Role of serotonin on behavioral responses to alarm substance in zebrafish: A putative model for panic disorder

Rhayra Xavier do Carmo Silva[1,2]

Sueslene Prado Rocha[3]

Maryana Pereira Pyterson[1]

Dainara Pereira dos Santos Sousa[1]

Gabriela Cristini Vidal Gomes[1]

Monica Gomes Lima[3]

Anderson Manoel Herculano[4]

Denis Broock Rosemberg[5]

Caio Maximino[1]

[1] Laboratório de Neurociências e Comportamento,

Universidade Federal do Sul e Sudeste do Pará

[2] Programa de Pós-Graduação em Neurociências e Biologia Celular,

Universidade Federal do Pará

[3] Laboratório de Neurofarmacologia e Biofísica, Universidade do Estado do Pará

[4] Laboratório de Neurofarmacologia Experimental, Universidade Federal do Pará

[5] Laboratório de Neuropsicobiologia Experimental, Universidade Federal de Santa Maria

Abstract

Panic disorder (PD) is a crippling condition associated with the occurrence of panic attacks and the appearance of avoidance strategies to stimuli associated with previous attacks. Evidence points towards serotonin (5-HT) as a key neurotransmitter system in the control of fear responses and in the mediation of panic-like symptoms. Animal models for PD suffer either from a lack of etiological validity or from a lack of behavioral detail, and rely mainly on mammalian species. In this Registered Report we propose the use of zebrafish (*Danio rerio* Hamilton 1822) behavioral reactions to a conspecific alarm substance (CAS) as a model for PD. We propose analyzing responses to CAS during exposure as a model for panic attack, and the "residual" post-stimulation effects as a model for PD. We also propose analyzing the effects of CAS exposure on brain monoamine oxidase activity as a surrogate of 5-HTergic activity, and the use of 5-HTergic drugs (fluoxetine and metergoline) to assess the role of this neurotransmitter in the model. We hypothesize that CAS will increase erratic swimming during exposure, and both freezing and geotaxis after exposure. Alterations of phasic serotonin release (i.e., fluoxetine), but not of serotonergic tone (i.e., metergoline), are expected expected to block the effects of CAS both during and after exposure. Finally, CAS is expected to increase serotonergic activity by inhibiting monoamine oxidase activity.

Keywords: Zebrafish, Panic disorder, Serotonin

Role of serotonin on behavioral responses to alarm substance in zebrafish: A putative model for panic disorder

Panic disorder (PD) is a crippling mental disorder that is characterized by the occurrence of unexpected and recurrent panic attacks, persistent apprehension, and avoidance of situations and places associated with past panic attacks (American Psychiatric Association, 2013). The occurrence of this disorder is quite high, with lifetime prevalences estimated as high as 22.7% for PD without agoraphobia (Kessler et al., 2006). The efficacy of pharmacotherapy for PD is relatively low, with chronic selective serotonin reuptake inhibitors (SSRIs) producing a better outcome (rate of participants free from panic attacks at the endpoint) than alprazolam (Caldirola et al., 2017). Knowledge of the neurochemical underpinnings of PD is still in its infancy, and could benefit drug discovery in the long term.

There is some indication of the participation of serotonin (5-HT) in phenotypes associated with PD. For example, treatment of PD patients with *m*-chlorophenylpiperazine (mCPP, a piperazine with high affinity for 5-HT_2 receptors; http://unc.live/2EBzSnQ) precipitates panic attacks and induces cortisol responses (René S Kahn et al., 1988; Rene S. Kahn et al., 1988). The Deakin/Graeff hypothesis of PD proposes that different subpopulations of 5-HTergic neurons innervate forebrain and brainstem structures modulating responses to aversive, fear-inducing stimuli, and that dysfunction in these circuits is a vulnerability to PD (Deakin and Graeff, 1991; Maximino, 2012; Paul et al., 2014). Specifically, the hypothesis predicts that serotonin inhibits panic-like responses in the periaqueductal gray area (PAG) and enhances anxiety-like responses in the forebrain.

Behavioral models for PD in animals are needed to advance tests of this hypothesis (Moreira et al., 2013; Schenberg et al., 2014). Neuroanatomical approaches have been championed, in which drug or electrical stimulation of structures such as the PAG or the dorsomedial hypothalamus is used to elicit panic-like responses in rodents (Brandão et al., 2008; Johnson et al., 2008; Vianna et al., 2001). Brandão et al. (2008) pointed that freezing elicited from dorsal PAG stimulation has two stages – stimulation-associated freezing, and post-stimulation freezing – which can be dissociated pharmacologically and behaviorally. The authors suggested that PAG stimulation-associated freezing could be a model for a panic attack, while post-stimulation freezing would serve as a model for the symptoms of apprehension and conditioned avoidance observed in PD (Brandão et al., 2008). Interestingly, injection of a 5-HT_2 receptor agonist in the dorsal PAG decreases stimulation-induced freezing without affecting post-stimulation freezing (Oliveira et al., 2006).

