Antidepressant augmentation upon intranasal Galanin and Neuropeptide Y agonists enhanced ventral hippocampal proliferative and neurotrophic actions

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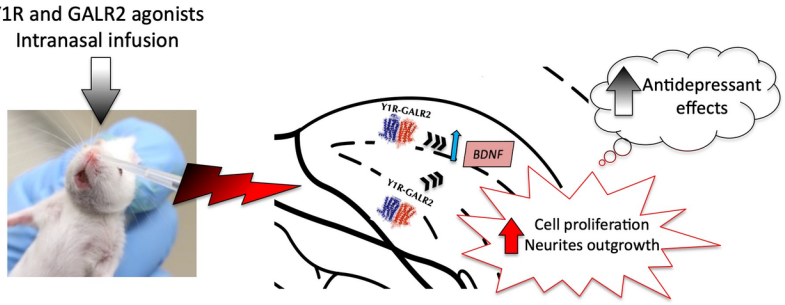
February 22, 2024

Abstract

Background and Purpose: Dysregulation of adult hippocampal neurogenesis is linked to major depressive disorder (MDD), with more than 300 million people diagnosed and worsened by the COVID-19 pandemic. Accumulating evidence for Neuropeptide Y (NPY) and galanin (GAL) interaction was shown in various limbic system regions at molecular-,cellular- and behavioral-specific levels. The purpose of the current work was to evaluate the proliferating role of GALR2 and Y1R agonists interaction upon intranasal infusion in the ventral hippocampus. Experimental approach: We studied their hippocampal proliferating actions using the proliferating cell nuclear antigen (PCNA) and the expression of of the brain-derived neurothrophic factor (BDNF). Moreover, we studied the formation of Y1R-GALR2 heteroreceptor complexes and analyzed morphological changes on hippocampal neuronal cells. Finally, the functional outcome of the NPY and GAL interaction on the ventral hippocampus was evaluated in the forced swimming test. Key Results: We demonstrated that the intranasal infusion of GALR2 and the Y1R agonists promotes cell proliferation in the DG of the ventral hippocampus and the induction of the neurotrophic factor BDNF. These effects were mediated by the increased formation of Y1R-GALR2 heteroreceptor complexes, which may mediate the neurites outgrowth observed on neuronal hippocampal cells. Importantly, BDNF action was found necessary for the antidepressant-like effects after GALR2 and the Y1R agonists intranasal administration. Conclusions & Implications: Our data may suggest the translational development of new heterobivalent agonist pharmacophores acting on Y1R–GALR2 heterocomplexes in the ventral hippocampus for the novel therapy of mayor depression disorder or depressive-affecting diseases.



Introduction

Neurogenesis is a process within neuronal plasticity, the capacity of the brain to reorganize its structure, function, and connections in response to extrinsic or intrinsic stimuli (Baptista & Andrade, 2018). In this regard, new neurons are produced in the brain via a manner known as adult hippocampal neurogenesis (AHN). Among the neurogenic areas studied in the human brain, hippocampal formation emerges as crucial, exerting a noteworthy role in brain function (Spalding et al., 2013; Toda, Parylak, Linker, & Gage, 2019). In the dentate gyrus (DG) of the hippocampus is located the neurogenic niche, aimed to maintain AHN and implicated in many processes under normal physiological conditions during adulthood. In fact, AHN is demonstrated to be preserved throughout human life, even until the ninth decade of life, being essential to keep related physiological functions (Boldrini et al., 2018; Moreno-Jimenez et al., 2019; Terreros-Roncal et al., 2022). Furthermore, a functional separation has been demonstrated in the hippocampus, where stress and modulation of emotional behavior is processed by the anterior portion in humans (ventral, in rodents) while cognitive functions and memory is related to the posterior part (dorsal, in rodents) (Fanselow & Dong, 2010; Kheirbek et al., 2013; Tanti & Belzung, 2013).

Intriguingly, dysregulation of AHN is related to several brain disorders, such as major depressive disorder (MDD), age-dependent cognitive decline, Alzheimer's disease (AD), amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, dementia with Lewy bodies, and frontotemporal dementia (I. B. Kim & Park, 2021; Martos, Tuka, Tanaka, Vecsei, & Telegdy, 2022; Moreno-Jimenez et al., 2019; Terreros-Roncal et al., 2022). MDD is one of the prominent mental health conditions in the world with more than 300 million people diagnosed. MDD is defined by a collection of behavioral, emotional and cognitive symptoms, and confer a challenge for the medical community by increasing the risk of suicidality and death. Suicide is considered the worst outcome or consequence of MDD, over 700,000 human lives lost every year (Elias, Zhang, & Manners, 2022). Moreover, during the COVID-19 pandemic, multiple challenges have arisen, such as loneliness or financial hardship, producing about 34% prevalence of depression in general population, with 5–15% suicidal ideation in that period (Giner, Vera-Varela, de la Vega, Zelada, & Guija, 2022).

The majority of current antidepressants target monoamines, with remarkable shortcomings such as adverse events and delayed onset of efficacy (Harmer, Duman, & Cowen, 2017). Notably, studies determine that approximately 50% of such patients fail to respond and about 65% of them fail to achieve remission, which clas-

sifies these patients with treatment-resistant depression (TRD) (Chen, 2019). Recently, the discovery of ketamine as an effective antidepressant led to the authorization of a nasal spray form of *esketamine* (Hashimoto, 2020). Esketamine provides fast-acting symptomatic relief in TRD patients, but the significant risks associated with esketamine limit its use for a broad patient population. Overall, nowadays MDD patients have no adequate treatment options, implicating that additional underlying mechanisms need to be considered in order to improve the efficacy of treatments. In recent decades, progress in the MDD field has been made possible in part through the use of rodent models (Planchez, Surget, & Belzung, 2019). Accordingly, boosting hippocampal neurogenesis in these patients emerges as a potential therapeutic approach (Colucci-D'Amato, Speranza, & Volpicelli, 2020; Miller & Hen, 2015). Critical components of AHN are cell proliferation, neuronal differentiation, and survival, strictly controlled by multiple intrinsic or extrinsic epigenetic factors that could promote or suppress neurogenesis (Toda et al., 2019). These factors confer a pivotal role in understanding the importance of adult neurogenesis on physiological and pathological conditions (Kempermann et al., 2018). In this way, the neurogenic-promoting effects of neurotransmitters/neuropeptides and neurotrophic factors are crucial regulators of neurogenic niche activities in health and disease (Kuhn, 2015).

