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

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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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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		

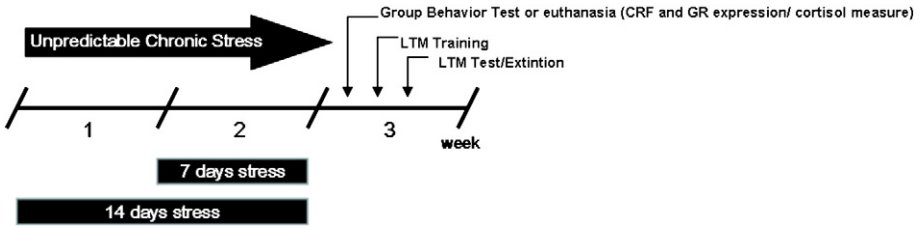

Fig. 1. Experimental design representative of the UCS protocol.

2007; Bencan et al., 2009). Fish position in the test tank was noted according to the following scores during 1-minute observations: 1 — only in the bottom third of the tank; 2 — preference for the lower two thirds of the tank; 3 — similar times exploring the 3 thirds; 4 — preference for the upper two thirds; 5 — only in the upper third.

2.3.1.2. Locomotion. Locomotor activity was used as a general index of behavioral excitation/inhibition. Activity was evaluated by comparison to control group using the following scores: 1 — virtually immobile; 2 — slower than normal; 3 — normal; 4 — increased locomotion; 5 — intense locomotion.

2.3.1.3. Color. Zebrafish change their color in response to certain stimuli. Fish that exhibit signs of fear (e.g. freezing or erratic movement) quickly become pale, especially when the background is light. When fish are more excited or aggressive, they become more chatoyant (Gerlai et al., 2000). Fish color was rated visually by comparing to control group and scored as follows: 1 — pale; 2 — lighter than normal but not pale; 3 — normal; 4 — darker than normal but not chatoyant with dark-blue stripes; 5 — chatoyant with dark-blue stripes.

2.3.1.4. Shoal cohesion. Zebrafish prefer swimming in groups and group aggregation is termed shoal cohesion (Engeszer et al., 2007; Miller and Gerlai, 2007; Saverino and Gerlai, 2008). This behavioral strategy is thought to be effective against predators in several fish species (Detrich et al., 1999; Gerlai et al., 2000). In contrast to other studies using only one fish during experiments, placing three in the test tank allows maintenance of their natural shoaling behavior. Shoal cohesion was measured by comparison to control group according to the following scores: 1 — complete lack of group cohesion or fish interaction; 2 — loose or partial shoaling behavior; 3 — normal distance and shoaling behavior; 4 — increased shoal cohesion.

2.4. Inhibitory avoidance protocol

Long-term memory was evaluated using an inhibitory avoidance (IA) protocol described in detail by Blank et al. (2009). Forty-eight hours after the end of the UCS protocol animals were individually trained and tested in a white/dark compartment IA apparatus, followed by memory extinction. Briefly, on each session animals were gently placed in the white tank compartment while the sliding partition between environment compartments was closed. After 1 min of habituation and orientation, the partition was raised, allowing fish to cross to the dark side of the tank through a 1 cm high opening. On training session, immediately after crossing to the dark compartment the sliding partition was closed and a pulsed electric shock of 3 ± 0.2 V AC administered for 5 s, after which animals were removed from the apparatus. Twenty-four hours after training animals were submitted to a test session that repeated the training protocol except that no shock was administered and the sliding partition was kept open, allowing animals to freely explore the apparatus for 1 min after crossing to the dark side for the first time. The test session was followed by identical extinction sessions with a 24-hour interval each. The latency to enter the dark compartment was measured in all sessions and the test and extinction latencies were used as an index of retention and extinction, respectively.

