Pharmacology, Biochemistry and Behavior 138 (2015) 58–69

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Adolescent rats are resistant to the development of ethanol-induced chronic tolerance and ethanol-induced conditioned aversion

Ricardo Marcos Pautassi [a,b,*], Juan Carlos Godoy [b], Juan Carlos Molina [a,b]

[a] Instituto de Investigación Médica M. y M. Ferreyra (INIMEC – CONICET – Universidad Nacional de Córdoba), Córdoba C.P 5000, Argentina
[b] Facultad de Psicología, Universidad Nacional de Córdoba, Córdoba C.P 5000, Argentina

ARTICLE INFO

Article history:
Received 8 April 2015
Received in revised form 17 August 2015
Accepted 15 September 2015
Available online 18 September 2015

Keywords:
Ethanol
Hypothermia
Tolerance
Adolescents
Adults
Rats

ABSTRACT

The analysis of chronic tolerance to ethanol in adult and adolescent rats has yielded mixed results. Tolerance to some effects of ethanol has been reported in adolescents, yet other studies found adults to exhibit greater tolerance than adolescents or comparable expression of the phenomena at both ages. Another unanswered question is how chronic ethanol exposure affects subsequent ethanol-mediated motivational learning at these ages. The present study examined the development of chronic tolerance to ethanol's hypothermic and motor stimulating effects, and subsequent acquisition of ethanol-mediated odor conditioning, in adolescent and adult male Wistar rats given every-other-day intragastric administrations of ethanol. Adolescent and adult rats exhibited lack of tolerance to the hypothermic effects of ethanol during an induction phase; whereas adults, but not adolescents, exhibited a trend towards a reduction in hypothermia at a challenge phase (Experiment 1). Adolescents, unlike adults, exhibited ethanol-induced motor activation after the first ethanol administration. Adults, but not adolescents, exhibited conditioned odor aversion by ethanol. Subsequent experiments conducted only in adolescents (Experiment 2, Experiment 3 and Experiment 4) manipulated the context, length and predictability of ethanol administration. These manipulations did not promote the expression of ethanol-induced tolerance. This study indicated that, when moderate ethanol doses are given every-other day for a relatively short period, adolescents are less likely than adults to develop chronic tolerance to ethanol-induced hypothermia. This resistance to tolerance development could limit long-term maintenance of ethanol intake. Adolescents, however, exhibited greater sensitivity than adults to the acute motor stimulating effects of ethanol and a blunted response to the aversive effects of ethanol. This pattern of response may put adolescents at risk for early initiation of ethanol intake.

1. Introduction

Repeated exposure to moderate and heavy ethanol exposure is widespread during adolescence. A study indicated that 85% of Argentinean male college students exhibited alcohol drinking during the last month, and 52% of the sample had 4 to 5 drinks per drinking occasion (Pilatti et al., 2014), which constitutes binge drinking (Courtney and Polich, 2009). It is thus not surprising that there is considerable interest in the effects of chronic ethanol exposure during adolescence; although the pre-clinical studies have often yielded mixed results, probably due to differences in methodology, route and length of ethanol dosing, among other factors (Swartzwelder et al., 2014). Protracted and continuous heavy ethanol exposure induces neuro-inflammation and neurotoxicity, and apparently to greater extent in adolescents, than in adults (Crews et al., 2000). On the other hand, moderate and intermittent ethanol exposure facilitates later ethanol consumption (Pascual et al., 2009), perhaps by facilitating the development of tolerance to the aversive and sedative effects of the drug. Tolerance is defined as decreased sensitivity to an effect of ethanol following exposure to the drug (Swartzwelder et al., 2014).

Age-related differences in sensitivity to ethanol-induced tolerance have been observed. Acute tolerance (i.e., a diminished response to ethanol's effects during the course of a single intoxication) to the sleep-inducing effect of ethanol was greater in infant and adolescent rats than in older counterparts (Silveri and Spear, 1998), and similar effects were found for ethanol-induced social impairment (Varlinskaya and Spear, 2006). On the other hand, rapid and chronic tolerance (a diminished response to ethanol's effects after a second ethanol administration or after repeated ethanol dosing across several days, respectively) to the sleep-inducing effect of ethanol are greater in adults than in adolescents (Silveri and Spear, 1999).

Tolerance can develop for some, but not for all effects of ethanol and seems to be related to the magnitude of the ethanol-induced disturbance. This is illustrated by a study in which infant rats were given ethanol every-other day from postnatal day (PD) 13 to 21

* Corresponding author at: Instituto de Investigación Médica M. y M. Ferreyra (INIMEC – CONICET), Friuli 2434, Córdoba C.P 5000, Argentina.
E-mail address: rpautassi@gmail.com (R.M. Pautassi).

http://dx.doi.org/10.1016/j.pbb.2015.09.012
0091-3057/

(Hunt et al., 1993). Pups exhibited ethanol-induced hypnosis readily at PD 13 but ethanol-induced hypothermia only at PD19. On the challenge at PD21 chronically treated pups exhibited, when compared to animals only given ethanol on PD21, reduced ethanol-induced hypnosis, but a similar fall in core body temperature. Subsequently, Silveri and Spear (2001) compared acute, rapid and chronic tolerance to the disrupting effect of ethanol on a swim task, in infant, adolescent and young adults (PD16–22, PD28–34 and PD54–60, respectively), after having equated the initial level of ethanol-induced perturbation across age. Under this condition, minimal age-related differences were found in either type of tolerance.

Other studies on chronic tolerance to ethanol in adults and adolescents have yielded mixed results. Tolerance to the hypothermic and sedative effects of ethanol has been reported in adolescents (Swartzwelder et al., 1998), yet others found no change in ethanol-sedative effects following chronic adolescent ethanol exposure (Matthews et al., 2008); and other work indicated comparable expression of the phenomena (assessed via alterations in social behavior) at both ages (Varlinskaya and Spear, 2007). A more recent study (Broadwater et al., 2011) employed a relatively high ethanol dose (4 g/kg, every other day for 10 days) and found an attenuated response to ethanol-induced sedation at challenge in adult, but not in adolescent, rats. Similar outcome (i.e., adults but not adolescents exhibiting ethanol-induced chronic tolerance) was reported in mice (Linsenbardt et al., 2009), and Ristuccia and Spear (2005) found no change in ethanol-induced hypothermia in adolescents after a week of ethanol vapor exposure. Overall, it seems that adolescents are less prone, or need higher or lengthier ethanol dosing, to develop chronic tolerance than adult counterparts; yet it is clear that more information is needed to understand age-related differences in the expression of chronic ethanol tolerance.

Another point that remains unclear is how chronic ethanol exposure affects subsequent ethanol-mediated motivational learning. Evidence suggests that adolescent rats may be more sensitive to the appetitive (Pautassi et al., 2008; Ristuccia and Spear, 2008) but less sensitive to the aversive effects of ethanol (Anderson et al., 2010) than adults. There is, however, little information on modulation of these age-related differences by chronic treatment likely to induce chronic tolerance, although early work suggested that chronic ethanol treatment may increase the net appetitive value of ethanol (Bozarth, 1990) by reducing the aversive consequences of ethanol (Cunningham et al., 2002). Also limited is the information on the relationship between chronic tolerance and ethanol intake. Broadwater et al. (2011) found greater consumption of ethanol in adolescent than in adults, which was unaffected by chronic ethanol exposure that resulted in the development of tolerance in adult, but not in adolescent, rats.

The present study examined chronic tolerance to ethanol, and the subsequent acquisition of ethanol-mediated conditioned odor aversion, in adolescent and adult Wistar rats. Tolerance was indexed via ethanol-induced hypothermia and ethanol-induced motor behavioral stimulation (Experiment 1), which indicates aversive and appetitive effects of ethanol, respectively. Ethanol-induced hypothermia regulates the acquisition of conditioned aversion by ethanol (Cunningham et al., 1992); and ethanol-induced behavioral stimulation is modulated by the same transmitter systems that modulate ethanol-induced appetitive conditioning. For instance, it was found that administration of naloxone (a general opioid antagonist) blocked conditioned place preference by ethanol and ethanol-induced motor activation in adolescent, Wistar rats (Pautassi et al., 2011). On the other hand, Cunningham et al. (1992) observed that exposure to high or low ambient temperature ameliorated or promoted, respectively, the expression of ethanol-induced conditioned taste aversion. After finding that adolescents did not exhibit signs of thermal tolerance after repeated ethanol exposure, we scrutinized mechanisms that may prevent adolescent animals from developing tolerance to ethanol's hypothermic effects. Experiment 2, Experiment 3 and Experiment 4 were conducted in adolescents only and manipulated context, length and predictability of ethanol administration.

2. Material and methods

2.1. Experimental designs

Experiment 1 was defined by a 2 (age: adolescence or adulthood) × 2 (treatment at experimental days 1, 3 and 5: ethanol or vehicle) × 2 (treatment at experimental day 7: ethanol or vehicle) × 2 [conditioned stimulus (CS) paired with ethanol during conditioning procedures: lemon or methyl salicylate]. Each of the 16 groups was composed by 8 subjects.

Experiment 2, Experiment 3 and Experiment 4 employed only adolescents. Experiment 2 used a 2 (treatment at experimental days 1, 3 and 5: ethanol paired or unpaired with exposure to the open field) × 2 (treatment at experimental day 7: ethanol or vehicle) factorial, with 9 subjects in each group.

A 2 (treatment at experimental days 1, 3, 5, 7, 9 and 11: ethanol paired or unpaired with exposure to the open field) × 2 (treatment at experimental day 13: ethanol or vehicle) × 2 (treatment at adulthood: ethanol or vehicle) factorial was employed in Experiment 3. Each group was composed by 11–12 subjects.

Experiment 4 had 3 groups, defined by the treatment at experimental days 1, 3, 5, 7, 9 and 11 [gradual (n = 9) or random (n = 9) ethanol administration, or vehicle (n = 7) administration].

2.2. General procedures

2.2.1. Subjects

A total of 307 Wistar male rats, representative of 77 L born and reared at the vivarium of the Psychology Department of the National University of Córdoba (Córdoba, Argentina) were employed (Experiment 1: 128 animals, 32 L; Experiment 2: 36 animals; 10 L; Experiment 3: 93 animals; 22 L; Experiment 4: 50 animals; 13 L). The rationale for using only males was that previous studies indicate that male rats are significantly more sensitive to ethanol-induced hypothermia than female rats (Taylor et al., 2009).

