Gender differences in zebrafish responses to cocaine withdrawal

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ABSTRACT

The acute responses to cocaine and its withdrawal contribute to cocaine dependence and potentiate relapse, with gender being one of the genetic factors affecting the outcome. Here we report that in both male and female zebrafish (*Danio rerio*, AB strain), an initial low-dose cocaine treatment (1.5 μM, immersion) does not acutely change their behavior. The cocaine withdrawal, however, is associated with an anxiety-like state that develops earlier in female zebrafish but is more robust and persistent in males, and can be acutely attenuated by cocaine administration. This is not a result of gender differences in the expression of anxiety-like state, since behavioral responses to an anxiogenic drug, FG-7142, are similar in male and female zebrafish. The basal brain dopamine (DA) levels and the expression of dopamine transporter mRNA (*zDAT*) show no significant sexual dimorphism. Acute cocaine exposure does not significantly change DA or *zDAT*. Withdrawal from repeated cocaine administration results in an overall reduction in *zDAT*, as well as an increase in DA levels. Neither treatment leads to significant gender differences in brain DA or *zDAT*. The common and gender-specific effects of cocaine on zebrafish, a well-characterized model of vertebrate development and genetics, should help in understanding the mechanisms involved in the anxiety associated with cocaine withdrawal and provide new opportunities in search for therapeutic solutions.

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1. Introduction

Genetic differences, including those associated with gender, can affect multiple aspects of responses to illicit drugs, including cocaine or its withdrawal, perpetuating a self-destructive behavior of drug abuse. Although the use of cocaine, current or throughout lifetime, is still substantially higher in males, the gender gap is getting narrower, judged by the higher rate of initiation among women [1]. It is thus increasingly important to address the potential gender dimorphism in the effects of cocaine. Indeed, the available data suggest that it may take less time for women to meet the criteria for dependence [2,3]. In seeming contradiction to this, women are reported to have delayed subjective effects of cocaine, less euphoria and dysphoria [4], longer periods of abstinence [5] and longer periods of use after relapse [6], leading to less frequent abstinence episodes in women, compared to men [7]. These differences cannot be explained by cocaine's pharmacokinetic and pharmacodynamic effects, since those were found to be similar in men and women [8]. The reasons for gender differences in the effects of acute and chronic cocaine intake, or its withdrawal, may be both psychological and physiological and require in-depth investigation.

Withdrawal (or abstinence) symptoms that follow acute euphoric responses to cocaine are part of the vicious cycle of drug abuse. During cocaine withdrawal, the spontaneous, stress- or cue-associated cocaine craving correlates with anxiety, anger, fear, and sadness [9,10], that are predictive of relapse [11]. In different studies, women were found to be more [12,13], less [14] or similarly reactive [15] to drug-associated cues during cocaine withdrawal, when compared to males. A contribution of these potential differences to sexual dimorphism in cocaine addiction remains unclear. However, it has been noted that while the subjective reports in females reflect increased stress-induced anxiety compared to males, the latter are emotionally and physiologically more reactive in the cue condition [16]. This is consistent with gender differences in baseline sensitivity of the hypothalamic–pituitary–adrenal (HPA) axis to stress, including that of drug withdrawal [17].

Studies in animal models have been invaluable in addressing the nature of drug addiction. Research in rodents (rats and mice) revealed gender differences in their responses to cocaine, including cocaine pharmacokinetics, behavioral alterations induced, motivation to receive cocaine, patterns of its self-administration and monoamine content in principal brain structures mediating reward [18–28]. Some of these studies suggest that female rodents might be more sensitive to the reinforcing effects of cocaine or more motivated to self-administer the drug. Together, studies in humans and animal models warrant further investigations into the gender differences in the effects of cocaine, in order to understand the potential mechanisms involved.

Among the major targets of cocaine is dopaminergic neurotransmission. Studies in both humans and animal models, including those that use newly-available imaging, functional genomic, or proteomic

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approaches [for review, 29,30], support this notion. It has been well established that a blockade of the dopamine transporter (DAT) by cocaine, resulting in the elevated extracellular dopamine (DA) levels [31], is responsible for a number of critical effects of the drug, including its stimulating and rewarding properties [32,33]. This is supported by the altered DAT protein levels or mutation in the *DAT* gene resulting in major modifications in rodents' responses to cocaine [34,35].

Further progress in understanding the mechanisms of acute and delayed cocaine-associated phenomena, including the principal symptoms of cocaine withdrawal, would benefit from the use of diverse high-throughput animal models in genetically tractable species. Among vertebrates, the zebrafish model appears to be well suited for such investigation, being responsive to the developmental [36] and rewarding effects [37–39] of drugs of abuse. This model provides unique opportunities for studying the genetic bases of drug-related phenomena [40].

We have recently described the behavioral symptoms of cocaine withdrawal in male zebrafish, consistent with the anxiety-like state and associated with hyperactivity and stereotypy [41]. These effects are augmented by increased environmental stimulation, simulated by an anxiogenic agent, and attenuated by acute cocaine or diazepam administration. The goal of this study was to determine whether zebrafish display gender differences in behavioral responses to acute cocaine administration and its withdrawal. We have also addressed whether brain DA levels and those of dopamine transporter (*zDAT*) mRNA at baseline and following acute cocaine administration or its withdrawal may display sexual dimorphism in zebrafish.

Here we report that, independent of gender, acute cocaine administration does not induce behavioral response, changes in brain DA levels or *zDAT* mRNA expression in zebrafish. In contrast, females show a more rapid onset but transient dynamics of the anxiety-like behavior during cocaine withdrawal, compared to males. Brain DA levels or *zDAT* mRNA expression show no significant gender differences at baseline, after acute cocaine administration or its withdrawal. However, for two genders combined, cocaine withdrawal results in significant increase in brain DA levels and reduction in *zDAT* mRNA expression in zebrafish. These results provide first evidence that zebrafish, a powerful model of vertebrate genetics and development, show sexual dimorphism in behavioral responses to cocaine, opening new opportunities to investigate the mechanisms involved in this phenomenon using a genetically well-characterized diurnal vertebrate.

2. Materials and methods

The treatment schedule, dose of cocaine used and behavioral observation conducted were similar to those described earlier [41], in order to provide consistent comparisons between the data collected in male and female zebrafish.

2.1. Animals and housing conditions

Adult male and female zebrafish (*Danio rerio*, AB wild type strain, 9± 1 months old, 5 fish/3-L tank) were housed in 14L:10D light:dark cycle, in a temperature (26.5 °C) and pH (7.0–7.4) controlled 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, USA).

Starting with the adaptation and throughout the entire experimental period, each fish remained in an individual 1-L tank (17×9×8 cm; 5 cm water depth), with uniform white, non-transparent walls, serving as both the animal's home and experimental tank. The environmental illumination and feeding schedule remained as in regular housing. Fish were fed equal amounts of pre-soaked decapsulated brine shrimp eggs (Brine Shrimp Direct, Ogden, Utah. USA), and 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 (Video-track, View Point Inc, France). Twenty individual home/experimental tanks, each containing one fish, were placed inside one recording chamber and monitored by two Video-Track systems and two cameras. The cameras were positioned 160 cm above the tanks, one camera per 10 tanks. This allowed us to reliably automatically monitor each fish track throughout the experiment and also maintain visual control of the track and its speed, with high and low speed tracks being of different color. The activity of fish in the control and treatment groups of both genders was documented in parallel, 5 fish per gender by treatment group. During recording, the walls of the individual tanks were uniformly white and non-transparent, with a back-light illuminated white floor [400 lx (lux) at the water surface level].

