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Weekly ethanol exposure alters dopaminergic parameters in zebrafish brain

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ABSTRACT

Binge drinking is defined as the infrequent consumption of excessive doses of alcohol in a short period of time. Zebrafish is a reliable model to investigate ethanol consumption impact on the CNS, including reward signaling like dopaminergic neurotransmission system. The aim of this study was to evaluate zebrafish brain dopaminergic parameters after intermittent weekly ethanol exposure (WEE), which mimics binge drinking. Thus, adult zebrafish were exposed to ethanol (1.4% *v*/v) for 30 min, once a week for three consecutive weeks. The groups were divided according to different time points after the third exposure and name as follow: immediately (WEE−I), two days (WEE-2), and nine days (WEE-9) after last exposure to ethanol. Brain dopaminergic function was assessed by the activity of the dopamine transporters (DAT); monoamine oxidase (MAO) activity; dopamine and noradrenaline levels by chromatography. The WEE-I and WEE-2 groups presented a significant increase in DAT activity. The MAO activity was decreased for WEE-2 and WEE-9 groups. The WEE-2 and WEE-9 groups presented an increase in brain dopamine levels, while noradrenaline levels were not affected. Therefore, dopaminergic parameters are still altered two and nine days after the last ethanol exposure in this binge experimental model, resulting in a modulatory event in this neurotransmission pathway.

1. Introduction

Alcohol use and abuse are primary public health concerns. The National Institute on Alcohol Abuse and Alcoholism (NIAAA) has established three main consumption patterns, varying according to the amount and frequency of alcohol ingestion. These are classified as, moderate, heavy, and binge drinking. Binge drinking is defined as the ingestion of excessive amounts of alcohol in a short period of time (NIAAA, 2015). This pattern of consumption can be found in both adolescents and adults, and is often associated with neurological and psychological changes, including cognitive impairment and a reduction in quality of life (Almeida and Campos, 2013; Holcombe et al., 2013). Unlike individuals who drink moderately, binge drinkers exhibit a strong response to the primary euphoric effects but are less sensitive to the sedative effects of alcohol. This indicates a pre-disposition to the development of alcohol addiction (Schuckit et al., 2008). After a series of exposures, alcohol withdrawal is debilitating and may promote increased anxiety, substance-seeking behavior, and seizures. The severity of these symptoms depends on the amount and speed of ingestion (Holcombe et al., 2013).

Ethanol is a small lipid-soluble substance which penetrates the blood-brain barrier and interacts with various neurotransmitter systems in the brain (Diamond and Gordon, 1997; Valenzuela, 1997). Ethanol can also affect the central nervous system (CNS) by causing an imbalance in excitatory and inhibitory neurotransmitters (De Witte, 2004). Dopamine is an important neurotransmitter and has previously been shown to be affected by altered synaptic plasticity related to alcohol misuse. (Chen et al., 2007). Ji et al. (2017) examined the relationship between alcohol consumption in binge drinking and timing-dependent plasticity in neurons of the nucleus accumbens (NAc) and found that repeated alcoholic binges modulated synaptic plasticity in medium spiny neurons of direct and indirect NAc pathways, implicating

Abbreviations: CNS, central nervous system; NAc, nucleus accumbens; CPP, conditioned place preference; WEE, weekly ethanol exposure; DAT, dopamine transporters; NETs, noradrenaline transporters; MAO, monoamine oxidase; cAMP, cyclic adenosine monophosphate

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dopamine as a promising target in the study of alcohol. Alcohol increases dopaminergic transmission and firing rate of dopaminergic neurons, thus increasing the release of dopamine (Ward et al., 2009). However, ethanol leads to an increase in this neurotransmitter only in the reward pathway (Boileau et al., 2003). This reward pathway is responsible for modulating the major physiological functions not only related to survival, such as ingestion of food, water, and sexual behavior, but also targets consumption of psychoactive substances including alcohol, cocaine, amphetamines, and opioids (Jones and Miller, 2008).

