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Intermittent ethanol binge exposure impairs object recognition but spares contextual and tone fear memory in adolescent rats

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Abstract

Adolescent brain development seems to be important for the maturation of brain structures and behavior. Intermittent binge ethanol drinking is common among adolescents, and this type of drinking can induce brain damage and cognitive deficits. In addition, emotional changes are frequently seen in alcoholics and rodents treated with ethanol. Considering the close relation between emotional arousal and cognitive responses, the present work investigates if intermittent ethanol binge exposure could differentially alter the performance of adolescent rats in aversive and non-aversive motivated tests. Male adolescent rats were submitted to ethanol treatment (2.5 or 5.0 g/Kg, o.a.) at 48-h intervals over postnatal day (PND) 30 to 60. Control animals were exposed to a similar administration protocol with saline administration. At PND61-PND63 animals were submitted to one-trial object recognition or contextual and tone fear conditioning paradigms. Binge ethanol drinking (at both 2.5 and 5.0 g/Kg) did not change freezing response in the contextual and tone fear conditioning. However, all doses impaired recognition rates 24h after training in object recognition test. In addition, despite a diminution of horizontal locomotion in the open field (only for the 5.0 g/Kg dose), no difference was detected regarding time in immobility, time in grooming and number of rearing in this paradigm. The present results show that the cognitive impairment resulting from intermittent binge ethanol exposure has a negative correlation with learning-associated emotional arousal **Keyword:** ethanol, binge drinking, adolescent rats, learning and memory, emotional arousal.

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Introduction

Heavy ethanol consumption has been reported to result in significant alterations of brain structure, physiology, and function. In fact, ethanol promotes brain injury and neurodegeneration in corticolimbic areas and results in memory impairment in both humans (Hildebrandt, Brokate, Eling, & Lanz, 2004; Kim, Ke, & Adkins, 2004; Ratti et al., 1999;Tedstone & Coyle, 2004) and rodents (Crews et al., 2004, 2006; Garcia-Moreno et al., 2002; Obernier, Bouldin, & Crews, 2002; Obernier, White, Swartzwelder, & Crews, 2002; Roberto, Nelson, Ur, & Gruol, 2002; Santucci et al., 2004).

Clinical and experimental studies have shown that the adolescent brain is more vulnerable to the neurodegenerative effects of ethanol (Crews, Braun, Hoplight, Switzer, & Knapp, 2000; Dahl, 2004; Spear, 2000; White & Swartzwelder, 2004), as well as to the functional consequences resulting from this neurodegenerative process, including learning and memory impairment (Acheson, Stein, & Swartzwelder, 1998; White & Swartzwelder, 2005).

Intermittent ethanol binge is common in adolescence. Differently from heavy ethanol drinking, relatively little is known about the functional consequences of this ethanol consumption pattern. Recently, Pascual and colleagues (2007) showed that intermittent ethanol binge promotes motor and cognitive impairment in adolescent rats as well as cell death in the neocortex, hippocampus, and cerebellum.

Besides learning and memory impairment, emotional changes have also been frequently reported in alcoholics (Grothues et al., 2008; Haynes et al., 2008) and in rodents submitted to ethanol consumption (Cabral et al., 2007; Läck, Diaz, Chappell, DuBois, & McCool, 2007). In addition,

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emotional tone is clearly relevant in learning and memory processes (for a review see McGaugh, 2006; Roozendaal, Barsegyan, & Lee, 2008). Curiously, the relation has not yet been described between the emotional arousal elicited by a task and the cognitively impairing effects of ethanol.

Thus, the aim of the present work was to assess whether intermittent ethanol binge exposure could differentially alter the performance of adolescent rats in aversive and non-aversive motivated tests. For this purpose we used object recognition test (a non-aversive motivated test) and fear conditioning (an aversive-motivated test). In the fear conditioning, an unconditioned aversive stimulus (foot-shock) was paired to a neutral conditioned stimulus (tone) in a specific context (equally neutral). After this association, the neutral stimuli acquired aversive properties and were thus able to promote fear responses in the animals (freezing). On the other hand, rodents naturally tend to approach and explore novel objects, even those objects which are assumed to have no natural meaning to the animal and which have never been paired with a reinforcing or aversive stimulus. Thus, differently from fear conditioning, the object recognition test is considered to be a poorly motivated task, involving low levels of arousal.

Considering the neurotoxicity of repeated ethanol withdrawal episodes, especially in the hippocampus, and the high anxiety levels during withdrawal, we hypothesized that intermittent ethanol binge exposures could impair the memory of animals in a non-aversive motivated test, while sparing the memory of animals in aversive motivated memory.

