Biomedicine & Pharmacotherapy 153 (2022) 113508

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Galanin N-terminal fragment (1−15) reduces alcohol seeking and alcohol relapse in rats: Involvement of mesocorticolimbic system

Noelia Cantero-García [a], Antonio Flores-Burgess [a], Juan Pedro Pineda-Gómez [a], Laura Orio [b], Antonia Serrano [c], Zaida Díaz-Cabiale [a,*], Carmelo Millón [a,b,**]

[a] Universidad de Málaga, Instituto de Investigación Biomédica de Málaga, Facultad de Medicina, Campus de Teatinos s/n, 29071 Málaga, Spain
[b] Departamento de Psicobiología y Metodología en Ciencias del Comportamiento, Facultad de Psicología, Universidad Complutense de Madrid, Spain
[c] Unidad de Gestión Clínica de Salud Mental e Instituto de Investigación Biomédica de Málaga, Hospital Regional Universitario de Málaga, Málaga 29010, Spain

ARTICLE INFO

Keywords:
Alcohol
Galanin
Galanin (1−15)
Alcohol seeking
Relapse

ABSTRACT

Alcohol Use Disorder (AUD) is among the most prevalent mental illnesses, and due to the low efficacy of the current medication, it is essential to find new biological targets that could modulate alcohol consumption. Since Galanin (1−15) [GAL(1−15)] produces a loss of motivational behaviour by an artificial reinforcer and decreases the preference an alcohol consumption in a voluntary alcohol intake, we have studied the role of GAL(1−15) in alcohol-seeking behaviour and the involvement of the corticomesolimbic system as well as the role of GAL(1−15) in context-induced alcohol relapse. In rats, we have studied GAL(1−15)-effects on alcohol-seeking in self-administration, in fixed-ratio (FR1) and progressive-ratio (PR), and the involvement of GAL receptors using siRNA GALR1 or GALR2 knockdown animals. We have analysed the transcriptional changes of C-Fos, dopamine receptors, GAL receptors and 5HT1A receptors in the corticomesolimbic system. Also, we have examined the effect of GAL(1−15) in context-induced alcohol relapse. GAL(1−15) substantially reduced alcohol-seeking behaviour in the operant self-administration model in an FR1 protocol and at the breaking point in a PR schedule. GALR1and GALR2 were involved in these effects, as indicated by the analysis by GALR2 antagonist and GALR1 and GALR2 knockdown animals. Notably, the mechanism of GAL(1−15)-mediated actions involved changes in C-Fos, Dopamine receptors and 5HT1A expression in the ventral tegmental area, accumbens nucleus and prefrontal cortex. Significantly, GAL(1−15) reduced the context-induced alcohol relapse. These results open up the possibility to use GAL(1−15) as a novel strategy in AUD.

1. Introduction

Alcohol Use Disorder (AUD) is among the most prevalent mental illnesses worldwide [1]. The total alcohol per capita consumption in the world's population over 15 years of age rose from 5.55.5 litres of pure alcohol in 2005–6.4 litres in 2016, resulting in some 3 million deaths (5.3% of all deaths) worldwide [2]. In 2016, 2.3 billion people were current drinkers.

Nowadays, the 2019 coronavirus disease (COVID-19) pandemic may also impact the population's alcohol use patterns [3–5]. Increased anxiety, depression, and stress in response to COVID-19 may be associated with increased alcohol use [5].

Another relevant factor of AUD patients is that more than 50% of new patients with alcohol abuse relapse within three months [6,7] being the main trigger the stressful events, the drug-associated cues and context, or the re-exposure to a small amount of alcohol [7,8].

However, AUD remains severely undertreated, with less than 10% of adults with AUD in Europe receiving pharmacotherapy and/or psychotherapy treatment [9,10]. Moreover, pharmacotherapies have seen limited use in the treatment of AUD, partially due to the low efficacy of the medication [10]. Therefore, it is essential to find new biological targets that could modulate alcohol consumption.

Recent studies indicate that neuropeptidergic signalling, including Galanin (GAL), is involved in alcohol consumption [11].

Galanin (GAL) is a neuropeptide [12] widely distributed in neurons within the central nervous system (CNS) [13]. Three GAL receptor (GALR1–3) subtypes with high affinities for GAL exist [14,15].

GAL participates in several central functions [11,15–17] and also it is

* Correspondence to: Departamento de Fisiología, Facultad de Medicina, Universidad de Málag, Campus de Teatinos s/n., 29080 Málaga, Spain.
** Correspondence to: Departamento de Fisiología, Facultad de Medicina, Universidad de Málaga, Campus de Teatinos s/n., 29080 Málaga, Spain.
E-mail addresses: zaida@uma.es (Z. Díaz-Cabiale), carmelomp@uma.es (C. Millón).

https://doi.org/10.1016/j.biopha.2022.113508
Received 1 June 2022; Received in revised form 25 July 2022; Accepted 30 July 2022
Available online 4 August 2022

involved in drug abuse and addiction [18], including alcohol intake and alcoholism [11,19–21].

Galanin (1−15) [GAL(1−15)] is an N-terminal fragment of GAL, which is active in the CNS [16,22–24]. GAL(1−15) shows a differential and specific role than the complete molecule of GAL in several central functions, including depression, anxiety and alcohol intake [23–25].

We have described that GAL(1−15) induced a solid anhedonia-like phenotype in non-operant and operant models, producing a loss of motivational behaviour caused by an artificial reinforcer [26]. This anhedonic-like effect of GAL(1−15) was accompanied by the dopaminergic mesolimbic system modulation [26].

Moreover, GAL(1−15) decreased, via central mechanisms, the preference and alcohol consumption in a voluntary alcohol intake model in rats [24]. GAL(1−15) induced a significant increase in immediate-early gene C-Fos in the striatum, indicating a high relevance of this nucleus in the GAL(1−15)-mediated effects on the voluntary alcohol intake [24]. Also, the critical role of the striatum as a target for GAL(1−15) was supported by the ability of GAL(1−15) to enhance the suppression of locomotor activity induced by ethanol [24].

Recently, we have also suggested using the combination of GAL (1−15) with the antidepressant SSRIs Escitalopram (ESC) in depression and AUD comorbidity. This combination reduces the ethanol intake in the alcohol self-administration paradigm and reverses the adverse ESC-mediated effects in depression-related behavioural tests [27].

The purpose of the current study was to assess the role of GAL(1−15) in alcohol-seeking behaviour using alcohol self-administration, a widely accepted tool for studying drug-seeking motivated behaviour. Moreover, GALR1 and GALR2 in GAL(1−15)-mediated effects in this test were analysed with the selective GALR2 antagonist M871 and using an in vivo model siRNA GALR1 or GALR2 knockdown rats. To investigate the mesocorticolimbic circuitry in the effect of GAL(1−15) in alcohol-seeking behaviour, we analysed transcriptional changes in the ventral tegmental area (VTA), accumbens nucleus (NAc) and prefrontal cortex [28] on the mRNA expression of dopamine transporters DAT and Vmat2; the C-Fos gene; the dopamine receptors D1, D2, D3, D5; the GAL receptors GALR1 and GALR2; and the serotonin receptor 5HT1A.