While neuroanatomical/pharmacological approaches to modeling PD in animals are valuable in the description of brain areas putatively associated with the disorder, the construct/etiological validity of such models is reduced. Complementary approaches include exposing animals to "etho-experimental" stimuli, such as natural predators or partial predator stimuli, to elicit panic-like responses (Blanchard et al., 2001; Graeff and Del-Ben, 2008; Moreira et al., 2013; Schenberg et al., 2002). In this sense, aiming to contribute to the available etho-experimental approaches used in rodents, the use of zebrafish (*Danio rerio* Hamilton 1822) has been proposed. Zebrafish is a cyprinid widely used in genetics and developmental biology that, in the latter years, gained visibility as a model in behavioral research (Gerlai, 2010a; Norton and Bally-cuif,

2010; Rinkwitz et al., 2011; Shams et al., 2018; Stewart et al., 2012). The premise is that zebrafish antipredatory responses represent certain advantages for complementary modeling of PD.

Among the different defensive responses observed in zebrafish, the alarm reaction is relatively well-characterized, and is relevant to PD models (Gerlai, 2013, 2010b; Jesuthasan and Mathuru, 2008). The alarm reaction is a response to substances released in the water by the damaged epidermis of conspecifics, and is characterized by dramatic and measurable changes in swimming patterns and well-defined physiological responses. The "conspecific alarm substance" (CAS) that is produced by damaging specialized epidermal cells ("club cells") has unknown composition (Døving and Lastein, 2009; Jesuthasan and Mathuru, 2008), but hypoxanthine 3-*N*-oxide and chondroitin fragments are found in it and are able to partially mimic the reactions (Mathuru et al., 2012; Parra et al., 2009).

During exposure to CAS, increases in shoal cohesion (Speedie and Gerlai, 2008) and the initiation of erratic swimming patterns (Mathuru et al., 2012; Speedie and Gerlai, 2008) are observed. CAS did not affect the time that the animal spends in the bottom of the tank during exposure (Speedie and Gerlai, 2008), but there are reports of increased bottom-dwelling after exposure (Cachat et al., 2011; Egan et al., 2009; Quadros et al., 2016). Moreover, it has been reported that CAS increases erratic swimming, but not freezing, during exposure (Speedie and Gerlai, 2008), while increases in freezing were observed after exposure (Egan et al., 2009; Quadros et al., 2016). These differences in stimulation-induced and post-stimulation behavioral changes are reminiscent of what is observed in PAG-stimulated animals (Brandão et al., 2008).

The alarm substance also produces an intense autonomic response, with increases in the plasma levels of glucose, norepinephrine, and epinephrine (Maximino et al., 2014); a neuroen-

docrine stress response is also observed, with increased whole-body cortisol levels (Schirmer et al., 2013). These behavioral and physiological adjustments simulate some important behavioral aspects and neurovegetative symptoms of panic attacks and PD, lending significant face validity to the model. The dissociation of responses elicited during exposure to CAS (erratic swimming, without increased bottom-dewlling) and after exposure to CAS (freezing and increased bottom-dwelling) is reminiscent of the distinction between freezing responses during and after electrical PAG stimulation in rodents (Brandão et al., 2008), and could be exploited in models of panic attack and PD, respectively.

The 5-HTergic system has also been implicated in alarm reactions in zebrafish. Acute fluoxetine inhibits behavioral and autonomic responses after CAS exposure (Maximino et al., 2014). WAY 100,635, a 5-HT_{1A} receptor antagonist, potentiated the effect of CAS on bottom-dwelling during exposure (Nathan et al., 2015), but did not affect freezing or dark preference after exposure (Maximino et al., 2014; Nathan et al., 2015). The drug, however, blocked the post-stimulation effect of CAS on nocifensive behavior (Maximino et al., 2014), suggesting a dissociation of behavioral effects through 5-HT receptors. The 5-HT_2 antagonist methysergide produced a similar effect on bottom-dwelling and freezing during exposure, but only higher doses potentiated the post-stimulation effects of CAS (Nathan et al., 2015). These results suggest that 5-HT_{1A} and 5-HT_2 receptor inhibit panic-like responses to CAS in zebrafish. The present Registered Report proposes the use of stimulation-induced and post-stimulation responses to CAS as a model for PD and panic attacks; the activity of CAS on brain monoamine oxidase activity; and the use of 5-HTergic drugs (fluoxetine, metergoline) to investigate the role of this neurotransmitter on a zebrafish model of PD.

Methods

1. Research Questions

1.1. What are the behavioral effects of conspecific alarm substance (CAS) on zebrafish behavior in the novel tank test during exposure?