Method

Subjects

Thirty male Wistar EPM-1 rats [30 days old (PND30), initial weight = 150 g] were kept on a standard light/dark cycle (12/12h) with lights on at 07:00 AM, with free access to rat chow pellets and tap water. The animal care and experimental protocols were conducted under protocols approved by the Animal Care and Use Ethics Committee of the Faculty, according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, 1996.

Apparatus

The open field and object recognition test was performed in a circular arena made of white wood (150 cm diameter), enclosed by stainless steel walls and divided in 19 squares by black lines. For the conditioning procedure in the fear conditioning, as well as in the contextual fear conditioning test, a passive avoidance apparatus from Ugo Basile (Italy) was used. Finally, for the tone fear conditioning, a cylindrical chamber of plexiglass (30 cm diameter x 60 cm height) was used.

Binge ethanol protocol

After a 4h water and chow pellets deprivation, the animals received administrations of ethanol (Synth, Brazil) every other day, orally [25% (v/v)] at doses of 3 or 5 g/Kg from PND 30 until PND60. Specifically, animals were treated at PND 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, and 60. Thus, each animal was exposed on 16 occasions to alcohol, simulating the binge, an intermittent drinking pattern characteristic of young adolescents (Tur, Puig, Pons, & Benito, 2003; White, Kraus, & Swartzwelder, 2006). The saline control group received a similar treatment with saline. Thus, the following experimental groups were formed: Saline (animals submitted to saline treatment, from PND30 – PND60; n = 8), Et2.5 (animals submitted to ethanol treatment at the dose of 2.5 g/Kg, from PND30 - PND60; n = 8) and Et5 (animals submitted to ethanol treatment at the dose of 5.0 g/Kg, from PND30 - PND60; n = 8).

Procedure

The object recognition test and the contextual and tone fear conditioning were performed in the same animals, at PND61 - PND63. In the morning (08:00h – 12:00h) rats were submitted to the object recognition test, and in the afternoon (14:00h – 17:00h) to the contextual and tone fear conditioning (see experimental design in Figure 1).

Experiment 1 – Open field and object recognition test

The object recognition test was performed according to Ennaceur and Delacour (1988), with some modifications. At PND61 all animals were given a single 10-min habituation session with no objects in the open field arena. This habituation is crucial to improve the interaction of the animals with the objects during training and for the object recognition test. During the first five minutes we recorded the number of squares crossed, rearing, and time spent in immobility and in grooming (Archer, 1973). These parameters were used as an index of emotionality and to exclude possible bias resulting from locomotor impairment. Twenty four hours after habituation (PND62), training was conducted by placing

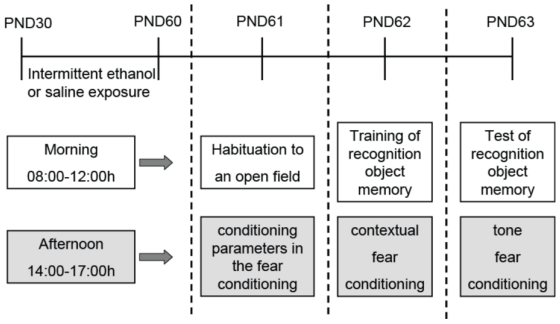

Figure 1. Illustration of experimental design. PND = postnatal day.

individual rats for 3 min into the field, in which two identical objects (A1 and A2; Lego toys) were positioned in two adjacent corners, 10 cm from the walls. After 24 h the test was conducted (PND63) when the same rats similarly explored the arena for 3 min in the presence of a familiar object A and a novel object B. A recognition index was calculated for each animal, and expressed by the ratio (time spent in interaction with object A1/time spent in interaction with objects A1 and A2, for training - and time spent in interaction with object A1/time spent in interaction with objects A and B, for 24 h retention). Both objects presented similar textures and sizes, but distinctive colors and shapes. In addition, between trials, the objects were washed with a 10% ethanol solution. Finally, exploration was defined as the time spent in sniffing or touching the object with the nose and/or forepaws. Sitting on the object was not considered exploration.

Experiment 2 – Contextual and tone fear conditioning

Conditioning parameters

The conditioning procedure was performed at PND61. The rat was individually confined in the dark compartment of the passive avoidance apparatus. Two minutes later, the conditioned stimulus (5 s long) sounded, and in the last second a foot-shock of 1mA and 1 s (unconditioned stimulus) was delivered, which finished together with the 70 dB tone (conditioned stimulus). The tone foot-shock pairing was repeated five times, 30 s apart. Thirty seconds after the last foot-shock, the animal was removed from the apparatus.