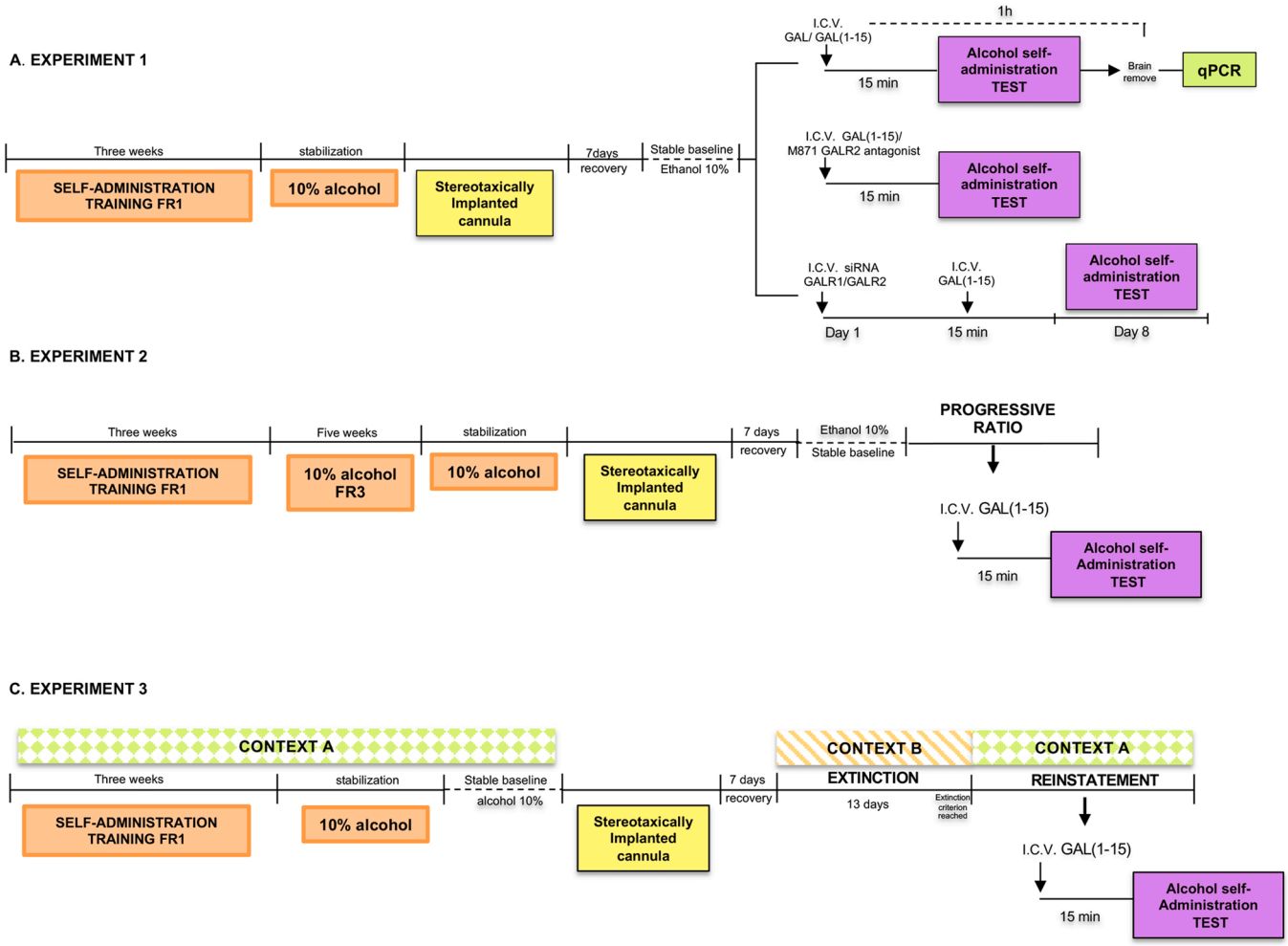

Fig. 1. Diagram of the complete experimental schedule. **A.** The animals were trained in the self-administration boxes to consume 10% alcohol under a fixed ratio 1 (FR1). Subsequently, stereotaxic surgery was performed. GAL and GAL(1−15) were injected icv 15 min before the alcohol self-administration test. The animals were euthanised by decapitation one hour after a single icv administration of GAL(1−15) or aCSF and the brains were used to perform qPCR. To study the receptors involved in the effect of GAL(1−15), GAL(1−15) 3nmol and the M871 GALR2 antagonist were injected icv 15 min before the alcohol self-administration test. Also, we have used knockdown rats for galanin receptors GALR1 or GALR2 in the alcohol self-administration test; siRNA GALR1, siRNA GALR2 or Delivery Media (DM) were injected icv eight days before the alcohol self-administration test. GAL(1−15) or aCSF were injected 15 min before the test. **B.** Animals were trained in the self-administration boxes to consume 10% alcohol under FR1 for three weeks and then under FR3 for five weeks. Subsequently, stereotaxic surgery was performed. GAL (1−15) was injected icv 15 min before the alcohol self-administration test to study the progressive ratio. **C.** The animals were trained in the self-administration boxes to consume 10% alcohol in context A under a FR1. Subsequently, stereotaxic surgery was performed. Extinction was carried out in context B. Alcoholic relapse was carried out in context A. GAL(1−15) was injected icv 15 min before the alcohol self-administration test to study the alcoholic relapse.; I.C.V: intracerebroventricular GAL: Galanin; GAL(1−15): Galanin (1−15); aCSF: artificial cerebrospinal fluid.

We have also studied the degree of motivation for alcohol after administering GAL(1−15) through a breaking point test in a Progressive Ratio schedule, and we have determined whether GAL(1−15) attenuates the context-induced alcohol relapse.

2. Material and methods

2.1. Animals

Male Sprague Dawley rats (body weight 225 – 250 g) were obtained from criffa and maintained in a humidity-controlled and temperature-controlled (20–22 °C) room. Rats were during the entire protocol maintained on a 12-hour reversed light/dark cycle (lights off at 9 am). All animal experimentation was conducted in accordance with the University of Málaga Guidelines for the Care and Use of Laboratory Animals (Ethic Code: 22/05/2017/066). To carry out the experiments, the animals were chosen at random.

Detailed descriptions are available in the supplementary material on the animal controlled-conditions, surgical preparation and icv injections.

2.2. Materials

GAL, GAL(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 the experimental design is shown in Fig. 1.

2.3.1. Experiment 1

We have analysed the effect of the administration of GAL(1−15) on the alcohol self-administration test and the GAL receptors involved (Fig. 1 A).

Rats were trained to self-administer 10% alcohol solution initially under fixed-ratio 1 (FR1) for three weeks, as previously described [29].

Three sets of experiments were conducted on the alcohol self-administration. In the first set of experiments, a dose-response curve of GAL(1−15) was performed. For this, groups of rats received icv GAL (1−15) 0.3 (n = 6), 1 (n = 11), 3 nmol (n = 9) or cerebrospinal fluid-injected (aCSF) (n = 13) 15 min before the test. In the second set of experiments, the effects of the alcohol self-administration test of GAL and GAL(1−15) were compared. For this, groups of rats received icv GAL 3 nmol (n = 9), GAL(1−15) 3 nmol (n = 11) or aCSF (n = 13) 15 min before the test.

In the last set of experiments, the GAL receptors involved in the effect of GAL(1−15) were studied; for this, groups of rats received icv GAL (1−15) 3 nmol (n = 10), M871 3nmol antagonist (n = 6), GAL(1−15) 3nmol combined with GALR2 antagonist or cerebrospinal fluid-injected (aCSF) (n = 15) 15 min before the test. In addition, we have used knockdown rats for galanin receptors GALR1 or GALR2 in the alcohol self-administration test; siRNA GALR1(n = 5), siRNA GALR2 (n = 6) or Delivery Media (DM) (n = 10) were injected icv eight days before the alcohol self-administration test. GAL(1−15) or aCSF were injected 15 min before the test. Treatments were administered between-subject.

2.3.2. Experiment 2

We have analysed the effect of the administration of GAL(1−15) on alcohol seeking-behaviour in a progressive ratio schedule (Fig. 1B).

Rats were trained to self-administer 10% alcohol solution initially under fixed-ratio FR1 for three weeks and then under FR3 for five weeks, as previously described [29,30] with minor modifications (see the supplementary material for details).

A progressive ratio (PR) schedule test was performed to evaluate the effect of GAL(1−15) 3nmol on the motivation to consume alcohol as described previously [31]. In this test, the effort necessary to obtain one reward (i.e. the number of presses on the active lever) was continuously increased after each reward delivery (1,2,3,4,5,6,7 etc.). During the one hour session, the maximum ratio value (breaking point) completed receiving a single reward of alcohol was measured and considered an index of motivation. For this, groups of rats received icv GAL(1−15) 3 nmol (n = 11) or aCSF (n = 14) 15 min before the alcohol self-administration test. Treatments were administered within subject and was seven days elapsed between treatments.

2.3.3. Experiment 3

We analysed the effect of the administration of GAL(1−15) on extinction and context-induced alcohol relapse (Fig. 1 C).

Rats were trained to self-administer 10% alcohol solution initially under fixed-ratio FR1 for three weeks, as previously described [29,31, 32] with minor modifications (see the supplementary material for details). After 24 days on FR1 (context A), context extinction was carried out. During extinction, the rats continued to be placed in the chambers for one hour daily, and operant responding was extinguished by removing all consequences of lever responding, i.e., cue-light, pump sound, grid and alcohol (context B). The extinction criterion was two consecutive sessions with the number of presses less than 20 per cent of the baseline, i.e. the mean of the two last sessions before the beginning of extinction [31]. As an extinction test, GAL(1−15) 3nmol was injected icv 15 min before the first extinction session (see the supplementary material for details).

When the extinction criterion was reached, the reacquisition model of relapse was performed, as described previously [31]. Alcohol, light, pump sound and grid were reintroduced in the self-administration boxes (context A). Rats were injected icv with GAL(1−15) 3nmol (n = 12) or aCSF (n = 11) 15 min before the one-hour alcohol self-administration test. All treatments were administered between-subject.

2.4. Behavioural assessment

2.4.1. Alcohol self-administration test

Alcohol seeking was assessed using the self-administration test, performed as described previously [29,31]. First, a pre-training phase was carried out in Experiments 1, 2 and 3 (see the supplementary information for details). After that, rats were trained to self-administered 10% alcohol in 30 min sessions on an FR1 schedule of reinforcement. Finally, all the animals reached a stable level of 10% alcohol responding. One lever was paired with the delivery of alcohol as a reward (active lever), whereas the other lever was paired with no reward (inactive lever). Active lever pressing was paired with a conditioned stimulus light adjacent to the lever was illuminated when the FR1 requirement was met and signalled to the rat that alcohol had been dispensed as a reward. During the test sessions, the responses on the active lever, inactive lever and number of alcohol reinforcements were recorded.

2.4.2. Generation of siRNA GALR1 and siRNA GALR2 rats by siRNA Accell Smartpool injection

Knockdown rats were generated as previously described [25,33]. Using real-time quantitative PCR, we have previously performed a time course of GALR1 or GALR2 mRNA in the dorsal hippocampus [25,33], and we had also performed a time course of GALR1 or GALR2 protein expression using quantification of immunohistochemical staining for GALR1 or GALR2 in the hippocampus [25,33]. The time course curve indicated a maximal reduction of GALR1 or GALR2 receptor protein expression 8 days after the injection [25,33].

Briefly, during the stereotaxic surgery, once the cannula is fixed, animals received an intracerebroventricular (icv) injection of 5 μg (0.35 nmol) of Accell Smart pool siRNA for GALR2 or GALR1 (Dharmacon, Lafayette, EEUU). Animals had a recovery period of 8 days before the behavioural test, the time required to reduce the levels of GAL receptors as previously described [25].

2.5. Genes expression in VTA, NAc and PFC

Rats from the alcohol self-administration test on experiment 1 were euthanised by decapitation 1 h after a single icv administration of GAL (1−15) 3 nmol or aCSF. The brains were rapidly removed and frozen until use. An experimental group of basal animals that had not previously consumed alcohol and not received any treatment was added. The dose and time selected were based on previous works, GAL(1−15) (3 nmol/rat) (n = 5–7), basal group (n = 4–5), aCSF (n = 4–8) [25,34]. The nuclei dissections were conducted as described [34] with modifications (see supplementary material for details).

