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Research report

Erysodine, a competitive antagonist at neuronal nicotinic acetylcholine receptors, decreases ethanol consumption in alcohol-preferring UChB rats

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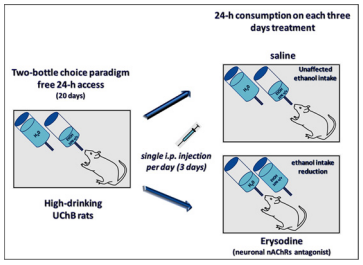
GRAPHICAL ABSTRACT

The erysodine administration (during three days of treatment) reduces ethanol consumption in an animal model of alcohol dependence (UChB rats) using two-bottle choice paradigm.



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ABSTRACT

Alcohol abuse is a worldwide health problem with high economic costs to health systems. Emerging evidence suggests that modulation of brain nicotinic acetylcholine receptors (nAChRs) may be a therapeutic target for alcohol dependence. In this work, we assess the effectiveness of four doses of erysodine (1.5, 2.0, 4.0 or 8.0 mg/kg/day, i.p.), a competitive antagonist of nAChRs, on voluntary ethanol consumption behavior in alcohol-preferring UChB rats, administered during three consecutive days. Results show that erysodine administration produces a dose-dependent reduction in ethanol consumption respect to saline injection (control group). The highest doses of erysodine (4 and 8 mg/kg) reduce (45 and 66%, respectively) the ethanol intake during treatment period and first day of post-treatment compared to control group. While, the lowest doses of erysodine (1.5 and 2 mg/kg) only reduce ethanol intake during one day of treatment period. These effective reductions in ethanol intake were 23 and 29% for 1.5 and 2 mg/kg erysodine, respectively. Locomotor activity induced by a high dose of erysodine (10 mg/kg) was similar to those observed with saline injection in control rats, showing that the reduction in ethanol intake was not produced by hypolocomotor effect induced by erysodine. This is the first report showing that erysodine reduces ethanol intake in UChB rats in a dose-dependent manner. Our results

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highlight the role of nAChRs in the reward effects of ethanol and its modulation as a potentially effective pharmacological alternative for alcohol dependence treatment.

1. Introduction

The reward system or mesocorticolimbic system is formed by dopamine (DA) neurons from ventral tegmental area (VTA) that projects their axons to nucleus accumbens (NAc) and prefrontal cortex (PFC) [1,2]. These neurons are regulated by several neurotransmitter systems that reduce the firing rate of VTA DA neurons (e.g. GABA) and other neurotransmitters (e.g. serotonin, glutamate and acetylcholine, between others) that stimulate them (see Fig. 4, Panel A) [3,4]. At neurochemical level, natural reinforcement (such as high-fat foods [5], sweetened solutions [6] and sex [7]) and drugs of abuse (such as amphetamine, cocaine, nicotine and alcohol [8]) increase the NAc DA release, activating reward system. However, when the NAc DA release induced by a natural reinforcement such as sucrose or saccharin is of lesser magnitude than that produced by ethanol [6,9].

Regard to alcohol and nicotine addictions, they are often treated as separate disorders, although ~60–80% of heavy drinkers smoke tobacco and common genes may be involved in the susceptibility to both dependence [10,11]. Although the rewarding properties of alcohol have long been studied, showing an increase in NAc DA release [8,12], the exact mechanism of action of alcohol has not yet been fully elucidated. Considerable evidence suggests that ethanol acts directly as a positive modulator of glycine and $GABA_A$ receptors and negative modulator of *N*-methyl-D-aspartate (NMDA) receptor [13–15]. In addition, it has also been shown that ethanol indirectly activates nicotinic acetylcholine receptors (nAChRs) through increasing extracellular levels of acetylcholine (ACh) in VTA [16–19] that promote NAc DA release [20,21]. In summary, nAChRs in reward system are activate by ethanol-induced acetylcholine and nicotine [8,11,19,22], and they represent a potential pharmacological target for the addiction to tobacco and alcohol.

Pharmacological studies seeking to establish a role for nAChRs in the modulation of the rewarding effects of ethanol have utilized mecamylamine (MEC), a noncompetitive antagonist of central and peripheral nAChRs [23–26]. In this sense, MEC decreases ethanol consumption in rats [17,25,26] and attenuates ethanol-induced DA release in the NAc [25–27]. The levels of ACh in the VTA and DA in the NAc are increased in animals consuming ethanol [16], suggesting that nAChRs may be involved in mediating the rewarding properties of ethanol. On the other hand, changes in NAc ACh levels have been suggested to be involved in modulating alcohol withdrawal [28].

