Drug and Alcohol Dependence

Binge ethanol self-administration at adolescence, in Wistar rats, promotes ethanol drinking at adulthood in a S1RA sensitive manner

--Manuscript Draft--

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Abstract:	Background: Binge drinking at adolescence is a risk factor for problematic alcohol (ethanol) consumption later in life, yet the murine studies that modelled this phenomenon via ethanol self-administration have provided mixed findings. Antagonism of the sigma-1 receptor (S1-R) system at adolescence modulates ethanol's motivational effects and intake. It is still unknown, however, whether this antagonism would protect against enhanced ethanol intake at adulthood after adolescent binge ethanol exposure. Methods: Exp. 1 and 2 tested adults male or female Wistar rats - exposed or not to ethanol self-administration at adolescence (postnatal days 31-49; nine 2-hour sessions of access to 8-10% ethanol)- for ethanol intake using 24-h two-bottle choice test (Exp. 1) or time restricted, single-bottle, tests (Exp. 2). Experiments 2-5 evaluated, in adolescent or adult rats, the effects of the S1-R antagonist S1RA on ethanol intake and on ethanol-induced conditioned taste or place aversion. Ancillary tests (e.g., novel object recognition, ethanol-induced locomotor activity) were also conducted. Results: Adolescent ethanol exposure promoted ethanol consumption at both the restricted, single-bottle, and at the two-bottle choice tests conducted at adulthood. S1RA administration reduced ethanol intake at adulthood and facilitated the development of ethanol-induced taste (but not place) aversion. Conclusions: S1RA holds promise for lessening ethanol intake after chronic and substantial ethanol exposure in adolescence that results in heightened ethanol exposure at adulthood. This putative protective effect of S1-R antagonism may relate to S1RA exacerbating the aversive effects of this drug.
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Title of Paper: Ethanol drinking at adulthood is sensitive to S1-R antagonism and is promoted by binge ethanol self-administration at adolescence
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Dear Editor,

On behalf of my co-authors, I submit the enclosed MS for consideration by *Drug and Alcohol Dependence*. The manuscript has not been published in this or a substantially similar form (in print or electronically, including on a web site), nor accepted for publication elsewhere, nor is it under consideration by another publication. As the corresponding author, I assure that all co-authors have made important contributions to this work and deserve to be in the by-line.

All authors have read and approved the MS, believe that the paper represents honest work and are able to verify the validity of the statements reported. Due care has been exercised by us to ensure the integrity of the study. We declare having no competing interests in this paper.

Sincerely,

Ricardo M Pautassi

RESPONSE TO EDITOR AND REVIEWERS

We were very pleased by the quick turnaround and the thoroughness and fairness of the opinions of the Reviewers. Those comments really helped streamlined the MS and make it a better paper. We now respond to each query. Please note that we conducted a full proof-read of the MS and fixed minor typos and grammar issues. We also deleted some unnecessary material to make room (i.e., word count) for the new material requested by the Reviewers.

REVIEWER 1

REVIEWER COMMENT: "The chemical composition of S1RA is missing.

OUR RESPONSE: Thank you very much for noting this. We now indicate (page 7, lines 1-3) "S1RA, whose chemical formula is 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy] ethyl] morpholine hydrochloride, was…"

REVIEWER COMMENT: Some limited introduction of the biology of S1R is recommended.

OUR RESPONSE: This was very helpful. We now indicate (see page 4, lines 15-20) "S1-R is an intracellular, Ca2+-sensing, protein chaperone. It is located in the endoplasmic reticulum, specifically in mitochondrial-associated membranes (Ryskamp et al., 2019), and is concentrated in brain areas related to motivation and learning, including hippocampus, olfactory bulb, and substantia nigra (Cobos et al., 2008). S1-R is involved in several cell functions, including lipid transport (Hayashi and Su, 2005) and the monitoring of the folding status of proteins inside the endoplasmic reticulum (Mori et al., 2013)."

REVIEWER COMMENT: In Materials and Methods authors only indicate: "S1RA was supplied byWelab (Barcelona, Spain)". Must be more specific. Note in Discussion: "These studies, however, employed S1-R antagonists other than S1RA [i.e., NE-100, Sabino et al. (2009b)"

OUR RESPONSE: Thanks for noting these issues. We now indicate (see page 6, line 23 to page 7, line 4) "S1RA was supplied in powder form by Welab (Barcelona, Spain), and used without further treatment except for dissolution in physiological saline. S1RA, whose chemical formula is 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy] ethyl] morpholine hydrochloride, was subcutaneously administered at a volume of 5 ml/kg, using 27G needles." Later on, we changed the writing in the noted sentence to make it read (see page 18, lines 18-22) "However, it should be taken into account that those studies did not employ S1RA, but instead tested other antagonists [i.e., NE-100, Sabino et al. (2009b); BD1063, Sabino et al. (2009a)] or tested immature [i.e., adolescent, (Ruiz-Leyva et al.,

2020)] subjects. The present results, therefore, suggest that S1RA may offer, when compared to other S1-R antagonists, a broader protection against ethanol intake."

REVIEWER COMMENT: Methods: Authors indicate: "Ethanol was administered i.p. at a volume of 0.015 ml/g" Was ethanol dissolved in saline or pure ethanol was administered?. This reviewer calculates that if pure ethanol was administered a 150 g rat would receive 2.25 ml of ethanol. This is unlikely.

OUR RESPONSE: We clarified this issue, now the sentence (page 6, lines 21-23) reads "Ethanol (Porta, Córdoba, Argentina) was dissolved in physiological saline and administered i.p. at 2.5 or 1.75 g/kg doses (21% or 14.8% v/v solution, respectively), at a volume of 0.015ml/g."

REVIEWER COMMENT: The style of presentation of results is odd and does not readily communicate the message It should be avoided. For example, RESULTS given for experiment 1 in page 10 only refer only to the statistical analysis and do not recall the question asked in experiment 1(indicated in page 7). It would help to communicate these data by referring to the title of the figure. For example… studies showed that binge ethanol exposure at adolescence heightens ethanol intake, which was tested via two-bottle choice, 24h sessions at adulthood.

OUR RESPONSE: This is a very important suggestion and one we completely agree with. We have added a few sentences to the results paragraphs of each experiment, so as to recall the question asked in each one. In Experiment 1 we indicate (page 10, lines 15-16) "In Experiment 1 the rats, males and females, were exposed to ethanol at adolescence, via nine 2-h, single bottle, sessions. The ANOVA for…", and later (following the request of Reviewer #2) we further comment on the drinking patterns observed in that experiment (page 10, line 19 to page 11, line 1). In the same experiment, the presentation of the drinking patterns at adulthood begins with (page 11, lines 5-6) "Our hypothesis was that ethanol exposure at adolescence would heighten ethanol intake at PD 87-97 (tested via two-bottle choice, 24h sessions). Absolute ethanol intake (g/kg) at adulthood was significantly…". In Experiment 2 and 3a we indicate "Ethanol intake at adulthood, and sensitivity of that behavior to S1-R antagonism, was tested via five restricted, 2h sessions conducted between PDs 111-119. Replicating results of Exp. 1 (see Figure 3B-D), rats that had binged at adolescence drank…" (page 13, lines 4-6) and "This experiment assessed if S1RA affected simultaneous ethanol induced TA, during adolescence. Saccharin consumption at the conditioning session was not…" (page 14, lines 7-8). Similar additions can be found for Experiments 3b, 4 and 5.

REVIEWER COMMENT: The conclusion that can be derived from the conditioned aversion to ethanol extinction is not clear (Fig 5A and 6). Authors indicate: S1RA…. enhances the aversive motivational effect of ethanol. This could represent a mechanism by which S1RA

reduces ethanol self-administration. However, such a high dose of ethanol is administered (1.75 and 2.5 g/kg) (generating an overt intoxication and malaise) that the aversion is likely nonspecific for ethanol. Have the authors carried out a control S1RA experiment where after saccharin the animals another aversive condition? (foot shock, lithium? etc). Such an experiment would not require a prior binge drinking

OUR RESPONSE: This is a very astute comment that helped us enhance the new version of the MS. Indeed, we had conducted a supplementary experiment analyzing, in adolescents, if S1RA affects lithium-chloride induced taste aversion. The results indicated that the S1-R antagonist had no effect on this learning. Therefore, in this new version we indicate, in section 2.5 (page 8, line 20 to page 9, line 2) "It is important to remark that we conducted, in adolescent rats, a separate, supplementary experiment, in which saccharin exposure was followed by the nausea-inducing agent lithium chloride (Jung et al., 2022). Pre-treatment with S1RA did not alter lithium-chloride induced TA. This experiment, and the associated data analysis, is described in the supp. Material.". The concluding supplementary material, then, provides a full description of the results of this additional experiment, including a figure and a corresponding caption. It should be noted that we are already over the word count required by the journal, thus including this a full experiment in the main MS was not an option. We did, however, called back these results in the new version of the discussion and indicate (page 19, lines 19-22) "It is important to highlight that the effect of S1RA on TA was, at least under the conditions tested in this study, specific for ethanol. CS ingestion at the conditioning (i.e., before US exposure) was not significantly affected by S1RA, nor the antagonist affected TA induced by LiCl."

REVIEWER 2

REVIEWER COMMENT: "It's hard to argue that S1RA's effect in figure 2 were specific to the binge-group; because there appears to be an effect in both the adolescent control and binge groups. Therefore, saying that S1RA "inhibits this promoting effect of binge ethanol exposure" isn't quite right - instead S1RA reduced alcohol drinking generally speaking, not specific to the promoting effect."

OUR RESPONSE: Thank you very much for noting this. We now changed the first sentence of Figure's 2 caption to "Binge ethanol exposure at adolescence enhances ethanol intake (tested via time-restricted, single-bottle, sessions) at adulthood, and pre-session treatment with S1RA reduces ethanol intake at adulthood."

REVIEWER COMMENT: Similarly, it is clear that S1RA is enhancing trace CTA in both adolescents and adults, but unfortunately the latter effect is not compared in adolescent-exposed vs adolescent control rats. Therefore, we are not able to assess whether S1RA is affecting the promoting effect specifically or has a more general effect on trace CTA.

