GALANIN RECEPTOR 2-NEUROPEPTIDE Y Y1 RECEPTOR INTERACTIONS IN THE AMYGDALA LEAD TO INCREASED ANXIOLYTIC ACTIONS.

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ABSTRACT

Galanin (GAL) and Neuropeptide Y (NPY) are neuropeptides involved in behaviors associated with anxiety. Both neuropeptides interact in several central functions. However, the potential behavioral and cellular interactions between them in anxiety are unknown. GAL was found to act through GAL receptor 2 (GALR2) to enhance NPYY1 receptor (NPYY1R) mediated anxiolytic behaviors in rats. Using receptor autoradiography, c-fos expression and in situ proximity ligation assay, the medial paracapsular intercalated nuclei of the amygdala were determined to be a key area in the interaction probably involving the formation of GALR2/NPYY1R heteroreceptor complexes. In cell cultures co-stimulation of GALR2 and NPYY1R induced changes in the functions of these receptors. The changes involved a potentiation of the decrease in the phosphorylation of CREB induced by NPYY1R and a delay in the internalization of NPYY1R. These results indicate that GALR2/NPYY1R interactions can provide a novel integrative amygdaloid mechanism in anxiety.

Keywords: Galanin, Galanin receptor 2, Neuropeptide Y, Neuropeptide Y Y1 receptor, anxiety

INTRODUCTION

Anxiety disorders have a lifetime prevalence of over 25%, making them the most common of psychiatric disorders (Mathew et al. 2008). Specific neuropeptide systems participate in the behaviors associated with anxiety. Long-term dysregulation of these systems contribute to the development of anxiety disorders, including panic disorder, and social anxiety disorders (Madaan and Wilson 2009).

Neuropeptide Y (NPY) is a neuropeptide widely distributed in the mammalian brain. Central NPY and its receptors, especially NPYY1R receptor (NPYY1R) participates in mood disorders, including depression and anxiety (Kask et al. 2002; Thorsell and Heilig 2002; Karlsson et al. 2008; Rotzinger et al. 2010).

NPY induces anxiolytic effects that involve the NPYY1R. The administration of the NPYY1R agonist [Leu^{31},Pro^{34}]NPY into the central nucleus of the amygdala produces anxiolytic behavior in the conflict test with similar potency as intracerebroventricular (i.c.v.) NPY (Heilig et al. 1993) and antisense inhibition or an NPYY1R antagonist blocks anxiolytic-like effects of NPY in the elevated plus-maze and on social interactions (Heilig 1995; Sajdyk et al. 1999). Studies on NPYY1R null mice validate that the NPYY1 receptor is necessary for anxiolytic-like effects of i.c.v NPY in the elevated plus-maze (Karlsson et al. 2008).

Galanin (GAL), is also widely distributed in the central nervous system (CNS) (see review (Jacobowitz et al. 2004)). GAL and Galanin receptors (GALR) participate in anxiety-like and depression-related behaviors via modulation of neuroendocrine and monoaminergic systems (Fuxe et al. 2012; Wrenn and Holmes 2006). The role of GAL in anxiety 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). For instance, i.c.v GAL produced an anxiolytic 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). Intra-amygdala administration of GAL resulted in anxiolytic-like effects only in animals tested under heightened stress conditions in the elevated plus maze (Morilak et al. 2003; Barrera et al. 2005), suggesting that GAL may be involved mainly under high-stress and silent otherwise (Karlsson et al. 2005). The varying effects of GAL on anxiety-related behaviors may partly stem from actions of GAL at differentially-distributed GALR subtypes (Holmes and Picciotto 2006; Bailey et al. 2007). GALR1 knockout mice exhibit increased anxiety in the elevated plus-maze (Holmes et al. 2003) and GARLR2 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 GALR3 produces anxiolytic-like effects in several behavioral tests (Swanson et al. 2005).

The presence of GAL and NPY and/or their receptors in many relevant brain regions related to their brain functions implies that GAL and NPY may balance the actions of one another (Leibowitz 2005; Diaz-Cabiale et al. 2006). We have obtained evidence of an antagonistic GALR modulation of NPYY1R mediated action in cardiovascular functions and food intake potentially involving GALR/ NPYY1R heteroreceptor complexes (Diaz-Cabiale et al. 2006; Parrado et al. 2007; Diaz-Cabiale et al. 2011; Fuxe et al. 2012). The formation of homodimers and heterodimers among neuropeptide receptors is known (AbdAlla et al. 2005). NPY Y1 receptors can exist as homodimers and/or as heterodimers with other members of the NPY receptor family (Gehlert et al. 2007) and GALR1 can form homodimers (Wirz et al. 2005) and heterodimers with 5HT1A receptors (Borroto-Escuela et al. 2010) and likely with other G-protein coupled receptors.

GALR and NPYY1R are also involved in behavioral functions and we predict that GALR/NPYY1R interactions can induce changes in anxiety-related behaviors in animal models. Using a combination of tools, including behavioral tests, GALR2 activation was found to enhance the anxiolytic effect induced by a NPYY1R agonist. Results were obtained indicating that the medial paracapsular

intercalated nuclei of the amygdala can be a key area in this interaction probably involving the formation of putative GALR2/NPYY1R heteroreceptor complexes. At the molecular level in cell cultures, co-stimulation of GALR2 and NPYY1R enhances the decrease in the phosphorylation of CREB induced by NPYY1R activation and delays the internalization of NPYY1R. Taken together, the results suggest that GALR2/NPYY1R receptor-receptor interactions in the intercalated nuclei of the amygdala are involved in the enhancement of NPYY1R-mediated anxiolytic related behaviors.

MATERIALS AND METHODS

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 behaviours 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).

Intracerebral Cannulations

The procedures of cannulation and postsurgical care have been described (Parrado et al. 2007; Diaz-Cabiale et al. 2011). 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 using the stereotaxic coordinates: +1.4mm lateral, -1mm posterior to the bregma, and 3.6mm below the surface of the skull (Paxinos G 1986). After surgery, animals were individually housed and allowed a recovery period of 7 days.

Intracerebroventricular Administration of Peptides

The methods of icv injections and preparation of aCSF have already been standarized in our laboratory (Parrado et al. 2007; Diaz-Cabiale et al. 2011). Cannulated rats were randomly allocated to different groups. Peptides were freshly prepared, dissolved in aCSF and injected into the right lateral ventricle. The total volume was 5 µl per injection with an infusion time of 1 min. Galanin (GAL), NPYY1 Receptor (NPYY1R) Agonist (Leu^{31}-Pro^{34}-NPY), the Galanin Receptor 2 (GALR2) Antagonist M871 and Galanin Receptor 1 (GALR1) agonist M617 were obtained from Tocris Bioscience (Bristol, United Kingdom). After experiments, brains were removed and frozen, testing the placement of the cannula for icv injection by cutting in the coronal plane in a Cryostat (HM550, Microm International).

Open field and Elevated Plus Maze

Behavioural experiments were performed between 09:00 and 14:00 hours and rats, once used, were not reemployed. 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. On the test day, groups of rats received GAL, the NPYY1R agonist (Leu^{31}-Pro^{34}- NPY), the GALR2 Antagonist M871, the GALR1 Agonist M617 at a dose of 3 nmol alone or in combination based on previous work (Bing et al. 1993; Broqua et al. 1995; Kuteeva et al. 2008). Rats were individually placed and allowed to freely explore, recording the behaviour over a 5min period by a ceiling-mounted video camera. Activity was analyzed using the video tracking software Smart2.5 (Panlab, SL). After each trial all surfaces were cleaned with a paper towel adding 70% ethanol solution. For the open-field (120x120x50cm) total time spent in and entries into the inner square were recorded. The elevated plus maze test was performed as previously described (Holmes, 2002), with two open arms (50×15cm), two closed arms (50×15×100cm), a common central platform (15×15cm) and elevated to a height of 100cm above floor level. Rats

were placed on the center square facing an open arm, and the percentage of entries to and the time spent in the open arms were recorded (an arm entry was defined as all four of the paws being placed in an arm of the plus-maze). All behavioral experiments were blinded.

Quantitative receptor autoradiography

The procedure used has been described previously (Dumont et al. 1996; Diaz-Cabiale et al. 2011). Fifteen minutes after icv injections with aCSF or GAL brains were rapidly removed, sectioned in coronal sections at 14µm thick (Bregma levels Amygdala:-1.80mm to -4.16mm) (Paxinos G 1986) and immediately processed for binding studies. Sections were preincubated for 1h at room temperature in a Krebs-Ringer phosphate buffer (KRP) at pH 7.4 and then incubated for 2h in KRP buffer supplemented with 0.1% BSA, 0.05% bacitracin, 25pM NPYY1R agonist [^{125}I]Leu^{31},Pro^{34}PYY (Perkin-Elmer, USA (Dumont et al. 1996). Non-specific binding was defined as the binding in the presence of NPY 1µM. After incubation, sections were washed four times (2 min each) in ice-cold KRP buffer, dipped in deionised water to remove salts, and rapidly dried under a stream of cold air. Sections were placed in X-ray cassettes and apposed against Hyperfilms for 6 days together with ^{125}I microscales (Amersham International) as reference standards.

Autoradiograms were analyzed as described previously (Parrado et al. 2007). Measurements, using the NIH image analysis system, were made bilaterally in the entire amygdala ($6.3^{\pm}$ 0.1mm^2). One observation per region and rat was obtained since the average of the measurements was calculated. Prefabricated ^{125}I-labeled polymer strips (Amersham Microscale, Amersham, Little Chalfont, UK) were used to convert the grey values into femtomole/milligram protein values

c-Fos immunohistochemistry

Anaesthetized rats with sodium pentobarbital (Mebumal; 100mg/kg, i.p.) were perfused with 4% Paraformaldehyde (wt/vol, Sigma) 90 minutes after injections (Diaz-Cabiale et al. 2011).

Animals were divided into five experimental groups: (1)aCSF: control group; (2)GAL: group pretreated with Galanin 3nmol; (3)Y1: group receiving the NPY1R agonist (Leu^{31}-Pro^{34}-NPY) 2.5 nmol; (4)GAL+Y1: group administered with both substances; (5)GAL+Y1+M871: group injected with GAL, NPYY1R agonist and the GALR2 antagonist M871 3 nmol.

Brains were coronally sliced and immunostained using previously published protocols (Diaz-Cabiale et al. 2011). For primary antibody, rabbit antibody to c-Fos protein (1:5000, Santa Cruz Biotech.sc-52, USA) was used. Appropriate biotinylated specific secondary antibody to rabbit (1:200; Vector Labs Inc, Burlingame, CA) was used. Using accepted cytoarchitectonic criteria (Swanson 1992), we then compared the position of ITC cell clusters on adjacent sections stained with 0,1 % cresyl violet. Sections were mounted on glass slides and the whole medial paracapsular intercalated (ITC) nucleus of the amygdala was analyzed using the optical fractionator method in unbiased stereological microscopy (Olympus BX51 microscope, Olympus, Denmark) as previously described (Diaz-Cabiale et al. 2011).

Proximity ligation assay (Duolink)

In situ proximity ligation assay (PLA) was performed as described previously (Borroto-Escuela et al. 2012). Free-floating brain sections were incubated with blocking (5% goat serum) and permeabilization (0.3% triton X100 in PBS) solutions during 60 min. Primary antibodies of different host directed to anti-GalR2 Rabbit (Alomone Lab, 1:100) and anti-NPYY1R Goat (Santa Cruz Biotecnology INC, EEUU, 1:200) were incubated for 24 hours at 4 ºC. PLA signals detection were performed according to manufacturer's instructions (Duolink *in situ* PLA detection kit (Olink, Sweden)) with PLA PLUS or MINUS probes for rabbit or goat antibodies. Sections were mounted on slides

with Fluorescent Mounting Medium (Dako) containing 4',6-diamidino-2-phenylindole (DAPI), 1:200, staining nuclei with blue colour. Control experiments used only one primary antibody in tissue. PLA signals were visualized by using a confocal microscope Leica TCS-SL confocal microscope (Leica).

