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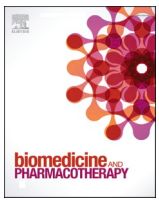

Galanin(1−15) and Naltrexone: A novel approach for alcohol use disorder in rats, involving the mesolimbic system

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ABSTRACT

Alcohol Use Disorder (AUD) is a highly prevalent psychiatric and represents a significant public health challenge. Naltrexone (NTX), a mu-opioid receptor antagonist widely used for AUD treatment, has limited efficacy due to side effects and variability in patient response. Interactions between the full-length GAL molecule and the opioid system have been demonstrated. In our recent studies, we showed that the Galanin (1−15) fragment [GAL (1−15)] decreased alcohol seeking along with alcohol consumption. This study aims to examine the effects of GAL(1−15)+NTX on alcohol-seeking behavior and alcohol consumption, as well as the involvement of the mesolimbic system. In rats, we assessed GAL(1−15)+NTX in reward-seeking and the role of GALR2 using the antagonist M871 in the self-administration test. In addition, GAL(1−15)+NTX effects were studied on voluntary alcohol using the two-bottle choice paradigm. Locomotor activity and stereotyped behaviors, along with dopamine release in the dorsal striatum following alcohol injections, were assessed. Moreover, we have analyzed the transcriptional changes of C-Fos, MOR, POMPC, and dopamine receptors in the ventral tegmental area, nucleus accumbens and the hypothalamus. GAL(1−15)+NTX combination reduced alcohol seeking in self-administration and two-bottle choice consumption, with GALR2 involved in the effect. In addition, GAL(1−15)+NTX attenuated alcohol-induced locomotor activity and stereotyped behaviors linked to reduced dopamine release in the dorsal striatum. Notably, these effects were associated with C-Fos, MOR, and dopamine receptor changes, suggesting that the mesolimbic pathway, including the opioid system, is involved in GAL(1−15)+NTX effects. These results open up the possibility of using GAL(1−15) with NTX as a novel strategy in AUD.

1. Introduction

Alcohol Use Disorder (AUD) is a highly prevalent psychiatric, chronically recurrent disorder that is associated with binge drinking, loss of control over drinking, and the emergence of a negative emotional state when alcohol is no longer available [1,2].

Alcohol consumption leads to 3 million deaths annually worldwide and accounts for over 7 % of health issues and premature deaths in Europe [3,4]. A growing concern is the rise in binge drinking, especially among youth aged 15–19 [5]. The COVID-19 pandemic has also contributed to increased alcohol consumption [6–9].

Naltrexone, one of the most used medications for the treatment of alcoholism, is a mu-opioid receptor antagonist, with an additional affinity for kappa and delta opioid receptors, that acts by attenuating alcohol-induced opioidergic activity, thereby reducing alcohol consumption by modulating the rewarding effects of alcohol via the mesolimbic dopaminergic system. Although some medications reduce cravings and alcohol consumption, their efficacy is debated due to issues like side effects, dependence, and varying effects among patients. As a result, pharmacotherapies for AUD have seen limited use [10]. This highlights the need for new treatments. Combining drugs to enhance effectiveness has recently emerged as a promising approach [10,11].

Recently, studies have shown the role of endogenous neurotransmitters and neuropeptides involved in drug abuse and addiction, such as the neuropeptide Galanin (GAL) [12,13]. The GAL and its receptors are implicated in alcohol consumption, as GAL administration increases ethanol consumption in rats [12,14,15].

GAL also has antagonistic interactions with the opioid system [16,17]; for example, the central administration of GAL decreases morphine preference in conditioned place experiments in rodents. In genetic

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studies, associations have been found between specific polymorphisms in the GAL gene and vulnerability in opioid use disorder [18,19]. Recent studies have identified the existence of functionally significant MOR-GALR1 heteromers in the ventral tegmental area (VTA) that determine the degree of activation of dopaminergic cells that may explain this interaction [18].

Not only the Galanin but also terminal fragments of Galanin, such as fragment 1–15 [(GAL(1−15)] have activity in the CNS as well as an essential role in mood disorders and alcohol-seeking behaviors, acting through GALR1-GALR2 heteroreceptor complexes [20–22]. Recently, our research group has shown that GAL(1−15) significantly reduces ethanol preference and consumption in rats through central mechanisms, focusing on the striatum, a key area for the rewarding and motivational effects of drugs, and this effect is different from the whole molecule of GAL [23]. Furthermore, icv administration of GAL(1−15) significantly reduces alcohol seeking-behavior in an operant model of self-administration as well as context-alcohol relapse induced [24]. In addition, the substantial reduction of alcohol-seeking behavior induced by GAL(1−15) involves the mesocorticolimbic pathway, a critical region in the reward effects of the drug [24].

Moreover, the combination of GAL(1−15) with the antidepressant escitalopram (ESC) is effective in reducing alcohol seeking-behavior in an operant model, being a novel treatment proposal for the treatment of alcoholism-depression comorbidity [25].

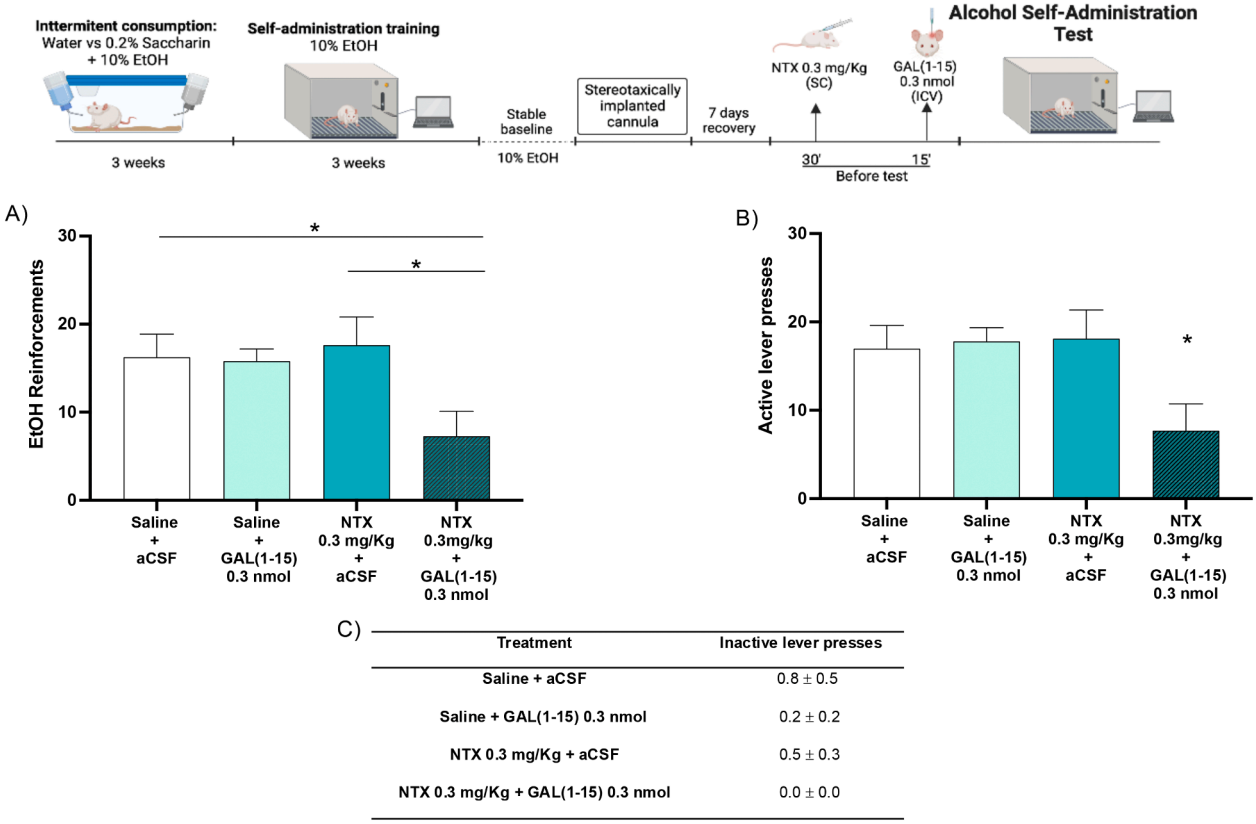
Given the above, GAL(1−15) can be proposed as a new strategy of combined therapy using with NTX for AUD. In the current study, we have analyzed the effect of GAL(1−15) combined with NTX to assess reward-seeking behaviors, the self-administration test, and we have surveyed the receptors involved in the effect of GAL(1−15) using the GALR2 antagonist, M871. In addition, we have performed alcohol consumption in a voluntary two-bottle model and the locomotor activity test preceded by a single intraperitoneal alcohol injection to observe whether there were changes in dopamine release after GAL(1−15) and NTX coadministration.

