OXFORD

RESEARCH ARTICLE

A Role for Galanin N-Terminal Fragment (1–15) in Anxiety- and Depression-Related Behaviors in Rats

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Abstract

Background: Galanin (GAL) plays a role in mood regulation. In this study we analyzed the action of the active N-terminal fragment [GAL(1–15)] in anxiety- and depression-related behavioral tests in rats.
Methods: The effect of GAL(1–15) was analyzed in the forced swimming test, tail suspension test, open field test, and light/dark test. The proximity of GAL_1 and GAL_2 receptors was examined with the proximity ligation assay (PLA). We tested the GAL receptors involved in GAL(1–15) effects with the GAL_2 receptor antagonist M871 and with an *in vivo* model of siRNA GAL_2 receptor knockdown or siRNA GAL_1 receptor knockdown rats. The effects of GAL(1–15) were also studied in the cell line RN33B.
Results: GAL(1–15) induced strong depression-like and anxiogenic-like effects in all the tests. These effects were stronger than the ones induced by GAL. The involvement of the GAL_2 receptor was demonstrated with M871 and with the siRNA GAL_2 receptor knockdown rats. The PLA indicated the possible existence of GAL_1 and GAL_2 heteroreceptor complexes in the dorsal hippocampus and especially in the dorsal raphe nucleus. In the siRNA GAL_1 receptor knockdown rats the behavioral actions of GAL(1–15) disappeared, and in the siRNA GAL_2 receptor knockdown rats the reductions of the behavioral actions of GAL(1–15) was linked to a disappearance of PLA. In the cell line RN33B, GAL(1–15) decreased 5-HT immunoreactivity more strongly than GAL.
Conclusions: Our results indicate that GAL(1–15) exerts strong depression-related and anxiogenic-like effects and may give the basis for the development of drugs targeting GAL_1 and GAL_2 heteroreceptor complexes in the raphe-limbic system for the treatment of depression and anxiety.

Keywords: anxiety, depression, galanin

Introduction

Mood disorders, including depression and anxiety, are among the most prevalent mental illnesses, with high socioeconomic impacts (Gelenberg, 2010; Wittchen et al., 2011). According to the World Health Organization, between 10% and 15% of the general population will experience a clinical depressive episode in their lifetime (Wittchen et al., 2011). Although the underlying

mechanisms have not yet been clearly defined in the last decade, the importance of the role of neuropeptides, including galanin, and/or their receptors in the treatment of stress-related mood disorders is becoming increasingly apparent (Kormos and Gaszner, 2013).

Galanin (GAL) is a neuropeptide (Tatemoto et al., 1983) widely distributed in neurons within the central nervous system (CNS; Jacobowitz et al., 2004). Three GAL receptor (GAL$_{1-3}$ receptors) subtypes with high affinities for GAL have been cloned (Branchek et al., 2000; Mitsukawa et al., 2008). GAL$_1$ and GAL$_2$ receptors are found in many regions of the CNS, as demonstrated with *in situ* hybridization, radioligand binding, and immunohistochemical studies (Jacobowitz et al., 2004). GAL$_1$ and GAL$_3$ receptors mainly activate inhibitory G proteins Gi/Go, while the GAL$_2$ receptor mainly couples to Gq/G11 to mediate excitatory signaling (Wang et al., 1997; Branchek et al., 2000)

The GAL$_{1-3}$ receptors are involved in a number of central functions modulating neuroendocrine levels, pain control, cardiovascular functions, addiction, and food intake (Mitsukawa et al., 2008; Diaz-Cabiale et al., 2010; Picciotto, 2010). GAL is also involved in mood regulation, including depression-related and anxiety-like behaviors (Juhasz 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 the forced swimming test (FST), indicative of depression-like behavior (Weiss et al., 1998; Kuteeva et al., 2005, 2007). Also, in a genetic rat model of depression, the Flinders Sensitive Line, an up-regulation of the GAL receptor binding sites, is found in the dorsal raphe nucleus and linked to high immobility in the FST (Bellido et al., 2002). However, intraperitoneal (i.p.) injections of GAL$_2$ receptor agonists exhibit anti-depressant–like effects, and two non-selective GALR agonists, galnon and galmic, decreased immobility time in the FST (Bartfai et al., 2004; Lu et al., 2005). These discrepancies may be attributed to the different physiological roles of the GAL receptor subtypes. Intracerebroventricular (i.c.v.) infusion of a GAL receptor ligand agonist with higher specificity for GAL$_1$ receptor (M617) or a GAL$_2$ receptor antagonist (M871) elevated immobility time, while the GAL$_2$/GAL$_3$ receptors agonist (AR-M1896) produced an antidepressant-like effect in the rat FST (Kuteeva et al., 2008). Furthermore, administration of GAL$_3$ receptor-selective antagonists to rats and mice produced antidepressant-like effects in both FST and the tail suspension test (TST; Swanson et al., 2005; Barr et al., 2006).

In view of these results, it has been proposed that activation of GAL$_1$ and GAL$_3$ receptors results in a depression-like behavior while stimulation of the GAL$_2$ receptor leads to anti-depressant–like effects.

The role of GAL in anxiety-like behaviors depends on the route and site of drug administration, and also on the intensity of stress conditions (Holmes et al., 2003; Holmes and Picciotto, 2006). I.c.v GAL produced an anxiolytic-like effect in rats in the Vogel conflict test (Bing et al., 1993), whereas intra-amygdala microinjection induced the opposite effect on the same task (Moller et al., 1999). The varying effects of GAL on anxiety-related behaviors may also depend on the differentially-distributed GAL receptor subtypes (Holmes and Picciotto, 2006; Bailey et al., 2007; Brunner et al., 2014). GAL$_1$ receptor knockout mice exhibit increased anxiety in the elevated plus-maze (Holmes et al., 2003), while GAL$_2$ receptor knockout mice show anxiety-like behavior or no effect, depending on the genetic background of the mutants (Bailey et al., 2007; Lu et al., 2008). In contrast, an antagonist of GAL$_3$ receptor produces anxiolytic-like effects in several behavioral tests (Swanson et al., 2005).

Not only GAL but also the galanin N-terminal fragments like GAL(1–15) are active in the CNS. Both GAL and GAL(1–15) molecules have specific roles in cardiovascular regulation and interact differently with other neuropeptides (Diaz-Cabiale et al., 2005, 2007). The three cloned receptors show a higher affinity for GAL than for GAL(1–15) (Branchek et al., 2000), but the demonstration of specific GAL(1–15) binding sites in the rat brain emphasizes the powerful role of GAL fragments in GAL communication, especially in the dorsal hippocampus, neocortex, and striatum (Hedlund et al., 1992). Only GAL(1–15), but not GAL, can antagonistically modulate the serotonin 5-HT1A receptors in the dorsal hippocampus, and this effect was blocked by the GAL receptor antagonist M35 (Hedlund et al., 1994). In the ventral limbic cortex, N-terminal GAL fragments can more strongly and more potently reduce postjunctional 5-HT1A receptor recognition than GAL, where high-affinity GAL receptors also exist (Diaz-Cabiale et al., 2000).

The purpose of the present study was to assess the role of GAL(1–15) in mood regulation using anxiety- and depression-related behavioral test in rats. Additionally, we used the proximity ligation assay (Trifilieff et al., 2011; Borroto-Escuela et al., 2013) to analyze the proximity of GAL$_1$ and GAL$_2$ receptors in the dorsal hippocampus and dorsal raphe nuclei showing specific GAL(1–15) binding (Hedlund et al., 1992) since GAL$_1$ and GAL$_2$ heteroreceptor complexes may exist in areas selectively enriched in GAL(1–15) binding sites (Fuxe et al., 2008, 2012). Therefore, we tested the involvement of the GAL$_2$ receptor in the GAL(1–15) effect with the selective GAL$_2$ receptor antagonist M871 and with an *in vivo* model of siRNA GAL$_2$ receptor knockdown rats. The role of the GAL$_1$ receptor in the behavioral actions of GAL(1–15) were also analyzed in siRNA GAL$_1$ receptor knockdown rats. The effects of GAL(1–15) versus GAL were analyzed on 5-HT immunoreactivity (IR) in the rat medullary raphe-derived cell line RN33B to compare the actions of these two GAL peptides on 5-HT mechanisms on the RN33B cells, which may be of relevance for their actions on the dorsal raphe 5-HT nerve cells.

Materials and Methods

Animals

Male Sprague Dawley rats (body weight 225–250 g, age 8 weeks) were obtained from Criffa and maintained in a humidity- and temperature-controlled (20–22°C) room under a 12:12 h light/dark cycle (lights on 08:00 and off 20:00 h). The animals had free access to food pellets and tap water. All animal experimentation was conducted in accordance with the University of Málaga Guidelines for the Care and Use of Laboratory Animals.