Births were examined daily and the day of parturition was considered PD0. Pups remained with their dam in maternity cages until weaning day at PD21. They were then housed in standard cages (45 × 30 × 20 cm, up to four animals per cage) with *ad libitum* access to water and food. Experimental procedures began at PD28 or PD70 (adolescence or adulthood, respectively). The colony was maintained on a 12 h. light/dark cycle (0800) at an ambient temperature of 22 ± 1 °C. Across experiments, no more than one subject per litter was assigned to the same experimental condition. This helped avoid litter effects (Zorrilla, 1997). During breeding and experimental procedures animals were treated according to the Guide for Care and Use of Laboratory Animals (National Research Council, 2011) and the guidelines indicated by the Institutional Ethics Committee.

2.2.2. Drug preparation and administration procedures

The ethanol doses of 0.0, 0.5, 1.0, 1.5, 2.0, 2.25, 2.5 and 3.0 g/kg were achieved by intragastrically (i.g.) administering 0.015 ml/kg of 0.0 (vehicle-treated control), 4.2, 8.4, 10.6, 16.8, 18.9, 21 and 25.2% ethanol solution (Porta Hnos., Cordoba, Argentina; vehicle: tap water). Intubations were executed through a section of PE 10 or PE 50 polyethylene tubing (Clay-Adams; length: 15 cm, internal width: 0.11 mm), for adolescents and adults, respectively, connected to a 5 cm^3 syringe mounted with a 27-1/2 gauge needle. The intragastric route was chosen to model the oral self-administration of ethanol normally observed in human adolescents and adults.

2.3. Specific procedures for Experiment 1

2.3.1. Measurement of ethanol-induced hypothermia and forward locomotion in an open field

In experimental days 1, 3 and 5, animals were given ethanol or vehicle (Experiment 1) administration. This pre-exposure or training

defined the induction phase of the experiment. Ethanol-induced hypothermia and forward locomotion were recorded during those days. Animals remained in their homecages, untreated, during days 2, 4, and 6. Core body temperature and forward locomotion after administration of ethanol or vehicle were measured on day 7 (challenge or test day). The induction-expression protocol has been widely employed for the assessment of ethanol-induced behavioral sensitization and tolerance in mice (Faria et al., 2008; Pastor and Aragon, 2006). Ethanol dose was 2.25 g/kg for adolescents and 2.5 g/kg for adults across induction and challenge days. According to previous studies (Pautassi et al., 2008), the intubation of ethanol doses >2.0 g/kg induces slight, yet significantly higher, peak blood ethanol concentrations in adolescent than in adult rats. Thus, the use of a slightly higher ethanol dose in the adults allowed equating level of intoxication across age.

More in detail, on days 1, 3, 5 and 7, the animals were removed from their maintenance cages, weighed to the nearest gram (Ohaus ls 2000) and individually returned to a holding cage (20 × 24 × 20 cm). They remained in these cages without access to water or food for 45 min, to allow body temperature return to its resting level and to eliminate alterations in body temperature due to huddling (Swartzwelder et al., 1998). Baseline temperature was then recorded, using a temperature monitor (Model 44,011, Yellow Spring, OH) equipped with a rectal probe. The probe was lubricated with mineral oil and then inserted in the rectum for about 45 s. The animals received an i.g. administration of ethanol immediately after baseline temperature recording and returned to the holding chambers.

Behavioral measurements were conducted at ethanol post-administration time 7–10, 75–78 and 150–153 min using a wooden open field (50 × 50 × 50 cm). During each 3 min interval, animals were videotaped to allow later assessment of forward locomotion, which was defined as the combined movement of the four paws in the horizontal plane (Nizhnikov et al., 2009). Total duration of this behavior during each 3 min interval (s) was measured *via* experimenter-operated stopwatches. The experimenter opened and closed the count in the stopwatch at the beginning and termination of each behavioral unit. In the intervals between behavioral recordings (i.e., ethanol post-administration time 0–6, 11–74 and 79–149 min) the animals were returned to a holding cage. Temperature was recorded at the end of each behavioral recording interval (i.e., at post-administration time 10, 78 and 153 min), immediately before the animals were returned to their holding cages or, after the last recording, to the maintenance cage in the vivarium.

2.3.2. Conditioning and testing procedures

Ethanol-induced odor conditioning began 48 h after termination of the chronic ethanol exposure (i.e., PD 36 and 78, for adolescents and adults, respectively). Adolescents and adults were trained in a single-trial, odor conditioning procedure, with ethanol as the unconditional stimulus (US). An odor stimulus (CS^-) followed a sham intragastric administration, whereas an alternative odor stimulus (CS^+) was paired with the effects of intragastrically delivered ethanol. Testing involved a sequential one-way odor preference tests.

Conditioning began by removing animals from their maintenance cages. They were weighed, given a sham intubation (i.e., the tubing was guided into the rat's stomach but no drug was delivered) and 15 min later placed in a clear acrylic chamber (20 × 25 × 25 cm). The chamber had a grid floor, and underneath it there was a lemon (1 ml of a 0.25% *v/v* solution, Montreal Co, Córdoba, Argentina) or metil salicilate (1 ml, Montreal Co, Córdoba, Argentina) scented cotton disk. During this first section of the conditioning session, half of the animals were exposed for 15 min to lemon odor and the remaining half to metil salicilate odor. Animals subsequently returned to their holding cages for 40 min. At that point, they were administered ethanol (2.25 g/kg or 2.5 g/kg, for adolescents and adults, respectively). At ethanol post-administration time 15 min they were gently placed in the acrylic chamber, for 15 min. The chamber was now odorized with the alternative odor stimulus. That is, those animals that had been originally stimulated with lemon odor were now placed in a box scented with metil salicilate. On the other hand, rats that had been exposed to metil salicilate odor after sham intubation were introduced in a lemon-scented cage.

Testing began 6 h after termination of conditioning and during this interval, that allowed clearance of ethanol, animals were kept in groups of four in standard maintenance chambers. The testing apparatus was a modified runway maze. It had a black, circular end chamber (diameter: 28 cm, height: 30 cm) connected to a white closed arm (length: 60 cm or 40 cm for adults and adolescents, respectively; width: 12 cm, height: 30 cm). Both sections had a grid floor. Animals were given a short, 3-min habituation to the apparatus. During the habituation, the apparatus was devoid of salient odor stimuli. Fifteen min after termination of the habituation, the animals were gently placed in the section of the white arm farthest from the black circle. This circle was now equipped with a cotton disk (placed underneath the grid floor) odorized with either lemon or metil salicilate. Animals explored the apparatus for three minutes, returned to their maintenance cages for fifteen minutes and then had another 3 min testing. During the second test, those animals that had been tested with lemon during the first test were exposed to metal salicilate and vice versa. This testing order was fully counterbalanced within each experimental group. The subject's behavior was videotaped for later analysis of odor preference or aversion patterns. Experimenters blind to the experimental treatments registered the latency (s) to enter into the black odorized section and the total time spent (s) in this area when the CS^+ or the CS^- were present.

2.4. Specific procedures for Experiment 2

Only adolescents were used in this and in subsequent experiments. All animals were given ethanol (2.25 g/kg, i.g.) during experimental days 1, 3 and 5 (training or induction phase). Half of the subjects (paired group, P) experienced the effects of ethanol in the open field, using the procedures of Experiment 1. The remaining half (unpaired group, UP) were exposed to the open field after a simulated, sham administration. These animals were intubated, yet no delivery of ethanol or vehicle was conducted.

Forward locomotion in the open field and core body temperature were measured at ethanol or sham post-administration time 7–10, 75–78 and 150–153 min, as described in Experiment 1. Unpaired animals were administered ethanol in the homecage, 90 min after termination of the behavioral and thermal measurements. On experimental day 7 (challenge or test), animals were given 2.25 g/kg ethanol or vehicle and assessed for thermal and behavioral response.

2.5. Specific procedures for Experiment 3

The rats were given 6 administrations of 2.25 g/kg ethanol, one daily every other day from PD28 to PD38 (i.e., induction phase: experimental days 1, 3, 5, 7, 9 and 11). P adolescents were given ethanol and shortly after exposed to an open field, whereas UP adolescents were exposed to the open field after sham administration and treated with ethanol 90 min after the last exposure to the open field. Forward locomotion and rectal temperature were measured as described in Experiment 1 and Experiment 2. On PD 40 (challenge: experimental day 13) animals were given 2.25 g/kg ethanol or vehicle and assessed for motor activity and rectal temperature.

2.6. Specific procedures for Experiment 4

Animals were given, instead of a fixed dose of ethanol, gradually increasing doses of ethanol (gradual group: 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g/kg) every other day from PD28 to PD38 (induction phase: experimental days 1, 3 5, 7, 9 and 11), or received during these days the same doses of ethanol in a randomly chosen order (random group: 1.5, 0.5, 2.5, 3.0, 1.0 and 2.0 g/kg, in days 1 to 11, respectively).

All animals in the random group received the doses in the same order. A relatively lower dose (1.5 g/kg), compared to that employed in Experiment 1, Experiment 2 and Experiment 3, was given during the subsequent challenge at PD40 (day 13), and animals were tested for ethanol intake in a three-bottle test at PD 42.

The ethanol intake test (duration: 2 h) was conducted in individual wire mesh cages equipped with three graduated glass intake tubes (volume capacity: 25 ml; graduation: 0.1 ml). Wistar rats are notoriously reluctant to ingest non-sweetened ethanol solutions. Therefore, testing was preceded by 22 h of liquid deprivation. During the tests adolescents had simultaneous availability of tap water, 3% *v*/*v* ethanol and 6% *v*/*v* ethanol. The vehicle of the ethanol solutions was tap water. Similar intake tests have been used in our lab and have allowed detection of sex and early ethanol exposure effects (Pepino et al., 2004; Ponce et al., 2004). Animals were weighed before the test and volume of intake (ml) from each tube was recorded after the test, to calculate g/kg of ethanol ingested and % preference for the ethanol solution.

2.7. Data analysis

Unless explicitly noted, the thermal scores under analysis across experiments were derived from subtracting the absolute temperature scores registered at 10, 78 and 153 min post-intubation from the baseline temperature measurement.

