Biomedicine & Pharmacotherapy 161 (2023) 114433

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Decreased medial prefrontal cortex activity related to impaired novel object preference task performance following GALR2 and Y1R agonists intranasal infusion

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ARTICLE INFO

Keywords:
Medial prefrontal cortex
Novel object preference task
GALR2
Y1R
Intranasal infusion

ABSTRACT

Different brain regions' interactions have been implicated in relevant neurological diseases, such as major depressive disorder (MDD), anxiety disorders, age-dependent cognitive decline, Alzheimer's disease (AD) and addiction. We aim to explore the role of the medial prefrontal cortex (mPFC) in the Neuropeptide Y (NPY) and Galanin (GAL) interaction since we have demonstrated specific NPY and GAL interactions in brain areas related to these brain diseases. We performed GALR2 and Y1R agonists intranasal infusion and analyzed the mPFC activation through c-Fos expression. To assess the associated cellular mechanism we studied the formation of Y1R-GALR2 heteroreceptor complexes with in situ proximity ligation assay (PLA) and the expression of the brain-derived neurotrophic factor (BDNF). Moreover, the functional outcome of the NPY and GAL interaction on the mPFC was evaluated in the novel object preference task. We demonstrated that the intranasal administration of both agonists decrease the medial prefrontal cortex activation as shown with the c-Fos expression. These effects were mediated by the decreased formation of Y1R-GALR2 heteroreceptor complexes without affecting the BDNF expression. The functional outcome of this interaction was related to an impaired performance on the novel object preference task. Our data may suggest the translational development of new heterobivalent agonist pharmacophores acting on Y1R–GALR2 heterocomplexes in the medial prefrontal cortex for the novel therapy on neurodegenerative and psychiatric diseases.

Data Sharing and Data Accessibility: The 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.

1. Introduction

Different brain regions interactions have been implicated in relevant neurological diseases, such as major depressive disorder (MDD), anxiety disorders, age-dependent cognitive decline, Alzheimer's disease (AD) and addiction [1–5]. These brain regions are conceived as a neuronal network including, but not limited to, cortical or subcortical brain regions, such as hippocampus, amygdala, dorsal raphe nucleus and medial prefrontal cortex [6–8].

Major depressive disorder (MDD) is described by a compilation of

Abbreviations: GALR2, Galanin Receptor 2; Y1R, Neuropeptide Y1 receptor.

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https://doi.org/10.1016/j.biopha.2023.114433
Received 23 December 2022; Received in revised form 10 February 2023; Accepted 20 February 2023
Available online 26 February 2023

behavioral, emotional and cognitive symptoms with more than 300 million people diagnosed in the world. Moreover, suicide is considered the worst outcome or consequence of MDD, over 700,000 human lives lost every year, conferring a challenge for the medical community [9]. Anxiety disorders are the most common of psychiatric disorders, showing a lifetime prevalence of over 25% [10]. Regarding AD, is the most common neurodegenerative disease with 35 million people diagnosed, representing about 70% of dementia cases worldwide [11]. Furthermore, the COVID-19 pandemic produced multiple challenges, such as loneliness or financial hardship, producing about 34% prevalence of depression in general population, with 5–15% suicidal ideation in that period [12]. Seriously, the COVID-19 infection was demonstrated to impair cognitive and psychiatric symptoms in these patients [13].

Nowadays there is no adequate treatment options for these neurological diseases, implicating that additional underlying mechanisms need to be considered in order to improve the efficacy of treatments. For example, main prescribed antidepressants target monoamines with noteworthy limitations, such as adverse events and delayed onset of efficacy [14]. Remarkably, 65% of patients present treatment-resistant depression (TRD), without achieving remission and 50% of such patients fail to respond [15]. Besides, no pharmacological treatments are available to cure or even significantly slow down the course of neurodegenerative diseases [11]. Neuropeptide systems and their receptors participating in these neurological disorders and its related brain regions have distinctive consideration as attractive therapeutic targets on neurodegenerative and psychiatric diseases [16–18].

Neuropeptide Y (NPY) is one of the most abundant neuropeptides in the mammalian brain. Central NPY and its receptors, especially NPY Y1 receptors (Y1R) are involved in basic biological and pathophysiological functions, such as mood regulation, neuronal excitability, neuroplasticity and memory [18,19]. Several evidences indicate that the anxiolytic activity of NPY is primarily mediated by Y1R [20–23]. Regarding MDD, reduced brain NPY was found not only preclinical models [24–26], but also in postmortem brains from MDD patients who committed suicide [27,28]. Recently was demonstrated that intranasal NPY and the Y1R agonist administration produced antidepressant effects in rodents [29,30] and in MDD patients [31]. Similarly, in AD patients decreased NPY expression was related to memory impairment in hippocampal and cortical regions [32,33], with reduced NPY levels in cerebrospinal fluid and plasma samples [34]. Overall, Y1R have been proposed as a critical target on neurodegenerative and psychiatric diseases in different brain regions [35,36].