Intracerebroventricular Injections

This protocol has been used previously (Parrado et al., 2007; Diaz-Cabiale et al., 2011). Briefly, the rats were anesthetized intraperitoneally with Equitesin (3.3 mL/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: 1.4 mm lateral and 1 mm posterior to the bregma, and 3.6 mm 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 stainless-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 1 min.

Solutions were prepared freshly and the peptides were dissolved in artificial cerebrospinal fluid (composition is 120 nM NaCl, 20 nM NaH2CO3, 2 nM KCl, 0.5 nM KH2PO4, 1.2 nM CaCl2, 1.8 nM MgCl2, 0.5 nM Na2SO4, and 5.8 nM D-glucose, pH 7.4). GAL was obtained from NeoMPS; GAL(1–15) and the GAL_2 receptor antagonist M871 were obtained from Tocris Bioscience.

Behavioral Assessment

Groups of rats were assessed in two behavioral tests: the open field and the forced swim 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 08:00 and 14:00 h.

Three sets of experiments were conducted. In the first set of experiments, a dose-response curve of GAL(1–15) was performed. For this, groups of rats received i.c.v GAL(1–15) 1 nmol, 3 nmol, 6 nmol, or vehicle 15 min before the test. In the second set of experiments, the effects in the behavioral test of GAL and GAL(1–15) were compared; for this, groups of rats received i.c.v GAL 3 nmol, GAL(1–15) 3 nmol, or vehicle 15 min before the test. In the last set of experiments, the role of the GAL_2 receptor was studied; for this, groups of rats received i.c.v GAL(1–15) 3 nmol combined with GAL_2 receptor antagonist M871 3 nmol 15 min before the test.

In a second group of experiments, GAL(1–15) at the effective dose obtained in the experiments described above was assessed in two more behavioral tests: the light/dark test and the tail suspension test.

Forced Swimming Test

Animals were individually placed in a vertical glass cylinder (50 cm height, 20 cm diameter) containing water (25ºC) 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 (Kuteeva et al., 2007). The total duration of immobility behavior and climbing were recorded during the second 5 min. Immobility was defined as floating passively in an upright position in water with only small movements necessary to keep the head above the water surface. Climbing was defined as forepaw movements directed toward the walls of the cylinder.

Open Field Test

Rats were individually placed and allowed to freely explore; their behavior was recorded over a 5 min 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 50 cm), total time spent in and entries into the inner square were recorded (Grivas et al., 2013; Narvaez et al., 2014).

Light/Dark Test

The light–dark box apparatus consisted of two chambers (26 x 25 x 27 cm) joined together lengthwise. One chamber was black and the other was white. The white chamber was illuminated by a 40W light bulb. Animals were individually placed in the center of the light box and were allowed to freely explore during 5 minutes. The latency to enter (with all four paws) the dark compartment, time spent in the light chamber, and the latency time for re-entering the light box were recorded (Grivas et al., 2013; Desikan et al., 2014).

Tail Suspension Test

In this test (Hinojosa et al., 2006), the rat was hung upside down using an adhesive tape to fix its tail to a rope through an eyebolt at 60 cm. The animal was considered immobile when it was not making any movements of struggling, attempting to catch the adhesive tape, body torsions, or jerks (Hinojosa et al., 2006; Yan et al., 2014).

Proximity Ligation *In Situ* Assay (Duolink)

Proximity ligation assay (PLA) and quantification were carried out as described previously using a Duolink *in situ* PLA detection kit (Borroto-Escuela et al., 2012, 2013). The primary antibodies of different species directed to the GAL_1 receptor (goat polyclonal, Santa Cruz Biotechnology Inc., EEUU, 1:250) and GAL_2 receptor (rabbit polyclonal, Alomone Lab, 1/250) were used. Sections were mounted on slides with fluorescent mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), staining the nuclei with blue color. The *in situ* PLA positive signals were visualized using a Leica SP5 confocal microscope and the Duolink Image Tool software.

For *in situ* PLA in raphe-derived RN33B cells used the same primary antibodies and the same procedure.

Double Immunofluorescence

The procedures have been previously used (Narváez et al., 2014) . Primary antibody rabbit anti-GAL_2 receptor (Alomone Lab, 1/250) was incubated for 12 hours at 4ºC and detected with the red secondary antibody mouse anti-rabbit DyLight 549 (Jackson inmunoResearch Laboratories, 1/100). Goat anti-GAL_1 receptor (Santa Cruz Biotechnology INC, EEUU, 1:250) was incubated 12 hours at 4ºC and detected with the secondary antibody rabbit biotynilated anti-goat (Vector Labs Inc.) and Alexa Fluor 488-conjugated Streptavidin (Jackson Laboratories InmunoResearch, 1:1000). Sections were mounted on slides with fluorescent mounting medium (Dako) and visualized using a Leica TCS-SL confocal microscope. The double immunolabeling was performed in the tissue of rats without treatment (Figure S1) or in knockdown rats of GAL_2 receptor (Figure S4).

Knockdown Rats of GAL_2 Receptor and GAL_1 Receptor Animals and Treatment

For knockdown rats, animals were operated with the same procedure as previously described (Nakajima et al., 2012). When the cannula was implanted, rats were intracerebroventricularly injected with 5 μg (0.35 nmol) of Accell Smart pool siRNA GAL_2 receptor or siRNA GAL_1 receptor (Dharmacon) or 5 μl of vehicle (Acell siRNA Delivery Media). Also, some animals were injected with siRNA-Control (Acell non-targeting pool, Dharmacon) to verify that the Acell system has no effect on animals. Previous to injection, the siRNAs were previously resuspended in 1X siRNA buffer (Dharmacon B-002000-UB-100), aliquotted, and stored in -80º, following the manufacturer's instructions. For the administration of siRNAs an aliquot was diluted in Acell siRNA Delivery Media (Dharmacon B-005000-100) at a concentration of 1 μg/μl (0.07 nmol/μl).

Time-course curve was performed and GAL_2 receptor protein expression was examined via immunohistochemical analysis (Figure S2) and the GAL_2 receptor and GAL_1 receptor mRNA reduction was examined with real-time quantitative polymerase chain reaction (RT-PCR; Figures S3 and S5).

These knockdown rats were used in the open field test (7 days after the Accell Smart pool siRNA GAL_2 receptor injection or siRNA GAL_1 receptor injection) and in the forced swimming test (9 days after the Accell Smart pool siRNA GAL_2 receptor injection or siRNA GAL_1 receptor injection) either alone or after receiving i.c.v. GAL(1–15) 3 nmol or vehicle 15 min before the test. The brain tissues of the knockdown rats of GAL_2 receptor were used for *in situ* PLA and double inmunofluorecence.

Immunohistochemical

At 4, 6, 8, 10, 12, and 14 days after injection of siRNA GAL_2 receptor, two rats per day were perfused transcardially. The brains were then dissected and serial, coronal free-floating sections (30 μm thick) were collected. The control group received an injection of Accel siRNA Delivery Media (vehicle). The sections were incubated with a primary antibody rabbit anti-GAL_2 receptor (Alomone Lab, 1/250) overnight at 4ºC. 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 GAL_2 receptor IR was performed under light microscopy (Nikon Optiphot-2) in the neuro-anatomical area of interest. Four sections of two animals per day were examined and captured by a video camera (Olimpus UC30) linked to a PC computer, and GAL_2 receptor IR was quantified by optical density using the computer software ImageJ (National Institutes of Health).

RT-PCR

At 4, 6, 8, and 14 days after injection of siRNA GAL_2 receptor or siRNA GAL_1 receptor, 4 animals treated with siRNA GAL_2 receptor or siRNA GAL_1 receptor were killed by decapitation. Four animals injected with vehicle followed the same process as controls.

The hippocampi of all animals were quickly extracted and frozen until use. Total RNA was isolated from the dorsal hippocampus using RNeasy Lipid Tissue Kit (Qiagen), followed by treatment to remove genomic DNA with Recombinant DNase I (RNase-free) (Takara biotechnology Co., TLD). cDNA was obtained using a Reverse Transcriptase Core kit (Eurogentec). These three steps were performed according to the manufacturer's instructions.

