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Research report

Modeling withdrawal syndrome in zebrafish

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ABSTRACT

The zebrafish (*Danio rerio*) is rapidly becoming a popular model species in behavioral neuroscience research. Zebrafish behavior is robustly affected by environmental and pharmacological manipulations, and can be examined using exploration-based paradigms, paralleled by analysis of endocrine (cortisol) stress responses. Discontinuation of various psychotropic drugs evokes withdrawal in both humans and rodents, characterized by increased anxiety. Sensitivity of zebrafish to drugs of abuse has been recently reported in the literature. Here we examine the effects of ethanol, diazepam, morphine and caffeine withdrawal on zebrafish behavior. Overall, discontinuation of ethanol, diazepam and morphine produced anxiogenic-like behavioral or endocrine responses, demonstrating the utility of zebrafish in translational research of withdrawal syndrome.

1. Introduction

Drug withdrawal is a common problem among both self-medicating abusers and chronically treated clinical patients [33,55,67]. Withdrawal syndrome has been reported for many psychoactive drugs, including ethanol [42,69], benzodiazepines [2], opioids [31,41], cocaine [22], nicotine [60], caffeine [30], phencyclidine [66], barbiturates [19] and cannabinoids [68]. Clinical symptoms of withdrawal include excessive perspiration, nausea, headache, hallucinations and, most commonly, anxiety [13,14,29,48,56,61,65,71].

In line with clinical findings, published rodent data describe anxiety-like behaviors evoked by acute withdrawal from ethanol [54], opioids [21,28,57], amphetamine [40] and nicotine [34]. In addition to robust behavioral effects of a single period of withdrawal, repeated administration and cessation of a drug treatment evokes strong withdrawal-like effects [32]. For example, increased anxiety-like behavior was reported in rodents following repeated withdrawal from ethanol [70] and morphine [72]. The importance of understanding neurobiological mechanisms requires innovative approaches to modeling withdrawal syndrome, including novel experimental paradigms, new biomarkers and alternative model species [18,51,58].

The zebrafish (*Danio rerio*) has gained prominence in recent years as a useful model species in experimental neuroscience and biological psychiatry [9,17,27,45]. Recent studies also suggest the potential of zebrafish as a model for drug reward and addiction [53]. For example, rewarding properties of different drugs, including amphetamine [53], salvinorin A [9], cocaine [15], morphine and heroin [4], have been reported in zebrafish. Mounting anatomical and genomic evidence further supports this notion, as zebrafish dopaminergic projections to the basal forebrain parallel the mammalian mesolimbic system implicated in drug addiction [16]. Likewise, chronic treatment of zebrafish with ethanol and nicotine alters the expression of multiple CNS genes, some of which have been identified as components of the addiction pathways in mammals [39]. Moreover, some evidence suggests sensitivity of zebrafish to drug withdrawal. For example, ethanol discontinuation disrupts zebrafish shoaling behavior [26], whereas

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cocaine withdrawal evokes marked alterations in their locomotion [45,46].

In addition to behavioral markers of withdrawal syndrome, both clinical and pre-clinical data implicate endocrine dysregulation in drug abuse and withdrawal [8,38,47,57]. Withdrawal-evoked anxiety strongly correlates with elevated blood or salivary cortisol in patients with heroin [44,59], opioid [6,73], nicotine [12], cocaine [24] and ethanol [37,50] addiction. Similarly, increased levels of brain and plasma corticosterone have been reported in rodents following morphine [57] or ethanol withdrawal [8], respectively. Taken together, this indicates that glucocorticoid abnormalities may represent important biological markers of withdrawal syndrome.

Our study aims to further validate the utility of zebrafish in modeling drug withdrawal syndrome. Here, we examine anxiety-like behavioral and cortisol responses elicited in adult zebrafish by withdrawal from a wide spectrum of psychotropic drugs, including ethanol, diazepam, caffeine, and morphine.

