THE CORRELATION OF NEURAL AND PHYSIOLOGICAL PHENOTYPES IN ZEBRAFISH MODELS OF STRESS

PC Hart*, CL Bergner**, RJ Egan**, JM Cachat*, PR Canavello*, B Bartels*, E Beeson*, S Elkhayat*, MF Elegante*, S Mohnot*, DH Tien*, AK Tien*, H Amri**, E Glasgow**, Z Zhukowska**, AV Kalueff*,**

* Pharmacology Dept., Tulane University Medical School, New Orleans, LA **Physiology and Biophysics Dept., Georgetown University Medical School, Washington, DC

Abstract

Zebrafish are becoming increasingly popular in genetic, neuroscience, and experimental biology research. To validate the experimental utility of zebrafish in stress research, the correlation between physiological and neural phenotypes is important. Behavioral data in the novel tank test translates stress/anxiety into quantifiable endpoints (latency to enter the top half of the tank, number of entries, time spent exploring the top half of the tank, fear-like erratic movements, and freezing bouts). As a corresponding physiological measure of stress or anxiety, cortisol levels provide a sensitive and reliable analysis of endocrine response. The juxtaposition of behavioral and physiological phenotypes is particularly useful when evaluating pharmacological manipulation in zebrafish. For example, chronic administration of the SSRI antidepressant fluoxetine [for 2 weeks] reduces anxiety behavior and lowers whole-body cortisol levels. To induce an anxiogenic response, zebrafish were subjected to a model of benzodiazepine withdrawal syndrome. After chronic administration of the benzodiazepine diazepam, drug treatment was halted for 3 days before novel tank testing and subsequent cortisol assessment. While behavioral data signified a strong anxiety-like phenotype, cortisol levels also tended to rise in these fish. Collectively, our experiments substantiate zebrafish as dependable and consistent subjects in anxiety research, as well as in studies focusing on drug dependency and withdrawal. Based on the strong correlation of behavioral data and cortisol analysis, zebrafish prove to be an excellent model organism for experimental stress research.

Introduction

A relatively simple vertebrae species, zebrafish (*Danio rerio*) is popular in biomedical research because its physiology is analogous to humans, permitting researchers to probe the mechanisms and pathways relevant to human disease and therapy.

The zebrafish nervous system possesses all of the "classical" vertebrate neurotransmitter systems, and contains a well-documented corticosteroid stress axis. Zebrafish are also an ideal animal model for laboratory research because they are low-maintenance and abundantly produce offspring (Gerlai et al. 2006). Together, this makes the zebrafish a premiere model to investigate principles of nervous system development, function, disease and behavior. Here we examine the zebrafish anxiety/fear-like behavior, and correlate these "affective" stress-evoked states with physiological phenotypes, such as the levels of stress hormone cortisol.

Methods

Novel tank test: Zebrafish were relocated from home-tanks to novel tanks. Latency to reach the upper portion of the tank, time spent in the upper portion of the tank, number of entries into the upper portion of the tank, erratic movements, freezing bouts, and freezing duration were recorded. Erratic movements were characterized as sharp changes in direction and velocity, or rapid darting behaviors. Anxiety response was measured as a significant decrease in exploration, including: longer latency to reach the top, shorter duration in upper half as a ratio to the number of entries to the top, an increase in freezing or erratic movements.

Alarm pheromone test: To damage the epidermal cells, 10-15 shallow cuts were made on one-side of the trunk of the sacrificed fish. The animal was then washed (for 5 min) in a Petri dish filled with 10 ml of distilled water. The distilled water in each Petri dish was transferred to 50ml tubes and kept refrigerated until use. To administer the pheromone to zebrafish, 7-10 ml were added to the novel tank and dispersed equally throughout the water.

Indian Leaf Fish exposure: The Indian Leaf Fish (*Nandus nandus*), is a natural predator of zebrafish, and its presence has been shown to elicit fear-like responses in zebrafish (Bass and Gerlai, 2008).

Acute exposure: Individual zebrafish were placed in a 1.5-L exposure tank, where the leaf fish was housed, for a period of 20 min. After exposure to the leaf fish, each zebrafish was transferred to a novel observation tank.

Chronic exposure: The Indian leaf fish was added to the home tank of a shoal of zebrafish for 48 h. Following the exposure period in the home tank, individual zebrafish were tested in the novel tank test.

Chronic diazepam exposure: To examine the effects of anxiolytic drugs on zebrafish behavior, diazepam was used at a dose of 5 μM. Following two weeks of drug administration, the fish were individually tested in the novel tank.

Diazepam withdrawal: At the end of the two week drug administration period the fish were not administered the drug for a period of three days. Following this withdrawal period the fish were individually tested in the novel tank diving paradigm for signs for distress based on standard endpoint measurement.

Chronic SSRI exposure: To investigate the anxiolytic effects of chronic antidepressant administration and its effect on zebrafish behavior, the selective serotonin reuptake inhibitor (SSRI) fluoxetine was administered at 5 μg/L/day (Brooks et al. 2003) for 2-4 weeks. Following the period of drug administration, the fish were individually tested in the novel tank.

