Zebrafish Behavioral Response to Chronic Drug Exposure

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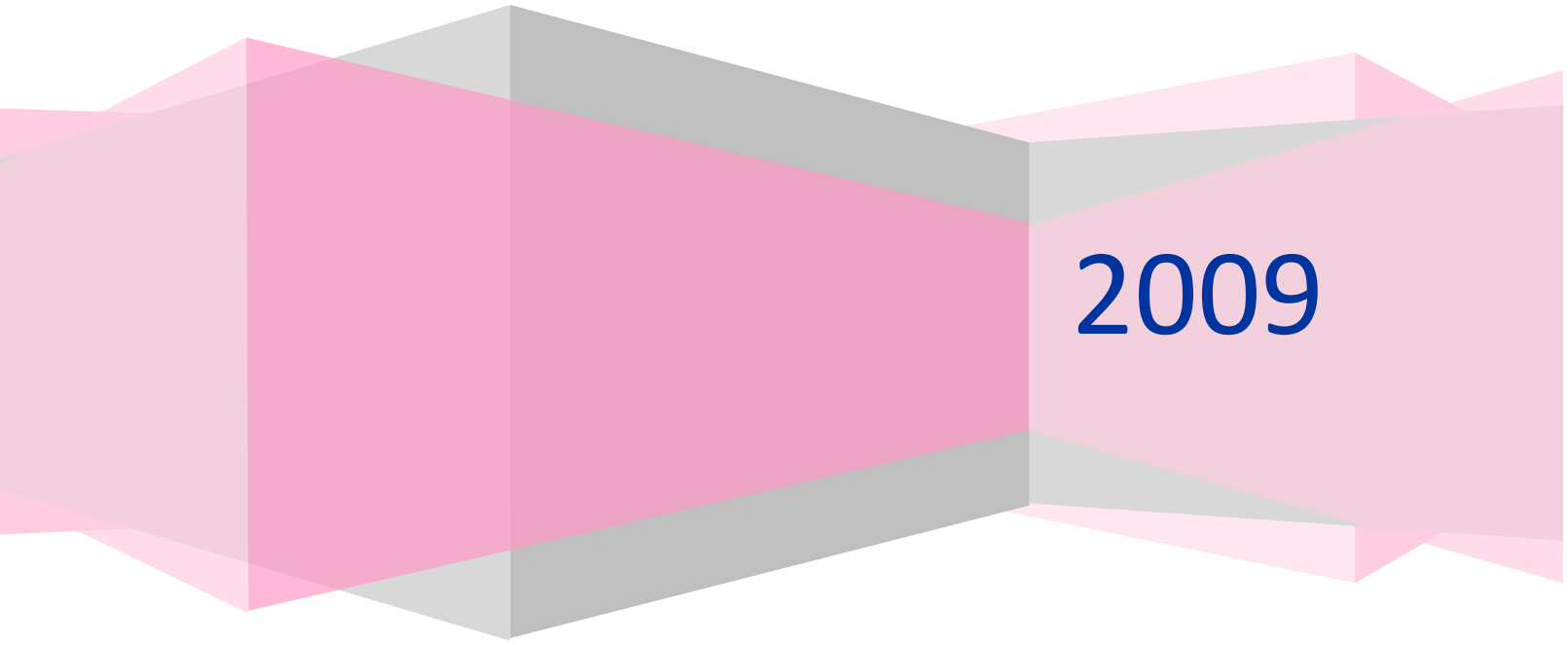

Abstract:

Zebrafish (*Danio rerio*) are tropical freshwater fish belonging to the Cyprinidae, the most species-rich vertebrate family (Nelson, 2006). There are currently approximately 44 million danionin species (Fang, 2001). Great populations of zebrafish are found across a wide range of geological localities in the northeast Indian states, West Bengal, Assam, Meghalaya, and Orissa (Engeszer *et al*., 2007) (Fig. 1). A wide variety of mechanisms have evolved to produce and interpret signals that can be used to convey information (Jesuthasan *et al.*, 2008). Zebrafish can also absorb various chemicals through their gills and skin. Here we study the effects of withdrawal from addictive substances to produce anxiogenic-like responses in zebrafish.

