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ON THE ROLE OF NEUROPEPTIDE Y RECEPTOR 1 (NPY1R)-GALANIN RECEPTOR 2 (GALR2) HETERORECEPTOR COMPLEXES IN ENHANCING ADULT HIPPOCAMPAL NEUROGENESIS AND COGNITIVE FUNCTION

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ABSTRACT

The extensive body of evidence supporting the existence of Neuropeptide Y receptor (NPYR) homo- and heteroreceptor complexes, alongside allosteric receptor-receptor interactions, has unveiled a pioneering frontier in molecular neuroscience within the Central Nervous System (CNS). These receptor complexes introduce a novel dimension to molecular neuroscience and brain function, representing a fundamental biological principle that integrates biological signals across various tissues. Through heteromerization and allosteric interactions, these complexes lead to modifications in receptor recognition, the emergence of new allosteric binding sites, alterations in pharmacology, signaling pathways, and receptor trafficking. This dynamic interplay shapes a diverse and biased signaling profile specific to each receptor heteromer.

Beyond NPYR-GPCR complexes, these receptor assemblies can include ion channel receptors, receptor tyrosine kinases (RTKs), groups of G protein-interacting proteins, and transmitter transporters, thereby enhancing their integrative capabilities. The localization of homo- and heteroreceptor complexes on synaptic or extrasynaptic regions of the plasma membrane is governed by various factors, including the density of participating receptor protomers and their affinity. The presence or absence of adapter proteins within heteroreceptor complexes significantly influences the affinity developed among receptor protomers.

The widespread distribution of heteroreceptor complexes, characterized by allosteric receptor-receptor interactions in the CNS, presents a groundbreaking integrative molecular mechanism within neuronal and glial cell plasma membranes. It is hypothesized that the molecular mechanism underlying learning and memory involves the reorganization of existing higher-order heteroreceptor complexes (including GPCRs) and the resetting of multiple allosteric receptor-receptor interactions within these complexes. Additionally, novel heteroreceptor complexes may form due to alterations in the patterns of synaptic and volume transmission signals. These molecular adjustments within heteromers, including changes in receptor-protein architecture on the postsynaptic membrane, potentially constitute the basis for short- and long-term memory.

This PhD thesis explores NPY1R heteroreceptor complexes and their allosteric receptor-receptor interactions within the CNS, focusing specifically on their roles in hippocampal neuronal cells. The research emphasizes the importance of adult neurogenesis under physiological conditions and the regulatory role of neuropeptides, specifically NPY and GAL. By further investigating the effects of NPY1R and GALR2 agonists on neurogenesis and cognition, the aim is to deepen understanding of these complex biological processes. Through an exploration of these complexes and their integration of neuronal

signals, this research seeks to enhance understanding of their involvement in various mental and neurological disorders, potentially offering promising therapeutic avenues for addressing age-related cognitive decline, early stages of cognitive impairment, and depression.

The thesis begins with an extensive review of prior research on allosteric receptor-receptor interactions, particularly in G protein-coupled receptors (GPCRs). In the early 1980s, researchers observed that neuropeptides could alter the affinity and density of monoamine agonist and antagonist binding sites in various CNS regions in a receptor subtype-specific manner. This discovery indicated neuropeptide-monoamine receptor-receptor interactions in the plasma membrane. Earlier work by Lefkowitz, Limbird, and colleagues had identified negative cooperativity in beta-adrenergic receptors, attributed to beta-adrenergic homodimers leading to site-site interactions. The first symposium on GPCR receptor-receptor interactions in Stockholm in 1986 proposed a broader field, including interactions among different classes of biologically active macromolecules. Receptor heteromerization was suggested as the molecular basis for these interactions in 1993. Initial observations of GPCR homodimerization date back to 1982, with further discoveries in 1987 of homodimerization upon epidermal growth factor stimulation. Validation of GABA B receptor heterodimerization a decade later supported early findings of receptor-receptor interactions in putative GPCR heteroreceptor complexes.

The exploration of allosteric interactions among receptors within homo- and heteroreceptor complexes in the Central Nervous System (CNS), particularly among G protein-coupled receptors (GPCRs), has significantly advanced our understanding of brain integration and neuropsychopharmacology. This field, extensively explored by researchers such as Fuxe, Lefkowitz, Milligan, and Borroto-Escuela, highlights how receptor oligomerization induces dynamic changes in receptor protomers, affecting recognition, pharmacology, signaling, and trafficking, and potentially creating novel allosteric binding sites. GPCR heteroreceptor complexes can include ion channel receptors, receptor tyrosine kinases (RTKs), G protein-interacting proteins, ion channels, and transmitter transporters, illustrating the intricate network of interactions in the CNS. These dynamic interactions occur in a coordinated spatio-temporal manner, contributing to learning and the formation of molecular engrams for short- and long-term memory. Understanding the molecular organization of receptor oligomers, their allosteric communication, and the features of the receptor interface remains a critical area for improvement.

Heteroreceptor complexes represent a fundamental principle for molecular integration in biology, involving GPCR interacting proteins. These discoveries have led to new treatment strategies for diseases such as Parkinson's disease (e.g., A2A and mGluR5 receptor antagonists), schizophrenia (e.g., A2A and mGluR5 agonists), depression (e.g., 5-HT1A agonists enhancing FGFR1 function), and cocaine addiction (e.g., A2A agonists).

Recent hypotheses propose that learning and memory involve the reorganization of

homo- and heteroreceptor complexes in synapses (pre- and postjunctional membranes of synapses), influencing receptor complexes to facilitate neurotransmitter release patterns. Long-term memory formation may involve parts of heteroreceptor complexes transforming into unique transcription factors, leading to the development of specific adapter proteins that consolidate these complexes into long-lived entities with preserved allosteric interactions. These homo-heteroreceptor complexes are dynamic assemblies shaped by integrated synaptic and volume transmission signals, essential for learning. They can transform into consolidated states with enduring allosteric communication, representing molecular engrams that profoundly modulate neuronal networks and influence behavioral and cognitive functions over time. For structural plasticity in dendritic trees and spines, the recruitment of RTKs to heteroreceptor complexes may result in synergistic increases in neurite densities and protrusions in primary neuronal cultures.

The significance of receptor-receptor interactions in enhancing receptor diversity was first highlighted in 1983/1985 through studies on neuropeptide/dopamine (DA) interactions. Neuropeptide-monoamine receptor-receptor interactions in the CNS have demonstrated the existence not only of GPCR monomers but also of GPCR homo- and heteroreceptor complexes, including receptor dimers, higher-order receptor complexes, and receptor-interacting proteins such as various adapter proteins and synaptic/non-synaptic proteins. The recent identification of the GPCR heterodimer network (GPCR-HetNet) underscores that allosteric receptor-receptor interactions significantly expand GPCR diversity and bias recognition and signaling, thereby enhancing signaling specificity. These interactions are reciprocal, dynamic, and substantially alter the signaling, trafficking, recognition, and pharmacology of the involved protomers. Modulations can enhance interactions with agonists or antagonists, switch G protein coupling, or promote β-arrestin recruitment.

The GPCR heterodimer network (www.gpcr-hetnet.com, last updated 2014) provides insights into direct interactions between GPCRs, revealing a scale-free model where a few protomers, such as the adenosine A2A receptor, dopamine D2R, and β2-adrenergic receptor, dominate connectivity. Experimentally verified interactions have been reported for 156 GPCR protomers, approximately 20% of the total putative human GPCR protomers. Despite the majority of identified protomers belonging to the rhodopsin-like superfamily, their interactions were largely incomplete. The Secretin-like and metabotropic glutamate receptor-like superfamilies exhibited higher interaction rates, with 33% and 60% of putative protomers involved in interactions, respectively. While more than 87% of identified protomers exist as homomers, the balance between homo- and heteromeric populations is crucial, potentially influencing diseases where GPCR dimerization plays a role. Intrafamily connections were significantly more prevalent than interfamily connections, possibly due to the co-evolution of protomer interfaces within subfamilies and diverse cell and tissue expression patterns. Further research into GPCR heterocomplex

specificities may reveal cross-family heterodimerization or intrafamily specificities, shedding light on the complex landscape of GPCR interactions.

Understanding the interface of GPCR dimers is particularly crucial for drug development in CNS diseases. In 2004, mass spectrometry and pulldown techniques demonstrated direct epitope-epitope electrostatic interactions between A2AR-D2R protomers, involving the third intracellular loop of D2R and the C-terminal tail of A2R. In 2010, Borroto-Escuela et al. showed that a serine point mutation in the C-terminal tail of A2AR diminished heteromerization and revealed for the first time that transmembrane helices were involved. In 2018, a structural model of the A2AR-D2R heterodimer was obtained by mapping its interface using computational and experimental methods. The receptor interface modeling employed peptides derived from transmembrane helices to investigate their impact on A2AR-D2R interactions using BRET and PLA assays, alongside modulation of D2R binding. Peptides from A2AR TM-IV and TM-V regions inhibited heteromer formation and attenuated A2AR agonist-induced allosteric inhibition of D2R affinity. Protein-protein docking generated a model of A2AR-D2R incorporating the TM-IV and TM-V interface, refined through molecular dynamics simulations. Mutations at this interface diminished allosteric D2R inhibition and reduced BRET signal, highlighting the method's potential for modeling GPCR heterocomplexes and aiding in novel neurological and psychiatric drug development.

Approximately one-third of non-odorant GPCRs remain orphan receptors, lacking identified ligands and displaying potential ligand-independent functions. GPCR members often modulate other receptors through heterodimerization. For instance, GPR50, an orphan GPCR, interacts with the melatonin MT1 receptor, influencing their signaling pathways. Similarly, GPR143 interacts with dopamine receptors D2R and D3R, suggesting implications for neurological conditions such as Parkinson's disease. Orphan receptors GPR18 and GPR55 heterodimerize with cannabinoid CB1 and/or CB2 receptors, showing negative cross-talk and bidirectional cross-antagonism, potentially involved in neurodegenerative diseases like Alzheimer's and Parkinson's.

Several methods have been employed to study receptor-receptor interactions and receptor-protein interactions. Fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET1) have been instrumental in studying protein homo- and heteromerization, including receptors, in live cells. These techniques involve constructing receptor constructs fused with donor and acceptor fluorescent proteins. In FRET, energy transfer occurs if donor and acceptor fluorophores are within 10 nm, detected by donor excitation and acceptor emission. While classical FRET faces challenges with plasma membrane protein interaction measurements due to intracellular protein localization, advancements such as cell surface FRET detection and total internal reflection fluorescence microscopy (TIRF) have enhanced plasma membrane GPCR heteromerization studies by providing precise excitation fields. Biomolecular fluorescence

complementation (BiFC) demonstrates protein dimerization by using proteins with complementary fluorescent protein halves that fluoresce upon dimerization. Similarly, BRET, utilizing Renilla luciferase with YFP or GFP2 for energy transfer, effectively detects receptor heteromers in artificial cell systems. BRET saturation assays are crucial for determining receptor complex oligomeric order and affinity, essential for understanding receptor interactions despite challenges like fluorescent protein-fused receptor requirements and potential overexpression artifacts. Combined BRET/BiFC assays have facilitated higher-order heteroreceptor complex detection, while sequential BRET-FRET techniques (SRET2) have demonstrated GPCR trimeric heteromer presence. These advanced methods are invaluable despite overexpression-related nonspecific receptor complex concerns, necessitating ongoing technique improvements for accurate native receptor oligomer detection.

The in situ Proximity Ligation Assay (PLA) has successfully identified native CNS heteroreceptor complexes. Using primary and secondary antibodies linked with oligonucleotides, PLA enables protein interaction detection and quantification via fluorescence microscopy. PLA has proven effective in demonstrating ex vivo heteroreceptor complexes like A2A-D2 and D2-5HT2A in brain tissues, contributing significantly to CNS heteroreceptor complex study, localization, and modulation. PLA advancements have revolutionized protein-protein interaction studies in brain disorders, enhancing understanding of neurological and psychiatric diseases. For instance, PLA identified A2AR-D2R heterocomplexes in the human striatum, providing insights into brain pathology and guiding human neuroimaging strategies. PLA applications in Alzheimer's disease (AD) have detected tau-ubiquitin complexes, highlighting protein distribution alterations associated with pathology. In Parkinson's disease (PD), PLA visualized alpha-synuclein (AS) oligomers in affected brain regions, shedding light on PD and multiple system atrophy (MSA) pathological mechanisms. PLA's ability to preserve spatial context while delivering molecular information enhances brain disorder protein interaction and modification understanding, especially in neurodegenerative and neuropsychiatric research.

Using in situ PLA techniques it has been possible to understand and study GPCR homo and heteroreceptor complexes vulnerabilities in neurodegenerative and mental disorsers. For instance, dysfunctional D2R heteroreceptor complexes may underlie brain circuit pathological changes, such as increased D2R activity altering glutamate prefrontal afferent function, potentially contributing to schizophrenia symptoms. Investigating these complexes in schizophrenia could lead to new treatments that mitigate current antipsychotics' D2R antagonist side effects. Recent discoveries of various D2R heteroreceptor complexes suggest potential optimized combination therapies or single heterobivalent drugs targeting these complexes in schizophrenia.

Understanding serotonin (5-HT) system-related heteroreceptor complexes is crucial

in major depression context. The serotonin hypothesis, dating to the 1960s, underscores midbrain 5-HT neuron projections to tel- and diencephalon involvement in depression. Biochemical studies on tryptophan and 5-HT, along with drugs like imipramine inhibiting 5-HT reuptake, established selective serotonin reuptake inhibitors (SSRIs) as antidepressants. Identification of 5-HT receptor subtypes categorized into GPCR families (5-HT1, 5-HT2, 5-HT4, 5-HT5, 5-HT6, and 5-HT7) furthered serotonin system understanding. Early indications suggested traditional antidepressants targeting specific 5-HT receptor subtypes. Current research supports activating 5-HT1A and 5-HT4 receptors while blocking 5-HT2A, 5-HT3, and 5-HT7 receptors for antidepressant effects. Exploration of selective agonists or antagonists for these subtypes, alongside drugs targeting multiple 5-HT receptors and serotonin transporter (SERT), enhances SSRI antidepressant efficacy. The imbalance in 5-HT receptor activity in major depression supports targeting these receptors for therapeutic benefit.

FGFR1-5-HT1A heteroreceptor complexes discovery in the hippocampus advanced 5-HT1A receptor role understanding in hippocampal plasticity. These complexes, activated with FGF2 and 5-HT1A agonist co-activation, enhance neurite density and potentially induce antidepressant effects by counteracting depression-induced hippocampal atrophy. FGFR1-5-HT1A complexes also modulate GIRK channels, promoting hippocampal pyramidal nerve cell firing towards prefrontal cortex and ventral striatum networks. Similar modulation is suggested in midbrain raphe, where FGFR1-5-HT1A autoreceptor complexes affect neuroplasticity and depression. FGFR1 activation reduces 5-HT1A autoreceptor function, restoring raphe 5-HT neuron firing and exerting potential antidepressant actions.

Oxytocin receptors (OXTR) co-distribute with serotonin networks in limbic regions and hypothalamus, forming heterocomplexes with GPCRs such as 5-HT2AR, 5-HT2CR, and D2R. Allosteric interactions in these complexes modulate receptor signaling and trafficking, crucial for social and cognitive behaviors. D2R-OXTR complexes enhance receptor function, offering a potential target to boost emotional networks via oxytocin receptor signaling in striatal regions. Conversely, 5-HT2AR and 5-HT2CR agonists inhibit OXTR signaling, potentially influencing depressive actions through allosteric modulation of oxytocin receptor signaling. Serotonin receptors, especially 5-HT2AR, interact with metabotropic glutamate receptors (mGluRs) like mGluR2 and mGlu5, influencing neuropsychiatric disorders like schizophrenia and depression. Disruptions in these complexes alter cellular signaling and behavioral responses, implicated in psychosis-like effects and altered locomotor activity. Understanding these interactions underscores their potential as therapeutic targets in neuropsychiatric diseases.

Neuropeptide Y receptors (NPYRs), comprising Y1, Y2, Y4, and Y5 subtypes, play essential roles in the CNS, modulating mood regulation, stress response, and homeostasis in key brain regions like the cortex, hippocampus, amygdala, and hypothalamus. Recent studies have elucidated NPYR homo- and heteroreceptor complex formation, revealing

intricate mechanisms underlying their functional diversity. These complexes influence NPY-mediated signaling pathways, neurotransmission, synaptic plasticity, and neuroendocrine functions critical for emotional balance and cognitive processes.

Experimental evidence highlights the involvement of NPYR subtypes (Y1, Y2, Y4, Y5) in depressive disorders, impacting neurotransmitter systems and stress responses. Y1 receptor activation attenuates depressive symptoms and anxiety-like behaviors, enhancing stress resilience via HPA axis and limbic system modulation. Conversely, Y2 receptors, through antagonism, increase NPY release, augmenting anxiolytic and antidepressant-like effects. The interplay between NPY and its receptors, along with their interactions with other neurotransmitter systems (e.g., serotonin, dopamine), underscores their broader impact on mood regulation beyond stress responses. NPYRs' role extends to neurogenesis and neuroprotection, critical processes disrupted in depression. Y2 and Y5 receptors contribute to neuroprotective mechanisms against excitotoxicity and neuronal survival promotion in neurological and psychiatric disorders. Targeting NPYRs offers potential treatments that alleviate depressive symptoms and reverse neuronal damage associated with chronic stress and depression. Modulating NPYRs could enhance synaptic plasticity and resilience, providing novel strategies for mood stability and emotional well-being.

The thesis also investigates the role of NPYR1-GALR2 heteroreceptor complexes in hippocampal neurogenesis, learning, and memory. The involvement of GPCR heteroreceptor complexes in brain plasticity, learning, and memory has been extensively explored. Initially discussed in 1993, the relationship between receptor-receptor interactions and neuronal plasticity underscores the requirement for multiple concurrent events or signals to induce lasting changes in neuronal function, such as long-term potentiation (LTP) and long-term depression (LTD). Intramembrane receptor-receptor interactions act as coincidence detectors, making them likely candidates for mediating these forms of neuroplasticity. For example, in the context of LTD, D2 receptor (D2R) activity promotes LTD in striato-pallidal GABA neurons. Interestingly, mGluR5 blockade counters this LTD despite its role in antagonizing D2R signaling through inhibitory receptor-receptor interactions in an extrasynaptic A2A-D2-mGluR5 heteroreceptor complex outside the glutamate synapse. This blockade underscores mGluR5 signaling's role in enhancing endocannabinoid production and release via extrasynaptic volume transmission (VT), thereby activating CB1 receptors on glutamate nerve terminals to inhibit glutamate release. This mechanism illustrates the crucial role of CB1-mediated endocannabinoid VT on glutamate nerve terminals in inducing D2R-mediated LTD at glutamate synapses on striato-pallidal GABA neurons. In cortico-striatal synapses, spike-timing-dependent plasticity dictates whether LTD or LTP occurs, with presynaptic activity preceding postsynaptic spiking leading to LTD, and vice versa inducing LTP. Notably, the presence of a D2R agonist like quinpirole shifts the outcome towards LTD even with the LTP-inducing protocol, highlighting D2R's potent ability to counteract mechanisms

favoring LTP and instead promoting LTD.

Regarding learning and memory, the formation of long-lived heteroreceptor complexes through intramembrane receptor-receptor interactions has been proposed as a potential mechanism. Learning in neuronal networks likely involves instructions altering synaptic weights (efficacies), possibly mediated by multiple interactions among plasma membrane receptors, forming higher-order heteroreceptor complexes or receptor mosaics via oligomerization at pre- and postsynaptic levels. These receptor assemblies, along with adapter proteins, G-proteins, and ion channels, form part of a complex molecular circuit on the plasma membrane. In the cytoplasmic part, protein kinases, phosphatases, and phosphoproteins capable of learning and storing information are included. The formation of engrams depends on resetting molecular circuits via the formation of new heteroreceptor complexes, facilitating the transduction of chemical messages through specific sets of G-proteins, ion channels, and other protein effectors. Short-term memory might involve the transient stabilization of receptor mosaics, adjusting synaptic weights accordingly.

Engram consolidation into long-term memory likely engages intracellular signals translocated to the nucleus, activating immediate early genes and forming adapter proteins that stabilize receptor mosaics as long-lived heteroreceptor complexes. Changes in ERK signaling are crucial for long-term memory, influencing activity-dependent modifications of histones and epigenetic processes. Transcriptional and epigenetic regulations participate in both Hebbian and non-Hebbian forms of plasticity, influencing learning and memory by forming and stabilizing molecular circuits with newly formed higher-order heteroreceptor complexes. These molecular changes, whether transient or long-lasting, can alter brain circuit output patterns, inducing transient and enduring changes in behaviors and cognitive functions. Structural plasticity in dendritic trees and spines, crucial for learning and memory, involves the recruitment of RTKs to novel heteroreceptor complexes, enhancing cell extensions and neurite densities.

Neurogenesis is a crucial aspect of neuronal plasticity, enabling the brain to reorganize its structure, function, and connections in response to both extrinsic and intrinsic stimuli. A fundamental process within this domain is adult hippocampal neurogenesis (AHN), where new neurons are continuously generated in the brain throughout adulthood. The hippocampus, particularly the dentate gyrus (DG), serves as a neurogenic niche, playing a vital role in maintaining AHN under normal physiological conditions.Despite its importance, the persistence of AHN in humans remains debated. Some studies suggest a decline in AHN with age, while others provide evidence supporting its continuation into late adulthood, emphasizing its significance in maintaining cognitive and emotional functions. The hippocampus is functionally divided, with the anterior portion (ventral in rodents) involved in stress and emotional behavior, and the posterior part (dorsal in rodents) associated with cognitive functions and memory.

Dysregulation of AHN is implicated in various neurological and psychiatric disorders, including major depressive disorder (MDD), age-related cognitive decline, Alzheimer's disease (AD), and other neurodegenerative diseases. MDD is a prevalent mental health condition affecting over 300 million people worldwide, characterized by a constellation of behavioral, emotional, and cognitive symptoms that significantly impact daily life and increase the risk of suicide, the most severe consequence of the disorder. The COVID-19 pandemic has exacerbated the prevalence of depression, with increased rates of loneliness and financial hardship contributing to a higher incidence of depressive symptoms and suicidal ideation.

Current antidepressants primarily target monoamines but often fall short due to adverse effects and delayed therapeutic onset. Approximately 50% of patients do not respond to these treatments, and 65% fail to achieve remission, resulting in treatment-resistant depression (TRD). The advent of ketamine as a rapid-acting antidepressant has offered new hope, but its significant risks limit widespread use (references 195, 196, 198, 199). This underscores the need for novel therapeutic approaches targeting different underlying mechanisms to improve treatment efficacy for MDD.

Enhancing hippocampal neurogenesis emerges as a promising therapeutic strategy for MDD. Neurogenesis in the hippocampus involves cell proliferation, neuronal differentiation, and survival, regulated by various intrinsic and extrinsic factors. Neuropeptides such as neuropeptide Y (NPY) and galanin (GAL), along with neurotrophic factors like brain-derived neurotrophic factor (BDNF), play crucial roles in modulating these processes. NPY, a highly conserved neurotransmitter, has demonstrated proneurogenic effects in the hippocampus, with reduced levels observed in MDD models and patients. Antidepressant treatments typically increase brain NPY levels, highlighting its potential therapeutic relevance. Additionally, GAL, particularly through the GAL2 receptor (GALR2), has shown proliferative and neuroprotective effects in the hippocampus, suggesting its role in antidepressant mechanisms.

Specific Aims of the Thesis:

AIM-1: This aim seeks to investigate whether intranasal administration of GALR2 and NPY1R agonists can stimulate adult neurogenesis in the ventral hippocampus and induce antidepressant-like effects. This involves assessing ventral hippocampal activation and proliferation through c-Fos expression and PCNA, identifying specific proliferating cell subpopulations using double immunolabeling, examining BDNF expression in the ventral hippocampal dentate gyrus, analyzing NPY1R-GALR2 heteroreceptor complex formation via in situ proximity ligation assays (PLA), studying morphological changes in hippocampal neurons, and evaluating functional outcomes in the ventral hippocampus using the forced swimming test (FST), with a particular focus on BDNF's role.

AIM-2: This aim aims to explore the impact of GAL on hippocampal neurogenesis, including its dose- and site-dependent effects on memory and implications for Alzheimer's

disease models. It also investigates interactions between NPY and GAL through NPY1R-GALR2 heteroreceptor complexes in various brain regions and assesses their potential therapeutic implications for age-related cognitive decline. This includes evaluating spatial memory, neuronal survival, and differentiation in adult rats following intranasal co-administration of GALR2 and NPY1R agonists, highlighting GALR2's significant role in these processes.

By addressing these aims, the research aims to advance our understanding of NPY1R-GalR2 complexes and their interactions and their underlying role in neurogenesis and cognition, potentially paving the way for the development of novel therapeutic approaches.

The thesis encompasses Two chapters, each dedicated to addressing the aims.

Chapter 1: This study demonstrates that intranasal infusion of GALR2 and Y1R agonists stimulates neurogenesis in the adult ventral hippocampus and produces antidepressant-like effects. Intranasal delivery offers a non-invasive alternative to intracerebroventricular (icv) infusion, bypassing the blood-brain barrier to deliver peptides and protein therapeutics directly to the CNS. This method is supported by substantial evidence from preclinical and clinical trials. Its advantages include reduced side effects compared to peripheral administration and the noninvasive nature of the application. For instance, intranasal esketamine, recently introduced as an antidepressant, faces limitations due to potential neurotoxicity, psychomimetic side effects, risk of abuse, and variability in treatment response.

Following intranasal administration of GALR2 and Y1R agonists, increased cell proliferation was observed in the ventral dentate gyrus (DG) of the hippocampus using PCNA as a marker. This aligns with previous findings indicating enhanced cell proliferation in the dorsal DG within 24 hours. Previous studies using icv infusion of GAL and Y1R agonists induced cell proliferation in the ventral hippocampus using 5-bromo-2-deoxyuridine. This study's strength lies in demonstrating a noninvasive route via intranasal delivery of specific GALR2 and Y1R agonists. Notably, genetic enhancement of neurogenesis in the ventral hippocampal DG has been shown to increase resilience in depression models. Similarly, the molecule P7C3, associated with increased cell proliferation in the hippocampal DG, has shown antidepressant effects in rodents and primates.

Our study also found that intranasal administration of the Y1R agonist alone increased cell proliferation in the ventral DG, but not in the dorsal DG. This highlights functional differences between the ventral and dorsal regions and suggests a differential role for NPY in these subregions of the hippocampus. In contrast, the GALR2 agonist alone did not affect cell proliferation in the ventral hippocampus. Previous studies indicated that GALR2/3 mediates the proliferative and trophic effects of GAL, with subsequent studies suggesting a role for GALR3. However, these findings were based on in vitro conditions, which can differ significantly from in vivo systems.

Furthermore, we identified that the combined administration of M1145 and Y1R agonist specifically stimulated the proliferation of neuroblasts (PCNA+/DCX+ cells) without affecting quiescent neural progenitors and astrocytes (PCNA+/GFAP+ cells). This aligns with previous reports showing that NPY promotes the proliferation of amplifying neural progenitors and neuroblasts.

Dysregulation of neurogenesis in the subventricular zone (SVZ) is a common feature in various neurodegenerative diseases. For instance, stem cell proliferation is reduced in Alzheimer's and Parkinson's diseases, whereas stroke and Huntington's disease enhance SVZ neurogenesis to aid in the repair of damaged areas. NPY has been reported to promote neurogenesis via Y1R on DCX-positive neuroblasts and to play a role in cell migration. Future research should investigate the potential of intranasally administered GALR2 and Y1R agonists in cell replacement strategies for neurodegenerative diseases affecting SVZ neurogenesis.

At the cellular level, the increased hippocampal cell proliferation following intranasal co-administration of GALR2 and Y1R agonists appears to be mediated by elevated BDNF expression in the ventral hippocampal DG. BDNF, a crucial neurotrophin, plays a significant role in promoting neurogenesis through its effects on cell proliferation and survival. Physical exercise has been shown to protect against depressive symptoms by increasing hippocampal neurogenesis and BDNF levels. Therapeutics that enhance the relationship between dentate neurogenesis and BDNF, like the combined GALR2 and Y1R agonists, may be key to treating depression. This is supported by previous evidence on the neuroprotective effects of NPY in neurodegeneration models.

In hippocampal neuronal cells, the co-administration of GALR2 and Y1R agonists increased the formation of GALR2/Y1R heteroreceptor complexes, as observed using in situ PLA. This effect was confirmed in previous studies in HEK cells and various limbic brain regions, including the amygdala and dorsal hippocampus. Additionally, we observed that coincubation of these agonists promoted neurite outgrowth in hippocampal neuronal cells, potentially mediated by BDNF, consistent with its known effects on dendritic outgrowth in primary hippocampal cultures and the hippocampus.

The functional outcome was validated by demonstrating enhanced antidepressant-like responses in the forced swim test (FST) 24 hours after intranasal administration of GALR2 and Y1R agonists. Previous studies have shown that intranasal infusion of Y1 agonist in rats or humans induces antidepressant effects for at least 24 hours. Similarly, single injections of the NMDA receptor antagonist Ketamine or the mGlu2/3 receptor antagonist LY341495 have been shown to produce antidepressant-like effects in the FST in rats at 24 hours. However, the GALR2 agonist alone did not exhibit antidepressant-like effects at 24 hours, suggesting that subchronic or chronic intranasal treatments may be required for long-lasting effects in pathological depression models. Notably, species-

specific differences in antidepressant responses between rats and mice have been reported. For instance, the intranasal infusion of a spexin-based GALR2 agonist showed antidepressant-like effects in mice within 2-3 hours. Recently, M39b, a stabilized GALR2 agonist, has shown promise in intranasal delivery studies in rats. Moreover, the GALR2 antagonist M871 counteracted the enhanced response observed, aligning with previous findings. These behavioral effects were independent of motor activity, as neither GALR2 nor Y1R agonists, nor their co-administration, affected locomotor activity. This is consistent with the involvement of the ventral hippocampus in the antidepressant effects of NPY in posttraumatic stress disorder. Thus, the enhanced antidepressant effects of Y1R and GALR2 agonists at 24 hours may be mediated by increased signaling of Y1R-GALR2 heterocomplexes in the ventral hippocampus, supported by BDNF, as observed in vivo and in vitro.

Overall, intranasal infusion of Y1R and GALR2 agonists promotes cell proliferation in the ventral hippocampal DG and induces BDNF expression. These effects are likely mediated by Y1R-GALR2 heteroreceptor complexes, leading to increased neurite outgrowth in hippocampal neurons and enhanced antidepressant effects. These findings suggest the potential for developing new therapeutic approaches targeting Y1R-GALR2 heterocomplexes for major depressive disorder (MDD) and related conditions. Future clinical trials could explore the efficacy of intranasally delivered Y1R and GALR2 agonists in these contexts.

