Behavioural Brain Research 298 (2016) 278–285

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Interaction between handling induced stress and anxiolytic effects of ethanol in zebrafish: A behavioral and neurochemical analysis

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HIGHLIGHTS

- Handling induced stress increases anxiety-like behavior and decreases 5-HIAA levels.
- Handling x ethanol interaction is detected for behavioral responses.
- Handling x ethanol interaction is detected for dopamine levels.

ARTICLE INFO

Article history:
Received 10 September 2015
Received in revised form 5 October 2015
Accepted 8 October 2015
Available online 28 November 2015

Keywords:
Zebrafish
Alcohol
Stress
Dopamine
Serotonin

ABSTRACT

Stress is often considered an important factor in the development of alcohol addiction. In rodents, various types of stressors have been shown to potentiate the effects of alcohol on behavioral responses, and to increase consumption of this substance. However, few have investigated the interaction between stress and alcohol in zebrafish. In the current study we present a repeated handling stress paradigm we developed for zebrafish, and examine whether stress alters alcohol induced behavioral and neurochemical responses. Our results show that repeated handling of zebrafish conducted for 2 consecutive days is sufficient to increase anxiety-like behavioral responses quantified 24 h post-stressor. Repeatedly handled zebrafish also exhibited a reduction in the levels of serotonin's metabolite, 5-hydroxyindole acetic acid (quantified by high precision liquid chromatography) compared to unhandled controls. A 60-min acute exposure to 1% ethanol was found to significantly increase locomotor activity and decrease anxiety-like behavioral responses in stressed zebrafish but not in controls. Furthermore, unhandled control zebrafish exhibited a significant increase in whole-brain dopamine levels following exposure to ethanol but the increase was not observed in repeatedly handled fish. Our findings suggest that ethanol induced locomotor activity and anxiolysis is potentiated by handling stress and may be partially mediated by changes in dopaminergic and serotonergic activity. Overall, we demonstrate the validity of our repeated handling stressor paradigm for zebrafish, which can be used to investigate the interaction between stress and ethanol.

1. Introduction

Recurrent stressful life events may increase alcohol (ethanol, ethyl alcohol) consumption for the well-established reason that alcohol's anxiolytic properties effectively reduce anxiety-like behaviors [15,30,14]. Consequently, stress-prone humans are at a higher risk of alcohol abuse since this drug is often consumed to alleviate symptoms of anxiety and depression [22,35]. Similarly, rats acutely exposed to various stressors show heightened sensitivity to anxiolytic drugs compared to non-stressed controls [17]. For example, low to moderate doses of ethanol following a stressor has been shown to mitigate stress-induced anxiety-like behaviors

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http://dx.doi.org/10.1016/j.bbr.2015.10.061
0166-4328/

in rodents [4,14]. Furthermore, mild stress in rats has also been shown to increase ethanol consumption [7]. However, alcohol also engages central and peripheral nervous system stress pathways, such as the hypothalamic pituitary axis (HPA) axis and extrahypothalamic systems, which results in it occasionally serving as a stressor [4,33]. Alcohol, like most drugs of abuse, also has the ability to stimulate locomotor activity, and this effect is well-characterized across multiple animal species [11,29,38]. In rats, modifying stress levels has been shown to alter their locomotor response to alcohol [1,2]. The relationship between stress and alcohol is complex and warrants further analysis.

The zebrafish (*Danio rerio*) is a relatively novel model organism in behavioral neuroscience. Nevertheless, the past few years have seen a rapid increase of interest in this species especially for the study of alcohol's effects on the vertebrate brain. For example, a one hour long acute exposure to ethanol was found to dose-dependently increase locomotion, with the highest motor excitation occurring during exposure to 1% v/v ethanol [38]. Exposure to this ethanol concentration induces a time-dependent increase in motor activity that peaks at approximately 20–30 min post-exposure. Beyond 30 min, motor activity plateaus and remains heightened up to 1 hour of ethanol exposure [39]. Acute exposure to 1% alcohol in zebrafish has also been shown to reduce anxiety-like behavioral responses, for example, it reduces jumping [27], increases time swimming near the surface of the tank [23], increases number of transitions to the top of the tank [32], reduces freezing [5], and reduces anti-predatory responses [10]. Moreover, acute ethanol exposure induced changes in brain function of zebrafish can also be detected at the neurochemical level with a number of changes in neurotransmitter levels occurring within 1 h post ethanol exposure, a time course which is in line with results obtained for multiple species [9,38,37,42]. The robust behavioral and neurochemical responses to alcohol found in zebrafish and the similarities of these responses with those seen in other animal species make zebrafish a valuable tool for investigating mechanisms underlying alcohol's effects on the brain.