Core body temperature and forward locomotion scores were separately analyzed through analyses of variance (ANOVAs). In Experiment 1, preliminary analysis indicated significant differences in absolute baseline temperature between adolescent and adult rats that did not interact with day of assessment, with adolescents having significantly lower baseline scores than adults in all days (Age effect, $F_{1,123} = 34.61$, $p < 0.001$) despite a significant fluctuation across time in animals of both ages (day effect, $F_{3,369} = 2.83$, $p < 0.05$). Mean and SEM absolute temperature scores (C°) during experimental days 1, 3, 5 and 7 were 37.76 ± 0.05, 37.76 ± 0.04, 37.80 ± 0.04 and 37.86 ± 0.06, respectively, for adolescent rats; and 38.15 ± 0.07, 38.09 ± 0.07, 38.22 ± 0.06 and 38.22 ± 0.06, respectively, for adult rats. Baseline locomotion (i.e., after vehicle) was also significantly greater in adolescents than in adults (Age effect, $F_{1,62} = 68.78$, $p < 0.001$), particularly during the first bin of day 1 (age × day × bin effect, $F_{4,128} = 16.09$, $p < 0.001$; Fig. 2). Due to these baseline differences between adolescent and adult rats, core body temperature scores and locomotion scores of Experiment 1 were analyzed separately at each age.

In Experiment 1, the thermal scores obtained during chronic treatment were analyzed through an ANOVA that considered time at test (10, 78 and 153 min) and day of assessment (days 1, 3 and 5) as within measures, and treatment (ethanol or saline) as the between-group factor. Thermal scores on the challenge (day 7) were separately analyzed in adults and adolescents through a 2 (Drug treatment during training: ethanol or vehicle) × 2 (drug treatment at challenge: ethanol or vehicle) × 3 (time at test) mixed ANOVA.

In Experiment 2 and Experiment 3, temperature and behavioral scores obtained at chronic treatment were analyzed through a 3-way [Condition at chronic treatment (open field paired or unpaired with ethanol administration) × day of assessment (Experiment 2: days 1, 3 and 5; Experiment 3: days 1, 3, 5, 7, 9 and 11) × time at test (10, 78 and 153 min)] ANOVA. In Experiment 2 and Experiment 3 thermal and behavioral scores at challenge day (experimental days 7 or 13, respectively) were analyzed through a 2 [Condition at chronic treatment × treatment at challenge (vehicle or ethanol) × 3 (time at test) mixed ANOVA.

In Experiment 4 thermal and behavioral scores were recorded during chronic treatment and during a challenge that took place on postnatal day 40. Scores during chronic treatment were analyzed *via* a 3-way [Condition at chronic treatment (gradually increasing or variable ethanol dosing; or vehicle treatment) × day of assessment (days 1, 3, 5, 7, 9 and 11) × Time at test (10, 78 and 153 min)] RM ANOVA. Thermal scores at challenge were analyzed through an ANOVA that considered Chronic treatment at PDs 30, 32, 34, 36, 38 and 40 (gradually increasing or variable ethanol dosing; or vehicle treatment), Whereas time at test (10, 78 and 153 min) was the within-measure. A two way-RM ANOVA was used to analyze absolute (g/kg) ethanol intake (within-variable: Ethanol concentration, 3 or 6%), whereas a one-way ANOVA was used to analyze percent ethanol intake and water intake (ml/100 g of body weight).

The development of tolerance to a given effect of ethanol is associated with the magnitude of the initial disturbance induced by the drug (Hunt et al., 1993). Therefore, and despite the significant baseline temperature differences between adolescent and adults, we deemed important to explicitly compare age-related differences in ethanol-induced hypothermia, during the initial exposure to the drug in Experiment 1. A mixed ANOVA (between factors: Age and Drug treatment, within factor: Time at test, 10, 78 and 153 min post-administration) was applied to the thermal scores registered in day 1 of this experiment.

The variables measured during the one-way odor preference tests (Experiment 1) were latency (s) to enter into the black odorized section and total time spent (s) in this area when the CS^+ or the CS^- were present. These scores were subtracted (i.e., latency to enter the circular compartment when the CS^+ was present – latency to enter the circular compartment when the CS^- was present; time spent in circular compartment when the CS + was present – time spent in the circular compartment when the CS^- was present) to obtain a measure of *latency to approach the* CS^+ and *time spent on* CS^+. A positive *latency score* is indicative of animals avoiding the CS predicting ethanol's effects, whereas a negative latency score reflects a higher propensity to approach the CS^+ in comparison with the CS^-. In regards with *time spent on* CS^+, positive and negative scores in this variable reflect relative preference and aversion, respectively, for the odor paired with ethanol's effects. These variables were analyzed through separate four-way factorial ANOVAs. The between factors were age (adolescence or adulthood), drug condition across training (ethanol or vehicle), drug condition at challenge (ethanol or vehicle), and odor paired with ethanol during conditioning (lemon or metal salicylate).

The loci of significant main effects or significant interactions were analyzed *via* Newman–Keuls *post hoc* comparisons. An alpha level of 0.05 was enforced for all the analyses.

3. Results

3.1. Experiment 1

This experiment assessed age-related differences in the development and expression of tolerance to ethanol's thermal effects; and the effects of repeated ethanol treatment on ethanol-induced motor stimulation. Another aim was to assess, after the chronic ethanol treatment, the motivational effects of the drug using an odor conditioning procedure.

3.1.1. Initial level of ethanol-induced hypothermia across age, on experimental day 1

The ANOVA indicated significant main effects of drug treatment and time at test, $F_{1,124} = 13.92$; $F_{2,248} = 111.28$, $p < 0.005$. Ethanol-treated animals exhibited, during the experimental day 1, significantly lower thermal scores than vehicle-treated subjects, an effect that was similar in adolescent and adult rats. Thermal scores were significantly lower at 78 and 153 min than at 10 min post-administration. These results suggest that the initial magnitude of ethanol-induced hypothermia was fairly similar across both age groups. These results are depicted on Fig. 1.

3.1.2. Adolescents

3.1.2.1. Thermal scores during chronic intermittent exposure on experimental days 1, 3 and 5. The ANOVA yielded significant main effects of treatment, day of assessment and time at test, $F_{1,62} = 20.45$; $F_{2,124} = 12.04$; $F_{2,124} = 147.47$; respectively, all p's < 0.05. The interaction between treatment and

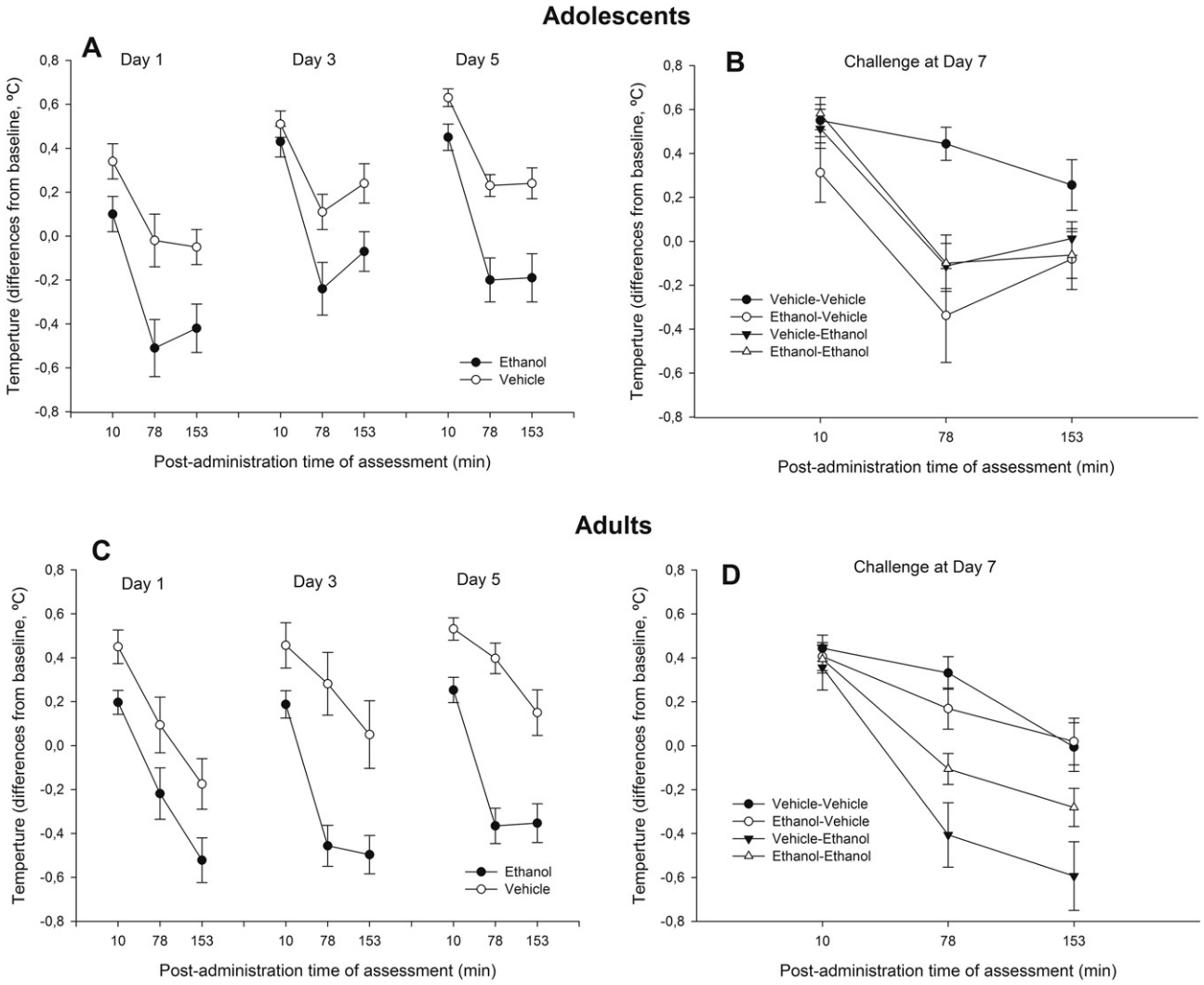

Fig. 1. Ethanol-induced hypothermia (differences from baseline temperature, °C) in adolescent and adult rats (upper and lower panels, respectively), during experimental days 1, 3 and 5 (pre-exposure or induction phase, Panels A and C) and during the challenge at day 7 (Panels B and D), in Experiment 1. During the induction animals received administrations of ethanol or vehicle. During the challenge, animals representative from the induction treatments received ethanol or vehicle. Four groups were thus defined: vehicle–vehicle, ethanol–vehicle, vehicle–ethanol and ethanol–ethanol. Temperature was recorded at post-administration times 10, 78 and 153 min) and during experimental days 2, 4 and 6 animals remained untreated in their homecages. Across induction and challenge ethanol dose was 2.25 and 2.5, for adolescent and adults, respectively. The vertical bars indicate the SEM. Please refer to the text for an account of significant differences across groups.

time at test also achieved significance, $F_{2,124} = 6.17$, $p < 0.005$. According to Newman–Keuls *post-hoc* tests, ethanol-treated adolescents exhibited lower thermal scores than vehicle-treated animals during the second and third evaluation bins. The magnitude of this drug-induced hypothermia, which has been depicted in Fig. 1A, was similar across days, although there was an overall increase in overall temperature scores from day 1 to days 3 and 5. There were no indications of the development of tolerance to ethanol's hypothermic effects.

