

ROLE OF THE GALANIN N-TERMINAL FRAGMENT (1-15) IN ANHEDONIA: INVOLVEMENT OF THE DOPAMINERGIC MESOLIMBIC SYSTEM

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ABSTRACT

BACKGROUND: Anhedonia is a core feature of depressive disorders. The Galanin N-terminal fragment (1-15) [GAL(1-15)] plays a role in mood regulation since it induces depression and anxiogenic-like effects in rats. In this study, we analysed GAL(1-15) actions in anhedonic-like behaviours in rats using operant and non-operant tests and the areas involved with these effects.

METHODS: GAL(1-15) effects were analysed in saccharin self-administration, sucrose preference, novelty-suppressed feeding and female urine sniffing tests. The areas involved in GAL(1-15)-mediated effects were studied with positron emission tomography (PET) for *in vivo* imaging, and we analysed the ventral tegmental area (VTA) and nucleus accumbens (NAc). GAL(1-15) had effects on the mRNA expression of the dopamine transporters Dat and Vmat2; the C-Fos gene; the dopamine receptors D1, D2, D3, D5; and the GALRs (GALR1 and GALR2).

RESULTS: GAL(1-15) at a concentration of 3 nmol induced a strong anhedonia-like phenotype in all tests. The involvement of GALR2 was demonstrated with the GALR2 antagonist M871 (3 nmol). The 18F-FDG PET images indicated the action of GAL(1-15) over several nuclei of the limbic system. GAL(1-15)-mediated effects also involved changes in the expression of Dat, Vmat2, D3 receptor and GALR in VTA as well as the expression of C-Fos, D1, D2 and D3 and TH immunoreactivity in the NAc.

CONCLUSIONS: Our results indicated that GAL(1-15) exerts strong anhedonic-like effects and that this effect was accompanied by changes in the dopaminergic mesolimbic system. These results may provide a basis for the development of novel therapeutic strategies using GAL(1-15) analogues for the treatment of depression and reward-related diseases.

INTRODUCTION

In 2016, the World Bank Group and the World Health Organization reported that mental disorders currently account for 30% of the nonfatal disease burden worldwide and that global cost is expected to reach \$6 trillion by 2030 (Marquez and Saxena, 2016). Major depression is the most frequent mood disorder, with a lifetime prevalence that has been reported to range from 7% to 21% (Albert et al., 2011). It is associated with substantial functional impairment, diminished quality of life, increased burden both for patients and caregivers, and a higher risk of mortality.

Although the underlying mechanisms have not yet been clearly defined in the last decade, the importance of the roles of neuropeptides, including Galanin (GAL), and/or their receptors in the treatment of stress-related mood disorders is becoming increasingly apparent (Kormos and Gaszner, 2013; Genders et al., 2018).

GAL is a neuropeptide (Tatemoto et al., 1983) that is widely distributed in neurons within the central nervous system (CNS) (Jacobowitz et al., 2004). Three GAL receptor (GALR1-3) subtypes with high affinities for GAL have been cloned (Branchek et al., 2000; Mitsukawa et al., 2008). GALR1 and GALR3 mainly activate the inhibitory G proteins Gi/Go while GALR2 mainly couples to Gq/G11 to mediate excitatory signalling (Branchek et al., 2000). GAL and its receptors evoke depression-related and anxiety-like behaviours (Juhász et al., 2014). GAL-overexpressing mice and rodents in which GAL was infused either intraventricularly (i.c.v) or into the ventral tegmental area showed an increase in immobility during a forced swimming test (FST) that was indicative of

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3 depression-like behaviour (Kuteeva et al., 2005; Kuteeva et al., 2007; Weiss et
4 al., 1998). Additionally, in a genetic rat model of depression, the Flinders
5 Sensitive Line, upregulation of the GAL receptor binding sites was found in the
6 dorsal raphe nucleus, which has been linked to high immobility in the FST
7 (Bellido et al., 2002). However, intraperitoneal (i.p.) injections of GALR2
8 agonists exhibited antidepressant-like effects and two non-selective GALR
9 agonists, galnon and galmic, decreased immobility times in the FST (Bartfai et
10 al., 2004; Lu et al., 2005). These discrepancies are attributed to the different
11 physiological roles of the GAL receptor subtypes (Swanson et al., 2005;
12 Kuteeva et al., 2008b; Barr et al., 2006). Thus, the activation of GALR1 and
13 GALR3 results in a depression-like behaviour while stimulation of GALR2 leads
14 to antidepressant-like effects (Bartfai et al., 2004; Lu et al., 2005; Kuteeva et al.,
15 2008a). Recently, it has also been described that, in humans, the GAL gene
16 and its receptor GALR3 are differentially methylated and expressed in brains of
17 major depression subjects in a region- and sex-specific manner (Barde et al.,
18 2016).

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41 Not only GAL but also N-terminal fragments similar to GAL(1-15) are active in
42 the CNS (Hedlund and Fuxe, 1996; Diaz-Cabiale et al., 2005; Diaz-Cabiale et
43 al., 2007; Diaz-Cabiale et al., 2010). Recently, we demonstrated that GAL(1-15)
44 induces strong depression-related behaviour in rats and these effects were
45 significantly stronger than the ones induced by GAL (Millon et al., 2015). We
46 analysed GAL(1-15)-mediated effects in two tests developed to screen
47 monoamine-based antidepressant drugs: the FST and the tail suspension test
48 (TST). Both assess the response to an inescapable stressor, provoking despair-
49 based behaviour/immobility and, in these tests, GAL(1-15) induced depression-
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3 like behaviour since significant increases in immobility were observed (Millon et
4 al., 2015). We demonstrated that GALR1-GALR2 heteroreceptor complexes in
5 the dorsal hippocampus and DR, areas rich in GAL(1-15) binding sites (Hedlund
6 et al., 1992) were involved in these effects (Millon et al., 2015) (Borrito-Escuela
7 et al., 2014).

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15 Moreover, GAL(1-15) also induced an anxiogenic-like effect in the open field
16 and the light/dark test (Millon et al., 2015). Since, in animal models, anxiety can
17 by considered an element of the depression, the fact that GAL(1-15) induced a
18 strong depression and anxiogenic-like effects in all the tests indicated a
19 potential role for GAL(1-15) in mood disorders, especially in depression.
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28 However, the role of GAL(1-15) in an endophenotype that represents a key
29 symptom of depression, such as reward-related and anhedonic behaviour, is
30 still unknown. Anhedonia is considered to be a core feature of major depressive
31 disorders as, for example, the *Diagnostic and Statistical Manual of Mental*
32 *Disorders*, fifth edition (DSM-V) requires that either a depressed mood or
33 anhedonia be present to propose this diagnosis. Furthermore, the lack of
34 reactivity and anhedonia are key diagnostic criteria for the *DSM-V* melancholic
35 subtype of major depression, and the presence of anhedonia has been shown
36 to be predictive of an antidepressant response (Gorwood, 2008).
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50 The purpose of the present study was to assess the role of
51 intracerebroventricular (i.c.v.) GAL(1-15) in anhedonic behaviour using a widely
52 accepted approach to assess reward seeking behaviour in operant and
53 nonoperant tests: saccharin self-administration, sucrose preference, novelty-
54 suppressed feeding and female urine sniffing tests. Moreover, the involvement
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of GALR2 in the effects induced by GAL(1-15) were analysed with the selective GALR2 antagonist M871. To investigate the areas involved in GAL(1-15)-mediated effects, positron emission tomography (PET) for *in vivo* imaging of metabolic processes was performed. We also analysed transcriptional changes in the ventral tegmental area (VTA) and nucleus accumbens (NAc), which are essential nuclei in reward processes, and the effect of GAL(1-15) on the mRNA expression of dopamine transporters Dat and Vmat2; the C-Fos gene; the dopamine receptors *D1*, *D2*, *D3*, *D5*; and the GALRs (*GALR1* and *GALR2*).

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (225–250 g) were obtained from Criffa and maintained in a humidity- and temperature-controlled room. All animal experimentation was conducted in accordance with the University of Málaga Guidelines for the Care and Use of Laboratory Animals.

Detailed descriptions are available in the supplemental information on the animal controlled-conditions, surgical preparation and i.c.v. injections.

Saccharin Self-administration

Training was achieved using a modification of the protocol reported by (Alen *et al.*, 2009). Briefly, rats were placed on a water restriction schedule for 2-4 days to facilitate training of lever pressing. The rats were trained to self-administer saccharin 0.2% (w/v) in 30-min daily sessions for two weeks on a fixed ratio 1 schedule of reinforcement in which each response resulted in the delivery of 0.1 ml of fluid. At this point, saccharin self-administration training continued until the

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3 animals reached stable baseline responding. During the test sessions, the
4 responses on the active lever and number of saccharin reinforcements were
5 recorded (see the supplemental information for details).
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10 Three sets of experiments were conducted in the saccharin self-administration
11 test. In the first set of experiments, a dose-response curve of GAL(1-15) was
12 generated. For this, groups of rats received i.c.v. 1 nmol or 3 nmol GAL(1-15) or
13 vehicle 15 min before the test. In the second set of experiments, the effects in
14 saccharin self-administration of GAL and GAL(1-15) were compared. Groups of
15 rats received i.c.v. 3 nmol GAL, 3 nmol GAL(1-15) or vehicle 15 min before the
16 test. In the last set of experiments, groups of rats received i.c.v. 3 nmol GAL(1-
17 15) was combined with GALR2 antagonist 3 nmol M871 15 min before the
18 measures to study the role of GALR2. The doses employed and the injection
19 protocol were based on those of previous studies (Millon et al., 2015).
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34 The general schema of the experimental design is shown in Figure 1A.
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36 37 38 **Sucrose Preference Test**

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40 Anhedonia was assessed using the sucrose preference test (SPT), performed
41 as described previously (Marco et al., 2017) with minor modifications (see the
42 supplemental information for details). Briefly, on the testing day, rats were
43 allowed free access to two-bottles: one containing 1% (w/v) sucrose solution
44 and the other containing tap water. After 2, 8 and 24 h, the bottles were
45 weighed to calculate the sucrose intake (g/kg) and sucrose preference (sucrose
46 preference=sucrose consumption / (water + sucrose consumption) x100), which
47 reflected the rats' anhedonia levels.
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3 In the experiment, groups of rats received i.c.v. 3 nmol GAL(1-15) or vehicle,
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5 based on a previous study (Millón et al., 2015), and the sucrose intake and
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7 sucrose preference were measured at 2, 8 and 24 h after injection.
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11 The general schema of the experimental design is shown in Figure 1B.
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14 **Novelty Suppressed feeding**

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17 The NSF test was carried out during the 10 min period as previously described
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19 (Pascual-Brazo et al., 2012) with some modifications (see the supplemental
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21 information for details). Briefly, all rats were deprived of food for 24 h before the
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23 test. The testing apparatus consisted of a plastic box (60x40x20 cm) with a
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25 single pellet of food in the centre of the box on white circular filter paper. In the
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27 experiment, groups of rats received i.c.v. 3 nmol GAL(1-15) or vehicle 15 min
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29 before the test, based on a previous study (Millón et al., 2015), and the latency
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31 of the first feeding episode was recorded.
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36 **Female Urine Sniffing Test**

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39 The female urine sniffing test (FUST) was conducted as described previously
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41 (Malkesman et al., 2012) (see the supplemental information for details). Briefly,
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43 the test had three phases: a single exposure to the cotton tip dipped in sterile
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45 water; an interval during which no applicator was presented to the animal; and a
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47 single exposure to a cotton tip infused with fresh urine or water, during which
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49 the time spent sniffing was measured.
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54 The animals receiving 3 nmol GAL(1-15) or vehicle were injected 15 min before
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56 exposure to the urine or water.
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The general schema of the experimental design of NSF and FUST is shown in Figure 1B.