For in situ PLA in transiently transfected HEK293T cells coexpressing 3xHA-Y1R and GALR2 were used primary monoclonal mouse anti-HA (Clon HA-7, H9658, Sigma Aldrich, 1:5000) and polyclonal rabbit anti-GALR2 (Affinity pure IgG, GALR21-A Alpha diagnostic, USA, 1:500).

Cloning of NPYY1R-EGFP, Cell culture and Transfection

NPYY1R-EGFP was made using standard molecular biology techniques employing PCR and fragment replacement strategies. Human NPYY1R coding sequences without their stop codons were amplified from 3xHA-Y1R-pcDNA vectors using sense and antisense primers harboring unique NheI and AgeI sites and fragments were subcloned in-frame into humanized pEGFP-N1 vector (PerkinElmer, Waltham, MA, USA).

Human embryonic kidney 293T (HEK293T) cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium supplemented with L-glutamine 2mM, penicillin/streptomycin 100units/ml, and FBS 10% (v/v) at 37°C and 5% CO_2. For transfection, cells were plated in 6-well dishes at a concentration of $1x10^6$ cells/well and cultured overnight before transfection. Cells were transiently transfected (cDNA molar ratio 1:1) using Fugene HD Transfection Reagent (Promega) and empty pcDNA3.1 vector DNA to maintain a constant total amount of DNA per well.

Receptor Internalization using Confocal Microscopy

Twenty-four hours prior to imaging, the FBS growth media was removed and replaced with serum-free media (Cellgro-Free, Mediatech). NPYY1R-EGFP

and GALR2 HEK293T coexpressing cells were incubated with NPY 1μM and/or GAL1μM, at different times. Antagonist studies were performed 15 min prior to the addition of agonist with NPYY1R antagonist BIBP3226 10μM or GALR2 antagonist M871 10 μM. Cells were fixed in 4% paraformaldehyde for 10 min, washed with Glycine 10 mM in PBS and mounted in a Vectashield immunofluorescence medium (Vector Laboratories, Burlingame, CA).

NPYY1R-EGFP endosomes seen as green fluorescent molecules were excited with a krypton/argon laser at 488 nm and are shown as a single z-scan image. Timed-interval images of different cell groups were acquired (63×, Leica TCS-SL confocal microscope) following agonist addition. Percentage of internalization was determined by Leica software analysis of total membrane fluorescence compared to total internal compartment fluorescence at the various time points.

Luciferase Gene Reporter Assay

Dual luciferase gene reporter assay was used to detect variations of cAMP levels or activation of PLC/PKC pathway in transiently transfected HEK293T cell treated with different compounds in a range of concentrations (typically 50nM–1μM) (Borroto-Escuela et al. 2010).

Cells were transfected as follows (per 6-well): 1μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p or pGL4-NFAT-luc2p; Promega, Stockholm, Sweden), 1μg of NPYY1R and/or GALR2 and 50ng *Renilla* luciferase-encoding internal control plasmid (phRG-B; Promega). Approximately 48h post transfection cells were treated for 4h with appropriate ligands (NPY 50nM, GAL 100nM, BIBP3226 1μM, M871 1μM, in presence of Forskolin 2μM). For NFAT-Luciferase cells were treated for 8 h without the presence of Forskolin. Cells were harvested with passive lysis buffer (Promega) and the luciferase activity was determined in a POLARstar Optima plate reader (BMG Labtech) using a 30-nm bandwidth excitation filter at 535 nm. Firefly luciferase was measured as firefly luciferase luminescence over a 15s reaction

period. The luciferase values were normalized against *Renilla* luciferase luminescence values. Transfection experiments were performed in quadruplicate and repeated at least three times.

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 $P<0.05$ (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

RESULTS

GAL enhances NPYY1R-mediated anxiolytic related behaviors

To assess the role of GAL in the regulation of NPYY1R-mediated anxiolytic related behaviors, we examined two anxiety-related behaviors in rats: the time spent in and numbers of entries into the central square in the open field and the percent of time and entries into the open arms in the elevated plus maze.

In both tests, the NPYY1R agonist at an effective dose of 3 nmol induces an anxiolytic effect as it significantly increased the time spent in ($p<0.05$) and the entries ($p<0.01$) into the central square in the open field (Figure 1a,b) and the percent of time ($p<0.05$) and entries ($p<0.01$) into the open arms in the elevated plus maze (Figure 1c,d). The dose of 0.1nmol of NPYY1R agonist was a threshold dose in both tests (Supplementary Figures 1 and 2).

GAL at 3 nmol lacks effect in all the parameters analyzed in both tests (Figure 1). However, an anxiolytic effect was observed after the coadministration of GAL (3nmol) and a threshold dose of NPYY1R (0.1nmol) agonist in both tests (Supplementary Figure 1,2). The strong enhancement of GAL on anxiolytic

NPYY1R agonist mediated actions was validated using an effective dose of the NPYY1R agonist. GAL (3nmol) significantly increased the time spent in ($p<0.001$) and the entries ($p<0.01$) into the central square induced by an effective dose of the NPYY1R agonist (3nmol) in the open field (Figure 1a,b).

In the elevated plus maze, the percentage of time in ($p<0.001$) and percentage of entries into ($p<0.001$) the open arms induced by the NPYY1R agonist, were also significantly increased by GAL (Figure 1c,d). These anxiolytic effects were independent of the locomotor activity, as the total distance reached and speed were equivalent between all the groups (Supplementary Table 1).

GALR2 participates in this interaction since the presence of the GALR2 antagonist M871 blocks significantly ($p<0.001$) the effect induced by the coadministration of GAL and NPYY1R agonist in both tests (Figure 1). No effect was observed with M871 alone (For open field= Time in inner square: 7.2±2, means sem; Entries to inner square: 3.8±0.4; For Elevated Plus Maze= % Time in Open Arms: 2.6±2; % Entries to Open Arms: 2.8±2). The specific GALR1 agonist M617 did not reproduce the GAL-mediated effect on NPYYR1-agonist action (Supplementary Table 2).

Involvement of the ITC nucleus of the amygdala

In the amygdala, a key area in mediating behavioral effects of NPYY1R agonist and GAL (Heilig et al. 1993; Morilak et al. 2003; Barrera et al. 2005), GAL is known to significantly increase the NPYY1R agonist binding using “in vitro” experiments (Parrado et al. 2007)

To analyze if this area was involved in the interactions observed in the open field and elevated plus-maze, we analyzed the binding of the NPYY1R agonist binding [I^{125}]$Leu^{31}Pro^{34}$-PYY fifteen minutes after the i.c.v injection of GAL 3nmol. We observed that GAL induced an increase in the amygdala of the NPYY1R agonist binding [I^{125}]$Leu^{31}Pro^{34}$-PYY by 22% ($p<0,05$) (Figure 2a,b) suggesting that GAL modifies NPYY1R recognition in this area.

To determine the subnuclei of the amygdala involved we studied by immunohistochemistry the expression of the immediate early gene Fos (c-Fos IR), an indirect marker of neural activity, ninety minutes after the i.c.v. administration of GAL and NPYY1R agonist alone or in combination (Parrado et al. 2007). We performed a stereological analysis in: Basal (BA), lateral (LA), Central [lateral capsular subdivision (CeC), lateral intermediate subdivision (CeI)] and the medial paracapsular intercalated (ITC) subnuclei of the amygdala.

We observed that GAL and NPYY1R agonist alone induced c-Fos IR in several of the nuclei analyzed. GAL, as previously reported (Blackshear et al. 2007), increased c-Fos IR in ITC and CeC (Figure 2c,f, Supplementary Table 3); the NPYY1R agonist induced an increase of c-Fos IR in BA and CeC, while a decrease in c-Fos IR was observed in LA and ITC (Figure 2c,g, Supplementary Table 3). In the ITC nucleus, the c-Fos IR induced by the NPYY1R agonist was colocalized with Glutamate Decarboxylase (GAD65/67) positive neurons (Supplementary Figure 3). No induction in c-Fos IR was observed in CeI (Supplementary Table 3). However, we observed specifically in the ITC nucleus a modification of the c-Fos expression after the coadministration of both peptides compared to the effect of GAL or the NPYY1R agonist alone. The coadministration of both peptides significantly decreased the c-Fos expression ($P<0,05$) induced by GAL or the NPYY1R agonist alone in this nucleus (Figure 2c-h). The presence of the GALR2 antagonist M871 completely reversed this decrease (Figure 2c,i) demonstrating the involvement of GALR2 in this interaction.

To analyze the possible formation of GALR2/NPYY1R heteroreceptor complexes in the ITC nucleus we performed in situ proximity ligation assay (PLA) supplemented with double immunolabeling to show colocation.

PLA-positive red clusters were found in large number of cells of the ITC nucleus (Figure 3), giving the indication that GALR2 and NPYY1R are in close proximity and may form GALR2/NPYY1R heteroreceptor complexes. The specificity was

demonstrated by the fact that no PLA clusters were observed in lateral corpus callosum, an area that seems to lack of GALR2 receptor (O'Donnell et al. 1999). These results were validated in PLA experiments on HEK cells (see below). In line with these results, extensive colocalization of GALR2 and NPYY1R immunoreactivities was observed in the nerve cells of ITC nucleus (Supplementary Figure 4).

Co-activation of GALR2 and NPYY1R enhances NPYY1R signaling and delays NPYY1R internalization in cellular models

The possible formation of GALR2/NPYY1R heteroreceptor complexes was examined in HEK-293 cells using PLA. We also examined if GALR2 stimulation affects NPYY1R signaling and internalization.

In transiently transfected HEK293T cells coexpressing NPYY1R 3xHA and GALR2 the results obtained in the ITC nucleus were validated. We observed PLA-positive red clusters in large number of cells (Supplementary Figure 5) which gives the indication that GALR2 and NPYY1R are in close proximity and may physically interact with the formation of GALR2/NPYY1R heteroreceptor complexes.

To examine the mechanism by which GALR2 may alter the signaling of NPYY1R we used the CRE-luciferase and NFAT-luciferase gene reporter assays in transiently transfected HEK293T cells coexpressing NPYY1R and GALR2 .

Both GALR2 and NPYY1R activation can, via the Gi/o-AC-PKA cascade (Fuxe et al. 2012; Parker et al. 2008), decrease the phosphorylation of CREB that subsequently leads to a reduction of the luciferase gene transcription. In GALR2-NPYY1 transfected cells; the forskolin-induced increase of luciferase activity through the direct activation of adenylate cyclase was significantly reduced by 50 nM NPY, which is fully counteracted by the selective NPYY1

antagonist BIBP3226 (1 μM) (Fig. 4a). In the same way, GAL (100 nM) significantly reduced to a similar degree the forskolin-induced increase of luciferase activity. The selective GALR2-antagonist M871 (1 μM) counteracted this effect. NPY (50 nM) together with GAL (100 nM) significantly enhanced the inhibition of the forskolin-induced increase of luciferase activity compared with either NPY or GAL stimulation alone.

We also measured NFAT transcriptional reporter activity, which was shown to be capable of monitoring Ca^{2+} mobilization and subsequent NFAT activation. GALR2 is also coupled to Gq/11 protein and induces an increase of inositol triphosphate accumulation and of intracellular Ca^{2+} (Fuxe et al. 2012). In GALR2-NPYY1 transfected cells GAL (100 nM) significantly increased the NFAT-luc ($p<0.001$), an effect that is fully counteracted by the selective GALR2-antagonist M871 (1 μM) (Fig. 4b). NPY (50 nM) together with GAL significantly inhibited the increase in NFAT-luc activity induced by GAL stimulation alone.

