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Modeling consequences of prolonged strong unpredictable stress in zebrafish: Complex effects on behavior and physiology

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ABSTRACT

Chronic stress is the major pathogenetic factor of human anxiety and depression. Zebrafish (*Danio rerio*) have become a novel popular model species for neuroscience research and CNS drug discovery. The utility of zebrafish for mimicking human affective disorders is also rapidly growing. Here, we present a new zebrafish model of clinically relevant, *prolonged* unpredictable strong chronic stress (PUCS). The 5-week PUCS induced overt anxiety-like and motor retardation-like behaviors in adult zebrafish, also elevating whole-body cortisol and proinflammatory cytokines - interleukins IL-1β and IL-6. PUCS also elevated whole-body levels of the anti-inflammatory cytokine IL-10 and increased the density of dendritic spines in zebrafish telencephalic neurons. Chronic treatment of fish with an antidepressant fluoxetine (0.1 mg/L for 8 days) normalized their behavioral and endocrine phenotypes, as well as corrected stress-elevated IL-1β and IL-6 levels, similar to clinical and rodent data. The CNS expression of the *bdnf* gene, the two genes of its receptors (*trkB, p75*), and the *gfap* gene of glia biomarker, the glial fibrillary acidic protein, was unaltered in all three groups. However, PUCS elevated whole-body BDNF levels and the telencephalic dendritic spine density (which were corrected by fluoxetine), thereby somewhat differing from the effects of chronic stress in rodents. Together, these findings support zebrafish as a useful in-vivo model of chronic stress, also calling for further cross-species studies of both shared/overlapping and distinct neurobiological responses to chronic stress.

1. Introduction

Chronic stress is the leading cause of anxiety and depression, the two most prevalent and debilitating neuropsychiatric disorders (Baxter et al., 2013; Ferrari et al., 2013; Malki et al., 2014; McEwen, 2004; Melchior et al., 2007; Slavich and Irwin, 2014). Paralleling clinical findings, animal models of chronic stress are widely used in translational research of affective disorders, demonstrating robust behavioral, neuroendocrine, neuroimmune and neuromorphological responses (Bondi et al., 2008; Goshen et al., 2008; Kreisel et al., 2014; Malki et al., 2014). Chronic stress may contribute to depressive illness in several ways, including the dysregulation of hypothalamic-pituitary-adrenal (HPA) axis, as well as the activation of neuroinflammation and neuronal apoptosis (Brown et al., 2004; Goshen et al., 2008; Herbert, 2013; Kreisel et al., 2014; Lauretti et al., 2016; McKim et al., 2016; Rossetti et al., 2016). While increased glucocorticoid secretion and deficits in glucocorticoid biofeedback cause both anxiety and depression pathogenesis, mounting recent evidence suggests that chronic stress also affects microglia and astrocyte functions, thereby promoting neuroinflammation and neurodegeneration (Biesmans et al., 2015; Calcia et al., 2016; Rossetti et al., 2016). However, the relationship between chronic stress, affective pathogenesis and neuroinflammation, remains unclear (Kreisel et al., 2014).

The importance of studying 'core', evolutionarily conserved

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Table 1
The five-week unpredictable chronic stress (PUCS) battery protocol used in this study (also see Fig. 1 for a general outline of its research design), modeled to parallel in zebrafish the well-established rodent long-term chronic unpredictable stress batteries (Kreisel et al., 2014; Liu et al., 2015a; Willner et al., 1987).