OUR RESPONSE: This is a very clever comment that we have taken into consideration. The discussion now indicates the limitation indicated by the Reviewer (page 20, lines 6-8):" …an important drawback of Experiment 3 is that the S1RA effect on ethanol-induced TA was not compared in ethanol-exposed adolescent vs adolescent control rats. Future studies should…"

REVIEWER COMMENT: These two limitations do not really detract from the findings of the paper, but the wording should be addressed more carefully. For example, in the abstract it is stated that "S1RA administration blocked this [promoting] effect of adolescent ethanol exposure", whereas it actually simply reduced alcohol intake. The title is technically correct but could similarly be reworded. The discussion section avoids this pitfall and more accurately describes the results. I do agree that this appears to be due to an enhancement of alcohol's aversive effects.

OUR RESPONSE: Thank you for the suggestion, which we have addressed. The abstract now states "S1RA administration reduced ethanol intake at adulthood and facilitated the development of ethanol-induced taste (but not place) aversion" and the title has been changed to "Ethanol drinking at adulthood is sensitive to S1-R antagonism and is promoted by binge ethanol self-administration at adolescence"

REVIEWER COMMENT: I don't understand the peak-valley-peak pattern of alcohol intake (fig. 1), some speculation of the cause of this might be helpful. "saccharin" is used in some places, "saccharine" elsewhere.

OUR RESPONSE: We have added a few sentences speculating on the emergence of the peak-valley-peak pattern (see page 10, line 19 to page 11, line 1). Specifically, we indicate "This pattern may relate to the rats developing mild taste aversion to ethanol (after achieving relatively high ethanol intake scores) that extinguishes during the next drinking session, thus allowing for the subsequent peak". We also changed to "saccharin" the few instances in which "saccharine" was used.

Author Disclosures: none

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

- Binge adolescent ethanol exposure heightened ethanol drinking at adulthood
- Recognition memory was preserved after binge ethanol exposure
- S1RA administration inhibited ethanol drinking at adulthood
- S1RA promoted ethanol-induced taste aversion
- S1RA did not affect ethanol-induced place aversion

Ethanol drinking at adulthood is sensitive to S1-R antagonism and is promoted by binge ethanol self-administration at adolescence

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Abstract: Background: Binge drinking at adolescence is a risk factor for problematic alcohol (ethanol) consumption later in life, yet the murine studies that modelled this phenomenon via ethanol self-administration have provided mixed findings. Antagonism of the sigma-1 receptor (S1-R) system at adolescence modulates ethanol's motivational effects and intake. It is still unknown, however, whether this antagonism would protect against enhanced ethanol intake at adulthood after adolescent binge ethanol exposure. **Methods**: Exp. 1 and 2 tested adults male or female Wistar rats -exposed or not to ethanol self-administration at adolescence (postnatal days 31-49; nine 2-hour sessions of access to 8-10% ethanol)- for ethanol intake using 24-h two-bottle choice test (Exp. 1) or time restricted, single-bottle, tests (Exp. 2). Experiments 2-5 evaluated, in adolescent or adult rats, the effects of the S1-R antagonist S1RA on ethanol intake and on ethanol-induced conditioned taste or place aversion. Ancillary tests (e.g., novel object recognition, ethanol-induced locomotor activity) were also conducted. **Results**: Adolescent ethanol exposure promoted ethanol consumption at both the restricted, single-bottle, and at the two-bottle choice tests conducted at adulthood. S1RA administration reduced ethanol intake at adulthood and facilitated the development of ethanol-induced taste (but not place) aversion. **Conclusions**: S1RA holds promise for lessening ethanol intake after chronic and substantial ethanol exposure in adolescence that results in heightened ethanol exposure at adulthood. This putative protective effect of S1-R antagonism may relate to S1RA exacerbating the aversive effects of this drug.

Keywords: ethanol aversion, ethanol intake, sigma-1 antagonism, sigma-1 receptor, S1RA

1. Introduction

Alcohol (ethanol, in pre-clinical studies) use is highly prevalent during adolescence. Pilatti et al. (2017) reported that, in the 6 months preceding data collection, 55% of a sample of college students from Argentina (n>4,000) ingested 4–5 drinks of alcohol in 2≤h (a pattern known as binge drinking). These patterns can heighten the risk of developing an alcohol use disorder (AUD) (Hingson and Zha, 2009; Rial Boubeta et al., 2018). Likewise, a fast progression from first drink to first drunkenness is associated with greater odds of alcohol use problems (Morean et al., 2018).

An influential review (Towner and Varlinskaya, 2020) indicated that greater ethanol intake after adolescent ethanol exposure is reliably observed in pre-clinical models that give alcohol via intraperitoneal (i.p.) or intragastric administration. These routes hardly mimic the voluntary nature of alcohol use of humans. According to the review, only a third of the studies that used voluntary ethanol self-administration at adolescence reported a promoting effect of such experience on ethanol drinking at adulthood. A critical issue is that voluntary ethanol intake in outbred rodents is not usually high. This can be circumvented by using lines genetically selected for exhibiting high ethanol consumption (Crabbe et al., 2006) or by using limited-access ethanol intake procedures (Thiele et al., 2014).

A study from our lab suggested that ethanol intake at adulthood in rats was significantly greater if the onset of alcohol exposure was adolescence, compared to adulthood (Salguero et al., 2020). That study, however, did not include a basic control condition (i.e., rats not exposed to ethanol at adolescence) to ascertain the magnitude of the promoting effect of early ethanol exposure, and exposed the adolescent rats to a combination of ethanol exposure protocols (e.g., limited-access intake procedures or i.p. administrations, plus two-bottle choice sessions). Thus, the study did not convincingly answer if self-administration of

ethanol at adolescence increases free-choice drinking at adulthood in rats. In the present study we addressed these issues by applying, in male and female Wistar rats, an adapted version of a binge-like, limited-access, ethanol self-administration procedure (Salguero et al., 2020). The rats, which drank ethanol throughout adolescence or were exposed to standard housing, were tested for free-choice drinking at adulthood.

The mechanisms underlying the effect of adolescent ethanol exposure on later ethanol use are yet to be clarified (Pautassi et al., 2020). Such exposure may alter development of neurocognitive systems that participate in ethanol seeking. A conclusive testing of this, or other, hypothesis is hampered by the lack of appropriate animal models (Brocato and Wolstenholme, 2021). In the present study, and to ascertain potential cognitive deficits associated with adolescent ethanol exposure, the rats were tested after termination of the adolescent self-administration procedure for exploratory behavior and for recognition memory. We also attempted to inhibit the effects of adolescent ethanol exposure on ethanol intake at adulthood via antagonism of the Sigma-1 Receptor (S-1R).

S1-R is an intracellular, Ca2+-sensing, protein chaperone. It is located in the endoplasmic reticulum, specifically in mitochondrial-associated membranes (Ryskamp et al., 2019), and is concentrated in brain areas related to motivation and learning, including hippocampus, olfactory bulb, and substantia nigra (Cobos et al., 2008). S1-R is involved in several cell functions, including lipid transport (Hayashi and Su, 2005) and the monitoring of the folding status of proteins inside the endoplasmic reticulum (Mori et al., 2013). S1R has been also implicated in the motivational effects of alcohol. For instance, administration of the S1-R agonist PRE-084 enhanced (Maurice et al., 2003) or reinstated (Bhutada et al., 2012) ethanol-induced conditioned place preference. S1-R agonism exacerbated operant responding for ethanol (Sabino et al., 2011; Valenza et al., 2020), an effect disrupted by BD-1063 (Sabino

et al., 2011). On the other hand, antagonism of S1-R inhibited ethanol self-administration in alcohol-preferring rats and in rats made dependent to ethanol, but not in non-dependent rats (Sabino et al., 2009a). We reported (Ruiz-Leyva et al., 2020) that the S1-R antagonists BD-1063 and S1RA inhibited binge ethanol drinking in outbred adolescent rats without a history of ethanol exposure. Altogether, these studies suggest S1-R antagonism is a promising strategy to treat risky drinking, either in the context of AUD or during ontogenetic periods in which high level of ethanol intake is normative, such as adolescence.

In the present study, after finding (Exp. 1) a reliable facilitatory effect of adolescent ethanol exposure on free-choice drinking at adulthood, we evaluated (Exp. 2) if adolescent ethanol exposure enhanced adult ethanol drinking in single bottle, limited-access intake sessions. We also tested if S1-R antagonism, induced by S1RA administrations before each drinking session at adulthood, would attenuate this effect. Experiments 3-5 analyzed motivational mechanisms underlying the effect of S1RA. We expected S1RA to promote the aversive motivational value of ethanol and, hence, to facilitate the emergence of ethanol-induced taste or conditioned place aversion.

2. Materials and Methods

2.1 Subjects, experimental design, and drugs

Wistar rats derived from 62 litters (12, 10, 24, 7 and 9 litters; Experiments 1-5, respectively) reared at INIMEC-CONICET-UNC (Argentina) were employed. The litters were culled to 10 offspring (5-6 males per litter) on PD1. Lights were turned on and off at 645AM and 645PM, respectively. Weaning was performed at PD21, and from that on the rats were housed in same-sex pairs. The procedures were certified by the local IACU

Committee, and complied with the Declaration of Helsinki, the ARRIVE guidelines, and the Guide for the Care and Use of Laboratory Animals.

Experiment 1 employed eighty-one (40 males and 41 females) rats. Litter effects were controlled by averaging male or female siblings assigned to the same group, resulting in a final dataset of 46 units of analysis. Litter effects in Exp. 2-5 were controlled by not assigning more than one male from each litter to each group. Experiment 1 revealed that the permissive effect of adolescent ethanol exposure, on adult ethanol intake, was statistically similar in males and females, albeit it was descriptively stronger in males. Hence, Exp. 2-5 were conducted in male rats only. Experiment 2 employed 60 males, whereas 46, 41, 28 and 33 males were used in Experiments 3a, 3b, 4 and 5, respectively. Data for 4 animals were lost in Experiment 2 due to technical problems, and not replaced.

