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# Anxiogenic effects of cocaine withdrawal in zebrafish

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**Abstract**

Continued usage of cocaine is determined by genetic, conditioned and homeostatic factors, while it is reinforced by drug-induced reward and the emotionally negative state of drug withdrawal, which includes anxiety. The molecular mechanisms of these long-term behavioral and physiological alterations have yet to be fully elucidated. Here we demonstrate that in zebrafish, a wide range of non-anesthetic cocaine doses, 0.015–15 μM, does not result in acute alterations in locomotor activity, in spite of the high brain cocaine levels induced (7–120 pg/μg protein). Conversely, cocaine withdrawal causes hyperactivity associated with stereotypy. The behavioral hyperactivity is progressively increased during the initial period of withdrawal (24–72 h) and is maintained for at least 5 days. Such effect of cocaine withdrawal is aggravated by environmental stimulation and attenuated in the home environment. Administration of cocaine (1.5 μM) or a non-sedative dose of diazepam (5 μM, immersion) acutely counteracts withdrawal-associated hyperactivity and stereotypy in zebrafish, with the magnitude of these effects positively correlating with the degree of prior increase in basal activity. Administration of an anxiogenic benzodiazepine inverse agonist, FG-7142, results in zebrafish behavior similar to that observed during cocaine withdrawal. Together, the results suggest that cocaine withdrawal produces long-lasting behavioral effects in zebrafish which are consistent with an anxiety-like state. Thus, zebrafish, a powerful model for the study of vertebrate genetics, could provide insights into the molecular mechanisms of drug withdrawal.


*Keywords:* Cocaine; Withdrawal; Locomotor activity; Zebrafish; Anxiety; Benzodiazepine

## 1. Introduction

Cocaine addiction is a chronic relapsing disorder. Genetic, homeostatic and conditioning factors define the course of addiction and therapeutic outcomes. The short-term rewarding effects of cocaine can be quickly replaced by homeostatic deregulation, which results in anxiety, dysphoria and negative somatic symptoms [1], for which a new dose of cocaine can be a fast and effective antidote. As a result, both continued cocaine use and relapse are promoted by the conditioning factors associated with its positive reinforcing properties and by the motivation to counteract the negative symptoms associated with cocaine withdrawal. Combined, these effects lead to compulsive drug-seeking behavior [2].

In spite of the in-depth human studies and sophisticated animal model approaches that have been used to study cocaine, there are still many questions that remain with regards to the nature and mechanisms of drug abuse [3,4]. For instance, the genetic factors that cause variability in the vulnerability to drugs of abuse in humans and the initial motivation to use such drugs have yet to be determined. Studies in mice [5] and flies [6] demonstrate that genetically-tractable species, which allow for high throughput behavioral studies, could assist in increasing our current understanding of the nature and mechanisms of drug addiction.

Zebrafish is another popular organism used to investigate vertebrate development and genetics and is subject to high throughput assays. Because zebrafish are also responsive to the rewarding properties of drugs of abuse, including cocaine [7], they are useful models in the investigation of the biological mechanisms of drug addiction [7–10]. However, the extent to which drug responses in zebrafish are similar to those in other

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species is largely unknown, as is whether they experience the drug withdrawal symptoms at all.

Here we show that in adult zebrafish cocaine withdrawal results in increased and stereotypic basal locomotor activity which is maintained for prolonged periods and aggravated by environmental stimulation. Such anxiety-like behavior in zebrafish is also caused by an established anxiogenic drug, FG-7142, and is acutely counteracted by cocaine or diazepam (DzP). This finding establishes the zebrafish as a new model for elucidating the drug withdrawal phenomenon.

## 2. Materials and methods

### 2.1. Animals and housing conditions

Adult male zebrafish (*Danio rerio*, AB wild-type strain, 9± 1 months old, 5 fish/3 L tank) were maintained on a constant 14L:10D light:dark cycle, in a temperature-controlled (26.5 °C) multi-tank re-circulating water system (Aquaneering, San Diego, CA, USA). Animals were fed twice a day with live brine shrimp (Brine Shrimp Direct, Ogden, Utah. USA), and flake food (TetraMin, Tetra Blacksburg, VA. Germany).

Starting with the adaptation and throughout the entire experimental period, each fish remained in an individual 1-L tank (17×9×8 cm), which served as both the animal's home and experimental tank. Throughout the experimental period, the fish were not able to see each other. The environmental illumination and feeding schedule remained as in regular housing. Fish were fed the pre-soaked decapsulated brine shrimp eggs (Brine Shrimp Direct, Ogden, Utah. USA). Tank water was changed an hour after feeding.

### 2.2. Locomotor activity recordings

Individual zebrafish locomotor activity was continuously documented using automatic image-analysis software (Videotrack, View Point Inc, France). Twenty individual home/experimental tanks, each containing one fish, were placed (or maintained) inside the recording chamber. The activity of fish in the control and treatment groups was documented in parallel. During recording, the walls of the individual tanks were uniformly white and non-transparent with a back-light illuminated white floor (400 lx at the water surface level).

The data acquisition speed was set at 30 frames/s, with integration period of 30 s. The distance traveled (total, at low speed or high speed) was documented for the entire tank and each of the tank areas defined. The high speed threshold was defined as above 15 cm/s. Low speed was defined as between 0.1 and 15 cm/s. Consistent environmental conditions and thorough pre-recording calibration assured lack of recording artifact. In the figures, mean distance traveled in one minute is presented.

Automatic recordings and visual observations throughout the experimental periods allowed the documentation of the presence or absence of stereotypy. The stereotypy was defined as unvarying, repetitive behavioral patterns with no obvious function [11] maintained for longer than 1 min. In the majority of the AB strain zebrafish used in this study, the stereotypic behavior was characterized by continuous fast pacing back and forth along one side of the tank. By outlining several specific areas of the tank, we have documented fish activity within the peripheral areas, 1.5 cm from each wall of the tank. The time spent moving or distance traveled in the periphery was an objective measure of stereotypy. Based on the visual observations of the fish image on the computer monitor, during such stereotypic behavior zebrafish was typically pacing next to the bottom of the tank, as reported earlier [9]. However, since we have used only one camera positioned above the fish tank for the automatic recordings, such behavior could not be objectively documented.

### 2.3. Treatments

Cocaine hydrochloride (supplied by NIDA) was dissolved in water and administered directly into the fish tank water in a 100–200 μl volume of working treatment solution in order to achieve different doses. In all experiments, the fish were treated with cocaine (or vehicle, i.e., water, during cocaine withdrawal), for 1 h 15 min, at the same time of day, starting at ZT4 (zeitgeber time, ZT0 = lights on time). The acute treatment doses were 0.015; 0.15; 1.5; 15; and 150 μM. These doses were also used in pilot repeated treatment experiments. For the repeated treatments presented in this paper, a medium dose (1.5 μM) was chosen based on its consistent efficacy to change zebrafish behavior and increase brain cocaine content. Recorded in parallel, control fish received the same volume of water (vehicle).

Diazepam (DzP, Abbott Laboratories, Chicago, IL, USA) working solution was 17.5 mM and contained 10% ethanol in water. Pilot experiments were employed in order to select the dose of DzP that resulted in minimal, if any, behavioral effects in naive zebrafish. Once chosen, this DzP dose (5 μM final concentration in the fish tank), was administered at ZT4 to treat the cocaine-exposed and control fish. The control solution contained ethanol, with the final 0.003% ethanol concentration in the fish tank water.

The benzodiazepine receptor inverse agonist, *N*-methyl-β-carboline-3-carboxamide (FG-7142, Tocris Cookson Inc., Ellisville, MO, USA), was dissolved in 10% ethanol to produce a 3.5mM working solution. A range of doses (0.25–1.0 μM, final concentration in tank water) were tested in naive zebrafish. Independent of the FG-7142 dose (or control), ethanol concentration in the tank water was maintained at 0.003% during treatment.

