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(*R*)-Salsolinol, a product of ethanol metabolism, stereospecifically induces behavioral sensitization and leads to excessive alcohol intake

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ABSTRACT

Ethanol is oxidized in the brain to acetaldehyde, which can condense with dopamine to generate (*R*/*S*)-salsolinol [(*RS*)-SAL]. Racemic salsolinol [(*RS*)-SAL] is self-infused by rats into the posterior ventral tegmental area (VTA) at significantly lower concentrations than those of acetaldehyde, suggesting that (*RS*)-SAL is a most active product of ethanol metabolism. Early studies showed that repeated intraperitoneal or intra-VTA administration of (*RS*)-SAL (10 mg/kg) induced conditioned place preference, led to locomotor sensitization and increased voluntary ethanol consumption. In the present study, we separated the (*R*)- and (*S*)-enantiomers from a commercial (*RS*)-SAL using a high-performance liquid chromatography with electrochemical detection system fitted with a β-cyclodextrin-modified column. We injected (*R*)-SAL or (*S*)-SAL (30 pmol/1.0 μl) into the VTA of naïve UChB rats bred as alcohol drinkers to study whether one or both SAL enantiomers are responsible for the motivated behavioral effects, sensitization and increase in voluntary ethanol intake. The present results show that repeated administration of (*R*)-SAL leads to (1) conditioned place preference; (2) locomotor sensitization; and (3) marked increases in binge-like ethanol intake. Conversely, (*S*)-SAL did not influence any of these parameters. Overall, data indicate that (*R*)-SAL stereospecifically induces motivational effects, behavioral sensitization and increases ethanol intake.

Keywords Ethanol, motivation, (*R*)-salsolinol, (*RS*)-salsolinol, (*S*)-salsolinol, sensitization.

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INTRODUCTION

Acetaldehyde generated from ethanol can react with dopamine to form (*R*)-salsolinol [(*R*)-SAL] and (*S*)-salsolinol [(*S*)-SAL] (King, Goodwin & Sandler 1974; Bates, Bagheri & Vertino 1986), two planar molecules where the methyl group derived from the acetaldehyde moiety can be above or below the planar structure (Supporting Information Fig. S1). A number of studies have shown that chronic administration of ethanol to animals leads to increases in brain racemic (*RS*)-SAL (Sjöquist, Liljequist & Engel 1982; Myers *et al.* 1985a,b; Matsubara, Fukushima & Fukui 1987; Starkey *et al.* 2006). Increases in (*RS*)-SAL were found in the brain of alcoholics intoxicated at the time of death (Sjöquist, Perdahl & Winblad 1983). In *ex vivo* studies, ethanol led to the synthesis of SAL in ventral tegmental area (VTA) slices only if the synthesis of dopamine was intact; indeed, SAL synthesis was blocked by the dopamine synthesis inhibitor α-methyl-*p*-tyrosine (Melis *et al.* 2015).

There is still debate as to whether (*R*)-SAL and (*S*)-SAL are formed non-enzymatically (King *et al.* 1974; Bates *et al.* 1986) or enzymatically (Naoi *et al.* 1996). Regretfully, such is not yet settled and the steady levels of these enantiomers reflect not only the rates of synthesis but also their degradation rates. Rojkovicova *et al.* (2008), using highly specific chromatographic methods, demonstrated an increase of both (*R*)- and (*S*)-SAL in the brain of rats following chronic ethanol drinking, in line with non-enzymatic synthesis.

Earlier animal studies showed that (*RS*)-SAL infused chronically into the brain ventricles promoted ethanol

consumption in rats (Myers & Melchior 1977; Duncan & Deitrich 1980). Further studies indicated that (*RS*)-SAL has reinforcing properties per se; alcohol-preferring (P) rats were shown to self-administer (*RS*)-SAL into the posterior ventral tegmental area (pVTA) (Rodd *et al.* 2008). The concentrations that elicited (*RS*)-SAL self-administration (30–100 nM) were one to three orders of magnitude lower than those required for acetaldehyde (23 μM) self-administration (Rodd-Henricks *et al.* 2002) and were significantly lower than those required for ethanol self-administration into the same brain area (17 mM) (Rodd *et al.* 2005).

