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STUDY OF G PROTEIN-COUPLED RECEPTOR (GPCR) HOMO- AND HETERORECEPTOR COMPLEXES INTERACTIONS IN THE CENTRAL NERVOUS SYSTEM THROUGH PROXIMITY LABELLING TECHNIQUES

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ABSTRACT

This PhD thesis is focused on investigating GPCR receptor-receptor interactions in the central nervous system (CNS), with a specific emphasis on the adenosine A2AR and dopamine D2-likeR homo and heteroreceptor complexes. The thesis utilizes various methods and techniques, including biochemical binding studies, bioluminescence resonance energy transfer (BRET), and proximity ligation assay (PLA), to study these complexes.

Adenosine, an endogenous nucleoside, functions as a neuromodulator in many regions of the CNS. Its intracellular concentration depends on the breakdown and synthesis of ATP, which is metabolized to AMP. Adenosine is then formed from AMP through the action of a 5'-nucleotidase, and its intracellular and extracellular concentrations are balanced by equilibrative transporters. The main sources of extracellular adenosine are likely intracellular adenosine released from active cells in response to increased metabolic demand. Adenosine acts as a VT signal due to its presence in and release into the ECF, coupled with the existence of extrasynaptic adenosine receptors.

The two major adenosine receptors in the CNS are the adenosine A1 and A2A receptors. A1R receptors are widely distributed in the brain but are predominantly expressed in the hippocampus, cerebellum, and neocortical areas. On the other hand, A2AR receptors have a more restricted brain distribution, with the highest density found in the striatum. Within the striatum, A2AR receptors are particularly concentrated in the GABAergic striato-pallidal neurons, alongside the D2R receptors. Conversely, D1R receptors are predominantly found in the direct pathway, striato-entopeduncular, and striato-nigral GABAergic neurons.

For many years, it has been postulated that adenosine-dopamine interactions primarily occur via receptor-receptor interactions in A2AR-D2R and A1R-D1R heteromers, located perisynaptically at glutamate synapses on the striato-pallidal and striato-entopeduncular/nigral GABAergic neurons, respectively. Although such interactions may be relatively infrequent, it is possible that adenosine, through VT into dopamine synapses, directly modulates synaptic dopamine transmission via synaptic A2AR-D2R and A1R-D1R heteromers.

The thesis begins by providing an overview of previous research on allosteric receptor-

receptor interactions in brain dopamine and adenosine transmission, highlighting the A2AR-D2R and A2AR-D3R interactions. It also discusses the existence of A1R-D1R interactions in the basal ganglia and the formation of A2AR-D2R, D2R-D4R, and D1R-D2R heteroreceptor complexes. Additionally, it explores the interactions between 5-HT receptor subtypes and DA receptor subtypes, particularly the D2R-5-HT2AR and D2R-5-HT1A heteroreceptor complexes.

The thesis also delves into the field of neuropeptide-monoamine receptor-receptor interactions in the CNS, suggesting the presence of not only GPCR monomers but also GPCR homo and heteroreceptor complexes, including receptor dimers, higher-order receptor complexes, and receptor-interacting proteins. It reviews biochemical binding studies that provide evidence for the existence of allosteric receptor-receptor interactions in brain dopamine and adenosine transmission, particularly in the basal ganglia. The thesis also discusses the findings from BRET and FRET studies that support the existence of A2AR-D2R and A1R-D1R heterocomplexes, which play a role in modulating specific neural pathways. Furthermore, it highlights the interaction between different DA receptor subtypes, such as D2R-D4R and D1R-D2R heteroreceptor complexes, particularly in the basal ganglia. These interactions involve bidirectional modulation of receptor recognition, signaling, and trafficking, including high-order heteroreceptor complexes.

The field of receptor-receptor interactions has opened up new avenues for drug development, and several strategies can be explored based on receptor-receptor interactions in receptor heteromers. Higher-order receptor heteromers, also known as receptor mosaics, offer additional targets for drug development. Novel drugs may be developed to modify the composition, topography, order of activation, and allosteric regulation of receptor mosaics. Drugs may impact various aspects, including the synthesis and release of receptor oligomeric building blocks, their insertion into the plasma membrane, the internalization of receptor mosaics, and the adapter and scaffolding proteins organizing the receptor mosaics. Additionally, allosteric modulators that affect the functional state of individual receptors within receptor mosaics may also be developed.

The thesis emphasizes the importance of developing allosteric modulators and their potential effects on the integrative function of receptor mosaics, receptor mosaics assembly, G protein and β-arrestin coupling, and receptor recognition. The discovery of an allosteric D2R antagonist, homocysteine (Hcy), which interacts with the third intracellular loop (IC3) of the D2R and modulates its binding and function, exemplifies the potential of allosteric modulation as a therapeutic approach. Such allosteric ligands can target different

GPCR oligomers, including receptor mosaics, and can provide new opportunities for selective drug development.

The thesis also explores the development of A2AR antagonists targeting A2AR-D2R heteroreceptor complexes in the dorsal striato-pallidal GABA pathway. Studies have shown that A2AR antagonists can increase locomotor activity in Parkinson's disease (PD) models when combined with sub-threshold doses of L-DOPA and D2R agonists in reserpinized mice. These antagonists have also been found to reverse Parkinsonian deficits in non-human primates and rodents. Notably, tolerance development is not observed with subchronic A2A antagonist treatment, making them suitable for clinical development. The hypothesis suggests that A2AR antagonists enhance D2R signaling in A2AR-D2R heteroreceptor complexes, which are constitutive and exist even in the absence of agonist activation. Therefore, it is unlikely that A2A antagonist treatment disrupts these heteroreceptor complexes, but it may induce conformational changes that alter their integrative activity. Clinical studies using the A2AR antagonist istradefylline (KW-6002) have shown symptomatic improvement, particularly in advanced PD patients with dyskinetic complications. Additionally, A2AR blockade may improve bradykinesia, muscle rigidity, and resting tremor in PD patients.

The thesis proposes that A2AR antagonists should be administered in combination with close-to-threshold doses of L-DOPA and/or D2R agonists to optimize their effects in PD patients. By targeting A2AR-D2R heteroreceptor complexes, A2AR antagonists enhance D2R signaling at the soma-dendritic level, leading to a reduction in the activity of the striato-pallidal GABA/enkephalin pathway. This reduction in motor inhibition restores the motor drive to some extent. Combined treatment with L-DOPA restores D1R activity in the direct pathway, aiding motor initiation. The integration of the direct and indirect pathways in the globus pallidus interna and zona reticulata of the substantia nigra inhibits GABA projections to the motor thalamus, allowing for the removal of GABA inhibition on the excitatory glutamate thalamo-cortical pathway and the restoration of movements.

