Galanin (GAL) and Neuropeptide Y (NPY) elicit antidepressant activity linked to neuronal precursor cells of the dentate gyrus in the ventral hippocampus through GAL 2/NPY Y1 receptor interactions.

Dasiel O. Borroto-Escuela 1,2,3; Mariana Pita 1,4,5; Ramón Fores1,4; Miguel A. Barbancho 4; Kjell Fuxe 1 and Manuel Narváez*1,4.

1 Department of Neuroscience, Karolinska Institute, Stockholm, Sweden,

2 Department of Biomolecular Science, Section of Physiology, University of Urbino, Urbino, Italy,

3 Grupo Bohío-Estudio, Observatorio Cubano de Neurociencias, Yaguajay, Cuba,

4 Instituto de Investigación Biomédica de Málaga, Facultad de Medicina, Universidad de Málaga, Málaga, Spain,

5 Departamento de Neurogenética. Instituto de Neurología y Neurocirugía. La Habana. Cuba.

*Correspondence:

Manuel Narváez, mnarvaez@uma.es

Introduction

According to the World Health Organization (WHO), major depressive disorder (MDD) is the most prevalent mood disorder and the leading cause of disability worldwide, with more than 300 million patients affected (WHO, 2017). MDD is defined by a constellation of behavioral, emotional and cognitive symptoms, and confer a challenge for the medical community. Traditional antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) or tricyclic antidepressants (TCAs) have several flaws, as their therapeutic efficacy is often limited due to side effects and delayed onset of effect, from weeks to months (Harmer et al., 2017). Furthermore, those who failed standard antidepressant treatment are regarded as having treatment-Resistant Depression (TRD), as studies determine that approximately 50% of such patients fail to respond to such treatment, and approximately 65% of them fail to achieve remission (Chen, 2019).

These drawbacks emphasize a need for new antidepressants that can produce a faster and more efficient therapeutic responses. For these reasons, the elucidation of the pathophysiology of MDD, as well as the search for novel antidepressants, are two of the foremost challenges in mental health research now, to expand the understanding of MDD and its treatment. Presently only electroconvulsive treatment and infusion of the NMDA receptor antagonist ketamine (DiazGranados et al., 2010) are available for TRD treatment.

MDD, as a heterogeneous disease, may have a contribution to its etiology from multiple neural regions, and the hippocampus has been one of the most broadly considered (Miller and Hen, 2015). The hippocampus exhibits a functional segmentation, where different neural pathways distribute to an anterior portion (ventral, in rodents) or a posterior portion (dorsal, in rodents). The dorsal hippocampus primarily establishes cognitive functions and participates in declarative memory, spatial navigation, and contextual learning (Fanselow and Dong, 2010; Tanti and Belzung, 2013). The ventral hippocampus plays a significant role in stress and modulation of emotional behavior. Immature adult-born neurons also present this ventrodorsal differentiation and, as a result, neurogenesis in the ventral hippocampus might actively participate in limbic functions and influence MDD pathophysiology (Baptista and Andrade, 2018).

Adult neurogenesis is a complex multistep process, occurring particularly in the

dentate gyrus, not in other hippocampal subregions. The precursor cells, from which adult neurogenesis originates, reside in a narrow band of tissue between the granule cell and the polymorphic layers, called the subgranular zone (SGZ). The first critical step is the precursor cell proliferation, followed by neuronal differentiation and cell survival to be included in the final steps. These three crucial elements of neurogenesis can be modulated, being subject to ‘‘control’’ and ‘‘regulation’’ by multiple factors (Kempermann et al., 2015). The existence of neurogenesis in humans was first demonstrated by Eriksson et al.(Eriksson et al., 1998). Their findings have been supported (Spalding et al., 2013; Boldrini et al., 2018; Moreno-Jimenez et al., 2019) but also questioned (Sorrells et al., 2018; Cipriani et al., 2018) by other studies. However, the limited availability of accurately preserved human brain tissue samples, together with the heterogeneity of tissue processing methodologies, is considered to have contributed to this disagreement (Kempermann et al., 2018).

The Role of hippocampal neurogenesis in mediating aspects of MDD is widely recognized with supporting pieces of evidence in rodents, non-human primates, and humans (Miller and Hen, 2015). As key regulators of cell precursors, the neuropeptides and their receptors have received special consideration, especially the NPY neuron system, as attractive therapeutic targets in emotional disorders, including depressive behaviors (Kormos and Gaszner, 2013; Zaben and Gray, 2013).

The Neuropeptide Y (NPY) is a 36 amino acid polypeptide neurotransmitter broadly distributed in the mammalian central nervous system, with high expression in several limbic regions. In the hippocampus, a subset of GABAergic interneurons in the polymorphic layer of the DG co-releases NPY, which innervates the granule cell layer mainly via volume transmission (VT) (Fuxe et al., 1991) , close to the SGZ (Sperk et al., 2007). NPY has a physiological role in modulating the granule cell excitability and enhances the proliferation of neuronal precursor cells both *in vitro* (Howell et al., 2003; Howell et al., 2007) and *in vivo* (Decressac et al., 2011). *In vitro* evidence suggests a proliferative effect, specifically involving the NPY Y1 receptor (NPYY1R), which is mediated by the extracellular-regulated kinases (ERK) 1/2 signaling (Cheung et al., 2012). *In vivo* administration of NPY validated its proliferative role and a preferential differentiation of newly generated cells towards a neuronal lineage trough NPYY1R (Decressac et al., 2011; Geloso et al., 2015).

Furthermore, increased NPY and NPYY1R expression in DG is related to decreased immobility in the forced swimming test (FST) in rats, implying the participation of the NPY-NPYY1R system in the pathophysiology of MDD (Catena-Dell'Osso et al., 2013). Conversely, genetic and experimental 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 a neuropeptide extensively distributed in the central nervous system. GAL effects on hippocampal precursor cells were not clarified until the in vitro demonstration that the GalR2/3 agonist galanin 2–11 produces proliferation of hippocampal precursor cells and appears to produce granule cell neurons of the dentate gyrus (Abbosh et al., 2011). In agreement, the GAL receptor 2 (GALR2) is widely expressed in the rat brain with the hippocampus present among the areas of the highest expression (Branchek et al., 2000). The activation of GALR2 leads to decreased immobility in the FST, and the increased expression of GALR2 in the ventral hippocampus is related to antidepressant effects (Kuteeva et al., 2008; Luo et al., 2019). In line with these results, GalR2-knockout mice manifest depression-like behaviors (Lu et al., 2008).

We have described several subtypes of GALR and NPYY1R interactions in distinct regions of the limbic system, with behavioral, region-specific, cellular and molecular correlations (Diaz-Cabiale et al., 2011; Narvaez et al., 2016; Narvaez et al., 2018; Narvaez et al., 2015). In the dentate gyrus of the dorsal hippocampus, a facilitatory GALR/NPYY1R interaction was shown, involving the formation of GALR2/NPYY1R heteroreceptor complexes. Moreover, the activation of GALR2 enhanced NPYY1R-mediated short-term antidepressant actions in the FST (Narvaez et al., 2016).

The purpose of the current research is to assess the sustained antidepressant effects induced by the coactivation of GALR2 and NPYY1R and their neurochemical correlates. Different behavioral determinations were conducted in the FST, together with the evaluation of cell proliferation through 5-Bromo-2'-deoxyuridine (BrdU) expression study within the dentate gyrus of the ventral hippocampus. We employed in situ proximity ligation assay (PLA) to manifest the formation of GALR2/NPYY1R heteroreceptor complexes and their dynamics under the different treatments. Additionally, the expression pattern of GALR2 was examined and the activation of the ERK pathway after GALR2 and NPYY1R co-stimulation in cell cultures.

Materials and Methods

Animals

Male Sprague-Dawley rats from CRIFFA (Barcelona; 200-250gr; 6-8 weeks) had free access to food pellets and tap water. They were maintained under the standard 12h dark/light cycle, with controlled temperature ($22^{\pm}2^{o}$C) and relative humidity (55-60%). All procedures concerned with housing, maintenance and experimental treatment of the rats were approved by the Local Animal Ethics, Care and Use Committee for the University of Málaga, Spain. Guidelines for animal experiments were carried out following EU Directive 2010/63/EU and Spanish Directive (Real Decretory 53/2013) recommendations. A detailed description of animal intracerebral cannulations is available in Supplement material.

