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.

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Abstract

Galanin N-terminal fragment (1-15) [GAL(1-15)] is associated with depression-related and anxiogenic-like effects in rats. In this study, we analyzed the ability of GAL(1-15) to modulate 5-HT1Areceptors (5-HT1AR), a key receptor in depression.

GAL(1-15) enhanced the antidepressant effects induced by the 5-HT1AR agonist 8-OH-DPAT in the forced swimming test. These effects were stronger than the ones induced by GAL. This action involved interactions at receptor level since GAL(1-15) affected the binding characteristics and the mRNA levels of 5-HT1AR in the dorsal hippocampus and dorsal raphe. The involvement of the GALR2 was demonstrated with the GALR2 antagonist M871. Proximity ligation assay experiments indicated that 5-HT1AR are in close proximity with GALR1 and GALR2 in both regions and in raphe RN33B cells.

The current results indicate that GAL(1–15) enhances the antidepressant effects induced by 8-OH-DPAT acting on 5-HT1AR operating as postjunctional or as autoreceptors. These results may give the basis for the development of drugs targeting potential GALR1-GALR2-5-HT1A heteroreceptor complexes linked to the raphe-hippocampal 5-HT neurons for the treatment of depression.

Key words: Galanin, Galanin (1-15), 5-HT1A receptors, heteroreceptor complexes, depression

INTRODUCTION

Depression is a heterogeneous disorder that will become the second cause of disability worldwide.

Decreased serotoninergic (5-HT) activity is involved in the development and expression of major depression and most drugs used for treating depression increase 5-HT levels (Blier and de Montigny 1994; Jans et al. 2007). Of the 5-HT1 receptors, the role of the 5-HT1A subtype in depression is well-established (Albert et al. 1996; Albert et al. 2011; Fuxe et al. 2008; Artigas et al. 1996; Artigas 2013). The 5-HT1A receptor (5-HT1AR) is an inhibitory G-protein coupled receptor expressed both as an autoreceptor on dorsal raphe (DR) neurons, and as a postsynaptic receptor in many brain regions such as hippocampus where it participates in mediating the antidepressant effects of 5-HT (Hamon et al. 1990; Barnes and Sharp 1999; Artigas 2013).

Several studies reported abnormalities in 5-HT1AR in patients with major depression. Postmortem and neuroimaging studies suggest an increased density of 5-HT1A autoreceptor in patients with major depression (Stockmeier et al. 1998; Blier 2010). In animal models, transgenic mice with a reduction of 5-HT1A autoreceptors report antidepressant-like responses (Richardson-Jones et al. 2010) and acute 5-HT1A autoreceptor knockdown mice increased antidepressant responses (Ferres-Coy et al. 2013).

Contrary to 5-HT1A autoreceptors, the response to activation of postsynaptic hippocampal 5-HT1AR indicated antidepressant efficacy. PET studies on 5-HT1A binding demonstrated a reduced postsynaptic hippocampal 5-HT1AR and cortical regions in major depression (Drevets et al. 2000; Drevets et al. 2007; Savitz et al. 2009). In preclinical studies, chronic antidepressant treatments tonically activated hippocampal 5-HT1AR (Haddjeri et al. 1998) and selective 5-HT1AR agonists showed antidepressant-like activity (Cryan et al. 2005).

All these results indicate an opposing role of 5-HT1A autoreceptors and postsynaptic 5-HT1AR in depression. Combinations of serotonin reuptake inhibitors (SSRI) and a mixed 5-HT1AR agonist-antagonist to accelerate the clinical effect of SSRI were introduced. However, pharmacological approaches were hampered by the difficulty in separating effects on autoreceptors from

effects on postsynaptic 5-HT1AR (Artigas 2013). Moreover, the partial 5-HT1AR agonist pindolol enhanced the SSRI effects but the non-selective 5-HT1AR antagonist DU-125530 did not cancel the effects of the SSRI indicating that postsynaptic 5-HT1A sites are involved but not fully required for antidepressant effects (Scorza et al. 2012).

Galanin (GAL) is a neuropeptide (Tatemoto et al. 1983) widely distributed in neurons within the Central Nervous System (CNS). To date, three GAL receptor (GALR) subtypes, GALR1-3, with high affinity for GAL have been cloned (Branchek et al. 2000; Mitsukawa et al. 2008). GALR1 and GALR3 mainly activate inhibitory G proteins Gi/Go, while GALR2 mainly couples to Gq/G11 to mediate excitatory signaling (Wang et al. 1997; Branchek et al. 2000).
GAL participates in mood regulation and depression (Weiss et al. 1998; Bellido et al. 2002; Juhasz et al. 2014). The activation of GALR1 and GALR3 results in a depression like behavior while stimulation of GALR2 leads to anti-depressant-like effects (Bartfai et al. 2004; Lu et al. 2005; Kuteeva et al. 2008).
Moreover, GAL modulates 5-HT1AR function at autoreceptor and postsynaptic level in the brain. In the DR, intracerebroventricular (icv) GAL induced a time-dependent reduction in affinity and an increase in the 5-HT1AR autoreceptor density (Razani et al. 2000). At post-synaptic level GAL reduced the affinity of the 5-HT1AR in the ventral limbic cortex (Fuxe et al. 1988; Hedlund and Fuxe 1996). Moreover, in hypothermia, locomotor activity and passive avoidance, icv GAL blocked post-synaptic 5-HT1AR function (Misane et al. 1998; Razani et al. 2001; Kehr et al. 2002). Recent results indicate that this interaction can in part be due to the existence of GALR1-5-HT1AR heteroreceptor complexes in discrete brain regions (Borroto-Escuela et al. 2010).

Not only GAL but also the N-terminal fragments like GAL(1-15) are active in the CNS (Hedlund and Fuxe 1996; Diaz-Cabiale et al. 2005; Diaz-Cabiale et al. 2007; Diaz-Cabiale et al. 2010). Recently we described that GAL(1-15) induces strong depression-related and anxiogenic-like effects in rats and these effects were significantly stronger than the ones induced by GAL (Millon et al. 2015). The GALR1-GALR2 heteroreceptor complexes in the dorsal hippocampus and especially in the DR, areas rich in GAL(1-15) binding sites (Hedlund et al. 1992)

were involved in these effects (Millon et al. 2015) and demonstrated also in cellular models (Borroto-Escuela et al. 2014). Also, N-terminal GAL fragments interacts with 5-HT1AR in the dorsal hippocampus (Hedlund et al. 1994) and in the ventral limbic cortex (Diaz-Cabiale et al. 2000).

The purpose of the current study was to assess the ability of GAL(1-15) to modulate 5-HT1AR at the autoreceptor and postsynaptic receptor level in rats. We have analyzed the effect of GAL(1-15) on the 5-HT1AR-mediated response in a behavioral test of depression and the involvement of the GALR2 in these effects. Moreover, the effects of GAL(1-15) were determined on the binding characteristics and mRNA levels of 5-HT1AR in the DR and dorsal hippocampus. Furthermore, with the proximity ligation assay (PLA), the proximity of the 5-HT1AR with GALR1 and GALR2 was analized to evaluate the existence of GALR1-5-HT1AR and GALR2-5-HT1AR heteroreceptor complexes in these two brain regions and in the rat medullary raphe-derived cell line RN33B. The effects of GAL(1-15) on 5-HT1A immunoreactivity were also analyzed in the raphe RN33B cells.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats from CRIFFA, Barcelona (200-250gr) were maintained in temperature/humidity-controlled conditions. Experimental procedures were approved by the Institutional Animal Ethics Committee of the University of Málaga, Spain.

Detailed descriptions are available in Supplemental Information 1 on animal controlled-conditions, surgical preparation, icv injections, forced swim test (FST) and behavioral scores.

Behavioral Assessment

Groups of rats were assessed in the FST. Three sets of experiments were conducted.

In the first set of experiments, a dose-response curve of the 5-HT1AR agonist 8-OH-DPAT was performed. For this, groups of rats received subcutaneously (s.c) 8-OH-DPAT 0.125mg/Kg, 0.25mg/Kg or vehicle 60 min before the tests. In the second set of experiments, in order to evaluate the interactions of 8-OH-DPAT and GAL(1-15) at behavioral level, groups of rats received s.c. 8-OH-DPAT (0.25mg/Kg) 60 minutes before the tests and icv GAL(1-15) (1nmol) 15 before the tests alone or in combination based on previous work. In this set of experiments, other groups of rats received s.c. 8-OH-DPAT (0.125mg/Kg), icv GAL(1-15) (1nmol) and icv the GALR2 antagonist M871 (3nmol) alone or in combination.

In the third set of experiments, the interactions in the behavioral test of GAL and GAL(1–15) with 8-OH-DPAT were compared; for this, groups of rats received s.c 8-OH-DPAT (0.125mg/kg) 60 minutes before the test and icv GAL (1 nmol) or GAL(1–15) (1nmol) 15 min before the test.

Saline (s.c) plus cerebrospinal fluid (CSFa) (icv) injected rats were used as the control group.

The experimental groups are detailed in Table S1 A. The locomotor activity was analyzed in the open field test (see Supplemental Information 1 for details).

Autoradiography and in situ hybridization

The procedure to perform receptor autoradiography and *in situ* hybridization was described previously (Razani et al. 2000; Razani et al. 2001). Coronal sections were obtained at the dorsal hippocampus and DR level (see Supplemental Information 1 for details).

Quantitative autoradigraphy

Saturation experiments were performed using [3H]-8-OH-DPAT. The sections were preincubated for 30 minutes at room temperature in 50mM Tris-HCl buffer (pH 7.6), containing 4mM $CaCl_2$, 0.01% ascorbic acid and 10mM pargyline. The sections were then incubated for 60 minutes at room temperature with [^{3}H]-8-OH-DPAT in the same solution as above. Film exposure time for sections was 6 weeks (see Supplemental Information 1 for details).

In situ hybridization:

After prehybridization in a humidified chamber with the prehybridization buffer for 2 h at 37°C the sections were hybridized under coverslips to $1x10^6$ cpm RNA probe/100µl of the hybridization mix for 16 h at 55°C. Following hybridization, the sections were rinsed 4 x 20 min each in 1x SSC at 55ºC. Finally, sections were rinsed in autoclaved water for 10 s, dehydrated in alcohol and air-dried. Film exposure time for sections was 2 weeks (for riboprobe information see Supplemental Information 1)

Image analysis

Measurements were made in the Dentate Gyrus (DG) and CA1 of the hippocampus (0.15 mm^2 square). The ventral part of the midline area of the DR was analyzed using a square as a sampling field (0.09mm2) (see Supplemental Information 1 for details).

The experimental groups are detailed in Table S1 B.

Proximity Ligation In Situ Assay (Duolink)

The PLA study and the quantification were carried out as described previously using a Duolink kit (Olink, Sweden) (Borroto-Escuela et al. 2012; Narvaez et al. 2014; Millon et al. 2015). The primary antibodies directed to GALR1 (goat polyclonal (C-20), Santa Cruz Biotechnology Inc, Dallas, TX; 1:250), GALR2 (rabbit polyclonal, Alomone Labs, Israel; 1/250) and 5-HT1AR (mouse monoclonal (MAB11041) Millipore corp, USA, 1:400) were used (see Supplement 1 for details)

Statistical Analysis

Data are presented as the means ± SEM and samples number (n) are indicated in figure legends. All data were analyzed using GraphPad PRISM 4.0 (GraphPad Software, La Jolla, CA).

