



Research report

Voluntary alcohol intake after noise exposure in adolescent rats: Hippocampal-related behavioral alterations

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ABSTRACT

Different physical or chemical agents, such as noise or alcohol, can induce diverse behavioral and biochemical alterations. Considering the high probability of young people to undergo consecutive or simultaneous exposures, the aim of the present work was to investigate in an animal model if noise exposure at early adolescence could induce hippocampal-related behavioral changes that might be modified after alcohol intake.

Male Wistar rats (28-days-old) were exposed to noise (95–97 dB, 2 h). Afterwards, animals were allowed to voluntarily drink alcohol (10% ethanol in tap water) for three consecutive days, using the two-bottle free choice paradigm. After that, hippocampal-related memory and anxiety-like behavior tests were performed.

Results show that whereas noise-exposed rats presented deficits in habituation memory, those who drank alcohol exhibited impairments in associative memory and anxiety-like behaviors. In contrast, exposure to noise followed by alcohol intake showed increases in exploratory and locomotor activities as well as in anxiety-like behaviors, unlike what was observed using each agent separately. Finally, lower levels of alcohol intake were measured in these animals when compared with those that drank alcohol and were not exposed to noise.

Present findings demonstrate that exposure to physical and chemical challenges during early adolescence might induce behavioral alterations that could differ depending on the schedule used, suggesting a high vulnerability of rat developing brain to these socially relevant agents.

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1. Introduction

Developing Central Nervous System (CNS) can be affected by different exogenous agents (Guerra and Pascual, 2010; White and Swartzwelder, 2005; Wille-Bille et al., 2017; Willing and Juraska, 2015). In particular, consumption of alcohol and other recreational drugs usually begins during adolescence, a developmental stage defined in humans as the second decade of life (Chan et al.,

2016). In rodents, adolescence has a number of similarities with that of humans, being a highly vulnerable period likely to be modified by environmental challenges. In consequence, the use of an experimental model of adolescent rats might be useful to study the effects of alcohol intake at this stage (Bell et al., 2017; Spear, 2000). In this species, the time interval from weaning to early adulthood runs between postnatal day (PND) 21 and PND56, in which the lapse between PND21 and PND28 corresponds to pre-adolescence, between PND28 and PND34 to early adolescence, between PND34 and PND46 to mid-adolescence and between PND46 and PND56 to late adolescence (García-Burgos et al., 2009; Han et al., 2012; Lupien et al., 2009; Spear, 2015).

Ethanol, a type of alcohol that is a common ingredient of different drinks, is a chemical agent that has been considered to be especially hazardous because of its link with various health conditions (Chan et al., 2016). The 2007 National Survey on Drug Abuse and

Abbreviations: CNS, Central Nervous System; EPM, Elevated plus maze; IA, Inhibitory avoidance; OF, Open Field; PND, Postnatal Day; Trx1, Thioredoxin-1.

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Health in the USA reported that 16% of adolescents between 12 and 17 years were considered ethanol consumers (Amodio et al., 2017). *In vitro* and *in vivo* animal studies reported different effects of alcohol on various tissues depending on the period of development in which it is administered, the form of administration (continuous or intermittent) and the levels of alcohol intake (Desikan et al., 2014; Evrard et al., 2006; Fernandez et al., 2016; Little et al., 1996; Odeon et al., 2015; Sircar and Sircar, 2005; White et al., 2002; White and Swartzwelder, 2005). These studies provided evidence of the vulnerability of the young brain to this drug as well as of the behavioral consequences derived from its excessive consumption (Carpenter-Hyland and Chandler, 2007; Chandler, 2003; Crews et al., 2000; Pascual et al., 2007; Spear, 2000).

On the other hand, environmental noise is a physical agent that could be dangerous for human health, although its effects have been largely underestimated. Certainly, individuals coexist with and are influenced by a wide variety of noise sources coming from their surroundings including traffic, work background, as well as entertainment places (e.g., discos, concert venues, etc.). Estimations of the World Health Organization (WHO, 1999) showed that noise coming from the traffic above 65 dB of intensity –a level that is close to the minimum considered harmful (80 dB)– affects approximately 20% of the population and may cause different disorders that can alter not only auditory (Cappaert et al., 2000; Hu and Zheng, 2008; Saunders et al., 1985), but also extra-auditory structures located in the nervous, endocrine and/or cardiovascular systems (Basner et al., 2014; de Souza et al., 2015; Lenzi et al., 2003; Rabat, 2007; Turner et al., 2005; Uran et al., 2010, 2012, 2014).

Given that it should not be ignored that human adolescents drink alcohol before and throughout their stay in the discos, where the noise level is excessive and uncontrolled, experimental animal studies are required in order to elucidate whether combined exposures to noise and alcohol during adolescence could induce changes that would differ from those that might be produced if the agents would be presented individually.