Chapter 2: Our study demonstrates that co-administration of GALR2 and NPY1R agonists via intranasal delivery enhances spatial memory and promotes neuronal survival and differentiation in the dentate gyrus of the dorsal hippocampus. Previously, we observed short-term improvements in object-in-place memory consolidation following intracerebroventricular (icv) administration of GALR2 and NPY1R agonists 169. However, administering M1145 or the NPY1R agonist individually via icv did not yield significant memory improvements, suggesting potential allosteric enhancement between GalR2 and NPY1R with combined treatment. Our findings underscore a synergistic effect at both the transmembrane and cytoplasmic levels, potentially enhancing spatial memory consolidation through intranasal co-administration of these agonists. Our results emphasize the importance of neuronal age, specifically reaching three weeks, for achieving functional hippocampal integration in rats.

Our cellular analysis revealed a significant increase in BrdU-immunoreactive profiles within the subgranular zone of the dentate gyrus following combined treatment with these neuropeptides, indicating enhanced neurogenesis closely linked to learning and memory. Furthermore, most newly generated cells differentiated into mature neurons, supported by increased BrdU+ cells and detection of neuronal-specific nuclear protein (NeuN) after NPY1R-GALR2 administration, a marker for mature neurons. Interestingly, the proportion of BrdU-doublecortin (DCX) co-expressing cells, despite DCX's role as a

marker for neurogenesis, was relatively low, suggesting that memory enhancements may primarily stem from synergistic signaling between NPY1R and GalR2, potentially forming heteroreceptor complexes that aid in memory consolidation and support integration of newly matured neurons.

To account for sex differences in adult hippocampal neurogenesis, our study utilized male rats, as earlier noted by Yagi et al., who observed higher densities of BrdU-ir cells in males at earlier time points compared to females 248. However, by the third week, these differences diminished, resulting in comparable densities between sexes. Additionally, while the maturation rate of adult-born neurons was initially higher in males at two weeks, it equalized by the third week. This informed our choice of male rats to mitigate sex-related disparities during earlier neurogenesis stages, although future studies should explore potential sex differences across various conditions and timeframes.

Moreover, our research identified dendritic morphological changes in DCX-labeled cells post-treatment, indicating enhanced functional integration of these neurons into existing circuits. Given DCX's role in neuritic growth cone formation and synapse development, these changes suggest that dendritic complexity and length crucial for neuronal functionality may be influenced by DCX activity 180, 184, 235, 236. Further research is necessary to elucidate the interplay between cellular survival, maturation, and neuronal integration.

While our immunocytochemistry findings offer valuable insights into GALR2 and NPY1R agonists' effects on hippocampal neurogenesis, advanced techniques such as transcriptomic analysis could provide deeper insights into activated molecular pathways. Additionally, electrophysiological studies could reveal how newly generated neurons functionally integrate into neural circuits, while in vivo imaging techniques like two-photon microscopy could offer real-time insights into neuronal development and integration following agonist administration. These advanced methodologies promise to enhance our understanding of neuropeptide receptor agonists' roles in neurogenesis and cognitive functions.

In summary, our results suggest a promising approach for enhancing memory and neuronal maturation through intranasal co-administration of GALR2 and NPY1R agonists. Further investigations are crucial to elucidate the molecular mechanisms underlying this synergistic effect and its potential therapeutic applications for learning and memory.

Our study provides compelling evidence that intranasal administration of GALR2 and NPY1R agonists effectively stimulates adult neurogenesis in the ventral hippocampus, thereby exerting robust antidepressant-like effects. Using a comprehensive approach, we demonstrated significant increases in cell proliferation within the ventral dentate gyrus, indicated by enhanced PCNA expression. Moreover, our findings underscore the specificity of this effect, showing that co-administration of GALR2 and Y1R agonists selectively increased the proliferation of neuroblasts without impacting quiescent neural progenitors or

astrocytes, highlighting the nuanced role of NPY in hippocampal subregions. At the cellular level, our study revealed a mechanistic link between GALR2 and NPY1R agonists and increased BDNF expression in the ventral hippocampal DG, suggesting that BDNF-mediated neurotrophic signaling may significantly contribute to the observed antidepressant effects. This aligns with previous literature implicating BDNF in promoting neurogenesis and dendritic outgrowth, reinforcing its crucial role in hippocampal plasticity and mood regulation. Additionally, our investigation into NPY1R-GALR2 heteroreceptor complexes via in situ PLA provided novel insights into the molecular mechanisms underlying the synergistic actions of these agonists in the hippocampus. Functionally, our behavioral assessments using the forced swim test (FST) demonstrated enhanced antidepressant-like responses following intranasal co-administration of GALR2 and Y1R agonists, corroborating our cellular findings. These effects were specific to the ventral hippocampus, suggesting targeted therapeutic potential for treating depression-related disorders. Importantly, our study contributes to the growing body of evidence supporting intranasal delivery as a viable method for bypassing the blood-brain barrier and delivering therapeutic agents directly to the CNS, thereby minimizing peripheral side effects and improving patient compliance. Our findings not only elucidate the neurobiological mechanisms through which GALR2 and Y1R agonists exert antidepressant effects but also highlight their therapeutic potential for developing novel treatments targeting neurogenic deficits associated with mood disorders. Future research should further explore the long-term effects and translational potential of these findings in clinical settings, with a focus on optimizing therapeutic strategies for enhancing hippocampal neurogenesis and BDNF-mediated signaling pathways in depression.

A second goal of this work aimed to explore the influence of Galanin (GAL) on hippocampal neurogenesis, focusing on its dose- and site-dependent effects on memory and implications for Alzheimer's disease models. Additionally, we investigated interactions between Neuropeptide Y (NPY) and GAL through NPY1R-GALR2 heteroreceptor complexes in various brain regions, assessing their potential therapeutic implications for age-related cognitive decline. The findings indicate that intranasal co-administration of GALR2 and NPY1R agonists significantly enhances spatial memory and promotes neuronal survival and differentiation in the adult rat hippocampus. These results underscore the critical role of GALR2 in these processes and suggest potential therapeutic avenues for combating cognitive decline. Our experimental evidence demonstrates that rats treated with the combination of GALR2 and NPY1R agonists showed marked improvements in spatial memory, as assessed by the object-in-place memory task. This enhancement was not observed when either agonist was administered alone, highlighting a synergistic effect. Further, the combined treatment led to a significant increase in the number of BrdU-immunoreactive cells in the dentate gyrus, specifically in the subgranular zone. This suggests an enhanced rate of neurogenesis, which was diminished by the GALR2

antagonist, reinforcing the critical role of GALR2 in this process. Most newly generated cells differentiated into mature neurons, as indicated by the increased co-labeling of BrdU with NeuN, a marker for mature neurons. The increase in DCX-positive cells, particularly those with more mature dendritic structures, suggests enhanced neuronal maturation and integration into existing hippocampal circuits. These morphological changes imply that the newly generated neurons are functionally integrating into hippocampal circuits, potentially enhancing cognitive functions.

Our study provides compelling evidence that targeting GALR2 and NPY1R receptors through intranasal co-administration of their agonists can significantly enhance hippocampal neurogenesis and spatial memory, suggesting a viable therapeutic approach for age-related cognitive impairments and Alzheimer's disease. Further research is warranted to unravel the molecular mechanisms underlying these effects and to explore their potential clinical applications.

RESUMEN EN ESPAÑOL

Esta tesis de doctorado se llevó a cabo entre el año 2020-2024 en el Laboratorio de Receptómica y Enfermedades Mentales, Departamento de Fisiología Humana, Educación Física y del Ejercicio, de la Facultad de Medicina, Universidad de Málaga y el Laboratorio de Neurobiología Molecular y Celular del Departamento de Neurociencias del Instituto Karolinska de la Suecia. La tesis se centra en la investigación de las interacciones de los receptores acoplados a la proteína G (GPCRs) en el sistema nervioso central (SNC). Con un particular énfasis en la existencia e implicaciones funcionales de los complejos heteroreceptor del receptor de neuropéptido Y tipo 1 (NPYR1) y el receptor de galanina 2 (GalR2) en enfermedades mentales como la depresión, empleando modelos de ratas. La tesis utiliza diversos métodos y técnicas, como estudios de comportamiento animal, histoquímica, transferencia de energía de resonancia bioluminiscente (BRET), modelos matemáticos y/o bioinformáticos y ensayo de ligadura de proximidad (in situ PLA), etc.

Existen un extenso cuerpo de evidencias experimentales que respaldan la existencia de los complejos de homo-receptores y hetero-receptores del receptor de Neuropeptido Y (NPYR). Estas interacciones y el estudio de sus regulaciones alostéricas han contribuido significativamente a la comprensión de los mecanismos de integración de la señal neuronal. A través de la heteromerización y las interacciones alostéricas, estos complejos producen modificaciones en el reconocimiento del receptor, permiten la creación de nuevos sitios de unión alostérica, y conducen a alteraciones en la farmacología, las vías de señalización y tráfico de receptores. Por tanto, modelándose o dándose lugar a un perfil de señalización mucho más diverso y específico para cada heterómero de receptores.

Los NPYR no sólo interactúan con otros GPCRs, sino que también pueden interactuar y asociarse a otras proteínas o receptores de membranas, como pueden ser los canales iónicos, receptores de tirosina cinasa (RTKs), grupos de proteínas que interactúan con proteínas G y transportadores de neurotransmisores. Esta amplia diversidad de interacciones contribuye a la mejora de su capacidad integrativa de la señalización celular. La localización de los complejos homorreceptores y heterorreceptores en regiones sinápticas o extrasinápticas de la membrana plasmática está regulada por varios factores, incluyendo la densidad de los protómeros participantes y su afinidad. También se ha demostrado y observado que la presencia o ausencia de proteínas adaptadoras dentro de los complejos heterorreceptores influye significativamente en la afinidad desarrollada entre los protómeros receptores.

La amplia distribución de complejos heterorreceptores, caracterizados por interacciones alostéricas receptor-receptor en el SNC, presenta un innovador mecanismo molecular integrador dentro de las membranas plasmáticas de las células neuronales y gliales. Se postula que el mecanismo molecular subyacente al aprendizaje y la memoria implica la reorganización de complejos heterorreceptores existentes (incluyendo GPCR) y el reajuste de múltiples interacciones alostéricas receptor-receptor dentro de estos complejos. Además, podrían formarse nuevos complejos heterorreceptores debido a alteraciones en los patrones de señales de transmisión sináptica y volumétrica. Estos ajustes moleculares dentro de los heterómeros, incluyendo cambios en la arquitectura receptor-proteína en la membrana pre and postsináptica, potencialmente constituyen la base para la memoria a corto y largo plazo.

Esta tesis doctoral tiene como objetivo más global, explorar los complejos heterorreceptores del NPY1R y sus interacciones alostéricas receptor-receptor dentro del SNC, centrándose específicamente en sus roles en las células neuronales del hipocampo. La investigación enfatiza la importancia de la neurogénesis adulta en condiciones fisiológicas y el papel regulador de los neuropéptidos, específicamente NPY y GAL. Al investigar más a fondo los efectos de los agonistas de NPY1R y GALR2 en la neurogénesis y la cognición, el objetivo es profundizar en la comprensión de estos complejos procesos biológicos y determinar hasta qué punto la formación de un complejo GalR2-NPYR1 es determinante en estos procesos neurofisiológicos. A través de la exploración de estos complejos y su integración de señales neuronales, esta investigación busca mejorar la comprensión de su participación en diversos trastornos mentales y neurológicos, potencialmente ofreciendo promisorias vías terapéuticas para abordar el declive cognitivo relacionado con la edad, los primeros estadios del deterioro cognitivo y la depresión.

La tesis comienza con una amplia revisión de investigaciones previas sobre interacciones alostéricas receptor-receptor, particularmente en receptores acoplados a proteínas G (GPCRs). A principios de la década de 1980, los investigadores observaron que los neuropéptidos podían alterar la afinidad y la densidad de los sitios de unión de agonistas y antagonistas de monoaminas en varias regiones del SNC de manera específica para un subtipo determinado de receptor. Este descubrimiento indicó interacciones alostéricas receptor-receptor entre neuropéptidos y receptores de monoaminas en la membrana plasmática. Trabajos anteriores de Lefkowitz, Limbird y colegas identificaron la existencia de mecanismos de cooperatividad negativa en receptores beta-adrenérgicos, atribuida a la formación de complejos homodímericos de los receptores beta-adrenérgicos. El primer simposio sobre interacciones receptor-receptor de GPCRs se celebró en Estocolmo en el año de 1986. En este simposium se propuso que los mecanismos de integración de la señal de los GPCR eran mucho más

amplio que el modelo de un ligando-un receptor-una proteína G, que dada la complejidad de los modelos estudiados se proponía tener en consideración la existencia y formación de complejos de receptores de mayor magnitud, no solo considerarlos como monómeros sino considerar las interacciones entre diferentes clases de macromoléculas biológicamente activas. La heteromerización de receptores se sugirió como la base molecular de estas interacciones en 1993. Las observaciones iniciales de homodimerización de GPCR datan de 1982, con descubrimientos posteriores en 1987 de homodimerización tras la estimulación del factor de crecimiento epidérmico. La validación de la heterodimerización del receptor GABA B una década después apoyó los hallazgos iniciales de interacciones receptor-receptor en complejos heterorreceptores de GPCR y abrió una nueva era de investigaciones en el campo de los GPCR y la farmacología.

La exploración de las interacciones alostéricas entre receptores dentro de complejos homorreceptores y heterorreceptores en el Sistema Nervioso Central (SNC), especialmente entre los receptores acoplados a proteínas G (GPCRs), ha avanzado significativamente nuestra comprensión de la integración cerebral y la neuropsicofarmacología. Este campo, ampliamente explorado por investigadores como Fuxe, Lefkowitz, Milligan y Borroto-Escuela, resalta cómo la oligomerización de receptores induce cambios dinámicos en los protómeros de los receptores, afectando el reconocimiento, la farmacología, las vías de señalización y el tráfico, y potencialmente creando nuevos sitios de unión alostérica. Los complejos heterorreceptores de GPCR pueden incluir receptores de canales iónicos, receptores de tirosina quinasa (RTKs), proteínas que interactúan con proteínas G, canales iónicos y transportadores de neurotransmisores, ilustrando la intrincada red de interacciones que pueden tener lugar en la membrana plasmáticas de neuronas y otras células del SNC. Estas interacciones dinámicas ocurren de manera coordinada espaciotemporalmente, contribuyendo al aprendizaje y la formación de engramas moleculares para la memoria a corto y largo plazo. Comprender la organización molecular estos oligómeros de receptores, su comunicación alostérica y las características de la interfaz del receptor sigue siendo un área crítica para mejorar. Además, estos descubrimientos han llevado a nuevas estrategias de tratamiento para enfermedades como la enfermedad de Parkinson (por ejemplo, antagonistas de los receptores A2A y mGluR5), esquizofrenia (por ejemplo, agonistas de los receptores A2A y mGluR5), depresión (por ejemplo, agonistas de 5-HT1A que potencian la función de FGFR1) y adicción a la cocaína (por ejemplo, agonistas de los receptores A2A).

Recientemente se ha propuesto que los mecanismos moleculares del aprendizaje y la memoria pueden implicar la reorganización de complejos homo- y heterorreceptores en sinapsis (membranas pre- y postsinápticas de sinapsis), y con

ello facilitar patrones de liberación de neurotransmisores. La formación de memoria a largo plazo puede implicar partes de complejos heterorreceptores que se transforman en factores de transcripción únicos, conduciendo al desarrollo de proteínas adaptadoras específicas que consolidan estos complejos en entidades duraderas con interacciones alostéricas conservadas. Estos complejos homo-heterorreceptores son ensamblajes dinámicos moldeados por señales integradas de transmisión sináptica y volumétrica, esenciales para el aprendizaje. Pueden transformarse en estados consolidados con comunicación alostérica duradera, representando engramas moleculares que modulan profundamente las redes neuronales e influyen en funciones conductuales y cognitivas con el tiempo. Para la plasticidad estructural en árboles dendríticos y espinas, el reclutamiento de RTKs a complejos heterorreceptores puede resultar en aumentos sinérgicos en densidades de neuritas y protrusiones neuronales.

La importancia de las interacciones receptor-receptor en la mejora de la diversidad de receptores fue destacada por primera vez en 1983/1985 a través de estudios sobre interacciones de neuropeptidos/dopamina (DA). Las interacciones receptor-receptor entre neuropeptido y monoamina en el SNC han demostrado la existencia no solo de monómeros de GPCR, sino también de complejos homorreceptores y heterorreceptores de GPCR, incluidos dímeros de receptores, complejos de receptores de orden superior y proteínas que interactúan con receptores como diversas proteínas adaptadoras y proteínas sinápticas/no sinápticas. La identificación reciente de la red de heterodímeros de GPCR (GPCR-HetNet) subraya que las interacciones alostéricas receptor-receptor amplían significativamente la diversidad de GPCR y la especificidad de reconocimiento y señalización, mejorando así la especificidad de señalización. Estas interacciones son recíprocas, dinámicas y alteran sustancialmente las señalizaciones, el tráfico, el reconocimiento y la farmacología de los protómeros involucrados. Las modulaciones pueden mejorar las interacciones con agonistas o antagonistas, cambiar el acoplamiento a proteínas G o promover el reclutamiento de β-arrestina. La red de heterodímeros de GPCR (www.gpcr-hetnet.com, última actualización en 2014) proporciona información sobre las interacciones directas entre GPCRs, revelando un modelo de red libre de escala donde algunos protómeros, como el receptor de adenosina A2A, el receptor de dopamina D2 y el receptor β2-adrenérgico, dominan la conectividad. Se han reportado interacciones verificadas experimentalmente para 156 protómeros de GPCR, aproximadamente el 20% del total de GPCRs en el genoma humano. A pesar de que la mayoría de los protómeros identificados pertenecen a la superfamilia de tipo rodopsina, aún existen un amplio abanico de GPCRs para los que no se han reportado, estudiado o identificado interacciones algunas. Es válido recordar que más de un 60% de los GPCRs del genoma humano corresponden aún a receptores huérfanos. Por otra parte el estudio de la Hetnet revela que para la familia B y C de la superfamilia de los GPCRs (Secretina

y metabotrópico glutamato receptor-like) estas exhiben tasas de interacción más altas que las reportadas para la familia A, con un 33% y un 60% de protómeros involucrados en interacciones, respectivamente. Mientras que más del 87% de los protómeros identificados existen como homómeros, el estudio del equilibrio entre poblaciones homoméricas y heteroméricas es crucial, para poder comprender el papel de estas interacciones en diversas enfermedades o patologías. Del análisis de la Hetnet se desprende además que las conexiones intrafamiliares fueron significativamente más prevalentes que las conexiones interfamiliares, posiblemente debido a la coevolución de interfaces de protómeros dentro de subfamilias y los diferentes patrones de expresión celular y tisular. Nuevas investigaciones sobre las especificidades de los heterocomplejos de GPCR podrían revelar un mayor alcance de las heterodimerización cruzada entre familias o especificidades intrafamiliares, y arrojar más luz sobre el complejo panorama de las interacciones de GPCR.

Por otra parte, comprender la interfaz de los dímeros de GPCR es especialmente crucial para el desarrollo de fármacos en enfermedades del SNC. En 2004, mediante espectrometría de masas y las técnicas de pull-down se demostró que las interacciones electrostáticas directas epítopo-epítopo entre protómeros A2AR-D2R, involucrando el tercer bucle intracelular de D2R y la cola C-terminal de A2R jugaban un papel muy importante en la formación del hterodímero de A2AR-D2R. En 2010, Borroto-Escuela et al. mostraron que una mutación puntual de serina en la cola C-terminal de A2AR disminuyó la heteromerización y reveló por primera vez que las hélices transmembrana estaban involucradas. En 2018, se obtuvo un modelo estructural del heterodímero A2AR-D2R mapeando su interfaz utilizando métodos computacionales y experimentales. El modelado de la interfaz del receptor empleó péptidos derivados de las hélices transmembrana para investigar su impacto en las interacciones A2AR-D2R utilizando ensayos BRET y PLA, junto con la modulación de la unión de D2R. Péptidos de las regiones TM-IV y TM-V de A2AR inhibieron la formación del heterómero y atenuaron la inhibición alostérica de la afinidad de D2R inducida por agonistas de A2AR. El acoplamiento proteína-proteína generó un modelo de A2AR-D2R que incorpora la interfaz TM-IV y TM-V, refinado mediante simulaciones de dinámica molecular. Las mutaciones en esta interfaz disminuyeron la inhibición alostérica de D2R y redujeron la señal BRET, destacando el potencial de este método para modelar heterocomplejos de GPCR y ayudar en el desarrollo de fármacos neurológicos y psiquiátricos novedosos.

Como se mencionó más arriba, aproximadamente un tercio de los GPCRs no olfativos permanecen como receptores huérfanos, careciendo de ligandos identificados y mostrando funciones independientes de la unión de un ligando. ¿Cómo se logra ser funcional sin la unión de un ligando?. Pues mediante los mecanismos de modulaciones alostéricas que tienen lugar una vez formado un

homodímero o un heterodímero. Los miembros de los GPCR a menudo modulan otros receptores a través de la heterodimerización. Por ejemplo, GPR50, un GPCR huérfano, interactúa con el receptor de melatonina MT1, influenciando sus vías de señalización. De manera similar, GPR143 interactúa con los receptores de dopamina D2R y D3R, modulando su función. Los receptores huérfanos GPR18 y GPR55 heterodimerizan con los receptores cannabinoides CB1 y/o CB2, mostrando un antagonismo cruzado negativo y antagonismo cruzado bidireccional, potencialmente involucrados en enfermedades neurodegenerativas como el Alzheimer y el Parkinson.

El uso de varias técnicas ha sido fundamental para estudiar las interacciones receptor-receptor y receptor-proteína. La transferencia de energía por resonancia de fluorescencia (FRET) y la transferencia de energía por resonancia de bioluminiscencia (BRET1) han sido de gran ayuda en el estudio de la homo- y heteromerización de proteínas, incluidos los receptores, en células vivas y en tejido. Estas técnicas implican la construcción de constructos de receptores fusionados con proteínas fluorescentes donadoras y aceptoras. En FRET, la transferencia de energía ocurre si los fluoróforos donadores y aceptores están dentro de 10 nm, detectada por excitación del donador y emisión del aceptor. Si bien el FRET clásico enfrenta desafíos con las mediciones de interacción de proteínas de membrana plasmática debido a la localización intracelular de proteínas, avances como la detección de FRET en la superficie celular y la microscopía de fluorescencia de reflexión interna total (TIRF) han mejorado los estudios de heteromerización de GPCR en la membrana plasmática proporcionando campos de excitación precisos. La complementación de fluorescencia biomolecular (BiFC) demuestra la dimerización de proteínas utilizando proteínas con mitades de proteínas fluorescentes complementarias que fluorescen al dimerizarse. De manera similar, BRET, utilizando luciferasa de Renilla con YFP o GFP2 para la transferencia de energía, detecta eficazmente heterómeros de receptores en sistemas celulares artificiales. Los ensayos de saturación de BRET son cruciales para determinar el orden oligomérico y la afinidad del complejo receptor, esenciales para comprender las interacciones receptoriales a pesar de desafíos como los requisitos de receptores fusionados con proteínas fluorescentes y posibles artefactos de sobreexpresión. Los ensayos combinados de BRET/BiFC han facilitado la detección de complejos de heterorreceptores de orden superior, mientras que las técnicas secuenciales de BRET-FRET (SRET2) han demostrado la presencia de heterómeros trímeros de GPCR. Estos métodos avanzados son invaluables a pesar de las preocupaciones relacionadas con la sobreexpresión de complejos de receptores no específicos, lo que requiere mejoras continuas en las técnicas para la detección precisa de oligómeros de receptores nativos.

Más recientemente, mediante ensayos de proximidad (PLA) se ha identificado con éxito un gran número de complejos de heterorreceptores en el SNC. Utilizando

anticuerpos primarios y secundarios vinculados con oligonucleótidos, la técnica del PLA permite la detección y cuantificación de interacciones proteína-proteína mediante microscopía de fluorescencia. El PLA ha demostrado ser efectivo al mostrar complejos de heterorreceptores ex vivo como 5-HT1A-FGFR1, A2A-D2 y D2-5HT2A en tejidos cerebrales, contribuyendo significativamente al estudio, localización y modulación de complejos de heterorreceptores en el SNC. Los avances en PLA han revolucionado los estudios de interacción proteína-proteína en trastornos cerebrales, mejorando la comprensión de enfermedades neurológicas y psiquiátricas. Por ejemplo, el PLA identificó heterocomplejos A2AR-D2R en el estriado humano, proporcionando información sobre la patología cerebral y orientando estrategias de neuroimagen humana. Las aplicaciones del PLA en la enfermedad de Alzheimer (EA) han detectado complejos de tau-ubiquitina, destacando alteraciones en la distribución de proteínas asociadas con la patología. En la enfermedad de Parkinson (EP), el PLA visualizó oligómeros de alfa-sinucleína (AS) en regiones cerebrales afectadas, arrojando luz sobre los mecanismos patológicos de EP y la atrofia de múltiples sistemas (AMS). La capacidad del PLA para preservar el contexto espacial mientras proporciona información molecular mejora la comprensión de la interacción y modificación de proteínas en trastornos cerebrales, especialmente en investigación neurodegenerativa y neuropsiquiátrica.

El uso de técnicas de PLA in situ ha permitido comprender y estudiar las vulnerabilidades de los complejos de heterorreceptores de GPCR en trastornos neurodegenerativos y mentales. Por ejemplo, complejos disfuncionales de D2R heterorreceptores pueden subyacer a cambios patológicos en circuitos cerebrales cerebrales, como el aumento de la actividad de D2R que altera la función aferente glutamatérgica prefrontal, potencialmente contribuyendo a los síntomas de esquizofrenia. Investigar estos complejos en la esquizofrenia podría conducir a nuevos tratamientos que mitiguen los efectos secundarios de los antagonistas de D2R de los antipsicóticos actuales. Descubrimientos recientes de varios complejos de D2R heterorreceptores sugieren terapias combinadas optimizadas o fármacos heterobivalentes individuales dirigidos a estos complejos en la esquizofrenia.

Comprender los complejos heterorreceptor relacionados con el sistema de serotonina (5-HT) es crucial en el contexto de la depresión mayor. La hipótesis de la serotonina, que data de la década de 1960, subraya las proyecciones de neuronas 5-HT del mesencéfalo hacia el telencéfalo y el diencéfalo involucradas en la depresión. Estudios bioquímicos sobre triptófano y 5-HT, junto con fármacos como la imipramina que inhiben la recaptación de 5-HT, conllevaron al desarrollo de los inhibidores selectivos de la recaptación de serotonina (ISRS) como antidepresivos. La identificación de los subtipos de receptores 5-HT categorizados en familias de GPCR (5-HT1, 5-HT2, 5-HT4, 5-HT5, 5-HT6 y 5-HT7) ha ampliado la comprensión del

sistema serotoninérgico. Indicaciones tempranas sugirieron que los antidepresivos tradicionales apuntaban a subtipos específicos de receptores 5-HT. La investigación actual apoya la activación de los receptores 5-HT1A y 5-HT4 mientras se bloquean los receptores 5-HT2A, 5-HT3 y 5-HT7 para obtener efectos antidepresivos. La exploración de agonistas o antagonistas selectivos para estos subtipos, junto con fármacos que afectan múltiples receptores 5-HT y el transportador de serotonina (SERT), mejora la eficacia de los ISRS como antidepresivos. El desequilibrio en la actividad de los receptores 5-HT en la depresión mayor respalda el objetivo de estos receptores para beneficio terapéutico.

El descubrimiento de complejos heterorreceptores FGFR1-5-HT1A en el hipocampo ha avanzado la comprensión del papel de los receptores 5-HT1A en la plasticidad del hipocampo. Estos complejos, activados por la coactivación de FGF2 y agonistas de 5-HT1A, aumentan la densidad de neuritas e inducen potencialmente efectos antidepresivos al contrarrestar la atrofia del hipocampo inducida por la depresión. Los complejos FGFR1-5-HT1A también modulan los canales GIRK, promoviendo la actividad de las células nerviosas piramidales del hipocampo hacia las redes del córtex prefrontal y el estriado ventral. Se sugiere una modulación similar en el mesencéfalo del rafe, donde los complejos autoreceptores FGFR1-5-HT1A afectan la neuroplasticidad y la depresión. La activación de FGFR1 reduce la función de los autoreceptores 5-HT1A, restaurando la actividad neuronal de las neuronas 5-HT del rafe y ejerciendo posibles acciones antidepresivas.

Por otra parte, es sabido que los receptores de la oxitocina (OXTR) se distribuyen junto con las redes serotoninérgicas en regiones límbicas e hipotálamo, formando heterocomplejos con GPCR como 5-HT2AR, 5-HT2CR y D2R. Las interacciones alostéricas en estos complejos modulan la señalización y el tráfico de receptores, siendo cruciales para los comportamientos sociales y cognitivos. Los complejos D2R-OXTR mejoran la función del receptor, ofreciendo un objetivo potencial para potenciar las redes emocionales a través de la señalización del receptor de oxitocina en regiones estriatales. Por otro lado, los agonistas de 5-HT2AR y 5-HT2CR inhiben la señalización de OXTR, potencialmente influyendo en acciones depresivas a través de la modulación alostérica de la señalización del receptor de oxitocina. Los receptores de serotonina, especialmente 5-HT2AR, interactúan con receptores metabotrópicos de glutamato (mGluR) como mGluR2 y mGluR5, influyendo en trastornos neuropsiquiátricos como la esquizofrenia y la depresión. Las interrupciones en estos complejos alteran la señalización celular y las respuestas conductuales, implicadas en efectos similares a la psicosis y la actividad locomotora alterada. Comprender estas interacciones subraya su potencial como objetivos terapéuticos en enfermedades neuropsiquiátricas.

Los receptores de neuropeptido Y (NPYR), que comprenden los subtipos Y1, Y2, Y4 y Y5, desempeñan roles esenciales en el SNC, modulando la regulación del estado de ánimo, la respuesta al estrés y la homeostasis en regiones clave del cerebro como la corteza, el hipocampo, la amígdala y el hipotálamo. Estudios recientes han elucidado la formación de complejos heterorreceptores de NPYR, revelando mecanismos intrincados que subyacen a su diversidad funcional. Estos complejos influyen en las vías de señalización mediadas por NPY, la neurotransmisión, la plasticidad sináptica y las funciones neuroendocrinas, críticas para el equilibrio emocional y los procesos cognitivos.