Contextual fear conditioning

The test was carried out at PND62. The animal was individually placed in the dark compartment of the apparatus, with the sliding door closed, where it remained for 5 min. Unconditioned or conditioned stimuli were not delivered. The freezing time in each minute during the total 5 min was registered.

Tone fear conditioning

The test was carried at PND63. Each animal was individually placed in the cylindrical chamber (new context), where it remained for 8 min. The chamber had been previously placed in another room to avoid spatial cues. The conditioned stimulus was presented 5 times at 30-s intervals, beginning at the end of the third minute. The freezing time was measured, minute-by-minute, during the first 3 min (before the tone) and during the final 5 min (during and after tone).

Statistical analysis

The data obtained in the contextual and tone fear conditioning were analyzed by one-way ANOVA for repeated measures followed by Newman-Keuls post hoc. The indexes of recognition in the training and 24 h retention, as well as the results from the open field test, were analyzed by one-way ANOVA followed by Newman-Keuls post hoc. Differences with $p < .05$ were considered significant.

Results

Experiment 1

Open field test

The results of the open field test are depicted in Table I. The animals treated with ethanol at the dose of 5.0 g/Kg crossed a smaller number of squares, when compared to both the Saline and Et2.5 groups [$F(2,21) = 6.41$; $p < .01$ and $p < .05$, respectively]. However, no differences were seen between the experimental groups regarding time in immobility [$F(2,21) = 0.53$; $p = .59$], time in grooming [$F(2,21) = 1.57$; $p < .01$], and rearing [$F(2,21) = 0.38$; $p = .69$].

Table 1. Effects of ethanol treatment over behaviour in the open field test.

Groups	Squares crossed	Time in immobility (s)	Number of rearing	Time in grooming (s)
Saline (N = 8)	81.02 ± 9.13	112.75 ± 11.79	1.51 ± 0.71	37.02 ± 10.57
Et2.5 (N = 8)	58.21 ± 5.36	131.02 ± 13.18	1.91 ± 0.77	33.42 ± 13.18
Et5 (N = 8)	47.78 ± 5.03** #	113.22 ± 17.91	1.11 ± 0.42	18.01 ± 6.08

Data expressed as means ± S.E.M. ** $P < 0.01$ in comparison to Saline group. # $P < 0.05$ in comparison to Et2.5 group (ANOVA followed by Newman-Keuls).

Object recognition test

Figure 2 shows the results of the object recognition test. No differences were seen between the experimental groups regarding the recognition index in the training session [F(2,21) = 0.09; p = .92]. However, 24 h after training, a significant decrease in the recognition index was seen for both the Et2.5 and Et5 groups, when compared to the saline-treated group [F(2,21) = 4.17; p < .05].

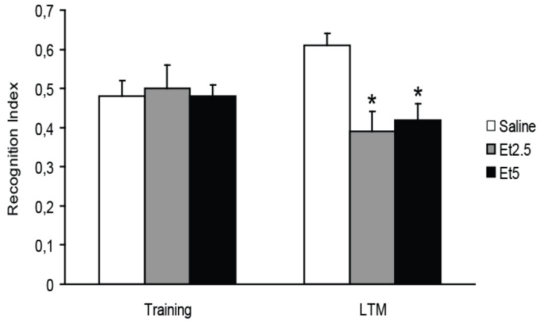

Figure 2. Recognition index performed by Saline (N=8), Et2.5 (N=8) and Et5 (N=8) groups during training (PND62) and long-term memory - LTM (PND63). Recognition index was expressed as means ± S.E.M. and calculated as a ratio [time of interaction with object A1 divided by the time of interaction with object A1+A2, for training], [time of interaction with above presented object (A) divided by the time of interaction with object A and a new object (B), for LTM]. LTM=long-term memory. * P<0.05 in comparison to Saline group.

Experiment 2

Contextual fear conditioning

The results obtained in the contextual fear conditioning are depicted in Figure 3. One-way ANOVA for repeated measures detected significant differences in the minute factor [F(2,21) = 8.8; p < .01], but not in the treatment factor [F(2,21) = 0.37; p = .69]. In addition, no interaction was seen between factors [F(2,21) = 1.22; p = .29]. Newman-Keuls post hoc revealed that all animals (despite of treatment) spent less time in freezing during the first minute of the test, when compared to the other minutes.