Galanin (GAL), is also a neuropeptide broadly distributed in the central nervous system [37]. The GAL role in anxiety mainly depends on the route and site of administration [38,39]. Concerning MDD, preclinical data demonstrated prodepressive-like effects for GAL, while antidepressant-like effects following GALR2 activation [40–43]. Recently, the intranasal infusion of modified GALR2 agonists induced antidepressant-like effects in rodents [44]. Moreover, GALR2 receptors were shown to mediate memory-improving and hippocampal toxicity-inhibiting effects in a preclinical model of AD [45,46].

We have demonstrated Neuropeptide Y (NPY) and Galanin (GAL) interactions through specific Y1R-GALR2 heteroreceptor complexes in interconnected brain regions, such as the amygdala, ventral and dorsal hippocampus or different hypothalamic regions with specific actions related to neurological disorders discussed above [47–52]. In this regard, we have recently described a facilitatory interaction between NPY and GAL through the formation of GALR2/Y1R heteroreceptor complexes on the dorsal and the ventral hippocampus. Associated augmented cell proliferation and spatial memory performance was found on dorsal hippocampus with great potential for AD, while enhanced antidepressant-like effects related to increased cell proliferation was observed in the ventral hippocampus [51,52].

Nowadays is crucial the accurate assessment of the implicated neural circuit network in psychiatric or neurocognitive disorders for developing future therapeutic strategies and understanding the pathology. Based on the distribution and actions of NPY and GAL related with MDD and AD, it would interesting to determine the involvement of both peptides in cortical regions. In this respect, we aim to explore the role of the NPY and GAL interaction on the medial prefrontal cortex (mPFC) since it plays an essential role in cognitive process, regulation of emotion, motivation, and sociability and is implicated in MDD, anxiety disorders and AD [53].

We performed an innovative method to deliver potential therapeutics to the brain, the intranasal infusion. Consequently, following GALR2 and Y1R agonists intranasal administration, we analyzed the mPFC activation using through c-Fos expression. To assess the associated cellular mechanism we studied the formation of Y1R-GALR2 heteroreceptor complexes with in situ proximity ligation assay (PLA) and the expression of the brain-derived neurotrophic factor (BDNF). Moreover, the functional outcome of the NPY and GAL interaction on the mPFC was evaluated in the novel object preference task.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats were acquired from CRIFFA (Barcelona; 200–250gr; 6–8 weeks) had ad-libitum food and water access. They were preserved under the standard 12 h dark/light cycle, with controlled relative humidity (55–60%) and temperature ($22^{\pm}2$ °C). Guidelines for preclinical experiments were performed following EU Directive 2010/63/EU and Spanish Directive (Real Decretory 53/2013) approvals. All dealings concerned with an experimental treatment, housing and maintenance of the rats were permitted by the Local Animal Ethics, Care, and Use Committee for the University of Málaga, Spain (CEUMA 45–2022-A).

2.2. Drugs used

Diluted peptides were freshly prepared in distilled water, that was used as control. Galanin receptor 2 agonist (M1145), Y1R receptor agonist [Leu^{31}, Pro^{34}] NPY, GALR2 Antagonist M871 were acquired from Tocris Bioscience (Bristol, UK). A detailed report is accessible in Supplement material on intranasal infusion of peptides.

2.3. Assessment of medial prefrontal cortex neuronal activity after intranasal infusion

Animals were randomly distributed into five experimental groups: [1] Control: distilled water; [2] Y1R agonist-treated group receiving the Y1R agonist [Leu^{31}- Pro^{34}] NPY (132 μg); [3] M1145- treated group (132 μg); [4] Y1R+M1145: group administered with both substances; [5] Y1R+M1145 +M871: group treated with M1145, [Leu^{31}- Pro^{34}] NPY and the GALR2 antagonist (M871; 132 μg) (n = 6 in each group). The doses indicated are based on previously published protocols [30,52].

Twenty-four hours after the after the intranasal infusion, 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 medial prefrontal cortex (3.20–2.70 Bregma) according to Paxinos & Watson atlas coordinates [54].

We used the c-Fos immunohistochemistry, as an indirect marker of neuronal activity. Free-floating sections were incubated for antigenic retrieval at 65 °C during 90 min in saline sodium citrate buffer (pH 6; 10 nM sodium citrate). After this procedure the slices were treated 30 min in 0.6% H_2O_2 to remove endogenous peroxidases. Then, slices were incubated at 4 °C overnight with a primary antibody mouse anti-c-Fos protein (sc-271243, 1:800, 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 SLU, 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 [49,50,52] (see Supplementary Materials for details).

