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CELLULAR AND MOLECULAR BIOLOGY

Effects of moderate alcohol consumption on behavior and neural systems of Wistar rats

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Abstract: Chronic alcohol consumption affects various neurotransmitters, especially those implicated in the transitioning to alcohol use disorders (particularly dopaminergic and CRFergic systems). Few studies have investigated moderate alcohol consumption and its harmful consequences. The objective of this work was to analyze behavioral and neurochemical (dopaminergic and CRFergic systems) alterations during chronic moderate alcohol consumption. Twelve male Wistar rats were submitted to an intermittent alcohol ingestion protocol (alcohol group) for four weeks. The control group consisted of six rats. Open Field and Elevated Plus Maze tests were used for analysis of motor and anxiety-like behaviors. Immunohistochemistry analysis was performed in dopaminergic and CRFergic systems. Animals exposed to alcohol consumed moderate doses, chronic and intermittently. Behavioral tests detected fewer fecal *boli* in the alcohol exposed group, and immunohistochemical analysis indicated fewer dopamine-immunoreactive cells in the ventral tegmental area, and more CRF-immunoreactive cells in the anterior cingulate cortex and dorsolateral septum in this group. Thus we concluded that Wistar rats that consumed moderate doses of alcohol voluntarily and chronically showed a discreet anxiolytic effect in behavior, and a hypodopaminergic and hyperCRFergic neurochemical condition, which together are strong inducers of alcohol consumption predisposing to the development of alcohol use disorder (AUD).

Key words: alcohol, corticotrophin-releasing factor, dopamine, moderate consumption.

INTRODUCTION

According to the World Health Organization (World Health Organization 2018), 43% of the world population is currently drinkers (drink any amount of alcohol). Heavy episodic drinking (more than 60 grams of pure alcohol, or 6 standard alcoholic drinks, on one occasion or at least once a month) was prevalent in 18.2% of the world population, and 39.5% among drinkers in 2018 (World Health Organization 2018). This consumption pattern is related to extensive damage to the organism (Rehm et al. 2010). For a long time, low or moderate doses were seen as not-harmful or even protective in certain conditions (Lichtenstein et al. 2006), although recent research indicated that there is not a clear threshold between safe and harmful alcohol ingestion. Alcohol consumption even at low doses, accordingly to Global Burden of Disease Study 2016 (GBD 2016 Alcohol Collaborators 2018), can induce deleterious consequences to the organism (Amodeo et al. 2017, Burton & Sheron 2018).

Alcohol effects in the brain involve several neurotransmitter systems (Roberto & Varodayan 2017). The initial pleasurable response (positive reinforcement by consumption; stimulatory effect) is orchestrated mainly by the

dopaminergic system through the activation of the mesolimbocortical pathway that comprises the ventral tegmental area (VTA), nucleus accumbens (NAc), and distinct regions of the prefrontal cortex (PFC). Additionally, alcohol can help to cope with negative emotions (i.e. anxiety, dysphoria, etc.) that arise from environmental stress or periods of heavy alcohol withdrawal (negative reinforcement by consumption; anxiolytic effect). In this case, alcohol acts on the stress response system, which has the corticotrophin release factor (CRF) peptide as its main activator. Consequently, disbalances on the CRFergic system are critical to the emergence of alcohol use disorders (AUD) (Heilig 2007, Koob et al. 2014, Koob & Volkow 2016).

The dopaminergic superstimulation during alcohol consumption can promote impairments of the system after prolonged use and prompt compulsive alcohol consumption (Söderpalm & Ericson 2011). Moreover, the activation of CRF system by alcohol consumption can be trigered acutely and chronically, having long term effects (Allen et al. 2011). CRF activates the hypothalamic-pituitary-adrenal axis (classically known as the HPA axis) for the neuroendocrine stress response. But beyond that, different areas with extra-hypothalamic CRFergic neurons seem to contribute to behavioral and mental responses to stressors. Some of these areas have been linked to alcohol consumption such as the lateral septum (responsible for emotional reactivity), the amygdala (one of the centers of the stress response that is associated with the intensification of consumption), the bed nucleus of stria terminalis (BNST), and portions of PFC, such as the anterior cingulate cortex (ACC) (both involved in relapse) (Wscieklica et al. 2016).

The literature still lacks studies investigating low to moderate alcohol consumption. Therefore, to explore the effects of such doses of alcohol is important because the misconception that moderate drinking does not have detrimental consequences to the organism persists in society. The identification of biological effects during moderate drinking can help the development of preventive strategies before the subject advances to heavier drinking and AUD onset. Hence, we hypothesize that drinking moderate doses of alcohol could induce subtle alterations in brain circuits involved in posterior complications such as AUD. Thus, the objective of this work was to evaluate behavioral and neurophysiological responses in the dopaminergic and CRFergic systems during moderate chronic alcohol consumption in an animal model.

MATERIALS AND METHODS

Subjects

Eighteen male Wistar rats (CEDEME - Centro de Desenvolvimento de Modelos Experimentais para Biologia e Medicina, Universidade Federal de São Paulo - UNIFESP, Brazil), about 60 days old, weighing 240-310g, were kept under controlled environmental conditions (21 ± 1°C, dark-light cycle - 12h on-off cycle, 7:00 AM to 7:00 PM lights on) with food and water *ad libitum*. The experimental protocols were developed according to principles adopted by Brazilian Society of Neuroscience and Behavior, which are based on the conditions stated in "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources 1996) and approved by UNIFESP Research Ethics Committee (number 3482080218).

Animals were sorted into one per cage (for precise liquid ingestion control) in translucent cages side by side constituting the following groups:

Control group (CO): six rats were not subjected to alcohol ingestion, comprising the control group. Measurements of body mass,

water, and food ingestion were coincident with the other (experimental) group.