The data acquisition speed was set at 30 frames/s, with integration period of 30 s. The Video-Track software automatically records the time and overall distance traveled by individual fish, as well as distance traveled at low and high speed. The thresholds defining speed range were set manually, when the recording protocol is originally designed. For all the recordings, the high speed threshold was defined as above 15 cm/s. Low speed was defined as between 0.1 and 15 cm/s. Any minor changes in position with the speed less than 0.1 cm/s were scored as inactivity. The distance traveled (total, at low speed and at high speed) was documented for the entire tank and for each of the tank areas defined, including the center area and that of the periphery of each tank. Consistent environmental conditions and thorough pre-recording calibration assured lack of recording artifact.

Automatic activity recordings and visual observations throughout each experimental period allowed the documentation of the presence or absence of stereotypy. The stereotypy was defined as unvarying, repetitive behavioral patterns with no obvious function [12], 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 the periphery of the tank area for automatic activity recording, 15 mm from each wall of the tank, we have automatically documented the time spent moving and distance traveled at the periphery and this served as an objective measure of stereotypy. Based on the visual observations of the fish image on the computer monitor, during such stereotypic behavior zebrafish were typically moving near the bottom of the tank, as reported earlier [41]. However, since we 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 to 10 mM working solution. It was then administered directly into the fish tank water to achieve the 1.5 μM final cocaine concentration (dose) that did not significantly affect water quality, including pH (0.02 pH change following treatment). The dose of cocaine used was based on the dose-dependence studies conducted earlier, showing that it induced consistent behavioral effects and changes in brain cocaine levels in male zebrafish [41]. In all behavioral experiments, the fish were treated with cocaine (or water during cocaine withdrawal) for 1 h 15 min, at the same time of day, starting at ZT4 (zeitgeber time, ZT0=lights on time). Recorded in parallel, control fish received the same volume of water serving as cocaine vehicle.

An anxiogenic 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 3.5 mM working solution. A range of doses (0.25–0.75 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 of the recording system for 1 h/day, for 5 consecutive days. On the first day of the experimental period, the baseline behavior was documented. After that, a treatment period was initiated. Cocaine or control treatment was administered repeatedly and zebrafish behavior was documented daily, before and after treatment, including the days of cocaine withdrawal. The only exception was on day 6, when no testing or treatment was conducted in order to maintain the same protocol that was used earlier to study the responses in male zebrafish [41]. Intermittent repeated treatment involved three cocaine administrations, with the schedule presented in Fig. 1A.

As illustrated in Fig. 1(B,C), on each day, zebrafish activity was recorded for two 15-min periods. The first period served to assess individual basal activity levels before-daily-treatment (BDT). Fish were then treated with cocaine or a vehicle (water). An hour later, their behavior was documented during the second 15-min period, referred to as after-daily-treatment (ADT) recording. The results obtained during this second period reflected the effect of cocaine or vehicle administration to cocaine groups on treatment and withdrawal days, respectively, and the vehicle administered to the control groups of male and female zebrafish each day.

Since the effects of cocaine can be significantly influenced by the level of environmental stimulation, two principal cocaine treatment designs were used that either involved handling the fish immediately before the behavioral assessment or not [41]. 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 daily water change procedures. In contrast, according to the high environmental
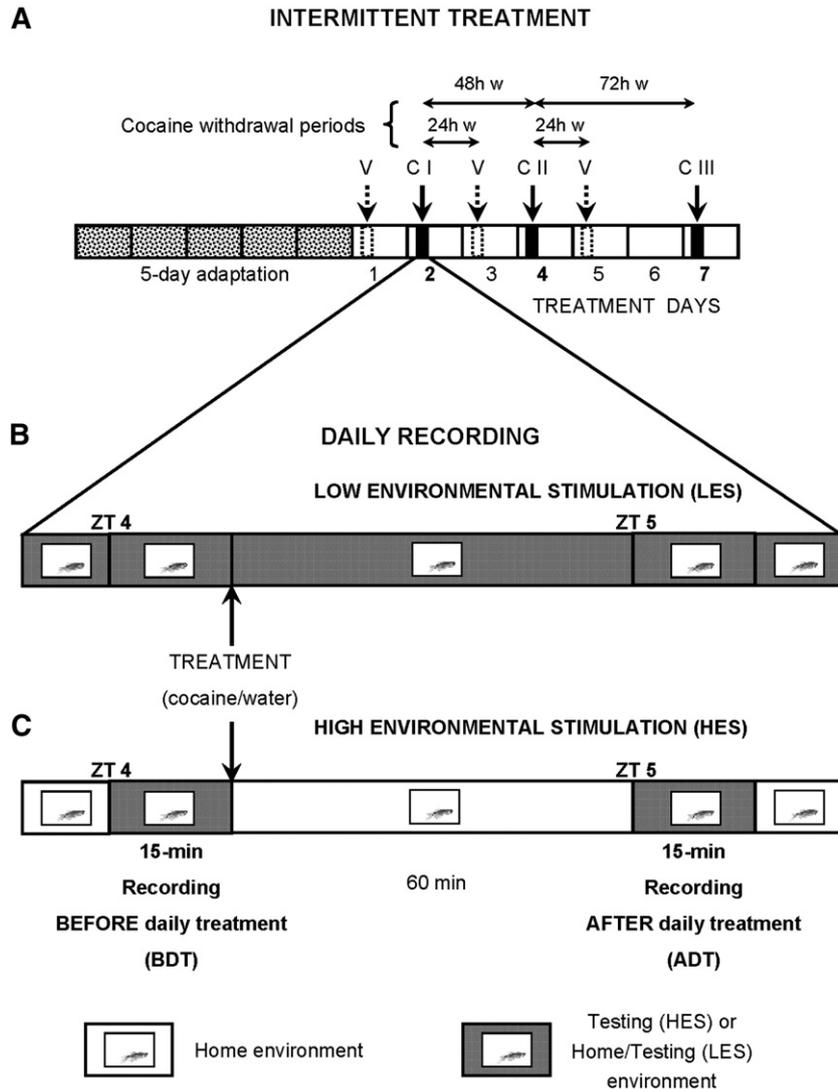
Fig. 1. Protocol schematics. (A) Intermittent repeated cocaine treatment schedule: following 5-day adaptation, adult male and female zebrafish were repeatedly treated with cocaine (black). During 24–72 h withdrawal periods, both cocaine and control groups received control (vehicle) treatment (white). (B) Daily activity recording protocol with low environmental stimulation (LES). Treatment and behavioral recordings were conducted in home environment. (C) Daily activity recording protocol with relatively high environmental stimulation (HES). Animals were moved to the test environment twice a day immediately before the behavioral recordings, while remaining in their home tanks. The 15-min daily activity recordings were scheduled before (BDT) and an hour after (ADT) daily 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.

stimulation (HES) design, fish were maintained in the regular housing room and moved to the experimental room (while remaining in their individual home/experimental tanks) for daily BDT recordings. They were then returned to the housing room, where they received their treatment. An hour later, fish were once again moved to the recording system for an ADT activity recording of the day, while remaining in the treatment solution (Fig. 1C). Tank water was 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 about 10 min, on average, adding to the time spent in cocaine solution.

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, 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). All the procedures, except for type of treatment in the control zebrafish, were identical between the experimental groups tested in parallel.