Recent studies in alcohol-preferring Wistar-derived UChB rats (ethanol naïve) showed that repeated microinjections of (*RS*)-SAL into the pVTA led to (1) a strong increase in voluntary 'binge-like' ethanol intake and (2) the development of place preference for the physical setting where (*RS*)-SAL was administered, thus indicating marked motivational effects of SAL. In addition, (3) SAL significantly increased locomotor activity (Quintanilla *et al.* 2014). The possibility exists that only one, the (*R*)- or (S)-SAL enantiomer, might induce motivational effects, locomotor sensitization and promotion of ethanol consumption.

To address the above possibility, in the present study, (*R*)-SAL and (*S*)-SAL were purified from a commercially available (*RS*)-SAL. Complete separation of (*R*)-SAL and (*S*)-SAL was achieved by high-performance liquid chromatography (HPLC) on a β-cyclodextrin-modified column, which has the ability to separate these enantiomers (Baum & Rommelspacher 1994; Quan *et al.* 2005; Huang, Quan & Liu 2009). Studies were conducted to determine the differential effects of intra-VTA administration of (*R*)- or (*S*)-SAL on the generation of (1) conditioned place preference (CPP); (2) locomotor sensitization; and (3) voluntary ethanol consumption.

MATERIALS AND METHODS

Animals

Female Wistar-derived rats selectively bred as alcohol consumers at the University of Chile for over 90 generations (University of Chile Bibulous; UChB) (Mardones & Segovia-Riquelme 1983; Quintanilla *et al.* 2006), weighing 200–250 g at the time of experiments, were used in the study. Rats were individually housed and maintained on a normal 12-hour light/dark cycle (lights off at 7:00 PM). Food and water were freely available. Animal experimental procedures were approved by the Ethics Committee for Studies with Laboratory Animals at the Faculty of Medicine (Protocol CBA#0507, FMUCH) and by the Chilean Council for Science and Technology Research (CONICYT).

Drugs and solutions

Racemic (*RS*)-SAL, free of isosalsolinol (Quintanilla *et al.* 2014), was purchased from Santa Cruz Biotechnology (sc-215838; reporting 98 percent purity) (Dallas, TX, USA). Ammonium acetate was from Merck (Darmstadt, Germany) and triethylamine was from Sigma (St. Louis, MO, USA). For intracerebral administration, SAL was diluted with artificial cerebrospinal fluid (aCSF, containing 120.0 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 10 mM D-glucose and 0.2 mM ascorbate, pH 6.5) for slow injection (1.0 μl) into the VTA (30 pmol/μl), according to Hipólito *et al.* (2010, 2011).

Drug microinjection procedures

Rats were anesthetized (1.8 percent isoflurane in 0.2 l/minute air flow), placed in a stereotaxic apparatus and implanted with a 22-gauge guide cannula (Plastics One, VA, USA) aimed 1.0 mm above the *left* posterior VTA (anteroposterior, −5.6 mm; lateral, −0.5 mm, dorsoventral, −7.4 mm; from bregma, according to Paxinos & Watson 1998). Cannula assemblies were secured in place with dental cement, anchored to the skull with screws. Following surgery rats were individually housed with free access to food and water for at least 7 recovery days. Drug microinjections were carried out with a 28-gauge stainless steel injector (Plastics One), extending 1.0 mm below the tip of the guide cannula and connected with a polyethylene tube to a 2.0-μl Hamilton syringe. A syringe pump (model 55-5920, Harvard Apparatus, South Natick, MA, USA) delivered 1.0 μl of vehicle (aCSF) or of (*R*)-SAL or (*S*)-SAL) in 100 seconds (0.6 μl/minute) and injectors remained in place for 30 seconds after the injection procedure was completed in order to prevent backflow through the cannula. A note on laterality: Our previous studies (Karahanian *et al.* 2011, 2015; Quintanilla *et al.* 2012; Tampier *et al.* 2013) suggest that at a higher brain level, differences in (right – left) input appear integrated as one. Thus, clear physiological effects are observed when only one side is injected. In line with this view, in a previous study (Quintanilla *et al.* 2014), we injected unilaterally the same dose of (*RS*)-SAL (30 pmol) that (Hipólito *et al.* 2010, 2011) had injected bilaterally, obtaining the same results on locomotor activity and CPP.