Previously published results from our laboratory demonstrated that immature PND7 and PND15 animals were vulnerable to noise, affecting different behaviors (Molina et al., 2016a,b, Uran et al., 2010, 2012, 2014). However, no data of hippocampal-related behavioral effects of noise on adolescent rats have been obtained yet. Moreover, the effect of a combined exposure to noise with other agents (e.g., alcohol) has not been assessed until now.

Therefore, given the elevated susceptibility of the immature CNS to environmental and chemical agents and considering the high chance of human adolescents to be subject to simultaneous and/or consecutive exposures to noise and alcohol, the aim of this work was to investigate in an experimental model of adolescent animals if these agents could be able to modify different behavioral aspects, in particular diverse types of hippocampal-related memory and anxiety-like behaviors. The finding of alterations could be relevant not only to understand the mechanisms involved but also to contribute to the development of new therapeutic practices aimed to improve human health, especially of children and adolescents.

2. Results

2.1. Daily alcohol intake in alcohol-only and noise + alcohol groups

When total alcohol intake was measured, a significant decrease (in grams of alcohol taken per kg body weight per day, g/kg/day) was found in rats that were pre-exposed to noise when compared with alcohol-only rats (Fig. 2, $t = 2.2$, $p < .05$).

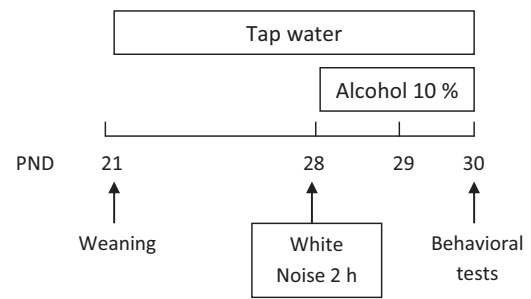


Fig. 1. Experimental design timeline. PND: postnatal day.

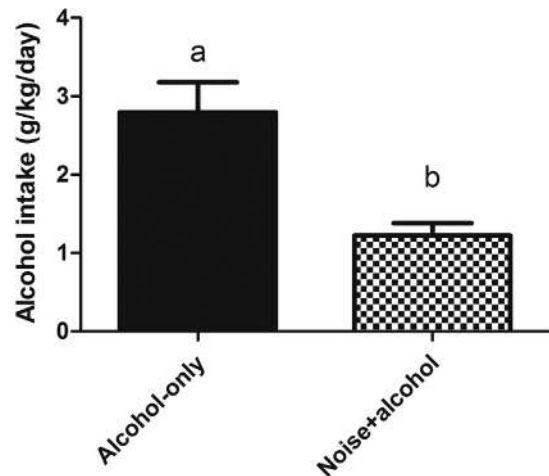


Fig. 2. Alcohol intake in alcohol-only and noise + alcohol groups. Filled bar: Alcohol-only group; dark-dotted bar: Noise + alcohol group. Total daily alcohol intake of PND28 animals pre-exposed to noise was significantly reduced when compared with non-exposed animals. Different letters (a and b) mean significant differences ($p < .05$). Data are expressed as a mean of grams of alcohol intake per body weight per day (g/kg/day) $\pm$ SEM, $n = 18$ for each group.

2.2. Number of lines crossed in the first and second sessions of the open field (OF) task

The number of lines crossed in an OF throughout two sessions of 5 min, separated by an interval of 1 h, might be taken as an index of short-term habituation to a new environment. Data show that rats from the alcohol-only and noise + alcohol groups as well as animals that did not receive alcohol and were not exposed to noise (sham-none group), reduced the number of lines crossed in the second session of the OF, whereas rats exposed to noise-only crossed the same number of lines in both sessions of the task (Fig. 3: Three-way ANOVA, $F_{7,43} = 5.03$, $p < .01$; Between factors: exposure (sham or noise), $F_{1,43} = 8.17$, $p < .01$; substance administered (none or alcohol), NS; within factor: session (1st or 2nd): $F_{1,43} = 20.25$, $p < .01$. Interactions were non-significant; post hoc comparisons: 1st session vs 2nd session: sham, noise + alcohol and alcohol-only, $p < .05$; noise, NS). Additionally, when the number of lines crossed in the first session was computed to quantify ambulation, a significant increase was found in noise + alcohol group when compared with the other groups (One-way ANOVA, $F_{3,19} = 4.76$, $p < .05$; post hoc comparisons, noise + alcohol vs. sham, noise or alcohol, $p < .05$).