All PCR were conducted in triplicate using Power SYBR Green PCR Master Mix (Applied Biosystems) in a 7500 RT-PCR system (Applied Biosystems). The primer sequences used in this study are: GAPDH-Forward: 5´-GCTCTCTGCTCCTCCCTGTTC; GAPDH-Reverse: 5´-GAGGCTGGCACTGCACAA; GAL2 RECEPTOR-Forward: 5´-AACAGGAATCCACAGACC; GAL2 RECEPTOR-Reverse: 5´-CCCTTTGGTCCTTTAACAAG; GAL1 RECEPTOR-Forward: 5´-AAAACTGGACAAAACTTAGCC; and GAL1 RECEPTOR-Reverse: 5´-GGATACCTTTGTCTTTGCTC. The data were analyzed using the comparative Ct method and normalized to measures of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Cell Culture and Immunocytochemistry

RN33B cells (a CNS raphe-derived neuronal precursor cell line; Lundberg et al., 2002; American Type Culture Collection) were grown in DMEM/F12 supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 10% (v/v) fetal bovine serum at 37°C in an atmosphere of 5% CO2. RN33B cells were treated for 1 h with GAL 100 nM, GAL(1–15) 100 nM, or control,

followed for 1 h by recuperation. Cells were stained with rabbit anti-5-HT monoclonal antibody (1/5000; Sigma-Aldrich). The secondary antibody used was Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:2000; Invitrogen). 5-HT immunoreactivity semiquantification is expressed as the 5-HT immunoreactivity 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.

Statistical Analysis

Data are presented as the means ± standard error of the mean and sample numbers (n) are indicated in figure legends. All data were analyzed using GraphPad PRISM 4.0 (GraphPad Software).

For comparing two experimental conditions, student's unpaired *t*-test statistical analyses were performed. Otherwise, one-way analyses of variance (ANOVAs) followed by Newman-Keuls comparison post-tests were performed. Differences were considered significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

Behavioral Studies

GAL(1–15) Induced a Depression-Related and Anxiogenic-Like Effect in the Behavioral Tests

In the FST, rats were pre-exposed to water for 15 minutes. Twenty-four hours later, their immobility and climbing during a second 5 min exposure to water were analyzed as signs of depression-like behavior.

GAL(1–15) at 3 nmol significantly increased the immobility ($t = 4.071$, $p < 0.001$, df = 13) and decreased the climbing ($t = 3.857$, $p < 0.01$, df = 12) behavior by 44% and 46%, respectively (Figure 1A and B). These results indicate that GAL(1–15) evokes a strong depression-like behavior. Similar results were obtained at the dose of 6 nmol: GAL(1–15) also significantly increased the immobility ($t = 4.97$, $p < 0.001$, df = 13) and decreased the climbing ($t = 4.815$, $p < 0.01$, df = 12) behavior (Figure 1A and B).

In the tail suspension test, GAL(1–15) at the dose of 3 nmol significantly increased the immobility behavior recorded during the 6 min of testing (($t = 2.02$, $p < 0.05$, df = 12; Table 1)

To assess the role of GAL(1–15) in the regulation of anxiety-related behaviors, the time spent in and numbers of entries into the central square were examined in the open field.

GAL(1–15) at the dose of 3 nmol induced an anxiogenic-like effect, since it significantly decreased the entries ($t = 3.379$, $p < 0.01$, df = 16; Figure 1C) and the time spent ($t = 2.318$, $p < 0.05$, df = 13; Figure 1D) in the central square in the open field by 42% and 39%, respectively. GAL(1–15) lacked effects at the doses of 1 nmol or 6 nmol (Figure 1C and D).

These anxiogenic-like effects were independent of the locomotor activity, since the total distance reached and speed were equivalent between all the groups (Supplementary Table 1).

The effects of GAL(1–15) at 3 nmol on performance in the light/dark test are described in Table 1. GAL(1–15) significantly decreased the total time spent in the light compartment ($t = 2.56$, $p < 0.05$, df = 9) and the latency to enter in the dark chamber compared with controls ($t = 2.66$, $p < 0.05$, df = 9; Table 1). Moreover, the latency time for reentering to light was significantly increased after GAL(1–15) treatment ($t = 2.74$, $p < 0.05$, df = 8; Table 1).
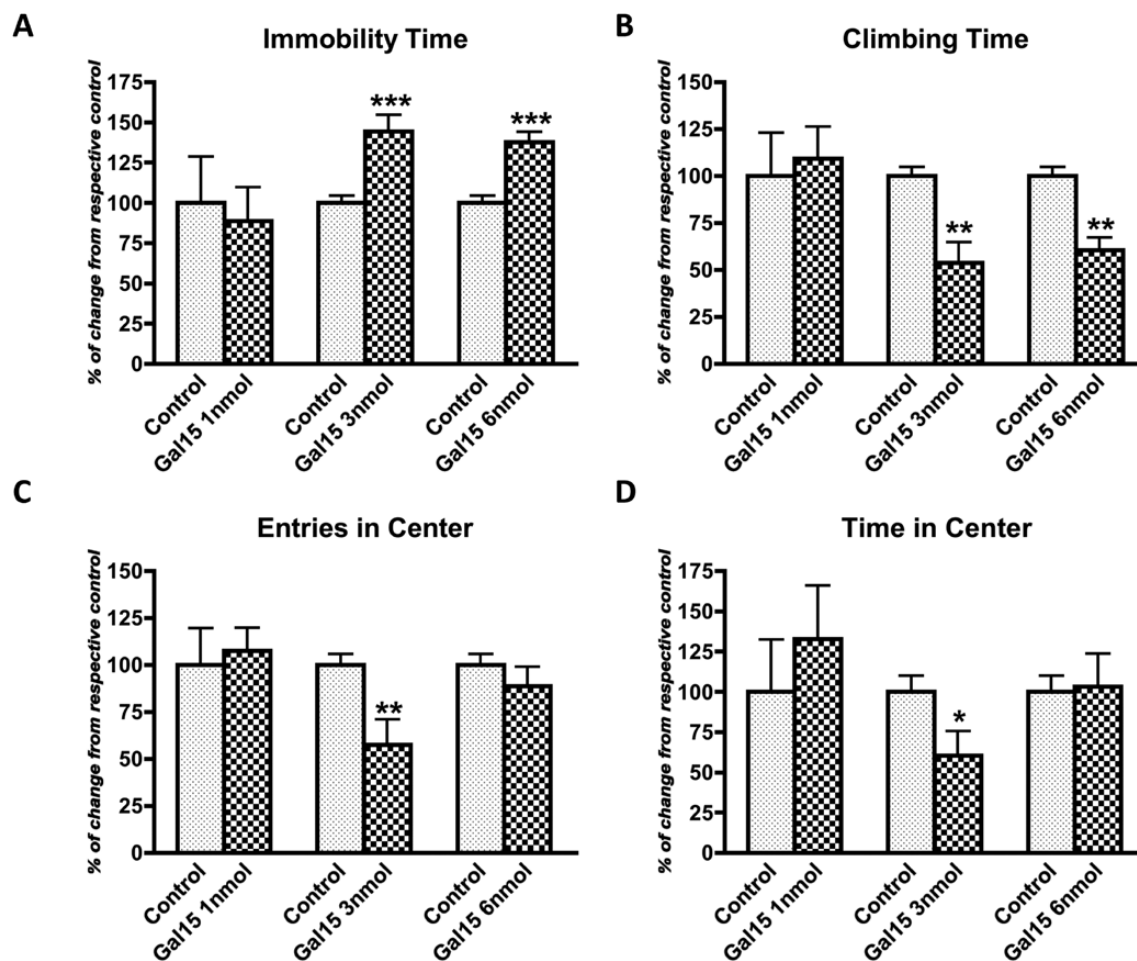
Figure 1. Dose-response curve of galanin(1–15) [GAL(1–15)] in the forced swimming test (A and B) and open field test (C and D) in rats. GAL(1–15) (at 1, 3, or 6 nmol/rat) was administered intracerebroventricularly 15 min before the tests. Cerebrospinal fluid–injected rats were used as the control group. Vertical bars represent mean ± standard error of the mean of the percentage of change from the respective controls in the time of immobility (A) or climbing (B) and the number of entries (C) or time in the central square (D) during a 5 min test period (6–12 animals per group). $*p < 0.05$; $**p < 0.01$, and $***p < 0.001$ versus respective control according to student's t-test.

Table 1. Effect of Gal(1–15) at 3 nmol in the Light/Dark Test and Tail Suspension Test.

Parameters Measured	Light/Dark Test			Tail Suspension Test
	Latency to enter in dark chamber (s)	Time spent in light chamber (s)	Latency time for re-entering to light (s)	Immobility (s)
Control	32.6 ± 7.8	92 ± 6.9	24.9 ± 5.9	185.8 ± 11.03
Gal(1–15) 3nmol	11.5 ± 3.3*	44.1 ± 15.9*	138.3 ± 40.9*	215 ± 7.7*

Group of animals (n = 5–8) were injected intracerebroventricularly with Gal(1–15) or cerebrospinal fluid as the vehicle 15 min before the test. Values are indicated as mean ± standard error of the mean. $*p < 0.05$ vs control according to student´s t-test.