Experiment 1. Preparation of purified (*R*)-SAL and (*S*)-SAL from (*RS*)-AL

(*R*)- and (*S*)-SAL were separated from the racemic solution as described previously, with some modifications (Liu *et al.* 2000; Juricic *et al.* 2012). Briefly, a solution of commercial (*RS*)-SAL–HCl (2×10^{-2} M), free of isoSal (Santa Cruz Biotechnology), was prepared in distilled and deionized water. Then, 50 μl of this solution was injected

onto an HPLC system fitted with: (1) a NUCLEODEX β-cyclodextrin-modified column 200 × 8 mm i.d. packed with silica gel modified with β-cyclodextrin (Macherey-Nagel, Düren, Germany) kept at 30°C; (2) an isocratic pump adjusted to 0.80 ml/minute (Shimadzu LC-10AD, Kyoto, Japan); and (3) an LC-4C BAS amperometric detector (ED) set at a potential of 0.7 V. The mobile phase was 100 mM ammonium acetate containing 10 mM triethylamine (pH 4.0). Under these conditions and using a similar chiral HPLC system, it was reported that (*S*)-SAL was the first to elute (Baum & Rommelspacher 1994; Deng *et al.* 1995; Naoi *et al.* 1996; Liu *et al.* 2000; Tóth *et al.* 2001; Quan *et al.* 2005; Cai & Liu 2008; Rojkovicova *et al.* 2008; Huang *et al.* 2009; Lee *et al.* 2010).

Once an (*RS*)-SAL sample was injected onto the HPLC, the enantiomers were separated and collected after disconnecting the electrochemical detector in order to avoid sampling oxidized elution fractions. Thus, (*S*)-SAL and (*R*)-SAL were sampled and selected according to their corresponding elution time, 1 minute elapsing between samples. The procedure was repeated to obtain sufficient amounts of the corresponding enantiomers. In order to eliminate the mobile phase from each collected sample, the fractions were lyophilized at −54°C for 6 hours, dissolved in aCSF achieving a concentration of 30 μM (for intracerebral administration) and injected onto the HPLC system to check their purity and for their quantification using (*RS*)-SAL as standard.

Experiment 2. Effect of repeated intra-VTA injections of (*R*)-SAL or (*S*)-SAL on CPP

One week after surgery, ethanol-naïve UChB rats were tested ($n = 4$ rats/group) to determine whether CPP was induced by the intra-VTA microinjection of (*R*)-SAL, (*S*)-SAL or sCSF as control. Animals were handled every day for 4 days to familiarize them with the experimenter, the experimental room and the manipulation procedure. The place-conditioning test was performed as described previously (Quintanilla & Tampier 2011) with minor modifications, as described below.

Pre-conditioning phase

CPP was tested in a biased two-chambered apparatus. During the pre-conditioning phase, the 60 percent of animals preferred spontaneously the white compartment with perforated stainless steel floor (882 ± 39 seconds), while forty percent spent more time in the black compartment with smooth floor (848.7 ± 54 seconds).

Rats had access to the entire chamber (two compartments, one with black walls and smooth floor and the other with white walls and perforated stainless steel floor, separated by a removable barrier) during three pre-conditioning sessions for 20 minutes (days 1, 2 and 3) and behavior was recorded using a digital camera. This pre-conditioning session was conducted to determine the initially less preferred compartment of the apparatus for each animal.

Conditioning phase

Three groups of rats ($n = 4$ rats/group) were microinjected intra-VTA with either: (1) (*R*)-SAL (30 pmol/1.0 μl aCSF) or (2) (*S*)-SAL (30 pmol/1.0 μl aCSF) on days 4, 6 and 8, and placed in the less preferred compartment for 30 minutes. On days 5, 7 and 9, all rats were injected with vehicle (aCSF) and placed in their initially preferred compartment. The control group ($n = 4$) received aCSF (intra-VTA) in both compartments (aCSF/aCSF), first in the less preferred side (on days 4, 6 and 8) and thereafter in the preferred side (on days 5, 7 and 9), to match the schedule of the drug groups.

Post-conditioning phase

The post-conditioning phase started after the last conditioning session. Each animal was tested for place preference by placing it in the open doorway of the barrier, allowing the animal to move between compartments for 20 minutes. A video camera including a recorder was used for data acquisition, quantifying the time spent in each compartment (seconds).

Experiment 3. Effect of repeated intra-VTA injections of (*R*)-SAL or (*S*)-SAL on (a) locomotor activity and (b) voluntary ethanol intake

Naïve rats ($n = 5$ for each group) were used to determine the effect of repeated intra-VTA injections of (*R*)-SAL, (*S*)-SAL or vehicle on the locomotor activity. Following the assessment of locomotor activity, the same animals were used to determine their voluntary ethanol consumption.