2.3. Number of rearings made in the first session of the OF task

Exploratory activity was assessed in the OF by means of the number of elevations in the hind limbs (rearings) made in the first

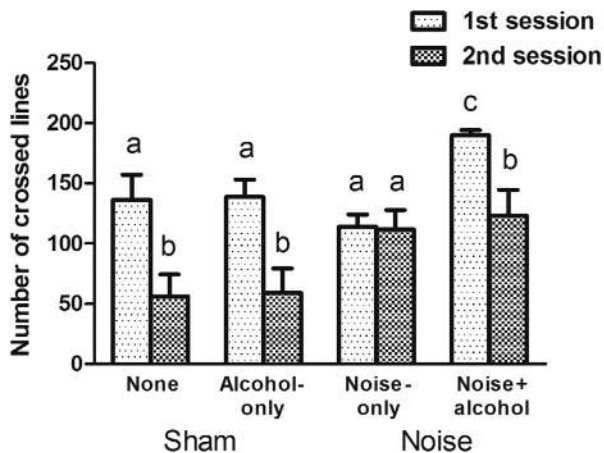


Fig. 3. Number of lines crossed measured in the OF task by sham and noise-exposed animals. Effect of alcohol intake. Clear-dotted bars: first session; dark-dotted bars: second session. PND28 animals exposed to noise-only did not reduce the number of lines crossed in the second session of the OF, whereas in the other groups this measurement was significantly decreased ($p < .05$). Different letters (a, b, c) mean significant differences ($p < .05$). Data are mean of the number of lines crossed $\pm$ SEM, $n = 6$ for each group.

session. Rats from the alcohol-only and noise-only groups made the same number of rearings than sham-none animals. However, when rats were subjected to the two-bottle free choice paradigm with 10% alcohol after exposure to noise (noise + alcohol group), a significant increase in the number of rearings was observed when compared with sham-none and alcohol-only intake group (Two-way ANOVA, $F_{3,19} = 11.27$, $p < .01$; Between factors: exposure (sham or noise), $F_{1,19} = 23.05$, $p < .01$; substance administered (none or alcohol), NS; post hoc comparisons, noise + alcohol vs. sham-none, $p < .01$; noise + alcohol vs. alcohol-only, $p < .01$, noise-alcohol vs. noise-only, $p < .01$, Fig. 4).

2.4. Latency to enter and number of entries to the open arms in the elevated plus maze (EPM) task

Open arms-related parameters measured in the EPM, such as the decrease in the latency to enter and an increase in the number of entries, are thought to be associated with a reduction of anxiety-

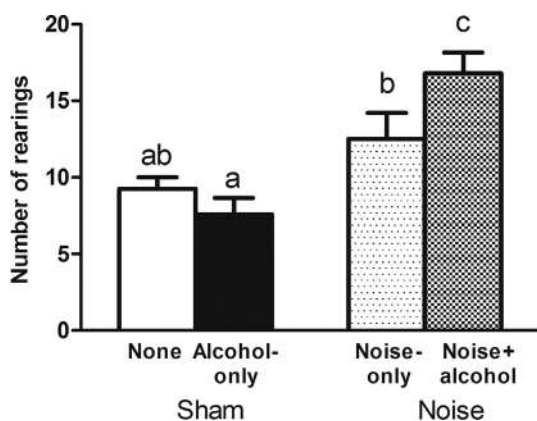


Fig. 4. Number of rearings (exploratory activity) measured in the OF task made by sham and noise-exposed rats. Effect of alcohol intake. PND28 rats exposed to noise-only or alcohol-only made similar number of rearings than sham animals that did not receive alcohol. In contrast, noise + alcohol animals significantly increased the number of rearings when compared to either rats exposed to noise-only or to alcohol-only. Open bars: sham-none; filled bars: alcohol-only; clear-dotted bars: noise-only; dark-dotted bars: noise + alcohol. Different letters (a, b, c) mean significant differences ($p < .05$). Data are mean $\pm$ SEM of the number of rearings, $n = 6$ for each group.

like behaviors. Fig. 5a shows a significant main effect on the latency to enter the open arms of the EPM (Two-way ANOVA, $F_{3,16} = 5.45$, $p < .01$, between factors: exposure (sham or noise), $F_{1,16} = 6.67$, $p < .05$; substance administered (none or alcohol), NS). As a significant interaction between these independent variables was observed ($F_{1,16} = 6.64$, $p < .05$), a simple effect statistical analysis was performed for sham (none and alcohol-only) and noise (noise-only and noise + alcohol) groups. Data show that animals from the alcohol-only group significantly decreased the latency to enter the open arms of the EPM task when compared with sham-none rats ($F_{1,5} = 101.56$, $p < .01$), whereas a significant increase in this measurement was observed in animals which drank alcohol after noise exposure (noise + alcohol group) when compared with noise-only animals ($F_{1,10} = 5.94$, $p < .05$; noise + alcohol vs. noise-only, $p < .05$). On the other hand, post hoc analyses showed no changes in this parameter in the noise-only group in comparison with sham-none animals, whereas data from noise + alcohol rats significantly differed from those of the alcohol-only group ($p < .01$). Fig. 5b shows a significant main effect on the number of entries to the open arms of the EPM (Two-way ANOVA, $F_{3,15} = 3.36$, $p < .05$, between factors: exposure (sham or noise), NS; substance administered (none or alcohol), NS). As a significant interaction between these independent variables was observed ($F_{1,15} = 6.96$, $p < .05$), a simple effect analysis was performed. Data showed that animals from the alcohol-only group made the same number of entries to the open arms of the EPM than sham-none animals. In contrast, those animals exposed to noise that afterwards drank alcohol (noise + alcohol group) showed a significant decrease in the number of entries to the open arms when compared to rats exposed to noise-only ($F_{1,8} = 12.44$, $p < .01$; noise + alcohol vs. noise-only, $p < .01$). Finally, post hoc comparisons showed that the number of entries to the open arms accomplished by animals that drank alcohol after noise exposure significantly differed from that of the animals from alcohol-only group ($p < .01$).