Alcohol group (AL): twelve rats had intermittent access to ethanol solution (20%, v/v) with the two-bottle choice paradigm (Carnicella et al. 2014, Wscieklica et al. 2016) on Mondays, Wednesdays, and Fridays during a 24h period (12h light and 12h dark cycle) during 4 weeks. The placement of alcohol bottle in the cage was switched with water bottle in every session, thus avoiding the influence of placement bias. Between sections of alcohol availability, two bottles of water were offered per cage. Fluids were administered in 200ml bottles with hermetically sealed lids, tightly secured on the cage walls. The difference in the sample sizes is to account for the variability of drinking behavior (Momeni & Roman 2014). The administration method is illustrated in Figure 1.

To control the aforementioned variables, every week (on Mondays and Fridays) each animal was weighed. Food ingestion was controlled by offering a known amount of food in the beginning of the week and weighing the residual at the end of the week. Water and alcohol were measured daily by weighing, before and after their administration. Solutions (alcohol and water) were double-checked by gauging its volumes also before and after their administration.

Procedures

Behavioral Tests

- Open Field (OF)

The Open Field (OF) test was performed on a round arena (60 cm of diameter), with the floor radially divided in inner and outer sections and also divided into 12 equal parts, its walls were 50 cm high, the apparatus was made of plexiglass. Tests were performed under 60 lux illumination and the apparatus was located in a separate room. Animals were briefly habituated to the testing room, out of the apparatus, for about 10 minutes before the test, subsequently, each animal was placed in the center of the apparatus for testing.

All the animals were subjected to OF in the morning (8:00 AM to 11:00 AM) after the last hours of the last session of the alcohol administration protocol. The test was performed to access motor parameters (line crossing) allowing inferences on anxiety-like behaviors, such as if the animals were more active in the center or the periphery

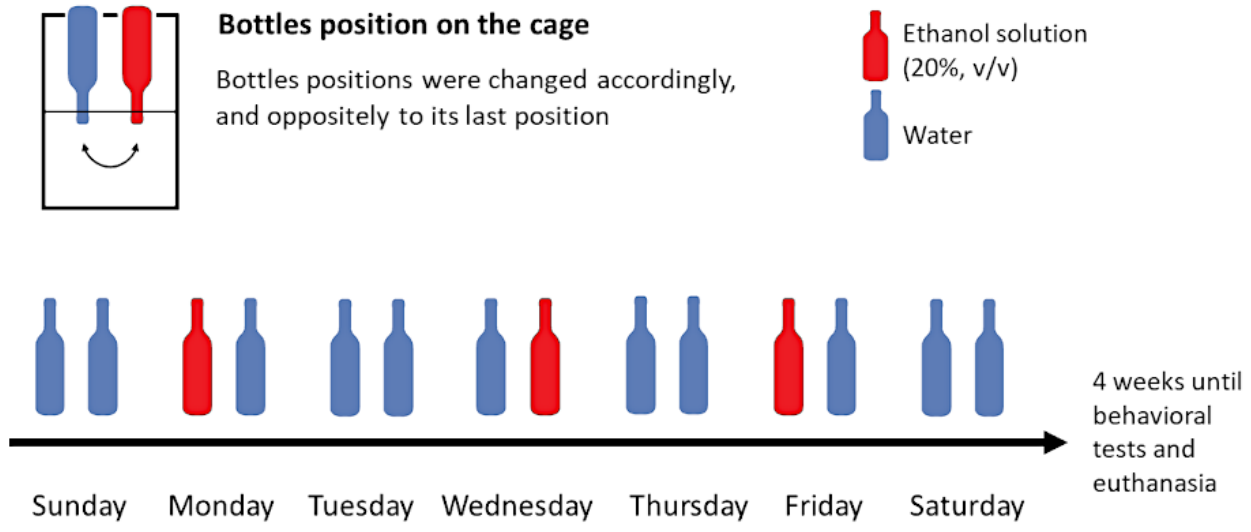

Figure 1. Representation of the alcohol ingestion protocol.

of the arena (the latter related to anxiety-like behavior), as well as the largest number of fecal *boli* (physiological parameter), rearing (standing on their hind legs, indicating vertical exploratory activity), and grooming (cleaning their own body, a stereotypical behavior) represent a greater degree of anxiety (Kliethermes 2005). The procedures were videorecorded and later assessed by a researcher blind to the assigned group of each rat.

- *Elevated Plus Maze (EPM)*

After the motor activity test, all animals were submitted to Elevated Plus Maze (EPM) test to evaluate the possible effects of chronic alcohol ingestion on the anxiety state of the animals and its relation to stress-related brain areas (Kliethermes 2005). The EPM is based on the principle that the animal avoids open areas and the confinement in the closed arms of the maze can be related to a state of anxiety. Therefore, the overall exploration of the maze was analyzed by the following behaviors. Number of entries and time spent on the closed or open arms, look outs (projecting only the head out of the maze arm), transitions between the arms, rearing, head dips (dipping of the head below the level maze floor), and grooming, (Pellow et al. 1985). The apparatus was located in another similar room where animals underwent 10 minutes of habituation before the test. At the beginning of the test each animal was placed in the center of the apparatus that was made of wood, painted in white, and each arm had the same dimensions (50 cm x 12 cm), both of the arms were enclosed by a 40 cm high wall.

In both tests, that occurred after the last session of alcohol exposure, each animal was evaluated for 5 minutes and entered the respective apparatus in random order, and after each animal finished the test the apparatuses were cleaned with alcohol 70% v.v. The procedures were videorecorded and later assessed by a researcher blind to the assigned group of each rat.

