Contents lists available at ScienceDirect

Progress in Neuropsychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com

Short-term selection for high and low ethanol intake yields differential sensitivity to ethanol's motivational effects and anxiety-like responses in adolescent Wistar rats

Macarena Soledad Fernández[a, b], Bárbara Báez[a], Ana Bordón[a], Laura Espinosa[a], Eliana Martínez[a], Ricardo Marcos Pautassi[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

Keywords:
Short-term selective breeding
Adolescents
Ethanol aversion
Ethanol intake

ABSTRACT

Alcohol use disorders are modulated by genetic factors, but the identification of specific genes and their concomitant biological changes that are associated with a higher risk for these disorders has proven difficult. Alterations in the sensitivity to the motivational effects of ethanol may be one way by which genes modulate the initiation and escalation of ethanol intake. Rats and mice have been selectively bred for high and low ethanol consumption during adulthood. However, selective breeding programs for ethanol intake have not focused on adolescence. This phase of development is associated with the initiation and escalation of ethanol intake and characterized by an increase in the sensitivity to ethanol's appetitive effects and a decrease in the sensitivity to ethanol's aversive effects compared with adulthood. The present study performed short-term behavioral selection to select rat lines that diverge in the expression of ethanol drinking during adolescence. A progenitor nucleus of Wistar rats (F_0) and filial generation 1 (F_1), F_2, and F_3 adolescent rats were derived from parents that were selected for high (STDRHI) and low (STDRLO) ethanol consumption during adolescence and were tested for ethanol intake and responsivity to ethanol's motivational effects. STDRHI rats exhibited significantly greater ethanol intake and preference than STDRLO rats. Compared with STDRLO rats, STDRHI F_2 and F_3 rats exhibited a blunted response to ethanol in the conditioned taste aversion test. F_2 and F_3 STDRHI rats but not STDRLO rats exhibited ethanol-induced motor stimulation. STDRHI rats exhibited avoidance of the white compartment of the light-dark box, a reduction of locomotion, and a reduction of saccharin consumption, suggesting an anxiety-prone phenotype. The results suggest that the genetic risk for enhanced ethanol intake during adolescence is associated with lower sensitivity to the aversive effects of ethanol, heightened reactivity to ethanol's stimulating effects, and enhanced innate anxiety.

1. Introduction

The literature suggests that 50% of the variability of alcohol use disorders (AUDs) is attributable to genetic factors (Dick and Agrawal, 2008; Ducci and Goldman, 2008, 2012). Seminal studies indicated that alcoholism runs in families. Children of alcoholics are 3- to 5-times more likely to be diagnosed with AUD than children of non-alcoholic parents (Cotton, 1979). Dozens of studies have indicated that a positive family history of AUD (FH +) is a risk factor for AUD.

Alcohol use disorder does not follow a simple dominant or recessive pattern of inheritance but instead appears to be polygenic (i.e., it is caused by the independent and interactive effects of several genes); (Rietschel and Treutlein, 2013) and impacted by environmental modulation. The identification of specific genes and their concomitant biological changes that are associated with a higher risk of AUD has been difficult. Genetic alterations in enzymes that metabolize alcohol (hereinafter referred to as ethanol) were shown to be associated with differential degrees of AUD, a finding that opened the door to promising therapies (Ocaranza et al., 2008; Rivera-Meza et al., 2012). Genome-wide association studies (Adkins et al., 2017) and copy number variation studies (Bae et al., 2012) have helped pinpoint promising target genes. One alternative to these technically demanding experimental approaches is to identify biobehavioral correlates of AUD that are linked to the genetic predisposition to AUD, even iB60n subjects that do not fully express the disease (Schuckit, 1994).

FH + subjects perceive the autonomic and subjective effects of moderate ethanol doses significantly differently from those who are not

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

http://dx.doi.org/10.1016/j.pnpbp.2017.06.027
Received 1 April 2017; Received in revised form 25 May 2017; Accepted 24 June 2017
Available online xxx
0278-5846/

at risk. Several studies (Conrod et al., 1997a; Conrod et al., 2001) reported heightened psychomotor stimulation in FH + individuals than in their FH- counterparts during the rising limb of the blood ethanol curve. Other studies (Schuckit et al., 2004) suggest that FH + individuals exhibit a blunted response to the aversive and sedative effects of ethanol. Thus, alterations in the sensitivity to the motivational (e.g., appetitive, aversive, and anxiolytic) effects of ethanol may be one way by which genes modulate the initiation and escalation of ethanol intake. The latter is an intriguing hypothesis that has been investigated in preclinical studies using rats that are selectively bred for high and low ethanol consumption, such as alcohol-preferring (P) and alcohol-non-preferring (NP) rats (Bell et al., 2008), Universidad de Chile Abstinent and Bibulous (UChA and UChB, respectively) rats (Quintanilla and Tampier, 2011), and Marchigian Sardinian alcohol-preferring (msP) rats (Ciccocioppo et al., 2006). P rats exhibit lower sensitivity to the aversive effects of ethanol (Stewart et al., 1996) but heightened sensitivity to the motor-stimulating effects of ethanol (Waller et al., 1986), which are considered proxies of the positive rewarding effects of the drug. UChB but not UChA rats exhibit ethanol-induced conditioned place preference (CPP) after preexposure to free-choice ethanol drinking (Quintanilla and Tampier, 2011).

Strains that are selectively bred for high ethanol intake are valuable animal models. They are generated by crossing males and females with high preference for 10% alcohol during adulthood for 30, 40, or 70 generations. Still unknown, however, are the ways in which ethanol intake and ethanol-induced appetitive and aversive responses diverge or converge across the initial generations. Phillips et al. (2005) reported lower ethanol-induced conditioned taste aversion (CTA) in the second generation of female but not male mice that were short-term selected for high ethanol intake compared with their counterparts that were selected for high ethanol intake. Several other studies with selectively bred or heterogeneous rats and mice (Doremus et al., 2005) have yielded a negative correlation between ethanol-induced CTA and ethanol drinking [for review and references, see (Green and Grahame, 2008)]. For instance, adolescent rats usually drink significantly more ethanol than adult counterparts (Doremus et al., 2005; Vetter et al., 2007) and, unlike their mature counterparts, are relatively insensitive to ethanol-induced CTA (Vetter-O'Hagen et al., 2009). Overall, this supports the hypothesis that insensitivity to ethanol's aversive effects is a factor in the vulnerability to enhanced ethanol consumption. The intriguing study by Phillips et al. (2005) also found greater ethanol-induced CPP in a short-term line that was selected for high ethanol consumption compared with their low ethanol consumption counterparts (hereinafter referred to as STDRHI and STDRLO, respectively). Ethanol-induced CPP is notoriously difficult to observe in genetically heterogeneous rats, yet it has been found in Marchigian Sardinian alcohol-preferring (Ciccocioppo et al., 1999) and other, genetically selected, alcohol-preferring rats. These studies further suggest a genetic relationship between ethanol's motivational effects and ethanol intake. The association between ethanol-induced CPP and ethanol intake is, however, much more variable than that found between ethanol-induced CTA and ethanol intake (Green and Grahame, 2008).

Other short-term selection programs for ethanol intake have been used to map quantitative trait loci (Belknap et al., 1997) and analyze differences in behavioral traits other than ethanol responses. STDRHI mice exhibited deficits in response inhibition (Wilhelm et al., 2007), a component of the broader construct of impulsivity that is linked to the vulnerability to ethanol intake during adolescence (Pilatti et al., 2017). An exacerbated anxiety response is another innate trait that can promote ethanol drinking via negative reinforcement mechanisms. msP rats are less prone than non-selected rats to explore the open, potentially dangerous, arms of the elevated plus maze and the central area of the open field (OF; Roman et al., 2012). Roman high-avoidance rats exhibit greater anxiety and consume more ethanol than inbred Roman low-avoidance rats (Manzo et al., 2012). Moreover, our lab recently reported significantly higher intake of ethanol in female, adolescent, rats with high levels of inborn anxiety than in counterparts with standard levels of anxiety (Acevedo et al., 2016). Together, this evidence suggests that an "anxious" phenotype may facilitate the sustained engagement in ethanol intake in this line (Ciccocioppo et al., 2006).

To our knowledge, a selective breeding program has not been performed for low and high levels of ethanol drinking during adolescence in rats or mice. Early and highly influential typological accounts of alcoholism differentiated between type I alcoholism that emerges later in life (i.e., after years of heavy drinking) and type II alcoholism that emerges during adolescence, predominantly in males, and is driven by the appetitive, rewarding effects of ethanol (Cloninger et al., 1996; Sigvardsson et al., 1996). At the epidemiological level, the time course of ethanol intake is initiated, peaks, and is almost normative during adolescence in western youth (Pinsky et al., 2010a). By the end of high school, more than half of adolescents engage in heavy episodic drinking patterns every time they drink, and a similar percentage have engaged in at least one binge drinking episode within the past year (Pilatti et al., 2013). Epidemiological and preclinical studies have shown that the earlier initiation and escalation of ethanol drinking is associated with a higher probability of problematic ethanol intake later in life. Still unknown, however, is whether both events are casually linked or whether they are both symptoms of a third variable, namely genetic vulnerability. A previous study of college students found that the frequency of drunkenness and other ethanol-related consequences was related to the age of onset of ethanol use but only in FH + individuals (Pilatti et al., 2014). Preclinical studies have consistently indicated that adolescent rats exhibit patterns of ethanol responsiveness that may facilitate the initiation and escalation of ethanol use. Compared with adult counterparts, adolescent rats are more sensitive to the appetitive (Pautassi et al., 2008) and social-facilitating effects of ethanol (Varlinskaya and Spear, 2015) and the acute cognitive deficits that are induced by ethanol (Swartzwelder et al., 2014). Moreover, adolescent rats are less sensitive to the aversive and sedative effects of ethanol that serve as natural barriers to sustained engagement in ethanol drinking (Spear and Swartzwelder, 2014). These and other studies have changed the concept of AUDs, which are now considered developmental conditions that have etiological roots in adolescence (NIH, 2008).

The breeding of rats that are selected for high and low ethanol consumption during adolescence would facilitate analyses of the mechanisms by which genes increase the likelihood of AUD. Such selective breeding may reveal the putative relationship between motivational sensitivity to ethanol and ethanol drinking (Green and Grahame, 2008) or detect preexisting (i.e., before any contact with ethanol) differences in innate anxiety or other traits between adolescents that are derived from high- and low-ethanol progeny. Anxiety-related disorders usually begin during adolescence (Cunningham et al., 2002) and are significantly associated with the emergence of AUD (Hobbs et al., 2011). This breeding strategy could help uncover endophenotypes, stable heritability, and behavioral traits that are linked to the pathophysiology of AUD (Hines et al., 2005; Klee et al., 2012) during a key developmental stage for the initiation of ethanol use.

The present study produced rat lines that diverged in the expression of ethanol drinking during adolescence through short-term behavioral selection (Belknap et al., 1997; Linsenbardt and Boehm, 2013). A progenitor, F_0, nucleus of genetically heterogeneous Wistar rats and filial generation 1 (F_1), F_2, and F_3 STDRHI and STDRLO offspring that derived from the selective mating of animals with high and low ethanol intake were tested for ethanol intake throughout adolescence (postnatal days 32–57 [PD32–57], Exp. 1) or for responsiveness to ethanol's motivational effects. Our hypothesis was that selection pressure would yield significant differences between STDRHI and STDRLO rats in ethanol-induced motor stimulation (Exp. 2a), basal innate anxiety (Exp. 2b), and ethanol-induced motivational learning (measured by CTA and place conditioning; Exp. 3 and 4, respectively). The measurement of saccharin intake during CTA conditioning allowed us to evalu-

ate the relationship between the predisposition to ethanol and saccharin intake. Sweet liking has been suggested to facilitate the transition from heavy drinking to AUD in young adults (Kampov-Polevoy et al., 2014). Blood ethanol concentrations (BECs) in F_3 rats were measured in Exp. 5.

2. Materials and methods

2.1. Experimental design

The selective breeding project (Experiment 1) included two groups in the F_0 generation (male and female, $n = 40$ per sex). In the following generations Experiment 1 used a 2 (Sex) × 2 (Line: STDRHI vs. STDRLO) × 2 (Generation: F_1 vs. F_2) design ($n = 20$ per group, except for $STDRLO_2$ females [$n = 17$]). Exp. 2 employed a 2 (Sex) × 2 (Line: STDRHI vs. STDRLO) × 2 (Generation: F_2 vs. F_3) × 3 (Dose [ethanol treatment prior to motor activity test]: 0.0, 1.25, and 2.5 g/kg) factorial design ($n = 7$–10 per group). A similar design was used for Exp. 3 that assessed ethanol-induced CTA ($n = 6$–9 per group), but ethanol treatment consisted of only two conditions (0.0 and 2.5 g/kg). Exp. 4 assessed ethanol-induced place conditioning in F_2 rats and employed a 2 (Sex) × 2 (Line: STDRHI vs. STDRLO) × 2 (Dose [ethanol treatment during conditioning]: 0.0 and 2.5 g/kg) factorial design ($n = 8$–12 per group). Exp. 5 assessed BECs in male and female F_3 rats (STDRHI and STDRLO) that were given 1.25 or 2.5 g/kg ethanol ($n = 5$–7 per group).