2.5. Latency to enter the dark compartment in the inhibitory avoidance (IA) task in retention and training sessions

In the IA task, T1 is defined as the time required to enter the dark compartment (i.e., the side in which an electric shock, an aversive stimulus, was delivered) in the training session and T2 is the time required to enter the same compartment in the retention session, after an interval of 1 h. T2/T1 ratio is the relationship between time in retention and training sessions and it might be taken as an index of associative memory. Fig. 6 shows a significant reduction in the ratio T2/T1 recorded in the IA task in animals from the alcohol-only group when compared with sham-none rats (Two-way ANOVA, $F_{1,17} = 3.25$, $p < .05$; between factors: exposure (sham or noise), NS; substance administered (none or alcohol), $F_{1,17} = 6.37$, $p < .05$; post hoc comparisons: alcohol-only vs. sham, $p < .05$).

2.6. Correlation between alcohol intake and behavioral outcomes

Correlation analyses were run between the levels of alcohol intake in all groups and the different behavioral parameters measured. Data showed a significant and negative correlation with the T2/T1 ratio ($R^2 = 0.23$, $p = .05$), whereas no significant correlations were observed with the other behavioral measurements (Supplementary Fig. 1).

3. Discussion

Present data demonstrate that PND30 rats exposed to noise at PND28 (95–97 dB, 2 h) experienced a deficit in habituation mem-

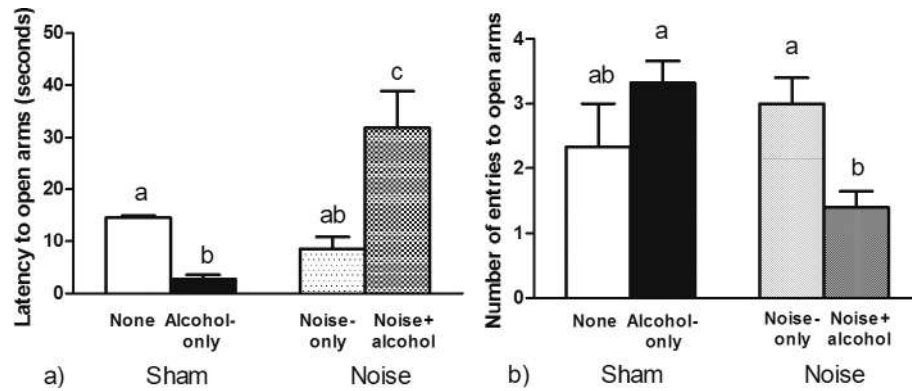


Fig. 5. Latency to enter and number of entries to the open arms measured in the EPM task in sham and noise-exposed rats. Effect of alcohol intake. a) PND28 alcohol-only animals significantly decreased the latency to enter the open arms of the EPM task when compared with sham-none rats. In contrast, a significant increase was observed in noise + alcohol animals when compared to either alcohol-only or noise-only animals. b) PND28 noise + alcohol animals significantly decreased the number of entries to the open arms when compared to rats exposed to either alcohol-only or noise-only. Open bars: sham-none; filled bars: alcohol-only; clear-dotted bars: noise-only; dark-dotted bars: noise + alcohol. Different letters (a, b, c) mean significant differences ($p < .05$). Data are mean $\pm$ SEM of the latency to enter the open arms or entries to open arms, $n = 6$ for each group.