PET studies

The ^{18}F -FDG PET imaging was performed at Unidad de Imagen Molecular, CIMES, Spain in a dedicated small animal Philips MOSAIC tomograph (Cleveland, OH, USA) system as previously described (Prieto et al., 2011). Animals were administered icv with CSFa and, after 3-4 days, with Gal(1-15) (3 nmol). The doses employed and the injection protocol were based on previous works (Millon et al., 2015). Immediately after the treatment, the ^{18}F -FDG (43.6 ± 5.5 MBq in 0.3 ml of 0.9% NaCl) was injected through the tail vein. After an awake uptake period of 25 minutes to ensure the incorporation of ^{18}F -FDG during drug-induced behavioural effects (Prieto et al., 2011), the animals were anaesthetized and placed prone on the PET scanner bed to perform static acquisition for 30 min at three different timepoints (30, 60 and 90 min) after tracer administration (see the supplemental information for details).

Statistical analysis of PET images

Differences in ^{18}F -FDG uptake between aCSF- and GAL[1-15]-injected animals were assessed using software package SPM8 under the general lineal model framework and whole brain contrast analysis. A t-test was performed for each timepoint (30, 60 and 90 min), and the differences between the groups were represented by a map of t-statistics. The contrast images were yielded based on a voxel-level height threshold of $p < 0.01$ (uncorrected) and a cluster-extend threshold of 100 voxels. Resultant clusters are shown on a SAMIT rat RM template in Paxinos and Watson space.

Genes expression in VTA and NAc

Rats from NSF and FUST experiments (Figure 1B) were euthanized by decapitation 1 h after a single i.c.v. administration of GAL(1-15) 3 nmol or vehicle and the brains were rapidly removed and frozen until use. The dose and time selected were based by previous works (Millón et al., 2015; García-Perez et al., 2016). The nuclei dissections were conducted as described (García-Perez et al., 2016) with modifications (see supplemental information for details).

RNA isolation and quantitative real-time polymerase chain reaction analysis

The procedure to perform RNA isolation and real-time polymerase chain reactions (RT-PCRs) was described previously (Millon et al., 2015; Millon et al., 2017a; Flores-Burgess et al., 2017) (see the supplemental information for details).

The primer sequences used to evaluate the mRNA expression levels of the genes *Gapdh*, *C-Fos*, *Dat*, *Vmat2*, *D1*, *D2*, *D3*, *D5*, *GALR1* and *GALR2* are shown in Table S1.

Tyrosine Hydroxylase immunohistochemistry

Rats from the NSF and FUST experiment (Figure 1B) were perfused transcardially 90 min after a single i.c.v. administration of 3 nmol GAL(1-15) or vehicle. The TH immunohistochemistry was conducted in NAc and CPu with modifications as previously described (Cadoni et al., 2017) (see the supplemental information for details).

Double immunofluorescence

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3 The procedures have been used previously (Millon et al., 2015). Primary
4 antibodies directed to GALR2 (rabbit polyclonal, Alomone Lab, 1/250) and
5 GALR1 (goat polyclonal, Santa Cruz Biotechnology INC, EEUU, 1:250) were
6 used (see supplemental information for details). Double immunolabelling was
7 performed in sections of the striatum and VTA of rats without treatment (Figure
8 S1).
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10 11 12 13 14 15 16 17 18 **Statistical Analysis**

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20 Data are presented as the mean \pm standard error of the mean, and sample
21 numbers (n) are indicated in figure legends. All data were analysed using
22 GraphPad PRISM 6.0 (GraphPad Software). For comparing two experimental
23 conditions, Student's unpaired t-tests were performed. Otherwise, one-way or
24 two-way analysis of variance (ANOVA) followed by Fisher's least significant
25 difference (LSD) comparison post-test was performed. Differences were
26 considered significant at $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).
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38 **RESULTS**

39 40 41 **Behavioural Studies**

42 43 44 **GAL(1-15) induced an anhedonic-like effect.**

45 46 47 *Effects of GAL(1-15) in the saccharin self-administration test*

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50 GAL(1-15) at a concentration of 3 nmol significantly decreased the number of
51 reinforcements of saccharin self-administration (one-way ANOVA, $F_{2,26}=5.743$,
52 $p < 0.01$, Fisher's LSD *post hoc* test: $p < 0.01$) by 43% (Figure 2A) fifteen minutes
53 after administration. GAL(1-15) at a concentration of 1 nmol lacked the effect on
54 the number of saccharin reinforcements.
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3 In the comparison between GAL and GAL(1-15) in the saccharin self-
4 administration test, one-way ANOVA revealed a significant difference between
5 the N-terminal fragment GAL(1-15) and GAL. GAL(1-15) significantly reduced
6 the number of saccharin reinforcements compared with GAL (one-way ANOVA,
7 $F_{2,28}=6.068$, $p<0.01$, Fisher's LSD *post hoc* test: $p<0.01$) (Figure 2B). GAL
8 lacked an effect on the number of saccharin reinforcements compared with the
9 control group (Figure 2B).

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20 GALR2 antagonist M871 blocked GAL(1-15)-mediated effects in the saccharin
21 self-administration test. The GALR2 antagonist M871 significantly blocked the
22 decrease in the number of saccharin reinforcements (one-way ANOVA,
23 $F_{3,28}=3.449$, $p<0.05$, Fisher's LSD *post hoc* test: $p<0.05$) induced by GAL(1-15)
24 administration (Figure 2C). M871 alone at the dose of 3 nmol lacked an effect
25 on the number of saccharin reinforcements.
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34 *Effects of GAL(1-15) in the sucrose preference test (SPT)*

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38 GAL(1-15) at a concentration of 3 nmol decreased the sucrose intake (two-way
39 ANOVA (group x time), $F_{2,26}=4.8836$, $p<0.05$) at 8 hours (Fisher's LSD *post hoc*
40 test $p<0.05$) and 24 hours (Fisher's LSD *post hoc* test $p<0.01$) after
41 administration (Figure 3A). Moreover, i.c.v. injection of 3 nmol GAL(1-15)
42 induced a **not significant** decrease in sucrose preference (two-way ANOVA
43 (Group), $F_{1,13}=4.039$, $p=0.0656$) at 2 h, 8 h and 24 h (Figure 3B).
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52 Regarding water intake (Table S2), no differences (two-way ANOVA (Time x
53 Group), $F_{2,26}=0.2915$, $p=0.7494$) were found at any timepoint after GAL(1-15)
54 i.c.v. administration.
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Effects of GAL(1-15) in the novelty suppressed feeding (NSF) test

In the NSF, GAL(1-15) at the dose of 3 nmol significantly increase the latency to eat ($t_{15}=5.147$, $p<0.001$; Figure 4A) by 80%.

Effects of GAL(1-15) in the female urine sniffing test (FUST)

In this test, the overall one-way ANOVA revealed a significant difference between the sniffing duration of water cotton and female urine cotton (one-way ANOVA, $F_{2,23}=14.39$, $p<0.001$, Fisher's LSD *post hoc* test: $p<0.001$ urine-CSFa; $p<0.01$ urine-GAL(1-15); Figure 4B). Moreover, 3 nmol GAL(1-15) induced a significant decrease in sniffing duration over urine cotton to compare with the urine-CSFa group (one-way ANOVA, $F_{2,23}=14.39$, $p<0.001$, Fisher's LSD *post hoc* test $p<0.05$; Figure 4B).

No differences were found between the water-CSFa and water-GAL(1-15) groups in sniffing duration ($t_{10}=1.480$, $p=0.084$; Figure 3C.)

PET Studies

The GAL(1-15) treatment induced a significant decrease in [^{18}F]FDG uptake in the hippocampus (bilateral) and thalamus at 30 min. This reduction was maintained in both nuclei at 60 min and 90 min (Figure 5). In addition, a reduced uptake was also observed in the striatum (caudate putamen and external globus pallidum) at 30 min. Moreover, the administration of the GAL fragment induced an increase in [^{18}F]FDG uptake after 30 min and 60 min in the prefrontal cortex and in the piriform cortex after 60 min (Figure 5).

Neurochemical Studies

GAL(1-15) affects the mRNA expression of the dopamine transporters *Dat* and *Vmat2* and of *C-Fos* in the ventral tegmental area (VTA) and the accumbens nucleus (NAc).

As shown in Figure 6, GAL(1-15) at a dose of 3 nmol produced a significant decrease in the mRNA levels of *Dat* ($t_6=1.988$, $p<0.05$; Figure 6A) and *Vmat2* ($t_6=2.480$, $p<0.05$; Figure 6B) in VTA 1 hour after its administration. However, GAL(1-15) did not affect *C-Fos* mRNA expression in the nucleus (Figure 6C). In the NAc, 3 nmol i.c.v. GAL(1-15) induced a significant decrease in the expression of *C-Fos* mRNA ($t_7=2.329$, $p<0.05$; Figure 6D) 1 hour after administration.

Additionally, *C-Fos* mRNA expression was measured in other regions, such as the dentate gyrus in the hippocampus, where GAL(1-15) produced a significant increase compared with the control group (mRNA expression: Control: 1.39 ± 0.2 ; GAL(1-15): 2.60 ± 0.2 ; $t_6=3.352$, $p<0.01$).

GAL(1-15) affects the mRNA expression of dopamine receptors in the ventral tegmental area (VTA) and the accumbens nucleus (NAc)

In the VTA, the administration of GAL(1-15) induced a significant increase in the mRNA expression of *D3* 1 hour after injection ($t_7=1.981$, $p<0.05$; Figure 7C). No differences were found in *D1*, *D2* and *D5* receptor expression after GAL(1-15) administration in the VTA (Figure 7A,B, and D).