Comparison Between GAL and GAL(1–15)

In the FST, the overall one-way ANOVA revealed a significant effect of treatment for immobility (one-way ANOVA, $F_{2,26} = 12.8$, $p < 0.001$). The increase in the immobility induced by GAL(1–15) was significantly higher than the one induced by GAL (Newman-Keuls post-hoc test: $p < 0.01$; Figure 2A). In the climbing behavior we observed the same pattern of response (one-way ANOVA, $F_{2,26} = 7.6$, $p < 0.01$): GAL(1–15) induced a significantly stronger decrease in climbing response compared with GAL (Newman-Keuls post-hoc test: $p < 0.05$; Figure 2B).

In the open field, in agreement with other authors (Narvaez et al., 2014) the dose of 3 nmol of GAL did not affect the time spent in and number of entries into the central square in the open field (Figure 2C and D). In view of the fact that GAL(1–15) at 3 nmol significantly decreased the time ($t = 2.318$, $p < 0.05$, df = 13) and number ($t = 3.379$, $p < 0.01$, df = 16) of entries in the open field, our results indicate than only GAL(1–15) and not GAL modifies the anxiety parameters in the present model.

GAL$_2$Receptor Antagonist M871 Induced Blockade of the Behavioral Effects of GAL(1–15)

In the FST, GAL$_2$ receptor antagonist M871 significantly blocked the increased the immobility (one-way ANOVA, $F_{3,32} = 10.38$, $p < 0.001$, Newman-Keuls post-hoc test: $p < 0.01$; Figure 3A) and decreased
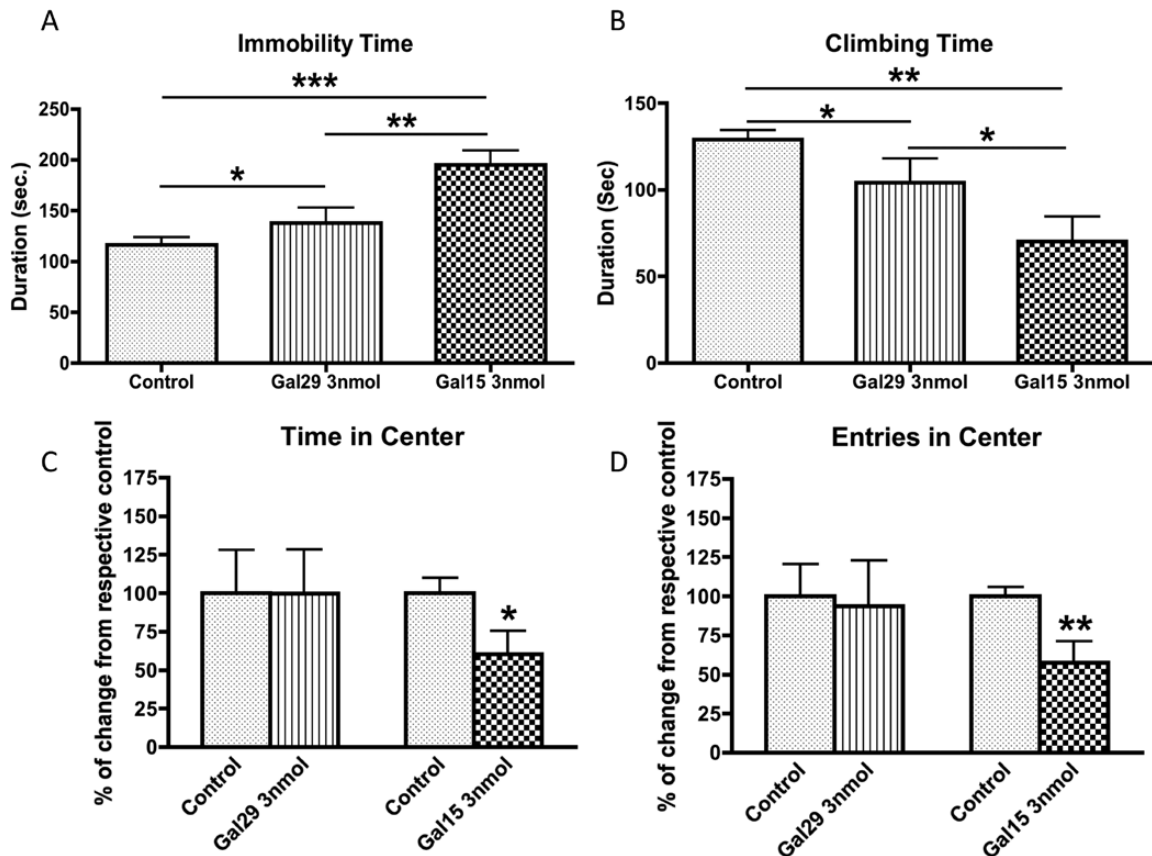
Figure 2. Behavioral effect of administration of galanin (GAL29) and galanin(1–15) [GAL(1–15)] in the Forced Swimming Test (A,B) and Open Field Test (C,D) in rats. GAL29 (3 nmol/rat) and GAL(1–15) (3 nmol/rat) were administered intracerebroventricularly 15 min before the test. Cerebrospinal fluid–injected rats were used as the control group. Values, indicated as mean ± standard error of the mean, represent time of immobility (A) and climbing (B) in the forced swimming test and percentage of change from respective controls in entries (D) and time in the center in the open field test (C) during a 5 min test period (6–15 animals per group). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, according to (A and B) one-way analyses of variance followed by Newman Keuls Multiple Comparison Tests and (C and D) versus respective controls according to student's *t*-tests.

the climbing (one-way ANOVA, $F_{3,32} = 4.535$, $p < 0.01$, Newman-Keuls post-hoc test: $p < 0.05$) induced by GAL(1–15) (Figure 3B).

The GAL$_2$ receptor antagonist M871 alone in the dose of 3 nmol lacked effects with respect to immobility or climbing time (Figure 3A and B).

In the open field the same type of change was observed. GAL$_2$ receptor participates in the effect induced by GAL(1–15), since the GAL$_2$ receptor antagonist M871 significantly decreased the number of entries (one-way ANOVA, $F_{3,29} = 10.6$, $p < 0.001$, Newman-Keuls post-hoc test: $p < 0.05$; Figure 3C) and time spent in the central square (one-way ANOVA, $F_{3,26} = 4.03$, $p < 0.05$, Newman-Keuls post-hoc test: $p < 0.05$) mediated by GAL(1–15) (Figure 3D). In this test, M871 significantly increased the number of entries (Newman-Keuls post-hoc test: $p < 0.01$) but not the time spent in the central square of the open field (Figure 3C).

Neurochemical Studies

GAL$_1$ and GAL$_2$Receptors in the Dorsal Raphe Nucleus and Dorsal Hippocampus

Positive results were obtained with the *in situ* PLA indicating the formation of GAL$_1$ and GAL$_2$ heteroreceptor complexes. This was supported by studies using double immunolabeling, which demonstrated colocation of GAL$_1$ and GAL$_2$ receptors IR in the nerve cells of the dorsal raphe nucleus and of the dorsal hippocampus (Supplementary Figure S1A and B).

In Figure 4A, large numbers of the Ammon´s horn 1, 2, and 3 (CA1, CA2, and CA3) and the dentate gyrus of the dorsal hippocampus and the dorsal raphe show a significant number of red clusters (blobs) representing positive PLA signals. The quantification of PLA in these areas demonstrate that the highest number of PLA clusters are present in the cytoplasm of nerve cells of the dorsal raphe (one-way ANOVA, $F_{4,35} = 26.57$, $p < 0.001$, Newman-Keuls post-hoc test: $p < 0.001$; Figure 4B).

The specificity was demonstrated by the fact that no PLA clusters were observed in the lateral corpus callosum (Figure 4A), an area that seems to lack the GAL$_2$ receptor (O'Donnell et al., 1999). The fact that we did not observe a PLA positive signal in the molecular layer of the dentate gyrus confirms the specificity of the signal (Figure 4A). The results obtained in the dorsal raphe were validated in PLA experiments on raphe RN33B cells (see below).

siRNA GAL$_2$ and GAL$_1$ Receptor Knockdown Validates the Involvement of Heteroreceptor Complexes

To verify the Acell system employed, we generated siRNA-Control rats (Acell Non-targeting pool) and vehicle rats (Acell siRNA Delivery Media). We did not observe any differences between these groups compared to artificial cerebrospinal fluid normal rats in any behavioral tests (data not shown).