2.4. Heteroreceptor complexes analysis by in situ proximity ligation assay

The in situ proximity ligation assay (in situ PLA) (NaveniFlex GR, Navinci, Sweden) was performed to uncover the presence of GALR2-Y1R heteroreceptor complexes on the medial prefrontal cortex in free-floating sections as described previously [51,55]. The in situ PLA Technology enables visualization of protein-protein interaction using one primary antibody for each target protein, without the need to disrupt the tissue microenvironment. Moreover, this method allow the precise localization of the target (Y1R-GALR2 heteroreceptor complexes) with intact tissue morphology and to study the dynamics of the interaction induced by an specific treatment, useful for biomarkers discovery.

Briefly, slices were treated with blocking buffer for 60 min at 37ºC in a pre-heated humidity chamber. Slices were then incubated with the primary antibodies diluent in a suitable concentration at 4 °C overnight. 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). Then, slices were washed three times, and the Navenibodies proximity probe mixture (Navenibody goat and Navenibody rabbit, were 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 at room temperature under gentle agitation and the sections were incubated with the Enzyme A in a humidity chamber at 37 °C for 60 min, followed by Enzyme B incubated in a humidity chamber at 37 °C for 30 min. The excess of connector oligonucleotides was removed by washing at room temperature under gentle agitation and the rolling circle detection mixture (Enzyme C, Tex615) was added to the slices and incubated in a humidity chamber at 37 °C for 90 min. Then, the slices were mounted on a microscope slide and a drop of appropriate mounting medium (e.g., Duolink Mounting Medium with DAPI, Sigma-Aldrich) was applied and sealed with nail polish. The slides were protected against light and stored for several days at − 20 °C before confocal microscope analysis. 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. Acquisition of microscopy images and in situ PLA data analysis was performed as previously described [56].

2.5. Assessment of brain-derived neurotrophic factor- (BDNF) induction on mPFC

The followed procedure was performed as described for c-Fos immunohistochemistry. Briefly, 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 rabbit anti-BDNF (Chemicon, Sigma-Aldrich, AB1534SP, 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-rabbit IgG, 1:200, B8895, 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 SLU, Darmstadt, Germany). BDNF-labeled cells were studied using the optical fractionator method in unbiased stereological microscopy (Olympus BX51 Microscope, Olympus, Denmark), as described above.

2.6. Behavioural testing

2.6.1. Novel object preference task

The novel object preference task was developed to evaluate instinctive tendency to explore novel items [57]. The novel object preference (Object recognition), in which the rats' exploration of a novel object is compared with that of a familiar object. Rats were exposed to the task to assess memory consolidation at 24 h using a plastic open field, 100 × 100 × 60 cm (length × width × height), under dim light. Rats were single-housed during the behavioral period. The task trials contain three phases: habituation, training, and test [58,59] as follows (Fig. 1a):

2.7. Habituation

Animals were handled for two days, then familiarized to the empty arena for 10 min (1 trial, 10 min).

2.8. Training

Every animal was placed in the middle of the arena 24 h after the habituation. Rats were allowed to explore duplicate objects placed near 2 corners in the arena. Each subject was allowed a total of 3 min of object investigation. After the exploration, all objects were cleaned with 5% ethanol.

2.9. Test

The test session was performed 24 h post-training, in which one of the objects was the third copy of the object used at acquisition and the other varied in shape and colour with similar weight and size. The animal was replaced in the arena, presented with objects in the same positions and were allowed to examine the objects (1 trial, 3 min). Exploration was described as time spent sniffing or touching the object with the nose or forepaws. If novel object recognition memory is intact subjects spend more time investigating the novel object. The discrimination capacity was represented by the time spent investigating the new object (N) compared with the time spent exploring the familiar same object (F). A discrimination ratio was calculated as DI= (N–F)/(N + F). An overhead video camera monitored and recorded the animal's behaviour, which was scored and analyzed blind to the treatment, using the Raton Time 1.0 software (Fixma S.L., Valencia, Spain). We also examined the locomotor activity using the video-tracking software EthovisionXT (Noldus, Wageningen, Nederland). Between trials, object position was counterbalanced between rats and the arena and the objects were carefully cleaned with 5% ethanol. The intranasal treatments were administered 24 h before the test phase. In addition, the total exploration time and the locomotor activity between the experimental groups were controlled to demonstrate that treatment did not affect the exploration ability of the rats.