The thesis also considers the balance between A2AR homomers, D2R homomers, and A2AR-D2R heteroreceptor complexes and the potential interactions between several GPCRs and alpha-synuclein. Prolonged L-DOPA treatment may disrupt this balance, leading to increased A2AR signaling compared to D2R signaling. This disruption may contribute to the reduction of therapeutic effects of L-DOPA and the emergence of L-DOPA-induced dyskinesias. The hypothesis suggests that L-DOPA-induced co-internalization of A2AR-D2R heteromers and D2R homomers results in compensatory up-

regulation of A2AR and an increase in A2AR homomers. This up-regulation of A2AR leads to increased inhibition of PP-1 and protein phosphorylation, potentially stabilizing pathological receptor mosaics formed under excessive D2R activation induced by L-DOPA. These receptor mosaics may contribute to the development of dyskinesias. This hypothesis provides an explanation for the observed anti-dyskinetic effect of A2AR antagonists, which cannot be solely attributed to the enhancement of D2R signaling in the heteromer.

Moreover, the thesis mentions the existence of A2AR-D3R and A2AR-D4R heteroreceptor complexes and emphasizes the importance of studying GPCR homo and heteroreceptor complexes. The interaction between A2AR and D3R is demonstrated through FRET experiments and the antagonistic nature of their interaction is confirmed in membranes prepared from stably transfected CHO cell lines. Similarly, evidence suggests the existence of A2AR-D4R heteroreceptor complexes based on the interaction between the arginine-rich epitope in the D4R and negatively charged epitopes in the A2AR carboxyl terminus. The A2AR and D4R may be codistributed especially in the island (striosome, patch) striato-nigral GABAergic system at the soma-dendritic level, where we demonstrated that A2AR-D4R heteroreceptor complexes exist. The striatal island system is involved in cognitive, reward and motivational functions, which may be modulated by the discovered A2AR-D4R heteroreceptor complexes. These heteroreceptor complexes may have functional implications in the nucleus accumbens and the striato-nigral GABAergic system, respectively.

The thesis then delves into the novel methods and techniques used to study GPCR homo and heteroreceptor complexes, aiming to provide a deeper understanding of their molecular aspects and allosteric receptor-receptor interactions in the CNS. One of the key techniques discussed is Bioluminescence Resonance Energy Transfer (BRET), which are powerful tool for determining receptor oligomeric order, assessing ligand-induced signals, and studying affinity relationships. These techniques offer valuable insights into the complex dynamics of GPCR complexes.

In addition to BRET, the thesis introduces the in situ Proximity Ligation Assay (in situ PLA), a cutting-edge technique used to visualize the proximity of interacting molecules within a biological sample. In the context of GPCR research, in situ PLA enables the detection and quantification of native heteroreceptor complexes in the CNS. The assay involves the use of primary antibodies that recognize the receptor complexes, followed by secondary antibodies linked to oligonucleotides. When the two antibodies bind to the

receptor complex, the oligonucleotides join through enzymatic ligation, forming a DNA circle strand that serves as a template for amplification and detection through hybridization with fluorescent oligonucleotides. This technique enables the visualization of heteroreceptor complexes by fluorescence microscopy, facilitating the study of their number, localization, and modulation in brain tissue. The use of in situ PLA provides significant advantages in terms of its ability to detect and quantify heteroreceptor complexes ex vivo in brain tissue samples, thus enhancing our understanding of their distribution and functional implications.

To advance our knowledge of adenosine A2AR and dopamine D2R heteroreceptor complexes in vivo and ex vivo, it is crucial to employ innovative techniques. One approach involves the use of proximity ligation assays to identify and analyze the heteroreceptor complexes. By examining these complexes in animal models of neurological and mental diseases such as Parkinson's disease and schizophrenia, we can gain insights into their roles in disease pathogenesis and potential therapeutic interventions. In parallel with in vivo studies, biochemical in vitro work is essential for unravelling the pharmacology of various adenosine and dopamine heteroreceptor complexes. This work can be facilitated by computerized modelling of the homo and heteroreceptor complexes, as well as bioinformatic analysis of the participating receptors. By combining these approaches, researchers can identify adenosine and dopamine receptor interfering drugs that preferentially interact with distinct heterodimers, opening up new possibilities for targeted therapeutic interventions.

Lastly, the thesis emphasizes the importance of establishing and characterizing the role of allosteric modulators in adenosine and dopamine heteroreceptor complexes, as well as their integration with receptor-receptor interactions to modulate the orthosteric sites and the coupling of receptors to G proteins and beta-arrestins. Understanding these integrative processes is crucial for drug development in the field of neuropsychopharmacology.

We are currently witnessing a revolutionary period in neurobiology, driven by remarkable advances in proteomic and transcriptomic technology, as well as the progress made in computer science. These advancements enable us to identify specific factors and protein/receptors responsible for human brain disorders. Moreover, analysing their physical interactions and allosteric receptor-receptor interactions has given us a powerful toolkit to comprehend the functioning of the nervous system in both health and disease.

Recent breakthroughs in this field include molecular-systems methods that allow us to dynamically measure protein/receptor products in a highly parallel manner, coupled with a

comprehensive understanding of the organization of these products. This integrated approach gives us unprecedented insights into nervous system function. By examining receptor-receptor interacting complexes in relation to others, we can explore previously uncharted territories of biology, generate new hypotheses, and evaluate these hypotheses through quantitative reasoning.

To identify membrane heteroreceptor complexes, we employ diverse techniques such as whole brain mapping of receptor complexes through in situ PLA and interacting proteins association studies. Additionally, single-cell analysis helps us measure various forms of protein complex products and study their post-transcriptional regulation. We have at our disposal a wealth of allied methods for investigating their regulation, along with various biochemical and biophysical tools. In vitro modelling and advances in model organism genetics provide valuable experimental platforms for neurobiological research and hypothesis testing, both in terms of low and high throughput. Finally, we have access to multiple high-throughput assays, ranging from arrayed and pooled CRISPR screening approaches to multiple parallel reporter assays, which enable wide-ranging functional screening of receptors.

In this thesis, we will introduce the analysis of GPCR homo and heteroreceptor complexes using three different proximity ligation assays approaches. Moreover, we will address the significant challenges in integrating various levels of analysis, from genes to pathways to cells and circuits, to comprehend how variations in heteroreceptor complexes contribute to behavioural and cognitive phenotypes.

The overall aim of this thesis is to provide insights into the molecular aspects of adenosine A2AR and dopamine D2R heteroreceptor complexes and their allosteric receptor-receptor interactions in the CNS. As a proof of concept, the thesis optimizes and utilizes in situ Proximity Ligation Assay methods to study A2AR and D2R isoforms, homo and heteroreceptor complexes. By employing these advanced techniques and exploring the intricate dynamics of GPCR complexes, this research aims to advance our understanding of GPCR signaling in the CNS and open new avenues for therapeutic interventions in neurological and mental disorders.