1.2. What are the behavioral effects of CAS on zebrafish behavior in the novel tank test after exposure?

1.3. What is the effect of acute fluoxetine on CAS-elicited behavior during exposure?

1.4. What is the effect of acute fluoxetine on CAS-elicited behavior after exposure?

1.5. What is the effect of acute metergoline on CAS-elicited behavior during exposure?

1.6. What is the effect of acute metergoline on CAS-elicited behavior after exposure?

1.7. What is the effect of CAS on brain monoamine oxidase activity?

2. Hypotheses and predictions

2.1. Based on findings reported in the literature (Egan et al., 2009; Maximino et al., 2014; Quadros et al., 2016; Speedie and Gerlai, 2008), CAS will increase erratic swimming during exposure, and both freezing and geotaxis after exposure.

2.2. Since CAS also increases autonomic activity in zebrafish (Maximino et al., 2014), and since melanosome movement is highly controlled by the autonomic system (Nguyen et al., 2014), we hypothesize that CAS will induce melanosome aggregation. This response will be blocked by pCPA pre-treatment.

2.3. Alterations of phasic serotonin release (i.e., fluoxetine), but not of serotonergic tone (i.e., metergoline, pCPA), are expected expected to block the effects of CAS both during and after exposure.

2.4. CAS is expected to increase serotonergic activity by inhibiting monoamine oxidase activity.

Sampling Plan

3. Existing data

3.1. Data collected prior to registration: As of the date of submission, data on monoamine oxidase activity exists, though no analysis has been conducted related to the research plan.

3.2. Data collected after registration: Behavioral data on the effects of CAS, as well as data on the effects of fluoxetine and metergoline on these effects, have not been collected as of the date of submission.