For comparing two experimental conditions, Student's unpaired t-test statistical analysis was performed. Otherwise, One-way analysis of variance (ANOVA)

followed by Newman-Keuls comparison post-test was performed. Differences were considered significant at P<0.05 (*P<0.05, **P<0.01, ***P<0.001).

The data from the saturation experiments were analyzed by non-linear regression analysis for the determination of the dissociation constant (Kd) and the total number of agonist binding sites (Bmax) using GraphPad PRISM 4.0

RESULTS

GAL(1-15) enhances 8-OH-DPAT-mediated antidepressant-like behaviors

Dose-response effects of 8-OH-DPAT

As previously described, the 5-HT1AR agonist 8-OH-DPAT at 0.25mg/Kg induced antidepressant-like effects in the FST as it significantly decreased the immobility (t_{10}=2.476,p<0.05) and the climbing behavior (t_{11}=2.519,p<0.05) while an increased swimming behavior by 140% (t_{11}=4.550,p<0.001) was observed (Table S2-A). This dose modifies the locomotor parameters, since the total distance travelled (t_{10}=5.811,p<0.001) and the mean speed (t_{10}=5.811,p<0.001) were significantly reduced during a 5 min test period (Table S2-B).

The dose of 0.125mg/Kg of 8-OH-DPAT was a threshold dose in this test (Immobility:t_8=0.378,p=0.357;Climbing:t_9=0.858,p=0.206;Swimming:t_9=0.281,p=0.392). Moreover, this dose lacked effects in the locomotor parameters (total distance:t_{11}=1.612,p=0.067;mean speed:t_{11}=1.612,p=0.067) (Table S2).

GAL(1-15) and 8-OH-DPAT interactions

The threshold dose of GAL(1-15) 1nmol enhanced the antidepressant-like effects mediated by the effective dose of the 8-OH-DPAT (Figure S1). GAL(1-15) significantly decreased the immobility ($F_{3,26}$=11,75,p<0.001;post hoc,p<0.05) and the climbing ($F_{3,24}$=10.20,p<0.001;post hoc,p<0.05) behavior induced by an effective dose of the 8-OH-DPAT in the FST (Figure S-1). Moreover GAL(1-15) increased the swimming behavior induced 8-OH-DPAT by 20% ($F_{3,29}$=47,56,p<0.001;post hoc,p<0.01) (Figure S-1).

To avoid the locomotor activity of the 8-OH-DPAT we analyzed the effects of the coadmistration of 8-OH-DPAT (0.125mg/Kg) and GAL(1-15) (1nmol) at threshold doses. A strong antidepressant effect was observed after the coadministration of GAL(1-15) and 8-OH-DPAT (0.125mg/Kg). Thus, a significant decrease appeared in the immobility ($F_{3,19}$=6.974,p<0.01;post hoc,p<0.01) and the climbing ($F_{3,22}$=5.969,p<0.01;post hoc,p<0.01) behavior by 50 and 60% respectively versus the 8-OH-DPAT group (Figure 1). Moreover an increase of the swimming behavior of about 80% versus 8-OH-DPAT group was also observed ($F_{3,21}$=13.17,p<0.001;post hoc,p<0.001) (Figure 1). These antidepressant effects were independent of the locomotor activity, as the total distance reached and speed were equivalent between all the groups (Table S-3).

Effects of the GALR2 antagonist

GALR2 participates in this interaction since the GALR2 antagonist M871 blocked significantly the immobility ($F_{3,19}$=6.974,p<0.01;post hoc,p<0.01), climbing ($F_{3,22}$=5.969,p<0.01;post hoc,p<0.05) and the swimming ($F_{3,21}$=13.17,p<0.001;post hoc,p<0.001) effects induced by the coadministration of GAL(1-15) and 8-OH-DPAT in the FST (Figure 1).

The GAL2 receptor antagonist M871 alone in the dose of 3nmol lacked effects with respect to immobility (88.6±1 sec.), climbing (92±1 sec.) or swimming time (84.6±8 sec.).

Comparison between the effect of GAL(1–15) and GAL on 8-OH-DPAT-mediated antidepressant-like behaviors

In the FST, the overall one-way ANOVA revealed a significant effect of treatment for immobility ($F_{3,19}$=5.771,p<0.01). The decrease in the immobility induced by the coadministration of GAL(1–15) and 8-OH-DPAT (0.125mg/kg) was significantly higher than the one induced by GAL and 8-OH-DPAT (0.125mg/kg) (post hoc,p<0.05;Figure 2A). In the climbing behavior we observed the same pattern of response ($F_{3,21}$=8.995,p<0.001): 8-OH-DPAT (0.125mg/Kg)+GAL(1-15) induced a significantly stronger decrease in climbing response compared with GAL+8-OH-DPAT (0.125mg/kg) (post hoc,p<0.05;Figure 2B). Importantly, in the swimming behavior GAL(1–15)+8-

DPAT (0.125mg/kg) also induced a significantly stronger increase in swimming response compared with 8-OH-DPAT (0.125mg/Kg)+GAL ($F_{3,29}$=27.47,*p*<0.001;post hoc,*p*<0.01; Figure 2C).

GAL(1–15) modifies the binding characteristics and mRNA levels of 5-HT1AR in the dorsal hippocampus and DR

10 min time-interval

5-HT1AR autoradiography in dorsal hippocampus

Icv. GAL(1-15) (3nmol/rat) produced a time dependent effect on the binding of [3H]-8-OH-DPAT in CA1/DG areas of the dorsal hippocampus (Figure 3). 10 minutes after icv GAL(1-15), that corresponds with the time point of the FST performance, GAL(1-15) produced a significant increase in the Kd (t_9=1.959,p<0.05) and Bmax (t_9=2.022,p<0.05) values of the [^{3}H]8-OH-DPAT binding sites in the CA1 area by about 90% and 85% ,respectively. In the DG the same pattern of response was observed. GAL(1-15) induced a significant increase in the Kd (t_9=3.291,p<0.01) and Bmax (t_9=3.685,p<0.01) values of the [^{3}H]8-OH-DPAT binding sites by about 150% and 95%, respectively (Figure 3B). Representative autoradiograms with a high radioligand concentration (7 nM) illustrate the increase of labelling (increase in Bmax values) in both areas, CA1 and DG 10 minutes after icv. GAL(1-15) (Figure 3C).

The coinjection of the GALR2 antagonist M871 (3nmol/rat) blocked significantly the increase in the Kd and Bmax values of the [^{3}H]8-OH-DPAT binding sites induced by GAL(1-15) in both areas, CA1 and DG. This established the involvement of GALR2 in the effect (Table 1).

2h and 5h time interval

5-HT1AR autoradiography in dorsal hippocampus

These effects on the Kd and Bmax values of the [^{3}H]8-OH-DPAT binding sites were maintained at 2 h after icv GAL(1-15) in the CA1 (Kd: t_9=2.091,p<0.05; Bmax:t_{10}=1.877,p<0.05) and also in the DG (Kd:t_{10}=2.158,p<0.05; Bmax:t_{10}=2.799,p<0.01) (Figure 3). However, 5 hours after icv GAL(1-15) the

only significant change remaining was the increase of the density of [^{3}H]8-OH-DPAT binding sites in the DG (t_{10}=2.203,p<0.05) (Figure 3).

In situ hybridization of 5-HT1AR mRNA levels in dorsal hippocampus

2 hours after icv. GAL(1-15) administration a substantial increase was observed in the 5-HT1AR mRNA level by 53% and 43%, in the CA1 (t_8=3.545,p<0.01) and DG (t_8=3.003,p<0.01), respectively (Figure 5A,C). However, 5 hours after GAL(1-15), the effects had disappeared (Table D, in Figure 5).

10min to 5h time interval

5-HT1AR autoradiography in DR

GAL(1-15) also produced a time dependent effect on the binding of [^{3}H]-8-OH-DPAT in the DR (Fig 4). As seen in Figure 4A GAL(1-15), 2 hours after its icv administration, produced no changes in the K_D values of [^{3}H]8-OH-DPAT binding sites in the DR (t_{10}=0.462,p=0.326) but caused instead a decrease in the Bmax values by about 20% (t_9=2.530,p<0.05). No effect was observed at 10 minutes (Kd: t_{19}=0.815,p=0.212; Bmax: t_{19}=0.397,p=0.347) nor at 5 hours (Kd: t_{10}=0.090,p=0.464; Bmax: t_{10}=0.315,p=0.379) after icv GAL(1-15) (Figure 4). Representative autoradiograms illustrate the reduction of labelling (decrease in Bmax values) 2 hours after icv. GAL(1-15) (Figure 4C).

In situ hybridization of 5-HT1AR mRNA levels in DR

The results from the *in situ* hybridization studies also showed GAL(1-15) 2 hours after administration produced a substantial decrease by 30% in the 5-HT1AR mRNA in the DR (t_6=3.161,p<0.01) (Figure 5B). However, 5 hours after icv. galanin the effects had disappeared (Table D, in Figure 5).

GALR1-5-HT1AR and GALR2-5-HT1R are in close proximity in the Dorsal Hippocampus and DR Nucleus

Positive and specific PLA blobs were obtained with the in situ PLA giving the indication that GALR1 and 5-HT1AR are in close proximity and may form GALR1-5-HT1AR heteroreceptor complexes in the areas studied. Moreover, positive and specific PLA blobs were also obtained for the GALR2 and 5-

HT1AR in these regions indicating the existence also of GALR2-5-HT1AR heteroreceptor complexes within them.

In Figure 6, CA1, CA2, and CA3 and the DG of the dorsal hippocampus and the DR show a significant number of red clusters (blobs) per cell The quantification of PLA in these areas demonstrated that the highest number of GALR1-5-HT1AR PLA positive clusters among these areas were found in the CA3 area ($F_{4,33}$=29,08,p<0.001;post hoc,p<0.001). Instead the highest number of GALR2-5-HT1AR positive PLA clusters was present in the cytoplasm of nerve cells of the DR ($F_{4,35}$=12.49,p< 0.001;post hoc,p<0.01;Figure 6A).

The specificity was demonstrated by the fact that no PLA clusters were observed in the lateral corpus callosum (Figure 6), an area that seems to lack the GALR1 and GALR2 (O'Donnell et al. 1999).

The results obtained in the DR were validated in PLA experiments on raphe RN33B cells (Figure S2). In these cells, where GALR1, GALR2 and 5-HT1AR exist (Millon et al. 2015), PLA-positive clusters were observed both with regard to GALR1-5-HT1AR PLA and GALR2-5-HT1AR PLA (Figure S2).