With these results we can propose a schematic diagram of the mechanism of agonist-mediated inhibition of the CRE-luciferase reporter assay under the control of the GALR2-NPYY1R heteroreceptor complex in Fig. 4 (right panel). The GALR2 upon coactivation through the NPYY1R-GALR2 interactions in the heteroreceptor complex switches from a Gq to a Gi/o coupling. In this way both GALR2 and NPYY1R become coupled to Gi/o and produce additive effects on inhibition of adenylate cyclase upon coactivation

The GALR2 effects were also examined on the NPY induced NPYY1R internalization by immunofluorescence microscopy in transiently cotransfected HEK 293T cells with GALR2 and EGFP-tagged NPYY1R. HEK 293T cells coexpressing NPYY1-EGFP and GALR2 were incubated in the presence of NPY with or without the GALR2 agonist GAL at 37º for 60 min to monitor receptor internalization (Figure 5).

Addition of NPY induced a rapid decrease in the cell surface expression of NPYY1-EGFP that slowly recovered during the 70-min measuring period (Figure 5). We observed a maximum of internalization of 80% three minutes after the NPY stimulation (Figure 5). However, combined treatment with GAL and NPY induced a delay in the internalization of NPYY1-EGFP, with a maximum of internalization thirty minutes after the co-stimulation. The specific GALR2 antagonist M871 abolished this delay in internalization of NPYY1-EGFP (Figure 5), suggesting that this effect was mediated through the coactivation of GALR2 and NPYY1R. This delay in NPYY1R internalization may contribute to an increase in NPYY1R signaling in this period.

DISCUSSION

GAL was demonstrated to act via GALR2 to enhance NPYY1R mediated signaling leading to enhanced anxiolytic actions thereby linking GALR2-NPYY1R interactions to the neuronal networks of fear and anxiety. In the amygdala this interaction takes place at the receptor level probably involving the formation of GALR2-NPYY1R heteroreceptor complexes. Importantly, our behavioral data were supported by findings at the cellular level. Thus, we demonstrated in the ITC nucleus of the amygdala that coactivation of GALR and NPYY1R enhances the NPYY1R mediated reduction in the total number of c-Fos immunoreactive profiles in this nucleus in spite of the fact that GAL alone increases the total number of such profiles. The GABA neurons within the ITC nucleus seem to be involved in the interaction since c-Fos IR-induced by NPYY1 agonist was colocalized with the GABA neuron marker Glutamate Decarboxylase. Such an increase in inhibition of GABAergic medial paracapsular ITC nerve cells can lead to disinhibition of more medially located GABAergic paracapsular ITC cells projecting to and inhibiting the medial efferent subdivision of the central amygdaloid nucleus (Palomares-Castillo et al. 2012). This results in a reduction of the efferent anxiogenic outflow from the

amygdala and may give the mechanism at the brain circuit level for the enhanced anxiolytic activity observed upon cotreatment with GAL and the NPY Y1 agonist. In line with these findings GALR and NPYY1R were co-expressed in the medial paracapsular ITC nucleus where also PLA positive clusters of these two receptors were observed indicating the presence of GALR2-NPYY1R heteroreceptor complexes in this nucleus.

The studies in transiently GALR2 and NPYY1R cotransfected HEK cells using forskolin-induced CRE-luciferase and NFAT-luciferase gene reporter assays give indications of the changes in receptor function. This gives a cellular basis for the enhanced anxiolytic activity observed upon cotreatment with GAL and the NPYY1 agonist. Thus, the results in the NFAT-luciferase and CRE-luciferase assays indicate that the GALR2 upon activation of NPYY1R through the NPYY1R-GALR2 interactions in the heteroreceptor complex switches from a Gq to a Gi/o coupling. In this way both GALR2 and NPYY1R become coupled to Gi/o and produce inhibition of adenylate cyclase, which is shown to lead to additive effects on inhibition of adenylate cyclase upon coactivation. Furthermore, a delay in the NPYY1R internalization was observed upon coactivation of GALR2 and NPYY1R in the cotransfected HEK cells, which may involve a reduction of beta-arrestin recruitment to the heteroreceptor complex.

However, we can´t exclude that these interactions take place independently of the formation of GALR2-NPYY1R heteroreceptor complexes. The effects we observed could be also explained by an interaction between GALR2 and NPYY1R at a postsynaptic level without the formation of heteroreceptors.

These observations on the signaling of the GALR2-NPYY1R receptor interaction in cellular models may explain the enhancement of the NPYY1R mediated anxiolytic actions observed upon GAL and NPYY1 agonist cotreatment in the behavioral tests. All these findings based on the enhancing GALR2-NPYY1R interaction on Gi/o signaling and its existence in the medial

paracapsular ITC nucleus provide a novel integrative anxiolytic mechanism in the anxiety networks of the amygdala.

Anxiolytic responses were observed in both the open field and in a plus-maze following cotreatment with GAL and threshold doses of NPYY1R agonist. When administered alone, neither of these treatments affected performance in these tests, indicating that GAL and the NPYY1R agonist interact to provoke the anxiolytic responses. Thus, based on the cellular findings the NPYY1R agonist via an allosteric receptor-receptor interaction may switch the GALR2 from Gq towards Gi/o coupling. The fact that a threshold dose of GAL was able to facilitate the anxiolytic effect of an effective dose of the NPYY1R agonist indicates that GAL via GALR2 also can enhance the NPYY1R mediated action. The behavioral change could not be attributed to an enhancement of motor activity, as entries into the closed arms of the plus-maze were unaffected by the treatments.

The results obtained with the NPYY1R agonist and GAL alone agrees with previous studies and confirms the behavioral models used (Broqua et al. 1995; Rotzinger et al. 2010)**.** The fact that GAL 3nmol lacks a behavioral effect 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). The GALR1 agonist M617 and the GALR2 antagonist M871 alone did not have any effect and validated the absence of effect of GAL in these tests (Khoshbouei et al. 2002).

The GAL receptor involved in this GAL/NPYY1R agonist interaction in vivo is GALR2, since the GALR2 antagonist M871 blocked the GAL-induced enhancement of NPYY1 agonist produced anxiolytic effects in the open field and in the elevated plus maze. This interaction was not mediated via GALR1, since we did not reproduce the GAL-mediated effect with the specific GALR1 agonist M617 in these tests. The specific role for GALR2 in GAL induced

anxiety is not well characterized. In fact, GALR2 knockout mice showed 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 GALR2 in the GAL/NPYY1R interaction as an enhancer of the NPYY1R agonist induced anxiolytic effect.

The results of the current study from receptor autoradiography experiments indicated that the GAL receptor interacts at the membrane level with the NPYY1R in the amygdala. In fact, GAL caused an increase of the NPYY1R agonist [^{125}I] Leu^{31}, Pro^{34}PYY binding.
The demonstrated increase by GAL of the NPYY1R agonist binding using receptor autoradiography indicates the existence of a GAL receptor which upon activation can induce an increase of NPYY1R affinity in the amygdala. Thus, the concentration of the NPYY1R agonist used (25pM) is in the range of the Kd value, where mainly affinity changes affect the binding level (Dumont et al. 1996). This effect may be explained on the based that GAL through GALR2 can cause a conformational change in the NPYY1R that leads to an increase of NPYY1R recognition. Based on the demonstration that GAL in GALR2 and NPYY1R cotransfected HEK cells reduced NPYY1R internalization, such a mechanism can also contribute to the increase in NPYY1 binding observed in the amygdala.

Taken together, our results indicate that GAL acted through GALR2 to enhance NPYY1R mediated signaling and anxiolytic behaviors thereby linking GALR2/NPYY1R interaction to the response to anxiety. This interaction appears to take place in the medial paracapsular ITC nerve cells. The receptor-receptor interactions within these complexes switches the G protein coupling of GALR2 from Gq to Gi/o. GALR2 signaling therefore adds to the Gi/o signaling of NPYY1R leading to an increased inhibition of the AC-PKA-CREB pathway. This results in an enhanced inhibition of their neuronal activity as seen from the

enhanced reduction of the number of c-Fos immunoreactive profiles in the medial paracapsular ITC upon cotreatment. Such an increase in inhibition of the medial paracapsular ITC nerve cells**,** probably involving GABAergic neurons, result in a reduction of the efferent anxiogenic outflow from the central amygdala. Our data offer a novel integrative anxiolytic mechanism in the anxiety networks of the amygdala based on the enhancing GALR2-NPYY1R interaction on Gi/o signaling and its existence in the medial paracapsular ITC nucleus.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Analysis of Galanin and the Neuropeptide Y Y1 receptor Agonist mediated anxiety-related behaviors in the open field and in the elevated plus maze. The increase induced by GAL on the Y1 mediated anxiolytic-related behaviors is blocked by the GALR2 antagonist. n=6-9 animals in each test group. Data represents mean ± SEM (a) Time spent in the inner square of the open field. (b) Entries to the inner square of the open field. In (a): $*P$ <0.05 versus GAL 3nmol. $***P$ <0,001 versus the rest of the groups. In (b): $\neq P$ <0.05 versus Y1 3nmol and GAL+Y1+M871. ** P <0.01 versus GAL+Y1 3nmol. $\neq\neq\neq P$ <0.001 versus GAL 3nmol according to one-way ANOVA followed by Newman Keuls Multiple Comparison Test. (c) Percentage of time in the open arms of the elevated plus maze. (d) Percentage of entries to the open arms of the elevated plus maze.In (c): $*P$ <0.05 versus GAL 3nmol. $***P$ <0,001 versus the rest of the

groups. In (d): **P* <0.05 versus GAL 3nmol. ** *P* <0.01 versus GAL 3nmol and GAL+Y1 3nmol. ≠≠≠*P* <0.001 versus GAL 3nmol and GAL+Y1+M871 according to one-way ANOVA followed by Newman Keuls Multiple Comparison Test.
GAL = Galanin 3 nmol; Y1 3nmol = NPY Y1 receptors agonist [Leu^{31}-Pro^{34}]NPY 3nmol; GAL+Y1 3nnmol= Coadministration of GAL and Y1 3nmol; GAL+Y1+M871= Coadministration of GAL, Y1 3nmol and GALR2 antagonist M871 3nmol.

Figure 2. Effect after 15 minutes of icv administration of Galanin on the NPY Y1 receptor agonist binding in the Amygdala. (**a**) Values, indicated as mean ± SEM, represent the specific binding of [125I]Leu31,Pro34PYY using a concentration of 25pM. Non-specific labelling (in the presence of 1 μM NPY) was digitally substracted from all readings. n=6 animals in each test group. *P <0.05 versus control group according to Student's t-test. (**b**) Representative autoradiograms from coronal sections of the rat brain at Bregma -3.5 mm showing in the Amygdala the increase of the NPY Y1R agonist binding following the icv administration of GAL 3nmol. aCSF= Basal binding of the NPY Y1 agonist [125I]-Leu31Pro34PYY. GAL= Galanin 3 nmol.
Effects of Galanin and NPY Y1 receptor agonist alone or in combination with the GALR2 antagonist (M871) on c-Fos expression in Medial Intercalated paracapsular amygdala (ITC) neurons. (**c**) The quantification of total c-Fos IR nerve cell bodies was performed in the whole ITC nucleus. Data, indicated as mean ± SEM show the differences between groups after intracerebroventricular injections of aCSF, GAL, NPY Y1 agonist, the coadministration of both peptides and M871. The coadministration of GAL and the Y1 agonist decreased the c-Fos expression compared with both peptides alone and the aCSF group. Moreover, the effect of GAL and Y1 agonist coadministration is counteracted by M871. n=4 in each test group. * P <0,05 versus Y1 and GAL+Y1+M871. **P <0,01 versus Y1 and GAL+Y1+M871. ***P <0,001 versus the rest of the groups. ≠≠≠ P <0,001 versus aCSF according to one-way ANOVA followed by

Newman Keuls Multiple Comparison Test. (**d**) Representative photomicrograph illustrating the Medial Intercalated paracapsular amygdala (ITCp) nucleus, located between Lateral (LA), Basal (BA), Lateral capsular (CeC), Lateral intermediate (CeI) and Medial (CeM) subdivisions of Central nuclei of the Amygdala (Bregma: -2.5mm) counterstained with cresyl violet. (**e-i**) Magnified views from dashed box in Fig.2d show the immunodetection for c-Fos positive cells (revealed with DAB plus nickel; black-purple reaction as indicated with arrowheads). The coadministration of both peptides GAL and Y1 agonist (**h**) decreased the c-Fos expression compared with the NPY Y1 agonist alone (**g**), Galanin alone (**f**) and the control group (**e**). The effect of the GAL and Y1 agonist coadministration is counteracted by M871 (**i**). GAL = Galanin 3 nmol; Y1 2.5nmol = NPY Y1 receptors agonist [Leu31-Pro34]NPY 2.5nmol; GAL 3nmol+Y1 2.5nnmol= Coadministration of GAL 3nmol and Y1 2.5nmol; GAL+Y1+M871= Coadministration of GAL, Y1 2.5nmol and GALR2 antagonist M871 3nmol

Figure 3. Detection of Galanin receptor 2 (GALR2) and Neuropeptide Y Y1 (NPYY1R) heteroreceptor complexes by *in situ* proximity ligation assay (PLA). **(a)** Red filled circle indicate the positive PLA regions (Medial Intercalated paracapsular amygdala (ITCp) nucleus, Basolateral and Central Amygdala) at Bregma: -2.5mm. Blue filled circles indicate negative PLA region (Corpus callosum) **(b-d)** Constitutive GALR2-NPY-Y1 heteroreceptor complexes are detected by in situ PLA (seen as red clusters) in the ITCp nucleus but not in the corpus callosum **(e).** Nuclei appear as a blue color in all panels and the white arrows indicate the red cluster formation (PLA signal).