2.2. Subjects

The ethanol intake and behavioral tests were conducted with a total of 608 Wistar rats (80, 80, 248, and 200 from the F_0, F_1, F_2, and F_3 generations, respectively). The number of male and female animals that were used in each experiment are shown in Table 1, which also serves as a visual aid to understand the procedures conducted in each filial generation. Please note that the ethanol intake tests were conducted in F_0, F_1 and F_2 rats only. F_3 rats were not tested for ethanol intake but instead were tested in several behavioral assays. The animals were born and reared in one of the vivariums at the Instituto de Investigaciones Médicas M. y M. Ferreyra (INIMEC-CONICET-UNC, Córdoba, Argentina). The vivarium, a producer of specific pathogen-free animals, was maintained under a 12 h/12 h light/dark cycle (lights on at 8:00 AM) at 22–24 °C. All of the procedures followed the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), as adopted and promulgated by the NIH and the EU, were certified by the Institutional Animal Care and Use Committee at INIMEC-CONICET-UNC and the experiments were carried out in accordance with the Declaration of Helsinki.

Female rats were mated with a single male to provide subjects for the study. These couples were maintained in standard maternity cages that were lined with corn cob bedding. The animals had ad libitum access to food and water. Births were checked daily. The day of birth was considered PD0. The offspring were kept with the parental couple up to weaning on PD21 when they were transferred to maintenance cages in same-sex groups of five each.

2.3. Procedures

2.3.1. Ethanol intake tests and criteria for selection and production of high and low ethanol consumption lines of rats (i.e., Experiment 1, selection breeding process)

The foundational nucleus (i.e., F_0) was composed of 120 (60 male and 60 female) genetically heterogeneous Wistar rats that were derived from 12 litters. Eighty of these animals were randomly selected and tested for ethanol intake and preference throughout adolescence (PD32–57) using an intermittent-access intake protocol (three sessions per week, 24 h sessions). Between sessions, the rats were pair-housed in same-sex pairs and had ad libitum access to food and water.

During the intake sessions, the animals were individually housed in half of their homecage, separated from their conspecific by a Plexiglas divider. They had ad libitum access to food and were exposed to two bottles, one of which always contained water. The other bottle was filled with 4% ethanol during the first week (PD32, PD34, and PD36) and 5% ethanol during the second week (PD39, PD41, and PD43). During the last 2 weeks (PD46, PD48, PD50, PD53, PD55, and PD57), the animals were given a choice between the water bottle and a 6% ethanol bottle. The rationale for using this relatively low ethanol concentration is that it is similar to the ethanol content of beverages that are preferred by adolescents. Beer, which typically contains 3–8% ethanol, accounts for more than half of the ethanol that is ingested by adolescents (Pinsky et al., 2010b). Beer was also the beverage of choice for 67% of adolescents aged 18–20 years who engaged in binge drinking in the United States (Naimi et al., 2007). Moreover, uninitiated adolescent Wistar rats ingest very little ethanol at concentrations ≥ 6%. We have had some success with higher concentrations when mixed with sucrose or when provided after substantial water and food deprivation (Ponce et al., 2011). In the present study, however, we preferred to avoid the confounding effects of the caloric surplus of sucrose and stress associated with dehydration.

Before and after each session, the bottles were weighed to the nearest 0.01 g (Ohaus L2000, Ohaus, Pine Brook, NJ, USA). These measurements were used to calculate ethanol intake (g/kg) and the percent preference of ethanol intake ([ethanol consumption/overall fluid consumption] × 100), which in turn were used to classify the animals as STDRHI or STDRLO. Specifically, 12 males and 12 females that exhibited the highest absolute ethanol intake during the last two weeks (i.e., average g/kg during the three sessions of week 3 and the three sessions of week 4) were classified as high ethanol consumers and mated together. Endogamic mating was avoided whenever possible. For an animal to be selected as STDRHI, it also had to be within the 12 males and 12 females that exhibited the highest average percentage of ethanol preference (during the three sessions of week 3 and the three sessions of week 4), and this average had to be > 50%. Similar criteria, yet taking into account the lowest absolute and percent ethanol intake scores, were used for low ethanol consumers. The selection process resulted in STDRHI F_0 rats that exhibited daily ethanol intake > 4.0 g/kg and percent ethanol preference > 50% by the end of the protocol. Conversely, daily ethanol intake and percent ethanol preference in STDRLO F_0 rats were around 1.0 g/kg and 20%, respectively. These values were similar to those exhibited by other line pairs of rats that were selectively bred for divergent ethanol preference and consumption, such as Sardinian alcohol-preferring (sP) and -non-preferring (sNP) rats (Colombo et al., 2006a; Colombo et al., 2006b).

The F_1 rats in this study were generated from crossing male and female F_0 STDRHI rats, and male and female F_0 STDRLO rats. These couples produced 120 F_1 STDRHI rats and 120 F_1 STDRLO rats. Of these animals, 40 F_1 STDRHI rats and 40 F_1 STDRLO rats, randomly selected, were tested for ethanol intake. The 12 F_1 STDRHI males and 12 F_1 STDRHI females that exhibited the highest ethanol intake during the last two weeks were classified as high ethanol consumers and mated together. Similarly, the 12 F_1 STDRLO males and 12 F_1 STDRLO females that exhibited the lowest ethanol intake during the last two weeks were classified as low ethanol consumers and mated together. These 24 couples (i.e., 12 STDRHI and 12 STDRLO) produced 120 F_2 STDRHI rats and 120 F_2 STDRLO rats. The selection process was repeated in 40 F_2 STDRHI rats and 37 F_2 STDRLO rats. This is, the F_3 rats were the offspring generated from crossing male and female F_2 STDRHI rats, and male and female F_2 STDRLO rats.

F_3 rats were not tested for ethanol intake, but instead were assessed for ethanol-induced motor activity, basal anxiety response, ethanol-induced conditioned taste aversion and blood ethanol concentrations. Similarly, those animals from the F_1 and F_2 generations that were not

Table 1
Number of male and female Wistar rats employed in each Experiment, as a function of selected line (high or low-ethanol drinkers, STDRHI and STDRLO, respectively) and generation [F0 or progenitors and filial generations 1, 2 and 3 (F_1, F_2 and F_3, respectively)]. The numbers italicized between parentheses correspond to animals that had been part of a bigger group. For instance, the 7, F_2 animals, assessed for anxiety response in Exp. 2b are rats that had been among the 21 rats treated with vehicle during the assessment of ethanol-induced motor activity in Experiment 2a. Data for the F_0 is presented for the group of eighty animals originally tested for ethanol intake and preference (i.e., overall group) and for the sub-groups classified as STDRHI or STDRLO, that were mated together to produce the F_1 offspring.

Generation		F_0	F_1	F_2					F_3			
Experiment & line		Exp. 1 Ethanol intake tests		Exp. 1 Ethanol intake tests	Exp. 2a Ethanol induce motor activity	Exp. 2b Basal anxiety response	Exp. 3 Ethanol-induced conditioned taste aversion	Exp. 4 Ethanol-induced place conditioning	Exp. 2a Ethanol-induced motor activity	Exp. 2b Basal anxiety response	Exp. 3 Ethanol-induced conditioned taste aversion	Exp. 5 Measurement of blood ethanol levels
F0 (overall group)	♂	39	–	–	–	–	–	–	–	–	–	–
	♀	41	–	–	–	–	–	–	–	–	–	–
STDRHI rats	♂	(12)	20	20	21	*(7)*	15	20	25	*(10)*	14	12
	♀	(12)	20	20	21	*(7)*	15	21	28	*(10)*	13	12
STDRLO rats	♂	(12)	20	20	27	*(9)*	13	25	25	*(8)*	10	11
	♀	(12)	20	17	27	*(7)*	14	24	27	*(8)*	12	10
Total		80	80	248					200			

subjected to the ethanol intake protocol were submitted to behavioral assays, or they were discarded. The 12 F_2 couples and 12 F_3 couples provided three and two parturitions, respectively. Across experiments, litter effects (Zorrilla, 1997) were avoided by not including more than one male and one female from each litter in a given group.

Some differences with previous selective breeding studies should be noted. Unlike the continuous access paradigm that has been employed in most studies, we used an every-other-day access ethanol intake protocol (with a 2-day rest every week). The rationale was to mimic the normative pattern of consumption in adolescents who, compared with adults, drink fewer grams of ethanol per month (Carvajal and Lerma-Cabrera, 2015) but consume about twice as much ethanol per drinking occasion (SAMSA, 2007), which is a consequence of concentrating their ethanol intake on a few days per week. Our intermittent access protocol also helped mitigate isolation-induced stress because the animals were reunited with a peer between sessions. In most, if not all, rodent studies that selectively bred animals for alcohol consumption, the mice or rats were housed individually for 1 or more months. This procedure imposes unwanted stress exposure that can significantly alter the selection process.

2.3.2. Ethanol administration (Exp. 2–5)

Ethanol doses of 1.25, 2.0, or 2.5 g/kg were achieved by administering 0.015 ml/g of a 10.5%, 16.8%, or 21% ethanol solution (v/v, Porta Hnos, Córdoba, Argentina), respectively. Intragastric administration in Exp. 2 (ethanol-induced motor activity), Exp. 3 (CTA), and Exp. 5 (BECs) was performed by introducing an approximately 8 cm length of polyethylene 10 tubing (Clay Adams) into the oral cavity, which was then gently guided to the stomach. This intubation procedure, which mostly avoids orosensory stimulation, was chosen because it mimics the oral route commonly used by humans. The dose and mode of administration were selected based on prior studies from our laboratory that indicated that these combinations yield reliable ethanol-induced acute stimulant effects or CTA in adolescent rats (Acevedo et al., 2010; Acevedo et al., 2013; Pautassi et al., 2011). The tubing was connected to a 10 ml BD syringe that was mounted with a 27-gauge needle. In Exp. 4, we switched to intraperitoneal injections to allow better contiguity between ethanol's pharmacological effects at a dose of 2.0 g/kg and exposure to the place conditioning chambers.

Assessment of basal and ethanol-induced motor activity (Exp. 2a) and assessment of basal anxiety (Exp. 2b). A subset of male (n = 48) and female (n = 48) F_2 animals that were naive to ethanol intake and a subset of F_3 animals (50 males and 55 females) were assessed for ethanol-induced stimulant effects on PD32. This is, ethanol-induced distance traveled was assessed in F_2 and F_3, but not in F1, rats. Ethanol (or vehicle) was administered via intragastric administration by trained researchers. The animals that were treated with vehicle during the motor activity test (30 F_2 rats and 36 F_3 rats) underwent the light-dark box (LDB) test on PD39 to assess anxiety reactivity.

Ethanol-induced motor activity was tested in opaque (60 cm × 60 cm × 60 cm) open field (OF) chambers that were made of Plexiglas and equipped with photocell beams. The rats were treated with 0.0, 1.25, or 2.5 g/kg ethanol. Five minutes later, they were placed in the dimly lit (~ 50 lx) OF. Beam breaks were recorded by an activity monitoring system (ITCOMM, Córdoba, Argentina) that provided a measure of horizontal distance traveled (cm) and frequency of rearings (number of beam breaks of a photocell that was placed ~ 15 cm above the floor of the OF) during the 12-min test (i.e., post-administration time 5–16 min).

Patterns of anxiety-like behavior were tested in the LDB test. The apparatus was made of high-impact acrylic. It featured two compartments. One compartment (24.5 cm × 25 cm × 25 cm) was white and brightly lit by a 60 W white bulb that generated 400 lx illumination. The other compartment (17.5 cm × 25 cm × 25 cm) was black and not illuminated (0 lx). Illumination was determined by a digital lux meter (LX1010B). The compartments were separated by a divider with an opening at floor level. The test lasted 5 min. The animals were initially placed in the white compartment, facing away from the black compartment. The test was videotaped and subsequently analyzed by a trained observer who recorded the following parameters: latency (in seconds) to enter the dark compartment, time (in seconds) spent in the white compartment, and number of transitions between compartments.

2.3.3. Ethanol-induced CTA (Exp. 3)

Taste aversion induced by ethanol's pharmacological effects was tested in a subset of F_2 and F_3 animals. A 7-day protocol was adapted from one that is commonly used in our laboratory. In this procedure, animals ingest a novel taste (saccharin) and, immediately after removal of the taste they are intubated (i.e., i.g. administered) with the drug. The tubing is gently guided to the stomach, to avoid stimulation with ethanol's orosensory properties. In other words, the taste of saccharin is the conditioned stimulus (CS) and the pharmacological, post-absorptive, effect of ethanol is the unconditional stimulus (US). In contrast to our previous CTA studies, we did not water-deprive the animals before conditioning day 1. This provided a basal, unconfounded measure of saccharin intake, which was important for analyzing the relationship between sweet preference and ethanol intake. The animals were subjected to moderate water deprivation before conditioning session 2. This ensured motivation to drink the sapid conditioned stimulus.