### 2.4. Treatment designs

Prior to the initiation of experimental procedures, fish were adapted to their individual home/experimental tanks and testing environment by spending 1 h per day in the recording system during a 5-day adaptation period. After that, the baseline behavior was documented for 1 day and a treatment period was initiated. Cocaine or control treatment was administered either once or repeatedly and zebrafish behavior was documented daily, before and after treatment, including up to 5 days of cocaine withdrawal. Intermittent repeated treatment involved seven cocaine administrations, with the schedule represented in Fig. 1a.

On each day of the experimental period, zebrafish activity was recorded for two 15-min periods. The first period is referred to as "before-daily-treatment" (BDT) recording and the results reflect the basal activity on a specific day. The second recording occurred an hour later, following cocaine or control treatment, and is referred to as "after-daily-treatment" (ADT) recording (Fig. 1b, c). The results obtained during this second period reflect the effect of cocaine or control treatment (water) administered to cocaine group on withdrawal days, and the control treatment administered to the control group of zebrafish.

To address the role of environmental stimulation in the behavioral effects of cocaine, two principal cocaine treatment designs were used. According to the "low environmental stimulation" (LES) design (Fig. 1b), fish were continuously maintained in the recording system throughout the experimental period and were briefly disturbed only during the regular water change procedures. In contrast, according to the "high environmental stimulation" (HES) design, fish were maintained in the regular housing room and were moved (while remaining in their individual home/experimental tanks) to the experimental room for a daily 15-min basal activity recording (BDT). They were then moved back to the housing room, where they received their treatment. An hour later, they were once again moved to the recording system for an ADT activity recording of the day (Fig. 1c). Tank water was immediately changed after the second daily recording (ADT) in both HES and LES designs. Moving the 20 fish tanks from one room to another, placing them into the recording system and confirming the system calibration took 10 min, on average.

Thus, according to both LES and HES designs, fish received cocaine treatment or were exposed to cocaine withdrawal in their home tanks and home environment. However, their locomotor BDT and ADT activity was documented either in the comfort of their home environment (LES) or, alternatively,

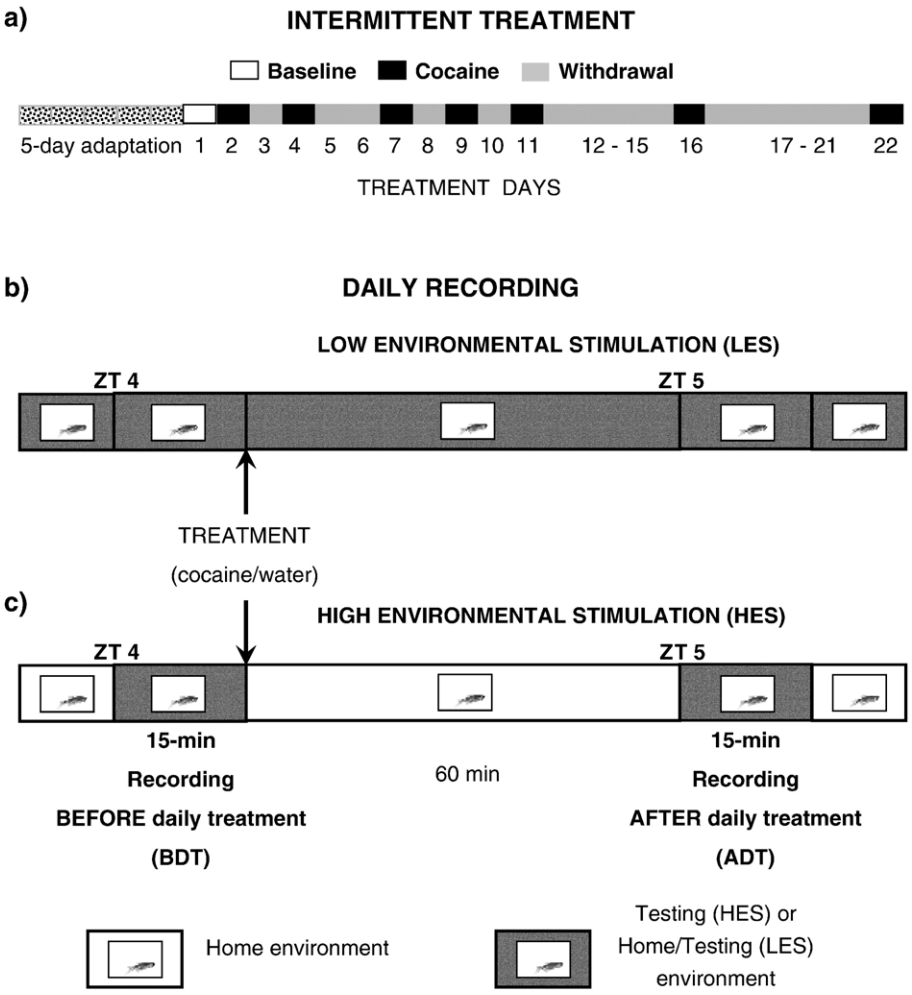


Fig. 1. Protocol schematics. (a) Intermittent repeated cocaine treatment schedule: following 5-day adaptation, zebrafish were intermittently treated with cocaine (black). During 1–5 day withdrawal periods, both cocaine and control groups received control treatment (gray). (b) Daily activity recording protocol with low environmental stimulation (LES). Fish were continuously maintained in test environment which also served as their home environment, i.e., treatment and behavioral recordings were conducted in home environment. (c) Daily activity recording protocol with relatively high environmental stimulation (HES). Fish received treatment in their home environment but were moved to the test environment twice a day immediately before the behavioral recording, while remaining in their home tanks. In both (b) and (c), 15-min daily activity recordings were scheduled before (BDT) and an hour after (ADT) treatment, when zebrafish were exposed to 1.5 μM cocaine (cocaine group) or water (control group every day; cocaine group during withdrawal periods). The arrows indicate cocaine or control treatment time.

in an environment that, while familiar, was one that they were exposed to only periodically, and to which they were moved right before the activity recording (HES).

DzP treatment was administered 72-h after a single or repeated intermittent cocaine (or control) administration in LES or HES conditions. The activity recordings were conducted continuously for 30 min before and 30 min after DzP administration.

FG-7142 treatment was administered to naive animals that had previously adapted to the testing environment. After 30-min baseline recording, a single dose of FG-7142 (or control) was administered (0.25–1.0 μM final concentration in tank water) and locomotor activity recording continued.

### 2.5. Evaluation of potential anesthetic effects of cocaine

Cocaine is known to have anesthetic properties [12]. To assess whether the cocaine doses used have anesthetic effects in zebrafish, sensitivity threshold to repeated electric stimulation before and at intervals after administration of cocaine was evaluated. Individual zebrafish were placed in a tank containing flat stainless steel electrodes along the tank sides. Following 5-min adaptation, fish were stimulated with an increasing current (in 0.1 mA steps, with at least 1 min between steps; starting with 0.3 mA; Master-8cp, A.M.P. Instruments, Jerusalem, Israel), until a consistent minimal startle or escape response to the stimulation was documented, defined as immediate initiation of fast activity following stimulation, typically associated with a bend and change in prior heading of around 30°. The data were based on subjective scoring, with each fish evaluated by three independent researchers in parallel. At each current level, the stimulation was repeated three times, following 1-min interval, whether the response was observed or not, to exclude false-positive or false-negative result.

Following basal sensitivity threshold evaluation, fish were treated with a range of cocaine concentrations/doses (0.5, 1.5, 3.0 and 18 μM, with each dose administered for 5 min) or control solution. Sensitivity threshold to electric stimulation was tested at intervals (2 and 5 min) after each dose administration. The effect of a 3.0 μM dose of cocaine was also tested 90 min after continuous exposure. All treatments were administered into the tank water, as described above.