Among them, *neuropeptide Y* (NPY) is one of the most abundant neuropeptides in the nervous system. NPY is a 36 amino acid polypeptide neurotransmitter highly conserved in mammals and involved in basic biological and pathophysiological functions, such as neuroendocrine secretions, mood regulation, feeding behavior, circadian rhythms, neuronal excitability, neuroplasticity and memory (Kormos & Gaszner, 2013; Zaben & Gray, 2013). Regarding hippocampal neurogenesis, a pro-neurogenic role of NPY on hippocampal stem cells has been evidenced both in vitro (Howell et al., 2003; Howell et al., 2007) and in vivo (Decressac et al., 2011; Geloso, Corvino, Di Maria, Marchese, & Michetti, 2015). In preclinical models, it was found reduced brain NPY in genetic and environmental models of MDD (Cohen, Vainer, Zeev, Zohar, & Mathe, 2018; Jimenez Vasquez, Salmi, Ahlenius, & Mathe, 2000; Jimenez-Vasquez, Overstreet, & Mathe, 2000). Reliable with the animal data, brain NPY is reduced in postmortem brains from MDD patients who committed suicide (Kautz, Charney, & Murrough, 2017; Sah & Geracioti, 2013). With regard to treatment effects, all antidepressant procedures tested preclinically to date increase brain NPY (Bjornebekk, Mathe, & Brene, 2006; Husum, Mikkelsen, Hogg, Mathe, & Mork, 2000). In line with these findings, transgenic rats overexpressing hippocampal NPY show decreased depression-like behaviors (Thorsell et al., 2000). Furthermore, recently was demonstrated that intranasal NPY and the NPY *Y1 receptor* (Y1R) agonist administration had antidepressant effects in rodents (Nahvi et al., 2021; Serova, Mulhall, & Sabban, 2017) and in MDD patients (Mathe, Michaneck, Berg, Charney, & Murrough, 2020). In this respect, the Y1R have been proposed as a critical target by mediating dentate neurogenesis and antidepressant effects (Rana et al., 2022).

Brain-derived neurotrophic factor (BDNF) is a pivotal molecule involved in the neuroprotective effects of the antidepressants by regulating different neurogenic processes in the hippocampus (Castren & Kojima, 2017; Colucci-D'Amato et al., 2020; Miranda, Morici, Zanoni, & Bekinschtein, 2019). Moreover, a reduction in BDNF was reported in the hippocampus of post-mortem brain tissues of MDD and suicide victims (Dwivedi, 2012; Pandey et al., 2008) while injection of BDNF in the hippocampus reduces depression-like behavior in rodents (Hoshaw, Malberg, & Lucki, 2005). Besides, BDNF was significantly increased 24 h following treatment with NPY in the trimethyltin (TMT)-induced model of hippocampal neurodegeneration (Corvino et al., 2014).

We have demonstrated NPY and *galanin* (GAL) interactions in different limbic system regions at molecular-, cellular-, and behavioral-specific levels (Mirchandani-Duque et al., 2022; Narvaez et al., 2016; Narvaez et al., 2018; Narvaez et al., 2015). GAL is broadly distributed in the central nervous system contributing to numerous physiological effects (Katsetos et al., 2001). Regarding hippocampal neurogenesis, the *GAL2 receptor* agonist, *GAL 2–11* was involved in proliferative and trophic actions in vitro (Abbosh, Lawkowski, Zaben, & Gray, 2011). Concerning depression, GALR2 activation induced antidepressant effects in rodents and the increased GALR2 expression in the ventral hippocampus was related to antidepressant effects (Kuteeva et al., 2008; Luo et al., 2019). Moreover, GalR2-knockout mice displayed depression-like behaviors (Lu, Ross, Sanchez-Alavez, Zorrilla, & Bartfai, 2008). Recently, we described a facilitatory interaction between NPY and GAL through the Y1R-GALR2 heteroreceptor complexes formation. Intranasally combined

GALR2 and Y1R agonists improved spatial memory performance related to increased cell proliferation in the dentate gyrus of the dorsal hippocampus (Borroto-Escuela et al., 2022).

The purpose of the current work was to assess the role of the NPY and GAL interaction in the neurogenic actions on the ventral hippocampus. Following GALR2 and Y1R agonists intranasal administration, we analyzed the ventral hippocampal activation and their proliferating actions through c-Fos expression and proliferating cell nuclear antigen (PCNA). To examine the associated cellular mechanism we assessed the expression of the brain-derived neurothrophic factor (BDNF) on the ventral hippocampal dentate gyrus (DG). Moreover, we studied the formation of Y1R-GALR2 heteroreceptor complexes with in situ proximity ligation assay (PLA) and analyzed morphological changes on hippocampal neuronal cells. Finally, the functional outcome of the NPY and GAL interaction on the ventral hippocampus was evaluated in the forced swimming test, employing the intranasal procedure that takes advantage of a direct nose-to-brain transport of therapeutics.

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 Malaga, Spain. Guidelines for animal experiments were carried out following EU Directive 2010/63/EU and Spanish Directive (Real Decretory 53/2013) recommendations.

Drugs used

Solutions were freshly prepared in distilled water. Galanin receptor 2 agonist (*M1145*), Y1R receptor agonist*[Leu^{31}, Pro^{34}]NPY*, GALR2 Antagonist*M871* were purchased from Tocris Bioscience (Bristol, UK) and TrkB antagonist (ANA-12, 5.06304) from Sigma Aldrich (St. Louis, MO, USA). Detailed descriptions are available in Supplement material on intranasal (i.n.) infusion of solutions.

Assessment of ventral hippocampus activation after intranasal infusion

Animals were randomly allocated into five experimental groups: (1) Control: distilled water; (2) M1145-treated group (132 μg); (3) Y1R agonist-treated group receiving the Y1R agonist [Leu^{31}- Pro^{34}]NPY (132 μg); (4) M1145+Y1R: group administered with both substances; (5) M1145+Y1R+M871: group treated with M1145, [Leu^{31}- Pro^{34}]NPY and the GALR2 antagonist (M871; 132 μg) (N=4 in each group). The doses indicated are based on previously published protocols (Borroto-Escuela et al., 2022; Serova et al., 2017).