Figure 4. Analysis of agonist-induced Neuropeptide Y Y1 receptor (NPYY1R) and Galanin 2 receptor (GALR2) activation in a forskolin-induced CRE-luciferase and NFAT-luciferase reporter gene assays. **(a)** The costimulation with NPY and Galanin enhanced the inhibition of the CRE-Reporter expression

compared with GAL and NPY alone. The specifics antagonist for NPYY1R and GALR2 M871 counteracted the inhibition of CRE-expression. Light emission is expressed as a percentage of the control forskolin-induced value. Data represent the means ± S.E.M. of three independent experiments performed in triplicate. ***P <0,001 versus the control group. +++P <0,001 versus NPY+BIBP3226. ≠≠≠P <0,001 versus GAL+M871. ≠P <0,05 versus NPY and GAL groups. **(b)** The costimulation with NPY and Galanin decreased the NFAT-Reporter expression induced by GAL alone. The specific antagonist for GALR2 M871 counteracted the increased NFAT-expression. Light emission is expressed as a percentage of the over control value. Data represent the means ± S.E.M. of three independent experiments performed in triplicate. ***P <0,001 versus the rest of the groups. *P <0,05 versus control, NPY, NPY+BIBP3226 and GAL+M871 groups. **(c)** Schematic cross-talk signaling pathway proposed for GALR2 and NPY-Y1 heteroreceptor complex with a positive modulation on CREB while an inhibition on NFAT signalling . Control= (a): Forskolin 1 μM, (b): Basal medium; NPY= Neuropeptide Y 50nM; BIBP3226= NPY-Y1 Receptor Antagonist 1 μM; GAL = Galanin 100nM; M871= GALR2 antagonist 1 μM.

Figure 5. Analysis of Y1-EGFP agonist-induced receptor internalization after Galanin 2 and NPY Y1 Receptor costimulation. **(a-c)** Representative laser-scanning confocal micrographs showing the distribution of Y1-GFP in HEK 293 cells cotransfected with Galanin 2 and Y1-EGFP receptors . Following the costimulation with 1 μM GAL and 1 μM NPY **(b)** was a delay of the fluorescence translocation to the interior of the cell after 1, 3, 8, 15 and 40 minutes, compared with NPY alone **(a)**. This effect is counteracted with the GALR2 antagonist M871 (10 μM) at 3 (upper) and 40 (bottom) minutes **(c)**. **(d)** Representation of time course quantitation of Y1-GFP internalization. Data, indicated as mean ± SEM show the maximal internalization after NPY stimulation alone at 3 minutes, compared with the maximal effect after NPY and

Galanin costimulation at 30 minutes. Micrographs are representative images of multiple cells imaged on three independent experiments.

Figure 1-1
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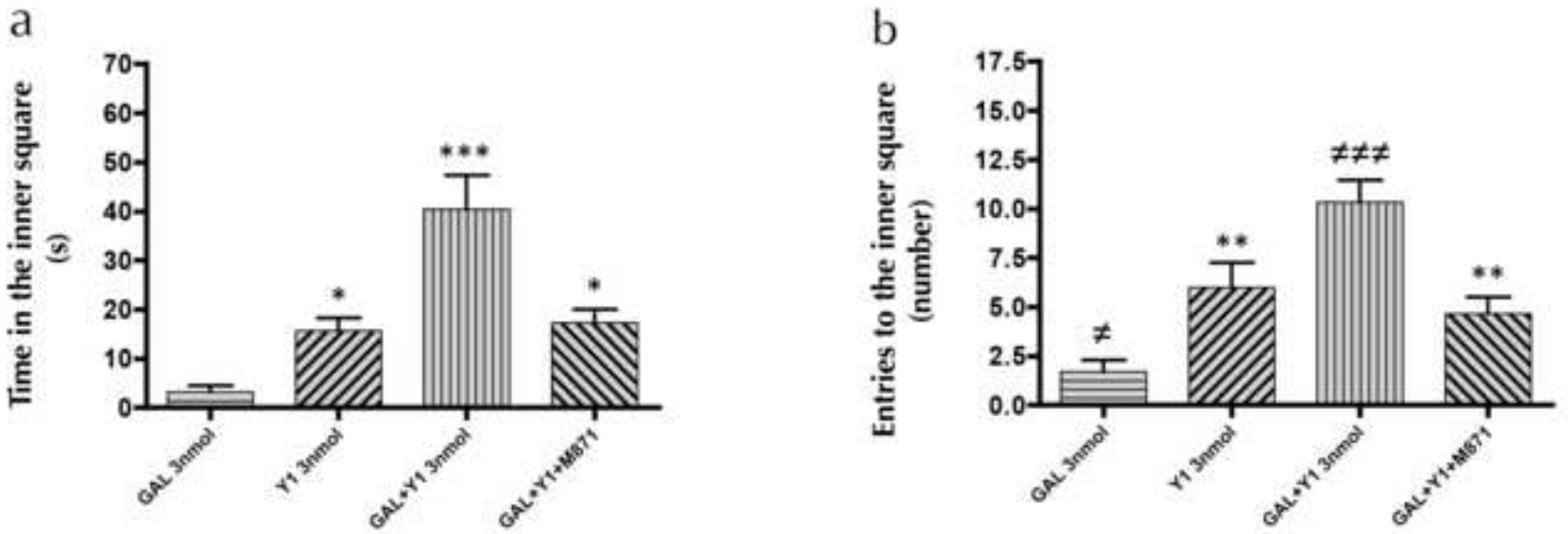

Figure 1

Figure 1-2
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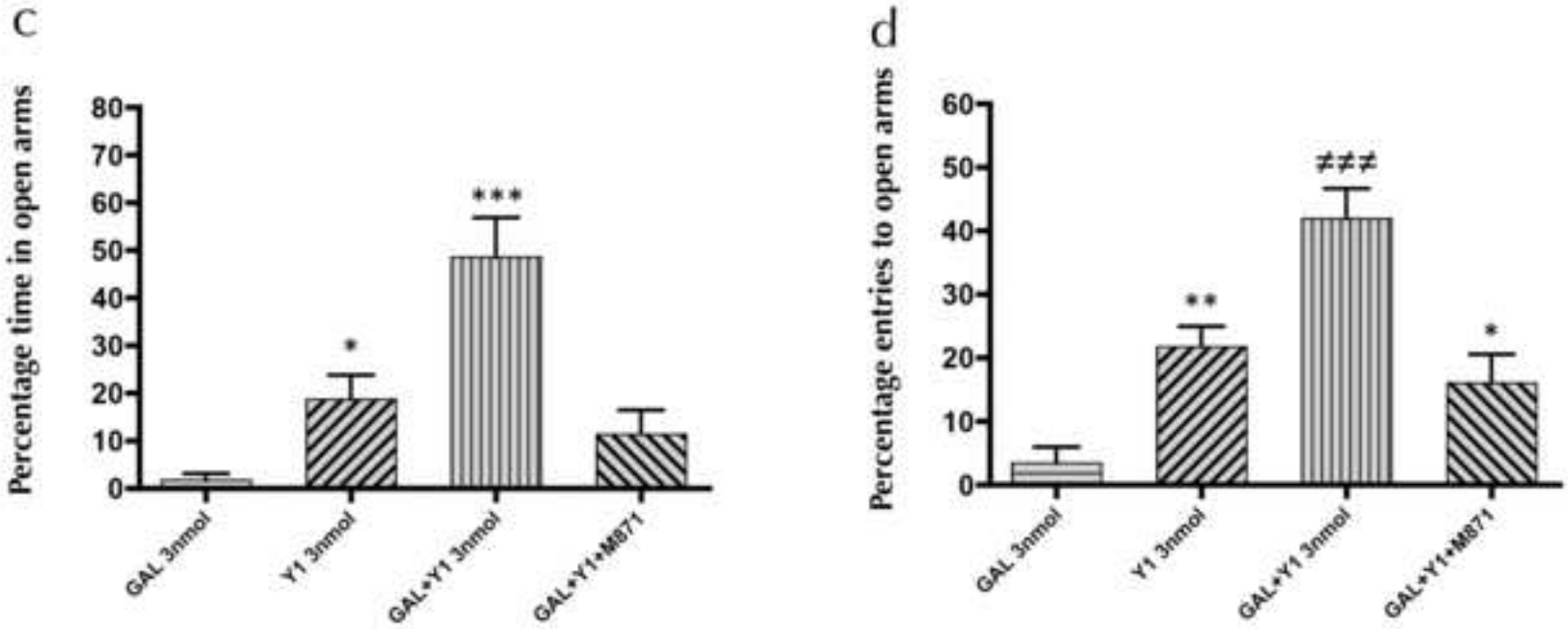