A 2 (Sex) x 2 (ethanol treatment at adolescence: binge-like ethanol intake or standard housing: Binge and Control groups, respectively) factorial was employed in Exp. 1. Each of the four groups had, after averaging scores for pairs of same-sex siblings subjected to the same treatment, 11 datapoints. Exp. 2 employed a 2 (ethanol treatment at adolescence) x 3 (S1RA treatment), with 8-10 subjects per group. Exp. 3a, 3b and 4 had 7-8 rats in each group (except for group 0 ethanol–16 mg/kg S1RA of Exp. 3b, n=6), whereas Exp. 5 had 8-9 subjects per group. Exp. 3a, 3b, 4 and 5 employed factorial designs, with ethanol treatment (Exp. 3a, 3b and 5: 0, 2.5 g/kg; Experiment 4: 0, 1.75 g/kg) and S1RA treatment (Exp.2 and 3a: 0, 4, 16 mg/kg; Exp. 3b, 4 and 5: 0, 16 mg/kg) as factors.

Ethanol (Porta, Córdoba, Argentina) was dissolved in physiological saline and administered i.p. at 2.5 or 1.75 g/kg doses (21% or 14.8% v/v solution, respectively), at a volume of 0.015ml/g. S1RA was supplied in powder form by Welab (Barcelona, Spain), and

used without further treatment except for dissolution in physiological saline. S1RA, whose chemical formula is 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy] ethyl] morpholine hydrochloride, was subcutaneously administered at a volume of 5 ml/kg, using 27G needles. The following sections provide a summary of the methods and statistical analyses employed. Descriptive diagram and full descriptions of these can be found in the Supplemental Material.

2.2 Repeated exposure to self-administered ethanol at adolescence (Exp. 1 and 2) and Novel object recognition test (NOR, Exp.1)

These procedures followed those of Salguero et al. (2020). In Exp. 1 and 2 the rats were exposed thrice a week for 3 weeks (PDs 31-49) to a bottle of ethanol (8 or 10% v/v). In each session the water bottle was replaced at 300PM by an ethanol bottle, until 500PM. Control rats were left undisturbed. In Exp. 1, between PD52 and PD54, the rats were tested for distance travelled in an open field test and for recognition memory via the NOR test.

2.3 Two-bottle choice ethanol intake tests conducted at adulthood (Exp.1)

In Exp. 1 the rats, exposed or not to binge drinking at adolescence, were tested for ethanol intake at adulthood via 5 two-bottle ethanol intake tests (length: 24 h, described in Salguero et al., 2020), that took place every-other-day between PDs 87 and 97. Ethanol intake (g/kg) and percent preference of ethanol intake [(ethanol consumption/overall fluid consumption) x100] were measured.

2.4 Time-restricted, single-bottle, ethanol intake testing at adulthood (Exp. 2)

Exp. 2 assessed, akin to Exp. 1, the effects of ethanol exposure at adolescence on ethanol intake patterns at adulthood. In Exp. 2 the intake tests at adulthood involved time-restricted, single-bottle, sessions. The rats were tested for ethanol intake in five, 2 h limited-access sessions at late adulthood (five every-other-day sessions, between PDs 111-119). Exp. 2 also assessed the effects of S1-R antagonism upon ethanol intake. Thirty min before each session rats received S1RA (0, 4, or 16 mg/kg). These doses were selected based on prior studies (Ruiz-Leyva et al., 2020).

On PD122 the Binge rats and 6 Controls underwent a 1-h ethanol intake session and were subsequently sacrificed. These rats had been given, the day before the test, 50% of the water they usually drank. A colorimetric enzymatic method was used to analyze the blood samples for BELs (Marengo et al., 2023).

2.5 Effect of S1RA on ethanol-induced taste aversion (TA) at adolescence, with the conditioned (CS) and unconditioned stimulus (US) overlapping (Experiment 3a)

A TA conditioning [adapted from Salguero et al. (2022)] was conducted between PD29 and 36. Briefly, the rats were injected S1RA (0, 4 or 16 mg/kg) and, 30 min later, exposed to a 0.1% saccharin bottle, which was immediately followed by the administration of the US (0 or 2.5 g/kg ethanol). Testing for ethanol-induced TA occurred on PD32, 34 and 36 (i.e., extinction sessions 1-3, 60 min each). It is important to remark that we conducted, in adolescent rats, a separate, supplementary experiment, in which saccharin exposure was followed by the nausea-inducing agent lithium chloride (Jung et al., 2022). Pre-treatment

with S1RA did not alter lithium-chloride induced TA. This experiment, and the associated data analysis, is described in the Supp. Material.

2.6 Effect of S1RA on ethanol-induced TA acquired with a trace between CS and US, at adolescence (Exp. 3b) and adulthood (Exp. 4); and effect of S1RA on Ethanol-induced place aversion conditioning (Exp. 5)

Experiment 3a indicated that, in adolescents, S1RA did not modulate ethanol-induced TA, when the CS and the US overlapped. Experiments 3b and 4 replicated, in adolescent and adult rats respectively, the TA conditioning of Exp. 3a yet imposed a 30 min temporal trace between the CS and the US. This procedure is, compared to the TA procedure of Exp. 3a, more demanding, requiring bridging of the gap between the stimuli (Beylin et al., 2001). The ethanol dose was 2.5 and 1.75 g/kg (Exp. 3b and 4, respectively; schematic diagram and full description of these procedures in the Supp. material). The rationale for the use of a lower dose in Exp. 4, is that adult rats are, vs adolescents, more sensitive to ethanol's aversive effects (Saalfield and Spear, 2016). The rats of Experiment 3b were also assessed (PD39) for ethanol-induced (2.5 g/kg) locomotor activity.

Place conditioning induced by ethanol at adolescence, and S1RA (16 mg/kg) modulation of this conditioning, was assessed through procedures (fully described in the Supp. Material) similar to those of Fernandez et al. (2017).

2.7 Statistical analysis

The data derived from the intake sessions were analyzed via repeated measures (RM) Analyses of Variance (ANOVAs) that, in Exp. 1 and 2, included sex, ethanol exposure or (in Exp. 2) S1RA treatment as between factors. Data derived from the NOR test were analyzed via factorial ANOVAs that considered sex and ethanol exposure as between factors. Consumption of saccharin (ml/100 g of body weight; Exp. 3a, 3b and 4) and time spent (s) and preference (%) for the sandpaper texture paired with the effects of ethanol (Exp. 5), were analyzed via RM ANOVA, with S1RA treatment and ethanol treatment as between factors.

The significant main effects or interactions were scrutinized via Newman-Keul's *post hoc* tests. Data is informed as mean±SE. Planned comparisons were run when supported by *a priori* hypotheses, and the alpha level was ≤0.05.

3. Results

3.1 Experiment 1

3.1.1 Ethanol intake at the 2h sessions conducted at adolescence (Fig. 1A).

In Experiment 1 the rats, males and females, were exposed to ethanol at adolescence, via nine 2-h, single bottle, sessions. The ANOVA for ethanol intake (g/kg) yielded a significant main effect of Session ($F_{8,160}$=17.30, p<0.01, η^2p=0.43). The *post-hoc* tests indicated a peak-valley-peak pattern, with drinking scores being significantly higher in sessions 2, 4 and 6 than in sessions 1, 3 and 5, respectively. This pattern may relate to the rats developing mild taste aversion to ethanol (after achieving relatively high ethanol intake scores) that extinguishes during the next drinking session, thus allowing for the subsequent

peak. Ethanol intake was low and stable at sessions 7-9 and the scores at these sessions were significantly lower than those registered at sessions 1-4 and 6.

FIGURE 1 HERE

3.1.2 Ethanol intake patterns at adulthood.

Our hypothesis was that ethanol exposure at adolescence would heighten ethanol intake at PD 87-97 (tested via two-bottle choice, 24h sessions). Absolute ethanol intake (g/kg) at adulthood was significantly greater in females vs. males ($F_{1,40}$=77.65, p<0.001, η^2p=0.66), and females drank more in the first or last test than in the second or fourth test (significant sex x session interaction: $F_{4,160}$=3.00, p<0.05, η^2p=0.07). Consistent with the hypothesis, the rats that had binged at adolescence drank significantly more than controls (significant main effect of treatment: $F_{1,40}$=12.36, p<0.001, η^2p=0.24). These data are shown in Figure 1B and 1D. The ANOVA on ethanol percent predilection vs water (Fig. 1C and 1E) yielded similar results. Ethanol preference was greater in females than in males ($F_{1,40}$=21.60, p<0.001, η^2p=0.35), in the first than in the following sessions ($F_{4,160}$=35.09, p<0.001, η^2p=0.46) and in rats that binged at adolescence than in controls ($F_{1,40}$=9.38, p<0.001, η^2p=0.19). Visual inspection of Fig. 1 suggests that the permissive effect of adolescent ethanol exposure was greater in males than in females, with males with a history of adolescent ethanol exhibiting a 3-fold increase vs. controls in sessions 2 or 4. The treatment x sex or the treatment x sex x sessions interactions, however, did not achieve significance.

Water intake at sessions 1, 2 and 4 (ml/100 g of body weight, Table 1) was significantly lower in rats that had binged at adolescence than in controls (significant treatment x session interaction: $F_{4,160}$=3.03, p<0.05, η^2p=0.07), and significantly lower across groups in the first than in the last session ($F_{4,160}$=14.21, p<0.05, η^2p=0.26). The ANOVA on

overall liquid intake (ml/100 g of body weight, Table 1) yielded significant main effects of sex and session ($F_{1,40}$=80.27, p<0.001, η^2p=0.67 and $F_{4,160}$=17.60, p<0.001, η^2p=0.31, respectively), and a significant sex x session interaction ($F_{4,160}$=9.18, p<0.001, η^2p=0.19). The *post hoc* tests indicated that female rats drank more fluid than the male rats, an effect that was significantly greater at the first two-bottle intake session.

TABLE 1 HERE

3.1.3 Motor activity in the OF and recognition memory scores (NOR test).

Distance travelled (cm) in the OF (Fig. 2A) was significantly less in BINGE than in CONTROL rats (significant main effect of treatment: $F_{1,40}$=7.44, p<0.01, η^2p=0.16). Distance travelled and time spent locomoting (Fig. 2B) was reduced in females vs. males (significant effect of sex: $F_{1,40}$=22.82, p<0.001, η^2p=0.36 and $F_{1,40}$=29.41, p<0.001, η^2p=0.42, respectively).