Twenty-four hours after the after the i.n. administration, rats were deeply anesthetized with pentobarbital (Mebumal, 100 mg/kg, i.p.) and transcardially perfused with 4% PFA (para-formaldehyde (wt./vol, Sigma Aldrich, St. Louis, MI, USA)). Using a Cryostat (HM550, Microm International, Walldorf, Germany) the brains were coronally sliced (30 μm-thick) through the ventral hippocampus (anterior in primates) (-5.20 to -6.72 Bregma; (Paxinos & Watson, 2006)).

We used the c-Fos immunohistochemistry, as an indirect marker of neuronal activation. Free-floating sections were incubated for antigenical retrieval at 65 °C during 90 min in saline sodium citrate buffer (pH 6; 10 nM sodium citrate). After this procedure to remove endogenous peroxidases, the slices were treated 30 min in 0.6% H_2O_2. Then, slices were incubated at 4 °C overnight with a primary antibody mouse anti-c-Fos protein (1:800 sc-271243, Santa Cruz Biotechnology, CA) in 2.5% donkey serum. After several washes with PBS, the slices were incubated with a secondary antibody for 90 min (biotinylated anti-mouse IgG, 1:300, B8520, Sigma, St. Louis, MO, USA). Then, ExtrAvidin peroxidase (1:100, Sigma, St. Louis, MO, USA) was used to amplify the specific signal for one hour at room temperature in darkness. Detection was performed with 0.05% diaminobenzidine (DAB; Sigma) and 0.03% H_2O_2 in PBS. After several washes, slices were

mounted on gelatin-coated slides, dehydrated in graded alcohols, and cover-slipped with DePeX mounting medium (Merck Life Science S.L.U., Darmstadt, Germany). C-Fos-labeled cells were studied using the optical fractionator method in unbiased stereological microscopy (Olympus BX51 Microscope, Olympus, Denmark), as previously described (Borroto-Escuela et al., 2022; Mirchandani-Duque et al., 2022; Narvaez et al., 2018) (see Supplementary Materials for details).

Evaluation of ventral Hippocampal Cell Proliferation and brain-derived neurotrophic factor-(BDNF) induction

Different free-floating sections were incubated for antigenical retrieval at 65 °C during 90 min in saline sodium citrate buffer (pH 6; 10 nM sodium citrate). After this procedure to remove endogenous peroxidases, the slices were treated 30 min in 0.6% H2O2. Then, a set of slices were incubated at RT overnight with a primary antibody mouse anti-PCNA (1:1500, P8825, Sigma, St. Louis, MO, USA) or a different one with mouse anti-BDNF (Abcam, ab205067, 1:500) in 2.5% donkey serum. After several washes with PBS, the slices were incubated with a secondary antibody for 90 min (biotinylated anti-mouse IgG, 1:200, B8520, Sigma, St. Louis, MO, USA). Then, ExtrAvidin peroxidase (1:100, Sigma, St. Louis, MO, USA) was used to amplify the specific signal for one hour at room temperature in darkness. Detection was performed with 0.05% diaminobenzidine (DAB; Sigma) and 0.03% H_2O_2 in PBS. After several washes, slices were mounted on gelatin-coated slides, dehydrated in graded alcohols, and cover-slipped with DePeX mounting medium (Merck Life Science S.L.U., Darmstadt, Germany). PCNA and BDNF-labeled cells were studied using the optical fractionator method in unbiased stereological microscopy (Olympus BX51 Microscope, Olympus, Denmark), as described above.

Hippocampal Cell Culture and conditions

Rat primary hippocampal neuronal cells were purchased from QBM Cell Science (Montreal, Canada) and cultured in Neuro basal medium supplemented with 10% FBS, 2 mM GlutaMAX-1, 1 mM sodium pyruvate, 100 U/ml penicillin G, and 100 µg/ml streptomycin and 2% B-27 supplement at 37°C in a humidified 10% CO_2 environment according to manufacturer's instructions. Half part of the medium was changed every 3 days. The cells were grown under the above conditions (control condition) for 7 d. Cultured hippocampal neurons were grown and treated for 24 hours under specific pharmacologic conditions. Treated hippocampal cells were divided into experimental groups: (1) Control group; (2) M1145-treated group (100nM); (3) Y1R agonist-treated group receiving an NPYY1R agonist [Leu^{31},Pro^{34}]NPY (100nM); (4) GAL+Y1R: Group administered with both substances; (5) GAL+Y1+M871: Group injected with GAL, [Leu^{31},Pro^{34}]NPY and the GALR2 antagonist (M871; 1µM). Cells were grown on poly-D-lysine-coated glass coverslips and fixed with 4% formaldehyde solution for 20 min followed by two washes with PBS containing 20 mM glycine to quench the aldehyde groups.

In situ proximity ligation assay and analysis of neurite length

To study the GALR2-Y1R heteroreceptor complexes, the *in situ*proximity ligation assay (*in situ* PLA) was performed as described previously (Borroto-Escuela et al., 2021; Narváez et al., 2021). After permeabilization with PBS containing 0.2% Triton X-100 for 5 min, cells were treated with PBS containing 1% bovine serum albumin. The hippocampal cells were then incubated with the primary antibodies diluted in a suitable concentration in the blocking solution at 4 °C overnight. Then, cells were washed twice, and the proximity probe mixture (Duolink PLA probe anti-goat MINUS and Duolink PLA probe anti-rabbit PLUS, Sigma-Aldrich, Stockholm, Sweden) was applied to the samples 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 oligonucleotides (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 A (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 cells and incubated in a humidity chamber at 37 °C for 100 min. Then, the cells were incubated with the detection solution in a humidity chamber at 37 °C for 30 min. In a last step, the cells were washed twice in the dark, for 10 min each, with the washing buffer B (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 coverslips were put on a microscope slide and a drop of appropriate mounting medium (e.g., Duolink Mounting Medium, Sigma-Aldrich) was applied and sealed with nail polish. The slides were protected against light and stored for several days at -20 degC before confocal microscope analysis. The in situ PLA experiments were performed using the following primary antibodies: rabbit anti GALR2 (Alomone Lab, 1:100) and goat anti NPYY1R (sc-21992 Santa Cruz Biotechnology INC, CA, 1:200). Furthermore, cells were labelled with Neuro-Chrom Pan Neuronal Marker primary antibody (ABN2300, 1:100, Sigma-Aldrich; Merck Life Science S.L.U.) for 1 h, extensively washed, and stained with the green fluorescence secondary antibody goat anti-rabbit DyLight 488 (Jackson Laboratories InmunoResearch, 1:100). Cell nuclei were counterstained with DAPI (blue) contained in the mounting medium. 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). Acquisition of microscopy images, *In situ* PLA data analysis and morphometric quantifications were performed as previously described (Narvaez et al., 2020).