Strategies to investigate the neural mechanisms related to alcohol dependence are critical for the development of new therapies for alcohol dependence. The use of zebrafish as a model for the investigation of alcohol abuse is increasing. Previous studies have focused on describing the structural neuroanatomical organization and neurochemistry of the monoaminergic system and its connections with other aminergic systems to better understand the role of monoamines in the zebrafish CNS (Kaslin and Panula, 2001; Panula et al., 2010). The dopaminergic system and its components have been identified as key homologues of evolutionarily conserved reward pathways such as selective attention and impulse control (Filippi et al., 2010; Parker et al., 2013). Zebrafish solute carrier family 6 member 3 gene (*slc6a3 or dat*) is related with dopamine transport and highly homologous to human SLC6A3 (Bai and Burton, 2009). However, there is no evidences regarding functionality of dopamine transporters (DAT) in zebrafish brain. Four subtypes of dopaminergic receptor have been identified in zebrafish corresponding to the mammalian D1, D2, D3, and D4 genes (Li et al., 2007; Boehmler et al., 2004, 2007). As such, neuromodulatory and behavioral influences may be conserved among the species. The zebrafish is often proposed as an alternative to mammalian models when researching the molecular basis of addiction. Zebrafish are attractive for a number of reasons, one such reason being the advantage of genetic tractability which could identify novel candidates for subsequent investigation in higher order models and ultimately provide insight into human behavior (Darland and Dowling, 2001; Ninkovic et al., 2006; Liu et al., 2017).

An increasing number of studies have demonstrated that acute and chronic ethanol exposure affects a variety of zebrafish behaviors. Similar to rodent behavioral paradigms, conditioned place preference (CPP) has been used in zebrafish to assess the behavioral consequences of ethanol exposure (Kily et al., 2008; Mathur et al., 2011a; Mathur et al., 2011b; Parmar et al., 2011). Furthermore, the biphasic alcohol dose response in adult and fry zebrafish mimics what has been found in mammals (Addicott et al., 2007; Tsang et al., 2019). It has been shown that ethanol in the range of 0.25 to 1% v/v influences behavior in a dose-dependent manner, in many cases, reflecting an inverted U-shaped function. Low to intermediate concentrations result in hyperactivity compared to higher concentrations, which result in hypoactivity (Gerlai et al., 2000; Gerlai, 2003). To better understand human consumption patterns in binge drinking, an intermittent heavy (1.4% v/v) alcohol exposure protocol has also been evaluated in zebrafish (Holcombe et al., 2013).

Considering that: (i) ethanol mediates its actions through several excitatory or inhibitory neurotransmitter systems; (ii) dopaminergic signaling plays a role in mediating cellular and behavioral effects of ethanol; (iii) zebrafish possess suitable attributes for studies related to dependence-related psychotropic drugs including ethanol; and (iv) intermittent and heavy ethanol exposure modulates addiction behavioral phenotypes in experimental models, the present study aimed to evaluate the effect of intermittent weekly ethanol exposure on various dopaminergic parameters in the zebrafish brain.

2. Materials and methods

2.1. Reagents

Ethanol (C_2H_6O; CAS number 64-17-5) was purchased from Merck (Darmstadt, Germany), and L-[^{3}H]dopamine (specific activity 30 Ci mmol^{-1}) was purchased from Perkin Elmer (Madrid, Spain). All other reagents used were of analytical grade. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

Adult zebrafish (*Danio rerio*, 4 month-old) from the outbreed wild-type short-fin phenotype (~50:50 male:female ratio) were obtained from Federal University of Rio Grande do Sul (UFRGS). All fish were acclimated to their new environment for at least two weeks in 50 L tanks conditioned at 27 ± 1 °C, pH 7.0–7.2. Room illumination by ceiling-mounted fluorescent lamps on a 14/10 light/dark photoperiod (lights on at 8:00 a.m.). The animals were fed four times a day with a commercial flake fish food (Alcon BASIC, Alcon, Brazil) and nauplii of brine shrimp (*Artemia salina*). After experiments, fish were anesthetized in water at 4 °C and then euthanized by section of the spinal cords. All procedures were in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. The Ethics Committee of University of Southern Santa Catarina (UNESC) approved the protocol under the number 034/2017-1.