Erysodine is a competitive antagonist of α4β2 nAChR and chemically it is an alkaloid obtained from *Erythrina falcata* related to dihydro-β-erythroidine (DHβE) [29]. Erysodine displaces [^{3}H]-cytisine, a partial agonist with high affinity binding for α4β2 nAChR, in a concentration-dependent manner ($Ki = 5 \pm 1$ nM) and inhibits ACh-stimulated current ($IC_{50} = 20$ nM) [30]. Animal studies showed that erysodine (s.c.) reduce nicotine self-administration in a dose-dependent manner in rats [31]. Considering that nAChRs would be modulating ethanol intake [32,33], we hypothesize that erysodine, a potent and competitive antagonist of nAChRs, could reduce the rewarding effects of ethanol intake in alcohol preferring rats UChB rats. Therefore, we evaluated the effect of erysodine on voluntary ethanol intake and ethanol preference using the two-bottle free choice ethanol access paradigm. In addition, we measured erysodine-induced locomotor activity to discriminate that the effects on ethanol intake should not be due to motor impairment.

2. Materials and methods

2.1. Animals

The ethanol intake studies were performed in male Wistar-UChB rats (n = 25) selectively bred for their high alcohol consumption [34,35]. Rats of the UChB line satisfy the essential criteria proposed for an animal model of alcoholism [36] and have been used previously as a tool for screening alcoholism medications [32,37–39]. All experiments to measure ethanol intake were conducted at the Faculty of Medicine, Universidad de Chile. The locomotor activity experiments were performed in male Sprague-Dawley rats (n = 15) at the Faculty of Science, Universidad de Valparaíso. In their respective vivariums, animals were housed in a temperature-controlled room (21 ± 2 °C) under a 12-h light cycle with lights on at 08:00 h with food and water *ad libitum*. All experimental procedures were approved by Ethics Committee of the Universities of Chile and Valparaíso, and the Science Council (FONDECYT) of Chile.

2.2. Drugs and drinking solutions

Erysodine was isolated from the seeds of *Erythrina falcata* Benth. Purity and structure of Erysodine were established by high-resolution one- and two-dimensional ^{1}H and ^{13}C-NMR and was typically 98 to 100%, such as previously reported [40]. Methylphenidate hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). Erysodine and methylphenidate (10 mg/kg) doses were dissolved in physiological saline solution and administered according to the body weight of the animal (dose range between 1.5–10 mg/kg). Ethanol solution (10% $^{v}/_{v}$) was prepared by mixing absolute ethanol (Merck Darmstadt, Germany) with tap water and this concentration was chosen based on prior studies performed in UChB rats [32,34,35,37].

2.3. Assessment of voluntary ethanol consumption

Adult male UChB rats were used for ethanol intake studies (250–300 g). Prior the experiments, rats were tested for their voluntary ethanol consumption in the following way: Two-month-old ethanol naïve UChB rats were housed in individual cages in temperature- and humidity-controlled rooms under a 12-h light cycle. After one week of acclimation animals were exposed continuously (24 h/day) for 25 days to a choice between a 10% ($^{v}/_{v}$) ethanol solution and water. All fluids were presented in 50-mL graduated glass cylinders with glass drinking spouts, which had been previously tested to ensure that they did not spill fluid. The placement of the ethanol bottle was alternated daily to avoid side preferences. Food was provided *ad libitum* and the volume of water and ethanol solution consumed was recorded daily.

Baseline ethanol intake was obtained by averaging last three drinking days (day 23–25) before saline or erysodine administration (day 26–28). Ethanol consumption for each rat was expressed as grams per kilogram of body weight per day (g/kg/day).

2.4. Erysodine effects on voluntary ethanol consumption by 2-bottle choice paradigm for 24-hour free access period

On day 25 of free-choice, UChB rats (n = 25) were randomly divided into five experimental groups to evaluate the effect of erysodine administration (3 days of treatment) on voluntary ethanol intake. Since we have previously showed that the intraperitoneal administration of cytisine (1.5 mg/kg), for 3 consecutive days, was effective in reducing

ethanol intake [32], we assessed if the intraperitoneal (i.p.) administration of increasing doses of erysodine from 1.5 to 8.0 mg/kg, could reduce ethanol intake in a dose dependent manner.

Control group received the physiological saline solution (1 mL/kg i.p., n = 5) and four groups of 5 rats per group received administration of erysodine (Group 1: 1.5 mg/kg i.p.; Group 2: 2.0 mg/kg i.p.; Group 3: 4.0 mg/kg i.p.; Group 4: 8.0 mg/kg i.p.) per day for three consecutive days. Water and ethanol intake was reading daily at 14:00 h. for 6 days, 3 days for treatment (day 26–28) and 3 days for post-treatment (day 29 to 31).