On experimental days 1 and 2 (PD32–33), the animals were individually housed (22 cm × 20 cm × 30 cm cages) with ad libitum access to food and water. Daily water consumption (in milliliters) was measured. The first conditioning session occurred on day 3 (PD34). At 9:00 AM, the animals were weighed and returned to their housing chamber, which was now equipped with a graded tube that contained 0.1% saccharin (Parker Davis, Buenos Aires, Argentina). The graded tube was removed after 30 min. At that moment, saccharin intake (in milliliters) was measured, and the rats received 2.5 g/kg ethanol or vehicle. They were then returned to the housing chamber with ad libitum access to food and water. Saccharin consumption on this day provided a measure of basal reactivity to this sweet tastant (i.e., before ethanol administration and before water deprivation).

On experimental day 4, the animals had access to only 50% of the water they would usually drink to ensure motivation to consume the conditioned stimulus in the subsequent conditioning session, which occurred on day 5 (PD36). The second conditioning session followed identical procedures as those described for experimental day 3. On experimental day 6 (PD37), the rats were water-restricted (50%). Testing for ethanol-induced CTA occurred on the morning of day 7 (PD38). The animals were given 60-min access to a graded tube that contained 0.1% saccharin.

2.3.4. Ethanol-induced place conditioning (Exp. 4)

Ethanol-induced place conditioning was tested in a subset of STDRHI and STDRLO F_2 animals. During the conditioning sessions, the apparatus featured one compartment that was lined with a copolymer of ethylene vinyl acetate (EVA) and one compartment that was lined with 60-grit sandpaper (SAND). A guillotine door, which was raised during the habituation and test sessions, separated both compartments. During the habituation and test sessions, the apparatus featured an intermediate zone with a black Plexiglas floor.

The procedures began on PD32, with a 10 min pretest (i.e., an habituation session that yielded preconditioning preference scores). Four daily conditioning sessions were conducted on PD33–36, in which the animals experienced pairings between the effects of ethanol or vehicle and the distinctive compartments. At the beginning of the conditioning session, all of the animals were treated with vehicle (saline; volume: 1 ml/g body weight, 0.89% v/v) and subsequently placed in the EVA compartment for 12 min. Twenty minutes later, the rats were administered ethanol (experimental group: 2.0 g/kg, volume: 1 ml/g body weight, i.p.) or vehicle (control group) and immediately placed for 12 min in the SAND compartment. For the experimental animals, EVA

and SAND served as excitatory and inhibitory (CS^+ and CS^-, respectively) stimuli that were conditioned to ethanol's aversive effects. The control groups were exposed to both stimuli, always after vehicle injections. The test session was conducted on PD37 and was similar to the habituation session. The time spent in each compartment was recorded.

2.3.5. Determination of BECs (Exp. 5)

The animals that were used for BEC assessment were male and female STDRHI F_3 and STDRLO F_3 adolescent rats, 32 days old and naive to any experimental manipulation until they were injected with 1.25 or 2.5 g/kg ethanol on PD37. The rats were individually placed for 20 min in cages that were lined with corn cob bedding. Immediately afterward, the animals were decapitated, and a 1 ml trunk blood sample was placed in a heparinized capillary tube. The blood samples were transferred to a 2.5 ml Eppendorf tube and kept at − 20 °C for subsequent processing via head-space gas chromatography (Hewlett-Packard 5890 series II, Hewlett-Packard, Wilmington, DE, USA). BECs are expressed as milligrams of ethanol per deciliter of blood (mg/dl = mg%).

2.4. Statistical analysis

Ethanol intake (g/kg and percent preference) and overall fluid intake (ml/100 g body weight) in the progenitor group (i.e., F_0, Exp. 1) were analyzed using independent two-way mixed analysis of variance (ANOVA). The dependent variables are expressed as average weekly consumption. Average absolute ethanol intake in week 1 was calculated as the following: (g/kg ingested in session 1 + g/kg ingested in session 2 + g/kg ingested in session 3)/3. In the ANOVA, Sex (male, female) was the between-subjects fact, and Week (1, 2, 3, and 4) was the within-subjects factor. A subsequent ANOVA of ethanol intake included Line (STDRHI, STDRLO) as an additional factor to confirm that the separation into low and high ethanol drinkers resulted in statistically well-differentiated groups. Data from three subjects were lost because of procedural errors. These data were not replaced.

Ethanol intake (g/kg and percent preference) and overall fluid intake in the F_1 and F_2 generations (Exp. 1) were analyzed using repeated-measures ANOVA (Sex × Line × Generation × Week). The analysis of overall fluid intake was conducted to assess if the predicted greater intake of STDRHI rats was specific for ethanol or also generalized to the total quantity of fluids consumed.

Heritability (h^2; i.e., the amount of variance in g/kg ethanol scores [average consumption in weeks 3 and 4] that was attributable to genetic differences) was calculated in F_1 and F_2 STDRHI rats, for the overall sample, and separately for females and males using previously described methods (Falconer and Mackay, 1996). Following prior literature (Oberlin et al., 2011) a mean heritability estimate was obtained for each sex, by averaging the heritability estimates derived for each generation. We also analyzed, separately for each sex and generation of STDRHI rats, the correlation (Pearson product-moment) between offspring intake scores (i.e., average consumption in weeks 3 and 4) vs. the average of parental intake scores (i.e., mid-parent phenotypic value), as described in Wray (2008).

The selection for high or low ethanol consumption was conducted on the average ethanol consumption during the third and fourth week of intake. It was thus important to further analyze whether, in each generation, the selected lines actually differed significantly on this primary outcome. Planned comparisons were conducted between STDRHI and STDRLO rats, one for each generation (i.e., F_0, F_1 and F_2). The dependent variable was the average ethanol consumption (g/kg) on weeks 3 and 4.

The total distance traveled (in centimeters) and frequency of rearings in the OF test (Exp. 2a) were analyzed using independent ANOVAs, with Sex, Line, Dose (0.0, 1.25, and 2.5 g/kg), and Generation (F_2 and F_3) as between-subjects factors. The variables that were recorded in Exp. 2b (i.e., the latency to exit and time spent in the white compartment of the LDB box and number of transitions between compartments) were analyzed using factorial ANOVAs (Sex × Line × Generation).

Saccharin intake scores (ml/100 g body weight, Exp. 3) during the 30-min conditioning sessions were analyzed using repeated-measures ANOVA, with Generation, Line, Sex, and Treatment (0.0 and 2.5 g/kg) as between-subjects factors and Conditioning Session (1 and 2) as the within-subjects factor. Saccharin intake during the 60-min test session was analyzed using factorial ANOVA, with Generation, Line, Sex, and Treatment as between-subjects factors.

In Exp. 4, habituation scores during place conditioning were analyzed using three-way repeated-measures ANOVA, with Line and Sex as between-subjects factors and absolute time spent in each compartment as the within-subjects factor. This analysis was intended to reveal possible innate, preconditioning preference for the chambers that were used during training. Test scores (i.e., absolute [in seconds] or percent preference for the SAND CS^+ compartment) were analyzed using ANOVA, with Sex, Line, and Treatment (0.0 and 2.5 g/kg ethanol) as factors. Blood ethanol concentrations (mg/dl) in Exp. 5 were analyzed using factorial ANOVA, with Sex, Line, and Treatment (1.25 and 2.5 g/kg ethanol) as factors.

We also analyzed weight scores and basal reactivity to the different stimuli and procedures that were used during the experiments in STDRHI and STDRLO rats. Body weight on PD32 (i.e., the beginning of intake and most of the behavioral procedures) was analyzed using repeated-measures ANOVA (Sex × Line × Generation [F_0, F_1, F_2, and F_3]). This analysis was conducted for STDRHI and STDRLO F_0, F_1, and F_2 rats that subsequently underwent the intake tests and F_3 rats that underwent the motor activity test. Independent factorial ANOVAs were used to analyze the distance traveled in the OF (Exp. 2a) and saccharin consumption during the first conditioning day of Exp. 3 in vehicle-treated male and female rats from both lines of the F_2 and F_3 generations. Distance traveled in the OF in these control groups reflected the response to novelty, whereas saccharin intake provided a measure of sweet preference.

Significant main effects and interactions in the ANOVA were analyzed using Duncan's post hoc test. The alpha level was 0.05. Cohen's partial eta squared (η^2p) was used to describe effect sizes. Given the complexity of depicting significant main effects and interactions that spanned several groups and conditions, some of the significant differences between groups are not depicted in the figures with asterisks or other signs. In those cases, a description of these significant differences is found in each figure legend.

3. Results

3.1. Ethanol intake in F_0 rats (Exp. 1)

Table 2 shows the weekly averages of g/kg ethanol intake, percent preference, and overall fluid intake (ml/100 g body weight) in F_0 rats, as a function of sex and line. Weekly averages of g/kg ethanol intake and percent preference are also depicted, with data collapsed across sex, in Fig. 1 (panels A-B).

The repeated-measures ANOVA of g/kg ethanol intake yielded significant main effects of Sex and Week ($F_{1,74} = 18.61$, $p < 0.001$, $\eta^2p = 0.20$, and $F_{3,222} = 8.14$, $p < 0.001$, $\eta^2p = 0.10$, respectively). The post hoc tests indicated that intake scores significantly increased in weeks 3–4 compared with weeks 1–2. Females consumed more ethanol than males throughout testing. The ANOVA of percent ethanol preference only yielded a significant main effect of Sex ($F_{1,74} = 7.61$, $p < 0.001$, $\eta^2p = 0.09$). Overall fluid intake was significantly greater in females than in males and significantly greater in the first week than in the subsequent weeks (significant main effects of Sex and Week: $F_{1,74} = 9.71$, $p < 0.005$, $\eta^2p = 0.12$, and $F_{3,222} = 29.47$, $p < 0.001$, $\eta^2p = 0.28$, respectively).

Table 2
Ethanol intake (g/kg and % preference) and overall fluid intake (ml/100 g of body weight), expressed as weekly averages, in the foundational or progenitor generation (F_0). Data is presented for the group of eighty animals originally tested for ethanol intake and preference (i.e., overall group including all F_0 rats) and for the sub-groups classified as high- or low-ethanol consumers (STDRHI F_0 and STDRLO F_0, respectively) that were mated together to produce the F_1 offspring. Values express mean ± SEM.

	Week	F_0 (all rats)		STDRHI F_0 rats		STDRLO F_0 rats	
		♂	♀	♂	♀	♂	♀
Ethanol intake (g/kg)	1	1.95 ± 0.25	3.19 ± 0.31	2.97 ± 0.51	3.25 ± 0.65	1.24 ± 0.30	2.64 ± 0.51
	2	1.58 ± 0.29	3.55 ± 0.37	2.87 ± 0.69	5.02 ± 0.65	0.52 ± 0.10	2.28 ± 0.54
	3	2.71 ± 0.39	4.70 ± 0.42	5.23 ± 0.59	7.41 ± 0.53	0.47 ± 0.08	1.66 ± 0.33
	4	2.39 ± 0.35	3.94 ± 0.41	4.77 ± 0.45	7.00 ± 0.45	0.37 ± 0.16	1.28 ± 0.35
Ethanol preference (%)	1	30.86 ± 4.36	42.27 ± 4.31	46.66 ± 9.10	42.95 ± 7.74	19.62 ± 5.17	35.20 ± 7.65
	2	22.02 ± 4.21	42.74 ± 4.48	37.84 ± 8.83	62.53 ± 8.04	7.02 ± 1.59	28.20 ± 7.04
	3	32. 11 ± 4.82	46.18 ± 4.20	62.50 ± 6.43	68.99 ± 5.00	4.84 ± 1.01	17.85 ± 5.04
	4	34. 14 ± 5.14	43.12 ± 4.26	4.47 ± 2.06	71.90 ± 3.68	4.47 ± 2.06	15.09 ± 3.85
Overall fluid intake (ml/100 g of body weight)	1	21. 36 ± 0.81	25. 08 ± 0.91	21. 15 ± 1.21	24. 53 ± 1.67	20. 96 ± 1.07	24. 82 ± 1.21
	2	18. 92 ± 0.86	22. 09 ± 0.96	18. 79 ± 1.17	22. 24 ± 1.60	19. 54 ± 1.44	20. 96 ± 1.19
	3	19.45 ± 0.68	22. 49 ± 0.91	17. 60 ± 0.62	23. 06 ± 1.02	20. 74 ± 1.17	22. 43 ± 1.74
	4	17.05 ± 0.67	20. 04 ± 0.81	15. 24 ± 0.98	21. 14 ± 1.02	19. 35 ± 1.11	18. 49 ± 1.39