3.1.2.2. Thermal scores during challenge at experimental day 7 (Fig. 1B). The ANOVA revealed significant main effects of drug condition during training and time at test, $F_{1,60} = 5.25$; $F_{2,120} = 51.78$, p's < 0.05. The two-way interactions between drug treatment at training and at challenge, drug treatment at training × time and test, and drug treatment at test × time at test achieved significance, $F_{1,60} = 5.35$, $F_{2,120} = 3.70$, $F_{2,120} = 3.50$, all $p < 0.05$. The ANOVA also yielded a significant three-way interaction between drug treatment at training, drug treatment at challenge and time at test, $F_{2,120} = 3.53$, $p < 0.05$. Subsequent *post-hoc* tests revealed similar thermal scores across groups during the initial testing at 10 min. As testing progressed (i.e., at 78 and 153 min post-intubation) animals given ethanol during the challenge exhibited significantly lower thermal scores than the basal control group (i.e., animals given vehicle during training and at the challenge). This hypothermia was similar in animal with or without a prior history of ethanol exposure, thus indicating that repeated ethanol exposure did not facilitate the development of tolerance in adolescent rats. The post-hoc also revealed that animals chronically treated with ethanol and given vehicle at the challenge (group ethanol-vehicle) exhibited, when compared to the basal control condition (group vehicle–vehicle), significant hypothermia during the second testing bin. Thermal scores in this group were statistically similar to those exhibited by animals given ethanol during the challenge.

3.1.2.3. Locomotion scores during chronic intermittent exposure on experimental days 1, 3 and 5. The ANOVA for locomotion scores during chronic intermittent exposure indicated a significant main effect of day and bin of assessment, [$F_{2,124} = 107.12$; $F_{2,124} = 131.12$, both $p < 0.001$] as well as significant day × bin interaction, $F_{4,248} = 34.91$, $p < 0.001$. The interaction between bin and treatment also achieved significance, $F_{2,124} = 16.17$, $p < 0.001$; whereas the bin × treatment × day interaction

neared significance, $F_{4,248} = 2.28$, $p = 0.06$. As depicted in Fig. 2A and revealed by *post-hoc* tests, adolescent rats given ethanol exhibited greater locomotor activity than vehicle-treated controls during the first testing bin of the first day of testing.

3.1.2.4. Locomotion scores during the challenge at experimental day 7. The ANOVA indicated a significant main effect of bin of assessment, and a significant drug treatment at challenge × bin of assessment interaction, $F_{2,124} = 40.52$; $F_{2,124} = 3.65$; both p's < 0.05. *Post-hoc* tests indicated a significant decrease in locomotor activity across bins, and revealed – despite the significant drug treatment at challenge × bin interaction – the absence of significant differences between the groups during any testing bin. These results are shown in Fig. 2B.

3.1.3. Adults

3.1.3.1. Thermal scores during chronic intermittent exposure on experimental days 1, 3 and 5. The ANOVA revealed significant main effects of treatment and time at test, $F_{1,62} = 28.19$; $F_{2,124} = 153.51$; respectively, $p < 0.05$. The bin × treatment interaction achieved significance, $F_{2,124} = 12.99$, $p < 0.000$. As shown in Fig. 1C, ethanol induced significant hypothermia and this effect, which was similar across days, was greater in bins 2 and 3 than in bin 1, according to the Newman–Keuls test.

3.1.3.2. Thermal scores during challenge at experimental day 7 (Fig. 1D). The ANOVA revealed significant main effects of time at test and drug condition at challenge, $F_{2,120} = 108.80$; $F_{1,60} = 14.85$; $p < 0.005$. The two-way interaction between these factors achieved significance [$F_{2,120} = 17.02$, $p < 0.0001$] and the three-way interaction (time at test × drug condition at chronic treatment × drug treatment at challenge) neared significance, [$F_{2,120} = 2.62$, $p = 0.07$]. *Post-hoc tests* revealed similar thermal scores during the initial testing at 7 min. At 78 and 153 min post-intubation, animals given ethanol during the challenge exhibited significantly lower thermal scores than animals given vehicle. Newman–Keuls tests, conducted between groups given ethanol at the challenge, indicated a borderline difference ($p = 0.07$) between the animals that experienced chronic ethanol treatment vs. those treated with vehicle, during the second testing bin. Specifically, those given chronic ethanol treatment exhibited higher thermal response than their vehicle-treated counterparts. This difference suggests the development of tolerance to ethanol-induced hypothermia.

3.1.3.3. Locomotion scores during chronic intermittent exposure on experimental days 1, 3 and 5 (Fig. 2, panel C). The ANOVA for locomotion scores during experimental days 1, 3 and 5 revealed significant main effects of day and bin of testing, $F_{2,124} = 46.43$; $F_{2,124} = 77.83$; respectively, both p's < 0.001. The day × bin interaction, and the three-way interaction (day × bin × treatment), also achieved

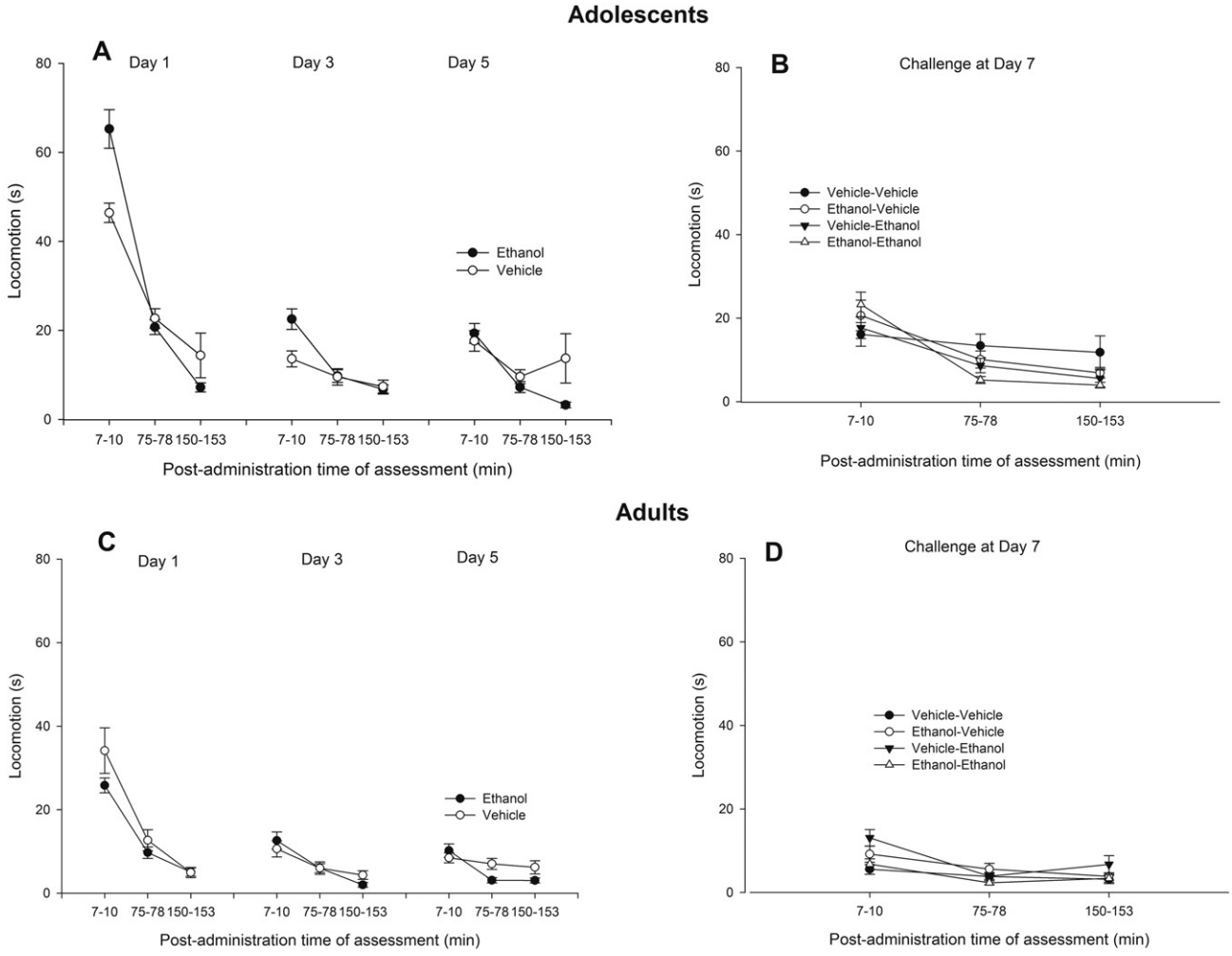

Fig. 2. Ethanol-induced forward locomotion (seconds) in adolescent and adult rats, during experimental days 1, 3 and 5 (pre-exposure or induction phase, Panels A and C) and during the challenge at day 7 (Panels B and D), in Experiment 1. During the induction animals received administrations of ethanol or vehicle. During the challenge, animals representative from the induction treatments received ethanol or vehicle. Four groups were thus defined: vehicle–vehicle, ethanol–vehicle, vehicle–ethanol and ethanol–ethanol. Locomotion was at post-administration times 7–10, 75–78 and 150–153 min) and during experimental days 2, 4 and 6 animals remained untreated in their homecages. Ethanol dose was 2.25 and 2.5, for adolescent and adults, respectively. The vertical bars indicate the SEM. Please refer to the text for an account of significant differences across groups.

significance, $F_{4,248} = 21.45$, $p < 0.001$; $F_{4,248} = 2.72$, both $p < 0.05$. *Post-hoc tests* revealed that overall locomotion was greater during testing bin 1 than in testing bins 2 or 3, and that this effect was significantly greater on the first day. Ethanol-treated adults exhibited significantly less motor activity than their saline-treated controls, during bin 3 of day 2 and during bin 2 of day 3. No activating effect of ethanol was found across days or bins.

3.1.3.4. Locomotion scores during the challenge at experimental day 7 (Fig. 2, panel D). The ANOVA yielded a significant main effect of bin and a significant drug treatment across training × drug condition at challenge, $F_{2,120} = 20.63$; $F_{1,60} = 9.50$; both $p < 0.005$. As indicated by *post-hoc* tests, locomotion was significantly greater during the first than during the second or third testing bins. *Post-hocs* also revealed that ethanol treatment at challenge enhanced locomotion among animals that had been treated with vehicle during training. This stimulating effect of ethanol was not observed in animals chronically treated with ethanol, which exhibited similar locomotion scores regardless treatment with ethanol or vehicle at challenge.