3. Statistical analysis

The data obtained are showed as mean ± SEM, and sample number (n) is detailed 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 Tukey's multiple comparisons test

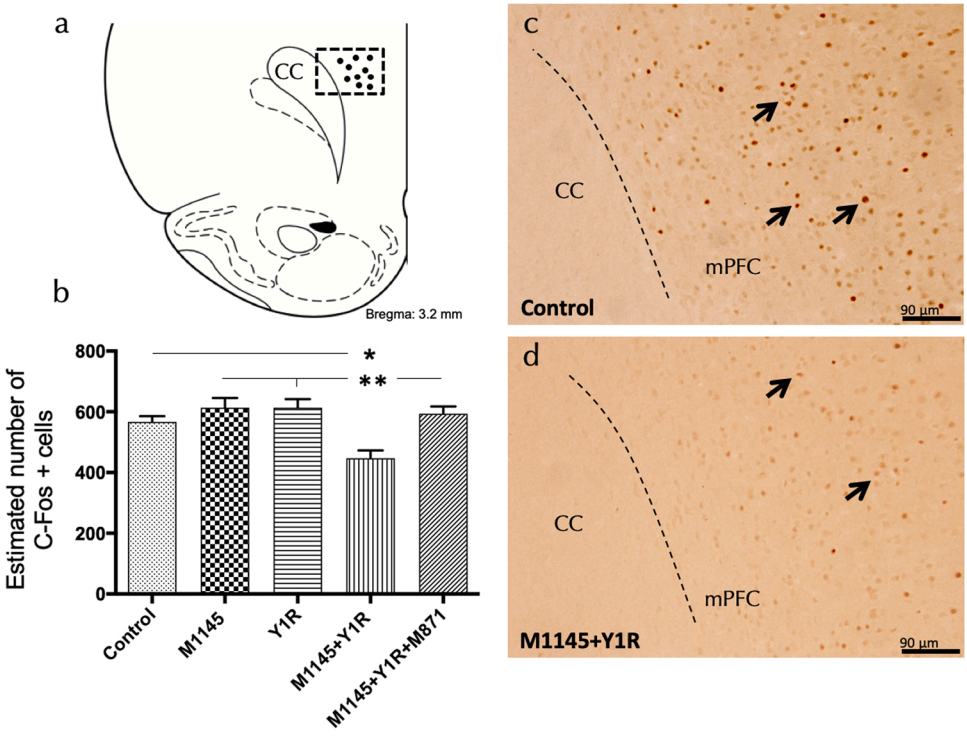

Fig. 1. Medial prefrontal cortex is inactivated under Y1R and GALR2 agonists intranasal delivery. c-Fos immunohistochemistry was used as an indirect marker of neuronal activity. C-Fos-labeled cells were studied using the optical fractionator method in unbiased stereological microscopy (Olympus BX51 Microscope, Olympus, Denmark). Effects of the intranasal (in) administration of Y1R receptor agonist ([Leu^{31}-Pro^{34}] NPY) and Galanin 2 receptor agonist (M1145), either alone or in combination the GAL 2 receptor antagonist (M871) on c-Fos expression in the medial prefrontal cortex. (a,d) C-Fos-IR profiles were specifically located medially to the corpus callosum. (Bregma: 3.2 mm; according to the Paxinos and Watson stereotaxic atlas (2006)). (b) Quantification of the total number of c-Fos IR nuclei within the medial prefrontal cortex. 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 decreased the c-Fos expression in the medial prefrontal cortex compared to the rest of the groups. Moreover, this effect was counteracted by the GALR2 antagonist M871. *P < 0.05 vs control; ** P < 0.01 vs M1145, Y1R and M1145 + Y1R +M871 according to one-way ANOVA followed by Tukey post-hoc test (n = 6 in each group). Data are expressed as mean ± SEM. Inter-group comparisons are indicated by the vertical lines from the horizontal line above bars. Intranasal coadministration of M1145 and Y1R agonist (d) decreased the c-Fos-IR nuclei in medial prefrontal cortex compared with the control group (c). Arrows indicate examples of c-Fos-IR nuclei. Dashed lines outline the corpus callosum. 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.

was performed to analyze the results. Within-group analyses to study the discrimination ability between the objects of the animals in the novel object preference task was achieved with paired Student's t-test (two-tailed). Differences were considered significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4. Results

4.1. Reduced c-Fos expression in the medial prefrontal cortex after Y1R and GALR2 agonists intranasal infusion

To demonstrate if medial prefrontal cortex is involved on the GALR2 and the Y1R agonists actions we evaluated the c-fos induction, a marker of neuronal activation, following their intranasal infusion.