2.5. CRF and GR expression

Zebrafish were cryoanaesthetized and euthanized 24 h after the UCS protocol (Wilson et al., 2009), and brains were removed by dissection. The expression of CRF and GR genes were analyzed by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. To standardize the RNA extraction, all animals were euthanized at the same time of day (12:30 pm). Total RNA from zebrafish brain was isolated using the TRIzol® reagent (Invitrogen) in accordance with the manufacturer's instructions. The purity of the RNA was spectrophotometrically quantified by calculating the ratio between absorbance values at 260 and 280 nm and its integrity was confirmed by electrophoresis through a 1% agarose gel. Afterwards, all samples were adjusted to 160 ng/μl and cDNA species were synthesized using SuperScript III™ First-Strand Synthesis SuperMix Kit (Invitrogen, USA), following the supplier's instructions.

PCR reactions were performed (total volume of 25 μl) using a concentration of 0.1 μM primers, 0.2 μM dNTP, 2 mM $MgCl_2$ and 0.5 U platinum Taq DNA polymerase (Invitrogen). Conditions for CRF PCR were as follows: initial 1-minute denaturation step at 94 °C, 1-minute at 94 °C, 1-minute annealing step at 60 °C, 1-minute extension step at 72 °C for 25 cycles and a final 10-minute extension at 72 °C. Conditions for GR PCR were as follows: initial 1-minute denaturation, step at 94 °C, 1-minute at 94 °C, 1-minute annealing step at 62 °C, 1-minute extension step at 72 °C for 35 cycles and a final 10-minute extension at 72 °C. Conditions for β-actin PCR were as follows: initial 1-minute denaturation step at 94 °C, 1-minute at 94 °C, 1-minute annealing step at 54 °C, 1-minute extension step at 72 °C for 35 cycles and a final 10-minute extension at 72 °C. Zebrafish sequence encoding to CRF and GR were retrieved from GenBank database (NM_001007379 and EF_436284, respectively) and used for searching specific primers, which were designed using Oligos 9.6 program. In order to confirm primers specificity, each primer was compared with zebrafish genome and was able to recognize only its specific target sequence. Thus, the strategy adopted to construct the primers did not allow cross-amplification. The following set of primers were used: for CRF: forward 5′-TCG TCA CCA CGG TGG CTC TGC TCG-3′ and reverse 5′-CAG ATG AAA GGT CAG ATC TAG GGA AAT CG-3′; for GR: forward: 5′-AAC ATG CTG TGT TTC GCT CC-3′ and reverse: 5′-CTG CAA GCA TTT CGG GAA AC-3′; for β-actin: forward 5′-GTC CCT GTA CGC CTC TGG TCG-3′ and reverse 5′-GCC GGA CTC ATC GTA CTC CTG-3′.The amplification products were: CRF 383 bp, GR 401 bp and β-actin 383 bp. In order to confirm cDNA sequences amplified in RT-PCR reactions, the putative gene products were sequenced in both directions using MegaBase 1000 automatic sequencer. Resulting chromatograms were analyzed and cDNA sequences were also blasted using NCBI-BLAST searches of GenBank, confirming the identity and specificity of each sequence amplified (data not shown). PCR products were submitted to electrophoresis using a 1% agarose gel and the relative abundance of mRNA versus β-actin was determined by densitometry using freeware ImageJ 1.37 for Windows.

2.6. Measurement of cortisol

The extraction and measurement of cortisol from zebrafish have been described in detail by Barcellos et al. (2007). Briefly, fish were captured and immediately frozen in liquid nitrogen and stored at −80 °C until cortisol extraction. Each zebrafish was weighed, and a pool of three fish were minced and placed into a disposable stomacher bag with 2 ml of phosphate buffered saline (PBS, pH 7.4) for 6 min. The contents were transferred to a 10 ml screw top disposable test tube and 5 ml of laboratory grade ethyl ether was added. The tube was vortexed for 1 min and centrifuged for 10 min at 3000 rpm. The tube was then immediately frozen at liquid nitrogen and the unfrozen portion (ethyl ether containing cortisol) was decanted. The ethyl ether was transferred to a new tube and completely evaporated under a gentle stream of nitrogen for 2 h, yielding a lipid extract containing the cortisol. The extract was stored at −20 °C until the ELISA was conducted on the samples suspended with 1 ml of PBS buffer. In order to prevent a possible stress response induced by manipulation, the time elapsed between capture and killing was less than 10 s. Whole-body cortisol was measured in duplicate samples of tissue extract with a commercially available High Sensivity Salivary Cortisol — enzyme immunoassay kit (Salimetrics®, USA). The specificity of the test was evaluated by comparing the parallelism between the standard curve and serial dilutions of the tissue extracts in PBS (pH 7.4). The standard curve constructed with the human standards ran parallel to that obtained using serial dilutions of zebrafish tissue extracts. In the linear regression test, high positive correlation ($R2 = 0.9818$) was found between the curves. The intra-assay coefficient of variation was 3.33–3.65%.