As shown in the second part of Figure 7, GAL(1-15) 3 nmol significantly increased the mRNA expression of *D1* ($t_7=2.019$, $p<0.05$; Figure 7E), *D2* ($t_7=2.008$, $p<0.05$; Figure 7F) and *D3* ($t_6=3.045$, $p<0.05$ Figure 7G) in the NAc,

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3 1 hour after its administration. GAL(1-15) lacked an effect on mRNA expression
4 of *D5* compared with the control group in the NAc.
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8 ***GAL(1-15) effects in mRNA expression of Galanin receptors in the Ventral***
9 ***Tegmental Area (VTA) and Accumbens nucleus (NAc).***
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14 Using double immunolabelling, we observed a colocation of GALR1 and GALR2
15 immunoreactivity in the nerve cells of the VTA (Figure S1A), in both the core
16 (NAcC; Figure S1B) and shell (NAcSh; Figure S1C) of the NAc and in the dorsal
17 striatum (caudate putamen; CPu) (Figure S1D).
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24 The administration of GAL(1-15) modified *GALR2* and *GALR1* expression in the
25 VTA, producing a significant increase in *GALR2* mRNA levels ($t_7=1.970$,
26 $p<0.05$; Table 1) and a slight increase in *GALR1* expression ($t_7=0.980$, $p=0.17$;
27 Table 1), suggesting the involvement of GALR in GAL(1-15)-mediated effects.
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34 ***GAL(1-15) decreased TH immunoreactivity in the striatum.***
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37 As shown in Table 2, i.c.v. administration of GAL(1-15) reduced TH
38 immunoreactivity in the striatum. GAL(1-15) induced a significant decrease in
39 TH immunoreactivity in the NAc core ($t_4=2.694$, $p<0.05$; Table 2) 90 min after its
40 administration. Moreover, the injection of GAL(1-15) induced a slight decrease
41 in TH immunoreactivity in the NAc Shell. In the dorsal striatum (CPu); the same
42 response pattern was observed in that GAL(1-15) produced a slight reduction in
43 TH immunoreactivity 90 min after its injection (Table 2).
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57 **DISCUSSION**
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3 In the present study, we demonstrated for the first time that GAL(1-15) induced
4 a strong anhedonia-like phenotype in non-operant and operant models: the
5 sucrose preference test (SPT), the novelty suppressed feeding (NSF) test, the
6 female urine sniffing test (FUST) and the operant saccharin self-administration
7 test. GALR2 was involved in this effect since the specific GALR2 antagonist
8 M871 blocked GAL(1-15) mediated action in saccharin self-administration.
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18 ¹⁸F-FDG PET images demonstrated that GAL(1-15) induced modifications in
19 FDG uptake *in vivo* in limbic nuclei, such as the hippocampus, the thalamus,
20 striatum, the prefrontal cortex and the piriform cortex. Importantly, the
21 mechanism of the GAL(1-15) behaviour-mediated effect **was accompanied by**
22 changes in the VTA and NAc, which are the key nuclei of the dopaminergic
23 mesolimbic circuit and are critical for the neurobiological bases of anhedonia
24 (Der-Avakian and Markou, 2012). Changes in the expression of the dopamine
25 transporters DAT and Vmat2 and in the D3 and Galanin receptors in the VTA
26 were observed. Changes in the immediate-early gene C-Fos and in the D1, D2
27 and D3 receptors in NAc were also observed. The relevance of the mesolimbic
28 circuit as a target for GAL(1-15) was also supported by the effect of GAL(1-15)
29 on the TH immunoreactivity in the striatum.
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46 In the operant self-administration models used as a tool for the study of reward-
47 seeking motivated behaviour (Sanchis-Segura and Spanagel, 2006; Ettenberg,
48 2009), GAL(1-15) induced a strong reduction in the number of reinforcements in
49 saccharin self-administration, suggesting that GAL(1-15) induced a loss of
50 motivational behaviour induced by an artificial reinforcer.
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3 In this work, we also studied the differential role of GAL(1-15) and GAL in
4 reward-seeking motivated behaviour using the saccharin self-administration
5 paradigm. GAL(1-15), but not GAL, induced a strong reduction in the number of
6 reinforcements, suggesting that only GAL(1-15) reduces the motivation to
7 consume the reward. We have previously described a different action of GAL
8 and GAL(1-15) in behaviour functions (Millon et al., 2015; Millon et al., 2016;
9 Millon et al., 2017b; Millon et al., 2017a). GAL(1-15) induces depression-related
10 and anxiogenic-like effects in rats, and these effects were significantly stronger
11 than the one induced by GAL (Millon et al., 2015). Furthermore, GAL(1-15)
12 produced a strong reduction in the alcohol intake and preference in a model of
13 voluntary alcohol intake, showing a different role than that of the complete GAL
14 molecule (Millon et al., 2017a). The differential action between GAL and
15 GAL(1-15) was observed not only in behavioural functions but also in central
16 cardiovascular regulation (Diaz-Cabiale et al., 2005; Diaz-Cabiale et al., 2010).

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19 Therefore, the fact that the effects of GAL(1-15) in the saccharin self-
20 administration were different than the effects induced by GAL validated and
21 extended the view of a specific role of GAL(1-15) in reward-related and
22 anhedonic behaviour.

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25 We demonstrated that GALR2 is involved in the anhedonia-like effects induced
26 by GAL(1-15) since the administration of the specific GALR2 antagonist M871
27 blocked the effects of GAL(1-15). The formation of heterodimers between
28 GALR1/GALR2 has been proposed to be the main mechanism of action of
29 GAL(1-15) (Fuxe et al., 2008; Fuxe et al., 2012; Millon et al., 2015; Borroto-
30 Escuela et al., 2014). Therefore, the anhedonia-like effects induced by GAL(1-
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3 15) are mediated by GALR2, confirming that GAL(1-15) acts through the
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5 heterodimer GALR1/GALR2.
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9 Not only in the operant model but also in non-operant models using several
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11 reinforcements, such as the natural reinforcer sucrose (SPT) (Willner, 2005;
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13 Walser, 2011; Der-Avakian and Markou, 2012), the food (NSF) (Walser, 2011;
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15 Ferres-Coy et al., 2016) and the sexual motivation behaviour (FUST)
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17 (Malkesman et al., 2010; Malkesman et al., 2012), GAL(1-15) induced a
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19 significant anhedonic-effect, confirming an important role for this neuropeptide
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21 in anhedonia, which is a core feature of Major Depressive Disorders.
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25 We have used ¹⁸F-FDG Positron Emission Tomography for *in vivo* imaging of
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27 metabolic processes (Eidelberg, 2009) as a powerful tool to study the effect of
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29 GAL(1-15). GAL(1-15) treatment altered glucose metabolism in several nuclei of
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31 the limbic system, decreasing FDG uptake in the hippocampus and thalamus or
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33 in several areas of the striatum and increasing the uptake in the prefrontal and
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35 piriform cortexes. These results are in agreement with previous works in which
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37 the hippocampus, the striatum or the prefrontal cortex were targeted for GAL(1-
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39 15) (Millon et al., 2015; Millon et al., 2016; Millon et al., 2017a) (Flores-Burgess,
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41 2018), which are areas rich in GAL-fragment binding sites (Hedlund et al., 1992).
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43 However, we cannot disregard GAL(1-15) action in other small nuclei such as
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45 the dorsal raphe (Millon et al., 2015), VTA or NAc because of the limited
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47 resolution of PET scans (Prieto et al., 2011).
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53 We further investigated GAL(1-15) effects on the key mesolimbic reward nodes,
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55 the VTA and the NAc, by evaluating the effects of GAL(1-15) treatment on the
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57 expression of genes that regulate dopaminergic activity, which is critical for the
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3 reward system and anhedonia (Russo and Nestler, 2013; Nestler and Carlezon,
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5 2006; Bressan and Crippa, 2005). GAL(1-15) induced a strong reduction in the
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7 expression of the dopamine transporters DAT and Vmat2 in the VTA. Since
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9 DAT regulates extracellular DA levels in the extracellular space into presynaptic
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11 DA terminals after DA release and since Vmat2 packages monoamines into
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13 synaptic vesicles for subsequent release (Garcia-Perez et al., 2016), our results
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15 suggest that GAL(1-15) affects the production and release of DA. In addition,
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17 GAL(1-15) also increased D3 receptor expression in the VTA, which is a
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19 receptor localized not only in the postsynaptic region but also in the presynaptic
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21 region, where it modulates both dopamine synthesis and release, thereby
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23 acting as an autoreceptor (Leggio et al., 2013; Diaz et al., 2000).
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29 Moreover, the colocalization, as determined by procedures previously used
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31 (Millon et al., 2015), of GALR1 and GALR2 in the VTA (Figure S1A) and the
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33 increased GALR1 and GALR2 expression induced by GAL(1-15) in this area
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35 confirmed that the VTA is a target of GAL(1-15). Since GAL(1-15) acts through
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37 a GALR1/GALR2 heteroreceptor complex that inhibits the Gq/G11-mediated
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39 signalling of the GALR2 protomer and switches it towards Gi/o-mediated
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41 signalling, thereby enhancing Gi/o-mediated signalling of GALR1 in the
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43 GALR1–GALR2 heterodimer (Millon et al., 2015; Borroto-Escuela et al., 2014).
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45 This enhancement of the Gi/o-mediated signal reduces DA formation in this cell,
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47 which could explain the functional results found in this work.
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53 In the NAc, GAL(1-15) reduced C-Fos mRNA expression and TH
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55 immunoreactivity and increased the numbers of the D1, D2 and D3 receptors.
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57 The reduction of DA release suggested by our experiments would explain the
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3 reduction in neural activity (Manning et al., 2017) in the NAc and can explain
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5 the increase in DA receptor expression as a compensatory effect.
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8 Altogether, these modifications induced by GAL 1-15 suggested that its main
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10 effect on the mesolimbic pathway is the ~~structural and~~ functional reorganization
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12 of the dopaminergic system, which likely leads to its functional inhibition.
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16 The results of the present work are in agreement with previous work that
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18 showed that the effect of GAL(1-15) in EtOH consumption was directly related
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20 to the dopaminergic system and the striatum (Millon et al., 2017a).
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24 In conclusion, our results indicate that GAL(1-15) induces a strong anhedonia-
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26 like phenotype in rats, likely due to the involvement of the dopaminergic
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28 mesolimbic system, which is critical for the reward system and anhedonia
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30 (Russo and Nestler, 2013; Nestler and Carlezon, 2006; Gold et al., 2018;
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32 Bressan and Crippa, 2005). Motivation is a brain function that is based on this
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34 dopaminergic system, which is critically compromised in several
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36 neuropsychiatric diseases/conditions, including depression. Our results may
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38 help to better understand the complex problem of impaired motivation (i.e.,
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40 anhedonia) and may provide a basis for the development of novel therapeutic
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42 strategies using GAL(1-15) analogues for the treatment of depression and
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44 reward-related diseases.
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REFERENCES

- Albert PR, Le Francois B and Millar AM. (2011) Transcriptional dysregulation of 5-HT_{1A} autoreceptors in mental illness. *Mol Brain* 4: 21.
- Alen F, Santos A, Moreno-Sanz G, et al. (2009) 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): 465-473.
- Barde S, Ruegg J, Prud'homme J, et al. (2016) Alterations in the neuropeptide galanin system in major depressive disorder involve levels of transcripts, methylation, and peptide. *Proc Natl Acad Sci U S A* 113(52): E8472-E8481.
- Barr AM, Kinney JW, Hill MN, et al. (2006) A novel, systemically active, selective galanin receptor type-3 ligand exhibits antidepressant-like activity in preclinical tests. *Neurosci Lett* 405(1-2): 111-115.
- Bartfai T, Lu X, Badie-Mahdavi H, et al. (2004) Galmic, a nonpeptide galanin receptor agonist, affects behaviors in seizure, pain, and forced-swim tests. *Proc Natl Acad Sci U S A* 101(28): 10470-10475.
- Bellido I, Diaz-Cabiale Z, Jimenez-Vasquez PA, et al. (2002) Increased density of galanin binding sites in the dorsal raphe in a genetic rat model of depression. *Neurosci Lett* 317(2): 101-105.
- Borroto-Escuela DO, Narvaez M, Di Palma M, et al. (2014) Preferential activation by galanin 1-15 fragment of the GalR1 protomer of a GalR1-GalR2 heteroreceptor complex. *Biochem Biophys Res Commun*.
- Brancheck TA, Smith KE, Gerald C, et al. (2000) Galanin receptor subtypes. *Trends Pharmacol Sci* 21(3): 109-117.
- Bressan RA and Crippa JA. (2005) The role of dopamine in reward and pleasure behaviour--review of data from preclinical research. *Acta Psychiatr Scand Suppl* (427): 14-21.
- Cadoni C, Pisanu A, Simola N, et al. (2017) Widespread reduction of dopamine cell bodies and terminals in adult rats exposed to a low dose regimen of MDMA during adolescence. *Neuropharmacology* 123: 385-394.
- Der-Avakian A and Markou A. (2012) The neurobiology of anhedonia and other reward-related deficits. *Trends Neurosci* 35(1): 68-77.
- Diaz J, Pilon C, Le Foll B, et al. (2000) Dopamine D₃ receptors expressed by all mesencephalic dopamine neurons. *J Neurosci* 20(23): 8677-8684.
- Diaz-Cabiale Z, Parrado C, Fuxe K, et al. (2007) Receptor-receptor interactions in central cardiovascular regulation. Focus on neuropeptide/alpha(2)-adrenoreceptor interactions in the nucleus tractus solitarius. *J Neural Transm* 114(1): 115-125.