2.5. Locomotor activity

Locomotor activity studies were performed in male Sprague-Dawley rats according to our previous work [41,42]. Briefly, rats were divided into 3 experimental groups (n = 5 per group): First group was given erysodine (10 mg/kg i.p.), second group methylphenidate (10 mg/kg i.p.) and third group physiological saline solution (1 mL/kg i.p.). Methylphenidate (an inhibitor of DA and noradrenalin transporters) was used as positive control, since it increases the locomotor activity *in vivo* through an increase of extracellular levels of DA in NAc [43]. Saline administration was used as a negative control of locomotor activity. Animals were placed in a test cage (44 cm long × 22 cm height × 28 cm wide) and for the first 30 min basal locomotor activity were recorded. At 30 min, each animal was injected with a dose of erysodine, methylphenidate or saline and its locomotor activity was recorded during 60 min. The locomotor activity was recorded by internet protocol cameras (Model LX-C202, Lynx Security, China) fixed above the each test cage and connected to a computer in another room. Videos were analyzed with ANY-maze™ video tracking system (Stoelting Co., IL, USA) and test cages were wiped and cleaned with 5% ethanol solution after each trial. The locomotor activity was expressed as distance traveled (m) every 5 min and as a cumulative locomotor activity in 30 and 60 min.

2.6. Statistical analysis

Data were expressed as mean ± SEM. One-way ANOVA followed by Dunnett post hoc test was used to compare the mean of ethanol intake in each day of treatment and post-treatment periods with the mean of respective baseline (Fig. 1). Multiple t-test Sidak-Bonferroni method was used to determine eventual significant differences per day between ethanol intake in erysodine groups with saline injection. Figs. 2 and 3B were analyzed by one-way ANOVA followed by Dunnett post hoc test to compare saline versus treatment groups. Two-way ANOVA followed by Bonferroni post hoc test was used to determine differences in locomotor activity in erysodine, methylphenidate and saline injection (Fig. 3A).

3. Results

3.1. Erysodine administration induced a dose-dependent reduction of ethanol intake

Fig. 1 (Panels A, B, C and D) shows the effect of erysodine administration (1.5; 2.0; 4.0 or 8.0 mg/kg i.p.) on the voluntary ethanol intake of UChB rats. Ethanol intake in control rats was not affected by saline injection during the treatment period and the following 3 days compared to baseline [$F_{(}6,28_{)} = 0.1133$, $P = 0.9941$] (Fig. 1).