The procedure for RNA isolation and RT-PCRs was described previously [25,26,33] (see the supplementary material for details).

The primer sequences used to evaluate the mRNA expression levels of the genes C-Fos, DAT, Vmat2, D1, D2, D3, D5, 5HT1A and GALR1 and GALR2 are shown in the supplementary material.

2.6. Statistical and data analysis

Data are presented as the mean ± standard error of the mean, and sample numbers (n) are indicated in figure legends. All data were analysed using GraphPad PRISM 8.0 (GraphPad Software, San Diego, CA, USA). For comparing two experimental conditions, Student's unpaired t-tests were performed. For comparing more than two groups, one-way analysis of variance (ANOVA) was performed. 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 $p \leq 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

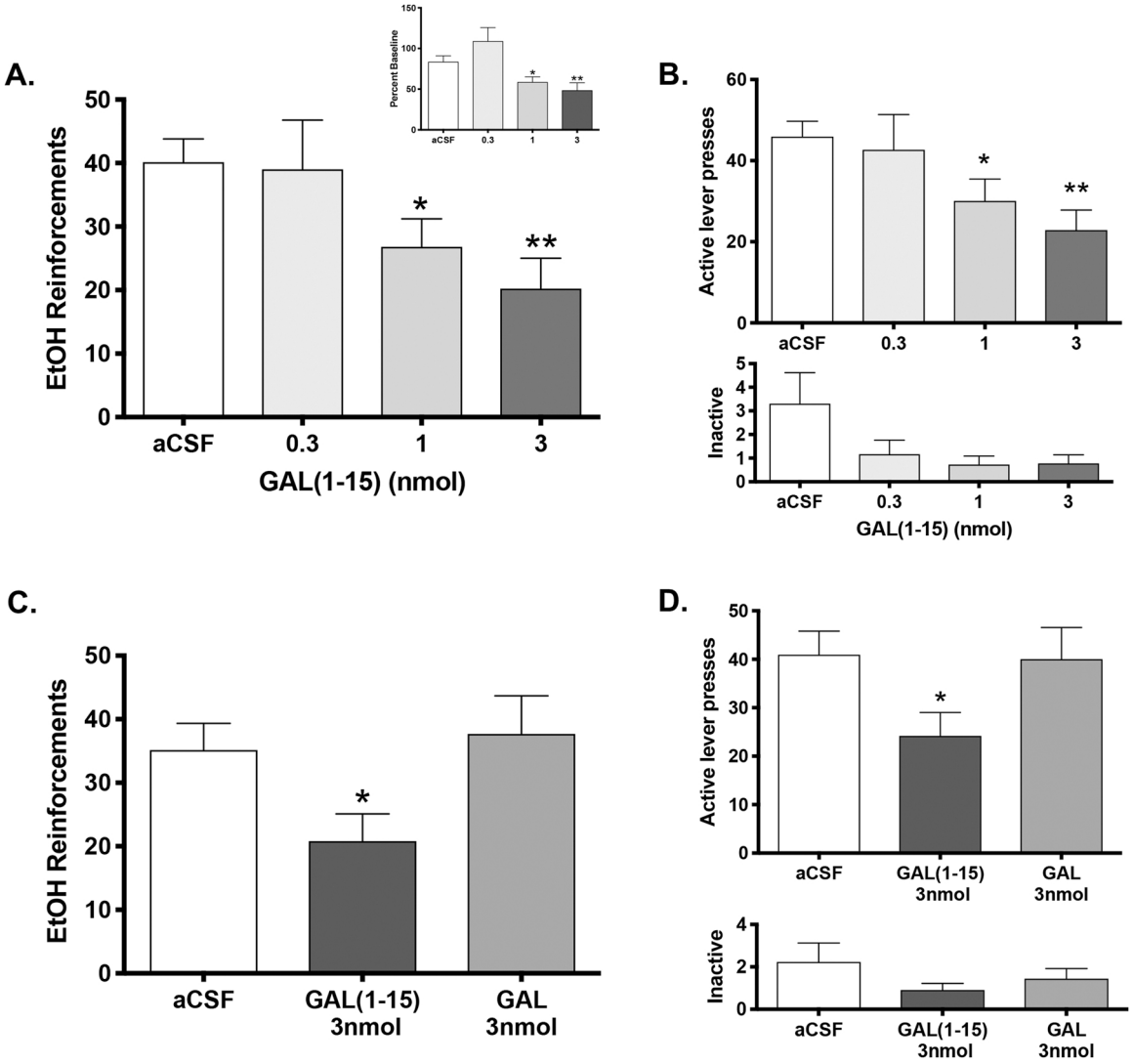

Fig. 2. Effect of the administration of Galanin(1−15) [GAL(1−15)] on the alcohol self-administration test. A-B. GAL(1−15) 0.3nmol (n = 6), 1nmol (n = 11), 3nmol (n = 9) or artificial cerebrospinal fluid (aCSF) (n = 13) were administered icv 15 min before the test. **C-D.** GAL(1−15) 3nmol (n = 11), GAL 3nmol (n = 9) or artificial cerebrospinal fluid (aCSF) (n = 13) were administered icv 15 min before the test. aCSF injected rats were used as control group. **A.** Vertical bars represent a mean ± standard error of the mean of the number of alcohol reinforcements and in the upper graph the number of alcohol reinforcements according to Percent Baseline during the test period. * $p < 0.05$; ** $p < 0.01$ vs control group, according to one way ANOVA followed by Fisher Multiple Comparison Test. **B.** Vertical bars represent a mean ± standard error of the mean of the active lever and inactive lever presses. * $p < 0.05$; ** $p < 0.01$ vs control group, according to one way ANOVA followed by Fisher Multiple Comparison Test. **C.** Vertical bars represent a mean ± standard error of the mean of the number of alcohol reinforcements during the test period. *$p < 0.05$ vs the rest of the groups, according to one way ANOVA followed by Fisher Multiple Comparison Test. **D.** Vertical bars represent a mean ± standard error of the mean of the active lever and inactive lever presses. * $p < 0.05$ vs the rest of the groups, according to one way ANOVA followed by Fisher Multiple Comparison Test.

3. Results

3.1. GAL(1−15) substantially reduced the alcohol-seeking behaviour on the alcohol self-administration test. siRNA GALR1 and siRNA GALR2 knockdown rats demonstrated the role of GALR1 and GALR2 in this effect

3.1.1. Dose-response curve of GAL(1−15) on the alcohol self-administration test

In alcohol self-administration test, GAL(1−15) 3nmol induced a significant reduction in the number of reinforcements by 50% (one-way ANOVA, $F_{3.35} = 4.11$, $p = 0.01$, Fisher's LSD post hoc: $p < 0.01$; Fig. 2 A) and in the active lever presses by 48% (one-way ANOVA, $F_{3.35} = 4.29$, $p = 0.01$, Fisher's LSD post hoc: $p < 0.01$; Fig. 2B) compared with control animals. GAL(1−15) (1nmol) induced a less strong but significant reduction in the number of reinforcements ($p < 0.05$) and active lever presses ($p < 0.05$) compared with control animals. GAL(1−15) at 0.3nmol lacked an effect in the alcohol self-administration test compared with controls animals.

GAL(1−15) did not affect the number of inactive lever presses in the alcohol self-administration at all doses (Fig. 2B).

3.1.2. GAL(1−15) but not GAL reduced the alcohol-seeking behaviour

In the alcohol self-administration test, GAL(1−15) 3nmol significantly reduces the number of reinforcements (one-way ANOVA, $F_{2.30} = 3.59$, $p = 0.04$, Fisher's LSD post hoc: $p < 0.05$; Fig. 2 C) and the active lever presses (one-way ANOVA, $F_{2.30} = 3.15$, $p = 0.06$, Fisher's LSD post hoc: $p < 0.05$; Fig. 2D) compared with GAL.

The complete GAL molecule does not affect the number of reinforcements or the active lever presses compared with control animals (Fig. 2 C, D).

There are no differences between the experimental groups in the inactive lever (Fig. 2D).