In order to determine whether basal sensitivity threshold and/or a potential anesthetic effect of cocaine might be affected by prior history of repeated cocaine exposure, the design employed the zebrafish withdrawn for 72-h from intermittent cocaine treatment (1.5 μM, 5 treatments total) or control solution. Each of these groups was further divided into two subgroups that received either control solution or cocaine after the evaluation of their basal sensitivity threshold. Such design also allowed us to control for the potential dynamics in the sensitivity threshold depending on the prior cocaine or control treatment history of the zebrafish.

### 2.6. Measurements of brain cocaine levels

Zebrafish males, matched in age and size to those behaviorally tested, were used for measuring brain tissue cocaine levels at intervals following a single cocaine treatment using the range of doses specified above. Fish were sacrificed after different intervals of cocaine exposure (20, 40, 60 or 120 min) by fast immersion in liquid nitrogen. Brain tissue was dissected while frozen, homogenized in buffer (0.1 M Sodium Acetate and Sodium Fluoride 1%) and stored at −20 °C. Each experimental sample contained four brains of identically-treated fish, due to low cocaine levels in individual brains. An aliquot of each duplicate sample was used for protein quantification (BCA Protein Assay, Pierce Biotechnology, Rockford IL, USA) for further normalization of cocaine levels measured. The samples were then sent on dry ice to the Center of Human Toxicology (University of Utah) for cocaine measurements. There, cocaine levels and those of major cocaine metabolites were assessed using 0.5 ml of each sample, followed by addition of 0.5 ml of blank plasma and the deuterated internal standards (cocaine-$d_3$, benzoylecgonine-$d_3$, and ecgonine methyl ester-$d_3$). Fish samples, along with the calibration standards and quality control samples, were analyzed by solid-phase extraction, followed by liquid chromatography-tandem mass spectrometry (LC/MS/MS) using electrospray ionization and selected-reaction monitoring [13] with modifications [14]. The resulting lower limit of quantification for each analyte was 2 ng/ml.

### 2.7. Statistical analysis

Using the SAS Proc Mixed procedure, a mixed-model analysis was conducted to examine the effects of repeated intermittent cocaine administration. Total distance, as well as distance at high and low speed, were analyzed over the entire 22-day treatment period. The same statistical method was used to analyze the effect of the cocaine-withdrawal duration on zebrafish locomotor activity and to evaluate the acute effects of DzP.

In addition, two-way analysis of variance (ANOVA) was used to compare the effect of cocaine treatment in different conditions (LES and HES) at two specific time points (BDT and ADT). The two factors compared were time and environment. A post-hoc multiple comparison Tukey test was employed (Sigma Stat software, SPSS).

A two-way repeated measures ANOVA test (Sigma Stat software, SPSS) was used to analyze the potential anesthetic properties of cocaine for each dose used, with the history of cocaine treatment (control vs. cocaine withdrawal) and time as the factors. The same statistical method was used to analyze the acute effects of FG-7142.

The differences in brain cocaine content at different time points after cocaine administration were analyzed by using a one-way ANOVA test. If significant differences were detected between more than two groups, a Tukey post-hoc comparison was employed.

All the statistical effects presented in the text and/or graphs reflect individual representative experiments (out of 2–3 replicates) and show groups of fish recorded and treated in parallel. Unless otherwise indicated, the significance level in all the tests was set at $P<0.05$.

## 3. Results

### *3.1. Cocaine administration through immersion significantly and dose-dependently augments brain cocaine levels in adult zebrafish*

The brain cocaine content following different cocaine doses used was consistently detectable following the 0.15–15 μM treatment (final concentration in tank water) and was within the range from 7.0±0.1 to 120.4±20.1 pg/μg protein, respectively. Based on these measurements and pilot behavioral experiments, we chose the 0.015–150 μM cocaine doses for studying the acute effects of cocaine and the 1.5 μM dose for the repeated treatments and detailed behavioral assessments of cocaine withdrawal. To synchronize our behavioral recordings with the time point at which brain cocaine levels are maximal, the zebrafish brain cocaine levels were evaluated at 4 different time points (20, 40, 60 and 120 min after administration) following a single 1.5 μM dose of cocaine. The one-way ANOVA showed significant time dependence for the brain cocaine levels following cocaine administration ($F_{3,9}=9.029$; $P=0.04$; Fig. 2). The post-hoc Tukey test revealed the highest cocaine levels achieved 60 min after treatment ($P=0.011$ and $P=0.02$, compared to 20 min or 40 min, respectively). After 120 min, the brain cocaine content remained high, similar to that after 60 min and different from the 20-min measurement ($P=0.013$, 120 min vs. 20 min).

### *3.2. Basal locomotor activity in zebrafish is increased during cocaine withdrawal and the change correlates with the withdrawal duration*

Continuous recording of locomotor activity following a single administration of a range of cocaine doses (0.015–150 μM) revealed no significant changes in activity level and did not result in behavioral stereotypy on the day of treatment (e.g., for 1.5 μM dose, Fig. 3). This lack of an acute initial effect following cocaine administration was consistent between the animals tested according to the HES or LES treatment design.

When fish were withdrawn from cocaine for several days, their activity was found to be increased after a single (Mixed

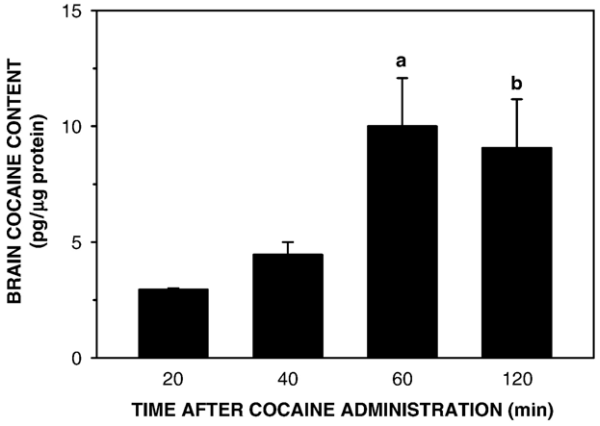


Fig. 2. Brain cocaine levels in zebrafish at intervals after 1.5 μM cocaine treatment. Brain cocaine levels (pg/μg protein) in zebrafish immersed in 1.5 μM cocaine solution for 20, 40, 60 or 120 min. Each column represents the mean± SEM of 5 samples (4 brains/sample). [a]$P<0.05$, relative to both 20 and 40 min; and [b]$P<0.05$ relative to 20 min after cocaine administration.