T-tests for NOR scores (Fig. 2C) indicated the rats exhibited a predilection for the novel object significantly higher than 50% (M=0.59, t_{43}=5.85, p<0.001; ethanol group: M=0.61, t_{22}=5.98, p<0.001; control group: M=0.57, t_{22}=2.88, p<0.01). An ANOVA indicated that this effect, suggestive of short-term memory, was not significantly affected by sex or ethanol treatment.

FIGURE 2 HERE

3.2 **Experiment 2**

3.2.1 Ethanol intake at the 2h sessions conducted at adolescence and at adulthood.

Akin to Exp. 1, the exposure to ethanol at adolescence resulted in the rats self-administering 2.2-3 g/kg in the first four sessions and 1.4-2.3 g/kg in the last 5 sessions

(Figure 3A). The ANOVA for ethanol intake yielded a significant effect of session ($F_{8,224}$=9.41, p<0.001, η^2p=0.25), with scores at sessions 1-6 and 9 being significantly higher than those registered at sessions 7-8 and 3, respectively.

Ethanol intake at adulthood, and sensitivity of that behavior to S1-R antagonism, was tested via 5 restricted, 2h sessions conducted between PDs 111-119. Replicating results of Exp. 1 (see Figure 3B-D), rats that had binged at adolescence drank significantly more than controls ($F_{1,50}$=28.77, p<0.001, η^2p=0.37); with the difference being maximal at session 1 (0.78 vs 0.22 g/kg). Administration of S1RA induced a significant decrease in ethanol ingestion, $F_{2,50}$=11.75, p<0.001, η^2p=0.32, that was statistically similar in rats with or without exposure to ethanol at adolescence. The S1RA treatment x session interaction was significant, ($F_{8,200}$=2.91, p<0.005, η^2p=0.10, Figure 3B). The *post hoc* tests indicated that rats administered 16 mg/kg S1RA exhibited significantly lower ethanol intake than controls in sessions 2 to 5, whereas those given 4 mg/kg S1RA consumed significantly less ethanol than controls only in session 4. A planned comparison indicated that ethanol intake in Binge rats given 16 mg/kg S1RA (Figure 3D) was statistically similar (p>0.05) to that found in Control rats given 0 mg/kg S1RA (Figure 3C). This corroborated the hypothesis that S1RA inhibited the promoting effect of adolescent ethanol exposure.

FIGURE 3 HERE

3.2.2 Blood ethanol levels after a 1-h session of ethanol drinking

At PD122, 12 rats (6 binge, 6 controls) underwent a 1-h intake session, in which they were exposed to 10% ethanol. Ethanol intake (g/kg) was significantly greater in the Binge than in control rats (1.05±0.36 vs 0.59±0.24; t_{10}=2.63, p<0.05). The control rats exhibited BELs below the detection range whereas mean BEL (mg/dl) in Binge rats was 40.8±32.7

(range 14-86), which were significantly correlated with ethanol intake scores (r=0.95, p<0.05).

3.3 **Experiment 3**

3.3.1 S1RA does not affect ethanol-induced TA at adolescence, when CS and US overlap (Exp. 3a)

This experiment tested if S1RA affected simultaneous ethanol induced TA, during adolescence. Saccharin consumption at the conditioning session was not affected by group assignment (Table 2, p>0.05). The ANOVA for test intake scores yielded significant main effects of Ethanol treatment and Day of Assessment ($F_{1,44}$=21.80, p<0.001, η^2p=0.33 and $F_{2,88}$=63.25, p<0.001, η^2p=0.59); with their interaction achieving significance, $F_{2,88}$=14.23, p<0.001, η^2p=0.24. The *post-hoc* tests indicated a significantly lower saccharin intake in ethanol- than in vehicle-treated rats in the first and second, but not in the third, tests. These differences (see Fig. 4), suggestive of ethanol-induced TA that persisted for two extinction tests, were not significantly affected by S1RA.

FIGURE 4 AND TABLE 2 HERE

3.3.2 Ethanol-induced TA at adolescence, acquired with a trace between CS and US, is only expressed when S1RA precedes conditioning (Exp. 3b)

This experiment resembled Exp. 3a, yet trained the adolescent rats in a TA procedure featuring a temporal trace between the CS and the US. The rationale was to increase the level of complexity of the procedure, to leverage the possibility of S1RA modulating ethanol-induced TA. The acceptance of the CS at the conditioning session was not significantly

affected by group assignment (p>0.05). In contrast, the ANOVA on saccharin intake at the extinction tests revealed significant main effects of S1RA and extinction tests ($F_{1,26}$=6.86, p<0.05, η^2p=0.21, and $F_{2,52}$=19.58, p<0.001, η^2p=0.43, respectively). The interaction between extinction tests and ethanol treatment, and the interaction between extinction tests, ethanol treatment and S1RA treatment also achieved significance ($F_{2,52}$=9.85, p<0.001, η^2p=0.27, and $F_{2,52}$=3.57, p<0.05, η^2p=0.12, respectively). The *post-hoc* tests indicated the absence of TA in rats spared from S1RA treatment. This is, among rats given 0 mg/kg S1RA CS drinking was similar between ethanol and vehicle-treated groups. In contrast, in extinction test 1 rats given S1RA and ethanol exhibited significantly less saccharin drinking than rats given S1RA and vehicle. A similar pattern was found in extinction test 2, yet the difference between the groups failed to achieve significance. These results are in Fig. 5A.

FIGURE 5 HERE

Seventy-two hours after extinction 3 the rats were given 2.5 g/kg ethanol and tested (10 min) for distance traveled in the OF (Fig 5 B-C). Distance travelled was significantly lower in rats given 16 mg/kg S1RA, compared to those given 0 mg/kg S1RA and, in parallel, was significantly greater treated in rats given ethanol at the TA procedure, than in vehicle-treated controls. The significant effects yielded by the corresponding ANOVA and a description of BELs as a function of S1RA treatment can be found in the Supp. material.

3.4 Experiment 4

This experiment tested if S1RA allowed the emergence of ethanol-mediated TA at adulthood, when a temporal trace exists between CS and US. Saccharin intake at conditioning (Table 2)

was similar across groups (p>0.05). Inspection of Figure 6 suggests that ethanol-induced TA was stronger in rats given 16 mg/kg S1RA than in those given 0 mg/kg S1RA. The ANOVA revealed significant main effects of ethanol and S1RA treatment (i.e., lower CS intake in ethanol-treated rats vs. controls and in rats given S1RA vs. controls, $F_{1,24}$=28.26, p<0.001, η^2p=0.54 and $F_{1,24}$=10.65, p<0.005, η^2p=0.31 respectively). There was a significant main effect of tests ($F_{2,48}$=53.42, p<0.001, η^2p=0.69) and the interaction between tests and S1RA was borderline ($F_{2,48}$=3.16, p=0.051, η^2p=0.12). Based in our *a priori* hypothesis we conducted, among ethanol-treated rats, planned comparisons between the groups given 0 or 16 mg/kg S1RA. CS intake across tests was significantly lower in rats given 16 mg//kg S1RA, than in controls ($F_{1,24}$=10.02, p>0.005), a difference that was significant when focusing on extinctions days 1 ($F_{1,24}$=7.00, p>0.05), 2 ($F_{1,24}$=6.69, p>0.05) or 3 ($F_{1,24}$=9.05, p>0.05).

INSERT FIGURE 6 HERE

3.5 **Experiment 5**

Experiments 3 and 4 suggested that S1-R antagonism may exacerbate ethanol-induced aversion. Experiment 5 further probed this possibility, via place conditioning. At the habituation the time spent and preference in sandpaper (the texture associated with ethanol's effects) were not significantly affected by group assignment. The ANOVAs for the extinction showed a significant main effects of ethanol ($F_{1,29}$=19.95, p<0.001, η^2p=0.41 and $F_{1,29}$=14.81, p<0.001, η^2p=0.34, respectively). Across tests, sandpaper absolute or preference scores were significantly lower in ethanol- vs vehicle-treated rats (see Table 3). This aversion was similarly exhibited after 0 or 16 mg/kg S1RA treatment.

TABLE 3 HERE

Discussion

Preclinical models of the "early ethanol exposure effect" have provided mixed results (Towner and Varlinskaya, 2020) particularly when the methods to induce the first contact with ethanol involve forced, bolus drug delivery, or when the rats or mice are given continuous exposure to ethanol and water that result in low BELs. Varlinskaya et al. (2017) found that exposure to ethanol at adolescence (3.5 g/kg, i.g., PD25-PD45) did not alter ethanol intake at adulthood. Likewise, others (Tambour et al., 2008) failed to see a substantial effect of age at drinking onset on 2-bottle preference testing. These studies illustrate the lack of reliable preclinical preparations to model the effects of early alcohol exposure, which has hampered the testing of putative treatments. The present study addresses some of these gaps.

Experiment 1 described an ethanol exposure model that induced high levels of ethanol drinking. Consistent with studies that used a sharply different voluntary ethanol intake method [i.e., consumption-off-the floor (Truxell et al., 2007)], the maximal drinking scores occurred in early adolescence (≈3 g/kg/2h) and declined thereafter. Binge-like exposure at adolescence, in turn, heightened ethanol intake at adulthood when testing involved either 24-h, free-choice tests (Exp. 1), or time-restricted single-bottle tests (Exp. 2).

The effect of early ethanol exposure was statistically similar in males and females, albeit less variable and of greater magnitude in males. In Exp. 1 the adolescent females exhibited, in congruence with Vetter-O'Hagen et al. (2009), significantly greater ethanol intake than males. This could have represented a ceiling effect that prevented further enhancement of ethanol intake. Intriguingly, we have already reported that ethanol intake is more likely to be enhanced by treatments given at adolescence in male than in female rats. Specifically,

repeated amphetamine treatment during adolescence enhanced ethanol intake during late adolescence in male, but not in female rats (Ruiz et al., 2018).