3.1.4. Odor conditioning in adolescent and adult rats

As depicted in Fig. 3 (lower panel), time spent on the CS^+ signaling ethanol's effects was similar across adolescent and adults exposed to most of the drug treatment conditions. This did not seem to be the case, however, for animals given vehicle during training but administered ethanol during the challenge. The ANOVA confirmed this impression, yielding significant two-way interactions between age × drug treatment at challenge, and drug treatment at training × drug treatment at challenge, $F_{1,112} = 4.05$, $F_{1,112} = 4.19$; $p < .05$. The three-way interaction (age × drug treatment at training × drug treatment at challenge) also achieved significance, $F_{1,112} = 9.46$, $p < .005$. The Newman–Keuls *post-hoc* tests indicated that adults treated with vehicle across days 1, 3 and 5 but ethanol at challenge spent significantly less time on the ethanol-paired CS + than any other group (all ps < 0.05).

The ANOVA for latency to approach the CS^+ (Fig. 3, upper panel) revealed a significant drug treatment at challenge × age interaction, $F_{1,112} = 7.65$, $p < 0.01$. Subsequent Newman–Keuls *post-hoc* tests, however, indicated the absence of significant differences between the groups (all ps > .08).

The ANOVA indicated that odor conditioning was not affected by whether salicylate or lemon was used as CS^+.

3.2. Experiment 2

Experiment 1 indicated that adolescents chronically treated with ethanol and given vehicle at challenge exhibited a decrease in thermal reactivity when compared to pertinent controls. It is conceivable that re-exposure to the context where ethanol was experimented triggered a conditional response (CR) that resembled the acute effect of the drug. Under this framework, this isodirectional conditioned response could prevent adolescent animals from developing tolerance to ethanol's hypothermic effects.

Experiment 2 was conducted with adolescent animals only and aimed at disrupting the development of the putative, isodirectional, ethanol-induced CR. Animals were administered ethanol in an intermittent basis and experienced the effects of the drug in the open field or were exposed to the open field after sham administrations and only 90 min later were given ethanol administration in the familiarity of their homecage. At the end of training all animals were given ethanol or its vehicle in the open field and assessed for motor and thermal reactivity.

3.2.1. Thermal scores during chronic intermittent exposure on experimental days 1, 3 and 5

The ANOVA revealed significant main effects of treatment and time at test (10, 78 and 153 min), $F_{1,34} = 30.43$, $p < .001$; $F_{2,68} = 80.80$, $p < .001$. The *post-hoc* revealed that temperature scores were significantly lower in the second and third testing bins than in the first bin. Furthermore, animals given ethanol immediately prior to placement in the open field exhibited significantly less temperature than control animals given only sham and this difference was similar across testing bins and across experimental days 1, 3 and 5 (see Fig. 4A). These results indicate that tolerance was not evident in the adolescents during the induction phase of the experiment.

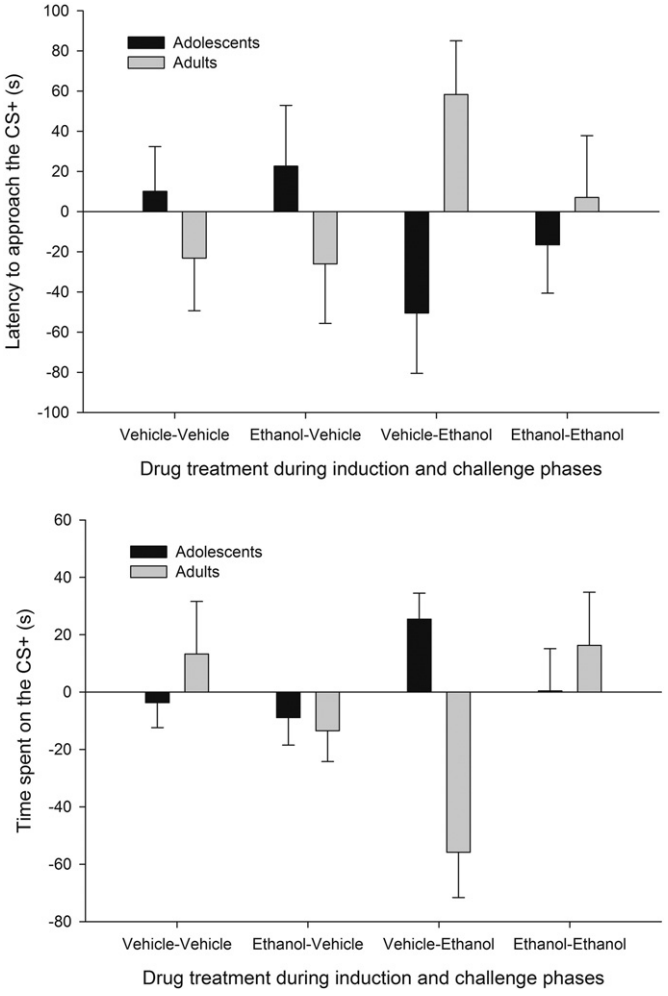

Fig. 3. Ethanol-induced odor conditioning in adolescent and adult rats as a function of treatment during experimental days 1, 3 and 5 (pre-exposure or induction phase: ethanol or vehicle) and during the challenge at day 7 (ethanol or vehicle). The four groups defined by these treatments (i.e., vehicle–vehicle, ethanol–vehicle, vehicle–ethanol and ethanol–ethanol) were subsequently given pairings of a distinctive odor (excitatory conditioned stimulus, CS^+) with the effects of ethanol administration (2.25 g/kg or 2.5 g/kg, for adolescents and adults, respectively). Another odor (inhibitory conditioned stimulus, CS^-) was presented following a sham intubation. Six hours after conditioning, animals were tested for preference or aversion towards each odorant, in a modified runway test. The measures were latency (s) to enter into the odorized goal chamber and the total time spent (s) in this area, when the CS^+ or the CS^- were present. These scores were subtracted to obtain a measure of *latency to approach the* CS^+ and *time spent on* CS^+ (upper and lower panels, respectively). A positive *latency score* is indicative of animals avoiding the CS predicting ethanol's effects, whereas a negative latency score reflects a higher propensity to approach the CS^+, in comparison with the latency exhibited for the CS^-. The vertical bars indicate the SEM. Please refer to the text for an account of significant differences across groups.

3.2.2. Thermal scores during challenge at experimental day 7 (Fig. 4B)

Significant main effects of time at test and drug treatment at challenge were found, $F_{2,64} = 68.35$; $F_{1,32} = 27.07$; $p < 0.001$. The interaction between these factors achieved significance, $F_{2,64} = 11.01$; $p < 0.001$. *Post-hocs* tests revealed that all adolescents exhibited similar temperature values during the initial bin of assessment. During the second and third

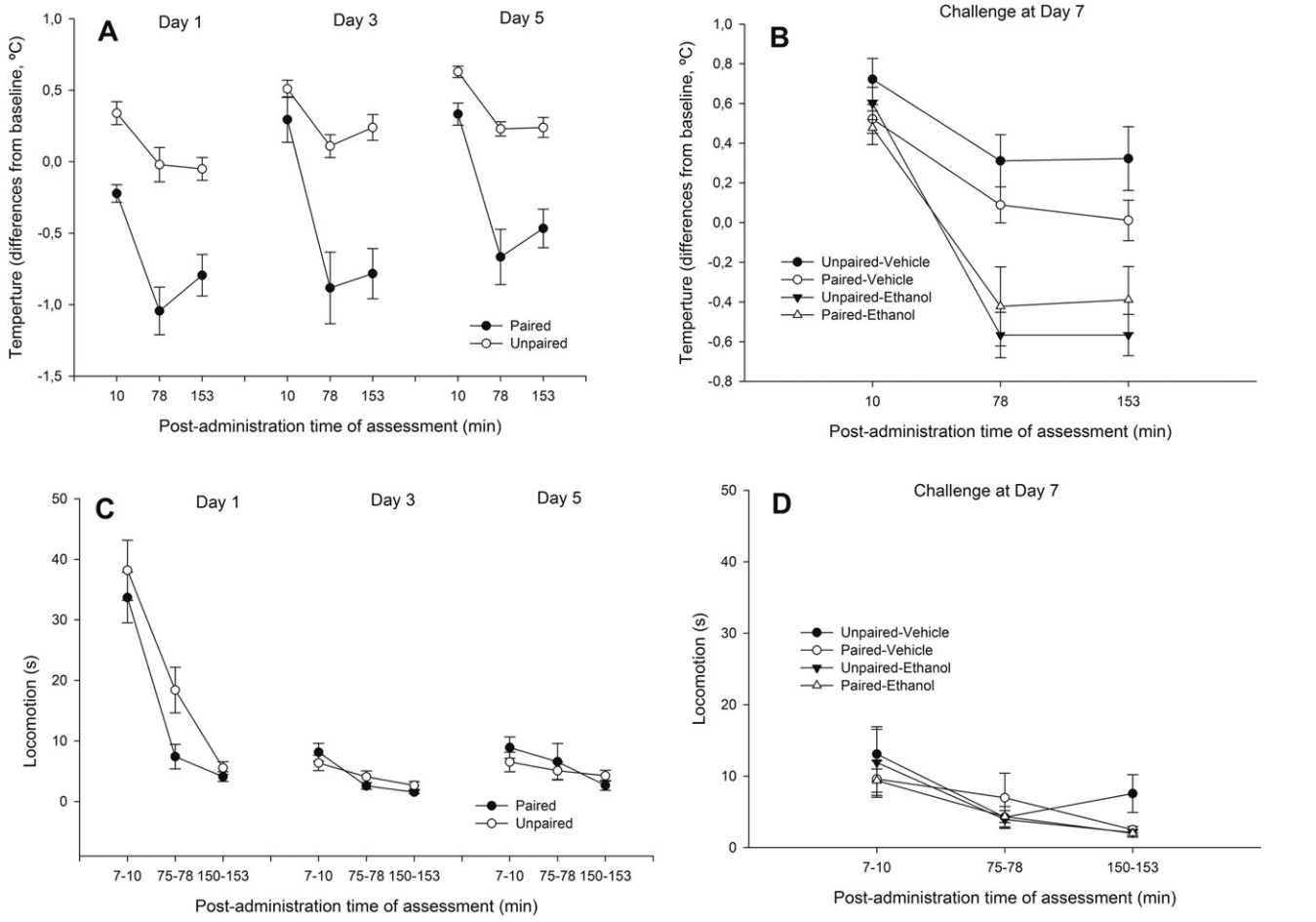

Fig. 4. Ethanol-induced hypothermia (differences from baseline temperature, °C, upper panels A and B) and ethanol-induced forward locomotion (seconds, lower panels C and D) in adolescent rats during experimental days 1, 3 and 5 (pre-exposure or induction phase, Panels A and C) and during the challenge at day 7 (Panels B and D), in Experiment 2. During the induction, the paired group experienced the effects of the drug in an open field. Unpaired animals were exposed to the open field after a simulated, sham administration; and administered ethanol in their homecage, 90 min after termination of the behavioral and thermal measurements. On experimental day 7 (challenge or test), animals from both groups were given 2.25 g/kg ethanol or vehicle and assessed for ethanol-induced hypothermia and ethanol-induced forward locomotion. The vertical bars indicate the SEM. Please refer to the text for an account of significant differences across groups.

testing bin adolescents given ethanol at challenge exhibited significantly reduced temperature scores when compared to groups given vehicle. There was no significant main effect of chronic ethanol treatment nor was this factor involved in any significant interaction.