2.7. Statistical analysis

Behavioral scores in GBT were expressed as medians [interquartile ranges] and evaluated by Kruskal–Wallis non-parametric analysis of variance; comparisons between two specific groups were performed by Mann–Whitney U-test. Inhibitory avoidance data were expressed as mean ± standard error of mean (S.E.M) and analyzed by independent *t*-test. GR and CRF expression and cortisol levels were expressed as mean ± S.E.M and analyzed by ANOVA following Duncan post hoc test. In all comparisons, significance was set at $p<0.05$. All data were evaluated by SPSS 18.0 for Windows.

3. Results

UCS produced behavioral alterations in all parameters of the Group Behavior Task, as shown in Fig. 2. Both 7 and 14 days of UCS significantly reduced height in the tank ($p<0.001$). Locomotor activity was lower after 14 days of stress and color score increased both at 7 and 14 days of stress. Moreover, shoal cohesion was distinctly altered by 7 and 14 days of UCS, being increased at day 7 and decreased after 14 days of stress ($p<0.001$).

In the inhibitory avoidance task (Fig. 3), control and 7-day-stressed animals showed a significant retention when training and test sessions were compared ($p<0.01$), but stressed animals showed worse performance when compared with control test latencies ($p<0.01$). Both control and 7-day-stressed fish were able to extinct aversive memory 3 days after training, when latencies reached levels as low as those of training session. The UCS protocol during 14 days significantly impaired the natural tendency of animals to go to dark environments, evaluated in the training session ($p<0.05$), which prevented a reliable assessment of memory in this group. Nevertheless, at the 3rd day of extinction, animals recovered and showed low latencies to go to the dark part of the tank.

The effects of UCS during 7 and 14 days on CRF (Fig. 4A) and GR (Fig. 4B) expression pattern were analyzed. Both 7 and 14 days of UCS significantly increased ($p<0.001$) CRF expression and decreased ($p<0.001$) GR expression. Cortisol levels were significantly higher in zebrafish submitted to UCS protocol ($p<0.01$) when compared to control group (Fig. 5).

4. Discussion

Although there are many animal models of chronic stress in rodents (Willner, 2005; Mineur et al., 2006; Yalcin et al., 2008; Piato et al., 2010), the study of this condition in zebrafish is still superficial. Here we presented a detailed description of a realistic chronic stress protocol, mimicking some behavioral and physiological modifications triggered both in rodents and humans. As expected, the stress protocol used in this study enhanced anxiety levels, disturbed social interaction, activated the HPI axis and increased cortisol levels.

The GBT is a protocol that allows simultaneous observation of several behaviors in zebrafish. All 4 parameters in this task were affected in UCS protocol. Both 7 and 14 days of stress produced a reduction of height in the tank and darker color, but social cohesion and locomotion were distinctively affected. After 7 days of stress, shoal cohesion was increased and locomotion was unchanged, whereas after 14 days both social cohesion and locomotion were reduced.

