Behavioural Brain Research 378 (2020) 112264

Contents lists available at ScienceDirect

Behavioural Brain Research

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

Tryptophan alleviates neuroendocrine and behavioral responses to stress in zebrafish

Ana C.V.V. Giacomini[a,b,c,*], Audren S. Piassetta[a], Rafael Genario[a], Carla D. Bonan[d,e], Angelo Piato[c,f], Leonardo J.G. Barcellos[b,g,h], Murilo S. de Abreu[a,c,*]

[a] Bioscience Institute, University of Passo Fundo (UPF), Passo Fundo, RS, Brazil
[b] Postgraduate Program in Environmental Sciences, University of Passo Fundo (UPF), Passo Fundo, Brazil
[c] The International Zebrafish Neuroscience Research Consortium (ZNRC), Slidell, LA, USA
[d] Postgraduate Program in Cellular and Molecular Biology, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil
[e] Postgraduate Program in Medicine and Health Sciences, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil
[f] Postgraduate Program in Neurosciences, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil
[g] Postgraduate Program in Bio-Experimentation, University of Passo Fundo (UPF), Passo Fundo, Brazil
[h] Postgraduate Program in Pharmacology, Federal University of Santa Maria (UFSM), Santa Maria, Brazil

ARTICLE INFO

Keywords:
Cortisol
Anxiety
Behavior
Fluoxetine
Zebrafish

ABSTRACT

Stressful experiences are related to the triggering of anxiety and mood disorders. Tryptophan (amino acid precursor of serotonin synthesis) emerges as important treatment of these disorders. Here, we evaluate the effects of pre-treatment with tryptophan (300 mg/L) and fluoxetine (50 µg/L) in response to acute stress in zebrafish. Overall, acute stress decreased the distance traveled, entries and time in top of tank, as well as increased the cortisol levels, demonstrating an anxiogenic behavior. Tryptophan and fluoxetine prevented anxiogenic effects. This study showed the importance of tryptophan and fluoxetine in the regulation of stress and anxiety-like behavior in adult zebrafish. Collectively, our data support tryptophan effects on stress responses in zebrafish and reinforce the growing utility of this aquatic model to screen CNS therapies.

Stress situations trigger mood and anxiety disorders [1]. Anxiety disorders have been increased worldwide reaching 264 millions of people, elevating nearly 14.9% - from 2005 to 2015 [2]. Anxiety disorders have been treated mainly with anxiolytics, which act mainly on the serotonergic, noradrenergic, dopaminergic and GABAergic systems [3]. Although anxiolytics present a reasonable clinical response, many patients do not respond to treatment [4], as well as induce various adverse effects (e.g., sleepiness, dizziness, ataxia, nystagmus), contributing to poor adherence [5]. Therefore, new therapeutic approaches are necessary to the treatment of central nervous system (CNS) disorders. For instance, new therapies include the use of regular exercise (e.g., running [6]), herbal medicines (e.g., *Valeriana officinalis* [7]) and dietary interventions (e.g., increased dietary fiber [8]). Considering the involvement of serotonin in the pathophysiology of stress and anxiety disorders [9], a possible therapeutic intervention is food supplementation with tryptophan (TRP), an essential amino acid precursor of serotonin synthesis. The use of TRP supplementation in the diet has been demonstrated as an important therapy to control of stress response, emotional and cognition phenotypes in humans and fishes [9]. However, there are no reports of the effect of TRP pre-treatment on the acute stress response in zebrafish. Here we evaluate the effects of pre-treatment with TRP and fluoxetine on response to acute stress in adult zebrafish.

A total of 72 adults zebrafish (*Danio rerio*), ~50/50 male/female ratio of the wild-type short-fin (SF) strain were housed 1 fish/L in 30-L tanks equipped with biological filters, under constant aeration and a photoperiod (14 h light:10 h dark). Water temperature was maintained at 27 ± 0.6 °C; pH 7.0 ± 0.2, with dissolved oxygen kept at 6.0 ± 0.1 mg/L, total ammonia at < 0.01 mg/L, total hardness at 6 mg/L, and alkalinity at 22 mg/L $CaCO_3$. Animal experimentation reported here was approved by the Institutional Animal Care Committee (Protocol #18/2017, University of Passo Fundo, Passo Fundo, Brazil) and fully adhered to National and International guidelines on animal experimentation. Animals were exposed to fluoxetine ($C_{17}H_{18}F_3NO$, fluoxetine hydrochloride, Nova Quimica, São Paulo, Brazil) at concentration 50 µg/L, or TRP amino acid ($C_{11}H_{12}N_2O_2$, Infinity Pharma, Campinas, Brazil) at concentration 300 mg/L. The concentrations used in the study were chosen due to their effective anxiolytic response in