A limitation of this study is the lack of brain measurements, which precludes the analysis of underlying mechanisms. Behavioral measurements, however, indicated the lack of recognition memory deficits, after the adolescent ethanol exposure. A study reported that intermittent ethanol exposure at adolescence increased permeability of the blood-brain-barrier, in areas related to ethanol-induced reward (Vore et al., 2022). These alterations were observed in males but not in females, thus mirroring the greater vulnerability of males to the promoting effects of early ethanol exposure, shown in the present study. Future studies should analyze if exposure to binge ethanol, in the timing and dosing of this study, also results in sexually dimorphic alterations of the blood-brain-barrier.

Another relevant finding of the present study is that ethanol intake at adulthood was significantly reduced by S1RA treatment (Exp. 2), both in Binge and in Control rats. It was somehow novel to see this effect in the adult controls that remained unexposed to ethanol at adolescence. Previous studies suggest that antagonism of S1-R inhibits ethanol self-administration when genetic (Sabino et al., 2009b), ontogenetic (Ruiz-Leyva et al., 2020) or environmental factors (Sabino et al., 2009a) promote high levels of intake, but may not affect ethanol intake in naïve adults. However, it should be taken into account that those studies did not employ S1RA, but instead tested other antagonists [i.e., NE-100, Sabino et al. (2009b); BD1063, Sabino et al. (2009a)] or tested immature [i.e., adolescent, (Ruiz-Leyva et al., 2020)] subjects. The present results, therefore, suggest that S1RA may offer, when compared to other S1-R antagonists, a broader protection against ethanol intake.

Experiments 3a, 3b and 4 explored mechanisms by which S1RA might affect ethanol-induced motivation. The strategy was to generate a labile ethanol-induced TA conditioning to test if S1RA would modulate such motivational learning. The strategy was not successful in Experiment 3a, which used a delayed conditioning [i.e., in which the US immediately followed the CS, Bangasser et al. (2006)] that induced potent ethanol-induced TA that was insensitive to S1RA. We then switched to trace conditioning, defined by the presence of a temporal trace between CS and US. This preparation is more challenging than delay, for instance requiring more sessions to induce conditioning (Beylin et al., 2001). Under these conditions, ethanol-mediated TA was not expressed at adolescence. Intriguingly, preconditioning S1RA administration allowed the emergence of such TA. Exp. 4 revealed that adults, unlike adolescents, were able to acquire ethanol-induce TA when trained via trace conditioning, a result consistent with studies showing that adolescents are less likely to acquire conditioned responses when the training involves trace conditioning (Hunt and Barnet, 2016; Misanin et al., 2002). Besides this ontogenetic difference, it is notable that the expression of ethanol-induced TA, in Exp. 4, was still substantially improved after S1RA.

Altogether the results of Experiment 3 suggest that S1-R antagonism in adolescents and adults, at a dose that seem to lack motivational effects of its own, enhances the aversive motivational effect of ethanol. This could represent a mechanism by which S1RA reduces ethanol self-administration. It is important to highlight that the effect of S1RA on TA was, at least under the conditions tested in this study, specific for ethanol. CS ingestion at the conditioning (i.e., before US exposure) was not significantly affected by S1RA, nor the antagonist affected TA induced by LiCl.

Consistent with our proposals, a study of our lab (Salguero et al., 2022) reported attenuated ethanol-induced TA after PRE-084, and Valenza et al. (2016) described enhanced ethanol-induced TA in mice knock-out for S1-R. Experiment 5 suggests, however, that we should be cautious when discussing the possibility of S1RA enhancing ethanol's aversive effects, as no S1-R modulation of ethanol-induced place aversion was evident. It is possible that the strength of the place aversion prevented such modulation. Also, an important drawback of Experiment 3 is that the S1RA effect on ethanol-induced TA was not compared in ethanol-exposed adolescent vs adolescent control rats. Future studies should assess these possibilities and also scrutinize the effects of S1-R modulation on ethanol's appetitive effects. It has been shown that antagonism of S1-R dose-dependently inhibited ethanol-induced behavioral activation in an open field (Maurice et al., 2003), a result consistent with the findings of Exp. 3b, in which ethanol-induced distance travelled was lower in subjects that, several days ago, had received S1RA. This result suggests that S1RA may induce plastic changes in the neural systems that process ethanol-induced motivational effects.

Shortcoming of the present study are the lack of dose-response curves, the use of only males from Experiment 2 onwards and the recourse to different ethanol access schedules in adulthood, in Experiments 1 and 2. The rationale is that we aimed to investigate whether adolescent binge-like ethanol intake could have a lingering effect on different patterns of ethanol intake. Despite the limitations, the study represent progress towards understanding the consequences of adolescent exposure to ethanol. The study cements the notion that early alcohol exposure can have a causal role on later drinking, and provides evidence that S1-R antagonism can prove a valuable treatment -probably because it effects on ethanol-induced aversion- to reduce ethanol intake, even that promoted by early ethanol exposure.

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Figure Legends

Figure 1. Binge ethanol exposure at adolescence heightens ethanol intake (tested via two-bottle choice, 24h sessions) at adulthood. A. Ethanol intake (g/kg) in adolescent, male and female, Wistar rats as a function of binge intake session (1–9). The rats self-administered 8% (v/v, first three sessions) or 10% ethanol (fourth and subsequent session) during 2 h, three times a week (Monday, Wednesday, and Friday) during postnatal days (PDs) 31–49. Ethanol intake was significantly higher in sessions 2, 4 and 6 than in sessions 1, 3 and 5, respectively, and significantly lower in sessions 7-9 than in sessions 1-4 and 6 (effects denoted by the asterisk and the pound signs, respectively). **B-C.** Ethanol intake (g/kg) and % preference in male and female adult Wistar rats as a function of group assignment (Binge, Control) at the ethanol exposure at adolescence. The rats were assessed on intermittent, two-bottle, 24h tests on PDs 87-97. Ethanol intake (g/kg) and preference was significantly greater in Binge than in Control rats and in female vs. male rats as shown by the ampersand and "$" sign, respectively. **D-E.** Same data as B-C but averaged across intake sessions. The data are expressed as mean±SEM.

Figure 2. Locomotive behavior, but not recognition memory, are affected by binge ethanol exposure at adolescence. A-B. Distance travelled (cm) and time spent locomoting (s) in an open field in Wistar rats as a function of sex and binge ethanol exposure at adolescence (Binge, Control). Time spent locomoting was significantly reduced in female than in male Wistar rats, whereas rats exposed to binge ethanol exposure exhibited significantly less distance travelled than controls. These effects are indicated by the

ampersand and "$" signs, respectively. **C.** Preference for a novel object, as tested in a novel object recognition test, was significantly greater than chance (an effect denoted by the percentage sign) but not affected by sex or adolescent binge-like ethanol exposure. The data are expressed as mean ± SEM, and the tests were conducted during PDs 52–54.

Figure 3: Binge ethanol exposure at adolescence enhances ethanol intake (tested via time-restricted, single-bottle, sessions) at adulthood, and pre-session treatment with S1RA reduces ethanol intake at adulthood. A. Ethanol intake (g/kg) in adolescent male Wistar rats as a function of binge intake session (1–9). The rats self-administered 8% (first three sessions) or 10% ethanol (fourth and subsequent session) during 2 h, three times a week (Monday, Wednesday, and Friday) during postnatal days (PDs) 31–49. Ethanol intake at sessions 1-6 was significantly higher than at sessions 7-8, and significantly greater at session 9 vs. session 2. These effects are denoted by the asterisk and the pound signs. **B.** Ethanol intake at adulthood as a function of session and S1RA treatment. The rats, that had been exposed or not to adolescent binge ethanol intake, were administered the sigma-1 receptor antagonist S1RA (0, 4 or 16 mg/kg) 30 min before being tested for ethanol intake in five, 2 h limited-access sessions (PDs 111-119). The rats administered 16 mg/kg S1RA exhibited significantly lower ethanol intake than those given 0 mg/kg in sessions 2 to 5, whereas those given 4 mg/kg S1RA consumed significantly less ethanol than controls in session 4. These effects are denoted by the pound signs and the asterisk sign, respectively. **C-D.** Same data as in B but shown as a function of S1RA treatment and adolescent binge exposure (adolescent binge ethanol intake and control groups given standard rearing at adolescence). As shown by the asterisk sign, rats that binged at adolescence drank significantly more ethanol at adulthood than controls. A planned comparison indicated that ethanol intake in Binge rats

given 16 mg/kg S1RA was statistically similar ($p>0.05$) to that found in Control rats given 0 mg/kg S1RA. The data are expressed as mean ± SEM.

Figure 4: S1RA administration does not affect ethanol-induced taste aversion at adolescence, when the conditioned and unconditioned stimulus overlap. A. Saccharin intake (ml/100 g of body weight, bw) at extinction tests 1, 2 and 3 (PDs 32, 34 and 36, respectively), as a function of ethanol treatment (0 or 2.5 g/kg, i.p.). On PD30 the rats underwent a conditioning session in which S1RA (0, 4 or 16 mg/kg, s.c.) was administered right before a 30 min exposure to saccharin, which in turn was immediately followed by the ethanol or vehicle injection. The statistical analysis revealed a significantly lower saccharin intake in ethanol- than in vehicle-treated rats in the first and second tests (as denoted by the asterisk sign). **B.** Same data as A but depicted as a function of ethanol and S1RA treatment. The latter treatment did not significantly affect ethanol-induced TA patterns. Data are expressed as mean ± SEM.