To assess the acute effects of cocaine on brain *zDAT* and DA levels, using QPCR and HPLC measurements described below, groups of male and female zebrafish were sacrificed by fast immersion in liquid nitrogen at intervals after the first cocaine treatment at ZT4. To evaluate the potential changes in brain *zDAT* and DA during cocaine withdrawal, a separate set of fish was exposed to repeated cocaine (or control) treatment and its withdrawal, according to the HES design (Fig. 1C). Fish were immersed in liquid nitrogen 30 min after cocaine administration, since changes in brain DA levels can be fast and no acute behavioral responses were observed within 5–120 min following acute cocaine administration to naive animals, as part of earlier studies (data not shown). To assess the effects of cocaine withdrawal, fish were immersed in liquid nitrogen 72 h after the second cocaine administration, immediately prior to the time of behavioral testing.

FG-7142 treatment was administered to naive animals that had previously adapted to the testing environment. After a 30-min baseline recording, a single dose of FG-7142 (or control) was administered (0.25–0.75 µM final concentration in tank water) and locomotor activity recording continued. In all the experiments described above, the control fish of both genders were tested in parallel and received a vehicle (water).

2.5. Measurements of brain cocaine levels

Zebrafish males and females, matched in age and size to those behaviorally tested, were used for measuring brain-tissue cocaine levels at intervals following a single cocaine treatment, 1.5 µM final concentration in the fish tank. Fish were sacrificed by fast immersion in liquid nitrogen, 20 min or 120 min after cocaine administration. The duration of exposure was based on the prior data [41] showing the onset of detectable brain cocaine levels in male zebrafish at 20-min post-treatment and plateau in peak brain cocaine levels maintained at 60–120 min after administration.

Prior to dissection, frozen fish were thoroughly washed out in ice-cold water to remove any cocaine from their body surface. Brain tissue was carefully 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). 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-d3, benzoylecgonine-d3, and ecgonine methyl ester-d3). 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 [42] with modifications [43]. The resulting lower limit of quantification for each analyte was 2 ng/ml.

2.6. Real-time quantitative RT-PCR (QPCR)

Fish were dissected on dry ice and total RNA was extracted from one hemisphere of each individual brain using RNeasy kit for high lipid content tissue (Qiagen, Chatsworth, CA, USA), according to the manufacturer's protocol. The quantity and quality of RNA was determined spectrophotometrically, at 260 nm and 260/280 nm. The same amount of RNA from each sample was converted into cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instruction. QPCR was performed using a TaqMan® Universal PCR Master Mix and ABI Prism 7300 Real Time PCR System (ABI, Foster City, CA, USA). The TaqMan® primers and probes {5' FAM, 3' TAMRA} for dopamine transporter (*zDAT*) were designed based on previously reported sequences of zebrafish genes and obtained from ABI: Forward, 5'-GTCTGGAAGATCTGCCCCATATT-3', Reverse, 5'-CACATACAGCGAGATCAGGATCAC-3'; {AAGCCCACGCCTTTG}. Gene expression was normalized using β-*actin* expression level for each individual fish sample. Relative mRNA expression level was calculated using the standard comparative delta-Ct method. Male and female brain samples were collected and processed in parallel, and the expression was measured within the same microplate, each sample in triplicate. The data are presented as fold change, relative to mean male control group result, shown as "1" on Y-axis.

2.7. HPLC-brain dopamine (DA) levels

Tissue samples containing one brain hemisphere (the other one being used for QPCR) were sonicated in 200 µl of cold 0.1 N HClO$_4$ and centrifuged at 14,000 g for 6 min at 4 °C. DA was separated on a C18 column (100×2.0 mm, 3 µm particle size; Phenomenex, Torrance, CA, USA) and eluted with a mobile phase consisting of 32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM ethylenediaminetetraacetic acid (Na$_2$-EDTA), 0.215 mM octyl sodium sulfate and 3% methanol (pH 3.8). DA was detected with an LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA) with a glassy carbon working electrode maintained at a potential of +0.670 V relative to an Ag/AgCl reference electrode. Data were recorded using the EZ Chrom (Scientific Software, Pleasanton, CA, USA) software package. Concentrations were expressed as pg/µg protein. Protein content was determined by method of Bradford [44].

2.8. Statistical analysis

A mixed-model analysis was used to examine the effects of repeated intermittent cocaine administration, as well as the gender differences (SPSS 15.0; SPSS Inc., Chicago, IL, USA). The Bonferroni confidence interval adjustment was employed for multiple comparisons. Total distance, distance at high and low speed, differences in locomotor activity following cocaine administration or its withdrawal, as well as stereotypy, were compared. A two-way ANOVA test was used to compare brain cocaine, DA and *zDAT* levels in male and female zebrafish at intervals after treatment. If significant differences were detected, 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. The significance level was set at $P<0.05$ or reduced for multiple comparisons (Bonferroni adjustment).

3. Results

3.1. Cocaine administration through immersion significantly augments brain cocaine levels in adult zebrafish

Based on the earlier study [41], showing consistent effects of the 1.5 μM cocaine dose on brain cocaine levels in male zebrafish and their time dependence, the effect of this dose was evaluated at 20 min and 120 min after treatment. The absolute cocaine levels in females were found to be similar to those in male zebrafish. Time-dependent increases were present in both males and females ($F_{3,12}=16.997$, $P<0.001$, two-way ANOVA), with a significant increase in cocaine levels (per protein) by 20 min post-treatment (2.95 ± 0.05 pg/μg and 2.25 ± 0.25 pg/μg for males and females, respectively) and peak levels (9.88 ± 1.52 pg/μg and 10.00 ± 2.20 pg/μg, in males and females) at 120 min for both genders ($P=0.006$ and $P<0.001$, respectively, for 120-min versus 20-min, Tukey

test). There was no significant gender difference in brain cocaine content at either time point. The levels of cocaine metabolites in the samples were too low to produce meaningful results and thus not reported here.

3.2. Gender differences in the dynamic pattern of hyperactivity in zebrafish withdrawn from cocaine: transient changes in females versus escalating changes in males

