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NOS-2 participates in the behavioral effects of ethanol withdrawal in zebrafish

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Abstract

Nitric oxide has been implicated in symptoms of ethanol withdrawal in animal models. Zebrafish have been used as models to study neurobehavioral effects of ethanol (EtOH) withdrawal, but the mechanisms associated with these effects are not yet clear. Adult zebrafish were treated with 1% EtOH for 20 min per day for 8 days, injected with the nitric oxide synthase 2 (NOS-2) inhibitor aminoguanidine (50 mg/kg), and allowed to experience withdrawal (WD) in their hometanks for 7 days. EtOH WD increased anxiety-like behavior in the novel tank test, an effect that was blocked by aminoguanidine. EtOH WD also increased brain levels of nitrite, an effect that was partially blocked by aminoguanidine. These results underline a novel mechanism by which NOS-2 controls anxiety-like responses to ethanol withdrawal, with implications for the mechanistic study of symptoms associated with chronic ethanol abuse.

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Data and scripts: https://github.com/lanec-unifesspa/etoh-withdrawal/tree/master/NOS2

Keywords: Nitric oxide; Oxidative stress; Ethanol withdrawal; Anxiety

1. Introduction

Nitric oxide synthase 2 (NOS-2), an inducible isozyme which participates in the biosynthesis of nitric oxide under conditions of stress and neuroinflammation [1], has been shown to be involved in the formation of peroxynitrite and other nitrogen reactive species [2]. Due to the fact that this isozyme is not constitutionally expressed and has calcium-independent activity, it has been implicated in stress sensibilization mechanisms: in situations of sustained physiological stress, the expression of NOS-2 is induced, and continually produces nitric oxide as long as there is available subtrate and the enzyme is not degraded. Nitric oxide has been implicated in the mechanisms of withdrawal syndrome and alcoholic abstinence syndrome, characteristic dysphoric states that follows the abrupt cessation of drug and/or alcohol use; in preclinical research, inhibiting nitric

oxide synthesis diminishes withdrawal-like symptoms in rats treated with ethanol [3], and ethanol withdrawal (EtOH-WD) activates nitric oxide-producing neurons in anxiety-related areas [4]. We have previously confirmed, through meta-analysis and a conceptual replication, that EtOH-WD increases anxiety-like behavior in adult zebrafish, an effect that is accompanied by decreased activity of the antioxidant enzyme catalase in the brain [5]. Moreover, EtOH-WD has been shown to induce oxidative imbalance in the zebrafish brain, and the antioxidant *N*-acetylcysteine blocks both effects [6]. These results suggested that oxidative and nitrosative stress can be involved in the pathophysiology of EtOH-WD. In zebrafish, NOS-2 is coded by two different genes, *nos2a* and *nos2b*, which are expressed in the central nervous system and upregulated after inflammatory stimuli [7], suggesting that these are also inducible isoforms. In the present work, we test the hypothesis that EtOH-WD-elicited anxiety is also mediated by NOS-2 in zebrafish. This manuscript is a complete report of all the studies performed to test the effect of aminoguanidine on anxiety-like behavior in zebrafish after EtOH-WD.

2. Materials and methods

42 adult (~4 mo., standard length 23.1 - 34.8 mm) zebrafish from the longfin phenotype were acquired in a local aquarium shop and kept in collective tanks (40 L, 5 animals/L) for at least two weeks before experiments begun, and fed twice daily on commercial dry feed (Alcon® Gold Fish Colour). Animals used in the experiments were of mixed sexes, and kept in an approximate proportion of 50 females:50 males, based on body morphology. Animals were randomly drawn from the tank and allocated to experimental conditions (below); randomization was achieved by using a random number generator (http://www.jerrydallal.com/random/random_block_size_r.htm), with each subject randomized to a single treatment using random permuted blocks. One PI attributed a random letter to treatment (e.g., “A” for control, “B” for withdrawal) and a random integer for drug dose (e.g., “1” for 50 mg/kg, “2” for 0 mg/kg [vehicle]), and combinations for