La evidencia experimental destaca la participación de los subtipos de NPYR (Y1, Y2, Y4, Y5) en trastornos depresivos, impactando en sistemas de neurotransmisores y respuestas al estrés. La activación del receptor Y1 atenúa los síntomas depresivos y los comportamientos tipo ansiedad, mejorando la resiliencia al estrés mediante la modulación del eje hipotálamo-hipofisario-adrenal y el sistema límbico. Por el contrario, los receptores Y2, a través de la antagonización, aumentan la liberación de NPY, potenciando efectos ansiolíticos y similares a los antidepresivos. La interacción entre NPY y sus receptores, junto con sus interacciones con otros sistemas de neurotransmisores (por ejemplo, serotonina, dopamina), subraya su impacto más amplio en la regulación del estado de ánimo más allá de las respuestas al estrés. El papel de los NPYRs se extiende a la neurogénesis y la neuroprotección, procesos críticamente interrumpidos en la depresión. Los receptores Y2 y Y5 contribuyen a mecanismos neuroprotectores contra la excitotoxicidad y la promoción de la supervivencia neuronal en trastornos neurológicos y psiquiátricos. El objetivo de los NPYRs ofrece tratamientos potenciales que alivian los síntomas depresivos y revierten el daño neuronal asociado con el estrés crónico y la depresión. La modulación de los NPYRs podría mejorar la plasticidad sináptica y la resiliencia, proporcionando estrategias novedosas para la estabilidad del estado de ánimo y el bienestar emocional.

En esta tesis también se investiga el papel de los complejos heteroreceptores NPYR1-GALR2 en la neurogénesis del hipocampo, el aprendizaje y la memoria. El papel de los complejos heteroreceptores de GPCR en la plasticidad cerebral, el aprendizaje y la memoria ha sido extensamente explorado. Inicialmente discutida en 1993, la relación entre las interacciones receptor-receptor y la plasticidad neuronal subraya la necesidad de múltiples eventos o señales concurrentes para inducir cambios duraderos en la función neuronal, como la potenciación a largo plazo (LTP) y la depresión a largo plazo (LTD). Las interacciones receptor-receptor intramembranosas actúan como detectores de coincidencia, haciéndolos candidatos probables para mediar estas formas de plasticidad neuronal. Por ejemplo, en el contexto de la LTD, la actividad del receptor D2 (D2R) promueve la LTD en

neuronas GABA estriato-palidales. Curiosamente, el bloqueo de mGluR5 contrarresta esta LTD a pesar de su papel en antagonizar la señalización de D2R a través de interacciones inhibidoras receptor-receptor en un complejo heteroreceptor A2A-D2-mGluR5 extrasináptico fuera del sinapsis de glutamato. Este bloqueo subraya el papel de la señalización de mGluR5 en mejorar la producción y liberación de endocannabinoides a través de la transmisión de volumen extrasináptica (VT), activando así los receptores CB1 en terminales nerviosas de glutamato para inhibir la liberación de glutamato. Este mecanismo ilustra el papel crucial de VT de endocannabinoides mediados por CB1 en terminales nerviosas de glutamato para inducir LTD mediada por D2R en sinapsis de glutamato en neuronas GABA estriato-palidales. En sinapsis cortico-estriatales, la plasticidad dependiente del tiempo de espiga dicta si se produce LTD o LTP, con la actividad presináptica precediendo a la espiga postsináptica que conduce a LTD, y viceversa induciendo LTP. Notablemente, la presencia de un agonista de D2R como quinpirole desplaza el resultado hacia LTD incluso con el protocolo inductor de LTP, destacando la capacidad potente de D2R para contrarrestar los mecanismos que favorecen LTP y en cambio promueven LTD.

En cuanto al aprendizaje y la memoria, se ha propuesto la formación de complejos heteroreceptores de larga duración a través de interacciones receptor-receptor intramembranosas como un mecanismo potencial. El aprendizaje en redes neuronales probablemente implica instrucciones que alteran los pesos sinápticos (eficacias), posiblemente mediados por múltiples interacciones entre receptores de la membrana plasmática, formando complejos heteroreceptores de orden superior o mosaicos de receptores mediante oligomerización a niveles presinápticos y postsinápticos. Estas asambleas receptoras, junto con proteínas adaptadoras, proteínas G y canales iónicos, forman parte de un circuito molecular complejo en la membrana plasmática. En la parte citoplásmica se incluyen quinasas de proteínas, fosfatasas y fosfoproteínas capaces de aprender y almacenar información. La formación de engramas depende de la reconfiguración de circuitos moleculares mediante la formación de nuevos complejos heteroreceptores, facilitando la transducción de mensajes químicos a través de conjuntos específicos de proteínas G, canales iónicos y otros efectores proteicos. La memoria a corto plazo podría implicar la estabilización transitoria de mosaicos de receptores, ajustando los pesos sinápticos en consecuencia.

La consolidación del engrama en la memoria a largo plazo probablemente involucra señales intracelulares translocadas al núcleo, activando genes tempranos inmediatos y formando proteínas adaptadoras que estabilizan mosaicos de receptores como complejos heteroreceptores de larga duración. Los cambios en la señalización de ERK son cruciales para la memoria a largo plazo, influenciando las modificaciones dependientes de la actividad de histonas y procesos epigenéticos. Las regulaciones transcripcionales y epigenéticas participan en formas de plasticidad

tanto Hebbianas como no-Hebbianas, influyendo en el aprendizaje y la memoria mediante la formación y estabilización de circuitos moleculares con complejos heteroreceptores de orden superior recién formados. Estos cambios moleculares, ya sean transitorios o duraderos, pueden alterar los patrones de salida del circuito cerebral, induciendo cambios transitorios y duraderos en comportamientos y funciones cognitivas. La plasticidad estructural en árboles dendríticos y espinas, crucial para el aprendizaje y la memoria, implica el reclutamiento de RTKs a nuevos complejos heteroreceptores, mejorando las extensiones celulares y las densidades de neuritas.

La neurogénesis es un aspecto crucial de la plasticidad neuronal, que permite al cerebro reorganizar su estructura, función y conexiones en respuesta a estímulos tanto extrínsecos como intrínsecos. Un proceso fundamental dentro de este dominio es la neurogénesis adulta del hipocampo (AHN), donde se generan continuamente nuevas neuronas en el cerebro a lo largo de la adultez. El hipocampo, especialmente el giro dentado (DG), sirve como un nicho neurogénico, desempeñando un papel vital en el mantenimiento de AHN bajo condiciones fisiológicas normales. A pesar de su importancia, la persistencia de AHN en humanos sigue siendo objeto de debate. Algunos estudios sugieren un declive en AHN con la edad, mientras que otros proporcionan evidencia que respalda su continuación hasta la edad avanzada, enfatizando su importancia en el mantenimiento de las funciones cognitivas y emocionales. El hipocampo está funcionalmente dividido, con la porción anterior (ventral en roedores) involucrada en el estrés y el comportamiento emocional, y la parte posterior (dorsal en roedores) asociada con funciones cognitivas y memoria.
La desregulación de AHN está implicada en varios trastornos neurológicos y psiquiátricos, incluyendo el trastorno depresivo mayor (MDD), el deterioro cognitivo relacionado con la edad, la enfermedad de Alzheimer (AD) y otras enfermedades neurodegenerativas. MDD es una condición prevalente que afecta a más de 300 millones de personas en todo el mundo, caracterizada por una constelación de síntomas conductuales, emocionales y cognitivos que impactan significativamente la vida diaria y aumentan el riesgo de suicidio, la consecuencia más grave del trastorno. La pandemia de COVID-19 ha exacerbado la prevalencia de la depresión, con un aumento en las tasas de soledad y dificultades financieras que contribuyen a una mayor incidencia de síntomas depresivos e ideación suicida.

Los antidepresivos actuales se dirigen principalmente a los monoaminas pero a menudo son insuficientes debido a efectos adversos y un inicio terapéutico retrasado. Aproximadamente el 50% de los pacientes no responden a estos tratamientos y el 65% no logra la remisión, resultando en depresión resistente al tratamiento (TRD). El advenimiento de la ketamina como antidepresivo de acción rápida ha ofrecido nuevas esperanzas, pero sus riesgos significativos limitan su uso

generalizado. Esto subraya la necesidad de enfoques terapéuticos novedosos que apunten a diferentes mecanismos subyacentes para mejorar la eficacia del tratamiento para MDD.

El aumento de la neurogénesis hipocampal emerge como una estrategia terapéutica prometedora para el trastorno depresivo mayor (MDD). La neurogénesis en el hipocampo involucra la proliferación celular, diferenciación neuronal y supervivencia, reguladas por diversos factores intrínsecos y extrínsecos. Neuropeptidos como el neuropeptido Y (NPY) y la galanina (GAL), junto con factores neurotróficos como el factor neurotrófico derivado del cerebro (BDNF), desempeñan roles cruciales en la modulación de estos procesos. El NPY, un neurotransmisor altamente conservado, ha demostrado efectos pro-neurogénicos en el hipocampo, con niveles reducidos observados en modelos de MDD y pacientes. Los tratamientos antidepresivos típicamente aumentan los niveles de NPY en el cerebro, destacando su relevancia terapéutica potencial. Además, la GAL, especialmente a través del receptor GAL2 (GALR2), ha mostrado efectos proliferativos y neuroprotectores en el hipocampo, sugiriendo su papel en los mecanismos antidepresivos.

En esta tesis nos hemos propuesto como objetivo específico:

Primero, investigar si la administración intranasal de agonistas de GALR2 y NPY1R puede estimular la neurogénesis adulta en el hipocampo ventral e inducir efectos similares a los antidepresivos. Esto incluye evaluar la activación y proliferación del hipocampo ventral mediante la expresión de c-Fos y PCNA, identificar subpoblaciones específicas de células proliferativas mediante doble inmunomarcación, examinar la expresión de BDNF en el giro dentado hipocampal ventral, analizar la formación de complejos heteroreceptores NPY1R-GALR2 mediante ensayos de proximidad de ligación in situ (PLA), estudiar cambios morfológicos en neuronas hipocampales y evaluar resultados funcionales en el hipocampo ventral utilizando la prueba de nado forzado (FST), con un enfoque particular en el papel de BDNF.

El segundo propósito ha sido explorar el impacto de la GAL en la neurogénesis hipocampal, incluyendo sus efectos dependientes de la dosis y el sitio en la memoria y las implicaciones para los modelos de enfermedad de Alzheimer. También investiga las interacciones entre NPY y GAL a través de los complejos heteroreceptores NPY1R-GALR2 en varias regiones cerebrales y evalúa sus posibles implicaciones terapéuticas para el deterioro cognitivo relacionado con la edad. Esto incluye evaluar la memoria espacial, la supervivencia neuronal y la diferenciación en ratas adultas tras la coadministración intranasal de agonistas de GALR2 y NPY1R, destacando el papel significativo de GALR2 en estos procesos.

Al abordar estos objetivos, la investigación busca avanzar en nuestra comprensión de los complejos NPY1R-GalR2 y sus interacciones, así como su papel subyacente en la neurogénesis y la cognición, potencialmente allanando el camino para el desarrollo de enfoques terapéuticos novedosos.

La tesis comprende dos capítulos, cada uno dedicado a abordar los objetivos mencionados.

En el capítulo 1 se demuestra que la infusión intranasal de agonistas de GALR2 y Y1R estimula la neurogénesis en el hipocampo ventral adulto y produce efectos similares a los antidepresivos. La administración intranasal ofrece una alternativa no invasiva a la infusión intracerebroventricular (icv), evitando la barrera hematoencefálica para administrar péptidos y terapéuticos proteicos directamente al sistema nervioso central (SNC). Este método está respaldado por evidencia sustancial de ensayos preclínicos y clínicos. Sus ventajas incluyen efectos secundarios reducidos en comparación con la administración periférica y la naturaleza no invasiva de la aplicación. Por ejemplo, la esketamina intranasal, recientemente introducida como antidepresivo, enfrenta limitaciones debido a la neurotoxicidad potencial, efectos secundarios psicomiméticos, riesgo de abuso y variabilidad en la respuesta al tratamiento.

Después de la administración intranasal de agonistas de GALR2 y Y1R, se observó un aumento en la proliferación celular en el giro dentado (DG) ventral del hipocampo utilizando PCNA como marcador. Esto concuerda con hallazgos previos que indican una mayor proliferación celular en el DG dorsal en 24 horas. Estudios anteriores utilizando infusión icv de GAL y agonistas de Y1R indujeron la proliferación celular en el hipocampo ventral utilizando 5-bromo-2-desoxiuridina. La fortaleza de este estudio radica en demostrar una ruta no invasiva mediante la entrega intranasal de agonistas específicos de GALR2 y Y1R. Es importante destacar que el aumento genético de la neurogénesis en el DG ventral del hipocampo ha demostrado aumentar la resistencia en modelos de depresión. De manera similar, la molécula P7C3, asociada con un aumento en la proliferación celular en el DG hipocampal, ha mostrado efectos antidepresivos en roedores y primates.

Nuestro estudio también encontró que la administración intranasal del agonista de Y1R solo aumentó la proliferación celular en el DG ventral, pero no en el DG dorsal. Esto resalta las diferencias funcionales entre las regiones ventral y dorsal y sugiere un papel diferencial para NPY en estas subregiones del hipocampo. En contraste, el agonista de GALR2 solo no afectó la proliferación celular en el hipocampo ventral. Estudios anteriores indicaron que GALR2/3 media los efectos proliferativos y tróficos de GAL, con estudios posteriores sugiriendo un papel para GALR3. Sin embargo, estos hallazgos se basaron en condiciones in vitro, que pueden diferir significativamente de los sistemas in vivo.

Además, identificamos que la administración combinada de M1145 y agonista de Y1R estimuló específicamente la proliferación de neuroblastos (células PCNA+/DCX+) sin afectar a los progenitores neurales quiescentes y los astrocitos (células PCNA+/GFAP+). Esto concuerda con informes previos que muestran que NPY promueve la proliferación de progenitores neurales amplificadores y neuroblastos.

La disfunción de la neurogénesis en la zona subventricular (SVZ) es una característica común en diversas enfermedades neurodegenerativas. Por ejemplo, la proliferación de células madre se reduce en enfermedades como el Alzheimer y Parkinson, mientras que el accidente cerebrovascular y la enfermedad de Huntington aumentan la neurogénesis de SVZ para ayudar en la reparación de áreas dañadas. Se ha informado que NPY promueve la neurogénesis a través de Y1R en neuroblastos positivos para DCX y juega un papel en la migración celular. Futuras investigaciones deberían investigar el potencial de los agonistas de GALR2 y Y1R administrados por vía intranasal en estrategias de reemplazo celular para enfermedades neurodegenerativas que afectan la neurogénesis de SVZ.

A nivel celular, el aumento de la proliferación celular en el hipocampo tras la coadministración intranasal de agonistas de GALR2 y Y1R parece estar mediado por la elevada expresión de BDNF en el giro dentado (DG) ventral del hipocampo. El BDNF, un neurotrofino crucial, desempeña un papel significativo en la promoción de la neurogénesis a través de sus efectos en la proliferación y supervivencia celular. Se ha demostrado que el ejercicio físico protege contra los síntomas depresivos al aumentar la neurogénesis hipocampal y los niveles de BDNF. Terapias que potencian la relación entre la neurogénesis dentada y el BDNF, como los agonistas combinados de GALR2 y Y1R, podrían ser clave para el tratamiento de la depresión. Esto está respaldado por evidencia previa sobre los efectos neuroprotectores del NPY en modelos de neurodegeneración.

En las células neuronales del hipocampo, la coadministración de agonistas de GALR2 y Y1R incrementó la formación de complejos heteroreceptores GALR2/Y1R, como se observó utilizando ensayos de proximidad de ligación in situ (PLA). Este efecto fue confirmado en estudios previos en células HEK y varias regiones límbicas del cerebro, incluyendo la amígdala y el hipocampo dorsal. Además, observamos que la coincubación de estos agonistas promovió el crecimiento de neuritas en las células neuronales del hipocampo, posiblemente mediado por BDNF, lo cual es consistente con sus efectos conocidos sobre el crecimiento dendrítico en cultivos primarios de hipocampo y en el hipocampo.

El resultado funcional se validó al demostrar respuestas mejoradas similares a las de los antidepresivos en la prueba de nado forzado (FST) 24 horas después de la administración intranasal de agonistas de GALR2 y Y1R. Estudios previos han

mostrado que la infusión intranasal de agonista Y1 en ratas o humanos induce efectos antidepresivos durante al menos 24 horas. De manera similar, inyecciones únicas del antagonista del receptor NMDA ketamina o del antagonista del receptor mGlu2/3 LY341495 han demostrado efectos similares a los antidepresivos en la FST en ratas a las 24 horas. Sin embargo, el agonista de GALR2 solo no mostró efectos similares a los antidepresivos a las 24 horas, sugiriendo que podrían ser necesarios tratamientos intranasales subcrónicos o crónicos para efectos duraderos en modelos de depresión patológica. Es importante destacar que se han reportado diferencias específicas de especie en las respuestas antidepresivas entre ratas y ratones. Por ejemplo, la infusión intranasal de un agonista de GALR2 basado en spexina mostró efectos similares a los antidepresivos en ratones en 2-3 horas. Recientemente, el agonista de GALR2 estabilizado M39b ha mostrado promesa en estudios de administración intranasal en ratas. Además, el antagonista de GALR2 M871 contrarrestó la respuesta mejorada observada, alineándose con hallazgos previos. Estos efectos conductuales fueron independientes de la actividad motora, ya que ni los agonistas de GALR2 ni de Y1R, ni su coadministración, afectaron la actividad locomotora. Esto es consistente con la participación del hipocampo ventral en los efectos antidepresivos del NPY en el trastorno por estrés postraumático. Por lo tanto, los efectos antidepresivos mejorados de los agonistas de Y1R y GALR2 a las 24 horas podrían estar mediados por un aumento en la señalización de los heterocomplejos Y1R-GALR2 en el hipocampo ventral, apoyados por BDNF, como se observó in vivo e in vitro.

En resumen, la infusión intranasal de agonistas de Y1R y GALR2 promueve la proliferación celular en el DG ventral del hipocampo e induce la expresión de BDNF. Estos efectos probablemente estén mediados por complejos heteroreceptores Y1R-GALR2, lo que conduce a un aumento en el crecimiento de neuritas en las neuronas del hipocampo y efectos antidepresivos mejorados. Estos hallazgos sugieren el potencial para desarrollar nuevos enfoques terapéuticos dirigidos a los heterocomplejos Y1R-GALR2 para el trastorno depresivo mayor (MDD) y condiciones relacionadas. Futuros ensayos clínicos podrían explorar la eficacia de los agonistas de Y1R y GALR2 administrados por vía intranasal en estos contextos.

En un segundo capítulo se demuestra que la coadministración de agonistas de GALR2 y NPY1R mediante entrega intranasal mejora la memoria espacial y promueve la supervivencia y diferenciación neuronal en el giro dentado del hipocampo dorsal. Previamente, observamos mejoras a corto plazo en la consolidación de la memoria objeto-en-lugar tras la administración intracerebroventricular (icv) de agonistas de GALR2 y NPY1R. Sin embargo, la administración individual de M1145 o del agonista de NPY1R por icv no produjo mejoras significativas en la memoria, sugiriendo una posible potenciación alostérica entre GALR2 y NPY1R con el tratamiento combinado. Nuestros hallazgos subrayan

un efecto sinérgico tanto a nivel transmembranal como citoplasmático, potencialmente mejorando la consolidación de la memoria espacial a través de la coadministración intranasal de estos agonistas. Nuestros resultados enfatizan la importancia de la edad neuronal, específicamente alcanzando las tres semanas, para lograr una integración funcional del hipocampo en ratas.

Nuestro análisis celular reveló un aumento significativo en los perfiles inmunoreactivos de BrdU dentro de la zona subgranular del giro dentado tras el tratamiento combinado con estos neuropeptidos, indicando una neurogénesis mejorada estrechamente vinculada con el aprendizaje y la memoria. Además, la mayoría de las células recién generadas se diferenciaron en neuronas maduras, respaldado por el aumento de células BrdU+ y la detección de la proteína nuclear específica neuronal (NeuN) después de la administración de NPY1R-GALR2, marcador de neuronas maduras. Curiosamente, la proporción de células coexpresoras de BrdU-doblecortina (DCX), a pesar del papel de DCX como marcador de neurogénesis, fue relativamente baja, lo que sugiere que las mejoras en la memoria pueden derivar principalmente de la señalización sinérgica entre NPY1R y GalR2, potencialmente formando complejos heterorreceptores que ayudan en la consolidación de la memoria y apoyan la integración de neuronas recién maduras.

Para tener en cuenta las diferencias de sexo en la neurogénesis del hipocampo adulto, nuestro estudio utilizó ratas macho, como fue señalado anteriormente por Yagi et al., quienes observaron mayores densidades de células BrdU-ir en machos en puntos temporales más tempranos en comparación con las hembras. Sin embargo, para la tercera semana, estas diferencias disminuyeron, resultando en densidades comparables entre los sexos. Además, aunque la tasa de maduración de las neuronas recién nacidas en adultos fue inicialmente mayor en machos a las dos semanas, se igualó para la tercera semana. Esto informó nuestra elección de ratas macho para mitigar disparidades relacionadas con el sexo durante las etapas tempranas de la neurogénesis, aunque estudios futuros deberían explorar posibles diferencias de sexo en diversas condiciones y marcos temporales.

Además, nuestra investigación identificó cambios morfológicos dendríticos en células etiquetadas con DCX después del tratamiento, lo que indica una integración funcional mejorada de estas neuronas en circuitos existentes. Dado el papel de DCX en la formación de conos de crecimiento neurítico y el desarrollo de sinapsis, estos cambios sugieren que la complejidad y longitud dendrítica, cruciales para la funcionalidad neuronal, pueden estar influenciados por la actividad de DCX. Se requiere más investigación para dilucidar la interacción entre la supervivencia celular, la maduración y la integración neuronal.

Si bien nuestros hallazgos de inmunocitoquímica ofrecen información valiosa sobre los efectos de los agonistas de GALR2 y NPY1R en la neurogénesis del hipocampo,

técnicas avanzadas como el análisis transcriptómico podrían proporcionar una comprensión más profunda de las vías moleculares activadas. Además, los estudios electrofisiológicos podrían revelar cómo las neuronas recién generadas se integran funcionalmente en circuitos neurales, mientras que técnicas de imagenología in vivo como la microscopía de dos fotones podrían ofrecer información en tiempo real sobre el desarrollo e integración neuronal después de la administración de agonistas. Estas metodologías avanzadas prometen mejorar nuestra comprensión del papel de los agonistas de receptores de neuropeptidos en la neurogénesis y las funciones cognitivas.

En resumen, nuestros resultados sugieren un enfoque prometedor para mejorar la memoria y la maduración neuronal mediante la coadministración intranasal de agonistas de GALR2 y NPY1R. Investigaciones adicionales son cruciales para dilucidar los mecanismos moleculares subyacentes a este efecto sinérgico y sus posibles aplicaciones terapéuticas para el aprendizaje y la memoria.

Nuestro estudio proporciona evidencia convincente de que la administración intranasal de agonistas de GALR2 y NPY1R estimula efectivamente la neurogénesis adulta en el hipocampo ventral, ejerciendo robustos efectos similares a los antidepresivos. Mediante un enfoque integral, demostramos aumentos significativos en la proliferación celular dentro del giro dentado ventral, indicado por una mayor expresión de PCNA. Además, nuestros hallazgos subrayan la especificidad de este efecto, mostrando que la coadministración de agonistas de GALR2 y Y1R aumentó selectivamente la proliferación de neuroblastos sin afectar a progenitores neurales quiescentes o astrocitos, destacando el papel matizado de NPY en las subregiones del hipocampo. A nivel celular, nuestro estudio reveló una conexión mecanicista entre los agonistas de GALR2 y NPY1R y el aumento de la expresión de BDNF en el DG ventral del hipocampo, sugiriendo que la señalización neurotrófica mediada por BDNF puede contribuir significativamente a los efectos antidepresivos observados. Esto coincide con la literatura previa que implica a BDNF en la promoción de la neurogénesis y el crecimiento dendrítico, reforzando su papel crucial en la plasticidad del hipocampo y la regulación del estado de ánimo.

Además, nuestra investigación sobre los complejos heterorreceptores NPY1R-GALR2 mediante la técnica PLA in situ proporcionó nuevas ideas sobre los mecanismos moleculares que subyacen a las acciones sinérgicas de estos agonistas en el hipocampo. Funcionalmente, nuestras evaluaciones conductuales utilizando la prueba de nado forzado (FST) demostraron respuestas mejoradas similares a los antidepresivos después de la coadministración intranasal de agonistas de GALR2 y Y1R, corroborando nuestros hallazgos celulares. Estos efectos fueron específicos del hipocampo ventral, sugiriendo un potencial terapéutico dirigido para tratar trastornos relacionados con la depresión.

Es importante destacar que nuestro estudio contribuye al creciente cuerpo de evidencia que respalda la entrega intranasal como un método viable para eludir la barrera hematoencefálica y administrar agentes terapéuticos directamente al SNC, minimizando así los efectos secundarios periféricos y mejorando la adherencia del paciente. Nuestros hallazgos no solo elucidan los mecanismos neurobiológicos a través de los cuales los agonistas de GALR2 y Y1R ejercen efectos antidepresivos, sino que también destacan su potencial terapéutico para desarrollar nuevos tratamientos dirigidos a los déficits neurogénicos asociados con los trastornos del estado de ánimo. Investigaciones futuras deberían explorar más a fondo los efectos a largo plazo y el potencial translacional de estos hallazgos en entornos clínicos, con un enfoque en optimizar estrategias terapéuticas para mejorar la neurogénesis del hipocampo y las vías de señalización mediadas por BDNF en la depresión.

ABBREVIATION LIST

5-HT1A	Serotonin receptor subtype 1A
AC	Adenylyl cyclase
A2AR	Adenosine A2A receptor
ATP	Adenosine 5'-triphosphate
BRET	Bioluminescence resonance energy transfer
BDNF	Brain-derived neurotrophic factor
cAMP	Adenosine 3',5'-cyclicmonophosphate
DA	Dopamine
DMSO	Dimethylsulfoxide
EDTA	Ethylendiaminetetraacetic acid
ER	Endoplasmatic reticulum
ERK-1/2	Extracellular regulated kinase-1/2
FGFR1	Fibroblast growth factor receptor 1
FRET	Fluorescence resonance energy transfer
GalR2	Galanin receptor 2
GRK	G-protein coupled receptor kinase
GTP	Guanosine 5'-triphosphate
GFP	Green fluorescent protein
In situ PLA	In situ Proximity Ligation Assay
MAPK	Mitogen-associated protein kinase
NPYR1	Neuropeptide Y receptor 1
PKC	Protein kinase C
PLC	Phospholipase C
Rluc	Renilla luciferase
SSRI	Selective serotonin reuptake inhibitor
TRK	Receptor tyrosine kinase
TrkB	Tropomyosin receptor kinase B
YFP	Yellow fluorescent protein

LIST OF PUBLICATIONS

This PhD thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. GALR2 and Y1R agonists intranasal infusion enhanced adult ventral hippocampal neurogenesis and antidepressant-like effects involving BDNF actions. **Alvarez-Contino JE**, Díaz-Sánchez E, Mirchandani-Duque M, Sánchez-Pérez JA, Barbancho MA, López-Salas A, García-Casares N, Fuxe K, Borroto-Escuela DO, Narváez M. J Cell Physiol. 2023 Feb;238(2):459-474. doi: 10.1002/jcp.30944. Epub 2023 Jan 4.

II. Enhancement of neurogenesis and cognition through intranasal co-delivery of galanin receptor 2 (GALR2) and neuropeptide Y receptor 1 (NPY1R) agonists: a potential pharmacological strategy for cognitive dysfunctions. Sánchez-Varo R, López-Salas A, Beltran-Casanueva R, Díaz-Sánchez E, **Alvarez-Contino JE**, Barbancho-Fernández MA, Serrano-Castro P, Fuxe K, Borroto-Escuela DO, García-Casares N, Narváez M.Behav Brain Funct. 2024 Mar 28;20(1):6. doi: 10.1186/s12993-024-00230-5.PMID: 38549164

RELATED WORK BY THE AUTHOR

I. Díaz-Sánchez E, López-Salas A, Mirchandani-Duque M, **Alvarez-Contino JE**, Sánchez-Pérez JA, Fuxe K, Borroto-Escuela DO, García-Casares N, Narváez M. Decreased medial prefrontal cortex activity related to impaired novel object preference task performance following GALR2 and Y1R agonists intranasal infusion. Biomed Pharmacother. 2023 May;161:114433. doi: 10.1016/j.biopha.2023.114433.

II. Mirchandani-Duque M, Barbancho MA, López-Salas A, **Alvarez-Contino JE**, García-Casares N, Fuxe K, Borroto-Escuela DO, Narváez M.Galanin and Neuropeptide Y Interaction Enhances Proliferation of Granule Precursor Cells and Expression of Neuroprotective Factors in the Rat Hippocampus with Consequent Augmented Spatial Memory. Biomedicines. 2022 Jun 1;10(6):1297. doi: 10.3390/biomedicines10061297.

III. Borroto-Escuela DO, Ambrogini P, Narvaez M, Di Liberto V, Beggiato S, Ferraro L, Fores-Pons R, **Alvarez-Contino JE**, Lopez-Salas A, Mudò G, Díaz-Cabiale Z, Fuxe K. Serotonin Heteroreceptor Complexes and Their

Integration of Signals in Neurons and Astroglia-Relevance for Mental Diseases. Cells. 2021 Jul 27;10(8):1902. doi: 10.3390/cells10081902.

IV. Borroto-Escuela DO, Ferraro L, Beggiato S, Narváez M, Fores-Pons R, **Alvarez-Contino JE**, Wydra K, Frankowska M, Bader M, Filip M, Fuxe K. The coming together of allosteric and phosphorylation mechanisms in the molecular integration of A2A heteroreceptor complexes in the dorsal and ventral striatal-pallidal GABA neurons. Pharmacol Rep. 2021 Aug;73(4):1096-1108. doi: 10.1007/s43440-021-00314-3.

INTRODUCTION

1. Introduction to the field of GPCR homo-and heteroreceptor complexes

In the early 1980s, it was observed that neuropeptides could alter the affinity and density of monoamine agonist and antagonist binding sites in different CNS regions in a receptor subtype-specific manner [1, 2]. This indicated neuropeptide-monoamine receptor-receptor interactions in the plasma membrane. Lefkowitz, Limbird, and colleagues had earlier discovered negative cooperativity in beta-adrenergic receptors, explained by beta-adrenergic homodimers leading to site-site interactions [3, 4]. This can be explained based on the existence of beta-adrenergic homodimers leading to site–site interactions. The first symposium on GPCR receptor-receptor interactions, held in Stockholm in 1986, proposed a broader field including interactions among different classes of biologically active macromolecules[5]. Receptor heteromerization was suggested in 1993 as the molecular basis for these interactions[6]. Early observations of GPCR homodimerization date back to 1982 [7, 8], with further discoveries in 1987 of homodimerization upon epidermal growth factor stimulation [9]. The validation of GABA B receptor heterodimerization ten years later supported early findings of receptor-receptor interactions in putative GPCR heteroreceptor complexes (see [10-12]).