The specific aims of the thesis are outlined as follows:

AIM-1: Adenosine, through its four receptor subtypes (A1R, A2AR, A2BR, A3R), plays a critical role in various physiological processes, including neuronal signaling, astrocytic function, learning and memory, motor function, and normal aging. However, adenosine is also implicated in several neuropathologies, such as epilepsy, schizophrenia, addiction, and

Parkinson's disease. Of particular interest are A1R and A2AR, which are highly expressed in many brain regions and serve as central components of the global GPCR heterodimer network, forming numerous heteroreceptor complexes. Despite our understanding of this integrative mechanism in the plasma membrane, little is known about the formation of adenosine A2A isoreceptor complexes. Therefore, investigating the distribution patterns of A2AR homoreceptor complexes and A1R-A2AR isoreceptor complexes in the hippocampus and other forebrain areas using the proximity ligation assay (PLA) has become a compelling objective.

AIM-2: The identification of adenosine and dopamine receptor mosaics (higher-order receptor heteromers) in the striatum has provided new insights into adenosine-dopamine interactions within the central nervous system (CNS). Initial discoveries revealed the presence of A2AR-D2R and A1R-D1R heterodimers in the striatum, followed by indications of striatal A2AR-D3R and A2AR-D4R heterodimers. A2AR and D4R co-localization in the dorsal striatum suggests their fundamental involvement in complex behaviors and motor function. The existence of A2AR-D4R heteroreceptor complexes in several regions of the rat brain will be explored using in situ proximity ligation assays (in situ PLA).

AIM-3: Notably, A2ARs are known to modulate the aggregation of alpha-synuclein, a protein associated with Parkinson's disease pathology. A2AR antagonists have been shown to inhibit the late stages of alpha-synuclein aggregation, reducing its toxicity. Therefore, our aim is to investigate potential interactions between alpha-synuclein and several GPCRs, including A2AR, D2R, and D4R. In situ proximity ligation assay (PLA) will be employed in the BSSG animal model to examine these interactions. To serve as a positive control, DAT-alpha-synuclein interactions will also be assessed. Additionally, we will explore how the expression of alpha-synuclein (both wild type and A53T mutant) modulates A2AR-D2R and A2AR-D4R heteroreceptor complexes using in situ PLA in cell lines and the BSSG model.

By addressing these aims, we aim to advance our understanding of the distribution, formation, and functional significance of adenosine receptor complexes, dopamine receptor complexes, and their interactions with alpha-synuclein. These investigations will provide crucial insights into the underlying mechanisms of neurological disorders and may open avenues for the development of novel therapeutic approaches.

The thesis comprises three chapters that address these aims. Chapter 1 optimizes several variants of the PLA method for the detection and quantification of adenosine isoreceptor

complexes. Chapter 2 provides evidence for the existence of A2AR-D4R heteroreceptor complexes in the rat brain. Chapter 3 focuses on understanding alpha-synuclein-GPCR heteroreceptor complexes in a rat model of Parkinson's disease, highlighting interactions between alpha-synuclein and D2-like GPCRs.

Chapter 1 of the thesis delves into comprehensive studies on adenosine isoreceptor complexes, shedding light on the pivotal role of adenosine and its receptor subtypes in modulating various physiological processes and their involvement in neuropathologies. The distribution patterns of A2AR homoreceptor complexes and A1R-A2AR isoreceptor complexes in the hippocampus and other forebrain areas are investigated using a combination of PLA and immunohistochemistry techniques. The findings reveal the highest densities of A2AR-A1R isoreceptor complexes and A2AR-A2AR homoreceptor complexes in specific regions of the hippocampus, indicating a delicate balance between these complexes in regulating adenosine A2AR signaling and neuroinflammation within the hippocampus.

Furthermore, the current study reports the existence of A2A-A2A homoreceptor and A1-A2A heteroreceptor complexes in the dorsolateral cerebral cortex and the piriform cortex, exhibiting similar distribution patterns using the PLA technique. These intriguing findings open up new avenues for understanding the brain circuits modulated by adenosine receptor complexes and their relevance to learning, memory, and psychiatric diseases.

Through bioinformatics analysis of the A1-A2A heteromers, it is revealed that their receptor interface differs from that of A1-D1 and A2A-D2 receptor heteromers. The A1-A2A heteromer exhibits unique AKS and KSL amino acid homologies, which are absent in the A1-D1 and A2A-D2 heteromers, indicating different types of homologies. These results emphasize the specificity of the heteromerization process and suggest that adenosine receptor heteromers formed by interacting with other receptors using different transmitter/modulator ligands can have distinct receptor interfaces.

The highest densities of A1-A2A heteroreceptor complexes are found in the pyramidal cell layers of CA1-CA3 and the polymorphic cell layer in the hilus of the dentate gyrus in the hippocampus. Previous research has demonstrated the co-expression of adenosine A1 and A2A receptors in many hippocampal glutamatergic pyramidal nerve cells and in glutamate nerve terminals. Additionally, indications have been obtained through co-immunoprecipitation and biochemical binding experiments that A1-A2A heteroreceptor complexes may be present on striatal glutamate nerve terminals. These observations suggest that antagonistic A2A-A1 receptor-receptor interactions can occur in these

complexes, where A2A receptor activation reduces the affinity of the A1 agonist binding sites. Based on these findings, it is proposed that the A2A-A2A and A1-A2A PLA blobs in the pyramidal cell layer of the hippocampus may exist in the soma-dendrites of the pyramidal cells and in local glutamate collaterals of the CA3 and CA2 regions. The precise localization of these adenosine receptor complexes within specific hippocampal regions suggests their involvement in regulating glutamate signaling and synaptic plasticity.

The intricate balance between A1-Gi/o and A2A-Gs/olf mediated receptor signaling in the hippocampus, which exert opposing actions on the AC-PKA-CREB signaling pathway to the nucleus, glutamate release, and brain function pathology such as seizures, is facilitated by a dynamic equilibrium of adenosine receptor signaling within A2A-A2A, A1-A1 homoreceptor complexes, and A1-A2A heteroreceptor complexes. This balance occurs within individual pyramidal cells and between pyramidal cells with different ratios of these major complexes mediating adenosine signaling. The importance of this balance is demonstrated by the ability of optogenetic activation of hippocampal A2A receptor signaling through phosphorylation of CREB to impair memory. Similarly, A2A inactivation enhances working memory and reversal learning. The proposed reorganization of homo and heteroreceptor complexes in the postsynaptic membrane provides a possible molecular basis for learning and memory. In learning, the transient reorganization of sets of homo and heteroreceptor complexes in postsynaptic and associated extrasynaptic membranes enables the acquisition of a new temporal pattern of neurotransmitter release, forming a short-term memory of the novel pattern. This short-term memory can be converted into long-term memory through newly formed adapter proteins that stabilize the receptor complexes and link them to the cytoskeleton. Disruption of the balance of signaling within adenosine homo and heteroreceptor complexes can lead to errors in the formation of molecular engrams at hippocampal glutamate synapses and impair spatial memory performance.

