ZEBRAFISH
Volume 14, Number 1, 2017

DOI: 10.1089/zeb.2016.1295

Neurochemical and Behavioral Responses to Unpredictable Chronic Mild Stress Following Developmental Isolation: The Zebrafish as a Model for Major Depression

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Abstract

Unpredictable chronic mild stress (UCMS) and developmental social isolation are often utilized in laboratory animals to mimic unpredictable life stressors and early life adversity that may contribute to the development of major depressive disorder in humans. Zebrafish (*Danio rerio)* have been used to examine the effects of both developmental social isolation and UCMS. However, anxiety-like behavioral responses, social behavior, and neurochemical changes induced by stressors have not been well characterized. Furthermore, the possible interaction between UCMS and developmental isolation remains unexplored. In this study, we analyzed the effect of UCMS on developmentally isolated and socially reared zebrafish. The UCMS procedure entailed delivering unpredictably varying mild stressors twice a day for 15 consecutive days. To quantify social and anxiety-like behaviors, we measured the zebrafish's behavioral and neurochemical (dopaminergic and serotonergic) responses to an animated image of conspecifics in a novel tank. Our results suggest that UCMS increased anxiety-like behavioral responses, whereas developmental isolation altered motor responses during stimulus presentation. We also found that UCMS diminished weight gain and reduced whole-brain levels of dopamine and serotonin's metabolite 5-HIAA in developmentally isolated, but not socially reared zebrafish. Our findings reinforce the utility of combining developmental isolation with UCMS in zebrafish to model depressive-like behavior in humans.

Keywords: anxiety, depression, fear, unpredictable mild chronic stress

Introduction

Psychiatric disorders continue to be of major interest to academic researchers and the pharmaceutical industry. The most prevalent psychiatric disorder today is depression—a debilitating condition affecting >21% of the world's population.[1,2] Chronic stress can trigger the onset and recurrence of major depression disorder.[3–7] Although depression may develop without notable chronic life stressors as well, exposure to life stress is one of the most consistently reported precipitating factors in the development of a depressive episode.[8] Research suggests that both unpredictability and variability of stressors are primary precursors for the development of depressive-like behaviors.[9,10] In contrast with the effect of chronic stressors in adulthood, early life adversities—such as lack of attention from a caregiver, abuse, and neglect—are well-established risk factors in the development of depression in humans.[11,12] Animal models of depression lack certain observable characteristics that patients suffering from depression typically display, such as low self-esteem and suicidal tendencies.[13] However, human studies of depression are limited by ethical considerations as well as by reduced ability to control environmental and genetic factors. Over the past several decades, animal models of depression using unpredictable chronic mild stress (UCMS) and developmental social isolation paradigms have been validated due to the development of behavioral assays that quantify stress- and anxiety-related behavioral responses.[14–16] Furthermore, UCMS and developmental social isolation in animals has been shown to alter neurotransmitter systems, including dopaminergic and serotonergic systems, which have been implicated in the etiology of human depression.[8,17]

Current evidence suggests that animal models of UCMS can properly mimic unpredictable life stressors that may lead to depression in humans.[18,19] In rodents, the procedure typically involves prolonged and repeated exposure to an array of unpredictable microstressors, for example, over a range of 10 days to 8 weeks (see Hill *et al.* for a review).[8] UCMS in rodents has been shown to increase anxiety- and depressive-like behavioral responses,[20] as well as alter dopamine (DA)

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and serotonin (5-HT) levels in specific brain areas.[21] In contrast to UCMS, developmental social isolation is thought to represent significant early life adversity.[22,23] In rodents, developmental isolation or isolated rearing may involve individually isolating pups for a period between postnatal day 0–28 (see Fone and Porkess for a review).[17] In rodents, isolated rearing postweaning, from postnatal day 21–28, has been shown to increase anxiety-like behavioral responses[24] as well as to alter the levels of dopamine, serotonin, and their metabolites in different brain regions—specifically, the prefrontal cortex, nucleus accumbens, hippocampus, and midbrain—suggesting these neurotransmitter systems play a role in regulating early social experiences.[25,26] For example, isolation was found to significantly increase levels of dopamine in the nucleus accumbens and midbrain, DOPAC in the midbrain, and 5-HIAA in the hippocampus, whereas serotonin levels increased in all brain areas, except the raphe nuclei, but these changes were age and stress level dependent.[26] Further analysis showed that in the amygdala and midbrain, isolation significantly enhanced monoamine biosynthesis, with monoamine turnover remaining unchanged.[25] Studies have also examined developmental isolation before weaning, which typically involves separation of the pup from the dam after birth, and requires an artificial rearing setup leading to lack of maternal care.[27,28] The behavioral effects associated with UCMS and social isolation in animals correspond well with depression and anxiety-like behaviors in humans.[18,17,23] A number of studies have also started to examine the effect of UCMS on developmental isolation. For example, long-term social isolation has been shown to potentiate the anxiety- and stress-related effects of UCMS in mice and rats.[22,23] Although rodent models of UCMS and social isolation have been successfully utilized in modeling depressive-like behaviors, they require significant infrastructure and labor-intensive procedures.

Behavioral responses are one of the most complex products of the central nervous system. The zebrafish has become a powerful organism in behavioral brain research due to their easily quantifiable behavioral responses.[29] Zebrafish offer translational relevance due to their comparable homology with mammals, including humans, at the genetic and neural levels.[29] In addition, zebrafish are advantageous for biomedical research due to their fully sequenced genome, amenability for high-throughput assays, moderately easy husbandry, and short generation times.[29,30]

Similarly to humans and rodents, zebrafish are innately social and display a quantifiable social behavior called shoaling, which is the aggregation of individuals to form a tight group.[29,31,32] UCMS paradigms using zebrafish have recently been established with effects showing impaired shoaling behavior,[33] altered adenosine metabolism,[34] impaired avoidance learning,[35] and increased anxiety- and mood disorder-related phenotypes.[36] In contrast to the effects of UCMS, the effect of developmental isolation in zebrafish has not been extensively characterized. Zebrafish may be uniquely well suited to study developmental isolation since embryos can be immediately isolated, and development occurs externally without the need for parental care. In zebrafish, developmental isolation has been shown to impair locomotor activity in larvae[37] and decrease cell proliferation in sensory areas of the brain in adults.[38] Although some studies have been conducted on UCMS and developmental isolation in zebrafish, changes in social behavior, anxiety-like responses, and neurochemical responses have not been well characterized. Furthermore, the possible interaction between UCMS and developmental isolation remains unexplored.