Zebrafish naturally go to the bottom of a novel environment (e.g., a test tank) and then gradually explore the upper portions of the tank (Levin et al., 2007; Egan et al., 2009). Thus, a reduction of this exploratory behavior towards upper zones can be interpreted as an index of anxiety, similar to thigmotaxis in rodents. Anxiolytic drugs such as buspirone and fluoxetine increased, whereas anxiogenic drugs such as caffeine decreased time on the top of the tank (Bencan et al.,

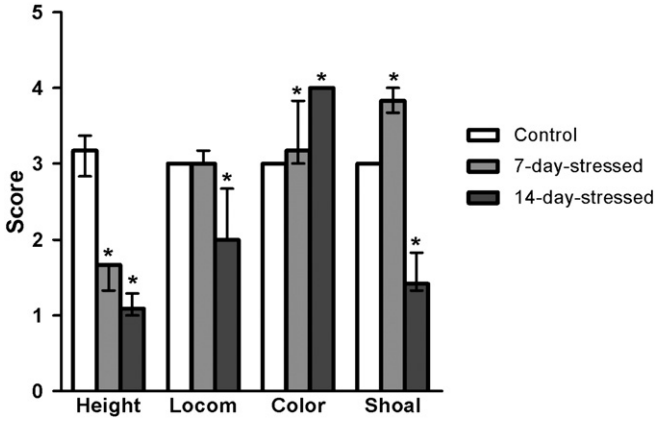

Fig. 2. Effects of 7 and 14 days of UCS protocol on height in the tank, locomotion, color and shoal cohesion parameters in zebrafish. Data are expressed as median ± interquartile interval. n = 12. *$p<0.01$ × control group. Kruskal–Wallis/Mann–Whitney.

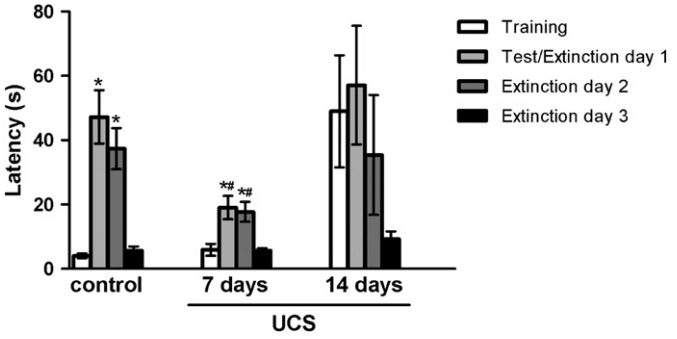

Fig. 3. Effects of 7 and 14 days of UCS protocol on latency to cross to dark compartment in training and long-term memory test sessions in the inhibitory avoidance task. Data are expressed as mean ± S.E.M. *$p<0.01$ × training session within group (ANOVA/Tukey). #$p<0.01$ × respective control (independent *t*-test). n = 12–20.

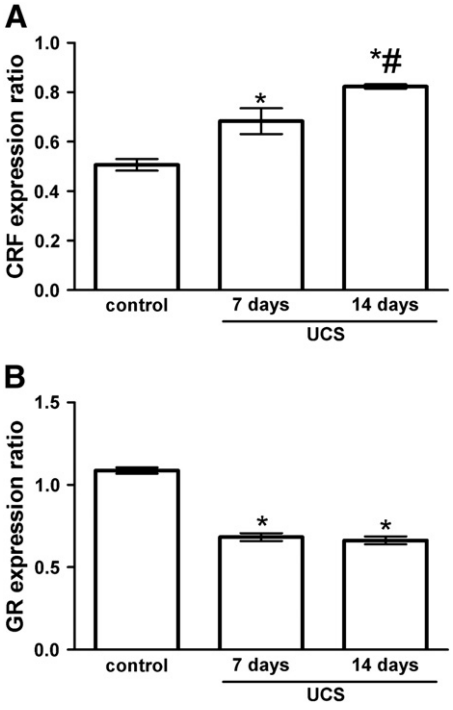

Fig. 4. Effects of 7 and 14 days of UCS protocol on CRF (A) and GR (B) expression. Data are expressed as mean ± S.E.M. *$p<0.01$ × control. #$p<0.01$ × 7-day-stressed group. ANOVA/Duncan post hoc test. n = 3.