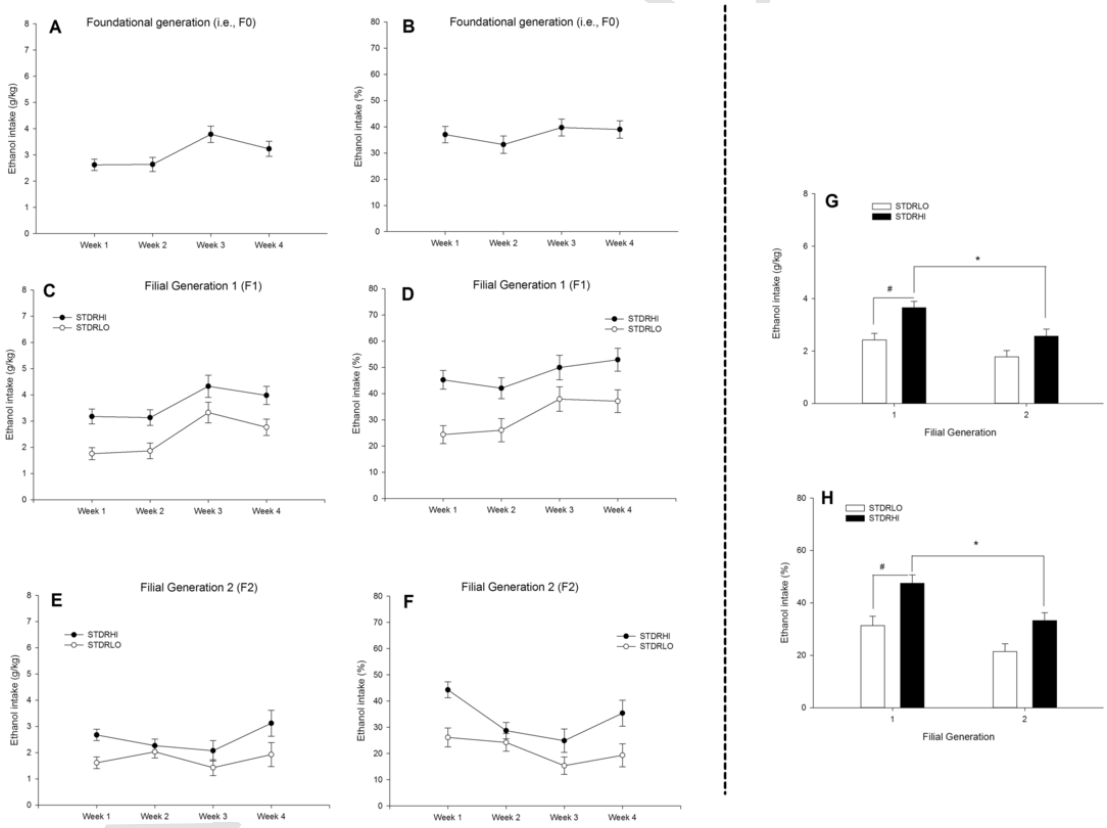

Fig. 1. (A–F) Mean ethanol intake (g/kg) (A, C, E) and percent preference (B, D, F) in male and female Wistar rats as a function of filial generation (F_0, F_1 and F_2) of short-term lines selected for high and low ethanol consumption (STDRHI and STDRLO, respectively) during weeks 1, 2, 3, and 4 of the intake session protocol (Exp. 1). F_0 are rats of the foundational nucleus. Two-bottle intake sessions (ethanol vs. plain water) were conducted on Monday, Wednesday, and Friday (session length: 24 h) for 4 weeks, beginning on postnatal day 32 (PD32) and ending on PD57. The ethanol concentrations were 4% (week 1, intake sessions 1–3), 5% (week 2, intake sessions 4–6), and 6% (weeks 3 and 4, intake sessions 7–12). The data are expressed as the average weekly intake of ethanol (± SEM), collapsed across sex. The latter factor did not significantly alter ethanol intake patterns. (G, H) Same as C—F, collapsed across the 4 weeks of testing. The statistical analysis revealed that, regardless of generation, STDRHI rats drank significantly more ethanol (g/kg and percent preference) than STDRLO rats, and F_1 rats drank significantly more ethanol (g/kg and percent preference) than F_2 rats, regardless of line. These significant main effects are depicted with asterisks and pound signs, respectively.

The three-way ANOVA (Sex, Line, and Week) revealed a significant effect of Line on g/kg ethanol intake ($F_{1,42} = 117.89$, $p < 0.001$, $\eta^2p = 0.74$) and percent ethanol preference ($F_{1,42} = 91.31$, $p < 0.001$, $\eta^2p = 0.68$) and a significant Line × Week interaction (g/kg: $F_{3,126} = 23.54$, $p < 0.001$, $\eta^2p = 0.36$; % preference: $F_{3,126} = 15.82$, $p < 0.001$, $\eta^2p = 0.27$). For both variables, the post hoc tests revealed similar ethanol consumption in STDRHI and STDRLO rats during the first week but significantly greater ethanol consumption in STDRHI rats than in STDRLO rats in weeks 2, 3, and 4. STDRHI rats exhibited an increase in ethanol intake across weeks, whereas ethanol intake in STDRLO rats remained constant across weeks. Sex did not significantly interact with the remaining factors, and overall fluid intake was fairly similar across lines.

3.2. Ethanol intake in F_1 and F_2 generations (Exp. 1)

As shown in Fig. 1, STDRHI rats exhibited greater absolute and percent ethanol intake than STDRLO rats, an effect that persisted in both generations, although F_2 rats drank markedly less ethanol than F_1 rats during weeks 3 and 4 of testing. The inferential analysis confirmed these impressions. Specifically, the repeated-measures ANOVA yielded significant main effects of Line, Generation, and Week on g/kg ethanol intake ($F_{1,150} = 15.76$, $p < 0.001$, $\eta^2p = 0.10$, $F_{1,150} = 12.44$, $p < 0.001$, $\eta^2p = 0.08$, and $F_{3,450} = 6.11$, $p < 0.001$, $\eta^2p = 0.03$, respectively) and percent ethanol preference ($F_{1,150} = 19.54$, $p < 0.001$, $\eta^2p = 0.12$, $F_{1,150} = 14.47$, $p < 0.001$, $\eta^2p = 0.08$, and $F_{3,450} = 3.24$, $p < 0.001$, $\eta^2p = 0.02$). The Generation × Week interaction was also significant (g/kg: $F_{3,450} = 10.66$, $p < 0.001$, $\eta^2p = 0.07$; percent preference: $F_{3,450} = 14.00$, $p < 0.001$, $\eta^2p = 0.09$). These patterns of results were fairly similar in males and females.

The analysis of overall fluid intake (for descriptive statistics, see upper section of Table 3) revealed significant main effects of Sex, Line, and Week ($F_{1,150} = 50.07$, $p < 0.001$, $\eta^2p = 0.25$, $F_{1,150} = 11.08$, $p < 0.005$, $\eta^2p = 0.07$, and $F_{3,450} = 32.03$, $p < 0.001$, $\eta^2p = 0.18$, respectively) and a significant Generation × Week interaction ($F_{3,450} = 31.72$, $p < 0.001$, $\eta^2p = 0.17$). The post hoc tests revealed that overall fluid intake was significantly greater in STDRHI rats than in STDRLO rats and significantly greater in females than in males. Moreover, F_1 rats consumed significantly more fluid in week 1 of testing but significantly less fluid in weeks 2 and 4 than their F_2 counterparts.

3.3. Heritability estimates and planned comparisons of ethanol consumption on weeks 3–4

The planned comparisons, one for each generation, indicated significantly greater ethanol intake (g/kg, average of weeks 3–4), in STDRHI than in STDRLO rats [F_0, $F_{1,192} = 69.66$, $p < 0.001$; F_1, $F_{1,192} = 5.60$, $p < 0.05$ and F_2, $F_{1,192} = 4.38$, $p < 0.05$].

Heritability (h^2) of the selected trait was 0.24 after the first generation (i.e., 24% of the between-line difference in absolute ethanol intake could be attributed to genetic differences). However, h^2 after two generations was − 0.35. This means that the selection response was minimal after F_1. The percentage of variance of the selected trait appeared to be driven by males. Heritability in males was 0.61 and 0.10 in the first and second generations, respectively. Heritability in females was − 0.02 and − 0.66 in the first and second generations, respectively. Thus, the mean heritability estimate across generations was 0.37 and − 0.34 for males and females, respectively. These estimates suggest that the proportion of trait variance that was influenced by genetic factors was greater in males than in females. These heritability scores were calculated following methods described by Falconer and Mackay (1996). The bivariate correlations also indicated high and low heritability after the first and second generation, respectively. Specifically, we conducted Pearson correlations, one for each sex and generation, between STDRHI offspring intake values and mid-parent phenotypic values. The correlations were significant in the F_1 ($r = -0.45$ and 0.50, male and female rats, respectively) but not in the F_2 ($r = -0.04$ and 0.17, male and female rats, respectively) generation.

Fig. 2 presents a graphical depiction of heritability. Specifically, the figure depicts mean ethanol intake (g/kg and percent preference, average of testing sessions of weeks 3 and 4) of the founding F_0 population, of the selected parents for each generation, and of F_1 and F_2 selected STDRHI and STDRLO offspring.

3.4. Ethanol-induced motor activation and anxiety response (Exp. 2)

Fig. 3 depicts distance traveled (in centimeters) as a function of sex, generation, line and ethanol dose. The ANOVA for this variable indicated a significant main effect of Dose ($F_{2,177} = 10.64$, $p < 0.001$, $\eta^2p = 0.11$), a significant Dose × Line interaction ($F_{2,177} = 3.26$, $p < 0.05$, $\eta^2p = 0.04$), and a significant Dose × Line × Sex interaction ($F_{2,177} = 3.10$, $p < 0.05$, $\eta^2p = 0.05$). The post hoc tests revealed a

Table 3
Upper section. Overall fluid intake (ml/100 g of body weight, expressed as weekly average) in filial generations 1 and 2 (F_1 and F_2, respectively) as a function of selected line (high or low-ethanol consumers, STDRHI and STDRLO, respectively), and sex. Values express mean ± SEM. Lower section. Frequency of rearing (i.e., vertical) behaviors during the assessment of ethanol-induced motor activity in Experiments 2a and 2b, in filial generations 2 and 3 (F_2 and F_3, respectively), and as a function of selected line (high or low-ethanol consumers, STDRHI and STDRLO, respectively), and sex. Values express mean ± SEM.

	Generation	Week	STDRHI rats		STDRLO rats	
			♂	♀	♂	♀
Overall fluid intake (ml/100 g of body weight)	F_1	1	19. 60 ± 0.64	23. 94 ± 1.08	20. 65 ± 0.92	25. 39 ± 1.00
		2	17. 74 ± 0.97	20. 40 ± 0.96	18. 92 ± 0.85	20. 91 ± 0.96
		3	16. 88 ± 0.55	20. 30 ± 0.82	18. 58 ± 0.98	21. 88 ± 0.65
		4	14. 18 ± 0.44	18. 03 ± 0.63	15. 80 ± 0.74	18. 70 ± 0.65
	F_2	1	17. 05 ± 0.51	21. 91 ± 0.99	21. 91 ± 0.99	18. 53 ± 0.83
		2	17. 97 ± 0.63	22. 80 ± 1.23	22. 80 ± 1.23	20. 80 ± 1.04
		3	16. 64 ± 0.60	19. 68 ± 0.99	19. 68 ± 0.99	20. 15 ± 0.65
		4	16. 30 ± 0.59	22. 32 ± 1.17	22. 32 ± 1.17	19. 94 ± 0.87
Rearing behaviors	Generation	Dose				
	F_2	0.0 g/kg	227.57 ± 56.04	201.42 ± 45.48	154.30 ± 28.42	101.20 ± 14.50
		1.25 g/kg	257.85 ± 53.60	147.00 ± 41.01	215.25 ± 39.31	153.77 ± 35.10
		2.5 g/kg	219.85 ± 62.17	70.00 ± 19.68	291.66 ± 50.91	183.00 ± 53.83
	F_3	0.0 g/kg	196.87 ± 44.73	141.22 ± 27.97	323.22 ± 59.66	205.55 ± 42.71
		1.25 g/kg	176.20 ± 26.67	136.33 ± 29.63	218.66 ± 39.69	219.37 ± 34.29
		2.5 g/kg	196.87 ± 44.73	80.28 ± 31.17	199.77 ± 38.80	230.62 ± 46.85