In the current study, we examined the effects of developmental social isolation and UCMS on anxiety-like behavior and social responses as well as dopaminergic and serotonergic responses in zebrafish. We compared isolated and socially reared zebrafish that were exposed to UCMS in adulthood with isolated and socially reared unstressed controls. To examine social as well as anxiety- and stress-like behaviors, we quantified behavioral changes in response to being exposed to handling and a novel test tank as well as to animated stimulus presentation of conspecifics in the novel tank. To examine changes in dopaminergic and serotonergic responses, we quantified whole-brain tissue levels of dopamine, its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin, and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) using high-precision liquid chromatography (HPLC).

Materials and Methods

Animals and housing

Fifty-three adult zebrafish (5 months old, mixed sexes) of the AB strain were used for this study. Zebrafish were bred at the University of Toronto Mississauga vivarium and housed on a single recirculating filtration aquaculture system rack equipped with mechanical and biological as well as activated carbon filtration and a UV sterilizing unit (Aquaneering, Inc.). Water quality was monitored daily and the water was maintained at an ideal temperature ($\sim$27°C), pH (6.0–8.0), and conductivity ($\sim$300 μS). Zebrafish were kept on a 12-h light–12-h dark cycle with lights turning on at 9:00 h. From 5 to 30 days postfertilization (dpf), larvae were fed twice daily with Larval AP100, Larval Food Supplement (microparticle size, <100 μm). Following 30 dpf, zebrafish were fed nauplii of *Artemia salina* (brine shrimp) and a mixture of Tetramin and spirulina flakes.

Following fertilization, individual embryos in the developmental isolation condition were placed in 1.8-L Plexiglas tanks with no visual, tactile, or olfactory access to conspecifics. The same procedure was followed for subjects in the social rearing condition, with the exception that they were placed in 2.8-L Plexiglas tanks with five embryos per tank. Gray polycarbonate dividers were placed between all tanks to prevent visual access to neighboring fish tanks. Water flow on the system rack was turned off until 8 dpf to control for possible olfactory cues that may be associated with kin recognition.[39,40] To ensure proper quality, manual water changes were performed daily using water from an isolated reservoir without fish until 8 dpf, after which water flow and central filtration was turned on. Zebrafish were raised to adulthood and were exposed to UCMS at $\sim$5 months of age.

Unpredictable chronic mild stressors

Stressors were randomized to ensure unpredictability. A total of six stressors were employed and subjects were exposed daily to two stressors at day-specific predetermined hours for 15 consecutive days (Table 1). The stressors were selected based on previous acute stress and UCMS paradigms

Table 1. The UCMS Schedule Included Total of Six Mild Stressors That Were Delivered to Fish Assigned to the Stressed Groups (Social or Isolated)

	UCMS schedule					
Day	*Dorsal body exposure*	*Tank change*	*Chase with net*	*Elevate with net*	*Water change*	*Restrain in tube*
Monday					13:30	14:45
*Tuesday	14:45			16:45		
*Wednesday					13:15	11:00
*Thursday	13:15	15:00				
*Friday			16:15	18:15		
*Saturday		12:45	17:00			
Sunday	19:00			17:30		
Duration or frequency of stressor	2 min	6 times	2 min	2 min	3 times	60 min

The stressors were delivered at the time of day indicated. Note that the different starting times of stress delivery ensured unpredictability. Subjects were randomly assigned to one of five cohorts (each cohort contained approximately equal number of fish from the isolated and social fish). The starting day for the stress procedure for a given cohort is indicated with an *asterisk*. Fish received two stressors each day for a consecutive 15-day period. The order of stressors can be deciphered from the table. For example, fish of cohort 1 received the first stressor (dorsal body exposure) on a Tuesday, which was followed by elevation by the net stressor and the next day (Wednesday) by the water change and restrain tube stress. The weekly stress cycle for this group completed with the restrain tube stress delivered on Monday, which was followed by the next weekly cycle starting with the Tuesday stressors.

UCMS, unpredictable chronic mild stress.

as follows: (1) water levels in housing tanks were lowered resulting in exposure of the dorsal part of the fish's body for 2 min[33,36]; (2) tanks were changed as subjects were transferred from one tank to another, six consecutive times[36]; (3) individual subjects were chased with a net for 8 min[33,36]; (4) subjects were elevated from their tanks and exposed to atmospheric air with a net for 2 min[41]; (5) water in housing tanks was replaced (three quarters of tank water), three consecutive times while subjects remained in tanks[33]; and (6) subjects were individually restrained for 60 min in 2-mL Eppendorf tubes with perforations at both ends to allow free water flow.[36] With the exception of the tank change stressor, all stressors took place in each subject's own housing tank. Subjects in the ''Social'' condition were only separated for the duration of the net and restraint stressors and were returned to their groups immediately posttreatment. Each fish of each stress (social or isolated) group, including fish in the social group, received the stress treatment. All fish of the stress treatment groups received the same number and kind of stress treatment overall. Following the last stressor, zebrafish were placed back in their respective home tanks, and behavioral testing commenced on the following day.

Behavioral apparatus and testing

Since zebrafish are diurnal, all test trials took place during the light phase (between 9:00 and 21:00 h). Subjects were individually netted from their housing tanks and placed in a novel 37-L testing tank ($51 \times 27 \times 19$ cm) filled with the same water as their housing tanks (25 cm high). The testing tank was illuminated from above by a 50 cm long Aquarium Spectrum florescent (15 W) lamp. The novelty of the testing tank is expected to be aversive to zebrafish especially for the first 3 min of exposure, allowing for quantification of anxiety-like behavioral responses.[42,43] Upon exposure to the novel testing tank, subjects experienced a 10-min habituation period, followed by an animated stimulus presentation of conspecifics for an additional 5 min, for a total testing time of 15 min. The animated images were displayed on one of two computer monitors flanking the testing tank that were connected to laptop computers (image presentation side was randomized). The stimulus was presented using a custom software application developed in our laboratory.[44] The animated stimulus displayed five independently moving zebrafish, similar in size to that of the experimental fish, which has been shown to induce a robust reduction of distance from the stimulus.[45,46] The reduction of distance from the stimulus has been found to be comparable when animated images of zebrafish are shown or when live conspecifics are presented,[44] and it has been used as a measure of the strength of shoaling.[45,47] The behavioral setup is shown in Figure 1.