The intranasal infusion of M1145 and Y1R agonists significantly decreased the number of c-Fos-IR profiles in the medial prefrontal cortex compared to the control group (one-way ANOVA, F4, 25 = 7.001, $p < 0.001$, Tukey post-hoc test: $p < 0.05$) (Fig. 1a-d) and the GALR2 and Y1R agonists administered alone (Tukey post-hoc test: $p < 0.01$). The cotreatment with the GALR2 antagonist M871 specifically blocked this reduced c-Fos expression in the medial prefrontal cortex (Tukey post-hoc test: $p < 0.01$) (Fig. 1b), indicating the participation of GALR2 in the Y1R-M1145 agonists interaction to inhibit c-fos induction. Conversely, the intranasal administration of the GalR2 agonist M1145 or the Y1R agonist given alone lacked effects on the numbers of c-Fos positive cells (Fig. 1b) compared with the control group (Fig. 1b, c). No c-Fos-IR profiles were observed in the corpus callosum.

4.2. Y1R and GALR2 agonists interaction decreased Y1R/GALR2 heteroreceptor complexes without affecting BDNF expression on medial prefrontal cortex

To study the cellular mechanisms at receptor level related to the observed c-Fos effects we performed in situ proximity ligation assay (PLA) on medial prefrontal cortex region. This procedure allowed to analyze the GALR2/Y1R heteroreceptor complexes formation after Y1R and/or M1145 agonist intranasal infusion. PLA-positive red clusters were found specifically in the membrane and cytoplasmatic region of medial prefrontal cortex cells (Fig. 2a-d). Quantification of PLA demonstrated a decrease in the density of the PLA-positive red clusters after Y1R and GALR2 agonists intranasal infusion compared to the rest of the groups (one-way ANOVA, F4,25 = 4.608, $p < 0.05$, Tukey post-hoc test: $p < 0.05$) (Fig. 2b-d). Upon intranasal administration of either Y1R agonist or M1145 alone no effects on Y1R-GALR2 heteroreceptor complexes were observed. Moreover, the specific GALR2 antagonist M871 counteracted this effect (Tukey post-hoc test: $p < 0.05$) (Fig. 2b), demonstrating that this interaction was mediated through the coactivation of GALR2 and Y1R. Lack of Y1R-GALR2 heteroreceptor complexes was observed in the corpus callosum.

Moreover, we study the brain-derived neurotrophic factor (BDNF) expression on the mPFC after M1145 and/or Y1R agonist intranasal administration. BDNF-positive cells were found specifically in the mPFC, while no cells were observed in the corpus callosum (Fig. S1). The intranasal infusion of Y1R and GALR2 agonists lacked effects on the number of BDNF-positive cells in the mPFC (one-way ANOVA, F4, 25 = 0.871, $p > 0.05$) (Fig. S1).