(a) *Locomotor activity*. It was evaluated as previously described (Quintanilla *et al.* 2014) in an open-field chamber (75 × 75 × 30 cm). Before testing locomotor activity, animals were handled for 5 minutes per day for 4 days. Prior to drug administration, the rats were habituated to the open field (20-minute session) for 1 day. After habituation, the animals were assigned to three groups ($n = 5$ rats/group) receiving three times the same dose of either (1) (*R*)-SAL (30 pmol in 1.0 μl of aCSF); (2) (*S*)-SAL (30 pmol in of 1.0 μl aCSF); or (3) vehicle (aCSF 1.0 μl) on days 1, 3 and 5. Locomotor activity (measured as centimeter of the traveled distance for 20 minutes) was evaluated immediately after each drug administration.

(b) *Voluntary ethanol intake*. On day 13, the animals of the four groups received a last dose of either (*R*)-SAL,

(*S*)-SAL or vehicle. On day 14, animals were given limited access to 10 percent and 30 percent ethanol solutions simultaneously for 1 hour every day (from 14:00 to 15:00 o'clock in the light cycle) for 7 consecutive days. Food and water were freely available (24 hours/day). Ethanol intake was expressed as grams of total ethanol consumed per kilogram of body weight per 60 minutes. Water intake was measured daily (every 24 hours).

Histology

Cannula position was verified according to the method described by Deehan *et al.* (2013). Rats were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) and bromophenol blue (1 percent; 0.5 µl) was injected into the VTA. Then, rats were killed by decapitation and brains rapidly removed, mounted on a CO_2 microtome and sectioned (40 µm each section). All sections were examined using a light microscope to verify the placement of the cannula.

Statistical methods

Statistical analysis was performed using GraphPad Prism (GraphPad, San Diego, CA, USA). One-way ANOVA was used for assessing the effects of (*R*)-SAL and (*S*)-SAL on place preference, locomotor activity and ethanol consumption followed by Tukey's *t*-test for *post hoc* comparisons. The level of significance was set at $P < 0.05$.

RESULTS

Supporting Information Fig. S2 depicts cannula placements in the posterior VTA of animals used in place-conditioning experiments (gray squares) and of those used in locomotor activity and ethanol consumption studies (black circles). Only one rat had the cannula placed outside the VTA (not shown). Data for this animal were not used in the statistical analyses.

Experiment 1. Preparation of purified (*R*)-SAL and (*S*)-SAL from (*RS*)-SAL

Figure 1a shows that the β-cyclodextrin-modified column was able to separate the two SAL enantiomers successfully after the injection of 50 µl of (*RS*)-SAL. The elution time of the enantiomers was approximately 6.2 minutes for (*S*)-SAL and 7.4 minutes for (*R*)-SAL (Fig. 1a), which is in line with the elution patterns described by other authors using a similar HPLC system (Baum & Rommelspacher 1994; Deng *et al.* 1995; Naoi *et al.* 1996; Liu *et al.* 2000; Tóth *et al.* 2001; Quan *et al.* 2005; Cai & Liu 2008; Rojkovicova *et al.* 2008; Lee *et al.* 2010). This elution pattern is also supported by compu-