2.3. Ethanol exposure procedure

For weekly exposure to ethanol, fish were introduced to three tanks (10 L) and received ethanol (1.4% v/v) on the 7th, 14th and 21st days as previously described by Holcombe et al. (2013). After the initiation of the alcoholic binge model, animals were separated into different groups: Control, immediately after weekly ethanol exposure (WEE-I); two days after weekly ethanol exposure (WEE-2); nine days after weekly ethanol exposure (WEE-9). All groups were handled equally and animals were kept in identical tanks. All groups, including control, underwent the same daily procedures with the exception of ethanol administration. Considering that three different techniques (Dopamine uptake, monoamine content, and MAO activity) were employed, 192 animals were used in this study.

2.4. Dopamine uptake

2.4.1. Tissue preparation

The animals were anesthetized and subsequently euthanized before brain dissection. Brains were excised into petri dishes with Hank's balanced salt solution (HBSS) containing: 137 mM NaCl; 0.63 mM Na_2HPO_4; 3.0 mM $NaHCO_3$; 5.36 mM KCl; 0.44 mM KH_2PO_4; 1.26 mM $CaCl_2$; 0.90 mM $MgSO_4$; 5.55 mM glucose; and 20 mM HEPES, pH 7.2. Each brain was separated and transferred to 24-well culture plates containing 0.5 mL of HBSS-HEPES buffer. Only one total brain was transferred to each well and all plates were maintained at 37 °C throughout the experiments.

2.4.2. Uptake assay

Dopamine uptake assay was performed as previously described by Rico et al. (2010). Total dopamine uptake was measured with the addition of 20 μL of 0.35 μCi mL − 1 L-[^{3}H] dopamine labeled solution corresponding to 10 nM L [^{3}H] dopamine and 0.75 nM unlabeled dopamine in the incubation medium warmed to 37 °C. These experiments were performed at the following times: 3, 5, 7, 10, and 15 min to identify the condition in which the reaction activity has a linear relationship with time. In order to assess the functionality of dopamine transporters (DAT), we added bupropion, known to inhibit DAT and NET function and is associated with increases in extracellular levels. After 15 min preincubation in HBSS-HEPES buffer, medium was removed and the brain was incubated for a further 15 min with 0.3 mL of HBSS buffer in the absence (control group) or presence of bupropion at final concentrations of 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM.

In all trials, the uptake was stopped with two subsequent washes

after a 5 min incubation (1 mL ice-cold HBSS-HEPES buffer) to ensure the complete removal of dopamine. Total brain tissue was immediately transferred to 0.5 N NaOH and incubated overnight, producing a homogenate. Protein content was measured using aliquots of homogenate (10 µL) following the method described by Peterson (1977). Radioactivity was measured by liquid scintillation.

2.5. Monoamine estimation

The content of dopamine, and noradrenaline in the zebrafish brain was estimated using high-performance liquid chromatography (HPLC; De Benedetto et al., 2014). The samples were stored at −80 °C until analysis. After homogenization in 400 µL of 0.2 M perchloric acid containing 3 mM cysteine, the samples were centrifuged and the supernatant was used for HPLC analysis followed by fluorimetric detection. The system comprised an Ascentis® C18 chromatography column (250 mm × 2.1 mm, 5 µm; Supelco®, Texas city, USA), an LC-20AT pump coupled to an SIL-20AHT autosampler and an RF-20A fluorescence detector, and LC Solution software (Shimadzu, Kyoto, Japan). The column temperature was maintained at 35 °C and a flow rate of 0.3 mL/min was used. The mobile phase consisted of acetate buffer (pH 3.5, 12 mM acetic acid, 0.26 mM EDTA) and methanol (86:14, v/v). The fluorescence was monitored at excitation and emission wavelengths of 279 and 320 nm, respectively. Peaks were identified by comparing their retention time in the sample (tissue extract) solution with that of the standard solution. The samples had an injection volume of 20 µL. Results were expressed as ng/mg of protein.