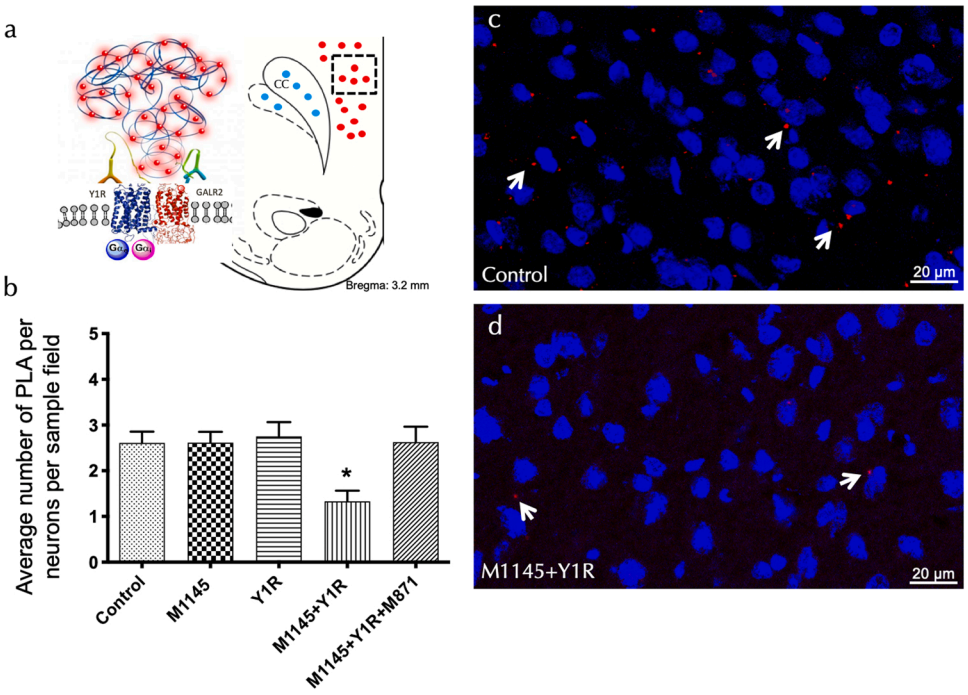

Fig. 2. Demonstration of Y1R-GALR2 heteroreceptor complexes by in situ PLA on medial prefrontal cortex region. The in situ PLA Technology enables visualization of protein-protein interaction using one primary antibody for each target protein (a) The diagram shows on the left the experimental procedures for the in situ PLA Technology. Briefly, two primary antibodies bind to their target epitopes and secondary antibodies are conjugated to oligonucleotide arms. Only if they are in close proximity the oligos can generate a DNA circle, amplified by a polymerase, and detected by fluorescent labeled probes generating fluorescence dots. On the right is illustrated the presence of positive red PLA signals (red circles) mainly in the medial prefrontal cortex region. Blue-filled circles indicate a negative PLA signal in the corpus callosum (CC) (Bregma: 3.2 mm; according to the Paxinos and Watson (2006) stereotaxic atlas). (b) Quantification of PLA signals in the medial prefrontal cortex 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 the rest of the groups according to one-way ANOVA followed by Tukey post-hoc test (n = 6 in each group). Data are expressed as mean ± SEM. (c,d) Representative microphotographs of the significant decrease in the density of Y1R-GALR2 heteroreceptor complexes (PLA clusters) after M1145 and Y1R agonists treatment (d) compared with the control group (c). Receptor complexes are shown as red PLA blobs (clusters, indicated by white arrows) found in medial prefrontal cortex region using confocal laser microscopy. The nuclei are shown in blue by DAPI staining. 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.

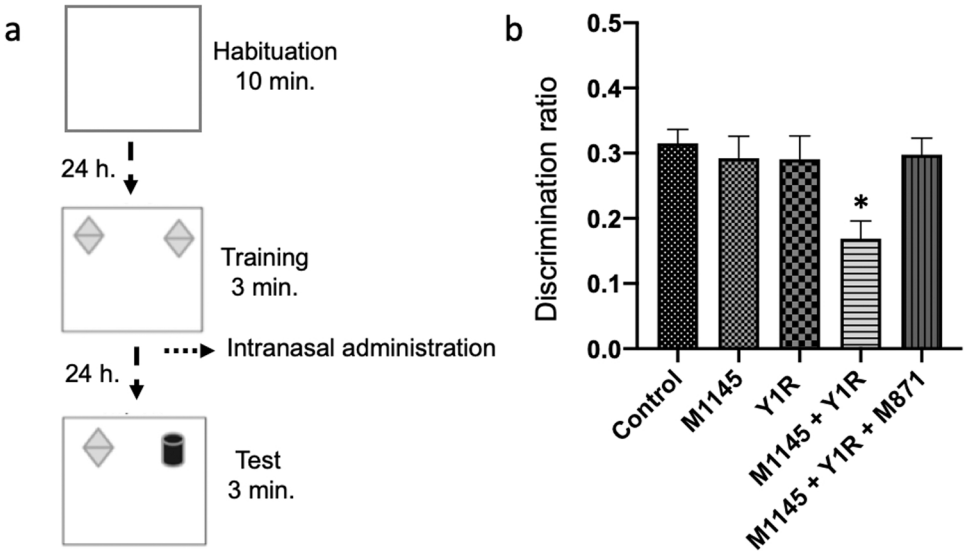

Fig. 3. Object recognition assessment after Y1R and the GALR2 agonists intranasally combined in the novel object preference task. (a) Schematic representation of the trials completed in the novel object preference task. The animals performed the task in three phases, divided 24 h from each other. In the habituation phase, animals explored freely for ten minutes without objects. In the training phase two identical objects are explored for three minutes. Finally, the test phase consist in three minutes of exploration with the same familiar object (F) and one different new object (N). To achieve recognition performance the pharmacological treatments were infused intranasally to the different groups of animals 24 h before the testing phase. Discrimination ratio index (DI) was calculated as DI= (N–F)/(N + F) (b) Performance on the novel object preference task showing the ability of rats to discriminate the new object at 24 h post-training after the intranasal infusion of Y1R and GALR2 agonists. An impairment in the novel object preference performance was observed after M1145 and Y1R co-administration following a 24 h delay. Besides, this effect is counteracted by the GAL 2 receptor (GALR2) antagonist M871. Data are presented as the mean ± SEM of the discrimination ratio on the test phase. n = 7 animals in each group. *p < 0.05 vs. the rest of the groups according to one-way ANOVA followed by Tukey post-hoc test. 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.

4.3. Impairment of novel object preference after Y1R and GALR2 agonists infusion

We performed the novel object preference task to achieve the functional outcome related to the findings on the medial prefrontal cortex after Y1R and GALR2 agonists co-administration. Rats explore freely for ten minutes during the habituation phase without objects and for three minutes in the training phase with both identical objects. Twenty-four hours after the intranasal infusion, animals were exposed to the test phase for three minutes with one different object to assess drug effects on object recognition ability (Fig. 3a).