The exploration of allosteric interactions among receptors within homo- and heteroreceptor complexes in the Central Nervous System (CNS), particularly among G protein-coupled receptors (GPCRs), has significantly advanced our understanding of brain integration and neuropsychopharmacology [1, 6, 13-21]. This field, explored extensively by researchers such as Fuxe, Lefkowitz, Milligan and Borroto-Escuela, highlights how receptor oligomerization induces dynamic changes in receptor protomers. These changes affect recognition, pharmacology, signalling, and trafficking, potentially creating novel allosteric binding sites [16, 21-24]. GPCR heteroreceptor complexes can include ion channel receptors, receptor tyrosine kinases (RTKs), G protein-interacting proteins, ion channels, and transmitter transporters, illustrating the intricate network of interactions in the CNS [18, 22, 25-31]. These dynamic interactions occur in a coordinated spatio-temporal manner, contributing to learning and the formation of molecular engrams for short- and long-term memory [32-35]. Understanding the molecular organization of receptor oligomers, their allosteric communication, and the features of the receptor interface remains a critical area for improvement [22, 36-43].

Heteroreceptor complexes appear to be a fundamental principle for molecular integration in biology, involving GPCR interacting proteins. These discoveries have led to new treatment strategies for diseases such as Parkinson's disease (e.g. A2A and mGluR5 receptor antagonists) [44], schizophrenia (e.g.A2A and mGluR5 agonists) [45, 46], depression (e.g. 5-HT1A agonists enhancing FGFR1 function) [29] and cocaine addiction (e.g.A2A agonists) [47].

Recent hypothesis propose that learning and memory involve the reorganization of homo- and heteroreceptor complexes in synapses (pre- and postjunctional membrane of synapses), impacting receptor complexes to facilitate neurotransmitter release patterns [34, 48-51]. Long-term memory formation may involve parts of heteroreceptor complexes transforming into unique transcription factors, leading to the development of specific adapter proteins that consolidate these complexes into long-lived entities with preserved allosteric interactions [34, 52]. These homo-heteroreceptor complexes are dynamic assemblies shaped by integrated synaptic and volume transmission signals, essential for learning. They can transform into consolidated states with enduring allosteric communication, representing molecular engrams that profoundly modulate neuronal networks and influence behavioural and cognitive functions over time [34, 49, 50]. For structural plasticity in dendritic trees and spines, the recruitment of RTKs to heteroreceptor complexes may result in synergistic increases in neurite densities and protrusions in primary neuronal cultures [53, 54].

1.1 GPCR-GPCR heteroreceptor complexes.

The significance of receptor-receptor interactions in enhancing receptor diversity was first introduced in 1983/1985 through studies on neuropeptide/dopamine (DA) interactions [1, 55, 56]. Neuropeptide-monoamine receptor-receptor interactions in the CNS have shown the existence of not only GPCR monomers but also GPCR homo- and heteroreceptor complexes, including receptor dimers, higher-order receptor complexes, and receptor-interacting proteins like various adapter proteins and synaptic/non-synaptic proteins Recent identification of the GPCR heterodimer network (GPCR-HetNet) underscores that allosteric receptor-receptor interactions significantly expand GPCR diversity and bias recognition and signaling, leading to increased signaling specificity[57]. These interactions are reciprocal, dynamic, and substantially alter the signaling, trafficking, recognition, and pharmacology of the involved protomers. Modulations can enhance interactions with agonists or antagonists, switch G protein coupling, or promote β-arrestin recruitment [48, 58-60].

The GPCR heterodimer network (www.gpcr-hetnet.com, last updated 2014 [57]) provides insights into direct interactions between GPCRs, revealing a scale-free model where a few protomers, such as the adenosine A2A receptor, dopamine D2R, and B2-adrenergic receptor, dominate connectivity. Experimentally verified interactions have been reported for 156 GPCR protomers, approximately 20% of the

total putative human GPCR protomers. Despite rhodopsin-like superfamily representing the majority of identified protomers, their interactions were most incomplete. The Secretin-like and metabotropic glutamate receptor-like superfamilies exhibited higher interaction rates, with 33% and 60% of putative protomers involved in interactions, respectively. While more than 87% of identified protomers exist as homomers, the balance between homo- and heteromeric populations is crucial, potentially influencing diseases where GPCR dimerization plays a role. Intrafamily connections were significantly more prevalent than interfamily connections, possibly due to the co-evolution of protomer interfaces within subfamilies and diverse cell and tissue expression patterns. Further research into GPCR heterocomplexes specificities may reveal cross-family heterodimerization or intrafamily specificities, shedding light on the complex landscape of GPCR interactions.

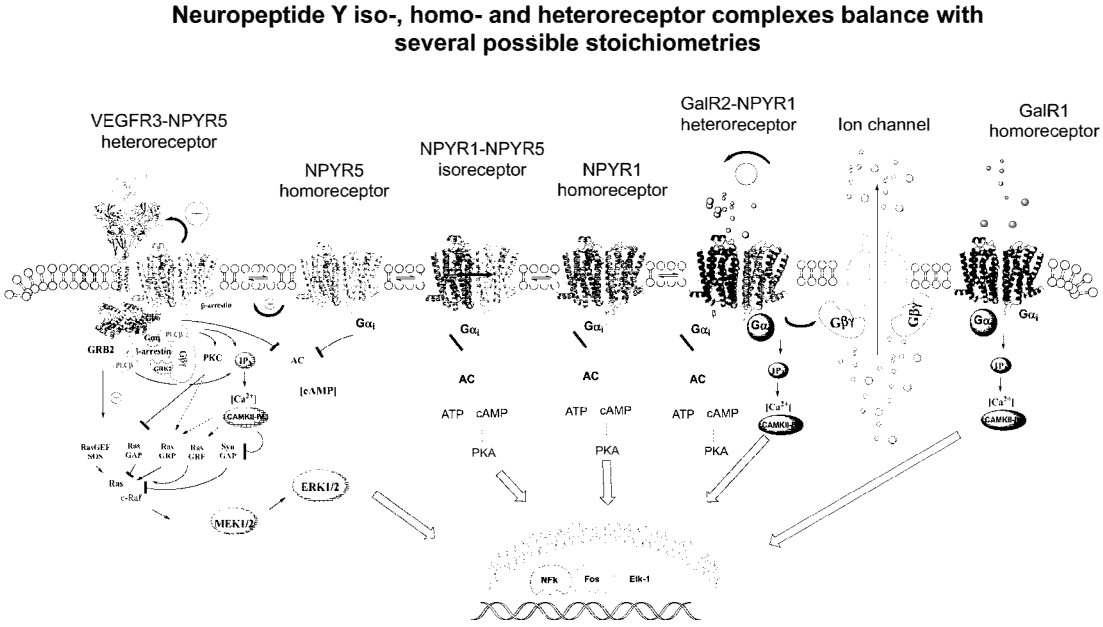


Figure 1. Overview of Neuropeptide Y Receptor 1 (NPYR1) Iso-, Homo-, and Heteroreceptor Complexes. *This figure illustrates the various configurations of NPYR1 iso-, homo-, and heteroreceptor complexes. It highlights the balance between NPYR1 homoreceptor complexes and their heteroreceptor counterparts, emphasizing the allosteric receptor-receptor interactions within these complexes. The nature of these interactions is depicted in the top part of each receptor complex: antagonistic allosteric modulation is marked with (-), and facilitatory allosteric modulation with (+). The term "isoreceptor" refers to the NPYR1-NPYR5 heteromer, given that both types of NPY receptors are activated by the same neurotransmitter, Neuropeptide Y (NPY). NPYR1 heteroreceptor complexes are proposed to be located mainly in extrasynaptic regions, although they can also be found in synaptic sites. These complexes are thought to modulate synaptic glutamate transmission in pyramidal neurons and synaptic GABA transmission in inhibitory GABA interneurons within the hippocampus. However, the specific roles of these NPYR1 heteroreceptor complexes in regulating hippocampal networks are yet to be fully characterized.*

Understanding the interface of GPCR dimers is particularly important for drug development in CNS diseases [61]. In 2004, mass spectrometry and pulldown techniques demonstrated direct epitope-epitope electrostatic interactions between A2AR-D2R protomers, involving the third intracellular loop of D2R and the C-terminal tail of A2R. In 2010, Borroto-Escuela et al. [62] showed that a serine point mutation in the C-terminal tail of A2AR diminished heteromerization and revealed for the first time that transmembrane helices were involved. In 2018, a structural model of the A2AR-D2R heterodimer was obtained through mapping its interface using computational and experimental methods [63]. The modeling of receptor interface regions was performed using peptides from the transmembrane helices and their effects on A2AR-D2R interactions were studied using BRET and PLA, along with modulation of D2R binding. Peptides from TM-IV and TM-V of A2AR counteracted heteromer formation and antagonized A2AR agonist-induced allosteric inhibition of D2R affinity. Protein-protein docking provided a model of A2AR-D2R containing the TM-IV and TM-V interface, improved by molecular dynamic simulation. Mutations in this receptor interface reduced allosteric inhibition of the D2R protomer and decreased the BRET signal, suggesting the utility of this approach for modeling other GPCR heterocomplexes and aiding in the development of novel drugs for neurological and mental diseases.

Approximately one-third of the 400 nonodorant GPCRs remain orphans [64, 65], with unidentified ligands and potential ligand-independent functions. Members of the GPCR family often modulate other receptors through heterodimerization. For instance, GPR50, an orphan GPCR, interacts with the melatonin MT1 receptor, influencing their signaling pathways [66]. Similarly, GPR143 interacts with dopamine receptors D2R and D3R, suggesting implications for neurological conditions such as Parkinson's disease [65]. Orphan receptors GPR18 and GPR55 heterodimerize with cannabinoid CB1 and/or CB2 receptors, exhibiting negative cross-talk and bidirectional cross-antagonism, suggesting their involvement in neurodegenerative diseases like Alzheimer's and Parkinson's [67-70].

1.2 GPCR-ionotropic receptor heteromers

Ion channel and GPCR interactions are critical for cellular signaling and physiological processes. In 2002, Dr. Fang Liu and colleagues expanded the understanding of receptor interactions by identifying D1R-NMDA receptor complexes [71, 72]. They found that two regions of the D1R carboxyl tail interact with NMDA receptor subunits NR1-1a and NR2, leading to dual regulation of NMDAR through current inhibition and reduced excitotoxicity via a PI-3 mechanism [71, 73]. This discovery enhanced our knowledge of receptor interactions, particularly following earlier observations that l-Glutamate decreased high-affinity D2R agonist

binding sites in striatal membranes [74]. In 2006, it was shown that D2R directly interacts with the NR2B subunit at the postsynaptic membrane of glutamate synapses on striatal neurons [75]. This interaction blocked Ca(2+)/calmodulin-dependent protein kinase II (CaMKII) association with the NR2B subunit, reducing phosphorylation and inhibiting NMDAR currents. This suggests a potential reciprocal allosteric antagonistic interaction between D2R and NMDAR in ventral striato-pallidal GABA neurons, possibly affecting anti-reward mechanisms [48, 75].

An interaction between neurotensin receptor 1 (NTS1) and NMDAR has also been observed [76]. Perroy et al. demonstrated a direct physical interaction between mGlu5a and NMDAR, leading to reciprocal inhibition of their functions. This interaction in hippocampal neurons suggests a higher degree of target-effector specificity and subcellular signaling localization than previously understood. Deletion of the mGlu5a C terminus abolished this interaction, highlighting its importance in mediating functional cross-talk between these receptors. Marino et al. showed that muscarinic M1 receptors (M1Rs) potentiate NMDA receptor currents in hippocampal pyramidal cells, suggesting roles in learning and memory. Colocalization of M1Rs and NR1a NMDA receptor subunits indicates a spatial relationship that allows physiological interactions, with implications for neurodegenerative diseases like Alzheimer's [77].

A study by Liu et al. [17] uncovered a selective complex formation between GABA(A) ligand-gated channels and D5 receptors. This interaction occurs through direct binding between the D5 receptor carboxy-terminal domain and the second intracellular loop of the GABA(A) gamma2 (short) receptor subunit, facilitating mutually inhibitory functional interactions, suggesting a previously unknown mechanism for regulating synaptic strength and implications for psychomotor disease states.

Voltage-gated calcium channels, crucial regulators of calcium homeostasis, are finely tuned by cellular signaling pathways activated by GPCRs. GPCRs not only regulate calcium channel activity via second messengers but can also physically associate with calcium channels to directly influence their functions and trafficking [78, 79]. Specific populations of ion channels are directly controlled by G proteins, while others are modulated indirectly through G protein-dependent phosphorylation events and lipid metabolism. These modifications affect ion channel activities and spatiotemporally regulate membrane potentials and intracellular calcium concentrations. G protein-gated inwardly rectifying potassium channels (Kir3 or GIRK), expressed in the brain, heart, and endocrine tissues, stably associate with several different GPCRs, forming macromolecular ion channel-GPCR signaling complexes [80-82]. The molecular determinants that mediate and maintain GPCR-GIRK channel complexes are not well understood but are crucial for determining synaptic

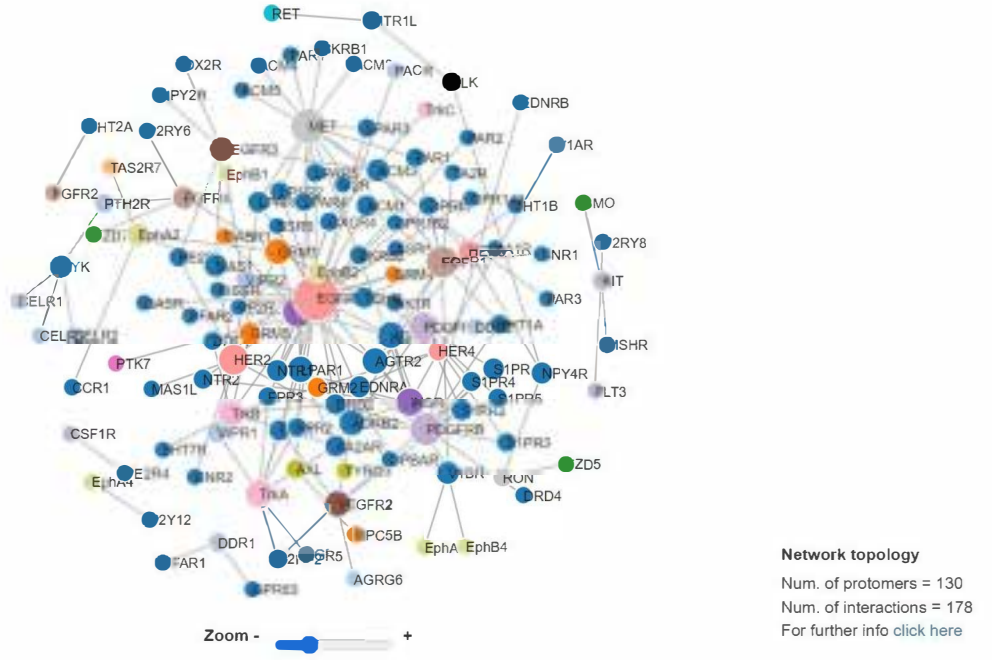
response times and the extent of GPCR "crosstalk" in GIRK-mediated inhibitory synaptic transmission [81].

Interactions between nicotinic acetylcholine receptors (nAChRs) and dopamine receptors illustrate complex mechanisms underlying synaptic modulation and neuronal excitability [83]. nAChRs, ligand-gated cationic channels composed of various α and β subunits, exist as α7-containing (α7nAChRs) and non-α7 nAChRs. Dopamine receptors, including D2 dopamine receptors (D2ARs), co-localize with nAChRs in dopamine neurons within the ventral tegmental area (VTA) and substantia nigra (SN). Specifically, α6-containing nAChRs are highly expressed in midbrain dopamine neurons, where their activation increases neuron firing, a process antagonized by D2ARs. Quarta et al. [84] demonstrated that nicotine-induced dopamine release in the striatum is modulated by D2 autoreceptors and non-α7 nAChRs. Co-immunoprecipitation experiments revealed physical interactions between β2 subunits of non-α7 nAChRs and D2 autoreceptors, suggesting the formation of heteromeric dopamine autoreceptor complexes that modulate dopamine release. These findings highlight significant crosstalk between GPCRs and ligand-gated ion channels in dopaminergic nerve terminals. Activation of nAChRs induces morphological remodeling in dopamine neurons, dependent on functional DA D3 receptors (D3Rs). Evidence suggests the existence of D3R-nAChR heteromers, with direct interaction between D3R and the β2 subunit of nAChR. Disruption of these heteromers by interfering peptides targeting intracellular loops reduces nicotine-induced neurotrophic effects on dopamine neurons, emphasizing the functional significance of the D3R-nAChR heteromer in mediating nicotine's effects.

1.3 GPCR-RTK heteroreceptor complexes

The concept of receptor tyrosine kinase (RTK) and GPCR heteromers was introduced in 2007, particularly focusing on FGFR1-5-HT1AR heteroreceptor complexes [25]. The following year, a direct physical interaction between FGFR1 and A2AR was demonstrated using the yeast two-hybrid method, revealing a novel connection where FGF acted as a cotransmitter to the A2AR protomer, enhancing synaptic plasticity both morphologically and functionally [26, 85].

Figure 1. GPCR-RTK heteroreceptor complexes network (www.gpcr-hetnet.com). *A recent report on the complex network of interactions between different GPCR-RTK pairs, encompassing around 178 GPCR-RTK receptor-receptor interactions, highlights the extensive crosstalk and signal integration occurring between these signaling pathways.*



In 2012, the discovery of FGFR1-5-HT1AR heteroreceptor complexes highlighted their potential as targets for antidepressant drugs by restoring activity and tropism in hippocampal neuronal circuits affected by depression [29]. In the dorsal raphe, FGFR1 forms a complex with the 5-HT1A auto-receptor. Combined treatment with 5-HT1AR agonist and FGF2 increased the density of these complexes in the hippocampus, enhancing FGFR1 signaling linked to antidepressant actions. These findings integrate the serotonin and neurotrophic hypotheses of major depression. Disturbances in FGFR1-5-HT1AR heterocomplexes in the raphe-hippocampal 5-HT neuronal system have been observed in a genetic rat model of depression (Flinders sensitive line rat) [86]. These deficits may involve a failure of combined agonist treatment to uncouple the 5-HT1A auto receptor from GIRK channels in raphe 5-HT neurons, increasing hyperpolarization and reducing firing [87]. This could relate to a reduced ability of the FGFR1 protomer to mitigate 5-HT1A auto-receptor signaling via allosteric interactions. Neurochemical and electrophysiological analyses demonstrated the existence of astrocytic FGFR1-5-HT1AR heterocomplexes in the hippocampus. Using in situ proximity ligation assay combined with immunohistochemistry, FGFR1-5-HT1AR heterocomplexes were localized in astrocytes, marked by GFAP immunoreactivity [87]. Acute treatment with 8-OH-DPAT alone or with FGF2 significantly increased FGFR1-5-HT1AR heterocomplexes in GFAP-positive cells, especially in the dentate gyrus polymorphic layer and CA3 area. Structural plasticity changes were also observed in astrocytes upon these treatments. FGFR1-5-HT1AR heterocomplexes in astrocytes modulate astroglial structure and function in the hippocampus, potentially affecting gamma oscillations.

Further research in 2017 revealed that FGFR can form heteroreceptor complexes with muscarinic acetylcholine receptors (mAChR) [54], promoting neurite outgrowth in neural hippocampal cultures [54]. GPCR and RTK trophic interactions are also suggested to involve functional crosstalk in intracellular pathways, such as the interaction between GABAB and insulin growth factor 1 [88]. Later research revealed A2AR also forms heteroreceptor complexes with TrkB receptors in the dorsal hippocampus [89], found in high densities in the pyramidal cell layers of the CA1-CA3 regions, but not in the dentate gyrus's molecular and granular cell layers. These complexes may have implications for hippocampal plasticity, which declines with age.

Also, a recent report on the complex network of interactions between different GPCR-RTK pairs, encompassing around 181 GPCR-RTK receptor-receptor interactions, highlights the extensive crosstalk and signal integration occurring between these signaling pathways (https://www.gpcr-hetnet.com/?network=rtk). This diversity in GPCR-RTK heteroreceptor complexes illuminates the intricate ways in which cells coordinate responses to various stimuli, offering valuable insights into the complexities of cellular signaling. These findings have significant implications for drug development and therapeutic strategies aimed at modulating GPCR-RTK interactions to treat various diseases and disorders.

1.4 GPCR interacting proteins and their receptor-protein interactions.

In the late 1990s, the study of GPCR interacting proteins (GIPs) began [90-92], significantly enhancing our understanding of GPCR function through their oligomerization. Key discoveries included beta-arrestin's role in forming beta2 adrenergic receptor-Src protein kinase complexes [93] and activating MAPK via inverse agonists, which revealed distinct active conformations for GPCRs [94]. Proteins containing PDZ domains, which participate in cellular signalling, were found to bind to GPCRs [95, 96], such as the beta2 adrenergic receptor and the 5-HT2C receptor [97]. Homer/Vesl proteins were also shown to bind to group 1 metabotropic glutamate receptors, regulating their signalling and synaptic plasticity [98]. Modulation of GPCR signalling by GIPs involves interactions with various structural features on the receptors, such as PDZ domains, proline-rich motifs, and specific phosphorylation sites. These interactions influence receptor clustering, anchoring, signalling duration, and endocytic trafficking. For example, β-arrestins and GRKs regulate GPCR desensitization and internalization, while other GIPs like GASP and SNX1 selectively promote receptor trafficking to lysosomes. This complex regulatory network ensures that GPCR-mediated signalling is finely tuned and adapted to specific cellular contexts.

By 2003, over 50 GPCR interacting proteins had been identified, highlighting the importance of the C-terminal tail of GPCRs as an anchorage for functional protein networks [91, 92, 99]. These proteins, serving as scaffolding or adapter proteins, modulate receptor-receptor interactions in heteromers [100] and help target and anchor receptor protomers to the plasma membrane [101]. This intricate network of interactions fine-tunes GPCR function by impacting receptor trafficking, localization, and pharmacological properties. GIPs are crucial throughout the GPCR lifecycle, aiding in proper folding, signalling, and degradation, thus playing a significant role in maintaining receptor function and preventing pathological condition [102-104].

Recent research has focused on targeting GPCR-GIP interactions for therapeutic purposes in neurological and mental diseases [99, 102]. Proteins like p11, which modulates 5-HT1B receptor function [105]. . In depression-like states it has been found to change 5-HT1B receptor function, modulating its plasma membrane expression [105]. p11 also participates in interactions with the 5-HT4 receptor contributing to the behavioural effects of 5-HT4 activation [106]. Spinophilin interacts with D2 and mu-opioid receptors, showing its potential as drug targets [107]. Additionally, PICK1, interacting with mGluR7 [184,221], has implications for epilepsy treatment. These examples underscore the clinical relevance of GPCR-GIP interactions and their potential for developing novel therapeutic strategies by targeting allosteric receptor-receptor interactions within heteroreceptor complexes.

2. Methodologies for Studying receptor-receptor and receptor-protein Interactions.

Fluorescence resonance energy transfer (FRET [108, 109]) and bioluminescence resonance energy transfer (BRET[1]) have been pivotal in studying the homo and heteromerization of proteins, including receptors, in living cells. These methods involve creating receptor constructs fused with 'donor' and 'acceptor' fluorescent proteins. In FRET, energy transfer occurs between the donor and acceptor fluorophores if they are within 10 nm of each other, indicated by donor excitation and acceptor emission [110]. Despite its usefulness, classical FRET faces challenges in measuring protein interactions at the plasma membrane when proteins are located in intracellular stores [108]. However, advancements like cell surface FRET detection technologies and total internal reflection fluorescence microscopy (TIRF) have improved the study of GPCR heteromerization at the plasma membrane by providing more defined fields of excitation. Biomolecular fluorescence complementation (BiFC [111, 112]) is another method used to demonstrate protein dimerization. It involves proteins carrying complementary halves of a fluorescent protein that, upon dimerization, form a fluorescent complex. Similarly, BRET operates on a principle

akin to FRET but uses Renilla luciferase and YFP or GFP2 for energy transfer, proving beneficial in detecting receptor heteromers in artificial cell systems [85, 113, 114]. BRET saturation assays help determine the oligomeric order of receptor complexes and their affinity, crucial for understanding receptor interactions. Despite some inherent issues like the need for fluorescent protein-fusion receptors and potential overexpression artifacts, these methods are integral in analyzing GPCR dimerization [114]. Combined BRET/BiFC assays have enabled the detection of higher-order heteroreceptor complexes, while sequential BRET-FRET techniques ($SRET^2$) help demonstrate trimeric heteromers of GPCRs. These advanced methods are valuable despite the challenges associated with overexpression-mediated non-specific receptor complexes. Improving these techniques is essential for detecting native receptor oligomers accurately [115].

The in situ Proximity Ligation Assay (PLA) has proven effective in establishing native heteroreceptor complexes in the CNS [29, 116-118]. This technique involves using primary and secondary antibodies linked with oligonucleotides, enabling the detection and quantification of protein interactions through fluorescence microscopy. In situ PLA has successfully demonstrated heteroreceptor complexes ex vivo, such as A2A-D2 and D2-5HT2A complexes in brain tissues, making it a valuable tool for studying the localization and modulation of CNS heteroreceptor complexes [29, 59, 116-121].

PLA has revolutionized the study of protein-protein interactions in brain disorders, advancing our understanding of neurological and psychiatric diseases [122]. For example, PLA has identified A2AR-D2R heterocomplexes in the human striatum [122], providing insights into brain pathologies and aiding in the strategic planning of human neuroimaging. PLA has also been used to study Alzheimer's disease (AD) by detecting tau-ubiquitin complexes in affected brains [123], revealing significant alterations in protein distribution associated with pathology. In Parkinson's disease (PD), PLA has visualized alpha-synuclein (AS) oligomers and their role in neurodegeneration [124]. The technique has demonstrated the accumulation of AS oligomers in brain regions affected by PD and multiple system atrophy (MSA) [125], providing insights into the pathological mechanisms of these diseases. The ability of ex-vivo PLA to maintain spatial knowledge while delivering detailed molecular information enhances our understanding of protein interactions and modifications in brain disorders, highlighting its significance in neurodegenerative and neuropsychiatric research.

3. Dysregulation of GPCR heteroreceptor complexes is implicated in the development of brain disorders.

The dysfunction of D2R heteroreceptor complexes can form the molecular basis for pathological changes in brain circuits. For example, increased D2R activity can alter the function of glutamate prefrontal afferents, leading to schizophrenia symptoms [126]. Investigating these complexes and their dysfunctions in schizophrenia could lead to new treatments and reduce the side effects of current antipsychotics, which often act as D2R antagonists [43, 127]. Recent discoveries of various D2R heteroreceptor complexes suggest the potential for optimized combination therapies or single heterobivalent drugs targeting these complexes in schizophrenia [16, 43, 128-131].

Numerous 5-HT1A iso and heteroreceptor complexes, such as 5-HT1A-5-HT7, FGFR1-5-HT1A, and the potential GalR1-GalR2-5-HT1A trimer complex, have deepened our understanding of the molecular basis of major depression based on the serotonin hypothesis [28, 29, 86, 132-134]. Postjunctional 5-HT1A receptors significantly contribute to the antidepressant effects of serotonin [135]. Receptor-interacting proteins, like the scaffolding protein p11 and the adaptor protein DISC1 in D2R heteroreceptor complexes, also play crucial roles [136].

Allosteric interactions in heteroreceptor complexes can alter the function of receptor protomers. This can change receptor recognition (affinity and density) and G protein coupling strength, affecting signalling cascades and ion channel activity like G protein-coupled inwardly rectifying potassium channels. These interactions can modify G protein selectivity, for example from Gi to Gq, and favor signalling over beta-arrestin pathways, leading to biased signalling. Changes in one receptor protomer can affect the recognition and signalling balance of multiple receptor protomers within higher-order complexes. This can filter incoming signals to one protomer based on changes in another, facilitating complex signal modulation. Receptor-receptor interactions can create novel receptor subtypes, like the GABAB receptor, and change the pharmacology of orthosteric binding sites [10, 11]. Dysfunctional protomers may form under pathological conditions through inappropriate interactions, potentially recognizing novel transmitters [137].

These interactions impact receptor cotrafficking [138], including maturation, cell surface expression, and internalization, which are crucial for receptor sensitization and desensitization. Studies have analyzed colocation, cotrafficking, coclustering, and cointernalization in complexes such as A1-D1 and A2A-D2 [139-141].

The regulation and dysregulation of GPCR heteroreceptor complexes play a critical role in brain function and disorders, offering potential pathways for therapeutic intervention in Parkinson's disease, schizophrenia, substance use disorder, anxiety and depression opening a new field in neuropsychopharmacology [142-144]. Innovative strategies for addressing heteroreceptor complexes in CNS diseases encompass combined drug treatments that target two receptor protomers, exploration of dual-acting drugs targeting two protomers within the receptor complex, and the

development of dual-acting pro-drugs, presents a potential and novel multi-target approach for treating CNS diseases [145, 146]. Heterobivalent drugs also offer an additional option for selectively targeting heterodimers.

3.1. Role of GPCR Heteroreceptor Complexes in Major Depression

GPCR heteroreceptor complexes are pivotal in the context of major depression, particularly within the serotonin (5-HT) system [147, 148]. The 5-HT hypothesis, originating in the 1960s, highlighted the role of 5-HT neurons originating from the midbrain and their projections to tel- and diencephalon in depression [149-151]. Biochemical studies on tryptophan and 5-HT, along with the discovery of 5-HT reuptake inhibition by drugs like imipramine, laid the foundation for selective serotonin reuptake inhibitors (SSRIs) as antidepressants. The subsequent identification of various 5-HT receptor subtypes, categorized into six families of G protein-coupled receptors (5-HT1, 5-HT2, 5-HT4, 5-HT5, 5-HT6, and 5-HT7), further enriched our understanding of the serotonin system.

Early indications suggested that traditional antidepressants might target specific 5-HT receptor subtypes [152]. Current knowledge supports the efficacy of activating 5-HT1A and 5-HT4 receptors, while blocking 5-HT2A, 5-HT3, and 5-HT7 receptors for antidepressant effects [153]. This insight has led to the exploration of selective agonists or antagonists for these receptor subtypes, alongside drugs targeting multiple 5-HT receptors and the serotonin transporter (SERT), to enhance SSRI antidepressant effects [154]. The hypothesis that major depression involves an imbalance in 5-HT receptor activity underscores the potential of targeting these receptors for therapeutic benefit.