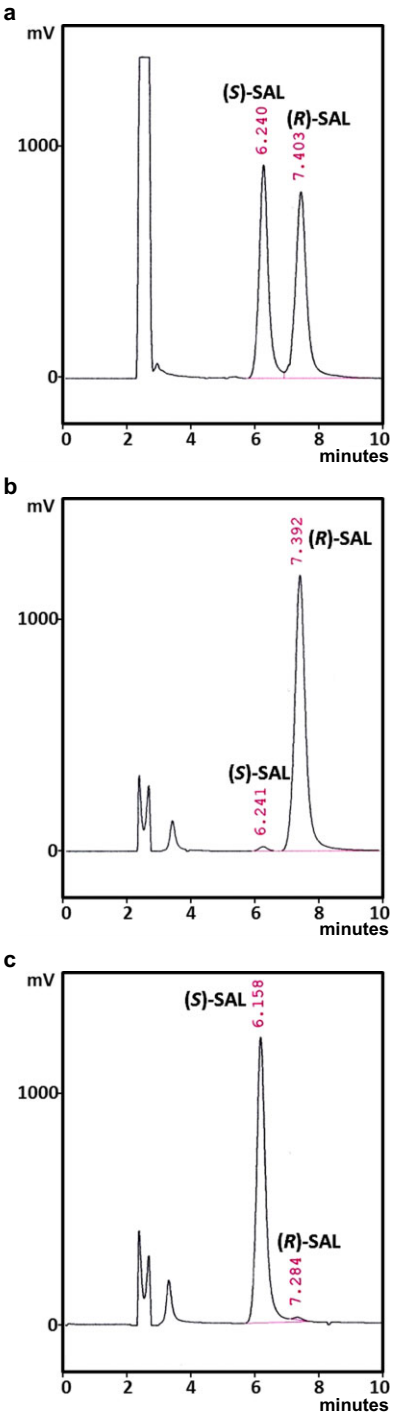

Figure 1 High-performance liquid chromatography (HPLC) determination of racemic (*RS*)-SAL and purified (*R*)-SAL or (*S*)-SAL. Representative HPLC chromatograms of: (a) commercial (*RS*)-SAL hydrochloride (Santa Cruz Biotechnology) (50 µM) in artificial cerebrospinal fluid (aCSF); (b) purified (*R*)-SAL (30 µM) dissolved in aCSF; (c) purified (*S*)-SAL (30 µM) dissolved in aCSF. The HPLC system was fitted with a stainless steel NUCLEODEX column 200 × 8 mm i.d. packed with silica modified with β-cyclodextrin (Macherey-Nagel, Düren, Germany), an isocratic pump adjusted to 0.80 ml/minute and an electrochemical detector. The mobile phase was 100 mM ammonium acetate containing 10 mM triethylamine (pH 4.0)

tational modeling, evaluating the total energy for stabilization, demonstrating that the complex formed by (*R*)-SAL and β-cyclodextrin is stronger than the complex of (*S*)-SAL and β-cyclodextrin. Therefore, (*S*)-SAL elutes before (*R*)-SAL (Huang *et al.* 2009). Figure 1b and c depicts the chromatograms obtained by injecting 50 μl of purified (*R*)-SAL and (*S*)-SAL, respectively, dissolved in aCSF, as used for their intracerebral administration. These chromatograms indicate that both solutions of the (*S*)- and (*R*)-SAL contained >97 percent of enantiomerically pure (*S*)-SAL or (*R*)-SAL, respectively.

Experiment 2. Effect of repeated intra-VTA injections of (R)-SAL or (S)-SAL on CPP

To compare the ability of (*R*)-SAL or (*S*)-SAL to induce CPP, naïve UChB rats ($n = 4$ for each group) were employed. The control group received only vehicle (aCSF) in both compartments. ANOVA of groups in Fig. 2 [control (aCSF), (*R*)-SAL and (*S*)-SAL groups] indicated significant effect of treatment ($F_{\text{treatment } (2,18)} = 5.4$; $P < 0.003$). *Post hoc* analysis revealed that conditioning with (*R*)-SAL increased significantly the time spent in the SAL-paired compartment during the post-conditioning phase relative to their respective pre-conditioning phase (Tukey's $P < 0.05$) and also relative to the post-conditioning phase of the controls rats ($P < 0.05$), which indicates that (*R*)-SAL elicits CPP. Conversely, *post hoc* analysis indicated that conditioning with (*S*)-SAL did not significantly change the time that the animals spent in the SAL paired side during the post-conditioning phase relative to the pre-conditioning phase, nor relative to the post-conditioning phase of the controls rats (Tukey's $P > 0.05$). Thus, data indicate that (*R*)-SAL but not the (*S*)-*SAL* was able to induce conditioned place preference.