Key words: Zebrafish, Withdrawal, Cortisol, Anxiety, Stress, Novel Tank test, Addiction

Introduction

In the early 1970's, Dr. George Streisinger, a University of Oregon scientist, began utilizing zebrafish as an exceptional model for studying vertebrate development and genetics (Angier, 1991). Zebrafish (*Dano rerio*) are small, 4-cm long freshwater species that can be easily kept in the laboratory (Gerlai *et al*., 2008) (Fig. 2). A single female can produce up to 200 eggs per spawning (every other day) (Speedie & Gerlai, 2007), which reach maturity within 2 to 3 months. Since they are easy to replicate and house in the laboratory, they can be kept efficiently in large numbers (Westerfield, 2000) in order to do validated research.

Zebrafish are vertebrates, which means that they are more closely related to humans than commonly used invertebrate models in today's laboratories; therefore, many are being utilized in research for human brain disorders (Speedie & Gerlai, (2007). Zebrafish organs are functionally and morphologically similar to humans, so it was hoped that the genetic screenings of zebrafish could unveil mechanisms and pathways directly relevant to human disease and therapy (Driever *et al*., 1996).

Zebrafish are popular in most biological laboratories as model organisms in genetics, developmental biology, neurophysiology and biomedicine (Vascotto *et al*., 1997; Grunwald & Eisen, 2002; Rubinstein, 2003; Amsterdam & Hopkins, 2006). In addition, mounting evidence suggests zebrafish are emerging as ideal model species for analysis of genetic and behavioral mechanisms of drug addiction because they are responsive to the rewarding properties of drugs of abuse (Lopez-Patino *et al*., 2007). Moreover, these observations can be translated to clinical research in human disease, stress, anxiety, and drug withdrawal, thereby making zebrafish a valuable model to study stress responses.

This research project was performed in the summer of 2009 in the Kalueff lab

(www.kaluefflab.com) at Pharmacology Department of Tulane University Medical School. Here, we examine the responses of zebrafish to serveral common drugs, such as caffeine and ethanol, in order to enhance knowledge of this vertebrate specie as a correlate to human-like behavior and response to addictive substances. This paper discusses the use of zebrafish as a model for withdrawal through analysis of the patterns of stress and psychological behavior, resulting from an acute or prolonged exposure to some of these drugs.

Methods

Animals and Housing:

A total of 250 adult (3-5 months old) male and female zebrafish were obtained from local community distributors (50 Fathoms, Metairie, LA). All fish were given 10 days to adjust to the laboratory and housed in groups of 20-30 fish per 40-L tanks. All tanks were filled with deionized water treated with Prime Freshwater and Saltwater Concentrated Conditioner (Seachem Laboratories, Inc., GA). Water temperature was maintained at 25-27 degrees Celsius. The fish were fed Tetramin Tropical Flakes. All fish used in this study were experimentally naïve. Following behavioral testing, the animals were euthanized in 500mg/L Tricane (Sigma-Aldrich, USA; buffered to pH=7), and immediately dissected for further analysis.

Our study examined the effects of ethanol (0.3% EtOH vol/vol. Pharmco-AAPER. USA), morphine (1.5 mg/L single withdrawal, 1.0 mg/L repeated withdrawal, Mallinckrodt, MO) and caffeine (1.5 mg/L, Sigma-Aldrich, USA). The zebrafish were treated with drugs in their home tanks before inducing withdrawal. Drugs were dissolved in tank water before adding zebrafish. Caffeine, morphine, and ethanol were administered daily for 1 week.