The dose of 1.5 mg/kg i.p. of erysodine (Fig. 1A) was ineffective in

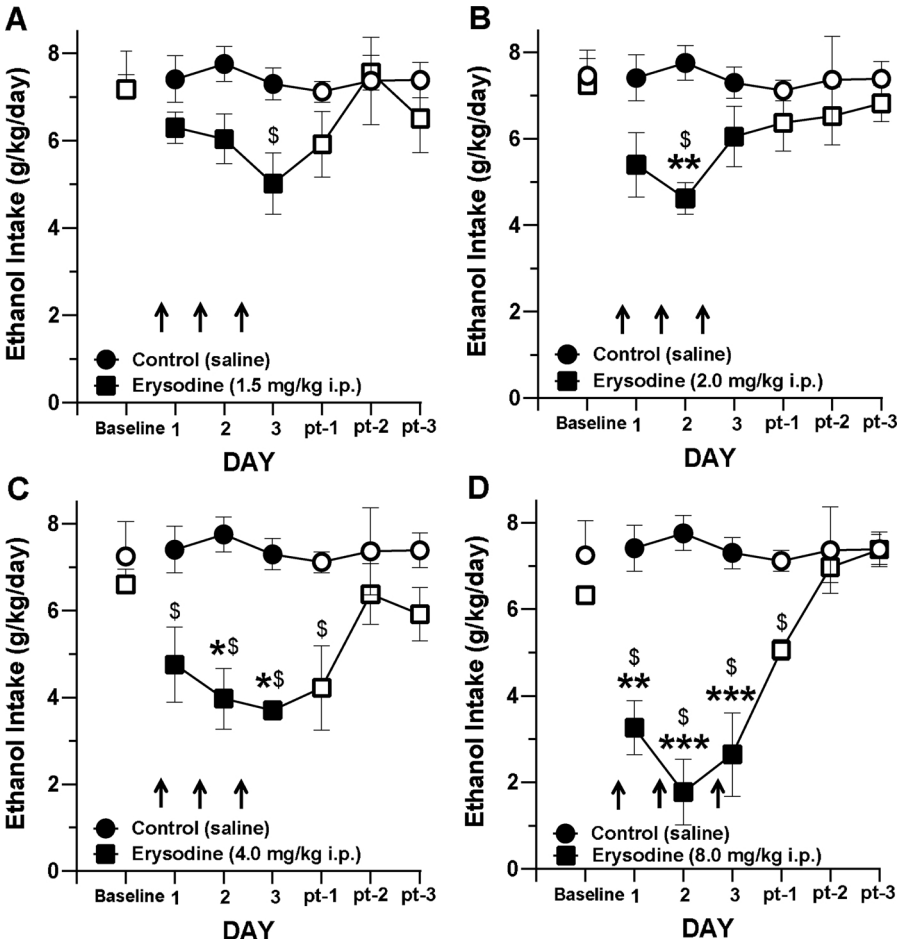

Fig. 1. Influence of three-day erysodine treatments on voluntary ethanol intake of high drinking UChB rats under 2-bottle choice paradigm free 24-h access. The baseline in ethanol consumption for each experimental group is the average of last three drinking days before the treatment. Erysodine doses (black squares) or saline (black circles) was daily administered by i.p. injection at 15:00 h and ethanol consumption recorded at 14:00 h of next day. **A)** erysodine 1.5 mg/kg i.p., **B)** erysodine 2.0 mg/kg i.p., **C**) erysodine 4.0 mg/kg i.p., **D**) erysodine 8.0 mg/kg i.p. or saline (1 mL/kg i.p.) were administered to rats (n = 5 per group) by single and daily i.p. injection during 3 consecutive days. Data are expressed as mean ± SEM of ethanol consumption g/kg/day and analyzed by one-way ANOVA followed by Dunnett post hoc test to compare ethanol consumption in treatment and post-treatment days to baseline (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Multiple t test Sidak-Bonferroni method was used to compare the effect of different erysodine doses in reduce ethanol intake *versus* saline control group at the same time ($ < 0.05). Arrows indicate the moment of i.p injection with different erysodine doses or saline. pt: post-treatment day.

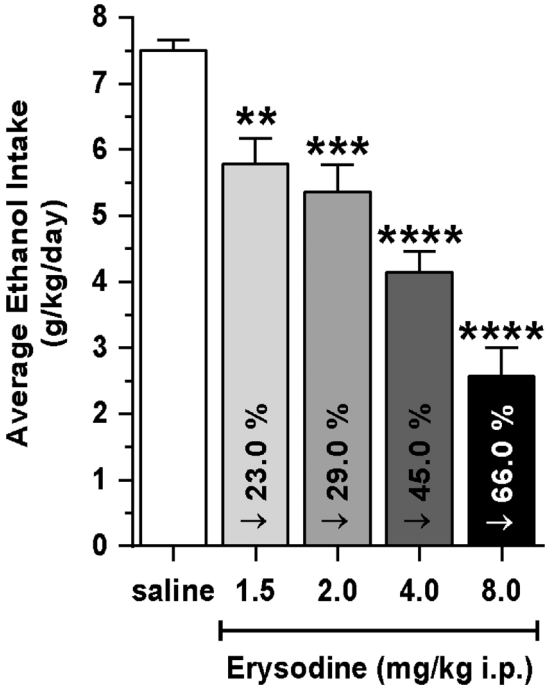

Fig. 2. Dose-dependent decrease in ethanol intake induced by erysodine (8, 4, 2, 1.5 mg/kg i.p.) or saline (1 mL/kg i.p.) administration. Each bar represents the average of ethanol intake during three days treatment period in high drinking UChB rats (n = 5 per group). Data are expressed as mean ± SEM (g/kg/day) and analyzed by one-way ANOVA followed by Dunnett post hoc test (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). The number inside each bar indicated the percentage of reduction in ethanol intake respect saline control group.

reducing ethanol consumption compared to baseline [$F_(6,28_) = 2.070$, $P = 0.0892$] (Fig. 1A) and ethanol preference [$F_(6,28_) = 0.1486$, $P = 0.9878$] (Table 1). However, only a statistically significant reduction in ethanol intake was observed on the third day of treatment when compared with the respective saline administration [$P = 0.0079$]. The dose of 2.0 mg/kg i.p. of erysodine (Fig. 1B) significantly reduced ethanol consumption [$F_(6,28_) = 2.563$, $P = 0.0418$] and ethanol preference [$F_(6,28_) = 2.492$, $P = 0.0465$] (Table 1) on the second day of treatment compared to baseline. In addition, the dose of 2.0 mg/kg i.p. of erysodine produced a significant reduction in ethanol intake compared with the saline administration at the same day [$P = 0.0003$]. The doses of 4 and 8 mg/kg i.p. of erysodine were the most effective doses in reducing ethanol intake and ethanol preference in UChB rats. The dose of 4 mg/kg i.p. of erysodine reduced alcohol consumption during the second and third day of treatment compared to baseline [$F_(6,28_) = 3.165$, $P = 0.0169$] (Fig. 1C). In addition, the ethanol preference was significantly reduced from baseline during second and third day of treatment and the first day of post-treatment [$F_(6,28_) = 3.450$, $P = 0.0112$] (Table 1). While the dose of 8 mg/kg i.p. of erysodine reduced ethanol intake throughout the treatment period [$F_(6,28_) = 15.56$, $P < 0.0001$] (Fig. 1D) and ethanol preference during all days of treatment and the first day of post-treatment [$F_(6,28_) = 7.148$, $P = 0.0001$] (Table 1).