Tone fear conditioning

Figure 4 illustrates the data of the tone fear conditioning. One-way ANOVA for repeated measures detected significant differences in the minute factor [F(2,21) = 26.43; p < .01], but not in the treatment factor [F(7,147) = 0.17; p = .84]. In addition, no interaction was seen between factors [F(7,147) = 1.58; p = .09]. Newman-Keuls post hoc revealed that all animals (despite of treatment) showed greater freezing time in minutes 4, 5, and 6 (i.e. the period in which the conditioned stimulus was presented) when compared to minutes 1, 2, 3 (i.e. the period prior to the conditioned stimulus presentation), 7, and 8 (i.e. period after the conditioned stimulus presentation).

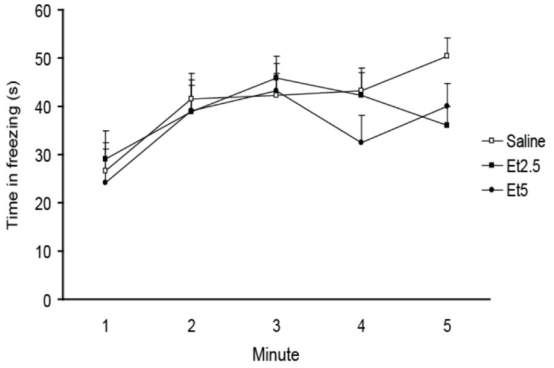

Figure 3. Performance of Saline (N=8), Et2.5 (N=8) and Et5 (N=8) groups during 5min of contextual fear conditioning. Each point represents the time in freezing. Data expressed as mean ± S.E.M.

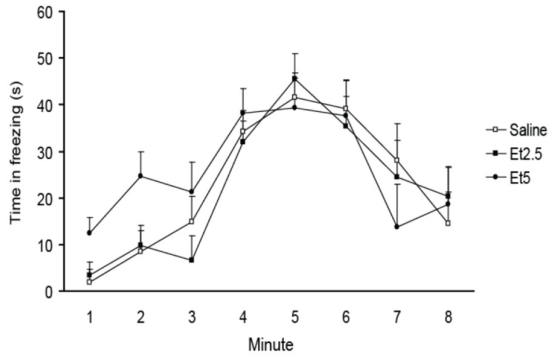

Figure 4. Performance of Saline (N=8), Et2.5 (N=8) and Et5 (N=8) groups in tone fear conditioning before, during and after tone presentation. The period from 1 to 3 min corresponds to freezing responses before tone presentation, and the period from 6 to 8 min corresponds to freezing responses after tone presentation. The tone was presented 5 times at 30-s intervals, beginning at the end of the 3rd minute. Data expressed as mean ± S.E.M.

Discussion

The results of the present work show that intermittent ethanol binge exposure, impaired the memory for object recognition while sparing contextual and tone fear conditioning. Although a diminution of squares crossed was seen in the animals treated with ethanol at 5.0 g/Kg, the other parameters evaluated in the open field (rearing, time in immobility and in grooming) did not change. Thus, the differences encountered in the object recognition test are not related to a possible motor impairment induced by the ethanol. In addition, all behavioural tests were initiated 24 h after the last ethanol exposure, in a drug-free state. Obviously, emotional aspects could play an important role in the decrease in the number of squares crossed. In fact, high anxiety levels intensify the conflict between the drive to explore a new environment and the protection instinct, resulting in decreased exploratory activity (for a review see Lister, 1990 and Treit, 1985). It is important to mention that other variables, such as augment of grooming activity, are also related

to anxiety. In addition, although in the Et5 group we observed a tendency to increased freezing response during the period before tone presentation in the tone fear conditioning (a good indicative of anxiety levels), this difference did not reach statistical significance ($p = .18$). Finally, the decrease in exploratory activity could be due to an anhedonic state in which the animals do not have sufficient motivation to explore a new environment. This hypothesis is in accordance with unpublished results from our laboratory that show a long-lasting increase of immobility in rats exposed to ethanol at a dose of 5.0 g/Kg (but not 2.5 g/Kg). Thus, it is possible that the decrease in the number of squares crossed could be related to alterations of mood regulation.