Chapter 2 of the thesis focuses on investigating the existence of A2AR-D4R heteroreceptor complexes in the rat brain. Cellular models and in situ PLA techniques are employed to detect these complexes. The formation of A2AR-D4R heteroreceptor complexes is confirmed through BRET1 techniques in HEK cells. This study further explores the allosteric receptor-receptor interactions within A2AR-D4R heteromers, revealing antagonistic interactions between the orthosteric binding sites of the corresponding heteromers. Analysis of the Gi/o-AC-PKA-CREB pathway using the CREB luciferase reporter gene assay demonstrates that the presence of an A2AR-D4R heteroreceptor

complex reduces the potency of the D4R-specific agonist PD-168077 in inhibiting the CREB signal. The antagonistic allosteric receptor-receptor interactions significantly inhibit Gi/o-mediated D4R protomer signaling. These findings indicate the significant role of A2AR-D4R heteroreceptor complexes in neuroplasticity. In situ PLA experiments identify these receptor complexes in high densities, mainly in the ventral striatum, the prefrontal cortex, and the pyramidal cell layer of the hippocampus. They appear as red clusters ranging in size from 0.5 to 2 μm, located in the soma outside the nuclei. These complexes are constitutive and have also been demonstrated in cellular models using BRET techniques.

Early studies have indicated that A2AR can form heteroreceptor complexes with D4R based on their interface, which involves electrostatic interactions similar to those described for the A2AR-D3R heteromer. It has been demonstrated that the D4R is enriched in the striosomes but also found in the matrix of the dorsal striatum. Therefore, the observation of A2AR-D4R heteroreceptor complexes in the dorsal and ventral striatum using proximity ligation assays is highly significant. In the dorsal striatum, the A2AR-D4R complexes are primarily located in the striosomes, which utilize GABA as a transmitter and directly project to nigral DA neurons, inhibiting their activity. Hence, the presence of A2AR-D4R heteroreceptor complexes in the striosomal GABA neurons is particularly intriguing. It is plausible that the antagonistic allosteric receptor-receptor interaction in this complex leads to a reduced Gi/o-mediated inhibitory D4R signaling, resulting in enhanced activity of the striosomal GABA neurons projecting directly to nigral DA cells. Consequently, a reduction in the activity and turnover of dopamine should occur in the DA nerve cells projecting to the striosomes.

Moving on to Chapter 3, this chapter investigates the interactions between alpha-synuclein and D2-like GPCRs (D2R and D4R), as well as adenosine A2AR, in the rat brain, particularly in the dorsal and ventral striatum. In situ PLA and BRET techniques are employed to provide evidence for the existence of these complexes. For the first time, this study reveals the presence of D2R-alpha-synuclein and D4R-alpha-synuclein interactions in the striatum of the rat brain. Additionally, the study confirms A2AR-alpha-synuclein and DAT-alpha-synuclein interactions using in situ PLA. These complexes are observed in both Sprague-Dawley (SD) control rat brains and in the BSSG rat model of Parkinson's disease (PD), and they are enriched in the dorsal and ventral striatum. These findings align with previous observations of alpha-synuclein interacting with several membrane receptors, such as NMDAR 2B, B2-adrenergic, mGluR5, M5R, and EGFR. Mass spectrometry, co-

immunoprecipitation, affinity-capture-western, and two-hybrid studies have demonstrated that alpha-synuclein can interact with over 500 proteins, both intracellular and membrane-associated. This complexity highlights the intricacies of synucleinopathies. The in situ PLA experiments in the rat brain also reveal the capacity of alpha-synuclein to interact with several proteins involved in dopamine signaling and neurotransmission, including D2R, D4R, and DAT.

Notably, a significant increase in receptor-alpha-synuclein interactions is observed for D2R and DAT in the BSSG model compared to the SD control group, supporting the hypothesis. Conversely, no significant difference is found in the alpha-synuclein-D4R interactions between the BSSG model of PD and the SD control groups. The increased densities of D2R-alpha-synuclein and DAT-alpha-synuclein complexes in the BSSG animal model may be attributed to the preferential affinity of the D2R and DAT for a specific conformational state of alpha-synuclein protein. Certain oligomeric forms of alpha-synuclein, which are present in the BSSG rat model and replicate human PD alpha-synuclein forms, may contribute to these changes. The variations in the densities of each complex in the BSSG model could also be influenced by cell type vulnerability, such as presynaptic dopamine terminals (varicosities) versus postsynaptic striatopallidal GABA neurons and/or astrocytes, as well as differences in the striatal compartments (matrix versus striosomes). The identification of such complexes represents a significant step towards a deeper understanding of the molecular pathophysiology underlying PD, offering great potential for therapeutic and diagnostic advancements. The study further explores the modulatory effects of receptor agonists and antagonists on these interactions, highlighting their potential therapeutic implications in Parkinson's disease.

Future studies should delve into how the signal transduction pathways of the involved GPCRs are modified in response to complex formations. This will provide insights into how GPCR functions are altered in the presence of alpha-synuclein (forming GPCR-alpha-synuclein complexes) and shed light on the resulting cellular effects and pathology of PD. For instance, conducting a cAMP-Glo assay to measure cAMP levels upon GPCR activation (Gs/olf) and blockade (Gi/o) could help understand the extent to which allosteric receptor-alpha-synuclein interactions contribute to these effects. A plausible hypothesis is that alpha-synuclein binding to the D2R and DAT antagonizes these receptors, leading to reduced D2R signaling and DAT activity, ultimately resulting in diminished dopamine activity. This exacerbates the dopamine depletion characteristic of PD. Furthermore, investigating the impact of GPCR protomer activation (agonist) or blockade (antagonist)

within the GPCR-alpha-synuclein complexes on alpha-synuclein distribution, function, and post-translational modifications (e.g., oligomerization) could offer valuable insights into potential novel therapeutic approaches and reveal the molecular mechanisms of such drugs in new contexts.

Overall, this thesis contributes to our understanding of molecular aspects of adenosine A2AR and dopamine D2R heteroreceptor complexes and their allosteric receptor-receptor interactions in the CNS. Advanced techniques such as PLA and BRET are used to visualize and characterize these complexes, providing insights into their role in normal physiological processes and neuropathology's. The findings have implications for the development of targeted therapeutic interventions for neurological disorders associated with GPCR dysregulation.

RESUMEN EN ESPAÑOL

Esta tesis de doctorado se llevó a cabo entre el año 2018-2023 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 Suecia. La tesis se centra en la investigación de las interacciones de los receptores acoplados a proteína G (GPCRs) en el sistema nervioso central (SNC). Con un particular énfasis en la existencia e implicaciones funcionales de los complejos homo y heteroreceptor de adenosina A2AR y de dopamina D2-likeR en enfermedades mentales y neurodegenerativas, empleando modelos de ratas. La tesis utiliza diversos métodos y técnicas, como estudios de unión bioquímica, histoquímica, transferencia de energía de resonancia bioluminiscente (BRET), modelos matemáticos y/o bioinformáticos y ensayo de ligadura de proximidad (in situ PLA), para estudiar estos complejos.