* Corresponding authors at: Bioscience Institute, University of Passo Fundo, Passo Fundo, Brazil.
E-mail addresses: anacvg@upf.br (A.C.V.V. Giacomini), abreu_murilo@hotmail.com (M.S. de Abreu).

https://doi.org/10.1016/j.bbr.2019.112264
Received 13 May 2019; Received in revised form 25 September 2019; Accepted 26 September 2019
Available online 27 September 2019
0166-4328/

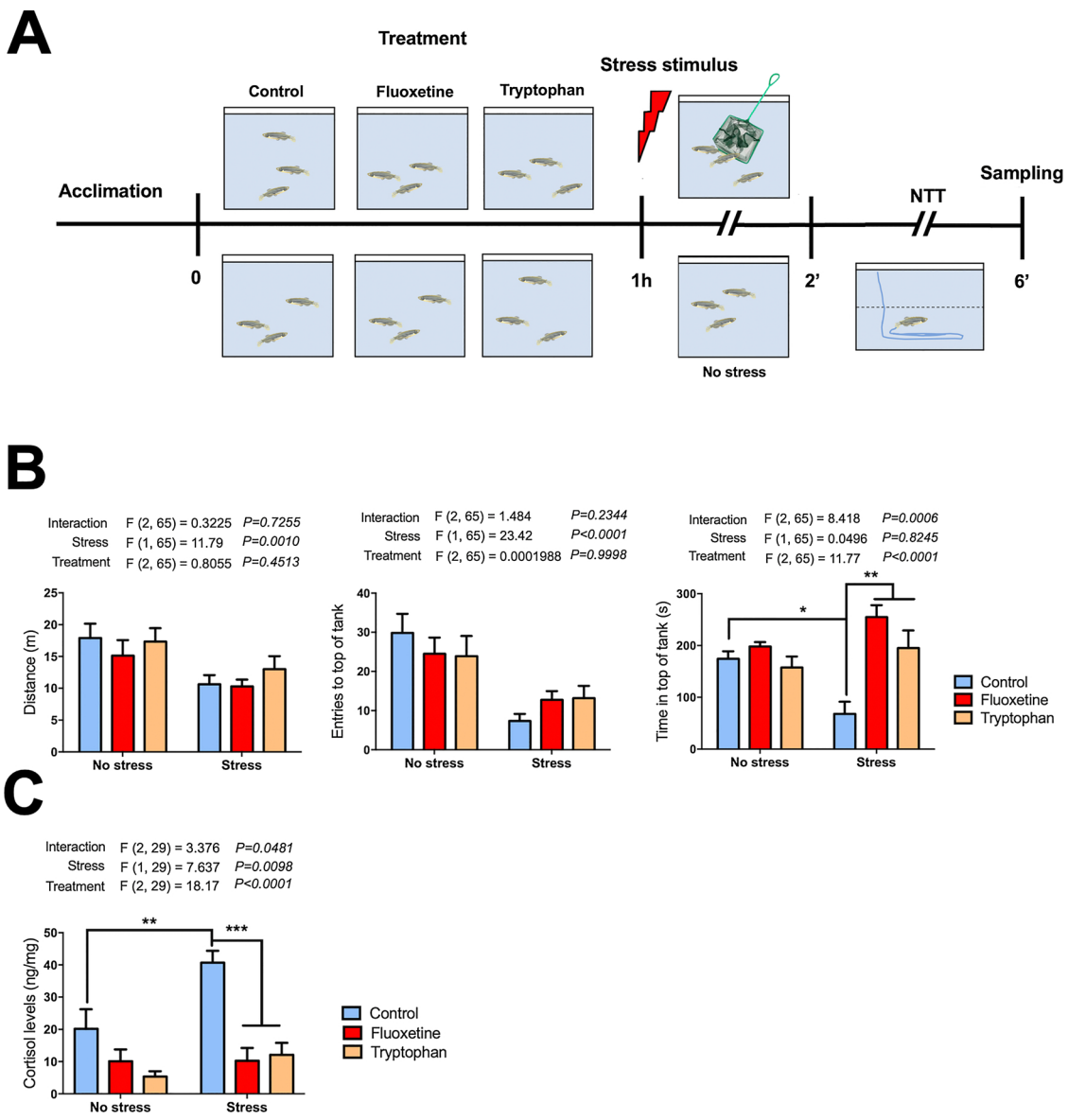

Fig. 1. Effects of tryptophan and fluoxetine on stress responses in zebrafish. Panel A shows a schematic summary of the study experimental design (stress stimulus was fish exposure to net chasing by 2 min). Panel B shows the behavioral effects of acute stress and treatment (fluoxetine or tryptophan) tested in the 6-min novel tank test (n = 11–12 per group). Panel C shows whole-body cortisol effects of acute stress and treatment (fluoxetine or tryptophan) (n = 5–6 per group (each "n" stands for a pool of two fish). Data are expressed as mean + standard error of mean (SEM) and assessed by Two-way ANOVA followed by the Tukey test and significance as *p < 0.05, **p < 0.01, ***p < 0.001, respectively.

adult zebrafish (e.g., fluoxetine [10] and TRP (assessed in a concentration-response curve, data not shown)). Drugs were administrated in water and fish were exposed in group (containing 3 fish per tank, n = 12) for 1 h for both treatments, and immediately behavior was quantified. After behavioral tests, zebrafish were anesthetized by cold water (2–4 °C) and after the cessation of opercular movements, they were euthanized by decapitation to evaluate whole-body cortisol levels.

Animals were initially assigned to two experimental groups (non-stressed or stressed), both groups were randomly subdivided into untreated (freshwater) and treated groups (fluoxetine and TRP), totaling 6 experimental groups: control; fluoxetine; TRP; control + stress; fluoxetine + stress; and TRP + stress (Fig. 1A). The stress protocol consisted of chasing fish for 2 min [10,12]. After chasing, fish from all six groups were individually tested in the novel tank test to evaluate their anxiety-like behavior. Fluoxetine has been used as a positive control due its effects on decreasing anxiety-like behavior and whole-body cortisol levels in zebrafish [10,12]. The novel tank test was selected here as one of the most sensitive aquatic paradigms for measuring zebrafish anxiety behavior. Fish from all experiments were individually tested in the novel tank apparatus (24 width × 8 depth × 20 height cm). Animals were recorded for 6 min using a Logitech HD Webcam C525 camera (Logitech, Switzerland). The videos were then analyzed offline using the ANY-maze® software (Stoelting Co, Wood Dale, USA), calculating the following