GALANIN RECEPTOR 2-NEUROPEPTIDE Y Y1 RECEPTOR INTERACTIONS IN THE DENTATE GYRUS ARE RELATED WITH ANTIDEPRESSANT-LIKE EFFECTS.

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Abstract

Galanin (GAL) and the NPYY1 agonist play a role in mood regulation and both neuropeptides interact in several central functions. The present study examined the interaction between Galanin receptor 2 (GALR2) and Neuropeptide Y Y1 receptor (NPYY1R) in the dentate gyrus (DG) of the Hippocampus in relation to depression-like behaviour. Using receptor autoradiography, in situ hybridization and in situ proximity ligation assay an interaction between GALR and NPYY1R was demonstrated in the DG probably involving the formation of GALR2-NPYY1R heteroreceptor complexes. These complexes were specifically observed in the polymorphic and subgranular subregions of the DG, where both receptors were found to colocalize. Moreover, this GALR2/NPYY1R interaction was linked to an enhancement of the antidepressive-like behaviour mediated by NPYY1R in the forced swimming test. Specific cells populations within DG subregions may be involved in this behavioural effect since the coactivation of GALR2 and NPYY1R enhances the NPYY1R-mediated reduction in the number of c-Fos immunoreactive nuclei in the polymorphic region. These results indicate that GALR2/NPYY1R interactions can provide a novel integrative mechanism in DG in depression-related behavior and may give the basis for the development of drugs targeting GALR2/NPYY1R heteroreceptor complexes in the DG of the hippocampus for the treatment of depression.

Keywords: Galanin, Neuropeptide Y, interaction, depression.

INTRODUCTION

The hippocampal formation is critically involved in various normal brain functions such as spatial, contextual, episodic memory (Burgess et al. 2002) and the response to stress (McEwen and Magarinos 1997); but also in

neuropsychiatry conditions as depression (Campbell and Macqueen 2004). Moreover, in recent years different neurotransmitters and substances related to neurotransmitters have been described to modulate short-term depressive-like actions in the hippocampus (Hiroaki-Sato et al. 2014; Bettio et al. 2014). Among hippocampal subregions, the dentate gyrus (DG) is a key region for the regulation of these functions and it is built of molecular and granular cell layers and polymorphic cell region (or hilus) (Amaral et al. 2007). Although the DG receives inputs mainly from the entorhinal cortex, the polymorphic layer of the DG receives noradrenergic input from the locus coeruleus (Blackstad et al. 1967) and serotoninergic input from the midbrain raphe nuclei that targets preferentially calbindin-, calretinin-, somatostatin-, and neuropeptide Y (NPY)-containing cells (Catena-Dell'Osso et al. 2013; Sperk et al. 2007).

Novel treatment strategies for psychiatric disorders are inter alia focused on DG as a potential brain target (Hagihara et al. 2013). The neuropeptides and their receptors have received special attention in this area, especially the NPY neuron system, which is an attractive therapeutic target in emotional disorders, including depressive behaviors (Holmes et al. 2003; Kormos and Gaszner 2013).

The NPY is widely distributed within the nervous system, with high concentration in several limbic and cortical regions. In the hippocampus, the largest population of NPY-immunoreactive (IR) cells is contained in the polymorphic layer of the DG**,** being all GABAergic neurons (Kohler et al. 1986; Sperk et al. 2007). The NPY Y1 (NPYY1R) and Y2 receptors are also expressed in the hippocampus, althought the NPYY1R are abundantly found throughout the entire DG (Dumont et al. 1996; Paredes et al. 2003). Furthermore, increased NPY and NPYY1R expression in DG is directly correlated with decreased immobility in the forced swimming test (FST) in rats, suggesting the participation of NPY-NPYY1 system in the pathophysiology of depression (Jimenez-Vasquez et al. 2007; Bjornebekk et al. 2010; Catena-Dell'Osso et al. 2013). Thus, intracerebroventricular (icv) infusion of NPY or

the NPYY1R agonist [Leu^{31},Pro^{34}]NPY decrease the immobility in the FST, which are blocked by coadministration of a NPYY1R antagonist (Redrobe et al. 2002; Stogner and Holmes 2000). In this way, the enhancement of the NPYY1 receptor mediated NPYergic transmission is one of the mechanisms that underlies antidepressants and electroconvulsive therapy (Caberlotto et al. 1999; Madsen et al. 2000). Conversely, genetic and environmental rat models of depression show decreased levels of NPY and NPYY1R in the DG (Mathe et al. 1998; Jimenez-Vasquez et al. 2007).

Galanin (GAL), is also a neuropeptide widely distributed in the central nervous system (Jacobowitz et al. 2004). The three receptors of GAL (GALR) are involved in depression-related behaviors via modulation of neuroendocrine and monoaminergic systems, with a different role depending on the GALR subtype (Fuxe et al. 2012; Wrenn and Holmes 2006). Thus, icv infusion of GAL or stimulation of GALR1 increased immobility in the FST and GALR3 antagonist results in decreased immobility in the tail suspension test and also in FST (Barr et al. 2006; Kuteeva et al. 2007; Kuteeva et al. 2008). However, the activation of the GALR2, which is mainly expressed in the DG (O'Donnell et al. 1999), leads to decreased immobility in the FST (Kuteeva et al. 2007; Kuteeva et al. 2008; Millon et al. 2014; Borroto-Escuela et al. 2014). These results suggest a differential role of GAL receptors in the regulation of depression-like behaviour.

We have previously shown that GAL interacts with NPYY1R in several regions including the amygdala nuclei, a key region in stress-related behaviors in rats (Diaz-Cabiale et al. 2011; Narvaez et al. 2015). We described that GAL through GALR2 enhanced NPYY1R mediated anxiolytic actions. This facilitatory GALR2/NPYY1R interaction takes place in the amygdala at receptor and cellular levels involving the formation of GALR2/NPYY1R heteroreceptor complexes (Narvaez et al. 2015). Moreover, intracellular signalling studies indicated that GALR2 in the GALR2/NPYY1R heteroreceptor complex switches from a Gq to a Gi/o coupling with both receptors operating

via Gi/o, leading to additive inhibitory effects on adenylate cyclase (Narvaez et al. 2015).

The purpose of the current work was to evaluate by quantitative receptor autoradiography and *in situ* hybridization the interactions between GALR and NPYY1R in the DG. Therefore, the presence of GALR2/NPYY1R heteroreceptor complexes was analyzed in the subregions of the DG with *in situ* proximity ligation assay (Borroto-Escuela et al. 2013). A GALR2/NPYY1R interaction was found in depression-like behaviour in the forced swimming test in rats. Additionally, specific cell activation was found with c-Fos immunohistochemistry in subregions of the DG induced by the GALR2/NPPYY1R interaction.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats from CRIFFA (Barcelona; 200-250gr) had free access to food pellets and tap water. They were maintained under the standard 12h dark/light cycle, in controlled temperature (22±2^{o}C) and relative humidity (55-60%). 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).

Detailed descriptions are available in Supplement 1 on animal intracerebral cannulations.

Drugs used

Solutions were freshly prepared and the peptides were dissolved in artificial cerebrospinal fluid (aCSF, composition is 120nM NaCl, 20nM NaH_2CO_3, 2nM KCl, 0.5nM KH_2PO_4, 1,2nM $CaCl_2$, 1,8nM $MgCl_2$, 0,5nM Na_2SO_4, and 5,8nM D-glucose, pH 7.4). Galanin (GAL), NPYY1 Receptor (NPYY1R) Agonist

[Leu^{31},Pro^{34}]NPY and Galanin Receptor 2 (GALR2) Antagonist M871 were obtained from Tocris Bioscience (Bristol, UK).
Detailed descriptions are available in Supplement 1 on intracerebroventricular administration of peptides.

Quantitative receptor autoradiography

The procedure and the dose of GAL are based on previous work (Diaz-Cabiale et al. 2011; Narvaez et al. 2015). Fifteen minutes after the icv injections of aCSF or GAL (3nmol) (n=6 per group) rats were killed by decapitation and their brains were rapidly removed from the skull and frozen in -40 °C isopenthane. Coronal sections (14 µm thick) were cut in a cryostate (HM550, Microm International) at bregma levels according to the atlas of Paxinos and Watson (Paxinos G 1986) (Bregma levels hippocampal DG: -2.12mm to -4.52mm) and thaw-mounted on gelatin-coated slides. 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 1 µM. 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 exposed against Hyperfilms (Kodak Biomax MR film, Kodak, Rochester, NY) for 6 days together with ^{125}I microscales (Amersham International) as reference standards.
Autoradiograms were analyzed in the DG of the hippocampus (0.15 mm^2 square) as previously described (Parrado et al. 2007) (see Supplement 1 for details).