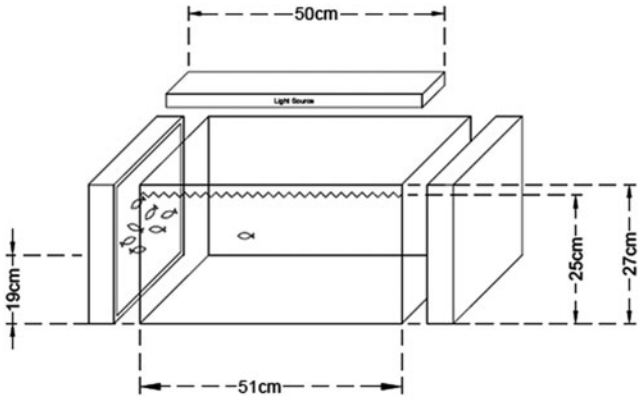

FIG. 1. The behavioral test apparatus was a 37-L tank flanked by two computer screens on each side. Experimental fish were placed in this novel environment singly and their responses were recorded using a video camera placed in front of the tank as described in the Materials and Methods section.

Quantification of behavior

A video camera (Sony HDR-CX430V) was placed in front of the testing tank to record the behavior of the experimental fish. The digital recordings were transferred to an external hard drive and analyzed using the automated video-tracking software EthoVision XT 8.5 (Noldus). We quantified absolute turn angle, total distance traveled, freezing duration (movement slower than 0.5 cm/s), and distance to bottom, which have all previously been used to interpret behavioral responses or states related to anxiety in zebrafish.[33,48–51] To examine social behavior, we quantified the mean distance zebrafish swam from the screen showing the animated conspecific images, the stimulus.[44–47] In addition, we also examined the variance of distance to stimulus screen, which provides a measure of within- or intraindividual temporal variability in an experimental subject's distance to the stimulus, a measure that quantifies how consistently close the experimental fish stayed to the stimulus.

Quantification of neurochemicals

Five minutes after the conclusion of behavioral recording, experimental subjects were removed from their test tank and were decapitated to quantify whole-brain neurochemicals ($n = 10$–11 per group). Zebrafish were weighed and whole brains were dissected on dry ice and stored at –40°C until processing. Brain samples were thawed and sonicated in 20 μL of artificial cerebrospinal fluid containing 25 μM ascorbic acid. One microliter of the sonicate was assayed to determine protein concentration using the Bio-Rad Protein Assay Reagent (Bio-Rad) as suggested by the manufacturer's instructions. Samples were centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was extracted. One microliter of 0.5 N perchloric acid was added to the sample and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was extracted and stored at –40°C until HPLC analysis. The levels of DA, DOPAC, serotonin, and 5-HIAA were quantified from the supernatant through HPLC using a modified protocol (see Chatterjee and Gerlai).[52] Ten microliters of the supernatant was injected into the HPLC system and analyzed using a BAS 461 MICROBORE-HPLC system with electrochemical detection (Bioanalytical Systems, Inc.). The stationary phase consisted of a UnigetC18 reversed phase microbore column (Cat No. 8949; BASi). The mobile phase consisted of a buffer (0.1 M monochloroacetic acid, 0.5 mM Na-EDTA, 0.15 g/L sodium octyl sulfate, and 10 nM sodium chloride, pH 3.4), acetonitrile, and tetrahydrofuran at a ratio of 97:2.3:0.7. Known concentrations of dopamine hydrochloride, DOPAC, serotonin hydrochloride, and 5-HIAA (Sigma-Aldrich) were used as standards to identify and quantify peaks on the chromatograph. The levels of each neurochemical was standardized and expressed as ng/mg protein.

Statistical analysis

The experimental design of this study was a 2×2 factorial design with isolation (two levels: isolation vs. social) and UCMS (two levels: stress vs. control) as the between-subject factors. A total of 53 zebrafish were used for behavioral testing ($n = 11$–14 fish per group). Three separate two-way analysis of variance (ANOVA) with UCMS and isolation as the between-subject factors were conducted to examine behavioral responses during—first time interval (first 3 min, reflecting handling and novelty-induced anxiety), the second time interval (minutes 6–10, during which the stimulus was off, reflecting a more habituated state), and the third time interval (minutes 11–15, during which the animated conspecific images, the stimulus, was on). In case of a significant stress × isolation interaction, Tukey's *post hoc* honest significant difference (HSD) tests were employed to compare all four experimental groups, with significance accepted when $p \leq 0.05$. For neurochemical analysis, outliers were removed by box plot analysis according to Williamson and Kendrick,[53] which reduced the final sample size to 8–11 fish per group and ANOVA and Tukey's HSD tests were conducted as described above.

Results

Effect of developmental isolation and UCMS on behavioral responses

Table 2 summarizes the detailed results of statistical analyses of the behavioral variables. Figures 2 and 3 show behavioral responses at different time periods of the recording session. To examine anxiety-like behavior, we analyzed behavioral responses in the first 3 min of exposure to the novel environment as described in previous studies.[42,43] Note that during this period no stimulus is presented. ANOVA revealed a significant main effect of UCMS for absolute turn angle [$F(1, 49) = 0.016$, $p = 0.016$; Fig. 2A], distance to bottom [$F(1, 49) = 4.451$, $p = 0.040$; Fig. 2D], freezing [$F(1, 49) = 4.171$, $p = 0.047$; Fig. 2G], total distance traveled [$F(1, 49) = 12.811$, $p = 0.001$; Fig. 3A], and variance of distance to stimulus screen [$F(1, 49) = 10.031$, $p = 0.003$, Fig. 3G], but not for distance to stimulus screen (Fig. 3D, $p > 0.05$). ANOVA also found a significant isolation × UCMS interaction for variance of distance to stimulus screen [$F(1, 49) = 7.053$, $p = 0.011$; Fig. 3G]. Tukey's HSD test confirmed that isolated control fish exhibited a significantly lower variance of distance to stimulus screen compared with all other groups ($p \leq 0.036$; Fig. 3G). However, there was no main effect of isolation and the isolation × UCMS interaction was also nonsignificant for any of these behavioral measures ($p > 0.05$).