3.1.3. GALR1 and GALR2 are involved in GAL(1−15)-mediated effect on alcohol-seeking behaviour

We have tested the involvement of GALR in the GAL(1−15) effect

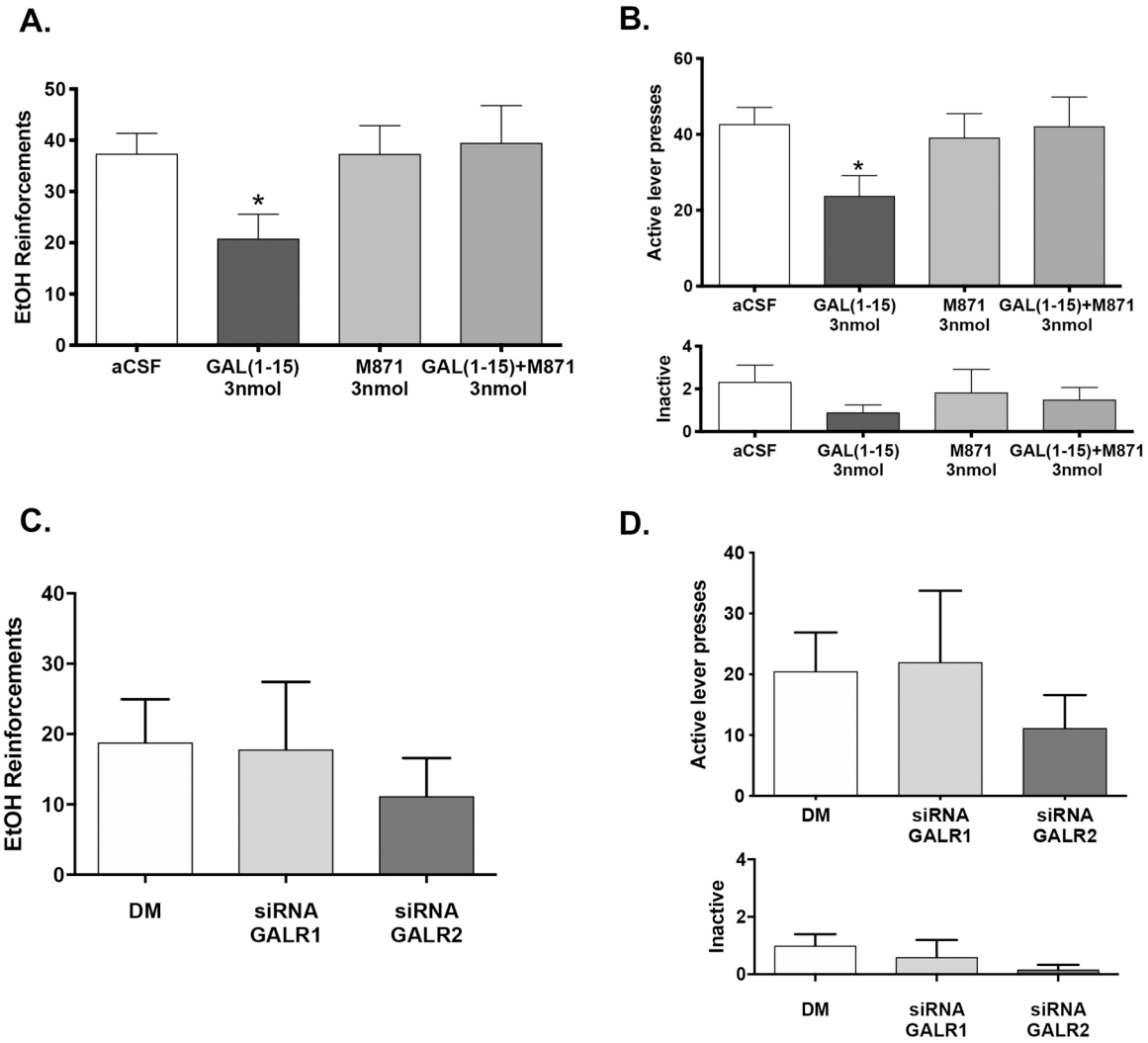

Fig. 3. GAL receptor subtype involved in Galanin(1−15) [GAL(1−15)] effect on motivated alcohol use. GAL(1−15) 3 mol (n = 10), GALR2 M871 antagonist (n = 6), GAL(1−15) 3 nmol combined with GALR2 antagonist M871 3nmol (n = 6) or cerebrospinal fluid-injected (aCSF) (n = 15) were injected icv 15 min before the test. For knockdown rats, Delivery Media (DM) (n = 10), siRNA GALR1 (n = 5), siRNA GALR2 (n = 6) were injected icv 8 days before the test. aCSF and DM were used as control group. **A.** Vertical bars represent a mean ± standard error of the mean of the number of alcohol reinforcements during the test period. *$p < 0.05$ vs the rest of the groups, according to a one-way analysis of variance (ANOVA) followed by Fisher's least significance difference test. **B.** Vertical bars represent a mean ± standard error of the mean of the active lever presses and inactive lever presses. *$p < 0.05$ vs aCSF, GAL(1−15) + M871 3nmol, according to a one-way analysis of variance (ANOVA) followed by Fisher's least significance difference test. **C.** Vertical bars represent a mean ± standard error of the mean of the number of alcohol reinforcements during the test period. **D.** Vertical bars represent a mean ± standard error of the mean of the active lever presses and inactive lever presses. There are no differences according to a one-way analysis of variance (ANOVA) between the experimental groups neither in the number of reinforcements or lever presses.

with the GALR2 antagonist M871 and using two in vivo rat models: siRNA GALR1 knockdown or siRNA GALR2 knockdown rats.

The GALR2 antagonist M871 3nmol significantly blocked the GAL (1−15)-induced reduction in the number of reinforcements of alcohol self-administration (one-way ANOVA, $F_{3.33} = 3.03$, $p = 0.04$ Fisher's LSD post hoc: $p < 0.05$; Fig. 3 A) and in the number of active lever presses (one-way ANOVA, $F_{3.33} = 2.78$, $p = 0.05$, Fisher's LSD post hoc: $p < 0.05$; Fig. 3B).

The GALR2 antagonist M871 injected alone in the dose of 3nmol lacked effects in the number of reinforcements of alcohol self-administration and the number of active lever presses (Fig. 3 A,B).

In addition, to study the receptors involved in the effect of GAL (1−15), we have used two in vivo rat models: siRNA GALR1 knockdown or siRNA GALR2 knockdown rats in the alcohol self-administration test.

Downregulation of GALR1 or GALR2 by siRNA did not affect any parameter in the alcohol self-administration test (Table S2). However, the decrease in GALR1 or GALR2 receptors by siRNA was sufficient to block the effect of GAL(1−15) in the alcohol self-administration test (Fig. 3C-D).

Thus, GAL(1−15) at the dose of 3nmol lacked effect in the number of reinforcements (one-way ANOVA, $F_{2.18} = 0.34$, $p = 0.71$), active lever presses (one-way ANOVA, $F_{2.18} = 0.52$, $p = 0.670$) neither inactive lever presses (one-way ANOVA, $F_{2.18} = 1.07$, $p = 0.36$) in the siRNA GALR1 knockdown or siRNA GALR2 knockdown rats (Fig. 3C-D).

3.2. Effect of GAL(1−15) on gene expression in VTA, NAc and PFC in an operant model of alcohol consumption

3.2.1. GAL(1−15) increased the mRNA expression of C-Fos in the VTA, the NAc and the PFC

In animals under chronic alcohol consumption by self-administration, GAL(1−15) 3 nmol produced a significant increase in the mRNA levels of C-Fos in the VTA (one-way ANOVA, $F_{2.13} = 11.1$, $p = 0.001$; Fisher's LSD post hoc: $p < 0.01$; Fig. 4 A), NAc (one-way ANOVA, $F_{2.12} = 7.38$, $p = 0.008$; Fisher's LSD post hoc: $p < 0.05$; Fig. 4B) and PFC (one-way ANOVA, $F_{2.14} = 5.18$, $p = 0.02$; Fisher's LSD post hoc: $p < 0.05$; Fig. 4 C) compared with aCSF group.

3.2.2. GAL(1−15) modified the mRNA expression of dopamine receptors in the VTA, NAc and PFC

We have analysed the mRNA expression of the dopamine receptors D1, D2, D3 and D5 in VTA, NAc and PFC.

As seen in Table 1, the administration of GAL(1−15) 3 nmol modified the mRNA expression of D3 in the VTA and NAc. In VTA, GAL (1−15) produced a significant increase in D3 mRNA levels compared with basal animals (one-way ANOVA, $F_{2.14} = 4.16$, $p = 0.04$; Fisher's LSD post hoc: $p < 0.05$; Table 1) while in the NAc, GAL(1−15) 3 nmol ($p < 0.01$) and aCSF ($p < 0.01$) induce a significant decrease in D3 mRNA expression compared with basal animals (one-way ANOVA, $F_{2.11} = 11.41$, $p = 0.002$; Fisher's LSD post hoc: $p < 0.01$; Table 1).

In PFC, GAL(1−15) increased D1 mRNA expression compared with basal ($p < 0.001$) and aCSF ($p < 0.01$) animals (one-way ANOVA, $F_{2.12} = 12.83$, $p = 0.001$; Fisher's LSD; Table 1).

GAL(1−15) lacked an effect on dopamine receptors D2 and D5 in the three areas analysed.

We have also studied the mRNA expression of dopamine transporters, Vmat2 and DAT, in the VTA. We observed that self-administered alcohol rats [aCSF or GAL(1−15)] showed an increased expression of Vmat2 compared to baseline animals (one-way ANOVA, $F_{2.12} = 7.03$, $p = 0.009$; Fig. S1) while there was no difference between the experimental group in the DAT mRNA levels (Fig. S1).

3.2.3. GAL(1−15) changed mRNA expression of 5HT1A in the VTA and PFC

In VTA, GAL(1−15) 3 nmol induced a significant increase of 5HT1A mRNA expression compared with basal group ($p < 0.01$), while aCSF also increased of 5HT1A expression ($p < 0.05$) (one-way ANOVA, $F_{2.12} = 8.59$, $p = 0.005$; Fisher's LSD; Table 1). In the PFC, both the administration of GAL(1−15) 3nmol ($p < 0.05$) and aCSF ($p < 0.05$) decreased 5HT1A mRNA levels compared with basal animals (one-way ANOVA, $F_{2.13} = 4.63$, $p = 0.03$; Fisher's LSD post hoc: $p < 0.05$).

No effect was observed in the NAc (Table 1).

3.2.4. GAL(1−15) lacked effect in the mRNA expression of GALR1 and GALR2 in the VTA, NAc and PFC

The administration of GAL(1−15) lacked an effect on GALR2 and GALR1 expression in the three areas analysed (Table 1).