- 1
2
3 Diaz-Cabiale Z, Parrado C, Narvaez M, et al. (2010) Neurochemical modulation of
4 central cardiovascular control: the integrative role of galanin. *EXS* 102: 113-
5 131.
6
7 Diaz-Cabiale Z, Parrado C, Vela C, et al. (2005) Role of galanin and galanin(1-15) on
8 central cardiovascular control. *Neuropeptides* 39(3): 185-190.
9 Eidelberg D. (2009) Metabolic brain networks in neurodegenerative disorders: a
10 functional imaging approach. *Trends Neurosci* 32(10): 548-557.
11 Ettenberg A. (2009) The runway model of drug self-administration. *Pharmacol*
12 *Biochem Behav* 91(3): 271-277.
13
14 Ferres-Coy A, Galofre M, Pilar-Cuellar F, et al. (2016) Therapeutic antidepressant
15 potential of a conjugated siRNA silencing the serotonin transporter after
16 intranasal administration. *Mol Psychiatry* 21(3): 328-338.
17
18 Flores-Burgess A, Millon C, Gago B, et al. (2017) Galanin (1-15) enhancement of the
19 behavioral effects of Fluoxetine in the forced swimming test gives a new
20 therapeutic strategy against depression. *Neuropharmacology* 118: 233-241.
21
22 Flores-Burgess A, Millon, C., Gago, B., García-Durán, L., Cantero-García, N., Coveñas,
23 R., Naváez, JA., Fuxe, K., Santín, L., Díaz-Cabiale, Z. . (2018) GALANIN(1-15)-
24 FLUOXETINE INTERACTIONS IN THE PREFRONTAL CORTEX:
25 INVOLVEMENT OF GALR1-GALR2-5-HT1A HETERORECEPTOR
26 COMPLEXES. *Neuropharmacology* (Submitted).
27
28 Fuxe K, Borroto-Escuela DO, Romero-Fernandez W, et al. (2012) On the existence
29 and function of galanin receptor heteromers in the central nervous system.
30 *Front Endocrinol (Lausanne)* 3: 127.
31
32 Fuxe K, Marcellino D, Rivera A, et al. (2008) Receptor-receptor interactions within
33 receptor mosaics. Impact on neuropsychopharmacology. *Brain Res Rev*
34 58(2): 415-452.
35
36 Garcia-Perez D, Nunez C, Laorden ML, et al. (2016) Regulation of dopaminergic
37 markers expression in response to acute and chronic morphine and to
38 morphine withdrawal. *Addict Biol* 21(2): 374-386.
39
40 Genders SG, Scheller KJ and Djouma E. (2018) Neuropeptide modulation of
41 addiction: Focus on galanin. *Neurosci Biobehav Rev*.
42
43 Gold MS, Blum K, Febo M, et al. (2018) Molecular role of dopamine in anhedonia
44 linked to reward deficiency syndrome (RDS) and anti- reward systems.
45 *Front Biosci (Schol Ed)* 10: 309-325.
46
47 Gorwood P. (2008) Neurobiological mechanisms of anhedonia. *Dialogues Clin*
48 *Neurosci* 10(3): 291-299.
49
50 Hedlund PB and Fuxe K. (1996) Galanin and 5-HT1A receptor interactions as an
51 integrative mechanism in 5-HT neurotransmission in the brain. *Ann N Y*
52 *Acad Sci* 780: 193-212.
53
54 Hedlund PB, Yanaihara N and Fuxe K. (1992) Evidence for specific N-terminal
55 galanin fragment binding sites in the rat brain. *Eur J Pharmacol* 224(2-3):
56 203-205.
57
58 Jacobowitz DM, Kresse A and Skofitsch G. (2004) Galanin in the brain:
59 chemoarchitectonics and brain cartography--a historical review. *Peptides*
60 25(3): 433-464.
61
62 Juhasz G, Hullam G, Eszlari N, et al. (2014) Brain galanin system genes interact
63 with life stresses in depression-related phenotypes. *Proc Natl Acad Sci U S A*
64 111(16): E1666-1673.

- 1
2
3 Kormos V and Gaszner B. (2013) Role of neuropeptides in anxiety, stress, and
4 depression: from animals to humans. *Neuropeptides* 47(6): 401-419.
- 5 Kuteeva E, Hokfelt T and Ogren SO. (2005) Behavioural characterisation of
6 transgenic mice overexpressing galanin under the PDGF-B promoter.
7 *Neuropeptides* 39(3): 299-304.
- 8 Kuteeva E, Hokfelt T, Wardi T, et al. (2008a) Galanin, galanin receptor subtypes
9 and depression-like behaviour. *Cell Mol Life Sci* 65(12): 1854-1863.
- 10 Kuteeva E, Wardi T, Hokfelt T, et al. (2007) Galanin enhances and a galanin
11 antagonist attenuates depression-like behaviour in the rat. *Eur*
12 *Neuropsychopharmacol* 17(1): 64-69.
- 13 Kuteeva E, Wardi T, Lundstrom L, et al. (2008b) Differential role of galanin
14 receptors in the regulation of depression-like behavior and
15 monoamine/stress-related genes at the cell body level.
16 *Neuropsychopharmacology* 33(11): 2573-2585.
- 17 Leggio GM, Salomone S, Bucolo C, et al. (2013) Dopamine D(3) receptor as a new
18 pharmacological target for the treatment of depression. *Eur J Pharmacol*
19 719(1-3): 25-33.
- 20 Lu X, Barr AM, Kinney JW, et al. (2005) A role for galanin in antidepressant actions
21 with a focus on the dorsal raphe nucleus. *Proc Natl Acad Sci U S A* 102(3):
22 874-879.
- 23 Malkesman O, Austin DR, Tragon T, et al. (2012) Acute D-serine treatment
24 produces antidepressant-like effects in rodents. *Int J Neuropsychopharmacol*
25 15(8): 1135-1148.
- 26 Malkesman O, Scattoni ML, Paredes D, et al. (2010) The female urine sniffing test: a
27 novel approach for assessing reward-seeking behavior in rodents. *Biol*
28 *Psychiatry* 67(9): 864-871.
- 29 Manning CE, Williams ES and Robison AJ. (2017) Reward Network Immediate
30 Early Gene Expression in Mood Disorders. *Front Behav Neurosci* 11: 77.
- 31 Marco EM, Ballesta JA, Irala C, et al. (2017) Sex-dependent influence of chronic
32 mild stress (CMS) on voluntary alcohol consumption; study of
33 neurobiological consequences. *Pharmacol Biochem Behav* 152: 68-80.
- 34 Marquez PV and Saxena S. (2016) Making Mental Health a Global Priority.
35 *Cerebrum* 2016.
- 36 Millon C, Flores-Burgess A, Castilla-Ortega E, et al. (2017a) Central administration
37 of galanin N-terminal fragment 1-15 decreases the voluntary alcohol intake
38 in rats. *Addict Biol*.
- 39 Millon C, Flores-Burgess A, Narvaez M, et al. (2017b) The neuropeptides Galanin
40 and Galanin(1-15) in depression-like behaviours. *Neuropeptides* 64: 39-45.
- 41 Millon C, Flores-Burgess A, Narvaez M, et al. (2016) Galanin (1-15) enhances the
42 antidepressant effects of the 5-HT_{1A} receptor agonist 8-OH-DPAT:
43 involvement of the raphe-hippocampal 5-HT neuron system. *Brain Struct*
44 *Funct* 221(9): 4491-4504.
- 45 Millon C, Flores-Burgess A, Narvaez M, et al. (2015) A role for galanin N-terminal
46 fragment (1-15) in anxiety- and depression-related behaviors in rats. *Int J*
47 *Neuropsychopharmacol* 18(3): 1-13.
- 48 Mitsukawa K, Lu X and Bartfai T. (2008) Galanin, galanin receptors and drug
49 targets. *Cell Mol Life Sci* 65(12): 1796-1805.
- 50 Nestler EJ and Carlezon WA, Jr. (2006) The mesolimbic dopamine reward circuit in
51 depression. *Biol Psychiatry* 59(12): 1151-1159.
- 52
53
54
55
56
57
58
59
60

- 1
2
3 Pascual-Brazo J, Castro E, Diaz A, et al. (2012) Modulation of neuroplasticity
4 pathways and antidepressant-like behavioural responses following the
5 short-term (3 and 7 days) administration of the 5-HT(4) receptor agonist
6 RS67333. *Int J Neuropsychopharmacol* 15(5): 631-643.
7
8 Prieto E, Collantes M, Delgado M, et al. (2011) Statistical parametric maps of
9 (1)(8)F-FDG PET and 3-D autoradiography in the rat brain: a cross-
10 validation study. *Eur J Nucl Med Mol Imaging* 38(12): 2228-2237.
11
12 Russo SJ and Nestler EJ. (2013) The brain reward circuitry in mood disorders. *Nat*
13 *Rev Neurosci* 14(9): 609-625.
14
15 Sanchis-Segura C and Spanagel R. (2006) Behavioural assessment of drug
16 reinforcement and addictive features in rodents: an overview. *Addict Biol*
17 11(1): 2-38.
18
19 Swanson CJ, Blackburn TP, Zhang X, et al. (2005) Anxiolytic- and antidepressant-
20 like profiles of the galanin-3 receptor (Gal3) antagonists SNAP 37889 and
21 SNAP 398299. *Proc Natl Acad Sci U S A* 102(48): 17489-17494.
22
23 Tatemoto K, Rokaeus A, Jornvall H, et al. (1983) Galanin - a novel biologically active
24 peptide from porcine intestine. *FEBS Lett* 164(1): 124-128.
25
26 Walser SMA DJM. (2011) Mouse Models of Depression. In: Uehara DT (ed)
27 *Psychiatric Disorders-Trends and Developments*. In Tech.
28
29 Weiss JM, Bonsall RW, Demetrikopoulos MK, et al. (1998) Galanin: a significant
30 role in depression? *Ann N Y Acad Sci* 863: 364-382.
31
32 Willner P. (2005) Chronic mild stress (CMS) revisited: consistency and
33 behavioural-neurobiological concordance in the effects of CMS.
34 *Neuropsychobiology* 52(2): 90-110.
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Figure 1. Experimental design of the procedures for saccharin self-administration (A), the sucrose preference test (SPT) (B), and the novelty suppressed feeding test (NSF) and female urine sniffing test (C). i.c.v., intracerebroventricular.