2.6. Monoamine oxidase activity

MAO activity was measured as previously described Anichtchik et al. (2006), with some modifications. In this assay, 4-aminoantipyrine is oxidized and condensed with vanillic acid to produce a red quinoneimine dye. The zebrafish brains were homogenized 1:40 (*w/v*) in ice-cold homogenizing buffer, containing 10 mM K_2HPO_4, 1 mM EDTA, pH 7.6. The homogenates were then centrifuged at 1000 ×g for 5 min. The supernatant was separated and frozen at −80 °C until use. The chromogenic solution, used in the assay, included final concentrations of 1 mM vanillic acid, 500 µM 4-aminoantipyrine, and 4 U/mL horseradish peroxidase type II in 0.2 M potassium PB, pH 7.6. The assay was performed in the 96-well plate. Each well contained 10 µL brain homogenate, 50 µL of H 2 0 and 50 µL chromogenic solution. Controls included 50 µL of inhibitors in wells, to deduct the inherent absorbance of the sample in the total activity. The 96-well plate was heated during 30 min at 28 °C. At that time, 100 µL of tiramine substrate was added to all the wells. The color result of the reaction was read on a microplate reader equipped with a 492-nm filter, at 28 °C for 2 h (kinetic method). The protein amount of each sample was measured by Peterson (1977) method and the final result was divided by this content.

2.7. Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M). Statistical analysis of data was carried out by one-way analysis of variance (ANOVA) followed by a post hoc Tukey test. Statistical significance was assumed at $p < 0.05$.

3. Results

The effect of intermittent weekly ethanol exposure on MAO activity and monoamine levels was assessed. The analyses were performed immediately, two days or nine days after the last ethanol exposure. In order to assess the influence of time on dopamine uptake in total encephalic tissue, a L-[^{3}H] dopamine uptake assay was carried out over 3 to 15 min. Incubation time (7 min) assays were chosen in order to ensure linear uptake of labeled dopamine over time (data not shown).

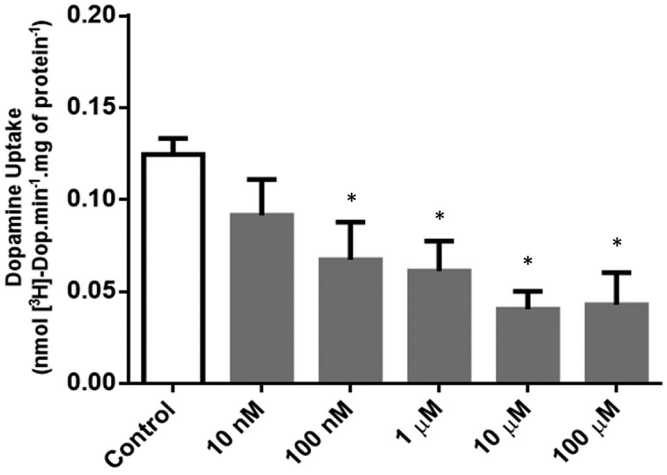

Fig. 1. Effects of different bupropion concentrations (10 nM to 100uM) on L-[^{3}H]dopamine uptake in zebrafish brain. Data were expressed as means ± standard error of mean (S.E.M.) using $n = 6$ per group. The data were analyzed by one-way ANOVA followed by Tukey post hoc test, considering the statistical differences at a $P \leqslant 0.05$ level. * Significant difference from control group.

Bupropion is an antidepressant and in micromolar concentrations inhibits dopamine transporters (Ferris and Cooper, 1993; Miller et al., 2002). To ensure specificity of dopamine uptake through DAT, we tested bupropion in vitro using different concentrations (10 nM–100 µM). The effects of bupropion on dopamine uptake are depicted in the Fig. 1. Significant reductions of 45.4 ± 3%, 50.63 ± 4%, 67.98 ± 3%, and 65.6 ± 3% for dopamine uptake were observed in the brain after exposure to concentrations of 100 µM, 1 µM, 10 µM, and 100 µM, respectively. This dose-response curve indicates that bupropion at 100 µM is an appropriate positive control for the following experiments.