5-HT1AR and c-FOS immunoreactivities in raphe RN33B cells

The semiquantitative measurements of immunoreativities showed that after 1h of GAL and GAL(1–15) incubation significant changes in 5-HT1AR ($F_{2,177}$=9.134,p<0.05) and c-Fos ($F_{2,177}$=30.75,p<0.001) immunoreactivities were observed. The incubation with GAL caused a significant decrease in the 5-HT1AR (post hoc,p<0.05) and c-Fos (post hoc,p<0.001) immunoreactivity in RN33B cells (Figure S3). GAL(1–15) also significantly decreased the 5-HT1AR (post hoc,p<0.001) and c-Fos (post hoc,p<0.001) immunoreactivity in these cells. This reduction was significantly stronger than the one induced by GAL (post hoc,p<0.05;Figure S3).

DISCUSSION

In the current study we describe for the first time that GAL(1-15) enhances the antidepressant effects induced by the 5-HT1AR agonist 8-OH-DPAT in the FST. These effects were significantly stronger than the ones induced by GAL.

Importantly, the mechanism of this action involved interactions at the receptor level in the plasma membrane with changes also at the transcriptional level. Thus, GAL(1-15) affected the binding characteristics as well as the mRNA levels of 5-HT1AR in the dorsal hippocampus and DR. GALR2 was involved in these effects, since the specific GALR2 antagonist M871 blocked GAL(1-15) mediated actions at behavioral and receptor level. PLA experiments indicated that 5-HT1AR are in close proximity with GALR1 and GALR2 in the dorsal hippocampus and DR suggesting the formation of GALR1-5-HT1AR and GALR2-5-HT1AR heteroreceptor complexes in these areas as previously proposed (Fuxe et al. 2012). The effects of GAL(1-15) on 5-HT1AR were supported by studies in RN33B cells, where GAL(1–15) decreased 5-HT1A-IR more strongly than GAL.

The FST is used as a behavioral test to predict the efficacy of antidepressant treatments. The reduced immobility time is interpreted as behavioral despair and indicates an antidepressant drug effect and the enhancement of 5HT neurotransmission is related to swimming (Detke et al. 1995a; Detke et al. 1995b). Compounds with high affinity for 5-HT1AR decreased immobility in the FST; 5-HT1AR agonist 8-OH-DPAT reduces the immobility and increases the swimming in the FST (Detke et al. 1995a; Detke et al. 1995b). The results obtained with the 8-OH-DPAT agonist alone agree with previous studies and validates the behavioral model used (Detke et al. 1995a; Detke et al. 1995b; Cervo and Samanin 1987). The locomotion alteration was avoided by performing the experiments one hour after the 8-OH-DPAT injections (Cervo and Samanin 1987).

In this paper, it is demonstrated that GAL(1-15) involves GALR2 to enhance 5-HT1AR antidepressant effects in the FST. A strong decrease in immobility behavior and an 80% increase of swimming behavior were observed following cotreatment with threshold doses of GAL(1-15) and 8-OH-DPAT. When administered alone, neither of these treatments affected performance in the test, indicating that GAL(1-15) and the 5-HT1AR agonist interact to provoke the antidepressant responses. Since GAL(1-15) at the effective dose induced a pro-depressive effect (Millon et al. 2015), the antidepressant effect could only be due to the enhancement of 5-HT1AR action. Moreover, the strong

enhancement by GAL(1-15) of the antidepressant 5-HT1AR agonist action was validated using an effective dose of the 5-HT1AR agonist.

Furthermore, the effects of GAL(1–15) on the 5-HT1AR mediated actions in the FST were significantly stronger than the corresponding behavioral effects induced by GAL. In previous work, N-terminal GAL fragments reduced post-junctional recognition of the 5-HT1AR more strongly than GAL (Diaz-Cabiale et al. 2000). Also GAL(1–15) induces strong depression-related and anxiogenic-like effects and again these effects were significantly stronger than the ones induced by GAL (Millon et al. 2015). The current results validate and extend the view of a specific role of GAL(1–15) in brain communication.

The antidepressant-like effects of 5-HT1AR agonists in the FST may involve effects on 5-HT1AR located at postsynaptic or autoreceptor sites. A postsynaptic site of action is supported by findings that pretreatment with the 5-HT synthesis inhibitors did not affect the behavior in the FST on its own (Schreiber and De Vry 1993), nor did it alter the immobility-reducing effects of 8-OH-DPAT. However, several other findings suggest that the immobility-reducing effect can be mediated via reducing the activation of DR 5-HT1A autoreceptors (Schreiber and De Vry 1993; Garcia-Garcia et al. 2014; Cervo and Samanin 1987).

In this work, GAL(1-15) produced a time-dependent modulation of 5-HT1AR postsynaptically and at the autoreceptor in the DR. 10 minutes after icv GAL(1-15) a significant increase in the Kd and Bmax values of the [3H]8-OH-DPAT binding sites was observed in the CA1 and DG areas of the hippocampus. 2 hours after GAL(1-15) administration, a marked increase in the Kd and the density of the 5-HT1AR agonist binding sites were still observed in both areas associated with an increase in the mRNA levels of 5-HT1AR. Moreover, at the autoreceptor level GAL(1-15) also modified 5-HT1AR. In the DR GAL(1-15) reduced the density of 5-HT1AR and its mRNA levels two hours after its administration. Since GAL(1-15) induced the enhancement of the antidepressant-like effects of 5-HT1AR agonists in the FST at 10 minutes when the only changes in the 5-HT1AR described were in the hippocampus, it may be that the CA1 and DG were the key regions in mediating these antidepressant effect induced by GAL(1-15). However it should be considered that a potential enhancement in the firing rate of the ascending 5-HT DR neurons was induced

by GAL(1-15) by counteracting the 8-OH-DPAT induced autoreceptor signaling in spite of lack of effects on 5-HT1A autoreceptor recognition. Future studies on 5-HT1A autoreceptor signaling appear necessary to solve this issue.

The effects of GAL(1-15) on 5-HT1AR in the hippocampus and the DR are different from the ones induced by GAL. GAL lacks effects on the [^{3}H]8-OH-DPAT binding characteristics in the hippocampus (Hedlund et al. 1994; Razani et al. 2000) and in the DR a time-dependent reduction in affinity and an increase in the 5-HT1A autoreceptor density was observed after GAL treatment (Razani et al. 2001). An antagonistic GALR-5-HT1AR has been proposed to explain GAL-mediated effect in the limbic forebrain and also in the DR (review (Fuxe et al. 2012)). Such interactions may be due to the existence of GALR1-5-HT1AR heteroreceptor complexes demonstrated in cellular models (Borroto-Escuela et al. 2010). Within these complexes antagonistic allosteric receptor-receptor interactions exist which counteract the 5-HT1AR induced activation of Gi/o mediated signaling at least in certain 5-HT1AR signaling cascades (Borroto-Escuela et al. 2010).

In the current work the effect of GAL(1-15) on 5-HT1AR at both autoreceptor and postsynaptic sites may involve receptor-receptor interactions in postulated GALR1-GALR2-5-HT1AR heteroreceptor complexes in the raphe-hippocampal 5-HT neuron system and its neuronal targets (Borroto-Escuela et al. 2010; Fuxe et al. 2012; Fuxe et al. 2008). GAL(1-15) alone acts through GALR1-GALR2 heteroreceptor complexes which preferentially bind GAL(1–15) (Fuxe et al. 2012; Fuxe et al. 2008; Millon et al. 2015; Borroto-Escuela et al. 2014). These heteroreceptor complexes were observed in the nerve cells of the dorsal hippocampus and DR (Millon et al. 2015). These complexes could operate via an allosteric GALR1-GALR2 interactions that inhibits the Gq/G11-mediated signaling of the GALR2 protomer and switches it towards Gi/o-mediated signaling (Borroto-Escuela et al. 2014; Millon et al. 2015).

The results on the PLA in this work give support to the possible existence of GALR1-GALR2-5-HT1AR heteroreceptor complexes since positive PLA were obtained for both GALR1-5-HT1AR and GALR2-5-HT1AR complexes in the DR and hippocampus. Moreover the studies on RN33B cells, where GALR1, GALR2 and 5-HT1AR exist (Millon et al. 2015), also showed PLA-positive clusters indicating the existence of GALR1-5-HT1AR and GALR2-5-HT1AR

complexes in these cells. Such results can also be explained by the existence of the heterotrimeric complexes and future work may establish their existence in cellular models with sequential BRET-FRET and fluorescent complementation assays in combination with BRET (Carriba et al. 2008; Cabello et al. 2009).

The existence of receptor mosaics of GAL fragment preferring receptors and 5-HT1AR were proposed previously (Fuxe et al. 2012; Fuxe et al. 2008). Such heteroreceptor complexes could work as integrative nodes in 5-HT neurotransmission and as major targets for the antidepressant effects of GAL(1-15) at the autoreceptor and postjunctional level of 5-HT transmission of the raphe-hippocampal neurons (Fuxe et al. 2012; Fuxe et al. 2008). In fact, the presence of GALR2 facilitates the interactions of the GALR1-5-HT1AR heteroreceptor complex (Borroto-Escuela et al. 2010).

The differential effects observed with GAL(1-15) in the modulation of the 5-HT1AR recognition and 5-HT1AR mRNA levels in the dorsal hippocampus vs the DR may be explained by differences in the composition of the 5-HT1AR heteroreceptor complexes involved in the two regions. This can lead to alterations in the allosteric receptor-receptor interactions and in the signaling of the heteroreceptor complexes with consequences also for 5-HT1AR gene expression. It should be noticed that e.g. in the anterior raphe 5-HT1AR especially interact with $G\alpha_{i3}$ (Mannoury la Cour et al. 2006) while they appear to be mainly coupled to $G\alpha_{o}$ in the hippocampus (Mannoury la Cour et al. 2006). Such a difference may help explain opposing changes induced by GAL(1-15) on 5-HT1AR mRNA levels. Also the adapter proteins in the heterocomplexes may differ between the two regions with consequences for the allosteric receptor-receptor interactions. Such a difference may contribute to the absence of affinity modulation of the 5-HT1AR binding sites in the DR in contrast to the hippocampus where also rapid allosteric induced increases in 5-HT1AR Bmax values were observed with GAL(1-15).

There can also be differences in the balance of putative GALR1-GALR2-5-HT1AR heterocomplexes formed versus 5-HT1AR-5-HT1AR and GALR1-GALR1 and GALR2-GALR2 homoreceptor complexes. Previous work has indicated that the formation of GALR1-GALR2-5-HT1AR heterocomplexes by adding GALR2 can increase their cell surface expression and thus increase

their trafficking to the plasma membrane (Borroto-Escuela et al. 2010). Therefore, the demonstrated differential reduction of the Bmax value of the 5-HT1AR in the dorsal raphe 2 h after GAL(1-15) may involve a reduced hetero/homoreceptor receptor ratio which can lead to enhanced internalization of the 5-HT1AR protomer.

In conclusion, our results indicate that GAL(1–15) enhances the antidepressant effects induced by the 5-HT1AR agonist 8-OH-DPAT acting on 5-HT1AR protomers of the above receptor complexes located at postjunctional sites and at the soma-dendritic level. The results open up the possibility that GALR1-GALR2-5-HT1AR heteroreceptor complexes may exist on the raphe-hippocampal 5-HT neurons. The development of novel drugs specifically targeting these heteroreceptor complexes may offer a novel strategy for treatment of depression.