Anesthesia, Blood-Analysis, Perfusion, and Microtomy

Each animal, immediately after the behavioral test, was perfused in accordance with the protocol of Wscieklica et al. (2019). This procedure was performed after the last session of alcohol exposure. The animals were anesthetized by intraperitoneal injection of a dose of 1 ml/kg of ketamine (40 mg/kg), xylazine (20 mg/kg), fentanyl (0.3 to 0.5 mg/kg) and acepromazine (1ml/kg) mixture. Immediately before the perfusion, blood samples were collected from the heart of the animals (left ventricle) utilizing a syringe and transferring the blood samples to heparinized tubes. The blood alcohol concentration (BAC) was analyzed utilizing the Multiple Reagent Test NAH-ADH (Sigma Aldrich, EUA), exactly as described by Conte et al. (2019), hence assessing the alcohol availability in the blood. Subsequently, perfusion was performed with a 4% paraformaldehyde solution (from formaldehyde heated to 60-65 °C) in H_2O at 4°C, pH 9.5, for approximately 25 min. The brains were post-fixed for 1h in the same fixative solution and then stored in a solution of 30% sucrose for cryoprotection at 4 °C. Regularly sampled series (1 in 5) of 30µm-thick frozen sections were cut in the coronal plane and stored in ethylene glycol-based cryoprotectant at -20 °C.

Immunohistochemistry for Dopamine or CRF

To investigate immunoreactivity to dopamine, in its main producing area, the mesolimbocortical system, the ventral tegmental area (VTA; bregma: –5.04) was analyzed (Dahlstroem & Fuxe 1964). For analysis of immunoreactivity to CRF the main brain areas associated with emotional stress responses in the extra-hypothalamic system were analyzed: the amygdala and its divisions

(central, medial and basolateral; bregma: –2.76); bed nucleus of stria terminalis (BNST) (bregma: –0.60); lateral septum and its divisions (dorsal, intermediate, and ventral; bregma: +0.60); and anterior cingulate cortex (ACC) (bregma: +0.60) (Sawchenko et al. 2010). The ACC was used as a representative area of PFC in stress response since functional alterations in this area are a predictor of relapses in alcohol consumption (Zakiniaeiz et al. 2016).

Brain sections were subsequently submitted to anti-dopamine or anti-CRF immunohistochemistry protocol based on Wscieklica et al. (2016). Slices were incubated with anti-dopamine polyclonal antibody (rabbit; 1:500; MyBioSource; catalog number: 2007234, San Diego, USA), or with anti-CRF polyclonal (rabbit; 1:1000; Bioss, catalog number: bs-0246R-HRP, Massachusetts, USA). The slices were rinsed in a solution with hydrogen peroxide (0.3%; Sigma, St. Louis, MO, USA) before incubation with the primary antibody, to avoid interference of endogenous peroxidase. Tissue incubation with the primary antibody occurred in Triton-X solution to increase neuronal membrane permeability and normal goat serum to avoid nonspecific binding. In sequence, the slices were incubated with biotinylated anti-rabbit secondary antibody (goat; 1:1000; Vector Laboratories, Burlingame, CA, USA). Avidin-biotin (Vectastain Elite Vector Laboratories, Burlingame, CA, USA) were added and finally, the diaminobenzidine chromogen (DAB; 0.05%; Sigma) was applied to the tissue and amplified using nickel ammonium sulfate. After the immunoperoxidase, the tissue was mounted on gelatin-coated glass slides and dehydrated for microscopy.

Results were analyzed with a Zeiss – Axio Observer D1 microscope. Dopamine or CRF positive cells were blind analyzed utilizing Image Pro-Plus™ v. 6.0 software. The color spectrum utilized in the measurements was adjusted from dark brown to black and a fixed bi-dimensional area (approximately 500µm x 500µm) was adopted for each region of interest.

Statistical analysis

Group analyses of quantitative results were performed with the unequal variance *t*-test (Welch´s test) as recommended by Ruxton (2006), as it accounts for unequal variances and is equally robust as a Student-t test when variances are equal, or one-way or two-way analysis of variance (ANOVA). Whenever the normality assumption was violated, the Mann Whitney U test (or the appropriate correction) was performed (software JAMOVI v.1.2). P values lower than 0.05 ($p<0.05$) were considered statistically significant. Results are reported as mean ± standard deviation (SD) and effect size when applicable (Cohen´s d or eta-square).

RESULTS

Alcohol, blood alcohol concentration, food, water ingestion, and body weight

The mean alcohol consumption during the experimental period of the alcohol group was: 2.89 ± 1.15 g/kg/24h. The sphericity assumption was violated as indicated by Mauchly's test ($p = 0.045$); thus, the repeated measures one-way ANOVA underwent the Greenhouse-Geisser correction to verify if there were significant differences along time. No statistically significant variation along time was observed $F(1.85, 20.34) = 0.37$; $p = 0.67$; $\eta^2 = 0.01$.

Regarding the mean blood alcohol concentration, it was observed 77.70 ± 28.30 mg/dl in the AL group.

Concerning food intake during the experiment, the average consumption (per week) of CO and AL groups were, respectively: 199.75 ± 24.90 g, and 187.75 ± 23.42 g. Repeated measures two-way ANOVA only indicated an effect of time

[F (3, 48) = 11.08; p < 0.001; η^2: 0.25]. Tukey post-hoc indicated differences along all the weeks (p < 0.05), although no particular trend was observed.

Mean water consumption (per day) during the experiment was: 37.60 ± 5.88 ml, and 37.15 ± 12.33 ml for CO and AL groups, respectively. Repeated measures two-way ANOVA detected a significant difference along time [after the Greenhouse-Geiser correction, as Mauchly´s W test indicated a violation of the sphericity assumption, p = 0.01; F (1.95, 31.12) = 5.71; p = 0.008; η^2 = 0.15]. Tukey post-hoc identified the difference between the first (33.8 ± 4.69 ml), and last week (45.3 ± 15.4 ml; p = 0.009); and second (32.6 ± 8.54 ml; p = 0.002) and last weeks.