Assessment of depression-like behavior in rats

Depression-like behavior was assessed in the FST, originally proposed as a model of stress-induced depression-like behavior (Porsolt, Le Pichon, & Jalfre, 1977). FST is broadly used for the early screening of novel molecules with putative antidepressant-like (AD) effects since immobility is commonly qualified as "despair" and considered to reflect depression-like states. Remarkably, the immobility response in the FST can be prevented by various types of AD treatments, including tricyclic antidepressants, monoamine oxidase inhibitors, SSRIs, and NA reuptake inhibitors (Planchez et al., 2019).

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 and assigned randomly to the experimental groups. Peptides were freshly prepared and intranasal treatments were administered 24 hours before the test phase (20 µl total volume). Doses for Galanin receptor 2 agonist (M1145), Y1R receptor agonist [Leu^{31}, Pro^{34}]NPY, GALR2 Antagonist M871 and ANA-12 were chosen based on previous published protocols (Borroto-Escuela et al., 2022; Ribeiro et al., 2020; Serova et al., 2017). We performed dose-response curves to determine effective doses. Separate group of rats were randomly allocated into six experimental groups: (1) Control: distilled water; (2) M1145- treated group (132 µg); (3) Y1R agonist-treated group receiving the Y1R agonist [Leu^{31}- Pro^{34}]NPY (132 µg); (4) M1145+Y1R: group administered with both substances; (5) M1145+Y1R+M871: group treated with M1145, [Leu^{31}- Pro^{34}]NPY and the GALR2 antagonist (M871; 132 µg); (6) M1145+Y1R+ANA-12: group treated with M1145, [Leu^{31}-Pro^{34}]NPY and the TrkB antagonist (ANA-12, 5.06304, Sigma, St. Louis, MO, USA; 0.5 mg/kg, i.p.) (N=6 in each group).

Swimming sessions were conducted by placing individually the rats in cylinders containing water (25±0.2°C), 30 cm deep. Two sessions were conducted: an initial 15 min pretest followed 48 h later by a 5-min test. The water in the cylinders was changed after every trial. The FST was performed according to previously reported methods (Borroto-Escuela et al., 2021; Koike & Chaki, 2014). The total duration of floating (immobility) and swimming 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. 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.

Statistical Analysis

Data are presented as mean ± SEM, and sample number (n) is indicated in figure legends. GraphPad PRISM 8.0 (GraphPad Software, La Jolla, CA, USA) was used to analyze all data. 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).

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in*http://www.guidetopharmacology.org*, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

Results

Ventral dentate gyrus is activated under GALR2 and the Y1R agonists intranasal infusion

The assessment of the successful delivery of M1145 and the Y1R agonists to the brain via the i.n. route was confirmed by c-fos induction. The i.n. administration of the Y1R agonist induced a significant increase in the number of c-fos-IR profiles in the granular region of the ventral dentate gyrus (one-way ANOVA, $F4, 15 = 12.04$, $p < 0.001$, Newman-Keuls post-hoc test: p<0.05) (Figure 1 a,b) compared with the control group. Conversely, the administration of the GalR2 agonist M1145 alone lacked effects on the numbers of c-fos positive cells (Figure 1 b) compared with the control group (Figure 1 b, c).

Furthermore, M114545 and Y1R agonist coinjection significantly increased the number of c-Fos-IR profiles in the granular region of the ventral dentate gyrus compared with the M1145 and the control groups (Newman-Keuls post-hoc test: p<0.001) and with the Y1R agonist alone group (Newman-Keuls post-hoc test: p<0.05) (Figure 1 b, d). The cotreatment with the GALR2 antagonist M871 specifically blocked this M1145 and Y1R agonists coadministration effects in the ventral dentate gyrus (Newman-Keuls post-hoc test: p<0.05) (Figure 1 b), indicating the participation of GALR2 in the Y1R-M1145 agonists interaction to stimulate c-fos induction.

GALR2 and the Y1R agonists intranasally administered increased cell proliferation in the ventral Hippocampus

We evaluated the impact of GALR2 agonist M1145 and the Y1R agonist intranasally co-injected on adult ventral hippocampal cell proliferation by using the proliferating cell nuclear antigen (PCNA). M1145 and the Y1R agonist coadministration significantly increased cell proliferation, as demonstrated by the number of PCNA-IR cells, specifically in the sub-granular zone (Sgz) of the dentate gyrus compared to control (one-way ANOVA, $F4,15 = 12.38$, p<0.001, Newman–Keuls post-hoc test: p<0.001) (Figure 2 a,b,d), M1145 (Newman–Keuls post-hoc test: p<0.001) and Y1R agonist groups (Newman–Keuls post-hoc test: p<0.05) (Figure 2 a,b,d). The addition of GALR2 antagonist M871 completely blocked the M1145 and the Y1R agonist effects in the dentate gyrus (Newman–Keuls post-hoc test: p<0.01) (Figure 2 b), validating the participation of GALR2 in the Y1R/GALR2 agonist interaction to stimulate cell proliferation on the ventral hippocampus.

Moreover, the intranasal administration of the Y1R agonist alone induced an increase in the number of PCNA positive cells in the subgranular zone (Sgz) of the ventral hippocampus (Figure 2 a,b) compared with the control and M1145 groups Newman-Keuls post-hoc test: p<0.05) (Figure 2 a,b). However, the intranasal delivery of M1145 alone lacked effects on the numbers of PCNA-IR profiles (Figure 2b) compared with the control group (Figure 2 a–c).

Enhanced cell proliferation is related to increased brain-derived neurotrophic factor (BDNF) upon M1145 and Y1R agonist agonist coactivation

To study the cellular mechanism related to the observed effects on cell proliferation, we study the BDNF expression on the ventral hippocampal dentate gyrus (DG) after M1145 and/or Y1R agonist administration.

BDNF-positive cells were found specifically in the subgranular zone (Sgz) of the ventral hippocampus, and some scattered cells were observed in the polymorphic layer (P) of the ventral DG (Figure 3a). Stereological quantification of BDNF positive cells demonstrated a significant increase after the intranasal coinjection of M1145 and YR1 agonist compared to control (one-way ANOVA, F4, 15 = 11.12, $p < 0.001$, Newman-Keuls post-hoc test: $p<0.001$), M1145 (Newman-Keuls post-hoc test: $p<0.001$) or the YR1 agonist alone (Newman-Keuls post-hoc test: $p<0.05$) (Figure 3 a-d).