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Conflict of interest

No conflict of interest

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FIGURE LEGENDS

Figure 1. Behavioural effects of the coadministration of threshold doses of 5-HT1AR agonist 8-OH-DPAT (0.125mg/Kg) and Galanin(1-15) [GAL(1-15)] (1nmol) and the coadministration with GALR2 antagonist M871 (3nmol) in the Forced Swimming Test (FST). 8-OH-DPAT was administered subcutaneously 60 min before the tests and GAL(1-15) and GAL(1-15)+M871 were injected icv 15 min before the test. Saline + cerebrospinal fluid (CSFa) injected rats were used as control group. Data represents mean ± SEM of immobility, climbing and swimming time in FST during the 5 min test period (n=5-8 rats per group). *p<0.05, **p<0.01 and ***p<0.001 versus rest of the group according to one-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

Figure 2. Behavioural effects of the coadministration of the 5-HT1AR agonist 8-OH-DPAT (0.125mg/Kg) and Galanin (GAL) (1nmol) and the coadministration of 8-OH-DPAT (0.125mg/Kg) and Galanin(1-15) [GAL(1-15)] (1nmol) in the Forced Swimming Test (FST). 8-OH-DPAT was administered subcutaneously 60 min before the tests and GAL or GAL(1-15) were injected icv 15 min before the test. Saline + cerebrospinal fluid (CSFa) injected rats were used as control group. Data represents mean ± SEM of immobility (A), climbing (B) and swimming (C) time in FST during the 5 min test period (n=5-7 rats per group). *p<0.05, **p<0.01 and ***p<0.001 according to one-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

Figure 3. Effects of Galanin (1-15) [GAL(1-15)] on the binding characteristics of 5-HT1AR agonist [^{3}H]-8-OH-DPAT binding sites in the dorsal hippocampus

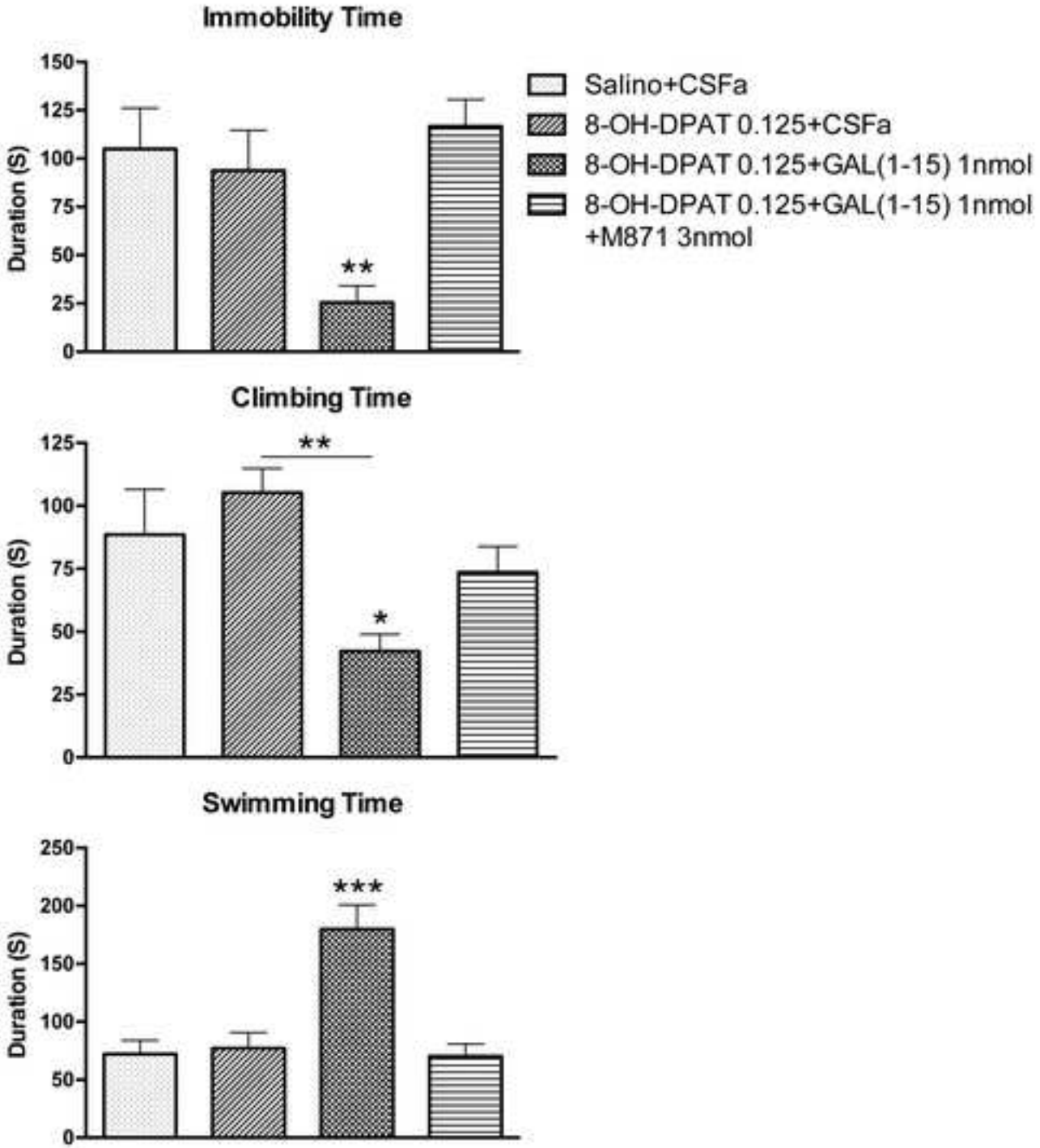
(CA1 (**A**) and DG (**B**)). Saturation experiments with ten concentrations of [^{3}H]-8-OH-DPAT (0.26-10nM) were performed in sections from dorsal hippocampus. Non-specific binding was defined as the binding in the presence of 10μM serotonin. The values shown are percentages of respective CSFa values. K_d (nM) value (100%) for the pooled CSFa groups (10min, 2h and 5h after icv. administration) in the CA1 was 3,27±0,9 (n=18) and in the DG was 1,73±0,2 (n=18). The B_{max} (fmol/mg protein) value (100%) for the pooled CSFa groups (10 min, 2h and 5h after icv. administration) in the CA1 was 751,9±70 (n=18) and in the DG was 696±49,5 (n=18). The K_d values and the B_{max} values are shown as mean± SEM (n=6 per group). *p<0.05; **p<0.01 vs respective CSFa group (Student's t-test). (**C**) Representative autoradiograms from dorsal hippocampus sections of rat showing the increase of the 5-HT1AR agonist binding in the CA1 and DG at 10 min with a high concentration of the radioligand [^{3}H]-8-OH-DPAT (7.2 nM) after the GAL(1-15) (3nmol, icv.) administration. Scale bar, 2mm. (**D**) Schematic drawing showing the areas analyzed in coronal sections of the rat brain at Bregma -3,60mm using quantitative receptor autoradiography.

Figure 4. Effects of Galanin (1-15) [GAL(1-15)] on the binding characteristics of 5-HT1AR agonist [^{3}H]-8-OH-DPAT binding sites in the dorsal Raphe (DR). (**A,B**) Saturation experiments with ten concentrations of [^{3}H]-8-OH-DPAT (0.26-10nM) were performed in DR sections. Non-specific binding was defined as the binding in the presence of 10μM serotonin. The values shown are percentages of respective CSFa values. The K_d (nM) value (100%) for the pooled CSFa groups (10min, 2h and 5h after icv. adminsitration) in the DR was 2,54±0,3 (n=18). The B_{max} (fmol/mg protein) value (100%) for the pooled CSFa groups (10 min, 2h and 5h after icv. administration) in the DR was 1262±132,5 (n=23). The K_d values and the B_{max} values are shown as mean± SEM (n=6 per group). *p<0.05 vs respective CSFa group (Student's t-test).(**C**) Representative autoradiograms from DR sections of rat showing the decrease of the 5-HT1AR agonist binding with a high concentration of the radioligand [^{3}H]-8-OH-DPAT (7.2 nM) 2 h after the GAL(1-15) (3nmol, icv.) administration. Scale bar, 1mm. (**D**) Schematic drawing showing the areas analyzed in coronal sections of the rat brain at Bregma -8,00mm using quantitative receptor autoradiography.

Figure 5. Effects of Galanin (1-15) [GAL(1-15), 3nmol] on 5-HT1AR mRNA levels in dorsal hippocampus (CA1 and DG) (**A**) and Dorsal Raphe nucleus (DR) (**B**) 2 h and 5 h (**D**) after icv. administration in comparison with the CSFa groups. The values shown are percentages of respective CSFa values. Optical density (O.D.) values are shown as mean± SEM (n=6 per group). **p<0.01 vs CSFa group according to Student's t-test. Representative autoradiograms showing the mRNA levels coding for 5-HT1AR 2 h after the administration of GAL(1-15) (3nmol, icv.) compared with CSFa-treated group in the dorsal hippocampus (CA1 and DG) (**C**) and DR (**F**) determined by *in situ* hybridization. Scale bar, 2mm.

Figure 6. Close proximity between GALR1-5-HT1AR (**A**) and between GALR2-5-HT1AR (**B**) is detected by *in situ* proximity ligation assay (PLA) (seen as red clusters indicated by arrows) in dorsal rat Hippocampus and Dorsal Raphe nucleus (DR). *In situ* PLA was performed using primary antibodies of different species directed to GALR1 and 5-HT1AR and to GALR2 and 5-HT1AR followed by PLA reagents. (**A,B**) Quantification of red clusters/positive PLA cells. Quantification was made in 10-20 cells per photo and performed on 8 photos per brain zone. The data represent the mean of clusters per positive cell (mean±SEM) for 80-160 cells per zone. ***p<0.001 versus rest of the groups, **P<0.01 versus rest of the groups according to one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. (**C**) Representative photographs for positive GALR1-5-HT1AR and GALR2-5-HT1AR PLA regions [Ammon's horn 1, 2 and 3 (CA1, CA2 and CA3) Dentate Gyrus (DG) and Dorsal Raphe nucleus (DR)] (Scale bar, 10 μm) and for negative PLA signal region Corpus Callosum (CC) (Scale bar, 50 μm). Nuclei are shown in blue (DAPI).

Figure 1.