In situ hybridization

In situ hybridization was carried out as previously described (Bjornebekk et al. 2010). Briefly, five hours after the icv injections of aCSF or GAL (3nmol) (n=6 per group) coronal brain sections (14 µm) were cut on a cryostat and sections were thawed onto glass slides. The hybridization cocktail contained 50% formamide, 4x SSC (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1x Denhardt's solution, 1% Sarcosyl, 0.02 M Na3PO4, pH 7.0, 10% dextran sulfate, 0.06 M dithiothreitol and 0.1 mg/ml sheared salmon sperm DNA. Single-stranded oligonucleotide probes specific for NPY-Y1R were used. The probes were labeled with ^{33}P-dATP (Perkin-Elmer, Boston, MA). Hybridization was performed for 18 h in a humidified chamber at 42ºC. Following hybridization, the sections were rinsed 4 x 20 min each in 1x SSC at 60ºC. Finally, sections were rinsed in autoclaved water for 10 s, dehydrated in alcohol and air-dried. Thereafter, the slides were exposed to film (Kodak Biomax MR film, Kodak, Rochester, NY) for 7 days before being developed. Films were scanned and optical density values were quantified in the distinctly labelled region within DG (see Supplement 1 for details).

In situ proximity ligation assay

In situ Proximity Ligation Assay (PLA) was performed as described previously (Borroto-Escuela et al. 2013; Narvaez et al. 2015). Untreated rats (n=3) were perfused with 4% paraformaldehyde, brains were removed and sections at the DG level were obtained. Free-floating sections were incubated with blocking (5% goat serum) and permeabilization (0.3% triton X100 in PBS) solutions during 60 min each. Primary antibodies of different hosts directed against GALR2 (rabbit, Alomone Lab, 1:100) and NPYY1R (goat, sc-21992 Santa Cruz Biotecnology INC, CA, 1:200) were incubated for 24 hours at 4ºC. PLA signal detection was 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. PLA signals were visualized by using a TCS-SL confocal microscope (Leica).

Forced Swimming Test

Depression-like behavior was assessed in the FST, originally proposed as a model of stress-induced depression-like behavior (Porsolt et al. 1977). Importantly, the immobility response in the FST can be prevented by various types of antidepressant treatments, including tricyclic antidepressants, monoamine oxidase inhibitors, SSRIs, and NA reuptake inhibitors (Petit-Demouliere et al. 2005; Kuteeva et al. 2008).

Animals were adapted to handling and were taken into the experimental room (80-90 lux) for at least 1 hour to reach habituation before the icv peptide administration. Rats were individually placed in a cylindrical tank measuring 50 cm in height and 20 cm in width, containing water (25±0.5°C) to a height of 30 cm. The animals were forced to swim for a 15-min period (pre-test) and 24 h later were subjected to a 5-min swimming session (test) 15 min after the administration alone or in combination of GAL, the NPYY1R agonist [Leu^{31},Pro^{34}]NPY and the GALR2 antagonist M871 (n=6-8 animals in each group). Effective doses for GAL, the NPYY1R agonist and for GALR2 antagonist M871 were chosen based on previous dose-response curves (Diaz-Cabiale et al. 2011; Millon et al. 2014; Narvaez et al. 2015). The total duration of floating (immobility), swimming, and climbing periods were scored during the 5 min test. Rats were considered to show immobility when they floated without struggling, making only those movements necessary to keep their heads above the water. Swimming was scored when they actively swam around the cylinder, while climbing was defined as vigorous forepaw movements directed toward the walls of the cylinder. Following swimming sessions, the rats were removed from the tank, carefully dried in heated

cages, and then returned to their home cages. Behavioural experiments were always performed between 09:00 and 14:00 hours.

c-Fos immunohistochemistry

Animals were divided into five experimental groups: (1) aCSF: control group; (2) GAL 3nmol: group treated with GAL 3nmol; (3) Y1 2,5nmol: group receiving the NPYY1R agonist [Leu^{31},Pro^{34}]NPY 2.5 nmol; (4) GAL 3nmol+Y1 2,5nmol: group administered with both substances; (5) GAL+Y1+M871 3nmol: group injected with GAL, [Leu^{31},Pro^{34}]NPY and the GALR2 antagonist M871 3nmol (n=4 in each group). All doses indicated above are based on previous published protocols (Parrado et al. 2007; Kuteeva et al. 2008; Diaz-Cabiale et al. 2011).

Anaesthetized rats with sodium pentobarbital (Mebumal; 100mg/kg, i.p.) were perfused with 4% Paraformaldehyde (wt/vol, Sigma) 90 minutes after icv injections and brains were coronally sliced and immunostained using previously published protocols (Diaz-Cabiale et al. 2011). As primary antibodies, an antibody against c-Fos protein (1:5000, sc-52, Santa Cruz Biotechnology, CA), revealed with DAB plus nickel, was used as an indirect marker of neural activity. The antibody to Calbindin-D28k (1:1000, Santa Cruz Biotecnology, CA), revealed with DAB, was used to outline the granular region since it marks mainly hippocampal granule cells, although there are some rare calbindin-immunoreactive interneurons in the hilus (Scharfman et al. 2002). Appropriate biotinylated specific secondary antibodies were used. Sections were mounted on glass slides and the different layers of the Dentate Gyrus were analyzed using the optical fractionator method in unbiased stereological microscopy (Olympus BX51 microscope, Olympus, Denmark) as previously described (Diaz-Cabiale et al. 2011) (see Supplement 1 for details).

Statistical Analysis

Data are presented as mean ± SEM and sample number (n) are indicated in figure legends. All data were analyzed using GraphPad PRISM 6.0 (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 the 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 increases NPYY1R binding and NPYY1R mRNA expression in the DG.

The icv injection of GAL 3nmol induced an increase in the NPYY1R agonist [I^{125}]-[Leu^{31},Pro^{34}]PYY binding at 25 pM in the DG by 20% ($t=1.924$, $p<0,05$, $df=10$) (Figure 1a) suggesting that GAL increases NPY1R recognition in this area. This increase in the [I^{125}]-[Leu^{31},Pro^{34}]PYY agonist binding in the DG is illustrated in representative autoradiograms in Figure 1b. The NPYY1 binding is mainly found in the molecular and granular layers.

Moreover, GAL affected not only NPYY1R binding but also the NPYY1R mRNA expression in the granular layer of DG. Thus, 5 hours after icv GAL 3nmol administration we found an increase of the NPYY1R mRNA expression in the granular layer of DG by 31% ($t=5.327$, $p<0,001$, $df=10$) (Figure 2a,b).

GALR2 and NPYY1R form PLA positive clusters in specific subregions of the DG.

Within the DG, PLA-positive red clusters were found specifically in the polymorphic layer and subgranular zone of the DG (Figure 3a-e). These PLA clusters indicate that GALR2 and NPYY1R are in close proximity and may form GALR2/NPYY1R heteroreceptor complexes in these regions. The specificity of the signal was confirmed by the fact that no PLA clusters were observed neither in the molecular layer of the DG nor in the corpus callosum, which is an area that seems to lack GALR2 (O'Donnell et al. 1999) (Figure 3 a,b,f).

In line with these results, extensive colocalization of GALR2 and NPYY1R immunoreactivities was observed specifically in the polymorphic layer and the subgranular zone of the hilus (Supplementary Figure 1a-f).

GAL enhances NPYY1R-mediated antidepressant-like behaviour

In the FST, rats were pre-exposed to water for 15 minutes. Twenty-four hours later, the immobility, swimming and climbing parameters were measured during a second 5 min exposure to water to find signs of depression-like behavior. The icv administration of the NPYY1R agonist (3 nmol) induces an antidepressive-like effect in the FST as it significantly decreased immobility (one-way ANOVA, $F_{4,30} = 44.81$, $p < 0.001$, Newman-Keuls post-hoc test: $p<0,001$; Figure 4a) and increased the swimming behaviour (one-way ANOVA, $F_{4,30} = 48.25$, $p < 0.001$, Newman-Keuls post-hoc test: $p<0,001$; Figure 4b). Besides, a significant decrease in climbing behaviour (one-way ANOVA, $F_{4,30} = 14.42$, $p < 0.001$, Newman-Keuls post-hoc test: $p<0,001$; Figure 4c) was observed.