behavioral parameters: the total distance traveled (m), number of entries and time spent (s) in the top zone of the tank, similar to [13]. Whole-body cortisol was extracted using the method described previously [14]. The accuracy was tested by calculating the recoveries from samples spiked with known amounts of cortisol (50, 25 and 12.5 ng/mL), the mean detection of spiked samples was 95%. All cortisol values were adjusted for recovery with the following equation: cortisol value = measured value × 1.05. Whole-body cortisol levels were measured using the commercially available enzyme-linked immunosorbent assay kit (EIAgen CORTISOL test, BioChem Immunosystems). In this study, animal groups were compared using two-

way ANOVA (factor 1: treatment (fluoxetine or TRP) and factor 2: stress or not), followed by the Tukey post-test for significant data. P was set as < 0.05 in all analyses. All animals tested were included in final analyses, without the attrition or outlier removal. The data normality was assessed by the Kolmogorov-Smirnov test.

Zebrafish exposure to acute stress decreased the distance traveled, entries and time in the top of the tank compared to not stressed animals (Fig. 1B). Interestingly, zebrafish treated with TRP or fluoxetine prevented the anxiogenic effect - a decrease of time in top of tank - induced by acute stress (Fig. 1B). The acute stress increased cortisol levels; whereas TRP and fluoxetine prevented this effect (Fig. 1C). Overall, TRP and fluoxetine present an anxiolytic effect, alleviating behavioral and endocrine effects of acute stress. We demonstrated for the first time that pre-treatment with TRP reduces anxiety-like behavior in zebrafish exposed to acute stress, similarly to fluoxetine. Acute stress increases anxiety-like behavior and aggression, while social interaction is reduced in zebrafish [10]. In addition, these behavioral phenotypes are modulated by fluoxetine [10]. Acute stress has also been demonstrated by reduced locomotor activity (e.g., distance traveled) in zebrafish [15]. Fluoxetine and TRP did not prevent the locomotor effects induced by acute stress on distance traveled and entries to the top of the tank, similar to zebrafish chronically exposed to fluoxetine [16]. Fluoxetine and TRP prevented the increase of cortisol levels caused by acute stress. Zebrafish exposure to stress (e.g., unpredictable chronic stress (UCS), social isolation) reduced levels of serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) [17]. Meanwhile, zebrafish treated with fluoxetine prevented behavioral and endocrine effects caused by UCS [16], as well as zebrafish treated with the 5-HTP demonstrated anxiolytic effect, elevating serotonin levels in the brain [11].