2. Methods

2.1. Animals and housing

Adult 4–6-month-old male and female zebrafish (~50:50%) of heterozygous "wild type" short-fin strain were obtained from local commercial distributors (Petco, Rockville, MD and 50 Fathoms, Metairie, LA). All fish were given at least 10 days to acclimate to the laboratory environment and housed in groups of 20–30 fish per 40-L tank. All tanks were filled with deionized water before introducing the fish. The room and water temperatures were maintained at 25–27 °C. Illumination (1010 ± 88 lx) was provided by ceiling-mounted fluorescent light tubes on a 12-h cycle (on: 6.00 h, off: 18.00 h). Fish were fed Tetramin Tropical Flakes (Tetra USA, VA). All fish used in this study were experimentally naïve. Following behavioral testing, the animals were euthanized in 500 mg/L Tricaine (Sigma–Aldrich, USA), immediately dissected and stored at −80 °C for further physiological analysis.

2.2. Pharmacological manipulations

Using a total of 265 fish, our study examined the effects of ethanol (0.3% EtOH v/v, Pharmco-AAPER, USA), diazepam (72 mg/L, Sigma–Aldrich, USA), morphine (1.5 mg/L single withdrawal, 1.0 mg/L repeated withdrawal, Mallinckrodt, MO) and caffeine (50 mg/L, Sigma–Aldrich, USA). Fish were treated chronically with drugs in home tanks before inducing withdrawal. Drugs were dissolved in tank water before introducing zebrafish. All home tanks had specially designed glass covers, to prevent evaporation of water and drugs. Ethanol, morphine and caffeine were administered chronically for 1 week, and diazepam was administered for 2 weeks. The doses and the duration of chronic treatment and withdrawal were selected based on our own pilot data confirming the lack of non-specific toxic/sedative effects of these drugs. Our choice of withdrawal intervals was also based on known biological half-lives of drugs used here (diazepam ≫ morphine, caffeine > ethanol) and was similar to those used in other withdrawal studies in zebrafish [45,46].

For single (acute) withdrawal experiments, fish in the ethanol and caffeine groups were treated for 1 week in their respective home tanks, which were then filled with untreated water for 12 h before behavioral testing. Fish in the diazepam cohort were treated chronically for 2 weeks, followed by placement in drug-free water for 72 h prior to testing, to evoke withdrawal. For morphine withdrawal, fish were treated chronically for 1 week and then placed in drug-free water for 48 h.

Repeated withdrawal trials were performed in this study on zebrafish treated with ethanol or morphine. After 1-week chronic treatment, fish were placed into exposure tanks with fresh untreated water for 3 h at a time, twice per day for 1 week prior to testing. Drug-free control fish were placed into treated water with no drugs added, and chronic drug treatment groups were placed into water containing concentrations of the drug identical to the treatment. Following 3-h exposure trials, animals were returned to their respective drug-treated home tanks. After 1 week of repeated withdrawal, fish were taken from home tanks and placed in exposure tanks for a final 3-h withdrawal session prior to behavioral testing.

2.3. Apparatus and behavioral testing

Behavioral testing was performed using the novel tank diving test, representing a 1.5-L trapezoidal tank (15.2 cm height × 7.1 cm width × 27.9 cm top length × 22.5 cm bottom length; Aquatic Habitats, Apopka, FL) maximally filled with aquarium-treated water. Illumination of the novel tank area was similar (1170 ± 68 lx) to that in the animal holding room. Novel tanks rested on a level, stable surface and were divided into two equal horizontal portions, marked by a dividing line on the outside walls. Behavioral testing occurred between 10.00 and 17.00 h. Once each fish was individually transferred to a novel tank, its swimming behavior was recorded for 6 min by two trained observers (inter-rater reliability >0.85) recorded the following behavioral endpoints: latency (s) to reach the upper half (top) of the tank, time spent in the top (s), number of transitions (entries) to the top, number of erratic movements, and number and duration (s) of freezing bouts. Erratic movements were defined as sharp changes in direction and/or velocity and repeated rapid darting behaviors. Freezing was defined as a total absence of movement, except for the gills and eyes, for 2 s or longer. Collectively, a reduction in exploration (i.e., longer latency to reach the top half, fewer entries to the top, more freezing) or elevated erratic movements represent behavioral profiles indicative of high stress and anxiety [3,43]. We also calculated the average top entry duration (total time spent in top divided by the number of entries), as well as the top:bottom ratio for time spent by each fish, as additional endpoints reflecting the level of zebrafish anxiety (both indices are generally lower in anxious fish) [17].

