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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.

Preprint:

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

Keywords: Nitric oxide; NOS-2; Ethanol withdrawal; Zebrafish

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. We report how the sample size was determined, all data exclusions (if any), all manipulations, and all measures in the study.

2. Materials and methods

42 adult 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. Sample sizes were defined based on the standard mean difference derived from a metanalysis of EtOH-WD in zebrafish, considering 80% power to detect a similar effect [5]. Water conditions, housing, and feeding conditions were standardised as per recommendations for zebrafish [8,9]. Water quality parameters can be found online (https://github.com/lanec-unifesspa/lanec-welfare); experiments were made between October and December 2018. Animals were exposed to a regimen based on Mathur and Guo [10], with exposure to either water or 1% EtOH for 20 min per day for 8 days. EtOH was mixed in the water just before putting the fish in. After the end of the exposure period,

animals were injected with either vehicle (Cortland's salt solution) or the NOS-2 blocker aminoguanidine. 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 [11]; behavioral variables were defined as per Lima et al. [12]. Animals were then sacrificed in ice-cold water [13], decapitated, and their brains were dissected, forming pools of 5 brains. Nitrite content in these matrices were analyzed using a modified Griess protocol [14]. Data were analyzed using robust ANOVAs with permutation tests, with p-values adjusted for the false discovery rate. Data and analysis scripts were posted on GitHub (https://github.com/lanec-unifesspa/etoh-withdrawal/tree/master/NOS2).

3. Results

Main effects of treatment ($p = 0.0322$) and dose ($p = 0.045$) were found for time on bottom (Fig. 1A). A main effect of dose ($p = 0.0438$), but not of treatment ($p = 0.0884$), was found for freezing duration (Fig. 1C). Interaction effects were found for time on bottom ($p = 0.026$) and top ($p = 0.002$; Fig. 1B), but not for freezing frequency ($p = 0.1108$; Fig. 1D), but not for erratic swimming ($p = 0.1668$; Fig. 1E), thrashing ($p = 0.7626$; Fig 1F), freezing duration ($p = 0.0874$; Fig 1G), or total locomotion ($p = 0.6162$; Fig 1H). WD increased time on bottom, freezing duration, and erratic swimming, and AG blocked these effects. Main effects of treatment ($p = 6.45 \times 10^{-5}$), dose ($p = 0.00182$), and interaction ($p = 0.00757$) were found for nitrite levels (Table 1).

Group	NOx levels
Control + Vehicle	1.14 (95% CI[0.739, 1,54])
Control + Aminoguanidine	0.957 (95%CI[0.707, 1.21])
Withdrawal + Vehicle	3.38 (95%CI[2.03, 4.74])
Withdrawal + Aminoguanidine	1.57 (95%CI[1.18, 1.96])

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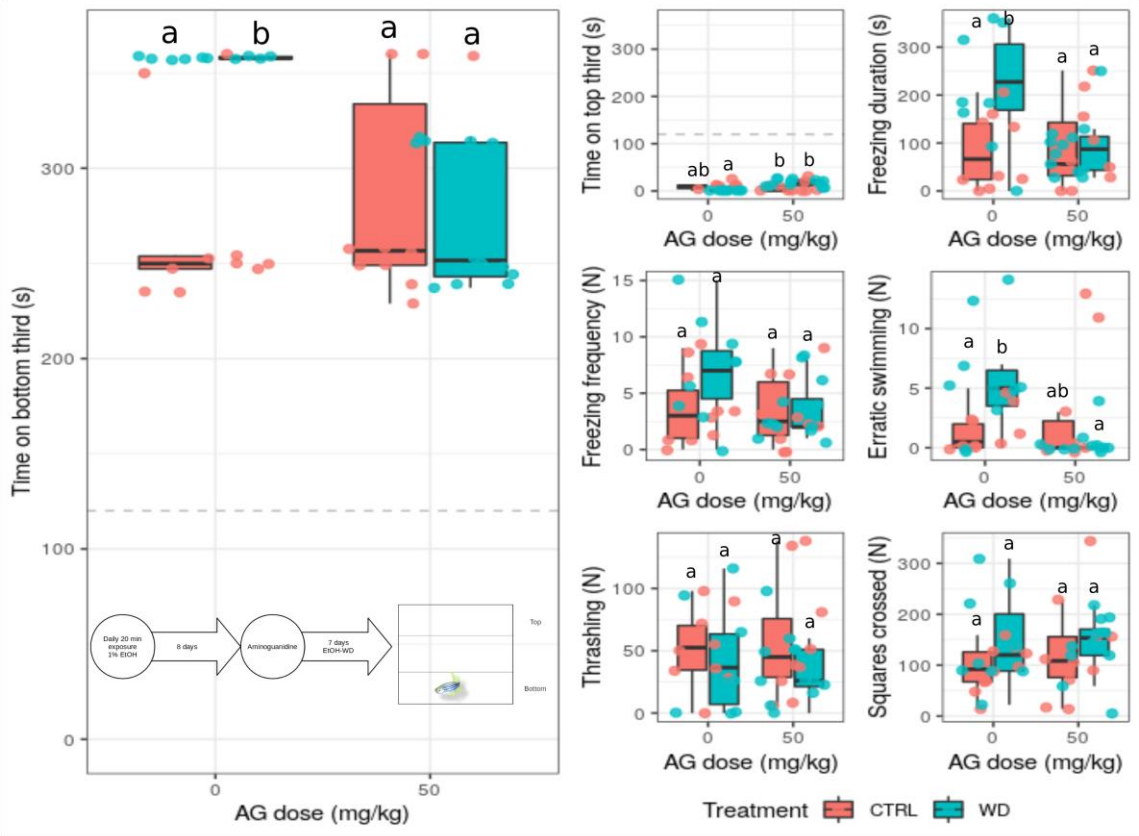

Figure 1: Aminoguanidine, a NOS-2 inhibitor, blocks the development of behavioral effects of ethanol withdrawal in zebrafish. (A) Time spent in the bottom of the tank. (B) Time spent in the top of the tank. (C) Freezing duration. (D) Freezing frequency. (E) Thrashing frequency. (F) Number of squares crossed. Inset: Experimental design. Data are presented as boxplots with Tukey hinges, overlapped with individual data points. Different letters indicate statistically significant differences.

4. Discussion

The increases in geotaxis, freezing, and erratic swimming after withdrawal is consistent with previous findings in EtOH WD in zebrafish [5], and are indicative of increased anxiety, stress, or arousal [15]. WD also increased nitrite levels in the brain, an effect that was blocked by treatment with aminoguanidine. Aminoguanidine, a NOS-2 blocker, 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], it is possible that the nitrite production observed here represents nitrosative stress. The present results suggest a novel mechanism by which ethanol withdrawal produces detrimental neurobehavioral effects: by up-regulating NOS-2 expression and,

consequently, activity, ethanol withdrawal initiates the production of nitric oxide and other reactive oxygen and nitrogen species, sensitizing neurons in regions associated with defensive behavior [16,17]. Further work is needed to understand how this NOS-2-mediated mechanism interacts with other known mechanisms of WD-sensitized behavior.

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59 Figure captions

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