The discovery of FGFR1-5-HT1A heteroreceptor complexes in the hippocampus has significantly advanced our understanding of 5-HT1A receptors' role in hippocampal plasticity [29]. Activation of these heterocomplexes enhances neurite density and may produce antidepressant effects when co-activated with FGF2 and a 5-HT1A agonist. FGFR1 activation counteracts depression-induced hippocampal atrophy by phosphorylating FGFR1, thereby promoting neuroplasticity [30]. Moreover, these complexes modulate GIRK channels, enhancing hippocampal pyramidal nerve cell firing towards the prefrontal cortex and ventral striatum networks. Similar modulatory effects are proposed in the midbrain raphe, where FGFR1-5-HT1A autoreceptor complexes are abundant, impacting neuroplasticity and depression. Activation of FGFR1 reduces 5-HT1A autoreceptor function, restoring raphe 5-HT neuron firing and promoting trophic effects, potentially exerting antidepressant actions [30, 155].

Oxytocin receptors (OXTR) co-distribute with serotonin networks in limbic regions and the hypothalamus, forming heterocomplexes with GPCRs such as 5-HT2AR, 5-

HT2CR, and D2R [156], [119, 157]. Allosteric interactions within these complexes modulate receptor signaling and trafficking, crucial for social and cognitive behaviors. Specifically, D2R-OXTR complexes [119, 156] enhance receptor functions, suggesting a potential target for enhancing emotional networks via oxytocin receptor signaling in striatal regions. Conversely, 5-HT2AR and 5-HT2CR agonists inhibit OXTR signaling, potentially influencing depressive actions through allosteric modulation of oxytocin receptor signaling [158, 159].

Serotonin receptors, particularly 5-HT2AR, interact with metabotropic glutamate receptors (mGluRs), such as mGluR2 and mGlu5, influencing neuropsychiatric disorders like schizophrenia and depression [160, 161]. Disruptions in these complexes alter cellular signaling and behavioral responses, implicated in psychosis-like effects and altered locomotor activity [161]. Understanding these interactions underscores their role as potential therapeutic targets in neuropsychiatric diseases.

3.2. Neuropeptide Y receptor complexes and depression

The neuropeptide Y receptors (NPYRs) form a subfamily of G-protein coupled receptors (GPCRs) that play essential roles throughout the CNS. Comprising four main subtypes—Y1, Y2, Y4, and Y5—these receptors are characterized by their distinct structural and functional properties, each interacting with neuropeptide Y (NPY) to modulate various physiological processes [162]. NPYRs are widely distributed in key brain regions involved in mood regulation, stress response, and homeostasis, including the cortex, hippocampus, amygdala, and hypothalamus. Their structural diversity allows for differential interactions with NPY and other ligands, influencing neurotransmission, synaptic plasticity, and neuroendocrine functions critical for maintaining emotional balance and cognitive processes.

Recent experimental evidence has shed light on the oligomerization and formation of homo- and heteroreceptor complexes involving neuropeptide Y receptors (NPYRs), revealing intricate mechanisms that underlie their functional diversity [57]. Studies utilizing advanced biochemical and biophysical techniques have identified that NPYRs, particularly Y1, Y2, Y4, and Y5 subtypes, can form dimers or higher-order oligomers both in vitro and in vivo [163-166]. These receptor complexes exhibit distinct pharmacological properties and signaling capabilities compared to individual receptors, influencing NPY-mediated signaling pathways and modulating neurotransmission and synaptic plasticity. Moreover, heteroreceptor complexes formed between different NPYR subtypes and other GPCRs or RTKs have been observed, suggesting cross-talk mechanisms that integrate diverse cellular signals (see, www.gpcr-hetnet.com). Such findings highlight the dynamic nature of NPYR interactions within the CNS, providing a foundation for further exploration into how

these receptor complexes contribute to neuropsychiatric disorders and their potential as therapeutic targets for novel pharmacological interventions.

The diverse subtypes of NPYRs—Y1, Y2, Y4, and Y5—have been implicated in various aspects of depressive disorders, influencing neurotransmitter systems and stress responses. Specifically, the Y1 receptor has garnered attention for its involvement in attenuating depressive symptoms [166, 167]. Animal studies have shown that Y1 receptor activation reduces anxiety-like behaviors and enhances resilience to stress, suggesting a potential antidepressant effect through modulation of the hypothalamic-pituitary-adrenal (HPA) axis and the limbic system. Furthermore, Y1 receptor agonists mimic NPY's anti-stress actions, pointing towards therapeutic strategies that could alleviate depressive symptoms by enhancing NPY signaling through Y1 receptors. In contrast, Y2 receptors have been associated with complex roles in depression, often exerting effects opposite to those of Y1 receptors. Preclinical evidence suggests that Y2 receptor antagonism enhances NPY release, thereby increasing its anxiolytic and antidepressant-like effects. This mechanism underscores the potential of Y2 receptor modulation as a novel approach for treating depression, potentially through augmentation of NPY's neuroprotective and stress-buffering properties. Moreover, studies exploring the interplay between NPY and its receptors have highlighted their interactions with other neurotransmitter systems implicated in depression, such as serotonin and dopamine, suggesting a broader impact on mood regulation beyond direct effects on stress responses.

The role of NPYRs extends beyond traditional neurotransmitter systems to encompass neurogenesis and neuroprotection, crucial processes disrupted in depression [168, 169]. Y2 and Y5 receptors have been linked to neuroprotective mechanisms that mitigate excitotoxicity and promote cell survival in models of neurological and psychiatric disorders. This neuroprotective role of NPYRs offers a promising avenue for developing treatments that not only alleviate depressive symptoms but also potentially halt or reverse neuronal damage associated with chronic stress and depression. Moreover, the ability of NPY to modulate synaptic plasticity and resilience in the face of stress underscores its therapeutic potential in depression, suggesting that targeting NPYRs could offer novel strategies for enhancing neuroadaptive processes critical for mood stability and emotional well-being.

4. GPCR heteroreceptor complexes in neuronal plasticity, learning and memory.

The role of GPCR heteroreceptor complexes in brain plasticity, learning, and memory has been extensively studied. Initially discussed in 1993[6], the connection between receptor-receptor interactions and neuronal plasticity underscores the

necessity of multiple concurrent events or signals for enduring changes in neuronal function, such as long-term potentiation (LTP) and long-term depression (LTD) [170]. Intramembrane receptor-receptor interactions act as coincidence detectors, making them plausible candidates for mediating these types of neuroplasticity.

For instance, in the context of LTD, D2 receptor (D2R) activity promotes LTD in striato-pallidal GABA neurons [170]. Interestingly, mGluR5 blockade counteracts this LTD despite its role in antagonizing D2R signaling through inhibitory receptor-receptor interactions in an extrasynaptic A2A-D2-mGluR5 heteroreceptor complex outside the glutamate synapse [171]. This blockade highlights the role of mGluR5 signaling in enhancing the production and release of endocannabinoids via extrasynaptic volume transmission (VT), thereby activating CB1 receptors on glutamate nerve terminals to inhibit glutamate release (reference 303). This mechanism illustrates the critical need for CB1-mediated endocannabinoid VT on glutamate nerve terminals to induce D2R-mediated LTD at glutamate synapses on striato-pallidal GABA neurons. In cortico-striatal synapses, spike-timing-dependent plasticity dictates whether LTD or LTP occurs, with presynaptic activity preceding postsynaptic spiking leading to LTD, and the reverse order inducing LTP [170]. Notably, the presence of a D2R agonist like quinpirole shifts the outcome towards LTD even with the LTP-inducing protocol, underscoring D2R's potent ability to counteract mechanisms favoring LTP and instead promoting LTD. This effect is achieved through various actions at striato-pallidal glutamate synapses, including antagonistic interactions between postsynaptic D2R and NMDA receptors within heteroreceptor complexes and inhibitory interactions between D2 and A2A receptors at the adenylate cyclase level [16, 172]. Of significant interest, co-administration of the A2AR agonist CGS21680 with quinpirole restores LTP at these glutamate synapses [170]. This restoration is attributed to antagonistic A2A-D2 receptor-receptor interactions in the extrasynaptic A2A-D2 heteroreceptor complex, which reduce D2R signaling [16, 44]. These findings underscore the pivotal role of heteroreceptor interactions in modulating neuroplasticity, shifting synaptic efficacy from LTD to LTP by reducing extrasynaptic D2R signaling and enhancing A2AR activity, which is crucial for NMDA receptor dependent LTP development. In models of Parkinson's disease (PD) characterized by low striatal dopamine levels, spike-timing-dependent protocols induce LTP in D2R-rich striato-pallidal GABA neurons, reflecting a shift in plasticity due to reduced D2R tone [170]. Again, A2AR activity plays a critical role, as LTP induction is blocked by A2AR antagonists. In this scenario, mechanisms may involve facilitatory interactions with alpha7 nicotinic receptors and A2AR homomer signaling via the AC-PKA cascade, enhancing glutamate release prejunctionally [172, 173]. The ligand for A2AR, adenosine, acts as a VT modulator originating from astroglial ATP metabolism, which underscores the role of VT signals in modulating

LTP and LTD in striato-pallidal GABA neurons. Thus, both receptor-receptor interactions and extrasynaptic VT significantly modulate bidirectional synaptic plasticity, crucial for Hebbian plasticity that coordinates presynaptic and postsynaptic activities precisely.

Regarding learning and memory, the formation of long-lived heteroreceptor complexes through intramembrane receptor-receptor interactions has been proposed as a potential mechanism [174]. Learning in neuronal networks likely involves instructions that alter synaptic weights (efficacies), possibly mediated by multiple interactions among plasma membrane receptors, forming higher order heteroreceptor complexes or receptor mosaics via oligomerization at pre- and post-junctional levels. These receptor assemblies, along with adapter proteins, G-proteins, and ion channels, form part of a complex molecular circuit on the plasma membrane, while the cytoplasmic part includes protein kinases, phosphatases, and phosphoproteins, capable of learning and storing information. The formation of engrams depends on resetting molecular circuits via the formation of new heteroreceptor complexes, facilitating the transduction of chemical messages through specific sets of G-proteins, ion channels, and other protein effectors. Short-term memory might involve transient stabilization of receptor mosaics, adjusting synaptic weights appropriately. Engram consolidation into long-term memory likely engages intracellular signals translocated to the nucleus, activating immediate early genes and forming adapter proteins that stabilize receptor mosaics as long-lived heteroreceptor complexes.

Changes in ERK signaling are crucial for long-term memory, influencing activity-dependent modifications of histones and epigenetic processes [175]. Transcriptional and epigenetic regulations participate in both Hebbian and non-Hebbian forms of plasticity, influencing learning and memory by forming and stabilizing molecular circuits with newly formed higher-order heteroreceptor complexes. These molecular changes, whether transient or long-lasting, can alter brain circuit output patterns, inducing transient and enduring changes in behaviors and cognitive functions. Structural plasticity in dendritic trees and spines, crucial for learning and memory, involves the recruitment of RTKs to novel heteroreceptor complexes, enhancing cell extensions and neurite densities [26, 29]. Additionally, A2ARs have been shown to recruit TrkB receptors to lipid rafts, suggesting a process of receptor heteromerization [176, 177]. The plasticity of presynaptic heteroreceptor complexes, activated by presynaptic and postsynaptic VT and synaptic signals, contributes significantly to learning new transmitter release patterns. This process is crucial for the transformation of short-term memories into long-lived heteroreceptor complexes and subsequently into long-term memories, involving local gene expression in nerve terminals [178]. These events underscore the role of prejunctional heteroreceptor complexes in regulating vesicular release and neurotransmitter modulation,

influenced by the pattern of action potentials reaching and depolarizing terminal membranes.

5. Enhancing Adult Hippocampal Neurogenesis for Therapeutic Applications in Neurological and Psychiatric Disorders through NPYR1 receptors.

Neurogenesis is a crucial aspect of neuronal plasticity, enabling the brain to reorganize its structure, function, and connections in response to both extrinsic and intrinsic stimuli. A fundamental process within this domain is adult hippocampal neurogenesis (AHN), where new neurons are continuously generated in the brain throughout adulthood [179-183]. The hippocampus, particularly the dentate gyrus (DG), serves as a neurogenic niche, playing a vital role in maintaining AHN under normal physiological conditions [184-186]. Despite its importance, the persistence of AHN in humans remains debated. Some studies suggest a decline in AHN with age, while others provide evidence supporting its continuation into late adulthood, emphasizing its significance in maintaining cognitive and emotional functions [180, 187-189].

The hippocampus is functionally divided, with the anterior portion (ventral in rodents) involved in stress and emotional behavior, and the posterior part (dorsal in rodents) associated with cognitive functions and memory [190]. Dysregulation of AHN is implicated in various neurological and psychiatric disorders, including major depressive disorder (MDD) [182, 183, 191, 192], age-related cognitive decline, Alzheimer's disease (AD) [187, 189], and other neurodegenerative diseases. MDD is a prevalent mental health condition affecting over 300 million people worldwide, characterized by a constellation of behavioral, emotional, and cognitive symptoms that significantly impact daily life and increase the risk of suicide, the most severe consequence of the disorder. The COVID-19 pandemic has exacerbated the prevalence of depression, with increased rates of loneliness and financial hardship contributing to a higher incidence of depressive symptoms and suicidal ideation [193].

Current antidepressants primarily target monoamines but often fall short due to adverse effects and delayed therapeutic onset [194-197]. Approximately 50% of patients do not respond to these treatments, and 65% fail to achieve remission, resulting in treatment-resistant depression (TRD). The advent of ketamine as a rapid-acting antidepressant has offered new hope, but its significant risks limit widespread use [195, 196, 198, 199]. This underscores the need for novel therapeutic approaches targeting different underlying mechanisms to improve treatment efficacy for MDD. Enhancing hippocampal neurogenesis emerges as a promising therapeutic strategy for MDD. Neurogenesis in the hippocampus involves cell proliferation, neuronal differentiation, and survival, regulated by various intrinsic and extrinsic factors. Neuropeptides such as neuropeptide Y (NPY) and galanin (GAL), along with

neurotrophic factors like brain-derived neurotrophic factor (BDNF), play crucial roles in modulating these processes [200-203].

NPY, a highly conserved neurotransmitter, has demonstrated proneurogenic effects in the hippocampus, with reduced levels observed in MDD models and patients [168, 169, 200, 204-207]. Antidepressant treatments typically increase brain NPY levels, highlighting its potential therapeutic relevance. Additionally, GAL, particularly through the GAL2 receptor (GALR2), has shown proliferative and neuroprotective effects in the hippocampus, suggesting its role in antidepressant mechanisms [166, 167, 208-210]. Previous studies have indicated a synergistic interaction between NPY and GAL through Y1R-GALR2 heteroreceptor complexes, which may mediate neurogenesis and antidepressant effects [166, 208]. However, invasive delivery methods like intracerebroventricular (icv) infusion limit their translational potential. Our recent work demonstrated that intranasal delivery of NPY and GAL agonists improves spatial memory and enhances cell proliferation in the hippocampus. The GAL 2/3 receptor agonist, GAL 2–11, has been shown to promote proliferation and trophism in progenitor cells. GAL's effects on memory are dose- and site-dependent, ranging from enhancing learning to having no effect or even inhibitory impacts [211]. GALR2 receptors mediate memory-enhancing and neuroprotective effects in Alzheimer's disease models. These findings offer promising therapeutic avenues for addressing age-related cognitive decline and early stages of cognitive impairment. By further exploring the effects of NPY1R and GALR2 agonists on neurogenesis and cognition, we aim to deepen our understanding of these complex biological processes.

AIMS

The extensive body of evidence supporting the existence of Neuropeptide Y receptor (NPYR) homo- and heteroreceptor complexes, alongside allosteric receptor-receptor interactions, has uncovered a novel frontier in molecular neuroscience within the Central Nervous System (CNS). These receptor complexes, through their heteromerization and allosteric interactions, introduce a new dimension to molecular neuroscience and brain function, representing a fundamental biological principle that integrates biological signals across various tissues. This dynamic interplay can lead to modifications in receptor recognition, the emergence of new allosteric binding sites, alterations in pharmacology, signalling pathways, and receptor trafficking, thereby shaping a diverse and biased signalling profile specific to each receptor heteromer.

Moreover, beyond NPYR-GPCR complexes, these receptor assemblies can incorporate ion channel receptors, receptor tyrosine kinases (RTKs), groups of G protein-interacting proteins, and transmitter transporters, thereby enhancing their integrative capabilities. The localization of homo- and heteroreceptor complexes on synaptic or extrasynaptic regions of the plasma membrane is governed by various factors, with the density of participating receptor protomers and their affinity playing critical roles. The presence or absence of adapter proteins within heteroreceptor complexes significantly influences the affinity developed among receptor protomers.

The widespread distribution of heteroreceptor complexes, characterized by allosteric receptor-receptor interactions in the CNS, presents a pioneering integrative molecular mechanism within neuronal and glial cell plasma membranes. It is hypothesized that the molecular mechanism underlying learning and memory involves the reorganization of existing higher-order heteroreceptor complexes (including GPCRs) and the resetting of multiple allosteric receptor-receptor interactions within these complexes. Additionally, novel heteroreceptor complexes may form due to alterations in the patterns of synaptic and volume transmission signals. These molecular adjustments within heteromers, including changes in receptor-protein architecture on the postsynaptic membrane, potentially constitute the basis for short- and long-term memory.

Therefore, the overarching aim to advance our understanding of NPY1R heteroreceptor complexes and their allosteric receptor-receptor interactions within the CNS, with a specific focus on their roles in hippocampal neuronal cells. Our research emphasizes the importance of adult neurogenesis under physiological conditions and

the regulatory role of neuropeptides, specifically NPY and GAL. By further exploring the effects of NPY1R and GALR2 agonists on neurogenesis and cognition, we aim to deepen our understanding of these complex biological processes. By delving into these complexes and their integration of neuronal signals, this research aimed to enhance our understanding of their involvement in various mental and neurological disorders. Our findings may offer promising therapeutic avenues for addressing age-related cognitive decline and early stages of cognitive impairment and depression.

The following specific aims were considered:

AIM-1: To investigate whether intranasal administration of GALR2 and NPY1R agonists stimulates adult neurogenesis in the ventral hippocampus and induces antidepressant-like effects. This included assessing ventral hippocampal activation and proliferation through c-Fos expression and PCNA, identifying specific proliferating cell subpopulations using double immunolabeling, examining BDNF expression in the ventral hippocampal dentate gyrus, analysing NPY1R–GALR2 heteroreceptor complex formation via in situ proximity ligation assays (PLA), studying morphological changes in hippocampal neurons, and evaluating functional outcomes in the ventral hippocampus using the forced swimming test (FST), with a focus on BDNF's role.

AIM-2: To explore the influence of GAL on hippocampal neurogenesis, including its dose- and site-dependent effects on memory and its implications for Alzheimer's disease models. The aim also involved investigating interactions between NPY and GAL through NPY1R-GALR2 heteroreceptor complexes in various brain regions and assessing their potential therapeutic implications for age-related cognitive decline. This included evaluating spatial memory, neuronal survival, and differentiation in adult rats following intranasal co-administration of GALR2 and NPY1R agonists, highlighting GALR2's significant role in these processes.

MATERIALES AND METHODS

I- RELATED TO SPECIFIC AIM 1 (see, Chapter 1)

GALR2 and Y1R agonists intranasal infusion enhanced adult ventral hippocampal neurogenesis and antidepressant-like effects involving BDNF actions.

Animals. Male Sprague–Dawley rats (200–250 g, aged 6–8 weeks) were obtained from CRIFFA. They had unrestricted access to food pellets and tap water and were kept under a standard 12-hour light/dark cycle, with the temperature controlled at 22 ± 2°C and humidity maintained at 55%–60%. The protocols for housing, maintenance, and experimental procedures were approved by the Local Animal Ethics, Care, and Use Committee of the University of Málaga, Spain, and complied with EU Directive 2010/63/EU and Spanish Directive (Real Decretory 53/2013).

Drugs Used. All solutions were freshly prepared using distilled water. GAL receptor 2 agonists (M1145), Y1R receptor agonist [Leu31, Pro34] NPY, GALR2 antagonist M871 were sourced from Tocris Bioscience, and the TrkB antagonist (ANA - 12, 5.06304) was acquired from Sigma - Aldrich. Detailed descriptions are provided in the Supporting Information: Material on intranasal infusion of solutions.

Intranasal administration of peptides. Galanin receptor 2 agonist (M1145), Y1R receptor agonist [Leu^{31}, Pro^{34}] NPY, GALR2 Antagonist M871 (Tocris Bioscience, Bristol, UK) were freshly dissolved in 20 µl distilled water. Each rat received 10 µl of them into each nostril with pipetteman and disposable plastic tip (1 mm in diameter) inserted no deeper than 1–1.5 mm into the nostril under light isoflurane anesthesia. Following the infusion, the head of the animal was held in a tilted back position for approximately 15 s to prevent loss of solution from the nares.

Assessment of Ventral Hippocampus Activation after Intranasal Infusion. Rats were randomly divided into five experimental groups: (1) Control group receiving distilled water; (2) M1145-treated group (132 µg); (3) Y1R agonist-treated group with [Leu31–Pro34]NPY (132 µg); (4) M1145 + Y1R group receiving both substances; (5) M1145 + Y1R + M871 group treated with M1145, [Leu31–Pro34]NPY, and the GALR2 antagonist M871 (132 µg) (N = 4 per group). Doses were based on previously published protocols [169]. Twenty-four hours post-

administration, the rats were deeply anesthetized with pentobarbital (mebumal, 100 mg/kg, ip) and perfused transcardially with 4% paraformaldehyde (wt/vol; Sigma‐Aldrich). Using a Cryostat (HM550; Microm International), the brains were coronally sectioned (30 μm thick) through the ventral hippocampus (−5.20 to −6.72 Bregma). We employed c-Fos immunohistochemistry as an indirect marker of neuronal activation. Free-floating sections were incubated for antigen retrieval at 65°C for 90 minutes in saline sodium citrate buffer (pH 6; 10 nM sodium citrate). After blocking endogenous peroxidases with 0.6% H2O2 for 30 minutes, the sections were incubated overnight at 4°C with a primary antibody mouse anti-c-Fos protein (1:800, sc-271243; Santa Cruz Biotechnology) in 2.5% donkey serum. After several washes with phosphate-buffered saline (PBS), the sections were incubated with a secondary antibody for 90 minutes (biotinylated anti-mouse immunoglobulin G [IgG], 1:300, B8520; Sigma‐Aldrich). ExtrAvidin peroxidase (1:100; Sigma‐Aldrich) was used to amplify the signal for 1 hour at room temperature (RT) in the dark. Detection was performed with 0.05% diaminobenzidine (DAB; Sigma‐Aldrich) and 0.03% H2O2 in PBS. After washing, the sections were mounted on gelatin-coated slides, dehydrated in graded alcohols, and coverslipped with DePeX mounting medium (Merck Life Science S.L.U.). C-Fos-labeled cells were analyzed using the optical fractionator method with unbiased stereological microscopy (Olympus BX51 Microscope), as previously described [168, 169]. (see Supporting Information: Materials for details).

Evaluation of Ventral Hippocampal Cell Proliferation and BDNF Induction. Different free-floating sections underwent antigen retrieval at 65°C for 90 minutes in saline sodium citrate buffer (pH 6; 10 nM sodium citrate). Endogenous peroxidases were blocked with 0.6% H2O2 for 30 minutes. One set of sections was incubated overnight at RT with a primary antibody mouse anti-PCNA (1:1500, P8825; Sigma‐Aldrich), and another set with mouse anti-BDNF (Abcam; ab205067, 1:500) in 2.5% donkey serum. After several PBS washes, the sections were incubated with a secondary antibody for 90 minutes (biotinylated anti-mouse IgG, 1:200, B8520; Sigma‐Aldrich). ExtrAvidin peroxidase (1:100; Sigma‐Aldrich) was used to amplify the signal for 1 hour at RT in the dark. Detection was done using 0.05% DAB (Sigma‐Aldrich) and 0.03% H2O2 in PBS. After washing, sections were mounted on gelatin-coated slides, dehydrated, and coverslipped with DePeX mounting medium (Merck Life Science S.L.U.). PCNA and BDNF-labeled cells were analyzed using the optical fractionator method with unbiased stereological microscopy (Olympus BX51 Microscope). For studying specific cell subpopulations, double immunolabeling was performed. The procedures for double immunohistochemistry followed previously described methods (Narváez et al.,

2016). PCNA immunostaining was conducted as described above and revealed with DAB plus 0.03% nickel (Sigma‐Aldrich) for a black-purple reaction. For the second primary antibody, rabbit anti-doublecortin (DCX; Abcam; ab18723, 1:2000) or rabbit anti-glial fibrillary acidic protein (GFAP; Abcam; ab7260, 1:1500), the chromogen used was DAB for a brownish reaction.

Hippocampal Cell Culture and Conditions. Rat primary hippocampal neurons were obtained from QBM Cell Science and cultured in Neurobasal medium with 10% fetal bovine serum, 2 mM GlutaMAX-1, 1 mM sodium pyruvate, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 2% B-27 supplement at 37°C in a humidified 10% CO2 environment, following the manufacturer's instructions. Half of the medium was changed every three days. Neurons were cultured under these conditions (control condition) for seven days before being treated for 24 hours under specific pharmacologic conditions. Experimental groups included: (1) Control; (2) M1145-treated group (100 nM); (3) NPY1R agonist-treated group with [Leu31, Pro34] NPY (100 nM); (4) GAL + NPY1R group receiving both substances; (5) GAL + Y1 + M871 group treated with GAL, [Leu31, Pro34]NPY, and the GALR2 antagonist M871 (1 µM). Cells were grown on poly-D-lysine-coated glass coverslips and fixed with 4% formaldehyde solution for 20 minutes, followed by two PBS washes with 20 mM glycine to quench the aldehyde groups.

In Situ PLA and Analysis of Neurite Length. To study GALR2-NPY1R heteroreceptor complexes, in situ PLA (proximity ligation assay) was performed as previously described [27, 117, 212]. After permeabilization with PBS containing 0.2% Triton X-100 for 5 minutes, cells were blocked with PBS containing 1% bovine serum albumin. Hippocampal cells were then incubated overnight at 4°C with primary antibodies diluted in blocking solution. Following washes, the proximity probe mixture (Duolink PLA probe anti-goat MINUS and Duolink PLA probe anti-rabbit PLUS; Sigma‐Aldrich) was applied and incubated for 1 hour at 37° C. Unbound proximity probes were removed by washing twice with blocking solution, and the samples were incubated with the hybridization-ligation solution (bovine serum albumin, 250 g/ml; T4 DNA ligase, final concentration of 0.05 U/µl; 0.05% Tween-20; 250 mM NaCl; 1 mM ATP; and circularization or connector oligonucleotides, 125–250 nM) for 30 minutes at 37°C. Excess connector oligonucleotides were removed by washing with washing buffer A (Sigma‐Aldrich; Duolink Buffer A) twice for 5 minutes each at room temperature under gentle agitation. The rolling circle amplification mixture (Duolink amplification red, DUO82011; Sigma‐Aldrich) was then added and incubated in a humidity chamber at 37° C for 100 minutes. Detection was carried out with the detection solution for 30 minutes at 37° C in a humidity chamber. Cells were washed twice with washing

buffer B (Sigma‐Aldrich; Duolink Buffer B) for 10 minutes each, followed by a final wash in 0.01X washing buffer B for 1 minute. Coverslips were mounted with Duolink In Situ Mounting Medium with DAPI (Sigma‐Aldrich). Fluorescent signals were visualized using an epifluorescence microscope (Olympus BX51) equipped with appropriate filters. Neurite length was measured using ImageJ (National Institutes of Health, USA) with the NeuronJ plugin. The results were expressed as the mean ± standard error of the mean (SEM) of three independent experiments.

Statistical Analysis. Data were analyzed using SPSS software version 25.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Differences among groups were assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test when necessary. Data from in situ PLA experiments showing cluster density (clusters per nucleus per sampled field) were analyzed using a one-way ANOVA followed by post-hoc Tukey's test. The number of rats (n) in each experimental condition is indicated in figure legends. Data are presented as mean ± SEM, and statistical significance was set at $P < 0.05$.

II- RELATED TO SPECIFIC AIM 2 (see, Chapter 2)

Enhancement of neurogenesis and cognition through intranasal co-delivery of galanin receptor 2 (GALR2) and neuropeptide Y receptor 1 (NPY1R) agonists: a potential pharmacological strategy for cognitive dysfunctions.

Animals. Male Sprague–Dawley rats, 6–8 weeks old and weighing between 200-250 g, were obtained from CRIFFA (Barcelona). The rats had free access to food and water and were maintained under a standard 12-hour dark/light cycle, with controlled relative humidity (55–60%) and temperature (22 ± 2 °C). All experimental protocols were approved by the Local Animal Ethics, Care, and Use Committee for the University of Málaga, Spain (CEUMA 45-2022-A), and conducted in accordance with EU Directive 2010/63/EU and Spanish Directive (Real Decretory 53/2013).

Drugs Used. The peptides used were freshly prepared in distilled water, which served as the control. Galanin receptor 2 agonist (M1145), NPY1R receptor agonist [Leu31, Pro34] NPY, and GALR2 Antagonist M871 were procured from Tocris

Bioscience (Bristol, UK). Peptides were administered once daily for a three-day duration, in line with the methods previously described [46, 47].

Intranasal administration of peptides. Galanin receptor 2 agonist (M1145), Y1R receptor agonist [Leu^{31}, Pro^{34}] NPY, GALR2 Antagonist M871 (Tocris Bioscience, Bristol, UK) were freshly dissolved in 20 µl distilled water. Each rat received 10 µl of them into each nostril with pipetteman and disposable plastic tip (1 mm in diameter) inserted no deeper than 1–1.5 mm into the nostril under light isoflurane anesthesia. Following the infusion, the head of the animal was held in a tilted back position for approximately 15 s to prevent loss of solution from the nares.

Behavioral Analysis (Assessment of Spatial Memory in Rats). To assess spatial hippocampal memory, we employed the object-in-place task, which is based on spontaneous object exploration behaviors. The advantage of this task over the Morris water maze task is its lesser stress on the rodents, which can interfere with learning and memory performance. Peptides were freshly prepared and administered intranasally three weeks prior to the testing phase (20 µl total volume). Animals were randomly divided into five groups: (1) Control: distilled water; (2) M1145-treated group (132 µg); (3) NPY1R agonist-treated group receiving the NPY1R agonist [Leu31, Pro34]NPY (132 µg); (4) M1145 + NPY1R: group administered with both substances; (5) M1145 + NPY1R + M871: group treated with M1145, [Leu31, Pro34]NPY and the GALR2 antagonist (M871; 132 µg) (n = 6 in each group). Dosage selection was based on prior research [165]. The object-in-place task trials were structured into three phases: habituation, training, and testing.