Repeated withdrawal experiments were performed on zebrafish treated with ethanol or morphine. After 1-week chronic treatment, fish were removed and placed into exposure tanks

with fresh untreated water for 3 h at a time, twice per day for one week before the behavioral testing. Control fish were placed into treated water containing concentrations of drug identical to the home tanks, while the experimental fish were put into water not containing psychoactive drugs in order to induce withdrawal. Three hours later, the fish were returned to their original tanks for at least 3 h between exposure trails. After 1-week of repeated withdrawal, fish were taken from home tanks and placed in exposure tanks for a final 3-h withdrawal prior to behavioral testing.

Apparatus and Behavioral Testing: Behavioral testing was performed using the novel tank diving test (Fig.3), representing a 1.5-L trapezoid tank (15.2 height x 7.1 width x 27.9 top length x 22.5 bottom length cm; Aquatic Habitats, FL) filled with aquarium treated water. Novel tanks were placed on a stable surface and were divided into 2 equal horizontal portions, marked by a dividing line on the outside walls. Lights were placed on both sides of the novel tank to insure a clear background and to easily see the fish. Behavioral testing took place between 10:00 to 17:00 h. Once each fish was individually transferred to a novel tank, it swimming behavior was video recorded for 6 min. During the video recording process, we took a manual record of the behavioral actions of the fish. This paradigm is called a novel test. Additionally, adult fish are very sensitive to stress caused by handled (Ninkovic, 2006; Lockwood, 2004) and great care has to be taken when behavioral assays are performed (Ninkovic, 2006). The following endpoints were recorded: latency to reach the upper portion of the tank, number of transitions to the upper portion of the tank, number of erratic movements, number of freezing bouts, and freezing duration. Erratic movements are defined as sharp changes in direction and/or velocity and repeated rapid darting behaviors. Freezing was shown as a total absence of movement, except for the gills and eyes, for period of 1s or longer. Significant decreases in exploration or

elevated erratic movements represent behavioral levels indicative of high stress and anxiety (Barcellos *et al*., 2007; Levin *et al*., 2007). In addition, the top: bottom ratio was calculated for each fish, reflecting the level of zebrafish anxiety.

Cortisol Assay: After behavioral testing, fish were tested for whole-body cortisol levels. Individual zebrafish were dissected into the brain, long body, and short body, and were homogenized in 750 uL of ice-cold 1X PBS buffer. The homogenizing rotor blades were washed with an additional 250uL of ice-cold 1X PBS and collected into a 2ml tube containing the homogenate. During this process, all samples were kept on ice. Samples were transferred to glass extract-O tubes and cortisol was extracted twice with 5mL of diethyl ether (Fisher Scientific, USA). The tubes were vortexed and centrifuged twice. After the ether evaporated, the cortisol was reconstituted in 1mL of 1X PBS. Once the ether evaporated over a 24 hour period the ELISA test was performed. To quantify cortisol concentrations, the ELISA was performed using a human salivary cortisol assay kit (Salmetric LLC, PA). ELISA plates were analyzed in a VICTOR-WALLAC plate reader. Cortisol levels are reported as relative concentrations based on the standardized sample used in the ELISA (Egan *et al*., 2009).

Statistical Analysis:

Experimental data were analyzed with a two-sample Wilcoxon U-test for comparison between control and experimental groups. Data is expressed as mean ± SEM. Significance was set at $P<0.05$.

Results:

The ethanol withdrawal group showed longer latency to enter the upper half of the novel tank, but lessened in total time and transition in the upper half (Fig. 4). The ethanol withdrawal showed more freezing bouts and had whole-body cortisol levels higher than their controls;

however, there was a low level of erratic movements.

Single morphine withdrawal did not induce anxiety-like behaviors; however, repeated morphine withdrawal produced strong anxiogenic responses. The repeated withdrawal group showed a significantly longer latency to enter the top, had fewer transitions to the top and spent less time there. Additionally, the repeated withdrawal group had a lesser top: bottom ratio, while erratic movements were increased and freezing bouts remained unaltered (Fig. 5).