letters and integers were randomized. For each experiment, animals were treated and tested in the order of allocation (i.e., randomly). In all experiments, experimenters and data analysts were blinded to drugs and treatment by using coded vials (with the same code used for randomization); blinding was removed only after data analysis. Experiments were always run between 08:00AM and 02:00 PM. Sample sizes were defined based on the standardized mean difference (SMD) derived from a metanalysis of the effects EtOH-WD on anxiety-like behavior in zebrafish (SMD = -0.18) for the main effect of withdrawal [5], and an effect size of 0.4 for the main effect of drug, considering 80% power to detect a similar effect, and were calculated based on a two-way ANOVA model using the R package 'pwr2' (v. 1.0) [8], resulting in a minimum sample size of 10 animals per group. 2 animals of each group (except animals treated with aminoguanidine and exposed to withdrawal) were removed from the experiment due to poor health. Thus, final sample sizes were n = 10/group for groups control+0 mg/kg, withdrawal + 0 mg/kg, and control + 50 mg/kg, and n = 12 for group withdrawal + 50 mg/kg. For the second experiment (nitrite levels), since the sensitivity of the Griess assay is relatively low [9], 3 brains needed to be pooled to produce sufficient signal; in order to reduce the amount of animals and limit suffering, only 5 pools were used per group (a total of 15 animals/group), and therefore sample sizes were n = 5/group. Animals from the second experiment were not the same as the animals from the first experiment. Water conditions, housing, and feeding conditions were standardized as per recommendations for zebrafish [10,11]. Water quality parameters can be found online (https://github.com/lanec-unifesspa/lanec-welfare); experiments were made between October and December 2018. Potential suffering of animals was minimized by controlling for the aforementioned environmental variables and scoring humane endpoints (clinical signs, behavioral changes, bacteriological status), following Brazilian legislation [10]. Experiments were approved by UEPA's IACUC under protocol 06/18.

Animals were exposed to a regimen based on Mathur and Guo [12], with exposure to either water or 1% EtOH in 2 L containers for 20 min per day (exposure at 10:00 AM) for 8 days. Individuals were

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randomly drawn from a single tank. EtOH was mixed in the water just before putting the fish in, and animals were exposed in groups of 6 animals, and group-housed in 5 L tanks (2 tanks per group). Tank effects were absent, based on effect sizes (average Cohen's d = 1.1865) between tanks of same groups on the variable "time on bottom". Immediately after the end of the exposure period (i.e., on day 8), animals were anesthetized in ice-cold water (11-12 ºC) [13], and injected intraperitoneally with either vehicle (Cortland's salt solution) or 50 mg/kg of the NOS-2 inhibitor aminoguanidine (AG). The injection was made based on standard protocols [14] using a 10 µL microsyringe (Hamilton® 701N syringe 701N, volume 10 µL, needle size 26s gauge at the cone tip), in a final volume of 5 µL. The dose was based on experiments made in rodents that showed that this dose blocks the anxiogenic effects of restraint stress [15] and the sensitizing effects of stress-restress [16]. After this period, animals were allowed to experience withdrawal from EtOH in their hometanks for 7 days. After that, animals were tested in the novel tank test [17], using a rectangular tank (15 cm X 24 cm X 22 cm, w X l X h), filled with 5 L tank water, that was freely explored for 6 min; behavioral variables were defined as per Lima et al. [18]. Tank zones were divided in a bottom third and a top third, producing a more strict definition of "tank top" and "tank bottom" [19]. Light levels above the tanks were measured using a handheld light meter, and ranged from 251 to 280 lumens (coefficient of variation = 3.399% between subjects).

For the second experiment, animals were treated as above, and then sacrificed in ice-cold water [13], decapitated, and their brains were dissected, forming pools of 3 brains. Nitrite content in these matrices were analyzed using a modified Griess protocol [20], and reported as micromoles per milligram of protein. Data were analyzed using robust ANOVAs with permutation tests [21], with p-values adjusted for the false discovery rate using the Benjamini-Hochberg procedure [22]. Post-hoc tests were made using pairwise permutation tests on Huber's M-estimators, with 5000 resamples, correcting p-values for the false discovery rate using the Benjamini-Hochberg procedure. As a distribution-free approach to ANOVA, reported p-values are not associated with an F-statistic, but

represent the fraction of permuted M-estimators that are at least as extreme than the original observed values [21]. Alpha was set at 0.05. Analyses were made using the package 'rcompanion' for R [23]. Data and analysis scripts were posted on GitHub (https://github.com/lanec-unifesspa/etoh-withdrawal/tree/master/NOS2). We report how the sample size was determined, all data exclusions (if any), all manipulations, and all measures in the study.