The injection of the Y1R agonist induced a significant increase in the number of BDNF-positive cells in the ventral dentate gyrus (Newman-Keuls post-hoc test: $p<0.05$) (Figure 3 b) compared with the control and the M1145 groups. However, the injection of M1145 alone lacked effects on the number of BDNF-positive cells in the ventral DG. Likewise to the PCNA-IR response described above, the presence of the GALR2 antagonist M871 completely blocked the increase induced by the coinjection (Newman-Keuls post-hoc test: $p<0.05$) (Figure 3 b), demonstrating the involvement of GALR2 in this interaction.

GALR2 agonist and Y1R agonist interaction enhanced GALR2/Y1R heteroreceptor complexes and neurite length on hippocampal neuronal cells

To study at receptor level the cellular mechanisms related to the observed in vivo effects we performed in situ proximity ligation assay (PLA) on hippocampal neuronal cells. This procedure allowed to analyze the GALR2/Y1R heteroreceptor complexes formation after M1145 and/or Y1R agonist incubation.

PLA-positive red clusters were found specifically in the membrane and cytoplasmatic region of hippocampal neuronal cells (Figure 4a-d). Quantification of PLA demonstrated an increase in the density of the PLA-positive red clusters after Y1R agonist incubation compared to control (one-way ANOVA, $F_{4,20} = 16.25$, $p < 0.001$, Newman-Keuls post-hoc test: $p<0.05$) or M1145 incubation (Newman-Keuls post-hoc test: $p<0.05$) (Figure 4 a,c-d). Moreover, upon incubation with M1145 and the Y1R agonist significantly increased the number of PLA-positive red clusters in the hippocampal neuronal cells (Figure 4a, d) compared to control (Newman-Keuls post-hoc test: $p<0.001$), M1145 alone (Newman-Keuls post-hoc test: $p<0.001$) and Y1R agonist alone (Newman-Keuls post-hoc test: $p<0.01$). Moreover, the specific GALR2 antagonist M871 counteracted this synergic effect (Newman-Keuls post-hoc test: $p<0.01$) (Figure 4a), demonstrating that this interaction was mediated through the coactivation of GALR2 and Y1R.

Due to the changes observed in the density of these complexes upon treatments, we decided to study the morphology and structural plasticity changes of hippocampal cultures upon these pharmacological treatments. The data show a significant synergistic increase of mean neurite length upon coactivation of M145 and the Y1R agonist for 24 hours compared to the YR1 agonist group alone (one-way ANOVA, F4, 20 = 16.91, $p < 0.001$, Newman-Keuls post-hoc test: $p<0.05$), control (Newman-Keuls post-hoc test: $p<0.001$) and M1145 alone (Newman-Keuls post-hoc test: $p<0.001$) (Figure 4b,c,d). The Y1R agonist incubated alone increased the neurite length compared to control and M1145 alone (Newman-Keuls post-hoc test: $p<0.01$) (Figure 4b). Furthermore, the presence of GALR2 antagonist M871 entirely blocks the synergic effects (Newman-Keuls post-hoc test: $p<0.05$) (Figure 4b).

Enhancement of antidepressant-like response by intranasally-administered GALR2 and Y1R agonists in the forced swimming test

We performed the forced swimming test (FST) to achieve the functional outcome related to the findings on the ventral hippocampus after the intranasal coadministration of GALR2 and Y1R agonists. Rats were pre-exposed to water for 15 minutes in the FST and twenty-four hours after the intranasal (i.n.) administration the immobility and swimming parameters were measured during the 5 min test phase to assess signs of depression-like behavior.

The dose response curve showed that the GALR2 agonist lacked effects at 68 μg and 132 μg in the FST. Regarding the Y1R agonist, the 68 μg dose was ineffective while the 132 μg induced a significant decrease in the immobility time (one-way ANOVA, $F_{4,25} = 3.79$, $p<0.05$, Supplementary Figure 1a) compared to the rest of the groups (Newman-Keuls post-hoc test: $p<0,05$; Supplementary Figure 1a). Moreover, an increase

in the swimming behavior (one-way ANOVA, $F_{4,25} = 3.57$, $p<0,05$; Supplementary Figure 1b) was observed compared to the rest of the groups (Newman-Keuls post-hoc test: $p<0,05$; Supplementary Figure 1b).

Upon the M1145 and the Y1R i.n. coadministration a significant decrease in the immobility time (one-way ANOVA, $F_{5,30} = 8.96$, $p<0.001$, Figure 5a) was observed compared with control animals (Newman-Keuls post-hoc test: $p<0,001$; Figure 5b), M1145 (Newman-Keuls post-hoc test: $p<0,001$; Figure 5b) and Y1R agonist alone (Newman-Keuls post-hoc test: $p<0,01$; Figure 5b). Moreover, an increase in the swimming behavior (one-way ANOVA, $F_{5,30} = 10.58$, $p<0,001$; Figure 5b) was observed after the combined treatment compared to the control animals (Newman-Keuls post-hoc test: $p<0,001$; Figure 5b), M1145 (Newman-Keuls post-hoc test: $p<0,001$; Figure 5b) and Y1R agonist alone (Newman-Keuls post-hoc test: $p<0,05$; Figure 1 b). GALR2 contribution in this result was confirmed since the addition of the GALR2 antagonist M871 counteracted the synergistic effects on immobility (Newman-Keuls post-hoc test: $p<0,01$; Figure 5b) and swimming (Newman-Keuls post-hoc test: $p<0,05$; Figure 5b) induced by the coadministration of M1145 and the Y1R agonist in the FST. Importantly, we confirmed the BDNF influence in this action since the addition of the TrkB antagonist ANA-12 blocked the synergistic on immobility (Newman-Keuls post-hoc test: $p<0,01$; Figure 5b) and swimming (Newman-Keuls post-hoc test: $p<0,05$; Figure 5b) induced by the coadministration of M1145 and the Y1R agonist in the FST.

The i.n. administration of the Y1R agonist decreased the time of immobility compared to the control (Newman-Keuls post-hoc test: $p<0,05$; Figure 5b) and the M1145 (Newman-Keuls post-hoc test: $p<0,05$; Figure 5b) animals; while induced an increase in the swimming behavior compared to the control (Newman-Keuls post-hoc test: $p<0,05$; Figure 5b) and the M1145 (Newman-Keuls post-hoc test: $p<0,05$; Figure 5b) groups. However, the administration of the GalR2 agonist M1145 alone lacked effects on the FST (Figure 5b) compared with the control group.