Under the conditions of relatively high environmental stimulation (HES), animals were treated in the home environment, but were moved to the experimental environment for 15-min recordings before-daily-treatment (BDT) and an hour after-daily-treatment (ADT), whether they received cocaine or a vehicle on that day (Fig. 1). Under such conditions, the first cocaine treatment did not result in significant acute (within 1 h) changes in locomotor activity in either gender.
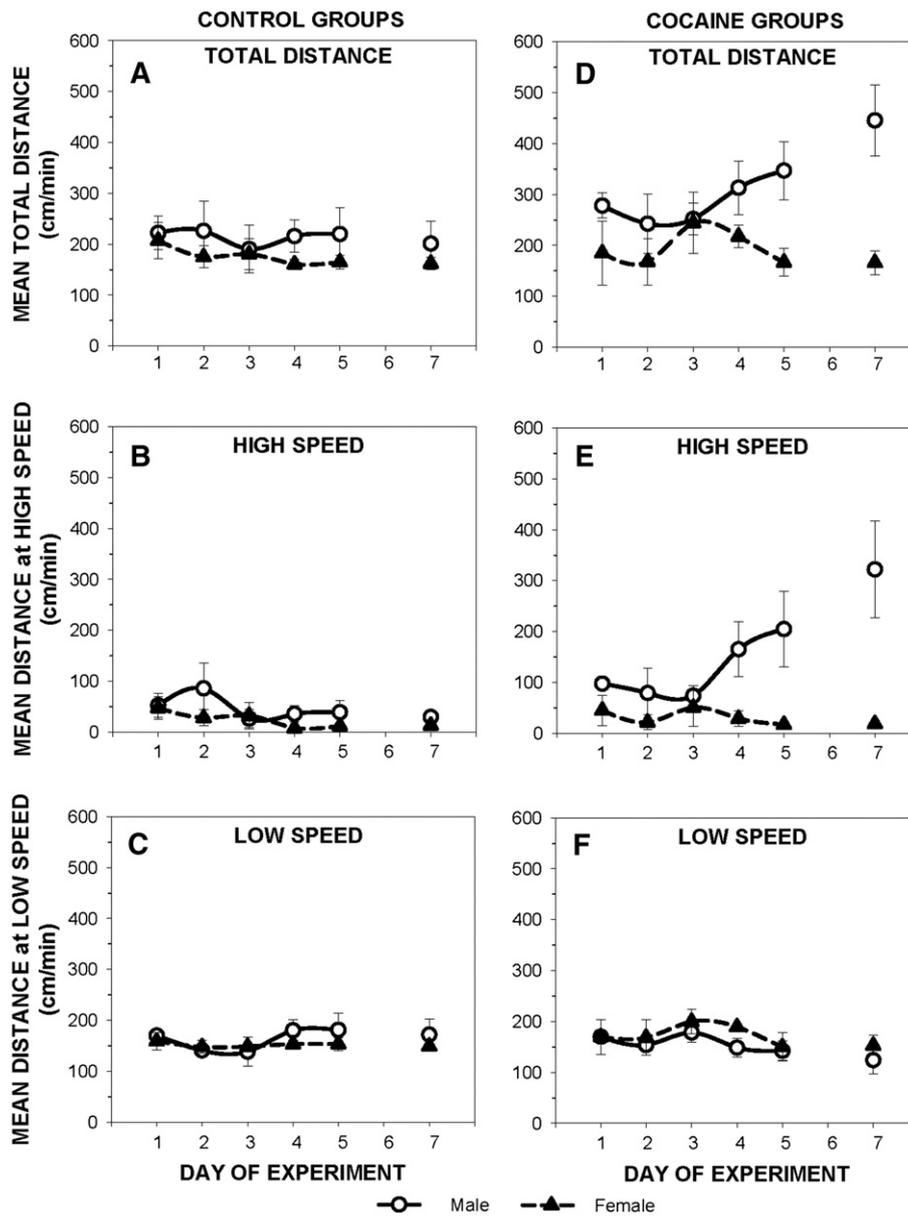
Fig. 2. Basal locomotor activity levels over the course of experiment in control and cocaine-treated zebrafish of both genders. Each data point reflects mean distance traveled (cm/min) by zebrafish of control and treatment groups during the first 15-min recording of each day, i.e., basal or before-daily-treatment (BDT) activity: (A–C) control groups, (D–F) cocaine groups. (A) and (D) total distance; (B) and (E) distance traveled at high speed; (C) and (F) distance traveled at low speed. Open circles and solid line — males, black triangle and dashed line — females, in both control and cocaine groups. Cocaine (1.5 μM) was administered during the experimental days 2, 4 and 7, following BDT recording (see Fig. 1A). Data are presented as mean±SEM for the representative groups recorded in parallel ($n=5$/group).
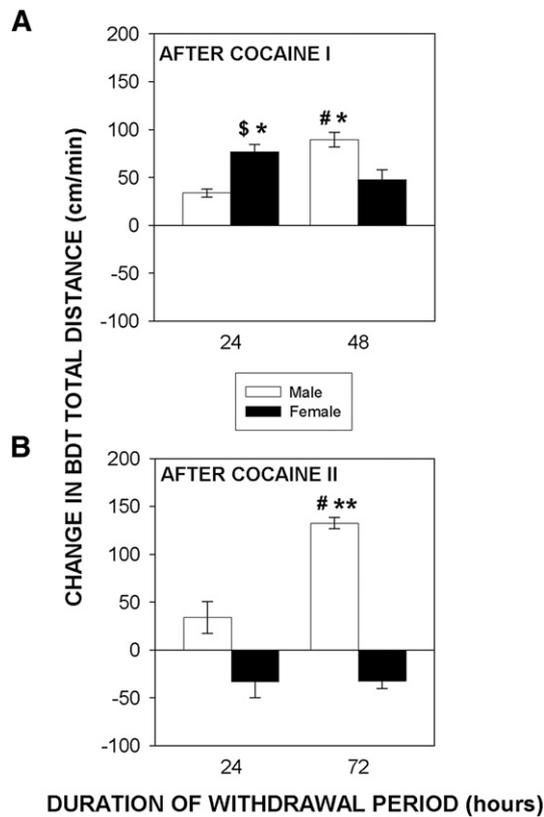
Fig. 3. The dynamics of behavioral response to repeated cocaine treatment and its withdrawal shows gender difference in zebrafish. Data are presented as difference in daily basal activity (BDT, total distance traveled) between the day of the first (A) or second (B) cocaine treatment and following 24, 48 or 72 h withdrawal period. Male — open bars and female — black bars. #: $P<0.01$ relative to 24-h withdrawal, $: $P<0.05$ relative to 48-h, * $P<0.05$ and ** $P<0.01$ relative to the corresponding data in another gender; mean±SEM for the groups recorded in parallel ($n=5$/group).

Fig. 2 presents the temporal pattern of activity levels at BDT in the Control (left column) and Cocaine-treated (right column) groups of male and female zebrafish on each day of the 7-day experimental period under HES conditions. In contrast to lack of significant changes over time in the Control groups of either gender (Fig. 2A–C), there were dynamic gender-specific changes in locomotor activity patterns in cocaine-treated fish for both total distance and that traveled at high speed (Fig. 2D–F).

Fig. 3 illustrates the changes in distance traveled at BDT at intervals after the first (Fig. 3A) and second (Fig. 3B) cocaine administration, relative to BDT immediately prior to the last cocaine treatment (i.e., on days 2 and 4, respectively). After both treatment days, this change was progressively increased in males, showing positive correlation with longer withdrawal periods, i.e., at 48- or 72-h, compared to corresponding 24-h withdrawal (Mixed model, $F_{1,47}=51.526$, $P<0.001$ and $F_{1,52}=104.751$ $P<0.001$, respectively). In contrast, in females, the largest change in activity at BDT was observed at 24-h after the first cocaine administration (Fig. 3A) and declined thereafter (Mixed model, $F_{1,8}=8.223$, $P=0.046$, for 24-h vs. 48-h withdrawal). When the magnitude of these changes was compared between the genders, the effects were higher in females soon after the initial treatment, at 24-h withdrawal, but were higher in males after prolonged withdrawal periods, 48-h or 72-h (Fig. 3A, B). This resulted in significant Gender by Withdrawal Duration interaction (Mixed model, $F_{1,52}=11.796$, $P=0.001$).

The observed gender differences in the effects of cocaine withdrawal were more robust when the distance traveled at high speed was considered (Fig. 2E). In males but not females, such high speed

activity during cocaine withdrawal (but not at baseline) was associated with stereotypy (Fig. 4). Typically, such stereotypic movements manifested as fast traverse along the wall of the tank and, less frequently, as circular movements in one of the tank areas. In both cases, the animals displaying stereotypy tended to move close to the bottom of the tank, as reported earlier for anxiety-like behavior in male zebrafish [41,45]. An objective measure of such behavior was increase in distance traveled in the periphery of the tank, that significantly correlated with cocaine treatment (Mixed model, $F_{1,12}=5.586$, $P<0.036$) and experimental day ($F_{1,12}=5.39$, $P<0.043$), being progressively increased in males after repeated cocaine administrations and its withdrawal ($F_{1,12}=5.188$, $P<0.042$ for Day by Treatment interaction). Since cocaine-treated females typically did not display stereotypy, there was also a significant Day by Treatment by Gender interaction ($F_{23,168}=6.051$, $P<0.001$) observed. Increase in stereotypic behavior illustrated in Fig. 4 shows gender differences in the distance traveled at the periphery of the tank at 72-h withdrawal following second cocaine administration in cocaine-treated versus control groups (Mixed model, $F_{1,8}=25.788$, $P=0.001$ and $F_{1,8}=10.979$, $P=0.011$ for the Treatment and Gender as main factors; $F_{1,8}=14.913$, $P=0.005$ for the Treatment by Gender interaction). The latter reflected that only the cocaine-treated male zebrafish showed a significant increase in distance traveled at the tank's periphery when compared to the respective control group ($P<0.001$). In contrast to fish receiving cocaine, the control groups of male and female zebrafish had consistent activity levels throughout the experimental period and did not display stereotypy.