3.2.3. Locomotion scores during chronic intermittent exposure on experimental days 1, 3 and 5 (Fig. 4C)

Locomotion was significantly higher during day 1 than in days 3 or 5, and this was more pronounced during the first and second bins. These effects were not affected by treatment during training. Significant main effects of days of training and bin of testing were found, $F_{2,68} = 53.58$ and $F_{2,68} = 75.82$; $p < 0.001$. The interaction between these factors was significant, $F_{4,136} = 35.82$.

3.2.4. Locomotion scores during the challenge at experimental day 7 (Fig. 4D)

Locomotion was greater during the first bin than during bins 2 and 3 and this effect was similar across groups. The ANOVA revealed a main effect of bin of testing, $F_{2,64} = 12.97$, $p < 0.001$.

3.3. Experiment 3

Experiment 1 and Experiment 2 revealed that adolescent animals did not develop tolerance to ethanol-induced hypothermia. It can be argued that the length of the pre-exposure treatment was too to short promote neuroadaptations leading to tolerance. Experiment 3 replicated the procedures of Experiment 2 but used a significantly longer training phase. Adolescents were given ethanol administrations every other day, from postnatal day 28 to 38. Half of the animals experienced ethanol's effects paired with the open field whereas half were exposed to the open field after sham intubations (i.e., unpaired group).

3.3.1. Thermal scores during chronic intermittent exposure on experimental days 1, 3, 5, 7, 9 and 11 (Fig. 5, left panel)

The ANOVA revealed significant main effects of treatment, day of assessment, and time at test (10, 78 and 153 min), $F_{1,44} = 55.44$, $p < .0001$; $F_{5,220} = 4.19$, $p < .005$; $F_{2,88} = 182.28$, $p < .0001$. The interactions between treatment × time at test, as well as the three-way interaction treament × day × time at test achieved significance, $F_{2,88} = 51.80$, $p < .0001$; $F_{10,440} = 3.26$, $p < .0005$. Separate day of assessment × time at test ANOVAs were conducted for each treatment condition, to understand the locus of the three-way interaction.

The ANOVA for unpaired subjects only revealed significant main effects of day and time at test, $F_{5,105} = 3.89$, $p < .005$; $F_{2,42} = 35.02$, $p < .0001$. The Newman–Keuls *post-hoc* tests revealed that temperature was significantly lower in the second and third testing bins than in the first one; and that temperature scores were higher in the last testing day than in the other days.

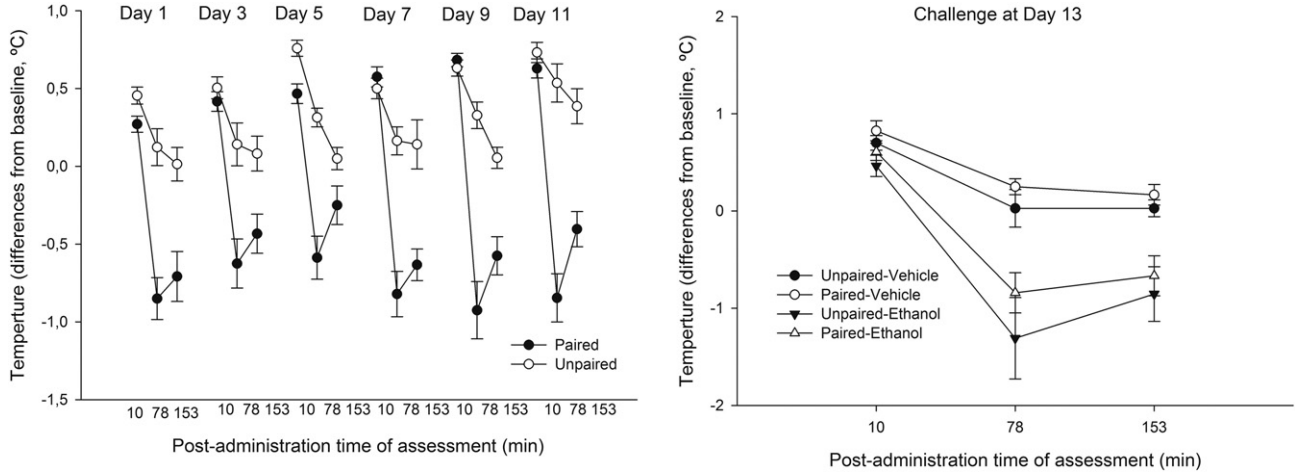

Fig. 5. Ethanol-induced hypothermia (differences from baseline temperature, °C) in adolescent rats, during experimental days 1, 3, 5, 7, 9 and 11 (pre-exposure or induction phase) and during the challenge at day 13, in Experiment 3. During the induction, the paired group experienced the effects of ethanol in an open field. Unpaired animals were exposed to the open field after a simulated, sham administration; and administered ethanol in their homecage, 90 min after termination of the behavioral and thermal measurements. On experimental day 7 (challenge or test), animals from both groups were given 2.25 g/kg ethanol or vehicle. Temperature was recorded at ethanol or sham post-administration times 10, 78 and 153 min, and during experimental days 2, 4 and 6, 8, 10 and 12 animals remained untreated in their homecages. The vertical bars indicate the SEM. Please refer to the text for an account of significant differences across groups.

The ANOVA for subjects given ethanol before exposure to the open field revealed a significant main effect of time at test and a significant interaction between this factor and day of assessment. *Post-hoc* tests indicated that, compared to the initial scores registered on day 1, temperature scores at 150 min exhibited a transient increase in experimental day 5. Temperature in days 7, 9 and 11 was similar to that observed during day 1.

3.3.2. Thermal scores at the challenge on experimental day 13 (Fig. 5, right panel)

Animals given ethanol immediately prior to placement in the open field exhibited significantly less temperature at 75 and 150 min than control animals given only vehicle and this difference was similar across animals that had been given or not chronic treatment with ethanol. The ANOVA yielded significant main effects of time and test and treatment at day 13 as well as a significant interaction between these factors, $F_{2,84} = 104.45$; $F_{1,42} = 22.90$, $F_{2,84} = 17.57$; $p < .005$.

3.4. Experiment 4

It is possible that the relatively high dose of ethanol employed in the previous Experiments precluded the development of neuroadaptations leading to tolerance. In Experiment 4 animals were given, instead of a fixed dose of ethanol, gradually increasing doses of ethanol every other day, or received the same doses of ethanol in a randomly chosen order. A moderate ethanol dose (1.5 g/kg) was given at the challenge, to avoid the possibility that the magnitude of temperature loss masked any tolerance that may have developed. Animals were tested for ethanol intake at the end of the protocol. The hypothesis was that animals given gradually increasing doses of ethanol would be more likely to develop tolerance than those given random ethanol.

3.4.1. Thermal scores during chronic intermittent exposure on experimental days 1, 3, 5, 7, 9 and 11 (Fig. 6A)

Significant main effects of chronic treatment and time of assessment were found, $F_{2,22} = 27.75$; $F_{2,44} = 74.32$; $p < .005$. The interaction between chronic treatment and day was significant [$F_{10,110} = 4.01$, $ps < .005$]. We compared, *via* Newman–Keuls *post-hoc* tests, temperature scores induced by 1.5, 2.0 and 3.0 g/kg ethanol, between animals given gradual and variable ethanol treatment, and between the thermal response of these groups at these doses and the scores observed in those particular days in the vehicle-control. Both the gradual and the random group display greater hypothermia than the saline control after 1.5, 2.0 and 3.0 g/kg ethanol and there were no significant differences between the gradual and the random group in the thermal responses induced by these doses.

3.4.2. Thermal scores at the challenge on experimental day 13 (Fig. 6B)

The ANOVA revealed a significant main effect of time at test and a significant interaction between time at test and chronic treatment, $F_{2,46} = 13.56$; $F_{4,46} = 3.68$; $p < .05$. Newman–Keuls *post-hoc* tests revealed a significant drop in temperature between the first and the last testing bins, in the groups given vehicle or random ethanol treatment during chronic treatment. This temperature decrease was not found in the gradual group. Temperature during the last bin, however, was similar across groups.

3.4.3. Ethanol and water intake

The ANOVAs for absolute (g/kg), percent ethanol intake and water intake (ml/100 g of body weight) indicated the lack of significant main effects or significant interactions. Mean and SEM ethanol intake (total amount of g/kg ingested and % preference of ethanol against water) across groups were 0.47 g/kg +/− 0.15 and 23.28% +/− 9.09, 0.51 g/kg +/− 0.19 and 22.56% +/− 9.07, and 0.47 g/kg +/− 0.21 and 21.55% +/− 10.11, for the vehicle-control, gradual and random groups, respectively. Mean and SEM water intake (ml/100 g) were 6.14 ml +/− 1.08, 6.27 ml +/− 1.02 and 6.66 ml +/− 1.19, for the vehicle-control, gradual and random groups, respectively.

4. Discussion

Adolescent and adult rats exhibited, during the induction or pre-exposure phase, a remarkably lack of tolerance to the hypothermic effects of ethanol. During the challenge, ethanol-experienced adults exhibited a trend towards a reduction in hypothermia after ethanol, when compared to peers given the drug for the first time (Experiment 1). No sign of ethanol-induced tolerance was found, however, in adolescent animals. Thus, a preliminary conclusion is that, under the present circumstances, adolescents seem to be markedly resistant to the expression of ethanol-induced tolerance, measured through changes in core body temperature.