Figure 2. Role of Galanin (1-15) [GAL(1-15)] on operant self-administration of saccharin 0,2% in rats. A) Dose-response curve of the effect GAL(1-15) on self-administration of saccharin. GAL(1-15) (n=7-10 animals) was administered intracerebroventricularly (i.c.v.) 15 min before the test session. Cerebrospinal fluid-injected rats were used as the control group (n= 12 animals). B) The effect of administration of Galanin (GAL) and GAL(1-15) on the self-administration of saccharin. GAL (3 nmol/rat) and GAL(1-15) (3 nmol/rat; n=8-9 animals) were injected i.c.v. 15 min before the test. Cerebrospinal fluid-injected rats were used as the Control group (n=14 animals). C) The effects of the co-administration of GALR2 receptor antagonist M871 (3 nmol/rat) and GAL(1-15) (n=7-9 animals) on the self-administration of saccharin. Treatments were injected i.c.v. 15 min before the test. Cerebrospinal fluid-injected rats were used as Control groups (n=9 animals). Vertical bars represent a mean \pm standard error of the mean of the Number of saccharin Reinforcements during the test period. *p<0.05 and **p<0.01 versus the rest of the groups according to a one-way ANOVA followed by Fisher's least significance difference test.

Figure 3. Effect of administration of Galanin (1-15) [GAL(1-15)] in the sucrose preference test with 1% sucrose concentration in rats. GAL(1-15) (3 nmol/rats; n= 8-9 animals per group) were administered intracerebroventricularly 2, 8 and 24 h before the measures. Cerebrospinal fluid-injected rats were used as the Control group (n= 7 animals). Vertical bars represent the mean \pm standard error of the mean of sucrose intake (g/kg) (A,) and preference (percent) (B) during the different period. *p<0.05 and **p<0.01 versus the Control group according to a repeated measures two-way ANOVA followed by Fisher's least significance difference test.

Figure 4. Effect of the administration of Galanin (1-15) [GAL(1-15)] in the novelty-suppressed feeding test (NSF) (A) and the female urine sniffing test (FUST) (B,C) in rats. (A) GAL(1-15) (3 nmol/rat; n= 9 animals per group) were administered intracerebroventricularly 15 min before the test. Cerebrospinal fluid-injected rats were used as the Control group (n= 8 animals). Vertical bars represent the mean \pm standard error of the mean of the latency period of the first feeding (s) during the test. ***p<0.001 versus Control group according to a Student's t-test. (B) GAL(1-15) (3 nmol/rat; n= 7 animals) were administered i.c.v. 15 min before the test. Cerebrospinal fluid-injected rats (CSFa) were used as the Urine-CSFa group (n= 6 animals). Vertical bars represent the mean \pm standard error of the mean of the sniffing duration of water (n= 14 animals) or oestrus female urine (s) during the test. ***p<0.001; **p<0.01 and *p<0.05 according to a one-way ANOVA followed by Fisher's least significant difference test. (C) GAL(1-15) (3 nmol/rat; n= 6 animals) were administrated icv 15 min before the test. Cerebrospinal fluid-injected rats (CSFa) were used as the Water-CSFa group (n= 6 animals). Data represent the mean \pm standard error of the mean of sniffing duration of water during the test. No differences were found according Student's t-test.

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4 **Figure 5.** Effects of GAL(1-15) on rat brain glucose metabolism. Statistical
5 parametric mapping (SPM) analysis results displayed on coronal, horizontal and
6 sagittal brain sections showing regions with significantly reduced
7 (hypometabolism, blue) and increased (hypermetabolism, red) uptake of
8 [¹⁸F]FDG at 30, 60 and 90 min after i.c.v. GAL(1-15) administration ($p < 0.01$
9 uncorrected, clusters with extended threshold $k = 100$).
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11 **Figure 6.** Effects of Galanin (1-15) [GAL(1-15)] in the ventral tegmental area
12 (VTA) mRNA expression of (A) *Dat*, (B) *Vmat2*, (C) *C-Fos* or nucleus
13 accumbens (NAc) mRNA expression of (D) *C-Fos* in rats. GAL(1-15) (3
14 nmol/rat) was injected intracerebroventricularly 1 hour before the measures.
15 Cerebrospinal fluid-injected rats were used as the Control group. Vertical bars
16 represent the mean \pm standard error of the mean ($n = 4-5$ animals per group).
17 * $p < 0.05$ versus Control group according to a Student's *t*-test.
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20 **Figure 7.** Effects of Galanin (1-15) [GAL(1-15)] in the expression of the
21 dopamine receptors *D1*, *D2*, *D3* and *D5* in the ventral tegmental area (VTA) (A-
22 D) or nucleus accumbens (NAc) (E-H) in rats. GAL(1-15) (3 nmol/rat) was
23 injected intracerebroventricularly 1 hour before the measures. Cerebrospinal
24 fluid-injected rats were used as the Control group. Vertical bars represent the
25 mean \pm standard error of the mean ($n = 4-5$ animals per group). * $p < 0.05$ versus
26 Control group according to a Student's *t*-test.
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Table 1. Galr1 and Galr2 mRNA expression.

	<i>Galr1</i>		<i>Galr2</i>	
	Control	GAL(1-15)	Control	GAL(1-15)
VTA	0.70±0.2	1.21±0.4	1.22±0.1	1.66±0.1*
NAc	0.72±0.2	0.47±0.1	0.99±0.1	0.98±0.1

Table 1. Effects of Galanin (1-15) [GAL(1-15)] in the expression of Galanin receptors *Galr1* and *Galr2* in the Ventral Tegmental Area (VTA) or Nucleus Accubens (NAc) in rats. GAL(1-15) (3nmol/rat) was injected intracerebroventricular 1 hour before the measures. Cerebrospinal fluid-injected rats were used as Control group. n=4-5 animals per group. *p<0.05 versus Control group according to Student's *t*-test.

Table 2. Tyrosine Hydroxylase immunoreactivity in the Striatum.

	Control	GAL(1-15)
NAc Shell	24.2±4.7	16.5±4.0
NAc Core	6.7±0.3	4.5±0.7*
Dorsal Striatum (CPu)	6.1±0.9	4.9±0.8

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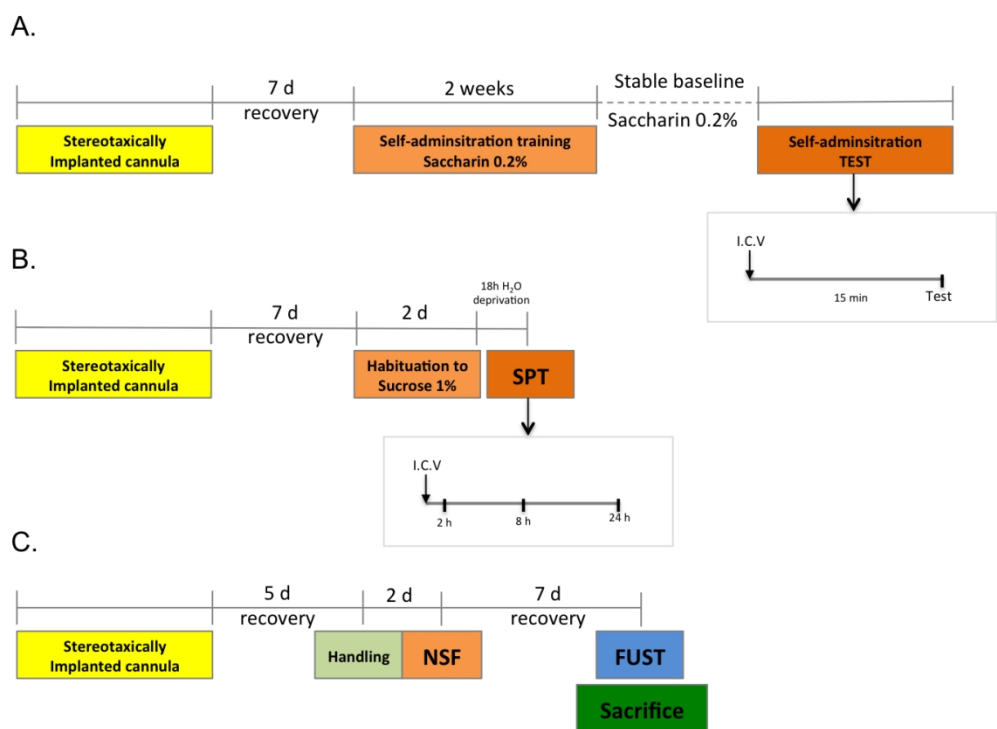


Figure 1

231x168mm (150 x 150 DPI)

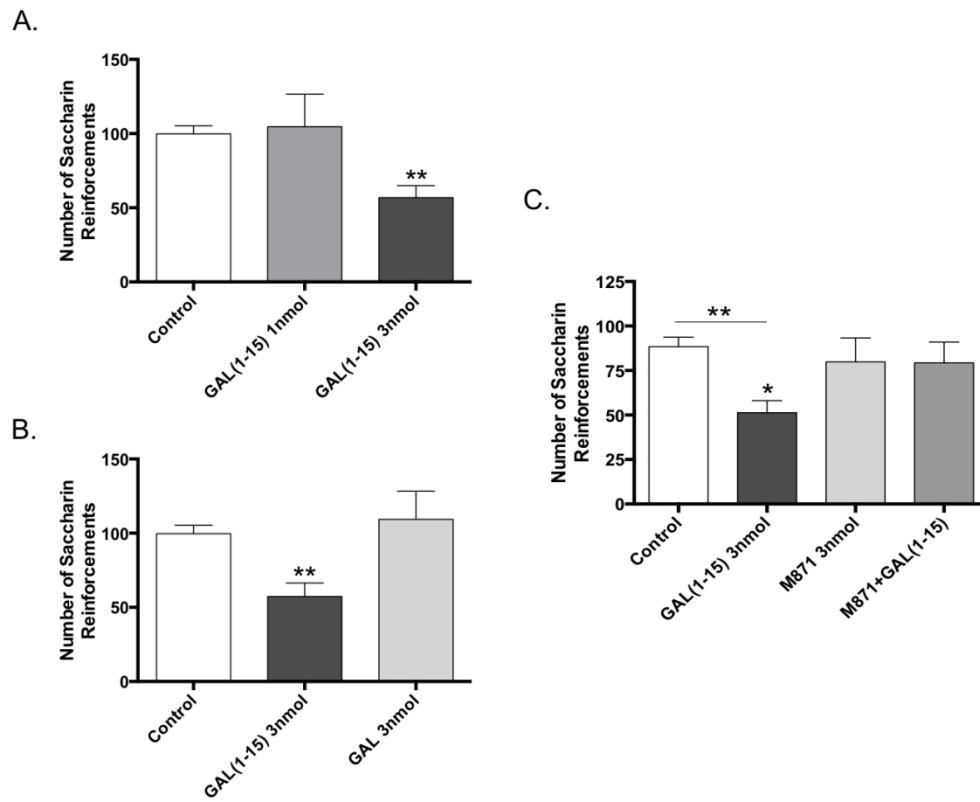


Figure 2

227x185mm (150 x 150 DPI)