Intermittent weekly ethanol exposure altered DAT activity with temporal differences in the zebrafish brain. An increase in DAT activity was observed in the WEE-I and WEE-2 groups ($F(3.21) = 45.42$; $p < 0.0001$) relative to control. However, for WEE-9, no significant difference was observed (Fig. 2). Dopamine levels in the brains of zebrafish intermittently exposed to ethanol were evaluated, and a significant increase in dopamine levels for WEE-2 and WEE-9 ($F(3.16) = 12.63$; $p = 0.0002$) in relation to control was found (Fig. 3A).

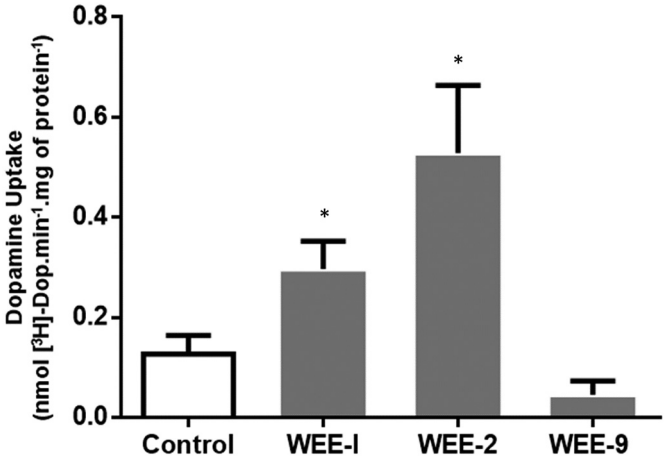

Fig. 2. Effect of weekly ethanol exposure on L-[^{3}H]dopamine uptake in zebrafish brain. The results represent the means ± S.E.M. (n = 6). The uptake activity values are expressed in nanomoles of dopamine labeled per minute per milligram of protein. The data were analyzed by one-way ANOVA followed by Tukey post hoc test, considering the statistical differences at a $P \leq 0.05$ level. * Significant difference from control group.

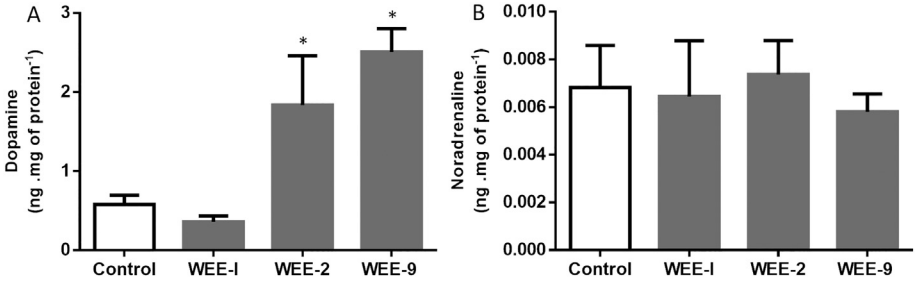

Fig. 3. Effect of weekly ethanol exposure on dopamine (A) and noradrenaline (B) levels in the zebrafish brain. Results expressed as mean ± S.E.M. (n = 6), each in triplicate, and are expressed in nanograms per milligram of protein. *p ≤ 0.05 compared with the control group (one-way ANOVA, followed by Tukey's post hoc test).

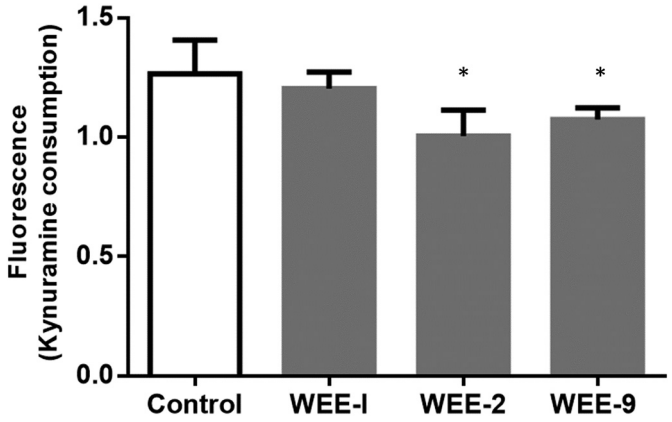

Fig. 4. Effect of weekly ethanol exposure on MAO activity in zebrafish brain. Results expressed as mean ± S.E.M. (n = 6), each in triplicate, and are expressed in nanograms per milligram of protein. The assay plate was incubated at 28 °C for 0.5–2 h, and the color result of the reaction was read on a microplate reader equipped with a 492-nm filter. *p ≤ 0.05 compared with the control group (one-way ANOVA, followed by Tukey's post hoc test).