La tesis comienza proporcionando una visión general de investigaciones anteriores sobre las interacciones alostéricas receptor-receptor en la transmisión de dopamina (DA) y adenosina en el cerebro, destacando específicamente las interacciones A2AR-D2R y A2AR-D3R. También se discute la existencia de interacciones A1R-D1R en el cuerpo estriado y la formación de complejos heteroreceptor A2AR-D2R, D2R-D4R y D1R-D2R. Además, se exploran las interacciones entre subtipos de receptores 5-HT y subtipos de receptores de DA, haciendo hincapié en la presencia de complejos heteroreceptor D2R-5-HT2AR y D2R-5-HT1A. Aborda también los estudios recientes que demuestran una interacción física entre los receptores de la familia de los GPCRs y los de la familia de los receptores de tipo tirosina quinasa (RTK). En particular se ejemplifican los trabajos de Marc Flajolet y Paul Greengard y el descubrimiento de la interacción entre el receptor de adenosina A2A y el FGFR1. Así como los trabajos de Dasiel O. Borroto-Escuela en los descubrimientos de las interacciones entre el FGFR1 y los receptores de serotonina de tipo 1A y 1B (5HT1A y 5HT1B respectivamente).

En la parte introductoria de la tesis se abordan además todas las evidencias experimentales (desde una perspectiva cronológica) sobre las interacciones de los receptores de los neuropéptidos y las monoaminas en el SNC , que en los 80-90 indicaron la existencia no solo de monómeros de GPCR, sino también de complejos homo y heteroreceptor de GPCRs. Hoy se ha podido confirmar con una serie de herramientas bioquímicas y biofísicas que estos receptores además de dímeros (homo y heterodímeros), pueden formar complejos de receptores de orden superior

(conocidos como oligómeros). Además, interactuar con una serie muy grande de proteínas citosólicas intracelulares (ejemplo, b-arrestina, GRK, calmodulina) y de membrana (receptor sigma 1, RAMP), así como con diferentes tipos de proteínas adaptadoras y proteínas sinápticas o no sinápticas.

Una gran cantidad de estudios en los años 80 se plantearon como objetivo la comprensión de la existencia de interacciones alostéricas receptor-receptor en la transmisión de dopamina (DA) y adenosina (ADA) en el cuerpo estriado. Todos ellos partían de estudios de unión bioquímica, especialmente en relación con las interacciones A2AR-D2R, pero también en los complejos A2AR-D3R y en la posible existencia de complejos A2AR-D4R. Los resultados de estos estudios arrojaron muestras fehacientes de la existencia de este tipo de modulaciones alostéricas y abrieron el camino a posteriores estudios en el resto de los miembros de la gran familia de los GPCRs. Postularon también, que los receptores de dopamina se encuentran principalmente en regiones extrasinápticas, lo que probablemente también sea cierto para estos heterocomplejos. En 1998 también se observaron interacciones antagonistas A1R-D1R en estudios de unión en el cuerpo estriado, reflejando interacciones alostéricas receptor-receptor en las regiones extrasinápticas entre estos dos tipos de receptores. Posteriormente, en 2003 y 2008, surgieron pruebas de estudios de BRET y FRET sobre la existencia de heterocomplejos A2AR-D2R y A1R-D1R, y se demostró que los complejos A2AR-D2R son los complejos que principalmente modulan las neuronas GABA estriatales-palidales, conocidas por inhibir la iniciación de los movimientos, y los complejos A1R-D1R los que principalmente modulan la vía GABA directa, conocida por potenciar los movimientos. Hoy se sabe, que los subtipos de receptores de dopamina también interactúan entre sí para formar, por ejemplo, isoreceptores, tales como los de D2R-D4R y D1R-D2R, especialmente en el cuerpo estriado. Las pruebas disponibles sugieren que las interacciones alostéricas receptor-receptor pueden involucrar la modulación bidireccional tanto del reconocimiento de los receptores como de la señalización y el tráfico, e incluir complejos heteroreceptor de alto orden.

Se finaliza este apartado introductorio con un resumen detallado de todos los métodos y técnicas novedosas que se utilizan para estudiar los complejos homo y heteroreceptor de GPCR. Se describen, por ejemplo, los principios y aplicaciones de la transferencia de energía de resonancia por fluorescencia (FRET) y bioluminiscente (BRET), destacando su capacidad para determinar el orden oligomérico de los receptores y evaluar las señales inducidas por ligandos y las relaciones de afinidad. También se presenta el ensayo de ligadura de proximidad in situ (in situ PLA), una técnica muy novedosa que visualiza la proximidad de las moléculas que interactúan dentro de una muestra biológica, lo que permite la detección y cuantificación de

complejos heteroreceptor nativos en el SNC. En esta sección, se destacan también las principales ventajas del uso de los ensayos por proximidad.

El empleo de los métodos de in situ PLA ha revolucionado el campo, permitiendo el análisis de las interacciones entre los diferentes tipos de receptores de membrana en su estado nativo o con niveles de expresión endógena no solo en células sino también en tejido. Este ensayo implica el uso de anticuerpos primarios que reconocen el dímero, seguidos de anticuerpos secundarios unidos a oligonucleótidos. Cuando los dos anticuerpos reconocen el dímero, los oligonucleótidos se unen mediante ligadura enzimática, formando una cadena de ADN circular que sirve como plantilla para la amplificación y detección a través de la hibridación con oligonucleótidos fluorescentes. El in situ PLA permite la visualización de los complejos heteroreceptor mediante microscopía de fluorescencia, lo que permite el estudio de su número, localización y modulación en tejido cerebral fijado en formalina. Los resultados indican que el in situ PLA se puede utilizar para demostrar complejos heteroreceptor ex vivo en tejido cerebral, como los complejos A2A-D2 heteroreceptor estriatal y los complejos D2-5HT2A heteroreceptor en la región acumbal y estriado dorsal.

Como se ha destacado anteriormente, el ensayo de ligadura de proximidad in situ (in situ PLA) es un método para visualizar la proximidad entre dos o tres moléculas que interactúan dentro de una muestra biológica. Es una mejora del ensayo de ligadura de proximidad (PLA), que originalmente se desarrolló para detectar interacciones proteicas in vitro. Esta importante técnica (PLA) fue desarrollada por Fredriksson, Gullberg, Söderberg y colegas. En el PLA, se utilizaron pares de aptámeros de ADN como sondas de afinidad para detectar homodímeros de proteínas específicas. Las sondas de PLA se extendieron con secuencias adicionales de ADN, y cuando los aptámeros se unían a sus proteínas objetivo en proximidad cercana, las secuencias adicionales de ADN podían hibridarse con un oligonucleótido conector común, lo que permitía la ligadura covalente. El producto de la ligadura se cuantificaba mediante PCR en tiempo real. En el in situ PLA, el método se adaptó para visualizar la proximidad entre proteínas endógenas utilizando pares de anticuerpos como sondas de afinidad. Cada anticuerpo se conjugó con un oligonucleótido, creando una sonda de PLA. Cuando un par de sondas de PLA se une a sus objetivos respectivos en proximidad cercana, tiene lugar la hibridación de dos oligonucleótidos adicionales para formar una molécula de ADN circular. Después de la ligadura por la ligasa T4, la molécula de ADN circular puede amplificarse mediante amplificación de círculos rodantes (RCA) utilizando la polimerasa de ADN phi29. Este proceso genera una larga molécula de ADN de cadena simple compuesta de repeticiones complementarias al círculo de ADN. El producto de RCA permanece unido al complejo de proteínas mediante una de las sondas de PLA. El círculo de ADN incluye una secuencia de detección que permite visualizar los productos de RCA