Discussion

The present study demonstrates, for the first time, the existence of an interaction between Galanin (GAL) and neuropeptide y (NPY) in the dentate gyrus (DG) of the ventral hippocampus upon the intranasal administration of GALR2 and Y1R agonists. Intranasal (i.n.) administration offers exciting potential to bypass the blood-brain barrier for the direct delivery of peptides and protein therapeutics rapidly into the CNS, supported by robust evidence in preclinical and clinical trials (Lochhead & Thorne, 2012; Rawal, Patel, & Patel, 2022). This method will be crucial as novel therapies continue to be studied in clinical trials and has several benefits, such as fewer side effects than peripheral administration and the comfort of non-invasiveness application (Chapman et al., 2013; Crowe & Hsu, 2022). Accordingly, intranasal esketamine has recently come into the market as an antidepressant, but its use is limited due to potential neurotoxicity, psychocomimetic side effects, potential abuse and interindividual variability in treatment response (Langmia, Just, Yamoune, Muller, & Stingl, 2022).

Following intranasal GALR2 and Y1R agonists co-administration we observed an increased cell proliferation in the ventral dentate gyrus (DG) of the hippocampus by using the proliferating cell nuclear antigen (PCNA). In agreement, we have recently observed the ability of the co-agonist treatment to enhance the cell proliferation in the DG of the dorsal hippocampus at 24 hours (Borroto-Escuela et al., 2022; Mirchandani-Duque et al., 2022). Interestingly, enhanced resilience in a model of depression was recently demonstrated by genetically boosting neurogenesis in the ventral hippocampal DG (Planchez et al., 2021). Furthermore, the molecule P7C3 increased cell proliferation in the hippocampal DG associated to antidepressant effects in rodents and primates (Bauman et al., 2018; Walker et al., 2015). Moreover, intranasal administration of the Y1R agonist alone increased cell proliferation in the ventral DG in this work. However, we observed that the Y1R agonist lacks effects on cell proliferation in the dorsal DG (Borroto-Escuela et al., 2022; Mirchandani-Duque et al., 2022). These results confirm the functional differences described between ventral and dorsal parts and a differential role for NPY in these subregions of the hippocampal formation (Baptista & Andrade, 2018; Lee, Kim, Cho, Kim, & Park, 2017). Regarding the intranasal administration of the GALR2 agonist alone, we observed no effects on ventral hippocampal cell proliferation. Previously, it was reported that GalR2/3 mediated the proliferative and trophic effects of GAL (Abbosh et al., 2011), indicating in subse-

quent studies a role for GALR3 (Khan, Khan, Runesson, Zaben, & Gray, 2017). However, these studies were performed *in vitro*conditions, exhibiting significant differences in systems *in vivo* .

At cellular level, these effects on hippocampal cell proliferation after GALR2 and Y1R agonists intranasally co-administered seem to be mediated by increased BDNF expression on the ventral hippocampal DG. BDNF, a member of neurotrophins, has a pivotal role in increasing neurogenesis through changes in proliferation and cell survival (Miranda et al., 2019). Recent evidences showed that physical exercise protects the brain from depressive symptoms through increasing hippocampal neurogenesis combined with BDNF (Murawska-Cialowicz et al., 2021). Thus, therapeutics that promote the close correlation between dentate neurogenesis and BDNF, as seen under the GALR2 and Y1R agonist combination, may be the key to preventing or curing depression. In this regard, our data are consistent with previous evidences on the BDNF-related neuroprotective effect of NPY in models of neurodegeneration (Corvino et al., 2012; Croce et al., 2011).

These cellular effects induced by GALR2 and the Y1R agonist were achieved in hippocampal neuronal cells by studying GALR2/Y1R heteroreceptor complexes upon agonist coactivation of both receptor protomers. We observed an increase of GALR2/Y1R heteroreceptor complexes by using *in situ* PLA upon combined co-incubation with GALR2 and Y1R agonists. We have previously reported the presence of GALR2/Y1R heteroreceptor complexes in HEK cells and several limbic brain regions, including the amygdala and the dorsal hippocampus (Borroto-Escuela et al., 2021; Narvaez et al., 2016; Narvaez et al., 2018; Narvaez et al., 2015). Additionally, we confirmed how 5HT1A-FGFR1 heteroreceptor complexes significantly stimulated hippocampal plasticity linked to antidepressant-like actions (Borroto-Escuela et al., 2012; Narvaez et al., 2020). Besides, GALR2 and Y1R agonist co-incubation promoted an increasement of the neurites length in hippocampal neuronal cells at 24 hours, where BDNF might be a common mechanism in our in vivo and in vitro experiments. In agreement, it was shown that BDNF exerted a promoting effect on dendritic outgrowth in primary hippocampal cultures and the hippocampus (H. I. Kim, Lim, Choi, Kim, & Choi, 2022; Park et al., 2016).

The functional outcome was certified by demonstrating the enhancement of the antidepressant-like response after GALR2 and Y1R agonists intranasal co-administration on the forced swimming (FST) test at 24 h. In previous reports, the intranasal infusion of the Y1 agonist (Serova et al., 2017) and NPY (Nahvi et al., 2021) in rats or NPY in humans (Mathe et al., 2020) induced antidepressants effects at least for 24 hours. In agreement, the N-methyl-d-aspartate receptor antagonist, Ketamine or the group II metabotropic glutamate (mGlu2/3) receptor antagonist, LY341495 were shown to exhibit antidepressant-like effects after a single injection in the FST in rats at 24hours (Gigliucci et al., 2013; Koike & Chaki, 2014). We found that the intranasal administration of the GALR2 agonist alone lacks antidepressant-like effects at 24 hours. Species-specific differences between rats and mice in antidepressant responses have been reported (Polis, Fitzgerald, Hale, & Watson, 2019). Thus, previous studies indicating that the intranasal infusion of an spexin-based GALR2 agonist induced antidepressant-like effects was performed on mice at 2-3 hours (Yun et al., 2019). Moreover, we found that the GALR2 antagonist M871 counteracted the enhanced response observed, as previously reported after intranasal GALR2 and Y1R agonists (Borroto-Escuela et al., 2022). In this previous work we confirmed that the behavioral effects were independent of the motor activity since GALR2, Y1R agonist or their intranasal coadministration not showed locomotor alterations. In agreement with our findings, ventral hippocampus was involved in the antidepressant effects of NPY in posttraumatic stress disorder (Sabban & Serova, 2018). Accordingly, we may speculate that the molecular mechanisms underlying the antidepressant-enhancing actions of the Y1R and GALR2 agonists at 24 hours might be mediated by enhancing the signaling of these two protomers in the Y1R–GALR2 heterocomplexes in the neurogenic zone of the ventral hippocampus. Moreover, our data support the contribution of BDNF in this mechanism, as observed *in vivo* and *in vitro*experiments. Besides, the TrkB antagonist ANA-12 was shown to counteract the antidepressant effects of ketamine at 24 hours (Ribeiro et al., 2020). Interestingly, running was shown to enhance BDNF signaling and neuronal proliferation on the ventral hippocampus related with antidepressant effects (Murawska-Cialowicz et al., 2021).