During minutes 6–10 (stimulus off), a period of time sufficient to allow intrasession habituation to occur,[42,43] there was a significant main effect of UCMS on total distance traveled [$F(1, 49) = 7.749$, $p = 0.008$; Fig. 3B]. However, there were no significant main effects of isolation, UCMS, or isolation × UCMS interaction for any other behavioral measures ($p > 0.05$); see Table 2.

In the following 5 min of the behavioral test (minutes 11–15), animated images of conspecifics were presented. For this period, there was a significant main effect of UCMS on absolute turn angle [$F(1, 49) = 6.295$, $p = 0.015$; Fig. 2C], as well as a main effect of isolation on total distance traveled [$F(1, 49) = 6.283$, $p = 0.016$, Fig. 3C]. In addition, there was a significant isolation × UCMS interaction found for absolute turn angle [$F(1, 49) = 6.295$, $p = 0.015$, Fig. 2C]. Tukey's HSD test confirmed that isolated control fish exhibited a significantly lower absolute turn angle compared with isolated stressed fish ($p = 0.015$; Fig. 2C).

TABLE 2. RESULTS OF THREE SEPARATE 2-WAY ANALYSIS OF VARIANCE (MINUTES 1–3; MINUTES 6–10; MINUTES 11–15) ARE SHOWN FOR DIFFERENT BEHAVIORAL MEASURES

	Minutes 1–3	
Absolute turn angle	Isolation	$F(1, 49) = 0.206$, $p = 0.652$
	UCMS	$\mathbf{F(1, 49) = 0.016}$, $\mathbf{p = 0.016}$
	Isolation × UCMS	$F(1, 49) = 0.314$, $p = 0.578$
Distance to bottom	Isolation	$F(1, 49) = 0.335$, $p = 0.565$
	UCMS	$\mathbf{F(1, 49) = 4.451}$, $\mathbf{p = 0.040}$
	Isolation × UCMS	$F(1, 49) = 0.903$, $p = 0.347$
Freezing	Isolation	$F(1, 49) = 0.042$, $p = 0.838$
	UCMS	$\mathbf{F(1, 49) = 4.171}$, $\mathbf{p = 0.047}$
	Isolation × UCMS	$F(1, 49) = 4.171$, $p = 0.966$
Total distance traveled	Isolation	$F(1, 49) = 0.620$, $p = 0.435$
	UCMS	$\mathbf{F(1, 49) = 12.811}$, $\mathbf{p = 0.001}$
	Isolation × UCMS	$F(1, 49) = 0.432$, $p = 0.514$
Distance to stimulus	Isolation	$F(1, 49) = 0.057$, $p = 0.813$
	UCMS	$F(1, 49) = 0.296$, $p = 0.589$
	Isolation × UCMS	$F(1, 49) = 0.048$, $p = 0.827$
Variance of distance to stimulus	Isolation	$F(1, 49) = 1.352$, $p = 0.251$
	UCMS	$\mathbf{F(1, 49) = 10.031}$, $\mathbf{p = 0.003}$
	Isolation × UCMS	$\mathbf{F(1, 49) = 7.053}$, $\mathbf{p = 0.011}$
	Minutes 6–10 (stimulus off)	
Absolute turn angle	Isolation	$F(1, 49) = 0.013$, $p = 0.909$
	UCMS	$F(1, 49) = 2.177$, $p = 0.146$
	Isolation × UCMS	$F(1, 49) = 0.223$, $p = 0.639$
Distance to bottom	Isolation	$F(1, 49) = 1.652$, $p = 0.205$
	UCMS	$F(1, 49) = 0.110$, $p = 0.741$
	Isolation × UCMS	$F(1, 49) = 1.214$, $p = 0.276$
Freezing	Isolation	$F(1, 49) = 0.198$, $p = 0.659$
	UCMS	$F(1, 49) = 2.706$, $p = 0.106$
	Isolation × UCMS	$F(1, 49) = 0.198$, $p = 0.659$
Total distance traveled	Isolation	$F(1, 49) = 1.300$, $p = 0.260$
	UCMS	$\mathbf{F(1, 49) = 7.749}$, $\mathbf{p = 0.008}$
	Isolation × UCMS	$F(1, 49) = 1.839$, $p = 0.181$
Distance to stimulus	Isolation	$F(1, 49) = 0.003$, $p = 0.960$
	UCMS	$F(1, 49) = 0.112$, $p = 0.740$
	Isolation × UCMS	$F(1, 49) = 1.913$, $p = 0.173$
Variance of distance to stimulus	Isolation	$F(1, 49) = 6.872$, $p = 0.012$
	UCMS	$F(1, 49) = 1.172$, $p = 0.284$
	Isolation × UCMS	$F(1, 49) = 0.261$, $p = 0.612$
	Minutes 11–15 (stimulus on)	
Absolute turn angle	Isolation	$F(1, 49) = 0.007$, $p = 0.932$
	UCMS	$\mathbf{F(1, 49) = 6.295}$, $\mathbf{p = 0.015}$
	Isolation × UCMS	$\mathbf{F(1, 49) = 4.292}$, $\mathbf{p = 0.044}$
Distance to bottom	Isolation	$F(1, 49) = 0.035$, $p = 0.853$
	UCMS	$F(1, 49) = 0.206$, $p = 0.652$
	Isolation × UCMS	$F(1, 49) = 0.947$, $p = 0.335$
Freezing	Isolation	$F(1, 49) = 1.114$, $p = 0.296$
	UCMS	$F(1, 49) = 1.114$, $p = 0.296$
	Isolation × UCMS	$F(1, 49) = 1.114$, $p = 0.296$
Total distance traveled	Isolation	$\mathbf{F(1, 49) = 6.283}$, $\mathbf{p = 0.016}$
	UCMS	$F(1, 49) = 0.827$, $p = 0.368$
	Isolation × UCMS	$F(1, 49) = 0.948$, $p = 0.335$
Distance to stimulus	Isolation	$F(1, 49) = 0.335$, $p = 0.565$
	UCMS	$F(1, 49) = 0.836$, $p = 0.365$
	Isolation × UCMS	$F(1, 49) = 0.388$, $p = 0.536$
Variance of distance to stimulus	Isolation	$F(1, 49) = 0.693$, $p = 0.409$
	UCMS	$F(1, 49) = 0.164$, $p = 0.687$
	Isolation × UCMS	$F(1, 49) = 0.223$, $p = 0.639$