2.4. Cortisol assay

In a separate study using 147 zebrafish, we analyzed their endocrine responses to single drug withdrawal, including chronic diazepam, morphine, ethanol and caffeine treatments (administered as described previously). The cortisol extraction procedure was performed using a modified protocol developed in our laboratory [17]. Briefly, individual body samples obtained from experimental and control cohorts were homogenized in 500 μL of ice-cold 1× PBS buffer. The homogenizing rotor blade was then washed with an additional 500 μL of PBS and collected in a 2-mL tube containing the homogenate. Samples were transferred to glass extract-O tubes and cortisol was extracted twice with 5 mL of diethyl ether (Fisher Scientific, USA). After ether evaporation, the cortisol was reconstituted in 1 mL of 1× PBS. To quantify cortisol concentrations, ELISA was performed using a human salivary cortisol assay kit (Salimetrics LLC, State College, PA). ELISA plates were measured in a VICTOR-WALLAC plate reader using the manufacturer's software package. Whole-body cortisol levels were determined using a 4-parameter sigmoid minus curve fit based on the absorbencies of standardized concentrations, and presented as relative concentrations per gram of body weight for each fish [17].

2.5. Statistical analysis

All experimental data was analyzed using the Kruskal–Wallis test, followed by a post-hoc Tukey HSD test, for significance between the groups. Data was expressed as mean ± SEM, and significance set at $P < 0.05$.

3. Results

As can be seen in Fig. 1A, single diazepam withdrawal produced mild anxiogenic responses in zebrafish, as the Kruskal–Wallis test revealed significant group effect only for the number of erratic movements ($H(2, 30) = 6.3$, $P < 0.05$), and a trend for the number of entries to the top ($H(2, 30) = 5.5$, $P = 0.06$). The withdrawal group produced significantly more erratic movements vs. the chronic drug group, which also showed a trend towards more transitions to the top, indicative of reduced anxiety. However, there were no differences between the three groups in the latency to top, time spent in top, average entry duration, freezing frequency and duration, as well as top:bottom duration ratio (NS). Likewise, no significant effects on zebrafish behavioral endpoints were observed following single ethanol withdrawal (Fig. 1B), as well as single caffeine and morphine withdrawal (data not shown).

In contrast, repeated morphine withdrawal produced robust anxiogenic effects on zebrafish behavior (Fig. 2A). The Kruskal–Wallis test revealed significant effects for the latency to enter the top ($H(2, 41) = 5.8$, $P < 0.05$), time spent in top ($H(2, 41) = 7.7$, $P < 0.05$), top:bottom duration ratio ($H(2, 41) = 7.7$, $P < 0.05$), average entry duration ($H(2, 41) = 9.4$, $P < 0.01$), and the number of erratic movements ($H(2, 41) = 8.5$, $P < 0.05$). There were also trends for the number of entries to the top ($H(2, 41) = 5.2$, $P = 0.07$) and freezing duration ($H(2, 41) = 4.3$, $P = 0.1$), but not for the number of freezing bouts (NS). As shown in Fig. 2A, the repeated morphine withdrawal cohort exhibited a significantly longer latency to enter the top, had fewer transitions to the top (although not significantly), and spent significantly less time in top, compared to the control group. Both average entry duration and top:bottom ratio were significantly reduced in the withdrawal group. Additionally, the repeated withdrawal group had significantly more erratic movements (compared to both control and