Taken together, the intranasal infusion of Y1R and GALR2 agonists may promote cell proliferation in the

ventral hippocampal dentate gyrus and the induction of the BDNF neurotrophic factor. These effects may be mediated by Y1R–GALR2 heteroreceptor complexes to mediate increased neurites outgrowth observed on neuronal hippocampal cells. Accordingly, these cellular effects may be linked to the enhanced antidepressant effects observed. In this way, through a reorganization of the signaling in this Y1R–GALR2 heteroreceptor complex, including the homo-receptor complex associated with altered hetero/homo-signaling might mediate the increased antidepressant actions. Our data may suggest the development of new heterobivalent o multi-targeting agonist pharmacophores acting on Y1R–GALR2 heterocomplexes in the ventral hippocampus for the novel therapy of mayor depression disorder or depressive-affecting diseases.

Data Sharing and Data Accessibility

T he data that support the findings of this study are openly available in Institutional repository of the University of Malaga (RIUMA) and from the corresponding author upon reasonable request.

Conflict of Interest

The authors have no conflict of interest to declare.

Acknowledgments

This research was funded by the UMA18-FEDERJA-100 (Junta de Andalucía), Proyecto Jovenes Investigadores (B1-2019-04), Proyecto puente (B4-2021-06), Universidad de Málaga, Spain, to M.N. Swedish Medical Research Council, Sweden (62X-00715-50-3), to K.F., by Stiftelsen Olle Engkvist Byggmästare to K.F., and by Hjärnfonden, Sweden (F02018-0286), Hjärnfonden, Swe-den (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. Ventral dentate gyrus is activated under GALR2 and the Y1R agonists intranasal coinjection. Effects of the intranasal (in) administration of Galanin 2 receptor agonist (M1145) and Y1R receptor agonist, either alone or in combination with or without the GAL 2 receptor antagonist (M871) on c-Fos expression in the granular layer of ventral dentate gyrus. **(a,d)** The majority of the c-Fos-IR profiles were located in the granular cell layer (Gcl). It is also indicated the polymorphic layer (P) of the dentate gyrus in the ventral hippocampus (Bregma: -5.6 mm; according to the Paxinos and Watson stereotaxic atlas (2006)). **(b)** Quantification of the total number of c-Fos IR nuclei within the dentate gyrus of the ventral hippocampus. Data, expressed as mean ± SEM, show the differences between groups after administration of Control, M1145, Y1R agonist [Leu^{31}-Pro^{34}]NPY, or the coadministration of both agonists with or without M871. The intranasal coadministration of M1145 and the Y1R agonist increased the c-Fos expression in the ventral hippocampus compared to the effects of both agonists given alone and the control group. Moreover, this effect was counteracted by the GALR2 antagonist M871.* $P<0.05$ vs control, M1145 and M1145+Y1R; δ $P<0.05$ vs M1145+Y1R; *** $P<0.001$ vs control and M1145 according to one-way ANOVA followed by Newman-Keuls post-hoc test (n=4 in each group). Inter-group comparisons are indicated by the vertical lines from the horizontal line above bars. Intranasal coadministration of M1145 and Y1R agonist**(d)** increased the c-Fos-IR nuclei in Gcl in the dentate gyrus compared with the control group **(c).** Arrows indicate examples of c-Fos-IR nuclei. Dashed lines outline the Gcl of the dentate gyrus. Abbreviations: Control= Distilled water; M1145 = Galanin 2 receptor agonist 132 μg; Y1R = Y1R receptor agonist [Leu^{31}-Pro^{34}]NPY 132 μg; M1145+ Y1R = Coadministration of M1145 and Y1R; M1145+ Y1R +M871 = Co-administration of M1145, Y1R and GALR2 antagonist M871 132 μg. Brain processing was performed 24 hours after treatments; see material and methods for further details.

Figure 2. Intranasal coadministration of Galanin receptor 2 and Y1R agonists increases cell proliferation in the ventral dentate gyrus of adult rats. Proliferating cell nuclear antigen immunolabelling (PCNA) immunolabelling (PCNA+) in the dentate gyrus of the ventral hippocampus, after the intranasal (in) administration of Galanin 2 receptor agonist (M1145) and Y1R receptor agonist, either alone or in combination with or without the GAL 2 receptor antagonist (M871). **(a,d)** The majority of the PCNA positive cells were 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 as clusters of 3–4 cells. (Bregma: -5.6 mm; according to the Paxinos and Watson stereotaxic atlas (2006)).**(b)** Quantification of total PCNA-IR cells in the dentate gyrus of the ventral hippocampus. Data represent mean ± SEM to show the differences between groups after administration of Control, M1145, Y1R agonist [Leu^{31}-Pro^{34}]NPY, or the coadministration of both agonists with or without M871. M1145 and the Y1R agonist coadministration increased the number of cells with PCNA+ expression in the ventral hippocampus compared to the effects of the two peptides given alone and the control group. Furthermore, this effect was counteracted by the GALR2 antagonist M871. *P <0.05 vs control, M1145 and M1145+Y1R; **P <0.01 vs M1145+Y1R; ***P <0.001 vs control and M1145 according to one-way ANOVA followed by Newman-Keuls post-hoc test. Inter-group comparisons are indicated by the vertical lines from the horizontal line above bars. N=4 in each group. M1145 and Y1R agonist intranasal coadministration**(d)** increased the PCNA immunolabelling in Sgz in the dentate gyrus compared with the control group **(c).** Arrows indicate examples of clusters of PCNA positive nerve cells. Dashed lines outline the Gcl of the dentate gyrus. Abbreviations: Control= Distilled water; M1145 = Galanin 2 receptor agonist 132 μg; Y1R = Y1R receptor agonist [Leu^{31}-Pro^{34}]NPY 132 μg; M1145+ Y1R = Coadministration of M1145 and Y1R; M1145+ Y1R +M871 = Co-administration of M1145, Y1R and GALR2 antagonist M871 132 μg. Brain processing was performed 24 hours after treatments; see material and methods for further details.