Finally, we have studied whether the GAL(1−15)+NTX combination modulates the mesolimbic pathway by analyzing RT-qPCR studies the expression of C-Fos mRNA, a marker of neuron activity, as well as different markers of the dopaminergic system (DAT, VTMAT2, D1, D2 and D3), the opioid system (MOR and POMC) and GAL receptors (GALR1, GALR2 and GALR3) in VTA, nucleus accumbens (NAc) and hypothalamic areas.

2. Material and methods

2.1. Animals

Male Sprague-Dawley rats (225–250 g) were obtained from Criffa and housed in a humidity- and temperature-controlled room (20–22 °C) on a 12-h reversed light/dark cycle (lights off at 9 am). All experiments followed the University of Málaga's Guidelines for the Care and Use of Laboratory Animals (Ethic Code: 22/05/2017/066). Rats were randomly assigned to experimental groups. Further details on controlled conditions, surgical procedures, and drug administration are available in the supplementary material.



Treatment	Inactive lever presses
Saline + aCSF	0.8 ± 0.5
Saline + GAL(1-15) 0.3 nmol	0.2 ± 0.2
NTX 0.3 mg/Kg + aCSF	0.5 ± 0.3
NTX 0.3 mg/Kg + GAL(1-15) 0.3 nmol	0.0 ± 0.0

Fig. 1. Effect of the administration of Galanin(1−15) [GAL(1−15)] and Naltrexone (NTX) on the alcohol self-administration test. NTX 0.3 mg/Kg or saline was administrated subcutaneous (SC) 30 min before the test, and GAL(1−15) 0.3 nmol or artificial cerebrospinal fluid (aCSF) was administered intracerebroventricular (ICV) 15 min before the test. Experimental groups: saline + aCSF (n = 8), saline + GAL(1−15) 0.3 nmol (n = 5), NTX 0.3 mg/Kg + aCSF (n = 8), NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol (n = 7). Vertical bars represent a mean ± standard error of the mean of the number of alcohol reinforcements and active lever presses. **(A)** *p ≤ 0.05 NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol vs saline + aCSF, NTX 0.3 mg/kg + aCSF. **(B)** *p ≤ 0.05 NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol vs the rest of the groups, according to one-way ANOVA followed by Fisher multiple comparison test. **(C)** Data represent mean ± standard error of the mean of inactive lever presses during the test period. There are no differences according to a one-way analysis of variance (ANOVA) between the experimental groups.

2.2. Materials

Naltrexone was obtained from Merck Life Science, Madrid, Spain. Galanin (1−15) and the GALR2 receptor antagonist M871 were obtained from Tocris Bioscience, Bristol, United Kingdom.

2.3. Experimental design

Three experimental procedures were carried out. The scheme of each experimental design is shown in Figs. 1–7.

2.3.1. Experiment 1: alcohol self-administration test

We analyzed the effect of the combination of GAL(1−15) and NTX on the alcohol self-administration test (Fig. 1) and the GAL receptors involved in this effect (Fig. 2).

Alcohol consumption was measured using a previously described self-administration test [24] (see supplementary information). Rats were trained to self-administer 10 % alcohol in 30-minute sessions on an FR1 schedule until stable alcohol intake was achieved. The active lever was paired with alcohol delivery and a conditioned light stimulus, while the inactive lever provided no reward. During each session, active lever presses, inactive lever presses, and alcohol reinforcements were recorded.

Two sets of experiments were conducted in the alcohol self-administration test. In the first set of experiments, groups of rats received a subcutaneous injection of NTX 0.3 mg/Kg or saline 30 min before the beginning of the test and one ICV injection of GAL(1−15) 0.3 nmol or aCSF 15 min before the test.

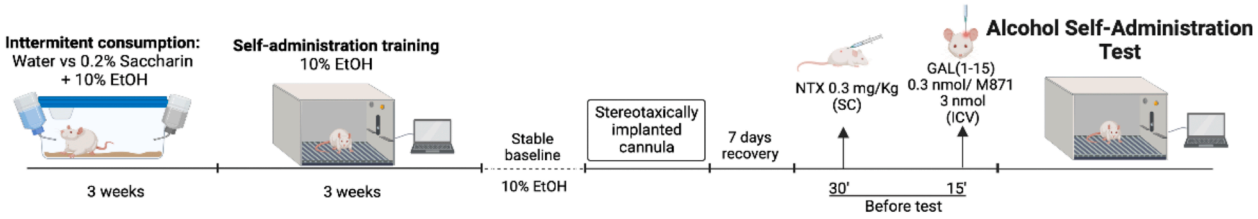
In the second set of experiments, the GAL receptors involved in the effect of GAL(1−15) were studied; for this, groups of rats received a subcutaneous injection of NTX 0.3 mg/Kg or saline 30 min before the test and M871 3 nmol antagonist, GAL(1−15) 0.3 nmol combined with GALR2 antagonist or aCSF 15 min before the test.

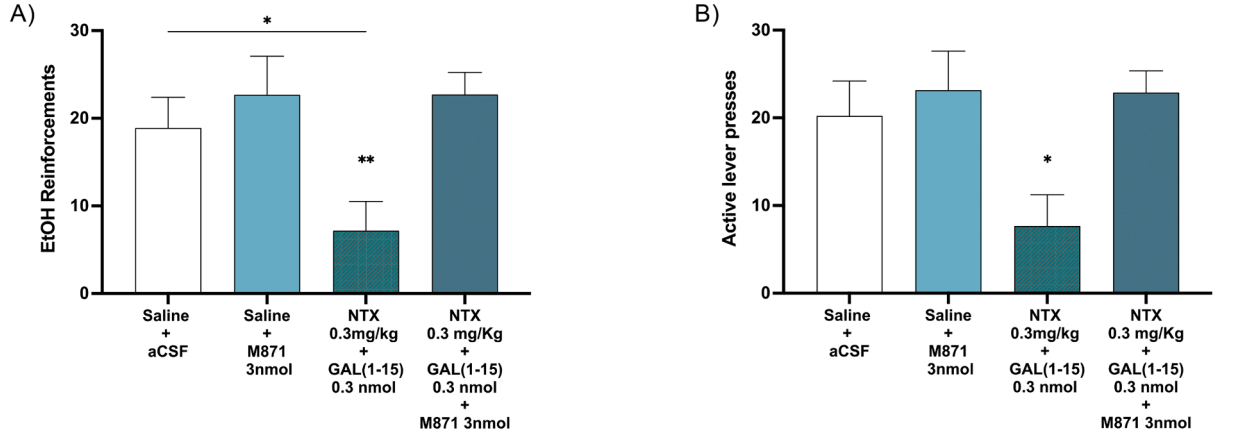
The general schema of the experimental design is shown in Figs. 1,2.