3. Results

Main effects of withdrawal status ($p = 0.0304$) and drug ($p = 0.0458$), as well as an interaction effect ($p = 0.0276$), were found for time on bottom (Fig. 1A, 1A'). No main effects of withdrawal status ($p = 0.172$) or drug ($p = 0.1488$) were found for time on top, but an interaction effect was found ($p = 0.001$; Fig. 1B, 1B'). No main effects of withdrawal status ($p = 0.1454$) or drug ($p = 0.0738$), nor interaction effects ($p = 0.1610$), were found for erratic swimming (Fig. 2A). No main effects of withdrawal status ($p = 0.3338$) or drug ($p = 0.583$), nor interaction effects ($p = 0.764$), were found for thrashing (Fig. 2B). No main effects of withdrawal status ($p = 0.5028$) or drug ($p = 0.0902$), nor interaction effects ($p = 0.1102$), were found for freezing frequency (Fig. 2C). No main effects of withdrawal status ($p = 0.0834$) nor a drug effect ($p = 0.918$) were found for freezing duration (Fig. 2D), but an interaction effect was found ($p = 0.0446$). No main effects of withdrawal status ($p = 0.244$) or drug ($p = 0.3268$), nor an interaction effect ($p = 0.6128$), were found for total locomotion (Fig. 2E). Main effects of withdrawal status ($p = 6.45 \times 10^{-5}$), drug ($p = 0.00182$), and interaction ($p = 0.00757$) were found for nitrite levels (Fig. 2F).

4. Discussion

The increases in geotaxis and freezing after withdrawal is consistent with previous findings in EtOH WD in zebrafish [5,24] (but see [25]), and are indicative of increased anxiety, stress, or arousal [26]. In a previous meta-analysis [5], we found that exposure duration and EtOH

concentration during exposure significantly affect the effects of EtOH-WD on anxiety-like behavior, which could explain differences in, for example, erratic swimming – which was not affected in the present experiment, but was altered in other studies [27]. In the present experiment, WD also increased nitrite levels in the brain, an effect that was partially blocked by treatment with aminoguanidine. Aminoguanidine, a NOS-2 inhibitor, also prevented the effects of WD on anxiety-like behavior in the NTT, suggesting that both the increases in nitrite and the anxiogenic-like effect of ethanol withdrawal are associated with this enzyme. Since EtOH-WD also produces oxidative imbalances in the zebrafish brain [5,6,24], it is possible that the nitrite production observed here represents nitrosative stress. Chronic ethanol exposure also produces oxidative stress, but not increased nitrite, in the zebrafish brain [28], suggesting that the effect on the nitrergic pathway is specific to withdrawal. Both oxidative stress [29,30] and nitric oxide [31,32] have been implicated in stress-induced anxiogenesis in zebrafish, suggesting that the nitrergic system is a common mediator of the behavioral effects of stressors. EtOH-WD has also been shown, in rodents, to sensitize neurons associated with defensive behavior [33,34]. Thus, the present results suggest that EtOH-WD increases nitric oxide production in the brain through NOS-2, and that this increased NO leads to increased anxiety. Further work is needed to understand how this NOS-2-mediated mechanism interacts with other known mechanisms of withdrawal-sensitized behavior.

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Figure captions

Figure 1 – Aminoguanidine (AG), a NOS-2 inhibitor, blocks the increases in bottom-dwelling after ethanol withdrawal in zebrafish. (A) Time spent in the bottom of the tank. (A') Detail of Figure 1A. (B) Time spent in the top of the tank. (B') Detail from Figure 1B. In A and B, the y axis represents the whole range of values, from minimum possible (0 s) to the whole session (360 s). To increase clarity and visualization of individual points, in A' and B', visualization is restricted to the region where actual values are observed. Data are presented as boxplots with Tukey hinges, overlapped with individual data points. Different letters indicate statistically significant differences.