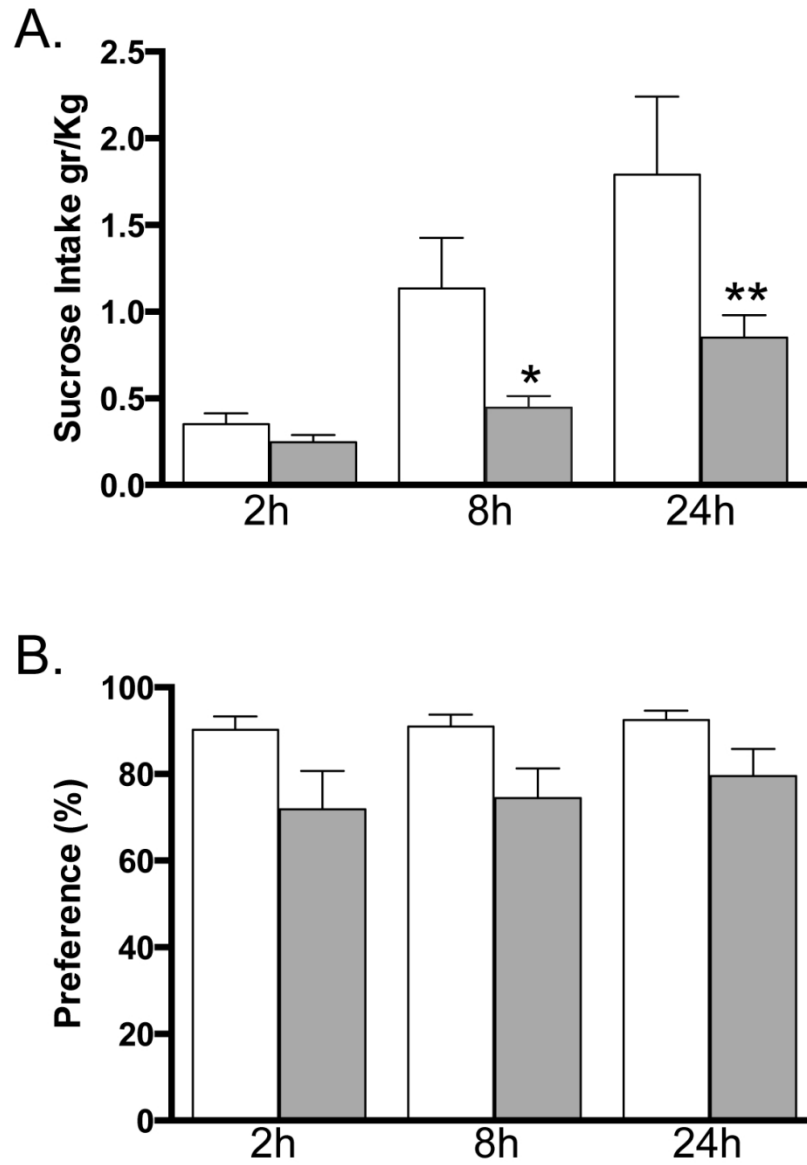


Figure 3 revised

99x140mm (300 x 300 DPI)

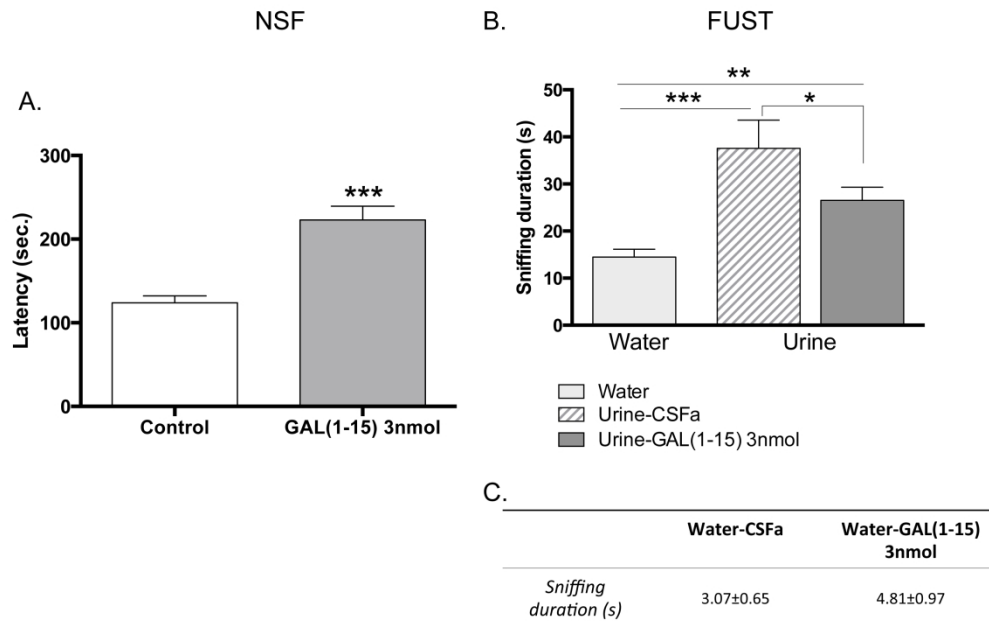


Figure 4 revised

249x156mm (300 x 300 DPI)

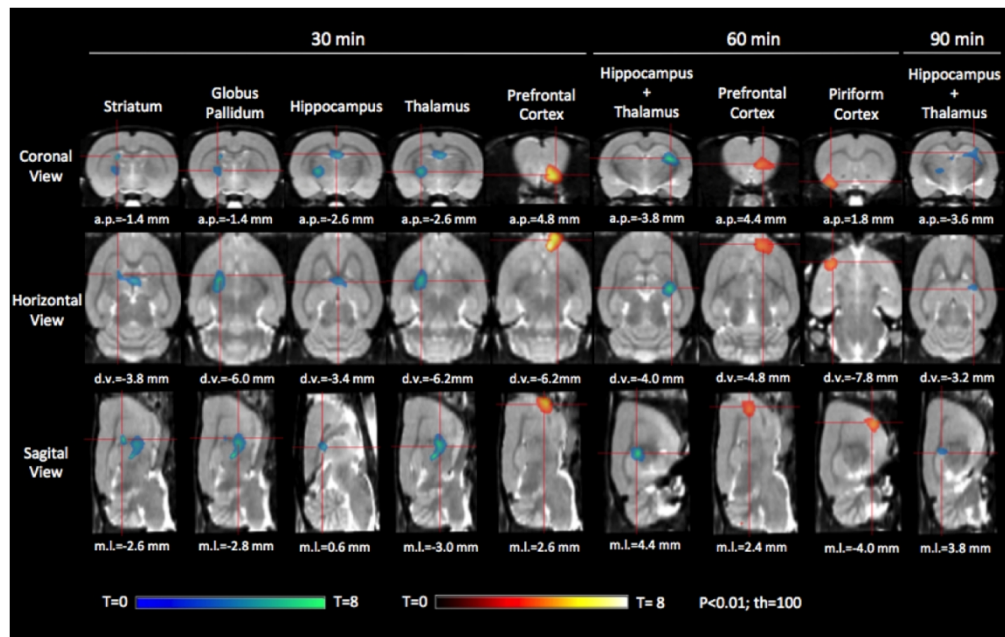


Figure 5

225x143mm (150 x 150 DPI)

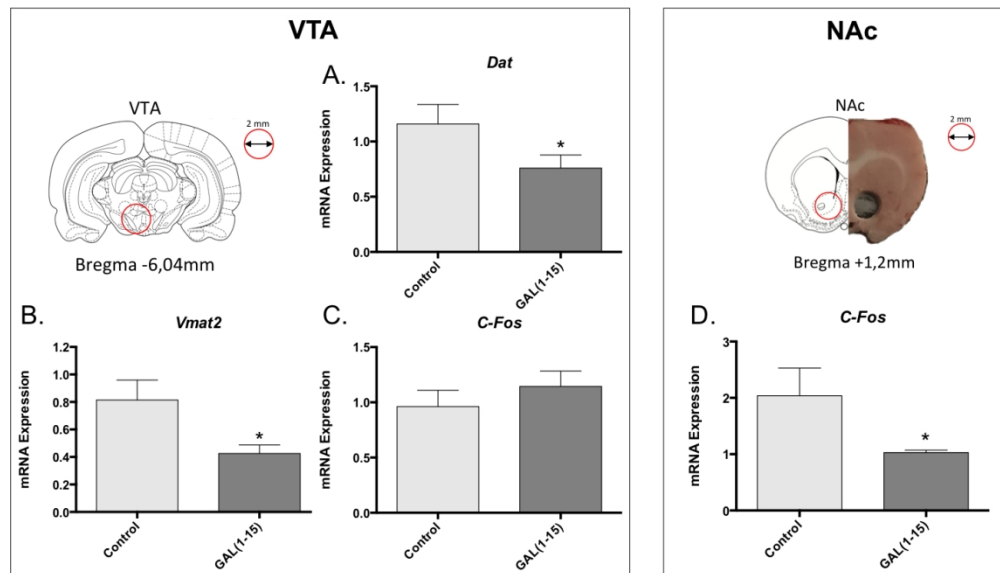


Figure 6

245x139mm (150 x 150 DPI)

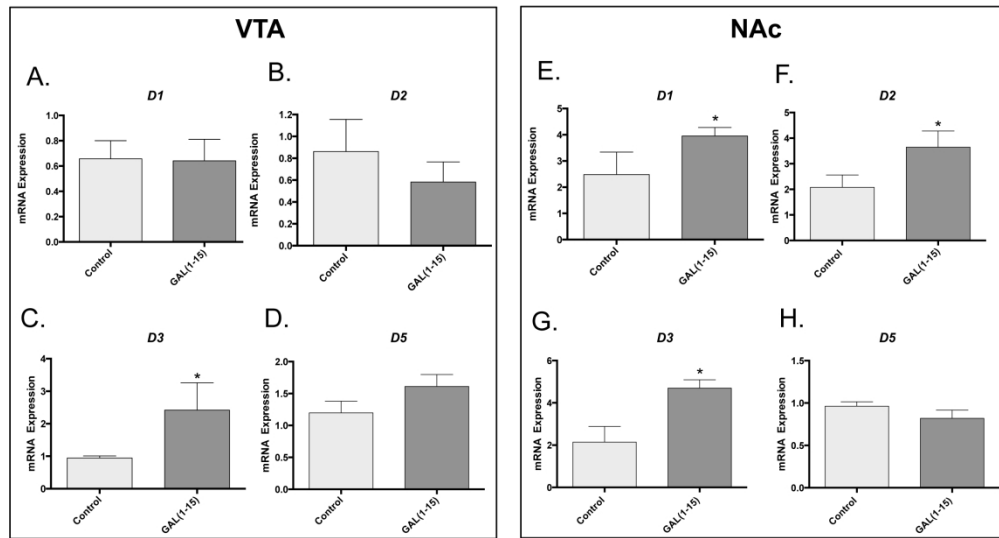


Figura 7 revised

244x130mm (300 x 300 DPI)

SUPPLEMENTARY MATERIAL AND METHODS

Animals

Male Sprague Dawley rats (body weight 225–250g, age 8 weeks) were obtained from Criffa and maintained in a humidity- and temperature-controlled (20–22°C) room. The rats in the saccharin self-administration and sucrose preference test were during the entire protocol maintained on a 12-hour reversed light/dark cycle (lights off at 10 am) whereas the other rats kept on 12-hour light/dark cycle. The animals had free access to food pellets and tap water. Experimental procedures were approved by the Institutional Animal Ethics Committee of the University of Málaga, in accordance with the European Directive (86/609/EEC) and Spanish Directive (Real Decretory 53/2013).