2.3.2. Experiment 2: two-bottle choice paradigm

We analyzed the effect of the GAL(1−15) and NTX combination on voluntary ethanol consumption using the two-bottle choice test [26]. Rats had continuous access to a 10 % ethanol solution as their only liquid for three days. After this forced exposure, they were given a choice between 10 % ethanol and water for four days, with bottle positions switched daily to avoid bias. Water and ethanol intake (g/Kg) and ethanol preference (%) were calculated. Evaporation and spillage were accounted for using control cages with water bottles and ethanol.

Groups of rats received a subcutaneous injection of NTX 1 mg/Kg or saline 30 min before the beginning of the test and one ICV injection of GAL(1−15) 1 nmol or aCSF 15 min before the test [23]. Measurements were collected at 2 and 24 h after the test.

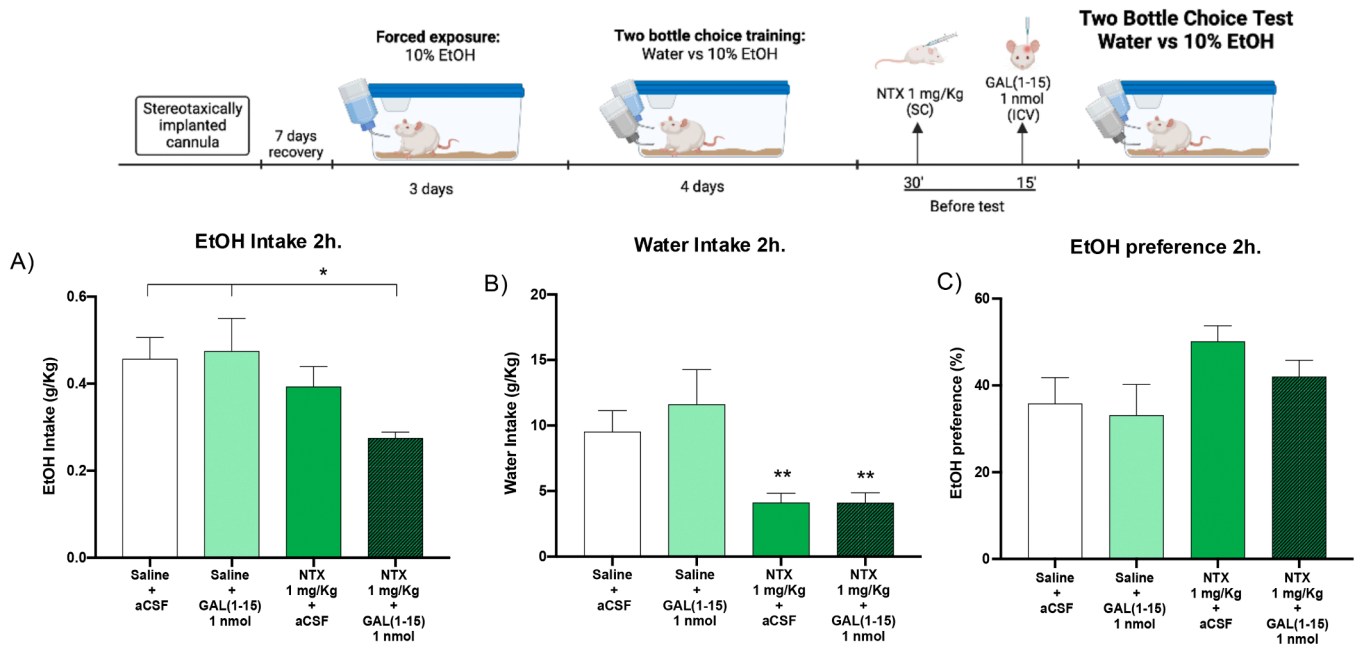




C)

Treatment	Inactive lever presses
Saline + aCSF	0.7 ± 0.5
Saline + M871 3 nmol	0.2 ± 0.2
NTX 0.3 mg/Kg + GAL(1-15) 0.3 nmol	0.0 ± 0.0
NTX 0.3 mg/Kg + GAL(1-15) 0.3 nmol + M871 3 nmol	0.4 ± 0.3

Fig. 2. GALR2 antagonist M871 in GAL(1−15)-NTX mediated effects in the alcohol self-administration test. NTX 0.3 mg/Kg or saline was administrated SC 30 min before the test, GAL(1−15) 0.3 nmol, GALR2 M871 antagonist 3 nmol or aCSF was injected by ICV 15 min before the test. Experimental groups: saline + aCSF (n = 9), saline + M871 3 nmol (n = 6), NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol (n = 6), NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol + M871 3 nmol (n = 7). Vertical bars represent a mean ± standard error of the mean of the number of alcohol reinforcements and active lever presses during the test period. **(A)** *p ≤ 0.05 saline + aCSF vs NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol, * *p < 0.01 vs saline + M871 3 nmol, NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol + M871 3 nmol, according to one-way ANOVA followed by Fisher multiple comparison test. **(B)** *p ≤ 0.05 vs saline + M871 3 nmol, NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol + M871 3 nmol. **(C)** Data represent mean ± standard error of the mean of inactive lever presses during the test period. There are no differences according to a one-way analysis of variance (ANOVA) between the experimental groups.



D)

Treatment	EtOH Intake (g/Kg)	Water Intake (g/Kg) 24h.	EtOH preference (%)
Saline + aCSF	2.5 ± 0.3	55.2 ± 7.7	33.2 ± 5.7
Saline + GAL(1-15) 1 nmol	2.6 ± 0.7	56.8 ± 19.2	39.1 ± 12.1
NTX 1 mg/Kg + aCSF	2.4 ± 0.5	39.4 ± 6.8	39.0 ± 5.4
NTX 1 mg/Kg + GAL(1-15) 1 nmol	2.1 ± 0.4	47.8 ± 6.6	30.0 ± 4.3

Fig. 3. Effect of GAL(1−15) and NTX combination in the two-bottle choice paradigm with 10 percent ethanol (EtOH) concentration in rats. NTX 1 mg/Kg or saline was administrated SC 30 min before the test, and GAL(1−15) 1 nmol or aCSF was administered ICV 15 min before the test. Experimental groups: saline + aCSF (n = 8), saline + GAL(1−15) 1 nmol (n = 6), NTX 1 mg/Kg + aCSF (n = 9), NTX 1 mg/Kg + GAL(1−15) 1 nmol (n = 7). **(A)** Vertical bars represent a mean ± standard error of the mean of EtOH intake (g/Kg). *p ≤ 0.05 NTX 1 mg/Kg + GAL(1−15) 1 nmol vs saline + aCSF, saline + GAL(1−15) 1 nmol, according to one-way ANOVA followed by Fisher multiple comparison test. **(B)** Vertical bars represent a mean ± standard error of the mean of water intake (g/Kg). * *p < 0.01 vs saline + aCSF, saline + GAL(1−15) 1 nmol, according to one-way ANOVA followed by Fisher multiple comparison test. **(C)** Vertical bars represent a mean ± standard error of the mean of EtOH preference (%). There are no differences according to a one-way analysis of variance (ANOVA) between the experimental groups. **(D)** Data represent mean ± standard error of the mean of EtOH intake (g/Kg), water intake (g/Kg) and EtOH preference (%) 24 h after the test. There are no differences according to a one-way analysis of variance (ANOVA) between the experimental groups.