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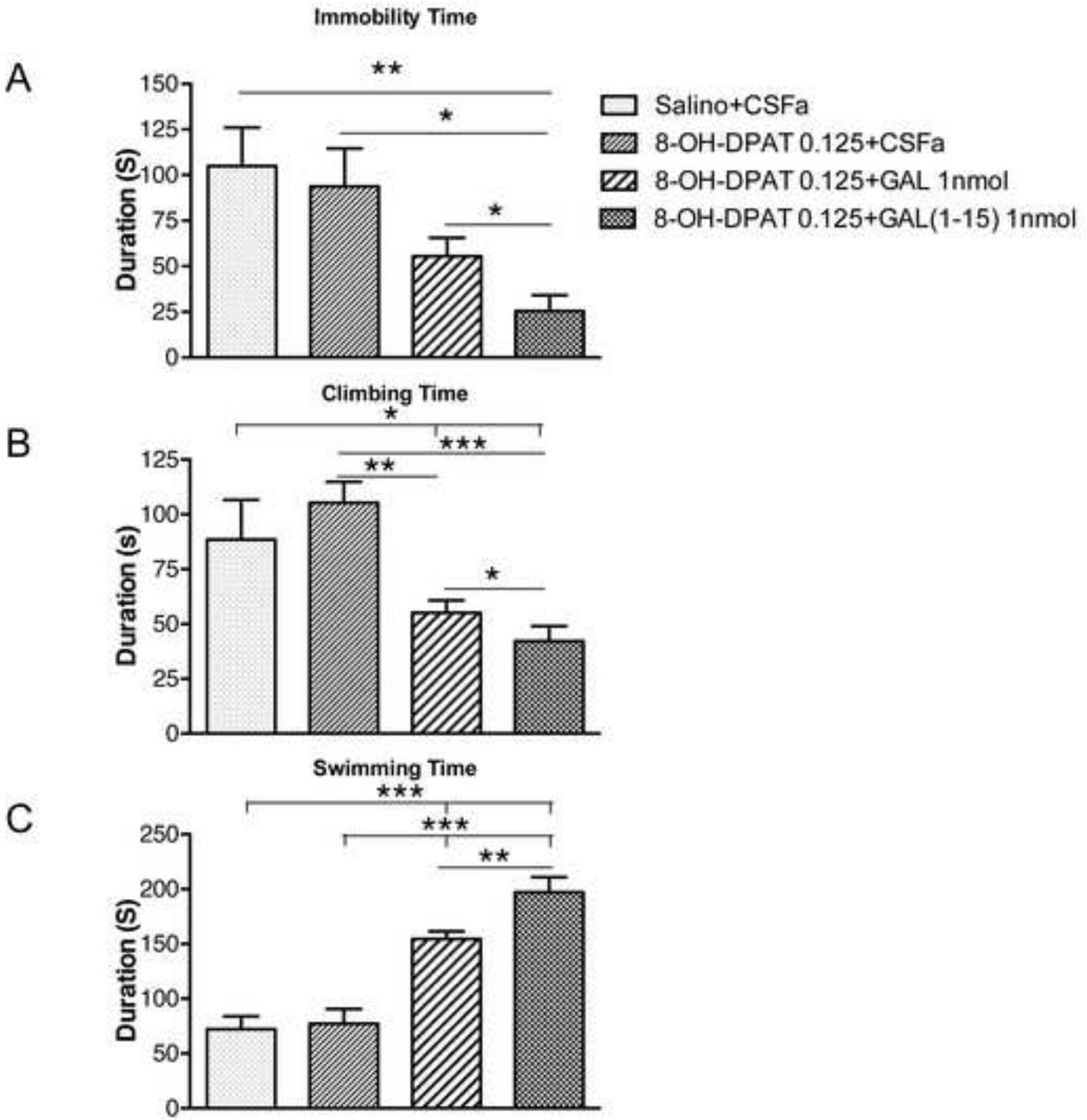

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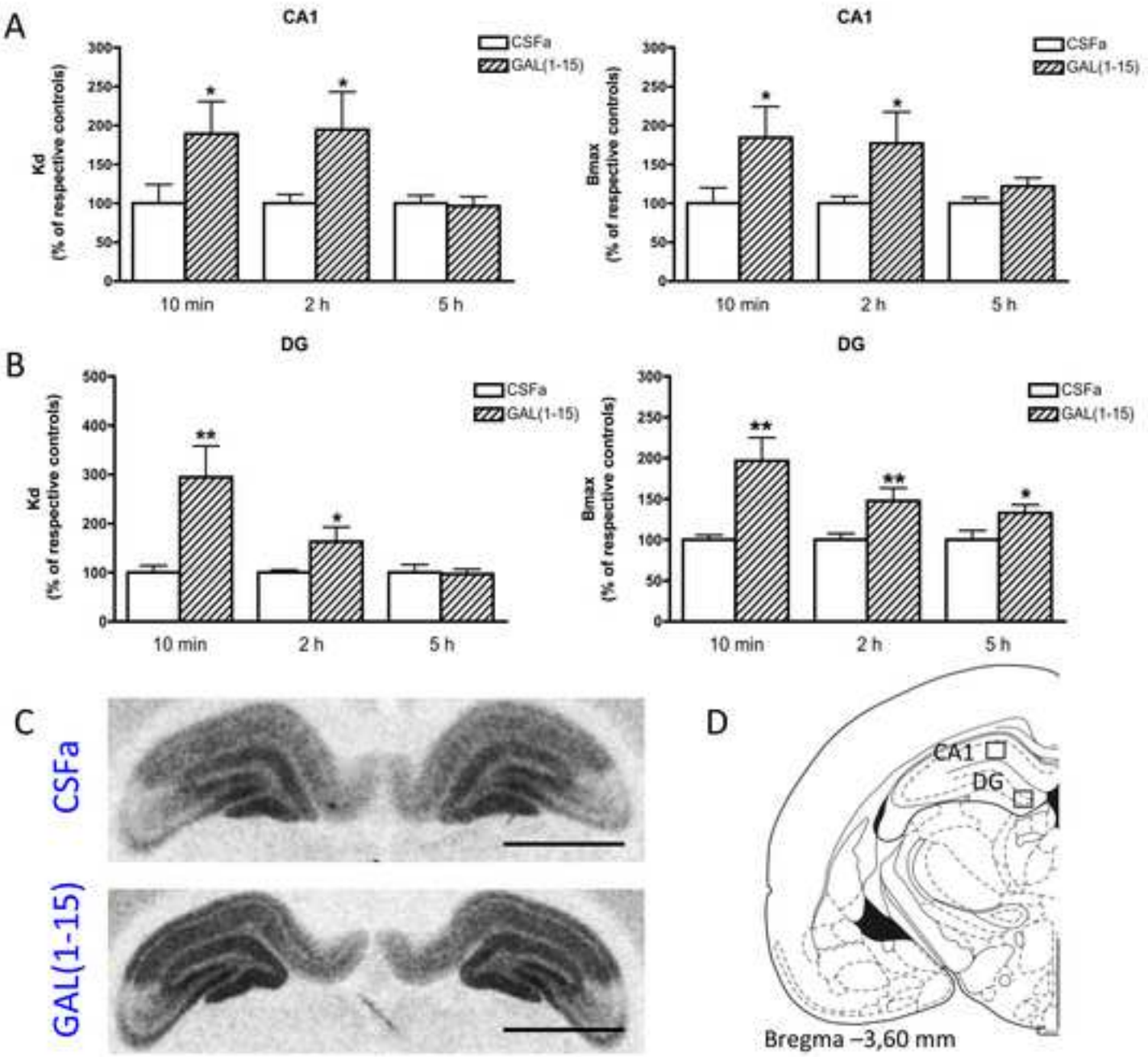

Figure

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Figure 4.