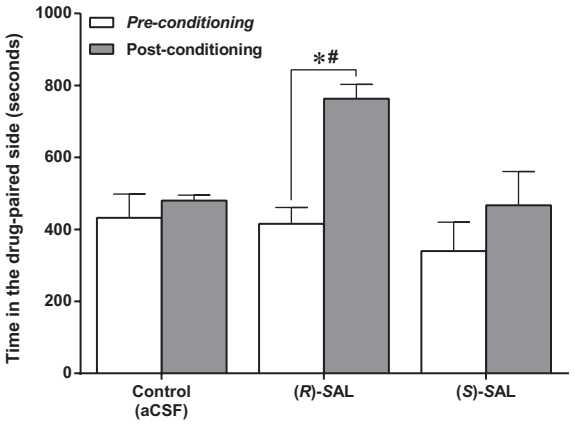

Figure 2 Conditioned place preference is induced by (*R*)-SAL, but not (*S*)-SAL, in ethanol-naïve rats ($n = 4$ animals/group). Rats were conditioned with (*R*)-SAL (30 pmol/1 μl), (*S*)-SAL (30 pmol/1 μl) or artificial cerebrospinal fluid (aCSF) (1 μl) microinjected intra-ventral tegmental area as described in the Materials and Methods section. The control group received vehicle (1 μl of aCSF) in both conditioning compartments. Conditioned place preference was assessed 1 day after the last conditioning session. Data are means ± SEM and represent the time spent in the drug-paired compartment (seconds; mean ± SEM, $n = 4$ for each group) during the *pre-* (white columns) and *post-conditioning* (gray columns) phases. Asterisk symbol ($^{*}P < 0.05$) indicates significant difference between the amount of time that the rats spent in the drug-paired side during the post-conditioning phase versus the value obtained during its pre-conditioning phase. A pound ($^{\#}P < 0.05$) indicates significant difference between the amount of time that (*R*)-SAL-treated rats spent in the drug-paired side during its post-conditioning phase and the post-conditioning phase of the control group

Experiment 3. Effect of repeated intra-VTA injections of (*R*)-SAL or (*S*)-SAL on (a) locomotor activity and (b) voluntary ethanol intake

(a) *Locomotor activity*. Locomotor activity (measured as centimeter of the traveled distance for 20 minutes) was evaluated immediately after each drug administration.

ANOVA of groups shown in Fig. 3 [(aCSF control), (*R*)-SAL and (*S*)-SAL groups] indicated significant effect of treatment ($F_{\text{treatment } (2,36)} = 15.0$; $P < 0.0001$). *Post hoc* analysis indicated that microinjection of (*R*)-SAL intra-VTA elicited higher locomotor activities 3 days of administration (days 1, 3 and 5) compared with the activity displayed by the control group the respective day (Tukey's $P < 0.05$). Conversely, locomotor activity of the rats microinjected with (*S*)-SAL was not significantly different than those of control group none of the 3 days (Tukey's $P > 0.05$). It is important to highlight that in relation to the first day of administration, the stimulation of locomotor activity induced by (*R*)-SAL increased significantly along the successive administration sessions (from day 1 to day 3) ($F_{\text{days } (2,13)} = 41.0$; $P < 0.0001$), but not in the (*S*)-SAL-treated group ($F_{\text{days } (2,13)} = 0.13$ $P = 0.87$). *Post hoc* analysis revealed that the locomotor activity induced by the third microinjection of (*R*)-SAL was significantly higher (Tukey's $P < 0.05$) than that induced by the first and the second (*R*)-SAL microinjections (Fig. 3), while the second microinjection elicited higher locomotor activity than that induced by the first (*R*)-SAL dose (Tukey's $P < 0.05$). Taken together, these results indicate that (*R*)-SAL but not the (*S*)-SAL was able to elicit sensitized locomotor activity.

(b) *Ethanol intake*. Figure 4 shows the ethanol intake (g/kg/60 minutes) of (*R*)-SAL, (*S*)-SAL and aCSF pre-exposed rats on days 14–20. ANOVA of all data in Fig. 4 [control (aCSF), (*R*)-SAL and (*S*)-SAL] indicated significant differences in the ethanol intake among the groups ($F_{\text{treatment } (2,84)} = 25.00$; $P < 0.0001$). *Post hoc* comparison indicated that the ethanol consumptions of the groups of animals pre-exposed to (*R*)-SAL were significantly higher than