In contrast, the icv GAL (3 nmol) significantly increased the immobility time (Newman-Keuls post-hoc test: $p<0,001$; Figure 4a) and decreased the climbing behaviour (Newman-Keuls post-hoc test: $p<0,001$; Figure 4c). However, a strong enhancement by GAL of the antidepressive-like actions of the NPYY1R agonist was observed after the coadministration of GAL and the

NPYY1R agonist. The icv injection of both substances significantly enhanced the decrease in the immobility (Newman-Keuls post-hoc test: p<0,001; Figure 4a) and the increase in the swimming behaviour (Newman-Keuls post-hoc test: p<0,001; Figure 4b) compared with the NPYY1R agonist alone. Moreover, a significant enhancement of the decrease in climbing behavior (Newman-Keuls post-hoc test: p<0,05; Figure 4c) was also observed.

The involvement of GALR2 in this interaction is confirmed since the presence of the GALR2 antagonist M871 counteracted the enhancement of the decrease in immobility (Newman-Keuls post-hoc test: p<0,01; Figure 4a) and in climbing behaviour (Newman-Keuls post-hoc test: p<0,05; Figure 4c) as well as the increase in swimming time (Newman-Keuls post-hoc test: p<0,001; Figure 4b) induced by the coadministration of GAL and NPYY1R agonist in the FST.

The GALR2 antagonist M871 alone in the dose of 3nmol lacked effects with respect to immobility (t=1.398, p 0,096, df=10, mean±SEM: 86.9±7), climbing behaviour (t=0.475, p 0,322, df=10, mean±SEM: 44.4±6) or swimming time (t=0.695, p 0,251, df=10, mean±SEM: 146±15) compared with control.

c-Fos activation pattern and cell populations involved after GAL and Y1 agonist coadministration in the subregions of DG.

As seen in Figure 5, the icv injection of [Leu^{31},Pro^{34}]NPY (2.5nmol) alone induced a decrease in the number of c-Fos IR profiles in the polymorphic region (one-way ANOVA, $F_{4,15} = 20.81$, $p < 0.001$, Newman-Keuls post-hoc test: p<0,05; Figure 5a,f), while an increase in c-Fos IR profiles in the granular cell layer of the DG was produced (one-way ANOVA, $F_{4,15} = 15.08$, $p < 0.001$, Newman-Keuls post-hoc test: p<0,01; Figure 5b,f). On the other hand, GAL (3nmol) alone significantly increased the number of c-Fos IR profiles in both areas, the polymorphic (Newman-Keuls post-hoc test: p<0,05) and granular cell layer of the DG (Newman-Keuls post-hoc test: p<0,01) (Figure

5a,b,e). No c-Fos IR was observed in the molecular layer after either GAL or NPYY1R agonist administration alone.

However, the coinjection of NPYYR1 agonist and GAL significantly modified the number of c-Fos IR profiles compared to the effect of GAL or the NPYY1R agonist alone in both subregions of the DG. Within the polymorphic region, the coadministration of GAL and [Leu^{31},Pro^{34}]NPY significantly decreased the c-Fos IR profiles compared with NPYY1R agonist (Newman-Keuls post-hoc test: $p<0,05$), GAL alone (Newman-Keuls post-hoc test: $p<0,001$) and the control group (Newman-Keuls post-hoc test: $p<0,001$) (Figure 5a,g). In this region the GABA interneurons could be involved in the interaction since c-Fos IR colocalized with a GABAergic marker (GAD65/67) after NPYY1R agonist injection (Supplementary Figure 2a-c).

Within the granular cells layer, GAL and NPYY1 agonist coadministration significantly increased c-Fos IR expression in the entire granular cell layer (Figure 5a,g) compared with GAL (Newman-Keuls post-hoc test: $p<0,05$) and [Leu^{31},Pro^{34}]NPY (Newman-Keuls post-hoc test: $p<0,01$) alone.

The cotreatment with the GALR2 antagonist M871 completely reversed the GAL contribution to the responses in both regions, the polymorphic and the granular layer of the DG (Figure 5a,b,h), demonstrating the involvement of GALR2 in the GAL actions.

DISCUSSION

The current study demonstrates for the first time the existence of an interaction between GALR2 and NPYY1R in the DG at receptor level, probably involving the formation of GALR2/NPYY1R heteroreceptor complexes based on the proximity ligation assay. These complexes were specifically observed in the polymorphic and subgranular subregions of the DG, where the expression of both receptors colocalized. Besides, this GALR2/NPYY1R interaction was linked to an enhanced antidepressive-like behaviour involving specific cell

populations within DG subregions. Thus, the coactivation of GALR2 and NPYY1R enhances the NPYY1R-mediated reduction in the number of c-Fos IR profiles in the polymorphic region. This strengthened response could be related with the increase of c-Fos IR profiles observed in the granular cells, and may provide the mechanism at circuits level for the enhancement of the antidepressant activity observed after the cotreatment with GAL and the NPY Y1 agonist.

We observed in receptor autoradiography experiments that GAL interacts at the membrane level with NPYY1R, as GAL caused an increase of the NPYY1R agonist [^{125}I]-[Leu^{31},Pro^{34}]PYY binding in the DG. This effect could be involved in producing the enhanced antidepressant activity observed after the cotreatment with GAL and the NPY Y1 agonist in the forced swim test through increased recognition and signaling of the NPYY1R in the GALR2-NPYY1R heteroreceptor complex. GALR2 may also switch from Gq to Gi/o mediated signalling in this receptor complex (Narvaez et al. 2015). We have previously described an increase of the NPYY1R agonist binding in the amygdala induced by the administration of GAL straightly associated to an enhancement of a NPYY1R induced anxiolytic-like effect (Narvaez et al. 2015).

Furthermore, we observed that GAL also increases the NPYY1R mRNA expression in the DG, which has been linked to antidepressant treatments. In this way, electroconvulsive therapy (ECT) in naive rats (Madsen et al. 2000) and ECT (Jimenez-Vasquez et al. 2007), running and escitalopram administration (Bjornebekk et al. 2010) in Flinders rats, a genetic animal model of depression, increase NPYY1R mRNA in the DG, correlated with antidepressive-like responses in the forced swim test. In view of these studies, the increase in NPYY1R mRNA induced by GAL in the DG showed in this work may be of relevance for depressive disorders and their treatment.

The current behavioral results demonstrated that GAL enhanced the antidepressive-like effects mediated by the NPYY1 agonist, decreasing the immobility and increasing swimming behaviors in the FST. The strong

increase in the swimming behavior observed could involve changes on extracellular levels of serotonin. In fact, it is well known that the selective SSRIs produced the increases in the swimming behaviors in the FST (Kuteeva et al. 2008). Since GAL modulate serotonin transmission in the hippocampus (Yoshitake et al. 2014) we cannot exclude that serotonin could be involved in the GAL-NPYY1R agonist antidepressant-like actions observed. Furthermore, GALR2 is involved in this GALR/NPYY1R interaction, since the GALR2 antagonist M871 counteracted the response observed and GALR2 has been previously proposed to mediate the antidepressive-like effects of GAL *in vivo* (Saar et al. 2013; Kuteeva et al. 2008). The results obtained after the administration of GAL or NPYY1 agonist alone agree with previous studies and confirm the prodepressive role of GAL and the antidepressive action of the NPYY1 agonist in the FST (Redrobe et al. 2002; Kotagale et al. 2013; Kuteeva et al. 2008). These behavioral effects observed in the FST were independent of the motor activity, since neither GAL, NPYY1 agonist nor the coadministration have shown locomotor alterations in different behavioral tasks (Narvaez et al. 2015).

Since the hippocampus participates in depressive-like behavior in the FST (Hiroaki-Sato et al. 2014; Bettio et al. 2014), our results indicate the specific participation of the DG. Within the DG subregions, colocalization of GALR2 and NPYY1R and the extensive presence of GALR2/NPYY1R heteroreceptor complexes were observed in the polymorphic layer and the subgranular region, where both receptors have previously been described (Depczynski et al. 1998; Paredes et al. 2003). GALR2/NPYY1R heteroreceptor complexes exist in several regions, including the amygdala (Narvaez et al. 2015). Thus, the integrated signalling of the GALR2/NPYY1R heteroreceptor complexes in the medial paracapsular nuclei of the amygdala enhanced the anxiolytic-related actions induced by the NPYY1 agonist and was linked to a decreasement of c-Fos IR at cellular level (Narvaez et al. 2015). Importantly, also in the current work, GALR2/NPYY1R heteroreceptor complexes in the DG

are correlated with the enhanced reduction of c-Fos profiles in the polymorphic layer. The mechanism for this interaction could be that the coactivation of the receptor protomers of the GALR2-NPYY1R heteroreceptor complex switches GALR2 from a Gq to a Gi/o coupling together with an enhancement of NPYY1 affinity (Narvaez et al. 2015). In this way, both GALR2 and NPYY1R become coupled to Gi/o and produce increased inhibition of AC-PKA-CREB pathway (Narvaez et al. 2015) and the decreased c-Fos IR observed in the hilus after the coadministration of GAL and NPYY1R agonist.