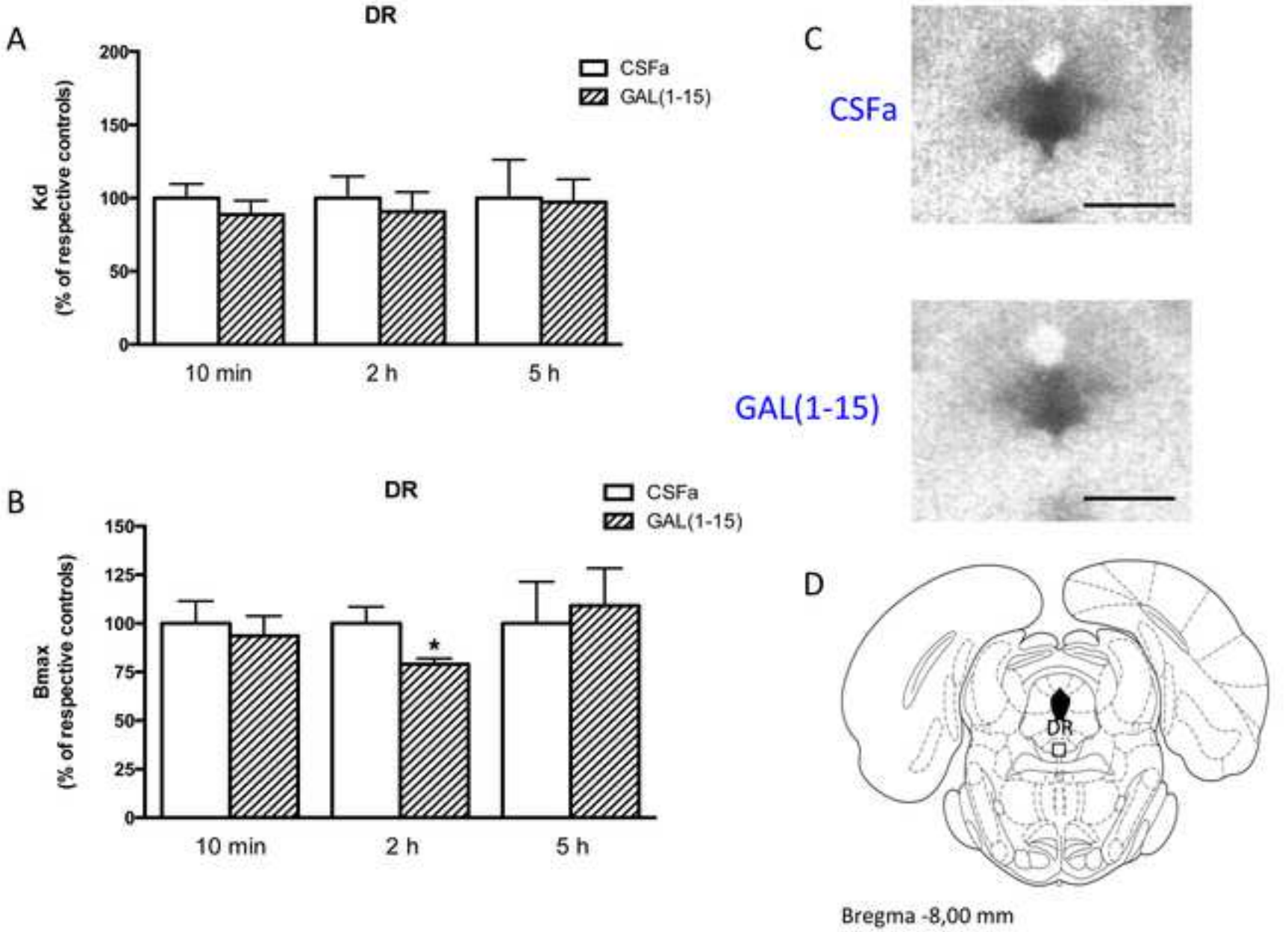

Figure 5

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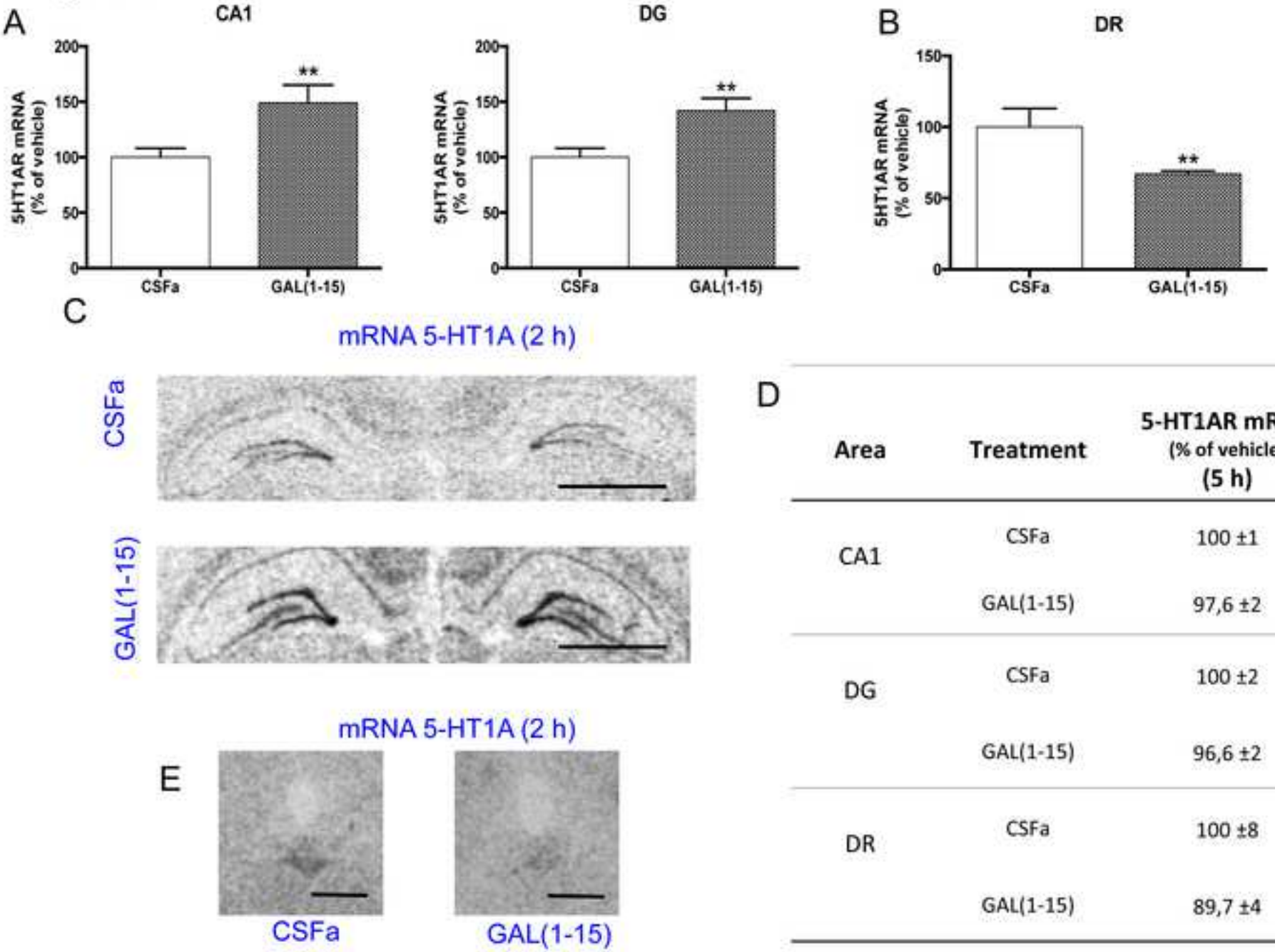
Figure 5.



D

Area	Treatment	5-HT1AR mRNA (% of vehicle) (5 h)
CA1	CSFa	100 ±1
	GAL(1-15)	97,6 ±2
DG	CSFa	100 ±2
	GAL(1-15)	96,6 ±2
DR	CSFa	100 ±8
	GAL(1-15)	89,7 ±4

Figure

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Figure 6.

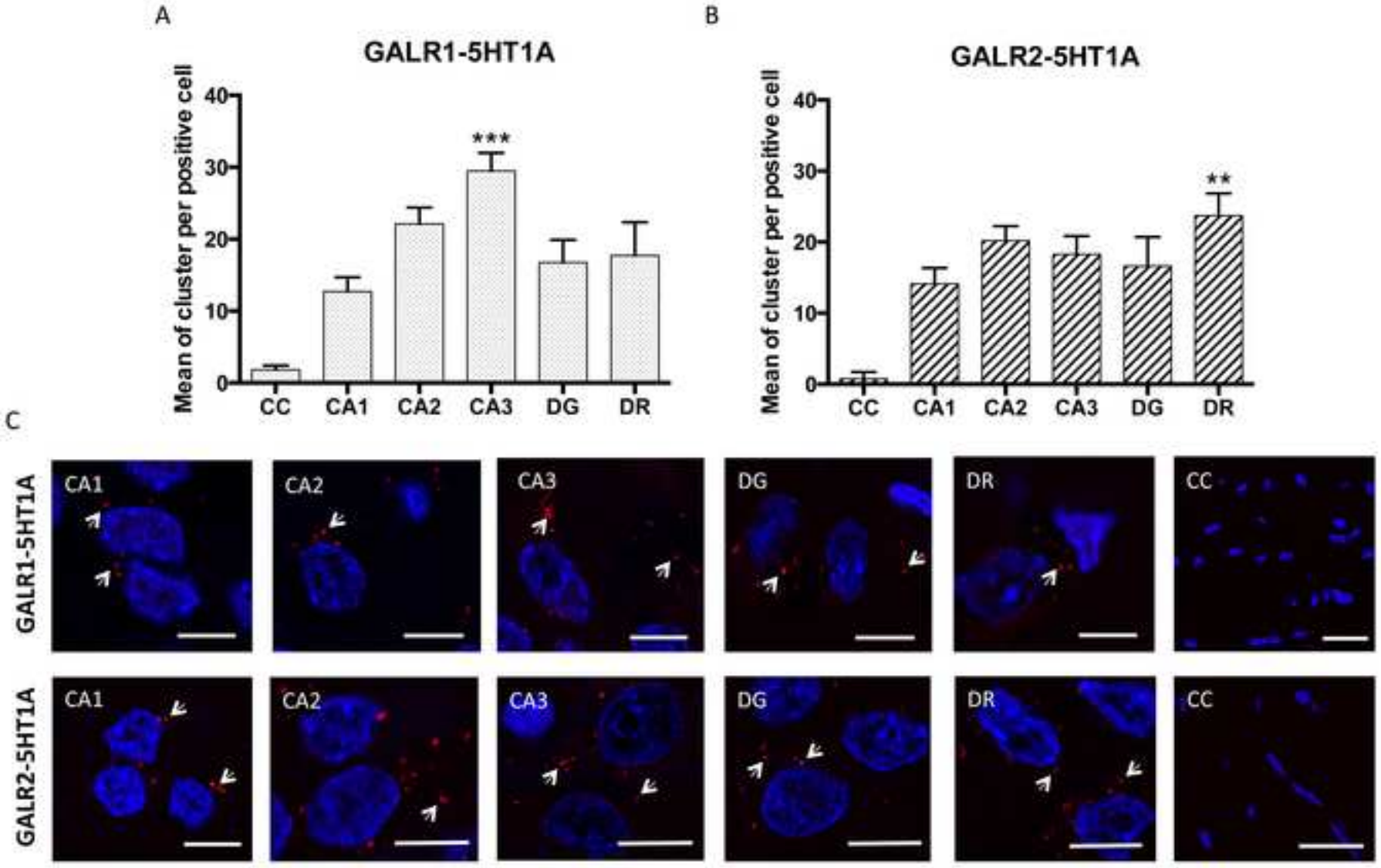

Table 1.

	Area	CSFa	GAL(1-15)	M871	GAL(1-15)+M871
K_d (nM)	CA1	3,28±0,53	7,05±0,8**	3,96±0,27	4,85±0,68
	DG	2,65±0,3	4,55±0,4[a]	3,3±0,3	3,3±0,73
B_{max} (fmol/mg protein)	CA1	1209±150,6	1874±127,4*	1541±114,3	1337±142,5
	DG	1493±118,4	2312±188,2[aa]	2013±124,2	1885±225,1

Table 1. Effects of GALR2 antagonist M871 on the GAL(1-15)-induced modulation of the 5HT1AR agonist [^{3}H]-8-OH-DPAT binding sites in the dorsal hippocampus (CA1 and DG). Saturation experiments with ten concentrations of [^{3}H]-8-OH-DPAT (0,26-10 nM) were performed in sections from dorsal hippocampus 10 min after i.c.v. administration of GAL(1-15) 3nmol alone, M871 3nmol alone, or GAL(1-15) and M871 together. Non-specific binding was defined as the binding in the presence of 10µM serotonin. The K_d values and the B_{max} values are shown as mean± SEM (n=6 per group). *p<0.05 vs CSFa and GAL(1-15)+M871; **p<0.01 vs CSFa and M871; [a]p<0.05 vs CSFa; [aa]p<0.01 vs CSFa according to one-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

Millón et al., 2015

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.

Supplemental Information

Supplementary Methods and Materials

Animals

Adult male Sprague-Dawley rats from CRIFFA, Barcelona (200-250gr) had free access to food pellets and tap water except during the test period. They were maintained under the standard 12h dark/light cycle, in controlled temperature (22±2ºC) and relative humidity (55–60%). Test behaviors were performed during the light phase of the diurnal cycle. 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

The procedures of cannulation and postsurgical care have been described elsewhere (Millón et al., 2014). Rats anesthetized intraperitoneally with Equitesin (3,3ml/Kg) were implanted with a chronic 22-gauge stainless-steel guide cannula (Plastics One In) into the right lateral cerebral ventricle (+1.4mm lateral, -1mm posterior to bregma, and 3.6mm below the surface of the skull). After surgery, animals were individually housed and allowed a recovery period of 7 days. 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 (CSFa). GAL was obtained from NeoMPS; GAL(1–15) and the GALR2 receptor antagonist M871 were obtained from Tocris Bioscience.

Forced Swimming Test

Rats were adapted to handling and were taken into the experimental room (80-90 lux) to habituate for at least 1 hour before the peptides administration. All tests were performed between 8:00 and 14:00.

Animals were individually placed in a vertical glass cylinder (50 cm height, 20 cm diameter) containing water (25 ºC) to a height of 30 cm. Two swimming sessions were conducted: a 15 min pre-test followed 24 h later by a 5 min test (Detke and Lucki, 1996; Kuteeva et al., 2007). The total duration of immobility, climbing and swimming behavior were recorded during the second, 5 min test.

Open Field Test

Rats were individually placed and allowed to freely explore; their behavior was recorded over a 5min period by a ceiling-mounted video camera. Their activity was analyzed using the video-tracking software EthovisionXT. After each trial, all surfaces were cleaned with a paper towel and 70% ethanol solution. For the open-field (100 x 100 x 50cm), total distance and mean speed as locomotor activity parameters were recorded (Narváez et al., 2014).