Fig. 2 shows the magnitude of reduction in ethanol intake in erysodine-treated UChB rats compared with the saline injection. The values represented in the graph correspond to the average ethanol intake in each group of UChB rats during the 3 days of treatment. The percentages of reduction in alcohol consumption were significant when comparing erysodine and saline administrations (23% in the 1.5 mg/kg i.p., 29% in the 2.0 mg/kg i.p., 45% in the 4.0 mg/kg i.p. and 66% in the 8.0 mg/kg i.p.) [$F_(4,70_) = 26.92$, $P < 0.0001$].

3.2. Erysodine administration does not affect locomotor activity

To determine if the erysodine administration affects locomotor activity, we measured distance traveled using a dose of erysodine 25% higher (10 mg/kg i.p.) than the highest dose used in experiments of ethanol consumption (8 mg/kg i.p.) and it used in other work to measure the effects of erysodine on reducing nicotine self-administration [31]. Equivalent dose of methylphenidate (10 mg/kg i.p.) and saline injection (1 mL/kg i.p.) were used such as positive and negative control, respectively. Fig. 3A shows the time course of locomotor activity measure each 5 min during 90 min (erysodine, methylphenidate and saline were injected at 30 min). Two-way ANOVA analysis did not show significant differences between saline vs erysodine (Interaction [$F_{(17,144)} = 1.38$, $P = 0.1558$], time [$F_{(17,144)} = 6.13$, $P < 0.0001$], treatment [$F_(1,144_) = 0.12$, $P = 0.7282$]). However, two-way ANOVA

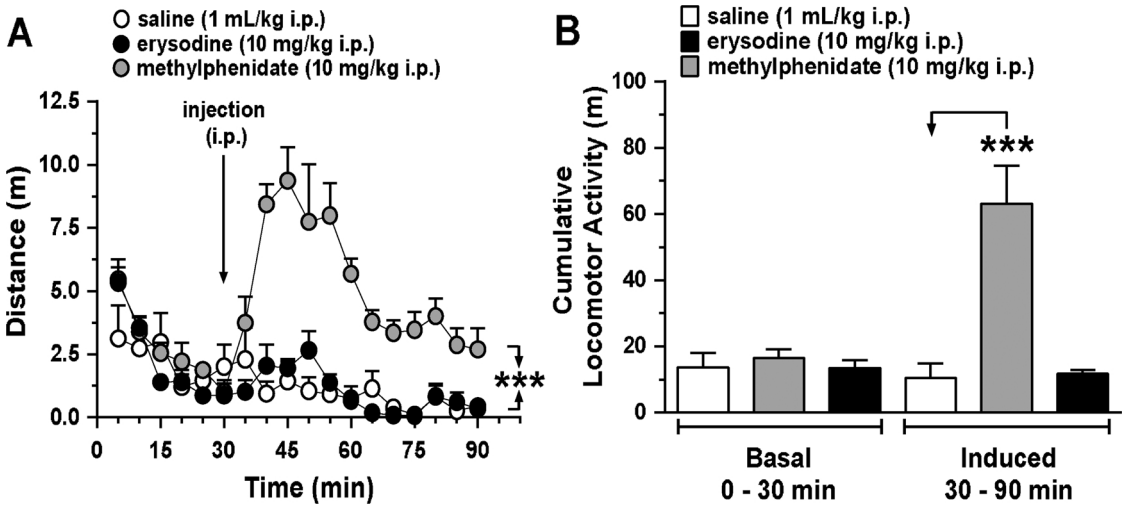

Fig. 3. Effect of erysodine (10 mg/kg i.p.), methylphenidate (10 mg/kg i.p.) or saline (1 mL/kg i.p.) administration on locomotor activity. **A**) Time course of locomotor activity was registered each 5 min. Results are expressed as the mean ± SEM of distance traveled (m) each 5 min and analyzed by two-way ANOVA followed by Bonferroni post hoc test (*$P < 0.05$). Arrow indicates the time of i.p. injection of erysodine, methylphenidate or saline. B) Cumulative locomotor activity induced by erysodine, methylphenidate or saline i.p. in adult male rats (n = 5 per group). Results are expressed as mean ± SEM of cumulative distance traveled (m) during first 30 min (basal) and following 60 min (induced by injection) and analyzed by one-way ANOVA followed by Dunnett post hoc test (***$P < 0.01$).

Table 1
Repeated administration of erysodine reduces ethanol preference in rats under continuous ethanol access.