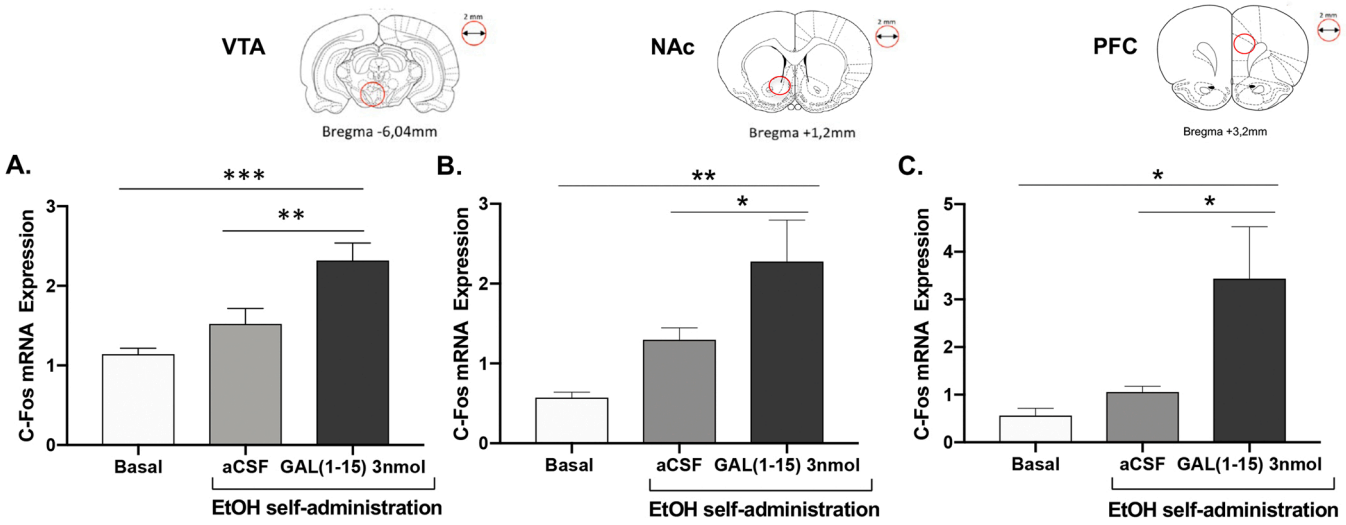

Fig. 4. Effects of Galanin(1−15) [GAL(1−15)] on C-Fos mRNA expression in the ventral tegmental area (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC). GAL(1−15) (3 nmol/rat) (n = 5–6) was injected icv one hour before the measures. Animals without previous alcohol consumption in self-administration was considered the basal group (n = 5). Cerebrospinal fluid-injected (aCSF) rats were used as the control group (n = 5–6). Vertical bars represent a mean ± standard error of the mean of the mRNA expression of C-Fos. **A.** ** $p < 0.01$ vs aCSF; ***$p < 0.001$ vs basal; B. *$p < 0.05$ vs aCSF; **$p < 0.01$ vs basal; C. *$p < 0.05$ vs aCSF, basal; according to a one-way analysis of variance (ANOVA) followed by Fisher's least significance difference.

Table 1
Effects of Galanin(1–15) [GAL(1–15)] on D1, D2, D3, D5, GALR1, GALR2 and 5HT1A mRNA expression in the ventral tegmental area (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC).

	VTA			*NAc*			*PFC*		
		EtOH self-administration			EtOH self-administration			EtOH self-administration	
	Basal	aCSF	GAL (1–15) 3 nmol	Basal	aCSF	GAL (1–15) 3 nmol	Basal	aCSF	GAL (1–15) 3 nmol
D1	1.23 ± 0.31	1.70 ± 0.29	1.73 ± 0.50	2.15 ± 0.11	1.42 ± 0.12	2.72 ± 0.64	**0.88 ± 0.17**	**0.93 ± 0.03**	**2.12 ± 0.2**$^{\diamond\diamond}$, ***
D2	3.33 ± 0.53	4.96 ± 1.09	4.38 ± 0.61	1.99 ± 0.21	1.47 ± 0.24	1.95 ± 0.35	0.97 ± 0.43	0.87 ± 0.19	0.93 ± 0.28
D3	**0.35 ± 0.05**	**0.95 ± 0.42**	**2.11 ± 0.57***	**5.26 ± 1.32**	**1.17 ± 0.12**$^{\#\#}$	**1.17 ± 0.29****	1.71 ± 0.40	3.74 ± 2.02	2.08 ± 0.58
D5	1.00 ± 0.17	1.05 ± 0.17	1.01 ± 0.24	1.97 ± 0.44	1.22 ± 0.14	2.93 ± 1.07	2.12 ± 0.32	1.28 ± 0.34	1.53 ± 0.20
GALR1	0.59 ± 0.15	1.29 ± 0.25	0.90 ± 0.28	0.72 ± 0.17	0.79 ± 0.09	1.42 ± 0.36	2.77 ± 0.59	2.73 ± 0.68	4.71 ± 0.93
GALR2	2.54 ± 0.41	4.24 ± 0.99	3.72 ± 0.60	0.35 ± 0.02	0.72 ± 0.18	0.69 ± 0.15	1.81 ± 0.47	1.06 ± 0.14	1.16 ± 0.16
5HT1A	**0.57 ± 0.15**	**0.93 ± 0.07**$^{\#}$	**1.19 ± 0.09****	1.62 ± 0.29	0.83 ± 0.05	1.58 ± 0.45	**2.50 ± 0.44**	**1.30 ± 0.28**$^{\#}$	**1.46 ± 0.15***

Effects of Galanin(1–15) [GAL(1–15)] on D1, D2, D3, D5, GALR1, GALR2 and 5HT1A mRNA expression in the ventral tegmental area (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC). GAL(1–15) (3 nmol/rat) (n = 5–7) was injected icv one hour before the measures. Animals without previous alcohol consumption in self-administration was considered the basal group (n = 4–5). Cerebrospinal fluid-injected (aCSF) rats were used as the control group (n = 4–8). VTA. D3: *p < 0.05 vs basal; 5HT1A: #p < 0.05 vs basal; **p < 0.01 vs basal; NAc. D3: $^{\#\#}$p < 0.01 vs basal; **p < 0.01 vs basal; PFC.D1: $^{\diamond\diamond}$p < 0.01 vs aCSF; ***p < 0.001 vs basal; 5HT1A: $^{\#}$p < 0.05 vs basal; *p < 0.05 vs basal; According to a one-way analysis of variance (ANOVA) followed by Fisher's least significance difference.

3.3. GAL(1−15) induced a substantial reduction in alcohol seeking-behaviour in a progressive ratio schedule

In the alcohol self-administration test, animals displayed a consistent preference for 10% v/v ethanol (active lever) over no reward (inactive lever) during the FR3 operant responding phase (Fig. 5 A).

In the alcohol self-administration, the animals injected icv with GAL (1−15) (3nmol) emitted significantly fewer presses during the progressive ratio session (Student t-test, t_{23} =1.72; p < 0.05, Fig. 5 C) and displayed a substantially lower breaking point compared with aCSF treated rats (Student t-test, t_{23} =1.71; p < 0.05, Fig. 5B).