Autoradiography and in situ hybridization

The rats were killed by decapitation and their brains were rapidly removed from the skull and frozen in -40 °C isopenthane. Coronal sections (14 µm thick) were cut in a cryostate at bregma levels according to the atlas of Paxinos and Watson (1986) (dorsal hippocampus: 3.14 mm –3.8 mm and DR: -7.3 mm to –8.3 mm) and thaw-mounted on gelatin-coated slides

Quantitative autoradigraphy

Saturation experiments were performed using [^{3}H]-8-OH-DPAT (specific activity: 187,4 Ci/mmol, Perkin Elmer) in concentrations ranging from 0.24 to 10.8 nM. In each saturation experiment, two groups of ten sections were prepared in order to perform the total binding and the non-specific binding. Non-specific binding was defined as the binding in the presence of 10^{-5}M of 5-HT (Sigma Aldrich, St. Louis, CA). The sections were preincubated for 30 minutes at room temperature in 50mM Tris-HCl buffer (pH 7.6), containing 4mM CaCl2, 0.01% ascorbic acid and 10mM pargyline. The sections were then incubated for

60 minutes at room temperature with [^{3}H]-8-OH-DPAT in the same solution as above.
Thereafter, the sections were washed twice for 5 minutes in the respective ice-cold buffer and rinsed briefly in ice-cold destilled water before being dried under a stream of cold air. The dried sections were exposed to [^{3}H]-hyperfilm (Kodak) for 6 weeks.

***Riboprobe preparation*:** The 5-HT1AR probe was prepared from a 910 bp-long cDNA fragment (*Bal*I - *PvU*III fragment) cloned into pGEM blue [20].
After labelling with [35S]-UTP (1000 Ci/mmol, Amersham Buckinghamshire, UK), as described earlier [22] the probe was purified on Nensorb columns (Dupont) and checked on a 5% polyacrylamide/8M Urea gel.

Image analysis
The autoradiograms from the receptor autoradiography and *in situ* hybridization experiments were analysed as described previously (Razani *et al.*, 2000, 2001) using a computer-assisted image analysis system. Briefly, measurements using the ImageJ system (NIH, USA) were made bilaterally in the distinctly labelled region within the Dentate Gyrus (DG) and CA1 of the hippocampus (0.15 mm^2 square). One observation per region and rat was obtained, since the average of the measurements was calculated. The ventral part of the midline area of the DR was analyzed using a square as a sampling field (0.09mm2).

Prefabricated [^{3}H]-labelled polymer strips (Amersham microscale, UK) were used to convert the grey values into fmol/mg protein values in the quantitative autoradiography. The semiquantitative evaluations of the *in situ* hybridization autoradiograms were analysed from the measured values of the specific optical density (O.D.).

Proximity Ligation In Situ Assay (Duolink)

The PLA study and the quantification were carried out as described previously using a Duolink in situ PLA detection kit (Olink, Sweden) (Borroto-Escuela et al., 2012,2013; Narváez et al., 2014; Millón et al., 2014). The primary antibodies of different species directed to GALR1 (goat polyclonal (C-20), Santa Cruz Biotechnology Inc, Dallas, TX; 1:250), to GALR2 (rabbit

polyclonal, Alomone Labs, Israel; 1/250) and 5HT1AR (mouse monoclonal (MAB11041) Millipore corp, USA, 1:400) were used. Sections were mounted on slides with Fluorescent Mounting Medium containing 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich), staining nuclei with blue colour .The in situ PLA positive signal were visualized using a Leica SP5 confocal microscope and the Duolink Image Tool software.

For in situ PLA in raphe RN33B cells were used the same primary antibodies and the same procedure.

Cell culture and double Immunocytochemistry

RN33B cells (a CNS-derived neuronal precursor cell line; Lundberg et al., 2002) American Type Culture Collection) were grown in DMEM/F12 supplemented with 2mM L-glutamine, 100U/ml penicillin/streptomycin, and 10% (v/v) fetal bovine serum at 37°C and in an atmosphere of 5% CO_2. RN33B cells were treated for 1 h with GAL 100nM, GAL(1-15) 100nM or Control, followed for 1h for recuperation. Cells were stained with rabbit anti-C-FOS polyclonal antibody (1:5000; Santa Cruz Biotechnology INC, EEUU) and mouse anti-5HT1AR receptor (1:400; Millipore corp, USA) . The secondary antibodies used were Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:2000; Invitrogen) and Alexa Fluor 546-conjugated goat anti-mouse IgG (1:2000; Invitrogen). The C-FOS and 5-HT1AR immunoreactivity (IR) semiquantification is expressed as the IR value of 30 positive cells per condition for each experiment. Two independent experiments were performed. The application settings were adjusted at the beginning of analysis and kept the same for all images in the experiment.

A	**Experiments**	**Groups**
Behavioural Assessment	Dose-response effects of 8-OH-DPAT	Saline (sc) + CSFa (icv) 8-OH-DPAT 0.125 mg/Kg (sc)+CSFa (icv) 8-OH-DPAT 0.25 mg/Kg (sc)+CSFa (icv)
	GAL(1-15) and 8-OH-DPAT Interactions. Effects of the GALR2 antagonist	Saline (sc) + CSFa (icv) Saline (sc) + GAL(1-15) 1 nmol (icv) 8-OH-DPAT 0.25 mg/Kg (sc)+CSFa (icv) 8-OH-DPAT 0.25 mg/Kg (sc)+GAL(1-15) 1 nmol (icv)
		Saline (sc) + CSFa (icv) 8-OH-DPAT 0.125 mg/Kg (sc)+CSFa (icv) 8-OH-DPAT 0.125 mg/Kg (sc)+GAL(1-15) 1 nmol (icv) Saline (sc) + M871 3 nmol (icv) 8-OH-DPAT 0.125 mg/Kg (sc)+GAL(1-15) 1 nmol (icv)+M871 3 nmol (icv)
	Comparison between the effects of GAL(1-15) and GAL on 8-OH-DPAT mediated antidepressant-like behaviors	Saline (sc) + CSFa (icv) 8-OH-DPAT 0.125 mg/Kg (sc)+CSFa (icv) 8-OH-DPAT 0.125 mg/Kg (sc)+GAL 1 nmol (icv) 8-OH-DPAT 0.125 mg/Kg (sc)+GAL(1-15) 1 nmol (icv)

B	**Experiments**	**Groups**
Autoradiography and in situ hybridization	5-HT1AR Autoradiography in Dorsal Hippocampus: 10 min, 2 h and 5 h	CSFa (icv) 10min; CSFa (icv) 2h; CSFa (icv) 5h GAL(1-15) 3nmol (icv) 10min; GAL(1-15) 3nmol (icv) 2h; GAL(1-15) 3nmol (icv) 5h
	5-HT1AR Autoradiography in Dorsal Raphe: 10 min, 2 h and 5 h	CSFa (icv) 10min; CSFa (icv) 2h; CSFa (icv) 5h GAL(1-15) 3nmol (icv) 10min; GAL(1-15) 3nmol (icv) 2h; GAL(1-15) 3nmol (icv) 5h
	In situ hybridization of 5-HT1AR mRNA levels in Dorsal Hippocampus: 2 h and 5 h	CSFa (icv) 2h; CSFa (icv) 5h GAL(1-15) 3nmol (icv) 2h; GAL(1-15) 3nmol (icv) 5h
	In situ hybridization of 5-HT1AR mRNA levels in Dorsal Raphe: 2 h and 5 h	CSFa (icv) 2h; CSFa (icv) 5h GAL(1-15) 3nmol (icv) 2h; GAL(1-15) 3nmol (icv) 5h

Table S1. Experimental groups conducted in Behavioural Assessments (A) and in Autoradiography and *in situ* hybridization experiments (B). Subcutaneously: sc; intracerebroventricular: icv.

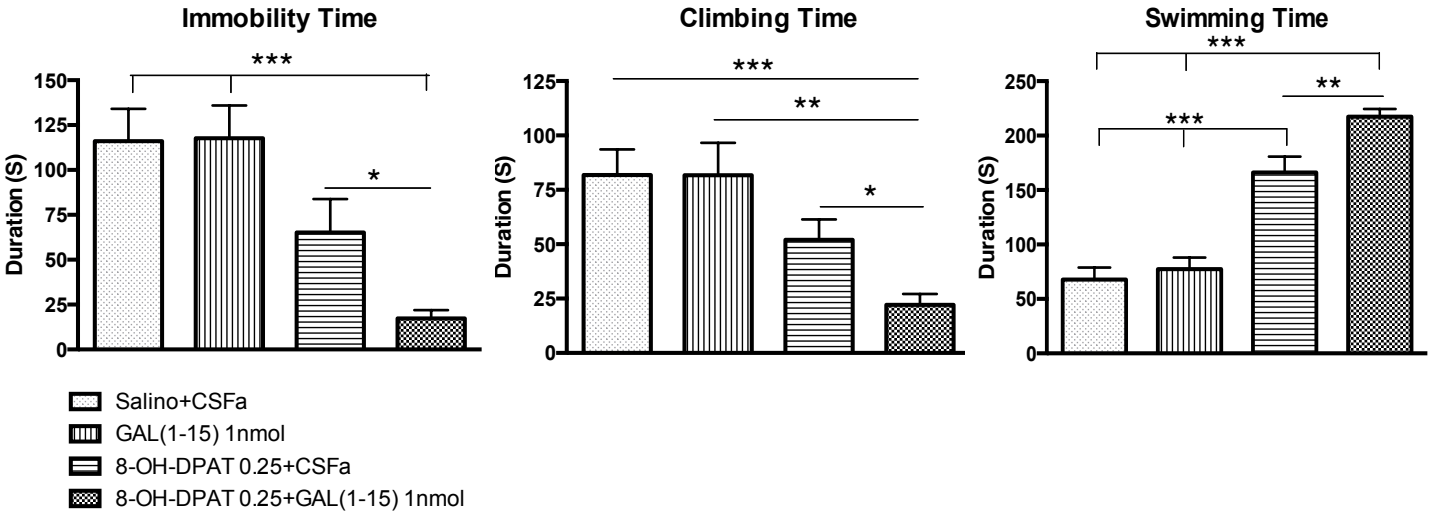

Figure S1. Behavioural effects of the coadministration of an effective dose of the 5-HT1AR agonist 8-OH-DPAT (0.25mg/Kg) and a threshold dose of GAL(1-15) (1nmol) in the Forced Swimming Test (FST). 8-OH-DPAT was administered subcutaneously 60 min before the tests and GAL(1-15) was injected icv 15 min before the test. Saline + cerebrospinal fluid (CSFa) injected rats were used as control group. Data represents mean ± SEM of immobility, climbing and swimming time in FST during the 5 min test period (n=6-11 rats per group). *p<0.05, **p<0.01 and ***p<0.001 according to one-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