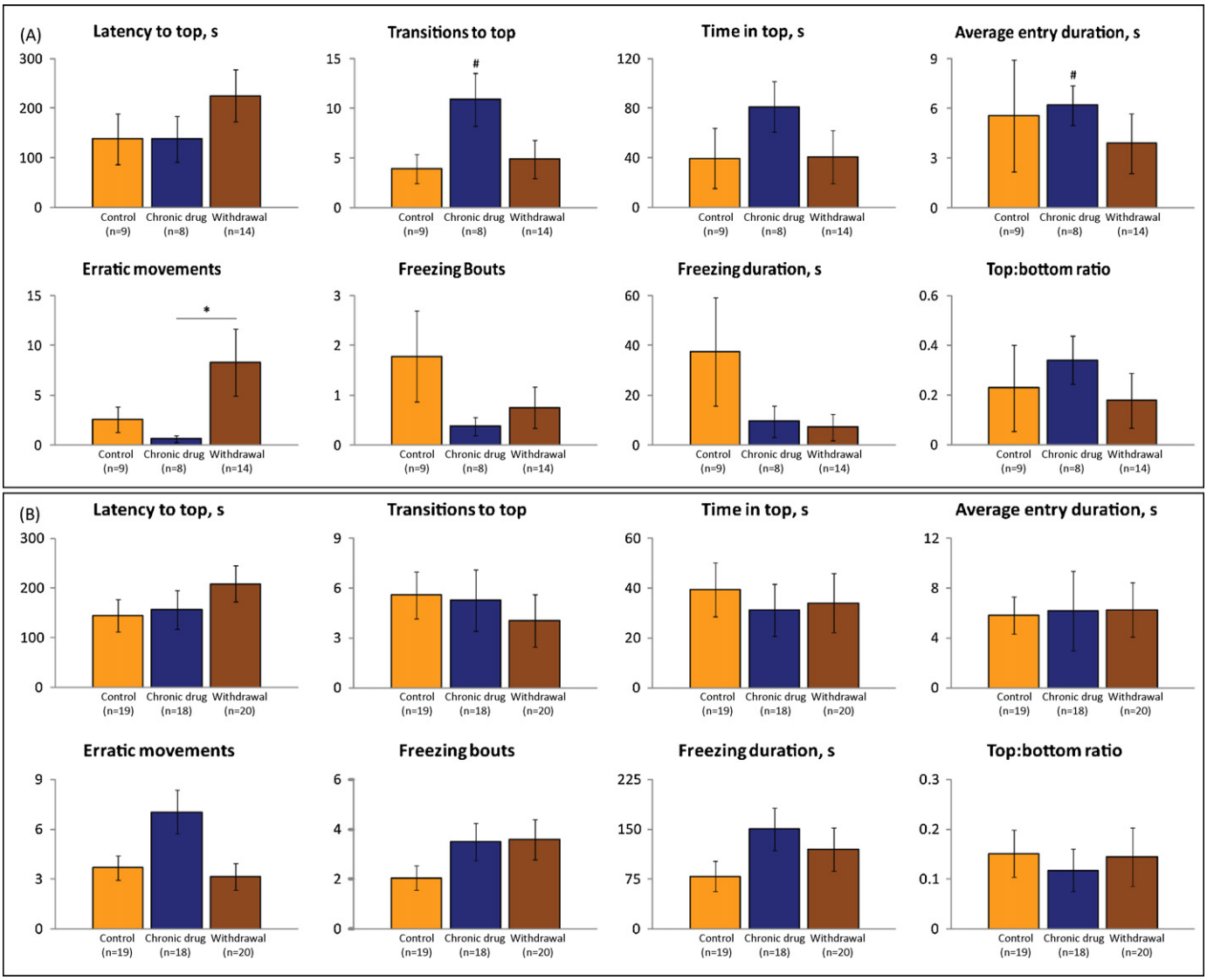

Fig. 1. Behavioral effects of single withdrawal from diazepam (A) and ethanol (B) treatments in adult zebrafish tested in the novel tank diving test. A – 72-h withdrawal from chronic diazepam (72 mg/L for 2 weeks); B – 12-h withdrawal from chronic ethanol (0.3%, v/v for 1 week); data are presented as mean ± SEM; $^{*}P < 0.05$, $^{\#}P = 0.05–0.1$ (trend); post-hoc Tukey test for significant Kruskal–Wallis data. Signs above data bars indicate significance/trends vs. control group, signs above horizontal lines indicate significance/trends between the respective experimental groups.

chronic drug groups), along with generally higher (although not significantly) freezing frequency and duration.