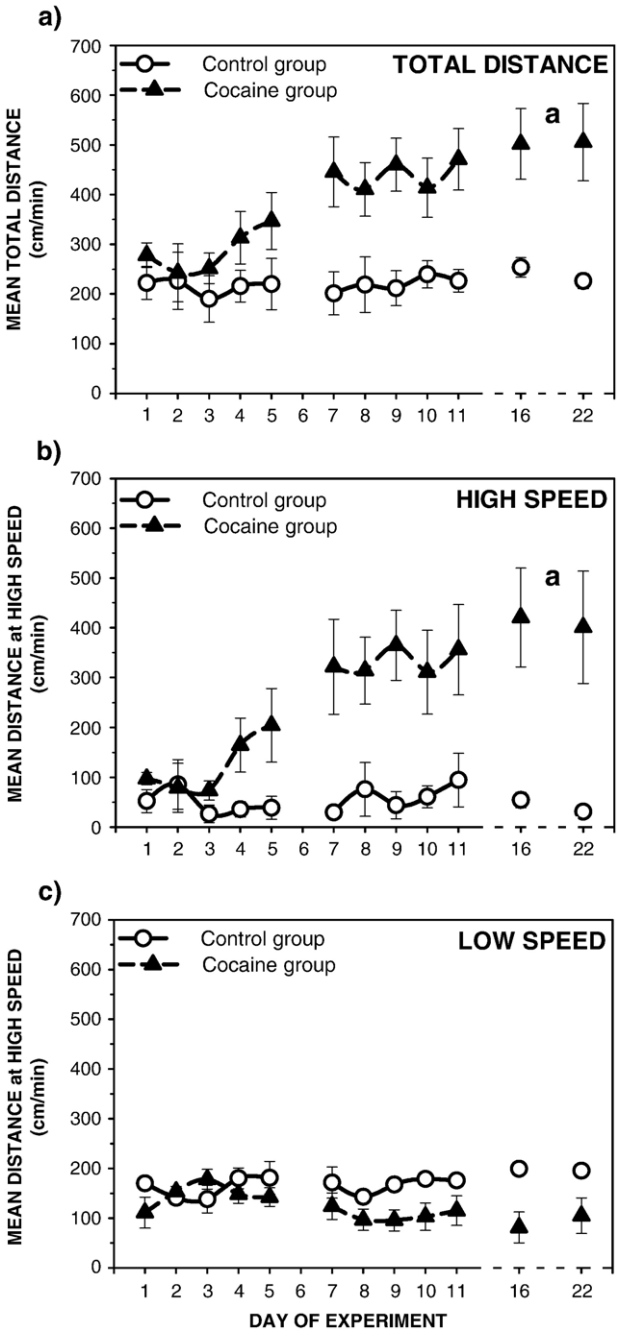


Fig. 3. Behavioral activation in zebrafish following intermittent cocaine treatment and cocaine withdrawal under conditions of relatively high environmental stimulation (HES). Distance traveled (cm/min) during the first 15-min recording of each day, i.e., before-daily-treatment recording (BDT), of basal activity: (a) total distance; (b) distance traveled at high speed; (c) distance traveled at low speed. Black triangle and dashed line — cocaine group; open circles and solid line — control group. Cocaine (1.5 μM) was administered during the experimental days 2, 4, 7, 9, 11, 16 and 22 (see Fig. 1a). Data for a representative experiment are presented as mean±SEM for the groups recorded in parallel ($n=5$/group). [a]$P<0.01$ relative to control group.

ANOVA model, $F_{1,2278}=8.343$, $P=0.004$) or repeated treatment. Withdrawal from intermittent repeated cocaine administration resulted in gradual increase in daily basal locomotor activity, i.e., before-daily-treatment (BDT). Fig. 3 illustrates the effect of 1.5 μM cocaine withdrawal on the total (a), high speed (b) and low speed (c) distance covered by zebrafish within 15 min of BDT in HES condition. The mixed ANOVA model revealed significant differences between treatment groups ($F_{1,8}=6.61$, $P=0.033$) for total distance covered. This effect was especially robust on distance traveled at high speed ($F_{1,8}=7.93$, $P=0.023$) and was not observed for low speed activity (Fig. 3b, c). Such progressive increase in the basal activity of the cocaine-withdrawal group under both HES (Figs. 3 and 5) and LES (Fig. 5) conditions was striking in contrast to the quite stable behavior of the control groups over the entire experimental period (Fig. 3). No significant difference between the behavior in the control LES and HES groups was found.

During the period of progressive increase in locomotor activity due to cocaine withdrawal, i.e. within the ascending part of the basal activity curve on Days 2–7 in Fig. 3, change in distance traveled significantly and positively correlated with the duration of cocaine withdrawal (Fig. 4). The mixed-model revealed statistically significant differences between experimental groups ($F_{1,153}=137.64$; $P<0.0001$), with higher activity in cocaine-withdrawal group. The withdrawal duration significantly affected basal locomotor activity ($F_{2,152}=9.13$; $P<0.0001$), with higher activity levels after 48-h and 72-h withdrawal, when compared to 24-h ($P<0.001$ for both). There was also a significant interaction between the treatment group and withdrawal duration ($F_{5,149}=78.24$; $P<0.0001$). The withdrawal duration was significantly different only in cocaine treated animals. The total distance traveled following a 72-h withdrawal period exceeded that after the 24-and 48-h withdrawal ($P<0.001$ for both comparisons) (Fig. 4). A significant difference was also found between the 24-h and 48-h withdrawal periods in cocaine-withdrawal group ($P<0.001$). Finally, significant differences were found when the control and cocaine-withdrawal groups were compared at any withdrawal period ($P=0.027$ for 24-h and $P<0.001$ for both 48-h and 72-h).

Further intermittent treatments scheduled 2–5 days apart (Days 9–22) did not significantly increase zebrafish basal activity level, though some increase after the 4–5 day withdrawal was observed (Days 16 and 22) in both total distance and distance traveled at high speed (Fig. 3a,b).

Comparison between the two test conditions, associated with either low (LES) or relatively high (HES) environmental stimulation, showed that the environment in which zebrafish were tested could modulate the behavioral effects of cocaine withdrawal, as shown for 72-h withdrawal in Fig. 5a. A two-way ANOVA revealed significant differences between the treatment groups tested under these two conditions ($F_{2,12}=31.638$; $P<0.001$). Total distance was higher in zebrafish tested under HES conditions (Tukey test; $P=0.007$ and $P<0.001$), when compared to LES or control groups, respectively.

During cocaine withdrawal under both LES and HES conditions, locomotor activity, especially for high speed movements, was associated with pronounced stereotypy. Fig. 5b shows the distance traveled in the periphery of the tank during baseline activity recording after a 72-h withdrawal period under HES conditions. Significant differences were found between cocaine-withdrawal and control groups ($P=0.023$, $t$-test), with the largest distance traveled in the periphery of the tank for cocaine-withdrawal group. Similar results were obtained when the experimental groups tested under LES conditions were compared (data not shown). In both cases, as per subjective visual observations (see Materials and methods), the animals displaying stereotypy tended to move close to the bottom of the tank, similar to that reported earlier for anxiety-like behavior in zebrafish [9,15].

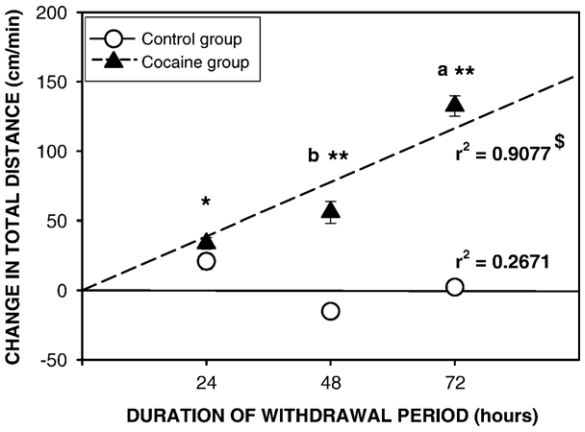


Fig. 4. Prolonged cocaine withdrawal enhances locomotor activation in zebrafish. Data presented as difference in basal activity (total distance traveled before-daily-treatment, BDT) before and after a certain duration of cocaine withdrawal (24, 48 and 72 h). Data for a representative experiment are presented as mean±SEM for the groups recorded in parallel ($n=5$/group). **$P<0.01$ and *$P<0.05$ relative to control; [a]$P<0.01$ relative to 24 and 48 h cocaine withdrawal; [b]$P<0.05$ relative to 24-h withdrawal; [$]$P<0.01$ relative to control group regression.