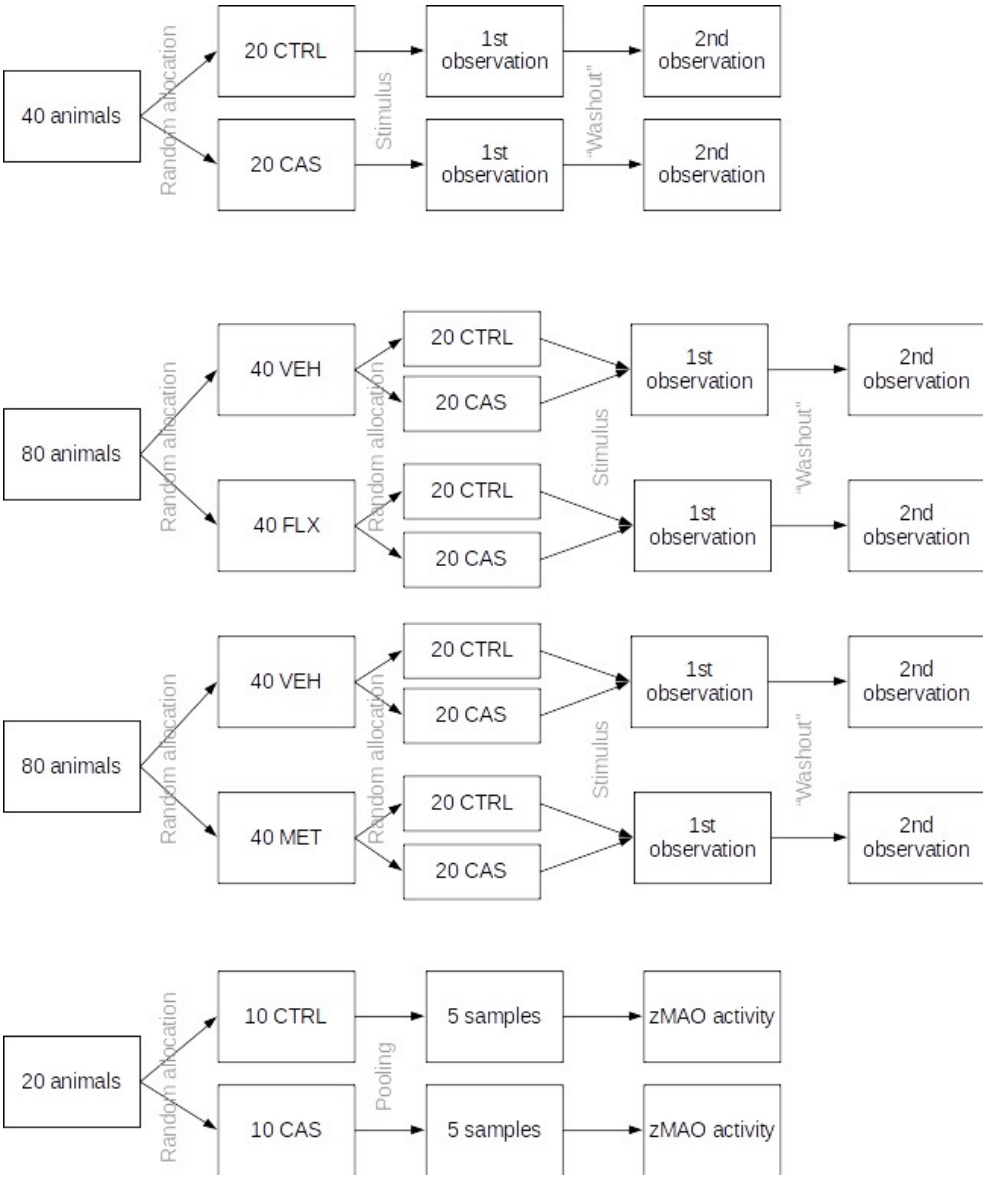
4. Explanation of existing data

4.1. While data on monoamine oxidase acitivity has already been collected, minimal processing took place (i.e., correction by protein levels in samples). Access to the data has been limited to P.I.s (CM and DBR), and no statistical analysis (including summary statistics and graphical representations) have yet been made.

5. Data collection procedures.

5.1. **Animals:** Zebrafish from the longfin phenotype will be used in all experiments. Outbred populations will be used due to their increased genetic variability, decreasing the effects of random genetic drift which could lead to the development of uniquely heritable traits (Parra et al., 2009; Speedie and Gerlai, 2008). Thus, the animals used in the experiments are expected to better represent the natural populations in the wild. Animals will be bought from a commercial vendor, and are expected to arrive in the laboratory with an approximate age of 3 months; animals will be quarantined for two weeks. The experiments will begin when animals reach an approximate age of 4 months (mean standard length = 23.0 mm). Animals will be kept in mixed-sex tanks during acclimation, with an approximate ratio of 50 male:50 female. Breeder should be licensed for aquaculture under Ibama's (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) Resolution 95/1993. Animals will be group-housed in 40 L tanks, with a maximum density of 25 fish per tank, for at least 2 weeks before experiments begin. Tanks are filled with non-chlorinated water at room temperature (28 °C) and a pH of 7.0-8.0. Lighting will be provided by fluorescent lamps in a cycle of 14-10 hours (LD), according to standards of care for zebrafish (Conselho Nacional de Controle de Experimentação Animal - CONCEA, 2017). Water quality parameters will be controlled as follows: pH 7.0-8.0; hardness 100-150 mg/L CaCO3; dissolved oxygen 7.5-8.0 mg/L; ammonia and nitrite < 0.001 ppm. Animals will be used for only one experiment, to reduce interference from apparatus exposure.

5.2. **CAS preparation:** CAS will be prepared as follows (cf. Maximino et al., 2014): one donor fish will be removed from the tank, cold-anesthetized, transferred to a Petri dish, and immediately decapitated with a surgical scalpel. The excess blood will be removed from the animal; after that, the donor fish will be transferred to another Petri dish, where 15 shallow cuts will be made on each side of the fish in order to rupture club cells. After that, 10 ml of distilled water will be used to wash the cuts, after which the carcass will be removed from the dish. Using a Pasteur pipette, scales and other impurities will be removed; the remaining solution will be transferred to a 50 ml beaker. 7 ml of this final solution (CAS) will be used as stimulus.



5.3. Behavioral assays: For Experiments 1 and 2, animals will be allocated to treatment groups, and their behavior will be observed during ("1st observation") and after ("2nd observation") stimulus exposure (**Figure 1**). For the 1st observation, animals will be individually transferred to a 1.5 L plastic tank for a 3-min acclimation interval. After that, the animal will be exposed to either 7 mL CAS

(CAS groups) or 7 mL distilled water (CTRL groups). Their behavior will be tracked for the entire duration of exposure (6 min). Immediately after exposure, animals will be subjected to a "washout", in which it is transferred to a 500 mL tank with clean water; after that step, animals will be individually transferred to another tank, filled with 7.5 L of clean water, for stimulus-free observation. Its behavior will be recorder for another 6 min. For both the 1st and the 2nd observation, the following variables will be recorded:

- **Geotaxis:** time spent on the bottom of the tank;
- **Erratic swimming:** frequency of rapid swimming with unpredictable, zig-zagging course;
- **Thrashing:** frequency of persistent swimming against the walls of the tank;
- **Freezing:** duration of complete movement cessation, with the exception of eye and opercular movements;
- **Squares crossed:** number of squares the animals crosses during the experiment;

These observations will be made by two trained experimenters which will be blind to treatment. Behavior will be recorded manually using X-Plo-Rat (https://github.com/lanec-unifesspa/x-plo-rat). Experiment 1 and 2 are expected to take one week to complete.