Mean mass of animals during the treatment period was: 302.00 ± 19.62 g, and 310.00 ± 30.87 g for CO and AL, respectively. Repeated measures two-way ANOVA revealed statistically significant differences through time [F (3,48) = 201.30; p < 0.001; η^2 = 0.45]. Tukey post-hoc indicated a progressive increase in their overall mass (first week: 266 ± 29.3 g, second week: 298 ± 27.8 g, third week: 316 ± 26.3 g, and fourth week: 337 ± 26.5 g; p < 0.001).

Behavioral tests: Open Field (OF) and Elevated Plus Maze (EPM)

Based on behavioral tests performed, the only statistically significant result was regarding the number of fecal *boli* during the OF test [CO group: 7.17 ± 3.19; AL group: 2.58 ± 2.43; Welch T (8.02) = 3.09; p = 0.01; d = 1.70]. The other parameters analyzed by these tests, such as stereotypical behaviors, were not statistically significant, as shown in Table I.

Table I. Parameters analyzed by the Open Field test and Elevated Plus Maze, in Control (CO) and Alcohol (AL) groups.

Test	Parameter	Control (n=6)	AL (n=12)
Open Field	Line Crossing	43.67 ± 15.05	40.08 ± 13.39
	Time on the periphery (sec.)	43.83 ± 49.41	31.50 ± 40.45
	Time on the center (sec.)	2.17 ± 0.75	2.58 ± 2.07
	Rearing	15.33 ± 7.68	20.25 ± 11.78
	Fecal *boli*	7.17 ± 3.19	2.58 ± 2.43*
	Grooming (sec.)	20.67 ± 17.91	19.50 ± 10.67
Elevated Plus Maze	Open arm entries	1.83 ± 2.78	3.58 ± 3.34
	Open arm look out	2.83 ± 4.02	4.67 ± 3.98
	Closed arm entries	8.33 ± 3.93	7.58 ± 3.42
	Closed arm look out	13.50 ± 3.93	12.33 ± 6.27
	Transitions	10.17 ± 6.49	11.17 ± 5.87
	Rearing	15.50 ± 5.20	16.42 ± 7.34
	Head dipping	0.66 ± 1.63	1.33 ± 1.78
	Time on open arm (sec.)	17.83 ± 29.97	32.58 ± 32.00
	Time on closed arm (sec.)	134.67 ± 143.85	193.67 ± 116.54
	Grooming (sec.)	15.33 ± 8.54	10.00 ± 11.78

Mean ± SD; * p < 0.05.

Dopamine and CRF immunoreactivity

Data from immunohistochemical analysis from dopaminergic neurons (dopamine-ir) of VTA, indicated that AL group showed significantly less immunoreactive cells than animals of CO group [528.00 ± 246.00 and 893.00 ± 341.00 respectively; Welch T (7.71) = 2.33; p = 0.04; d = 1.31], as can be observed in Figure 2.

Regarding the immunoreactive cells to CRF (CRF-ir) a significant increase was detected in the AL group when compared to CO group in the anterior cingulate cortex; CO: 226.00 ± 29.00, and AL: 567.00 ± 225.00; as Shapiro-Wilk test indicated a violation of normality (p = 0.001), Mann-Whitney U test was performed, U = 0; p = 0.002; d = - 1.78. Another region with similar result was the dorsolateral septum; CO: 176.0 ± 27.2, AL: 293.0 + 121.0; Welch T (13.29) = - 3.17; p = 0.007; d = - 1.12. These are shown in Figure 3. These analyses were performed with a control group sample of 5 due to mechanical damage during the immunohistochemical process.

DISCUSSION

In this model of non-invasive chronic alcohol intake, we aim to mimic alcohol consumption in moderate doses using a voluntary alcohol ingestion protocol in Wistar rats that are genetically heterogeneous and typically do not drink large amounts of alcohol (Palm et al. 2011). The animals consumed a stable and moderate amount of alcohol, then to exclude any animal due to high drinking behavior was not necessary (Leeman et al. 2010). The animals showed slightly higher levels of alcohol in the blood compared to other studies, an effect that can be explained by the fact that blood collection occurred at the end of the dark cycle, the phase of greatest consumption, and also by the often-inconsistent nature of these analyzes (Crabbe et al. 2011, Dilley et al. 2018). However, the mean BAC of 77.07 mg/dl (0.07%), (and consequently 2.89 g/Kg/24h of ingested alcohol) is comparable to the blood alcohol level considered legal for humans to drive in various countries. This BAC level, in humans, is related to euphoria, loss of inhibition and impairment of some motor skills (Dasgupta 2017). Therefore, alcohol intake levels