utilizando oligonucleótidos fluorescentes etiquetados, que se hibridan con la secuencia de detección repetida en el producto de RCA. Cada producto de RCA aparece como un punto brillante de aproximadamente 1 μm de tamaño cuando se visualiza mediante microscopía de fluorescencia. El gran número de fluoróforos por producto de RCA reduce la fluorescencia de fondo de la muestra en comparación con otros métodos como la inmunofluorescencia (IF) y FRET/BRET. Cada producto de RCA sirve como un marcador localizado de la proximidad de las dos sondas de PLA. Se pueden introducir sondas secundarias de PLA in situ, que consisten en pares de anticuerpos acoplados a oligonucleótidos de especies específicas, para permitir el uso de sondas generales de PLA in situ con anticuerpos primarios de diferentes especies.

Estamos en medio de una revolución en el campo de las neurociencias y la neurobiología que se basa en los avances en tecnología proteómica y transcriptómica, potenciados por los avances en ciencias de la computación. Además de identificar factores específicos y proteínas/receptores que causan trastornos cerebrales en humanos, el análisis de sus posibles interacciones físicas y las interacciones alostéricas receptor-receptor ahora proporcionan un conjunto de herramientas extraordinariamente poderosas para comprender la función del sistema nervioso en la salud y la enfermedad. Ha habido muchos avances importantes recientes. Estos incluyen métodos moleculares que de una forma sistemática permiten la medición de la expresión de RNA, microRNA, proteínas y/o receptores de membrana y sus cambios espaciotemporales en paralelo o al unísono (RNAscope, FISH, in situ PLA transcriptómica), proporcionando una comprensión más integrada de la función del sistema nervioso. Al permitirnos ver complejos receptores-receptores específicos en el contexto de todos los demás, podemos utilizar estos enfoques para descubrir una neurobiología previamente inexplorada y desarrollar nuevas hipótesis. Las herramientas específicas para la identificación de complejos homo y hetero-receptores de membrana incluyen el uso de métodos de ligación por proximidad (in situ PLA), el análisis de células individuales para medir virtualmente todos los complejos de proteínas, su regulación post-transcripcional, así como una gran cantidad de métodos bioquímicos y biofísicos.

En esta tesis, presentaremos el análisis de complejos homo y hetero-receptores de GPCR en modelos animales de rata mediante ensayos de ligadura de proximidad. Abordaremos algunos de los desafíos principales inherentes a la conexión de diferentes niveles de análisis, desde genes hasta vías celulares y circuitos, que se requieren para comprender cómo la variación de los complejos hetero-receptores conduce finalmente a fenotipos conductuales y cognitivos.

El objetivo general de la tesis es, por tanto, obtener conocimientos sobre los aspectos moleculares de varios complejos heteroreceptor de adenosina A2AR y dopamina D2R y sus interacciones alostéricas receptor-receptor en el sistema nervioso central, con

especial énfasis en el equilibrio entre sus complejos homo y heteroreceptor. Como prueba de concepto, hemos optimizado y utilizado los métodos de ensayo de ligadura de proximidad in situ (PLA) para estudiar los complejos A2AR y D2R isoformas, complejos homo y heteroreceptor.

Los objetivos específicos de la tesis se presentan de la siguiente manera:

OBJETIVO 1: La adenosina, a través de sus cuatro subtipos de receptor (A1R, A2AR, A2BR, A3R), desempeña un papel fundamental en varios procesos fisiológicos, como la señalización neuronal, la función astrocítica, el aprendizaje y la memoria, la función motora y el envejecimiento normal. Sin embargo, la adenosina también está implicada en varias neuropatologías, como la epilepsia, la esquizofrenia, la adicción y la enfermedad de Parkinson. De particular interés son los receptores A1R y A2AR, que se expresan en muchas regiones cerebrales y son componentes centrales de la red heterodimérica de GPCR global, formando numerosos complejos hetero-receptores. A pesar de nuestra comprensión de este mecanismo integrador en la membrana plasmática, se sabe poco sobre la formación de los complejos iso-receptores de A2A adenosina. Por lo tanto, investigar los patrones de distribución de los complejos de homoreceptores A2AR y los complejos de iso-receptores A1R-A2AR en el hipocampo y otras áreas del cerebro utilizando el ensayo de ligadura por proximidad (PLA) se ha convertido en un objetivo convincente.

OBJETIVO 2: La identificación de mosaicos de receptores de adenosina y dopamina (heterómeros de receptores de orden superior) en el cuerpo estriado ha proporcionado nuevos conocimientos sobre las interacciones adenosina-dopamina dentro del sistema nervioso central (SNC). Los descubrimientos iniciales revelaron la presencia de heterodímeros A2AR-D2R y A1R-D1R en el cuerpo estriado, seguidos de indicios de heterodímeros A2AR-D3R y A2AR-D4R estriatales. La co-localización de A2AR y D4R en el cuerpo estriado dorsal sugiere su participación fundamental en comportamientos complejos y la función motora. Se explorará la existencia de complejos hetero-receptores A2AR-D4R en varias regiones del cerebro de la rata utilizando ensayos de ligadura por proximidad in situ (PLA in situ).

OBJETIVO 3: Es importante destacar que se sabe que los receptores A2AR modulan la agregación de alfa-sinucleína, una proteína asociada a la patología de la enfermedad de Parkinson. Por ejemplo, se ha demostrado que los antagonistas de los receptores A2AR inhiben las etapas tardías de la agregación de la alfa-sinucleína, reduciendo su toxicidad. Por lo tanto, nuestro objetivo es investigar las interacciones potenciales entre la alfa-sinucleína y varios GPCR, incluidos los receptores A2AR, D2R y D4R. Se empleará el ensayo de ligadura por proximidad in situ (PLA) en el modelo animal BSSG para examinar estas interacciones. Además, exploraremos cómo la expresión de alfa-sinucleína (tanto el tipo salvaje como el mutante A53T)

modula los complejos heteroreceptores A2AR-D2R y A2AR-D4R utilizando PLA in situ en líneas celulares y en el modelo BSSG.

Al abordar estos objetivos, pretendemos mejorar nuestra comprensión de la distribución, formación y significado funcional de los complejos de receptores de adenosina, complejos de receptores de dopamina y sus interacciones con la alfa-sinucleína. Estas investigaciones proporcionarán conocimientos cruciales sobre los mecanismos subyacentes de los trastornos neurológicos y pueden abrir vías para el desarrollo de enfoques terapéuticos novedosos.