3.3. Rate-dependent effects of treatment on locomotor activity

During the second recording of the day (at ADT), locomotor activity was either similar to that at BDT or reduced. Fig. 5 illustrates the total distance traveled prior to (BDT) and following (ADT) each of three cocaine administrations in male and female zebrafish. In males, a progressive increase in basal (BDT) activity level after repeated cocaine exposure and its withdrawal was associated with cocaine effectively inhibiting their locomotion ($F_{1,10}=6.662$; $P=0.045$ for Period factor; Fig. 5C). Female zebrafish showed no significant change in activity immediately after cocaine treatment, resulting in significant gender differences (Mixed model, $F_{1,16}=23.036$, $P=0.001$ for Gender factor). Vehicle administration resulted in relatively small changes in activity of the control groups of both genders (Fig. 6A–D, Y-axis) and females withdrawn from cocaine (Fig. 6F,G Y-axis). Although
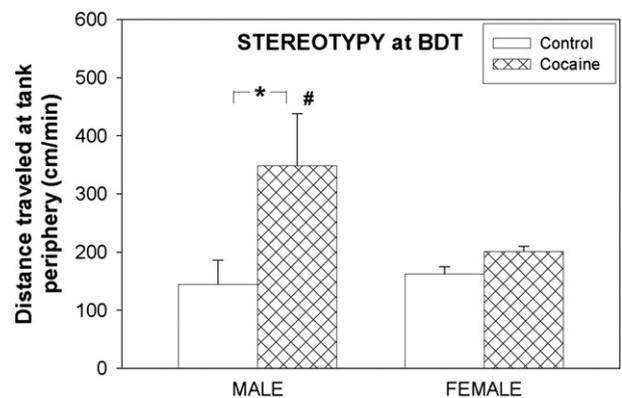
Fig. 4. Stereotypy accompanies hyperactivity during cocaine withdrawal in male but not female zebrafish. Following cocaine withdrawal (72-h), stereotypy, i.e., significantly increased distance traveled along the tank periphery, is observed in male but not in females zebrafish. * $P<0.01$ relative to control at before-daily-treatment (BDT); #: $P<0.05$ relative to cocaine-treated females at BDT. Each column represents the mean±SEM distance traveled at tank periphery for the representative groups recorded in parallel ($n=5$/group).

hyperactive male zebrafish reduced their activity following vehicle administration (Fig. 6F,G Y-axis), the effect of cocaine was significantly more pronounced (Mixed model, $F_{1,10}=41.887$, $P=0.007$ for cocaine vs. vehicle).

Fig. 6 also illustrates that the degree to which individual fish reduced their activity at ADT typically correlated with their basal level of activity on that day (at BDT). More active animals generally had more robust decline in locomotion. This rate-dependent reduction in activity was present in both control and treatment groups. There were, however, two exceptions observed. At 24-h withdrawal from the first cocaine administration, when females were hyperactive, relative to their baseline behavior, the rate-dependent effect of vehicle was inverted in such a way that more active females showed less change during ADT recording (Fig. 6F). Male zebrafish of Cocaine group tested in parallel did not have significant increase in BDT activity level at that time (Fig. 3A, 24 h) and retained the rate-dependent response to vehicle administration (Fig. 6F). However, after vehicle was administered to male fish at 24-h withdrawal from their second cocaine treatment, when their basal activity levels at BDT were augmented (Fig. 3B), the typical rate-dependent effect at ADT was abolished (Fig. 6G). Since a brief period of hyperactivity in female Cocaine group coincided with the day of vehicle treatment (day 3), no data on the effect of cocaine in hyperactive females were available. However, the data collected in hyperactive males of Cocaine group on day 7 provided evidence that the effect of cocaine continued to demonstrate the typical rate-dependence (Fig. 6H).

3.4. Low environmental stress has only minor effects on female zebrafish responses to cocaine

Earlier, we found that reduction in environmental stress can partially attenuate the responses to cocaine withdrawal in male zebrafish [41]. Indeed, the behavioral effects of cocaine under HES conditions, described above, were partially modified when animals were tested under the conditions of low environmental stimulation (LES), i.e., when zebrafish were continuously recorded and treated in home environment. In LES, similar to that in HES and independent of gender, zebrafish showed no acute behavioral response to the first cocaine administration, i.e., on day 2. Also, similar to HES data, the change in basal daily locomotor activity after a 24-h, but not 48-h, withdrawal from the first cocaine administration was significantly higher in females than in males (Fig. 7A). After the second cocaine administration, no gender difference was found at 24-h withdrawal,

while following the 72-h withdrawal period male zebrafish displayed significantly more change in BDT behavior than females (Fig. 7B).

These effects corresponded to increased locomotion in females on day 3, at 24-h withdrawal from the first cocaine treatment, and hyperactivity in males at 72-h withdrawal from the second cocaine administration, similar to that shown for HES in Fig. 2. However, when two conditions were compared at each time point, females were found to be more active in LES than in HES conditions at 72-h withdrawal period following second cocaine treatment (Mixed model, $F_{1,5}=20.060$; $P=0.007$), though remaining less active than males (Mixed model, $F_{1,8}=36.802$, $P=0.004$ for Gender factor). Overall a statistically significant Gender by Day interaction was documented in LES conditions (Mixed model, $F_{1,20}=13.856$, $P=0.006$), as it was in HES. No significant increase in stereotypic behavior was observed under LES conditions in either gender. The control zebrafish of both genders showed stable behavior under LES conditions and no difference from that in HES (data not shown).

3.5. Lack of gender difference in behavioral response to an anxiogenic drug in zebrafish

In male zebrafish, similarities between the effects of cocaine withdrawal and those of a known anxiogenic drug, the benzodiazepine receptor inverse agonist, FG-7142 [46–49], as well as rescue of cocaine effects by benzodiazepine treatment, allowed us to suggest that cocaine-induced behavior is related to anxiety [41]. Indeed, similar to cocaine withdrawal, FG-7142 can acutely induce hyperactivity and stereotypy in male zebrafish.