Figure 3. Effects induced by M1145 and Y1R agonist on hippocampal brain-derived neurotrophic factor immunoreactive (BDNF-IR) cells of the dentate gyrus (DG) hippocampal region. (a) BDNF-IR cells were located mainly in the subgranular zone (Sgz) of the dentate gyrus at the border of the granular cell layer (Gcl), some scattered cells were found in the polymorphic layer (P) of the dentate gyrus in the ventral hippocampus (Bregma: –5.6 mm; according to the Paxinos and Watson (2006) stereotaxic atlas). (b) Quantitative analysis of BDNF-IR cells of the DG. The intranasal coadministration of M1145 and the Y1R agonist significantly increased BDNF-IR cells in the ventral DG. This effect was blocked in the presence of the GALR2 antagonist M871. * P<0.05 vs control, M1145 and M1145+Y1R; δ P<0.05 vs M1145+Y1R; *** P <0.001 vs control and M1145 according to one-way ANOVA followed by Newman-Keuls post-hoc test (n=4 in each group). The vertical lines from the horizontal line above the bars indicate the inter-group comparisons. (c,d) Representative microphotographs showing the increase in the BDNF-positive cells in the DG after M1145 and Y1R agonist coinjection (d) compared with the control group (c). Black arrows point to BDNF-IR cells. Dashed lines outline the Gcl of the dentate gyrus. Abbreviations: Control= Distilled water; M1145 = Galanin 2 receptor agonist 132 μg; Y1R= Y1R receptor agonist [Leu^{31}-Pro^{34}]NPY 132 μg; M1145+ Y1R = Coadministration of M1145 and Y1R; M1145+ Y1R +M871 = Co-administration of M1145, Y1R and GALR2 antagonist M871 132 μg. Brain processing was performed 24 hours after treatments; see material and methods for further details.

Figure 4 . Demonstration of GALR2/Y1R heteroreceptor complexes by *in situ* PLA and morphological changes on hippocampal neuronal cells. **(a)** The presence of positive PLA signals (red circles) was determined by using the *in situ* proximity ligation assay (*in situ* PLA) on hippocampal neuronal cells after treatment with Galanin receptor 2 agonist (M1145, 100nM) and Y1R receptor agonist (100nM), either alone or in combination with or without the GALR2 receptor antagonist (M871, 1μM). M1145 and Y1R agonist incubation significantly increased PLA positive signals. Quantification of PLA signals was performed by measuring red PLA positive blobs per nucleus per sampled field by an experimenter blind to treatment conditions. This effect was blocked by treatment with the GALR2 antagonist M871. *P <0.05 vs control and M1145; **P <0.01 vs Y1R and M1145+Y1R+M871; ***P <0.001 vs control and M1145 according to one-way ANOVA 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. **(b)** GALR2 and Y1R agonists modulation of neurites length. The length of neurites per cell were determined after immunofluorescent labeling of neurons and neuronal nuclei (Pan Neuronal Marker (ABN2300) /neuronal nuclei (DAPI)). Quantification is shown in Figure 4b, where the data are presented as mean ± SEM. The combined M1145 and Y1R agonist group is significantly different from the rest of the groups. *P <0.05 vs Y1R and M1145+Y1R+M871; **P <0.01 vs control and M1145; ***P <0.001 vs control and M1145 according to

one-way ANOVA followed by Newman-Keuls post-hoc test. **(c,d)** Representative microphotographs of the significant increase in the density of GALR2/Y1R heteroreceptor complexes (PLA clusters) and neurites length per hippocampal neuronal positive cell after M1145 and Y1R agonist treatment**(d)** compared with the control group **(c)** . Receptor complexes are shown as red PLA blobs (clusters, indicated by white arrows) found in high densities per hippocampal neuronal cell using confocal laser microscopy. The nuclei are shown in blue by DAPI staining and the cells in green are hippocampal neurons-positive (Pan Neuronal Marker, ABN2300) using confocal laser microscopy. White arrowheads point to neurite extensions. Abbreviations: Control= Culture medium; M1145 = Galanin 2 receptor agonist 100 nM; Y1R agonist = Y1R receptor agonist [Leu^{31}- Pro^{34}]NPY 100nM; M1145+ Y1R = Coadministration of M1145 and Y1R; M1145+ Y1R +M871 = Coadministration of M1145, Y1R and GALR2 antagonist 1μM.

Figure 5. Behavioral actions induced by Galanin 2 receptor agonist (M1145) and the Neuropeptide Y (NPY) Y1 receptor agonist (Y1R agonist) alone and in combination in the forced swimming test (FST). An antidepressant-like effect in the FST was observed after M1145 and Y1R agonist intranasal co-administration following a 24 hours delay. Furthermore, this effect is counteracted by the GAL 2 receptor (GALR2) antagonist M871. Cumulative behavioral duration of Immobility**(a)** and swimming **(b)** time in the FST. Data represent mean ± SEM. N=6 animals in each group. For a: *P <0.05 vs Control and M1145; **P <0.01 vs Y1R agonist, M1145+Y1R+M871 and M1145+Y1R+ANA-12; ***P <0.001 vs Control and M1145. For b: *P <0.05 vs Control and M1145; P <0.05 vs Y1R agonist, M1145+Y1R+M871 and M1145+Y1R+ANA-12; ***P <0.001 vs Control and M1145 according to one-way ANOVA followed by Newman-Keuls post-hoc test. Inter-group comparisons are indicated by the horizontal and vertical lines above bars. Abbreviations: Control= Distilled water; M1145 = Galanin 2 receptor agonist 132 μg; Y1R = Y1R receptor agonist [Leu^{31}-Pro^{34}]NPY 132 μg; M1145+Y1R= Coadministration of M1145 and Y1R; M1145+Y1R+M871 = Coadministration of M1145, Y1R and GALR2 antagonist M871 132 μg; M1145+Y1R+ANA-12 = Coadministration of M1145, Y1R and TrkB antagonist ANA-12 0,5 mg/Kg.

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