Drugs used

Solutions were freshly prepared and the peptides were dissolved in artificial cerebrospinal fluid (aCSF, composition is (in mM) 120 NaCl, 20 NaH_2CO_3, 2 KCl, 0.5 KH_2PO_4, 1.2 $CaCl_2$, 1.8 $MgCl_2$, 0.5 Na_2SO_4, and 5.8 D-glucose, pH 7.4). Galanin (GAL), NPYY1 Receptor (NPYY1R) Agonist [Leu^{31},Pro^{34}]NPY, GALR2 Antagonist M871 and NPYY1R antagonist BIBP3226 were obtained from Tocris Bioscience (Bristol, UK). Detailed descriptions are available in Supplement material on intracerebroventricular (icv) administration of peptides.

Behavioral analysis

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).

Behavioral experiments were performed between 09:00 and 14:00 hours. 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. Doses for

GAL, the NPYY1R agonist [Leu^{31},Pro^{34}]NPY and for GALR2 antagonist M871 were chosen based on previous dose-response curves (Narvaez et al., 2015; Narvaez et al., 2016; Narvaez et al., 2018).

Swimming sessions were conducted by placing individually the rats in cylinders containing water (25±0.5°C), 30 cm deep. Two sessions were conducted: an initial 15 min pretest followed 48 h later by a 5-min test. Drugs were administered 24 hours before the test. The water in the cylinders was changed after every trial. The FST was performed according to a previously reported method (Koike and Chaki, 2014). The total duration of floating (immobility), swimming, and climbing periods were scored during the 5 min test, and analyzed using the Raton Time 1.0 software (Fixma S.L., Valencia, Spain). Floating in the water without struggling and only making movements necessary to keep its head above the water was regarded as immobility. 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. Behavioral experiments were carried out by observers blinded to all experimental conditions.

Evaluation of Hippocampal Cell Proliferation

For analysis of BrdU-positive cells, a different set of rats was administered 2 injections of 5′-Bromo-2′-deoxyuridine (BrdU, cat. no. B5002, Sigma, St. Louis, MO, USA) dissolved at 15 mg/mL in a sterile 0.9% NaCl solution. BrdU was administered intraperitoneally (i.p.) during the ad libitum feeding period at a dose of 50mg/kg body weight (every 2h after the icv treatments, starting at 9:00 AM). Twenty-four hours after the icv injection, rats were deeply anesthetized with pentobarbital (Mebumal; 100 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde (wt/vol, Sigma). Brains were coronally sliced (30 µm-thick) through the ventral hippocampus (anterior in primates) (-5.20 to -6.72 Bregma; Paxinos and Watson, 1998).

Animals were divided into five experimental groups: (1) aCSF: control group; (2) GAL- treated group (3nmol); (3) Y1-treated group receiving an NPYY1R agonist [Leu^{31},Pro^{34}]NPY (3nmol); (4) GAL+Y1: group administered with both substances; (5) GAL+Y1+M871: group injected with GAL, [Leu^{31},Pro^{34}]NPY and the GALR2 antagonist (M871; 3nmol) (N=4 in each group). Doses indicated above (Narvaez et al., 2015; Narvaez et al., 2016; Narvaez et al., 2018) and the BrdU procedure (Abrial

et al., 2014; Pilar-Cuellar et al., 2012) are based on previously published protocols.

Immunohistochemistry

Free-floating sections were incubated in saline sodium citrate buffer (pH 6; 10nM sodium citrate) for 90min at 65°C, followed by 30 min with 0,6% H2O2 to eliminate endogenous peroxidases. After 30 min in 2 M hydrochloric acid (HCl) to denature deoxyribonucleic acid (DNA), sections were incubated for neutralization with 0.1M sodium borate (pH 8). Then, sections were incubated at 4◦C overnight with a primary antibody against BrdU (1:1000, Abcam, USA) in 2,5% donkey serum. Sections were then washed with PBS and incubated with a secondary antibody for 90min (biotinylated anti-rabbit IgG, 1/200, Vector Laboratories), followed by amplification with ExtrAvidin peroxidase (Sigma, St. Louis, MO) diluted 1:1000 in darkness at room temperature for 1h. Immunolabeling was revealed with 0.05% diaminobenzidine (DAB; Sigma) and 0.03% H2O2 in PBS. After several rinses, sections were mounted on gelatin-coated slides, dehydrated in graded alcohols, and cover-slipped in DePeX mounting medium (VWR).

Counting Procedure

The number of BrdU-labeled cells was quantified with a light microscope (Olympus BX51 microscope, Olympus, Denmark) at 40x magnification on a series of every sixth section of the ventral hippocampus. About 5 sections in the ventral hippocampus were examined per animal. BrdU-labeled cells were counted through the rostrocaudal extensión, and the quantification was limited to the granular cell layer (GCL) and subgranular zone (SGZ),. The latter region was defined as a band-limited by three nuclei down from the border between the GCL and the hilar region (H), and cells that were located more than two cells away from the SGZ were omitted. To obtain the estimated total number of BrdU-labeled cells the resulting number of positive cells was multiplied by six (Pilar-Cuellar et al., 2012; Vega-Rivera et al., 2015).

In situ proximity ligation assay

To study the GALR2-NPYY1R heteroreceptor complexes the *in situ* proximity ligation assay (*in situ* PLA) was performed as described previously (Borroto-Escuela et al, 2016; Fuxe and Borroto-Escuela, 2018). Treated rats were divided

into experimental groups: (1) aCSF: control group; (2) GAL-treated group (3nmol); (3) Y1- treated group receiving an NPYY1R agonist [Leu^{31},Pro^{34}]NPY (3nmol); (4) GAL+Y1: group administered with both substances; (5) GAL+Y1+M871: group injected with GAL, [Leu^{31},Pro^{34}]NPY and the GALR2 antagonist (M871; 3nmol). (n=4 in each group). Animals were perfused with 4% paraformaldehyde 24 hours after icv injections, brains were removed and sections were obtained.

Free-floating sections were washed four times with PBS and quenched with 10 mM Glycine buffer for 20 min at room temperature. Then, after three PBS washes slices were permeabilized with a permeabilization buffer (10% fetal bovine serum (FBS) and 0.5% Triton X- 100 in Tris buffer saline (TBS), pH 7.4) for 30 min at room temperature. Again, the sections were washed twice, 5 min each, with PBS at room temperature and incubated with the blocking buffer (0.2% bovine serum albumin (BSA) in PBS) for 30 min at room temperature. The brain sections were then incubated with the primary antibodies (GALR2 rabbit, Alomone Lab, 1:100; NPYY1R goat, sc-21992 Santa Cruz Biotechnology INC, CA, 1:200) diluted in a suitable concentration in the blocking solution at 4 °C overnight. Then, the sections were washed twice, and the proximity probe mixture (Duolink PLA probe anti-mouse MINUS and Duolink PLA probe anti-rabbit PLUS, Sigma-Aldrich, Stockholm, Sweden) was applied to the sample and incubated for 1 h at 37 °C in a humidity chamber. The unbound proximity probes were removed by washing the slides twice, 5 min each time, with blocking solution at room temperature under gentle agitation and the sections were incubated with the hybridization-ligation solution (BSA, 250 g/ml), T4 DNA ligase (final concentration of 0.05 U/µl), 0.05% Tween-20, 250 mM NaCl, 1 mM ATP and the circularization or connector oligo- nucleotides (125–250 nM)) and incubated in a humidity chamber at 37 °C for 30 min. The excess of connector oligonucleotides was removed by washing twice, for 5 min each, with the washing buffer (Sigma-Aldrich, Duolink Buffer A (8.8 g NaCl, 1.2 g Tris Base, 0.5 ml Tween 20 dissolved in 800 ml high purity water, pH to 7.4) at room temperature under gentle agitation and the rolling circle amplification mixture (Duolink