Figure 5: S1RA facilitates the expression of ethanol-induced TA at adolescence, when the conditioned and unconditioned stimulus are separated by 30 min (i.e., trace conditioning), and exerts persistent effects upon locomotor activity in a novel open field. A. Saccharin intake (ml/100 g of body weight, bw) at extinction tests 1, 2 and 3 (PDs 32, 34 and 36, respectively), as a function of S1RA dose (0 or 16 mg/kg, s.c.) and ethanol treatment (0 or 2.5 g/kg, i.p.). On PD30 the rats underwent a conditioning session in which a 30 min exposure to saccharin was followed, 30 min later, by the ethanol injection. S1RA (0, 4 or 16 mg/kg, s.c.,) had been given immediately before the saccharin exposure. In extinction test 1 the rats given S1RA and ethanol treatment exhibited significantly less saccharin drinking

than rats given S1RA and vehicle, an effect indicated by the asterisk sign. **B-C.** Distance traveled (cm) by male Wistar rats in a 10 min open field test (conducted on postnatal day 39), which began immediately after a 2.5 g/kg ethanol (i.p.) challenge. The rats had been injected S1RA (0 or 16 mg/kg) or ethanol (0 or 2.5 g/kg) at the taste aversion conditioning on postnatal day 30. Panels B and C depict distance travelled averaged across ethanol or S1RA treatment, respectively. The analyses indicated that the rats treated with 16 mg/kg S1RA exhibited significantly reduced motor activity vs. 0 mg/kg controls, at minutes 1, 4 and 6; where rats with a history of ethanol exposure exhibited significantly greater ethanol-induced motor activity at minutes 1 and 4, compared to vehicle-treated controls. These effects are depicted by the asterisk sign. The data are expressed as mean ± SEM.

Figure 6: S1RA facilitates the expression of ethanol-induced TA at adulthood, when the conditioned and unconditioned stimulus are separated by 30 min (i.e., trace conditioning). Saccharin intake (ml/100 g of body weight, bw) at extinction tests 1, 2 and 3 (PDs 71, 73 and 75, respectively) as a function of S1RA dose (0 or 16 mg/kg, s.c.) and ethanol treatment (0 or 1.75 g/kg, i.p.). On PD69 the rats underwent a conditioning session in which a 30 min exposure to saccharin was followed, 30 min later, by the ethanol injection. S1RA (0 or 16 mg/kg, s.c.) had been given immediately before the saccharin exposure. The statistical analysis revealed lower saccharin intake in ethanol-treated rats vs. controls and in rats given S1RA vs. controls. Furthermore, among rats treated with ethanol, saccharin intake was significantly lower in rats given 16 mg/kg S1RA than in controls, on extinctions days 1, 2 and 3. These significant differences are indicated with the asterisk sign. The data are expressed as mean ± SEM.