Figure 1

Figure 2-1
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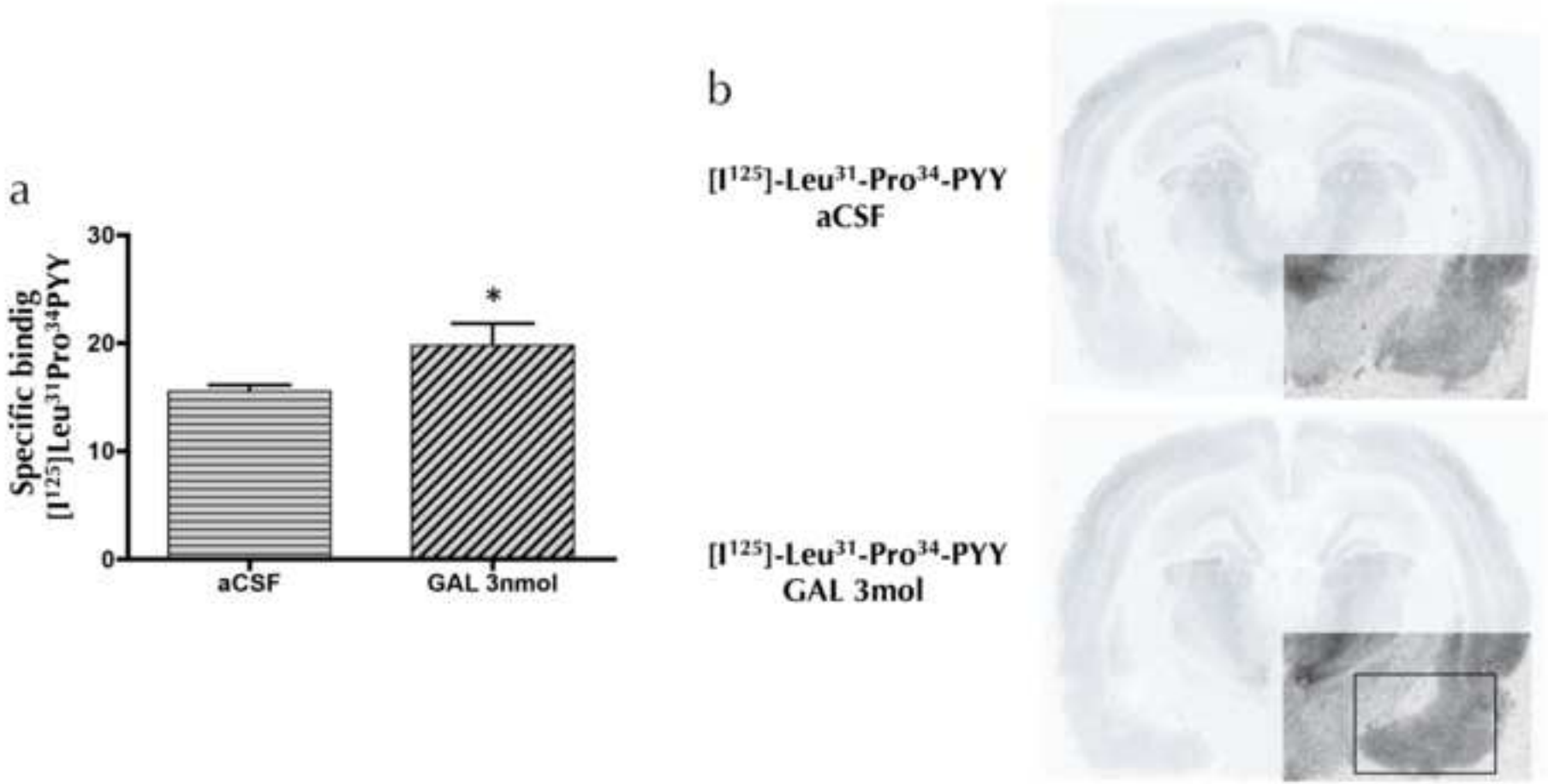

Figure 2

Figure 2-2
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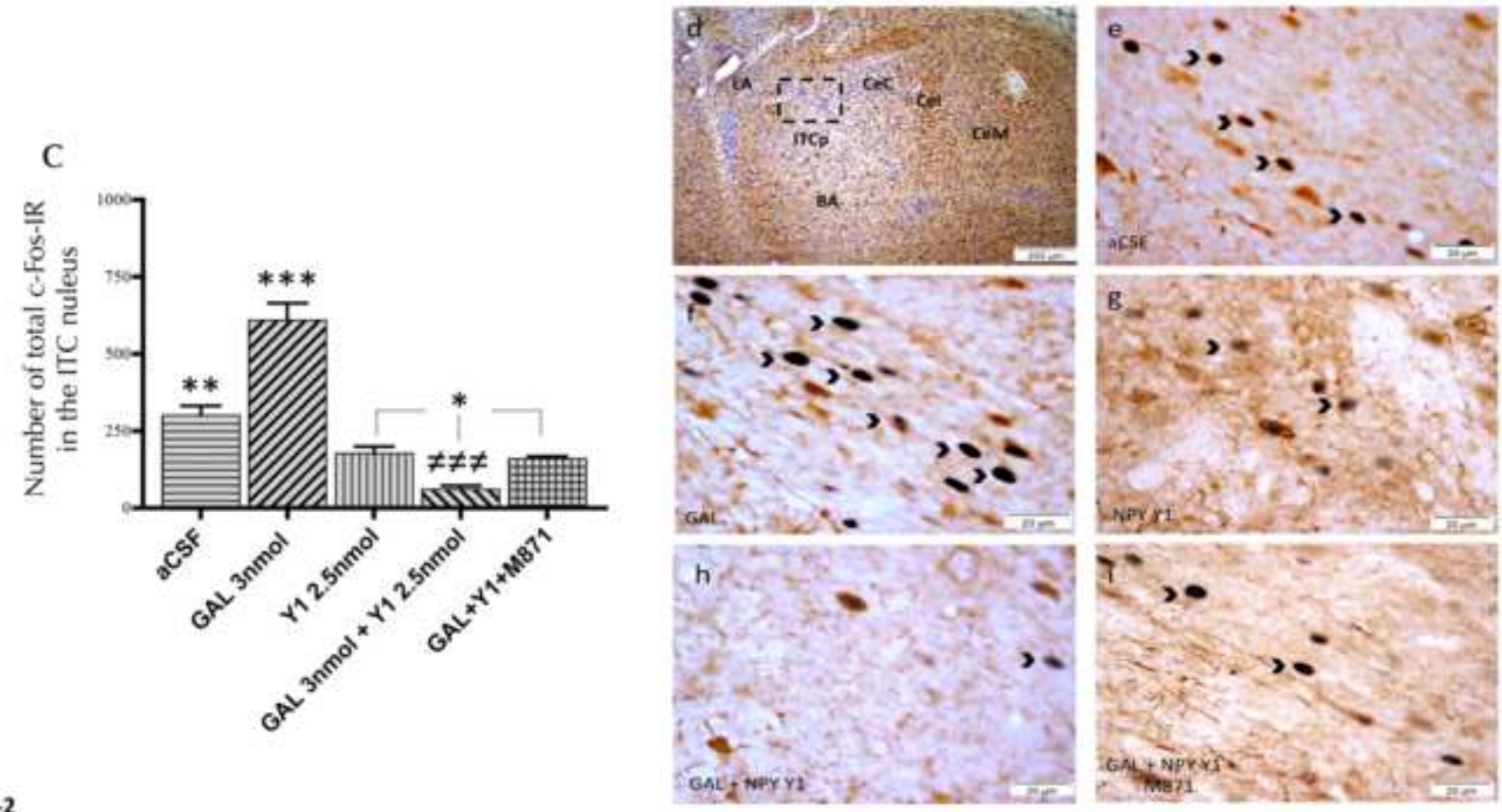

Figure-2

Figure 3
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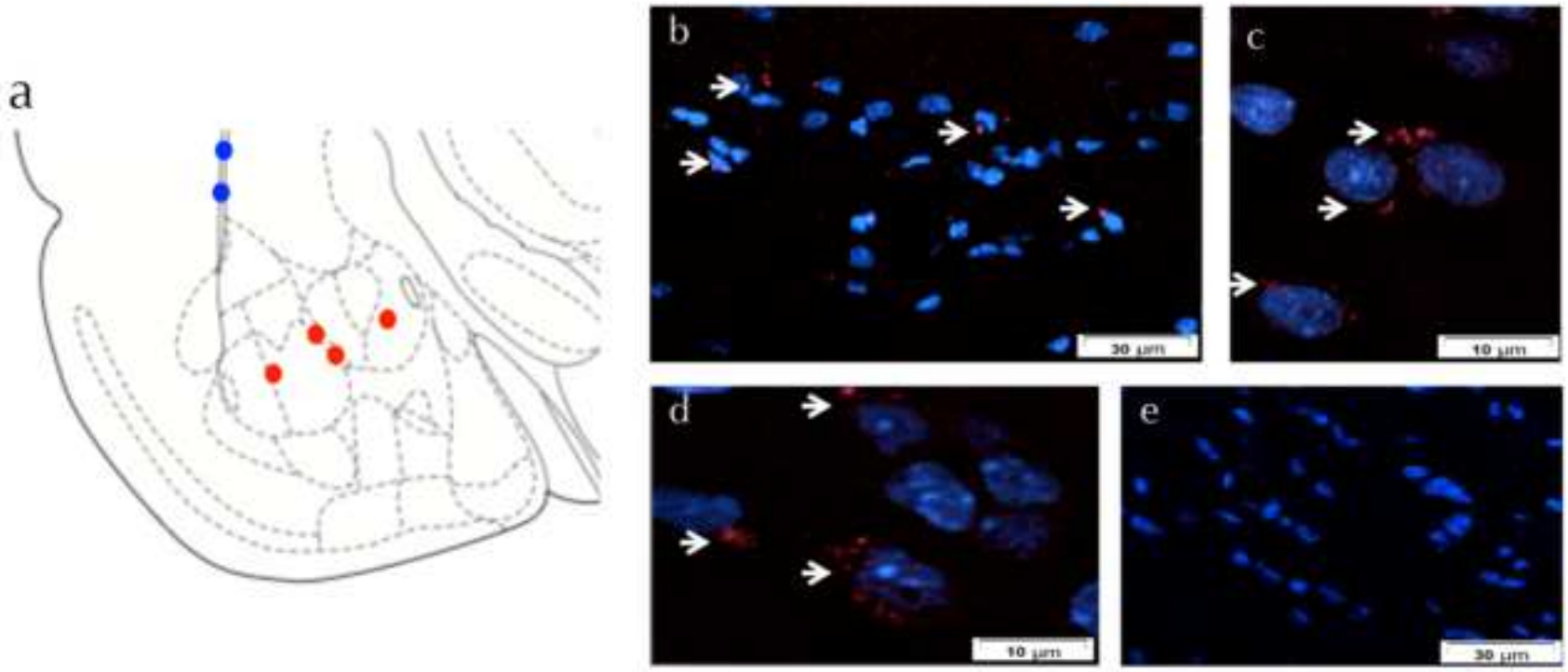

Figure 3

Figure 4
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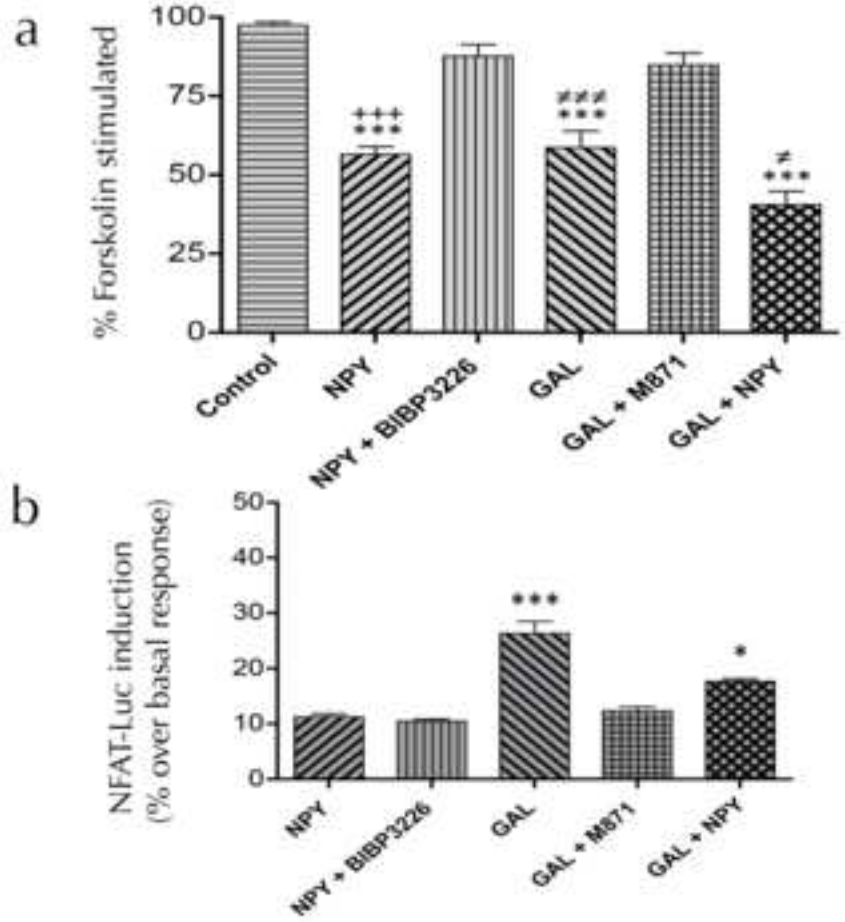