Groups	Ethanol preference (%) (mL ethanol intake x 100/ mL total fluid intake)						
	Baseline	Treatment days			Post-treatment days		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Saline (1.0 mL/kg)	81 ± 5	79 ± 3	82 ± 4	81 ± 3	78 ± 3	83 ± 8	82 ± 4
Erysodine (1.5 mg/kg)	88 ± 5	86 ± 7	81 ± 7	71 ± 10	78 ± 6	91 ± 3	77 ± 7
Erysodine (2.0 mg/kg)	82 ± 5	72 ± 9	52 ± 3**	78 ± 6	81 ± 8	79 ± 8	80 ± 6
Erysodine (4.0 mg/kg)	85 ± 4	65 ± 11	48 ± 6**	55 ± 7*	57 ± 12*	72 ± 8	86 ± 4
Erysodine (8.0 mg/kg)	86 ± 1	55 ± 12*	35 ± 13***	36 ± 9***	60 ± 11*	87 ± 1	85 ± 1

Percentage of ethanol preference versus total fluid intake is shown during baseline (average of day 23–25), 3 days of treatment (day 26 to 28) and during three post-treatment days (day 29 to 31) in saline and erysodine groups. Data are expressed as mean ± SEM (n = 5, for each group) and analyzed by one-way ANOVA followed by Uncorrected Fisher's LSD post hoc test to compare ethanol preference in treatment and post-treatment days to baseline (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

followed by Bonferroni's *post hoc* test comparisons showed significant differences between saline vs methylphenidate (Interaction [$F_{(17,144)} = 5.685$, $P < 0.0001$], time [$F_{(17,144)} = 4.945$, $P < 0.0001$], treatment [$F_{(1,144)} = 131.61$, $P < 0.0001$]), and erysodine vs methylphenidate (Interaction [$F_{(17,144)} = 5.28$, $P < 0.0001$], time [$F_{(17,144)} = 9.93$, $P < 0.0001$], treatment [$F_{(1,144)} = 158.31$, $P < 0.0001$]). Fig. 3B shows the cumulative locomotor activity that represents the total distance travelled by each animal during first 30 min (basal) and following 60 min (induced by injection). One-way ANOVA followed by Dunnett post hoc test did not show statistically significant differences between basal cumulative locomotor activities [$F_{(2,12)} = 0.2938$, $P = 0.7506$]. The administration of methylphenidate increased significantly cumulative locomotor activity vs saline and erysodine injection [$F_{(2,12)} = 17.59$, $P = 0.0003$] ($P < 0.001$).