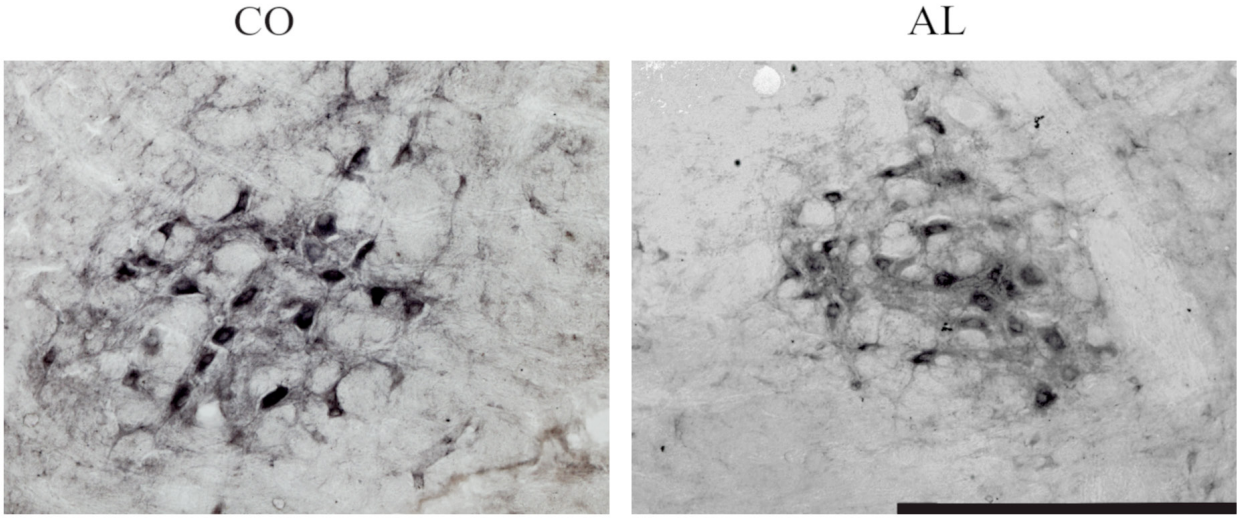

Figure 2. Dopamine immunoreactive cells in the VTA (black coloring) of animals in the following groups: Control (CO), and Alcohol (AL). Magnification: 200x; Scale bar: 300 µm.

and blood availability achieved in the current study are similar to those pursued by humans that drank moderately and avoided intoxication.

The food and water consumption, as well as animal weight, did not differ between the groups indicating any effect of alcohol consumption in these parameters. Although the differences in time were significant, they were expected. Also, the behavioral tests indicated fewer fecal *boli* in the alcohol group, which can be interpreted as an anxiolytic effect (Archer 1973). Alcohol has anxiolytic properties and all analyses were performed with animals under the chronic effects of the substance and not during the withdrawal phase as confirmed by the BAC levels. Motor behavior showed no difference between groups, which is in line with the defecation result of a mild anxiolytic effect (Lister 1990, Wegener et al. 1991, Palasciano et al. 1995). The other behavioral parameters could not detect any further alterations.

The mesocorticolimbic system undergo adaptative alterations after chronic alcohol exposure. During the ingestion of low or high doses of alcohol, there is an acute increase in the expression of dopamine in VTA (You et al. 2018), while chronic and intermittent high consumption (10/g/kg/day) can lead to decreased neuronal

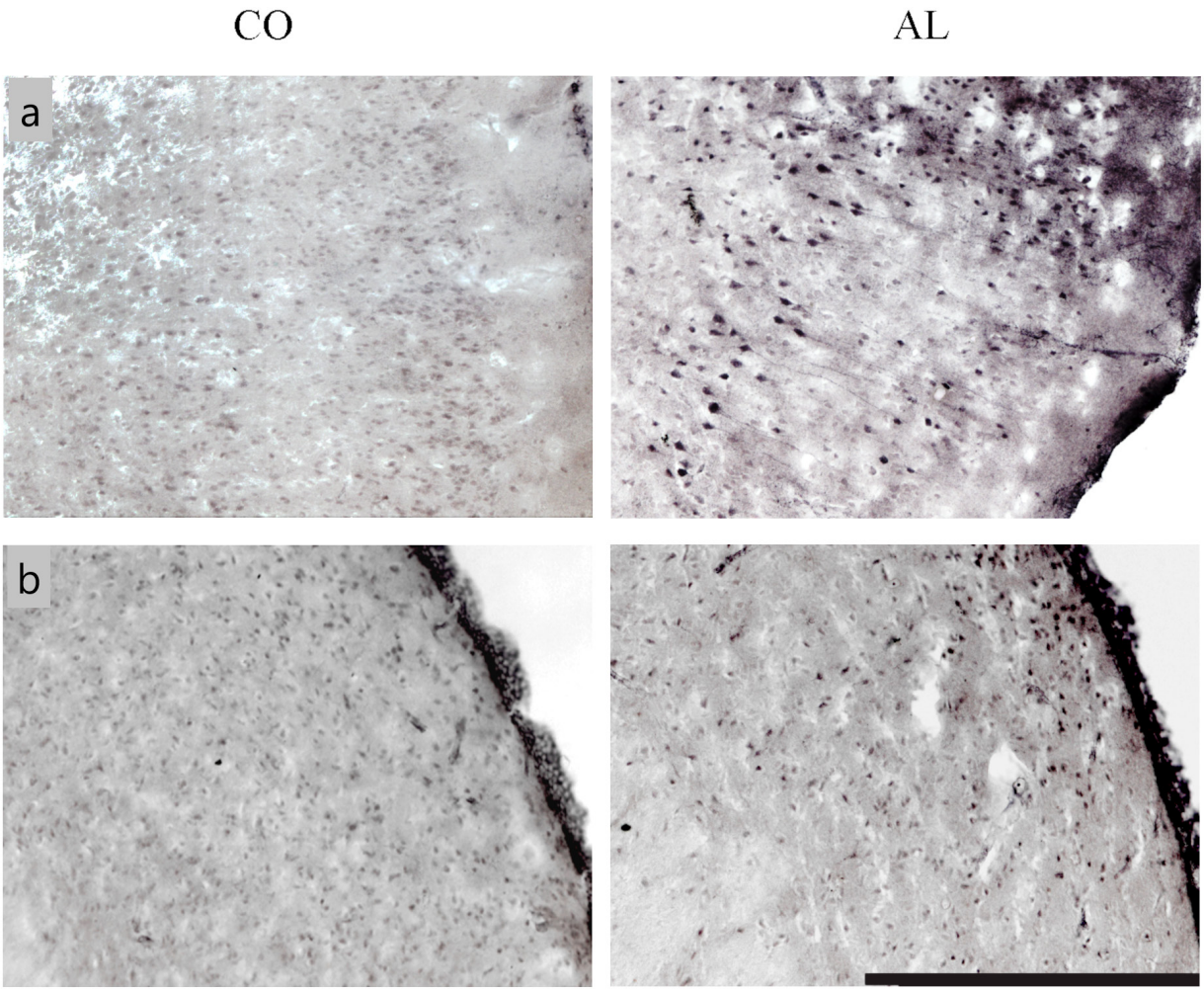

Figure 3. CRF immunoreactive cells (black coloring) in the following areas: (a) anterior cingulate cortex, (b) dorsolateral Septum, from animals in the following groups: Control (CO), and Alcohol (AL). Magnification: 200x.; Scale bar: 300 µm.