Y1R and GALR2 agonists infusion after the acquisition phase impaired novel object preference after a 24 h period compared to the rest of the groups (one-way ANOVA, F4, 30 = 4.132, $p < 0.01$; Tukey post-hoc test: $p < 0.05$; Fig. 3b). GALR2 participation in this effect was demonstrated since the addition of the GALR2 antagonist M871 neutralized the impaired object recognition performance (Tukey post-hoc test: $p < 0.05$; Fig. 3b) induced by the infusion of Y1R and GALR2 agonists in the novel object recognition task. Moreover, the infusion of either Y1R agonist or GALR2 agonist alone lacked effects on the novel object preference task (Fig. 3b) compared with the control group.

Moreover, the total exploration time was analyzed during the training and test sessions. We observed that the exploration ability of the animals was not affected by the treatments. Overall, the animals showed a significant preference for the novel object compared to the familiar object, as evidenced by within-group analyses: Control ($t = 9.65$; $df = 5$; $p < 0.001$), GALR2 agonist M1145 ($t = 8.78$; $df = 5$; $p < 0.001$), Y1R agonist ($t = 8.58$; $df = 5$; $p < 0.001$), M1145 + Y1R ($t = 6.48$; $df = 5$; $p < 0.01$), and GAL + Y1R + M871 ($t = 12.31$; $df = 5$; $p < 0.001$). In addition, spontaneous motor behavior was not affected by the different treatment.

5. Discussion

The current study demonstrates for the first time that Y1R and GALR2 agonists intranasal infusion decreases the medial prefrontal cortex (mPFC) neuronal activation related to Y1R-GALR2 heteroreceptor complexes-reduced performance in the novel object preference task.

Intranasal (IN) delivery offers an alternative to the small fraction of drugs able to cross the blood–brain barrier under physiological conditions [60,61]. Although the exact mechanisms, sites, and pathways of action of CNS-targeted IN therapeutics are not fully understood, recent evidence suggests that the perineural and/or perivascular spaces of the olfactory and trigeminal nerves are involved in brain delivery using volume transmission [62–64].

To demonstrate the participation of the mPFC after the intranasal delivery of GALR2 and the Y1R agonists we assess the c-fos induction, a marker of neuronal activation. Is this work, we observed a decreased c-Fos-IR profiles in the mPFC following intranasal infusion of GALR2 and Y1R agonists. The GALR2 activation was necessary since the presence of the GALR2 antagonist M871 blocked this effect. There are evidences supporting an increased mPFC activity associated with anxiety and depressive-like behaviors [65]. Besides, functional studies in treatment-resistant MDD patients have consistently shown hyperactivity in the mPFC [66,67]. In this way, functional imaging studies associate depression with hyperactivity in ventromedial PFC. Likewise, lesion studies demonstrate that ventromedial PFC lesions reduce depressive symptoms. Finally, brain stimulation studies of electrophysiological activity suggest electrical deep brain stimulation-mediated inhibition of ventromedial PFC [68]. Our findings may suggest an specific pharmacological strategy for the therapeutic manipulation of the mPFC activity on depressed patients. In this regard, our data argue in favor of anxiolytic and antidepressant-like effects mediated by Y1R and GALR2 agonists intranasal infusion since reduced c-Fos-IR in the mPFC was associated to decreased mPFC activity [69,70]. In agreement, we previously described reduced c-Fos expression in the amygdala, hypothalamus, periaqueductal gray matter and dentate gyrus of the hippocampus related to anxiolytic and antidepressant-like actions [47–49]. Moreover, previous studies suggest that neuronal hyperexcitability in mPFC is responsible for increased beta-amyloid deposition during early stage of Alzheimer's disease (AD) [71]. In this way, our findings following Y1R and GALR2 agonists intranasal administration might be beneficial to counteract functional and morphologic alterations in AD patients.

To examine the cellular effects following Y1R and GALR2 agonists intranasal infusion we studied the presence of Y1R-GALR2 heteroreceptor complexes on mPFC. We observed for the first time the existence of these Y1R-GALR2 heteroreceptor complexes on the mPFC. Moreover, we detected a decrease of these heteroreceptor complexes after the combined agonists activation of both receptor protomers on the mPFC. Here again, the GALR2 participation is necessary since the addition of the GALR2 antagonist M871 blocked the effect. Moreover, lacking of Y1R-GALR2 heteroreceptor complexes in the corpus callosum is in agreement with the absent GALR2 in this region [72]. We have previously described a modulatory increase of the Y1R-GALR2 heteroreceptor complexes related to augmented functional consequences in discrete brain regions [51,52]. Consequently, both agonists raised the integration in the intracellular signaling within the Y1R-GALR2 heteroreceptor complexes, as we found in the extracellular signal-regulated kinases (ERK) pathway through the SRE reporter assay [51]. Present data might involve reduced intracellular signaling on the mPFC cells related to decreased Y1R-GALR2 heteroreceptor complexes and the functional outcome observed. Furthermore, the assessment of these Y1R-GALR2 PLA numbers on the mPFC might be used as a new biomarker at single-cell resolution and to demonstrate drugs target engagement with local precision.