Down-regulation of the GAL$_2$ receptor by siRNA did not affect any parameter of the behavioral tests, neither in the FST nor in the open field (Figure 5A–E). However, the decrease in the GAL$_2$
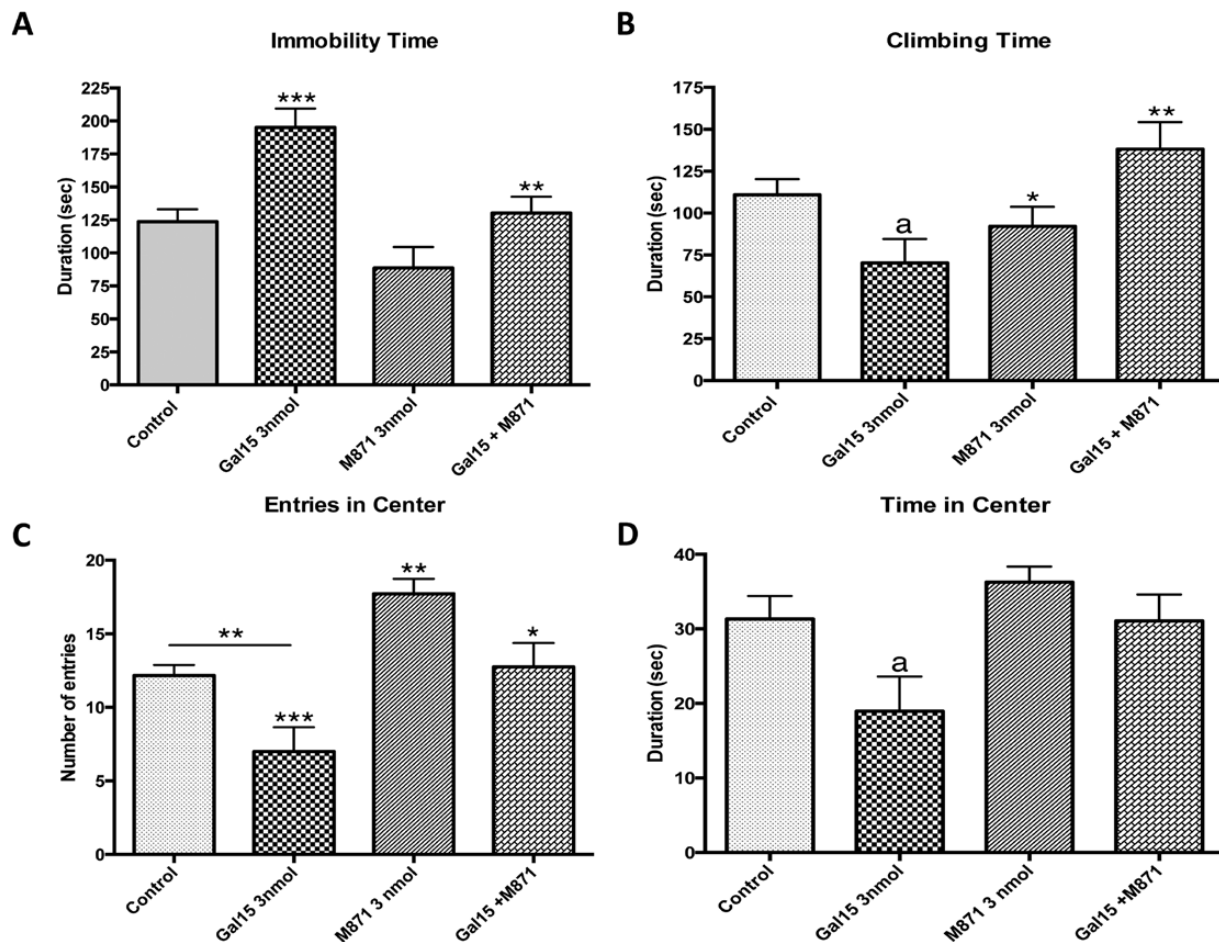
Figure 3. Behavioral effects of the co-administration of the GAL_2 receptor antagonist M871 (3 nmol/rat) and an effective dose of galanin(1–15) [GAL(1–15)] (3 nmol/rat) in the forced swimming test (FST; A and B) and open field test (C and D). Treatments were injected intracerebroventricularly 15 min prior the tests. Cerebrospinal fluid–injected rats were used as a control group. Data represents mean ± standard error of the mean of immobility (A) and climbing time (B) in the FST and entries (C) and time in the center in the open field test (D) during the 5 min test period (6–14 rats per group). (A,B) *$p < 0.05$ versus Gal15+M871; **$p < 0.01$ versus Gal15 group; ***$p < 0.001$ versus Control and M871 group; $^a p < 0.05$ versus Control group. (C,D) *$p < 0.05$ versus Gal15; **$p < 0.01$ versus Control and versus Gal15+M871; ***$p < 0.001$ versus M871; $^a p < 0.05$ versus rest of the groups according to one-way ANOVA followed by Newman Keuls Multiple Comparison Test.

receptor was sufficient to block the effect of GAL(1–15) in the siRNA GAL_2 receptor knockdown rats (Figure 5A–E). Thus, GAL(1–15) at the dose of 3 nmol lacked effect on the immobility (one-way ANOVA, F2,18 = 0.743, $p = 0.48$), climbing (one-way ANOVA, F2,18 = 0.426, $p = 0.65$), and swimming time (one-way ANOVA, F2,17 = 0.923, $p = 0.41$) in the FST (Figure 5A–C). The same effect was observed in the number of entries (one-way ANOVA, F2,17 = 0.517, $p = 0.6$) and time spent in the central square (one-way ANOVA, F2,16 = 0.253, $p = 0.77$) in the open field test (Figure 5D and E).

We observed the same pattern of response with the downregulation of the GAL_1 receptor by siRNA (Figure S5). The decrease in the GAL_1 receptor blocked the effect of GAL(1–15) in the siRNA GAL_1 receptor knockdown rats in all the parameters analyzed in the FST: immobility (one-way ANOVA, F2,20 = 0.737, $p = 0.49$), climbing (one-way ANOVA, F2,20 = 0.568, $p = 0.57$), and swimming time (one-way ANOVA, F2,20 = 0.026, $p = 0.97$; Figure 5S A–C). It also blocked the effects in the open field: the number of the entries (one-way ANOVA, F2,17 = 0.209, $p = 0.81$) and time spent in the central square (one-way ANOVA, F2,19 = 0.114, $p = 0.89$; Figure 5S D–E).

PLAs

In the siRNA GAL_2 receptor–treated animals, PLA-positive red clusters (blobs) were still observed in the dorsal hippocampus

and dorsal raphe (Figure 5F–H). However, the quantification of PLA shows a reduction of around 40% (t = 3.174, $p < 0.01$, df = 19) in nerve cells of the CA1 area and 60% (t = 7.325, $p < 0.001$, df = 14) of the dorsal raphe compared with the vehicle group.

These results confirm that the PLA signal obtained is specifically due to the GAL_1 and GAL_2 receptors complex.

In agreement with these results, the colocalization of GAL_1 and GAL_2 receptor immunoreactivities was reduced in the siRNA GAL_2 receptor in the dorsal raphe and also in the dorsal hippocampus (Supplementary Figure 4).

GAL(1–15) Decreased the 5-HT Immunoreactivity in Raphe RN33B Cells

The presence of GAL_1 and GAL_2 receptors in raphe RN33B cells was established with immunohistochemical techniques (data not shown). Moreover, PLA-positive clusters were detected within them, which supports the presence of GAL_1 and GAL_2 heteroreceptor complexes in these cells and the validity of these cells as a target for GAL(1–15) (Figure 6A).