activity in VTA 3 to 6 weeks after the end of treatment (Shen et al. 2007), which is in accordance with our results, although our data indicate this effect with moderate consumption. Therefore, the reduced dopaminergic activity in this region may be responsible for the transition to AUD, since the drug could be utilized to attenuate negative effects of unpleasant stimuli, thus coping with lower dopamine levels (Koob et al. 2014, Nutt et al. 2015). It seems that the dopaminergic system suffers a tolerance or overload effect after the initial stages of consumption even when consuming moderate doses, culminating to blunted levels of neurotransmitter. Thus, we can understand that after the acute phase, chronic intake leads to a hypodopaminergic condition, even, as we have shown in this study, in moderate doses. But this phenomenon requires further investigation (Charlet et al. 2011). However, it appears that the hypodopaminergic condition can promote the maintenance of alcohol consumption, even in moderate doses.

Studies show that activation of CRF system occurs during alcohol consumption, not only during withdrawal and during its adverse consequences (Quadros et al. 2016). Generally, alcohol seeking behaviors are induced, partly, by the activity of CRF system in specific regions of the brain involved in cognition and emotional control (Anthenelli 2012, Blaine & Sinha 2017). In this study, the CRFergic immunoreactivity was significantly higher in the anterior cingulate cortex of the alcohol group. This region is responsible for the integration of various circuits to perform executive functions, such as controlling impulsive behaviors (Vassena et al. 2017). It is particularly affected by alcohol consumption (Yang et al. 2016), as shown by recent work from our group, where we reported a volume reduction of this area in rats that consumed alcohol in high doses (Conte et al. 2019). This effect may be associated with the hyperregulation of stress related neurons of this area that in general has a degenerative effect on the nervous system. There is a considerable distribution of CRF receptors (Potter et al. 1994)

Table II. Number of immunoreactive cells to dopamine and CRF in the analyzed brain areas in Control (CO), and Alcohol (AL) groups.

Neurotransmitter System	Brain Areas	CO (n=6)	AL (n=12)
Dopamine	Ventral Tegmental Area	893.00 ± 341.00	528.00 ± 246.00*
CRF	Anterior Cingulate Cortex	226.00 ± 29.00	567.00 ± 225.00*
	Central Amygdala	686.00 ± 46.30	1068.0 ± 447.00
	Medial Amygdala	264.00 ± 75.00	363.00 ± 106.00
	Basolateral Amygdala	229.00 ± 60.9	340.00 ± 198.00
	Dorsolateral Septum	176.00 ± 27.20	293.00 ± 121.00*
	Intermediate Septum	191.00 ± 63.30	186.00 ± 66.60
	Ventrolateral Septum	214.00 ± 41.10	163.00 ± 81.70
	BNST	284.00 ± 123.20	385.00 ± 117.00

Mean ± SD; * $p < 0.05$.

and immunoreactive cells (Swanson et al. 1983) in the cingulate cortex, and few studies have analyzed this neurotransmitter in this brain area, during alcohol exposure, especially in animals consuming moderate doses. Data from the current study indicated a significant increase of the neuropeptide after moderate chronic alcohol consumption. It is important to point out that the analysis reflects the chronic alcohol intake phase and not the withdrawal phase or with high doses, as has been reported (Mohila & Onn 2005, Sommer et al. 2008, Roberto et al. 2017).

The dorsolateral septum also presented similar results to ACC. This region (lateral septum) is especially important in relation to affective and motivated behaviors, as it functions as a center for the integration of sensory and behavioral stimuli from cortical regions, amygdala, hippocampus, hypothalamus and mesocortical dopaminergic system, among others. Its role in anxiety, fear, mood, and substance use disorders are intricate (Sheehan et al. 2004) and there are few studies with this particular region, especially involving CRF system and substance use. The higher amount of immunoreactive CRF cells in this region is interesting as animals were ingesting alcohol voluntarily, and the region regulates motivational, contextual, and stress behaviors (Besnard et al. 2019). Conversely, the hyperactivation observed may be involved in drug seeking behavior, not only for the stressful effects of abstinence since our protocol is of chronic drug supply (Calfa et al. 2006, Singewald et al. 2011, Scalize Hirata et al. 2019), but by the drug effect itself in an area involved with motivated behaviors. This specific region also has shown responsiveness to anti-depressant treatments (Sheehan et al. 2004) indicating a role in mood control, which is related to alcohol consumption. The lack of studies about the regions made it difficult to come to further conclusions.

Limitations of this study include the use of only male rats, which prevented the evaluation of these effects on females. There was no temporal analysis performed, as this study was cross-sectional, and longitudinal studies are encouraged to address the progressive effects of moderate doses of alcohol. Also, analyzing different levels of alcohol voluntarily consumed may help to understand how changes in the brain dopaminergic and CRFergic systems are related to the amount of alcohol ingested.