5.4. Pharmacological treatments: In 8 groups of animals (Experiment 2), pharmacological treatment will take place 20 min before CAS exposure. Animals will be cold-anesthetized and transferred to a surgical bed, were continuous gill perfusion will be maintained (Kinkel et al., 2010). Immediately after fixation,

animals will be injected intraperitoneally with either vehicle (Cortland's salt solution; Wolf, 1963), 1 mg/kg metergoline, or 2.5 mg/kg fluoxetine. As a result, four metergoline groups will be formed as follows: control (distilled water) + vehicle, control + metergoline, CAS + vehicle, CAS + metergoline. Four fluoxetine groups will be formed as follows: control + vehicle, control + fluoxetine, CAS + vehicle, CAS + fluoxetine. Comparisons will not be made between drugs.

5.5. **Brain samples and MAO activity:** z-MAO activity was determined as reported previously (Müller et al., 2017). Two zebrafish brains were pooled per sample and homogenized in 0.5 mL of buffer solution containing 16.8 mM Na_2HPO_4 and 10.6 mM KH_2PO_4, pH 7.4, isotonized with sucrose. Samples (n = 5 per group) were centrifuged at 1.000 x *g* for 5 min, and the supernatants were kept on ice for the experiments. Protein samples (approximately 100 µg) were mixed with 460 µL of assay buffer (168 mM Na_2HPO_4 and 10.6 mM KH_2PO_4, pH 7.4, isotonized with KCl) and preincubated at 37°C for 5 min. The reaction started by adding 110 µM kynuramine hydrobromide in a final volume of 700 µL, and was stopped 30 min later with 300 µL 10% trichloroacetic acid. Reaction products were further centrifuged at 16.000 x *g* for 5 min and supernatants (800 µL) were mixed with 1M NaOH (1 mL). Fluorescence was measured using excitation at 315 nm and emission at 380 nm. Product formation (4-hydroxyquinoline) was estimated and enzyme activity was expressed as expressed as nmol 4-OH quinoline/min/mg protein.

5.6. **Melanophore responses:** Melanophore responses will be assessed based on a protocol used by Maximino et al. (2011). Briefly, animals will be exposed to either CAS or water, and then anesthetized with eugenol (up to 60 ppm) and transferred to a Petri dish under a stereomicroscope. Eugenol immersion will be maintained throughout the experiment. Photographs of the dorsal region will be taken within 2 min. Images will be analyzed using ImageJ 1.42q (National Institutes of Health, Bethesda, MD, USA) by thresholding a black-and-white image and measuring the area covered by the black vs. the white portion.

6. Quality control

6.1. **Behavioral data:** Quality control of samples will be maintained by periodic assessment of water quality and health parameters. All experimenters will be trained in the behavioral methods before experiments; training will include observation of all experiments by a PI (CM or MGL) on at least two occasions. After these observations, each trainee will perform two mock experiments, on a single subject each, while being observed by the PI. Additional practice assessments will be arranged as necessary. All protocols will be reviewed by all PIs. Behavioral records will be reviewed by at least one PI for administration/scoring accuracy, in order to ensure adherence to protocols and consistency across tests. Finally, a Cohen's κ of at least 0.8 will be used as marker of good inter-rater agreement.

6.2. **Biochemical data:** All experimenters were trained in the analytical method before experiments. Quality control was achieved periodically using Levey-Jennings charts for known concentrations of kynuramine, adopting a 1_{2s} rule.

7. Sample size

7.1. **Experiment 1:** 40 animals per group

7.2. **Experiment 2:** 10 animals per group

7.3. **Experiment 3:** 10 animals per group

8. Sample size rationale

8.1. **Experiment 1:** Power analysis for two-sample unpaired t-test. $\alpha = 0.05$, power = 0.8, expected effect size $d = 0.6$.

8.2. **Experiment 2:** Power analysis for 2-way ANOVA with interaction effects. $\alpha = 0.05$, $\beta = 0.8$, expected effect size $f = 0.25$ for each independent variable.

8.3. **Experiment 3:** Power analysis for two-sample unpaired t-test. $\alpha = 0.05$, power = 0.8, expected effect size $d = 1.5$.

Variables

In this section, we describe all variables (both manipulated and measured variables) that will later be used in the confirmatory analysis plan.

9. Manipulated variables

9.1. **Treatment –** variable with two levels: control (CTRL, animals exposed to distilled water) and CAS (CAS, animals exposed to conspecific alarm substance)

9.2. **Drug –** variable with two levels: vehicle (VEH, animals injected with Cortland's salt solution) and drug (either MET or FLX, depending on comparison)

10. Measured variables

10.1. **Behavioral outcomes:** Geotaxis; Erratic swimming; Thrashing; Freezing; Squares crossed.

10.2. **Biochemical outcome:** zMAO activity, corrected by protein levels.

Design Plan

In this section, we describe the overall design of your study.