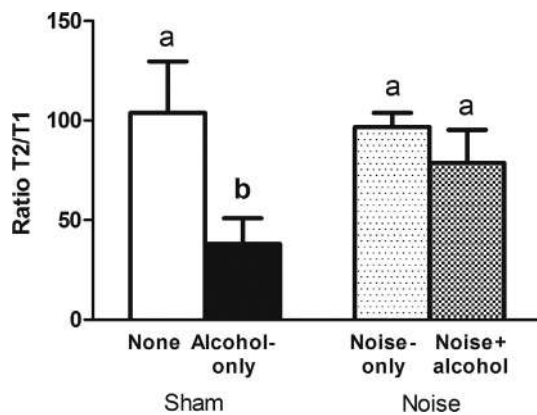


Fig. 6. Latency to enter the dark compartment measured in the IA task (ratio T2/T1) in sham and noise-exposed rats. Effect of alcohol intake. A significant reduction in the ratio T2/T1 recorded in the IA task in rats exposed to alcohol-only when compared to sham animals that did not receive alcohol was found. Open bars: sham-none; filled bars: alcohol-only; clear-dotted bars: noise-only; dark-dotted bars: noise + alcohol. Different letters (a, b) mean significant differences ($p < .05$). Data are mean $\pm$ SEM of the ratio T2/T1, $n = 6$ for each group.

ory, whereas *ad libitum* availability of 10% ethanol in drinking water for three consecutive days (between PND28 and PND30) triggered changes in associative memory and anxiety-related behaviors. Instead, exposure to noise at PND28 followed by voluntary alcohol intake for three days showed a different scenario from what was found in rats exposed to each agent separately: habituation and associative memory outcomes resulted similar to those of sham-none animals and significant increases in anxiety-like behavior and exploratory and ambulatory activities were observed in these animals. These changes could be related to the different levels of alcohol intake measured, as lower levels were observed in animals pre-exposed to noise when compared with alcohol-only rats. Of importance, the voluntary alcohol intake paradigm used in the present study results substantially less stressful in comparison with intragastric infusions or subcutaneous injections, which might interfere with the effects of alcohol during this critical period of development as well as with the behavioral assessments.

In particular, the behavior displayed in an OF task is considered to be the result of two conflicting drives: curiosity and fear, which might underlie habituation to a new environment. This behavior is recognized as one of the most basic forms of learning, in which

exploration and/or ambulation, that has been shown to be decreased as a function of a repeated exposure to the same environment, could be taken as an index of habituation memory. This type of memory is usually studied in two or more brief sessions in an OF and is known to depend on hippocampal integrity (Vianna et al., 2000). Previous results of our laboratory showed a significant deficit in habituation memory, an increase in anxiety-like behaviors and a better performance in an associative memory task when rats were exposed to noise at PND15 and tested at PND30, when compared with sham animals (Molina et al., 2016a; Uran et al., 2010, 2014), whereas no changes in these parameters were observed in rats exposed to noise at PND7 (Molina et al., 2016a). In contrast, in the present work, rats were exposed to noise at PND28 and only a deficit in habituation memory was found. Therefore, it could be suggested that this behavioral parameter might be used as a marker of susceptibility, given that the deficit did not become evident when animals younger than PND15 were exposed to noise and was retained even in animals exposed at PND28 (Zulma et al., 2017). In addition, it could be suggested that the auditory system, that becomes functional between PND7 and PND15, should be necessary to induce noise effects. Finally, a window of vulnerability could arise at about PND15, that might be delayed if the animals were exposed at later stages of development (McCormick et al., 2016). When PND28 rats were either subjected to voluntary alcohol intake or exposed to noise before alcohol drinking, no changes were induced in habituation memory when compared with sham-none rats. Therefore, it could be suggested that the presence of both agents might mask the alterations in habituation memory induced by noise-only exposure, probably because it seems to be a more adaptive behavior, as suggested by Rasmussen and Kincaid (2015). In consequence, alcohol could interfere with noise-induced impairment in habituation memory, even though it would have not produced changes (Beilharz et al., 2016; Kanoski and Davidson, 2010; Lanza et al., 2014).

Exploration is another behavior that might be measured in the OF. It is motivated by novel stimuli and consists of behavioral acts and postures that permit an animal to collect information about new aspects of the environment. However, caution should be put in the analysis of the results of exploratory activity and ambulation in the OF task as there are some controversies in the literature, exhaustively reviewed by Ennaceur (2014). Several authors suggested that an increased exploratory activity might imply reduced levels of anxiety-like behavior (Kalouda and Pitsikas, 2015; Prut

and Belzung, 2003), whereas others postulated that it may be interpreted as an anxiogenic-like behavior (Barnett and Cowan, 1976; Lamprea et al., 2008; Lever et al., 2006). In the present work, exploratory activity remained unchanged in PND28 animals exposed to either noise or alcohol separately when compared with sham-none animals. In contrast, an increase in this behavior was observed when rats were exposed to noise and then subjected to voluntary alcohol intake, supporting the results of increased anxiety-like behaviors observed in the EPM. Therefore, it could be suggested that the interest in the novelty of a new environment could be stressful enough to trigger anxiety-related behaviors, as proposed by Ennaceur (2014). Moreover, it would seem that the presence of noise before alcohol intake might unmask a behavioral anomaly that would have remained silenced otherwise (Molesworth et al., 2013). Given that the activity in the center of the OF remained unchanged in animals exposed to noise that drank alcohol when compared with the other groups (data not shown) and considering the increase in the number of lines crossed in the first session of the OF observed in this group (i.e., ambulation), it could be suggested that the rise in the number of rearings might be related to the increase in locomotor activity rather than to any anxiogenic- or anxiolytic-like effects (Speight et al., 2017; Thiel et al., 1999).