Lack of robust changes in activity and stereotypy in female zebrafish during cocaine withdrawal posed a question of whether the expression of anxiety might differ between the genders, masking the effects of cocaine withdrawal. To address this question, we characterized the effects of acute administration of a range of doses (0.25–0.75 μM) of FG-7142 in female zebrafish and found a dose-dependent increase in total distance traveled, similar to that in males (Mixed model $F_{3,23}=8.747$, $P<0.001$ Treatment group factor; Fig. 8). The 0.5 μM FG-7142 dose induced the most prominent hyperlocomotion in both females and males (Mixed model, $F_{3,13}=5.332$, $P=0.013$; $F_{3,10}=5.802$, $P=0.015$), when compared to their respective control groups. Moreover, in both genders, the effect of the drug showed an inverted U-shape dose-dependence, with the decline in the effect following high-dose treatment being more robust in females ($P=0.008$ for 0.5 μM vs. 0.75 μM comparison; Fig. 8). Unlike males [41], female
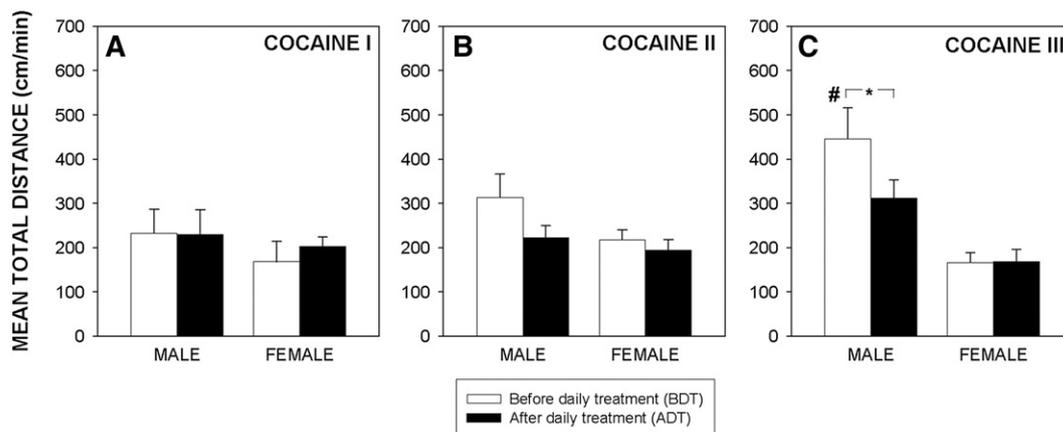
Fig. 5. Cocaine administration does not have acute behavioral effect in naive zebrafish of both genders but significantly inhibits locomotion in hyperactive male zebrafish withdrawn from cocaine. Each plot shows the mean total distance traveled before (BDT, white bar) and after (ADT, black bar) three repeated cocaine administrations on day 2 (A), day 4 (B) and day 7 (C), see Fig. 1 for schedule. No significant change in activity is observed in females on those days. In males, gradual increase in basal activity (BDT) is associated with the inhibitory effect of cocaine (at ADT), reaching significance after the third cocaine treatment on day 7, when basal activity was high (C). * $P<0.01$ relative to ADT; #: $P<0.01$ relative to females at BDT. Data are presented as mean±SEM ($n=5$/group, recorded in parallel).

zebrafish did not show significant stereotypy in response to FG-7142 (data not shown).

3.6. Changes in brain zDAT expression and DA levels following acute cocaine administration and its withdrawal

To address potential mechanisms that may underlie gender differences in response to cocaine, we assessed brain dopamine levels and expression of zDAT mRNA at baseline, after acute cocaine administration and during its withdrawal. Considering fast, within 20-min, increase in brain cocaine levels in both male and female zebrafish and lack of acute behavioral effects of cocaine for hours after

its first administration, as described above, we chose to document the acute effects of cocaine 30 min after treatment. In view of the behavioral effects during cocaine withdrawal, DA and zDAT expression levels were measured after a 72-h period of cocaine abstinence (day 7 at ZT 4, HES; Fig. 1).

In control zebrafish, zDAT expression was similar between the genders (Fig. 9A, Control). An acute exposure to cocaine did not induce significant changes in zDAT or DA in either male or female zebrafish, though some tendency toward reduction of both parameters was present in females (Fig. 9A, Acute). During cocaine withdrawal, a significant reduction in zDAT expression was documented for females and strong tendency toward reduction was present in males (Fig. 9A,
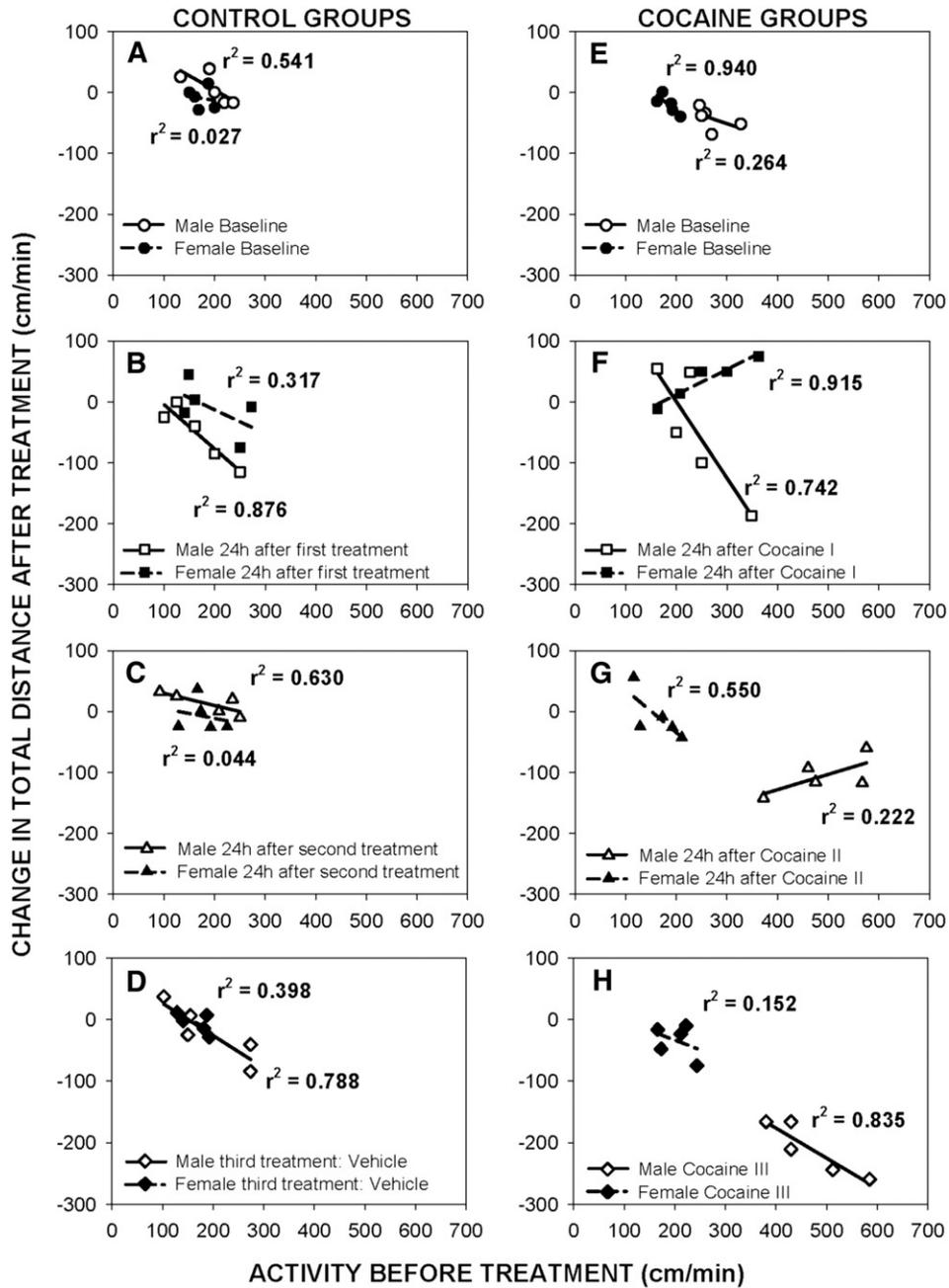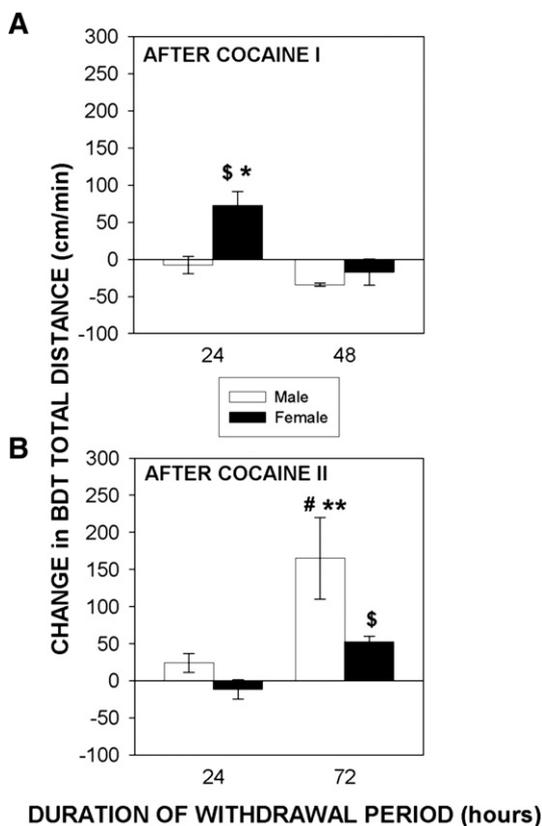