The increase of the number of c-Fos IR profiles observed in the granular cell layer after GAL and NPYY1 agonist coadministration may be mediated by a decreased activity on the polymorphic GABAergic interneurons in the hilus. In fact, the NPYY1 agonist induced an inhibition of GABAergic polymorphic nerve cells (Paredes et al. 2003), leading to a disinhibition and increased c-Fos expression in the granular cells of the DG (Andrews-Zwilling et al. 2012; Ledri et al. 2012). However, we could not exclude the involvement of projections from hilar mossy cell (MCs) to the granular cells in this effect, in fact, in vivo mossy cell axons excite inhibitory interneurons that inhibit activity in dentate granular cells and an inverse correlation in c-Fos IR profiles between MCs and granule cells has been described (Jinde et al. 2012; Duffy et al. 2013). Moreover, an indirect mechanism could also be involved, for example corticorterone induces a differential early decrease of c-fos, fosB and fra-1 transcripts in the DG compared to the other hippocampal subregions (Hansson and Fuxe 2008).

Previous reports show increased c-Fos expression in the granular cells of the DG after antidepressive therapies as acute imipramine administration (Li et al. 2013), reboxetine and mirtazapine combination (Masana et al. 2012) or running (Clark et al. 2011). Indeed, increased granular cell activity in the DG has been related with decreased immobility time in the FST (Jinde et al. 2012; Masana et al. 2012). This specific activation pattern-response within the DG

could explain the antidepressant-like behavior observed after the GAL and NPYY1 agonist coadministration.

Taken together, our study demonstrates the existence of an interaction between GALR2 and NPYY1R at the receptor level probably involving the formation of GALR2-NPYY1R heteroreceptor complexes in the DG, specifically in the polymorphic and subgranular subregions. This GALR2/NPYY1R interaction was linked to an enhanced antidepressive-like behavior involving specific cells populations within DG subregions. Therefore, our data offer a novel integrative anti-depressant mechanism based on the enhancing of a GALR2-NPYY1R interaction leading to increased Gi/o mediated signaling and increased NPYY1 recognition in the DG.

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Figure 1. Effect of Galanin after icv administration on the NPY Y1 receptor (NPYY1R) agonist [^{125}I]Leu^{31},Pro^{34}PYY binding in the dentate gyrus of the hippocampus. **(a)** Graphic showing values, expressed as mean ± SEM, of the specific binding of [^{125}I]Leu^{31},Pro^{34}PYY (25pM). Non-specific labelling (in the presence of 1 µM NPY) was digitally substracted from all readings. **P* <0.05 versus control group according to Student's t-test (n=6 per group) **(b)** Representative autoradiograms of [^{125}I]-[Leu^{31},Pro^{34}]PYY binding in the hippocampal dentate gyrus (Bregma -3.5 mm) showing a higher NPYY1R agonist binding following the icv administration of GAL 3nmol. Abbreviations: aCSF= Cerebrospinal fluid, showing basal binding of the NPY Y1 agonist [^{125}I]-Leu^{31}Pro^{34}PYY. GAL= Galanin.

Figure 2. Effect of Galanin after icv administration on the NPY Y1 receptor (NPYY1R) mRNA expression in the dentate gyrus of the hippocampus. **(a)** Graphic showing values, expressed as mean ± SEM, of the optical density of NPYY1R mRNA (probe concentration of 2,4pmol/µl, labelled with ^{33}P-dATP). ****P* <0.001 versus control group according to Student's t-test (n=6 per group).

(b) Representative autoradiograms from coronal sections of the rat brain (Bregma -3.5 mm) showing in the hippocampal dentate gyrus the increase of the NPYY1R mRNA expression following the intracerebroventricular administration of GAL 3nmol . Abbreviations: aCSF= Cerebrospinal fluid, showing basal optical density of the NPY Y1R mRNA. GAL= Galanin.

Figure 3. Detection of Galanin receptor 2 subtype (GALR2) and Neuropeptide Y Y1 receptor (NPYY1R) heteroreceptor complexes by *in situ* proximity ligation assay (PLA). **(a)** Red filled circle indicate the positive PLA regions mainly located in the polymorphic layer, but also in the subgranular zone of dentate gyrys (Bregma: -3.1mm). Blue filled circles indicate negative PLA signal in the molecular layer of dentate gyrus and corpus callosum) **(b-e)** Constitutive GALR2/NPYY1R heteroreceptor complexes are detected by *in situ* PLA (seen as red clusters) in the dentate gyrus but not in the corpus callosum **(f).** Magnified views from dashed boxes in Fig.3b and Fig.3c are shown in Fig.3c and Fig. 3d, e; respectively**.** Nuclei appear as blue in all panels and the white arrows indicate the red cluster formation (PLA signal).

Figure 4. Effect of Galanin and the Neuropeptide Y Y1 receptor (NPYY1R) agonist [Leu^{31}-Pro^{34}]NPY on depressive-related behaviors in the forced swimming test. The enhancement of the NPYY1R-mediated depressive-related behaviors by Galanin is blocked by M871, an antagonist of the Galanin receptor subtype 2 (GALR2). Graphics showing data, expressed as mean ± SEM, obtained in the forced swimming test: **(a)** immobility time, **(b)** time spent swimming, **(c)** time spent climbing. One-way ANOVA followed by Newman-Keuls multiple comparison test (n=6-8 animals in each group). In (a): ****P* <0,001 versus the rest of the groups; ≠≠≠*P* <0.001 versus aCSF and GAL+Y1 3nmol; a*P* <0.001 versus aCSF; ***P* <0.01 versus GAL+Y1 3nmol. In (b): ≠≠≠*P* <0.001 versus aCSF, GAL 3nmol, GAL+Y1 3nmol; ****P* <0,001 versus the rest of the groups. In (c): ****P* <0,001 versus aCSF; **P* <0.05 versus Y1 3nmol and

GAL+Y1+M871 3nmol. Abbreviations: GAL = galanin; Y1 = NPY Y1 receptor agonist [Leu^{31}-Pro^{34}]NPY; GAL+Y1 = coadministration of GAL and [Leu^{31}-Pro^{34}]NPY; GAL+Y1+M871 = coadministration of GAL, [Leu^{31}-Pro^{34}]NPY and GALR2 antagonist M871.

Figure 5. Effects of Galanin and NPY Y1 receptor (NPYY1R) agonist, alone or in combination with the antagonist M871 of the Galanin receptor subtype 2 (GALR2), on c-Fos expression in the polymorphic and granular layers of dentate gyrus. **(a-b)** Quantification of the total number of c-Fos IR nuclei within the polymorphic and granular layers. Data, expressed as mean ± SEM, show the differences between groups after the intracerebroventricular injection of aCSF, GAL, [Leu^{31}-Pro^{34}]NPY, or the coadministration of both peptides and M871. **(a)** 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 NPYY1R agonist coadministration is counteracted by M871.. **P*<0,05 versus aCSF; ≠*P*<0,05 versus aCSF, Y1 and GAL+Y1+M871; ****P*<0,001 versus Y1 and GAL+Y1+M871; ≠≠≠*P*<0,001 versus aCSF and GAL. **(b)** The coadministration of GAL and the NPYY1R agonist increased the c-Fos expression compared with both peptides alone and the aCSF group. Moreover, the effect of GAL and NPYY1R agonist coadministration is counteracted by M871. **P*<0,05 versus GAL+Y1 3nmol; ***P*<0,01 versus GAL, Y1 and GAL+Y1+M871; ****P*<0,001 versus aCSF; ≠≠*P*<0,01 versus Y1 and GAL+Y1+M871. One-way ANOVA followed by Newman-Keuls multiple comparison test (n=4 in each group).**(c)** Representative photomicrograph illustrating the dentate gyrus subregions: polymorphic (Pol), granular (Gc) and molecular (Mol) layers (Bregma: -3.5mm). **(d-h)** Immunodetection for c-Fos nuclei in polymorphic (indicated with arrowheads) and granule cells (indicated with arrows). The coadministration of both peptides GAL and NPYY1R agonist **(g)** decreased the c-Fos expression in the polymorphic layer while it increased c-Fos in granular neurons

compared with the NPYY1R agonist alone **(f)**, Galanin alone **(e)** and the control group **(d)**. The effect of the coadministration of GAL and [Leu^{31}-Pro^{34}]NPY is counteracted by M871 **(h)**. Abbreviations: aCSF = cerebrospinal fluid; GAL = Galanin; Y1 = NPY Y1 receptor agonist [Leu^{31}-Pro^{34}]NPY; GAL + Y1 = coadministration of GAL and [Leu^{31}-Pro^{34}]NPY; GAL+Y1+M871= Coadministration of GAL, [Leu^{31}-Pro^{34}]NPY and GALR2 antagonist M871.