The general schema of the experimental design is shown in Fig. 3.

2.3.3. *Experiment 3: locomotor activity and stereotyped behaviors*

The effects of the combination of GAL(1−15) and NTX on ethanol-induced locomotion and stereotyped behaviors were investigated.

Locomotor activity was registered in the open field (100 × 100 × 50 cm), where animals were individually placed and allowed to explore freely. Their behavior was recorded over 5 min by a ceiling-mounted video camera, and locomotor activity was analyzed using the video-tracking software Ethovision XT (Noldus, S.L). After each trial, all surfaces were cleaned with a paper towel and 70 percent ethanol solution. The total distance travelled (cm) and mean speed (cm/s) were recorded for the locomotor activity.

Stereotyped behaviors movements (grooming, body licking and rearing responses) were recorded in the home cage for 10 min and observed by trained blinded evaluators.

Groups of rats received a subcutaneous NTX injection (0.3 mg/Kg) or saline 30 min before the test, an ICV injection of GAL(1−15) (0.3 nmol) or aCSF 15 min before, and an intraperitoneal (ip) injection of ethanol (0.5 g/Kg) 5 min prior the test (see Figs. 4,5).

2.4. *Dopamine assay*

The concentration of dopamine in the dorsal striatum in the rats from the locomotor activity procedures in experiment 3 was analyzed. Rats were decapitated 1 h after administration of the treatments, and their brains were then removed, frozen in isopentane at −40 °C, and stored in a deep freezer until use. The nuclei dissections were conducted as described previously [24] with modifications (see supplementary material for details).

The dopamine assay was performed by following the recommended procedure for the dopamine enzyme-linked immunosorbent assay (ELISA) Kit (Catalog no.: E-EL-0046, Elabscience Biotechnology Co., Ltd., Wuhan, China). (see supplementary material for details).

2.5. *Genes expression in VTA, NAc and HYPO*

Rats from the locomotor activity procedures in experiment 3 were euthanized by decapitation 1 h after administration of the treatments. The brains were rapidly removed and frozen until use.

The nuclei dissections, the RNA isolation and the RT-PCR procedure were conducted as described [24] with modifications (see supplementary material for details).

The primer sequences used to evaluate the mRNA expression levels of the genes C-Fos, MOR, DAT, VMAT2, D1, D2, D3, GALR1, GALR2, GALR3 and POMC are shown in the supplementary material.

The general schema of the experimental design is shown in Figs. 6,7.

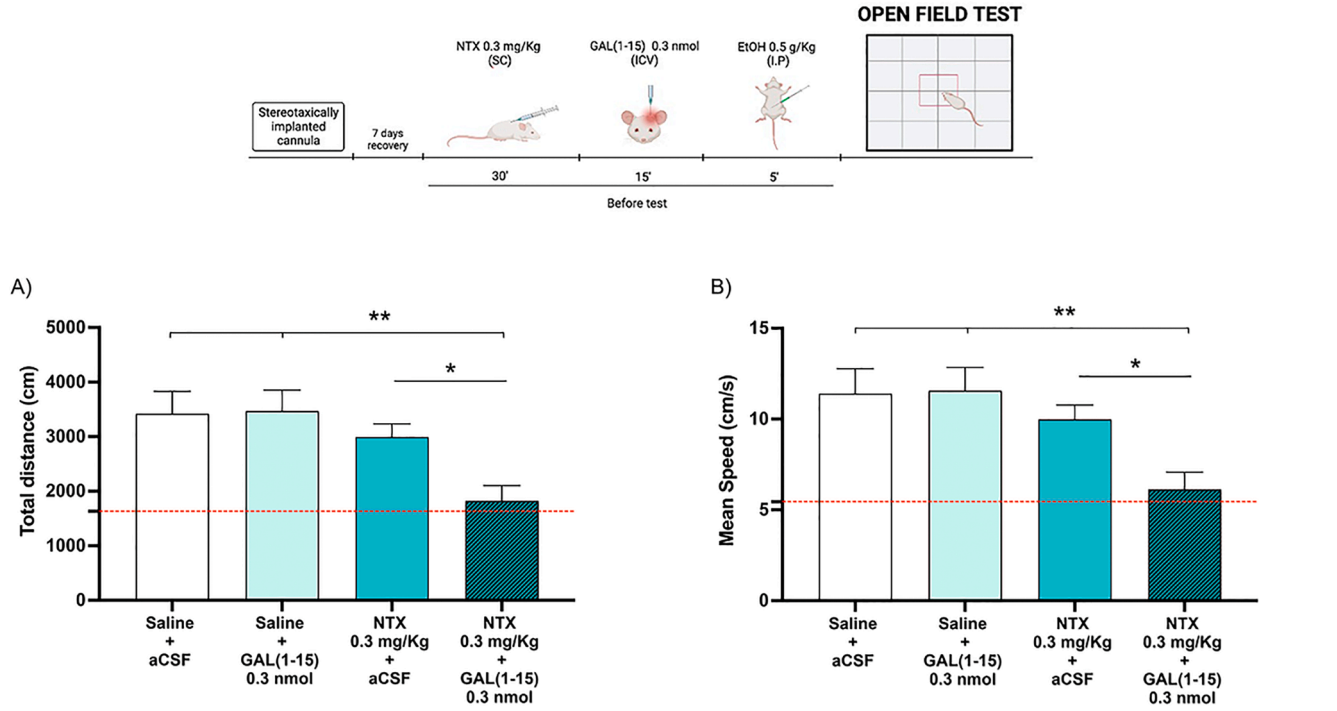


Fig. 4. Effect of the combination of GAL(1–15)-NTX on ethanol-reduced locomotion in the Open Field Test (OFT). NTX 0.3 mg/Kg or saline was administrated SC 30 min before the test, GAL(1−15) 0.3 nmol or aCSF was administered ICV 15 min before the test and alcohol (0.5 g/Kg) was administered intraperitoneally (ip) 5 min before the test. Experimental groups: saline + aCSF (n = 7), saline + GAL(1−15) 0.3 nmol (n = 7), NTX 0.3 mg/Kg + aCSF (n = 6), NTX 0.3 mg/Kg + GAL (1−15) 0.3 nmol (n = 6). Red line shows baseline of vehicle groups (rats without alcohol injection). **(A, B)** Vertical bars represent a mean ± standard error of the mean of distance travelled (cm) and mean speed (cm/s) in an open field test during the 5 min test period. * $p \leq 0.05$ NTX 0.3 mg/Kg vs NTX 0.3 mg/Kg + GAL (1−15) 0.3 nmol, * * $p < 0.01$ NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol vs saline+aCSF, saline+GAL(1−15) 0.3 nmol, according to one-way ANOVA followed by Fisher multiple comparison test.