The total number of inactive lever presses did not significantly differ

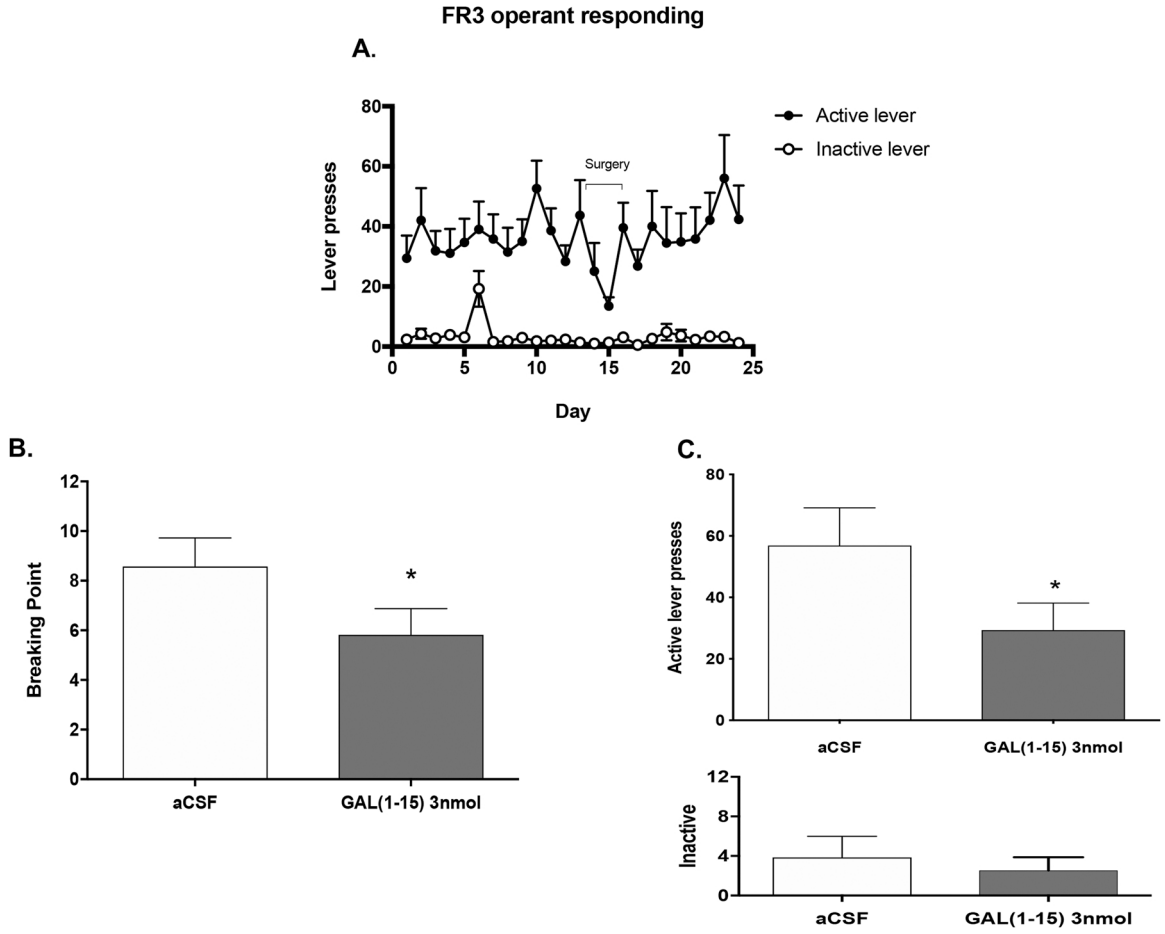

Fig. 5. Effect of the administration of Galanin(1−15) [GAL(1−15)] on alcohol seeking-behaviour (Progressive Ratio schedule). GAL(1−15) 3nmol (n = 11) or artificial cerebrospinal fluid (aCSF) (n = 14) were administered i.c.v 15 min before the test. aCSF injected rats were used as control group. **A.** Animals displayed a consistent preference for 10% (v/v) alcohol (active lever) over no reward (inactive lever) during the FR3 operant responding phase. **B.** Vertical bars represent a mean ± standard error of the mean of the breaking point during the test period. *p < 0.05 vs the control group, according to a Student t-test. **C.** Vertical bars represent a mean ± standard error of the mean of the active lever presses and inactive lever presses. * p < 0.05 vs control group, according to a Student t-test.

between groups (Fig. 5 C).

3.4. GAL(1−15) reduced alcohol relapse induced by the context in the alcohol self-administration test

In the alcohol self-administration (context A), animals displayed a consistent preference for 10% v/v ethanol (active lever) over no reward (inactive lever) during the FR1 operant responding phase (Fig. 6 A).

During the extinction period (context B), the animals progressively decreased the pressing for the active lever, reaching day 13 to the extinction criterion and almost the same level as inactive lever presses (Fig. 6B). On the first day of extinction, the animals were injected with icv GAL(1−15) 3nmol or aCSF. We didn't observe any differences in the number of active lever presses between the experimental groups that day (Table S3).

After context-induced reset (context A), we injected icv GAL(1−15) 3 nmol or aCSF, and we observed that GAL(1−15) 3 nmol reduced the response on the active lever compared to aCSF in alcoholic relapse (one-way ANOVA, $F_{2,43} = 40.79$, $p < 0.0001$, Fisher's LSD post hoc: $p < 0.05$; Fig. 6 C). Animals injected with GAL(1−15) 3nmol, or aCSF, increased active lever presses in the alcohol self-administration test compared to the extinction group ($p < 0.001$; Fig. 6 C).

4. Discussion

In the present study, we demonstrated that GAL(1−15) strongly reduced alcohol-seeking behaviour in the operant model of alcohol self-administration, not only in a fixed ratio protocol but also at the breaking point in a progressive ratio schedule. GALR1 and GALR2 were involved in these effects since the specific GALR2 antagonist M871 blocked the GAL(1−15) mediated action in alcohol self-administration, and the downregulation of GALR1 and GALR2 by siRNA was sufficient to block the GAL(1−15) effect. Importantly, the mesocorticolimbic circuitry participates in the mechanism of GAL(1−15) behaviour-mediated actions since we observed changes in the immediate-early gene C-Fos, dopamine receptor and 5HT1A in the VTA, NAc and PFC. Notably, GAL (1−15) significantly reduced context-induced alcohol relapse, expanding the role of the use of GAL(1−15) in AUD.

In the alcohol self-administration, an operant model widely used as a tool for studying drug-seeking motivated behaviour [35,36], GAL (1−15) 3 nmol induced a substantial reduction in the number of alcohol reinforcements suggesting that GAL(1−15) caused a loss of motivational behaviour induced by the alcohol. We confirmed the decrease in motivation for alcohol by analysing the Progressive Ratio (PR) schedule, which can be used to evaluate the reinforcing efficacy of drugs with potential abuse liability [37]. After specific training with PR, GAL (1−15) produced a significant reduction in the breaking point, which derived from a PR schedule is sensitive to pharmacological change [37].

The ability of GAL(1−15) to reduce motivation for ethanol is important because motivational dysregulation is a hallmark of addiction [31].

These results are in agreement with previous works, where the administration of GAL(1−15) induced a reduction of the motivation for different natural and artificial reinforcement including the saccharine

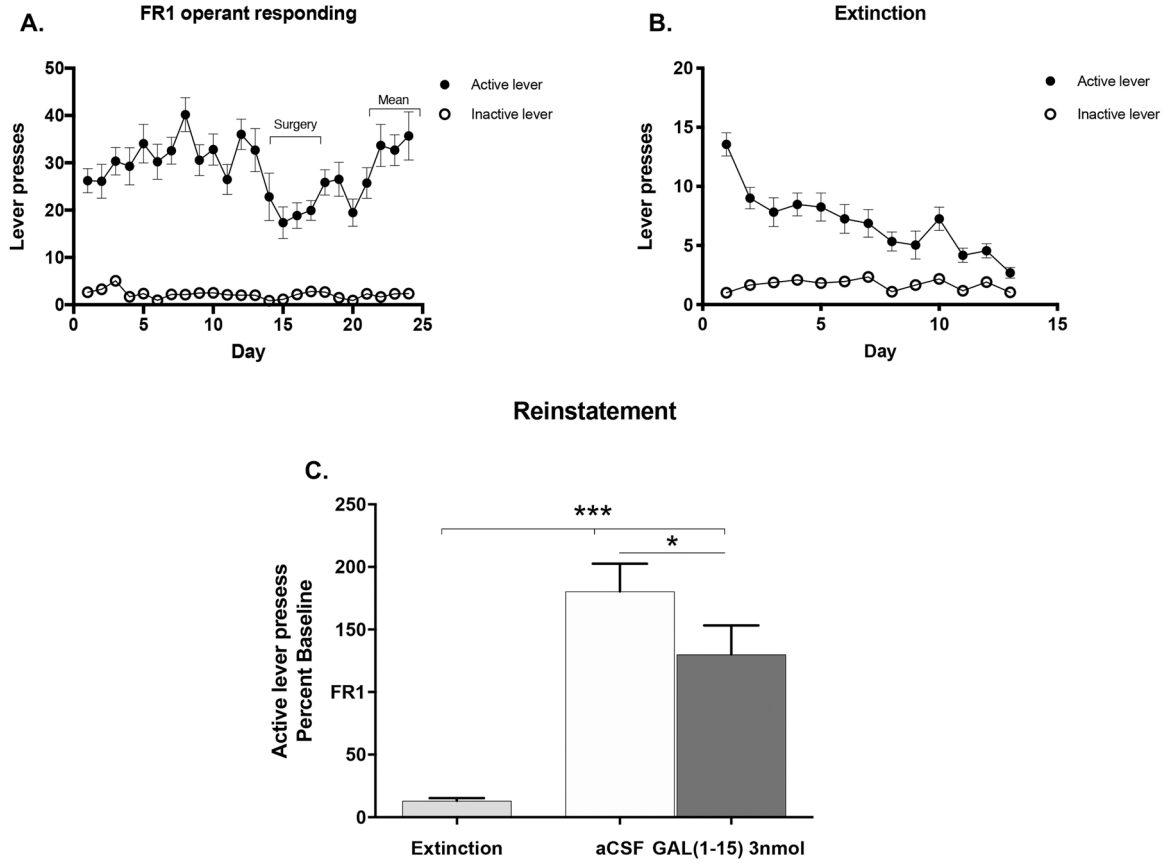

Fig. 6. Effect of the administration of Galanin(1−15) [GAL(1−15)] on alcoholic relapse. GAL(1−15) 3nmol (n = 12) or artificial cerebrospinal fluid (aCSF) (n = 11) were administered i.c.v 15 min before the test. aCSF injected rats were used as control group. **A.** Animals displayed a consistent preference for 10% (v/v) alcohol (active lever) over no reward (inactive lever) during the FR1 operant responding phase. **B.** Animals showed decreased pressing for the active lever over the 13 days extinction period. **C.** Vertical bars represent a mean ± standard error of the mean of the active lever presses according to Percent Baseline in FR1 during the test period. * $p < 0.05$ vs control group; *** $p < 0.001$ extinction period vs aCSF, GAL(1−15) 3nmol, according to one way ANOVA followed by Fisher Multiple Comparison Test.

self-administration [26] or directly, generating a strong decrease by 90% of preference and alcohol consumption in a voluntary alcohol intake model [24]. Moreover, the combination of GAL(1−15) with the antidepressant ESC reduces alcohol self-administration in rats and reverses the adverse ESC-mediated effects in depression-related behavioural tests [27].

Together, these results provide a new evidence of the involvement of GAL(1−15) in the motivation and reward-seeking process and open up the possibility to use the GAL(1−15) fragment as pharmacological therapy in AUD.

In this work, we also observed a differential role of GAL(1−15) compared with the complete molecule GAL in alcohol-seeking motivated behaviour. GAL(1−15) but not GAL induced a significant reduction in the number of alcohol reinforcements in the self-administration test, suggesting that only the GAL(1−15) fragment reduces the motivation to intake alcohol. We have previously described a different action of GAL and GAL(1−15) in several behavioural functions [16,25,38], including in the saccharine-seeking motivated behaviour [26] and the Alcohol voluntary consumption [24]. Therefore, the results of the present work validated a specific role of GAL(1−15) in brain communication.