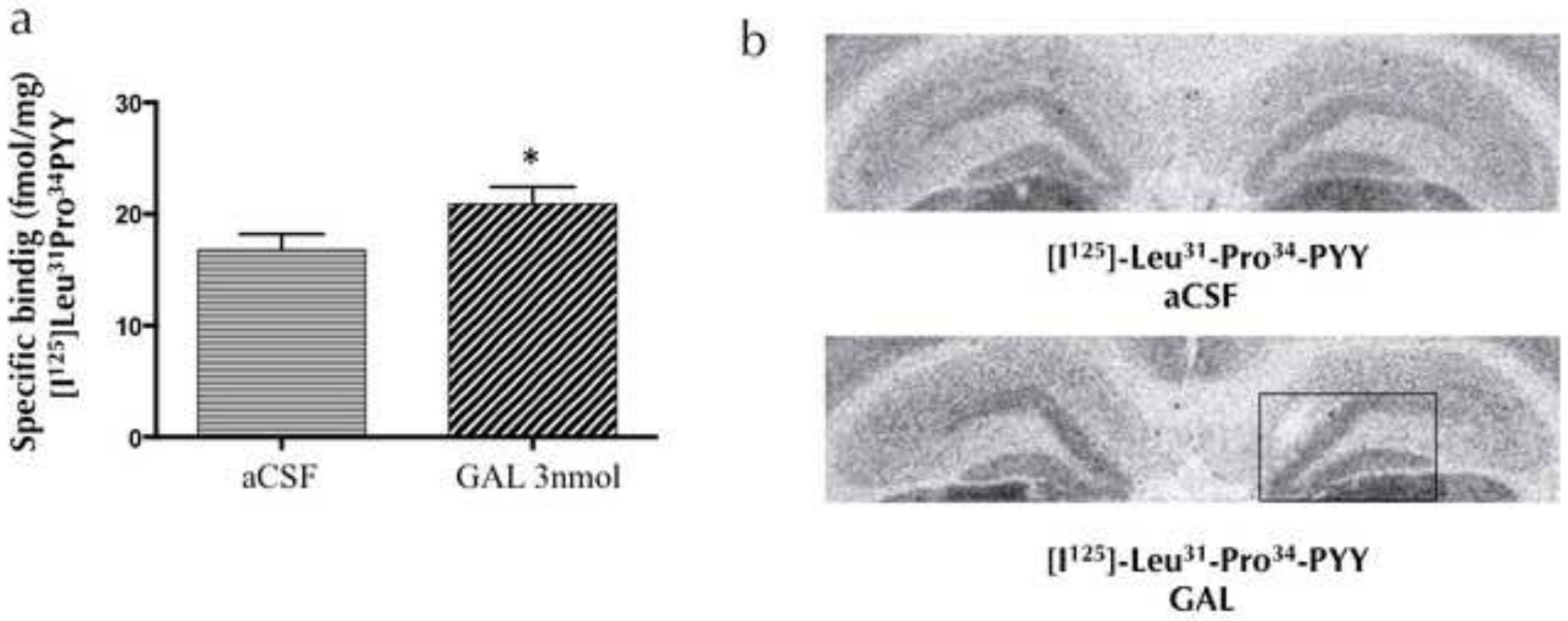

Figure 1.

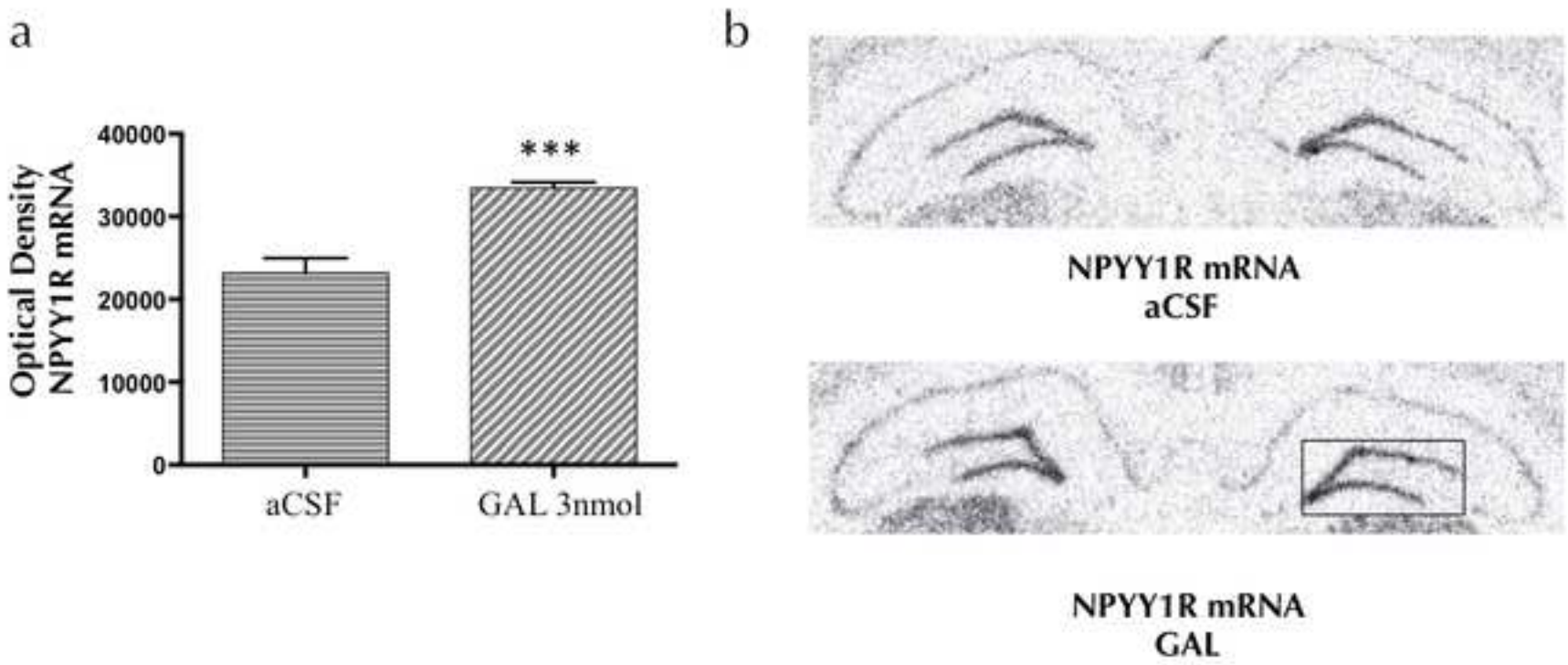

Figure 2.