In the semiquantitative image analysis, the one-way ANOVA showed a significant effect in 5-HT immunoreactivity after GAL and GAL(1–15) treatment (one-way ANOVA, F2,147 = 63.85,
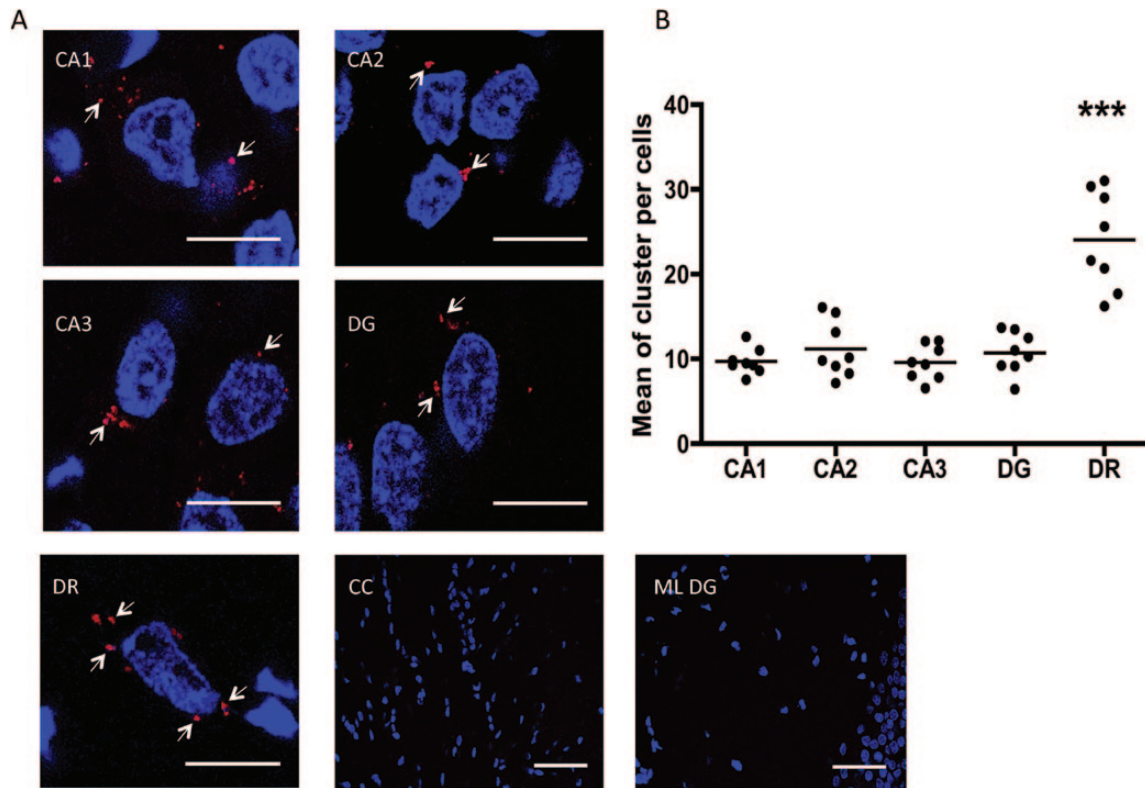
Figure 4. Close proximity between GAL_1 and GAL_2 receptors is detected by *in situ* proximity ligation assay (PLA; seen as red clusters indicated by arrows) in dorsal rat hippocampi and dorsal raphe nuclei. *In situ* PLA was performed using primary antibodies of different species directed to the GAL_1 receptor and GAL_2 receptor, followed by PLA reagents. (A) Representative photographs for positive PLA regions. (B) Quantification of red clusters/6-diamidino-2-phenylindole–positive nuclei. Quantification was made in 10–20 cells per photo and performed 8 photos per brain zone. Thus, each point in the scatter plot represents the mean between 10–20 cells (80–160 cells per zone). The line in each group represents the mean value. ***$p < 0.001$ versus the rest of the groups according to one-way analyses of variance followed by Newman Keuls Multiple Comparison Tests. Nuclei are shown in blue (6-diamidino-2-phenylindole). Scale bars are 10 μm and 50 μm in the CC and ML DG photographs. CA1, CA2 and CA3: Ammon's horn 1, 2, and 3; CC: corpus callosum; DG: dentate gyrus; DR: dorsal raphe nucleus; ML DG: molecular layer of dentate gyrus.

$p < 0.001$). The incubation with GAL caused a significant (Newman-Keuls post-hoc test: $p < 0.001$) decrease in the 5-HT immunoreactivity in RN33B cells (Figure 6B and C). GAL(1–15) also significantly decreased the 5-HT immunoreactivity in these cells (Newman-Keuls post-hoc test: $p < 0.001$). This reduction was significantly stronger than the one induced by GAL (Newman-Keuls post-hoc test: $p < 0.001$; Figure 6B and C).

Discussion

In the current study we describe for the first time how GAL(1–15) induces strong depression-related and anxiogenic-like effects in the FST, the tail suspension test, the open field test, and the light/dark test. Importantly, these effects were significantly stronger than the ones induced by GAL. PLA indications were also obtained for the existence of GAL_1 and GAL_2 heteroreceptor complexes in the dorsal hippocampus and especially in the dorsal raphe nerve cells, areas rich in GAL-fragment binding sites (Hedlund et al., 1992). Indications were also obtained for the involvement of this heteroreceptor complex in the actions of GAL(1–15), based on the use of the specific GAL_2 receptor antagonist M871 and i.c.v. injections of GAL_1 receptor siRNA or GAL_2 receptor siRNA, producing a reduction of the GAL_1 receptor or GAL_2 receptor, respectively. In the latter experiment, the reduction of the behavioral actions of GAL(1–15) was linked to a substantial disappearance of PLA-positive blobs in the nerve cells of the hippocampus and the dorsal raphe, and thus to a reduction in the GAL_1 and GAL_2 heteroreceptor complexes. Thus, the

current study supports the concept that GAL-fragment binding sites mainly exist on GAL_1 and GAL_2 heteroreceptor complexes which preferentially bind GAL(1–15) (Fuxe et al., 2008, 2012). The relevance of the dorsal raphe as a target for GAL(1–15) was supported by studies in rat medullary raphe-derived cell line RN33B, where GAL(1–15) decreased 5-HT IR more strongly than GAL.

In the behavioral tests we observed that GAL induced the same pattern of response as in previous studies, validating the conditions of our behavioral models. In the FST GAL infused either i.c.v or into the ventral tegmental area increased immobility (Weiss et al., 1998; Kuteeva et al., 2005, 2007). This effect was reproduced with i.c.v. GAL at 3 nmol in this test. Moreover, in the open field GAL at 3 nmol lacked behavioral effects (Narvaez et al., 2014). This indicates the absence of stress conditions in our model, since GAL produced anxiolytic-like effects only in animals tested under heightened stress conditions (Morilak et al., 2003; Barrera et al., 2005).