Finally, the caffeine withdrawal group showed fewer freezing bouts, displayed more erratic movements, and showed to lower in top: bottom ratio and time spent in the upper half (Fig. 6).

Discussion

For the past three decades, zebrafish have been increasingly important in biomedical research (Dooley & Zon, 2000; Shin & Fishman, 2002, Lieschke & Currrie, 2007), particularly as a model of human disease (Berghmans *et al.,* 2005; Guyon *et al.,*2006) and for screening of therapeutic drugs (Rubinstein, 2003, 2006). Furthermore, studies in mice (Pich & Epping-Jordan, 1998) demonstrate that genetically-tractable species, which allow for high throughput behavioral studies, could increase our current understanding of the nature and mechanisms of drug addiction (Lopez-Patino, 2007). In order to develop a deeper understanding of the correlation between zebrafish and humans, we attempted to study the physiological and behavioral responses in zebrafish elicited by drug withdrawal from a wide spectrum of psychotropic drugs, including ethanol, caffeine, and morphine.

Withdrawal results from the discontinuation of the use of an addictive substance and the physiological and mental readjustment that accompanies such discontinuation. Withdrawal can cause seizures, hallucinations, headaches, fatigue, irritability, depression, and poor concentration.

When the body is accustomed to an addictive substance, withdrawal can cause symptoms lasting from a few days to a month and may include nausea, vomiting, sweating, shakiness and anxiety depending on the length of time and amount of the substance used (Curtis & Lehart, 2008).

A spectrum of physical and behavioral symptoms following cessation from the continuous use of an addictive substance is known as the withdrawal syndrome. The discontinuation of an addictive substance can cause physiological effects notably high in cortisol levels. Our data shows that zebrafish can experience symptoms representative of stress and anxiety.

The experiments using repeated withdrawal of morphine and caffeine showed had an increase in certain anxiety-like behaviors, similar to effects of caffeine withdrawal–evoked anxiety in humans (Evans & Griffiths, 1999), and are consistent with anxiogenic-like effects.

Conclusion

By using the novel tank diving test, we were easily able to record the behaviors of the zebrafish and discover new endpoints to examine within the study. Repeated withdrawal gradually caused stress moving from freshwater tanks to exposure tanks. The endpoints of each group of fish were easily distinguished and identified. Through erratic movements of higher frequency, freezing bouts, latency to upper half, and time, the fish showed changes in behaviors correlating to anxiety-like behaviors demonstrating the occurrence of the withdrawal syndrome. With this progress more addictive substances can be used to model withdrawal syndrome in zebrafish and potentially draw correlations with humans in behavioral research.

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Publications resulting from this project:

1. Canavello, P.R., Cachat, J.M., Elegante, M.F., Bartels, B.K., Elkhayat S.I., Hart, P., Tien, D.T., Beeson, E., Mohnot, S. Laffoon, A.L., Haymore,W., Kalueff, A.V. Modeling Stress and Anxiety in Zebrafish. Zebrafish Behavioral Neurology. Eds: Kalueff, A.V., Cachat, J.M. Cambridge University Press, UK, 2010, in press.
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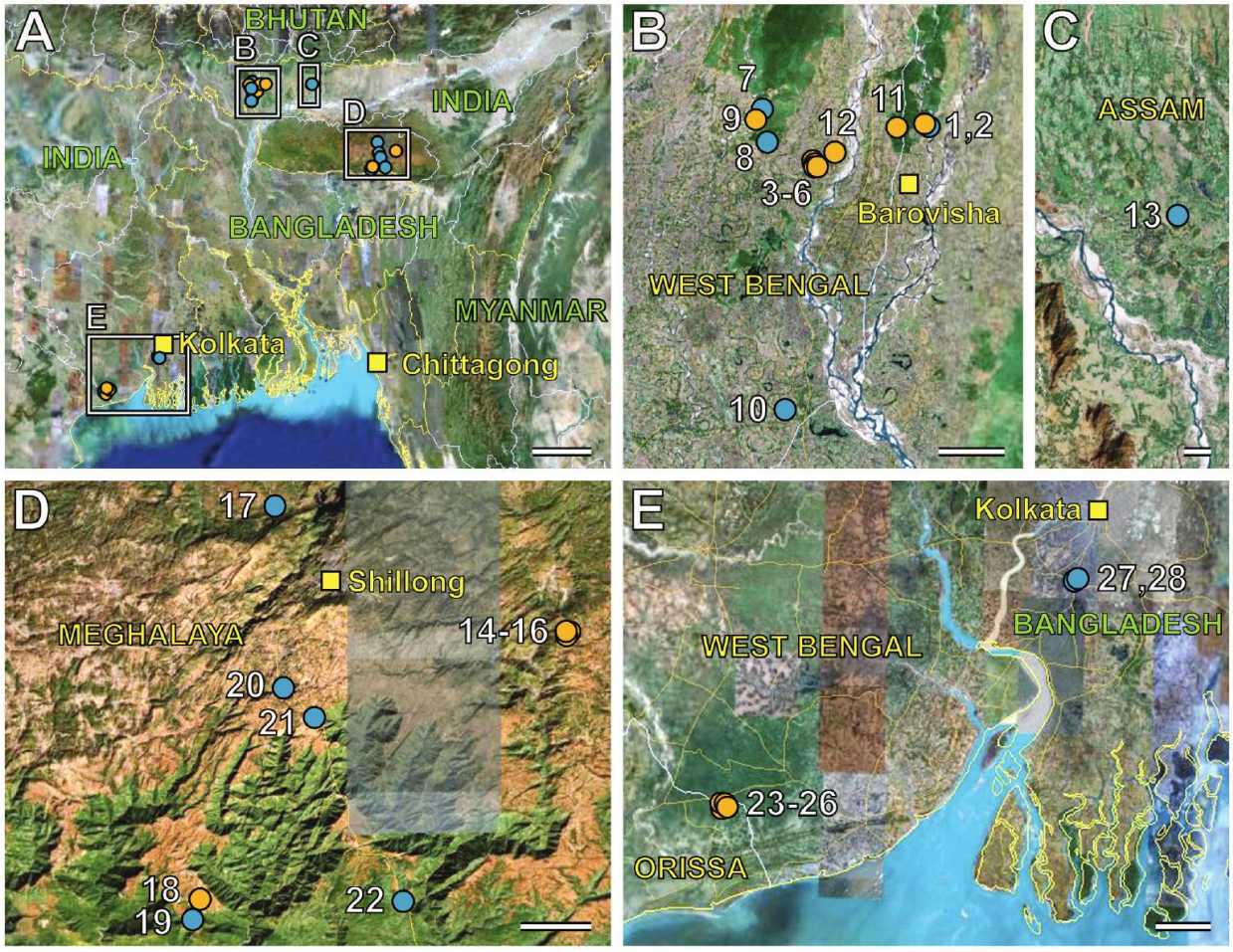

Figure 1. Localities visited during July 2006 survey of zebrafish habitat and natural history, including northeast India, bordered by Bhutan, Bangladesh, and Myanmar (Engeszer, 2007).

Figure 2. Two adult zebrafish (http:news-service.stanford.edu/news/2007/october17/gifts/zebrafish_600.jpg).