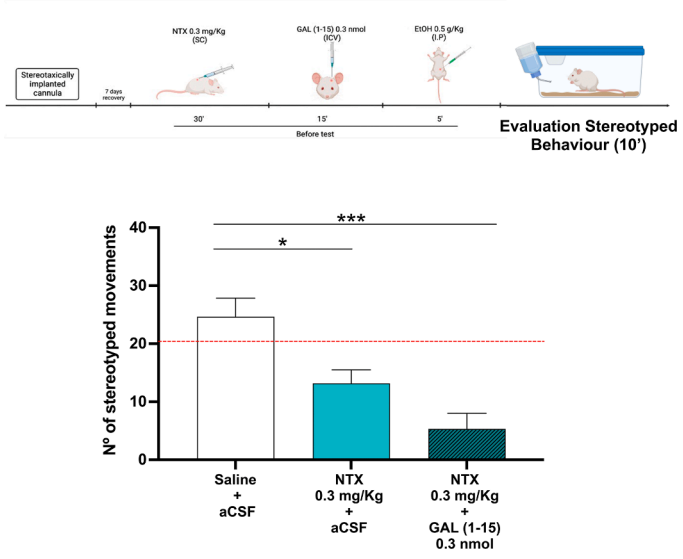


Fig. 5. Effect of the administration of GAL(1−15) and NTX in stereotypic behavior measured in the home cage. NTX 0.3 mg/Kg or saline was administrated SC 30 min before the test, GAL(1−15) 0.3 nmol or aCSF was administered ICV 15 min before the test and alcohol (0.5 g/Kg) was administered ip 5 min before the test. Experimental groups: saline + aCSF (n = 6), NTX 0.3 mg/Kg + aCSF (n = 6), NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol (n = 6). Red line shows baseline of vehicle groups (rats without alcohol injection). Vertical bars represent the mean ± standard error of the mean of the number of stereotyped movements (grooming, body licking and rearing responses) during the 10 min test period. * $p \leq 0.05$ saline + aCSF vs NTX 0.3 mg/Kg + aCSF, * ** $p < 0.0001$ saline + aCSF vs NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol, according to one way ANOVA followed by Fisher multiple comparison test.

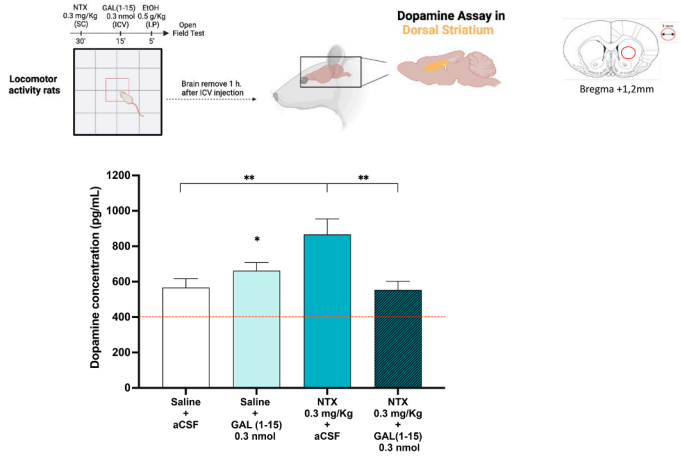
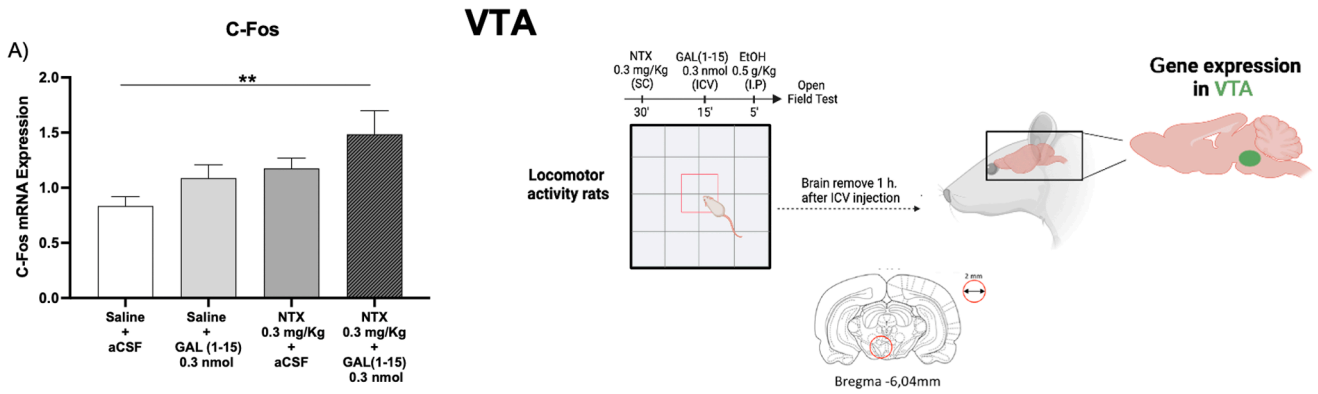


Fig. 6. Effect of the administration of GAL(1−15) and NTX in locomotor activity rats on striatal dopamine contents. The dopamine concentration (pg/mL) was measured 1 h after ICV injection. Experimental groups: saline + aCSF (n = 5), saline + GAL(1−15) 0.3 nmol (n = 5), NTX 0.3 mg/Kg + aCSF (n = 6), NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol (n = 5). Red line shows baseline of vehicle groups (rats without alcohol injection). Vertical bars represent a mean ± standard error of the mean of dopamine concentration (pg/mL). * $p \leq 0.05$ vs NTX 0.3 mg/Kg + aCSF, * * $p < 0.01$ NTX 0.3 mg/Kg + aCSF vs saline + aCSF, NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol.

2.6. Statistical and data analysis

Data are presented as the mean ± standard error of the mean. All data were analyzed using GraphPad PRISM 9.0 (GraphPad Software, San Diego, CA, USA). A one-way analysis of variance (ANOVA) was



VTA	Saline + aCSF	Saline + GAL(1-15) 0.3 nmol	NTX 0.3 mg/Kg + aCSF	NTX 0.3 mg/Kg + GAL(1-15) 0.3 nmol
DAT	1.4 ± 0.1	1.5 ± 0.5	1.2 ± 0.3	1.4 ± 0.7
VMAT2	1.2 ± 0.2	1.1 ± 0.2	0.9 ± 0.2	1.1 ± 0.2
D1	0.9 ± 0.1	0.8 ± 0.2	0.9 ± 0.0	0.8 ± 0.1
D2	1.0 ± 0.1	1.2 ± 0.2	0.8 ± 0.1	1.2 ± 0.3
D3	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.7 ± 0.1
GALR1	1.0 ± 0.1	1.1 ± 0.2	0.9 ± 0.1	0.8 ± 0.1
GALR2	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
GALR3	0.9 ± 0.1	0.7 ± 0.2	1.0 ± 0.1	1.2 ± 0.4

Fig. 7. Effect of the administration of GAL(1−15) and NTX on C-Fos, MOR, DAT, VMAT2, D1, D2, D3, GALR1, GALR2 and GALR3 mRNA expression in the ventral tegmental area (VTA). Measures were taken 1 h after ICV injection. The experimental groups have between 4 and 6 animals per group. Vertical bars represent a mean ± standard error of the mean of the mRNA expression of: **(A)** C-Fos. * * $p < 0.01$ saline + aCSF vs NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol, according to one-way ANOVA followed by Fisher multiple comparison test. **(B)** MOR. * $p \leq 0.05$ saline + aCSF vs NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol; saline + GAL (1−15) 0.3 nmol vs NTX 0.3 mg/Kg + aCSF; * * $p < 0.01$ saline + GAL(1−15) 0.3 nmol vs NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol, according to one way ANOVA followed by Fisher multiple comparison test. **(C)** Data represent mean ± standard error of the mean of the mRNA expression of DAT, VMAT2, D1, D2, D3, GALR1, GALR2 and GALR3. There are no differences according to a one-way analysis of variance (ANOVA) between the experimental groups.