GAL(1–15) induced a strong depression- and anxiogenic-like effect in all the tests, indicating a potential role of GAL(1–15) in mood disorders. The anxiogenic-like effect of GAL(1–15) at 3 nmol in the open field test is not present at 6 nmol. This rapid disappearance of the action at the higher concentration may reflect the depletion of a factor necessary for the formation of the GAL_1 and GAL_2 heteroreceptor complexes or the rapid development of a desensitisation mechanism at the GAL receptors. Similar biphasic concentration-response curves have also been observed for the GAL in its interaction with α2-adrenoreceptor (Diaz-Cabiale et al., 2005) and with the neuropeptide Y (Diaz-Cabiale et al., 2011).
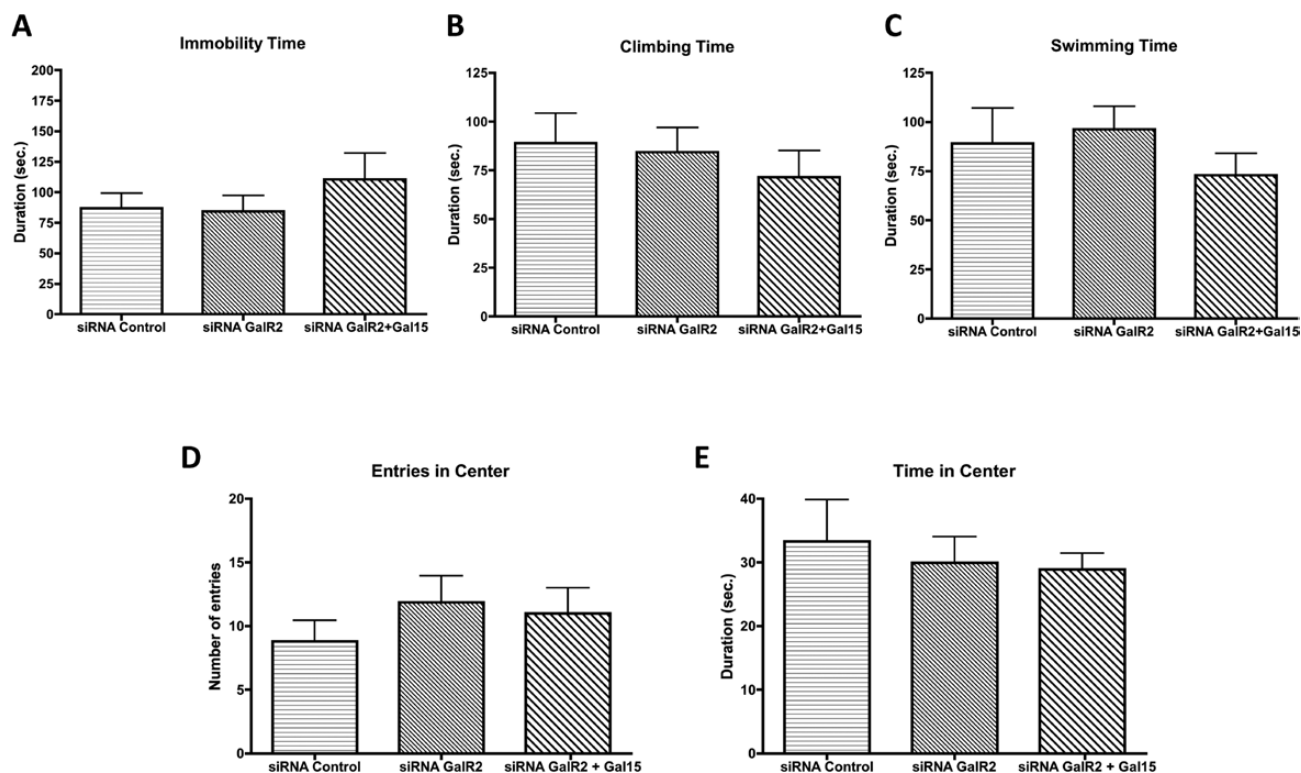
Figure 5. Analysis of galanin(1–15) [GAL(1–15)] in GAL$_2$ receptor knockdown rats in the forced swimming test (FST) and open field test or in the detection of close proximity between GAL$_1$ and GAL$_2$ receptors in rat brains. (A–E) Behavioral effects of an effective dose of GAL(1–15) (3 nmol/rat) in the GAL$_2$ receptor knockdown in FST and open field test. Treatments were injected intracerebroventricularly 15 min prior to the tests. siRNA control–injected rats were used as the control group. Data represents mean ± standard error of the mean of immobility (A), climbing (B), and swimming time (C) in the FST and entries (D) and time (E) in the center in the open field test during the 5 min test period (6–14 rats per group). No differences were found according to one-way analyses of variance. (F–H) Detection of close proximity between GAL$_1$ and GAL$_2$ receptors in the dorsal hippocampus and dorsal raphe in GAL$_2$ receptor knockdown rats by *in situ* proximity ligation assay (PLA). Quantification of red clusters/6-diamidino-2-phenylindole–positive nuclei in the dorsal hippocampus (CA1; F) and dorsal raphe (G) 9 days after a single intracerebroventricular injection of siRNA GAL$_2$ receptor or vehicle. Vertical bars represent mean ± standard error of the mean of percentage of change from respective controls. **$p < 0.01$ and ***$p < 0.001$ versus respective control according to student's *t*-test. (H) Representative photographs for decrease of detection of cytoplasmatic GAL$_1$ and GAL$_2$ receptors via *in situ* PLA (seen as red clusters indicated by arrows) in the dorsal hippocampus (CA1) and dorsal raphe in GAL$_2$ receptor knockdown rats. Nuclei are shown in blue (6-diamidino-2-phenylindole). Scale bar, 10 μm. CA1: Ammon's horn 1; DR: dorsal raphe nucleus.

Furthermore, the effects of GAL(1–15) in the FST and in the open field 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-HT1A receptor, known to be involved in mood disorders, more strongly and potently than GAL (Diaz-Cabiale et al., 2000). This was true not only for behavioral functions, but also for cardio-vascular functions. Thus, GAL(1–15) acts differentially from GAL. The N-terminal fragment GAL(1–15) antagonized the cardiovascular effects of GAL and GAL(1–15) but not GAL decreased baroreceptor reflex sensitivity (Diaz-Cabiale et al., 2005). GAL(1–15) also interacted differently from GAL with other neuropeptides, such as angiotensin or neuropeptide Y, involved in central cardiovascular functions (Diaz-Cabiale et al., 2005, 2010).

Our results at the behavioral level validate and extend the view of a specific role of GAL(1–15) in brain communication. The GAL$_2$ receptor is involved in the GAL(1–15) effects, since the GAL$_2$ receptor antagonist M871 blocked the GAL(1–15)-induced depression-like and anxiogenic effects.

The GAL$_2$ receptor has been involved in neuronal mechanisms underlying antidepressant-like effects, but the specific role for the GAL$_2$ receptor in anxiety is not well characterized. In fact, GAL$_2$ receptor knockout mice showed either anxiety-like behavior or no effect depending on the genetic background of the mutants (Bailey et al., 2007; Lu et al., 2008). However, our

results demonstrate the importance of the GAL$_2$ receptor in the anxiogenic-like effect induced by GAL(1–15).

In previous work, a GAL$_2$ and GAL$_3$ receptor agonist (AR-M1896) administered i.c.v. to rats decreased immobility time in the FST, indicating antidepressant effects (Kuteeva et al., 2008). Also, transgenic mice overexpressing the GAL$_2$ receptor demonstrated decreased immobility in the FST, indicative of antidepressant-like behavior (Le Maitre et al., 2011). Moreover, repeated fluoxetine treatment of rats increases the number of GAL$_2$ receptor binding sites in the dorsal raphe nucleus, which could contribute to the antidepressant effects of fluoxetine via the GAL$_2$ receptor in the dorsal raphe (Lu et al., 2005). These results indicate that the activation of the GAL$_2$ receptor induces antidepressant-like effects. Instead, the current results showing that the GAL$_2$ receptor antagonist blocks the depressant actions of the GAL fragment in the FST can be explained by our hypothesis that it targets the GAL$_1$ and GAL$_2$ heteroreceptor complex, which reduces the signaling from this complex, mediating the depression-like actions of GAL(1–15) (Fuxe et al., 2012).

In the current work, positive PLA blobs (puncta) formed from GAL$_1$ and GAL$_2$ receptors were, in fact, observed in the nerve cells of the dorsal hippocampus and dorsal raphe. This indicates that the distances between the two receptors are in a range compatible with the formation of GAL$_1$ and GAL$_2$ heteroreceptor complexes in these
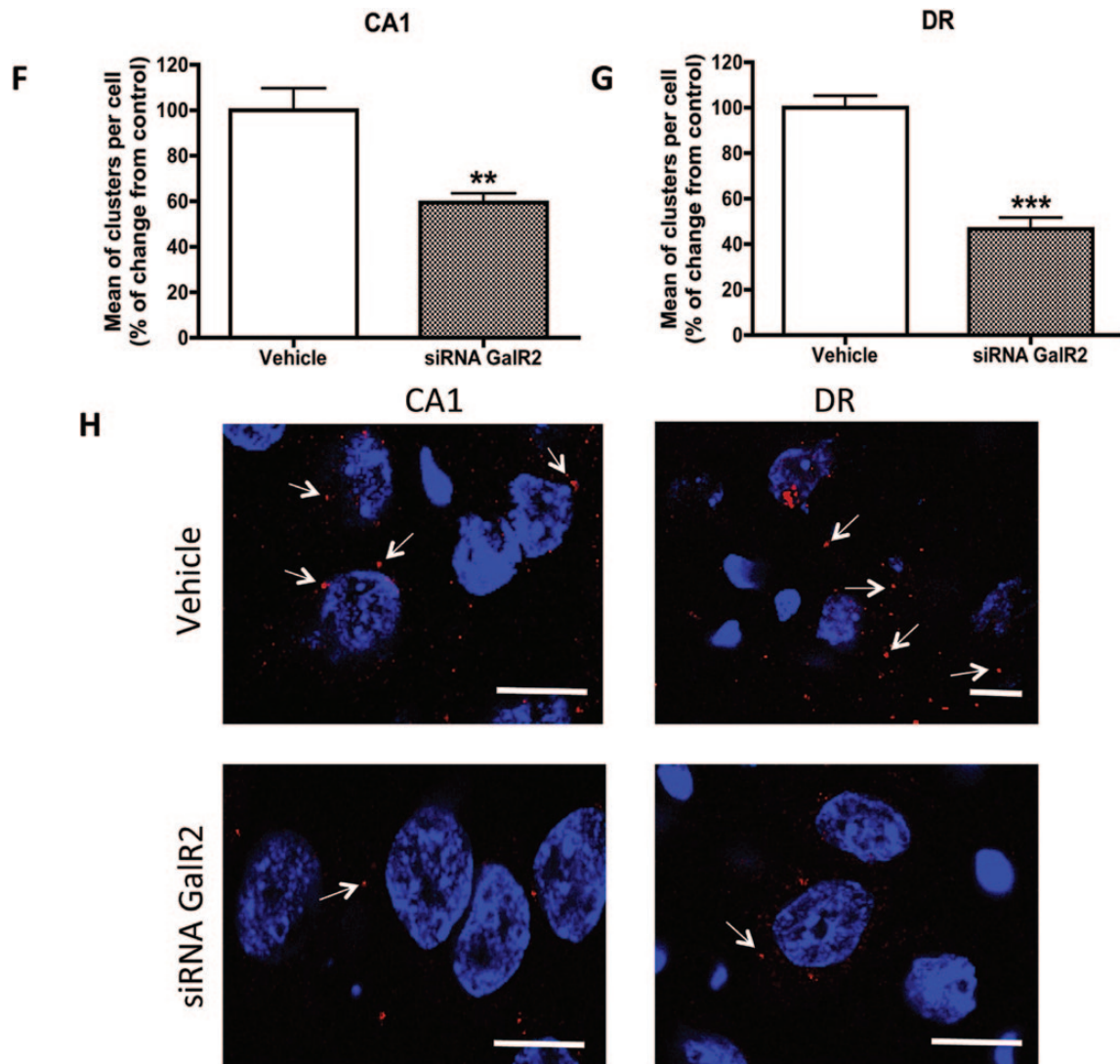
Figure 5. Continued