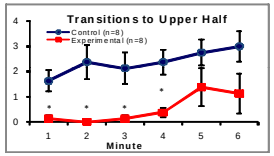
Cortisol extraction: After homogenizing each animal, samples were then transferred to glass tubes. Each sample was then extracted 2 times with 5mL of Ether. After the Ether had been allowed to evaporate (under a fume hood) the cortisol was reconstituted in 1mL of 1X PBS (Alsop and Vijayan, 2008).

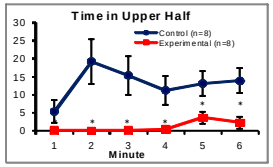
ELISA assay: ELISA was performed on cortisol samples using a cortisol assay kit (Salimetrics LLC, PA). Absolute cortisol levels (μg/dL) were assessed. Cortisol levels were then normalized based on the weight of the initial body sample.

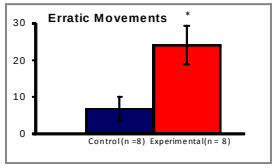
Statistical analysis of behavioral data: The data were analyzed using a two sample (Mann-Whitney) U-Test for significance between groups (control and experimental conditions). All data are expressed as Mean ± SEM. Significance was set at $p < 0.05$.

Results

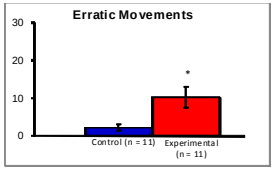
Alarm Pheromone: Alarm pheromone exposure reduced the number of top entries, and time spent at the top, indicating a robust anxiety response. Notably, the average number of total transitions was also significantly reduced, compared to the control group ($p < 0.005$). Alarm pheromone-exposed zebrafish also displayed a significantly higher number of erratic movements, again suggesting their increased anxiety.



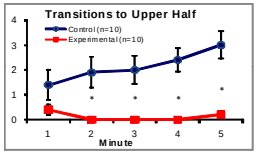


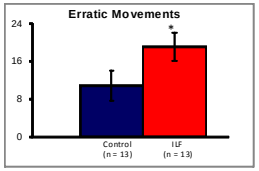


Chronic predator stress: In the 72-h Indian Leaf Fish exposure group, the average number of erratic movements was significantly elevated, indicating their strong fear-like response.



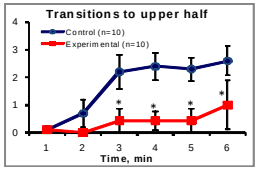
Acute predator stress: After acute exposure to the Indian Leaf Fish for 5 min, experimental zebrafish displayed significantly fewer transitions into the upper half, indicating high anxiety and stress. With the exception of minute 1, high statistical significance was achieved throughout testing, with an average of $p < 0.0005$. Likewise, erratic movements were significantly higher in the stressed group.





Diazepam exposure

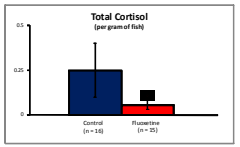
Withdrawal from anxiolytic drug diazepam elicited significant anxiogenic-like behavioral responses in zebrafish. This included markedly reduced exploration (transitions to the upper half), less time spent in the upper half, and longer latency to enter the upper half of the tank. Likewise, the diazepam withdrawal group also displayed significantly more erratic movements during the 6-min observational period ($P < 0.05$, data not shown), which is again in line with overall increased levels of anxiety and stress in this group.

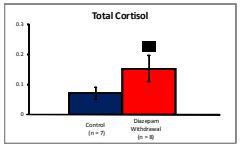


Chronic fluoxetine treatment: Exposure of zebrafish to chronic antidepressant drug fluoxetine consistently produced characteristic anxiolytic-like changes in zebrafish behavior. Overall, the amount of activity in the top of the tank was dramatically increased in zebrafish, suggesting the reduction of their anxiety (data not shown). The amount of freezing behavior and erratic movements was also markedly reduced in fluoxetine-treated group (compared to controls), consistent with anxiolytic-like responses. In general, locomotion of fluoxetine-treated zebrafish was strikingly different from controls, since drug-treated fish displayed a very "calm" swimming, with no freezing episodes or erratic movements. High statistical significance was achieved throughout testing for all behavioral endpoints, with an average of $p < 0.0005$.

Cortisol analysis

Overall, the whole-body cortisol level in chronically fluoxetine-exposed zebrafish was significantly lower ($p < 0.05$), indicating that antidepressant treatment effectively reduces stress and anxiety. In contrast, anxiogenic factors, such as diazepam withdrawal, predictably increased whole-body cortisol levels ($p = 0.07211$) in zebrafish, showing a good correlation between behavioral and endocrine stress responses.





Summary

These same attributes that give the zebrafish its power for genetics, drug discovery, and phenotyping research, make this species an ideal subject for studies of stress and anxiety. The similarity of its physiology to that of higher vertebrate species makes the zebrafish an good model for the development of more cost-effective models for behavioral pathogenesis. By comparing the robust behavioral phenotypes seen here with the physiological evidence from cortisol assays, we were able to demonstrate the consistency and reliability of this species as an experimental model for stress and anxiety.

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