### 3.3. Cocaine administration acutely counteracts the hyperactivity induced by cocaine withdrawal

In contrast to lack of the behavioral effect of the first dose of cocaine administered to naive animals, the acute effects of cocaine treatment were robust in zebrafish withdrawn from cocaine under both LES and HES conditions (two-way ANOVA; $F_{2,12}=6.703$; $P=0.014$). In HES conditions (Fig. 5a), the increased locomotor activity during cocaine withdrawal was significantly inhibited following cocaine administration (Tukey test; $P<0.001$ for total distance). The magnitude of this effect correlated with the degree of basal behavioral activation and thus with the day of treatment (Mixed-model; $F_{1,8}=6.91$, $P=0.03$; $F_{1,8}=7.08$, $P=0.0001$, respectively; Fig. 6a). Accordingly, the first and second cocaine administration (Fig. 6a, Days 2 and 4) resulted in only minor changes in the zebrafish behavior following treatment. Once the behavior was consistently activated following a 72-h withdrawal (Day 7), the effect reached significance (Tukey test; $P<0.001$; Fig. 5a; and Mixed-model; $F_{1,8}=7.08$, $P=0.0001$; Fig. 6a). In contrast, the control group showed no consistent changes

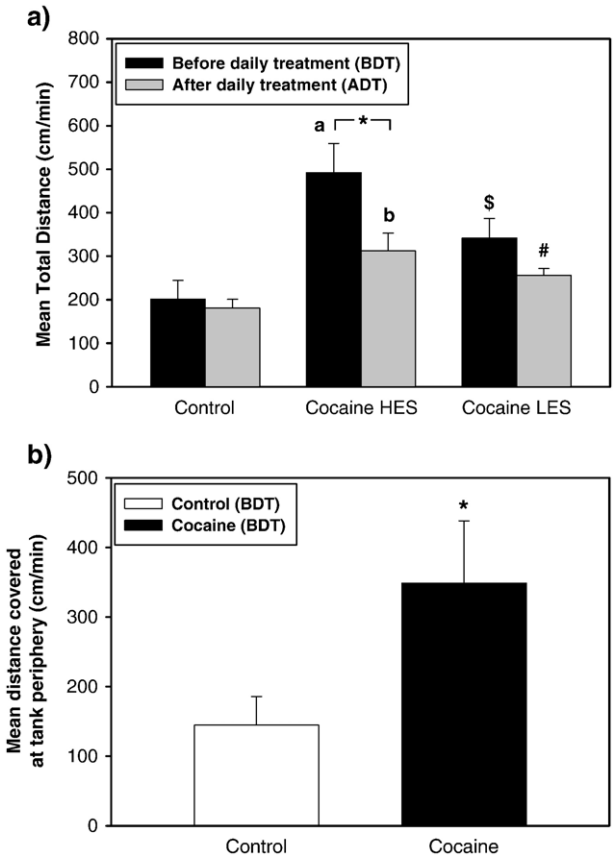


Fig. 5. Hyperactivity during cocaine withdrawal and acute inhibitory effect of cocaine administration thereafter is enhanced under conditions of high environmental stimulation (HES), compared to home environment (LES), and associated with behavioral stereotypy. (a) Cocaine withdrawal (72-h) following cocaine (1.5 μM) treatment results in increased daily basal activity (mean total distance traveled in cm/min, BDT) and this effect is more pronounced in HES, compared to LES test conditions (black bars). Cocaine (1.5 μM) administration after 72-h withdrawal (gray bars, ADT) acutely inhibits locomotor activity and this effect is also more robust under HES conditions (gray bars). No difference was found between the control groups in HES and LES conditions, thus the control data were pulled together for this graph. Each column represents the mean±SEM ($n=10$ in control group; $n=5$/group in cocaine/HES or cocaine/LES groups). *$P<0.01$ relative to after-daily-treatment (ADT); [a]$P<0.01$ relative to control and cocaine/LES groups; [b]$P<0.05$ relative to control and cocaine/LES; [$]$P<0.01$ relative to control; [#]$P<0.05$ relative to control. (b) Stereotypy, reflected in increased pacing along the tank wall (distance at tank periphery), is increased in zebrafish withdrawn from cocaine administration for 72-h (HES), compared to control. *$P=0.023$ ($t$-test; $n=5$/group).

between the BDT and ADT recordings, i.e., before and after the control treatment (Fig. 5a and 6a). Similarly, the control treatment during cocaine-withdrawal periods did not significantly affect the behavior of the cocaine-withdrawal group (Fig. 6a).

Fig. 6b illustrates that during ADT (on the days of cocaine or control treatment) locomotor activity levels decreased in those HES animals that had higher basal activity, whether they belonged to the control or cocaine-withdrawal group. However, positive correlation between the individual basal activity and its change during the ADT was present only in the control group ($r^2=0.778$), and in the cocaine-withdrawal group acutely treated with cocaine ($r^2=0.610$). No such dependence was observed in the cocaine-withdrawal group on the days of withdrawal, i.e., when they received the control treatment ($r^2=0.222$).

The direction of changes was similar between the animals tested under HES and LES conditions. However, while the magnitude of basal activity increase was significant, the degree

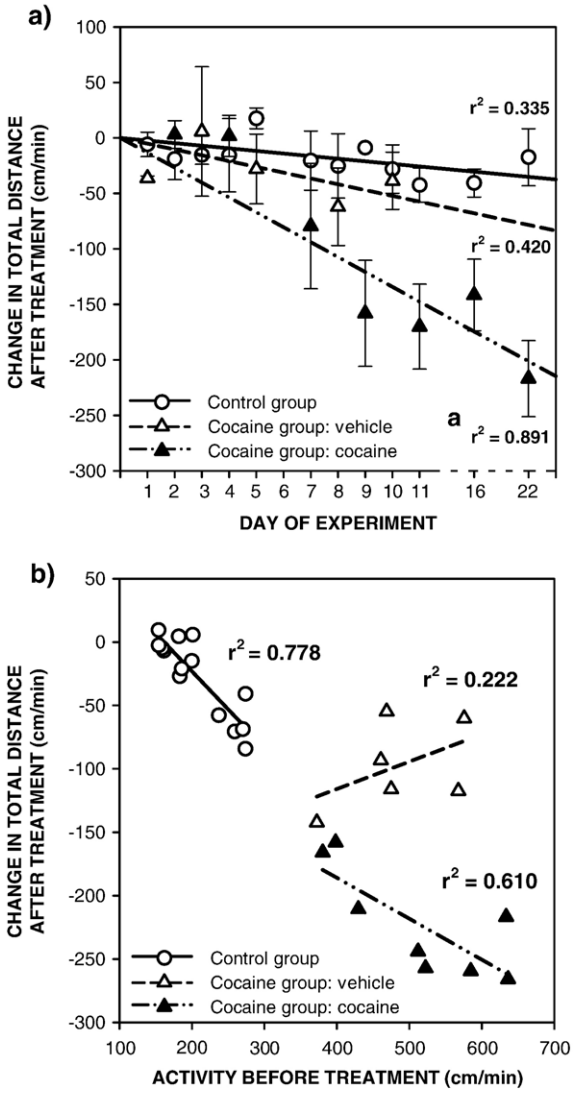


Fig. 6. Acute behavioral inhibition produced by cocaine treatment correlates with the behavioral activation during cocaine withdrawal. (a) Change in total distance traveled ($y$-axis) after cocaine administration (black triangle) in zebrafish withdrawn from cocaine correlates ($r^2=0.891$) with the duration of cocaine withdrawal, also associated with the increase in basal locomotor activity (see Fig. 3a). No such dependence is observed following control treatment in cocaine-withdrawal (open triangle) or control (open circle) groups of zebrafish. Data for a representative experiment in groups recorded in parallel are presented. Each data point is the mean±SEM ($n=5$/group, HES). (b) Change in total distance traveled ($y$-axis) before and after treatment (i.e., between BDT and ADT periods; HES), correlates with the basal activity (BDT) level in the control (open circle; $r^2=0.778$) fish and zebrafish receiving cocaine following a 72-h cocaine withdrawal (cocaine group: cocaine; black triangle; $r^2=0.610$), but not in cocaine-withdrawal fish that receive control treatment (cocaine group: control; open triangle; $r^2=0.222$). Data for a representative experiment in individual zebrafish recorded in parallel are presented. [a]$P<0.01$ relative to control group and cocaine group receiving control treatment.