In fact, anxiety-like behaviors can be more accurately assessed through the recording of the time an animal remains in the open arms of the EPM as well as the latency to enter and the number of entrances, as an increase in these measurements has been validated in the literature as a reduction in anxiety-like behaviors (Wright et al., 2011). Present results show that whereas a significant decrease was observed in anxiety-like parameters when rats were subjected to voluntary alcohol intake between PND28 and PND30 when compared with sham-none animals, a significant increase was found in rats pre-exposed to noise when compared with sham-none, noise-only or alcohol-only rats. These results demonstrate that although noise did not affect these behaviors when present alone, the presence of noise before alcohol intake could be able to interfere with the emotional mechanisms that would be affected by exposure to the chemical agent and might lead to an adjustment of anxiety-like behaviors in order to cope with the environmental challenges presented.

Last, the associative memory can be evaluated through the IA task by means of the ratio between the seconds taken to enter the dark compartment in the retention and the training sessions (T2/T1). Results presented here showed that although a deficit in associative memory was found in animals that drank alcohol, pre-exposure to noise was able to counteract this behavior. Therefore, it could be suggested that a consecutive exposure to noise and alcohol might mask the behavioral disturbances that one of them induced, which would result in a compensation of the observed changes. Finally, considering the significant correlation between alcohol intake and the T2/T1 ratio, it could be suggested that higher levels of daily alcohol intake could negatively influence associative behavior. Therefore, the lower levels of alcohol intake observed in noise + alcohol animals when compared with alcohol-only rats might account for the improvement in the performance observed in the associative memory task, being the measurement of the T2/T1 ratio a reliable predictor of alcohol intake. Caution should be put regarding the way alcohol intake was measured. Given that isolation might act as a confounding factor on anxiety-like behaviors (Leussis and Andersen, 2008), rats were pair-housed. In consequence, alcohol intake amounts were calculated per cage and the mean values were estimated per rat, not being able to assure that each rat ingested the same amount. Nevertheless, average values of alcohol intake measured in the present work were similar to those found elsewhere (Leeman et al., 2010), supporting this experimental setting.

Finally, we acknowledge that both the reduction in anxiety-like behaviors and the deficit in associative memory observed in the alcohol-only group are consistent with the ability of acute alcohol to be anxiolytic and amnesic and that pre-exposure to noise may occlude these effects. However, it should not be discarded that the higher level of alcohol intake observed in the alcohol-only group when compared with the noise + alcohol group might account for the different behavioral outcomes.

4. Conclusions

In summary, exposure of adolescent rats to either noise or voluntary ethanol intake is capable of generating different behavioral alterations. However, when noise exposure preceded the use of alcohol, other behavioral changes were observed and lower levels of alcohol intake were found when compared with unexposed rats. These pieces of evidence suggest the existence of an imminent risk to health when these physical and chemical agents are combined, in particular using a sequential schedule. Moreover, the medical community should be aware in order to develop protective strategies targeting adolescent humans, a group of people that could be often exposed to noise and chemical agents.

5. Experimental procedures

5.1. Animals

Healthy adult male and female albino Wistar rats were obtained from the animal facilities of the Biochemistry and Pharmacy School, University of Buenos Aires, Argentina. Pregnant rats were isolated and left undisturbed until delivery and only male rats were used for the different experimental procedures (in average, 4 per litter). To eliminate a possible confounding factor of litter on treatment effects, no more than one subject from a given litter was assigned to a particular treatment group, being the litter the experimental unit.

At least two male rats were placed in each cage with food and water *ad libitum*, on 12 h light-dark cycles (lights on at 7 A.M.) at $21 \pm 2^\circ\text{C}$ and mashed cornflower for bedding.

Sixty PND28 male littermates coming from 15 different litters were randomly divided into two groups, half were exposed to noise and the remaining animals were placed in the same box, but without being exposed (sham). In turn, these groups were subdivided into two subgroups, alcohol and none, configuring four different groups: sham-none, noise-only, alcohol-only and noise + alcohol. To comply with the reduction premise stated in the “3R principles in animal research” (Tannenbaum and Bennett, 2015), in some cases the same animal was used for a second behavioral experimental settings (sham and noise-only groups, $n = 12$ per group), whereas alcohol intake groups (i.e., alcohol-only and noise + alcohol animals, $n = 18$ per group) were tested in a single behavioral test with the aim of maintaining the same time interval between the last ethanol intake and the behavioral assessment. Therefore, in average six animals per behavioral task were used within each group.