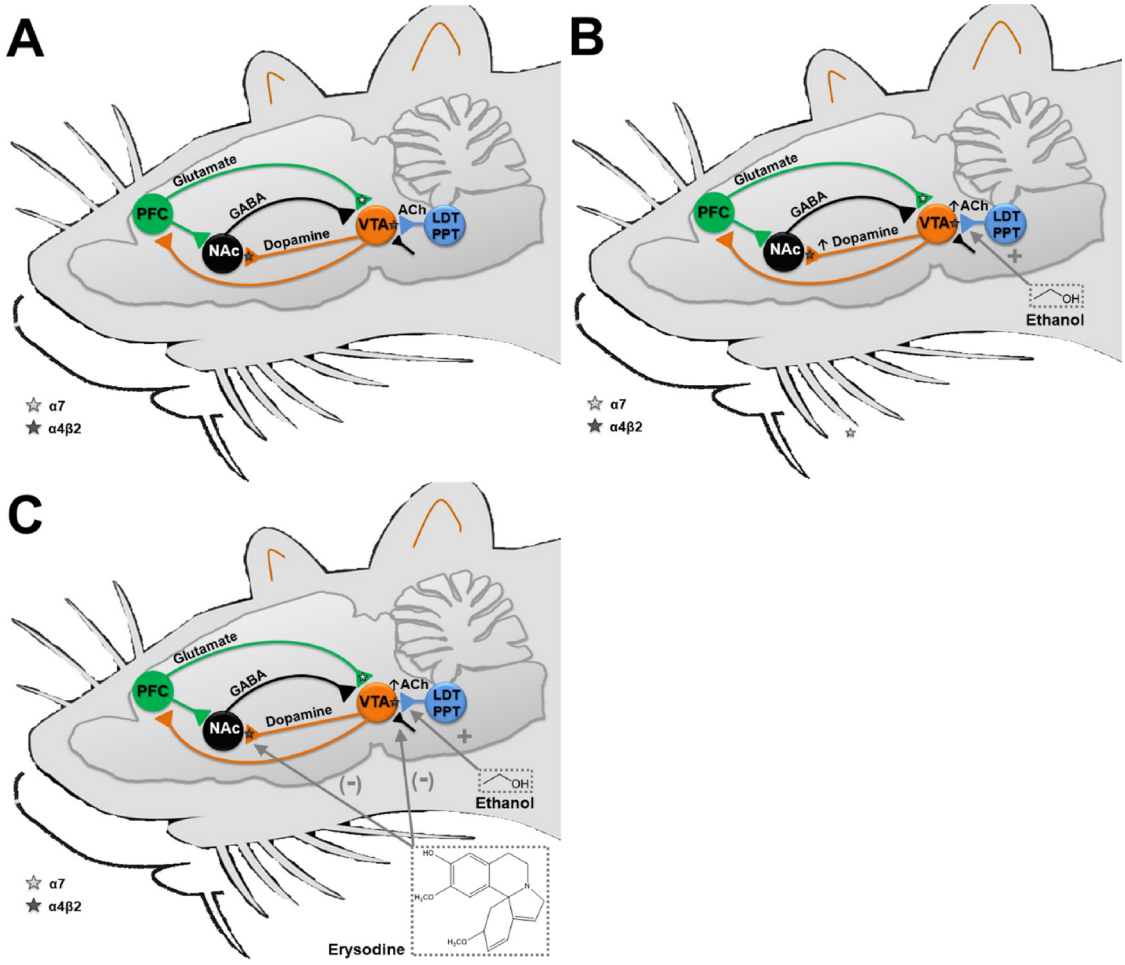

Fig. 4. Simplified figure of mesocorticolimbic pathway highlighting brain areas involved, neurons and the expression of nAChRs (Panel A). Fig. 4 Panel B shows the effect of ethanol on NAc DA release. Ethanol activates cholinergic neurons from LDT/PPT increasing VTA ACh release. ACh binds and activates nAChRs in DA cell bodies (α4β2) and glutamatergic terminal (α7) in VTA. This activation produces a depolarization in DA neurons that promote excitatory postsynaptic potential and NAc DA release. Fig. 4 Panel C shows the effect of erysodine (a competitive antagonist at α4β2 nAChR) in inhibiting the activation of DA neurons for ethanol-induced ACh release in VTA. **PFC:** Prefrontal Cortex; **NAc:** Nucleus Accumbens; **VTA:** Ventral Tegmental Area; **ACh:** Acetylcholine; **LDT/PPT:** laterodorsal and pedunculopontine tegmental nucleus.

4. Discussion

Our results show that erysodine elicited a dose dependent reduction in voluntary ethanol consumption and ethanol preference in high-alcohol-drinking UChB rats, an established animal model of high voluntary intake of alcohol [34–36,44]. This effect may not be due to malaise induced by erysodine because this compound did not affect locomotor activity. Furthermore, no other sign of discomfort were observed in the treated animals, further suggesting that changes in ethanol consumption pattern were brought about by erysodine treatment. Overall, our findings are in accord with studies showing that erysodine has low affinity for muscle-type nAChR and little effect on anxiety-like behavior [29]. Although the experiments of locomotor activity were performed in other strain of rats and these results are not completely extrapolated to alcohol intake data, it could be observed that the administration of erysodine did not affect the locomotor activity in rats at the same dose used in experiments to decrease the nicotine self-administration [31]. At neurochemical level, nAChRs expressed in VTA [45,46] are able to activate the mesolimbic DA pathway through depolarization of dopamine neurons and increasing its firing (Fig. 4, Panel A) [45]. In this context, ethanol intake promotes this process through an increase in extracellular levels of ACh in VTA [16,47,48] and a subsequent increase in NAc DA release (Fig. 4,Panel B) [49].

Previous studies have shown that erysodine blocks the discriminative and reinforcing actions of nicotine in rats in range effective doses of 0.32–10 mg/kg s.c. [31]. In this sense, low doses of erysodine are effective in reducing the nicotine self-administration and as we observed in this work higher doses of erysodine are required to reduce ethanol intake. This difference might be due to the fact that the effect of erysodine on nicotine self-administration was studied in sessions of only 20 min a day in length [31], while in the present study the effect of erysodine on alcohol consumption was studied in a long access condition (24 h a day). This discrepancy suggests a short plasma half-life of erysodine. By contrast, the finding that three consecutive doses of erysodine (4 or 8 mg/kg/day, i.p.) induced a decrease of ethanol intake 24 h after the last injection, suggesting a long-lasting inhibition of ethanol intake induced by consecutive doses of erysodine that blocks the activation of DA neurons for ethanol-induced ACh release in VTA (Fig. 4, Panel C). Probably the post-treatment effect of erysodine is due to changes in some pharmacokinetic parameters of erysodine, such as an increase in the maximum serum concentration (C_{max}) and area under the plasma concentration-time curve ($AUC_{0-\infty}$) after high consecutive doses of erysodine, favoring its pharmacological effect.