The blockade of GAL(1−15) mediated action in alcohol self-administration obtained with the siRNA GALR1 or siRNA GALR2 knockdown rats and the use of the GALR2 antagonist M871 confirm the critical role of both GALR1 and GALR2 in GAL(1−15) mediated action. These results are in consonance with our previous studies showing that GAL(1−15) preferentially binds to GALR1-GALR2 heteroreceptor complexes [25,38–40].

The mesocorticolimbic circuitry participates in GAL(1−15)-mediated effects in alcohol self-administration. In the key mesocorticolimbic reward nodes, VTA, NAc and PFC that are critical for developing and maintaining alcohol reinforcement [41] and drug addiction [42,43], GAL(1−15) induced an increase on the expression of immediate early gene C-Fos, a marker of behaviourally activated neurons in drug self-administration models [44].

In addition, GAL(1−15) modified the D3 expression in VTA and NAc. In the VTA, GAL(1−15) increased the expression of the D3 receptor, a receptor implicated in the motivation to self-administer drugs [45]. This increase in D3 receptors observed could indicate that GAL(1−15) could affect to production and release of DA since D3 receptors are expressed not only in the postsynaptic regions but also in dopaminergic cell bodies, where they may function as autoreceptors [46] with inhibitory effects on impulse flow, synthesis and DA release [47,48].

These results agree with previous works where the GAL(1−15)-mediated reduction of saccharine self-administration was accompanied by a significant increase of D3 receptor expression in VTA [26], suggesting a reduction in DA release [26].

Moreover, GAL(1−15) induced the D1 receptor expression to increase in PFC, where there is growing evidence that the prefrontal cortex modulates ethanol self-administration [43,49], confirming the GAL (1−15) modulation over the dopaminergic mesocorticolimbic system.

Interestingly, not only dopamine receptors but also the 5HT1A serotonin receptor expression was modified in VTA and PFC in GAL (1−15)-administered animals. The interaction between GAL(1−15) and 5HT1A receptor has been described both at the functional and the receptor level in recent years [28,33,38,50–52] and it appears as the possible mechanism for reducing the alcohol-seeking behaviour induced by GAL(1−15) and ESC combination [27]. Therefore, the present results confirm the modulation of GAL(1−15) over the 5HT1A receptor in this model, although future experiments should be performed to determine the role of 5-HT1A in alcohol-seeking behaviour.

Although the results indicate the implication of the mesocorticolimbic circuit in the behavioural effect of GAL(1−15), it will be necessary to perform additional experiments to determine the involvement of this circuit.

An essential result of the present work was the ability of GAL(1−15) to reduce context-induced alcohol relapse. Animals with a stable baseline in alcohol self-administration in context A were subjected to an extinction process in context B to subsequently induce an alcohol relapse by context A reintroducing, a paradigm widely used in the context-induced alcohol relapse [7]. The control group markedly increased the number of active lever presses for the previous extinction levels when reintroduced in context A, indicating a reinstatement of self-administration of alcohol, which confirms the validity of the experimental procedure [32]. However, animals injected with GAL (1−15) showed a significant reduction in this reinstatement of self-administration, indicating the ability of GAL(1−15) to reduce alcoholic relapse.

Alcohol reinstatement is a crucial factor in AUD, where more than 50% of new patients relapse within three months [6,7]; being the reintroducing to context-related alcohol consumption one of the behaviours that frequently culminates in relapse and therefore one of the main challenge of alcohol use disorders treatment [7]. Our results suggest the ability of GAL(1−15) to reduce alcohol relapse, which expands the profile of use in AUD treatment. However, a detailed study of this component should be carried out in the future.

In conclusion, our results indicate that Galanin (1−15) N-terminal fragment induces a strong reduction of alcohol-seeking behaviour with the involvement of the mesocorticolimbic pathway, a key region in the reward effects of drug, moreover showing a decrease of the context-induced alcohol relapse. These results open up the possibility of using GAL(1−15) as a novel strategy in AUD, being necessary to test the effects of the intranasal GAL(1−15) administration in future studies.

CRediT authorship contribution statement

Cantero-Garcia: Conceptualization, Methodology, Investigation, Formal analysis. **Flores-Burgess**: Investigation, Methodology, Formal analysis. **Pineda-Gomez**: Investigation, Methodology. **Orio**: Methodology. **Serrano**: Methodology. **Díaz-Cabiale**: Supervision, Writing – review & editing. **Millón**: Conceptualization, Methodology, Investigation, Formal analysis, Supervision, Writing – review & editing.

Conflict of interest statement

The authors declare no conflict of interest.

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.

Acknowledgements

This work was supported by grants awarded by Spanish Ministry of Economy PID2020-114392RB-100, PDC2021-121566-100 and by Junta de Andalucía P20-00026-R and PI-0083-2019.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113508.

References

[1] B.F. Grant, R.B. Goldstein, T.D. Saha, S.P. Chou, J. Jung, H. Zhang, R.P. Pickering, W.J. Ruan, S.M. Smith, B. Huang, D.S. Hasin, Epidemiology of DSM-5 alcohol use disorder: results from the National Epidemiologic Survey on Alcohol and Related Conditions III, JAMA Psychiatry 72 (8) (2015) 757–766.

[2] WHO, Global status report on alcohol and health 2018, World Health Organization, 2018.

[3] C. Kilian, A. O'Donnell, N. Potapova, H. López-Pelayo, B. Schulte, L. Miquel, B. Paniello Castillo, C.S. Schmidt, A. Gual, J. Rehm, J. Manthey, Changes in alcohol use during the COVID-19 pandemic in Europe: a meta-analysis of observational studies, Drug Alcohol Rev. (2022).

[4] J.M. Clay, M.O. Parker, Alcohol use and misuse during the COVID-19 pandemic: a potential public health crisis? Lancet Public Health 5 (5) (2020), e259.

[5] N.E. Charles, S.J. Strong, L.C. Burns, M.R. Bullerjahn, K.M. Serafine, Increased mood disorder symptoms, perceived stress, and alcohol use among college students during the COVID-19 pandemic, Psychiatry Res. 296 (2021), 113706.

[6] R. Sinha, New findings on biological factors predicting addiction relapse vulnerability, Curr. Psychiatry Rep. 13 (5) (2011) 398–405.

[7] E. Domi, A. Domi, L. Adermark, M. Heilig, E. Augier, Neurobiology of alcohol seeking behavior, J. Neurochem. 157 (5) (2021) 1585–1614.

[8] C.S. Hendershot, K. Witkiewitz, W.H. George, G.A. Marlatt, Relapse prevention for addictive behaviors, Subst. Abus. Treat. Prev. Policy 6 (2011) 17.

[9] J. Rehm, K.D. Shield, G. Gmel, M.X. Rehm, U. Frick, Modeling the impact of alcohol dependence on mortality burden and the effect of available treatment interventions in the European Union, Eur. Neuropsychopharmacol. 23 (2) (2013) 89–97.

[10] E.M. Burnette, S.J. Nieto, E.N. Grodin, L.R. Meredith, B. Hurley, K. Miotto, A. J. Gillis, L.A. Ray, Novel agents for the pharmacological treatment of alcohol use disorder, Drugs 82 (3) (2022) 251–274.

[11] S.G. Genders, K.J. Scheller, E. Djouma, Neuropeptide modulation of addiction: focus on galanin, Neurosci. Biobehav. Rev. 110 (2020) 133–149.

[12] K. Tatemoto, A. Rökaeus, H. Jörnvall, T.J. McDonald, V. Mutt, Galanin - a novel biologically active peptide from porcine intestine, FEBS Lett. 164 (1) (1983) 124–128.

[13] D.M. Jacobowitz, A. Kresse, G. Skofitsch, Galanin in the brain: chemoarchitectonics and brain cartography–a historical review, Peptides 25 (3) (2004) 433–464.

[14] T.A. Branchek, K.E. Smith, C. Gerald, M.W. Walker, Galanin receptor subtypes, Trends Pharmacol. Sci. 21 (3) (2000) 109–117.

[15] K. Mitsukawa, X. Lu, T. Bartfai, Galanin, galanin receptors and drug targets, Cell Mol. Life Sci. 65 (12) (2008) 1796–1805.

[16] Z. Díaz-Cabiale, C. Parrado, M. Narváez, C. Millón, A. Puigcerver, K. Fuxe, J. A. Narváez, Neurochemical modulation of central cardiovascular control: the integrative role of galanin, Exp Suppl 102 (2010) 113–131.

[17] R. Lang, A.L. Gundlach, F.E. Holmes, S.A. Hobson, D. Wynick, T. Hökfelt, B. Kofler, Physiology, signaling, and pharmacology of galanin peptides and receptors: three decades of emerging diversity, Pharmacol. Rev. 67 (1) (2015) 118–175.

[18] M.R. Picciotto, Galanin and addiction, Cell Mol. Life Sci. 65 (12) (2008) 1872–1879.

[19] M.J. Lewis, D.F. Johnson, D. Waldman, S.F. Leibowitz, B.G. Hoebel, Galanin microinjection in the third ventricle increases voluntary ethanol intake, Alcohol Clin. Exp. Res. 28 (12) (2004) 1822–1828.

[20] M.J. Lewis, P. Rada, D.F. Johnson, N.M. Avena, S.F. Leibowitz, B.G. Hoebel, Galanin and alcohol dependence: neurobehavioral research, Neuropeptides 39 (3) (2005) 317–321.

[21] B.L. Ash, T. Quach, S.J. Williams, A.J. Lawrence, E. Djouma, Galanin-3 receptor antagonism by SNAP 37889 reduces motivation to self-administer alcohol and attenuates cue-induced reinstatement of alcohol-seeking in iP rats, J. Pharmacol. Sci. 125 (2) (2014) 211–216.