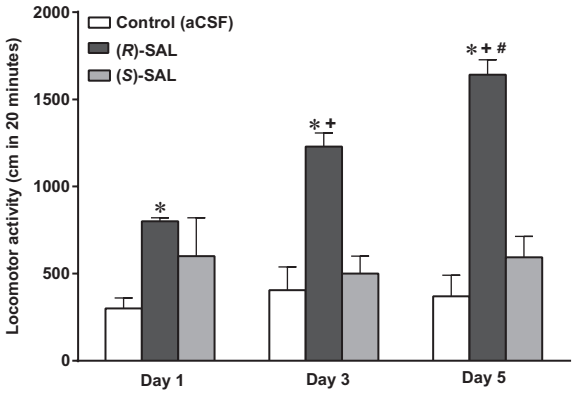

Figure 3 Locomotor sensitization is induced by repeated intraventral tegmental area administration of (*R*)-SAL but not (*S*)-SAL. Locomotor activity following repeated (*R*)-SAL (30.0 pmol/1 μl; black columns), (*S*)-SAL (30.0 pmol/1 μl; gray columns) or vehicle [1 μl of artificial cerebrospinal fluid (aCSF) control; white columns] ($n = 5$/group). Data are means ± SEM and represent distance traveled (cm) in 20 minutes. Asterisk symbol ($^{*}P < 0.05$) indicates significant differences of the groups microinjected with (*R*)-SAL versus the aCSF control group of the same day. A plus symbol ($^{+}P < 0.05$) indicates that the distance travelled is significantly higher compared to the value of the first day of treatment with (*R*)-SAL. A pound symbol ($^{\#}P < 0.05$) indicates that the distance travelled is significantly higher compared with the value of the second day of treatment with (*R*)-SAL

those of the control group (Tukey's $P < 0.05$). Conversely, the ethanol consumptions of rats pre-exposed to (*S*)-SAL were not significantly different than those of the control group (Tukey's $P > 0.05$). Furthermore, rats of the (*R*)-SAL groups drank significantly more ethanol than rats of the (*S*)-SAL group (Tukey's $P < 0.05$).

When all data of ethanol intake from each group were pooled over 7 days, the group pre-exposed to 30 pmol of (*R*)-SAL consumed an average of 2.480 ± 0.19 g of ethanol/kg/60 minutes (mean ± SEM), whereas the groups pre-exposed to 30 pmol of (*S*)-SAL or to aCSF consumed an average of 1.060 ± 0.140 and 1.093 ± 0.11 g of ethanol/kg/60 minutes, respectively. The one-way ANOVA presented significant differences in the ethanol intake among the groups ($F_{\text{treatment}\ (2,12)} = 29.0$; $P < 0.0001$). The *post hoc* comparisons showed that ethanol consumption of rats pre-exposed to 30 pmol of (*R*)-SAL was 2.3-fold that of the (*S*)-SAL and also that of the aCSF group (Tukey's $P < 0.05$).

DISCUSSION

This study shows for the first time that (*R*)-SAL enantioselectively induces motivational effects, sensitization and increases in ethanol consumption. (*R*)-SAL but not (*S*)-SAL repeatedly administered into the posterior VTA resulted in (1) conditioned place preference; (2) increased locomotor activity; and (3) increased voluntary ethanol consumption. Thus, the present study shows that the (*R*)-enantiomer rather than (*S*)-enantiomer may play a role in promoting voluntary ethanol consumption, even though both SAL enantiomers are increased in the brain of rats following chronic ethanol exposure (Rojkovicova *et al.* 2008). The finding by Rojkovicova *et al.* (2008) that both enantiomers are increased following ethanol consumption is in line with a spontaneous non-enzymatic synthesis of salsolinol (King *et al.* 1974; Deng *et al.* 1995). It is noted that in the present study (*R*)-SAL qualitatively replicates the effects that have been reported were produced by (*RS*)-SAL administered intra-VTA on conditioned place preference (Hipólito *et al.* 2011; Quintanilla *et al.* 2014), locomotor sensitization (Hipólito *et al.* 2010; Quintanilla *et al.* 2014) and voluntary ethanol intake (Quintanilla *et al.* 2014).

The possible mechanism underlying the reinforcing action of SAL involves stimulation of the dopamine reward pathway. Hipólito *et al.* (2011) reported that injection of (*RS*)-SAL into the VTA increased dopamine levels in the nucleus accumbens (shell). Further, Deehan *et al.* (2013) reported that microinjections of (*RS*)-SAL into the pVTA increased dopamine release by 300 percent in the nucleus accumbens (shell) at 0.3 μM, a concentration lower than that required of acetaldehyde to obtain the same effect (23 μM). Experimental evidence suggests that (*RS*)-SAL activates VTA dopaminergic neurons via opioid receptors located on GABA inhibitory interneurons. Xie *et al.* (2012), using the patch clamp technique, showed that the activating effect of SAL on VTA dopaminergic neurons is mediated through the activation of opioid receptors, which, in turn, inhibit GABAergic interneurons, resulting finally in a disinhibition of dopamine neurons. It remains to be determined if these actions are mimicked by (*R*)-SAL in the absence of (*S*)-SAL. The in *vivo* findings reported, showing that the behavioral effects were induced by (*R*)-SAL, but not (*S*)-SAL, should be of value in differentially dissecting or confirming the neurophysiological mechanisms of action of this metabolite of ethanol, as well as the possible association between different behavioral effects of drug treatment.

