish HPI axis controls the levels of circulating cortisol.

Abbreviations: UCS, unpredictable chronic stress; CRF, corticotrophin-releasing factor; GR, glucocorticoid receptor; HPI, hypothalamus–pituitary–interrenal; HPA, hypothalamus–pituitary–adrenal; ACTH, adrenocorticotropic hormone; COBEA, Brazilian Collegium of Animal Experimentation; CCAC, Canadian Council for Animal Care; GBT, Group Behavior Task; IA, inhibitory avoidance; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate buffered saline.

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doi:10.1016/j.pnpbp.2010.12.018

Activation of this system initiates at the hypothalamus, which receives inputs transmitted from central and peripheral nervous systems. A stressful signal stimulates secretion of hypothalamic corticotrophin-releasing factor (CRF). In response to CRF, the pituitary releases adrenocorticotropic hormone (ACTH) into the bloodstream, which reaches the head kidney of fish (homologous to the adrenal gland in mammals). Then, cortisol is secreted and binds to glucocorticoid receptor (GR), a ligand-activated nuclear transcription factor. GR regulates transcription of target genes related to glucose metabolism, immune function, and behavior (Mommsen et al., 1999; Bury and Sturm, 2007). This intricate signaling system resembles the human neuroendocrine system both in complexity and regarding cortisol utilization (as opposed to corticosterone in rodents), reinforcing the contribution of zebrafish to studies on the neurobiology of stress.

An extensive behavioral repertoire has been described for zebrafish. However, behavioral studies with this species are still elementary when compared to rodents (Gerlai, 2010a,b), and most of the behavioral research conducted so far has focused on analysis of natural and innate behaviors (Serra et al., 1999; Zhdanova et al., 2001; Yokogawa et al., 2007; Buske and Gerlai, 2010; Filby et al., 2010; Gerlai, 2010b).

Although there is evidence that zebrafish may be suitable to study the neurobiology of human psychopathologies, the assessment of stress in this model animal has been scarcely characterized (Egan et al., 2009; Champagne et al., 2010). Considering that zebrafish genome has already been assembled and genetic knowledge and tools are available, establishing a chronic stress protocol may be important to better understand the underlying mechanism of stress. Therefore, the purpose of this study was to develop a model of unpredictable chronic stress in zebrafish. We evaluated the effects of chronic stress on anxiety and exploratory behavior, memory, CRF and GR expression, and whole-body cortisol.

2. Material and methods

2.1. Animals and housing

A total of 255 adult male "wild type" (short fin) zebrafish (*D. rerio*) were obtained from a commercial supplier (Red Fish, Porto Alegre, Brazil). All fish were acclimated for at least two weeks in the experimental room and housed in groups of 20 fish in 15 l heated (28 ± 2 °C) tanks with constant aerated water. Fish were kept on a 14–10 h day/night cycle and fed three times a day with commercial flakes (TetraMin®) and supplemented with live brine shrimp. All protocols were approved by the Institutional Animal Care Committee (09/00126, CEUA-PUCRS) and followed Brazilian legislation, the guidelines of the Brazilian Collegium of Animal Experimentation (COBEA), and the Canadian Council for Animal Care (CCAC) guide on the care and use of fish in research, teaching, and testing.

2.2. Unpredictable chronic stress protocol (UCS)

Following a two-week habituation period, fish were submitted twice a day to one of the following stressors either during 7 or 14 days (Table 1): restraint stress, consisting of maintaining each animal for 90 min inside a small 2 ml microcentrifuge tube open in both ends to allow water flow; heating tank water up to 33 °C for 30 min; social isolation, maintaining animals alone for 45 min in a 250 ml beaker; cooling tank water up to 23 °C for 30 min; crowding of 10 animals for 50 min in a 250 ml beaker; exposition to predator (*Archocentrus nigrofasciatus* fish) in close proximity for 50 min but avoiding direct contact; low water level on housing tanks until animals' dorsal body wall were exposed for 2 min; tank water replacement, three consecutive times with animals inside; tank change, three consecutive times; and chasing animals for 8 min with a net.

Aeration and temperature were controlled during each stressor presentation (except during heating and cooling stress). To prevent habituation and maintain unpredictability, time and sequence of stressors' presentation were changed daily. A non-stressed control group remained in the same room during the equivalent 7- or 14-day period. Two separated sets of control and stressed fish were used to evaluate GR and CRF expression and cortisol levels. Despite the stressful conditions intermittently presented to the fish, no extreme suffering was caused nor abnormal number of deaths observed. The experimental design is shown in Fig. 1.

2.3. Behavioral apparatus

Twenty four hours after UCS protocol, a group of three fish were subjected during 10 min to the Group Behavior Task (GBT). GBT consists in simultaneously analyzing animals' locomotion, color, shoal cohesion and height on water column in a 2.7 l $24 \times 8 \times 20$ cm tank (length × width × height) with 15 cm of water level. Scores were attributed at minutes 1, 2, 3, 4, 5, and 10. The mean value of the scores during the 10 min period was calculated for each fish. All parameters in GBT were compared with control group and analyzed by two raters blinded. Water temperature was maintained with heaters. The lateral and back sides were visually blocked with white opaque self-adhesive plastic film to reduce the influence of the surrounding area and to facilitate observation. Before and after the test, oxygen levels in water were analyzed and remained adequate during the experiment (8 ppm, Labcom Test®, Camboriú, SC, Brazil). One week after stress, all fish were retested in the behavior apparatus with the aim of evaluating the potential residual effect of stress in the fish.

2.3.1. Behavioral scores in the Group Behavior Task

2.3.1.1. Height in the tank. The position (bottom × middle × upper levels) was considered an index of anxiety, similar to the position near the wall versus the center of an open field with rodents (Levin et al.,

Table 1
Procedure of the unpredictable chronic stress protocol in zebrafish.

Weeks	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week 1	9:00 am Restraint stress	10:00 am Social isolation	10:30 am Crowding	9:00 am Low water level	8:00 am Cooling	11:00 am Tank change	8:00 am Heating
	2:00 pm Heating	4:00 pm Cooling	1:30 pm Predator	3:00 pm Tank change	2:00 pm Crowding	5:30 pm Chasing	12:00 pm Social isolation
Week 2	10:00 am Tank change	11:00 am Predator	10:30 am Low water level	8:00 am Tank change	9:30 am Restraint stress	8:30 am Social isolation	9:00 am Tank change
	4:00 pm Tank change	2:30 pm Heating	3:00 pm Chasing	1:00 pm Crowding	5:00 pm Low water level	1:00 pm Cooling	0:30 pm Chasing
Week 3	0:30 pm Behavior test: GBT or euthanasia and collection of material	0:30 pm Behavior test: Training in the inhibitory avoidance apparatus	0:30 pm Behavior test: LTM in the inhibitory avoidance apparatus/extinction	0:30 pm Behavior test: Extinction	0:30 pm Behavior test: Extinction		