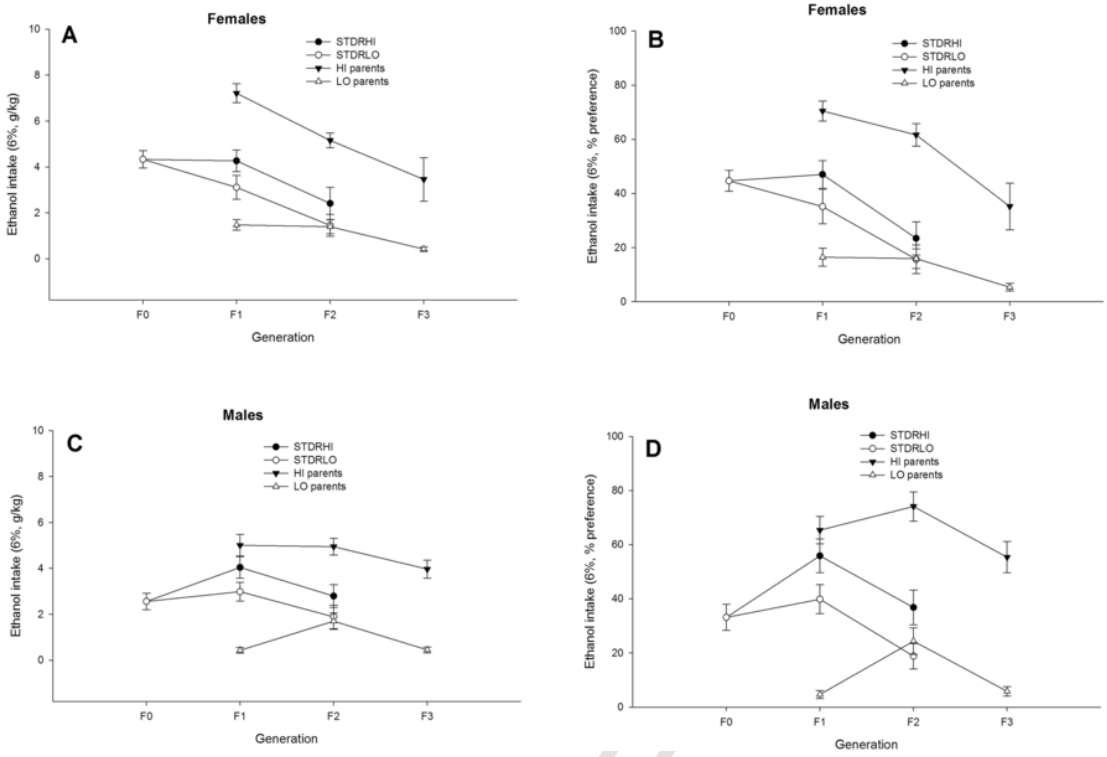

Fig. 2. Ethanol intake (6%, averaged across testing sessions of weeks 3 and 4), in males and females for the originating F0 population and generations F_1 and F_2 of the short-term selection for low (STDRLO) and high (STDRHI) ethanol drinking. Panels A and C depict absolute (g/kg) ethanol intake scores, and panels B and D depict percent ethanol predilection vs. vehicle (water). Data is shown as mean ± SEM. Also shown in each panel are the parental mean (± SEM) intakes for the F_1, F_2 and F_3 generations. These are the means for the parents selected to generate the offspring for each generation and line (high or low, HI and LO parents, respectively). Please note that the F_3 offspring was not tested for ethanol intake but instead was tested in several behavioral assays.

lack of ethanol-induced motor activity in STDRLO rats in both males and females. STDRLO rats of either sex that were given 2.5 or 1.25 g/kg ethanol traveled a similar distance as their same-sex STDRLO counterparts that were treated with vehicle. In contrast, male STDRHI rats that were given 2.5 but not 1.25 g/kg ethanol exhibited a significant increase in the distance traveled than STDRHI male or female controls that were given 0.0 g/kg ethanol. Male STDRHI rats that were given 2.5 g/kg ethanol also exhibited a significant increase in distance traveled than STDRLO males that were given 0.0, 1.25, or 2.5 g/kg ethanol. Female STDRHI rats that were given 2.5 or 1.25 g/kg ethanol exhibited a significant increase in distance traveled than STDRHI females that were given 0.0 g/kg ethanol. Overall, male and female STDRHI rats exhibited different dose-response patterns, but neither male nor female STDRLO rats were sensitive to the stimulatory effects of ethanol. These patterns were statistically similar across F_2 and F_3 rats.

Frequency of rearing behaviors can be found in the lower section of Table 3. The ANOVA for this variable yielded significant main effects of Sex, Line, and Dose ($F_{1,177} = 16.94$, $p < 0.001$, $\eta^2p = 0.09$, $F_{1,177} = 4.54$, $p < 0.05$, $\eta^2p = 0.03$, and $F_{2,177} = 5.49$, $p < 0.005$, $\eta^2p = 0.06$, respectively) and a significant Line × Generation interaction ($F_{1,177} = 5.65$, $p < 0.05$, $\eta^2p = 0.03$). The post hoc tests indicated that 2.5 g/kg ethanol significantly reduced the frequency of rearings compared with vehicle, and males exhibited a significant increase in the frequency of rearings than females. The post hoc tests also indicated a significant increase in the frequency of rearings in STDRLO rats than in STDRHI rats, although only in the F_3 generation.

Behavioral performance in the light-dark box test is shown in Fig. 4. Compared with STDRLO rats, STDRHI rats spent significantly less time in the light compartment of the LDB and made significantly fewer transitions between compartments (significant main effect of Line: $F_{1,62} = 9.91$, $p < 0.05$, $\eta^2p = 0.14$, and $F_{1,62} = 7.49$, $p < 0.001$, $\eta^2p = 0.11$, respectively). This pattern was indicative of higher baseline anxiety in animals that were selected for high ethanol consumption, which was statistically similar in both male and female F_2 and F_3 rats. The latency to enter the dark compartment was similar across groups (data not shown).

3.5. Conditioned taste aversion scores (Exp. 3)

Table 4 presents saccharin intake scores during the conditioning sessions, as a function of line, generation, sex an ethanol treatment (0.0 or 2.5 g/kg). The repeated-measures ANOVA indicated significant main effects of Treatment and Session ($F_{1,98} = 6.31$, $p < 0.05$, $\eta^2p = 0.06$, and $F_{1,98} = 157.22$, $p < 0.001$, $\eta^2p = 0.62$, respectively). The Treatment × Session interaction was significant ($F_{1,98} = 7.14$, $p < 0.01$, $\eta^2p = 0.07$). The post hoc tests indicated that saccharin intake was similar in ethanol- and vehicle-treated rats during conditioning session 1, but ethanol-treated rats drank less saccharin than controls in conditioning session 2. These results were similar in STDRHI and STDRLO rats.

Fig. 5 shows saccharin consumption (ml/100 g body weight) during the 60-min test in each group (upper panel) and across both filial generations (lower panel). Only STDRLO rats developed significant ethanol-induced CTA. The ANOVA revealed a significant effect of Treatment ($F_{1,101} = 26.72$, $p < 0.001$, $\eta^2p = 0.21$) and a significant Treatment × Line interaction ($F_{1,101} = 6.78$, $p < 0.05$, $\eta^2p = 0.06$). The post hoc tests revealed significantly less saccharin consumption in the test session in ethanol-treated STDRLO rats than in vehicle-treated STDRLO rats. This effect was absent in their STDRHI counterparts, which drank as much saccharin as vehicle-treated STDRHI controls (i.e., during the test session, STDRHI rats did not exhibit behaviors that were indicative of CTA). STDRLO rats that were treated with 2.5 g/kg

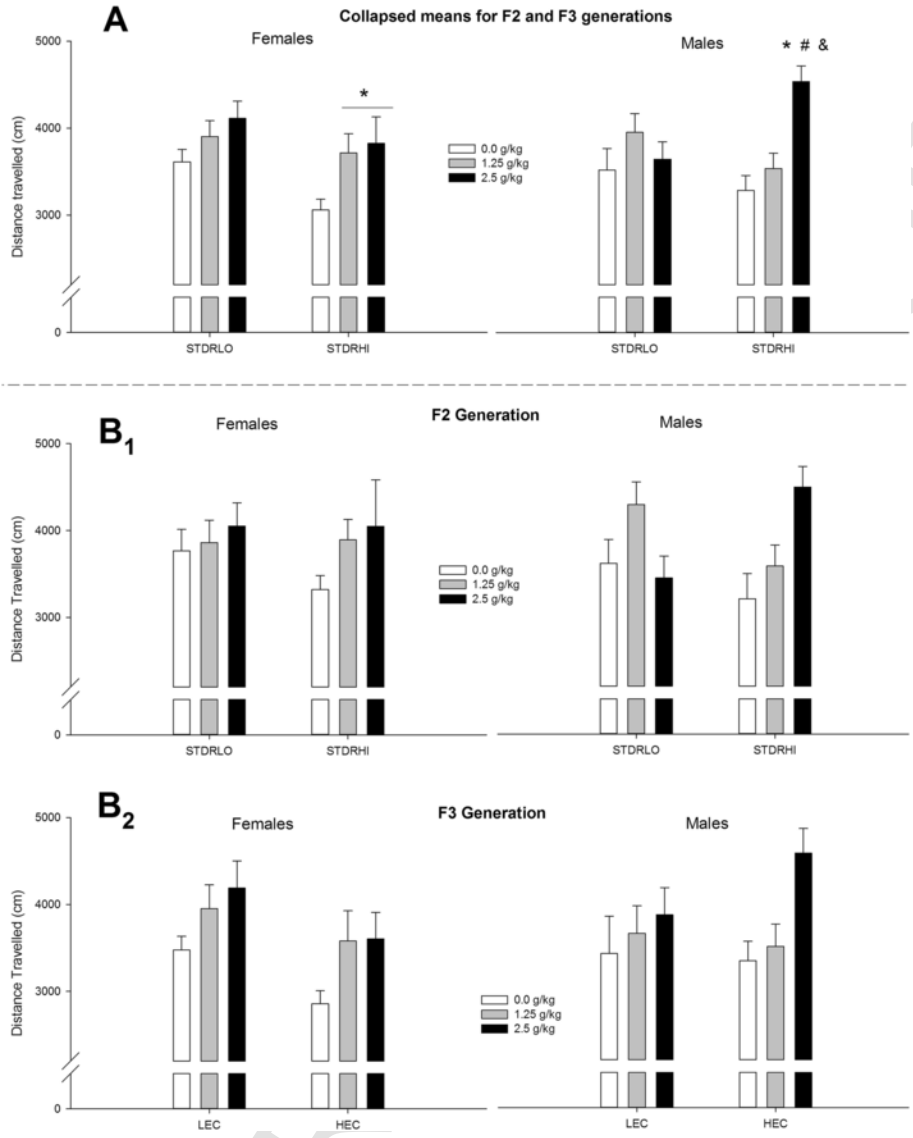

Fig. 3. Distance traveled (in centimeters) in the open field test in Exp. 2a in male and female Wistar rats as a function of filial generation (F_2 and F_3) of short-term lines selected for high and low ethanol consumption (STDRHI and STDRLO, respectively) and ethanol dose administered before the test (0.0, 1.25, and 2.5 g/kg). (A) Results collapsed across generation. The statistical analysis revealed that the ethanol dose did not significantly affect ethanol-induced locomotor activity (distance traveled). Male STDRHI rats that received 2.5 g/kg ethanol exhibited a significant increase in the distance traveled compared with male and female STDRHI rats that received 0.0 g/kg ethanol. These differences are indicated by asterisks and pound signs, respectively. Male STDRHI rats that received 2.5 g/kg also exhibited a significant increase in the distance traveled compared with male STDRLO rats that received 0.0, 1.25, and 2.5 g/kg ethanol. These significant differences are indicated by the ampersand. Female STDRHI rats that received 2.5 and 1.25 g/kg ethanol exhibited a significant increase in the distance traveled compared with female STDRHI rats that received 0.0 g/kg ethanol. These significant differences are indicated by the dollar sign. (B_1, B_2) The same data as in A but disaggregated by generation. The data are expressed as mean ± SEM.

ethanol also drank less saccharin than their counterparts in the STDRHI groups that received 0.0 or 2.5 g/kg ethanol.

3.6. Conditioned place aversion (Exp. 4)

The ANOVA of preconditioning scores (data not shown) revealed no main effects or interactions, suggesting that there was no innate preference (i.e., bias) for the chambers that were used for the CPP procedure. Absolute (s) time spent in the SAND CS^+ chamber during the test session was 229.80 ± 22.32 and 176.42 ± 36.24 in STRDLO rats treated with 0.0 g/kg and 2.5 g/kg ethanol, respectively; and 187.42 ± 20.60 and 100.06 ± 29.22 in STRDHI rats treated with 0.0 g/kg and 2.5 g/kg ethanol, respectively. Percent time spent in the SAND CS^+ chamber was 51.17 ± 4.33 and 36.90 ± 6.67 in STRDLO rats treated with 0.0 g/kg and 2.5 g/kg ethanol, respectively; and 43.81 ± 3.56 and 24.49 ± 6.24 in STRDHI rats treated with 0.0 g/kg and 2.5 g/kg ethanol, respectively. The ANOVA conducted on absolute and percent scores revealed a significant main effect of Treatment ($F_{1,82} = 4.39$, $p < 0.05$, $\eta^2p = 0.06$, and $F_{1,82} = 9.18$, $p < 0.005$, $\eta^2p = 0.11$, respectively). The post hoc tests revealed that ethanol-treated animals spent significantly less time in the SAND compartment than their vehicle-treated control counterparts. This effect was indicative of conditioned place aversion and was similar in STDRHI and STDRLO rats.