two brain regions (Trifilieff et al., 2011; Borroto-Escuela et al., 2012). The formation of GAL_1 and GAL_2 heteroreceptor complexes with high affinities for GAL(1–15) can help explain the fact that GAL(1–15) induces a stronger action than GAL at the behavioral level and in the reduction of 5-HT in RN33B cells. It is of interest that PLA blobs exist in higher number in the dorsal raphe nerve cells than in the dorsal hippocampal nerve cells, which indicates that the dorsal raphe nerve cells can be a special target for GAL(1–15). The meso-limbic 5-HT neurons are known to be dysfunctional in depression (Fuxe et al., 2008, 2012; Artigas, 2013). Therefore, the marked decrease in 5-HT IR induced by GAL(1–15) may indicate a mechanism contributing to the depression-like actions of GAL(1–15).

The GAL_2 receptor may induce antidepressant actions via Gq/G11-mediated GAL_2 receptor signaling. It seems possible that in the GAL_1 and GAL_2 heteroreceptor complex the GAL_2 receptor protomer signals in a different way upon activation by its preferred ligand, GAL(1–15), leading to its strong depression-like action. Alternatively, the GAL(1–15) activation of the GAL_1 receptor protomer may cause an allosteric receptor-receptor interaction that inhibits the Gq/G11-mediated signaling of the GAL_2 receptor protomer and switches it towards Gi/o-mediated signaling. In this way, both GAL_1 and GAL_2 receptor protomers become coupled to Gi/o, which may lead to the strong depression-like actions observed with GAL(1–15). The formation of homodimers and

heterodimers among neuropeptide receptors is known (AbdAlla et al., 2005). The GAL_1 receptor can form homodimers (Wirz et al., 2005) and heterodimers with 5HT1A receptors (Borroto-Escuela et al., 2010; Fuxe et al., 2012) and likely with other G-protein coupled receptors.

GAL_2 receptor siRNA was injected into the lateral ventricle of rats to reduce GAL_2 receptor expression and validate the role of the GAL_2 receptor in the formation of the PLA-positive blobs in the dorsal hippocampus and the dorsal raphe and in the GAL(1–15)-mediated behavioral actions. The time-course curve indicated a maximal reduction of GAL_2 receptor protein expression 8 days after the injection, and this was the timepoint selected for the behavioral tests. Moreover, in these animals the quantification of PLA showed a maximal reduction of around 40% in the CA1 area and 60% in the dorsal raphe compared with the vehicle group. These results strongly indicate that the PLA signals obtained are specific and represent the GAL_1 and GAL_2 heteroreceptor complex. This reduction of the PLA signal was sufficient to block the depression- and anxiogenic-like effects of GAL(1–15), linking them to its actions at the GAL_1 and GAL_2 heteroreceptor complex. Moreover, we also blocked the behavioral effects of GAL(1–15) in siRNA GAL_1 receptor knockdown rats, confirming that GAL(1–15) effects depend on the existence of GAL_1 and GAL_2 heteroreceptor complexes.
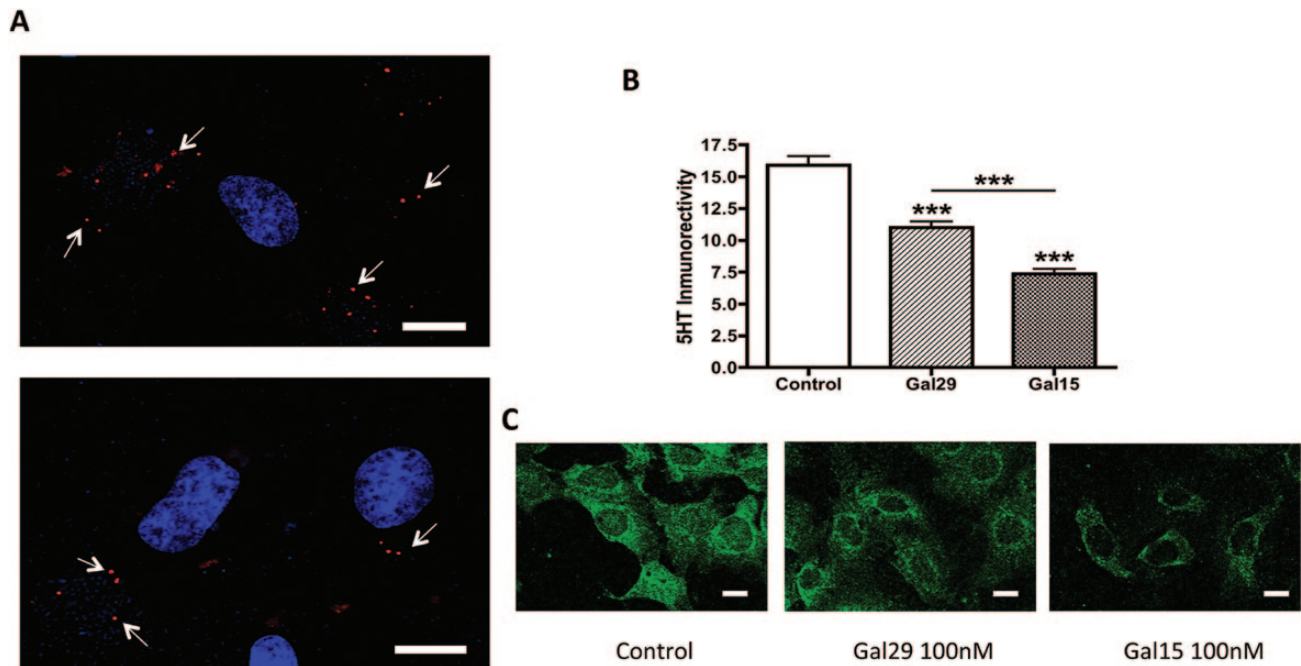
Figure 6. Close proximity between GAL$_1$ and GAL$_2$ receptors and their effects in 5-HT synthesis and storage in RN33B cells. (A) Detection of close proximity between GAL$_1$ and GAL$_2$ receptors (seen as red clusters indicated by arrows) in RN33B cells by *in situ* proximity ligation assay; nuclei are shown in blue (6-diamidino-2-phenylin-dole). (B) Quantification of 5-HT immunoreactivity (green color) was used as a marker for 5-HT synthesis and storage in RN33B cell cultures after incubation with GAL29 and GAL(1–15). RN33B brain-derived immortalized cells were treated for 1 h with GAL29 (100 nM), GAL(1–15) (100 nM), or control, followed for 1 h by recuperation. 5-HT immunoreactivity was determined after immunofluorescent labeling. The data are presented as mean ± standard error of the mean, n = 60 cells for two independent experiments. ***$p < 0.001$ vs. control according to one-way analyses of variance followed by Newman Keuls Multiple Comparison Tests. (C) Representative images of 5-HT stained RN33B cells under different conditions are presented. Scale bars, 10 μm.

In conclusion, our results give strong support to the view that GAL(1–15) acts at GAL$_1$ and GAL$_2$ heteroreceptor complexes in the raphe-limbic system to exert its strong depression-like and anxiogenic effects. These results may give the basis for the development of novel therapeutic drugs specifically targeting the GAL$_1$ and GAL$_2$ heteroreceptor complexes for treatment of depression and anxiety.

Supplementary Material

For supplementary material accompanying this paper, visit http://www.ijnp.oxfordjournals.org/

Acknowledgments

Statement of Interest

The authors declare no conflicts of interest.

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