amplification red, DUO82011, Sigma-Aldrich, Stockholm, Sweden) was added to the slices and incubated in a humidity chamber at 37 °C for 100 min. Then, the sections were incubated with the detection solution in a humidity chamber at 37 °C for 30 min. In a last step, the sections were washed twice in the dark, for 10 min each, with the washing buffer (Sigma-Aldrich, Duolink Buffer B (5.84 g NaCl, 4.24 g Tris Base, 26.0 g Tris-HCl.) dissolved in 500 ml high purity water, pH 7.5) at room temperature under gentle agitation. The free-floating sections were put on a microscope slide and a drop of appropriate mounting medium (Duolink Mounting Medium, Sigma-Aldrich) was applied. The coverslip was placed on the section and sealed with nail polish. The sections were protected against light and stored for several days at −20 °C before confocal microscope analysis. The mounting medium contained DAPI. DAPI (4′ ,6-diamidino-2-phenylindole) is a blue-fluorescent DNA stain that exhibits ~20-fold enhancement of fluorescence upon binding to AT regions of dsDNA. It is excited by the violet (405 nm) laser line and is commonly used as a nuclear counterstain in fluorescence microscopy.

The negative control consists in the omission of the species-specific primary antibody corresponding to the GALR2 in the presence of the two PLA probes. As a positive control of the PLA assay, a parallel analysis of the 5-HTR1A-5HTR2A isoreceptor complexes has been performed as previously documented (Borroto-Escuela et al., 2017). *In situ* PLA image acquisition and data analysis were performed as previously described (Narvaez et al., 2020).

Cloning of GALR2-GFP^2, Cell culture and Transfection

GALR2-GFP^2 was made using standard molecular biology techniques employing PCR and fragment replacement strategies. Human GALR2 coding sequences without their stop codons were amplified from GALR2-pcDNA vectors using sense and antisense primers harboring unique and sites and fragments were subcloned in-frame into humanized GFP^2 vector (PerkinElmer, Waltham, MA, USA) (Borroto-Escuela et al., 2014).

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. Cells were transfected with 1µg of GALR2-GFP^2 and NPYY1R.

GALR2-GFP^2 internalization using Confocal Microscopy

Twenty-four hours before imaging, the FBS growth media was removed and replaced with serum-free media (Cellgro-Free, Mediatech). GALR2-GFP^2/NPYY1R HEK293T coexpressing cells were incubated with GAL 1µM and/or NPY 1µM, at different times. Antagonist studies were performed 15 min before the addition of agonist with NPYY1R antagonist BIBP3226 10µM. 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) as previously described (Narvaez et al., 2015).

GALR2-GFP^2 endosomes seen as green fluorescent molecules were excited with a krypton/argon laser at 488nm 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. The percentage of internalization was determined by Leica software analysis of total membrane fluorescence compared to total internal compartment fluorescence at the various time points (Gehlert et al., 2007; Narvaez et al., 2015).

Luciferase Reporter Gene Assay

Dual-luciferase gene reporter assay was used to indirectly detect activation of extracellular signal-regulated kinases (ERK) pathway in transiently transfected

HEK293T cells treated with different compounds in a range of concentrations (typically 25 nM to 1 µM). (Borroto-Escuela et al., 2010; Narvaez et al., 2015).

For luciferase assays, 24 h before transfection, cells were seeded at a density of 1 × 10^6 cells/well in 6-well dishes and transfected with Fugene HD. Cells were co-transfected with plasmids corresponding to three constructs as follows (per 6-well): 1 µg firefly luciferase-encoding experimental plasmid (pGL4-SRE-luc2p; Promega, Stockholm, Sweden), 1µg of NPYY1R and/or GALR2 and 50 ng Renilla luciferase-encoding internal control plasmid (phRG-B; Promega). Approximately 36 h post-transfection, after the cells were treated for 4h with appropriate ligands (GAL 50nM; GAL 100nM; NPY 50nM; BIBP3226 1µM; M871 1µM) and harvested with passive lysis buffer (Promega), the luciferase activity of cell extracts was determined using a luciferase assay system according to the manufacturer's protocol 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 mean ± SEM and sample number (n) is indicated in figure legends. All data were analyzed using GraphPad PRISM 8 .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

Antidepressant-like behavior profile induced in the forced swimming test by

GALR2/NPYY1R interaction.

In the forced swimming test (FST), rats were pre-exposed to water for 15 minutes. Twenty-four hours after the intracerebroventricular (icv) administration, 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 at 3 nmol decreased the time of immobility (one-way ANOVA, $F_{4,30} = 53.62$, $p<0.001$, Newman-Keuls post-hoc test: $p<0,001$; Figure 1a) and the time of climbing behaviour (one-way ANOVA, $F_{4,30} = 13.97$, $p<0.001$, Newman-Keuls post-hoc test: $p<0,01$; Figure 1 c) compared with control animals. An increased time of swimming (one-way ANOVA, $F_{4,30} = 16.89$, $p<0.001$, Newman-Keuls post-hoc test: $p<0,05$; Figure 1 b) was observed after NPYY1R agonist injections.

In contrast, a significant increase in the immobility time (Newman-Keuls post-hoc test: $p<0.05$; Figure 1 a) was observed after the icv GAL administration at 3 nmol compared with control animals. A decreased time in climbing behavior (Newman-Keuls post-hoc test: $p<0,001$; Figure 1 c) was observed after GAL injection alone.

However, a specific increase in time reduction was observed after the coadministration of GAL and the NPYY1R agonist in the immobility (Newman-Keuls post-hoc test: $p<0,01$; Figure 1 a) and climbing behavior (Newman-Keuls post-hoc test: $p<0,05$; Figure 1 c), while an increased time in the swimming behavior (Newman-Keuls post-hoc test: $p<0,001$; Figure 1 b) compared with the NPYY1R agonist alone.

The involvement of GALR2 in this interaction was validated since the presence of the GALR2 antagonist M871 counteracted the enhancement of both, decreased immobility (Newman-Keuls post-hoc test: $p<0,01$; Figure 1a) and climbing time behavior (Newman-Keuls post-hoc test: $p<0,05$; Figure 1 c), as well as blocked the increased swimming time (Newman-Keuls post-hoc test: $p<0,001$; Figure 1 b) induced by the coadministration of GAL and NPYY1R agonist in the FST.

GAL and NPYY1R agonist coadministration increased cell proliferation in the ventral hippocampus

To investigate the neurophysiological mechanisms associated with the behavioral effects, we evaluated the impact of GAL and NPYY1R agonist coinjection on adult ventral hippocampal cell proliferation, by using the thymidine analog 5-Bromo-2'-deoxyuridine (BrdU).

The icv injection of the NPYY1R agonist alone induced an increase in the number of BrdU-IR profiles in the subgranular zone (Sgz) of the ventral hippocampus (one-way ANOVA, $F_{4, 15} = 75.37$, $p < 0.001$, Newman-Keuls post-hoc test: $p<0.001$) (Figure 2 b) compared with the control group. The injection of GAL alone lacked effects on the BrdU-IR profiles (Figure 2 b) compared with the aCSF control group (Figure 2 b, c).

However, GAL and NPYY1R agonist coinjection significantly increased the number of BrdU-IR profiles specifically in the Sgz of the ventral hippocampus compared with NPYY1R agonist alone (Newman-Keuls post-hoc test: $p<0.001$) (Figure 2 b, d). The cotreatment with the GALR2 antagonist M871 completely reversed the GAL contribution to the NPYY1R agonist response in the ventral hippocampus (Newman-Keuls post-hoc test: $p<0.001$) (Figure 2 b), demonstrating the involvement of GALR2 in the GAL/NPYY1R agonist interaction to promote cell proliferation.

GALR2/NPYY1R heteroreceptor complexes increase within ventral hippocampal dentate gyrus upon agonist coactivation of GALR2 and NPYY1R and their relationship to Doublecortin-expressing cells.