performed to compare more than two groups. Fisher's least significant difference (LSD) comparison post-test was performed only when the F ratio in the one-ANOVA was statistically significant. Differences were considered statistically significant at $p \leq 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3. Results

3.1. The combination of GAL(1−15) and NTX substantially reduced the alcohol-seeking behavior on the alcohol self-administration test. The GALR2 antagonist M871 blocked GAL(1−15) mediated effect

3.1.1. The combination of GAL(1−15) and NTX in the alcohol self-administration test decreased the alcohol reinforcements

In the alcohol self-administration, the administration of GAL(1−15) at the dose of 0.3 nmol or NTX 0.3 mg/Kg lacked an effect on the number of alcohol reinforcement and the number of active lever presses. However, the combination of GAL(1−15) 0.3 nmol and NTX 0.3 mg/Kg significantly decreased the number of alcohol reinforcements by 50 % (one-way ANOVA, $F_{3,24}= 2.86$, $p = 0.05$; Fig. 1A) compared with NTX and control groups ($p \leq 0.05$) and the number of active lever presses by 50 % (one-way ANOVA, $F_{3,24}= 2.98$, $p = 0.05$; Fig. 1B) compared with rest of the groups ($p \leq 0.05$).

However, the combination of GAL(1−15) and NTX did not affect the number of inactive lever presses in the alcohol self-administration test (Fig. 1C).

3.1.2. GALR2 are involved in GAL(1−15)-NTX mediated effect on alcohol-seeking behavior

The GALR2 antagonist M871 injected alone at the dose of 3 nmol lacked effects in the number of reinforcements of alcohol self-administration (Fig. 2A) and the active lever presses (Fig. 2B). However, The GALR2 antagonist M871 3 nmol significantly blocked the GAL (1−15)-NTX-induced reduction (one-way ANOVA, $F_{3,24}= 3.9$, $p = 0.02$) in the number of reinforcements of alcohol self-administration ($p < 0.01$) and the active lever presses (one-way ANOVA, $F_{3,24}= 3.4$, $p = 0.04$; $p \leq 0.05$).

No significant differences were observed between the experimental groups in the active and inactive lever (Fig. 2C).

3.2. GAL(1−15)- NTX induced a significant decrease in ethanol intake in the two-bottle choice test, a voluntary alcohol consumption model

The coadministration of GAL(1−15) 1 nmol and NTX 1 mg/Kg produced a significant decrease by 50 % in the ethanol intake at 2 h after their administration (one-way ANOVA, $F_{3,26} = 3.18$, $p = 0.04$; Fig. 3A) compared with the control group ($p \leq 0.05$).

In terms of water intake, the administration of NTX at a dose of 1 mg/Kg significantly reduced water consumption at the 2-h mark compared to the control group (one-way ANOVA, $F_{3,26}= 6.58$; $p = 0.002$; Fig. 3B). Post-hoc analysis revealed that this reduction was statistically significant ($p < 0.01$). Additionally, when GAL(1−15) was combined with NTX, the reduction in water intake was maintained at a similar level ($p < 0.01$).

No differences in EtOH preference were found 2 hs after the administration of GAL(1−15) and NTX combination (Fig. 3C).

In the two-bottle choice test at 24 hs, no differences were observed in any parameters after the administration of GAL(1−15)-NTX (Fig. 3D).

3.3. GAL(1−15)- NTX combination decreased the locomotor activity and the stereotyped behavior in rats with intraperitoneal alcohol injection

In the open field test, the ip administration of ethanol 0.5 g/Kg significantly increased the Total Distance by 50 % and the Mean Speed by 50 % compared with rats without alcohol injection (vehicle group) 5 min after its administration (see basal line; Fig. 4 A,B).

The administration of GAL(1−15) 0.3 mg/Kg or NTX 0.3 mg/Kg alone did not affect the increase in locomotor activity induced by the alcohol injection. However, the combination of GAL(1−15) with NTX induced a substantial reduction in Total Distance (one-way ANOVA, $F_{3,22}$= 4.69, p = 0.011; Fig. 4A) and Mean Speed (one-way ANOVA, $F_{3,22}$ = 4.59 p = 0.012; Fig. 4B) compared with NTX group ($p \leq 0.05$) and GAL(1−15) and control groups ($p < 0.01$) completely removing the effect produced by alcohol injection.

We also assessed the effects of the GAL(1−15)-NTX combination in the Stereotyped behavior following 0.5 g/Kg alcohol injection (Fig. 5).

In the 10 min-evaluation behavior, the administration of NTX 0.3 mg/Kg induced a significant reduction in the number of Stereotyped Movements (one-way ANOVA, $F_{2,15}$= 12.42, p = 0.0007), compared with the control group ($p \leq 0.05$) (Fig. 5). However, the coadministration of GAL(1−15) 0.3 nmol and NTX 0.3 mg/Kg induced a stronger effect, significantly reducing the number of Stereotyped Movements compared with the control group ($p < 0.001$) (Fig. 5).

3.4. The combination of GAL(1−15)-NTX reduced dopamine release in the dorsal striatum after an alcohol injection

Ethanol administration at a dose of 0.5 g/Kg increased dopamine concentration in the dorsal striatum compared with the non-alcohol group (see basal line; Fig. 6 A,B).

This increase was statistically significant in NTX (one-way ANOVA, $F_{3,17}$=5.42, p = 0.0084; Fig. 6), compared with GAL(1−15) ($p \leq 0.05$) and control group ($p < 0.01$). However, when GAL(1−15) and NTX were administered together, this increase in dopamine was reversed, suggesting that the combination of the two compounds reduced the effect produced by the alcohol injection.

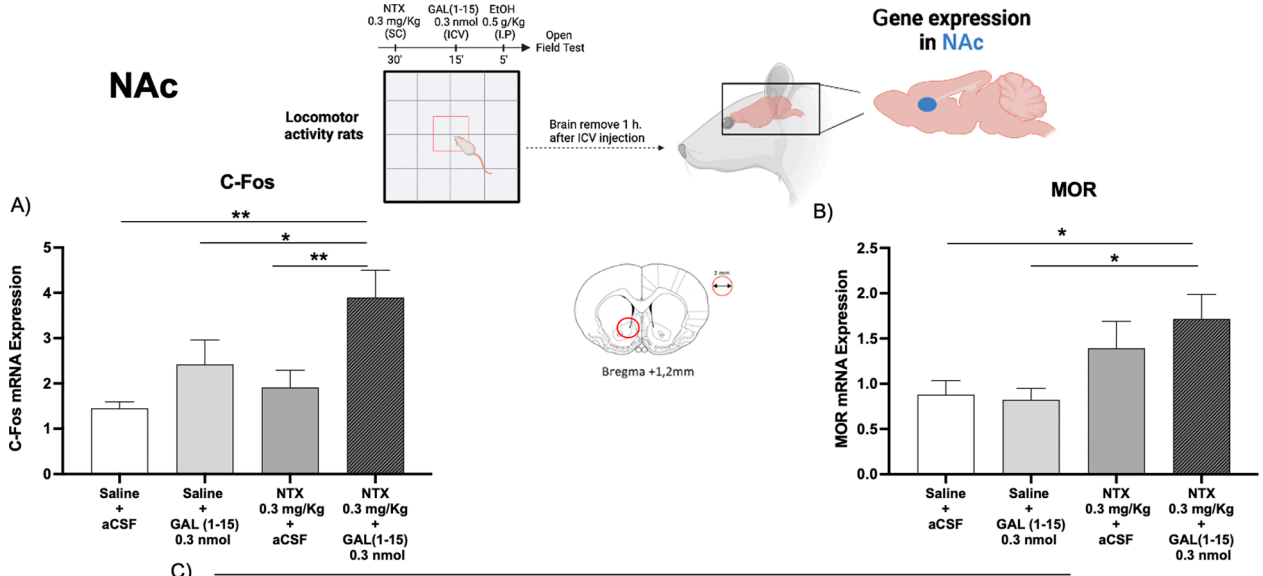
3.5. Effect of the combination of GAL(1−15) and NTX on gene expression in VTA, NAc and hypothalamus after an alcohol injection