Intermittent ethanol binge is common in adolescence. Differently from heavy ethanol drinking, relatively little is known about the functional consequences of this ethanol consumption pattern. Despite this, there is some evidence that memory problems develop after the cessation of prolonged alcohol intake rather than during drinking. In fact, Schandler, Clegg, Thomaz and Cohen (1996) found visuospatial learning to be more impaired in abstinent alcoholics than in those still intoxicated. In addition, memory deficits in rats were seen after withdrawal but not during alcohol intake (Farr, Scherrer, Banks, Flood, & Morley, 2005; Lukoyanov, Madeira & Paula Barbosa, 1999). Finally, alcoholics experiencing more withdrawal episodes had greater memory deficits (Duka, Townsend, Collier & Stephens, 2003). Thus, it is possible that intermittent ethanol binge promotes greater memory deficits as compared to daily ethanol exposure.

In the fear conditioning, an unconditioned aversive stimulus (foot-shock) was paired to a neutral conditioned stimulus (tone) in a specific context (equally neutral). After this association, the neutral stimuli acquired aversive properties and were thus able to promote the fear response in the animals (freezing behaviour). Therefore, fear conditioning is a strongly aversive motivated test.

Considering contextual fear conditioning, some authors described that acute ethanol administration diminishes the freezing response (Gould & Lommock, 2003; Land & Spear, 2004; Lattal, 2007; Wehner et al., 2004), while Bertotto, Bustos, Molina and Martijena (2006) showed that the discontinuation from chronic ethanol administration facilitates the formation of a new contextual fear memory concomitant with a marked resistance to being extinguished (evidenced by intense freezing response). However, in the present work no difference was seen between the experimental groups regarding the freezing response in contextual fear conditioning. Our result corroborates the findings obtained by Borlikova, Elbers and Stephens (2006), in adult rats submitted to repeated withdrawal from ethanol.

In relation to tone fear conditioning, Bergstrom, McDonald and Smith (2006) showed that chronic ethanol consumption impaired tone fear conditioning after 30 days of withdrawal in adolescent rats. Similar features were seen in human binge drinkers (Stephens et al., 2005). In contrast, Land and Spear (2004) described no differences in the freezing response of adolescent rats treated with ethanol just prior to training. Despite the differences between patterns of ethanol exposure, our results corroborated the findings obtained in the latter work.

It is consensus that the amygdala plays a crucial role in the fear responses to tone and contextual aspects of conditioning (Gentile, Jarrel, Teich, McCabe, & Schneiderman, 1986; Iwata, LeDoux, & Reis, 1986; Phillips & LeDoux, 1992). In addition, a review article showed that both lateral and central amygdaloid nuclei are essential in fear conditioning (Paré, Quirck, & LeDoux, 2004). Nevertheless, hippocampal lesions impair only contextual fear conditioning (Kim & Fanselow, 1992; Phillips & LeDoux, 1992; Kjelstrup et al., 2002; Phillips & LeDoux, 1992).

Rodents naturally tend to approach and explore novel objects, which are assumed to have no natural significance to the animal and which have never been paired with a reinforcing or aversive stimulus. Thus, different from fear conditioning, the object recognition test is considered to be a poorly motivated task, involving low levels of arousal. From this perspective, it is no surprise that evidence suggesting a role of the amygdala in object recognition is sparse (Roozendaal, Okuda, Van der Zee, & McGaugh, 2006), while strong evidence describes the hippocampal formation as playing a crucial role in this process (Bermudez-Rattoni, Okuda, Roozendaal, & McGaugh, 2005; Broadbent, Squire, & Clark, 2004; Clark, Zola, & Squire, 2000; de Lima, Luft, Roesler, & Schröder, 2006; Ennaucer & Aggleton, 1997; Winter & Bussey, 2005a, 2005b). Despite this, even the role of the hippocampus in the object recognition test remains controversial (for a review see Dere, Huston and de Souza Silva, 2007).

Published evidence suggests that ethanol impairs the performance in the object recognition test. In fact, impaired recognition was seen both after acute (Brooks, Hennebry, McAlpin, Norman, & Little, 2002) and chronic (Garcia-Moreno et al., 2002) ethanol administration. In addition, a recent article described that intermittent ethanol binge impaired object recognition in adolescent rats and promoted a significant increase in cell death in the neocortex, hippocampus and cerebellum (Pascual et al., 2007). Our results corroborate these previous findings, providing strong evidence that intermittent binge ethanol exposure disrupts object recognition memory. Considering that the hippocampal formation is necessary (although not sufficient) to normal behavioural responses in contextual fear conditioning and object recognition, it could be expected that intermittent ethanol binge exposure would result in similar impairments on both behavioural tests (but it did not). This discrepancy could be explained, at least in part, by the major role of

the amygdala in the former but not in the latter test.

To summarize, the cognitive impairment resulting from intermittent ethanol binge had a negative correlation with learning-associated emotional arousal.

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