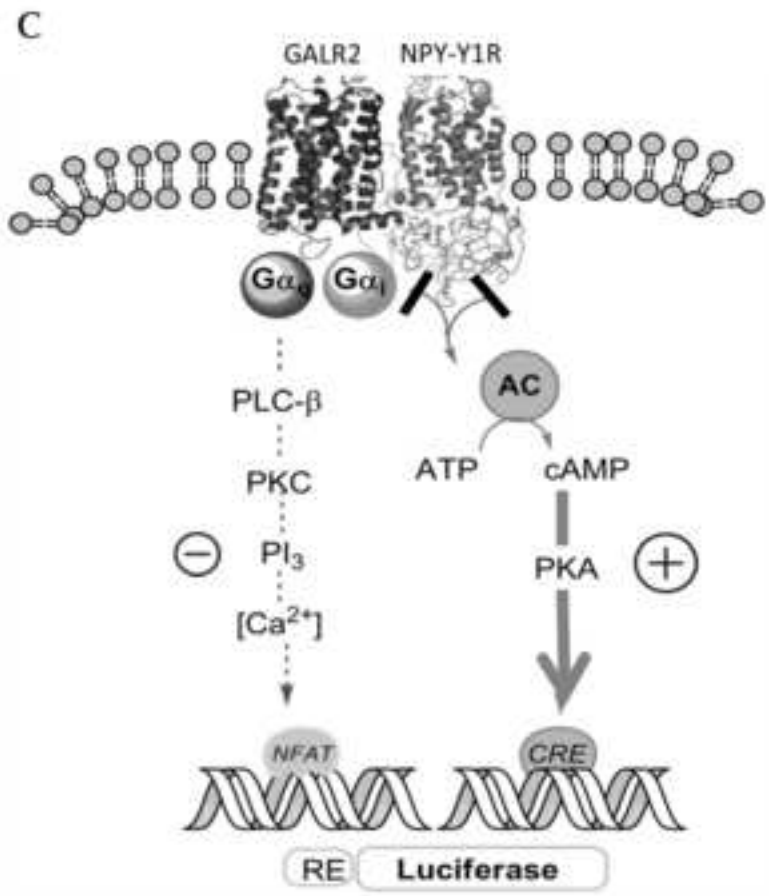

Figure 4

Figure 5-1
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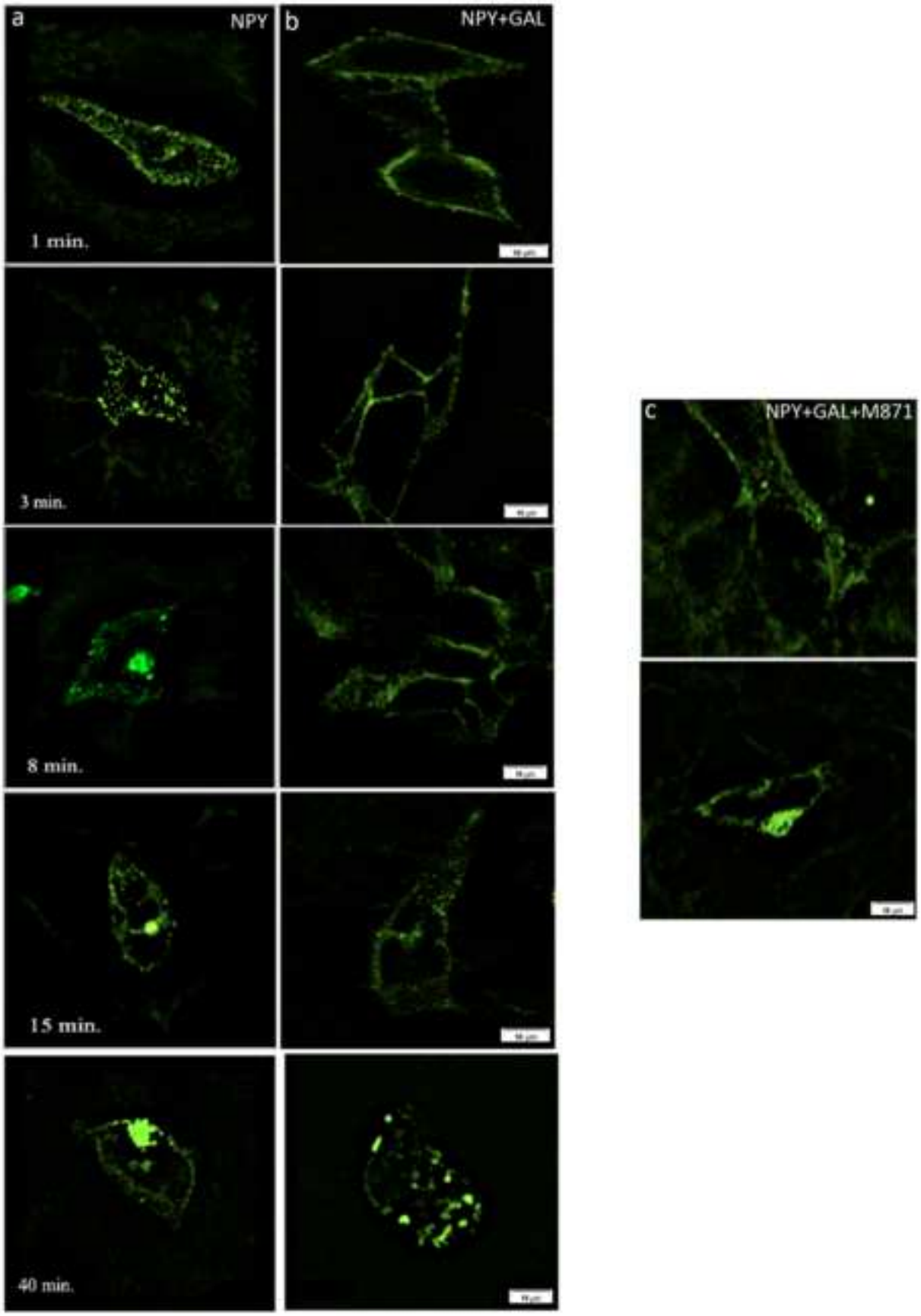

Figure 5-2
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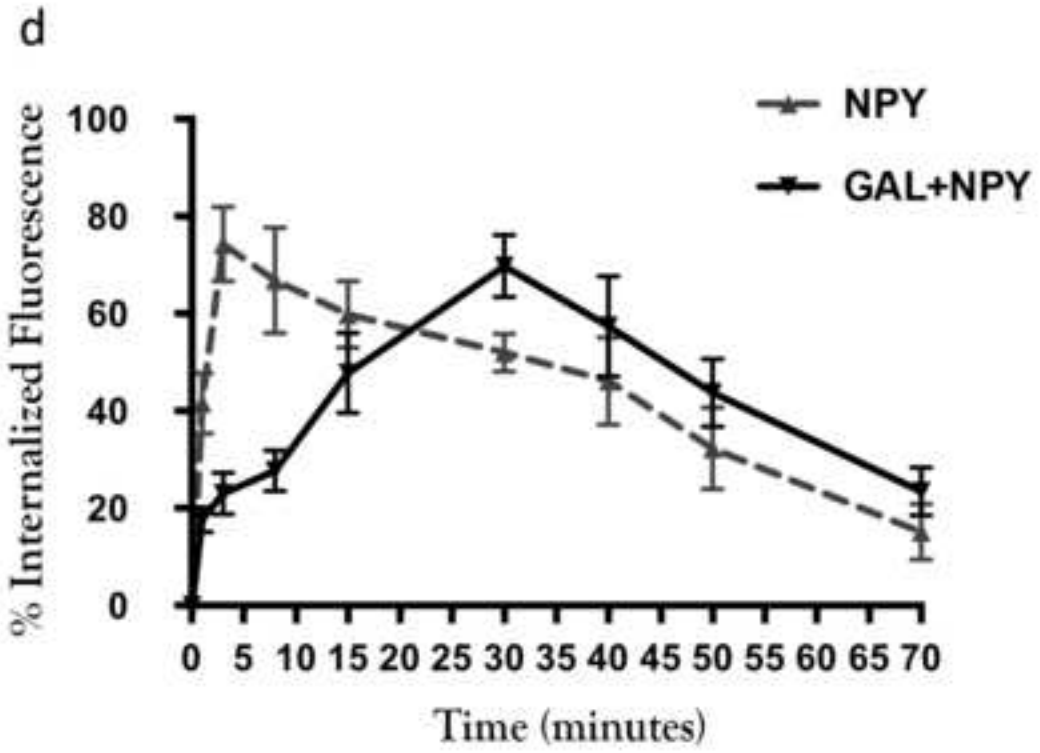

Figure 5

Electronic Supplementary Material
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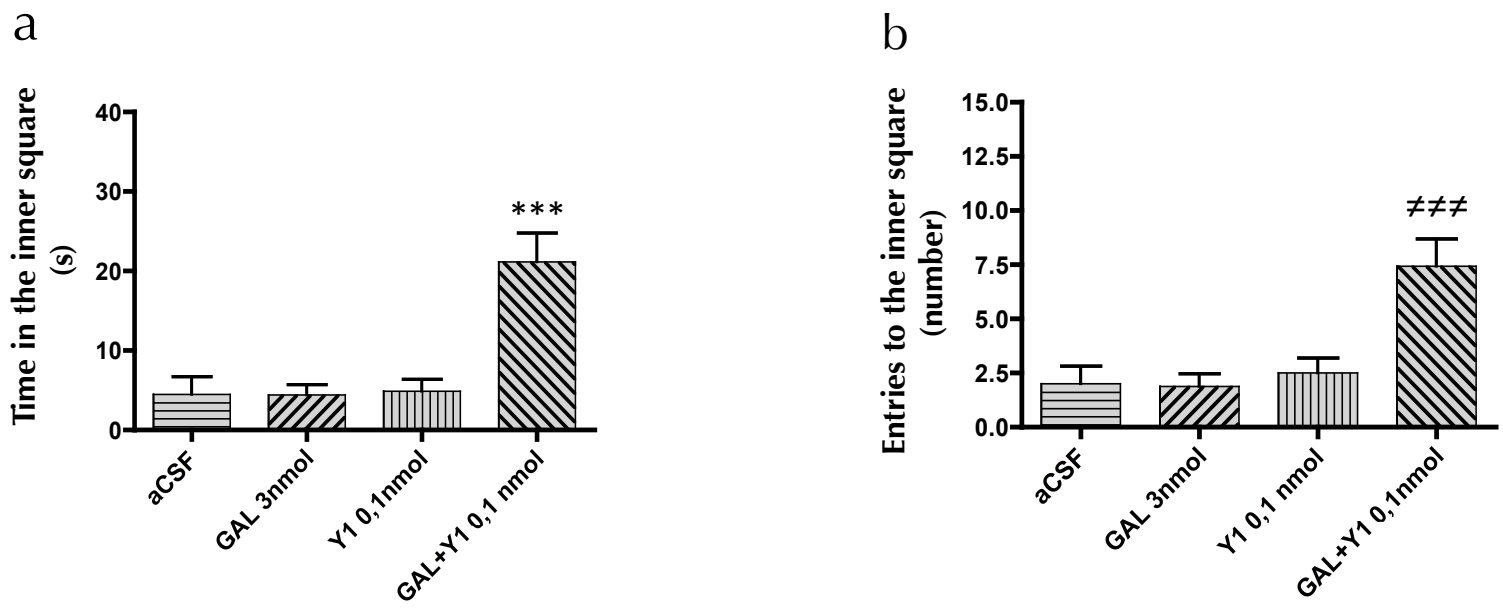
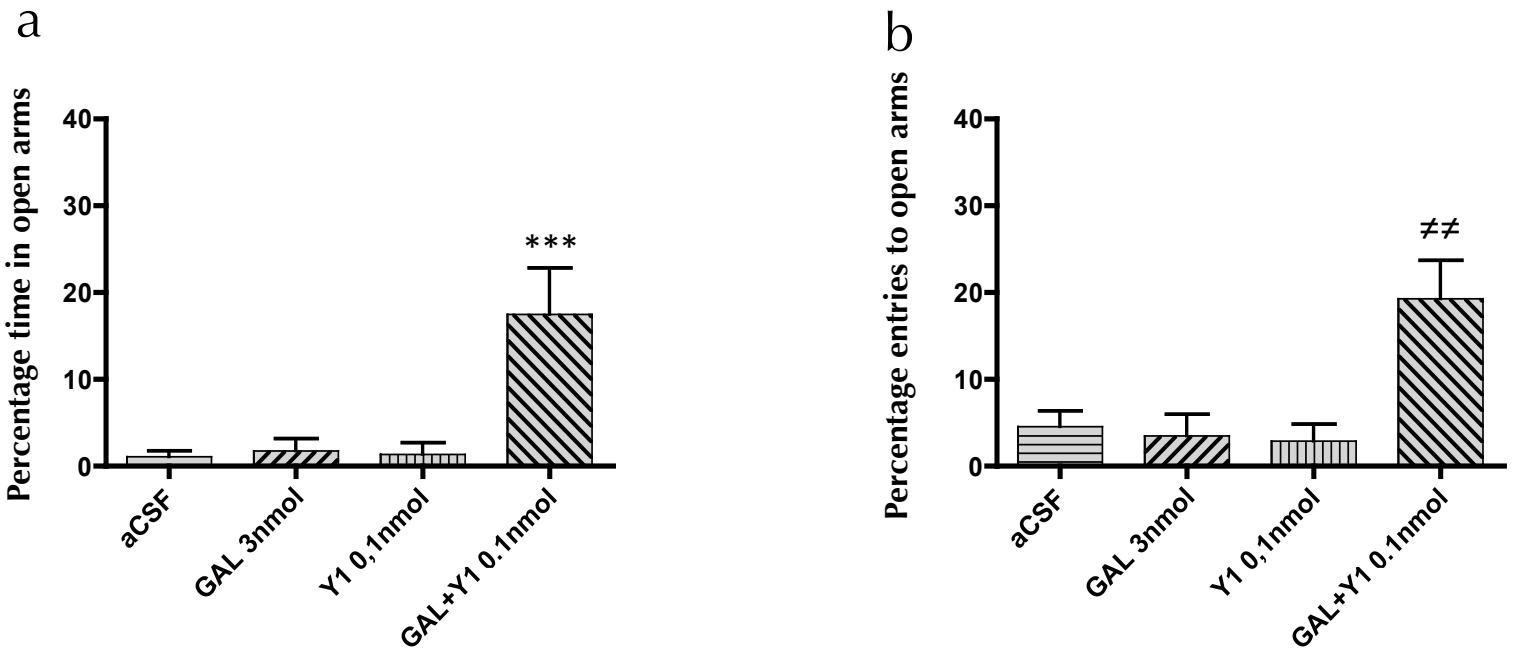