Animals were handled and sacrificed according to the Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, School of Medicine, University of Buenos Aires, Argentina). This Committee, under Resolution 2608/16, Exp 53.679/16, approved the present experimental protocol. The CICUAL adheres to the rules of the “Guide for the Care and Use of Laboratory Animals” (NIH) (2011 revision) and to the EC Directive 86/609/EEC (2010 revision) for animal experiments.

To avoid circadian rhythm alterations, noise exposures were performed in the intermediate phase of the light cycle, between

10 a.m. and 2 p.m. Moreover, behavioral tests were performed at the same time.

5.2. Noise exposure

PND28 male rats, kept in their home cages (dimensions: 40 cm × 25 cm × 16 cm), were introduced into an “ad hoc” wooden sound chamber of 1 m × 1 m × 1 m fitted with a ventilated top, as described in [Uran et al. \(2012\)](#). At least two rats per cage were exposed simultaneously. Animals were not handled throughout exposure period, as they were not removed from their home cages.

Computer software TrueRTA was chosen to produce white noise using a bandwidth from 20 Hz to 20,000 Hz in octave bands. For sound amplification, an active 2 way monitor (SKP, SK150A, 40 W RMS per channel) was used, located 30 cm above the animal cage placed in the sound chamber, that resulted in a flat response. Noise intensity was measured by using an omnidirectional measurement condenser microphone (Behringer ECM 8000) prior to animal exposure, by positioning the microphone in the sound chamber at several locations, and taking an average of the different readings.

Some animals were exposed to white noise at 95–97 dB(A) SPL (20–20,000 Hz) for two hours. Another group of animals was placed in the same box for the same period of time, but without being exposed (sham). Background noise level ranged between 50 and 55 dB SPL, being within the interval suggested by the WHO guidelines ([Rosenstock, 1998](#)) and by other authors ([Campeau et al., 2009](#); [Sasse et al., 2008](#)). Light was provided by a 20 W lamp located in the upper left corner of the sound chamber. In addition, the chamber was provided with a sound attenuation system made with Celotex™.

5.3. Alcohol administration (Two-bottle free choice paradigm)

Ethanol was administered at a concentration of 10% in tap water with *ad libitum* access ([Carnicella et al., 2014](#); [Nogales et al., 2014](#); [Penasco et al., 2015](#); [Sabino et al., 2013](#)). In the same cage, rats were given an additional bottle containing only tap water. This procedure was done for three consecutive days, starting on PND 28. In one group (noise + alcohol), the administration was initiated immediately after noise exposure. In the remaining cages (noise-only and sham groups), only tap water was provided. The amount of alcohol intake was calculated by subtracting the amount remaining after each 24 h period to the volume measured in each bottle at the beginning of the experiment.

Animals were weighed at the same time the volumes were measured to calculate g/kg/day of ethanol intake. Fluid intakes in milliliters were converted to grams (1 ml ethanol = 0.789 g) and expressed per kilogram of animal weight. The mean value of the total volume of fluid drank was calculated in each cage and a mean value per rat was estimated ([Bahí, 2017](#)). Only three drinking sessions were employed, given that long-lasting ethanol exposure during adolescence can alter social activity and anxiety-like behavior that might complicate assessment of social contributors to ethanol intake ([Varlinskaya et al., 2014](#); [Varlinskaya and Spear, 2014](#)). On the third day of voluntary alcohol intake (i.e., PND30), behavioral tasks were performed. A timeline for the experimental design is depicted in [Fig. 1](#).

In particular, it should be clarified that rats were pair-housed to avoid a possible additional source of stress induced by isolation, as suggested by several authors. In fact, stressful situations such as social isolation during early development could be a significant risk factor for future alcohol consumption ([Lopez and Laber, 2015](#)) and might have long-lasting effects on behavioral and biological responses in rodents ([Leussis and Andersen, 2008](#)). In addition, a recent study has shown that chronic social isolation during adoles-

cence resulted in higher voluntary ethanol intake in mice during adulthood ([Lopez et al., 2011](#)). For these reasons, animals were pair-housed, the total amount of alcohol drank was calculated per cage and a mean value per rat was obtained.

Finally, blood alcohol concentrations (BACs) were not measured because under continuous access conditions, animals drink in sporadic bouts that occur primarily during the dark phase of the circadian cycle. Since ethanol is quickly metabolized, it will be not enough to reach significant BACs. In fact, rats drink relatively low levels of alcohol (i.e., up to approximately 2–2.5 g/kg/day), which are unlikely to produce pharmacologically relevant BACs ([Leeman et al., 2010](#)).

5.4. Behavioral assessment

In all groups, behavioral tasks were performed at PND30.