As previous reports have indicated, MEC, a non-competitive and non-selective antagonist nAChRs that crosses blood-brain barrier (BBB) [50], had shown decreasing voluntary ethanol consumption in rodents by systemic administration [24,51,52]. However, hexamethonium, a nAChR blocker at autonomic ganglia that does not cross the BBB, it has no effect on ethanol intake [24], showing nAChR antagonists modulate ethanol consumption by inhibiting CNS nAChR. In addition, systemic administration of MEC reduced NAc DA release [23] and intra-VTA microinjections of MEC or hexametonium reduced NAc DA release associated to ethanol intake [25,26], evidencing that the blockade of nAChRs in VTA is an effective pharmacological target to treat alcohol addiction. In this sense, as it demonstrated by MEC, erysodine crosses the BBB [29] and exerts effects on nAChRs in CNS (specifically at the VTA level), which favor the reduction in nicotine self-administration [31] and ethanol intake.

Erysodine has shown to be 7 times more potent that DHβE in displace [^{3}H]-Cytisine in brain membrane fractions, showing a preferential antagonism on α4β2 nAChRs [29,40]. In addition, depolarizing currents induced by activation of α4β2 nAChRs are potentiated by ethanol [53,54], indicating that this subtype of nAChRs could be involved in rewarding effects of ethanol. In this sense, VTA DA neurons are activated by ethanol-induced ACh release from pedunculopontine/laterodorsal tegmental cholinergic neurons [16,55,56]. Despite binding studies show that erysodine (Ki = 5 nM) is more potent that DHβE (Ki = 35 nM) in displace [^{3}H]-cytisine of α4β2 nAChRs, *in vitro* studies show a similar potency in inhibiting DA release from rat striatal slices [29]. In addition, DHβE (0.5–2.0 mg/kg) is not able to reduce nicotine-induced ethanol intake in mice, while lobeline (a non-selective nAChR antagonist at β2* containing nAChRs) reduce it [57]. These facts suggest us that erysodine may reduce ethanol consumption through non-selective inhibition of nAChRs, such as α3β2, α4β2 and α6β2. Erysodine compared with MEC is an antagonist (competitive) more specific for α4β2 nAChR [29], while MEC is a non-selective, non-competitive antagonist of nAChRs [50]. In addition, the safety of erysodine for *in vivo* pharmacological studies is greater than MEC, since MEC is considered a ganglioplegic drug, affecting autonomic nervous system [58]. Regarding cytisine, a partial agonist of α4β2 nAChR, we have previously showed a reduction of ethanol intake using a similar protocol to use in this work, however the long-term cytisine administration (thirteen days) produce tolerance [32], a more gradual decrease in responsiveness to repeated administration of an agonist.

Regarding other nAChR subtypes that could be related with the erysodine effects observed in this work, it can be ruled out an important role of α7, since the antagonism of this receptor does not affect ethanol-related actions [47] and erysodine exhibits low affinity for α7 nAChRs subtype [29]. However, we cannot rule out that the increase in VTA ACh release induced by ethanol (Fig. 4, Panel B) [16] could be stimulate α7 nAChRs expressed in glutamatergic terminals of VTA [46], promoting glutamate release and depolarization of dopamine cell bodies through NMDA activation [59].

On the other hand, several nAChR subunits (α3, α4, α5, α6 and β2) are expressed in VTA [45], determining other active isoforms of nAChRs. In this sense, intra-VTA administration of α-conotoxin-MII, a selective antagonist of α3β2 and α6β2 nAChR, selectively blocks ethanol-associated conditioned reinforcement [60], reduces NAc DA release induced by ethanol and reduces ethanol intake [61]. In addition, MEC is more potent as inhibitor of currents evoked by nicotine induced by α3β2 than α4β2 nAChR activation (IC_{50} 0.28 vs 0.64 μM, respectively). Data about α3β2 nAChR subtype and alcohol consumption behavior indicate that we cannot rule out that the effects of erysodine on alcohol consumption (especially at high doses) could be mediated by the antagonism of this receptor. On the other hand, α6 subunit (related to α3) is other candidate to erysodine effects, since α6 subunit predominantly expressed in DA neurons [45,62] and it was observed an α6-dependent increment on rate firing and potentiation of VTA DA neurons induced by ethanol [63]. In summary, erysodine reduces ethanol consumption dose-dependent fashion and a new characterization is necessary to define its nAChR target related to drinking, probably to the antagonism of α4β2, α3β2 or α6β2 subtypes.

Because the inability to cease smoking is usually found in subjects with an alcohol-use disorder [64] erysodine may have a significant therapeutic effect in this population group. However, before its use in humans, it is necessary to test in preclinical studies if erysodine maintains its pharmacological effect in long-term treatments and if it can be administered orally.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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