Figure 1

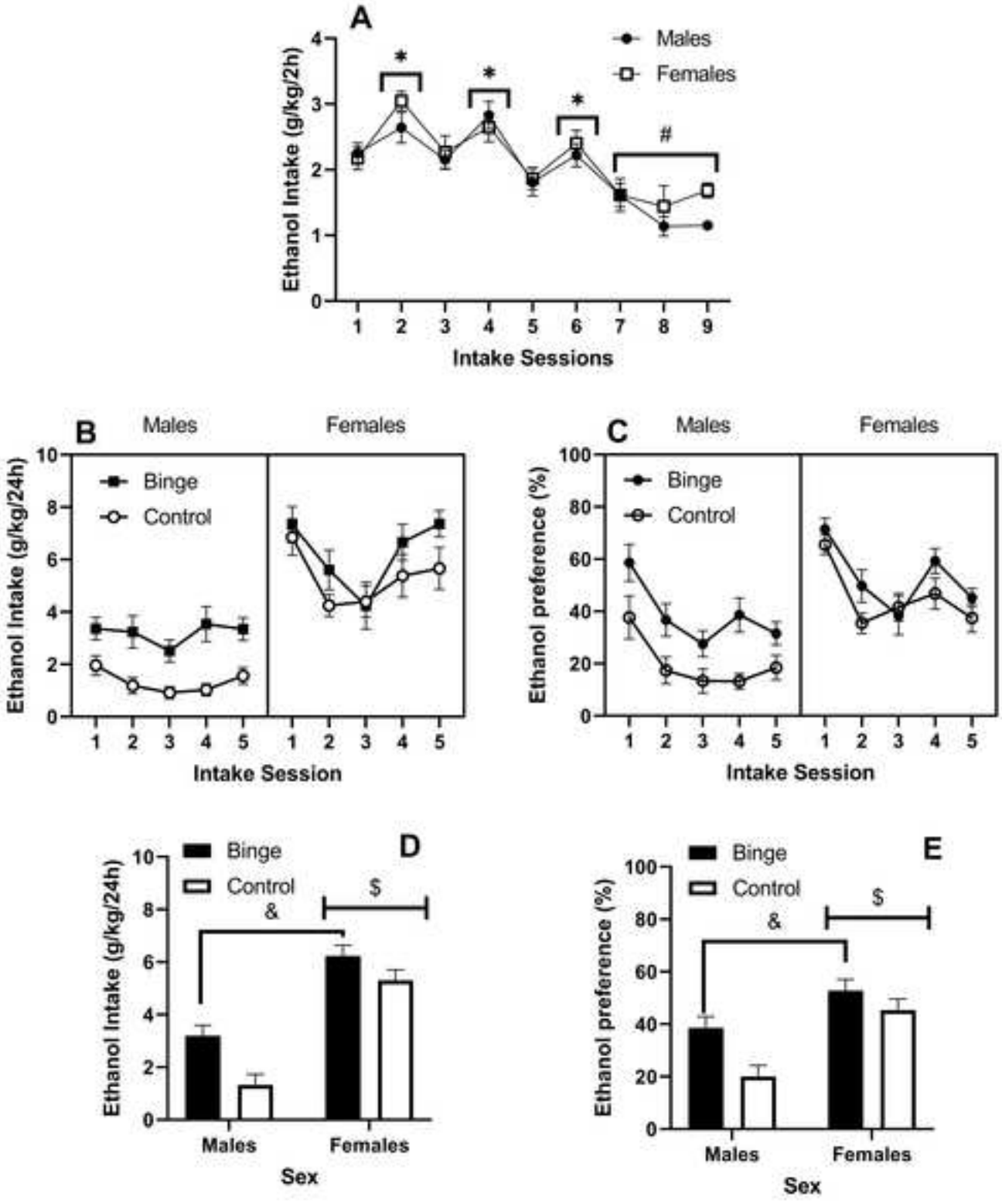

Figure 2

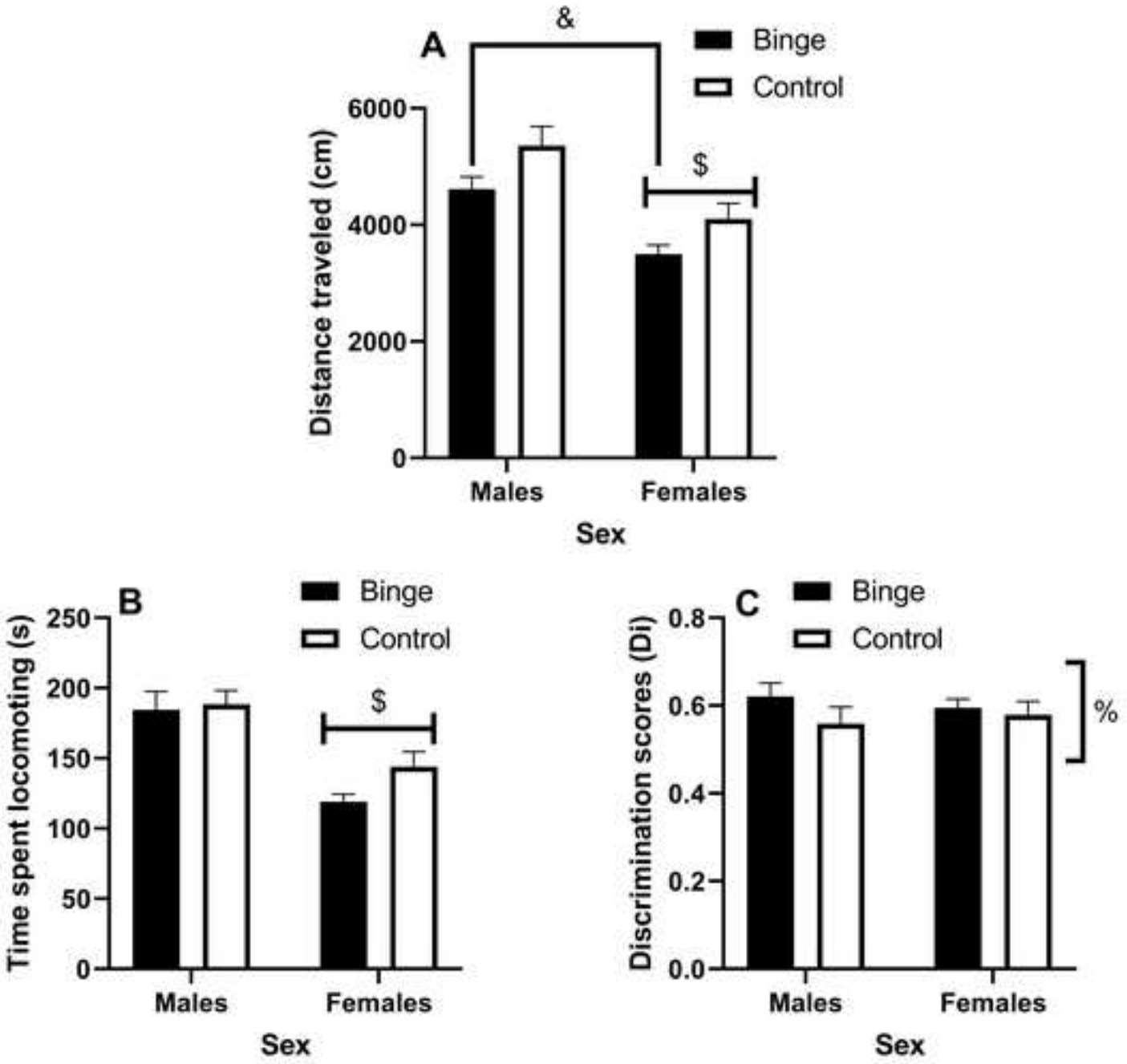

Figure 3

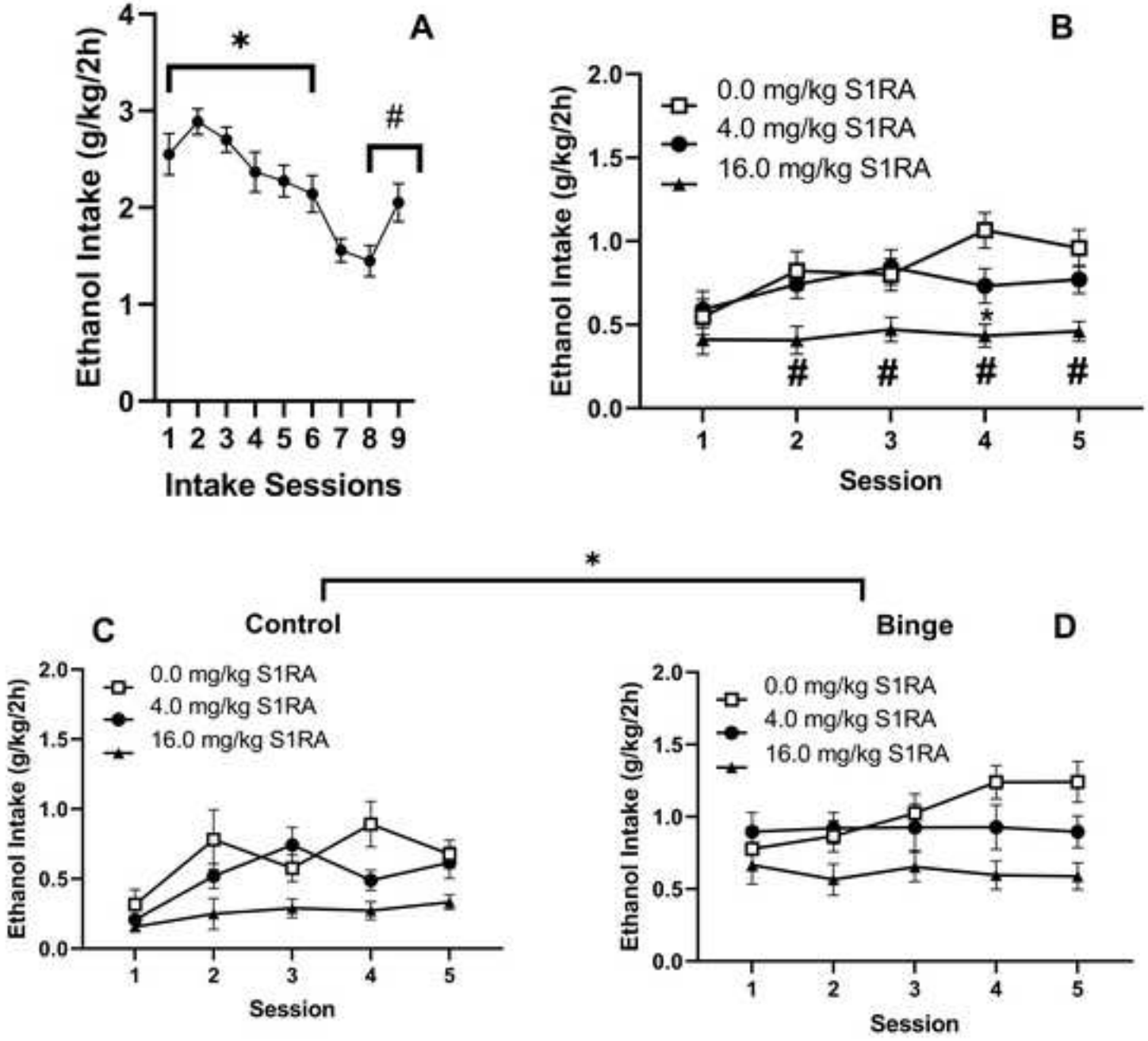

Figure 4

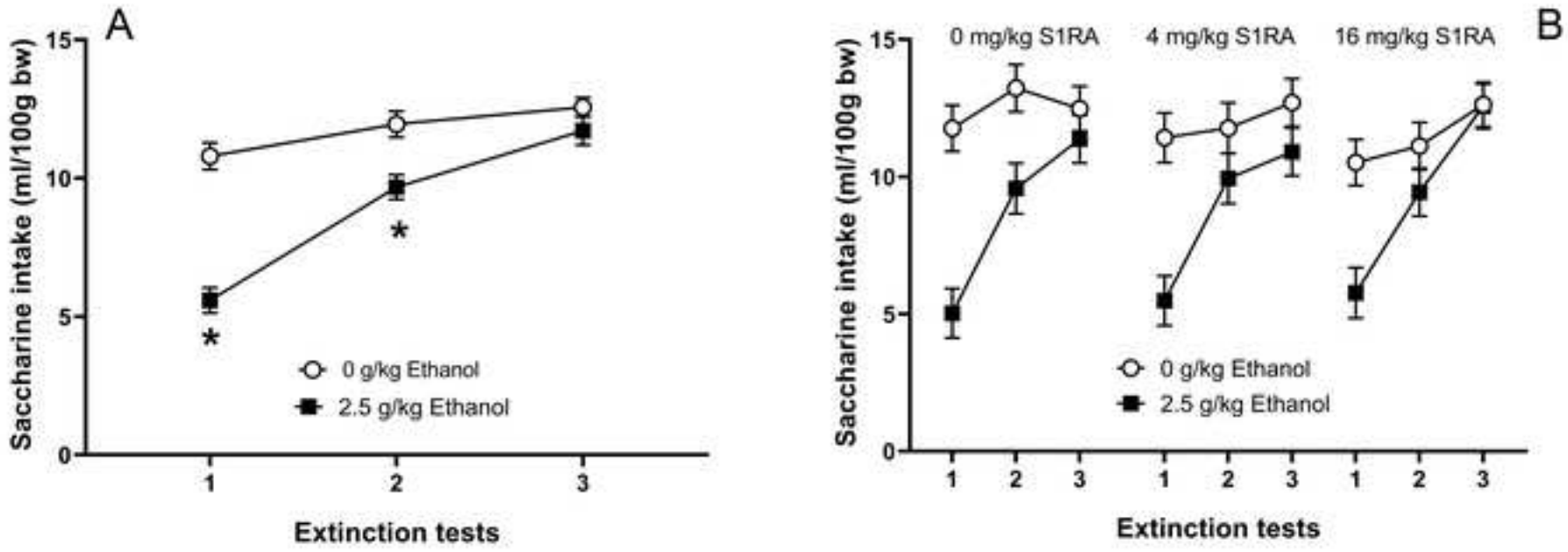

Figure 5

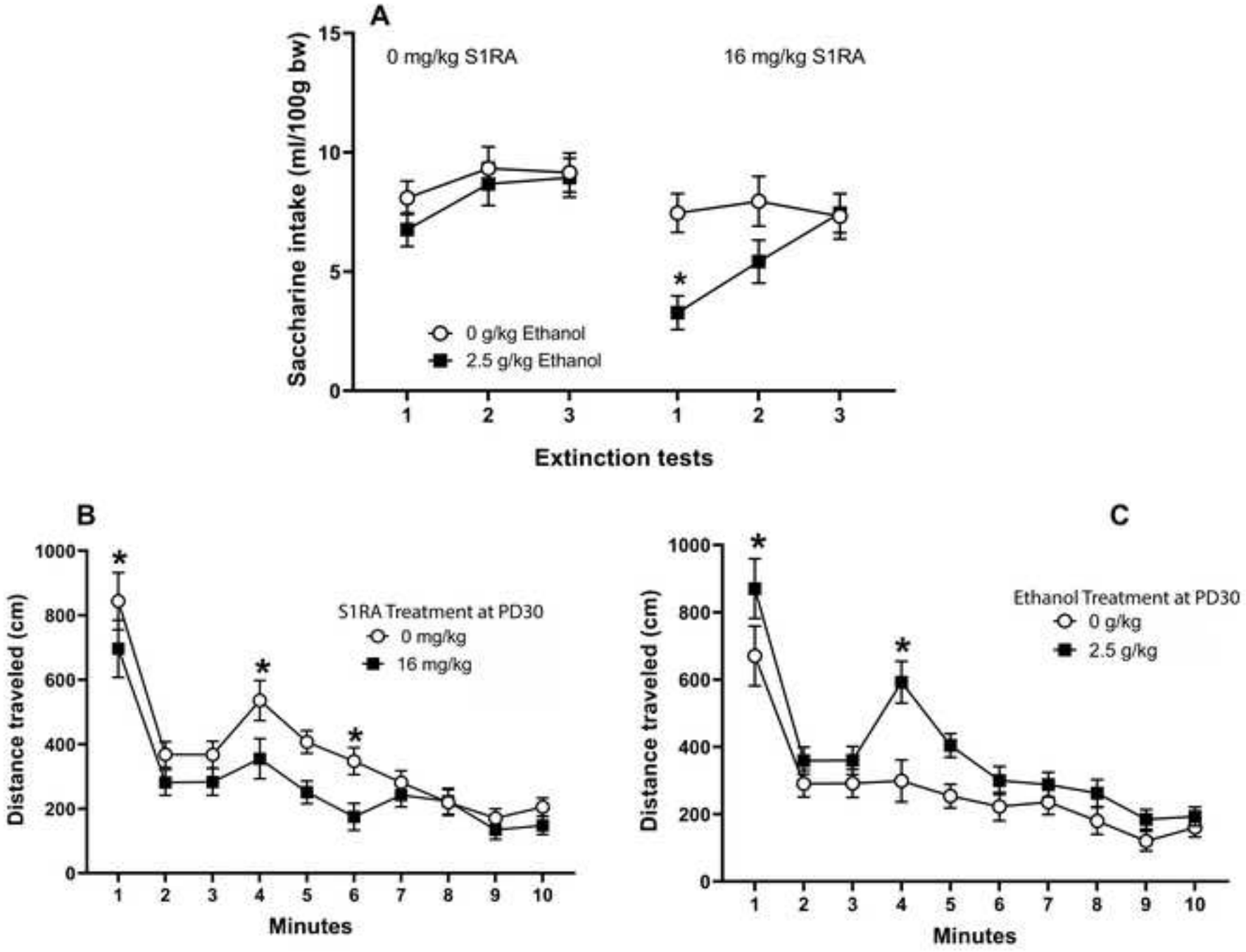

Figure 6

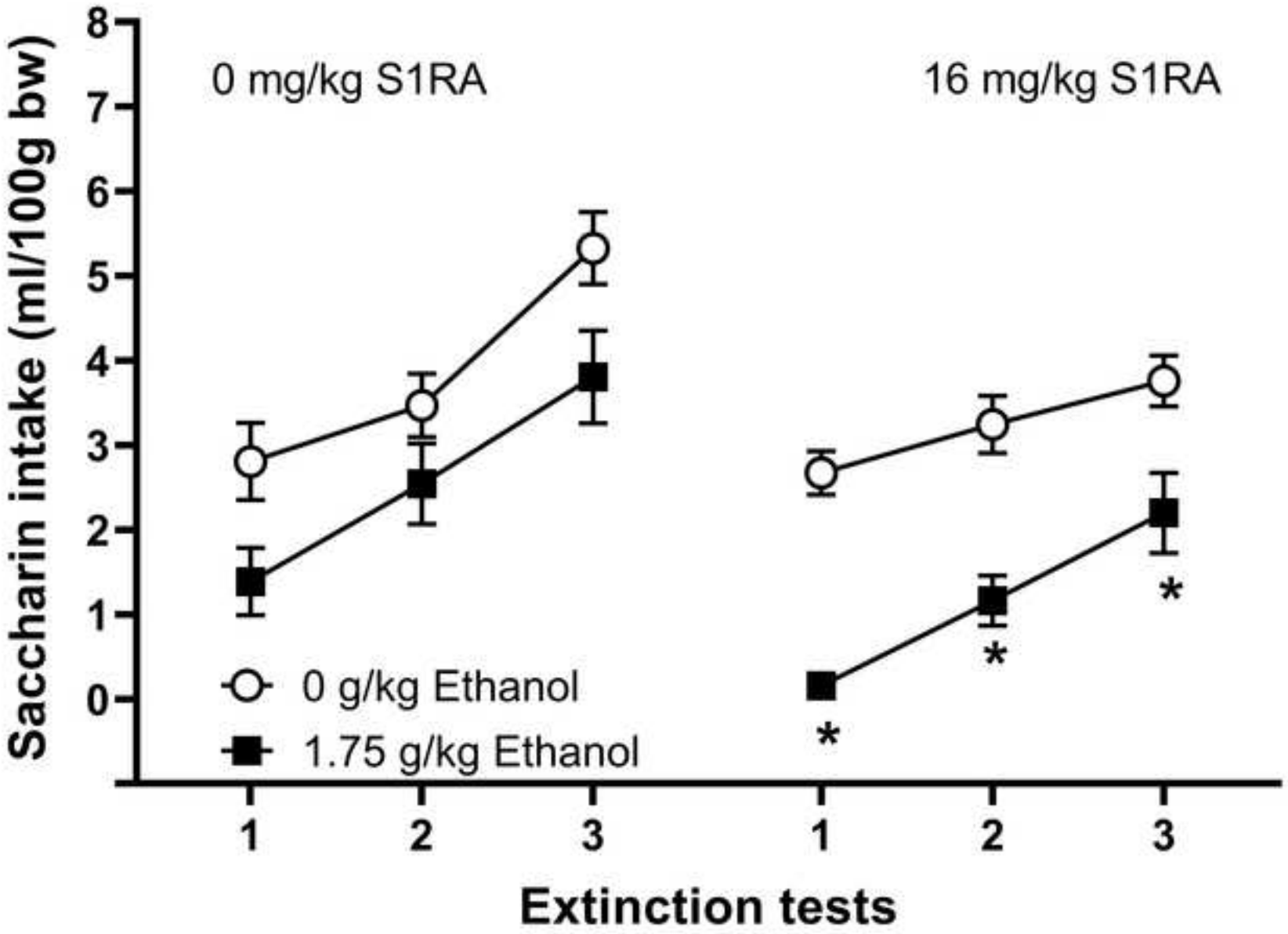

Table 1. Water and Overall fluid consumption [ml/100 g of body weight (bw)] during the 24-h two-bottle choice tests at adulthood (Exp. 1)

		Males		Females	
Intake sessions		Control	Binge	Control	Binge
1st (PD 87)	Water intake (ml/100 g of bw)	6.95 ±.95	4.75 ±.87	7.54 ±.99	5.64 ±1.06
	Overall liquid intake (ml/100 g of bw)	12.38 ±.87	11.83 ±.41	21.25 ±1.85	20.30 ±1.80
2nd (PD 89)	Water intake (ml/100 g of bw)	8.55 ±.76	7.35 ±1.10	10.08 ±1.20	6.96 ±.92
	Overall liquid intake (ml/100 g of bw)	10.43 ±.58	11.39 ±.73	15.39 ±1.06	13.97 ±.55
3rd (PD 91)	Water intake (ml/100 g body weight)	8.86 ±.61	8.49 ±.80	7.99 ±.94	8.06 ±.94
	Overall liquid intake (ml/100 g of bw)	10.02 ±.47	11.60 ±.70	13.48 ±.78	13.36 ±.58
4th (PD 94)	Water intake (ml/100 g of bw)	9.03 ±.71	7.10 ±.88	7.37 ±.76	5.73 ±.74
	Overall liquid intake (ml/100 g of bw)	10.65 ±.53	11.52 ±.70	14.09 ±.78	14.06 ±.70
5th (PD 96)	Water intake (ml/100 g of bw)	8.21 ±.79	7.99 ±.80	9.96 ±1.19	9.49 ±1.02
	Overall liquid intake (ml/100 g of bw)	9.77 ±.71	11.33 ±.57	15.62 ±1.10	16.86 ±.92

Note: Water and Overall fluid consumption (ml/100 g of bw) during 24-h, intermittent two-bottle ethanol intake tests which took place between PDs 87 and 97 in Experiment 1, as a function of sex and adolescent ethanol exposure [Control unexposed or Binge exposure (nine 2 h limited-access ethanol intake sessions between PDs 31-50)]. Values express mean ± SEM.

Table 2. Conditioned Stimulus (CS, saccharin) intake [ml/100 mg of body weight (bw)] at the taste aversion (TA) conditioning session in Experiments 3a, 3b and 4, as a function of S1RA dose and unconditioned Stimulus (US, ethanol) exposure.

Experiment 3a

	Control			Ethanol (2.5 g/kg)		
S1RA dose	0 mg	4 mg	16 mg	0 mg	4 mg	16 mg
Saccharin (0.1%) intake (ml/100 g of bw) (PD 30)	6.71 ±1.07	5.37 ±.76	5.36 ±1.02	5.74 ±.99	6.55 ±1.37	6.41 ±.94

Experiment 3b

	Control		Ethanol (2.5 g/kg)	
S1RA dose	0 mg	16 mg	0 mg	16 mg
Saccharin (0.1%) intake (ml/100 g of bw) (PD 30)	4.12 ±1.29	3.34 ±1.33	4.16 ±1.69	4.29 ±1.55

Experiment 4

US	Control		Ethanol (1.75 g/kg)	
S1RA dose	0 mg	16 mg	0 mg	16 mg
Saccharin (0.1%) intake (ml/100 g of bw) (PD 68)	3.22± 1.00	2.09 ±.29	3.15 ±.28	2.45 ±.39