Matsuzawa, Suzuki & Misawa (2000) observed that naloxone or β-funaltrexamine (an irreversible antagonist of μ-opioid receptors) blocked conditioned place preference induced by (*RS*)-SAL administered via intraperitoneal. Further, Hipólito *et al.* (2010) showed that β-funaltrexamine or naltrexone blocked the stimulation of locomotor activity induced by (*RS*)-SAL microinjected into the VTA. Recently, Quintanilla *et al.* (2014) showed that naltrexone microinjected directly into the VTA reduced the greater voluntary ethanol consumption

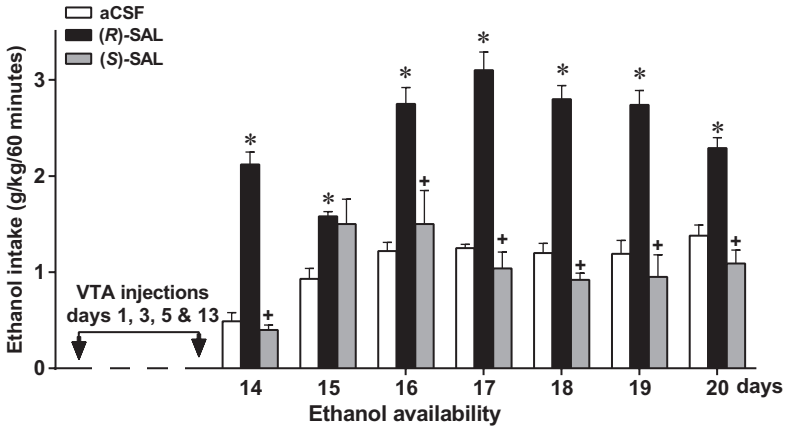

Figure 4 Prior repeated administration of (*R*)-SAL, but not of (*S*)-SAL, increases voluntary ethanol intake in ethanol-naïve rats. Rats were pretreated intra-ventral tegmental area with (*R*)-SAL (black columns), (*S*)-SAL (gray columns) or control artificial cerebrospinal fluid (aCSF) (white columns) on days 1, 3, 5 and 13 (*arrows*); then (from day 14) animals were exposed to ethanol for 1 hour/day. Asterisk symbol (*$P < 0.05$) indicates that the ethanol intake is significantly higher than that of the control (aCSF) group of the same day. Single plus ($^{+}P < 0.001$) indicates that the ethanol intake of (*S*)-SAL-treated animals is significantly lower that of the (*R*)-SAL group on the same day

induced by (*RS*)-SAL injected either intraperitoneally or intra-VTA.

The present study in Wistar-derived rats bred as high ethanol drinkers (UChB) is in line with the pioneering studies by Myers & Melchior (1977) and Duncan & Deitrich (1980) in Sprague-Dawley and Long-Evans rats, which showed that chronic infusion of (*RS*)-SAL into the rat cerebral ventricles led to marked increases in voluntary ethanol intake. These enhancements of ethanol consumption persisted for up to 10 months after the chronic SAL infusion, suggesting a protracted increase in the reinforcing effects of ethanol. It is important to highlight that in the present study the repeated administration of (*R*)-SAL to ethanol-naïve animals triggered the intake of highly intoxicating ethanol volumes, reaching 3 g ethanol/kg/60 minutes. Since the increased ethanol intake of rats pretreated with (*R*)-SAL was marked at the first ethanol access session, conditioning pairing with ethanol smell, taste or external cues unlikely play a role leading to the high ethanol intakes observed, pointing out to a biological rather than a learned effect. This observation should be of value in dissecting some of the elements that lead to uncontrolled drinking.

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Authors Contribution

MEQ, MRM and PBC were responsible for the study design and data collection. MRM and MHM contributed to stereotaxic experiments. PBC contributed to the separation and purification of (*R*)- and (*S*)-salsolinol enantiomers. MEQ and YI prepared the manuscript. All authors critically reviewed content and approved final version for publication. All authors contributed to editing the draft and critically reviewed and approved the final version submitted for publication.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Generation of (R)- and (S)-salsolinol by the non-enzymatic condensation of dopamine and acetaldehyde (from King *et al.* 1974)

Figure S2 Placement of the guide cannula