11. ***Study type:*** Experiment

12. ***Blinding:*** Experimenters will be blinded to treatment by coding drug vials or CAS containers. The data analyst will be blinded to phenotype by using coding to reflect treatments in the resulting datasets; after analysis, data will be unblinded.

13. Study design

13.1. **Experiment 1:** two-group, unpaired design.

13.2. **Experiment 2:** factorial design.

13.3. **Experiment 3:** two-group, unpaired design.

14. ***Randomization:*** In all experiments, animals will be randomly drawn from the holding tank immediately before testing, and treatment order will be randomized via the generation of random numbers using the tool at http://www.randomization.com/.

Analysis Plan

Only confirmatory analyses are described here; no exploratory analyses are planned and, if any take place, they will be clearly labeled as hypothesis generating.

15. Statistical models

15.1. **Experiment 1:** Differences between groups will be analyzed using Approximative Two-Sample Fisher-Pitman Permutation Tests 10,000 Monte-Carlo re-samplings, using the R package 'coin' (Hothorn et al., 2006). Behavioral variables will be included as primary outcomes, with treatment used as independent variable.

15.2. **Experiment 2:** Differences between groups will be analyzed using two-way analyses of variance with robust estimators on Huber's M-estimators, using the R package 'rcompanion' (Mangiafico, 2017). Behavioral variables will be included as outcomes, with treatment and drug used as independent variables; interaction between IVs will be assessed as the most important predictor. P-values will be adjusted for the false discovery rate.

15.3. **Experiment 3:** Differences between groups will be analyzed using Approximative Two-Sample Fisher-Pitman Permutation Tests 10,000 Monte-Carlo re-samplings, using the R package 'coin' (Hothorn et al., 2006). zMAO activity will be included as the primary outcome, with treatment used as independent variable.

16. Follow-up analyses

16.1. **Experiment 2:** Interaction effects will be followed by pairwise robust tests, with p-values adjusted for the false discovery rate.

17. ***Inference criteria:*** P-values will be used to make inferences, with a cut-off criterion based on a three-sigma rule (Colquhoun, 2014); all tests will be one-tailed, and multiple comparisons in Experiment 2 will be accounted for by adjusting p-values for the false discovery rate.

18. ***Data exclusion:*** For each group, the median deviation of medians (MDM) *within* the group will be applied; data points above or below the median ± 1 MDM will be excluded (Rousseeuw and Croux, 1993).

19. ***Missing data:*** Any missing data will be dealt with using the expectation maximization method, shown to be superior to the listwise deletion method for small to moderate sample sizes (Rubin et al., 2007).

Data availability

Datasets and scripts for all analyses will be made available from our GitHub repository (https://github.com/lanec-unifesspa).

Acknowledgments

Experiments reported are financed by a CNPq/Brazil grant to CMO (Edital Universal, Process number 400726/2016-5).

References

American Psychiatric Association, 2013. Diagnostic and Statistical Manual of Mental Disorders - Fifth Edition - DSM-5, 5th ed. American Psychiatric Publishing, Washington.

Blanchard, D.C., Griebel, G., Blanchard, R.J., 2001. Mouse defensive behaviors: Pharmacological and behavioral assays for anxiety and panic. Neurosci. Biobehav. Rev. 25, 205–18.

Brandão, M.L., Zanoveli, J.M., Ruiz-Martinez, R.C., Oliveira, L.C., Landeira-Fernandez, J., 2008. Different patterns of freezing behavior organized in the periaqueductal gray of rats: Association with different types of anxiety. Behav. Brain Res. 188, 1–13. doi:10.1016/j.bbr.2007.10.018

Cachat, J., Stewart, A., Utterback, E., Hart, P., Gaikwad, S., Wong, K., Kyzar, E., Wu, N., Kalueff, A. V, 2011. Three-dimensional neurophenotyping of adult zebrafish behavior. PLoS One 6, e17597. doi:10.1371/journal.pone.0017597

Caldirola, D., Grassi, M., Alciati, A., Riva, A., Sangiorgio, E., Daccò, S., Perna, G., 2017. Personalized medicine in panic disorder: where are we now? A meta-regression analysis. Pers. Med. Psychiatry 1, 26–38. doi:10.1016/j.pmip.2016.12.003

Colquhoun, D., 2014. An investigation of the false discovery rate and the misinterpretation of p-values. R. Soc. Open Sci. 1, Article 140216. doi:10.1098/rsos.140216

Conselho Nacional de Controle de Experimentação Animal - CONCEA, 2017. Diretriz brasileira para o cuidado e a utilização de animais para fins científicos e didáticos - DBCA. Anexo I. Peixes mantidos em instalações de instituições de ensino ou pesquisa científica. Brasil.

Deakin, J.F.W., Graeff, F.G., 1991. 5-HT and mechanisms of defense. J. Psychopharmacol. 5, 305–315.