Regarding BDNF expression we observed no modifications induced by the Y1R and/or GALR2 agonists intranasal infusion on the mPFC. Recently it was shown how the extinction of conditioned fear increases BDNF expression in ventral hippocampal neurons, but not in mPFC neurons [73]. According with the lack of BDNF expression on mPFC, we have recently observed an increase of BDNF expression on ventral dentate gyrus following intranasal infusion of Y1R and GALR2 agonists [74].

The functional validation of these findings was performed using the novel object preference task. We found a decrease in the discrimination index performance on this task after Y1R and GALR2 agonists intranasal delivery. Interestingly, there is controversy among researchers if the innate rodent exploratory behavior towards the new object is reflecting or not underlying memory discrimination or sensitivity [75]. However, this measure seems reliable since was described an empirical validation of the discrimination ratio as a measure of recognition memory sensitivity in humans, not bias [76]. Moreover, the authors suggest that preclinical data obtained with discrimination indexes are valid since interpreting them as an analogue of recognition memory sensitivity. These findings validate both the within-group analyses to detect the preference for the new object, and the inter-group comparisons for the level of discrimination [77]. We observed a preference for the new objects in all the groups. There are several evidences showing the involvement of the mPFC in the novel object preference task [78]. Moreover, neuroimaging studies have implicated the prefrontal cortex in recognition memory processes in humans [79] and monkeys [80,81]. Furthermore, c-Fos expressing in the mPFC was found when rodents perform the test phase of the novel object preference task [82,83]. Our results would reflect a recognition impairment in the novel object preference task related to decreased mPFC activity after the coadministration of both agonists. In agreement with our data, a recent study showed that chemogenetic inhibition of the mPFC after the sample trial, impaired object recognition performance tested 24 h later [84]. Based on the decreased c-Fos expression and the impaired recognition performance, we might determine the concern how NPY and GAL

interaction changed the mPFC activity for affecting the cognitive function in the novel object preference task. One limitation of the present study is that optogenetic and chemogenetic circuit dissection techniques would led to improved understanding of the local and global circuitry involved in depression-like behaviour and memory processing in animal models. Moreover, using pathological animal models of depression and neurodegenerative diseases would strength the present findings. In this way, further research is required to study NPY and GAL interactions on the mPFC in pathological models of neurodegeneration.

Taken together, the intranasal infusion of Y1R and GALR2 agonists may decrease neuronal activity on the medial prefrontal cortex. These effects may be mediated by Y1R–GALR2 heteroreceptor complexes without affecting the induction of the BDNF neurotrophic factor. Accordingly, these cellular effects may be linked to the impaired effects observed in the novel object preference task. In this way, through a reorganization of the signaling in this Y1R–GALR2 heteroreceptor complex might mediate the decreased object recognition actions. Our findings may provide some clinical contributions related to the development of new heterobivalent o multitargeting drugs, acting as agonist pharmacophores on Y1R–GALR2 heterocomplexes in the medial prefrontal cortex. We could speculate a future pharmacological strategy based on this heterocomplexes for the therapeutic manipulation of the mPFC activity on neurodegenerative diseases and/or psychiatric disorders. Besides, the assessment of the heteroreceptor complexes dynamics might be used as a new biomarker to determine drugs targeting with local precision. These outcomes warrant the design of upcoming clinical trials using intranasally delivered Y1R and GALR2 agonists.

Conflict of interest statement

The authors have no conflict of interest to declare.

Acknowledgments

This research was funded by the UMA18-FEDERJA-100 and ProyExcel_00613 (Junta de Andalucía, Spain), Proyecto Jovenes Investigadores (B1–2019-04), Proyecto puente (B4–2021-06) (Universidad de Málaga, Spain), to MN. Partial funding for open access charge: Universidad de Málaga/CBUA. Swedish Medical Research Council (Sweden) (62X-00715–50-3), to KF, by Stiftelsen Olle Engkvist Byggmästare (Sweden) to KF.Hjärnfonden (Sweden) (F02018–0286), (F02019–0296), Karolinska Institutet Forskningsstiftelser (Sweden), and EMERGIA (2020,39318) to D.O.B.-E. D.O.B.-E. belongs to the “Academia de Biólogos Cubanos” group, Cuba.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114433.

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