Fig. 6. Correlation between the daily basal activity level and post-treatment reduction in locomotion. (A–D): In control males and females, change in total distance traveled (y-axis) before and after treatment (i.e., between BDT and ADT), is stronger in those with high basal activity (BDT) level. A similar positive correlation is present in males and females of cocaine groups at baseline (E) and after cocaine administration (H). However, negative correlation between the change in activity after vehicle administration and prior basal activity of the day is observed when females of cocaine group are hyperactive (F) following 24-h withdrawal from the first cocaine administration (see Fig. 2D, day 3), and lack of positive correlation is observed when males of cocaine group are hyperactive (G) following 24-h withdrawal from the second cocaine administration (see Fig. 2D, day 5). Data are presented as mean±SEM for the representative groups recorded in parallel (n=5/group) under HES conditions; 95% confidence interval: males — open symbols, female — black symbols.

Withdrawal). Overall, when two genders were analyzed together, there was a significant treatment effect during cocaine withdrawal (Mixed model: $F_{1,17}=5.985$, $P=0.026$).
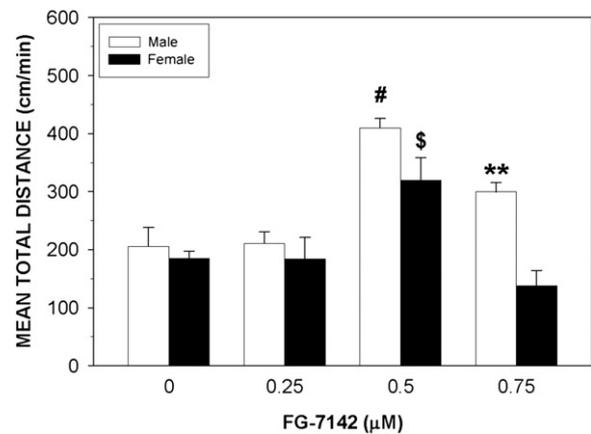
Brain DA levels tended to be higher in females at baseline and tended to decline after acute cocaine administration, though neither comparison reached significance (Mixed model, $F_{1,20}=2.770$, $P=0.112$ relative to males and $F_{1,16}=2.281$, $P=0.150$ for control vs. acute treatment respectively). During withdrawal, male zebrafish showed significantly higher brain DA levels than the control or males acutely treated with cocaine (Mixed model, $F_{1,13}=4.431$, $P=0.05$; $F_{1,9}=5.181$, $P=0.049$, respectively; Fig. 9B). In females, brain DA levels were also increased during withdrawal, being significantly different from those after acute cocaine administration (Mixed model, $F_{1,8}=10.451$, $P=0.012$), though did not reach significance relative to baseline (Mixed model, $F_{1,13}=2.921$, $P=0.111$). No significant Gender or Gender by Treatment interaction effect was documented for any of the experimental conditions, allowing us to combine the male and female data for further analysis. Such analysis showed that withdrawal from repeated cocaine administration resulted in significant increase in brain DA levels, compared to Control or acute cocaine exposure (Mixed model, $F_{1,19}=5.288$, $P=0.033$ and $F_{1,19}=6.662$, $P=0.018$, respectively) in adult zebrafish.

4. Discussion

The results of this study demonstrate that neither gender display acute behavioral responses to initial cocaine exposure in zebrafish.


Fig. 8. Similar dose-dependence of hyperactivity induced by an anxiogenic agent, FG-7142, in male and female zebrafish. The dose-dependence of the behavioral effect of FG-7142, augmenting the locomotor activity, is similar in male and female zebrafish, though its bi-modal pattern is more pronounced in females. # $P<0.05$ relative to vehicle and 0.25 μM dose; \$ $P<0.05$ relative to vehicle, 0.25 μM and 0.75 μM dose; ** $P<0.01$ relative to female. Data are presented as mean±SEM for the groups recorded in parallel ($n=5$/group).

Fig. 7. Under conditions of low environmental stimulation (LES), the dynamic behavioral response to withdrawal from repeated cocaine treatment differs in male and female zebrafish. Data are presented as difference in daily basal activity (BDT) between the day of the first (A) or second (B) cocaine treatment and the following 24, 48 or 72-h withdrawal period under LES conditions. Male — open bars and female — black bars. # $P<0.01$ relative to 24-h withdrawal, \$: $P<0.01$ relative to 48-h (A) and 24-h (B) withdrawal, * $P<0.05$ and ** $P<0.01$ relative to the corresponding data in another gender. Data are presented as mean±SEM total distance traveled for the representative groups recorded in parallel ($n=5$/group).

However, both males and females show behavioral, anxiety-like responses to cocaine withdrawal and these effects are gender-specific. A hyperactivity state during cocaine withdrawal develops more rapidly in female zebrafish, while being mild and transient. This is in contrast to delayed, intense and escalating hyperactivity, associated with stereotypy, induced by intermittent cocaine administration and its withdrawal in male zebrafish. Moreover, while high environmental stimulation aggravates the effects of cocaine withdrawal in male zebrafish, consistent with the reports in other species [for review, 50], it does not change the principal patterns of behavioral responses in females. In spite of these behavioral differences, the responses to cocaine or its withdrawal on the molecular level are largely similar between the genders. Once cocaine is administered repeatedly and then withdrawn for several days, brain DA levels increase and zDAT expression declines. This dynamic picture of response to cocaine administration and its withdrawal, manifesting at both behavioral and molecular levels, and some principal sex differences suggest that zebrafish offer a new, promising model to address a link between genetic, behavioral and neurochemical mechanisms of cocaine withdrawal.

Studies in humans, non-human primates and rodents convincingly show that anxiety is one of the principal symptoms of cocaine withdrawal [51–62]. The objective methods can depict cognitive, somatic, emotional and behavioral components of anxiety in experimental animals [for review, 63], even though some require cautious interpretation. In zebrafish, anxiety-related behaviors were described in males and, perhaps, due to different experimental approaches or the zebrafish strains used, reported both similar and distinct behavioral manifestations. Those included hyperactivity or, in some cases, freezing, erratic movements, and swimming close to the bottom or wall of the fish tank [38,41,45,64].