Statistical details are shown for the two main effects (UCMS and isolation), as well as the UCMS × isolation interaction. Significant results are *bold* ($p \leq 0.05$).

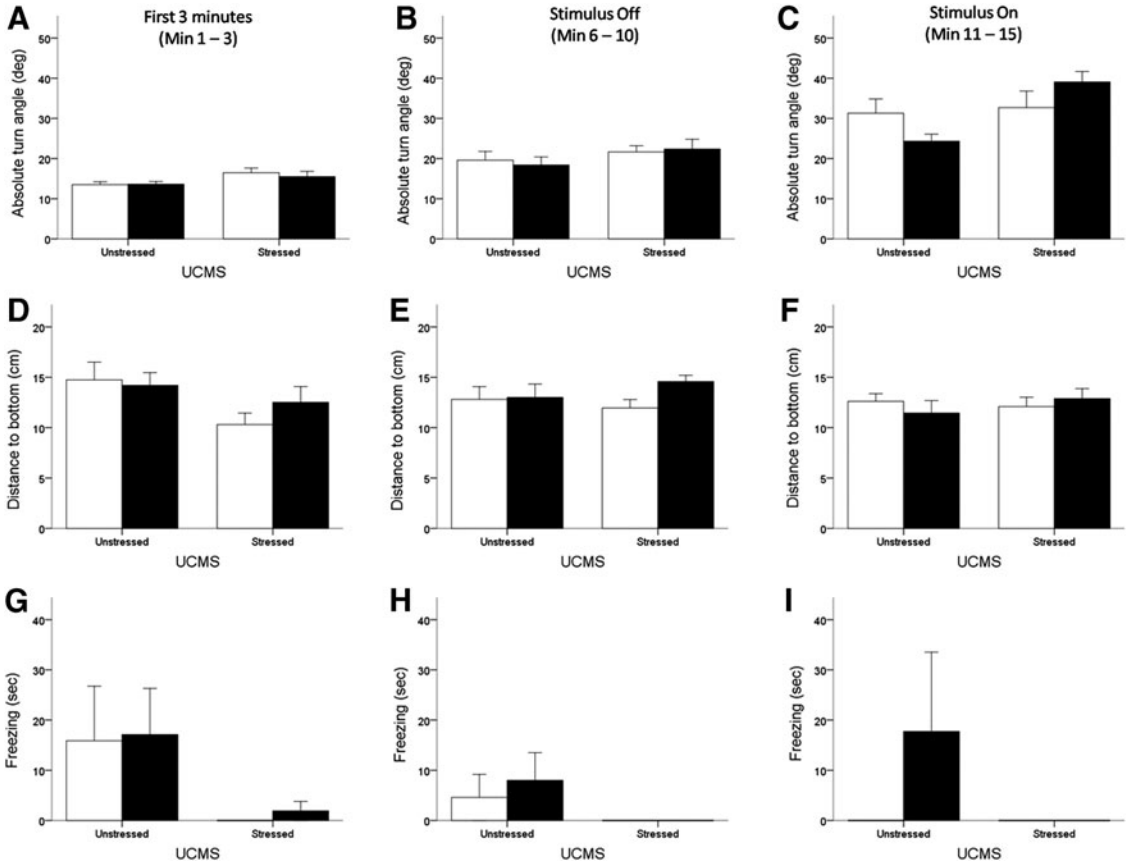

FIG. 2. Mean ± SEM absolute turn angle (panels **A–C**), distance to bottom (panels **D–F**), and freezing (panels **G–I**) are shown for control and stressed zebrafish in both social (*white bars*) and isolated (*black bars*) rearing conditions ($n = 11$–14 per group) during minutes 1–3 (first 3 min, panels **A, D, G**), minutes 6–10 (stimulus off, panels **B, E, H**), and minutes 11–15 (stimulus on, panels **C, F, I**).

Effect of developmental isolation and UCMS on body weight

Figure 4 shows the effect of isolation and UCMS on bodyweight. Two-way ANOVA found a significant main effect of isolation [$F(1, 39) = 29.385$, $p < 0.001$] and UCMS [$F(1, 39) = 4.896$, $p = 0.033$]. There was also a significant isolation × UCMS interaction [$F(1, 39) = 7.668$, $p = 0.009$]. Tukey's HSD test confirmed that isolated control fish weighed significantly more compared with all other groups ($p \leq 0.007$).

Effect of developmental isolation and UCMS on neurochemical levels

Analysis of whole-brain dopamine levels (Fig. 5A) revealed a significant effect of isolation [$F(1, 33) = 5.750$, $p = 0.022$], but no significant effect of UCMS was found [$F(1, 33) = 0.052$, $p = 0.821$], and the isolation × UCMS interaction also did not reach significance [$F(1, 33) = 3.818$, $p = 0.059$]. However, since ANOVA is known to be underpowered to detect significant interaction between main effects,[54] we conducted Tukey's HSD test. This test did show that socially reared stressed fish exhibited significantly higher whole-brain dopamine levels compared with isolated stressed fish ($p = 0.028$; Fig. 5A). Analysis of whole-brain dopamine levels (Fig. 5B) revealed no significant main effect of isolation [$F(1, 39) = 2.225$, $p = 0.144$] or UCMS [$F(1, 39) = 0.002$, $p = 0.962$], and the isolation × UCMS interaction was also nonsignificant [$F(1, 39) = 0.000$, $p = 0.997$].