Evaluation of Hippocampal Cell Survival. A different cohort of rats was used to examine BrdU-positive cells. Two injections of 5′ -Bromo-2′ -deoxyuridine (BrdU, cat. no. B5002, Sigma, St. Louis, MO, USA) dissolved at 15 mg/mL in a sterile 0.9% NaCl solution were administered intraperitoneally (i.p.) during the ad libitum feeding period at a dose of 50 mg/kg body weight (every 2 hours over three days, starting at 9:00 AM). The procedures used were based on previously published protocols. Three weeks after the intranasal infusion, rats were deeply anesthetized with pentobarbital (Mebumal, 100 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde (PFA; 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 dorsal hippocampus (−1.60 to −5.30 Bregma; Paxinos and Watson, 1998). These procedures are based on previously published protocols [165, 168, 169]. Rats were randomly distributed into five experimental groups: (1) Control: distilled water; (2) NPY1R agonist-treated group receiving the NPY1R agonist [Leu31, Pro34]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, [Leu31, Pro34]NPY and the GALR2 antagonist (M871; 132 μg) (n = 4 in each group).

Immunohistochemistry. Brain sections were incubated free-floating in saline sodium citrate buffer (pH 6; 10 nM sodium citrate) for 90 minutes at 65 °C, followed by 30 minutes with 0.6% H2O2 to remove endogenous peroxidases. After 30 minutes in 2 M hydrochloric acid (HCl) to denature deoxyribonucleic acid (DNA), sections were incubated for neutralization with 0.1 M sodium borate (pH 8). Then, slices were incubated at 4°C overnight with a primary antibody against BrdU (Abcam, ab152095, 1:1000) in 2.5% donkey serum. Following additional washes with PBS and incubation with a secondary antibody for 90 minutes (biotinylated anti-rabbit IgG, 1:200, Vector Laboratories), sections were amplified with ExtrAvidin peroxidase (Sigma, St. Louis, MO, USA) diluted 1:100 in darkness at room temperature for one hour. Immunolabeling was exposed with 0.05% diaminobenzidine (DAB; Sigma) and 0.03% H2O2 in PBS. After various washes, sections were mounted on gelatin-coated slides, dehydrated in graded alcohols, and cover-slipped in DePeX mounting medium (Merck Life Science SLU, Darmstadt, Germany). BrdU-labelled cells with morphological characteristics of glial precursors, i.e., small, irregularly shaped cell bodies, were excluded. Only round, regularly shaped BrdU-positive nuclei located in the dentate gyrus (DG) were counted, as new granule cells migrate approximately two cell body widths from the subgranular zone (SGZ) into the granule layer. As previously described, BrdU-labelled cells were analyzed using the optical fractionator method in unbiased stereological microscopy (Olympus BX51 Microscope, Olympus, Denmark).

Double Immunofluorescence. To study the fate of these newly generated cells, we performed double immunolabeling. Procedures for immunofluorescence were previously described [34, 35, 37–39]. Briefly, an initial incubation with blocking (5% goat serum) and permeabilization (0.3% Triton X-100 in PBS) solutions was performed for 60 minutes each. Pairs of primary antibodies rabbit anti-BrdU (Abcam, ab152095, 1:1000)/mouse anti-DCX (C-18, Santa Cruz, 1:500) or rabbit anti-BrdU (Abcam, ab152095, 1:1000)/mouse anti-NeuN (Abcam, ab1042241, 1:1000) were used to incubate the sections for 24 hours at 4 °C. Then, incubations were performed with appropriate secondary antibodies: Donkey anti-mouse AlexaFluor 488 (Abcam, ab150105, 1:200) and Donkey anti-rabbit AlexaFluor 647 (Abcam, ab150075, 1:200). Sections were mounted on slides with a fluorescent mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) to detect nuclei (Abcam, ab104139). BrdU/DCX- and BrdU/NeuN double-stained cells in the DG were quantified using z-scan confocal microscopy (Leica Stellaris 8) at 40× magnification. The entire length of the DG was assessed through the septo-temporal axis of the hippocampus, analyzing at least four representative 150 μm, evenly spaced sections per animal. All

analyses were conducted in sequential scanning mode to eliminate potential cross-bleeding between channels. Each cell was scrutinized using a multi-channel configuration, and colocalization with NeuN or GFAP was verified by examining multiple optical planes for each cell on the z-axis. Z-stacks were generated at 0.85 μm intervals throughout the 30 μm section to ensure accurate double-labeling of BrdU-IR cells.

Counting Procedure. BrdU and DCX-labeled cells were counted with an Olympus BX51 microscope, Olympus, Denmark interfaced with a computer and a colour JVC digital video camera. For stereological analysis, sampling of positive cells was performed throughout the dentate gyrus of the dorsal hippocampus in the rostrocaudal dimension using the optical fractionator, according to Paxinos & Watson atlas coordinates (Paxinos & Watson, 2006). This method combines the optical dissector with a fractionator sampling scheme to exclude volume divergences (Gundersen et al., 1988). Counterstaining with phase contrast allowed delineation of different areas in each section (Paxinos and Watson, 2006). Numbers of positive cells were quantified in at least six representative 150 μm, evenly spaced sections per animal (4 rats per group). A random set of sampling frames with a known area (α frame) was generated for each section using the C.A.S.T. Grid (Olympus; Albertslund, Denmark). After the objects were counted (ΣQ-) the total number of positive cells were estimated as: N = ΣQ- x fs x fa x fh, where fs is the numerical fraction of the section used, fa is the areal fraction and fh is the linear fraction of section thickness. The coefficient of error (CE) for each estimation and animal ranged from 0.05 to 0.1. The total CE of each group ranged from 0.07 to 0.08. Counting of labelled cells was set starting at 5 μm below the surface and focusing through the 20 μm section optical plane, and the number of counting frames used was 90-110 per animal. We have used this stereological procedure is previous studies [168, 169, 213].

Assessment of Hippocampal Doublecortin (DCX)-Labelled Newborn Neurons. In adult hippocampal neurogenesis, newly generated neuronal cells undergo a phase marked by transient DCX expression, which is evident during the initial stages of neuronal differentiation. This aligns with the understanding that DCX expression in adult hippocampal neurogenesis is confined to the neuronal lineage. Therefore, the assessment of DCX-positive cells can serve as an indicator of the neurogenic effects induced by a specific treatment [57, 58]. The procedure was performed as described for BrdU immunohistochemistry. Briefly, different free-floating sections were incubated for antigen retrieval at 65 °C for 90 minutes in saline sodium citrate buffer (pH 6; 10 nM sodium citrate). After this procedure to remove endogenous peroxidases, the slices were treated for 30 minutes in 0.6% H2O2. Then, a set of slices were incubated at room temperature overnight with a

primary antibody rabbit anti-DCX (Abcam, ab18723, 1:2000) in 2.5% donkey serum. After several washes with PBS, the slices were incubated with a secondary antibody for 90 minutes (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% H2O2 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). DCX-labeled cells were studied using the optical fractionator method in unbiased stereological microscopy (Olympus BX51 Microscope, Olympus, Denmark), as described above.

Categorization of Dendritic Morphology of DCX-Positive Cells. We categorized DCX-expressing cells into three categories according to the presence and the shape of apical dendrites and their presumed sequential order. Category "proliferative stage" were cells without dendrites or very short processes, less than one nucleus-wide (<10 μm). In category "Intermediate stage," the process was longer, reaching into the granule cell layer and even into the molecular layer. For "Postmitotic stage," cells acquire a more mature appearance. One thick dendrite reached into the molecular layer and showed comparatively sparse branching in the molecular layer; or the dendritic tree showed delicate branching and few major branches either near the soma or within the granule cell layer. To categorize the DCX-positive cells into the three distinct morphological subtypes outlined in our study, for each animal, a sample of 50 DCX-labeled cells was examined. The identified cells were then grouped based on their morphological characteristics, and the results were subsequently presented as the percentage of each subtype within the sampled population.

Statistical Analysis. Data are presented as mean ± SEM, and the sample number (n) is indicated in figure legends. GraphPad PRISM 8.0 (GraphPad Software, La Jolla, CA, USA) was used to analyse all data. One-way analysis of variance (ANOVA) followed by the Newman-Keuls comparison post-test was performed. To analyse the effects of different treatments (Factor A) and the dendrite categories (Factor B), two-way ANOVAs followed by Student—Newman Keuls post-hoc test were conducted. For comparing two experimental conditions, Student's unpaired t-test statistical analysis was achieved. Differences were considered significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

CHPATER 1
Results and Discussion

SPECIFIC AIM 1. *GALR2 and NPY1R agonists intranasal infusion enhanced adult ventral hippocampal neurogenesis and antidepressant-like effects involving BDNF actions.*

1.1. Background

Neurogenesis is a fundamental aspect of neuronal plasticity, the brain's ability to reorganize its structure, function, and connections in response to various stimuli, both extrinsic and intrinsic [192]. A critical process within this domain is adult hippocampal neurogenesis (AHN), where new neurons are generated in the brain throughout adulthood. The hippocampus, particularly the dentate gyrus (DG), serves as a neurogenic niche, playing a vital role in maintaining AHN under normal physiological conditions [188, 214]. Despite its importance, the persistence of AHN in humans remains a topic of debate. Some studies suggest that AHN declines with age [187, 215], while others provide evidence supporting its continuation into late adulthood, emphasizing its significance in maintaining cognitive and emotional functions [216, 217].

The hippocampus is functionally divided, with the anterior portion (ventral in rodents) involved in stress and emotional behavior, and the posterior part (dorsal in rodents) associated with cognitive functions and memory [183, 190]. Dysregulation of AHN is implicated in various neurological and psychiatric disorders, including major depressive disorder (MDD), age-related cognitive decline, Alzheimer's disease (AD), and other neurodegenerative diseases [189, 218].

MDD is a prevalent mental health condition affecting over 300 million people worldwide, characterized by a constellation of behavioral, emotional, and cognitive symptoms that significantly impact daily life and increase the risk of suicide, the most severe consequence of the disorder [194]. The COVID-19 pandemic has exacerbated the prevalence of depression, with increased rates of loneliness and financial hardship contributing to a higher incidence of depressive symptoms and suicidal ideation.

Current antidepressants primarily target monoamines but often fall short due to adverse effects and delayed therapeutic onset [197]. Approximately 50% of patients do not respond to these treatments, and 65% fail to achieve remission, resulting in

treatment-resistant depression (TRD). The advent of ketamine as a rapid-acting antidepressant has offered new hope, but its significant risks limit widespread use [196]. This underscores the need for novel therapeutic approaches targeting different underlying mechanisms to improve treatment efficacy for MDD [219].

Enhancing hippocampal neurogenesis emerges as a promising therapeutic strategy for MDD. Neurogenesis in the hippocampus involves cell proliferation, neuronal differentiation, and survival, regulated by various intrinsic and extrinsic factors [188]. Neuropeptides such as neuropeptide Y (NPY) and galanin (GAL), along with neurotrophic factors like brain-derived neurotrophic factor (BDNF), play crucial roles in modulating these processes [200, 220]. NPY, a highly conserved neurotransmitter, has demonstrated proneurogenic effects in the hippocampus, with reduced levels observed in MDD models and patients. Antidepressant treatments typically increase brain NPY levels, highlighting its potential therapeutic relevance [221]. Additionally, GAL, particularly through the GAL2 receptor (GALR2), has shown proliferative and neuroprotective effects in the hippocampus, suggesting its role in antidepressant mechanisms [211].

Previous studies have indicated a synergistic interaction between NPY and GAL through Y1R-GALR2 heteroreceptor complexes, which may mediate neurogenesis and antidepressant effects [169]. However, invasive delivery methods like intracerebroventricular (icv) infusion limit their translational potential. Our recent work demonstrated that intranasal delivery of NPY and GAL agonists improves spatial memory and enhances cell proliferation in the hippocampus [169].

This study aims to test the hypothesis that intranasal administration of GALR2 and Y1R agonists stimulates neurogenesis in the ventral hippocampus and exerts antidepressant-like effects. We assessed hippocampal activation, cell proliferation, and the specific cell populations involved through c-Fos and PCNA expression. Additionally, we examined the role of BDNF and the formation of Y1R-GALR2 heteroreceptor complexes in these processes. The functional outcome of NPY and GAL interactions in the ventral hippocampus was evaluated using the forced swimming test (FST), providing insights into the potential therapeutic benefits of non-invasive intranasal delivery for treating MDD.

1. 2. Results

Activation of the Ventral Dentate Gyrus Following Intranasal Administration of GALR2 and Y1R Agonists

To evaluate the intranasal delivery efficacy of GALR2 and NPY1R agonists across the blood-brain barrier, we measured c-fos expression, a marker of neuronal activation, in the ventral dentate gyrus (DG). Intranasal administration of the NPY1R

agonist significantly increased the number of c-fos-immunoreactive (IR) cells in the granular layer of the ventral DG (one-way ANOVA, $F(4, 15) = 12.04$, $p < 0.001$; Newman-Keuls post hoc test: $p < 0.05$) (**Figure 1a, b**) compared to the control group. In contrast, intranasal delivery of the GALR2 agonist M1145 alone did not affect the number of c-fos-positive cells (**Figure 1b, c**). Combined intranasal administration of M1145 and the Y1R agonist resulted in a significant increase in c-fos-IR cells in the granular region of the ventral DG compared to M1145 alone and control groups (Newman-Keuls post hoc test: $p < 0.001$) and compared to the Y1R agonist alone group (Newman-Keuls post hoc test: $p < 0.05$) (Figure 1b, d). This effect was specifically blocked by co-treatment with the GALR2 antagonist M871 (Newman-Keuls post hoc test: $p < 0.05$) (**Figure 1b**), indicating that GALR2 is involved in the interaction between NPY1R and M1145 agonists in stimulating c-fos induction.

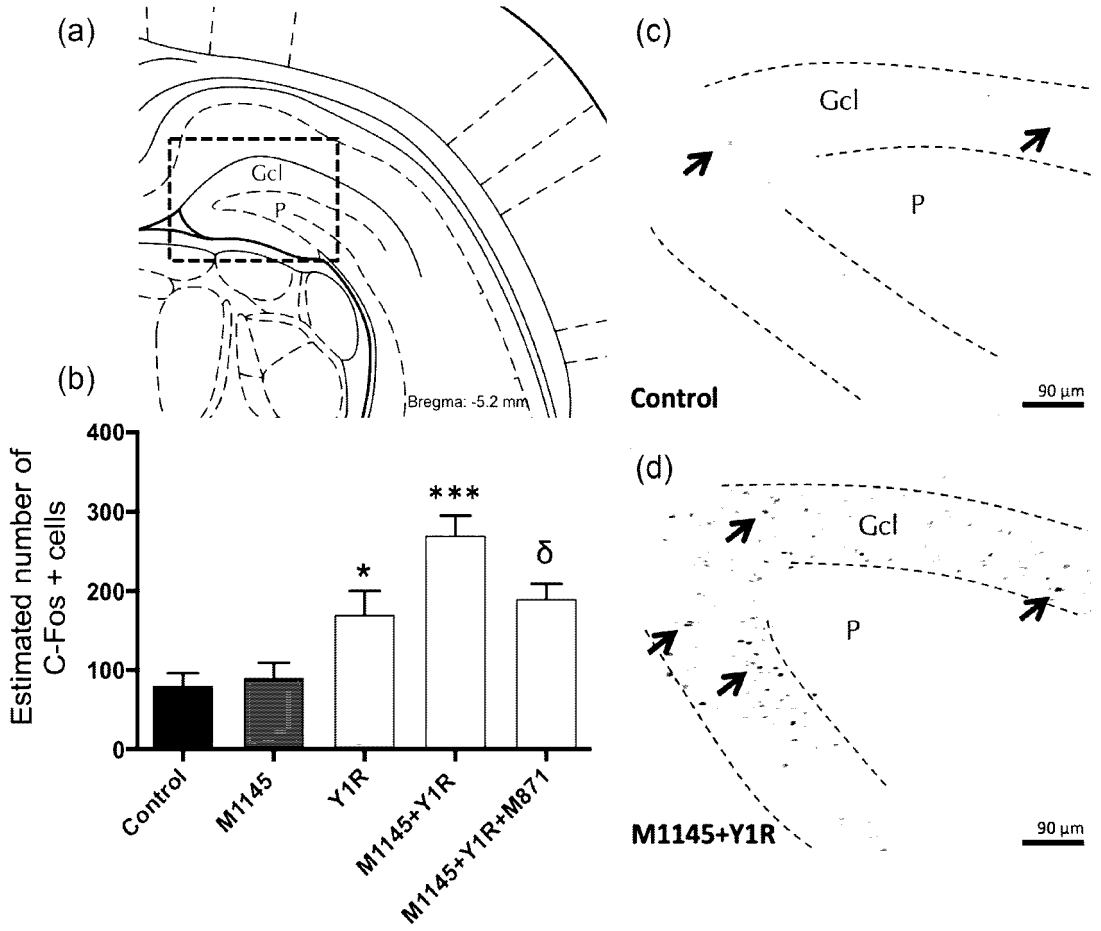


Figure 1: Activation of Ventral Dentate Gyrus by GALR2 and Y1R Agonists via Intranasal Delivery. *Effects of intranasally administered GALR2 agonist (M1145) and Y1R receptor agonist ([Leu31-Pro34]NPY), alone or in combination with or without GALR2 antagonist (M871), on c-Fos expression in the granular layer (Gcl) of the ventral dentate gyrus. (a, d) Immunoreactivity (IR) for c-Fos primarily observed in the Gcl. The polymorphic layer (P) of the ventral hippocampus is indicated (Bregma: −5.6 mm; Paxinos and Watson, 2006). (b) Quantification of c-Fos-IR nuclei in the dentate*

*gyrus. Data are presented as mean ± SEM, showing differences between groups after administration of control, M1145, Y1R agonist, or their coadministration with or without M871. *p < 0.05 versus control, M1145, and M1145 + Y1R; δp < 0.05 versus M1145 + Y1R; ***p < 0.001 versus control and M1145, by one-way ANOVA followed by Newman–Keuls post hoc test (n = 4/group). Vertical lines indicate intergroup comparisons above bars. (d) Increased c-Fos-IR nuclei in Gcl of dentate gyrus after coadministration of M1145 and Y1R agonist compared to control (c). Arrows indicate examples of c-Fos-IR nuclei. Dashed lines outline the Gcl of the dentate gyrus. Control, distilled water; M1145, GALR2 agonist 132 µg; M1145 + Y1R, coadministration of M1145 and Y1R; M1145 + Y1R + M871, coadministration of M1145, Y1R, and GALR2 antagonist M871 132 µg; Y1R, Y1R agonist [Leu31-Pro34]NPY 132 µg.*

Increased Neuroblast Proliferation in the Ventral Hippocampus Following Intranasal Administration of GALR2 and Y1R Agonists

To assess the impact of intranasally co-administered GALR2 agonist M1145 and Y1R agonist on adult ventral hippocampal cell proliferation, we utilized proliferating cell nuclear antigen (PCNA) as a marker. The combination of M1145 and the Y1R agonist significantly increased cell proliferation, indicated by the number of PCNA-IR cells in the subgranular zone (Sgz) of the DG, compared to control (one-way ANOVA, $F(4, 15) = 12.38$, $p < 0.001$; Newman-Keuls post hoc test: $p < 0.001$) (**Figure 2a, b, d**), M1145 alone (Newman-Keuls post hoc test: $p < 0.001$), and Y1R agonist alone groups (Newman-Keuls post hoc test: $p < 0.05$) (**Figure 2a, b, d**). The presence of GALR2 antagonist M871 completely blocked the proliferative effects of M1145 and Y1R agonist co-administration in the DG (Newman-Keuls post hoc test: $p < 0.01$) (**Figure 2b**), validating GALR2's role in mediating this interaction.

Intranasal administration of the NPY1R agonist alone increased the number of PCNA-positive cells in the Sgz of the ventral hippocampus (**Figure 2a, b**) compared to control and M1145 groups (Newman-Keuls post hoc test: $p < 0.05$) (Figure 2a, b). However, M1145 alone did not affect the number of PCNA-IR profiles (Figure 2b) compared to control (**Figure 2a–c**). We further identified the specific cell types affected by the co-administration by quantifying PCNA-labeled cells co-expressing either DCX (neuroblasts) or GFAP (quiescent radial stem cells) (**Figure 2e**). The number of PCNA+/DCX+ cells increased significantly after the intranasal infusion of M1145 and the Y1R agonist compared to the control group ($t = 6.063$, $df = 6$, $p < 0.001$) (**Figure 2f**). No significant change was observed in the number of PCNA+/GFAP+ cells in M1145-Y1R-treated animals compared to control ($t = 1.192$, $df = 6$, $p = 0.28$), indicating selective stimulation of neuroblast proliferation without affecting quiescent radial stem cells.

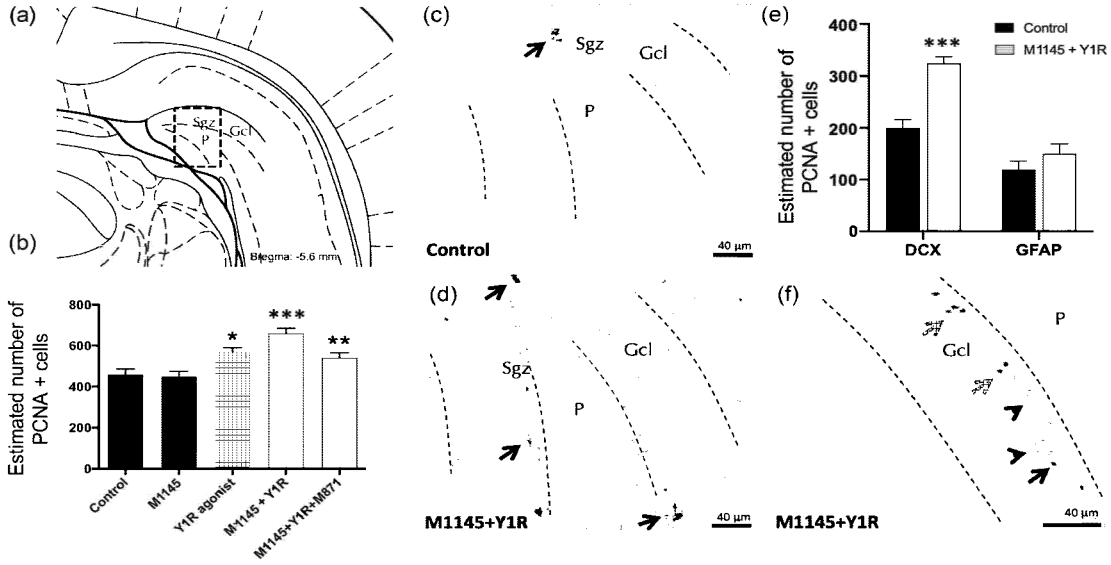


Figure 2: Increased Cell Proliferation in Ventral Dentate Gyrus by GALR2 and Y1R Agonists via Intranasal Delivery. *Proliferating cell nuclear antigen (PCNA) immunolabeling in the dentate gyrus of the ventral hippocampus following intranasal administration of GALR2 agonist (M1145) and Y1R receptor agonist, alone or in combination with or without GALR2 antagonist (M871). (a, d) PCNA-positive cells predominantly located in the subgranular zone (Sgz) at the Gcl and P border in the ventral hippocampus. Cells often appeared in clusters of 3–4 cells (Bregma: −5.6 mm; Paxinos and Watson, 2006). (b) Quantification of total PCNA-IR cells in the dentate gyrus. Data represent mean ± SEM, illustrating differences between groups after administration of control, M1145, Y1R agonist, or their coadministration with or without M871. *p < 0.05 versus control, M1145, and M1145 + Y1R; **p < 0.01 versus M1145 + Y1R; ***p < 0.001 versus control and M1145, by one-way ANOVA followed by Newman–Keuls post hoc test (n = 4/group). Vertical lines indicate intergroup comparisons above bars. (d) Increased PCNA immunolabeling in Sgz of dentate gyrus after coadministration of M1145 and Y1R agonist compared to control (c). Arrows indicate clusters of PCNA-positive cells. Dashed lines outline the Gcl of the dentate gyrus. (e) Quantification of PCNA-IR cells double-labeled with DCX or GFAP showed specificity of Y1R-GALR2 action on neuroblasts. Data represent mean ± SEM. ***p < 0.001 versus control, by Student's unpaired t-test. Representative photomicrograph (f) illustrating DCX+/PCNA+ cells (red arrows), DCX-/PCNA+ cells (red arrows), and DCX+/PCNA- cells (arrowheads) in M1145 and Y1R agonist group. Control, distilled water; DCX, doublecortin; GFAP, glial fibrillary acidic protein; IR, immunoreactivity; M1145, GALR2 agonist 132 µg; M1145 + Y1R, coadministration of M1145 and Y1R; M1145 + Y1R + M871, coadministration of M1145, Y1R, and GALR2 antagonist M871 132 µg; Y1R, Y1R agonist [Leu31-Pro34] NPY 132 µg.*

Enhanced Cell Proliferation Correlates with Increased BDNF Expression Following GALR2 and Y1R Agonist Coactivation

To investigate the cellular mechanisms underlying the observed proliferation, we analyzed BDNF expression in the ventral hippocampal DG following intranasal administration of M1145 and/or Y1R agonist. BDNF-positive cells were predominantly located in the Sgz of the ventral hippocampus, with some scattered cells in the polymorphic layer (P) (Figure 3a). Stereological quantification revealed a

significant increase in BDNF-positive cells after co-injection of M1145 and Y1R agonist compared to control (one-way ANOVA, $F(4, 15) = 11.12$, $p < 0.001$; Newman-Keuls post hoc test: $p < 0.001$), M1145 alone (Newman-Keuls post hoc test: $p < 0.001$), or Y1R agonist alone (Newman-Keuls post hoc test: $p < 0.05$) (**Figure 3a–d**). The Y1R agonist alone significantly increased BDNF-positive cells in the ventral DG (Newman-Keuls post hoc test: $p < 0.05$) (Figure 3b) compared to control and M1145 groups. M1145 alone did not affect BDNF-positive cell numbers in the ventral DG. Similar to PCNA-IR response, the GALR2 antagonist M871 completely blocked the co-injection-induced increase in BDNF expression (Newman-Keuls post hoc test: $p < 0.05$) (**Figure 3b**), confirming GALR2 involvement.

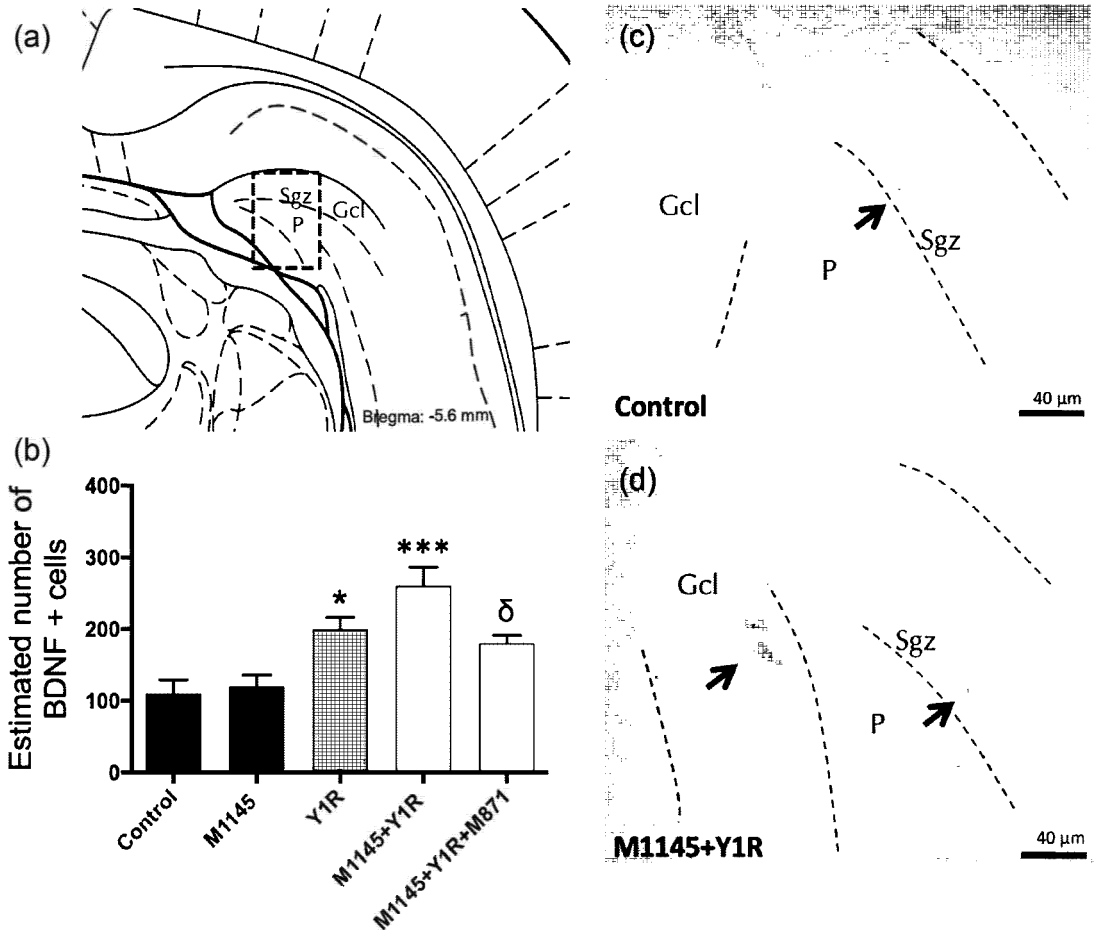


Figure 3: Enhancement of Brain-Derived Neurotrophic Factor (BDNF) Expression by GALR2 and Y1R Agonists in Dentate Gyrus. *BDNF-immunoreactive (IR) cells in the dentate gyrus (DG) of the ventral hippocampus following intranasal administration of GALR2 agonist (M1145) and Y1R receptor agonist, alone or in combination with or without GALR2 antagonist (M871). (a) BDNF-IR cells predominantly located in Sgz at the Gcl border, with some scattered cells in the P of the dentate gyrus (Bregma: −5.6 mm; Paxinos and Watson, 2006). (b) Quantitative analysis of BDNF-IR cells in DG. *$p < 0.05$ versus control, M1145, and M1145 + Y1R; $\delta p < 0.05$ versus M1145 + Y1R; ***$p < 0.001$ versus control and M1145, by one-way ANOVA followed by Newman–Keuls post hoc test (n =*

4/group). Vertical lines indicate intergroup comparisons above bars. (c, d) Representative microphotographs showing increased BDNF-positive cells in DG after M1145 and Y1R agonist coadministration (d) compared to control (c). Black arrows point to BDNF-IR cells. Dashed lines outline the Gcl of the dentate gyrus. Control, distilled water; M1145, GALR2 agonist 132 µg; M1145 + Y1R, coadministration of M1145 and Y1R; M1145 + Y1R + M871, coadministration of M1145, Y1R, and GALR2 antagonist M871 132 µg; Y1R, Y1R agonist [Leu31-Pro34]NPY 132 µg.