[22] A. Flores-Burgess, C. Millón, B. Gago, M. Narváez, D.O. Borroto-Escuela, G. Mengod, J.A. Narváez, K. Fuxe, L. Santín, Z. Díaz-Cabiale, Galanin (1-15) enhancement of the behavioral effects of Fluoxetine in the forced swimming test gives a new therapeutic strategy against depression, Neuropharmacology 118 (2017) 233–241.

[23] C. Millón, A. Flores-Burgess, M. Narváez, D.O. Borroto-Escuela, B. Gago, L. Santín, E. Castilla-Ortega, J. Narváez, K. Fuxe, Z. Díaz-Cabiale, The neuropeptides Galanin and Galanin(1-15) in depression-like behaviours, Neuropeptides 64 (2017) 39–45.

[24] C. Millón, A. Flores-Burgess, E. Castilla-Ortega, B. Gago, M. García-Fernandez, A. Serrano, F. Rodriguez de Fonseca, J.A. Narváez, K. Fuxe, L. Santín, Z. Díaz-Cabiale, Central administration of galanin N-terminal fragment 1-15 decreases the voluntary alcohol intake in rats, Addict. Biol. 24 (1) (2019) 76–87.

[25] C. Millon, A. Flores-Burgess, M. Narváez, D.O. Borroto-Escuela, L. Santin, C. Parrado, J.A. Narváez, K. Fuxe, Z. Diaz-Cabiale, A role for galanin N-terminal fragment (1-15) in anxiety-and depression-related behaviors in Ra, Int. J. Neuropsychopharmacol. 18 (3) (2015).

[26] C. Millón, A. Flores-Burgess, B. Gago, F. Alén, L. Orio, L. García-Durán, J. A. Narváez, K. Fuxe, L. Santín, Z. Díaz-Cabiale, Role of the galanin N-terminal fragment (1-15) in anhedonia: Involvement of the dopaminergic mesolimbic system, J. Psychopharmacol. 33 (6) (2019) 737–747.

[27] N. Cantero-García, A. Flores-Burgess, D. Ladrón de Guevara-Miranda, A. Serrano, L. García-Durán, A. Puigcerver, K. Fuxe, J. Narváez, L.J. Santín, Z. Díaz-Cabiale, C. Millón, The combination of Galanin (1-15) and Escitalopram in rats suggests a new strategy for alcohol use disorder comorbidity with depression, Biomedicines 10 (2) (2022).

[28] L. García-Durán, A. Flores-Burgess, N. Cantero-García, A. Puigcerver, J. Narváez, K. Fuxe, L. Santín, C. Millón, Z. Díaz-Cabiale, Galanin(1-15) potentiates the antidepressant-like effects induced by escitalopram in a rat model of depression, Int. J. Mol. Sci. 22 (19) (2021).

[29] F. Alén, A. Santos, G. Moreno-Sanz, G. González-Cuevas, E. Giné, L. Franco-Ruiz, M. Navarro, J.A. López-Moreno, Cannabinoid-induced increase in relapse-like drinking is prevented by the blockade of the glycine-binding site of N-methyl-D-aspartate receptors, Neuroscience 158 (2) (2009) 465–473.

[30] S. Alaux-Cantin, V. Warnault, R. Legastelois, B. Botia, O. Pierrefiche, C. Vilpoux, M. Naassila, Alcohol intoxications during adolescence increase motivation for alcohol in adult rats and induce neuroadaptations in the nucleus accumbens, Neuropharmacology 67 (2013) 521–531.

[31] S. Lebourgeois, M.C. González-Marín, J. Jeanblanc, M. Naassila, C. Vilpoux, Effect of N-acetylcysteine on motivation, seeking and relapse to ethanol self-administration, Addict. Biol. 23 (2) (2018) 643–652.

[32] K.E. Wilson, S. Limburg, M.K. Duggan, A.J. Lawther, S.J. Williams, A.J. Lawrence, M.W. Hale, E. Djouma, The galanin receptor-3 antagonist, SNAP 37889, inhibits cue-induced reinstatement of alcohol-seeking and increases c-Fos expression in the nucleus accumbens shell of alcohol-preferring rats, J. Psychopharmacol. 32 (8) (2018) 911–921.

[33] A. Flores-Burgess, C. Millon, B. Gago, M. Narváez, D.O. Borroto-Escuela, G. Mengod, J.A. Narváez, K. Fuxe, L. Santin, Z. Diaz-Cabiale, Galanin (1-15) enhancement of the behavioral effects of Fluoxetine in the forced swimming test gives a new therapeutic strategy against depression, Neuropharmacology 118 (2017) 233–241.

[34] D. García-Pérez, C. Núñez, M.L. Laorden, M.V. Milanés, Regulation of dopaminergic markers expression in response to acute and chronic morphine and to morphine withdrawal, Addict. Biol. 21 (2) (2016) 374–386.

[35] A. Ettenberg, The runway model of drug self-administration, Pharmacol. Biochem. Behav. 91 (3) (2009) 271–277.

[36] C. Sanchis-Segura, R. Spanagel, Behavioural assessment of drug reinforcement and addictive features in rodents: an overview, Addict. Biol. 11 (1) (2006) 2–38.

[37] N.R. Richardson, D.C. Roberts, Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy, J. Neurosci. Methods 66 (1) (1996) 1–11.

[38] C. Millón, A. Flores-Burgess, M. Narváez, D.O. Borroto-Escuela, L. Santín, B. Gago, J.A. Narváez, K. Fuxe, Z. Díaz-Cabiale, Galanin (1-15) enhances the antidepressant effects of the 5-HT1A receptor agonist 8-OH-DPAT: involvement of the raphe-hippocampal 5-HT neuron system, Brain Struct. Funct. 221 (9) (2016) 4491–4504.

[39] K. Fuxe, D.O. Borroto-Escuela, W. Romero-Fernandez, A.O. Tarakanov, F. Calvo, P. Garriga, M. Tena, M. Narvaez, C. Millón, C. Parrado, F. Ciruela, L.F. Agnati, J. A. Narvaez, Z. Díaz-Cabiale, On the existence and function of galanin receptor heteromers in the central nervous system, Front. Endocrinol. 3 (2012) 127.

[40] D.O. Borroto-Escuela, M. Narvaez, M. Di Palma, F. Calvo, D. Rodriguez, C. Millon, J. Carlsson, L.F. Agnati, P. Garriga, Z. Díaz-Cabiale, K. Fuxe, Preferential activation by galanin 1-15 fragment of the GalR1 protomer of a GalR1-GalR2 heteroreceptor complex, Biochem. Biophys. Res. Commun. 452 (3) (2014) 347–353.

[41] R.A. Gonzales, M.O. Job, W.M. Doyon, The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement, Pharmacol. Ther. 103 (2) (2004) 121–146.

[42] G.F. Koob, Neural mechanisms of drug reinforcement, Ann. N. Y. Acad. Sci. 654 (1992) 171–191.

[43] G.F. Koob, N.D. Volkow, Neurocircuitry of addiction, Neuropsychopharmacology 35 (1) (2010) 217–238.

[44] F.C. Cruz, F. Javier Rubio, B.T. Hope, Using c-fos to study neuronal ensembles in corticostriatal circuitry of addiction, Brain Res. 1628 (Pt A) (2015) 157–173.

[45] B. Le Foll, S.R. Goldberg, P. Sokoloff, The dopamine D3 receptor and drug dependence: effects on reward or beyond? Neuropharmacology 49 (4) (2005) 525–541.

[46] J. Diaz, C. Pilon, B. Le Foll, C. Gros, A. Triller, J.C. Schwartz, P. Sokoloff, Dopamine D3 receptors expressed by all mesencephalic dopamine neurons, J. Neurosci. 20 (23) (2000) 8677–8684.

[47] J.M. Tepper, B.C. Sun, L.P. Martin, I. Creese, Functional roles of dopamine D2 and D3 autoreceptors on nigrostriatal neurons analyzed by antisense knockdown in vivo, J. Neurosci. 17 (7) (1997) 2519–2530.

[48] G.M. Leggio, S. Salomone, C. Bucolo, C. Platania, V. Micale, F. Caraci, F. Drago, Dopamine D(3) receptor as a new pharmacological target for the treatment of depression, Eur. J. Pharmacol. 719 (1–3) (2013) 25–33.

[49] L.R. Halladay, A. Kocharian, P.T. Piantadosi, M.E. Authement, A.G. Lieberman, N. A. Spitz, K. Coden, L.R. Glover, V.D. Costa, V.A. Alvarez, A. Holmes, Prefrontal regulation of punished ethanol self-administration, Biol. Psychiatry 87 (11) (2020) 967–978.

[50] C. Millon, A. Flores-Burgess, M. Narváez, D.O. Borroto-Escuela, B. Gago, L. Santin, E. Castilla-Ortega, J. Narváez, K. Fuxe, Z. Diaz-Cabiale, The neuropeptides Galanin and Galanin(1-15) in depression-like behaviours, Neuropeptides (2016).

[51] A. Flores-Burgess, C. Millón, B. Gago, L. García-Durán, N. Cantero-García, R. Coveñas, J.A. Narváez, K. Fuxe, L. Santín, Z. Díaz-Cabiale, Galanin (1-15)-fluoxetine interaction in the novel object recognition test. Involvement of 5-HT1A receptors in the prefrontal cortex of the rats, Neuropharmacology 155 (2019) 104–112.

[52] A. Flores-Burgess, C. Millón, B. Gago, L. García-Durán, N. Cantero-García, A. Puigcerver, J.A. Narváez, K. Fuxe, L. Santín, Z. Díaz-Cabiale, GALANIN (1-15) enhances the behavioral effects of fluoxetine in the olfactory bulbectomy rat suggesting a new augmentation strategy in depression, Int. J. Neuropsychopharmacol. (2021).