5.4.1. Open field task (OF)

An OF device was used to analyze habituation memory and exploratory activity, known to depend on the hippocampus ([Barros et al., 2006](#); [Vianna et al., 2000](#)). It has been established that repeated exposure to the same environment can induce a decrease in locomotor activity that could be taken as a measure of preservation of habituation memory ([Pereira et al., 2011](#); [Vianna et al., 2000](#)). In addition, exploration and ambulation in the first session of the OF can be used to assess changes in emotionality induced by exposure to a novel environment. Therefore, vertical exploratory activity was quantified by recording the number and time spent doing rearings and climbing (i.e., standing on the hind limbs). Activity was recorded using a camcorder camera. The apparatus used consisted of a 50 cm × 50 cm × 50 cm dimly illuminated wooden box, with a floor divided into 25 equal squares by black lines. In the first session, prior to testing, the rat was individually placed in a cage inside the behavioral room and allowed to acclimatize for five minutes, to control different variables that could significantly alter physiological and behavioral indicators of stress ([Walf and Frye, 2007](#)). After that, the rat was removed from the cage, placed on the center rear quadrant of the OF box and allowed to freely explore the box for five minutes. The number of lines crossed was recorded over the session, as well as the number and time of rearings and climbings. In a second session (after 1 h inter-trial, housed in its home cage), the animal was left to explore the apparatus for other five minutes and the number of lines crossed was recorded again to evaluate habituation to the device ([Barros et al., 2006](#)).

5.4.2. Elevated plus maze (EPM)

This task was used to evaluate anxiety-related behaviors, dependent on the integrity of the hippocampus ([Brenes et al., 2009](#); [Montgomery, 1955](#); [Violle et al., 2009](#)). Open arms-related parameters measured in the EPM, as the decrease in the latency to enter and the increase in the number of entries, are thought to be associated with a reduction of anxiety-like behaviors. The wooden apparatus consisted of four arms of equal dimensions (50 cm × 10 cm) and raised 50 cm above the floor, with two arms enclosed by walls 40 cm high (i.e., closed arms) perpendicular to two open arms. Prior to testing, the rat was individually placed inside the behavioral room and allowed to acclimatize for five minutes, to control different variables that could significantly alter physiological and behavioral indicators of stress ([Walf and Frye, 2007](#)). After that, the rat was placed in one of the closed arms, facing towards the center of the maze. Next, its activity was recorded for five minutes using a camcorder camera and the latency to enter the open arms, as well as the number of entries and time spent on each arm type (open/closed), were calculated. Only few rats randomly

distributed across experimental groups fell down when walking in the open arms; these animals were excluded from the study.

5.4.3. Inhibitory avoidance task (IA)

Inhibitory avoidance task was used to quantify the memory of an aversive experience through the simple avoidance of a location in which the unpleasant incident occurred. This task is thought to depend on the integrity of the dorsal hippocampus and can be taken as an index of associative memory (Ennaceur and Delacour, 1988; Izquierdo and Medina, 1997). The apparatus consisted of a box (60 cm × 60 cm × 40 cm), divided into two compartments: one was illuminated while the other was equipped with a removable cover to allow it to be dark, as described by Roozendaal (2002). A removable partition divided both compartments. The floor of the dark compartment consisted of a stainless steel grid at the bottom, through which a continuous current can be delivered. In the habituation session, the rat was individually placed in a cage inside the behavioral room and allowed to acclimatize for five minutes, to control different variables that can significantly alter physiological and behavioral indicators of stress (Walf and Frye, 2007). After that, the rat was placed into the illuminated compartment of the box and allowed to freely explore the apparatus. Either after passing three times to the dark side or after three minutes spent in the dark side, the rat was removed from the apparatus. After ten minutes, the rat was placed again in the illuminated side and when it entered the dark side, the doors were closed and the rat was retained for ten seconds in this side. After 1 h, in the training session (T1), each rat was placed in the illuminated compartment, facing away from the dark side; the latency to move into the dark compartment was recorded. Thereafter, when the rat stepped with all four paws in the dark compartment, a foot shock (1.2 mA, 2 s) was delivered. The rat was then removed from the apparatus and returned to its home cage. An hour later, in the retention session (T2), the latency to move into the dark compartment was recorded and a similar procedure was followed, except that no electric shock was applied. The ratio between the latency to move into the dark compartment in the retention and the training sessions was taken as a measure of associative memory retention (T2/T1).

5.5. Statistical analysis

Normality test was performed for each group (KS-test). Significant differences between groups were analyzed through one, two or three way ANOVA test with LSD post hoc comparisons using the Infostat/L software. When interactions were significant, a simple effect analysis was performed. Results are expressed as mean values ± SEM and graphs were performed with Prism Graphpad software. Simple linear regression analyses were performed to examine the correlation between alcohol intake and the different behavioral measurements, obtaining the Pearson's R^2 index. A probability < .05 was accepted as significant.

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Competing interest

Authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.brainres.2017.11.001>.

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