3.7. Blood ethanol concentrations (Exp. 5)

The ANOVA revealed a significant main effect of Dose ($F_{1,37} = 12.18$, $p < 0.005$, $\eta^2p = 0.25$). Rats that received 2.5 g/kg ethanol had significantly higher BECs than rats that received 1.25 g/kg ethanol, an affect that was similar in STDRHI and STDRLO rats and in males and females. Mean ± SEM BECs in male STDRLO rats were 173.38 ± 78.08 mg/dl and 170.04 ± 29.44 mg/dl in the 1.25 and

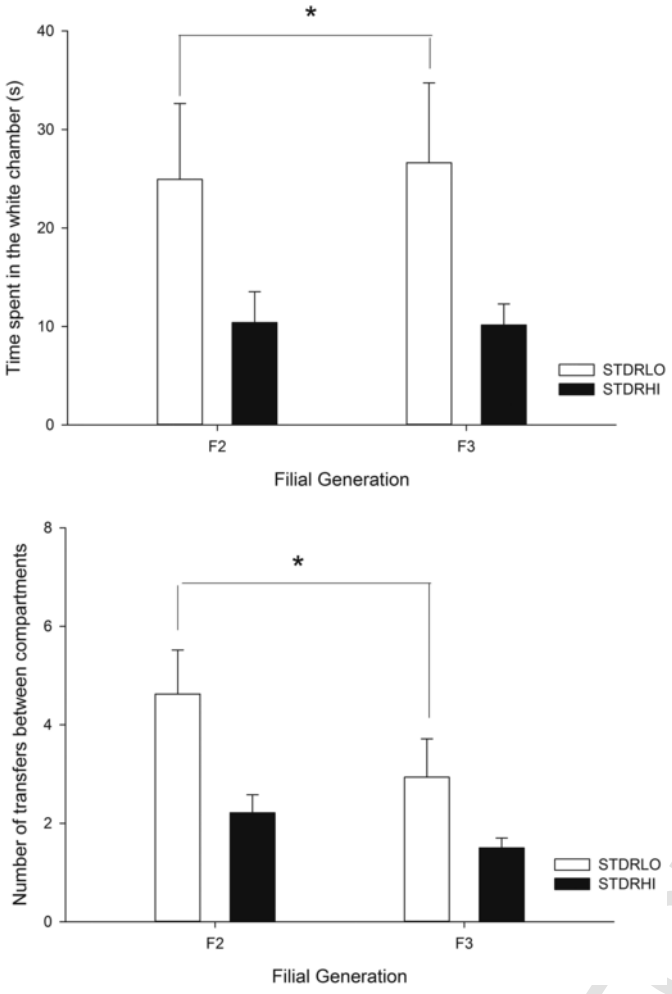

Fig. 4. Time spent in the white compartment and number of transitions between compartments in the light-dark box test in Exp. 2b in male and female Wistar rats as a function of filial generation (F_2 and F_3) of short-term lines selected for high and low ethanol consumption (STDRHI and STDRLO, respectively). The data (mean ± SEM) are collapsed across sex. The statistical analysis revealed that sex did not affect the time spent in the white compartment or number of transitions between compartments. STDRHI rats spent significantly less time in the white compartment and made significantly fewer transitions compared with STDRLO rats. These significant differences are indicated by asterisks.

2.5 g/kg groups, respectively. Mean ± SEM BECs in male STDRHI rats were 161.24 ± 25.19 mg/dl and 286.73 ± 46.86 mg/dl in the 1.25 and 2.5 g/kg groups, respectively. Mean ± SEM BECs in female STDRLO rats were 139.13 ± 28.26 mg/dl and 294.99 ± 19.58 mg/dl in the 1.25 and 2.5 g/kg groups, respectively. Mean ± SEM BECs in female STDRHI rats were 188.06 ± 31.71 mg/dl and 345.97 ± 37.02 mg/dl in the 1.25 and 2.5 g/kg groups, respectively.

3.8. Additional analyses of basal reactivity

Mean body weight was significantly higher in males than in females ($F_{1,274}$ = 149.11, $p < 0.001$, $\eta^2p = 0.35$; 131.49 ± 1.43 g and 111.59 ± 1.03 g, respectively). Body weights in F_2 STDRHI rats were higher than in STDRLO rats (significant Generation × Line interaction: $F_{3,274} = 3.12$, $p < 0.05$, $\eta^2p = 0.03$; 129.35 ± 2.34 g and 125.56 ± 2.35 g, respectively), although no significant differences were observed between STDRHI and STDRLO F_0 (115.95 ± 3.12 g and 116.95 ± 3.30 g, respectively), F_1 (137.05 ± 3.68 g and 127.56 ± 2.29 g, respectively), or F_3 (109.31 ± 2.57 g and 111.71 ± 2.40 g, respectively) rats. Thus, no systematic variations in body weight were attributable to the selection process.

We also analyzed the distance traveled in the OF in Exp. 2a in vehicle-treated animals only and saccharin intake during the first conditioning session in Exp. 3. STDRHI rats exhibited, when compared to STDRLO rats, significantly lower distance traveled ($F_{1,274}$ = 149.11, $p < 0.001$, $\eta^2p = 0.35$; 3175.39 ± 107.01 cm and 3564.43 ± 143.64 cm, respectively) and significantly lower saccharin intake ($F_{1,100} = 4.88$, $p < 0.05$, $\eta^2p = 0.05$; 2.62 ± 0.20 ml and 3.33 ± 0.26 ml, respectively). These effects were similar in males and females and in F_2 and F_3 rats.

4. Discussion

The present study performed short-term selection for differential ethanol intake during adolescence in rats. Our hypothesis was that adolescent rats that were derived from parents that consumed relatively more ethanol during adolescence would exhibit differential sensitivity to the motivational effects of ethanol compared with their peers that were derived from parents that consumed relatively less ethanol during adolescence. We were guided by studies that were conducted in human participants (Conrod et al., 1997b) and vertebrate (Phillips et al., 2005), and invertebrate (Klee et al., 2012) animals. Alterations in the motivational sensitivity to ethanol may be one mechanism by which genes influence ethanol intake. A novel aspect of our work was that both the selected trait and putative associated pleiotropic effects were evaluated during adolescence, a stage during which ethanol intake behaviors emerge and likely determine the magnitude of later ethanol-related problems.

Table 4
Saccharin Intake (ml/100 g of body weight) during conditioning sessions 1 and 2 of the conditioned taste aversion procedure (Experiment 3), in male and female adolescents rats as a function of ethanol treatment (0.0 or 2.5 g/kg) given immediately after each session, selected line (low- or high ethanol consumers, STDRLO or STDRHI, respectively) and filial generation (F_2 or F_3). Values express mean ± SEM.

	Filial generation					
	Ethanol dose and sex		F_1		F_2	
			Conditioning session 1	Conditioning session 2	Conditioning session 1	Conditioning session 2
STDRHI rats	0.0 g/kg	♂	2.04 ± 0.55	7.94 ± 0.59	2.07 ± 0.36	5.69 ± 1.07
		♀	2.75 ± 0.51	6.48 ± 0.63	2.53 ± 0.49	7.32 ± 1.12
	2.5 g/kg	♂	1.92 ± 0.38	4.33 ± 1.02	2.89 ± 0.38	7.12 ± 0.73
		♀	2.37 ± 0.62	4.52 ± 0.56	3.44 ± 0.42	5.94 ± 1.57
STDRLO rats	0.0 g/kg	♂	3.32 ± 1.31	6.81 ± 0.66	3.65 ± 0.69	6.63 ± 1.16
		♀	3.73 ± 0.57	6.39 ± 0.67	3.51 ± 0.88	8.04 ± 1.19
	2.5 g/kg	♂	3.21 ± 0.78	5.92 ± 0.67	3.65 ± 0.21	5.92 ± 1.20
		♀	2.58 ± 0.67	4.61 ± 0.87	2.84 ± 0.88	4.60 ± 1.15

Research Highlights

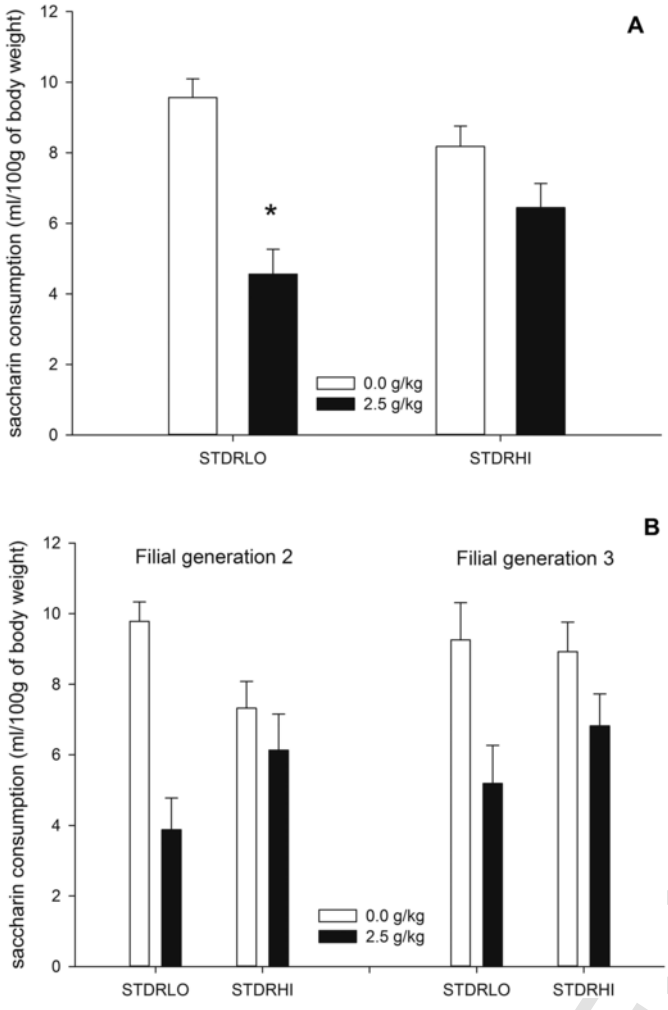

Fig. 5. Saccharin intake (ml/100 g body weight) during the 60-min conditioned taste aversion test session in Exp. 3 in male and female Wistar rats as a function of filial generation (F_2 and F_3) of short-term lines selected for high and low ethanol consumption (STDRHI and STDRLO, respectively) and ethanol dose administered during the conditioning sessions (0.0 or 2.5 g/kg). (A) The results (mean ± SEM) collapsed across generation and sex. The statistical analysis revealed that these factors did not significantly affect saccharin intake. The statistical analysis also revealed that STDRLO but not STDRHI rats that received 2.5 g/kg ethanol during conditioning exhibited significantly less saccharin intake than their 0.0 g/kg controls. This significant difference is indicated by the asterisk. (B) She same data as in A but disaggregated by filial generation.

Despite some shortcomings of our study, our main findings were that selective breeding rapidly generated lines with divergent and significant differences in free-choice ethanol drinking (Exp. 1). STDRHI rats were mostly, although not completely, insensitive to ethanol-induced CTA, which was reliably expressed in STDRLO rats (Exp. 3). STDRHI but not STDRLO adolescents exhibited ethanol-induced acute motor stimulation (Exp. 2a). Ethanol-induced conditioned place aversion, however, was similar across lines (Exp. 4). Other differences between the lines were also found, notably a greater baseline level of anxiety response in STDRHI rats than in STDRLO rats (Exp. 2b). These findings were not associated with significant differences in the blood ethanol levels recorded in Exp. 5.

Short-term selective breeding was generally successful. After only one crossing, STDRHI F_1 rats exhibited daily ethanol intake of 4.0 g/kg/day and ≥ 50% ethanol preference by weeks 3 and 4 of testing. These values were significantly higher than in STDRLO F_1 rats and similar to rat lines that were selectively bred for high alcohol preference and consumption during adulthood, such as sP rats (Colombo et al., 2006a). Increased ethanol drinking in STDRHI rats was accompanied by greater overall fluid intake, and F_2 rats drank significantly less ethanol than their F_1 counterparts, despite greater ethanol intake in STDRHI F_2 rats than in STDRLO F_2 rats. The latter pattern suggests substantial regression toward the mean during the time that selection pressure was applied. Regression to the mean is a phenomenon that occurs when subjects, akin to our STDRHI rats, are selected due to their extreme values in a given measurement. On a second measurement, these subjects (or, in our case, their offspring) will tend to exhibit values closer to the population average. This phenomenon was reflected in h^2 values and in the heritability scores obtained by correlating offspring and parental intake scores. Heritability was 0.24 after the first generation, indicating that 24% of the difference in ethanol intake between lines could be attributed to genetic differences, but it dropped to minimal values after two generations, although with notable sex differences (discussed below). Notably, the difference in drinking between the F_1 and F_2 generations did not translate into differences in behavioral reactivity to ethanol. Ethanol-induced motor stimulation and CTA were significantly different between STDRHI and STDRLO adolescents, and this pattern was similar across generations.