Døving, K.B., Lastein, S., 2009. The alarm reaction in fishes — Odorants, modulations of responses, neural pathways. Ann. N. Y. Acad. Sci. 1170, 413–423. doi:10.1111/j.1749-6632.2009.04111.x

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A. V, 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav. Brain Res. 205, 38–44. doi:10.1016/j.bbr.2009.06.022

Gerlai, R., 2013. Antipredatory behavior of zebrafish: Adaptive function and a tool for translational research. Evol. Psychol. 11, 591–605.

Gerlai, R., 2010a. High-throughput behavioral screens: The first step towards finding genes involved in vertebrate brain function using zebrafish. Molecules 15, 2609–2622. doi:10.3390/molecules15042609

Gerlai, R., 2010b. Zebrafish antipredatory responses: A future for translational research? Behav. Brain Res. 207, 223–231. doi:10.1016/j.bbr.2009.10.008

Graeff, F.G., Del-Ben, C.M., 2008. Neurobiology of panic disorder: From animal models to brain neuroimaging. Neurosci. Biobehav. Rev. 32, 1326–1335. doi:10.1016/j.neubiorev.2008.05.017

Hothorn, T., Hornik, K., van de Wiel, M.A., Zeileis, A., 2006. A Lego system for conditional inference. Am. Statician 60, 257–263. doi:10.1198/000313006X118430

Jesuthasan, S.J., Mathuru, A.S., 2008. The alarm response in zebrafish: Innate fear in a vertebrate genetic model. J. Neurogenet. 22, 211–229. doi:10.1080/01677060802298475

Johnson, P.L., Lowry, C.A., Truitt, W., Shekhar, A., 2008. Disruption of GABAergic tone in the dorsomedial hypothalamus attenuates responses in a subset of serotonergic neurons in the dorsal raphe nucleus following lactate-induced panic. J. Psychopharmacol. 22, 642–52. doi:10.1177/0269881107082900

Kahn, R.S., Asnis, G.M., Wetzler, S., van Praag, H.M., 1988. Neuroendocrine evidence for serotonin receptor hypersensitivity in panic disorder. Psychopharmacology (Berl). 96, 360–364. doi:10.1007/BF00216062

Kahn, R.S., Wetzler, S., van Praag, H.M., Asnis, G.M., Strauman, T., 1988. Behavioral indications for serotonin receptor hypersensitivity in panic disorder. Psychiatry Res. 25, 101–104. doi:10.1016/0165-1781(88)90163-1

Kessler, R.C., Chiu, W.T., Jin, R., Ruscio, A.M., Shear, K., Walters, E.E., 2006. The epidemiology of panic attacks, panic disorder, and agoraphobia in the National Comorbidity Survey Replication. Arch. Gen. Psychiatry 63, 415. doi:10.1001/archpsyc.63.4.415

Kinkel, M.D., Eames, S.C., Philipson, L.H., Prince, V.E., 2010. Intraperitoneal injection into adult zebrafish. J. Vis. Exp. 42, 2126. doi:10.3791/2126

Mangiafico, S., 2017. rcompanion: Functions to Support Extension Education Program Evaluation.

Mathuru, A.S., Kibat, C., Cheong, W.F., Shui, G., Wenk, M.R., Friedrich, R.W., Jesuthasan, S., 2012. Chondroitin fragments are odorants that trigger fear behavior in fish. Curr. Biol. 22, 538–544. doi:10.1016/j.cub.2012.01.061

Maximino, C., 2012. Serotonin and anxiety. Neuroanatomical, pharmacological, and functional aspects, SpringerBriefs in Neuroscience. Springer, New York, NY. doi:10.1007/978-1-4614-4048-2

Maximino, C., Lima, M.G., Costa, C.C., Guedes, I.M.L., Herculano, A.M., 2014. Fluoxetine and WAY 100,635 dissociate increases in scototaxis and analgesia induced by conspecific alarm substance in zebrafish (Danio rerio Hamilton 1822). Pharmacol. Biochem. Behav. 124C, 425–433. doi:10.1016/j.pbb.2014.07.003

Maximino, C., Lima, M.G., Oliveira, K.R.M., Picanço-diniz, D.L.W., Herculano, A.M., 2011. Adenosine A1, but not A2, receptor blockade increases anxiety and arousal in zebrafish. Basic Clin. Pharmacol. Toxicol. 109, 203–207. doi:10.1111/j.1742-7843.2011.00710.x

Moreira, F.A., Gobira, P.H., Viana, T.G., Vicente, M.A., Zangrossi Jr, H., Graeff, F.G., 2013. Modeling panic disorder in rodents. Cell Tissue Res. doi:10.1007/s00441-013-1610-1