To analyze the cellular mechanism related to the observed effects on cell proliferation, we performed in situ proximity ligation assay (PLA) on the ventral hippocampal dentate gyrus (DG), studying the GALR2/NPYY1R heteroreceptor complexes formation after GAL and/or NPYY1R agonist administration.

PLA-positive red clusters were found specifically in the subgranular zone and the polymorphic layer of the ventral DG (Figure 3 a). Quantification of PLA demonstrated an increase in the density of the PLA-positive red clusters after NPYY1R agonist injection compared to control (one-way ANOVA, $F_{4, 15} = 11.76$, $p < 0.001$, Newman-Keuls post-hoc test: $p<0.05$) or GAL group (Newman-Keuls post-hoc test: $p<0.05$)

(Figure 3 b,c-d). Moreover, the coinjection of GAL and NPYYR1 agonist significantly increased (Newman-Keuls post-hoc test: $p<0.05$) the number of PLA-positive red clusters in the ventral hippocampal DG (Figure 3 b,d-e) compared with NPYYR1 agonist alone. Similarly to the BrdU-IR profiles response described above, the presence of the GALR2 antagonist M871 completely blocked this increase (Newman-Keuls post-hoc test: $p<0.05$) (Figure 3 b), demonstrating the involvement of GALR2 in this interaction. The signal specificity was validated with no PLA clusters observed in the molecular layer of the DG, an area that seems to lack GALR2 (O'Donnell et al., 1999).

Furthermore, to study the specific cell population potentially involved in the results described above, we analyzed the neuroblast-expressing marker Doublecortin (Supplementary figure 1). We found a specific co-localization of GALR2 (Supplementary figure 1a) and NPYY1R (Supplementary figure 1b) with Doublecortin-expressing cells. Unexpectedly, GALR1 did not colocalize with Doublecortin-expressing cells, suggesting a specific role for GALR2/NPYY1R interaction on the neuroblasts located in the DG of the ventral hippocampus.

GALR2 and NPYY1R co-activation increased GALR2 availability on the plasma membrane and enhanced SRE/MAPK pathway signaling in cell cultures.

To study the cellular and molecular mechanisms involved in GALR2-NPYY1R effects we examined GALR2-GFP^2 expression and the activation of the mitogen-activated protein kinase (MAPK) pathway in HEK 293T cell cultures.

We analyzed the GAL/NPY-induced GALR2 internalization by immunofluorescence microscopy in transiently co-transfected HEK 293T cells with GALR2-GFP^2 and NPYY1R. HEK 293T cells coexpressing both receptors were incubated in the presence of GAL with or without NPY at 37º C for 36 hours to monitor receptor membrane expression (Figure 4).

The presence of GALR2-GFP^2 expression in the plasma membrane but also in the intracellular compartment indicated constitutive GALR2 internalization in basal state

without agonist addition (Supplementary Figure 2). The addition of GAL induced a rapid internalization of GALR2-GFP^2 expression, which slowly recovered during the 36 hours measuring period (Figure 4 a, d). However, combined treatment with GAL and NPY induced a GALR2-GFP^2 recruitment, moving up towards the plasma membrane. We observed a maximal effect at 30 minutes, compared to internalized GALR2-GFP^2 after GAL alone ($t = 7.842$, $p<0,001$, $df = 21$) (Figure 4 a,b,d). This effect was sustained for 24 hours ($t = 2.240$, $p<0,05$, $df = 20$) (Figure 4 d). At this time, GALR-GFP^2 expression was greater in the plasma membrane after GAL and NPY co-stimulation, compared to GAL alone ($t = 4.213$, $p<0,001$, $df = 21$) (Figure 4 a,b,d). The specific NPYY1R antagonist BIBP3226 abolished this recruitment of GALR2-GFP^2 expression (Figure 4 c,d), demonstrating that this effect was mediated through the coactivation of GALR2 and NPYY1R.

The extent of phosphorylation of extracellular signal-regulated kinases (ERK1/2) was studied upon the coactivation of GALR2 and NPYY1R using the principle of the SRE-luciferase gene reporter assay, in transiently transfected HEK293T cells co-expressing both receptors.

Treatment with NPY (50 nM) or GAL (100 nM) leads to a significant and similar increase in the mitogen-activated protein kinase (MAPK) pathway that subsequently leads to SRE activation in cells co-expressing GALR2 and NPYY1R receptors (Figure 5 a) (one-way ANOVA, $F7, 56 = 89.25$, $p < 0.001$, Newman-Keuls post-hoc test: $p<0.001$). These effects are fully counteracted by the corresponding selective antagonists, M871 (1 μM) for GALR2 and BIBP3226 (1 μM) for NPYY1R (Newman-Keuls post-hoc test: $p<0.001$) (Figure 5 a). GAL at 50 nM lacks effects on the SRE response.

Combined treatment with a threshold dose of GAL (50nM) and NPY (50nM) leads to a significant increment of SRE activity compared with NPY alone (Newman-Keuls post-hoc test:$p<0.001$). Furthermore, the effective dose of GAL (100nM) in combination with NPY (50nM) induce a significant increment of SRE activity compared with the observed when stimulated by GAL or NPY alone (Newman-Keuls post-hoc test: $p<0.001$) (Figure 5 a).

With these results we can propose a schematic diagram with possible direct and indirect interactions between GALR2-NPYY1R heteromer and RTK, known to be inter alia linked to the MAPK pathway (McKay and Morrison, 2007; Cheung et al., 2012; Luo et al., 2019). It can involve the Gβγ subunits modulating the activity of the Ras–Raf–MEK1–MAPK pathway to the SRE (Figure 5 b). The presence of an enhanced interaction in SRE activity upon coactivation of the two receptors suggests the existence of allosteric excitatory communication in the interface between the two receptors of the GALR2–NPYY1R heteroreceptor complex.

Taken together, the recruitment of GALR2 to the plasma membrane may allow the GALR2/NPPY1R complexes formation in neuroblastic cells in the ventral hippocampus. It allowed an excitatory modulation of the MAPK pathway to increase cellular proliferation and participation in the antidepressant effects observed.

Discussion

The current study demonstrates for the first time the existence of an interaction between GALR2 and NPYY1R in the dentate gyrus (DG) of the ventral hippocampus upon combined receptor agonist treatment. GAL was demonstrated to act via GALR2 to enhance NPYY1R-sustained effects at 24 hours in the forced swimming test (FST), reducing immobility and climbing time while increasing swimming time, that strongly support antidepressant actions. This antidepressant effect seems to be linked to the ability of the coagonist treatment to enhance the NPYY1R-mediated increase of the cell proliferation in the DG of the ventral hippocampus through 5-Bromo-2'-deoxyuridine (BrdU) expression analysis at 24 hours. This event may be determined by the increased presence of GALR2/NPYY1R heteroreceptor complexes, on Doublecortin-expressing neuroblasts, upon agonist coactivation of their two types of receptor protomers. Thus, it is proposed that the mechanism of how the GAL and NPY Y1 agonist co-injection underlies the antidepressant actions, is mediated by the increase of GALR2/NPYY1R heteroreceptor complexes leading to an enhancement of the neuronal proliferation in the ventral DG upon their receptor protomer coactivation. Accordingly, it was validated that boosting neural cell

proliferation in the hippocampal DG leads to antidepressants effects in rodents (Walker et al., 2015; Bauman et al., 2018).

The understanding of the cellular mechanisms was achieved by analyzing the GALR2 expression and the SRE–luciferase gene reporter response in transiently GALR2 and NPYY1R co-transfected HEK cells. Recruitment in the GALR2 expression to the plasma membrane was observed upon the coactivation of GALR2 and NPYY1R in the co-transfected HEK cells, presumably linked to the increase in GALR2/NPYY1R heteroreceptor complexes observed. These heteroreceptor complexes may allow enhanced effects on the activation of extracellular regulated Kinases (ERK) pathway, as observed in the SRE-luciferase assay after the agonist coactivation of GALR2 and NPYY1R.