In the repeated ethanol withdrawal experiment (Fig. 2B), we also found significant anxiogenic-like effects for the latency to enter the top ($H(2, 44) = 6.3$, $P < 0.05$), time spent in top ($H(2, 44) = 9.9$, $P < 0.01$), top:bottom duration ratio ($H(2, 44) = 10.0$, $P < 0.01$), average entry duration ($H(2, 44) = 9.8$, $P < 0.01$), the number of top entries ($H(2, 44) = 6.8$, $P < 0.05$) and freezing duration ($H(2, 44) = 6.2$, $P < 0.05$). The Kruskal–Wallis test also revealed trends for the number of freezing bouts ($H(2, 44) = 5.8$, $P = 0.06$) and erratic movements ($H(2, 44) = 0.94$). Compared to the control group, the repeated withdrawal group showed a trend to a longer latency to enter the upper half of the novel tank, fewer transitions to the upper half (NS), significantly less time spent in the top, reduced average entry duration, and a trend to lower top:bottom duration ratio (Fig. 2B). Furthermore, while erratic movements were unaltered in all groups, we observed significantly higher frequency and duration of freezing bouts, collectively indicating increased anxiety-like behavior in the withdrawal group.

Finally, cortisol levels were affected by drug withdrawal, including a significant treatment effects in ethanol withdrawal ($H(2, 41) = 6.6$, $P < 0.05$), a trend ($H(2, 45) = 5.2$, $P = 0.07$) for single morphine withdrawal, and a similar, although not significant, increase in single diazepam (NS) withdrawal group (Fig. 3). Overall, cortisol levels in these experiments were higher in the withdrawal groups compared to the other two cohorts. However, there were no significant differences on whole-body cortisol levels between the three groups in the single caffeine withdrawal experiment.

4. Discussion

In line with earlier reports on the behavioral effects of cocaine [45,46] and ethanol [26] withdrawal, our results demonstrate the ability of different psychotropic drugs to elicit withdrawal-related phenotypes in zebrafish. Single withdrawal from diazepam (Fig. 1A) produced mild anxiety-like behaviors in zebrafish, and a slight elevation of cortisol levels (Fig. 3). In the acute ethanol withdrawal paradigm, despite the lack of overt behavioral responses (Fig. 1B), cortisol levels were significantly elevated in the withdrawal cohort (Fig. 3), suggesting higher levels of stress and anxiety in the withdrawal fish. Although anxiety evoked by caffeine withdrawal has been reported in both humans [20] and rodents [5,35,52,63],