La tesis consta de tres capítulos que abordan estos objetivos. El Capítulo 1 optimiza varias variantes del método PLA para la detección y cuantificación de los complejos isorreceptores de adenosina. El Capítulo 2 proporciona evidencia de la existencia de complejos heteroreceptores A2AR-D4R en el cerebro de la rata. El Capítulo 3 se centra en comprender los complejos heteroreceptores de alfa-sinucleína-GPCR en un modelo de rata de la enfermedad de Parkinson, destacando las interacciones entre alfa-sinucleína y GPCR tipo D2.

El Capítulo 1 de la tesis explora de forma exhaustiva los complejos iso-receptores de adenosina, arrojando luz sobre el papel fundamental de la adenosina y sus subtipos de receptores en la modulación de varios procesos fisiológicos y su participación en neuropatologías. Se investigan los patrones de distribución de los complejos de homoreceptores A2AR y los complejos de isorreceptores A1R-A2AR en el hipocampo y otras áreas del cerebro utilizando una combinación de técnicas de PLA e inmunohistoquímica. Los hallazgos revelan las mayores densidades de complejos de isorreceptores A2AR-A1R y complejos de homoreceptores A2AR-A2AR en regiones específicas del hipocampo, lo que indica un delicado equilibrio entre estos complejos en la regulación de la señalización de la adenosina A2AR y la neuroinflamación dentro del hipocampo.

Además, estos estudios aportan información sobre la existencia de complejos de homoreceptores A2A-A2A y complejos de heteroreceptores A1-A2A en la corteza cerebral dorsolateral y la corteza piriforme, exhibiendo patrones de distribución similares mediante la técnica de PLA. Estos hallazgos intrigantes abren nuevas vías para comprender los circuitos cerebrales modulados por los complejos de receptores de adenosina y su relevancia para el aprendizaje, la memoria y las enfermedades psiquiátricas.

A través del análisis bioinformático de los heterómeros A1-A2A, se revela que su interfaz de receptor difiere de la de los heterómeros A1-D1 y A2A-D2. El heterómero A1-A2A muestra homologías únicas de aminoácidos AKS y KSL, ausentes en los heterómeros A1-D1 y A2A-D2, lo que indica diferentes tipos de homologías. Estos resultados enfatizan la especificidad del proceso de heteromerización y sugieren que los heterómeros de receptores de adenosina formados por la interacción con otros

receptores utilizando diferentes ligandos transmisores/moduladores pueden tener interfaces de receptor distintas.

Las mayores densidades de complejos de hetero-receptores A1-A2A se encuentran en las capas de células piramidales de CA1-CA3 y la capa de células polimórficas en el hilo del giro dentado en el hipocampo. Investigaciones previas han demostrado la co-expresión de los receptores A1 y A2A de adenosina en muchas células nerviosas piramidales glutamatérgicas del hipocampo y en terminales nerviosas de glutamato. Además, se han obtenido indicios a través de co-inmunoprecipitación y experimentos de unión bioquímica de que los complejos de heteroreceptores A1-A2A pueden estar presentes en terminales nerviosas estriatales de glutamato. Estas observaciones sugieren que pueden ocurrir interacciones antagonistas entre los receptores A2A y A1 en estos complejos, donde la activación del receptor A2A reduce la afinidad de los sitios de unión de agonistas A1. Basándose en estos hallazgos, se propone que los grupos de complejos de A2A-A2A y A1-A2A PLA en la capa de células piramidales del hipocampo pueden existir en los somas-dendritas de las células piramidales y en colaterales locales de glutamato de las regiones CA3 y CA2. La localización precisa de estos complejos de receptores de adenosina dentro de regiones específicas del hipocampo sugiere su participación en la regulación de la señalización de glutamato y la plasticidad sináptica.

El equilibrio intrincado entre la señalización de los receptores A1-Gi/o y A2A-Gs/olf en el hipocampo, que ejerce acciones opuestas en la vía de señalización AC-PKA-CREB hacia el núcleo, la liberación de glutamato y la patología cerebral como las convulsiones, se facilita mediante un equilibrio dinámico de la señalización de los receptores de adenosina dentro de los complejos A2A-A2A, complejos A1-A1 de homoreceptores y complejos A1-A2A de heteroreceptores. Este equilibrio ocurre dentro de las células piramidales individuales y entre células piramidales con diferentes proporciones de estos complejos principales que median la señalización de la adenosina A2A. La importancia de este equilibrio se demuestra por la capacidad de la activación optogenética de la señalización del receptor A2A en el hipocampo a través de la fosforilación de CREB para perjudicar la memoria. De manera similar, la inactivación del receptor A2A mejora la memoria de trabajo y el aprendizaje de inversión. La reorganización propuesta de los complejos de homo y heteroreceptores en la membrana postsináptica proporciona una posible base molecular para el aprendizaje y la memoria. En el aprendizaje, la reorganización transitoria de conjuntos de complejos de homo y heteroreceptores en las membranas postsinápticas y extrasinápticas asociadas permite la adquisición de un nuevo patrón temporal de liberación de neurotransmisores, formando una memoria a corto plazo del patrón novedoso. Esta memoria a corto plazo puede convertirse en memoria a largo plazo a través de nuevas proteínas adaptadoras que estabilizan los complejos de receptores y

los vinculan al citoesqueleto. La alteración del equilibrio de señalización dentro de los complejos de homo y heteroreceptores de adenosina puede llevar a errores en la formación de engramas moleculares en las sinapsis glutamatérgicas del hipocampo y afectar el rendimiento de la memoria espacial.

El Capítulo 2 de la tesis se centra en investigar la existencia de complejos de heteroreceptores A2AR-D4R en el cerebro de la rata. Se emplean modelos celulares y técnicas de PLA in situ para detectar estos complejos. La formación de complejos de heteroreceptores A2AR-D4R se confirma mediante técnicas de BRET1 en células HEK. Este estudio explora además las interacciones alostéricas receptor-receptor dentro de los heterómeros A2AR-D4R, revelando interacciones antagonistas entre los sitios de unión ortostéricos de los heterómeros correspondientes. El análisis de la vía Gi/o-AC-PKA-CREB utilizando el ensayo de luciferasa CREB demuestra que la presencia de un complejo de heteroreceptores A2AR-D4R reduce la potencia del agonista específico del receptor D4R, PD-168077, en la inhibición de la señal CREB. Las interacciones alostéricas receptor-receptor antagonistas inhiben significativamente la señalización protomérica del receptor D4R mediada por Gi/o. Estos hallazgos indican el papel significativo de los complejos de heteroreceptores A2AR-D4R en la neuroplasticidad. Los experimentos de PLA in situ identifican estos complejos de receptores en alta densidad, principalmente en el cuerpo estriado ventral, la corteza prefrontal y la capa de células piramidales del hipocampo. Aparecen como agrupaciones rojas de tamaño entre 0,5 y 2 µm, ubicadas en el soma fuera de los núcleos. Estos complejos son constitutivos y también se han demostrado en modelos celulares utilizando técnicas de BRET.