We previously determined how GAL and the NPYY1 agonist co-injection induced a short-term antidepressant effect in the FST (Narvaez et al., 2016). Our current behavioral results demonstrated that GAL elicits a sustained antidepressant effect mediated by the NPYY1 agonist at 24 hours, reducing the immobility and increasing swimming behaviors in the FST. In agreement, other drugs have also been shown to exhibit antidepressant effects after a single injection in the FST in rats at 24h, like the N-methyl-d-aspartate receptor antagonist, Ketamine or the group II metabotropic glutamate (mGlu2/3) receptor antagonist, LY341495 (Koike and Chaki, 2014; Gigliucci et al., 2013). Such results agree with the FST results obtained in our laboratory, finding the antidepressant effects of GAL and the NPYY1 agonist at 24 h after the co-administration. Importantly, our results support the relevance of GALR2 as an enhancer of the NPYY1R agonist activity through an allosteric enhancement of the receptor-receptor interactions within the GALR2/NPYY1R complex. Evidence was also found that the GALR2 antagonist M871 counteracted the enhanced response observed, as previously reported (Narvaez et al., 2015; Narvaez et al., 2016; Narvaez et al., 2018).

Furthermore, the effects achieved after the administration of GAL or NPYY1 agonist alone agree with the previous studies and support the pro-depressant role of GAL and the antidepressant actions of the NPYY1 agonist in the FST (Redrobe et al., 2002; Kuteeva et al., 2008; Kotagale et al., 2013). These behavioral effects observed

in the FST were independent of the motor activity since neither GAL and NPYY1 agonist nor their coadministration has shown locomotor modifications (Narvaez et al., 2015; Narvaez et al., 2016; Narvaez et al., 2018).

This behavioral effect may be linked to the increase of neuronal precursor cell proliferation in the ventral hippocampus observed through BrdU analysis, the precursor cells enriched in GALR2/NPYY1R heteroreceptor complexes. Multiple pieces of evidence in different species, including humans, established that neuronal cell proliferation is: altered in depression; impaired sufficiently to induce depression; modified by treatments for depression; required for antidepressant efficacy; increased to sufficiently treat depression (Miller and Hen, 2015). The rise of cell proliferation observed 24 hours after NPYY1 agonist injection is consistent with the findings of Decressac et al.,(Decressac et al., 2011), which demonstrated that the NPY administration increased dentate gyrus cell proliferation via NPYY1R activation. Furthermore, GAL alone was shown to induce neuronal cell proliferation *in vitro* exclusively through GAL2-11, a GALR2/3 agonist (Abbosh et al., 2011), in line with the lack of GAL to induce cell proliferation *in vivo*. Importantly, we observed that GAL, via GALR2, enhanced the cell proliferation induced by the NPYY1 agonist in the dentate gyrus of the ventral hippocampus. In agreement, the molecule P7C3, known to have neuroprotective activity, was shown to increase the neuronal cell proliferation associated with antidepressants effects in rodents and primates (Walker et al., 2015; Bauman et al., 2018).

GALR2/NPYY1R heteroreceptor complexes significantly increase in density as shown with PLA upon combined treatment with GAL and the NPYY1R agonist in the subgranular zone in the ventral hippocampus. The existence of GALR2/NPYY1R heteroreceptor complexes was described in several regions, including the amygdala and the dorsal hippocampus (Narvaez et al., 2015; Narvaez et al., 2016; Narvaez et al., 2018). Additionally, we described how heteroreceptor complexes, such as 5HT1A-FGFR1, promote significant hippocampal plasticity effects linked to hippocampal function changes, including antidepressant actions (Borroto-Escuela et al., 2012; Narvaez et al., 2020).

Our data support the hypothesis that GAL and the NPPYY1R agonist can act on the doublecortin (DCX)-cells expressing GALR2 and NPY1R in the ventral hippocampus with formation of GALR2/NPYY1R heteroreceptor complexes. DCX is expressed at the proliferative stage, first appearing at the type 2b stage of stem cell, widely used as a neuronal lineage marker (Kempermann et al., 2015). According to the results obtained, Decressac et al. confirmed that NPY-sensitive cells are the neuroblast expressing DCX, which selectively express the Y1R (Decressac et al., 2011).
Furthermore, we observed a reduction of the GALR2 internalization with this receptor mainly localized to the plasma membrane upon the coactivation of GALR2 and NPYY1R, during 24 hours. This effect may promote the formation of GALR2-NPYY1R heteroreceptor complexes formation since NPYY1R was also shown to mainly remain at the plasma membrane upon coactivation of the two receptors (Narvaez et al., 2015). Moreover, we recognized a constitutive internalization for GALR2, in line with previous results (Xia et al., 2005). This increased GALR2-NPYY1R interaction could lead to an increase integration in the intracellular signaling, as we observed in the extracellular signal-regulated kinases (ERK) pathway, through the SRE reporter assay. ERK kinases, as a group of mitogen-activated protein kinases (MAPK) are implicated in the control of cellular proliferation, including neural precursors in the hippocampus (Li et al., 2001). In agreement, both receptors GALR2 and NPYY1R were shown to provoke activation of the MAPK ERK1/2 pathway in the hippocampus (Cheung et al., 2012; Luo et al., 2019).

Taken together, changes in the formation, activation and trafficking of the GALR2 may promote the GALR2/NPYY1R heteroreceptor complexes formation in the ventral hippocampus. It may induce a transformation of cell proliferation towards a neuronal lineage by enhancement of MAP ERK 1/2 pathway. Thus, it may give the mechanism for the antidepressant behavioral observed upon cotreatment with GAL and the NPYY1R agonist. Therefore, our results may provide the basis for the development of heterobivalent agonist pharmacophors, targeting GALR2/NPYY1R

heteromers, especially in the neuronal precursor cells of the dentate gyrus in the ventral hippocampus for the novel treatment of depression.

Data Sharing and Data Accessibility

The data that support the findings of this study are openly available in: Narváez, Manuel (2020), “Raw data- Statistical analysis”, Mendeley Data, V2, doi: 10.17632/pcf28dx3z5.2

Conflict of Interest

The **authors have no conflict of interest to declare.**

Acknowledgements

The work was supported by the UMA18-FEDERJA-100 and Proyecto Puente UMA, Spain to MN. This work was also supported by the Swedish Medical Research Council, Sweden (62X-00715-50-3) to KF, by Parkinson Fonden, Sweden to KF, and by Hjärnfonden, Sweden (F02018-0286), Hjärnfonden, Sweden (F02019-0296) and Karolinska Institutet Forskningsstiftelser, Sweden to D.O.B-E. D.O.B-E belongs to the “Academia de Biólogos Cubanos” group, Cuba.

Figure 1. Behavioral effects produced by Galanin (GAL) and the Neuropeptide Y Y1 receptor (NPYY1R) agonist alone and in combination in the forced swimming test (FST). The response induced by GAL on the NPYY1-produced behaviors is blocked by the GAL 2 receptor (GALR2) antagonist M871. Cumulative behavioral duration of Immobility **(a),** swimming **(b)** and climbing **(c)** time in the FST. Data represent mean ± SEM. N=6-8 animals in each group. For a: *P <0.05 (GAL 3nmol vs aCSF); **P <0.01 (GAL+Y1 3nmol vs GAL+Y1+M871 3nmol); ***P <0.001 (aCSF vs Y1 3nmol;

GAL+Y1 3nmol and gal+Y1+M871 3nmol); ★★★ P <0.001 (GAL 3nmol vs Y1 3nmol; GAL+Y1 3nmol and gal+Y1+M871 3nm); δδP <0.01 (Y1 3nmol vs GAL+Y1 3nmol). For b: *P <0.05 (Y1 3nmol and GAL+Y1+M871 3nmol vs aCSF); **P <0.01 (Y1 3nmol vs GAL+Y1+M871 3nmol); ***P <0.001 (GAL+Y1 3nmol vs the rest of the groups); ★ P <0.05 (GAL 3nmol vs Y1 3nmol). For c: *P <0.05 (Y1 3nmol vs GAL+Y1 3nmol); ***P <0.001 (aCSF vs the rest of the groups); ★ P <0.05 (GAL+Y1 3nmol vs GAL+Y1+M871 3nmol) according to one-way ANOVA (Immobility: $F_{4,30} = 53.62$, $p<0.001$; Swimming: $F_{4,30} = 16.89$, $p<0.001$; Climbing: $F_{4,30} = 13.97$, $p<0.001$) followed by Newman-Keuls post-hoc test. Inter-group comparisons are indicated by the vertical lines from the horizontal line above bars. aCSF= control (artificial Cerebrospinal fluid); GAL 3nmol=Galanin 3 nmol; Y1 3nmol = NPY Y1 receptor agonist [Leu^{31}-Pro^{34}]NPY 3nmol; GAL+Y1 3nmol= Coadministration of GAL and Y1; GAL+Y1+M871 3nmol= Coadministration of GAL, Y1 and GALR2 antagonist M871 3nmol.