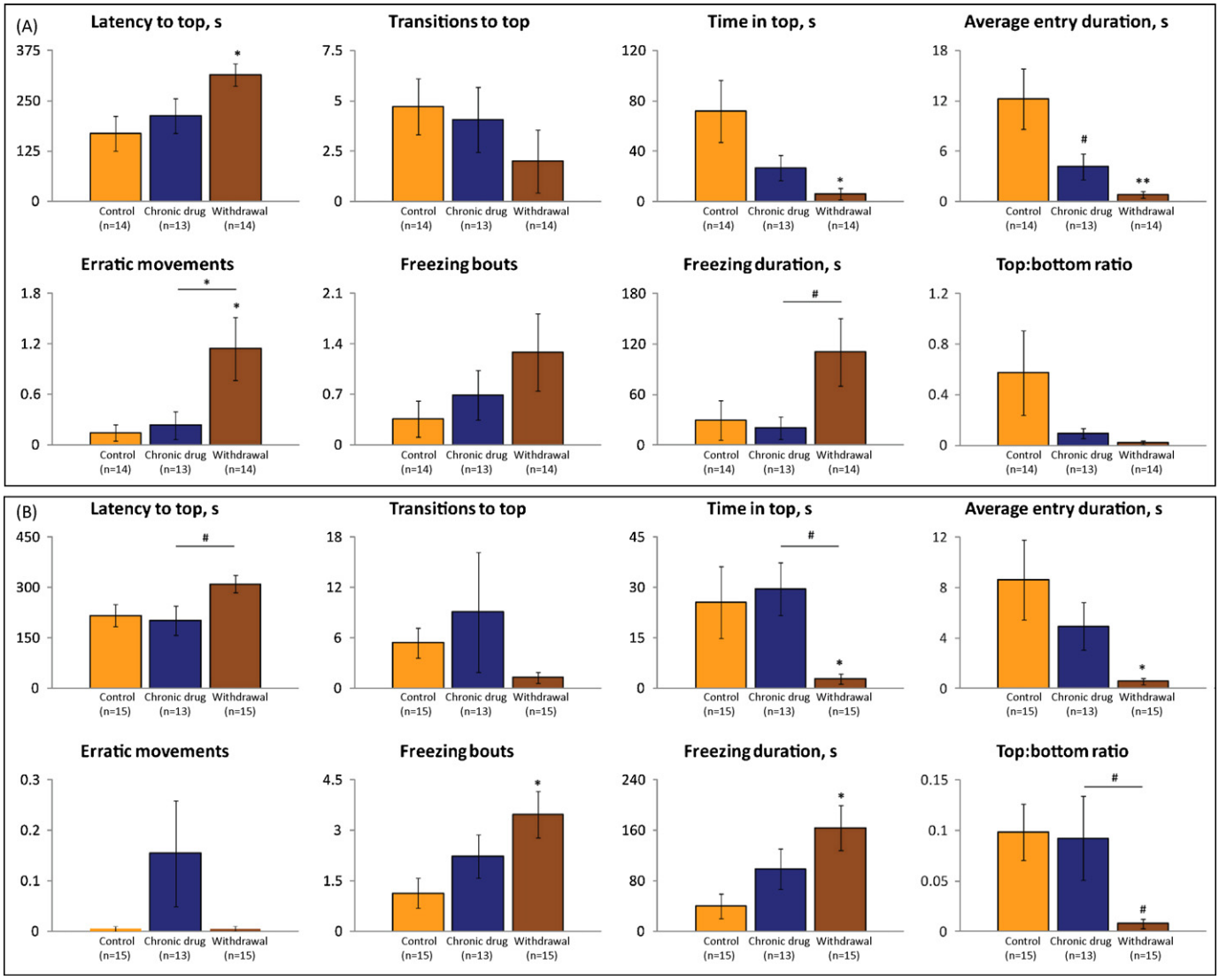

Fig. 2. Anxiogenic effects of repeated withdrawal (two 3-h withdrawal periods daily for 1 week) from chronic 1-week morphine (A; 1.0 mg/L) and ethanol (B; 0.3%, v/v) in adult zebrafish tested in the novel tank diving test. Data are presented as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.005$, $^{\#}P = 0.05$–0.1 (trend); post-hoc Tukey test for significant Kruskal–Wallis data. Signs above data bars indicate significance/trends vs. control group, signs above horizontal lines indicate significance/trends between the respective experimental groups.

our study failed to establish behavioral or endocrine alterations in zebrafish following single caffeine withdrawal. These results suggest that zebrafish models may be less sensitive to caffeine withdrawal, or that other paradigms and methodological approaches may be needed to better mimic this state in zebrafish.

Although single morphine withdrawal did not produce significant behavioral effects in this study, cortisol levels were significantly elevated in the withdrawal group (Fig. 3), indicating the possibility of higher anxiety in zebrafish under acute morphine withdrawal. The lack of strong behavioral effects in this experiment may be attributed to tolerance (particularly common for morphine), since zebrafish were exposed to the same daily dose for 1 week. Therefore, future studies may require gradually increasing the dose of daily morphine treatment, to counterbalance tolerance.