Our initial characterization of the effects of cocaine withdrawal in male zebrafish of the AB strain established that following repeated cocaine treatment and withdrawal they demonstrate hyperlocomotion, stereotypy and close-to-bottom movement during daily basal activity recordings, all consistent with an anxiety-like state [41]. Once the hyperactivity is developed during withdrawal, cocaine administration can acutely attenuate such effect, bringing male zebrafish behavior to close to normal levels, i.e., provide a partial rescue from withdrawal-induced behavior. Furthermore, such behavior in zebrafish is blocked by a non-sedative dose of diazepam [41].
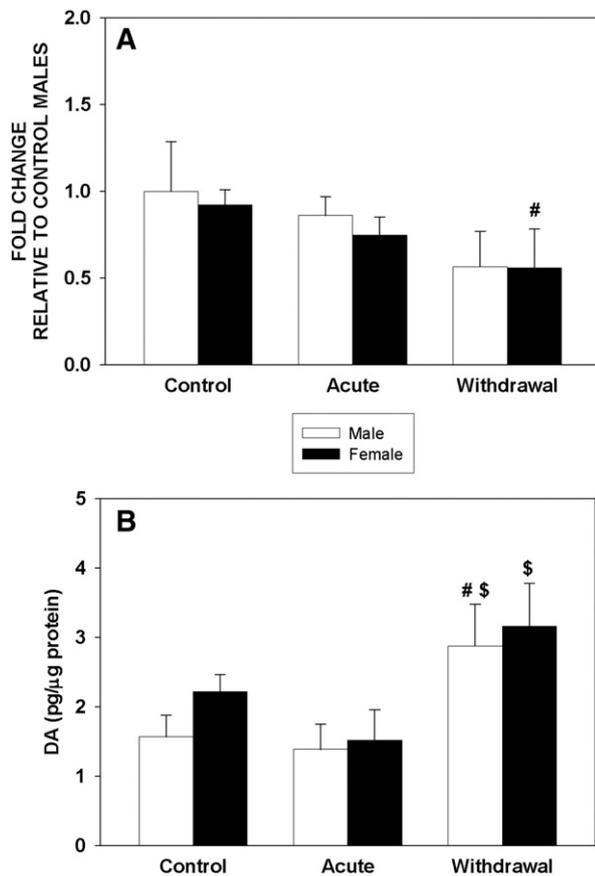
Fig. 9. Effects of acute cocaine administration or withdrawal from repeated cocaine treatment on brain *zDAT* mRNA expression and DA content in male and female zebrafish. (A) Brain *zDAT* expression show no significant gender difference at baseline, after a 30-min (acute) cocaine administration or following 72-h withdrawal from repeated cocaine administration. Females show significant reduction (# $P < 0.05$ relative to control females) and males show strong tendency toward reduction in *zDAT* after cocaine withdrawal, with significant treatment effect for two genders combined (see text). (B) Acute 30-min cocaine administration does not significantly modify brain DA levels for each gender and for both genders combined. At 72-h withdrawal after repeated cocaine administration, males show significant increase (# $P < 0.05$) and females a strong tendency toward increase in DA, relative to the control groups. Treatment effect is significant when two genders are combined (see text). The effect of withdrawal is significant for each gender ($ $P < 0.05$) and two genders combined (see text), relative to the acute treatment. Data are presented as mean±SEM for the groups tested in parallel ($n = 10$ per treatment group).

Similar to males, female zebrafish do not display acute changes in locomotor activity after initial cocaine administration, and demonstrate the effects of cocaine withdrawal. However, unlike males that typically develop the behavioral effects only following repeated cocaine administration, females become hyperactive within the first 24 h of cocaine abstinence and then tend to return to normal activity levels. We do not have a ready explanation for why the onset of hyperactivity happens earlier in females but suggest that it is important to understand the mechanisms involved in these initial events after cocaine is administered to naive individuals. We thus use several approaches to address the issue, some of which are described in this paper.

One of the ideas tested is that the behavioral expression of anxiety-like behavior might differ between genders, i.e., anxious female zebrafish might have low locomotor activity. However, such suggestion is contradicted by similar effects of FG-7142 in males and females, inducing comparable hyperactivity in both genders. That said, stereotypy after FG-7142 treatment is present only in males and a bi-modal pattern of FG-7142 dose-dependence is sharper in females.

Thus, some gender differences in the expression of anxiety in zebrafish are likely to exist and would benefit from further analysis.

We then hypothesized that the behavioral differences observed in male and female zebrafish during cocaine withdrawal could be related to sexual dimorphism in the dopaminergic system or its responses to cocaine. The catecholaminergic systems are well-developed in zebrafish [65–69] and an important role of DA, its receptors and its reuptake via DAT in the effects of cocaine and other drugs of abuse is well established [70]. Thus, gender differences in basal or drug-induced intracellular or extracellular DA levels, or in the expression of DAT, might underlie the gender variations in behavioral responses to drugs of abuse, including cocaine [71–74].

We do not observe significant gender differences in basal DA or *zDAT* mRNA levels or after acute cocaine administration in zebrafish. While we find brain DA levels to be increased and *zDAT* mRNA expression to be reduced following repeated cocaine administration and its withdrawal in this species, both responses are largely similar between the genders and thus do not explain the behavioral differences observed. It is likely that the reduction in *zDAT* mRNA that should result in the decline in DAT protein levels, contributes to the increase in brain DA. Decreased *zDAT* expression would reduce cytosolic DA concentrations and result in diminished feedback inhibition of DA synthesis and tyrosine hydroxylase activity. This would lead to an overall increase in DA [75], analogous to that observed after dopamine transporter inhibition with GBR12909 [76]. Consistent with this, are low basal DAT protein levels found in the reward-related brain structures of rodents following repeated cocaine treatment and its withdrawal [70]. Furthermore, cocaine administered after its withdrawal can have a bi-modal effect on *DAT* expression in rodents, first augmenting and then, within hours, inhibiting it [77]. The effects of cocaine challenge, i.e., acute cocaine administration following withdrawal, remain to be studied in zebrafish.

Thus, based on the data obtained so far, gender differences in behavioral responses to cocaine withdrawal in zebrafish are not due to different basal, acute cocaine-induced or post-withdrawal brain DA or *zDAT* expression. Further studies differentiating between the intracellular and extracellular DA levels in zebrafish, and those assessing DA and *zDAT* presence in specific brain structures or the effects of acute dopamine transporter inhibition, are needed to search for the mechanisms of this phenomenon. It is also plausible that the compensatory, rather than primary dopaminergic responses to cocaine differ between the genders, and those should also be addressed.

In this study, one dose of cocaine was used to explore the gender differences, based on the earlier dose-dependence study in male zebrafish [41]. We find no significant gender differences in brain cocaine levels at the time of its initial surge or peak, with brain cocaine metabolite levels being too low to provide useful data at those time points. Earlier studies in rodents suggested that while brain or plasma cocaine levels might not differ between the genders, sex-specific patterns of metabolite distribution can be detected, though may not explain the gender differences in the behavioral effects of cocaine [78]. Thus, further in-depth dose-dependence studies that would also address the timing and degree of involvement of specific brain structures, and assess metabolite levels in central and peripheral tissues are warranted.

In summary, this first report establishing gender differences in zebrafish responses to cocaine withdrawal opens new opportunities to study the role of genetic and endocrine gender-related factors in the development of cocaine withdrawal symptoms. The high-throughput capabilities of this vertebrate model, extensive knowledge of its genetics and molecular biology, as well as principal similarities between the catecholaminergic system in zebrafish and in mammals [see review, 79], is likely to provide valuable contribution to further understanding of the phenomenon of drug withdrawal syndrome and methods to oppose it.

Acknowledgements

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