CONCLUSIONS

Through this study, we concluded that Wistar rats that consumed moderate doses of alcohol voluntarily and chronically showed a discreet anxiolytic effect in behavior, and a hypodopaminergic and hyperCRFergic neurochemical condition, which together are strong inducers of alcohol consumption predisposing to the development of alcohol use disorder (AUD).

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REFERENCES

ALLEN CD, LEE S, KOOB GF & RIVIER C. 2011. Immediate and prolonged effects of alcohol exposure on the activity of the hypothalamic–pituitary–adrenal axis in adult and adolescent rats. Brain Behav Immun 25: S50-S60.

AMODEO LR, WILLS DN & EHLERS CL. 2017. Acute low-level alcohol consumption reduces phase locking of event-related oscillations in rodents. Behav Brain Res 330: 25-29.

ANTHENELLI RM. 2012. Overview: Stress and alcohol use disorders revisited. Alcohol Res Heal 34: 387-390.

ARCHER J. 1973. Tests for emotionality in rats and mice: a review. Anim Behav 21: 205-235.

BESNARD A ET AL. 2019. Dorsolateral septum somatostatin interneurons gate mobility to calibrate context-specific behavioral fear responses. Nat Neurosci 22: 436-446.

BLAINE SK & SINHA R. 2017. Alcohol, stress, and glucocorticoids: From risk to dependence and relapse in alcohol use disorders. Neuropharmacology 122: 136-147.

BURTON R & SHERON N. 2018. No level of alcohol consumption improves health. Lancet 392: 987-988.

CALFA G, VOLOSIN M & MOLINA VA. 2006. Glucocorticoid receptors in lateral septum are involved in the modulation of the emotional sequelae induced by social defeat. Behav Brain Res 172: 324-332.

CARNICELLA S, RON D & BARAK S. 2014. Intermittent ethanol access schedule in rats as a preclinical model of alcohol abuse. Alcohol 48: 243-252.

CHARLET K, BECK A & HEINZ A. 2011. The Dopamine System in Mediating Alcohol Effects in Humans. Curr Top Behav Neurosci 13: 461-488.

CONTE R, LADD FVL, LADD AABL, MOREIRA AL, LE SUEUR-MALUF L, VIANA MB & CÉSPEDES IC. 2019. Behavioral and stereological analysis of the prefrontal cortex of rats submitted to chronic alcohol intake. Behav Brain Res 362: 21-27.

CRABBE JC, HARRIS RA & KOOB GF. 2011. Preclinical studies of alcohol binge drinking. Ann NY Acad Sci 1216: 24-40.

DAHLSTROEM A & FUXE K. 1964. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. Acta Physiol Scand Suppl 232: 1-55.

DASGUPTA A. 2017. Alcohol a double-edged sword. Alcohol, Drugs, Genes Clin Lab, p. 1-21.

DILLEY JE, NICHOLSON ER, FISCHER SM, ZIMMER R & FROEHLICH JC. 2018. Alcohol Drinking and Blood Alcohol Concentration Revisited. Alcohol Clin Exp Res 42: 260-269.

GBD 2016 ALCOHOL COLLABORATORS. 2018. Alcohol use and burden for 195 countries and territories, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 6736: 1-21.

HEILIG MKGF. 2007. A key role for corticotropin-releasing factor in alcohol dependence. Trends Neurosci 30: 399-406.

INSTITUTE OF LABORATORY ANIMAL RESOURCES. 1996. Guide for the Care and Use of Laboratory Animals, Commission on Life Sciences. National Research Council.

KLIETHERMES CL. 2005. Anxiety-like behaviors following chronic ethanol exposure. Neurosci Biobehav Rev 28: 837-850.

KOOB GF ET AL. 2014. Addiction as a stress surfeit disorder. Neuropharmacology 76: 370-382.

KOOB GF & VOLKOW ND. 2016. Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatry 3: 760-773.

LEEMAN RF, HEILIG M, CUNNINGHAM CL, STEPHENS DN, DUKA T & O'MALLEY SS. 2010. Ethanol consumption: how should we measure it? Achieving consilience between human and animal phenotypes. Addict Biol 15: 109-124.

LICHTENSTEIN AH ET AL. 2006. Diet and lifestyle recommendations revision 2006: A scientific statement from the American heart association nutrition committee. Circulation 114: 82-96.

LISTER RG. 1990. Ethologically-based animal models of anxiety disorders. Pharmacol Ther 46(3): 321-340.

MOHILA CA & ONN SP. 2005. Increases in the density of parvalbumin-immunoreactive neurons in anterior cingulate cortex of amphetamine-withdrawn rats: evidence for corticotropin-releasing factor in sustained elevation. Cereb Cortex 15(3): 262-274.

MOMENI S & ROMAN E. 2014. Subgroup-dependent effects of voluntary alcohol intake on behavioral profiles in outbred Wistar rats. Behav Brain Res 275: 288-296.

NUTT DJ, LINGFORD-HUGHES A, ERRITZOE D & STOKES PRA. 2015. The dopamine theory of addiction: 40 years of highs and lows. Nat Rev Neurosci 16: 305-312.

PALASCIANO G, PORTINCASA P, CIAULA AD & PALMIERI V. 1995. Prolonged consumption of moderate doses of alcohol and in vitro gastro-duodenal and ileal contractility in the rat. Eur Clin Investig 25: 171-175.

PALM S, ROMAN E & NYLANDER I. 2011. Differences in voluntary ethanol consumption in Wistar rats from five different suppliers. Alcohol 45: 607-614.

PELLOW S, CHOPIN P, FILE SE & BRILEY M. 1985. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14: 149-167.

POTTER E, SUTTON S, DONALDSON C, CHEN R, PERRIN M, LEWIS K, SAWCHENKO PE & VALE W. 1994. Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. Proc Natl Acad Sci 91: 8777-8781.