Note: these experiments evaluated ethanol-induced TA US in adolescence or adulthood, with or without CS-US overlap. The conditioning session lasted 30 min. **Exp. 3a**: Saccharin intake (ml/100g of bw) during conditioning (PD 30) as a function of S1RA dose (0, 4 or 16 mg/kg) and US treatment (Ethanol 2.5 g/kg or vehicle). S1RA and Ethanol treatment were administered immediately before and after the conditioning session, respectively. **Exp. 3b and 4**: Saccharin intake (ml/100g of bw) during conditioning (PD 30 or PD 68 for Exp. 3b and 4 respectively) as a function of S1RA dose (0 or 16 mg/kg) and Ethanol treatment [vehicle or ethanol (2.5 for Exp.3a and 1.75 for Exp.4)]. S1RA dose was administered just after the intake session and Ethanol 30 min later. Values express mean ± SEM.

Table 3. Ethanol-induced conditioned place aversion in adolescent rats as a function of S1RA administration (Experiment 5). Absolute time spent (s) and % preference for the excitatory Conditioned Stimulus [CS+, Sandpaper texture (SAND)] at habituation and extinction tests.

		Control		Ethanol	
	S1RA dose	0 mg	16 mg	0 mg	16 mg
Habituation (PD 31)	Absolute time spent (s) on SAND	235.59 ±.41.92	184.63 ±17.26	180.92 ±39.49	180.76 ±27.54.
	SAND % preference	57.08 ±8.02	11.83 ±.41	42.64 ±8.39	40.62 ±7.31
1st Test (PD 36)	Absolute time spent (s) on SAND	198.28 ±42.00	190.63 ±25.84	55.15 ±15.62	100.63 ±24.08
	SAND % preference	49.80 ±8.32	11.39 ±.73	15.58 ±5.66	31.00 ±8.75
2nd Test PD (40)	Absolute time spent (s) on SAND	178.96 ±21.72	219.24 ±41.94	93.87 ±23.72	133.83 ±44.57
	SAND % preference	49.98. ±6.69	11.60 ±.70	30.41 ±7.36	36.78 ±9.08
3rd Test PD (41)	Absolute time spent (s) on SAND	176.83 ±41.82	177.73 ±28.25	94.10 ±20.53	119.04 ±16.20
	SAND % preference	43.83 ±7.42	11.52 ±.70	30.25 ±7.46	36.34 ±5.69

Note: Total time (s) spent and % preference for the CS+ SAND during the 10-minute tests (Habituation and Extinction Test 1, 2 and 3) as a function of S1RA dose (0 or 16 mg/kg) and ethanol treatment during conditioning (0 or 2.5 g/kg). During each conditioning session (PDs 32–35), the rats were administered saline (i.p) and then exposed to a smooth surface (CS-, EVA) for 12 minutes, then were administered S1RA (0 or 16 mg/kg; s.c.) and returned to the home-cage for 30 min. Then, they received an i.p. injection of vehicle or 2.5 g/kg Ethanol and immediately after were exposed to the SAND texture for 10 minutes. Extinction tests were conducted in PD36, 40 and 41. Values express mean±SEM.