It could be argued that the level of initial disturbance induced by 2.25 g/kg ethanol to the adolescents (i.e., a temperature drop nearing 0.5–0.6 °C) was not substantial to instantiate neuroadaptive changes

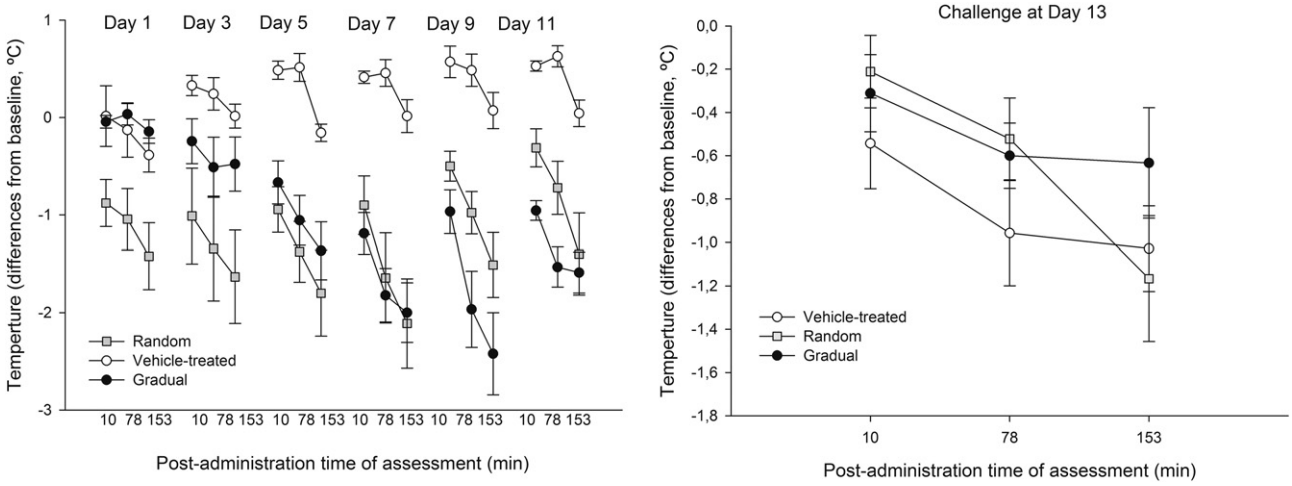

Fig. 6. Ethanol-induced hypothermia (differences from baseline temperature, °C) in adolescent Wistar, in Experiment 4. Animals were given gradually increasing doses of ethanol (gradual group: 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g/kg) every other day from postnatal days 28 to 38 (induction phase: experimental days 1, 3 5, 7, 9 and 11), or received during these days the same doses of ethanol in a randomly chosen order (random group: 1.5, 0.5, 2.5, 3.0, 1.0 and 2.0 g/kg, in days 1 to 11, respectively) or only vehicle. All animals in the random group received the doses in the same order. A 1.5 g/kg dose was given during the challenge. Temperature was recorded at ethanol or vehicle post-administration times 10, 78 and 153 min, ad during experimental days 2, 4 and 6, 8, 10 and 12 animals remained untreated in their homecages. The vertical bars indicate the SEM.

leading to tolerance. Among these, the changes induced by chronic ethanol in the $GABA_A$ receptor complex – which is involved in ethanol-induced hypothermia (Palmer et al., 2002) – have been studied at large (Grobin et al., 1998). Chronic ethanol exposure decreases the alpha-1 subunits of the $GABA_A$ receptor, an effect likely resulting from internalization of these subunits (Kumar et al., 2003). More specific to the present study, rats administered ethanol every other day from PD13 to 21 developed tolerance only for those measures in which ethanol exerted a significant disturbance (Hunt et al., 1993). This suggests that, for tolerance to develop to a given effect of ethanol, the acute initial disturbances induced by ethanol should be substantial [also see (Pohorecky et al., 1986)]. Under this framework, tolerance is a compensatory response to the detrimental effects of ethanol, which develops over time and counteracts the effect of the drug. It is interesting to note, however, that significant ethanol-induced hypothermia and conditioned aversion in rats has been found with lower doses [1.8 g/kg; (Cunningham et al., 1992)] than those used in our study. Moreover, in Experiment 1, the magnitude of the initial ethanol-induced hypothermic response (i.e., on day 1) was similar across the ages tested, although this analysis was confounded by the significant differences in baseline core body temperature between adolescent and adults.

Experiment 1 also hinted at a possible mechanism underlying the lack of tolerance in the adolescent rats. Ethanol-preexposed adolescents given vehicle at challenge exhibited as much hypothermia as ethanol-preexposed counterparts treated with ethanol during the challenge. This suggests that adolescents acquired a CR to the environment where ethanol was administered that led to the expression of a conditioned, hypothermic response at challenge. This CR may have partially accounted for the lack of tolerance at this age. Adolescents, unlike adults, exhibited ethanol-induced motor activation after the first ethanol intubation, a result consistent with previous work (Acevedo et al., 2013). Intriguingly, the adults given ethanol for the first time at the challenge – but not those given ethanol during the induction phase – exhibited ethanol-induced motor stimulation. This implies that both ages developed tolerance to the activating effects of ethanol, albeit with a somewhat different temporal pattern (induction vs challenge phase, in adolescents and adults, respectively).

It is not rare for ethanol to endow behavioral control to redundant, contextual cues that accompany drug intake or administration. Cunningham and Noble (1992) found behavioral activation in an ethanol-paired context and suggested that this CR may account for some of the failures to find conditioned place preference. Faria et al. (2008) gave adolescent and adult Swiss daily doses of ethanol (2.0 g/kg i.p.) in an open field or in the homecage; and found locomotor sensitization only in those adult mice treated in the open field. Adolescent mice, on the other hand, exhibited context-dependent tolerance. Moreover, contextual cues are important modulators of reinstatement of ethanol seeking and intake (Maccioni et al., 2007). We cannot discard, however, that the reduced thermal response exhibited by ethanol-experienced adolescents given vehicle at challenge obeys to withdrawal effects. Mice undergoing withdrawal exhibited significant hypothermia (Ritzmann and Tabakoff, 1976) and researchers suggest caution when interpreting measures taken within 24–48 of termination of chronic ethanol exposure, since they may reflect withdrawal consequences (Swartzwelder et al., 2014).

In Experiment 2 we inserted a significant delay between open field exposure and ethanol administration to adolescents, and expected this would preclude the development of an association between the context and ethanol's effects. The hypothesis was tolerance expression in adolescents pre-exposed to ethanol in the home cage and challenged with ethanol in the open field. Yet these animals exhibit significant ethanol-induced hypothermia at the challenge, similar to that exhibited by control animals. This indicates that the putative, context-evoked conditioned response, cannot be made fully accountable for the lack of tolerance in the adolescents.

In Experiment 4 we lowered the ethanol dose given at challenge, and during the induction we gave animals increasing doses of ethanol or a random dosing sequence that reduced the predictability of the effects of ethanol. In Experiment 3, in turn, we increased the length of ethanol pre-exposure. Yet none of these changes dramatically promoted tolerance in the youth. It is noteworthy mentioning that the random schedule of ethanol administration, which was meant to impede the development of an association between the open field context and the unconditional effects of the drug, had a subtle yet significant effect on the magnitude of ethanol-induced hypothermia. Adolescents given ethanol dosing at random during the induction exhibited significantly less ethanol-induced hypothermia than vehicle-treated controls. Albeit transient and admittedly subtle, this effect suggests that contextual cues may have some impact on the expression of ethanol-induced tolerance during adolescence; although this is just a hypothesis and more work will be need to test it.

Level of ethanol intake and preference in Experiment 4 was unaffected by history of alcohol exposure. Yet this measurement had several caveats that reduce the relevance of the null result: significant liquid deprivation

preceded the ethanol intake session, which likely encompassed stress-induced dehydration, and ethanol intake was measured for only 2 h. A more significant finding was that, unlike their adult counterparts, adolescents were sensitive to the motor stimulant effects of ethanol, which occurred during the rising limb of the ethanol-blood curve but not during later phases of the intoxication. Studies with humans indicate that the stimulant effects of ethanol are associated with positive mood state ratings, particularly in individuals at risk for exhibiting a positive familiar history of alcohol-related problems (Conrod et al., 2001). In line with previous studies indicating that adolescent rats do not develop ethanol-induced behavioral sensitization (Fabio et al., 2013), repeated administration of ethanol blunted this stimulant response and, if something, facilitated the emergence of sedative effects of ethanol.

Adults, but not adolescents, exhibited conditioned odor aversion by ethanol, in Experiment 1. The conditioned avoidance was only observed in animals that been exposed to ethanol once before conditioning, but not in animals devoid of ethanol exposure before conditioning or in animals given extensive exposure to ethanol. It seems that a brief, proximal pre-exposure to ethanol facilitated the expression of subsequent conditioned aversion by ethanol in the adults. This result meets the notion that adolescent rats may exhibit, when compared to adults, greater responsiveness to ethanol's appetitive effects but reduced responsiveness to the aversive effects of the drug. This hypothesis helps understand why adolescents drink, when compared to adults, greater amounts of ethanol per drinking occasion and exhibit a more rapid transition from controlled to problematic drinking. It has been observed that adolescent, but not adult, rats self-administered enough ethanol to induce tachycardia (Ristuccia and Spear, 2008) and exhibited conditioned tactile preferences induced by 0.5–2.0 g/kg ethanol (Pautassi et al., 2008).

It is important to contextualize the present results among previous studies on tolerance to ethanol across development. Acute tolerance to ethanol's sedative and narcotic effects exhibits a developmental decline, with two-week old rats exhibiting greater acute tolerance than adolescent or adult counterparts (Silveri and Spear, 1998). Findings on the ontogeny of chronic tolerance have been less consistent. Ristuccia and Spear (2005) found more rapid development of tolerance to the hypothermic effects of ethanol in adult than in adolescent rats. Yet adolescents showed greater tolerance to ethanol-induced sedation and hypothermia than adults in another study, which administered 4 g/kg ethanol i.p. twice a day during a 7-day induction phase (Swartzwelder et al., 1998). The latter found no initial age differences in the thermal reduction induced by ethanol, thus dispelling the possibility that age-specific tolerance development obeyed to differences in initial responsiveness to ethanol. It could be conceived that differences in ethanol dose or route of administration underlie these seemingly contradictory results. Another study (Broadwater et al., 2011) exposed adolescent and adults to a 4 g/kg dose of ethanol, once a day every 48-h and, unlike Swartzwelder et al. (1998) but similar to the results of the present study, found chronic tolerance in adults, but not in adolescents.