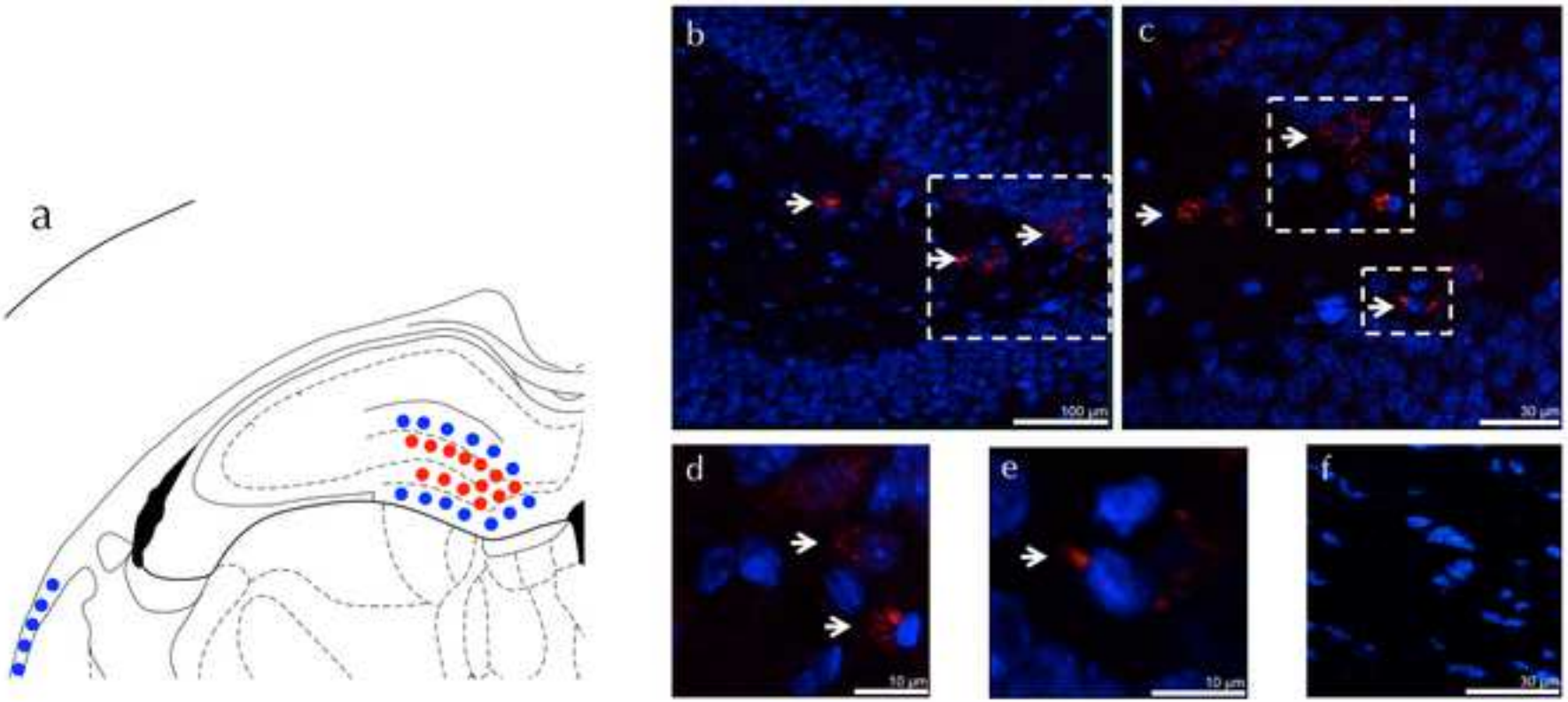

Figure 3.

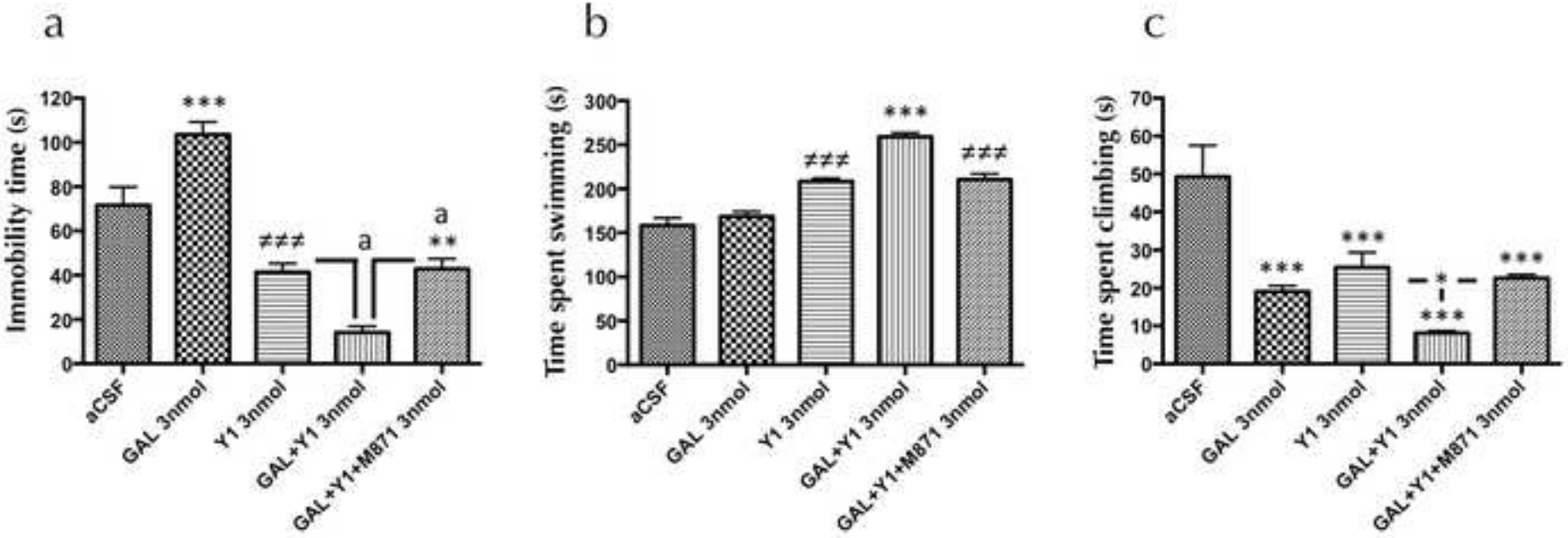

Figure 4.

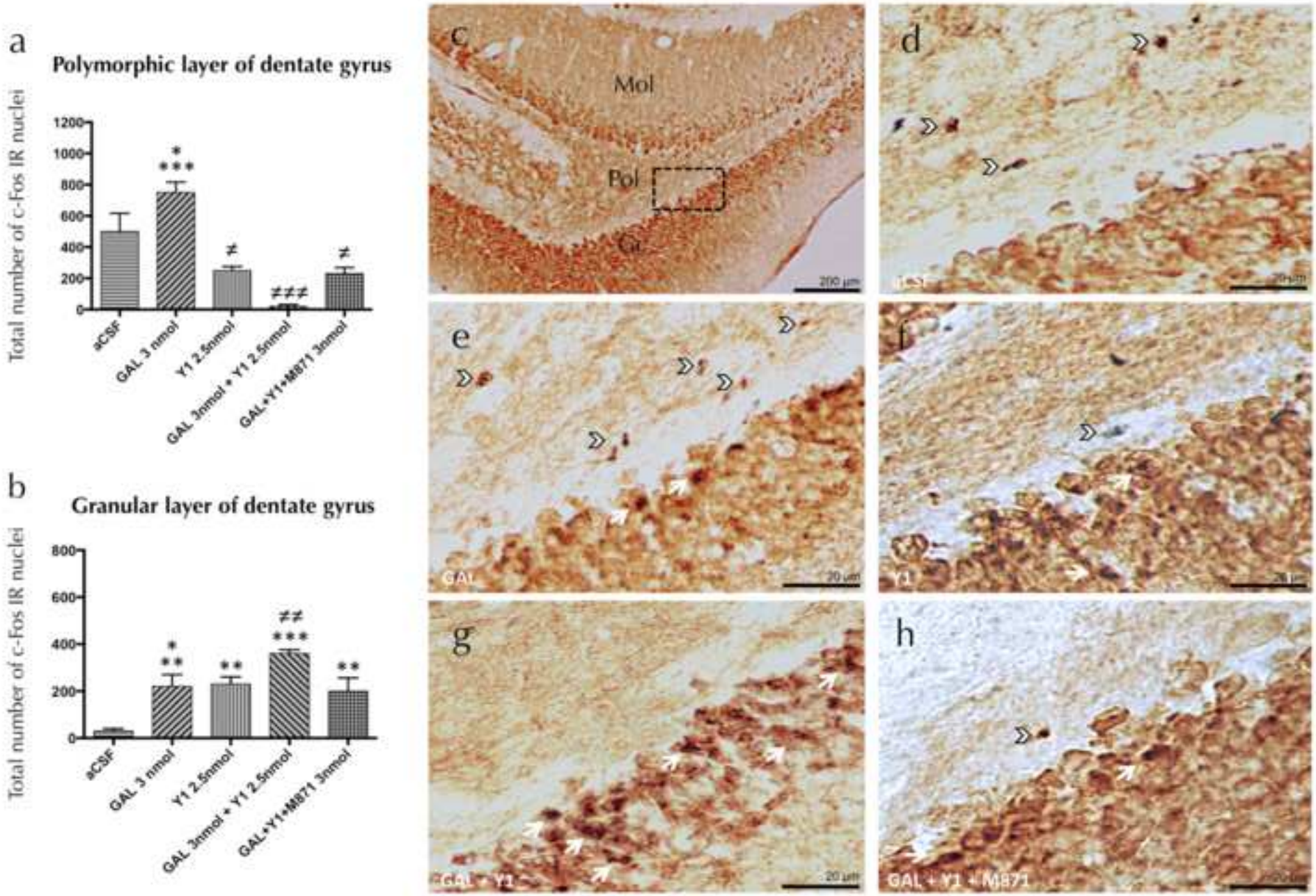

Figure 5.

Supplementary Information

Intracerebral cannulations

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 following stereotaxic coordinates: +1.4mm lateral, -1mm posterior to the bregma, and 3.6mm below the surface of the skull (Paxinos et al. 1985). After surgery, animals were individually housed and allowed recovery for 7 days. This method of cannulation and postsurgical care has been previously standarized (Diaz-Cabiale et al. 2011; Narvaez et al. 2015).