GALR2 and Y1R Agonists Synergistically Enhance GALR2/Y1R Heteroreceptor Complexes and Neurite Length in Hippocampal Neurons

To examine receptor-level mechanisms, we performed in situ proximity ligation assay (PLA) on hippocampal neurons to analyze GALR2/Y1R heteroreceptor complex formation following M1145 and/or Y1R agonist incubation. PLA-positive red clusters were detected in the membrane and cytoplasmic regions of hippocampal neurons (**Figure 4a–d**). Quantitative analysis showed a significant increase in PLA-positive 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 4a, c, d**). Co-incubation with M1145 and the Y1R agonist significantly increased PLA-positive clusters 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: $p < 0.01$). The GALR2 antagonist M871 blocked this synergistic effect (Newman-Keuls post hoc test: $p < 0.01$) (**Figure 4a**), demonstrating coactivation of GALR2 and Y1R mediates this interaction.

Given the changes in heteroreceptor complex density, we examined morphological and structural plasticity changes in hippocampal cultures following pharmacological treatments. Coactivation with M1145 and the Y1R agonist for 24 hours significantly increased mean neurite length compared to Y1R agonist alone (one-way ANOVA, $F(4, 20) = 16.91$, $p < 0.001$; 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–d). Y1R agonist alone also increased neurite length compared to control and M1145 alone (Newman-Keuls post hoc test: $p < 0.01$). GALR2 antagonist M871 entirely blocked these synergistic effects (Newman-Keuls post hoc test: $p < 0.05$).

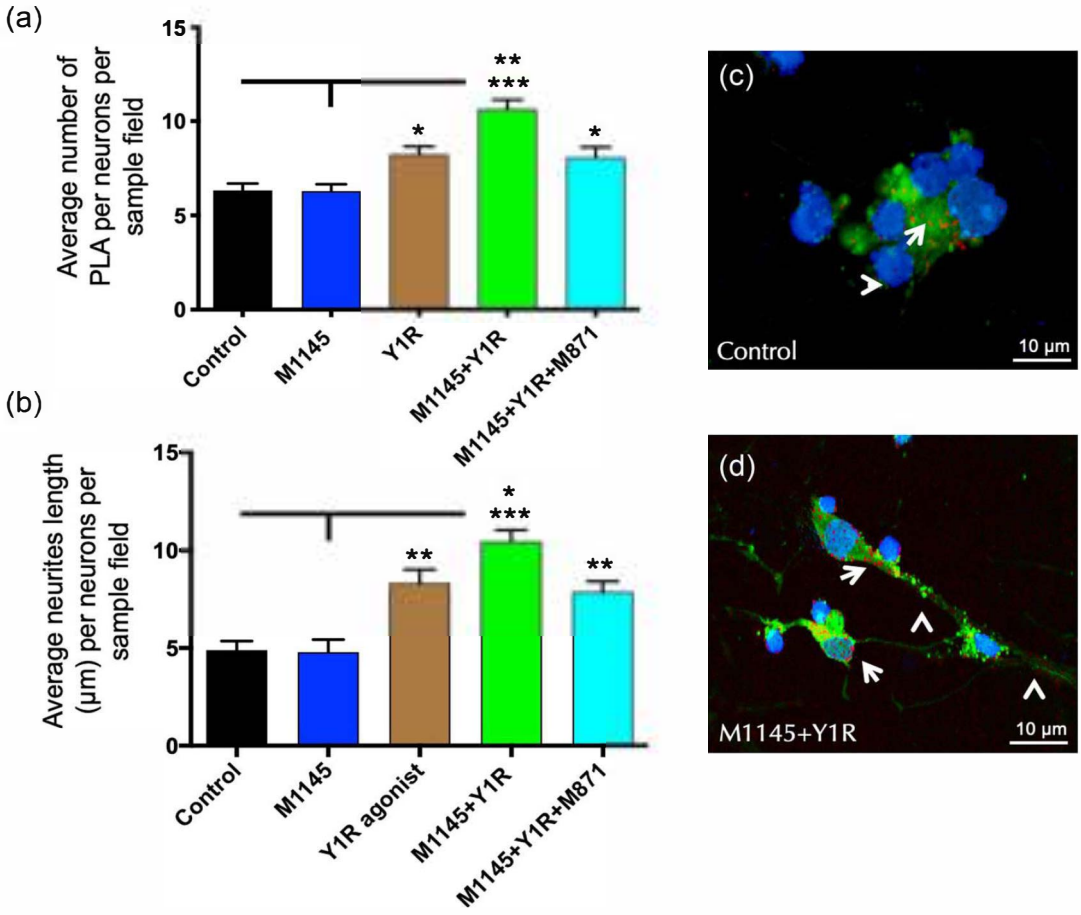


Figure 4: Demonstration of GALR2/Y1R Heteroreceptor Complexes and Neurite Modulation. *(a) GALR2 and Y1R agonists induce GALR2/Y1R heteroreceptor complexes shown by in situ proximity ligation assay (PLA) on hippocampal neurons, alone or in combination with or without GALR2 antagonist (M871). Quantification of PLA signals per nucleus per field. *p < 0.05 versus control and M1145; **p < 0.01 versus Y1R and M1145 + Y1R + M871; ***p < 0.001 versus control and M1145, by one-way ANOVA followed by Newman–Keuls post hoc test. Data are mean ± SEM. (b) GALR2 and Y1R agonists modulate neurite length per cell, determined by immunofluorescent labeling of neurons and neuronal nuclei (Pan Neuronal Marker/DAPI). Data are mean ± SEM. *p < 0.05 versus Y1R and M1145 + Y1R + M871; **p < 0.01 versus control and M1145; ***p < 0.001 versus control and M1145, by one-way ANOVA followed by Newman–Keuls post hoc test. (c, d) Representative microphotographs showing increased density of GALR2/Y1R heteroreceptor complexes (red PLA blobs) and neurite length per hippocampal neuronal cell after M1145 and Y1R agonist treatment (d) compared to control (c). Receptor complexes indicated by white arrows; neurite extensions by white arrowheads. Control, culture medium; DAPI, 4',6-diamidino-2-phenylindole; M1145, GALR2 agonist 100 nM; M1145 + Y1R, coadministration of M1145 and Y1R; M1145 + Y1R + M871, coadministration of M1145, Y1R, and GALR2 antagonist 1 µM; PLA, proximity ligation assay; Y1R, Y1R agonist [Leu31-Pro34]NPY 100 nM.*

Enhanced Antidepressant-like Response Following Intranasal Administration of GALR2 and Y1R Agonists in the Forced Swim Test (FST)

We conducted the FST to evaluate the functional outcomes of intranasal GALR2 and Y1R agonist administration on depression-like behaviour. Rats were pre-exposed to

water for 15 minutes and, 24 hours after intranasal administration, immobility and swimming parameters were measured during a 5-minute test phase.

Dose-response analysis indicated that intranasal GALR2 agonist alone had no effect at doses of 68 and 132 µg in the FST. The 68 µg dose of Y1R agonist was ineffective, while 132 µg significantly decreased immobility time (one-way ANOVA, $F(4, 25) = 3.79$, $p < 0.05$) compared to other groups (Newman-Keuls post hoc test: $p < 0.05$) (**Figure5a**), and increased swimming behavior (one-way ANOVA, $F(4, 25) = 3.57$, $p < 0.05$) compared to other groups (Newman-Keuls post hoc test: $p < 0.05$) (**Figure 5b**).

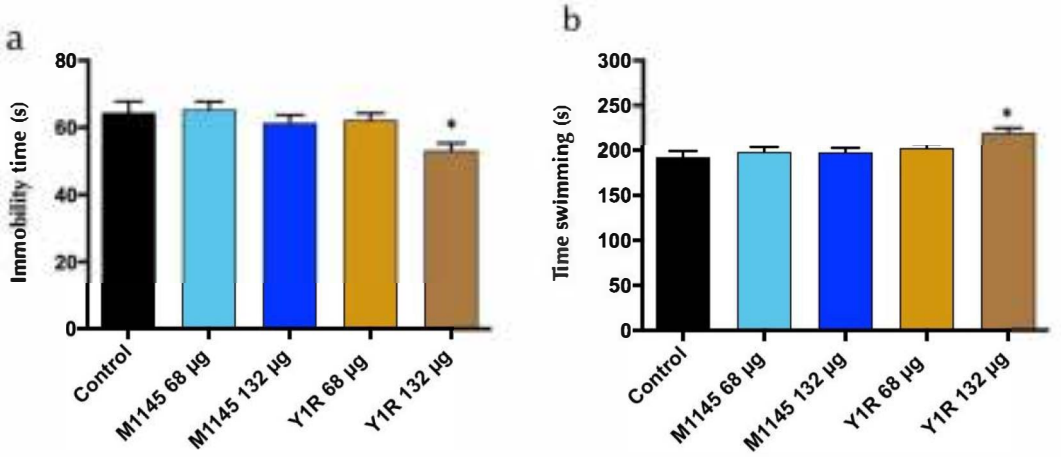


Figure 5: Dose-response and behavioral effects of GALR2 and Y1R agonists in the forced swimming test (FST). *Antidepressant-like effects observed in the FST after intranasal administration of Y1R receptor agonist ([Leu31–Pro34]NPY) at 132 µg following a 24-hour delay. Cumulative duration of immobility (a) and swimming (b) time in the FST. Data represent mean ± SEM. N = 6 animals per group. *P < 0.05 versus other groups according to one-way ANOVA followed by Newman-Keuls post-hoc test. Abbreviations: Control = Distilled water; M1145 68 µg = GALR2 agonist 68 µg; M1145 132 µg = GALR2 agonist 132 µg; Y1R 68 µg = Y1.*

Co-administration of M1145 and the Y1R agonist significantly decreased immobility time (one-way ANOVA, $F(5, 30) = 8.96$, $p < 0.001$) compared to control animals (Newman-Keuls post hoc test: $p < 0.001$), M1145 (Newman-Keuls post hoc test: $p < 0.001$), and Y1R agonist alone (Newman-Keuls post hoc test: $p < 0.01$) (**Figure 6a, b**). Swimming behavior was also significantly increased (one-way ANOVA, $F(5, 30) = 10.58$, $p < 0.001$) compared to control (Newman-Keuls post hoc test: $p < 0.001$), M1145 (Newman-Keuls post hoc test: $p < 0.001$), and Y1R agonist alone (Newman-Keuls post hoc test: $p < 0.05$) (**Figure 6b**). These synergistic effects on immobility and swimming were counteracted by the GALR2 antagonist M871 (Newman-Keuls post hoc test: $p < 0.01$ for immobility and $p < 0.05$ for swimming) (**Figure 6b**). The involvement of BDNF was confirmed as the TrkB antagonist ANA-12 blocked the synergistic effects on immobility (Newman-Keuls post hoc test: $p < 0.01$) and

swimming (Newman-Keuls post hoc test: $p < 0.05$) induced by co-administration of M1145 and the Y1R agonist in the FST.

The Y1R agonist alone reduced immobility time compared to control (Newman-Keuls post hoc test: $p < 0.05$) and M1145 groups (Newman-Keuls post hoc test: $p < 0.05$), and increased swimming behavior compared to control (Newman-Keuls post hoc test: $p < 0.05$) and M1145 groups (Newman-Keuls post hoc test: $p < 0.05$). However, M1145 alone had no effect on the FST compared to the control group (**Figure 6b**).

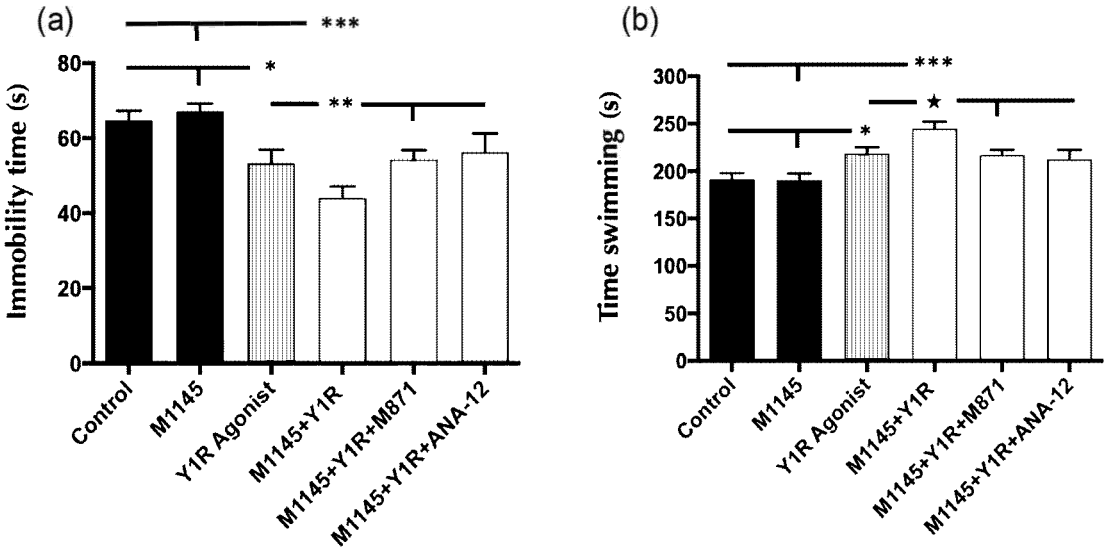


Figure 6: Behavioral effects of GALR2 and Y1R agonists in the forced swimming test (FST). *Antidepressant-like effects observed in the FST following intranasal coadministration of GALR2 agonist (M1145) and Y1R receptor agonist ([Leu31–Pro34]NPY) after a 24-hour delay. This effect is reversed by the GALR2 antagonist (M871). Cumulative duration of immobility (a) and swimming (b) time in the FST. Data represent mean ± SEM. N = 6 animals per group. For (a): *p < 0.05 versus control and M1145; **p < 0.01 versus Y1R agonist, M1145 + Y1R + M871, and M1145 + Y1R + ANA-12; ***p < 0.001 versus control and M1145. For (b): *p < 0.05 versus control and M1145; ★p < 0.05 versus Y1R agonist, M1145 + Y1R + M871, and M1145 + Y1R + ANA-12; ***p < 0.001 versus control and M1145 according to one-way ANOVA followed by Newman–Keuls post hoc test. Horizontal and vertical lines above bars indicate intergroup comparisons.*

1. 3. Discussion

This study is the first to demonstrate that intranasal infusion of GALR2 and Y1R agonists stimulates neurogenesis in the adult ventral hippocampus and produces antidepressant-like effects. Intranasal delivery presents a viable alternative to intracerebroventricular (icv) infusion, effectively bypassing the blood-brain barrier to deliver peptides and protein therapeutics directly to the CNS. This method is supported by substantial evidence from preclinical and clinical trials [222]. Its

advantages include reduced side effects compared to peripheral administration and the noninvasive nature of the application. For instance, intranasal esketamine, recently introduced as an antidepressant, faces limitations due to potential neurotoxicity, psychomimetic side effects, risk of abuse, and variability in treatment response [195].

Following intranasal administration of GALR2 and Y1R agonists, we observed increased cell proliferation in the ventral dentate gyrus (DG) of the hippocampus using PCNA as a marker. This aligns with our previous findings that coadministration of these agonists enhances cell proliferation in the dorsal DG at 24 hours [169]. Previous studies have shown that icv infusion of GAL and Y1R agonists induced cell proliferation in the ventral hippocampus using 5-bromo-2-deoxyuridine [208]. The current study's strength lies in demonstrating a noninvasive route via intranasal delivery of specific GALR2 and Y1R agonists. Notably, genetic enhancement of neurogenesis in the ventral hippocampal DG has been shown to increase resilience in depression models [223]. Similarly, the molecule P7C3, associated with increased cell proliferation in the hippocampal DG, has shown antidepressant effects in rodents and primates [224, 225].

Our study also found that intranasal administration of the Y1R agonist alone increased cell proliferation in the ventral DG, but not in the dorsal DG [168, 169]. This highlights functional differences between the ventral and dorsal regions and suggests a differential role for NPY in these subregions of the hippocampus [192]. In contrast, the GALR2 agonist alone did not affect cell proliferation in the ventral hippocampus. Previous studies indicated that GALR2/3 mediates the proliferative and trophic effects of GAL, with subsequent studies suggesting a role for GALR3 [211]. However, these findings were based on in vitro conditions, which can differ significantly from in vivo systems.

We further identified that the combined administration of M1145 and Y1R agonist specifically stimulated the proliferation of neuroblasts (PCNA+/DCX+ cells) without affecting quiescent neural progenitors and astrocytes (PCNA+/GFAP+ cells). This agrees with previous reports showing that NPY promotes the proliferation of amplifying neural progenitors and neuroblasts [206, 207].

Dysregulation of neurogenesis in the subventricular zone (SVZ) is a common feature in various neurodegenerative diseases. For instance, stem cell proliferation is reduced in Alzheimer's and Parkinson's diseases, whereas stroke and Huntington's disease enhance SVZ neurogenesis to aid in the repair of damaged areas [205-207]. NPY has been reported to promote neurogenesis via Y1R on DCX-positive neuroblasts and to play a role in cell migration [205, 206]. Future research should investigate the potential of intranasally administered GALR2 and Y1R agonists in cell replacement strategies for neurodegenerative diseases affecting SVZ neurogenesis.

At the cellular level, the increased hippocampal cell proliferation following intranasal coadministration of GALR2 and Y1R agonists appears to be mediated by elevated BDNF expression in the ventral hippocampal DG. BDNF, a crucial neurotrophin, plays a significant role in promoting neurogenesis through its effects on cell proliferation and survival (Miranda et al., 2019). Physical exercise has been shown to protect against depressive symptoms by increasing hippocampal neurogenesis and BDNF levels [226]. Therapeutics that enhance the relationship between dentate neurogenesis and BDNF, like the combined GALR2 and Y1R agonists, may be key to treating depression. This is supported by previous evidence on the neuroprotective effects of NPY in neurodegeneration models [204].

In hippocampal neuronal cells, the coadministration of GALR2 and Y1R agonists increased the formation of GALR2/Y1R heteroreceptor complexes, as observed using in situ PLA. This effect was confirmed in previous studies in HEK cells and various limbic brain regions, including the amygdala and dorsal hippocampus [169, 212, 213]. Additionally, we observed that coincubation of these agonists promoted neurite outgrowth in hippocampal neuronal cells, potentially mediated by BDNF, consistent with its known effects on dendritic outgrowth in primary hippocampal cultures and the hippocampus [227].

The functional outcome was validated by demonstrating enhanced antidepressant-like responses in the forced swim test (FST) 24 hours after intranasal administration of GALR2 and Y1R agonists. Previous studies have shown that intranasal infusion of Y1 agonist [228] in rats or humans induces antidepressant effects for at least 24 hours. Similarly, single injections of the NMDA receptor antagonist Ketamine or the mGlu2/3 receptor antagonist LY341495 have been shown to produce antidepressant-like effects in the FST in rats at 24 hours. However, the GALR2 agonist alone did not exhibit antidepressant-like effects at 24 hours, suggesting that subchronic or chronic intranasal treatments may be required for long-lasting effects in pathological depression models. Notably, species-specific differences in antidepressant responses between rats and mice have been reported. For instance, the intranasal infusion of a spexin-based GALR2 agonist showed antidepressant-like effects in mice within 2-3 hours. Recently, M39b, a stabilized GALR2 agonist, has shown promise in intranasal delivery studies in rats . Moreover, the GALR2 antagonist M871 counteracted the enhanced response observed, aligning with previous findings [169]. These behavioral effects were independent of motor activity, as neither GALR2 nor Y1R agonists, nor their coadministration, affected locomotor activity. This is consistent with the involvement of the ventral hippocampus in the antidepressant effects of NPY in posttraumatic stress disorder [228]. Thus, the enhanced antidepressant effects of Y1R and GALR2 agonists at 24 hours may be mediated by increased signaling of Y1R-GALR2 heterocomplexes in the ventral hippocampus, supported by BDNF, as

observed in vivo and in vitro. The TrkB antagonist ANA-12 was shown to counteract the antidepressant effects of ketamine at 24 hours [176]. Additionally, physical exercise enhances BDNF signaling and neuronal proliferation in the ventral hippocampus, contributing to antidepressant effects {Murawska-Cialowicz, 2021 #2533.

Overall, intranasal infusion of Y1R and GALR2 agonists promotes cell proliferation in the ventral hippocampal DG and induces BDNF expression. These effects are likely mediated by Y1R-GALR2 heteroreceptor complexes, leading to increased neurite outgrowth in hippocampal neurons and enhanced antidepressant effects. These findings suggest the potential for developing new therapeutic approaches targeting Y1R-GALR2 heterocomplexes for major depressive disorder (MDD) and related conditions. Future clinical trials could explore the efficacy of intranasally delivered Y1R and GALR2 agonists in these contexts.

CHPATER 2
Results and Discussion

SPECIFIC AIM 2. *Enhancement of neurogenesis and cognition through intranasal co-delivery of galanin receptor 2 (GALR2) and neuropeptide Y receptor 1 (NPY1R) agonists: a potential pharmacological strategy for cognitive dysfunctions.*

1. 1. Background

The longstanding belief that human adult neurogenesis continues throughout life has garnered significant scientific interest, particularly due to its critical role in various neurological conditions, such as Alzheimer's disease, Huntington's disease, Parkinson's disease, dementia with Lewy bodies, and frontotemporal dementia [189, 216, 217]. The urgent need for new therapeutic strategies is underscored by the prevalence of Alzheimer's disease, which accounts for approximately 70% of dementia cases worldwide, affecting nearly 35 million people. Enhancing hippocampal neurogenesis is a promising approach, especially given the exacerbation of cognitive and psychiatric symptoms in Alzheimer's patients following COVID-19 infection [219].

In the dentate gyrus of the hippocampus, a substantial number of neurons are generated, although only a few survive long enough for integration into neural networks, a process regulated by neural activity [186]. During this development phase, cells begin to express doublecortin (DCX), a protein crucial for neuronal differentiation, migration, and potentially synaptogenesis [229, 230]. DCX is uniquely expressed in neurogenesis-contributing cells in the dentate gyrus and marks an important developmental stage characterized by neurite elongation [230, 231].

Recent research by Canatelli-Mallat et al. has shown that in middle-aged rats, a decrease in immature (DCX-positive) neurons in the dorsal hippocampus does not affect performance in object recognition tasks but is significant for spatial memory tasks [232]. This highlights the specific role of the dorsal hippocampus in spatial context and object recognition, whereas other brain regions, such as the prefrontal cortex, are more involved in recognizing object features [233].

Mature granule cells do not express DCX, and its expression is transient, lasting about three weeks in the growth cones of dendrites and axons. This period coincides

with neuronal migration, a critical phase in neurodevelopment [180, 234]. Most new cells remain within the inner third of the granule cell layer, indicating a specific constraint in DCX-guided migration [191]. The cytoplasmic localization of DCX in immature neurons makes it a valuable marker for studying neuronal morphology through immunohistochemical methods [235, 236].

Emerging research has identified neuropeptides, specifically Neuropeptide Y (NPY) and Galanin (GAL), as key regulators of neurogenic niche activities. NPY and its receptors, particularly NPY Y1 receptors (NPY1R), play essential roles in mood regulation, neuronal excitability, neuroplasticity, and memory [200, 220]. Studies have demonstrated NPY's crucial role in promoting neurogenesis within hippocampal stem cells, both in vitro and in vivo [207]. Enhanced hippocampal NPY mRNA expression has been observed following spatial learning tasks, while aging rats show a decline in NPY expression correlated with memory deterioration and reduced neurogenesis [237]. Alzheimer's disease patients exhibit lower NPY receptor densities and NPY levels in cerebrospinal fluid and plasma, making NPY1R a target for enhancing dentate neurogenesis and spatial learning [238, 239].

Similarly, GAL, widely present in the central nervous system, impacts hippocampal neurogenesis [240]. The GAL 2/3 receptor agonist, GAL 2–11, has been shown to promote proliferation and trophism in progenitor cells [211]. GAL's effects on memory are dose- and site-dependent, ranging from enhancing learning to having no effect or even inhibitory impacts [241]. GALR2 receptors mediate memory-enhancing and neuroprotective effects in Alzheimer's disease models [242].

Previous research from our group has highlighted the interaction between NPY and GAL through specific NPY1R-GALR2 heteroreceptor complexes in interconnected brain regions, such as the amygdala, dorsal and ventral hippocampus, and various hypothalamic regions [166, 168, 243-245]. These interactions are significant for the neurological disorders mentioned earlier. Our study investigates whether NPY1R and GALR2 agonists stimulate proliferation in DG neuronal precursors in vivo within 24 hours.

We utilized a novel intranasal delivery method to infuse both peptides into rats. Intranasal drug administration has gained attention in neuroscience for its ability to deliver therapeutic agents directly to the central nervous system, bypassing the blood-brain barrier [246]. This non-invasabsorption andduces systemic side effects, ensures rapid absorption, and is advantageous for conditions requiring immediate intervention. Our study combines this method with BrdU labeling to analyze the phenotype of newly generated cells in the dentate gyrus three weeks post-treatment. This timeframe is based on research indicating that new granule neurons can contribute to hippocampal function as early as 2-3 weeks of age, although other

studies suggest a functional contribution around 6-8 weeks, with differences likely due to species variations [185, 247].

Our research emphasizes the importance of adult neurogenesis under physiological conditions and the regulatory role of neuropeptides, specifically NPY and GAL. These findings offer promising therapeutic avenues for addressing age-related cognitive decline and early stages of cognitive impairment. By further exploring the effects of NPY1R and GALR2 agonists on neurogenesis and cognition, we aim to deepen our understanding of these complex biological processes.

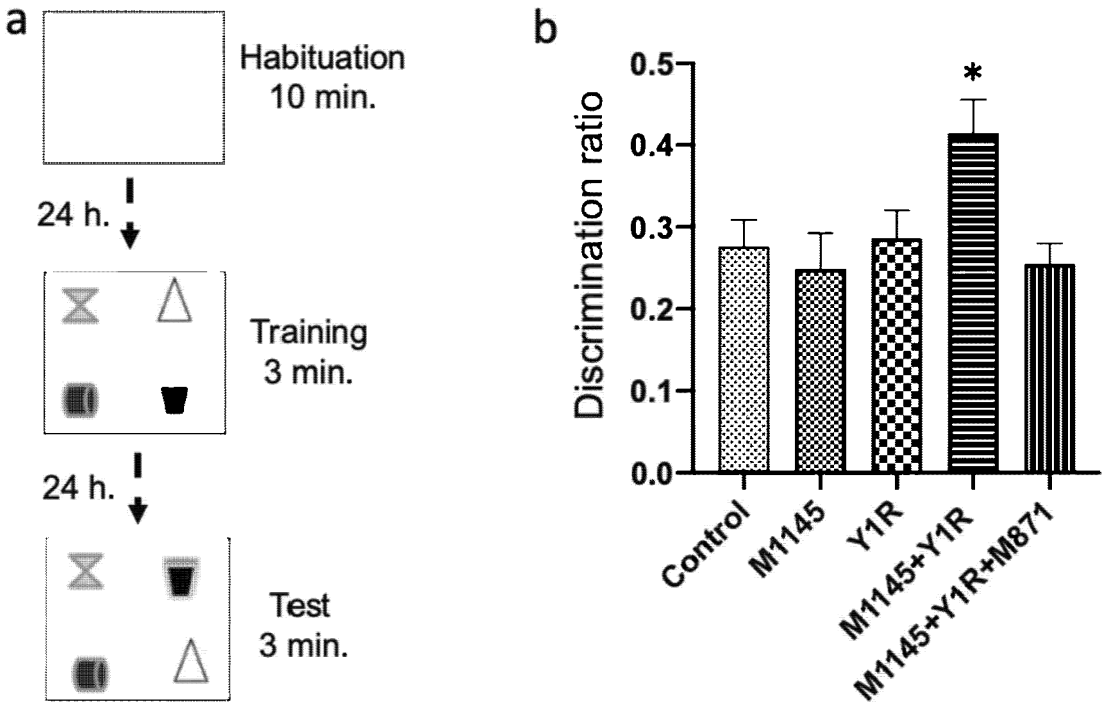
1. 2. Results

Enhanced spatial memory performance following GALR2 and NPY1R agonist intranasal infusion.

We conducted the object-in-place task three weeks after intranasal (i.n.) infusions. This test included a habituation phase where rats explored an empty arena for 10 minutes, a training phase involving four different objects, and a test phase where two objects swapped positions to evaluate memory performance (**Figure 1a**).

Intranasal infusion of the GalR2 agonist M1145 combined with the NPY1R agonist post-acquisition significantly enhanced object-in-place memory consolidation three weeks after treatment compared to other groups (one-way ANOVA, $F_{4, 25} = 3.60$, $p < 0.05$; Newman–Keuls post-hoc test: $p < 0.05$; Fig. 1b). However, administration of either M1145 or the NPY1R agonist alone did not affect object-in-place memory performance compared to the control group (**Figure 1b**). Our analysis of total exploration time during the training and test sessions indicated no significant changes in the animals' exploration capacity or spontaneous motor behavior following the treatments.

Figure 1. Assessing Spatial Memory in the Object-in-Place Task Following Intranasal Administration of NPY1R and GALR2 Agonists. *(a) Schematic of the object-in-place memory task, including the habituation phase (10 minutes of exploration without objects), the training phase (3 minutes with four different objects), and the test phase (3 minutes with two objects switched in position) conducted over 24-hour intervals. (b) Performance metrics showing improved memory in rats administered with a combination of M1145 (Galanin 2 receptor agonist, 132 µg) and NPY1R (Y1R receptor agonist [Leu31-Pro34] NPY, 132 µg). This enhancement was counteracted by the GALR2 antagonist M871 (132 µg). Results are expressed as the mean ± SEM of the discrimination ratio during the test phase, with 6 animals per group. *p < 0.05 indicates significant differences determined by one-way ANOVA and Newman-Keuls post-hoc test. Abbreviations: Control = Distilled water; M1145 = Galanin 2 receptor agonist 132 µg; Y1R = NPY1R receptor agonist [Leu31-Pro34]NPY 132 µg; M1145 + Y1R = Co-administration of M1145 and NPY1R; M1145 + Y1R + M871 = Co-administration of M1145, Y1R, and GALR2 antagonist M871 132 µg.*



GALR2 and NPY1R Agonist Co-Administration Enhances Survival of Mature Neurons in the Dorsal Hippocampus.

To explore the cellular mechanisms underlying these behavioral effects, we examined the impact of co-administering the GALR2 and NPY1R agonists intranasally on adult dorsal hippocampal cell proliferation using the thymidine analogue 5-Bromo-2'-deoxyuridine (BrdU) (**Figure 2a**). The intranasal co-administration of M1145 and the NPY1R agonist significantly increased the number of BrdU-IR profiles in the subgranular zone (Sgz) of the dentate gyrus compared to the control group and the groups receiving either M1145 or the NPY1R agonist alone (one-way ANOVA, $F_{4, 15} = 5.34$, $p < 0.01$, Newman-Keuls post-hoc test: $p < 0.05$) (**Figure 2b–d**). This increase was completely blocked when the GALR2 antagonist M871 was co-administered, indicating GALR2's involvement in the interaction between M1145 and the NPY1R agonist to stimulate cell proliferation. Further analysis revealed a significant increase in the number of BrdU+/NeuN+ cells after treatment with M1145 and the NPY1R agonist compared to the control group, indicating a preference for newly generated cells to differentiate into mature neurons.