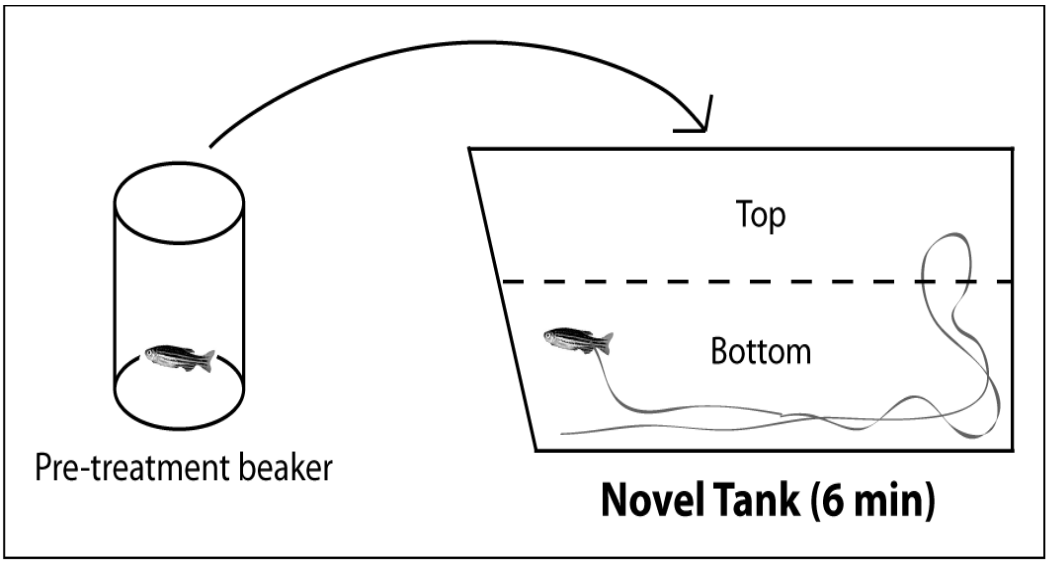

Figure 3. The novel tank diving test examines novelty-evoked anxiety in zebrafish. When the animal is exposed to a novel (potentially dangerous) environment, it initially dives to the bottom, and then gradually explores the top. Inhibited exploratory movement, reduced speed, and increased frequency of escape-like erratic behaviors are associated with higher levels of anxiety elicited by different stressors (Egan *et al.*, 2009).