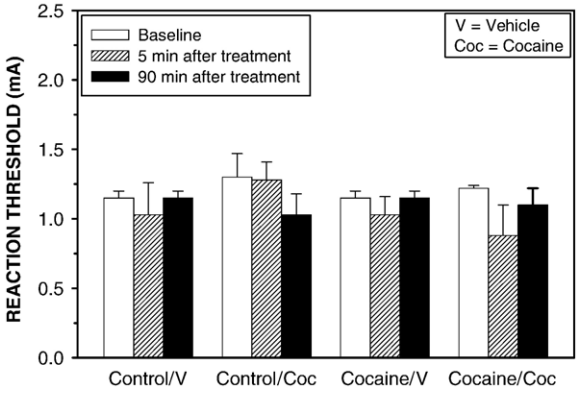


Fig. 7. The cocaine doses used in this study did not produce anesthetic effect. Sensitivity threshold following 3 μM cocaine exposure for 5 and 90 min, compared to baseline, in four groups: withdrawn for 72-h from repeated cocaine administration (Cocaine) versus control, and either acutely treated with cocaine (Coc) or vehicle (V, water) following baseline sensitivity threshold evaluation. No significant effect of treatment was detected (see Results).

of behavioral inhibition following cocaine administration was less pronounced in LES and did not reach the level of significance. Overall, both the LES and HES cocaine-treated groups were significantly different from control (two-way ANOVA; $F_{2,12}=31.638$; $P<0.001$) during both BDT (Tukey test; $P=0.004$, and $P<0.001$ for LES and HES, respectively) and ADT (Tukey test; $P=0.036$ for HES) recordings during cocaine withdrawal (Fig. 5a).

### 3.4. Lack of anesthetic effect of cocaine doses used in this study

The sensitivity thresholds to electric stimulation ranged from 0.88 to 1.30 mA in individual zebrafish. The baseline sensitivity threshold did not differ between the naive zebrafish and those with increased locomotor activity following a 72-h withdrawal from repeated cocaine administration (Fig. 7). Two-way repeated measures ANOVA showed no significant change in sensitivity threshold after the naive fish or those withdrawn from cocaine were acutely exposed to 0.5, 1.5, 3 or 18 μM doses of cocaine for 2–90 min, compared to the control group (i.e., no cocaine) or their individual baseline threshold levels ($F_{3,55}=0.324$, $P=0.809$). This was independent of the time after treatment ($F_{4,55}=1.582$, $P=0.195$), or the Group by Time interaction ($F_{15,55}=1.177$, $P=0.340$). These data demonstrated lack of anesthetic effect of cocaine in the doses employed in this study.

### 3.5. Diazepam counteracts the increase in basal locomotor activity during cocaine withdrawal in zebrafish

Mixed ANOVA model data analysis was employed to analyze the effects of a borderline dose of DzP (5 μM) on the locomotor activity levels. Treatment history (control vs. 72-h cocaine withdrawal), acute treatment (diazepam vs. control) and time were used as the factors. Fig. 8a illustrate the results obtained in zebrafish withdrawn from cocaine for 72-h following a single treatment (1.5 μM), resulting in significant increase in basal activity, compared to the control group ($F_{1,2278}=8.343$; $P=0.004$; mixed ANOVA model). There was a significant interaction between the treatment history group and DzP administration ($F_{1,2278}=70.926$; $P<0.001$). The control treatment (DzP vehicle) did not significantly change the activity in either the control or cocaine-withdrawal group. The DzP treatment also did not significantly change the control zebrafish behavior, although small decline in activity was observed within the first 15 min (Fig. 8a). In contrast, DzP significantly reduced locomotor activity in the cocaine-withdrawal group, compared to its baseline ($P<0.001$) and control ($F_{118,2278}=2.186$; $P<0.001$). Moreover, after DzP administration, the activity in the cocaine-withdrawal group became significantly lower than the activity of the control group ($P<0.001$) and remained such

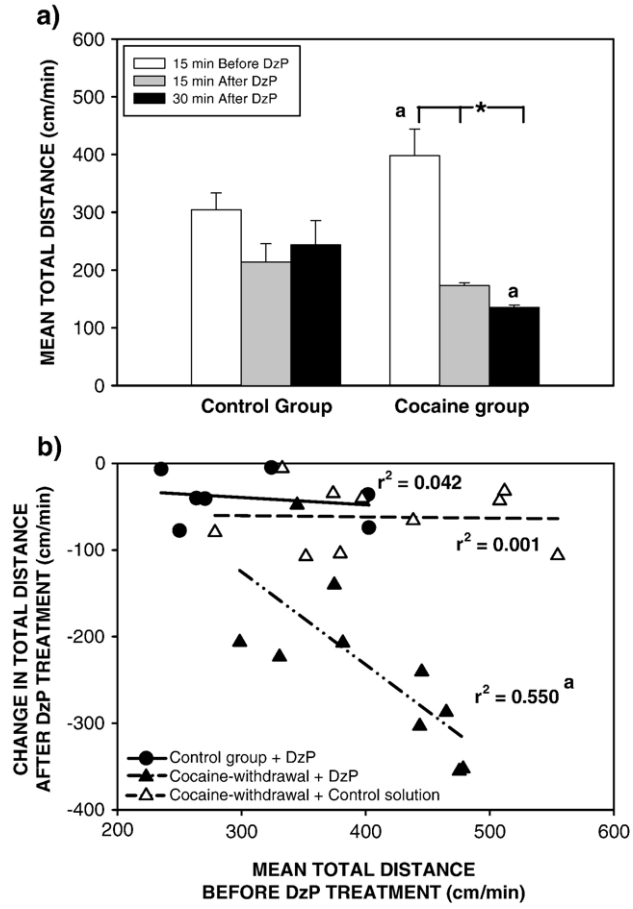


Fig. 8. Diazepam attenuates behavioral activation during cocaine withdrawal in zebrafish. (a) A borderline dose of DzP (5 μM) results in robust and prolonged inhibition of locomotor activity (total distance) in zebrafish withdrawn from cocaine for 72 h but causes only minor changes in the control group. Data for a representative experiment in groups recorded in parallel are presented in 15-min periods before treatment (open bar) and at 15 min (light gray) or 30 min (black) after DzP administration into the tank water. *$P<0.01$ relative to 15 min after and 30 min after DzP; [a]$P<0.01$ relative to control at the same time point. (b) The effect of DzP (change in total distance after treatment) correlates with the degree of prior behavioral activation in zebrafish withdrawn from cocaine for 72 h (black triangle; $r^2=0.55$). Zebrafish withdrawn from cocaine but receiving the control solution (DzP vehicle; open triangle; $r^2=0.001$) or control fish receiving DzP (black circle; $r^2=0.042$) show no such response to treatment. Data for a representative experiment in individual zebrafish recorded in parallel are presented. [a]$P<0.05$ relative to control group or cocaine-withdrawal+control solution group.