There was no significant difference in noradrenaline levels ($F(3.19) = 0.1750$, $p = 0.9120$) (Fig. 3B). We also evaluated MAO activity as it is responsible for dopamine degradation. MAO activity decreased in the WEE-2 and WEE-9 groups ($F(3.28) = 6.122$, $p = 0.0025$) when compared to control (Fig. 4).

4. Discussion

This is the first study that evaluates the cerebral dopaminergic system and how this is susceptible to the effects of the alcoholic binge model through intermittent weekly ethanol exposure in zebrafish. Dopamine transporters are the main target of several drugs of abuse, including ethanol. When dopamine is released in the synaptic cleft, it binds to specific presynaptic and postsynaptic receptors. The re-uptake of dopamine via DAT is the primary mechanism for the regulation of extracellular dopamine levels for dopaminergic receptors. In zebrafish, DAT-related genes have previously been identified (Holzschuh et al., 2001; Ryu et al., 2006; Bai and Burton, 2009). However, there are no studies analyzing the functionality of the transporters responsible for dopamine uptake. Pharmacologically, compounds can be used to identify and characterize this type of transporter. In cultured ventricular mesencephalic cells, dopamine uptake was performed and importantly potentially inhibited by the selective DAT blocker, GBR 12909 (Prasad and Amara, 2001). Another classic inhibitor of DAT is bupropion, which inhibits dopamine reuptake (Stahl et al., 2004). Therefore, we used bupropion to inhibit DAT and thereby standardize incubation conditions for dopamine uptake in the zebrafish brain. This is the first study to demonstrate the activity of DAT through the incubation of labeled dopamine uptake in the zebrafish brain, where bupropion was used as a positive control inhibiting dopamine uptake.

Weekly ethanol exposure altered dopamine transport in the zebrafish brain. Our study found an increase in DAT function in the WEE-I and WEE-2 groups relative to control. In contrast, WEE-9 showed a reduction, similar to the control group. These results indicate that DAT function changes with respect to time after intermittent exposure to ethanol. DATs are involved in removing synaptic dopamine and thereby, controlling the levels of ligand for dopaminergic receptors. The D1 receptor family are coupled to the stimulatory G protein (Gs), which in turn activates adenylyl cyclase (AC) leading to an increased cyclic adenosine monophosphate (cAMP) level. The D2 auto-receptor located at the cell membrane regulates dopamine release by calcium signaling (Missale et al., 1998). Our results suggest that this distinct time dependent response in dopamine uptake could reflect a possible neuronal modulation via dopaminergic transmission through respective dopamine receptors. To date, studies on acute and chronic ethanol exposure in rodents have shown a potential link between DAT and ethanol; however, these results remain controversial. Chronic exposure to ethanol has been associated with increased rates of dopamine uptake in the monkey brain striatum (Budygin et al., 2003) and striatum slices in the brains of rats exposed to ethanol vapor (Budygin et al., 2007). Furthermore, ethanol in vitro potentiates dopamine uptake and increases cell surface distribution of dopamine transporters expressed in SK-N-SH and HEK-293 cells. In contrast, in some models, ethanol results in a decrease (Lin and Chai, 1995; Robinson et al., 2005) or no change (Budygin et al., 2000; Yim and Gonzales, 2000; Gonzales et al., 2004) in the capacity of dopamine uptake. Although the results are conflicting, many factors may influence the dopaminergic system during ethanol exposure, such as the dose, time, and frequency of consumption, as well as the period of deprivation.