Despite the relationship between the serotonergic system and the stress reactivity is well established [18], the serotonergic system and the stress reactivity role of TRP supplementation are not yet clear. TRP is a precursor for 5-HT, and it also metabolized by kynurenine pathway which initiated by the TDO2 (tryptophan 2,3-dioxygenase) [19]. Mice with a genetic disruption of TDO2 present lower anxiety-like behavior [20], as well as TDO2 expression is increased in the hippocampus when administered pro-inflammatory mediators and stress hormones [21]. Similarly, TRP depletion causes anxiogenic response and increases the rate of panic attacks after CO_2 challenge in humans [22]. In addition, TRP supplementation demonstrated to act as a coadjuvant in anxiety disorders treatments, since mice exposed to TRP restriction and corticosterone supplementation showed anhedonia, reduced motivation to explore a novel environment, dopamine and serotonin levels in prefrontal cortex, as well as an increased hypothalamic brain-derived neurotrophic factor (BDNF) [23]. Thereby, TRP supplementation is a useful therapy to reduce cortisol levels, stress responsiveness and eating disorder (e.g., serotoninergic genetic disorder (polymorphism in 5-HT transporter gene (5-HTTLPR)) in neuroticism patients) [24].

Furthermore, the anxiolytic effect caused by TRP supplementation may result from modulation on the gut-brain axis [25]. The gut microbiota plays a role in tryptophan metabolism and consequently in the production of serotonin (e.g., enterochromaffin cells) [25]. In addition, zebrafish has been used as a useful experimental model in studies involving gut-brain relationship [26]. For example, zebrafish treated with diet containing the probiotic, *Lactobacillus rhamnosus,* increased the expression of tryptophan (*tph1a, tph1b, tph2*) and serotonin (5-hydroxytryptamine receptor 1A (*htr1aa*), solute carrier family 6 member 4 (*slc6a4a*) genes [27]. Likewise, supplemented with probiotic, *Lactobacillus plantarum*, also increased the expression of serotonergic (*slc6a4a*) gene in brain and demonstrated anxiolytic behavior [28].

In summary, there are several additional potential implications of our study. For instance, TRP and fluoxetine induced anxiolytic effects, as well as prevented behavioral and endocrine effects caused by acute stress (Fig. 1). Finally, our data are also in agreement with effects demonstrated by TRP and fluoxetine in rodents and humans such as therapies to stress disorders, suggesting zebrafish behavioral and endocrine models as a promising tool to drug discovery of stress disorders.

Declaration of Competing Interest

None.

Acknowledgments

A.C.V.V.G. is supported by fellowships 17/2551-001-269-0 from Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS). L.J.G.B. holds a CNPq research fellowship (303263/2018-0). The authors thank Mrs. Sirlei Cazarotto (University of Passo Fundo, Brazil) for her assistance during experimental procedures.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bbr.2019.112264.