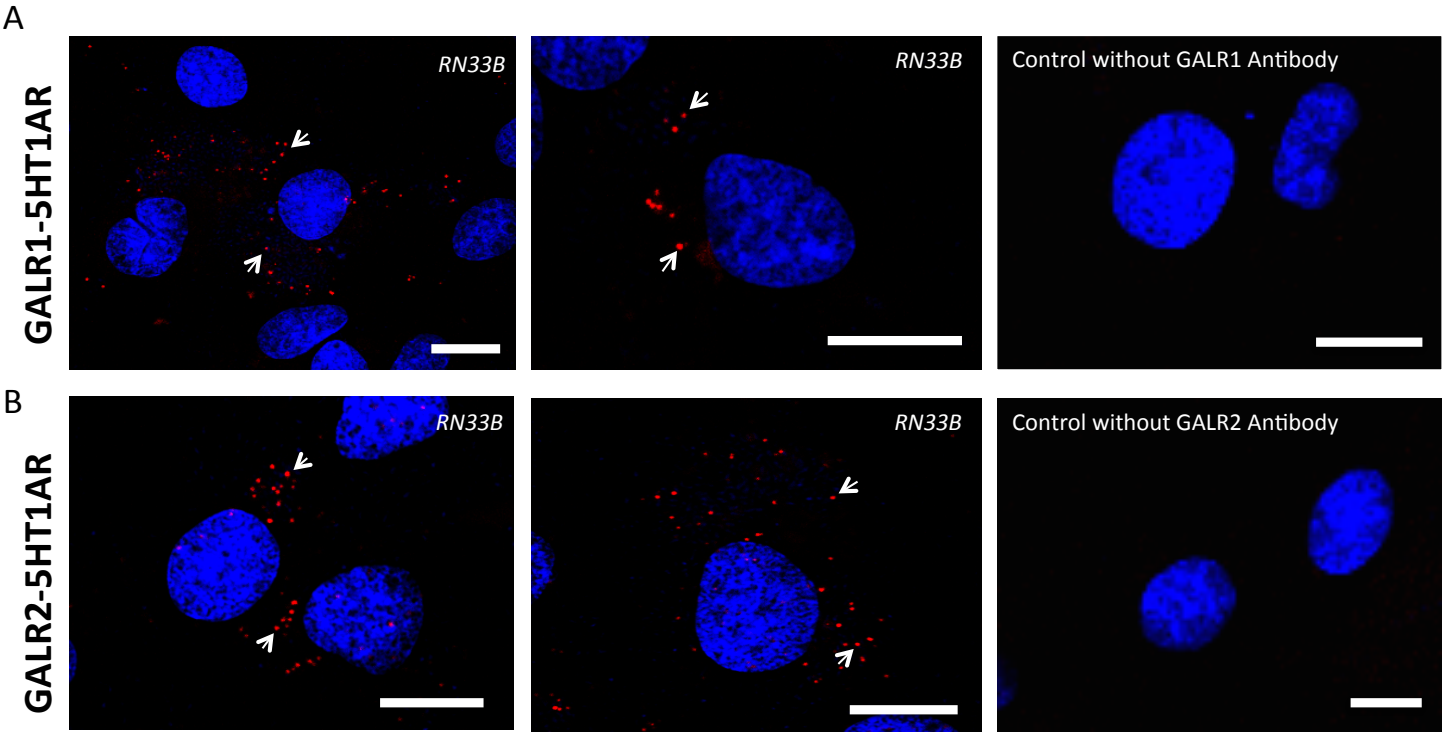

Figure S2. Close proximity between Galanin receptor 1 (GALR1) and 5HT1AR (**A**) and between Galanin receptor 2 (GALR2) and 5HT1AR (**B**) in RN33B cells. Detection of close proximity between GALR1-5HT1AR and GALR2-5HT1AR receptors (seen as red clusters indicated by arrows) in RN33B cells by *in situ* PLA. Control experiments employed only one primary antibody. Nuclei are shown in blue (DAPI). Scale bar, 10 μm.

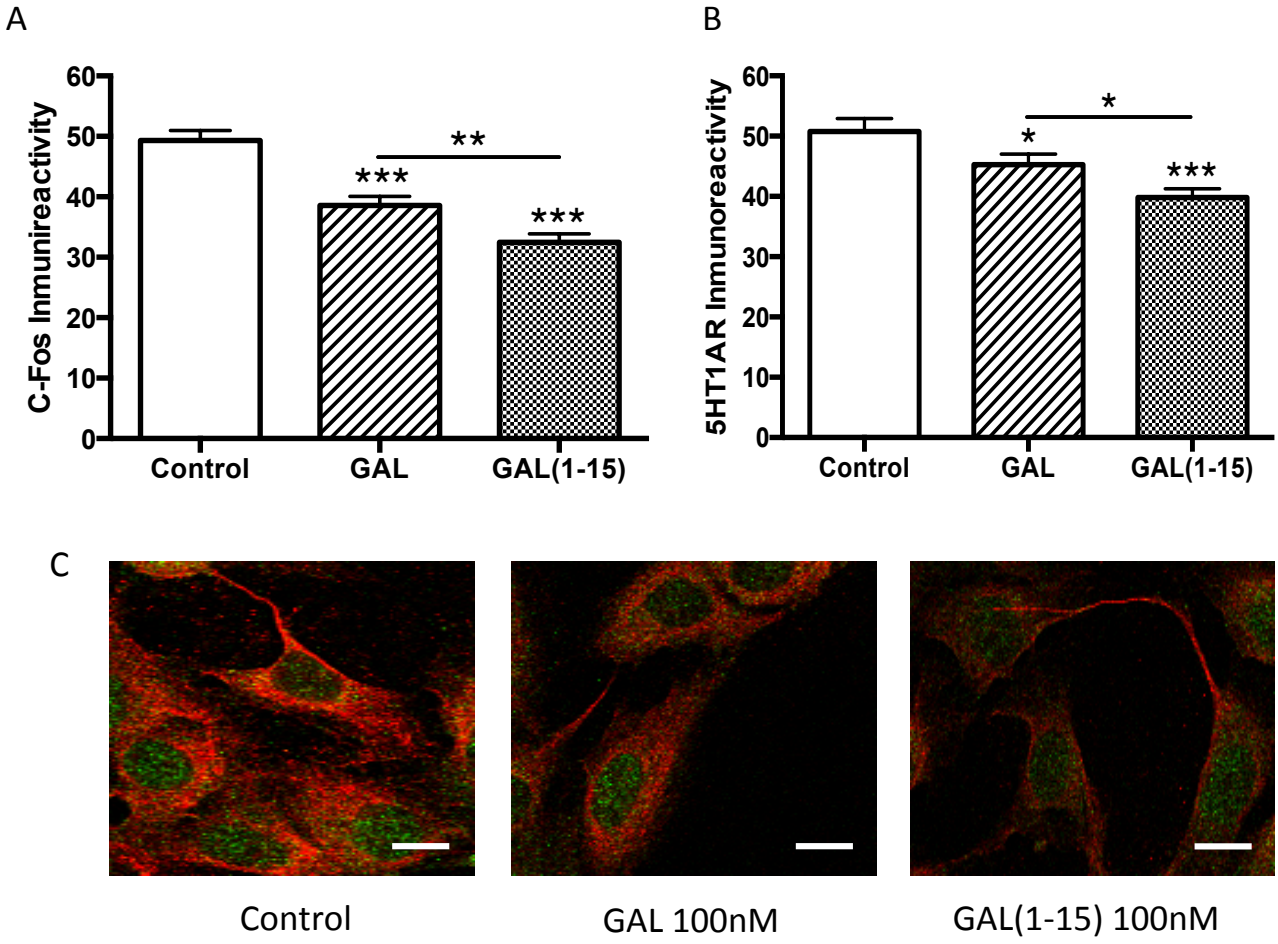

Figure S3. Effects of Galanin (GAL) and Galanin (1-15) [GAL(1-15)] in cellular activity and 5HT1AR expression in RN33B cells. Quantification of C-Fos immunoreactivity [C-Fos immunoreactivity (green color) was used as a marker for cellular activity] (**A**) and 5HT1AR immunoreactivity [5HT1AR immunoreactivity (red color) was used as marker for 5HT1AR expression] (**B**) in RN33B cell cultures after incubation with GAL and GAL(1-15). RN33B brain-derived immortalized cells were treated for 1h with GAL (100nM), GAL(1-15)(100nM) or control, followed for 1h for recuperation. The data are presented as mean ± SEM (n=60 cells for two independent experiments). *p<0.05 vs control or GAL(1-15); **p<0.01 vs GAL(1-15); ***p<0.001 vs control according to one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. (**C**) Representative images of C-Fos (green nucleus) and 5HT1AR (red cytoplasm) stained RN33B cells under different conditions are presented. Scale bar, 10 µm.

A

Parameters	Control Saline (sc)+CSFa(icv)	8-OH-DPAT 0,125 mg/Kg (sc) +CSFa (icv)	8-OH-DPAT 0,25 mg/Kg (sc) +CSFa (icv)
Immobility time (s)	104,9 ±21,21	93,7 ±20,9	44,1 ±14,3*
Climbing time (s)	88,6 ±18	105,3 ±9,6	42,7 ±9,2*
Swimming time (s)	72,2 ±11,6	77,3 ±13,3	166,2 ±14,5***

B

Locomotor parameters	Control Saline (sc)+CSFa(icv)	8-OH-DPAT 0,125 mg/Kg (sc) +CSFa (icv)	8-OH-DPAT 0,25 mg/Kg (sc) +CSFa (icv)
Total Distance Travelled (cm)	3827 ±255	3302 ±207,7	1854 ±223,2***
Mean Speed (cm/s)	12,7 ±0,8	11 ± 0,7	6,2 ±0,7***

Table S2. Dose-Response curve of 8-OH-DPAT in the Forced Swimming Test (FST) (**A**) and the locomotor activity parameters in the Open Field Test (**B**) in rats. 8-OH-DPAT (0.125 or 0.25mg/Kg) was administered subcutaneously 60 min before the test and cerebrospinal fluid (CSFa) was injected icv 15 min before the test. Saline + CSFa injected rats were used as control group. Data shown as mean ± SEM of time for immobility, climbing or swimming (**A**) and total distance travelled or mean speed (**B**) during a 5 min test period (n=5-8 animals per group). The control group represent a pooled of animals. *p<0.05 and ***p<0.001 vs respective control group according to Student's t-test.

Locomotor parameters	Control	8-OH-DPAT 0,125 mg/Kg + CSFa	8-OH-DPAT 0,125 mg/Kg + GAL(1-15) 1nmol
Total Distance Travelled (cm)	4188 ±181,6	3318 ±245	3295 ±244,8
Mean Speed (cm/s)	13,9 ±0,6	11,1 ±0,8	11 ±0.8

Table S3. Analysis of locomotor activity of the coadministration of the threshold doses of 8-OH-DPAT (0.125mg/Kg) and Galanin(1-15) [GAL(1-15), 1nmol] in the Open Field Test. 8-OH-DPAT was administered subcutaneously 60 min before the test and GAL(1-15) was injected icv 15 min before the test. Saline + cerebrospinal fluid (CSFa) injected rats were used as control group. Data shown as mean ± SEM (n=4-6 per group). No differences were found according to one-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

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