Similar to ethanol, repeated withdrawal of cocaine causes significant increases in dopamine levels in zebrafish as well as a decrease in the expression of the dopamine transporter gene. Decreases in *dat* expression could result in a decline in DAT protein levels and may lead to decreases in presynaptic dopamine, which could result in increased tyrosine hydroxylase activity as a compensatory process to restore neurotransmitter vesicles. Thus, it is possible that ethanol withdrawal induces a cascade of DAT-dependent alterations (López Patiño et al., 2008; Tran et al., 2015). In this context, the understanding of DAT-mediated extracellular dopamine homeostasis may be relevant in the study of heavy alcohol consumption and deprivation.

We found an increase in total dopamine levels in the WEE-2 and WEE-9 groups compared to the control group, whereas, norepinephrine levels did not show a significant difference. These findings show that during the alcohol withdrawal period there are relevant changes in dopamine levels. Previous studies have already described an increase in dopamine levels in the zebrafish brain when exposed acutely to ethanol at 1.00% for 60 min (Chatterjee and Gerlai, 2009; Chatterjee et al., 2014; Tran et al., 2017). Two studies (Chatterjee et al., 2014; Gerlai et al., 2009) have previously evaluated consecutive chronic consumption of alcohol in zebrafish (0.00% or 0.50%) followed by acute administration of 0.00%, 0.50%, or 1.00%. Zebrafish that were pretreated with fresh water and exposed to alcohol at 0.50% presented

higher levels of dopamine when compared to those that were pre-exposed to chronic alcohol and submitted to the same concentration of alcohol (0.50%) acutely. The attenuated effect of the highest acute (1.0%) alcohol concentration on this neurotransmitter, may also indicate alcohol adaption to chronic pre-treatment.

MAO activity decreased in in the WEE-2 and WEE-9 groups compared to the control group. To our knowledge this is the first study to evaluate the activity of this enzyme after intermittent ethanol exposure in zebrafish. The only study in zebrafish that evaluates the activity of MAO after alcohol is that of Chatterjee et al. (2014), where the fish were subjected to increasing acute concentrations of alcohol (0.0%, 0.5%, and 1.0%), and a slight decrease in MAO activity was found at alcohol 0.5%. Our results show that this decrease is observed on the second and ninth day after the last exposure with a 1.4% alcohol concentration using an intermittent exposure model, suggesting that even in a period of alcohol withdrawal, enzyme activity is reduced. The reduction of MAO activity was concomitant with an increase in dopamine levels for the WEE-2 and WEE-9 groups. Since MAO is responsible for the degradation of monoamines in the central nervous system, our findings suggest that the accumulation of dopamine can be attributed to the reduction in MAO activity.

Matthews et al. (2018), using rodents chronically exposed to ethanol vapor, assessed MAO activity immediately after exposure, acute withdrawal (24 h), and prolonged withdrawal (four days and three weeks). Chronic exposure to ethanol vapor significantly increased MAO-A activity in the pre-frontal and anterior cingulate cortex after acute withdrawal (24 h), suggesting a causal relationship between acute alcohol withdrawal and high levels of MAO-A activity. These increased protein levels have been associated with higher levels of dysphoria and depressive episodes (Chiuccariello et al., 2014; Matthews et al., 2014). Recognition of this imbalance in zebrafish may reveal novel strategies for effective pharmacotherapies for the treatment of alcohol abuse and alcoholism. Ethanol's mechanism of action, and regulation of these mechanisms through other processes, are important to better understand neurochemical pathways of control and neuroadaptation to chronic ethanol treatment and may be targeted for development of future therapeutic approaches. More detailed investigation of the intracellular integrators and modulators of dopamine signaling will allow for better understanding of the mechanisms that could be involved in the effects of ethanol withdrawal and other associated neuropsychiatric disorders.

5. Conclusion

To our knowledge, this is the first study showing the functional activity of dopamine transport in the zebrafish brain, offering a new perspective for the study of dopamine signaling in this organism. Furthermore, intermittent exposure to weekly ethanol was able to promote changes in dopaminergic neurotransmission in zebrafish through changes in dopamine levels, monoamine activity, and dopamine transporters. Our results suggest that some dopaminergic parameters are altered after two and nine days from the last exposure to ethanol, resulting in modulation of this pathway. Therefore, regulatory mechanisms may play an important role in understanding the time window after tolerance to weekly alcohol exposure.

Declaration of competing interest

None.

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