References

[1] P. Moreno-Peral, et al., Risk factors for the onset of panic and generalised anxiety disorders in the general adult population: a systematic review of cohort studies, J. Affect. Disord. 168 (2014) 337–348.
[2] O. World Health, Depression and Other Common Mental Disorders: Global Health Estimates, (2017).
[3] H.-N. Chan, et al., Pharmacological treatment approaches to difficult-to-treat depression, Med. J. Aust. 199 (6) (2013) 44–47.
[4] J.W.G. Tiller, Depression and anxiety, Med. J. Aust. 199 (6) (2013) 28–31.
[5] S. Taylor, J.S. Abramowitz, D.J.Joad. McKay, Non-adherence and non-response in the treatment of anxiety disorders, J Anxiety Disord 26 (5) (2012) 583–589.
[6] P.J. Carek, S.E. Laibstain, S.M.J.T.I.Jo.Pi.M. Carek, Exercise for the treatment of depression and anxiety, Int. J. Psychiatry Med 41 (1) (2011) 15–28.
[7] L. Liu, et al., Herbal medicine for anxiety, depression and insomnia, Curr. Neuropharmacol. 13 (4) (2015) 481–493.
[8] R.S. Opie, et al., The impact of whole-of-diet interventions on depression and anxiety: a systematic review of randomised controlled trials, Public Health Nutr. 18 (11) (2015) 2074–2093.
[9] E. Höglund, Ø. Øverli, S.J.Fi.E. Winberg, Tryptophan metabolic pathways and brain serotonergic activity: a comparative review, Front. Endocrinol. (Lausanne) 10 (2019) 158.
[10] A. Giacomini, et al., Fluoxetine and diazepam acutely modulate stress induced-behavior, Behav. Brain Res. 296 (2016) 301–310.
[11] A.M. Herculano, C.J.Pi.N.-P. Maximino, B. Psychiatry, Serotonergic modulation of zebrafish behavior: towards a paradox, Prog. Neuropsychopharmacol. Biol. Psychiatry 55 (2014) 50–66.
[12] M.S. de Abreu, et al., Diazepam and fluoxetine decrease the stress response in zebrafish, PLoS One 9 (7) (2014) 5.
[13] M. Marcon, et al., Environmental enrichment modulates the response to chronic stress in zebrafish, J. Exp. Biol. 221 (2018) jeb176735.
[14] T.D. Sink, R.T. Lochmann, K.A. Fecteau, Validation, use, and disadvantages of enzyme-linked immunosorbent assay kits for detection of cortisol in channel catfish, largemouth bass, red pacu, and golden shiners, Fish Physiol. Biochem. 34 (1) (2008) 95–101.
[15] R. Mocelin, et al., N-acetylcysteine prevents stress-induced anxiety behavior in zebrafish, Pharmacol. Biochem. Behav. 139 (2015) 121–126.
[16] M. Marcon, et al., Prevention of unpredictable chronic stress-related phenomena in zebrafish exposed to bromazepam, fluoxetine and nortriptyline, Psychopharmacology 233 (21-22) (2016) 3815–3824.
[17] N. Fulcher, et al., Neurochemical and behavioral responses to unpredictable chronic mild stress following developmental isolation: the zebrafish as a model for major depression, Zebrafish 14 (1) (2017) 23–34.
[18] J.R. Homberg, et al., The serotonin–BDNF duo: developmental implications for the vulnerability to psychopathology, Neurosci. Biobehav. Rev. 43 (2014) 35–47.
[19] R. Schwarcz, et al., Kynurenines in the mammalian brain: when physiology meets pathology, Nat. Rev. Neurosci. 13 (7) (2012) 465.
[20] S. Hattori, et al., Comprehensive behavioral analysis of tryptophan 2, 3-dioxygenase (Tdo2) knockout mice, Neuropsychopharmacol. Rep. 38 (2) (2018) 52–60.
[21] A.K. Brooks, et al., Interactions between inflammatory mediators and corticosteroids regulate transcription of genes within the Kynurenine Pathway in the mouse hippocampus, J Neuroinflammation 13 (1) (2016) 98.
[22] H. Miller, J. Deakin, I.J.T.B.Jo.P. Anderson, Effect of acute tryptophan depletion on CO 2-induced anxiety in patients with panic disorder and normal volunteers, Br. J. Psychiatry 176 (2) (2000) 182–188.
[23] F. Zoratto, et al., Neonatal tryptophan depletion and corticosterone supplementation modify emotional responses in adult male mice, Psychoneuroendocrinology 38 (1) (2013) 24–39.
[24] A.E.M. Capello, C.R. Markus, Effect of sub chronic tryptophan supplementation on

stress-induced cortisol and appetite in subjects differing in 5-HTTLPR genotype and trait neuroticism, Psychoneuroendocrinology 45 (2014) 96–107.
[25] S.M. O'Mahony, et al., Serotonin, tryptophan metabolism and the brain-gut-microbiome axis, Behav Brain Res 277 (2015) 32–48.
[26] M.S. de Abreu, et al., Modeling gut-brain interactions in zebrafish, Brain Res. Bull. 148 (2019) 55–62.
[27] L. Borrelli, et al., Probiotic modulation of the microbiota-gut-brain axis and behaviour in zebrafish, Sci Rep 6 (2016) 30046.
[28] D.J. Davis, et al., Lactobacillus plantarum attenuates anxiety-related behavior and protects against stress-induced dysbiosis in adult zebrafish, Sci. Rep. 6 (2016) 33726.