Analysis of whole-brain serotonin levels (Fig. 5C) also revealed no significant main effect of isolation [$F(1, 39) = 0.273$, $p = 0.604$], or UCMS [$F(1, 39) = 0.893$, $p = 0.350$], and the isolation × UCMS was also nonsignificant [$F(1, 33) = 0.074$, $p = 0.787$]. Finally, analysis of whole-brain 5-HIAA levels (Fig. 5D) revealed a significant effect of isolation [$F(1, 33) = 5.573$, $p = 0.024$], but no significant effect of UCMS was found [$F(1, 33) = 2.463$, $p = 0.126$], and the isolation × UCMS interaction also did not reach significance [$F(1, 33) = 2.156$, $p = 0.151$]. However, since ANOVA is underpowered to detect significant interactions, again we conducted Tukey's HSD tests, which revealed that socially reared stressed fish exhibited significantly higher levels of 5-HIAA compared with both isolated stressed fish ($p = 0.048$) and isolated control fish ($p = 0.032$).

Discussion

In the current study, we report for the first time the behavioral and neurochemical effects of UCMS on developmentally isolated and socially reared zebrafish. Using the novel tank test, our results suggest that UCMS increased anxiety-like behavioral responses during the first 3 min of

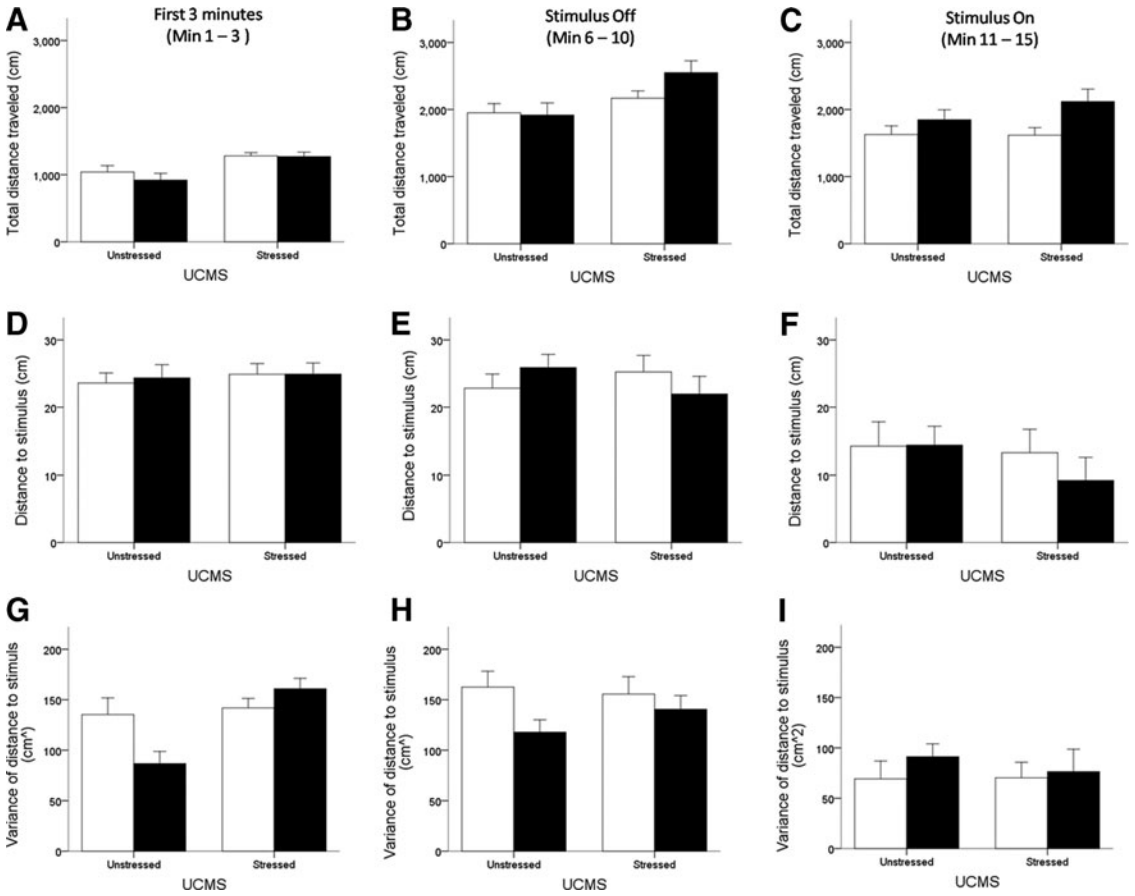

FIG. 3. Mean ± SEM total distance traveled (panels **A–C**), distance to stimulus (panels **D–F**), and variance of distance to stimulus (panels **G–I**) are shown for control and stressed zebrafish in both social (*white bars*) and isolated (*black bars*) rearing conditions ($n = 11–14$ per group) during minutes 1–3 (first 3 min, panels **A, D, G**), minutes 6–10 (stimulus off, panels **B, E, H**), and minutes 11–15 (stimulus on, panels **C, F, I**).

exposure to a novel environment. In contrast to UCMS, developmental isolation did not have a significant effect on behavioral responses during the first 3 min of the recording session, but instead was found to lead to some behavioral alterations observed during the stimulus presentation period.

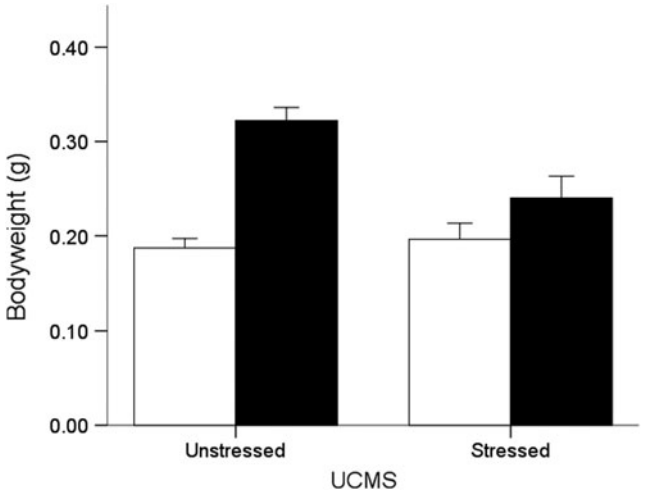

FIG. 4. Mean ± SEM body weight is shown for control and stressed zebrafish in both social (*white bars*) and isolated (*black bars*) rearing conditions ($n = 8–11$ per group).