Intracerebroventricular administration of peptides

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. The GAL, NPYY1R agonist [Leu^{31},Pro^{34}]NPY (K_i=0.39 nM for NPYY1R) and GALR2 antagonist M871 (Ki=13.1 and 420 nM for GALR2 and GALR1 respectively) were obtained from Tocris Bioscience (Bristol, UK). Experimental groups and the n size of each group is indicated in the different procedures. After the experiments, brains were removed, testing the placement of the cannula for icv injection by cutting the brain in the coronal plane in a Cryostat (HM550, Microm International). These procedures of intracerebroventricular (icv) injections and preparation of artificial cerebrospinal fluid (aCSF) have already been standarized in our laboratory (Diaz-Cabiale et al. 2011; Narvaez et al. 2015).

Image analysis of Autoradiography and *in situ* hybridization

The autoradiograms from the receptor autoradiography and in situ hybridization experiments were analysed as described previously (Parrado et

al. 2007; Razani et al. 2001) using a computer-assisted image analysis system. Briefly, measurements using the ImageJ system (NIH, USA) were made bilaterally in the distinctly labelled region within the DG of the hippocampus (0.15 mm^2 square). One observation per region and rat was obtained, since the average of the measurements was calculated. Duplicate sections were measured for autoradiography and triplicate sections for *in situ* hybridization for each treatment. Prefabricated ^{125}I-labeled polymer strips (Amersham Microscale, Amersham, Little Chalfont, UK) were used to convert grey values into femtomol/milligram protein values. The semiquantitative evaluations of the in situ hybridization autoradiograms were analysed from the measured values of the specific optical density (O.D.).

c-Fos immunohistochemistry stereological analysis

An Olympus BX51 microscope (Olympus, Denmark) was interfaced with a computer and a colour JVC digital video camera. For stereological analysis, sampling of c-Fos positive cells was performed throughout the dentate gyrus region (DG) in the rostrocaudal dimension using the optical fractionator. This method combines the optical dissector with a fractionator sampling scheme to exclude volume divergences (Gundersen et al. 1988). Counterstaining with phase contrast and calbindin-immunoreactive hippocampal granule cells allowed delineation of the DG area in each section (Paxinos et al., 1985) Sections were sampled every 150 μm, starting at the ventral part of the DG (approximately 2.12 mm posterior to Bregma). Sampling ended at approximately 4.52 mm posterior to Bregma at the dorsal part of the DG. Numbers of c-Fos IR were quantified in at least five representative 150 μm, evenly-spaced sections per animal (4 rats per group). A random set of sampling frames with a known area (α frame) was generated for each section using the C.A.S.T. Grid (Olympus; Albertslund, Denmark). After the objects were counted (ΣQ-) the total number of positive cells were estimated as: $N = \Sigma Q^- \times fs \times fa \times fh$ (Gundersen et al. 1988), where fs is the numerical

fraction of the section used, fa is the areal fraction and fh is the linear fraction of section thickness. The coefficient of error (CE) for each estimation and animal ranged from 0.05 to 0.1. The total CE of each group ranged from 0.07 to 0.08. Counting of labelled neurons was set starting at 5 μm below the surface and focusing through the 20 μm section optical plane. At least five sections of each region were counted per animal. The number of counting frames used was 90-110 per animal in DG, representing the 20% of the whole analyzed volume.

Double immunofluorescence

The procedures have been previously used (Narvaez et al. 2015). Untreated rats (n=3) were perfused with 4% paraformaldehyde, brains were removed and sections at the DG level were obtained. An initial incubation with blocking (5% goat serum) and permeabilization (0.3% triton X100 in PBS) solutions were done during 60 min each. Primary antibody rabbit anti-GALR2 (Alomone Lab, 1:100) was incubated for 48-72 hours at 4ºC and detected with the red secondary antibody mouse anti-rabbit DyLight 549 (Jackson inmunoResearch Laboratories, 1:100). Sections were also incubated with goat anti-NPYY1R (sc-21992 Santa Cruz Biotecnology INC, EEUU, 1:200) in a similar manner as described above, which was 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 TCS-SL confocal microscope (Leica). These antibodies have been previously used in our laboratory (Millon et al. 2014; Narvaez et al. 2015).

c-Fos/GAD double immunolabelling

The procedures for c-Fos and Glutamate Decarboxylase (GAD) 65/67 double immunohistochemistry have been previously described (Narvaez et al. 2015).

The primary antibody used for GAD65/67 has been validated to detect GABAergic neurons (1:1000, sc-7513; Santa Cruz Biotechnology, CA) (Papay, 2006). In fact, GAD 65/67 has been used for labelling GABAergic neurons in rat dentate hilus and subgranular zone (Muller et al. 2001). The chromogen used was 3-3´-diaminobenzidine tetrahydrochloride (DAB) (Sigma, Spain) for GAD 65/67 immunostaining in order to get a brownish reaction. The c-Fos immunostaining (revealed with DAB plus nickel) has been described in the materials and methods section of this work. Sections were mounted on glass slides and microphotographs were obtained (Olympus BX51 microscope, Olympus, Denmark).

Cell culture, transfection and immunofluorescence

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% CO2. For transfection, cells were plated in 6-well dishes onto slides 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. Cells were transfected with 1µg of NPYY1R or NPYY5R and 48h post transfection cells were fixed in 4% paraformaldehyde for 10 min. Incubations with blocking (5% goat serum) and permeabilization (0.3% triton X100 in PBS) solutions were done during 30 min each. Cells were incubated with goat anti-NPYY1R (sc-21992 Santa Cruz Biotecnology INC, EEUU, 1:100) in a similar manner as described above, which was 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). Slides with fluorescent mounting medium (Dako) were visualized by using a TCS-SL confocal microscope (Leica). Anti-

NPYY1R staining in cell cultures transfected with NPYY1R or NPYY5R is shown in supplementary figure 3.

Supplementary figure 1. (a,b) Representative laser scanning confocal micrographs illustrating the polymorphic (Pol), granular (Gc) and molecular (Mol) layers of the dentate gyrus (Bregma: -3.1mm). Nuclei are shown in blue (DAPI). Laser scanning confocal micrographs demonstrating the coexpression of endogenous Neuropeptide Y Y1 receptor (NPYY1R) (green) and Galanin receptor subtype 2 (GALR2) (red) **(c)** Representative laser scanning confocal micrograph showing the colocalization of NPYY1R and GALR2 in a subpopulation of neurons (dashed circles) in the polymorphic and subgranular zone of the dentate gyrus in a magnified view from dashed box in Supp. Fig. 1b. **(d)** Colocalization of NPYY1R and GALR2 is shown in dashed circles in a magnified view from dashed box in Supp. Fig. 1c.

Supplementary figure 2. Pattern of c-Fos expression in GAD-positive neurons within the dentate gyrus subregions after the intracerebroventricular injection of the NPYY1R agonist [Leu^{31}-Pro^{34}]NPY. **(a)** Representative photomicrograph illustrating the dentate gyrus subregions: polymorphic (Pol), granular (Gc) and molecular (Mol) layers (Bregma: -3.5mm) **(b-c)** Magnified views from dashed boxes in Supplementary Fig.2a showing c-Fos positive nuclei in GABAergic cells of polymorphic and subgranular zone (as indicated with arrows) and examples of c-Fos positive nuclei in granular region of the dentate gyrus (as indicated with arowheads).

Supplementary figure 3. Representative laser scanning confocal micrographs showing anti-NPYY1R antibody staining in transiently transfected cell cultures. Using the anti-NPYY1R antibody, specific positive green signal was detected in HEK293 cells expressing NPYY1R **(a)** while no specific signal was detected in HEK293 cells expressing NPYY5R **(b)**.