QUADROS IMH, MACEDO GC, DOMINGUES LP & FAVORETTO CA. 2016. An update on CRF mechanisms underlying alcohol use disorders and dependence. Front Endocrinol (Lausanne) 7: 134.

REHM J. 2010. Alcohol Consumption and Burden of Disease - an Overview. Addiction 105: 817-843.

ROBERTO M, SPIERLING SR, KIRSON D & ZORRILLA EP. 2017. Corticotropin-Releasing Factor (CRF) and Addictive Behaviors. Int Rev Neurobiol 136: 5-51.

ROBERTO M & VARODAYAN FP. 2017. Synaptic targets: Chronic alcohol actions. Neuropharmacology 122: 85-99.

RUXTON GD. 2006. The unequal variance t-test is an underused alternative to Student's t-test and the Mann-Whitney U test. Behav Ecol 17: 688-690.

SAWCHENKO PE, YUAN ZF, LAPLANTE F, RISSMAN RA & BITTENCOURT JC. 2010. Corticotropin-releasing hormone: Integration of adaptive responses to stress. Encycl Neurosci (in press): 239-245.

SCALIZE HIRATA RY, DOS SANTOS TB, DE ANDRADE JS, LE SUEUR MALUF L, ANTUNES HKM, BRITTO LRG, CÉSPEDES IC & VIANA MB. 2019. Chronic corticosterone increases ΔFOSB and CRFR1 immunoreactivity in brain regions that modulate aversive conditioning. Behav Brain Res 356: 107-119.

SHEEHAN TP, CHAMBERS RA & RUSSELL DS. 2004. Regulation of affect by the lateral septum: Implications for neuropsychiatry. Brain Res Rev 46: 71-117.

SHEN RY, CHOONG KC & THOMPSON AC. 2007. Long-Term Reduction in Ventral Tegmental Area Dopamine Neuron Population Activity Following Repeated Stimulant or Ethanol Treatment. Biol Psychiatry 61: 93-100.

SINGEWALD GM, RJABOKON A, SINGEWALD N & EBNER K. 2011. The modulatory role of the lateral septum on neuroendocrine and behavioral stress responses. Neuropsychopharmacology 36(4): 793-804.

SÖDERPALM B & ERICSON M. 2011. Neurocircuitry Involved in the Development of Alcohol Addiction: The Dopamine System and its Access Points, in: Brain Imaging in Behavioral Neuroscience, 127-161 p.

SOMMER WH, RIMONDINI R, HANSSON AC, HIPSKIND PA, GEHLERT DR, BARR CS & HEILIG MA. 2008. Upregulation of Voluntary Alcohol Intake, Behavioral Sensitivity to Stress, and Amygdala Crhr1 Expression Following a History of Dependence. Biol Psychiatry 63: 139-145.

SWANSON LWW, SAWCHENKO PEE, RIVIER J & VALE WWW. 1983. Organization of Ovine Corticotropin-Releasing Factor Immunoreactive Cells and Fibers in the Rat Brain: An Immunohistochemical Study. Neuroendocrinology 36: 165-186.

VASSENA E, HOLROYD CB & ALEXANDER WH. 2017. Computational models of anterior cingulate cortex: At the crossroads between prediction and effort. Front Neurosci 11: 1-9.

WEGENER M. 1991. Gastrointestinal transit of solid-liquid meal in chronic alcoholics. Dig Dis Sci 36(7): 917-923.

WORLD HEALTH ORGANIZATION. 2018. Global status report on alcohol and health 2018, Global status report on alcohol. Available in: https://www.who.int/substance_abuse/publications/global_alcohol_report/gsr_2018/en/, accessed in 23/06/2020.

WSCIEKLICA T, LE SUEUR MALUF L, PREARO L, CONTE R, VIANA MB & CÉSPEDES IC. 2019. Chronic intermittent ethanol administration differentially alters DeltaFosB immunoreactivity in cortical-limbic structures of rats with high and low alcohol preference. Am J Drug Alcohol Abuse 45(3): 264-275.

WSCIEKLICA T, VIANA MB, LE SUEUR MALUF L, POUZA KCP, SPADARI RC & CÉSPEDES IC. 2016. Alcohol consumption increases locomotion in an open field and induces Fos-immunoreactivity in reward and approach/withdrawal-related neurocircuitries. Alcohol 50: 73-82.

YANG X, TIAN F, ZHANG H, ZENG J, CHEN T, WANG S, JIA Z & GONG Q. 2016. Cortical and subcortical gray matter shrinkage in alcohol-use disorders: a voxel-based meta-analysis. Neurosci Biobehav Rev 66: 92-103.

YOU C, VANDEGRIFT B & BRODIE MS. 2018. Ethanol actions on the ventral tegmental area: novel potential targets on reward pathway neurons. Psychopharmacology 235: 1711-1726.

ZAKINIAEIZ Y, SCHEINOST D, SEO D, SINHA R & CONSTABLE RT. 2016. Cingulate cortex functional connectivity predicts future relapse in alcohol dependent individuals. NeuroImage Clin 13: 181-187.

Abbreviations

ACC – Anterior Cingulate Cortex
AL – Alcohol (group)
ANOVA – Analysis of Variance
AUD – Alcohol Use Disorders
BAC – Blood Alcohol Concentration
BNST – Bed Nucleus of Stria Terminalis
CEDEME – Centro de Desenvolvimento de Modelos Experimentais para Biologia e Medicina
CO – Control (group)
CRF – Corticotrophin Release Factor
DAB – Diaminobenzidine
EPM – Elevated Plus Maze
HPA – Hypothalamic-pituitary-adrenal (axis)
NAc – Nucleus Accumbens
OF – Open Field
PFC – Pre-Frontal Cortex
SD – Standard Deviation
VTA – Ventral Tegmental Area

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