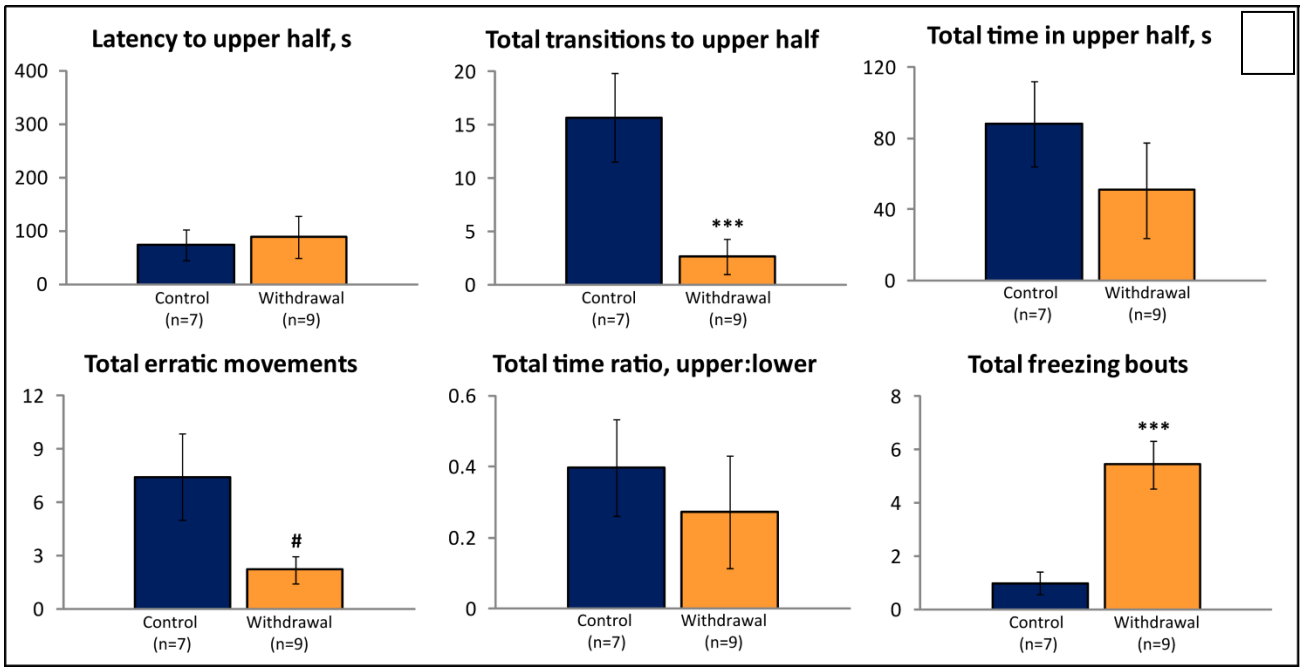

Figure 4. Anxiogenic effects of ethanol withdrawal in zebrafish tested in the novel tank diving test. 12-h withdrawal from chronic ethanol (0.3% vol/vol, 1 week). Data are presented as mean ± SEM; *P<0.05, **P<0.01, ***P<0.005, #P=0.05-0.1, U-test.