3.5.1. The combination of GAL(1−15) and NTX increased the gene expression of C-Fos and MOR in VTA

As shown in Fig. 7, in the VTA, the administration of GAL(1−15) 0.3 nmol or NTX 0.3 mg/Kg alone did not affect the C-Fos expression in animals after 0.5 mg/Kg ethanol injection. However, the combination of GAL(1−15) and NTX produced a significant increase in the mRNA level of C-Fos (one-way ANOVA, $F_{3,16}$= 3.33, p = 0.046; Fig. 7A) compared to the control group one h after its administration ($p < 0.01$).

In the same way, only the group treated with the combination of GAL (1−15)-NTX, and not the groups treated with GAL(1−15) or NTX alone, induced a significant increase in MOR opioid receptor expression (one-way ANOVA, $F_{3,15}$= 3.90, p = 0.031; Fig. 7B), compared to the control group in the VTA ($p \leq 0.05$).

Additionally, the expression levels of mRNA for the dopamine transporters DAT and VMAT2, the dopamine receptors D1, D2, and D3, as well as the Galanin receptors GALR1, GALR2, and GALR3, were studied, and no significant differences were found across the different



C)

NAc	Saline + aCSF	Saline + GAL(1-15) 0.3 nmol	NTX 0.3 mg/Kg + aCSF	NTX 0.3 mg/Kg + GAL(1-15) 0.3 nmol
D1	1.1 ± 0.3	1.3 ± 0.7	6.2 ± 2.1	8.3 ± 2.6*
D2	0.4 ± 0.1	0.9 ± 0.4[a]	1.9 ± 0.4**	1.0 ± 0.3
GALR1	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.6 ± 0.1
GALR2	0.7 ± 0.1	0.9 ± 0.2	0.7 ± 0.1	1.1 ± 0.2
GALR3	0.7 ± 0.1	1.2 ± 0.5	0.4 ± 0.0	0.9 ± 0.1

Fig. 8. Effect of the administration of GAL(1−15) and NTX on C-Fos, MOR, D1, D2, D3, GALR1, GALR2 and GALR3 mRNA expression in the nucleus accumbens (NAc). Measures were taken 1 h after ICV injection. The experimental groups have between 5 and 6 animals per group. Vertical bars represent a mean ± standard error of the mean of the mRNA expression of: **(A)** C-Fos. * $p \leq 0.05$ saline + GAL(1−15) 0.3 nmol vs NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol; * * $p < 0.01$ NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol vs saline + aCSF, NTX 0.3 mg/Kg + aCSF, according to one-way ANOVA followed by Fisher multiple comparison test. **(B)** MOR. * $p \leq 0.05$ NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol vs saline + aCSF, saline + GAL(1−15) 0.3 nmol, according to one-way ANOVA followed by Fisher multiple comparison test. **(C)** Data represent a mean ± standard error of the mean of the mRNA expression of D1: * $p \leq 0.05$ vs saline + aCSF, saline + GAL(1−15) 0.3 nmol. D2: [a] $p \leq 0.05$ vs NTX 0.3 mg/Kg + aCSF; * * $p < 0.01$ vs saline + aCSF, according to one-way ANOVA followed by Fisher multiple comparison test. GALR1, GALR2, GALR3: There are no differences according to a one-way analysis of variance (ANOVA) between the experimental groups.

treatments (Fig. 7C).

3.5.2. The combination of GAL(1−15) and NTX increased the gene expression of C-Fos, MOR and D1 receptors in NAc

We have analyzed the mRNA expression of C-Fos, Mu-Opioid receptor (MOR), dopamine receptors D1 and D2, and Galanin receptors GALR1, GALR2 and GALR3 in the NAc.

As seen in Fig. 8, the coadministration of GAL(1−15) 0.3 nmol and NTX 0.3 mg/Kg produced a significant increase in the C-Fos expression (one-way ANOVA, $F_{3,19}$= 5.03, p = 0.0098; Fig. 8A), compared to the control group ($p < 0.01$), while the single icv injection of GAL(1−15) 0.3 nmol or NTX 0.3 mg/Kg alone did not affect the mRNA level of C-Fos one h after de administration.

In the MOR mRNA expression, again only the combination of GAL (1−15)-NTX induced an increase in the MOR expression (one-way ANOVA, $F_{3,19}$= 3.37, p = 0.0403; Fig. 8B) compared with the control group ($p \leq 0.05$). The administration of GAL(1−15) or NTX alone did not modify the MOR expression.

In the dopamine receptors mRNA levels, the expression of the D1 receptor was significantly increased in the GAL(1−15)-NTX injected rats (one-way ANOVA, $F_{3,18}$= 3.76, p = 0.0296; Fig. 8C), compared to the control group ($p \leq 0.05$). The rest of the group did not affect the D1 expression.

In the D2 receptor, the administration of NTX 0.3 mg/Kg produced an increase in the levels of mRNA (one-way ANOVA, $F_{3,19}$= 4.15, p = 0.202; Fig. 8C), compared with the control group ($p < 0.01$) and the GAL(1−15) 0.3 nmol group ($p \leq 0.05$), but no differences were founded with the GAL(1−15)-NTX group.

Finally, in the analysis of Galanin receptor expression, the expression of GALR1, GALR2, or GALR3 was not modified by any treatment.

3.5.3. Effects of the combination of GAL(1−15)-NTX in the mRNA expression in the hypothalamus

As shown in Table 1, the administration of NTX 0.3 mg/Kg alone (one-way ANOVA, $F_{3,17}$= 5.72 p = 0.0068; $p < 0.01$) and the coadministration GAL(1−15)-NTX ($p \leq 0.05$) produced an increase in the C-Fos expression compared with the control group in animals after 0.5 mg/Kg ethanol injection. The administration of GAL(1−15) 0.3 nmol did not modify the C-Fos expression.

The expression of MOR or POMC was not modified by any treatment in the hypothalamus.

4. Discussion

In the present study, we demonstrated that the combination of GAL (1−15) with NTX induced a substantial reduction in alcohol-seeking behavior in the operant model of alcohol self-administration. Moreover, GAL(1−15) with NTX reduced the alcohol intake in the two-bottle choice test, a voluntary model of alcohol intake. GALR2 was involved in this effect since the specific GALR2 antagonist M871 blocked the GAL (1−15)+NTX mediated action in alcohol self-administration. Importantly, GAL(1−15) with NTX completely removed the increase of locomotor activity induced by the ip alcohol injection, as well as the stereotyped behavior. These effects were related to a reduction of dopamine release induced by administering GAL(1−15) and NTX in the dorsal striatum. The mesolimbic circuitry participated in the GAL (1−15)+NTX behavior-mediated actions since we observed changes in the immediate-early gene C-Fos, mu-opioid receptor (MOR) and dopamine receptors in VTA and NAc.