Supplementary Figure 1. Analysis of Galanin and the Neuropeptide Y Y1 receptor Agonist mediated anxiety-related behaviors in the open field. The coadministration of threshold doses of GAL and NPY Y1 agonist induced anxiolytic-related behaviors. **(a)** Time spent in the inner square of the open field. **(b)** Entries to the inner square of the open field. n=6-8 animals in each test group. Data represents mean ± SEM. In (a): ****P* <0,001 versus the rest of the groups. In (b): ≠≠≠*P* <0.001 versus the rest of the groups according to one-way ANOVA followed by Newman Keuls Multiple Comparison Test. GAL = Galanin 3 nmol; Y1 0,1nmol = NPY Y1 receptors agonist [Leu^{31}-Pro^{34}]NPY 0,1nmol; GAL+Y1 0,1nnmol= Coadministration of GAL and Y1 0,1nmol.

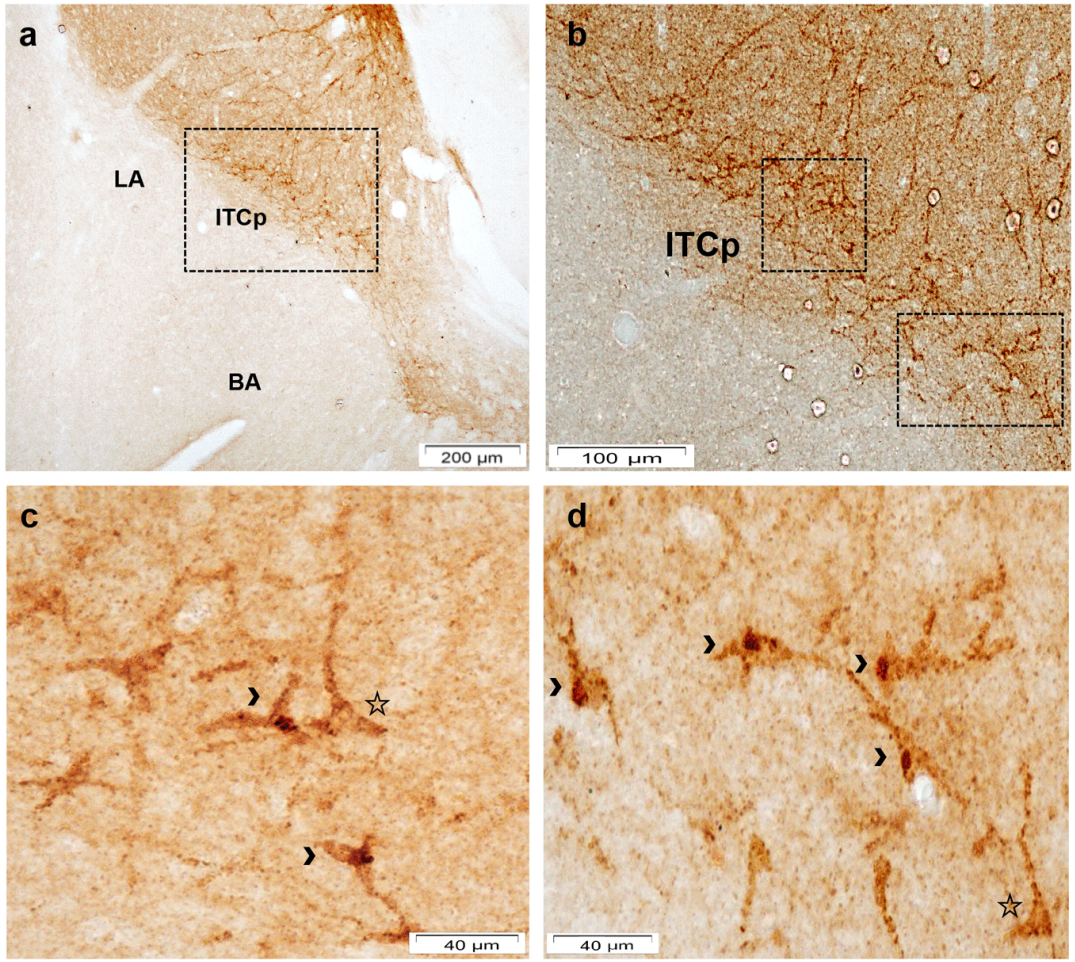


Supplementary Figure 2. Analysis of Galanin and the Neuropeptide Y Y1 receptor Agonist mediated anxiety-related behaviors in the elevated plus maze. The coadministration of threshold doses of GAL and NPY Y1 agonist induced anxiolytic-related behaviors. **(a)** Percentage of time in the open arms of the elevated plus maze. **(b)** Percentage of entries to the open arms of the elevated plus maze. n=5-8 animals in each test group. Data represents mean ± SEM. In (a): ****P* <0,001 versus the rest of the groups. In (b): ≠≠*P* <0.01 versus the rest of the groups according to one-way ANOVA followed by Newman Keuls Multiple Comparison Test GAL = Galanin 3 nmol; Y1 0,1nmol = NPY Y1 receptors agonist [Leu^{31}-Pro^{34}]NPY 0,1nmol; GAL+Y1 0,1nmol= Coadministration of GAL and Y1 0,1nmol.

SUPPLEMENTARY INFORMATION

c-Fos/GAD double labelling

The procedures have been described previously (Diaz-Cabiale et al. 2011). c-Fos immunostaining has been described in the materials and methods section. Double immunohistochemistry for c-Fos and Glutamate Decarboxylase (GAD) 65/67 for labelling GABAergic neurons in Central Nervous System has been used (Boothman L, Raley J, Denk F, Hirani E, Sharp T (2006) In vivo evidence that 5-HT(2C) receptors inhibit 5-HT neuronal activity via a GABAergic mechanism. Br J Pharmacol 149 (7):861-869. doi:10.1038/sj.bjp.0706935). Second primary antibody used (GAD65/67) has been validated to detect GABAergic neurons (1:1000, Santa Cruz Biotech.sc-7513, USA)(Papay R, Gaivin R, Jha A, McCune DF, McGrath JC, Rodrigo MC, Simpson PC, Doze VA, Perez DM (2006) Localization of the mouse alpha1A-adrenergic receptor (AR) in the brain: alpha1AAR is expressed in neurons, GABAergic interneurons, and NG2 oligodendrocyte progenitors. J Comp Neurol 497 (2):209-222. doi:10.1002/cne.20992). The chromogen used was 3-3´-diaminobenzidine tetrahydrochloride (DAB) (Sigma, Spain) for GAD 65/67 immunostaining in order to get a brownish reaction. Sections were mounted on glass slides and microphotographs were obtained (Olympus BX51 microscope, Olympus, Denmark).