It is important to compare the heritability estimates obtained in our adolescent selection vs. those obtained after adult selective breeding. Some adult selective breeding studies (Lê et al., 2001; Phillips et al., 2005) reported, similar to the present work, substantially greater heritability scores for ethanol intake in the first than in the second generations. Thus, a common denominator is that genetic variance at loci associated to ethanol intake seems to accumulate early, in the first generations. Despite this similarity, the mean heritability estimates, in studies conducted in adults and across species and phenotypes, are around 0.3 across generations, with little variation across sex. Lê et al. (2001) employed a within-family breeding procedure in rats and reported, after six generations, an estimated heritability of 0.25; whereas Grahame et al. (1999) reported a heritability of 0.26 after a bidirectional selection of alcohol drinking in mice. Li et al. (1993) reported heritability scores of 0.3–0.4 in high alcohol drinking rats. Therefore, it seems that the heritability scores found in the adolescent subjects of the present study are lower than those previously reported in adults. We can only speculate about the reasons underlying these differences in heritability between adolescent selection vs. adult selective breeding. It is possible that adolescents, compared to adults, exhibit more variability in traits associated with ethanol intake (e.g., novelty-seeking) or that ethanol exposure in the progenitors alters gene expression to a greater extent in adolescents than in adults. Future studies should test these and other possibilities. It is also unknown if the same patterns of ethanol intake and motivational reactivity to the drug would have been observed in adolescent offspring of rats that were high vs low drinker during their adult life cycle.

F_0 females drank more than F_0 males, a finding that is consistent with studies in mice (Middaugh et al., 1999) and rats (Berardo et al., 2016; Doremus et al., 2005). Selection pressure was associated with disappearance of this difference. We previously found that adolescent male rats were much more sensitive to treatments that facilitated ethanol drinking than adolescent females, a phenomenon that was associated with possible ceiling effects in terms of ethanol intake in females (Berardo et al., 2016). However, a robust sex difference was observed in the heritability of the selected trait. The h_2 index indicated a more robust response to selection in either the first or second generation of selection in males, a result that was different from findings in STDR mice (Phillips et al., 2005) but in agreement with several (Prescott, 2002) but not all (Rhee et al., 2003) epidemiological studies that reported a greater magnitude of genetic influence on ethanol-related problems in men than in women. Cloninger's theoretical account of alcoholism (Cloninger et al., 1996) indicates that the type of alcoholism that is associated with onset during adolescence is mainly restricted to males.

Compared with their STDRLO counterparts, STDRHI F_2 and F_3 rats exhibited a blunted response to ethanol-induced CTA. Conversely,

STDRHI but not STDRLO rats, with some sex differences, exhibited ethanol-induced motor stimulation. STDRLO F_2 and F_3 rats that received saccharin-ethanol pairings exhibited a two-fold reduction of saccharin intake compared with their counterparts that received vehicle. This significant difference was not observed in STDRHI animals of either generation. Conversely, STDRHI males and females that received 2.5 g/kg ethanol and STDRHI females that received 1.25 g/kg ethanol exhibited a significant increase in the distance traveled in the OF than their control counterparts that received 0.0 g/kg ethanol, an effect that was not observed in STDRLO animals. The relatively wider dose-response pattern that was observed in female STDRHI rats compared with male STDRHI rats is consistent with previous studies that reported greater sensitivity to these motor-stimulating effects of ethanol in female rats than in males (Erickson and Kochhar, 1985). In a previous study, female rats that were more sensitive to the activating effects of ethanol were also more sensitive to the facilitative effect of ethanol exposure on later ethanol intake (Acevedo et al., 2010).

Lower sensitivity to the sedative and aversive intoxicating effects of ethanol, combined with greater sensitivity to the stimulating and rewarding effects of ethanol, is suggested to be a risk factor for the development of AUD (Spear and Varlinskaya, 2010) and has been observed in the children of alcoholics (Schuckit et al., 2004), FH^+ individuals (Conrod et al., 2001), and rodent strains that were selectively bred for high ethanol intake during adulthood (McBride and Li, 1998). Moreover, a genome-wide association study linked alcohol consumption to a single-nucleotide polymorphism of the autism susceptibility candidate 2 (*AUTS2*) gene, and downregulation of the *AUTS2* homolog in *Drosophila* reduced the sensitivity to the sedative effects of ethanol (Schumann et al., 2011).

Numerous studies have reported an idiosyncratic pattern of responses to ethanol's effects in adolescent and adult heterogeneous rats and mice. For example, Vetter-O'Hagen et al. (2009) found that the lowest ethanol dose that induced CTA in adolescent rats was twice as high as the dose that induced CTA in adults. Our previous study found second-order conditioned place preference in adolescent but not adult rats (Pautassi et al., 2008). We also observed greater sensitivity to the stimulating effects of ethanol and less sensitivity to the sedative and hypnotic effects of ethanol in adolescents than in adults (Acevedo et al., 2013; Fernandez et al., 2016). The present study suggests that the adolescent-typical pattern of response to ethanol can be further differentiated in populations that are derived from parents that exhibited relatively high ethanol consumption during adolescence. As suggested by Spear and Varlinskaya (2010), the combination of idiosyncratic insensitivity to ethanol that is normally exhibited by adolescents and other risk factors (e.g., stress exposure, an early age of onset of first ethanol use, or specific genetic background) may constitute multi-level “whammies" that facilitate the initiation and escalation of ethanol use during adolescence.

Genetics can also facilitate ethanol drinking by altering more broad personality traits, such as impulsivity (Caswell et al., 2016), anxiety response (McCaul et al., 2017), and preference for basic tastants (Kampov-Polevoy et al., 2014). In the present study, STDRHI F_2 and F_3 rats exhibited lower basal exploration of the OF and LDB. These are indicators of a reluctance to explore new environments, although the dependence of these variables on overall activity levels lessens the specificity of these markers as indicators of anxiety. STDRHI F_2 and F_3 rats also exhibited a two-fold decrease in the time spent in the white compartment (i.e., a potentially dangerous area of the LDB) compared with their STDRLO peers. These data suggest that an elevated anxiety response may be yet another factor by which genes mediate the risk of ethanol use during adolescence. Unlike previous studies (Phillips et al., 2005; Terenina-Rigaldie et al., 2003), STDRHI rats exhibited lower basal saccharin consumption than STDRLO rats (i.e., before conditioning). This measurement, however, was performed in a single 30-min session. Therefore, the lower level of saccharin consumption in STDRHI rats may indicate a generalized neophobic response rather than specific avoidance of or low preference for the sweet taste. We suggest that these three indicators (i.e., lower locomotive patterns in the LDB and OF, avoidance of the white compartment of the LDB, and lower saccharin consumption) reflect an anxiety phenotype in adolescent STDRHI rats.

The present study has important limitations. The lines were not replicated and were terminated early, mainly because of the high costs of maintenance. We intend to perform an ongoing, longer follow-up study that will continue selection until the F_5 generation. We only used one ethanol dose in the place and taste conditioning experiments, which limits generalization of the results. Rats generally do not express ethanol-induced CPP unless they are given lengthy ethanol (Carrara-Nascimento et al., 2014) or stress (Yu et al., 2016) pre-exposure. One possibility is that place conditioning would switch from aversive to appetitive, with probable line differences, if a lower dose of ethanol is used. Notably, single-trial, ethanol-induced CTA was observed during the 30-min conditioning session 2, which was fairly similar in STDRHI and STDRLO rats. Unknown is why ethanol-induced CTA in session 2 was insensitive to selection pressure, but significant line differences emerged in the subsequent 60-min test session. The longer duration of the latter session may explain this disparate finding. Repeated exposure to stress that is associated with dehydration between sessions may differentially exacerbate the expression of CTA across lines. The initial lower saccharin consumption in STDRHI rats may be responsible for the reduction of ethanol-induced CTA in this line. However, both groups exhibited similar saccharin consumption and similar ethanol-induced CTA in conditioning session 2.

It is also important to note the BECs registered in Experiment 5 were highly variable, particularly among male STDRLO rats, and that BECs were measured once. The lack of repeated testing across the blood ethanol curve limits the conclusions of this experiment and leaves unanswered the question of whether STRDHI and STDRLO rats exhibit differences in ethanol metabolism.

5. Conclusion

Despite the limitations, the present results indicate that the interbreeding of animals that at a young age were high or low drinkers yields an offspring with differential reactivity to ethanol's pharmacological effects. These findings support the hypothesis that genetic risk of enhanced ethanol intake during adolescence is associated with lower sensitivity to the aversive effects of ethanol, heightened reactivity to ethanol's stimulating effects, and enhanced innate anxiety. Further ontogenetic animal studies of the genetic underpinnings of AUD are needed.

Declaration of absence of conflict of interest

We declare having no competing interest nor conflict of interest related to our MS or its results.

Authorship

Fernandez and Pautassi designed the study. Fernandez, Baez, Bordon, Espinosa, Martinez and Pautassi run the intake and behavioral tests and Pautassi and Fernandez run the statistical analysis. Pautassi and Fernandez wrote the first draft of the manuscript. All authors contributed to and approved the final manuscript.

Ethical statement

The experiments were carried out in accordance with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the NIH and the EU.

- We conducted a selective breeding for high- or low-ethanol drinking at adolescence.
- Rat lines with divergent differences in ethanol drinking were rapidly produced.
- STDRHI rats were mostly insensitive to ethanol-induced conditioned taste aversion.
- STDRHI but not STDRLO rats exhibited ethanol-induced acute motor stimulation.
- Baseline level of anxiety was greater in STDRHI rats than in STDRLO rats.

Acknowledgements

This work was supported by PICT 2012-0436 Agencia Nacional de Promoción Científica y Tecnológica (FONCyT), to RMP. The funders had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

References

Acevedo, M.B., Fabio, M.C., Fernandez, M., Pautassi, R.M., 2016. Anxiety response and restraint-induced stress differentially affect ethanol intake in female adolescent rats. Neuroscience

Acevedo, M.B., Molina, J.C., Nizhnikov, M.E., Spear, N.E., Pautassi, R.M., 2010. High ethanol dose during early adolescence induces locomotor activation and increases subsequent ethanol intake during late adolescence. Dev. Psychobiol. 52, 424–440.

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.

Adkins, A.E., Hack, L.M., Bigdeli, T.B., Williamson, V.S., McMichael, G.O., Mamdani, M., et al., 2017. Genomewide association study of alcohol dependence identifies risk loci altering ethanol-response behaviors in model organisms. Alcohol. Clin. Exp. Res.

Bae, J.S., Jung, M.H., Lee, B.C., Cheong, H.S., Park, B.L., Kim, L.H., et al., 2012. The genetic effect of copy number variations on the risk of alcoholism in a Korean population. Alcohol. Clin. Exp. Res. 36, 35–42.

Belknap, J.K., Richards, S.P., O'Toole, L.A., Helms, M.L., Phillips, T.J., 1997. Short-term selective breeding as a tool for QTL mapping: ethanol preference drinking in mice. Behav. Genet. 27, 55–66.

Bell, R.L., Rodd, Z.A., Toalston, J.E., McKinzie, D.L., Lumeng, L., Li, T.-K., et al., 2008. Autonomic activation associated with ethanol self-administration in adult female P rats. Pharmacol. Biochem. Behav. 91, 223–232.

Berardo, L.R., Fabio, M.C., Pautassi, R.M., 2016. Post-weaning environmental enrichment, but not chronic maternal isolation, enhanced ethanol intake during periadolescence and early adulthood. Front. Behav. Neurosci. 10, 195.

Carrara-Nascimento, P.F., Olive, M.F., Camarini, R., 2014. Ethanol pre-exposure during adolescence or adulthood increases ethanol intake but ethanol-induced conditioned place preference is enhanced only when pre-exposure occurs in adolescence. Dev. Psychobiol. 56, 36–48.

Carvajal, F., Lerma-Cabrera, J.M., 2015. Alcohol consumption among adolescents — implications for public health. In: Claborn, D. (Ed.), Topics in Public Health. INTECH.

Caswell, A.J., Celio, M.A., Morgan, M.J., Duka, T., 2016. Impulsivity as a multifaceted construct related to excessive drinking among UK students. Alcohol Alcohol. 51, 77–83.

Ciccocioppo, R., Economidou, D., Cippitelli, A., Cucculelli, M., Ubaldi, M., Soverchia, L., et al., 2006. Genetically selected Marchigian Sardinian alcohol-preferring (msP) rats: an animal model to study the neurobiology of alcoholism. Addict. Biol. 11, 339–355.

Ciccocioppo, R., Panocka, I., Froldi, R., Quitadamo, E., Massi, M., 1999. Ethanol induces conditioned place preference in genetically selected alcohol-preferring rats. Psychopharmacology 141, 235–241.