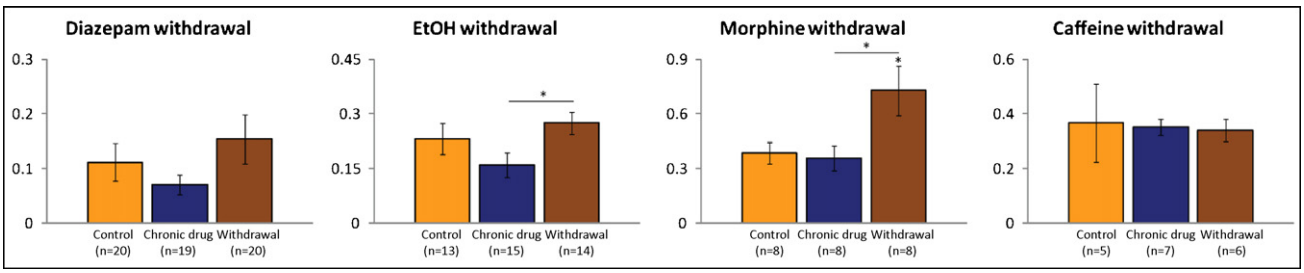

Fig. 3. Endocrine stress responses (whole-body cortisol, ng/g fish) in adult zebrafish to single withdrawal from chronic diazepam, chronic ethanol (see Figs. 1 and 2 legends for details), chronic morphine (24-h withdrawal from 1.5 mg/L morphine for 1 week) and caffeine (12-h withdrawal from chronic 50 mg/L for 1 week). Data are presented as mean ± SEM; $^{*}P < 0.05$, post-hoc Tukey test for significant Kruskal–Wallis data. Asterisks above data bars indicate significance/trends vs. control group, asterisks above horizontal lines indicate significance/trends between the respective experimental groups.

However, using the repeated withdrawal protocol, we were able to elicit increased anxiety-like behaviors (Fig. 2A), generally consistent with known anxiogenic-like effects of withdrawal from this drug [31,41].

Overall, there are several limitations of the present study, including the complexity of withdrawal phenotypes [13,14,29,48,56,61,65,71] and the difficulty with modeling withdrawal syndrome in animals [7,10,36]. Therefore, future studies using zebrafish models may focus on neurochemical alterations, neural circuits, as well as clinically relevant long-term consequences [44,49,59,73], of drug withdrawal. Albeit not explored here in detail, sex dimorphism in withdrawal-related behavior also warrants further investigation. This aspect of pathogenesis becomes particularly important, since sex differences in human [23] and rodent [1,11,62,64] withdrawal responses are strongly supported by recent zebrafish data [26,27]. Moreover, the strain differences also play a role in modulating zebrafish behavior (e.g., Refs. [17,25]), and may influence their withdrawal phenotypes. Since only one animal strain was used here, further analyses comparing drug withdrawal responses in different zebrafish strains may be necessary. Finally, altered gene expression in the zebrafish brain following chronic drug treatment and withdrawal [26,27,39] supports genomic profiling of zebrafish withdrawal as a promising direction of research in this field.

In summary, varying anxiogenic effects in zebrafish were produced by diazepam, morphine and ethanol withdrawal, as well as by repeated morphine and ethanol withdrawal (Figs. 1 and 2). Single caffeine withdrawal did not evoke anxiety-like responses, suggesting that different drugs have the potential to evoke different withdrawal states in zebrafish. Therefore, the modeling of withdrawal syndrome elicited by other drugs of abuse (e.g., nicotine, heroin, amphetamine and barbiturates) merits further study using zebrafish models.

The use of a human salivary cortisol assay provided physiological measures of the endocrine stress response in zebrafish (Fig. 3), generally consistent with their anxiety-like behaviors. Zebrafish cortisol responses have already been shown to correlate with anxiety behavior evoked by different non-pharmacological stressors [3,17]. Here we demonstrated that withdrawal may modulate zebrafish cortisol levels. Consistent with glucocorticoid dysregulations frequently reported in human and animal studies of withdrawal syndrome [8,37,47,57], our findings implicate cortisol abnormalities as an emerging endophenotype of drug withdrawal in zebrafish.

Our study also showed that zebrafish withdrawal-evoked behaviors can be easily distinguished and profiled using the novel tank diving test as a simple and high-throughput screen. However, adding new behavioral endpoints and using novel observation methods, such as automated video-tracking systems, may foster further withdrawal research in zebrafish. Overall, paralleling the anxiogenic effects of drug withdrawal in humans and rodents, our research strongly supports the utility of zebrafish to study the neurobiology of drug abuse and withdrawal.

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