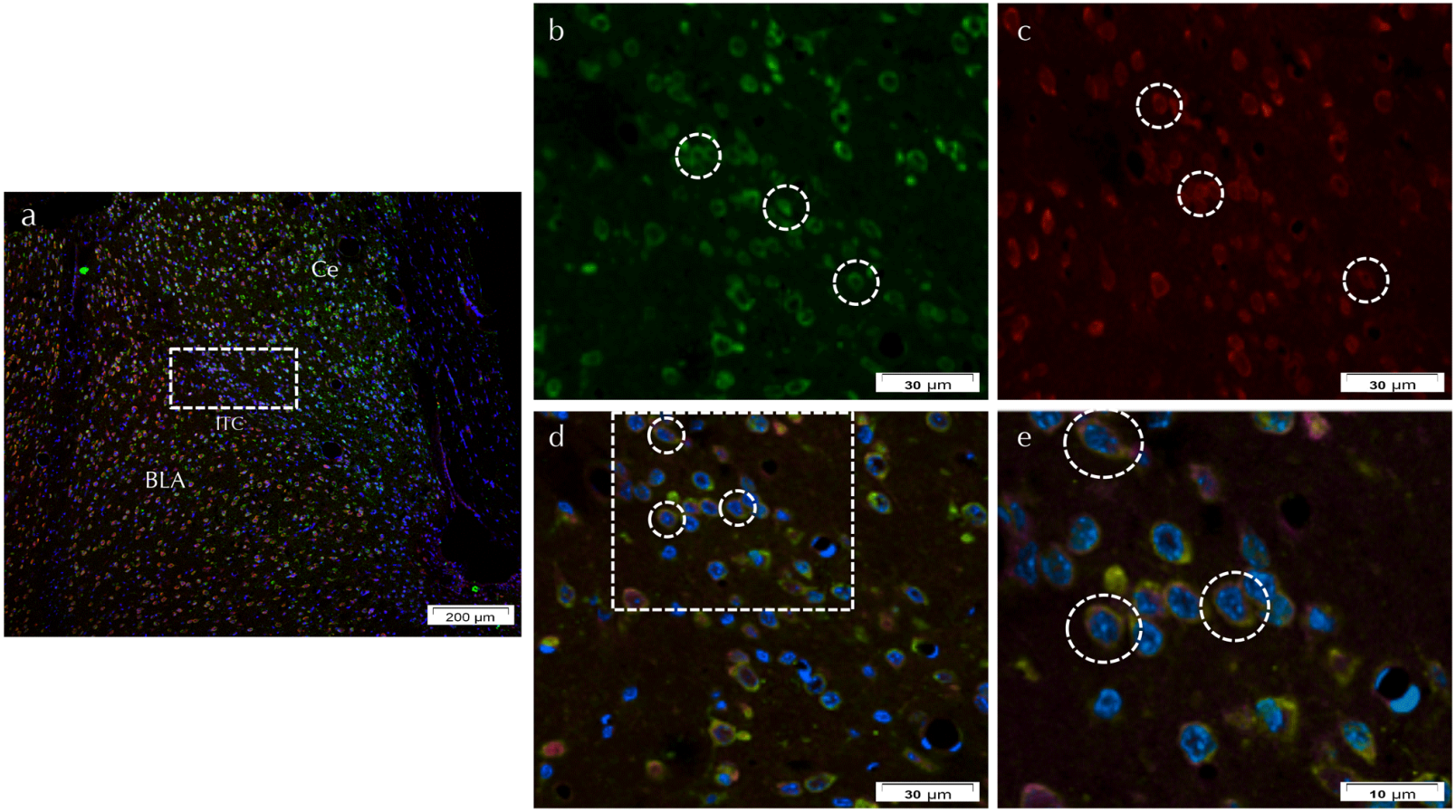


Supplementary Figure 3. C-Fos expression in GAD-positive neurons within the Medial Intercalated paracapsular amygdala (ITCp) nucleus after intracerebroventricular injections of NPY Y1 receptors agonist [Leu^{31}-Pro^{34}]NPY. **(a)** Representative photomicrograph illustrating the Medial Intercalated paracapsular amygdala (ITCp), Lateral (LA) and Basal (BA) nuclei of the Amygdala (Bregma: -3,3mm) **(b)** ITCp nucleus in a magnified view from dashed box in Supplementary Fig.3a **(c-d)** Magnified views from dashed boxes in Supplementary Fig.3b showing double immunodetection for c-Fos positive cells (revealed with DAB plus nickel; black-purple reaction) and GAD neurons (DAB; brown reaction) within ITCp nucleus. Arrowheads indicate examples of GAD neurons expressing c-Fos, whereas the stars represent examples of single-labelled GAD neurons.

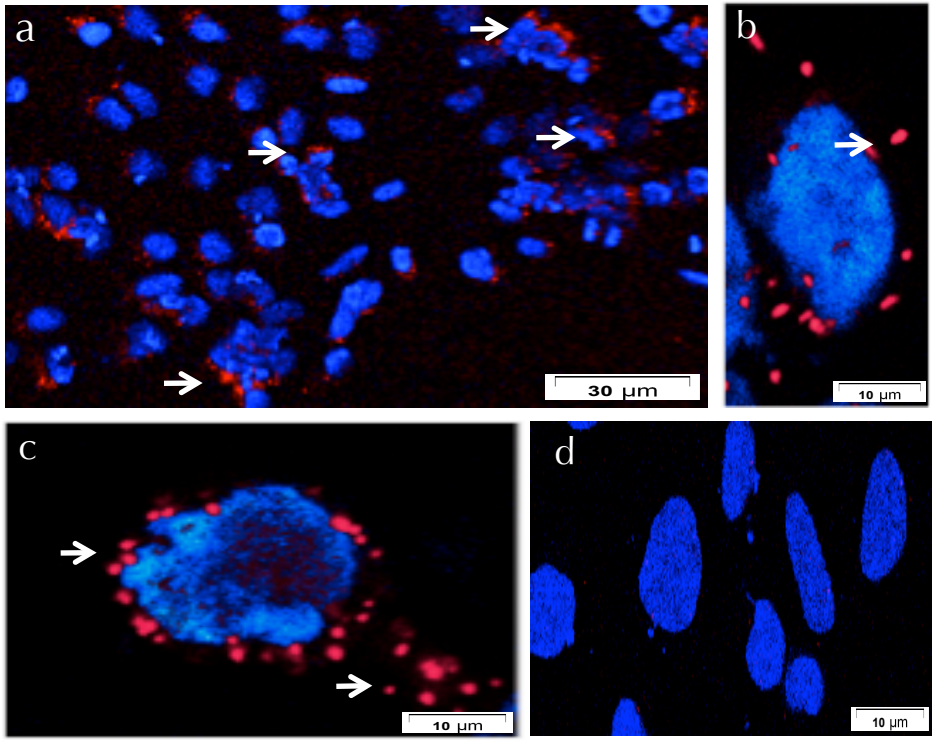
SUPPLEMENTARY INFORMATION

Double Immunofluorescence

The procedures have been previously used (Diaz-Cabiale et al. 2011). An initial incubation with blocking (5% goat serum) and permeabilization (0.3% triton X100 in PBS) were done during 60 min. Primary antibody Rabbit anti-GALR2 (Alomone Lab, 1:100) was incubated for 48-72 hours at 4 oC and detected with the red secondary antibody Mouse anti-rabbit DyLight 549 (Jackson inmunoResearch Laboratories, 1:100). Goat anti-NPYY1R (Santa Cruz Biotecnology INC, EEUU, 1:200) was incubated in a similar manner as described above and detected with the green secondary antibody mouse anti-goat DyLight 488 (Jackson Laboratories InmunoResearch, 1:100). Nuclei were detected with 4',6-diamidino-2-phenylindole (DAPI), 1:200. Sections were mounted on slides with Fluorescent Mounting Medium (Dako) and visualized by using a confocal microscope Leica TCS-SL confocal microscope (Leica).



Supplementary Figure 4. Representative laser scanning confocal micrographs illustrating the analyzed Medial Intercalated Paracapsular Amygdala nucleus (ITC), located between Basolateral nuclei (BLA) and central nuclei (Ce) of the Amygdala at Bregma: -2.5mm. Nuclei are shown in blue (DAPI). **(b-e)** Laser scanning confocal micrographs demonstrating the coexpression of endogenous Neuropeptide Y Y1 Receptor (Y1R) (green, dashed circles) **(b)** and Galanin Receptor 2 (GALR2) (red, dashed circles) **(c)** in 30 µm neuronal slice in a magnified view from dashed box in Supplementary Fig. 4a. **(d)** Representative laser scanning confocal micrograph showing the colocalization of Y1R and GALR2 in a subpopulation of neurons (dashed circles) in Medial Intercalated Paracapsular Amygdala nucleus. **(e)** The colocalization of Y1R and GALR2 is shown in a subpopulation of neurons (dashed circles) in a magnified view from dashed box in Supplementary Fig. 4d.



Supplementary Figure 5. Galanin receptor 2 (GALR2) and Neuropeptide Y Y1 (NPY-Y1) heteroreceptor complexes by *in situ* proximity ligation assay (PLA) performed in HEK293T transiently transfected cells **(a-c)** Detection of GALR2-NPY-Y1 heteroreceptor complexes (seen as red clusters indicated by arrows) in HEK293T cells by in situ PLA; nuclei are shown in blue (DAPI). **(d)** Lack of positive PLA signals in negative control taking away the GALR2 primary antibody.

Behavioral Test	Locomotor Parameters	aCSF	GAL 3nmol	Y1 0,1nmol	GAL+Y1 0,1nmol	Y1 3nmol	GAL+Y1 3mol	M871 3nmol	GAL +Y1+M871	M617 3nmol	M617+Y1 3nmol
Elevated Plus Maze	Total Distance Travelled (cm)	1117±46.7	1185±139.7	1195±67.2	1137±12	1158±42.5	1180±58.1	1131±34.4	1151±40.6	1125±59.9	1120±72.4
	Mean Speed (cm/s)	3.91±0.1	3.95±0.4	3.98±0.2	3.79±0.1	4.02±0.3	4.01±0.1	4.06±0.2	3.94±0.4	3.8±0.3	3.94±0.2
Open Field	Total Distance Travelled (cm)	3282±514.3	2924±128.5	3140±457.9	2878±132	2991±206.5	3252±205.8	3169±189.7	3345±145.3	3274±241.8	2740±256.9
	Mean Speed (cm/s)	10.94±1.7	9.72±0.4	10.47±1.5	9.59±0.4	10.01±0.6	9.02±0.1	11.42±0.6	10.19±0.2	10.93±0.8	9.13±0.8

Supplementary Table 1. Analysis of locomotor activity parameters in the open field and elevated plus maze. n=5-8 animals in each test group. Data shown as Mean ± SEM. No differences were found according to one-way ANOVA followed by Newman Keuls Multiple Comparison Test. aCSF= Control; GAL 3nmol = Galanin 3 nmol; Y1 0,1nmol = NPY Y1 receptors agonist [Leu^{31}-Pro^{34}]NPY 0,1nmol; GAL+Y1 0,1nnmol= Coadministration of GAL 3nmol and Y1 0,1nmol; Y1 3nmol = NPY Y1 receptors agonist [Leu^{31}-Pro^{34}]NPY 3nmol; GAL+Y1 3nnmol= Coadministration of GAL 3nmol and Y1 3nmol; M871 3nmol = GalaninR2 Antagonist 3 nmol; GAL+Y1+M871= Coadministration of GAL 3nmol, Y1 3nmol and M871 3nmol; M617 3nmol = GalaninR1 Agonist 3 nmol; M617+Y1 3nmol= Coadministration of M617 3nmol and Y1 3nmol.

Behavioral Test	Parameters	aCSF	M617 3nmol	Y1 3nmol	M617+Y1
Elevated Plus Maze	% Time in Open Arms	1.9±1.1	0.7±0.5	18.7±5 *	20.9±9.3 *
	% Entries to Open Arms	3±1	7.1±3.3	21.7±3.1 ≠	22.4±9.6 ≠
Open Field	Time in Inner Square	3.9±1.1	2.9±1	13.4±1.6 ***	8.0±1.3 *
	Entries to Inner Square	0.8±0.4	2.5±0.7	5.2±1.1 ≠	4.2±1.1 ≠

Supplementary Table 2. Analysis of Galanin and the Neuropeptide Y Y1 receptor Agonist mediated anxiety-related behaviors in the open field and elevated plus maze. The coadministration of the GALR1 agonist does not modify the NPY Y1 agonist anxiolytic-related behaviors. n=5-8 animals in each test group. Data shown as Mean ± SEM. In Elevated Plus Maze= *P<0,05 versus aCSF and M617 3nmol ≠P<0,05 versus aCSF. In Open Field= ***P <0,001 versus aCSF and M617 3nmol *P <0,05 versus the rest of the groups ≠P<0,05 versus aCSF according to one-way ANOVA followed by Newman Keuls Multiple Comparison Test. aCSF= Control; M617 = GalaninR1 Agonist 3 nmol; Y1 3nmol = NPY Y1 receptors agonist [Leu^{31}-Pro^{34}]NPY 3nmol; M617+Y1 3nmol= Coadministration of M617 3nmol and Y1 3nmol.

Nuclei Amygdala/ Group	Lateral	Basal	Lateral capsular subdivision of Central	Lateral intermediate subdivision of Central
aCSF	559.8±79.9	900.8±174.2	60±17.6	72±19.4
GAL 3nmol	533±78.5	880.5±163.5	212±39.9 ≠	112±27.7
Y1 2.5nmol	306.5±58.9 *	1751±106.3 **	176±31.3 ≠	92±12
GAL 3nmol+Y1 2.5nmol	213.2±16.9 **	2041±151 ***	185.6±34.5 ≠	67.2±11.7

Supplementary Table 3. Effects of Galanin and NPY Y1 receptor agonist alone or in combination on c-Fos expression in nuclei of amygdala complex. Quantification of total c-Fos IR nerve cell bodies data are indicated as mean ± SEM. n=4 in each test group. * *P* <0,05 versus aCSF and GAL 3nmol ≠*P* <0,05 versus aCSF ***P* <0,01 versus aCSF and GAL 3nmol ****P* <0,001 versus aCSF and GAL 3nmol according to one-way ANOVA followed by Newman Keuls Multiple Comparison Test. aCSF= Control; GAL 3nmol = Galanin 3 nmol; Y1 2.5nmol = NPY Y1 receptors agonist [Leu^{31}-Pro^{34}]NPY 2.5nmol; GAL 3nmol+Y1 2.5nnmol= Coadministration of GAL 3nmol and Y1 2.5nmol.