In summary, this study indicated that, when relatively low ethanol doses are given every-other day for a relatively short period, adolescents do not develop chronic tolerance to ethanol-induced hypothermia. Resistance to tolerance development could limit long-term maintenance of ethanol intake, and therefore could represent a protective factor for the development of alcohol-related problems. The relative importance of this factor, however, is lessened when other findings of the present study are considered. Adolescents exhibited greater sensitivity than adults to the acute motor stimulating effects of ethanol, which can be considered a proxy for the appetitive effects of the drug (Pautassi et al., 2011) and seemed to acquire ethanol-mediated context dependent learning likely to modulate subsequent ethanol seeking (Maccioni et al., 2007). They also showed a blunted response to the aversive effects of ethanol. The aversive effects of ethanol potentially serve as natural barriers, preventing excessive alcohol intake (Anderson et al., 2010). This idiosyncratic pattern of response may put adolescents at risk for early initiation of ethanol intake, which in turn favors the development of alcohol related problems, and the use of other drugs (Pilatti et al., 2014).

Acknowledgments

This work was supported by grants PICT 2012 (2012-0436) and PIP 2012 (11220120100223CO) to RMP.

References

Acevedo, M.B., Pautassi, R.M., Spear, N.E., Spear, L.P., 2013. Age-dependent effects of stress on ethanol-induced motor activity in rats. Psychopharmacology 230, 389–398.
Anderson, R.I., Varlinskaya, E.I., Spear, L.P., 2010. Ethanol-induced conditioned taste aversion in male Sprague–Dawley rats: impact of age and stress. Alcohol. Clin. Exp. Res. 34, 2106–2115.
Bozarth, M.A., 1990. Evidence for the rewarding effects of ethanol using the conditioned place preference method. Pharmacol. Biochem. Behav. 35, 485–487.
Broadwater, M., Varlinskaya, E.I., Spear, L.P., 2011. Chronic intermittent ethanol exposure in early adolescent and adult male rats: effects on tolerance, social behavior, and ethanol intake. Alcohol. Clin. Exp. Res. 35, 1392–1403.
Conrod, P.J., Peterson, J.B., Pihl, R.O., 2001. Reliability and validity of alcohol-induced heart rate increase as a measure of sensitivity to the stimulant properties of alcohol. Psychopharmacology 157, 20–30.
Courtney, K.E., Polich, J., 2009. Binge drinking in young adults: data, definitions, and determinants. Psychol. Bull. 135, 142–156.
Crews, F.T., Braun, C.J., Hoplight, B., Switzer, R.C., Knapp, D.J., 2000. Binge ethanol consumption causes differential brain damage in young adolescent rats compared with adult rats. Alcohol. Clin. Exp. Res. 24, 1712–1723.
Cunningham, C.L., Noble, D., 1992. Conditioned activation induced by ethanol: role in sensitization and conditioned place preference. Pharmacol. Biochem. Behav. 43, 307–313.
Cunningham, C.L., Niehus, J.S., Bachtold, J.F., 1992. Ambient temperature effects on taste aversion conditioned by ethanol: contribution of ethanol-induced hypothermia. Alcohol. Clin. Exp. Res. 16, 1117–1124.
Cunningham, C.L., Tull, L.E., Rindal, K.E., Meyer, P.J., 2002. Distal and proximal pre-exposure to ethanol in the place conditioning task: tolerance to aversive effect, sensitization to activating effect, but no change in rewarding effect. Psychopharmacology 160, 414–424.
Fabio, M.C., March, S.M., Molina, J.C., Nizhnikov, M.E., Spear, N.E., Pautassi, R.M., 2013. Prenatal ethanol exposure increases ethanol intake and reduces c-Fos expression in infralimbic cortex of adolescent rats. Pharmacol. Biochem. Behav. 103, 842–852.
Faria, R.R., Lima Rueda, A.V., Sayuri, C., Soares, S.L., Malta, M.B., Carrara-Nascimento, P.F., et al., 2008. Environmental modulation of ethanol-induced locomotor activity: correlation with neuronal activity in distinct brain regions of adolescent and adult Swiss mice. Brain Res. 1239, 127–140.
Grobin, A.C., Matthews, D.B., Devaud, L.L., Morrow, A.L., 1998. The role of GABA(A) receptors in the acute and chronic effects of ethanol. Psychopharmacology 139, 2–19.
Hunt, P.S., Molina, J.C., Rajachandran, L., Spear, L.P., Spear, N.E., 1993a. Chronic administration of alcohol in the developing rat: expression of functional tolerance and alcohol olfactory aversions. Behav. Neural Biol. 59, 87–99.
Kumar, S., Kralic, J.E., O'Buckley, T.K., Grobin, A.C., Morrow, A.L., 2003. Chronic ethanol consumption enhances internalization of alpha1 subunit-containing GABAA receptors in cerebral cortex. J. Neurochem. 86, 700–708.
Linsenbardt, D.N., Moore, E.M., Gross, C.D., Goldfarb, K.J., Blackman, L.C., Boehm, S.L., 2009. Sensitivity and tolerance to the hypnotic and ataxic effects of ethanol in adolescent and adult C57BL/6J and DBA/2J mice. Alcohol. Clin. Exp. Res. 33, 464–476.
Maccioni, P., Orru, A., Korkosz, A., Gessa, G.L., Carai, M.A., Colombo, G., et al., 2007. Cue-induced reinstatement of ethanol seeking in Sardinian alcohol-preferring rats. Alcohol 41, 31–39.
Matthews, D.B., Tinsley, K.L., Diaz-Granados, J.L., Tokunaga, S., Silvers, J.M., 2008. Chronic intermittent exposure to ethanol during adolescence produces tolerance to the hypnotic effects of ethanol in male rats: a dose-dependent analysis. Alcohol 42, 617–621.
National Research Council, 2011. Guide for the Care and Use of Laboratory Animals. 8th Edition. National Academies Press, Washington, DC.
Palmer, A.A., Moyer, M.R., Crabbe, J.C., Phillips, T.J., 2002. Initial sensitivity, tolerance and cross-tolerance to allopregnanolone- and ethanol-induced hypothermia in selected mouse lines. Psychopharmacology 162, 313–322.
Pascual, M., Boix, J., Felipo, V., Guerri, C., 2009. Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. J. Neurochem. 108, 920–931.
Pastor, R., Aragon, C.M., 2006. The role of opioid receptor subtypes in the development of behavioral sensitization to ethanol. Neuropsychopharmacology 31, 1489–1499.
Pautassi, R.M., Myers, M., Spear, L.P., Molina, J.C., Spear, N.E., 2008. Adolescent but not adult rats exhibit ethanol-mediated appetitive second-order conditioning. Alcohol. Clin. Exp. Res. 32, 2016–2027.
Pautassi, R.M., Nizhnikov, M.E., Acevedo, M.B., Spear, N.E., 2011. Naloxone blocks ethanol-mediated appetitive conditioning and locomotor activation in adolescent rats. Behav. Brain Res. 216, 262–269.
Pepino, M.Y., Abate, P., Spear, N.E., Molina, J.C., 2004. Heightened ethanol intake in infant and adolescent rats after nursing experiences with an ethanol-intoxicated dam. Alcohol. Clin. Exp. Res. 28, 895–905.
Pilatti, A., Caneto, F., Garimaldi, J.A., Vera Bdel, V., Pautassi, R.M., 2014. Contribution of time of drinking onset and family history of alcohol problems in alcohol and drug use behaviors in Argentinean college students. Alcohol Alcohol. 49, 128–137.

Pohorecky, L.A., Brick, J., Carpenter, J.A., 1986. Assessment of the development of tolerance to ethanol using multiple measures. Alcohol. Clin. Exp. Res. 10, 616–622.
Ponce, L.F., Pautassi, R.M., Spear, N.E., Molina, J.C., 2004. Nursing from an ethanol-intoxicated dam induces short- and long-term disruptions in motor performance and enhances later self-administration of the drug. Alcohol. Clin. Exp. Res. 28, 1039–1050.
Ristuccia, R.C., Spear, L.P., 2005. Sensitivity and tolerance to autonomic effects of ethanol in adolescent and adult rats during repeated vapor inhalation sessions. Alcohol. Clin. Exp. Res. 29, 1809–1820.
Ristuccia, R.C., Spear, L.P., 2008. Adolescent and adult heart rate responses to self-administered ethanol. Alcohol. Clin. Exp. Res.
Ritzmann, R.F., Tabakoff, B., 1976. Ethanol, serotonin metabolism, and body temperature. Ann. Acad. Sci. 273, 247–255.
Silveri, M.M., Spear, L.P., 1998. Decreased sensitivity to the hypnotic effects of ethanol early in ontogeny. Alcohol. Clin. Exp. Res. 22, 670–676.
Silveri, M.M., Spear, L.P., 1999. Ontogeny of rapid tolerance to the hypnotic effects of ethanol. Alcohol. Clin. Exp. Res. 23, 1180–1184.
Silveri, M.M., Spear, L.P., 2001. Acute, rapid, and chronic tolerance during ontogeny: observations when equating ethanol perturbation across age. Alcohol. Clin. Exp. Res. 25, 1301–1308.
Swartzwelder, H.S., Richardson, R.C., Markwiese-Foerch, B., Wilson, W.A., Little, P.J., 1998. Developmental differences in the acquisition of tolerance to ethanol. Alcohol 15, 311–314.
Swartzwelder, H.S., Hogan, A., Risher, M.L., Swartzwelder, R.A., Wilson, W.A., Acheson, S.K., 2014. Effect of sub-chronic intermittent ethanol exposure on spatial learning and ethanol sensitivity in adolescent and adult rats. Alcohol 48, 353–360.
Taylor, A.N., Tio, D.L., Bando, J.K., Truong, A.H., Prolo, P., 2009. Sex differences in ethanol-induced hypothermia in ethanol-naïve and ethanol-dependent/withdrawn rats. Alcohol. Clin. Exp. Res. 33, 60–69.
Varlinskaya, E.I., Spear, L.P., 2006. Ontogeny of acute tolerance to ethanol-induced social inhibition in Sprague–Dawley rats. Alcohol. Clin. Exp. Res. 30, 1833–1844.
Varlinskaya, E.I., Spear, L.P., 2007. Chronic tolerance to the social consequences of ethanol in adolescent and adult Sprague–Dawley rats. Neurotoxicol. Teratol. 29, 23–30.
Zorrilla, E.P., 1997. Multiparous species present problems (and possibilities) to developmentalists. Dev. Psychobiol. 30, 141–150.