Intracerebroventricular Injections

This protocol has been used previously (Millon et al., 2015; Diaz-Cabiale et al., 2011). Briefly, the rats were anesthetized intraperitoneally with equitiesin (3.3mL/kg body weight), and stereotaxically implanted with a unilateral chronic 22-gauge stainless-steel guide cannula into the right lateral cerebral ventricle using the following coordinates: 1.4mm lateral and 1mm posterior to bregma, and 3.6mm below the surface of the skull (Paxinos, 1986). After surgery, animals were individually housed and allowed a recovery period of 7 days. The injections in the lateral ventricle were performed using a 26-gauge stain less-steel injection cannula connected via a PE-10 tubing to a Hamilton syringe. The total volume was 5 µl per injection and the infusion time was 1min.

Solutions were prepared freshly and the peptides were dissolved in artificial cerebrospinal fluid (composition is 120nM NaCl, 20nM NaH₂CO₃, 2nM KCl, 0.5nM KH₂PO₄, 1.2nM CaCl₂, 1.8 nM MgCl₂, 0.5 nM Na₂SO₄, and 5.8 nM D-glucose, pH 7.4). GAL was obtained from NeoMPS, Strasbourg, France; GAL(1–15) and the GALR2 receptor antagonist M871 were obtained from Tocris Bioscience, Bristol, UK.

Saccharin Self-administration

Training and testing were conducted in standard operant chambers. Each chamber was equipped with a drinking reservoir (0.10 ml) positioned 4 cm above the grid floor in the center of the front panel of the chamber, and two retractable levers were located 3 cm to right and left of the drinking receptacle (one being the active lever, and the other being the inactive lever). Visual stimuli were presented via a light located on the front panel.

Sucrose Preference Test

Briefly, rats were habituated to 1% sucrose solution for two days with free access to water and sucrose in their home cage. To prevent possible effects of side preference in drinking behaviour, the position of the bottles was switched every 24h. After this adaptation, rats were deprived of water for 18 h. Food was available during the testing session.

Novelty Suppressed feeding

Novelty Suppressed feeding (NSF) is a conflict test that elicits competing motivations: the drive to eat and the fear of venturing into the center of the arena (Ferres-Coy et al., 2016). The testing apparatus consisted of a plastic box (60x40x20 cm), with its floor covered with wooden bedding (2 cm) and with a single pellet of food in the center of the box on white circular filter paper (10 cm in diameter). Each rat was placed in the corner of the testing arena and the latency of the first feeding episode was recorded. Non-feeding behaviours (e.g. smelling and touching) were ignored.

Female Urine Sniffing Test

The female Urine Sniffing Test (FUST) is a non-operant test for measuring reward-seeking behaviour in rodents based on interest in sniffing pheromones odours from opposite sex, and was conducted as described previously (Malkesman et al., 2012). Briefly, the test had three phases: (1) a single exposure (3 min) to the cotton tip dipped in sterile water, during which the time spent sniffing the applicator was measured; 2 an interval of 45 min during which no applicator was presented to the animal; and (3) a single exposure (3 min) to

a cotton tip infused with fresh urine collected from females of the same strain in oestrus or water, during which the time spent sniffing was measured.

The animals receiving GAL(1-15) 3nmol or vehicle were injected 15 min before to the exposure to the urine or water (phase 3).

PET Studies

Ten or eleven days after surgery, the animals ($n=8$; 335 ± 38 g) were fasted overnight and anaesthetized with isoflurane inhalation (5 % for induction and 2 % for maintenance in 100% oxygen gas). Animals were administered icv with aCSF and after 3-4 days with Gal(1-15) (3 nmol). Immediately thereafter the treatment, the ^{18}F -FDG (43.6 ± 5.5 MBq in 0.3 ml of 0.9% NaCl) was injected through the tail vein. After an awake uptake period of 25 minutes to ensure the incorporation of ^{18}F -FDG during drug-induced behavioural effects (Prieto et al., 2011), animals were anesthetized and placed prone on the PET scanner bed to perform a static acquisition of 30 min at three different time-points (30, 60 and 90 min) after tracer administration. The images were subsequently reconstructed using an iterative 3-D row action maximum likelihood algorithm (3-D RAMLA), with 2 iterations and a relaxation parameter of 0.024. Corrections for dead time, decay and random coincidences were applied. Images were reconstructed on a $128 \times 128 \times 120$ matrix, where the voxel size equals $1 \times 1 \times 1$ mm.

Blood glucose concentration (128 ± 24 mg/dl) was determined before tracer injection, using a glucose level meter with test strips (Accu Chek Aviva Nano, Roche, Mannheim, Germany).

Image preprocessing

The image preprocessing was performed using PMOD (PMOD Technologies, Zürich, Switzerland; <https://www.pmod.com/web/>), SPM8 (Wellcome Department of Imaging Neuroscience, London, UK; <http://www.fil.ion.ucl.ac.uk/spm/software/spm8/>), SAMIT (Small Animal Molecular Imaging Toolbox created by University Medical Center Groningen for SPM, Groningen, Netherlands; <http://mic-umcg.github.io/samit/>) and a custom-made software developed using MATLAB R2015b (Mathworks;

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3 https://es.mathworks.com/products/new_products/release2015b.html) to
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5 normalize the ^{18}F -FDG uptake values to a whole brain average value.
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8 The PET images were initially manually matched using PMOD Fusion
9 toolbox to a T2-MRI rat template of SAMIT (Technologies, 2016), which is in
10 Paxinos and Watson anatomical space (Schwarz et al., 2006; (UMCG). 2015).
11 This coregister included several rotations and translations in each of the three
12 dimensions (x, y and z) to match the location, and orientation of the T2 template.
13 Afterwards, the coregistered images were spatially normalized to a FDG rat
14 template provided by SAMIT (Vallez Garcia et al., 2015) in SPM8, skull stripped
15 (removing any extracerebral activity) and smoothed with a Gaussian kernel of
16 $1.2 \times 1.2 \times 1.2$ full width at half-maximum (FWHM) to remove any non-uniformities.
17 Finally, custom-made software was used to normalize the values of the ^{18}F -
18 FDG uptake in each image to the average brain uptake value.
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26 27 **Genes expression in VTA and NAc**

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29 Rats from NSF and FUST experiment (Fig. 1B) were killed by decapitation 1h
30 after a single i.c.v. administration of GAL(1-15) 3 nmol or vehicle and the brains
31 were rapidly removed and frozen until its use. The nuclei dissections were
32 conducted as described (Garcia-Perez et al., 2016) with modifications. Brains
33 were sliced on the brain matrix (1mm) and kept -20°C until each region of
34 interest comes into the cutting plane. For VTA analysis, one 1 mm coronal brain
35 section was obtained from bregma -6.04 mm (Paxinos, 1986). For NAc study,
36 two consecutive 1mm coronal slices were made corresponding to approximate
37 bregma $+1.2$ mm (Paxinos, 1986). Tissues of interest were dissected using a
38 punching device with 2 mm internal diameter. Bilateral punches of the NAc and
39 VTA (Fig. 4 and 6) were collected into Eppendorf tubes.
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49 **RNA isolation and quantitative real-time polymerase chain reaction** 50 **analysis**

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52 Total RNA was isolated from the punches of NAc and VTA using RNeasy Lipid
53 Tissue Kit (Qiagen, Hilden, Germany). cDNA was obtained using a Reverse
54 Transcriptase Core Kit (Eurogentec, Seraing, Belgium). These steps were
55 performed according to the manufacturer's instructions.
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All PCR were conducted in triplicate using FastStart essential DNA green master (Roche Diagnostics GmbH, Mannheim, Germany) in LightCycler 96 system (Roche Diagnostics GmbH, Mannheim, Germany).

The data were analysed using the comparative Ct method and normalized to measures of glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) mRNA.

Tyrosine Hydroxylase immunohistochemistry

Rats from NSF and FUST experiment (Fig. 1B) were perfused transcardially 90 min after a single i.c.v. administration of GAL(1-15) 3 nmol or vehicle. The brains were then dissected and serial, coronal free-floating sections (30 µm thick) were collected. The sections were incubated with a primary antibody rabbit anti-TH (Sigma, 1/2000) overnight at 4°C. An hour incubation with biotinylated, specific secondary antibodies (1:200; Vector Labs Inc) was performed at room temperature. The chromogen used was 0.03% 3-30-diaminobenzidine tetrahydrochloride (Sigma).

Analysis of TH Immunoreactivity was performed under light microscopy (Nikon Optiphot-2) in the neuro-anatomical area of interest. Three sections per animal were examined and captured by a video camera (Olimpus UC30) linked to a PC computer, and TH immunoreactivity was quantified by optical density using the computer software ImageJ (National Institutes of Health).

Double Immunofluorescence

The procedures have been previously used (Millon et al., 2015). Primary antibody rabbit anti-GAL2 receptor (Alomone Lab, 1/250) was incubated for 12 hours at 4°C and detected with the red secondary antibody mouse anti-rabbit DyLight 549 (Jackson ImmunoResearch Laboratories, 1/100). Goat anti-GAL1 receptor (Santa Cruz Biotechnology INC, EEUU, 1:250) was incubated 12 hours at 4°C and detected with the secondary antibody rabbit biotinylated anti-goat (Vector Labs Inc.) and Alexa Fluor 488-conjugated Streptavidin (Jackson Laboratories ImmunoResearch, 1:1000). Sections were mounted on slides with fluorescent mounting medium (Dako) and visualized using a Leica SP8 confocal microscope. The double immunolabeling was performed in the tissue of rats without treatment (Figure S1).