Interestingly, however, the distance to conspecific images was not affected by developmental isolation suggesting a lack of effect on shoaling behavior. We also report that UCMS decreased whole-brain dopamine and 5-HIAA levels, an effect that was only observed in isolated fish that received unpredictable chronic stress. In addition, we found that developmental isolation increased body weight, an effect that was abolished following 15 days of UCMS.

The novel tank test is one of the most frequently used behavioral tests for anxiety in zebrafish research.[42,43,48,55] Upon being placed in a novel environment, zebrafish exhibit a typical anxiety-like behavioral profile, which includes increased time spent at the bottom of the tank,[48,56] increased duration and frequency of freezing,[48] and erratic movement.[42] In our study, we found that UCMS reduced the average distance zebrafish swam from the bottom of the tank and increased absolute turn angle—indicative of increased anxiety-like behavioral responses, findings that corroborate previous results.[33,36] We also found that UCMS decreased freezing in the first 3 min in a novel environment, which appears to contradict our anxiety-like behavioral profile. However, various species, including zebrafish exhibit different coping styles in response to stressors.[57,58] The reduction of freezing in response to a stressor such as experimenter handling and exposure to a novel environment is often classified as a proactive coping style, which is often associated

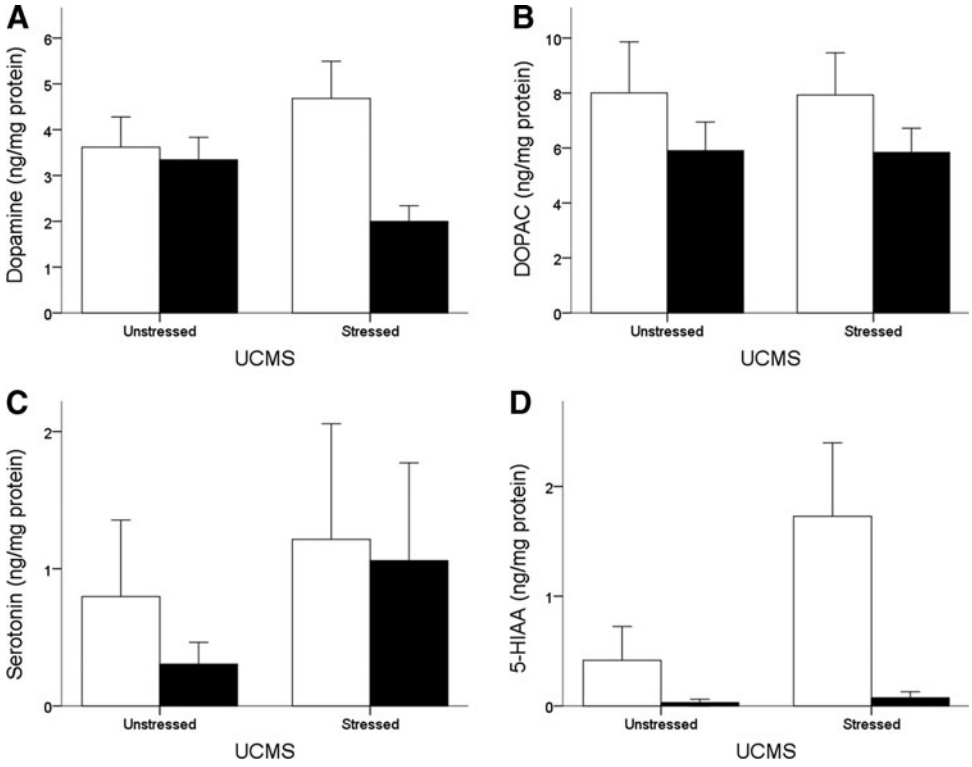

FIG. 5. Mean ± SEM whole-brain levels of dopamine **(A)**, DOPAC **(B)**, serotonin **(C)**, and 5-HIAA **(D)** are shown for control and stressed zebrafish in both social (*white bars*) and isolated (*black bars*) rearing conditions ($n = 8–11$ per group).

with increased locomotor activity and active avoidance responses. In contrast with proactive coping, reactive coping styles are environmentally cue driven, and are associated with increased immobility.[49,50] Our finding that UCMS decreased immobility and increased locomotor activity may suggest a proactive coping style in zebrafish. In support of our hypothesis, zebrafish lines bred for reactive coping styles also exhibit increased freezing behavior when tested in the novel tank diving test compared with zebrafish bred for proactive coping styles.[59] In our study, 15 days of UCMS may have led zebrafish to adapt to a proactive coping style in response to stress, which manifested as an increase in locomotor activity and a reduction in freezing behavior in response to a novel environment.

In contrast to our study, different UCMS protocols in zebrafish have also been shown to elicit a reactive coping style associated with increased freezing behavior and decreased locomotor activity.[33,36] Due to the heterogeneity of depression, humans exhibiting depression-like symptoms often exhibit either hyperactivity or hypoactivity.[60] Our finding that UCMS increases locomotor activity in contrast to others who found decreased locomotor activity is in line with this complex nature of human depression.