Figure 2. 5'-bromo-2'-deoxyuridine (BrdU) immunolabelling (BrdU+) in the dentate gyrus of the hippocampal ventral or anterior portion, after the intracerebroventricular (icv) administration of Galanin (GAL) and Neuropeptide Y Y1 receptor (NPYY1R) agonist, either alone or in combination with or without the GAL 2 receptor (GALR2) antagonist M871. **(a,d)** The majority of the BrdU-labeled cells was located in the subgranular zone (Sgz) of the dentate gyrus at the border between the granular cell layer (Gcl) and the polymorphic layer (P) of the dentate gyrus in the ventral hippocampus. They appeared to be nerve cells forming clusters of 3–4 cells. (Bregma: -5.6 mm; according to the Paxinos and Watson stereotaxic atlas (1998)). **(b)** Quantification of total BrdU IR cells at this Bregma level. Data represent mean ± SEM, to show the differences between groups after icv injection of aCSF, GAL, NPYY1 agonist [Leu^{31}-Pro^{34}]NPY, or the coadministration of both peptides with or without M871. GAL and the NPYY1 agonist coinjection increased the number of cells with BrdU expression in the ventral hippocampus compared with the effects of the two peptides given alone and the aCSF group. Furthermore, this effect was blocked by GALR2 antagonist M871. ***P <0.001 (Y1 3nmol vs aCSF and GAL 3nmol); ★★

★ P <0.001 (GAL+Y1 3nmol vs the rest of the groups); δδδ P <0.001 (GAL+Y1+M71 3nmol vs the rest of the groups) according to one-way ANOVA ($F_{4, 15} = 75.37$, $p < 0.001$) followed by Newman-Keuls post-hoc test. N=4 in each group. GAL and NPYY1R agonist coinjection **(d)** increased the BrdU immunolabelling in Sgz in the ventral hippocampus compared with the control group **(c).** Arrows indicate examples of clusters of BrdU positive nerve cells. Dashed lines outline the Gcl of the dentate gyrus. Abbreviations: aCSF= control (artificial Cerebrospinal fluid); GAL 3nmol=Galanin 3 nmol; Y1 3nmol = NPY Y1 receptor agonist [Leu^{31}-Pro^{34}]NPY 3nmol; GAL+Y1 3nmol= Coadministration of GAL and Y1; GAL+Y1+M871 3nmol= Coadministration of GAL, Y1 and GALR2 antagonist M871 3nmol. Treatments were performed 24 hours before brain processing, for further details see material and methods.

Figure 3. Detection of GALR2/NPYY1R heteroreceptor complexes by *in situ* PLA in the ventral hippocampal dentate gyrus (DG). **(a)** The diagram shows the presence of positive PLA signals (red circles) mainly in the subgranular zone (Sgz) of the dentate gyrus at the border between the granular cell layer (Gcl) and polymorphic layer (P) of the dentate gyrus in the ventral hippocampus. PLA positive signals were also observed in the polymorphic layer. Blue filled circles indicate negative PLA signal in the molecular layer (M). (Bregma: –5.6 mm; according to the Paxinos and Watson (1998) stereδtaxic atlas]). **(b)** Quantification of PLA signals in Sgz was performed by measuring red PLA positive blobs per nucleus per sampled field by an experimenter blind to treatment conditions. Sprague Dawley rats showed a significant increase in GALR2/NPYY1R heteroreceptor complexes (PLA blobs) in the Sgz after GAL and NPYY1R agonist coinjection. This effect was blocked by treatment with the GALR2 antagonist M871. *P<0.05 (aCSF and GAL 3nmol vs Y1 3nmol); ***P<0.001 (GAL+Y1 3nmol vs aCSF and GAL 3nmol); ★P<0.05 (Y1 3nmol vs GAL+Y1 3nmol); P<0.05 (GAL+Y1 3nmol vs GAL+Y1+M871 3nmol) according to one-way ANOVA ($F_{4, 15} = 11.76$, $p<0.001$) followed by Newman-Keuls post-hoc test. Inter-group comparisons are indicated by the vertical lines from the horizontal line above bars. Data are expressed as mean ± SEM, 4 rats per group, duplicates).

(c-f) Representative microphotographs of the significant increase in the density of GALR2/NPYY1R positive red PLA blobs in the Sgz subregion after GAL and NPYY1R agonist coinjection **(e)** compared with the control group **(c)**. Magnified views from dashed boxes in (c) and (e) are shown in **(d)** and **(f)**, respectively. GALR2/NPYY1R heteroreceptor complexes are shown as red PLA blobs (clusters) found in high densities per cell in a large number of nerve cells using confocal laser microscopy. White arrows point to PLA positive clusters. Dashed lines outline the Gcl of the dentate gyrus. The nuclei are shown in blue by DAPI. Abbreviations: aCSF= control (artificial Cerebrospinal fluid); GAL 3nmol=Galanin 3 nmol; Y1 3nmol = NPY Y1 receptor agonist [Leu^{31}-Pro^{34}]NPY 3nmol; GAL+Y1 3nmol= Coadministration of GAL and Y1; GAL+Y1+M871 3nmol= Coadministration of GAL, Y1 and GALR2 antagonist M871 3nmol. Treatments were performed 24 hours before brain processing, for further details see material and methods.

Figure 4. Analysis of the peak internalization values of Galanin receptor 2 (GALR2)-GFP^2 with GAL alone in the first hour period vs the combined treatment with NPY, and the co-agonist-induced disappearance of the internalization at the 24 h time interval. **(a-c)** Representative confocal micrographs show the distribution of GALR2-GFP^2 in GALR2-GFP^2/NPYY1R HEK293T coexpressing cells. Timed-interval images of different cell groups were acquired with the confocal microscope after GAL 1μM and/or NPY 1μM addition and washed with ice-cold PBS to stop the internalization, for further details see material and methods. Antagonist studies were performed 15 min before the addition of agonist with NPYY1R antagonist BIBP3226 10μM. Following co-stimulation with 1 μM GAL and NPY **(b)** there was an increased GALR2-GFP^2 presence in the plasma membrane at different time points, compared with GAL alone **(a).** This effect was counteracted by the NPYY1R antagonist BIBP3226 (10 μM). Representative confocal micrographs are showed at 1 and 24 hours **(c). (d)** Representation of time course of quantitation of GALR2-GFP^2 internalization. Data, as mean ± SEM, showed maximal internalization after GAL stimulation alone at 30 minutes. However, after GAL and NPY co-stimulation a

GALR2-GFP[2] recruitment was observed towards the plasma membrane. Internalization took place only after 60 minutes until 36 hours. *P <0.05 (t = 2.240, df = 20; 24 hours after GAL and NPY co-stimulation vs basal state); ***P <0.001 (t = 7.842, df = 21; 30 min. after GAL and NPY co-stimulation vs GAL alone); ★★★P <0.001 (t = 4.213, df = 21; 24 hours after GAL and NPY co-stimulation vs GAL alone) according to Student's unpaired t-test statistical analysis. Group comparisons are indicated by the lines. Micrographs are representative images of multiple cells imaged in three independent experiments.