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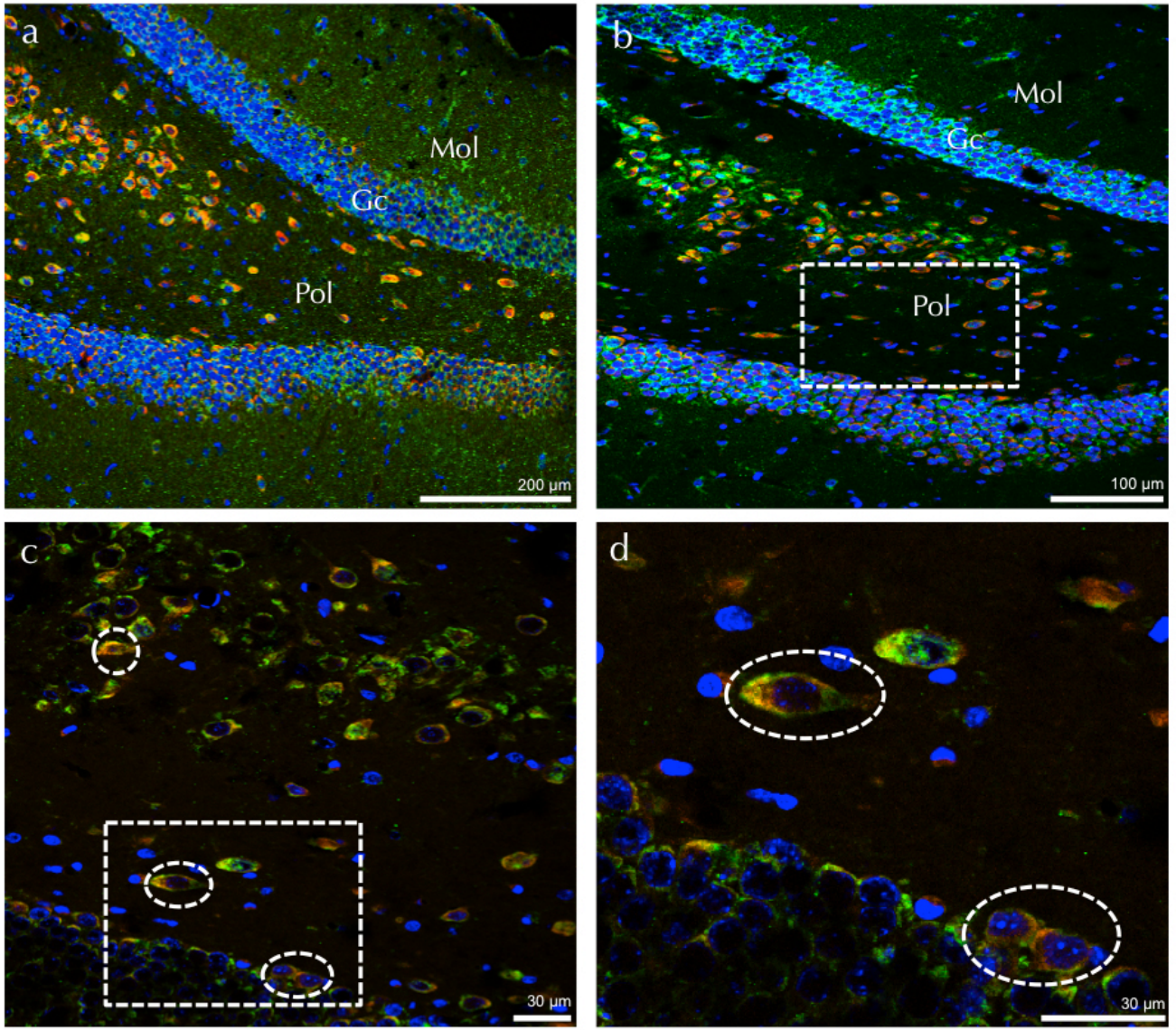
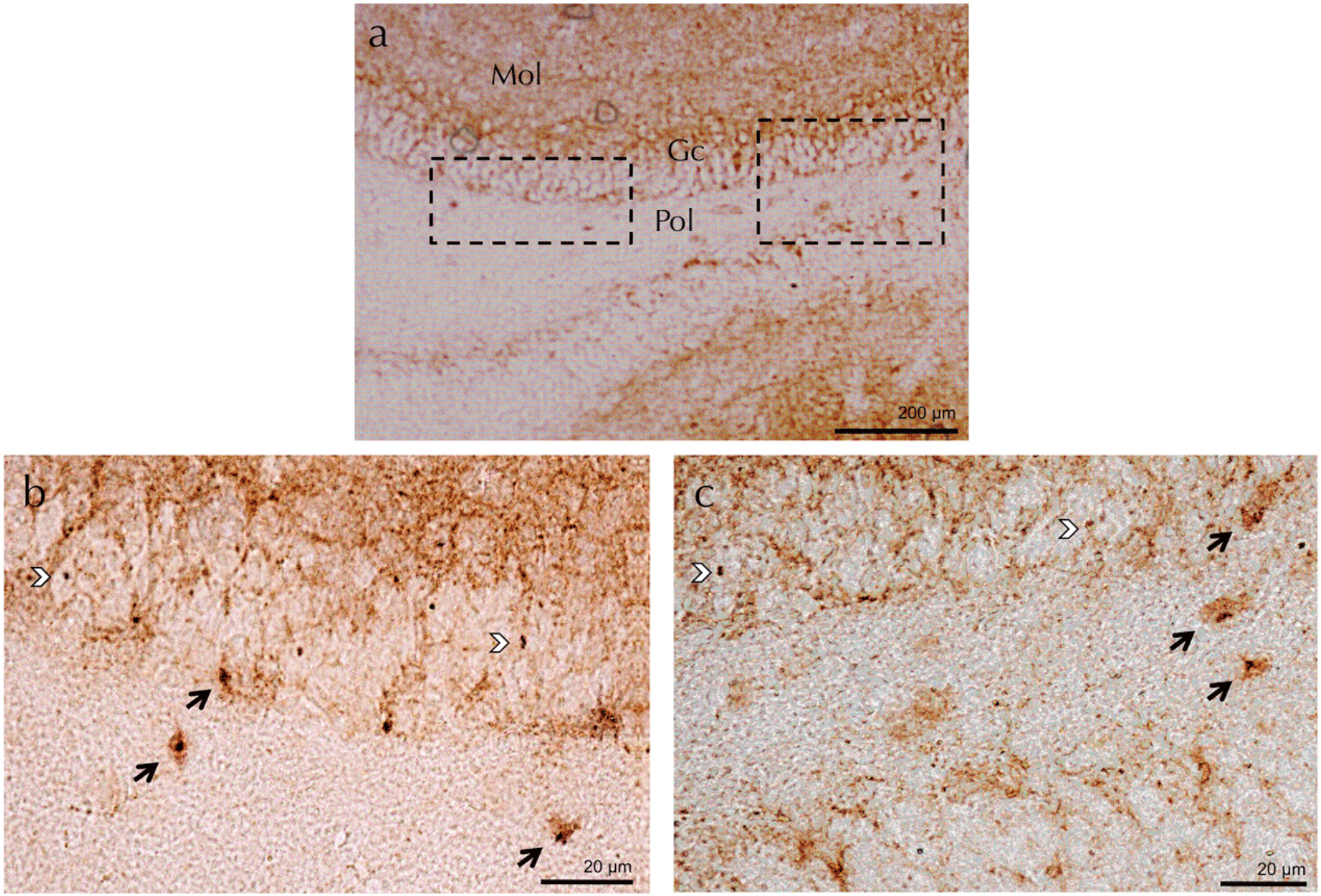
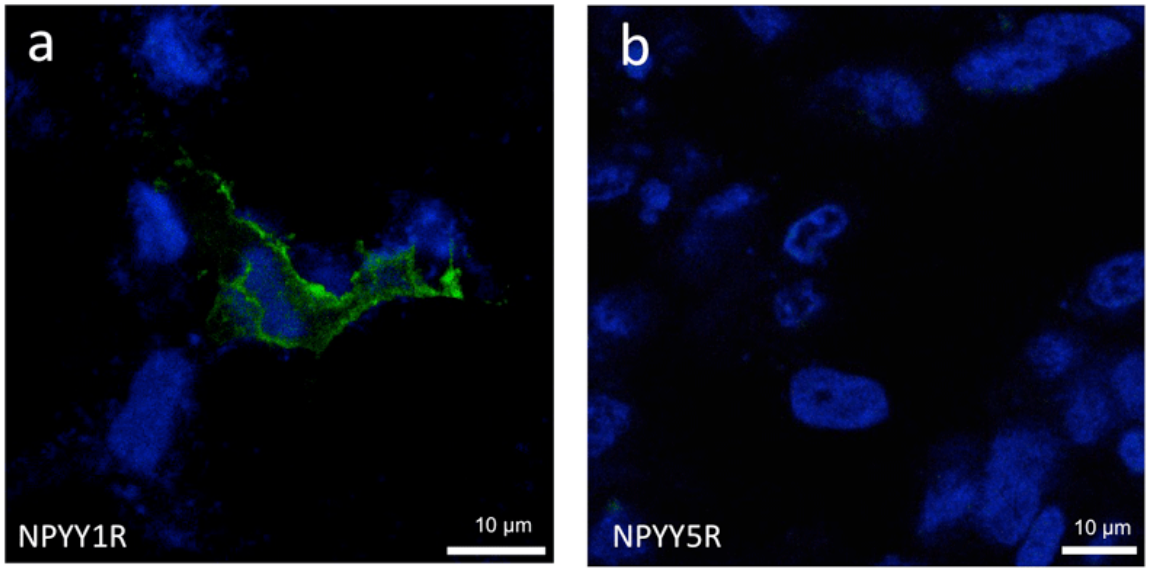


Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.