Although anxiety-like behavioral changes induced by UCMS in the first 3 min of exposure to the novel environment were not observed in developmentally isolated zebrafish, these animals exhibited a significant reduction in variance of distance to stimulus. Variance of distance to stimulus in the first 3 min can be considered a measure of exploration since a higher value would indicate that zebrafish actively change their distance from one side of the tank, indicative of active exploration. Our finding that developmental isolation reduced variance of distance to stimulus is suggestive of increased anxiety-like behavior. Similarly, perusal of Figure 3B indicates that developmentally isolated zebrafish exposed to UCMS continued to exhibit heighted locomotor activity even after 10 min of habituation, which may be associated with a proactive coping response to stress. Our observation of UCMS-induced anxiety-like behavioral responses in the first 3 min of exposure to the novel tank test, and the lack of significant effects following 10 min of habituation, highlights the importance of this initial period for the quantification of anxiety-like measures in zebrafish, confirming previous studies.[42,43]

In addition to examining the effect of anxiety-like behavioral responses, we also quantified changes in social behavior. Shoaling is the aggregation of individuals to form a tight group, and is one of the most studied behavioral responses in zebrafish.[31,33,44] To examine and quantify shoaling behavior, we used a behavioral paradigm that consists of presenting an animated image of five zebrafish conspecifics and measuring the distance of the experimental fish from the animated stimulus. Perusal of Figure 3E and F shows that in response to the presentation of the stimulus, all groups exhibit a reduction of distance to the stimulus as well as a reduction of variance of distance to stimulus (Fig. 3H, I) suggesting animals maintained their close proximity to the stimulus during the presentation period. However, there was no significant main effect of developmental isolation, UCMS, or a significant interaction.

Unlike socially reared fish, developmentally isolated fish exhibited a significantly higher total distance traveled (Fig. 3C) and lower absolute turn angle (Fig. 2C) during the stimulus presentation period. At the same time, developmentally isolated zebrafish exhibited a preference for the animated conspecific image similar to socially reared zebrafish. One possible explanation for this pattern of results is that although the shoaling response as defined by the

reduction of distance to stimuli may have been unaltered, the appearance of the images represented a novel stimulus[61] and developmental isolation altered responses to novelty itself. We also found that UCMS increased absolute turn angle in developmentally isolated, but not in socially reared zebrafish during the stimulus presentation period (Fig. 2C). Notably, the effect of UCMS on shoaling behavior have been inconsistent with Piato *et al.*[33] reporting reduced shoal cohesion and Chakravarty *et al.*[36] reporting increased shoal cohesion. In contrast to both of these studies, we found UCMS to not significantly alter the distance to stimulus, which has been shown to reflect shoal cohesion.[32] However, it is important to note that the artificial nature of our shoaling behavioral paradigm may still be different compared with live shoals.

In addition to characterizing anxiety-like and social behavior in zebrafish, we also examined changes in neurochemical responses. In depression, the dopaminergic system is often examined in the context of reward and hedonic processing.[62] Patients with depression have been reported to exhibit reduced dopamine D_1 receptor-binding in the striatum.[63,64] In rodents, UCMS has been shown to reduce dopamine levels in the frontal cortex[65] as well as in whole-brain tissue samples.[66] In contrast, the reported effect of isolation rearing on the brain tissue levels of dopamine and DOPAC has been inconsistent in rodents.[17] In our study, we found that only developmentally isolated zebrafish that experienced UCMS exhibited lower whole-brain tissue levels of dopamine following the presentation of a conspecific image. We have previously shown that zebrafish exhibit an increase in whole-brain dopamine levels in response to an animated image of conspecifics.[47] The decrease in whole-brain dopamine levels in isolated UCMS zebrafish may be related to their abnormal increase in absolute angle (Fig. 2C) and total distance traveled (Fig. 3C) in response to the conspecific image, which may be associated with altered social behavior.

The serotonergic system has been highly implicated in the pathophysiology of depression and anxiety disorders.[67] In contrast with dopaminergic changes, the effect of UCMS and isolated rearing on the serotonergic system has been extensively examined.[8,17] UCMS has been shown to reduce serotonin and 5-HIAA levels in rodent brain tissue[66] with notable decreases in the prefrontal cortex and nucleus accumbens.[68] Similarly, rats reared in isolation have been shown to have reduced serotonergic activity as well extracellular 5-HIAA levels primarily in the nucleus accumbens.[69] In our study, we found that developmental isolation reduced 5-HIAA levels without altering serotonin levels, and this effect appeared to be potentiated by UCMS (Fig. 5D). Our findings are similar to a previous study showing that increased anxiety-like behavioral responses are associated with reduced 5-HIAA brain tissue content.[70] It is notable that in rodents, UCMS-induced alterations to dopamine and serotonin have been attributed to altered monoamine oxidase (enzyme responsible for dopamine and serotonin breakdown) activity.[66,71] Although we did not examine momoamine oxidase (MAO) activity in this study, we have previously shown that changes in dopaminergic and serotonergic responses may be attributed to MAO activity in zebrafish as well.[72]

Finally, we examined the effect of UCMS and developmental isolation on body weight since depression in humans is often associated with both weight-gain and weight-loss.[73–75] In rodents, UCMS has been shown to attenuate weight gain,[76] whereas isolated rearing has been shown to increase weight gain.[77] In contrast, combining isolated rearing with UCMS has been shown to impair weight gain in rodents.[78] In our study, we found that developmentally isolated zebrafish gained more weight than socially reared controls. The increased weight in developmentally isolated zebrafish may be attributable to a number of different factors, including lower density housing, lack of social aggression, and competition for food. In contrast, UCMS was found to impair weight gain, but this was only observed in developmentally isolated zebrafish, and not socially reared fish. The lack of a UCMS effect on socially reared fish may be attributed to a flooring effect since they weighed less before the start of UCMS.

In conclusion, our results show that UCMS increased anxiety-like behavioral responses, whereas developmental isolation altered motor responses unrelated to shoaling during social stimulus presentation. We also found that UCMS impaired weight gain, dopaminergic and serotonergic responses in developmentally isolated but not socially reared zebrafish. Our behavioral and neurochemical results confirm the findings of previous UCMS studies[33,34,36] as well as developmental isolation studies in zebrafish,[37,38] reinforcing face and construct validity of the use of zebrafish in this behavioral paradigm for depression research. Predictive, that is, pharmacological, validity of the paradigm remains a question, and will require examination of the effect of anxiolytic and antidepressant drugs.

Acknowledgments

Supported by NSERC Canada (311637) and Brain Canada Foundation.

Disclosure Statement

No competing financial interests exist.

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