Figure 5. Agonist-induced Galanin 2 receptor (GALR2) and neuropeptide Y Y1 receptor (NPYY1R) activation in the serum response element (SRE)-luciferase reporter gene assay. HEK293T cells were transiently co-transfected with 1 μg firefly luciferase-encoding experimental plasmid (pGL4-SRE-luc2p), 1 μg of (GALR2 and/or NPYY1R) expression vectors and 50 ng Renilla luciferase-encoding internal control plasmid (phRG-B). Thirty-six hours after transfection, cells were treated for 6 hours with GAL and/or NPY or their specific antagonists (in presence of agonist). **(a)** The co-stimulation with GAL (50 and 100 nM) and NPY (50nM) substantially increased the induction of the SRE-Reporter expression compared with the increase found with GAL and NPY alone. The specific antagonists for GALR2 (M871) and NPYY1R (BIBP3226) counteracted the increase of SRE-expression found with effective doses of GAL and NPY, respectively. Data represent the mean ± SEM of three independent experiments performed in triplicate. ***P <0.001 (GAL 100nM vs Control, GAL 50 nM and GAL 100+M871); ★★★ P <0.001 (NPY 50nM vs control and NPY 50 + BIBP3226); δδδP <0.001 (GAL 50 + NPY 50 vs the rest of the groups); ☆☆☆P <0.001 (GAL 100 + NPY 50 vs the rest of the groups) according to one-way ANOVA one-way ANOVA, ($F_{7, 56} = 89.25$, $p < 0.001$) followed by Newman-Keuls post-hoc test. Inter-group comparisons are indicated by the vertical lines from the long horizontal line above bars. **(b)** Schematic cross-talk signaling pathway of GALR2-NPYY1R heteroreceptor complex with a positive modulation on SRE pathway signaling. There can be a putative involvement also of RTK in the GALR2-

NPYY1R heteroreceptor complex contributing to the enhancement of the SRE signaling. Control= basal medium; GAL 50nM= Galanin 500nM; GAL 100nM= Galanin 100nM; NPY 50nM= neuropeptide Y 50nM; BIBP3226 = NPYY1R antagonist 1μM; M871 = GALR2 antagonist 1μM.

Supplementary figure 1. Representative laser scanned confocal micrographs illustrating the polymorphic (P), granular (Gcl) and molecular (M) layers of the dentate gyrus (Bregma: –5.6 mm). Nuclei are shown in blue (DAPI). **(a)** Laser scanned confocal micrographs demonstrating the neuronal colocalization (yellow) of endogenous Galanin receptor subtype 2 (GALR2) (red)(Alomone, Rabbit, 1:100) and Doublecortin (green, microtubule associated protein(Gleeson JG, 1999))(C-18, Santa Cruz, 1:500) in the subgranular zone (Sgz) of the dentate gyrus (defined as a band-limited by three nuclei down from the apparent border between the Gc and the Pol region). **(b)** Laser scanned confocal micrographs demonstrating the colocalization (yellow) of endogenous Neuropeptide Y Y1 receptor (NPYY1R)(green)(Santa Cruz, Goat, 1:200) and Doublecortin (red)(Abcam, Rabbit, 1:500) in the Sgz of the dentate gyrus. **(c)** Laser scanning confocal micrographs demonstrating that endogenous Galanin receptor subtype 1 (GALR1)(red)(Alomone, Rabbit, 1:100) and Doublecortin (green)(C-18, Santa Cruz, 1:500) do not colocalize in the Sgz of the dentate gyrus.

Supplementary Figure 2. Analysis method and basal expression of GALR2-GFP^2 in GALR2-GFP^2/NPYY1R HEK293T coexpressing cells. **(a)** Representative laser-scanned confocal micrograph with regions of interest (ROI) used to measure fluorescence, representing total membrane (ROI1, green line) and total internal compartment (ROI2, purple line). The percentage of internalization was determined by total membrane fluorescence compared to total internal compartment fluorescence at the various time points showed in Figure 4. **(b)** Representative confocal micrograph showing presence of GALR2-GFP^2 in the internal compartment at a basal state without agonist addition.

World Health Organization. Depression and Other Common Mental Disorders: Global Health Estimates; World Health Organization: Geneva, Switzerland, 2017.

Abbosh C, Lawkowski A, Zaben M, et al. (2011) GalR2/3 mediates proliferative and trophic effects of galanin on postnatal hippocampal precursors. *J Neurochem* 117(3): 425-436.

Abrial E, Betourne A, Etievant A, et al. (2014) Protein kinase C inhibition rescues manic-like behaviors and hippocampal cell proliferation deficits in the sleep deprivation model of mania. *Int J Neuropsychopharmacol* 18(2).

Baptista P and Andrade JP (2018) Adult Hippocampal Neurogenesis: Regulation and Possible Functional and Clinical Correlates. *Front Neuroanat* 12: 44.

Bauman MD, Schumann CM, Carlson EL, et al. (2018) Neuroprotective efficacy of P7C3 compounds in primate hippocampus. *Transl Psychiatry* 8(1): 202.

Boldrini M, Fulmore CA, Tartt AN, et al. (2018) Human Hippocampal Neurogenesis Persists throughout Aging. *Cell Stem Cell* 22(4): 589-599 e585.

Borroto-Escuela DO, Li X, Tarakanov AO, et al. (2017) Existence of Brain 5-HT1A-5-HT2A Isoreceptor Complexes with Antagonistic Allosteric Receptor-Receptor Interactions Regulating 5-HT1A Receptor Recognition. *ACS Omega* 2(8): 4779-4789.

Borroto-Escuela DO, Narvaez M, Di Palma M, et al. (2014) Preferential activation by galanin 1-15 fragment of the GalR1 protomer of a GalR1-GalR2 heteroreceptor complex. *Biochem Biophys Res Commun* 452(3): 347-353.

Borroto-Escuela DO, Narvaez M, Marcellino D, et al. (2010) Galanin receptor-1 modulates 5-hydroxtryptamine-1A signaling via heterodimerization. *Biochem Biophys Res Commun* 393(4): 767-772.

Borroto-Escuela DO, Romero-Fernandez W, Mudo G, et al. (2012) Fibroblast growth factor receptor 1- 5-hydroxytryptamine 1A heteroreceptor complexes and their enhancement of hippocampal plasticity. *Biol Psychiatry* 71(1): 84-91.

Branchek TA, Smith KE, Gerald C, et al. (2000) Galanin receptor subtypes. *Trends Pharmacol Sci* 21(3): 109-117.

Catena-Dell'Osso M, Fagiolini A, Marazziti D, et al. (2013) Non-monoaminergic targets for the development of antidepressants: focus on neuropeptides. *Mini Rev Med Chem* 13(1): 2-10.

Chen P (2019) Optimized Treatment Strategy for Depressive Disorder. *Adv Exp Med Biol* 1180: 201-217.

Cheung A, Newland PL, Zaben M, et al. (2012) Intracellular nitric oxide mediates neuroproliferative effect of neuropeptide y on postnatal hippocampal precursor cells. *J Biol Chem* 287(24): 20187-20196.

Cipriani S, Ferrer I, Aronica E, et al. (2018) Hippocampal Radial Glial Subtypes and Their Neurogenic Potential in Human Fetuses and Healthy and Alzheimer's Disease Adults. *Cereb Cortex* 28(7): 2458-2478.

Decressac M, Wright B, David B, et al. (2011) Exogenous neuropeptide Y promotes in vivo hippocampal neurogenesis. *Hippocampus* 21(3): 233-238.

Diaz-Cabiale Z, Parrado C, Narvaez M, et al. (2011) Galanin receptor/Neuropeptide Y receptor interactions in the dorsal raphe nucleus of the rat. *Neuropharmacology* 61(1-2): 80-86.

DiazGranados N, Ibrahim LA, Brutsche NE, et al. (2010) Rapid resolution of suicidal ideation after a single infusion of an N-methyl-D-aspartate antagonist in patients with treatment-resistant major depressive disorder. *J Clin Psychiatry* 71(12): 1605-1611.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, et al. (1998) Neurogenesis in the adult human hippocampus. *Nat Med* 4(11): 1313-1317.

Fanselow MS and Dong HW (2010) Are the dorsal and ventral hippocampus functionally distinct structures? *Neuron* 65(1): 7-19.