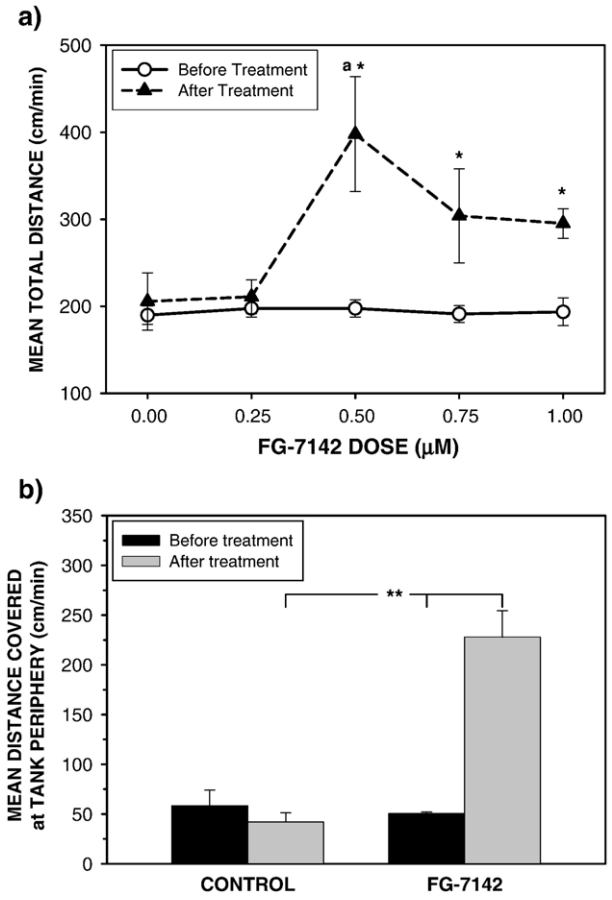


Fig. 9. An anxiogenic agent, FG-7142, increases locomotor activity and stereotypy in zebrafish. (a) The low doses of FG-7142 (0.5–1.0 μM), but not the control solution (vehicle), increase total distance traveled by adult zebrafish ($n$=10/group) within 15-min of treatment. *$P$<0.05 relative to baseline activity level in each treatment group and control group. [a]$P$<0.01 relative to vehicle and 0.25 μM dose. (b) Mean total distance traveled by zebrafish in the periphery of the tank before and after FG-7142 (or vehicle) treatment. **$P$<0.01 relative to baseline recording and control group after vehicle administration.

for at least 30 min of treatment (Fig. 8a). Similar effects of DzP treatment were observed in zebrafish withdrawn from repeated cocaine administration (data not shown).

Fig. 8b shows change in the activity level following DzP treatment relative to the basal activity in individual zebrafish of three groups: control group treated with DzP and the cocaine-withdrawal groups treated with either DzP or control solution (DzP vehicle). The regression comparison revealed significant differences between the groups (one-way ANOVA; $F_{2,24}$=5.842; $P$=0.009), with the highest DzP inhibitory effect observed in the cocaine-withdrawal group ($P$=0.036 and $P$=0.012 relative to the control group and cocaine-withdrawal group treated with the DzP vehicle, respectively). The magnitude of DzP inhibitory effect positively correlated with the basal locomotor activity in individual animals in the cocaine-withdrawal group treated with DzP ($r^2$=0.550). Neither control group treated with DzP ($r^2$=0.042), nor cocaine-withdrawal group treated with the DzP vehicle (i.e., 0.003% ethanol; $r^2$=0.001) showed such effect.

### *3.6. An anxiogenic drug imitates the behavioral effects of cocaine withdrawal*

Acute administration of a range of doses (0.25–1 μM) of the benzodiazepine receptor inverse agonist, FG-7142, resulted in significant increase in total distance traveled (two-way repeated measures; $F_{4,8}$=10.746, $P$=0.011; $F_{1,8}$=83.704, $P$<0.001 for main effects, i.e. treatment group and time respectively; and $F_{4,15}$=6.015, $P$=0.038 for the interaction between treatment group and time) (Fig. 9a). This drug is known to produce anxiogenic responses in humans, non-human primates, rodents and goldfish [16–19]. Although the behavioral effect of an acute low-dose FG-7142 treatment was less robust than following cocaine withdrawal, the principal characteristics of the response appeared to be similar. In addition to behavioral hyperactivity, zebrafish demonstrated stereotypic behavior, characterized by repetitive movements along the tank wall and preference of the bottom part of the tank. Fig. 9b illustrates distance traveled in the periphery of the tank before and after FG-7142 (0.5 μM) or vehicle administration. The analysis revealed a significant increase in locomotor activity in the periphery of the tank in the FG-7142 group after drug administration ($F_{3,11}$=111.50, $P$<0.001, ANOVA).

## 4. Discussion

### *4.1. Anxiogenic effects of cocaine withdrawal in zebrafish*

The results of this study demonstrate that zebrafish display cocaine-withdrawal symptoms, characterized by substantial and long-lasting increase in basal locomotor activity, which is associated with stereotypy. We submit that this behavior is an anxiety-like state and that cocaine withdrawal has an anxiogenic effect in zebrafish.

Anxiety, including its cognitive, somatic, emotional, and behavioral components [20], is one of the major symptoms of cocaine withdrawal, as reported in humans, non-human primates and rodents [21–31]. It can be related, in part, to an "ahedonic state" of the reward system [32] and to dysregulation of the stress system following repeated cocaine exposure [33]. While human self-reports leave no doubt about the existence of this phenomenon, the results obtained in animal models require interpretation.

In zebrafish, anxiety-related behavior has been addressed in only a limited number of studies, in which hyperactivity, freezing, erratic movements, swimming mainly close to the bottom or wall of the fish tank have been documented [8,15,34,35]. Our behavioral observations in several wild-type strains of zebrafish (unpublished) also suggest that there are distinct differences between individual zebrafish; some display "passive hiding" behavior in response to fear, while others react with hyperlocomotion. A tendency to display either of these behaviors is more or less prevalent in specific laboratory strains. In the AB strain, used in the present study, we find more individuals to have an active, hyperlocomotive, reaction to stressful, potentially anxiogenic situations, e.g., electric stimulation or social isolation.

Cocaine, at least in the doses used in this study, does not have an acute anxiogenic effect in zebrafish, with the first exposure neither reducing nor augmenting their activity, and not resulting in stereotypy. However, withdrawal from either single or repeated intermittent administration of cocaine dramatically changes zebrafish behavior. Daily recordings of locomotor activity reveal progressive behavioral activation during cocaine-withdrawal that does not occur in control zebrafish (Figs. 3 and 4). Such hyperlocomotion is coupled with a preference for the bottom of the tank and the walls, which results in stereotypic fast movement along the side of the tank. Similarly, treatment with a well-established anxiogenic drug, FG-7142, resulted in increased locomotor activity and stereotypy. Based on the earlier reports [15,35] and our observations mentioned above, we suggest that such behavior during cocaine withdrawal reflects an anxiety-like state in zebrafish.

### *4.2. Environmental stimulation potentiates the anxiogenic effects of cocaine withdrawal in zebrafish*

Multiple animal studies show that responses to cocaine depend on the individual or species-specific reaction to cocaine, behavioral procedures, treatment timing in relation to behavioral tests, dose levels, dose intermittency, and length of withdrawal period [20,36,37]. Additionally, testing environment is an especially important factor that affects animal behavior. Administration of cocaine in novel or stressful environment can augment drug effects [38,39], while administration of cocaine in a familiar or comfortable environment can blunt the responses [39–41].

In our study, cocaine was administered only in the animal's home environment. However, the test procedures were performed either in the same home/experimental environment (LES) or soon after the transition from the home to experimental environment (HES). This allowed us to explore whether such relatively mild environmental stress could alter the acute effects of cocaine or its withdrawal.