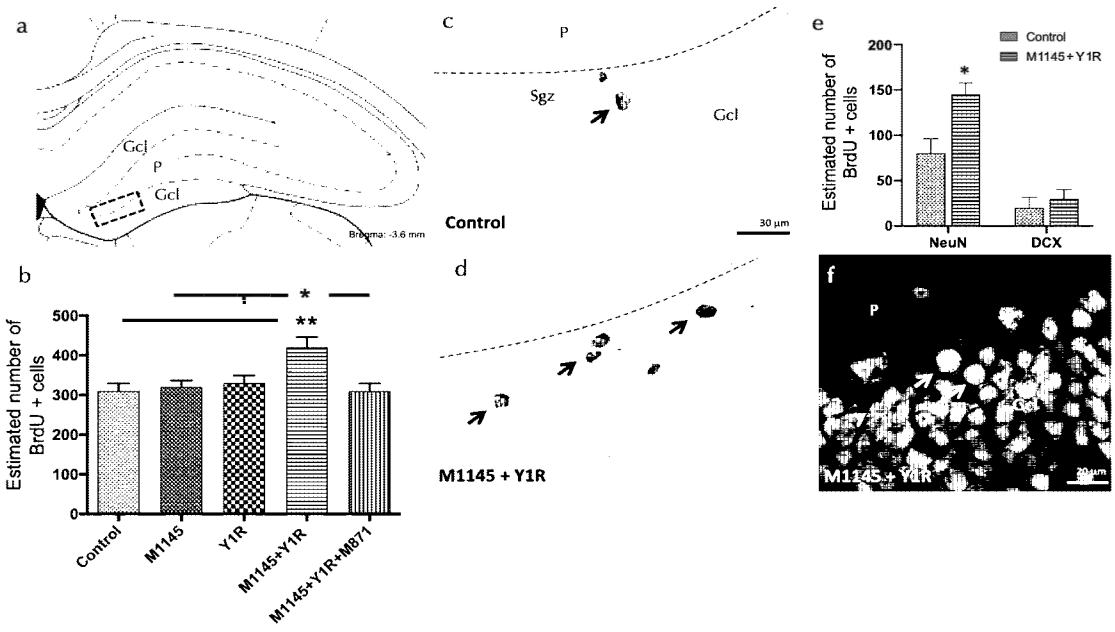
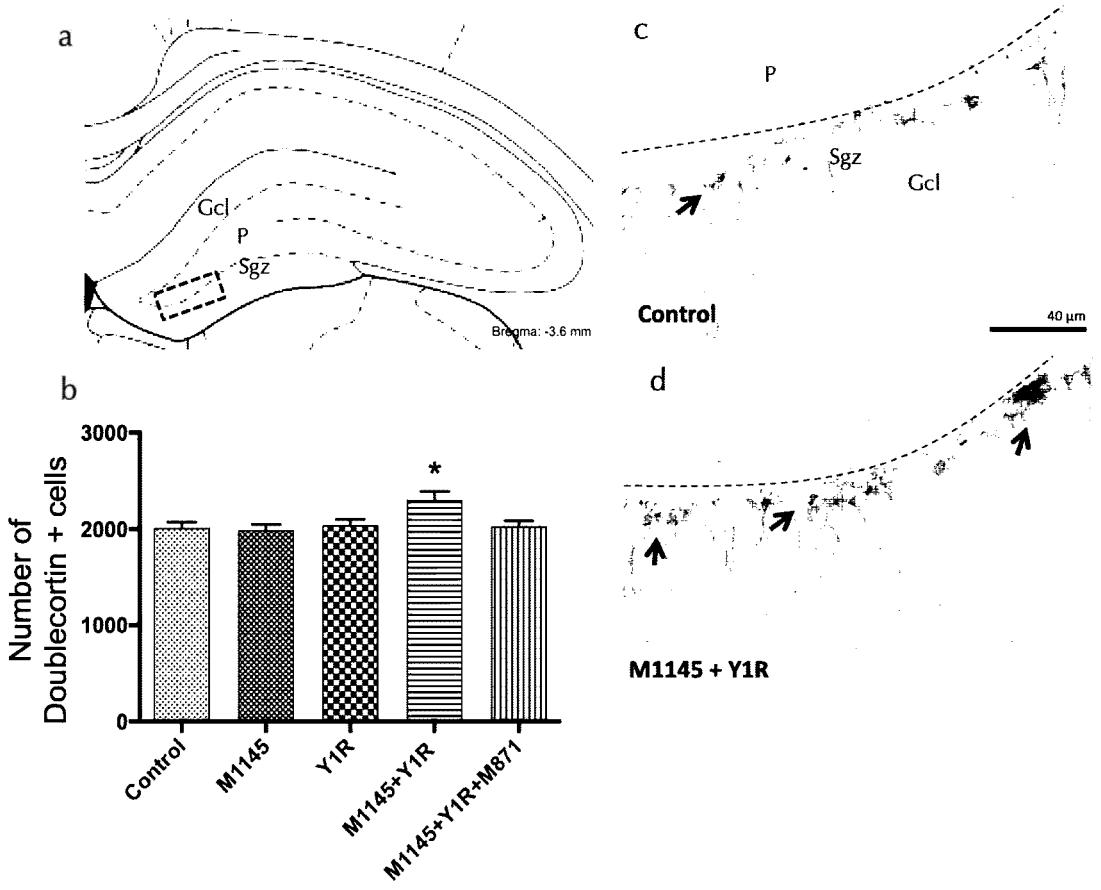


Figure 2. Enhanced Neuronal Survival in the Dorsal Dentate Gyrus Following Intranasal Co-administration of GALR2 and NPY1R Agonists. *(a, d) BrdU immunolabeling showing BrdU-positive cells in the granular cell layer (Gcl) of the dentate gyrus in the dorsal hippocampus, with round, regularly shaped nuclei (Bregma: -5.6 mm; Paxinos and Watson stereotaxic atlas, 2006). (b) Quantitative analysis of BrdU-immunoreactive (IR) cells in the dentate gyrus, comparing Control, M1145, NPY1R agonist [Leu31-Pro34] NPY, and co-administration of both agonists with or without M871. The combination of M1145 and the NPY1R agonist increased BrdU-positive cells, an effect nullified by the GALR2 antagonist M871. Data are mean ± SEM, with *P < 0.05 vs M1145, NPY1R, and M1145 + Y1R + M871: **P < 0.01 vs Control. (c) Arrows indicate BrdU-positive neurons, with dashed lines marking the Gcl of the dentate gyrus. (e) Quantification of BrdU-IR cells double-labeled with NeuN or DCX in control or M1145 + Y1R-treated rats, indicating neuronal maturation induced by Y1R-GALR2. Data are mean ± SEM, *P < 0.05 vs control by Student's t-test. (f) Representative photomicrograph showing BrdU+/NeuN+ cells (white arrows) and BrdU-/NeuN+ cells (white arrowheads) in the M1145 and NPY1R agonist group. Abbreviations: Control = Distilled water; M1145 = Galanin 2 receptor agonist 132 µg; Y1R = NPY1R receptor agonist [Leu31-Pro34] NPY 132 µg; M1145 + Y1R = Co-administration of M1145 and NPY1R; M1145 + Y1R + M871 = Co-administration of M1145, Y1R, and GALR2 antagonist M871 132 µg.*

Increase in Doublecortin-Positive Cells in the Dorsal Hippocampus Following NPY1R and GALR2 Agonist Intranasal Infusion.

We investigated the expression of doublecortin (DCX)-labeled newborn neurons in the dorsal hippocampal dentate gyrus (DG) following intranasal administration of M1145 and/or the NPY1R agonist. Results showed a significant increase in the number of DCX-labeled cells after co-administration of M1145 and the NPY1R agonist compared to other groups (one-way ANOVA, $F_{4, 15} = 3.79$, $p < 0.05$, Newman-Keuls post-hoc test: $p < 0.05$) (**Figure 3a–d**).

Figure 3. Increased Doublecortin (DCX) Expression in the Dorsal Dentate Gyrus After Intranasal Co-administration of GALR2 and NPY1R Agonists. *(a, d) DCX-positive cells located in the subgranular zone (Sgz) of the dentate gyrus, along the boundary between the granular cell layer (Gcl) and the polymorphic layer (P) (Bregma: -5.6 mm; Paxinos and Watson stereotaxic atlas, 2006). (b) Quantitative analysis of DCX-immunoreactive (IR) cells in the dentate gyrus, comparing Control, M1145, NPY1R agonist [Leu31-Pro34] NPY, and co-administration of both agonists with or without M871. The combination of M1145 and the NPY1R agonist significantly increased DCX-positive cells, an effect offset by the GALR2 antagonist M871. Data are mean ± SEM, with *P < 0.05 vs other groups. (c) Arrows indicate DCX-positive neurons, with dashed lines marking the Gcl of the dentate gyrus. Abbreviations: Control = Distilled water; M1145 = Galanin 2 receptor agonist 132 µg; Y1R = NPY1R receptor agonist [Leu31-Pro34] NPY 132 µg; M1145 + Y1R = Co-administration of M1145 and NPY1R; M1145 + Y1R + M871 = Co-administration of M1145, Y1R, and GALR2 antagonist M871 132 µg.*



NPY1R and GALR2 Agonist Co-Administration Promotes Neuronal Differentiation of DCX-Positive Cells in the Dorsal Hippocampus.

We examined whether M1145 and the NPY1R agonist altered dendrite organization in new neurons within the DG, categorizing DCX-labeled cells based on dendrite morphology into proliferative, intermediate, and post-mitotic phases (**Figure 4a**). Quantification revealed a decrease in DCX-labeled cells lacking dendrites or with dendrites shorter than soma size in M1145-NPY1R-treated rats compared to other

groups (two-way ANOVA, interaction $F_{6,36} = 30.60$ $p < 0.001$; row Factor $F_{2,36} = 436.5$ $p < 0.001$, Newman-Keuls post-hoc test: $p < 0.001$; Fig. 4b). Conversely, there was an increase in more mature cells in the M1145-NPY1R-treated rats compared to the vehicle group (Newman-Keuls post-hoc test: $p < 0.001$; Fig. 4b). Notably, the relative proportions of cells with dendrites larger than soma size were unaffected by the GALR2 and/or NPY1R agonists (**Figure 4b**).

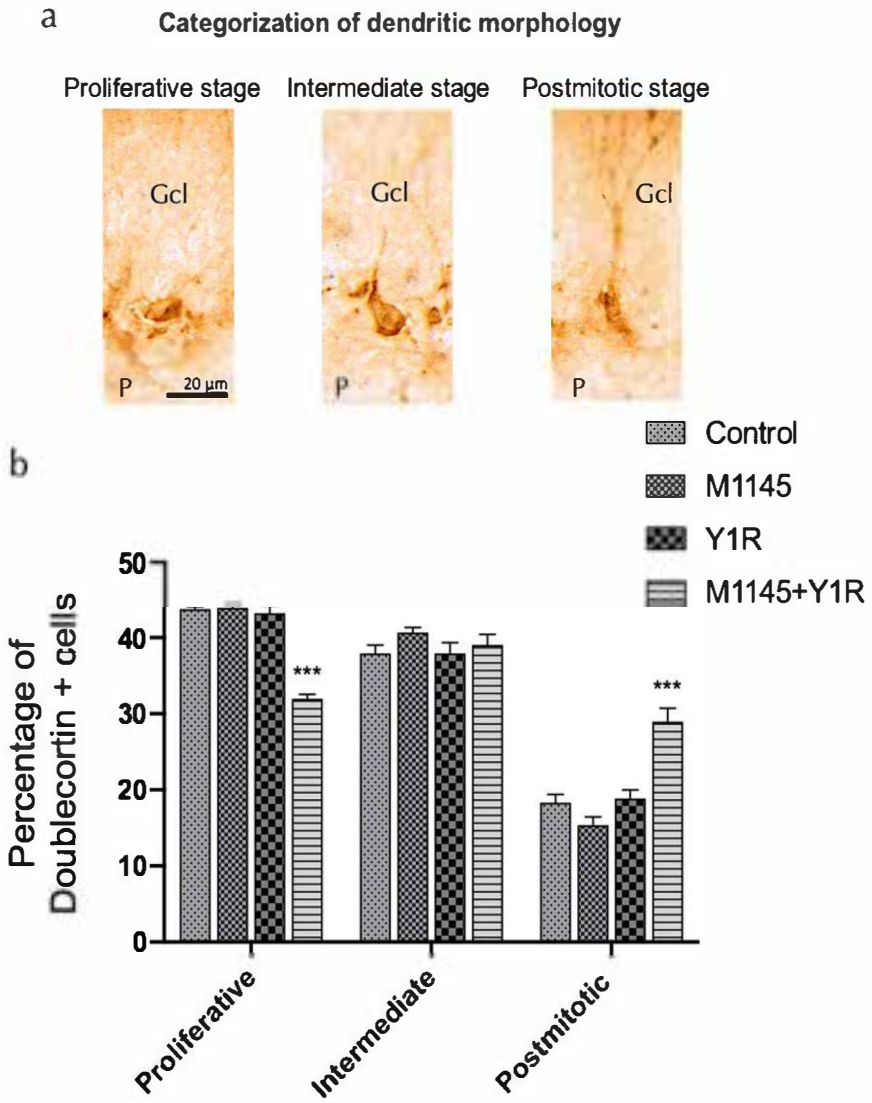


Figure 4. Neuronal Differentiation of DCX-Positive Cells in the Dorsal Hippocampus Induced by Intranasal Infusion of NPY1R and GALR2 Agonists. *(a) DCX-labeled cells categorized by dendritic morphology into proliferative, intermediate, and post-mitotic phases. (b) Quantitative analysis showing a reduction in DCX-labeled cells without dendrites or with short dendrites in rats treated with M1145 and NPY1R agonists, with an increase in mature cells compared to other groups. Data are presented as percentages, analyzed by two-way ANOVA (interaction $F_{6,36} = 30.60$, $p < 0.001$; row factor $F_{2,36} = 436.5$, $p < 0.001$, Newman-Keuls post-hoc test: $p < 0.001$). Abbreviations: Control = Distilled water; M1145 = Galanin 2 receptor agonist 132 µg; Y1R = NPY1R receptor agonist [Leu31-Pro34]NPY 132 µg; M1145 + Y1R = Co-administration of M1145 and NPY1R; M1145 + Y1R + M871 = Co-administration of M1145, Y1R, and GALR2 antagonist M871 132 µg.*

These findings suggest that M1145 and NPY1R agonist treatment impacts more mature neurons, resulting in a reduction of immature cells without dendrites and an increase in DCX-labeled cells with mature dendrite morphology.

1. 3. DISCUSSION

Our study demonstrates that the intranasal co-administration of GALR2 and NPY1R agonists enhances spatial memory and promotes neuronal survival and differentiation in the dentate gyrus of the dorsal hippocampus.

Previously, short-term improvements in object-in-place memory consolidation were observed following intracerebroventricular (icv) administration of GALR2 and NPY1R agonists [169]. However, individual icv administration of M1145 or the NPY1R agonist did not yield significant memory improvements. This suggests that the combined treatment might induce allosteric enhancement between GalR2 and NPY1R, leading to increased signaling and improved memory consolidation. Our findings highlight a synergistic effect between postjunctional GalR2 and NPY1R at both the transmembrane and cytoplasmic levels, potentially enhancing spatial memory consolidation through their combined intranasal administration. The importance of reaching a neuronal age of three weeks to achieve hippocampal functionality is emphasized by our results.

By the third week, a substantial population of active neurons can participate in hippocampal functions in rats [184, 185, 192, 236]. Our cellular analysis showed a significant increase in BrdU-immunoreactive profiles in the subgranular zone of the dentate gyrus following the combined treatment of the two neuropeptides, indicating enhanced neurogenesis, a process closely linked to learning and memory.

Moreover, the majority of these newly generated cells differentiated into mature neurons, as evidenced by the increase in BrdU+ cells and neuronal-specific nuclear protein (NeuN) detection after NPY1R-GALR2 administration. NeuN, a marker for mature neurons, confirmed this differentiation pattern, which was prominent three weeks post-injection. Interestingly, BrdU-doublecortin (DCX) co-expressing cells were relatively low, despite DCX being a marker for neurons, neural stem cells, and neurogenesis. Thus, the memory improvements observed may primarily result from enhanced synergistic signaling between NPY1R and GalR2, potentially forming a heteroreceptor complex that improves memory consolidation and supports the presence and integration of newly matured neurons.

Our study used male rats to account for sex differences in adult hippocampal neurogenesis. As noted by Yagi et al., male rats exhibit a higher density of BrdU-ir cells at earlier time points compared to females [248]. However, by the third week, these differences diminish, with both sexes showing comparable densities.

Additionally, the maturation rate of adult-born neurons is higher in males at two weeks but equalizes by the third week. This informed our choice of male rats to avoid sex-related disparities at earlier neurogenesis stages, though future studies should include both sexes to explore any differences across various conditions and timeframes.

Furthermore, our research observed dendritic morphological changes in DCX-labeled cells post-treatment, suggesting enhanced functional integration of these neurons into existing circuits. DCX's role in neuritic growth cone formation and synapse development indicates that dendritic complexity and length, crucial for neuronal functionality, may be influenced by DCX activity [180, 184, 235, 236]. Further research is needed to understand the interplay between cellular survival, maturation, and neuronal integration.

While our immunocytochemistry findings offer valuable insights into the effects of GALR2 and NPY1R agonists on hippocampal neurogenesis, advanced techniques could provide a deeper understanding of the underlying mechanisms. Transcriptomic analysis could elucidate molecular pathways activated by these agonists, while electrophysiological studies might reveal the functional integration of newly generated neurons into neural circuits. In vivo imaging techniques, such as two-photon microscopy, could offer real-time insights into neuronal development and integration following agonist administration. These advanced methodologies could enhance our understanding of neuropeptide receptor agonists' roles in neurogenesis and cognitive functions.

Overall, our results suggest a promising approach for memory enhancement and neuronal maturation through the co-administration of GALR2 and NPY1R agonists. Further investigations are essential to elucidate the molecular mechanisms behind this synergistic effect and its potential therapeutic applications for learning and memory.

CONCLUSIONS

I. Our.study provides compelling evidence that intranasal administration of GALR2 and NPY1R agonists effectively stimulates adult neurogenesis in the ventral hippocampus, thereby exerting robust antidepressant-like effects. By using a comprehensive approach, we demonstrated significant increases in cell proliferation within the ventral dentate gyrus, as indicated by enhanced PCNA expression. Moreover, our findings underscored the specificity of this effect, showing that coadministration of GALR2 and Y1R agonists selectively increased the proliferation of neuroblasts without impacting quiescent neural progenitors or astrocytes, highlighting the nuanced role of NPY in hippocampal subregions (Chapter 1).

II. At the cellular level, our study revealed a mechanistic link between GALR2 and NPY1R agonists and increased BDNF expression in the ventral hippocampal DG, suggesting that BDNF-mediated neurotrophic signaling may contribute significantly to the observed antidepressant effects. This aligns with previous literature implicating BDNF in promoting neurogenesis and dendritic outgrowth, reinforcing its crucial role in hippocampal plasticity and mood regulation. Additionally, our investigation into NPY1R-GALR2 heteroreceptor complexes via in situ PLA provided novel insights into the molecular mechanisms underlying the synergistic actions of these agonists in the hippocampus (Chapter 1).

III. Functionally, our behavioral assessments using the forced swim test (FST) demonstrated enhanced antidepressant-like responses following intranasal coadministration of GALR2 and Y1R agonists, corroborating our cellular findings. These effects were specific to the ventral hippocampus, suggesting a targeted therapeutic potential for treating depression-related disorders. Importantly, our study contributes to the growing body of evidence supporting intranasal delivery as a viable method for bypassing the blood-brain barrier and delivering therapeutic agents directly to the CNS, thereby minimizing peripheral side effects and improving patient compliance (Chapter 1).

IV. Our findings not only elucidate the neurobiological mechanisms through which GALR2 and Y1R agonists exert antidepressant effects but also highlight their therapeutic potential for developing novel treatments targeting neurogenic deficits associated with mood disorders. Future research should further explore the long-term effects and translational potential of these findings in clinical settings, with a focus on optimizing therapeutic strategies for enhancing

hippocampal neurogenesis and BDNF-mediated signalling pathways in depression (Chapter 1).

V. A second goal of this work, aimed to explore the influence of Galanin (GAL) on hippocampal neurogenesis, focusing on its dose- and site-dependent effects on memory and implications for Alzheimer's disease models. Additionally, we investigated interactions between Neuropeptide Y (NPY) and GAL through NPY1R-GALR2 heteroreceptor complexes in various brain regions, assessing their potential therapeutic implications for age-related cognitive decline. The findings indicate that intranasal co-administration of GALR2 and NPY1R agonists significantly enhances spatial memory and promotes neuronal survival and differentiation in the adult rat hippocampus. These results underscore the critical role of GALR2 in these processes and suggest potential therapeutic avenues for combating cognitive decline (Chapter 2).

VI. Our experimental evidence demonstrates that rats treated with the combination of GALR2 and NPY1R agonists showed marked improvements in spatial memory, as assessed by the object-in-place memory task. This enhancement was not observed when either agonist was administered alone, highlighting a synergistic effect. Further, the combined treatment led to a significant increase in the number of BrdU-immunoreactive cells in the dentate gyrus, specifically in the subgranular zone. This suggests an enhanced rate of neurogenesis, which was diminished by the GALR2 antagonist, reinforcing the critical role of GALR2 in this process (Chapter 2).

VII. Most newly generated cells differentiated into mature neurons, as indicated by the increased co-labeling of BrdU with NeuN, a marker for mature neurons. The increase in DCX-positive cells, particularly those with more mature dendritic structures, suggests enhanced neuronal maturation and integration into existing hippocampal circuits. These morphological changes imply that the newly generated neurons are functionally integrating into hippocampal circuits, potentially enhancing cognitive functions (Chapter 2).

VIII. Our study provides compelling evidence that targeting GALR2 and NPY1R receptors through intranasal co-administration of their agonists can significantly enhance hippocampal neurogenesis and spatial memory, suggesting a viable therapeutic approach for age-related cognitive impairments and Alzheimer's disease. Further research is warranted to unravel the molecular mechanisms underlying these effects and to explore their potential clinical applications (Chapter 2).

CONCLUSIONES EN ESPAÑOL

I. .En trabajos previos se indicó que el tratamiento agudo con el estabilizador de monoaminas OSU-6162 (5 mg/kg), debido a su alta afinidad por Sigma1R, aumentó significativamente la densidad de los complejos heteroreceptores D2R-Sigma1R y A2AR-D2R en la concha accumbal después de la autoadministración de cocaína. Las acciones ex vivo del agonista A2AR CGS 21680 también sugirieron la existencia de interacciones alostéricas antagonistas mejoradas A2AR-D2R en la autoadministración de cocaína después del tratamiento con OSU-6162. Sin embargo, el tratamiento durante tres días con OSU-6162 (5 mg/kg) no alteró los efectos conductuales de la autoadministración de cocaína. Para probar estos resultados y la relevancia de las interacciones de OSU-6162 (2.5 mg/kg) y/o A2AR (0.05 mg/kg), se realizaron tratamientos con estas bajas dosis de agonistas de los receptores en la autoadministración de cocaína y se estudiaron los efectos neuroquímicos y conductuales. No se demostraron efectos en la autoadministración de cocaína, pero se indujeron aumentos marcados y altamente significativos mediante el ensayo de ligadura de proximidad (PLA) en la densidad de los heterocomplejos A2AR-D2R en la concha del núcleo accumbens. También se observaron disminuciones significativas en la afinidad de los sitios de unión de agonistas D2R de alta y baja afinidad. Así, en dosis bajas, los efectos neuroquímicos altamente significativos observados con el co-tratamiento de agonistas de A2A y Sigma1R en los heterocomplejos A2AR-D2R y su mejora de la inhibición alostérica de la unión de alta afinidad de D2R no están vinculados a la modulación de la autoadministración de cocaína. La explicación podría estar relacionada con un aumento en la liberación de ATP y adenosina por la autoadministración de cocaína desde los astrocitos en la concha del núcleo accumbens.

II. El desarrollo de inhibidores de moléculas pequeñas y anticuerpos que apuntan a las interacciones receptor-receptor GPCR y las interacciones de los receptores tirosina quinasa (RTK), como el Factor de Crecimiento de Fibroblastos 1 (FGFR1), es de gran interés neurofarmacológico. Sin embargo, las técnicas bioquímicas convencionales utilizando lisados celulares o de tejidos y experimentos de coinmunoprecipitación para investigar complejos homoreceptores y heteroreceptores en el tejido cerebral no siempre son concluyentes. Además, los aspectos espacio-temporales de la actividad de RTK

y las interacciones receptor-receptor GPCR son esquivos. Se describe aquí un método elegante y relativamente simple, la técnica de ligadura de proximidad in situ (PLA in situ), que puede utilizarse para abordar estos problemas. Los datos experimentales resaltan la ventaja de la técnica PLA in situ para la visualización y cuantificación in situ de las interacciones dependientes de ligandos de FGFR1 en células intactas y en el tejido cerebral, así como para el estudio de las interacciones receptor-receptor GPCR en el tejido cerebral. La posibilidad de detectar receptores endógenos no modificados in situ y visualizar interacciones individuales con resolución espacial es la principal ventaja de esta técnica. También se proporciona evidencia de que se encuentran alteraciones en la activación de los complejos homoreceptores FGFR1 en el sistema 5-HT raphe-hipocampal en el modelo genético de depresión (ratas FSL). Estas observaciones ofrecen una nueva comprensión de la depresión mayor.

III. En el capítulo 3 nuestros hallazgos y evidencias experimentales brindan valiosas perspectivas sobre las complejas interacciones entre los receptores de serotonina (5HT2C) - oxitocina (OXTR) en el sistema nervioso central (SNC). La depresión mayor es un trastorno complejo y altamente prevalente con opciones de tratamiento limitadas. Las dificultades en el tratamiento de la depresión pueden estar relacionadas con la heterogeneidad molecular subyacente, ya que el fenotipo depresivo ampliamente entendido puede originarse a partir de diversas disfunciones celulares y moleculares. En este estudio, se evalúa el papel de los complejos heteroreceptores del Receptor Acoplado a Proteínas G (GPCR) en la depresión. Específicamente, se cuantifica la expresión del complejo heterodímero GPCR serotonina 2C - oxitocina (5-HT2C-OXTR) en el hipocampo de ratas, utilizando dos modelos separados de depresión. Los resultados sugieren una disminución en la expresión del heterodímero en el hipocampo de ratas deprimidas, con el modelo de la Línea Sensible de Flinders (FSL) mostrando disminuciones en todo el hipocampo, y el modelo de bulbectomía olfativa solo mostrando diferencias en la región CA1. Además, se evaluó el efecto del tratamiento con ketamina en ratas bulbectomizadas y se observó un aumento no significativo en la expresión del heterodímero en comparación con las ratas bulbectomizadas no tratadas. Estos hallazgos sugieren que una reducción en los heterodímeros 5-HT2C-OXTR en el hipocampo puede contribuir al desarrollo del fenotipo depresivo. Se demostró la desregulación del complejo 5-HT2C-OXTR en los modelos OBX y FSL de depresión mayor por primera vez, y este trabajo destaca la necesidad de un análisis más profundo de los heteroreceptores y su papel en esta enfermedad. La capacidad de los GPCRs para heterodimerizarse sigue siendo un dominio en gran parte inexplorado en

medicina y es quizás crucial en el campo de la neuropsiquiatría, donde la mayoría de los fármacos disponibles modulan sistemas que dependen de los GPCRs. Aunque es posible que el receptor 5-HT2C-OXTR sea un objetivo potencial para los antidepresivos, es necesario subrayar la necesidad más general de caracterizar los heterodímeros de los GPCR. Se ha descrito una amplia gama de heterodímeros en la literatura, pero la investigación es escasa en el contexto de la psicopatología o el tratamiento farmacológico. Hasta la fecha de esta redacción, no hay intervenciones farmacológicas que se dirijan selectivamente a los heterodímeros de los GPCR, y esto podría llevar a una nueva generación de compuestos con mayor eficacia y especificidad, lo cual es especialmente necesario en el tratamiento de la depresión mayor.

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Annexes

Annex 1

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RESEARCH ARTICLE

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GALR2 and Y1R agonists intranasal infusion enhanced adult ventral hippocampal neurogenesis and antidepressant-like effects involving BDNF actions

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Abstract

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 GAL2 receptor (GALR2) and Y1R agonists interaction upon intranasal infusion in the ventral hippocampus. We studied their hippocampal proliferating actions using the proliferating cell nuclear antigen (PCNA) on neuroblasts or stem cells and the expression of the brain-derived neurotrophic factor (BDNF). Moreover, we studied the formation of Y1R–GALR2 heteroreceptor complexes and analyzed morphological changes in hippocampal neuronal cells. Finally, the functional outcome of the NPY and GAL interaction on the ventral hippocampus was evaluated in the forced swimming test. We demonstrated that the intranasal infusion of GALR2 and the Y1R agonists promotes neuroblasts proliferation in the dentate gyrus 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. Our data may suggest the translational development of

Annex 2

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Enhancement of neurogenesis and cognition through intranasal co-delivery of galanin receptor 2 (GALR2) and neuropeptide Y receptor 1 (NPY1R) agonists: a potential pharmacological strategy for cognitive dysfunctions

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Abstract

Background Spatial memory deficits and reduced neuronal survival contribute to cognitive decline seen in the aging process. Current treatments are limited, emphasizing the need for innovative therapeutic strategies. This research explored the combined effects of intranasally co-administered galanin receptor 2 (GALR2) and neuropeptide Y1 receptor (NPY1R) agonists, recognized for their neural benefits, on spatial memory, neuronal survival, and differentiation in adult rats.

After intranasal co-delivery of the GALR2 agonist M1145 and a NPY1R agonist to adult rats, spatial memory was tested with the object-in-place task 3 weeks later. We examined neuronal survival and differentiation by assessing BrdU-IR profiles and doublecortin (DCX) labeled cells, respectively. We also used the GALR2 antagonist M871 to confirm GALR2's crucial role in promoting cell growth.

Results Co-administration improved spatial memory and increased the survival rate of mature neurons. The positive effect of GALR2 in cell proliferation was confirmed by the nullifying effects of its antagonist. The treatment boosted DCX-labeled newborn neurons and altered dendritic morphology, increasing cells with mature dendrites.

Conclusions Our results show that intranasal co-delivery of GALR2 and NPY1R agonists improves spatial memory, boosts neuronal survival, and influences neuronal differentiation in adult rats. The significant role of GALR2 is emphasized, suggesting new potential therapeutic strategies for cognitive decline.

Keywords Neurogenic enhancement, Cognitive enhancement, Intranasal administration, GALR2 agonists, NPY1R agonists, M1145, Spatial memory performance, Neuronal survival, Neuronal differentiation

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