Gehlert DR, Schober DA, Morin M, et al. (2007) Co-expression of neuropeptide Y Y1 and Y5 receptors results in heterodimerization and altered functional properties. *Biochem Pharmacol* 74(11): 1652-1664.

Geloso MC, Corvino V, Di Maria V, et al. (2015) Cellular targets for neuropeptide Y-mediated control of adult neurogenesis. *Front Cell Neurosci* 9: 85.

Gigliucci V, O'Dowd G, Casey S, et al. (2013) Ketamine elicits sustained antidepressant-like activity via a serotonin-dependent mechanism. *Psychopharmacology (Berl)* 228(1): 157-166.

Harmer CJ, Duman RS and Cowen PJ (2017) How do antidepressants work? New perspectives for refining future treatment approaches. *Lancet Psychiatry* 4(5): 409-418.

Howell OW, Scharfman HE, Herzog H, et al. (2003) Neuropeptide Y is neuroproliferative for post-natal hippocampal precursor cells. *J Neurochem* 86(3): 646-659.

Howell OW, Silva S, Scharfman HE, et al. (2007) Neuropeptide Y is important for basal and seizure-induced precursor cell proliferation in the hippocampus. *Neurobiol Dis* 26(1): 174-188.

Jimenez-Vasquez PA, Diaz-Cabiale Z, Caberlotto L, et al. (2007) Electroconvulsive stimuli selectively affect behavior and neuropeptide Y (NPY) and NPY Y(1) receptor gene expressions in hippocampus and hypothalamus of Flinders Sensitive Line rat model of depression. *Eur Neuropsychopharmacol* 17(4): 298-308.

Kempermann G, Gage FH, Aigner L, et al. (2018) Human Adult Neurogenesis: Evidence and Remaining Questions. *Cell Stem Cell* 23(1): 25-30.

Kempermann G, Song H and Gage FH (2015) Neurogenesis in the Adult Hippocampus. *Cold Spring Harb Perspect Biol* 7(9): a018812.

Koike H and Chaki S (2014) Requirement of AMPA receptor stimulation for the sustained antidepressant activity of ketamine and LY341495 during the forced swim test in rats. *Behav Brain Res* 271: 111-115.

Kormos V and Gaszner B (2013) Role of neuropeptides in anxiety, stress, and depression: from animals to humans. *Neuropeptides* 47(6): 401-419.

Kotagale NR, Paliwal NP, Aglawe MM, et al. (2013) Possible involvement of neuropeptide Y Y1 receptors in antidepressant like effect of agmatine in rats. *Peptides* 47: 7-11.

Kuteeva E, Wardi T, Lundstrom L, et al. (2008) Differential role of galanin receptors in the regulation of depression-like behavior and monoamine/stress-related genes at the cell body level. *Neuropsychopharmacology* 33(11): 2573-2585.

Li BS, Ma W, Zhang L, et al. (2001) Activation of phosphatidylinositol-3 kinase (PI-3K) and extracellular regulated kinases (Erk1/2) is involved in muscarinic receptor-mediated DNA synthesis in neural progenitor cells. *J Neurosci* 21(5): 1569-1579.

Lu X, Ross B, Sanchez-Alavez M, et al. (2008) Phenotypic analysis of GalR2 knockout mice in anxiety- and depression-related behavioral tests. *Neuropeptides* 42(4): 387-397.

Luo H, Liu Z, Liu B, et al. (2019) Virus-Mediated Overexpression of ETS-1 in the Ventral Hippocampus Counteracts Depression-Like Behaviors in Rats. *Neurosci Bull* 35(6): 1035-1044.

Mathe AA, Jimenez PA, Theodorsson E, et al. (1998) Neuropeptide Y, neurokinin A and neurotensin in brain regions of Fawn Hooded "depressed", Wistar, and Sprague Dawley rats. Effects of electroconvulsive stimuli. *Prog Neuropsychopharmacol Biol Psychiatry* 22(3): 529-546.

McKay MM and Morrison DK (2007) Integrating signals from RTKs to ERK/MAPK. *Oncogene* 26(22): 3113-3121.

Miller BR and Hen R (2015) The current state of the neurogenic theory of depression and anxiety. *Curr Opin Neurobiol* 30: 51-58.

Moreno-Jimenez EP, Flor-Garcia M, Terreros-Roncal J, et al. (2019) Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease. *Nat Med* 25(4): 554-560.

Narvaez M, Andrade-Talavera Y, Valladolid-Acebes I, et al. (2020) Existence of FGFR1-5-HT1AR heteroreceptor complexes in hippocampal astrocytes. Putative link to 5-HT and FGF2 modulation of hippocampal gamma oscillations. *Neuropharmacology* 170: 108070.

Narvaez M, Borroto-Escuela DO, Millon C, et al. (2016) Galanin receptor 2-neuropeptide Y Y1 receptor interactions in the dentate gyrus are related with antidepressant-like effects. *Brain Struct Funct* 221(8): 4129-4139.

Narvaez M, Borroto-Escuela DO, Santin L, et al. (2018) A Novel Integrative Mechanism in Anxiolytic Behavior Induced by Galanin 2/Neuropeptide Y Y1 Receptor Interactions on Medial Paracapsular Intercalated Amygdala in Rats. *Front Cell Neurosci* 12: 119.

Narvaez M, Millon C, Borroto-Escuela D, et al. (2015) Galanin receptor 2-neuropeptide Y Y1 receptor interactions in the amygdala lead to increased anxiolytic actions. *Brain Struct Funct* 220(4): 2289-2301.

O'Donnell D, Ahmad S, Wahlestedt C, et al. (1999) Expression of the novel galanin receptor subtype GALR2 in the adult rat CNS: distinct distribution from GALR1. *J Comp Neurol* 409(3): 469-481.

Petit-Demouliere B, Chenu F and Bourin M (2005) Forced swimming test in mice: a review of antidepressant activity. *Psychopharmacology (Berl)* 177(3): 245-255.

Pilar-Cuellar F, Vidal R and Pazos A (2012) Subchronic treatment with fluoxetine and ketanserin increases hippocampal brain-derived neurotrophic factor, beta-catenin and antidepressant-like effects. *Br J Pharmacol* 165(4b): 1046-1057.

Porsolt RD, Le Pichon M and Jalfre M (1977) Depression: a new animal model sensitive to antidepressant treatments. *Nature* 266(5604): 730-732.

Redrobe JP, Dumont Y, Fournier A, et al. (2002) The neuropeptide Y (NPY) Y1 receptor subtype mediates NPY-induced antidepressant-like activity in the mouse forced swimming test. *Neuropsychopharmacology* 26(5): 615-624.

Sorrells SF, Paredes MF, Cebrian-Silla A, et al. (2018) Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature* 555(7696): 377-381.

Spalding KL, Bergmann O, Alkass K, et al. (2013) Dynamics of hippocampal neurogenesis in adult humans. *Cell* 153(6): 1219-1227.

Sperk G, Hamilton T and Colmers WF (2007) Neuropeptide Y in the dentate gyrus. *Prog Brain Res* 163: 285-297.

Tanti A and Belzung C (2013) Neurogenesis along the septo-temporal axis of the hippocampus: are depression and the action of antidepressants region-specific? *Neuroscience* 252: 234-252.

Vega-Rivera NM, Fernandez-Guasti A, Ramirez-Rodriguez G, et al. (2015) Effect of sub-optimal doses of fluoxetine plus estradiol on antidepressant-like behavior and hippocampal neurogenesis in ovariectomized rats. *Psychoneuroendocrinology* 57: 113-124.

Walker AK, Rivera PD, Wang Q, et al. (2015) The P7C3 class of neuroprotective compounds exerts antidepressant efficacy in mice by increasing hippocampal neurogenesis. *Mol Psychiatry* 20(4): 500-508.

Xia S, Kjaer S, Zheng K, et al. (2005) Constitutive and ligand-induced internalization of EGFP-tagged galanin R2 and Rl receptors in PC12 cells. *Neuropeptides* 39(3): 173-178.

Zaben MJ and Gray WP (2013) Neuropeptides and hippocampal neurogenesis. *Neuropeptides* 47(6): 431-438.