2009; Egan et al., 2009). Thus, as expected, stress increased anxiety-like behavior in zebrafish, since fish remained most of the time at the bottom of the tank.

Locomotion and latency to move to the black zone in the inhibitory avoidance task were impaired after 14 but not after 7 days of UCS. Along with decreased shoal cohesion, these effects may be related with lower energy and/or decreased novelty seeking caused by extended stress period (Strekalova et al., 2004; Rygula et al., 2005; Salomons et al., 2010).

The color parameter may be related to different behavioral responses in zebrafish. In this protocol, stressed animals showed an increase in color intensity. Gerlai et al. (2000) mentioned that increased color intensity is associated with aggressiveness in zebrafish despite the background environment color while fear/anxiety was associated with decreased color intensity. Our data is not necessarily in contradiction since body color changes are rather unspecific responses observed in several conditions in zebrafish, ranging from fear to anxiety and aggressiveness. In the present study, UCS protocol induced an increase in the intensity of color in zebrafish, but aggression parameters were not directly evaluated. Importantly,

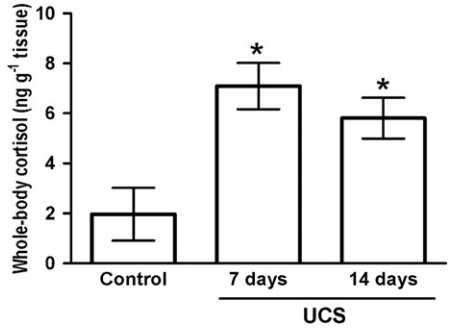

Fig. 5. Effects of 7 and 14 days of UCS protocol on cortisol levels. Data are expressed as mean ± S.E.M. *$p<0.01$ × control. ANOVA/Duncan post hoc test. n = 4.

Gerlai et al. did not discuss the conditions in which animals were evaluated in their study, preventing any direct comparison. The underlying mechanisms of body color changes in zebrafish are not yet completely understood, but could be related to general HPI activation.

Shoaling is a social and adaptive behavior observed in many species of fish (Pitcher and Parrish, 1993). Zebrafish show a marked tendency to form groups or shoals and this behavior is related with foraging, defense against predators, mating, and fear response (Rehnberg and Smith, 1988; Levin et al., 2007). The extension of the stress protocol determined the different responses obtained regarding shoal cohesion. Animals stressed for 7 days increased social cohesion, as a possible adaptive group behavior to respond more adequately to stressors (Landeau and Terborgh, 1986; Ryer and Olla, 1998; Wright et al., 2006). However, 14 days of UCS caused a substantial reduction in social interaction. One possible interpretation is that the ability to remain cohesive is a measure of resilience. Resilience, by definition, is the capacity of an organism to successfully cope with stress (Feder et al., 2009). The UCS protocols clearly produced anxiety, cognitive impairment and neuroendocrine disturbances. However, 14 days UCS protocol has also disrupted the social organization of this species that normally lives in a quite aggregate school. Such exhaustion of adaptive responses to long-lasting stress may reflect resilience loss and is compatible with a depressive-like behavior. Of note, there is a significant bidirectional relationship between loneliness and depression in humans (Heinrich and Gullone, 2006), and social separation and isolation per se can induce depressive-like behavior in rodents (Martin and Brown, 2010). The translational meaning of the shoaling behavior may be the traits known as behavioral inhibition or neuroticism, which are relevant for internalizing disorders (phobia, generalized anxiety, and depression) (Goldberg et al., 2009). These traits are known to be reduced by benzodiazepines and ethanol (McNaughton and Gray, 2000). We also observed that acute treatment with classical anxiolytics, such as benzodiazepines, or ethanol reduced shoal cohesion in the GBT (unpublished data). Gerlai et al. (2000) have also shown that shoal cohesion in zebrafish reduces with ethanol treatment.