Cloninger, C.R., Sigvardsson, S., Bohman, M., 1996. Type I and Type II Alcoholism: An Update Alcohol Health & Research World. vol. 20, 19–23.

Colombo, G., Lobina, C., Carai, M.A., Gessa, G.L., 2006. Phenotypic characterization of genetically selected Sardinian alcohol-preferring (sP) and -non-preferring (sNP) rats. Addict. Biol. 11, 324–338.

Colombo, G., Serra, S., Vacca, G., Carai, M.A., Gessa, G.L., 2006. Baclofen-induced suppression of alcohol deprivation effect in Sardinian alcohol-preferring (sP) rats exposed to different alcohol concentrations. Eur. J. Pharmacol. 550, 123–126.

Conrod, P.J., Petersen, J.B., Pihl, R.O., 1997. Disinhibited personality and sensitivity to alcohol reinforcement: independent correlates of drinking behavior in sons of alcoholics. Alcohol. Clin. Exp. Res. 21, 1320–1332.

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.

Conrod, P.J., Peterson, J.B., Pihl, R.O., Mankowski, S., 1997. Biphasic effects of alcohol on heart rate are influenced by alcoholic family history and rate of alcohol ingestion. Alcohol. Clin. Exp. Res. 21, 140–149.

Cotton, N.S., 1979. The familial incidence of alcoholism: a review. J. Stud. Alcohol 40, 89–116.

Cunningham, M.G., Bhattacharyya, S., Benes, F.M., 2002. Amygdalo-cortical sprouting continues into early adulthood: implications for the development of normal and abnormal function during adolescence. J. Comp. Neurol. 453, 116–130.

Dick, D.M., Agrawal, A., 2008. The genetics of alcohol and other drug dependence. Alcohol Res. Health 31, 111–118.

Doremus, T.L., Brunell, S.C., Rajendran, P., Spear, L.P., 2005. Factors influencing elevated ethanol consumption in adolescent relative to adult rats. Alcohol. Clin. Exp. Res. 29, 1796–1808.

Ducci, F., Goldman, D., 2008. Genetic approaches to addiction: genes and alcohol. Addiction 103, 1414–1428.

Ducci, F., Goldman, D., 2012. The genetic basis of addictive disorders. Psychiatr. Clin. N. Am. 35, 495–519.

Erickson, C.K., Kochhar, A., 1985. An animal model for low dose ethanol-induced locomotor stimulation: behavioral characteristics. Alcohol. Clin. Exp. Res. 9, 310–314.

Falconer, D.S., Mackay, T.F.C., 1996. Introduction to Quantitative Genetics, 4th ed. Longmans Green, Essex.

Fernandez, M.S., Fabio, M.C., Miranda-Morales, R.S., Virgolini, M.B., De Giovanni, L.N., Hansen, C., et al., 2016. Age-related effects of chronic restraint stress on ethanol drinking, ethanol-induced sedation, and on basal and stress-induced anxiety response. Alcohol 51, 89–100.

Grahame, N.J., Li, T.-K., Lumeng, L., 1999. Selective breeding for high andlow alcohol preference in mice. Behav. Genet. 29, 47–57.

Green, A.S., Grahame, N.J., 2008. Ethanol drinking in rodents: is free-choice drinking related to the reinforcing effects of ethanol?. Alcohol 42, 1–11.

Hines, L.M., Ray, L., Hutchison, K., Tabakoff, B., 2005. Alcoholism: the dissection for endophenotypes. Dialogues Clin. Neurosci. 7, 153–163.

Hobbs, J.D., Kushner, M.G., Lee, S.S., Reardon, S.M., Maurer, E.W., 2011. Meta-analysis of supplemental treatment for depressive and anxiety disorders in patients being treated for alcohol dependence. Am. J. Addict. 20, 319–329.

Kampov-Polevoy, A., Lange, L., Bobashev, G., Eggleston, B., Root, T., Garbutt, J.C., 2014. Sweet-liking is associated with transformation of heavy drinking into alcohol-related problems in young adults with high novelty seeking. Alcohol. Clin. Exp. Res. 38, 2119–2126.

Klee, E.W., Schneider, H., Clark, K.J., Cousin, M.A., Ebbert, J.O., Hooten, W.M., et al., 2012. Zebrafish: a model for the study of addiction genetics. Hum. Genet. 131, 977–1008.

Lê, A.D., Israel, Y., Juzytsch, W., Quan, B., Harding, S., 2001. Genetic selection for high and low alcohol consumption in a limited-access paradigm. Alcohol. Clin. Exp. Res. 25, 1613–1620.

Li, T.-K., Lumeng, L., Doolittle, D.P., 1993. Selective breeding for ethanolpreference and associated responses. Behav. Genet. 23, 163–170.

Linsenbardt, D.N., Boehm 2nd, S.L., 2013. Determining the heritability of ethanol-induced locomotor sensitization in mice using short-term behavioral selection. Psychopharmacology 230, 267–278.

Manzo, L., Gomez, M.J., Callejas-Aguilera, J.E., Fernandez-Teruel, A., Papini, M.R., Torres, C., 2012. Oral ethanol self-administration in inbred Roman high- and low-avoidance rats: gradual versus abrupt ethanol presentation. Physiol. Behav. 108, 1–5.

McBride, W.J., Li, T.K., 1998. Animal models of alcoholism: neurobiology of high alcohol-drinking behavior in rodents. Crit. Rev. Neurobiol. 12, 339–369.

McCaul, M.E., Hutton, H.E., Stephens, M.A., Xu, X., Wand, G.S., 2017. Anxiety, anxiety sensitivity, and perceived stress as predictors of recent drinking, alcohol craving, and social stress response in heavy drinkers. Alcohol. Clin. Exp. Res.

Middaugh, L.D., Kelley, B.M., Bandy, A.L., McGroarty, K.K., 1999. Ethanol consumption by C57BL/6 mice: influence of gender and procedural variables. Alcohol 17, 175–183.

Naimi, T.S., Brewer, R.D., Miller, J.W., Okoro, C., Mehrotra, C., 2007. What do binge drinkers drink? Implications for alcohol control policy. Am. J. Prev. Med. 33, 188–193.

Oberlin, B., Best, C., Matson, L., Henderson, A., Grahame, N., 2011. Derivation and characterization of replicate high- and low-alcohol preferring lines of mice and a high-drinking crossed HAP line. Behav. Genet. 41, 288–302.

Ocaranza, P., MaE, Quintanilla, Tampier, L., Karahanian, E., Sapag, A., Israel, Y., 2008. Gene therapy reduces ethanol intake in an animal model of alcohol dependence. Alcohol. Clin. Exp. Res. 32, 52–57.

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.

Phillips, T.J., Broadbent, J., Burkhart-Kasch, S., Henderson, C., Wenger, C.D., McMullin, C., et al., 2005. Genetic correlational analyses of ethanol reward and aversion phenotypes in short-term selected mouse lines bred for ethanol drinking or ethanol-induced conditioned taste aversion. Behav. Neurosci. 119, 892–910.

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.

Pilatti, A., Godoy, J.C., Brussino, S.A., Pautassi, R.M., 2013. Patterns of substance use among Argentinean adolescents and analysis of the effect of age at first alcohol use on substance use behaviors. Addict. Behav. 38, 2847–2850.

Pilatti, A., Fernández, C., Viola, A., Garcia, J., Pautassi, R.M., 2017. Reciprocal effect of impulsiveness and alcohol consumption in argentine teenagers. Health Addict. 17, 107–120.

Pinsky, I., Sanches, M., Zaleski, M., Laranjeira, R., Caetano, R., 2010. Patterns of alcohol use among Brazilian adolescents. Rev. Bras. Psiquiatr. 32, 242–249.

Pinsky, I., Zaleski, M., Laranjeira, R., Caetano, R., 2010. First national survey on patterns of alcohol consumption in the Brazilian population. Rev. Bras. Psiquiatr. 32, 214–215.
Ponce, L.F., Pautassi, R.M., Spear, N.E., Molina, J.C., 2011. Maternal care alterations induced by repeated ethanol leads to heightened consumption of the drug and motor impairment during adolescence: a dose-response analysis. Physiol. Behav. 103, 477–486.
Prescott, C.A., 2002. Sex differences in the genetic risk for alcoholism. Alcohol Res. Health 26, 264–273.
Quintanilla, M.E., Tampier, L., 2011. Place conditioning with ethanol in rats bred for high (UChB) and low (UChA) voluntary alcohol drinking. Alcohol 45, 751–762.
Rhee, S.H., Hewitt, J.K., Young, S.E., Corley, R.P., Crowley, T.J., Stallings, M.C., 2003. Genetic and environmental influences on substance initiation, use, and problem use in adolescents. Arch. Gen. Psychiatry 60, 1256–1264.
Rivera-Meza, M., Quintanilla, M.E., Tampier, L., 2012. Reduction of ethanol consumption in alcohol-preferring rats by dual expression gene transfer. Alcohol Alcohol. 47, 102–108.
Roman, E., Stewart, R.B., Bertholomey, M.L., Jensen, M.L., Colombo, G., Hyytiä, P., Badia-Elder, N.E., Grahame, N.J., Li, T.K., Lumeng, L., 2012. Behavioral profiling of multiple pairs of rats selectively bred for high and low alcohol intake using the MCSF test. Addict. Biol. 17, 33–46.
SAMSA, 2007. In: Administration SAaMHS (Ed.), National Survey on Drug Use and Health: National Findings. Rockville, SAMHSA.
Schuckit, M.A., 1994. Low level of response to alcohol as a predictor of future alcoholism. Am. J. Psychiatry 151, 184–189.
Schuckit, M.A., Smith, T.L., Kalmijn, J., 2004. Findings across subgroups regarding the level of response to alcohol as a risk factor for alcohol use disorders: a college population of women and Latinos. Alcohol. Clin. Exp. Res. 28, 1499–1508.
Schumann, G., Coin, L.J., Lourdusamy, A., Charoen, P., Berger, K.H., Stacey, D., et al., 2011. Genome-wide association and genetic functional studies identify autism susceptibility candidate 2 gene (AUTS2) in the regulation of alcohol consumption. Proc. Natl. Acad. Sci. U. S. A. 108, 7119–7124.
Sigvardsson, S., Bohman, M., Cloninger, C.R., 1996. Replication of the Stockholm Adoption Study of alcoholism. Confirmatory cross-fostering analysis. Arch. Gen. Psychiatry 53, 681–687.
Spear, L.P., Swartzwelder, H.S., 2014. Adolescent alcohol exposure and persistence of adolescent-typical phenotypes into adulthood: a mini-review. Neurosci. Biobehav. Rev. 45, 1–8.
Spear, L.P., Varlinskaya, E.I., 2010. Sensitivity to ethanol and other hedonic stimuli in an animal model of adolescence: implications for prevention science?. Dev. Psychobiol. 52, 236–243.
Stewart, R.B., Murphy, J.M., McBride, W.J., Lumeng, L., Li, T.K., 1996. Place conditioning with alcohol in alcohol-preferring and -nonpreferring rats. Pharmacol. Biochem. Behav. 53, 487–491.
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.
Terenina-Rigaldie, E., Moisan, M.P., Colas, A., Beauge, F., Shah, K.V., Jones, B.C., et al., 2003. Genetics of behaviour: phenotypic and molecular study of rats derived from high- and low-alcohol consuming lines. Pharmacogenetics 13, 543–554.
Varlinskaya, E.I., Spear, L.P., 2015. Social consequences of ethanol: impact of age, stress, and prior history of ethanol exposure. Physiol. Behav. 148, 145–150.
Vetter-O'Hagen, C., Varlinskaya, E., Spear, L., 2009. Sex differences in ethanol intake and sensitivity to aversive effects during adolescence and adulthood. Alcohol Alcohol. 44, 547–554.
Vetter, C.S., Doremus-Fitzwater, T.L., Spear, L.P., 2007. Time course of elevated ethanol intake in adolescent relative to adult rats under continuous, voluntary-access conditions. Alcohol. Clin. Exp. Res. 31, 1159–1168.
Waller, M.B., Murphy, J.M., McBride, W.J., Lumeng, L., Li, T.K., 1986. Effect of low dose ethanol on spontaneous motor activity in alcohol-preferring and -nonpreferring lines of rats. Pharmacol. Biochem. Behav. 24, 617–623.
Wilhelm, C.J., Reeves, J.M., Phillips, T.J., Mitchell, S.H., 2007. Mouse lines selected for alcohol consumption differ on certain measures of impulsivity. Alcohol. Clin. Exp. Res. 31, 1839–1845.
Wray, N.R., 2008. Estimating trait heritability. Nat. Educ. 1, 29.
Yu, L., Wang, L., Zhao, X., Song, M., Wang, X., 2016. Role of single prolonged stress in acquisition of alcohol conditioned place preference in rats. Life Sci. 151, 259–263.
Zorrilla, E.P., 1997. Multiparous species present problems (and possibilities) to developmentalists. Dev. Psychobiol. 30, 141–150.