We find that the acute effect of the first cocaine administration in the doses tested is absent independent of the environmental conditions used. The control zebrafish also behaved similarly under LES and HES conditions. In contrast, the cocaine withdrawal-associated increase in basal locomotor activity and stereotypy, present in both LES and HES, was more robust in HES (Figs. 4 and 5). Thus, even the mild stress of being moved from one room to another prior to the behavioral assessment (while staying in the home tank) provokes an amplified anxiogenic response in cocaine-withdrawn fish. Therefore, although home conditions do not prevent cocaine withdrawal-induced behavioral activation, they can attenuate it.

### *4.3. Anxiogenic effect of cocaine withdrawal in zebrafish is sensitive to anxiolytic effect of benzodiazepine*

In humans, benzodiazepines can attenuate the negative effects of cocaine withdrawal [42]. However, benzodiazepines have distinct dose-dependent effects in both humans and animal models. At lower doses, they act as anxiolytics, while increased doses lead to sedation or even general anesthesia. To avoid potential false-positive result by administering sedative doses of DzP to activated fish, we determined and then used a dose that produces little, if any, behavioral effect in naive zebrafish.

In contrast to the control fish, which only slightly respond to a borderline dose of DzP, zebrafish treated with the same dose of DzP during cocaine withdrawal show a dramatic decline in hyperlocomotion and stereotypy. Such reduction leads to prolonged inhibition of activity beyond the control level or that observed in these same fish at baseline, i.e., before they have been first exposed to cocaine. That said, DzP treatment does not result in total inactivity. Rather, it uniformly abolishes fast movement in the fish withdrawn from cocaine, as reflected in reduced intra-group variation in their activity levels. In agreement with this finding, the degree of zebrafish response to cocaine during drug withdrawal correlates with the prior behavioral activation and is maximal in zebrafish displaying the highest level of activity on withdrawal. This is in contrast to lack of such dependence in the DzP-treated control fish, even though their basal behavior differed within the group (Fig. 8b).

The robust effects of DzP in zebrafish with increased locomotor activity during cocaine withdrawal further support our hypothesis that the behavior displayed by these fish is likely to reflect an anxiety-like state. It should be noted, however, that DzP treatment is only capable of temporarily counteracting the behavioral effect of cocaine withdrawal. Once this drug is washed out, zebrafish gradually, within a few hours, return back to the hyperactive and stereotypic behavior. This suggests that although, benzodiazepine treatment might not be able to reverse cocaine withdrawal-associated anxiety in zebrafish, it can temporarily attenuate its symptoms. A similar effect of benzodiazepines during cocaine withdrawal has been observed in humans and in other animal models studied [43].

### *4.4. Is cocaine an anxiolytic in zebrafish withdrawn from cocaine?*

The bi-modal nature of cocaine effects in humans, with the subjectively positive effects immediately after intake and negative symptoms increasing over the initial period of drug withdrawal, that are alleviated by the next cocaine administration, could suggest that cocaine may display an acute anxiolytic effect during the drug withdrawal phase [44]. Indeed, we find that zebrafish with increased locomotion and stereotypy during cocaine withdrawal have more normal behavior following acute cocaine administration, with the declining activity and stereotypy. This is in contrast to lack of an acute effect of cocaine doses used here on the locomotor activity in naive animals. Moreover, the acute effect of cocaine on increased locomotion of cocaine withdrawal positively correlates with the behavioral activation induced by the withdrawal itself (Fig. 6). This can be interpreted as an anxiolytic effect of cocaine being greater in the fish that have a more pronounced anxiety-like state.

However, plausible alternative explanations need to be considered and addressed experimentally. For example, it is well-known that anxiety can manifest as two opposite behavioral states, hyperactivity/agitation or freezing, depending

on individual or species-specific behavioral features. Thus, reduction in anxiety, shifting behavior toward more normal one, could manifest as an increase or decrease in activity. Among the fish we have studied, none displayed reduction of activity during cocaine withdrawal compared to baseline. However, the AB strain of zebrafish, in general, tends to show an increase in activity under unfavorable conditions. In the future, it would be important to determine whether zebrafish strains that express anxiety with freezing behavior would respond similarly to cocaine administration after withdrawal period.

We find that zebrafish with higher rates of basal activity during cocaine withdrawal show more robust inhibition following cocaine administration. This is consistent with the described earlier rate-dependent effects of psychostimulants in both humans and animal models [45]. Accordingly, the stimulants are more likely to increase the low baseline rates of activity, rather than the high ones, and can reduce hyperactivity. Such effect of psychostimulants, e.g., amphetamine or methylphenidate, is utilized to mitigate the hyperactivity of attention deficit disorder (for review, [46]). The mechanisms of this intriguing phenomenon are not yet fully understood but should provide important insights into the role of the catecholaminergic transmission in different parts of the brain in structuring the behavioral responses [47]. Interestingly, comparison of the effects of amphetamine in diurnal and nocturnal non-human primates shows that low doses increase daytime activity in nocturnally-active monkeys but decrease activity in diurnal primates [48]. Similarly, studies in nocturnally-active rats show only increased locomotion in response to even the lowest doses of stimulants tested, as well as the high ones [49]. A diurnal life style of zebrafish thus may offer an additional advantage for this model in contributing to translational research on the rate-dependent effects of psychostimulants in diurnal vertebrates.

Another potential explanation of acute cocaine-induced behavioral inhibition following cocaine withdrawal could involve sensitization to cocaine's effect during withdrawal. This would, however, require the higher doses of cocaine administered acutely to the naive animals to produce a similar inhibitory effect. Based on our investigation, even increasing a dose of cocaine 100 times (150 μM), compared to 1.5 μM that induces behavioral inhibition when administered during cocaine withdrawal, does not result in either reduction or increase in locomotion in naive zebrafish. Thus, such explanation of the phenomenon observed does not seem to be plausible.

In contrast to lack of long-term changes in the behavior of the cocaine-withdrawal fish treated with DzP, the potentially anxiolytic short-term effects of acute cocaine administration during withdrawal are followed by aggravation of the withdrawal symptoms within the next 48–72 h and promote further increase in basal locomotion and stereotypy (Figs. 3 and 4). This is, however, observed only following the first few cycles of cocaine treatment-withdrawal. Later, the hyperactive behavior appears to reach a plateau, and only a tendency toward further increase is observed following more cocaine treatments and longer periods of withdrawal (Fig. 3, Days 16 and 22). Whether such plateau is dose-dependent or simply reflects a limit to which zebrafish behavior can be increased under the specific experimental conditions requires further investigation. Overall, while our current working hypothesis is that cocaine produces anxiolytic effects when administered after withdrawal, the nature of this effect or its combination with other effects of cocaine is likely to result in a fundamentally different process from that induced by other anxiolytic agents and requires in-depth investigation.

### *4.5. Nociceptive sensitivity threshold is not altered by acute or repeated cocaine administration in the doses tested*

Absorption of cocaine from the water surrounding fish may produce some peripheral effects that potentially affect their behavior. This concern is especially relevant considering the anesthetic properties of cocaine [12]. However, our nociceptive sensitivity threshold test revealed that the doses used in this study had no anesthetic effect in zebrafish over short or long periods of observation.

We also find that in zebrafish withdrawn from repeated cocaine administration, both basal nociceptive sensitivity and that following acute cocaine administration remain similar to the control fish. These results indicate that lack of an acute behavioral effect of the first dose of cocaine in zebrafish is not related to the potential anesthetic properties of this drug. Also, this means that the reduction in increased locomotion after cocaine is administered to zebrafish withdrawn from cocaine is not due to an anesthetic effect.

In summary, this study is the first to establish that zebrafish, a powerful model of vertebrate development and genetics, show cocaine withdrawal-associated behavioral alterations consistent with the anxiety-like state. This provides a new animal model for elucidating the mechanisms involved in cocaine withdrawal phenomenon. Considering high throughput capabilities of this animal model, it could also serve as an efficient model in the search for new therapeutic compounds to counteract the negative symptoms of cocaine withdrawal that promote relapse.

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