In this study, we have utilized two models to investigate addiction-related behaviors. Alcohol self-administration, a widely used model for studying drug-seeking motivated behavior [27,28], addresses motivational dysregulation, a defining feature of addiction [29]. The two-bottle choice paradigm, the most well-known variant of voluntary consumption models, also measures alcohol intake [30]. While an increase in alcohol consumption is a hallmark of alcoholism, this model does not capture the motivational component of behavior [31].

We have used NTX, an antagonist of the mu-opioid receptor, with additional affinity for kappa and delta opioid receptors [10] and the most effective medication currently available to treat AUD [32] (for review, see O'Malley & Froehlich [33]). NTX reduces heavy drinking in both alcohol-dependent and nondependent individuals, including young adults [34–36]. However, some studies report limited efficacy, with randomized trials showing no significant difference between NTX and placebo, partly due to adverse effects [37]. NTX blocks the therapeutic effects of opioid analgesics and may induce withdrawal in opioid-dependent patients [38]. It is also contraindicated for those with acute liver disease, restricting its use in alcohol-associated liver disease cases [10,39]. Overall, NTX has a moderate impact on reducing alcohol use [10].

Combining naltrexone with other medications is another approach to enhancing treatment response that has recently been successfully implemented in the treatment of addictions such as AUD [40]. Compared to monotherapies, combining therapies involving more than one medication enables drugs to be effective even at lower doses, reducing the adverse side effects that often hinder patient adherence to treatment [41].

This work demonstrates for the first time that the combination of non-effective doses of GAL(1−15) and NTX produces a substantial reduction in alcohol consumption and seeking behavior, as tested in alcohol self-administration and two-bottle choice. The combination of the two models used in this study-where both the motivational drive to consume alcohol and direct alcohol intake are examined-opens up the possibility of using the GAL(1−15) and NTX combination as a pharmacological therapy for AUD.

Previous studies have shown that GAL(1−15) effectively reduces alcohol motivation, both by decreasing alcohol consumption in a voluntary intake model [23] and by lowering alcohol-seeking behavior in an operant self-administration model [24]. We have licensed an international patent on GAL(1−15) and analogues thereof for use in the prevention and/or treatment of alcohol-related disorders and effects (WO2019/068948 A1). The results obtained in this work give new evidence of the GAL(1−15) implication on alcohol motivation and open the possibility of using it as pharmacological therapy for AUD.

The GALR2 receptor plays a key role in the effects of the GAL (1−15)+NTX combination on alcohol motivation, as shown by the fact that the GALR2 receptor antagonist M871 blocks the GAL(1−15)+NTX effects in the alcohol self-administration test. Previous studies have demonstrated, using siRNA for GALR1 and GALR2, that GAL(1−15) acts through the GALR1-GALR2 heterodimer [21,22,24,42,43]. Therefore, blocking GALR2 with the GALR2 antagonist M871 was sufficient to

Table 1
Effects of Galanin(1−15) and Naltrexone in the mRNA expression in the hypothalamus.

	Hypothalamus			
	Saline + aCSF	**Saline + GAL(1−15) 0.3 nmol**	**NTX 0.3 mg/Kg + aCSF**	**NTX 0.3 mg/Kg + GAL(1−15) 0.3 nmol**
C-Fos	**0.6 ± 0.1**	**0.7 ± 0.1[aa]**	**1.7 ± 0.3 * ***	**1.3 ± 0.2 ***
MOR	0.9 ± 0.2	1.0 ± 0.3	1.3 ± 0.4	0.9 ± 0.1
POMC	0.8 ± 0.1	0.8 ± 0.1	1.7 ± 0.5	1.0 ± 0.2

Effect of the administration of GAL(1−15) and NTX on C-Fos, MOR and POMC mRNA expression in the hypothalamus (HYPO). Measures were taken one h after the ICV injection. The experimental groups have between 5 and 6 animals per group. Data represent a mean ± standard error of the mean of the mRNA expression of C-Fos: * $p \leq 0.05$ vs saline + aCSF; * * $p < 0.01$ vs saline + aCSF; [aa] $p < 0.01$ vs NTX 0.3 mg/Kg + aCSF, according to one way ANOVA followed by Fisher multiple comparison test. MOR and POMC: There are no differences according to a one-way analysis of variance (ANOVA) between the experimental groups.

inhibit the reduction in alcohol-seeking behavior mediated by the GAL (1−15) + NTX interaction.

Alcohol administration modulates the locomotor activity and stereotyped behaviors through dopamine release in key regions such as the VTA and dorsal striatum [13,44,45]. In this study, intraperitoneal alcohol administration increased locomotor activity and dopamine release in rats, effects reversed by the GAL(1−15)+NTX combination, which reduced both locomotor activity and dopamine levels in the dorsal striatum. This combination also decreased stereotyped behaviors, highlighting dopamine's role in these effects. Alcohol's impact on motivation is closely linked to dopamine, a key neurotransmitter in the reward system and substance addiction [46].

The mesolimbic circuit is involved in GAL(1−15)+NTX combination mediated- effects. The combination of GAL(1−15) and NTX significantly increased immediate early C-Fos gene expression in the VTA, NAc and hypothalamus, indicating increased neuronal activity in key reward brain areas. The key mesolimbic reward nodes, VTA and NAc, are critical for developing and maintaining alcohol reinforcement [47] and drug addiction [48,49]. In addition, in NAc, GAL(1−15)+NTX increased the expression of the D1 receptor, a key role receptor in the brain reward system and alcohol abuse [50].

Interestingly, also the MOR receptor expression was modified in the VTA and NAc, central to positive reinforcement of alcohol. The dopamine release in the VTA is partially enhanced by stimulation of MOR located on inhibitory GABAergic interneurons in the VTA [51–53]. The data showed that only the combination of GAL(1−15)-NTX increased the MOR expression in both nuclei, suggesting a modulation of the opioid system.

Several studies have demonstrated an antagonistic interaction between Galanin and the opioid system [16,54,55]. Recently, MOR-GALR1 functional heteromers in the VTA were identified as regulators of dopaminergic cell activation [18,56], potentially explaining the previously observed GAL-opioid antagonism [18]. GAL(1−15) preferentially binds GALR1-GALR2 heteroreceptor complexes, which can form receptor mosaics like GALR1-GALR2–5HT1A [20,21,42,43,57–62]. This suggests GAL(1−15)+NTX effects on alcohol-related testing might involve a GALR1-GALR2-MOR mosaic, warranting further investigation.

In conclusion, our results indicate a potent effect of combining GAL (1−15) with NTX in reducing alcohol-motivated reward seeking, a process in which the mesolimbic pathway is actively involved by the modulation of the opioid system. These results open up the possibility of using GAL(1−15) in combination with NTX as a novel strategy in AUD.

CRediT authorship contribution statement

Millón Carmelo: Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Flores-Burgess Antonio:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Díaz-Cabiale Zaida:** Writing – review & editing, Supervision, Funding acquisition. **Pineda-Gómez Juan Pedro:** Methodology, Investigation. **Moh-Ahmed Amel:** Methodology. **Flores-Gómez Marta:** Methodology, Investigation, Formal analysis, Conceptualization. **Cantero-García Noelia:** Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118170.

Data availability

The data that support this study are openly available in RIUMA-University of Malaga at http://doi.org[doi], reference number [reference number] once the manuscript is accepted for publication.

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