Müller, T.E., Nunes, M.E., Menezes, C.C., Marins, A.T., Leitemperger, J., Gressler, A.C., Carvalho, F.B., de Freitas, C.M., Quadros, V.A., Fachinetto, R., Rosemberg, D.B., Loro, V.L., 2017. Sodium selenite prevents paraquat-induced neurotoxicity in zebrafish. Mol. Neurobiol. doi:10.1007/s12035-017-0441-6

Nathan, F.M., Ogawa, S., Parhar, I.S., Neurochemistry, J.O.F., 2015. Kisspeptin1 modulates odorant-evoked fear response via two serotonin receptor subtypes (5-HT<inf>1A</inf> and 5-HT<inf>2</inf>) in zebrafish. J. Neurochem. 133, 870–878. doi:10.1111/jnc.13105

Norton, W., Bally-cuif, L., 2010. Adult zebrafish as a model organism for behavioural genetics. BMC Neurosci. 11, 90.

Oliveira, L.C., Broiz, A.C., de Macedo, C.E., Landeira-Fernandez, J., Brandão, M.L., 2006. 5-HT2 receptor mechanisms of the dorsal periaqueductal gray in the conditioned and unconditioned fear in rats. Psychopharmacology (Berl). doi:10.1007/s00213-006-0653-3

Parra, K. V, Adrian Jr, J.C., Gerlai, R., 2009. The synthetic substance hypoxanthine 3-N-oxide elicits alarm reactions in zebrafish (*Danio rerio*). Behav. Brain Res. 205, 336–341. doi:10.1016/j.bbr.2009.06.037

Paul, E.D., Johnson, P.L., Shekhar, A., Lowry, C.A., 2014. The Deakin/Graeff hypothesis: Focus on serotonergic inhibition of panic. Neurosci. Biobehav. Rev. 46, 379–96. doi:10.1016/j.neubiorev.2014.03.010

Quadros, V.A., Silveira, A., Giuliani, G.S., Didonet, F., SIlveira, A.S., Nunes, M.E., Silva, T.O., Loro, V.L., Rosemberg, D.B., 2016. Strain- and context-dependent behavioural responses of acute alarm substance exposure in zebrafish. Behav. Processes 122, 1–11. doi:10.1016/j.beproc.2015.10.014

Rinkwitz, S., Mourrain, P., Becker, T.S., 2011. Zebrafish: An integrative system for neurogenomics and neurosciences. Prog. Neurobiol. 93, 231–243. doi:10.1016/j.pneurobio.2010.11.003

Rousseeuw, P.J., Croux, C., 1993. Alternatives to the median absolute deviation. J. Am. Stat. Assoc. 88, 1273–1283.

Rubin, L.H., Witkiewitz, K., St. Andre, J., Reilly, S., 2007. Methods for handling missing data in the behavioral neurosciences: Don't throw the baby rat out with the bath water. J. Undergrad. Neurosci. Educ. 5, A71–A77.

Schenberg, L.C., Bittencourt, A.S., Sudré, E.C.M., Vargas, L.C., 2002. Modeling panic attacks. Neurosci. Biobehav. Rev. 25, 647–659. doi:10.1016/S0149-7634(01)00060-4

Schenberg, L.C., Schimitel, F.G., Armini, R.D.S., Bernabé, C.S., Rosa, C.A., Tufik, S., Janaina, C., Müller, T., Quintino-dos-Santos, J.W., 2014. Translational approach to studying panic disorder in rats: Hits and misses. Neurosci. Biobehav. Rev. 46, 472–496. doi:10.1016/j.neubiorev.2014.10.002

Schirmer, A., Jesuthasan, S., Mathuru, A.S., 2013. Tactile stimulation reduces fear in fish. Front. Behav. Neurosci. 7, Article 167. doi:10.3389/fnbeh.2013.00167

Shams, S., Rihel, J., Ortiz, J.G., Gerlai, R.T., 2018. The zebrafish as a promising tool for modeling human brain disorders: A review based upon an IBNS Symposium. Neurosci. Biobehav. Rev. 75, 176–190.

Speedie, N., Gerlai, R., 2008. Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). Behav. Brain Res. 188, 168–177. doi:10.1016/j.bbr.2007.10.031

Stewart, A., Gaikwad, S., Kyzar, E., Green, J., Roth, A., Kalueff, A. V., 2012. Modeling anxiety using adult zebrafish: A conceptual review. Neuropharmacology 62, 135–143. doi:10.1016/j.neuropharm.2011.07.037

Vianna, D.M.L., Graeff, F.G., Brandão, M.L., Landeira-Fernandez, J., 2001. Defensive freezing evoked by electrical stimulation of the periaqueductal gray: Comparison between dorsolateral and ventrolateral regions. Learn. Mem. 12, 4109–4112.

Wolf, K., 1963. Physiological salines for freshwater teleosts. Prog. Fish-Culture 25, 135–140.