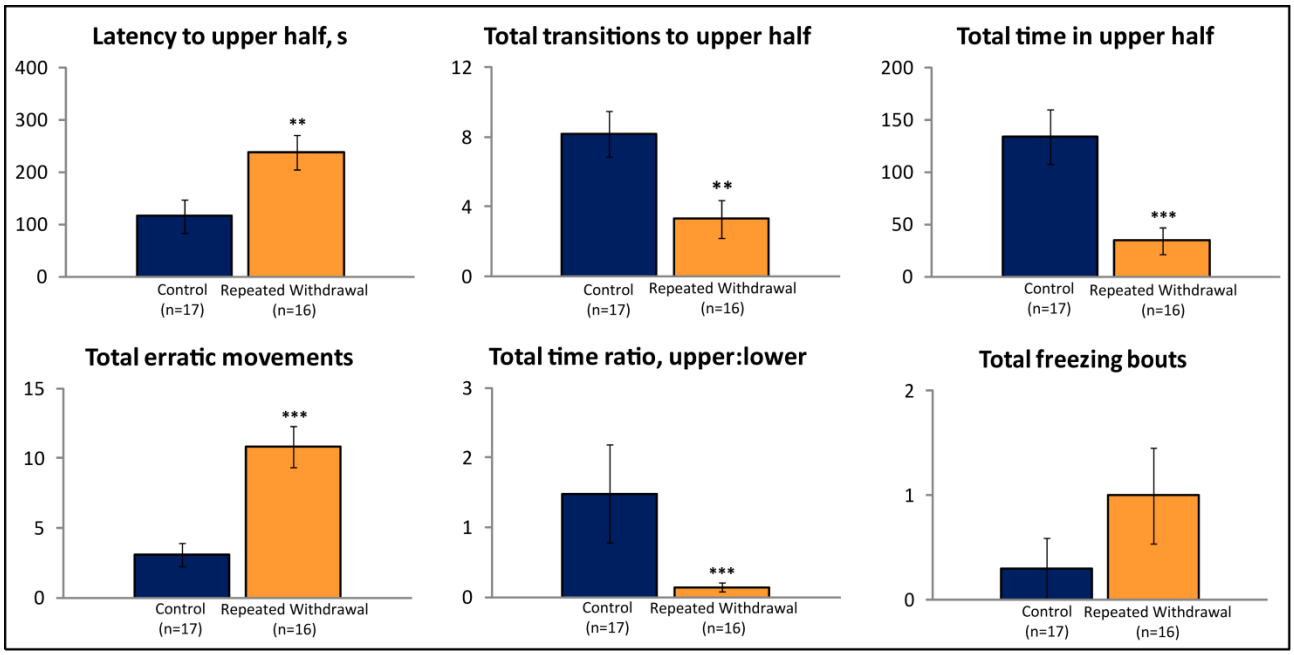

Figure 5. Behavioral effects of repeated morphine withdrawal (two 3-h withdrawal periods daily for 1 week) from chronic morphine (1.0 mg/L for 1-week) in zebrafish tested in the novel tank diving test. Data are presented as mean ± SEM, **P<0.01, ***P<0.005, U-test.

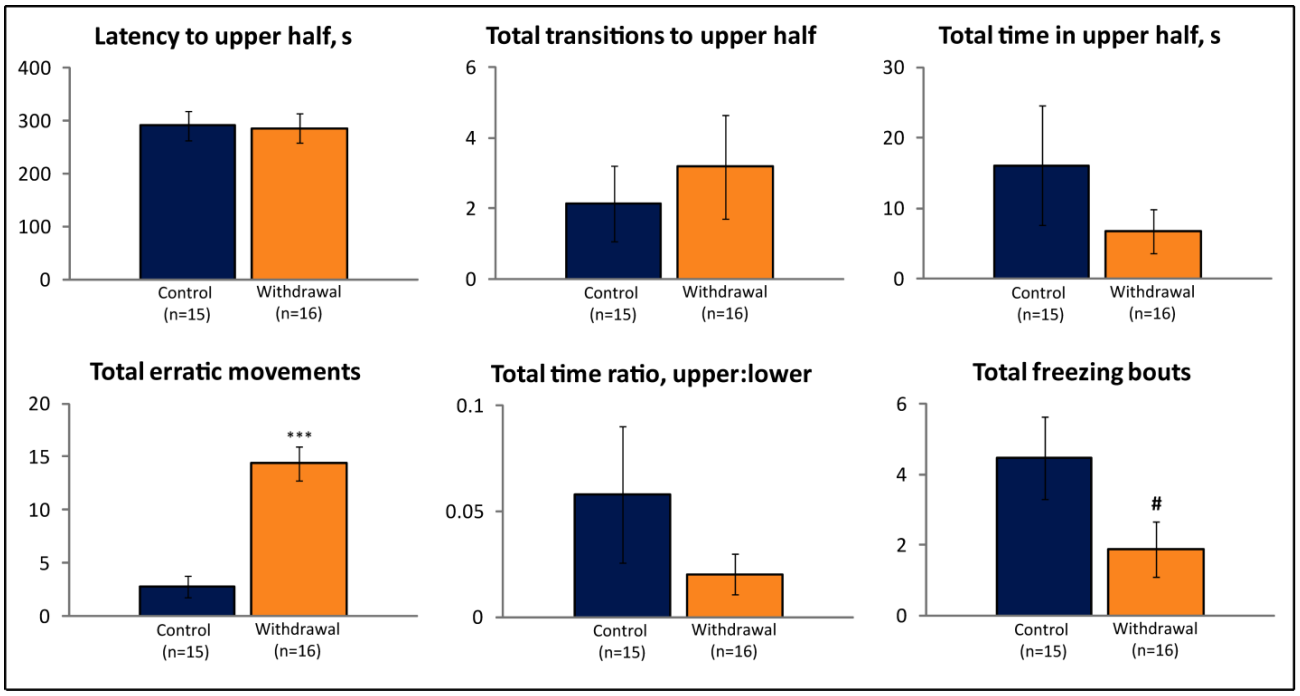

Figure 6. Behavioral effects of 12-h withdrawal from caffeine (50 mg/L for 1week) in zebrafish tested in the novel tank diving test. Data are presented as mean ± SEM, ***P<0.005, #P=0.05-0.1, trend, U-test.