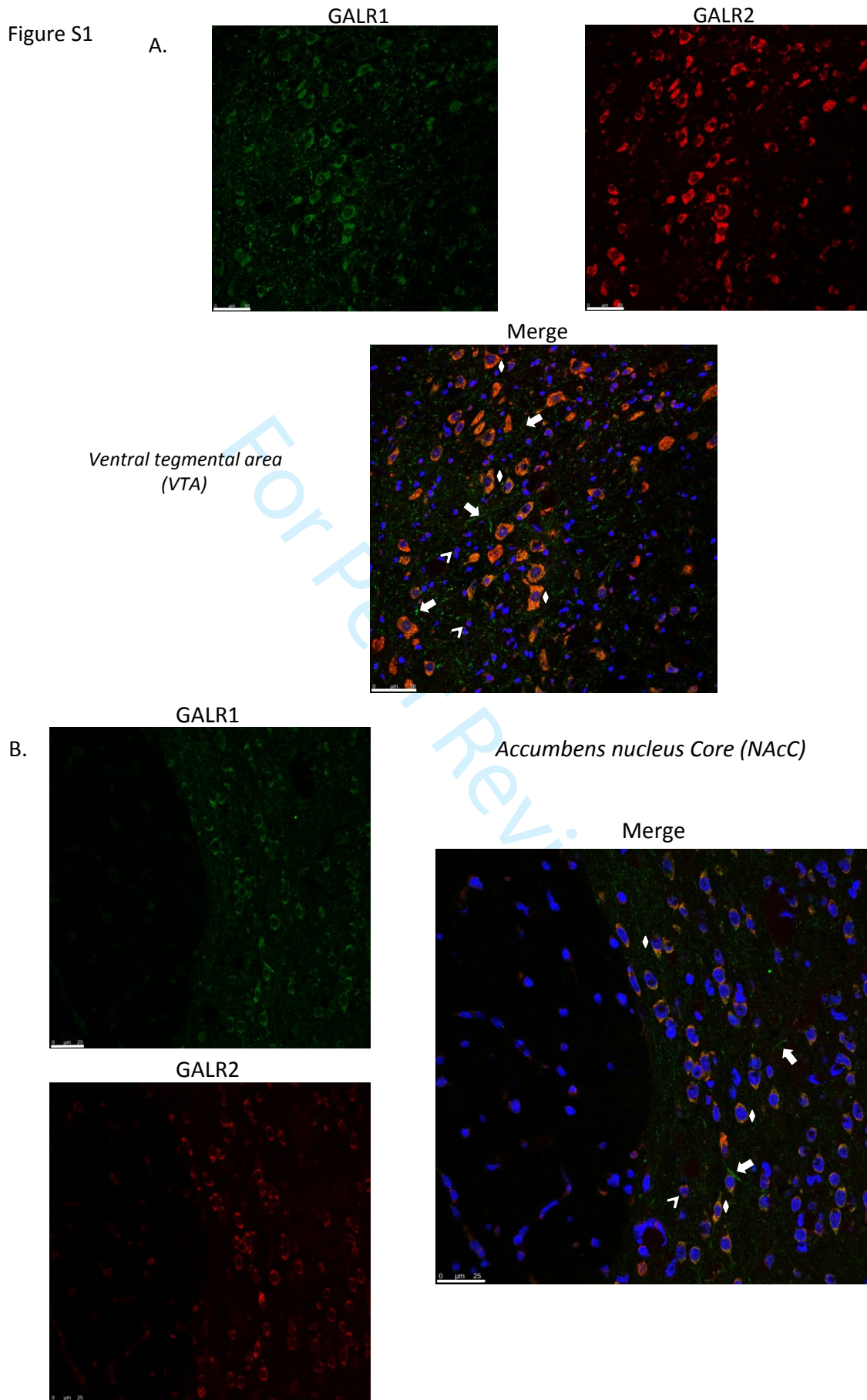
Table S1. Oligonucleotide primers used in qPCR.

Gene	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Dat</i>	NM_012694.2	ACGGTGGCATCTACGTCTTC	TCACTGAATTGCTGGACGCC
<i>Vmat2</i>	NM_013031.1	GGATGGGGCTATTGAGCTCTT	AAAGCAGATGGAGCCTGCA
<i>C-Fos</i>	NM_022197.2	AAACGGAGAATCCGAAGG	CGTCTTCAAGTTGATCTGTC
<i>Drd1</i>	NM_012546.3	AAACAAATAGGCCAAGAGTC	CTTTCCAATCTGAAAAGTGC
<i>Drd2</i>	NM_012547.1	AAGCGCCGAGTTACTGTCAT	GACCACAAAGGCAGGGTTG
<i>Drd3</i>	NM_017140.2	CATCTACATAGTCTGAGGC	CTGGCCCTTATTGAAAAGTGC
<i>Drd5</i>	NM_012768.1	AACTGTATGGTTCCTTTCTG	AATATGTCGAAGGTGGTCTC
<i>Galr1</i>	NM_012958.3	AAAAGTGGACAAAAGTTCAGCC	GGATACCTTTGTCTTTGCTC
<i>Galr2</i>	NM_019172.5	AACAGGAATCCACAGACC	CCCTTTGGTCTTTAACAAG
<i>Gapdh</i>	NM_017008.4	GCTCTCTGCTCCTCCCTGTTC	GAGGCTGGCACTGCACAA

Table S2. Water intake (g/Kg) in Sucrose Preference Test.

Treatment	2h	8h	24h
<i>Control</i>	3.13±0.77	7.46±1.49	9.97±1.64
<i>GAL(1-15) 3nmol</i>	8.55±2.58	13.47±3.12	16.58±2.90

Table S2. Effect of administration of Galanin (1-15) [GAL(1-15)] in the water intake in Sucrose Preference Test with 1% sucrose concentration in rats. GAL(1-15) (3nmol/rats; n= 8-9 animals per group) were administrated intracerebroventricular 2, 8 and 24 hours before the measures. Cerebrospinal fluid-injected rats were used as the Control group (n= 7 animals). Data represent the mean ± standard error of the mean of water intake (g/Kg). No significant differences were found by repeated measures two-way ANOVA.



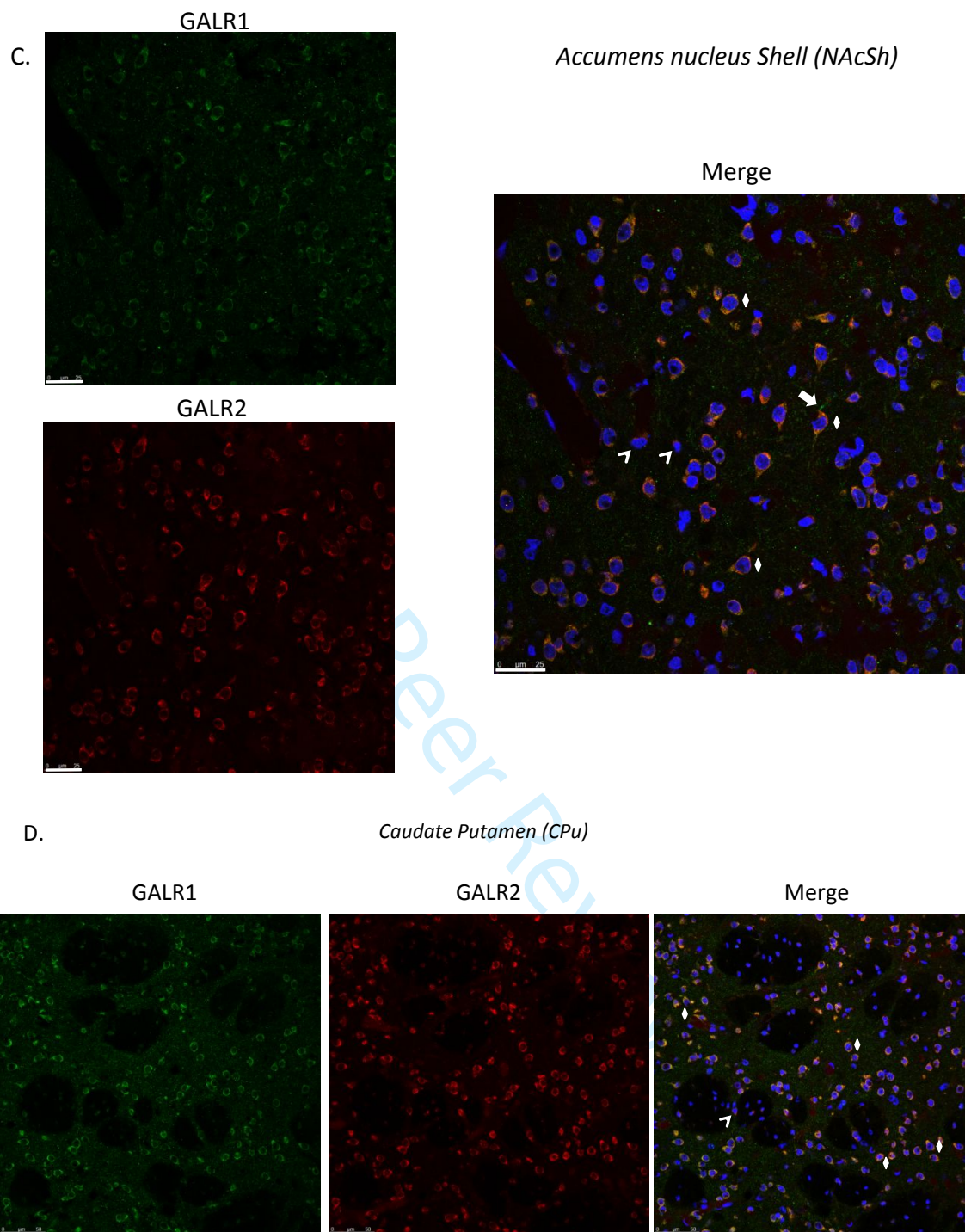


Figure S1. Galanin Receptor 1 (GALR1) and Galanin Receptor 2 (GALR2) colocalizes in neurons of rat striatum and Ventral Tegmental Area. Double-immunolabeled for GALR1 (green, arrows) and GALR2 (red, arrowhead) in (A) Ventral Tegmental Area (VTA), (B) Accumbens nucleus Core (NAcC), (C) Accumbens nucleus Shell (NAcSh) and (D) Caudate Putamen (Cpu). The colocalization of GALR1 and GALR2 is shown in the merge (orange-yellow, rhombus). Escal bar, 25-50 μ m.

REFERENCES

- (UMCG). NMaMI. (2015) Nuclear Medicine and Molecular Imaging (UMCG). SAMIT Introduction.
- Alen F, Santos A, Moreno-Sanz G, et al. (2009) 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): 465-473.
- Diaz-Cabiale Z, Parrado C, Narvaez M, et al. (2011) Galanin receptor/Neuropeptide Y receptor interactions in the dorsal raphe nucleus of the rat. *Neuropharmacology* 61(1-2): 80-86.
- Ferres-Coy A, Galofre M, Pilar-Cuellar F, et al. (2016) Therapeutic antidepressant potential of a conjugated siRNA silencing the serotonin transporter after intranasal administration. *Mol Psychiatry* 21(3): 328-338.
- Garcia-Perez D, Nunez C, Laorden ML, et al. (2016) Regulation of dopaminergic markers expression in response to acute and chronic morphine and to morphine withdrawal. *Addict Biol* 21(2): 374-386.
- Malkesman O, Austin DR, Tragon T, et al. (2012) Acute D-serine treatment produces antidepressant-like effects in rodents. *Int J Neuropsychopharmacol* 15(8): 1135-1148.
- Marco EM, Ballesta JA, Irala C, et al. (2017) Sex-dependent influence of chronic mild stress (CMS) on voluntary alcohol consumption; study of neurobiological consequences. *Pharmacol Biochem Behav* 152: 68-80.
- Millon C, Flores-Burgess A, Narvaez M, et al. (2015) A role for galanin N-terminal fragment (1-15) in anxiety- and depression-related behaviors in rats. *Int J Neuropsychopharmacol* 18(3): 1-13.
- Pascual-Brazo J, Castro E, Diaz A, et al. (2012) Modulation of neuroplasticity pathways and antidepressant-like behavioural responses following the short-term (3 and 7 days) administration of the 5-HT(4) receptor agonist RS67333. *Int J Neuropsychopharmacol* 15(5): 631-643.
- Paxinos G. (1986) *The rat Brain in the stereotaxic coordinates*.
- Prieto E, Collantes M, Delgado M, et al. (2011) Statistical parametric maps of (1)(8)F-FDG PET and 3-D autoradiography in the rat brain: a cross-validation study. *Eur J Nucl Med Mol Imaging* 38(12): 2228-2237.
- Schwarz AJ, Danckaert A, Reese T, et al. (2006) A stereotaxic MRI template set for the rat brain with tissue class distribution maps and co-registered anatomical atlas: application to pharmacological MRI. *Neuroimage* 32(2): 538-550.
- Technologies P. (2016) PMOD Image Fusion (PFUS). Version 3.8 ed.
- Vallez Garcia D, Casteels C, Schwarz AJ, et al. (2015) A standardized method for the construction of tracer specific PET and SPECT rat brain templates: validation and implementation of a toolbox. *PLoS One* 10(3): e0122363.