Neurons in the hippocampus and prefrontal cortex respond to repeated stress by showing atrophy that leads to memory impairment in mammals (McEwen, 2005; Song et al., 2006). Moreover, chronic stress is related with impaired declarative memory performance and smaller hippocampal volume in young and middle-aged adults with chronic posttraumatic stress disorder (Golier et al., 2006; Yehuda, 2009). Memory formation is known to be dependent on highly conserved synaptic molecular mechanisms and homologous neuroanatomical areas in a multitude of species (Barco et al., 2006). In teleost fish the lateral telencephalic pallial formation is considered homologous to mammals' hippocampus (Rodríguez et al., 2002; Wullimann & Mueller, 2004) and is enriched with glutamatergic NMDA receptors (Barnes and Henley, 1994), key mediators of long-term plasticity in this area (Nam et al., 2004) and critical to long-term memory formation in our inhibitory avoidance task (Blank et al., 2009). Here, 24 h after a single-trial learning session (training), fish increased their latency to enter the dark compartment (test), indicating memory retention. However, 7-day-stressed fish showed clear cognitive deficit in test sessions compared to controls. This result is in agreement with previous studies that reported cognition deficits in rats and mice after distinct stress protocols (Henningsen et al., 2009; Palumbo et al., 2010). Also, control and 7-day-stressed groups were able to extinct the aversive stimulus. In 14-day-stressed fish, normal training performance was impaired since fish did not cross to the dark compartment. As previously mentioned, this atypical result could be, at least in part, related with locomotor deficit and/or exhaustion of adaptive responses (resilience loss or decrease) due to extended stress protocol. Despite this abnormal profile, at 3 days after training and 5 days without UCS, the normal behavior of rapidly crossing to the dark zone was present.

In zebrafish, the HPI axis presents similar function and structure compared to mammalian HPA axis, coordinating the adaptive responses of an organism to any stressor agent. Activation of the stress system leads to behavioral and peripheral changes that improve the ability of the organism to adjust homeostasis and increase its chances of survival (Kyrou and Tsigos, 2007). The stress response is related with HPA activation through increase of CRF and cortisol release and is controlled by several loops that tend to normalize the time-integrated secretion of cortisol. This response to stress with the resultant activation of the HPA axis is meant to be acute or at least with limited duration. In this case, the response has an adaptive feature, without major damage to the body (McEwen, 2000, 2005). However, intense chronic stress overactivates, the HPA axis, prompting the development of a state of exhaustion that leads to dysregulation of stress mediators, pathologies and even death, as first proposed by Selye in 1936. Centrally, chronic stress differentially modulates expression of GR and CRF. While the expression of GR is decreased, the expression of CRF is increased (for review see Ulrich-Lai and Herman, 2009). Peripherally, chronic stress increases cortisol levels. This is underlined by the results of Sapolsky et al. (2006), who showed that elevated glucocorticoid levels cause GR-containing cell loss in the hippocampus, leading to the glucocorticoid-induced suppression of CRF neurons. Here, as expected, our UCS protocol increased CRF and decreased GR expression. Moreover, stressed fish presented increased cortisol levels. These results agree with previous studies of stress protocols in which stressed fish of different species showed increased CRF expression (Overli et al., 2002; Doyon et al., 2003; Bernier and Craig, 2005; Doyon et al., 2005; Ortega et al., 2005) and cortisol levels (Demers and Bayne, 1997; Rotllant and Tort, 1997; Overli et al., 2002; Doyon et al., 2003; Bernier and Craig, 2005; Doyon et al., 2005; Lankford et al., 2005; Ortega et al., 2005; Barcellos et al., 2007; Pérez-Casanova et al., 2008).

5. Conclusion

The major drawbacks of chronic mild stress in rodents is the practical difficulty of carrying out CMS experiments, which are labor intensive, space demanding, expensive and long lasting (Willner, 2005). The present UCS protocol in zebrafish may be a suitable alternative with a better cost/benefit relationship compared to other animal models for studying the neurobiology and the effects of chronic stress. This model seems to present good construct validity (same neurobiological basis as rodents and humans) for anxiety after 7 days and possibly for depression after 14 days of stress. Future studies should determine the predictive validity of this model using different classes of drugs.

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