Days	Stress procedures
1	Predator exposure (two 8-cm adult Blue marble gourami cichlids, *Trichogaster trichopterus*) for 24 h[a]
2	Conspecific exposure to a visually different zebrafish strain (20 pink same-age glofish) for 24 h[a]
3	Net chasing for 20 min → darkness for 24 h[a]
4	Crowding (10 fish/L in a 6-L small tank) for 6 h → shoaling test for 10 min (5 fish at a 1.5-L novel tank test) → 6 L of predator (gourami cichlid) hometank tank water added[a]
5	Crowding (10 fish/L)/novelty stress (placement in a 6-L red round plastic bucket) for 8 h in water containing alarm pheromone (30 mL extracted from 3 zebrafish[b]) → transfer to the hometank[a]
6	Food deprivation for 24 h with alarm pheromone (30 mL extracted from 3 zebrafish[b]) and 6 L of water from predator (gourami cichlid) hometank added[a]
7	Social isolation in small transparent 90-mL plastic cups for 8 h → 30% water change and darkness for 16 h in the hometank[a]
8	Shallow water exposure (20% of regular level) → darkness for 24 h[a]
9	The 3-cup crowding stress for 6 h (12 fish/0.5-L green, blue or orange cups, 2 h in each) → 60% water change (30 L of water from a conspecific tank) and novel objects (16 blue glass marbles) exposure for 18 h[a]
10	20-L light-dark box exposure (30 fish/L) for 8 h → shallow water (60% of regular level) and bright light exposure for 2 h → predator water exposure + food deprivation for 24 h[a]
11	Darkness for 24 h[a]
12	Predator exposure (2 gourami cichlids) for 24 h[a]
13	8-h light-dark box exposure for 12 h → shallow water stress (30% of regular level) for 12 h[a]
14	Social isolation for 8 h → food deprivation for 24 h[a]
15	Darkness for 24 h[a]
16	Alarm pheromone (50 mL extracted from 5 zebrafish[b]) exposure for 24 h[a]
17	Food deprivation for 24 h and 50% water change[a]
18	Shallow water (30% of regular level) exposure for 8 h → water change (30 L of gourami 'predator' water added)[a]
19	Predator exposure (2 gourami cichlids) for 24 h[a]
20	Crowding stress in red bucket (as above) for 8 h → transfer to the hometank[a]
21	Crowing stress (10 fish/L) in small 6-L tank with bright 40-wt light and shallow (10 cm deep) water for 8 h → 10 L predator (cichlid) water added[a]
22	Darkness and food deprivation for 24 h[a]
23	Predator exposure (2 gourami cichlids) for 24 h[a]
24	Shallow water (30% of regular level) and bright light exposure for 2 h → stress in a novel red bucket for 6 h → transfer to the hometank[a]
25	Shaking for 6 h (22 °C, 60 rpm) with 40 mL alarm pheromone added (extracted from 4 zebrafish[b]) under dim 10-wt light → transfer to the hometank[a]
26	Novel predator (2 small young 4-cm Oscar fish, *Astronotus ocellatus*) exposure for 18 h[a]
27	The 3-cup crowding stress for 6 h (as above) under bright 40-wt light → 50% shallow water exposure without aeration for 16 h; 50% cohort removal (fish cohort split in two separate sub-cohorts)[a]
28	Social isolation for 8 h → net chasing for 15 min → predator water (4 L, Oscar fish) exposure[a]
29	Exposure to air for 1 min/3 times with 10 min interval → predator (Oscar fish) exposure for 2.5 h → predator (gourami cichlid) exposure for 2.5 h → alarm pheromone exposure (10 mL, 7 times for 15 min[b]) → transfer to the hometank[a]
30	Darkness and food deprivation for 24 h[a]
31	Crowding (30 fish/L) and bright light stress for 8 h → novel non-predator heterospecific fish exposure (5-cm adult fathead rosy minnows, *Pimephales promelas*; at a 2 zebrafish:1 minnow ratio) for 14 h[a]
32	Net casing stress for 15 min → crowding stress (30-fish/L) and extra-bright light exposure (four 200-wt bulbs located 15 cm from the beaker) for 20 min → local hypothermia (adding 0.5 L of ice to the beaker) → shallow water (30%) stress for 24 h[a]
33	60% water change → 3-cup stress for 8 h → 10 L of conspecific (a different cohort of same-strain zebrafish) water added to the hometank[a]
34	Light–dark box exposure for 5 min (as above) → removal from water for 1 min thrice every 10 min → predator (gourami cichlid) exposure for 24 h[a]
35	Shallow water (30% of usual water level) for 18 h with exposure to alarm pheromone (10 mL, extracted from 10 zebrafish[b]) added 5 times with a 15-min interval[a]
36	Analyses: Behavioral testing, sacrifice and sample collection

[a] In the regular 46-L hometank.
[b] Alarm pheromone was extracted here from freshly euthanized adult zebrafish (10 mL/fish) by shallow cuts to their body skin.

Table 2
List of primers used in this study (obtained from Sangon Biotech Co., Ltd., Shanghai, China).

Primer names	Sequences
BDNF F	AACTCCAAAGGATCCGCTCA
BDNF R	GCAGCTCTCATGCAACTGAA
TrkB F	CCACCACTGGAGGACAGAGTTG
TrkB R	CCGAGGATGATGGCGTGTTGT
P75 F	TCTGTCAAGATTTCGATGCTCCT
P75 R	GCTCTCCGTAGGATTGTCCG
GFAP F	AATGTCAAACTGGCCCTGGAT
GFAP R	CTCTCCGTCACGGGTCTCAA
Actin F	CGAGCTGTCTTCCCATCCA
Actin R	TCACCAACGTAGCTGTCTTTCTG

trajectories of affective disordered mechanisms necessitates further comparative cross-species analyses of behavioral and physiological responses to chronic stress. The value of zebrafish (*Danio rerio*) for neuroactive drug discovery and biological psychiatry research is widely recognized (Fonseka et al., 2016; Kalueff et al., 2014; MacRae and Peterson, 2015; Stewart et al., 2015b). Although their utility for mimicking human affective disorders has emerged only recently, it is also growing rapidly (Fonseka et al., 2016; Stewart et al., 2015b)(Carr, 2015; Piato et al., 2011; Rambo et al., 2017; Teles et al., 2016; Wong et al., 2015). Here, we report a novel zebrafish model of *prolonged* 5-week strong unpredictable chronic stress (PUCS, Table 1), which can be particularly relevant clinically, since *long-term* recurrent stress commonly triggers affective pathogenesis in humans. This study aimed to comprehensively evaluate a wide spectrum of endocrine, morphological and behavioral phenotypes following chronic strong unpredictable stress in zebrafish. We also examined the potential for its therapeutic correction using chronic treatment with an antidepressant fluoxetine (which, like other selective serotonin reuptake inhibitors, SSRIs, is commonly utilized in experimental/rodent models of stress to validate/rescue the evoked affective states (Bondi et al., 2008; Song and Leonard, 2005). Additionally, the present study explored immune biomarkers in zebrafish exposed to prolonged unpredictable stress, including whole-body levels of several pro- and anti-inflammatory cytokines and neurotrophin brain-derived neurotrophic factor (BDNF), as well as brain expression of selected genes (Table 2). As we fully recognize that chronic unpredictable stress may differ pathobiologically from predictable stress (Barsy et al., 2010; Haile et al., 2001; Parihar