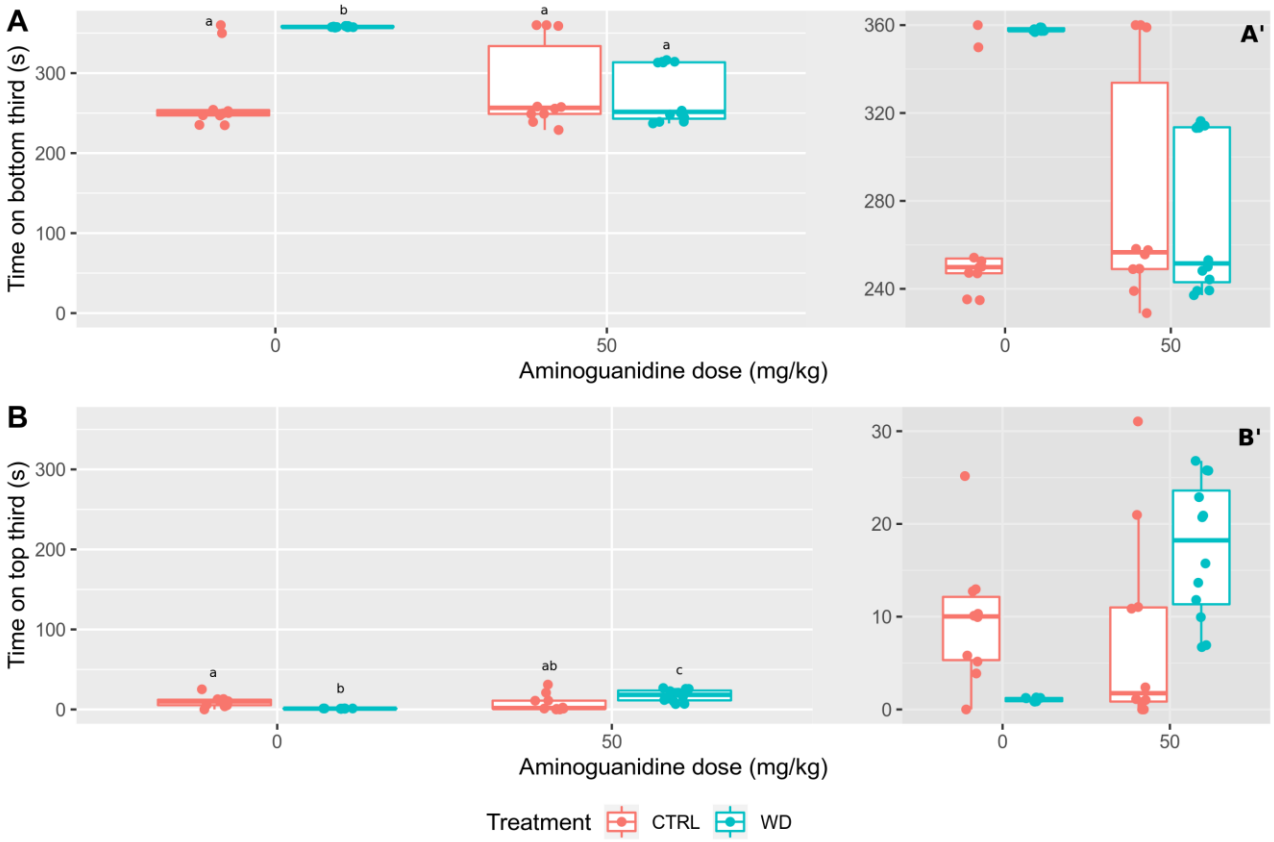

Figure 2 – Aminoguanidine (AG), a NOS-2 inhibitor, blocks the increase in freezing duration and brain nitrite levels after ethanol withdrawal in zebrafish. (A) Erratic swimming. (B) Thrashing. (C) Freezing frequency. (D) Freezing duration. (E) Number of squares crossed. (F) Brain nitrite levels. Data are presented as boxplots with Tukey hinges, overlapped with individual data points. Different letters indicate statistically significant differences.

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