Despite mounting evidence pointing toward an interaction between stress and alcohol in multiple species, few have examined this relationship in zebrafish. Recently, Parker et al. [28] found that individually housed zebrafish exhibit less anxiety-like behavioral responses compared to group housed zebrafish in the novel tank test, an effect that was attributed to a decrease in stress reactivity. Furthermore, these researchers found that exposure to 1% ethanol reduced anxiety-like behavioral responses in the novel tank diving test only in individually-housed zebrafish fish but not in group housed zebrafish, demonstrating an interaction between housing condition and the anxiolytic effects of alcohol. However, the relationship between stress and alcohol's locomotor stimulant and anxiolytic properties remains unclear in zebrafish. In the current study, we develop a novel repeated handling stress paradigm using zebrafish, and examine the locomotor stimulant and anxiolytic effects of alcohol on handled and unhandled zebrafish. In addition, since the dopaminergic and serotonergic system has been shown to respond to alcohol [9,38] and is also implicated in stress and anxiety in zebrafish [24,25], we quantify the levels of dopamine, serotonin, and their metabolites in zebrafish brains to examine changes in the dopaminergic and serotonergic systems.

2. Methods

2.1. Animal housing

104 sexually mature (10 month old) male and female zebrafish (*D. rerio*) of the AB strain were obtained from the University of Toronto Mississauga vivarium (Mississauga, Ontario, Canada). The AB strain was selected since it is most frequently studied and is genetically well-characterized, showing homozygosity at over 80% of its loci [16]. Animals were bred and raised in 37 L tanks (approximately 20 fish per tank) equipped with mechanical/chemical/biological filters. Zebrafish were kept on a 13 h light-dark cycle with lights turning on at 08:00 h and off at 21:00 h.Water quality parameters were maintained at optimal conductivity levels (100–300 microsiemens), temperature (28–30 °C), and pH levels (6.8–7.2). Upon hatching to 3 weeks post-fertilization, larvae were fed Larval AP 100 (Zeigler Bros, Inc). Subsequently, fish were fed twice a day alternating between brine shrimp (*Artemia salina*) and a mixture of 2 parts flake food (Scientific Hatcheries Diet) and 1 part powdered spirulina (Ocean Star International, Inc.).

2.2. Experimental design and procedure

The purpose of the experiment was to determine whether the stimulant and anxiolytic effects of ethanol are affected by acute stress. The experiment employed a 2×2 between subject factorial design with handling (handled or unhandled) and ethanol (0 or 1%) as the between subject factors. The testing procedure involved transferring animals between tanks which is a frequently used behavioral procedure in zebrafish research [23,6]. Since handling induces anxiety-like responses in zebrafish, a novel repeated handling stress paradigm was designed based on the testing procedure. The stress paradigm involved exposing zebrafish to handling and novel tank exposure at a frequency that was higher, at an inter-procedure interval that was shorter, and for a novel tank dwell time that was shorter than what we would normally employ for habituating the fish to their test environment. Briefly, instead of habituation, our goal was to sensitize the experimental subjects to the procedure. Prior to testing, zebrafish in the handled group ($n = 52$) underwent two handling trials once a day for 2 consecutive days. Individual zebrafish were placed in 1.5 L tanks containing system water for a period of 5 mins. They were then netted and immediately transferred to a second 1.5 L tank for an additional 5 mins and then returned to their home tanks. Unhandled control fish ($n = 52$) were not handled and remained in their housing tanks until testing.

Following the repeated handling trials, all fish were tested the subsequent day. Both handled and unhandled fish were individually transferred to 1.5 L tanks containing system water for 30 min to examine the effect of repeated handling on baseline (no ethanol) behavioral responses in a novel environment. Zebrafish were then immediately transferred into 1.5 L tanks containing either system water or 1% ethanol for a duration of 60 min. Behavioral responses were recorded during both exposure periods by camcorders positioned in front of the tanks. All testing tanks were made of transparent Plexiglas with the lateral and back sides covered with white corrugated plastic to provide a uniform environment and to reduce external visual cues during testing. In order to prepare the tanks for handling and testing trials, all tanks were filled with 1 L of system water supplemented with 100 mg/L of Instant Ocean Sea Salt. Water quality parameters were ensured to match those of the housing tanks (temperature, pH and conductivity). To set up the tanks containing ethanol, 100% anhydrous ethyl alcohol (Commercial Alcohols, Brampton, ON, Canada) was diluted to achieve a final concentration of 1% in the appropriate tanks.

2.3. Behavioral quantification

Behavioral responses during the last 10 mins of each exposure (baseline quantification and ethanol exposure) were quantified using EthoVision XT 8.0, an automated video-tracking software, to examine locomotor activity and anxiety-like behavior. We chose to analyze the last 10 mins of exposure since by this time, ethanol has