Estudios previos han indicado que A2AR puede formar complejos de heteroreceptores con D4R basándose en su interfaz, que involucra interacciones electrostáticas similares a las descritas para el heterómero A2AR-D3R. Se ha demostrado que el D4R está enriquecido en los estriosomas, pero también se encuentra en la matriz del cuerpo estriado dorsal. Por lo tanto, la observación de complejos de heteroreceptores A2AR-D4R en el cuerpo estriado dorsal y ventral mediante ensayos de ligadura por proximidad es altamente significativa. En el cuerpo estriado dorsal, los complejos de A2AR-D4R se encuentran principalmente en los estriosomas, que utilizan GABA como neurotransmisor y se proyectan directamente hacia las neuronas DA nigrales, inhibiendo su actividad. Por lo tanto, la presencia de complejos de heteroreceptores A2AR-D4R en las neuronas GABA estriosomales es particularmente intrigante. Es plausible que la interacción alostérica antagonista receptor-receptor en este complejo conduzca a una reducción de la señalización inhibidora mediada por Gi/o del receptor D4R, lo que resulta en una mayor actividad de las neuronas GABA estriosomales que se proyectan directamente hacia las células DA nigrales. En consecuencia, debería producirse una reducción en la actividad y el

recambio de la dopamina en las células nerviosas DA que se proyectan hacia los estriosomas.

En el Capítulo 3, se obtienen importantes resultados sobre los mecanismos moleculares subyacentes a la enfermedad de Parkinson (EP) al investigar las interacciones entre la alfa-sinucleína y los receptores acoplados a proteínas G (GPCR) tipo D2, es decir, los receptores D2R y D4R. Utilizando técnicas avanzadas como el ensayo de ligadura de proximidad in situ (PLA) y la transferencia de energía de resonancia bioluminiscente (BRET) hemos proporcionado, por primera vez, evidencia de la existencia de complejos de D2R y D4R con la alfa-sinucleína en el cerebro de ratas, específicamente en el cuerpo estriado dorsal y ventral. Además, el estudio confirma las interacciones A2AR-alfa-sinucleína y DAT-alfa-sinucleína utilizando PLA in situ. Estos complejos se observan tanto en los cerebros de ratas Sprague-Dawley (SD) de control como en el modelo de rata BSSG de la enfermedad de Parkinson (PD), y están enriquecidos en el cuerpo estriado dorsal y ventral. Estos hallazgos se alinean con observaciones previas de interacciones de la alfa-sinucleína con varios receptores de membrana, como NMDAR 2B, adrenérgicos B2, mGluR5, M5R y EGFR. Los estudios de espectrometría de masas, co-inmunoprecipitación, western-blot y de dos híbridos han demostrado que la alfa-sinucleína puede interactuar con más de 500 proteínas, tanto intracelulares como asociadas a membrana. Esta complejidad destaca las complejidades de las sinucleinopatías. Los experimentos de PLA in situ en el cerebro de la rata también revelan la capacidad de la alfa-sinucleína para interactuar con varias proteínas involucradas en la señalización de dopamina y la neurotransmisión, incluidos los receptores D2R, D4R y DAT.

Es importante destacar que se observa un aumento significativo en las interacciones receptor-alfa-sinucleína para D2R y DAT en el modelo BSSG en comparación con el grupo de control SD, respaldando la hipótesis. Por el contrario, no se encuentra una diferencia significativa en las interacciones alfa-sinucleína-D4R entre el modelo BSSG de la enfermedad de Parkinson y los grupos de control SD. Las mayores densidades de los complejos de D2R-alfa-sinucleína y DAT-alfa-sinucleína en el modelo animal BSSG pueden atribuirse a la afinidad preferencial del D2R y el DAT por un estado conformacional específico de la proteína alfa-sinucleína. Ciertas formas oligoméricas de alfa-sinucleína, que están presentes en el modelo de rata BSSG y replican las formas de alfa-sinucleína humana en la enfermedad de Parkinson, pueden contribuir a estos cambios. Las variaciones en las densidades de cada complejo en el modelo BSSG también podrían estar influenciadas por la vulnerabilidad del tipo de célula, como terminales presinápticas de dopamina (varicosidades) versus neuronas GABA estriatopallidales postsinápticas y/o astrocitos, así como diferencias en los compartimentos estriatales (matriz versus estriosomas). La identificación de tales complejos representa un paso significativo hacia una comprensión más profunda de la

fisiopatología molecular subyacente a la enfermedad de Parkinson, ofreciendo un gran potencial para avances terapéuticos y diagnósticos. El estudio también explora los efectos moduladores de los agonistas y antagonistas de los receptores sobre estas interacciones, destacando sus posibles implicaciones terapéuticas en la enfermedad de Parkinson.

Los estudios futuros deberían ahondar en cómo se modifican las vías de transducción de señales de los GPCR involucrados en respuesta a la formación de complejos. Esto proporcionará información sobre cómo se alteran las funciones de los GPCR en presencia de la alfa-sinucleína (formando complejos GPCR-alfa-sinucleína) y arrojará luz sobre los efectos celulares resultantes y la patología de la enfermedad de Parkinson. Por ejemplo, realizar un ensayo cAMP-Glo para medir los niveles de cAMP tras la activación (Gs/olf) y bloqueo (Gi/o) de los GPCR podría ayudar a comprender en qué medida las interacciones alostéricas receptor-alfa-sinucleína contribuyen a estos efectos. Una hipótesis plausible es que la unión de la alfa-sinucleína al D2R y DAT antagoniza estos receptores, lo que lleva a una reducción de la señalización del D2R y la actividad del DAT, lo que finalmente resulta en una disminución de la actividad de la dopamina. Esto exacerba el agotamiento de dopamina característico de la enfermedad de Parkinson.

Esta tesis doctoral proporciona una visión detallada de las interacciones de los receptores de GPCR en el sistema nervioso central, con un enfoque específico en los complejos homo y heteroreceptor de adenosina A2AR y de dopamina D2-likeR. A través del uso de diversas técnicas y métodos, se ha ampliado nuestra comprensión de estos complejos y su implicación funcional en el cerebro. Estos hallazgos tienen el potencial de tener un impacto significativo en el campo de la neurociencia y pueden allanar el camino para nuevas investigaciones y terapias dirigidas a trastornos neurológicos relacionados.

ABBREVIATION LIST

5-HT1A	Serotonin receptor subtype 1A
AC	Adenylyl cyclase
A2AR	Adenosine A2A receptor
ATP	Adenosine 5'-triphosphate
BRET	Bioluminescence resonance energy transfer
CAMK-II	Ca2+/calmodulin dependent protein kinase
cAMP	Adenosine 3',5'-cyclicmonophosphate
D2R	Dopamine D2 receptor
D2LR	Dopamine D2 long isoform receptor
D2SR	Dopamine D2 short isoform receptor
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
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
PKC	Protein kinase C
PLC	Phospholipase C
Rluc	Renilla luciferase
σ1R	Sigma 1 receptor
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. On the balance and distribution of adenosine A2A isoreceptor complexes in hippocampus and other areas of cerebral cortex operating as an integrative mechanism in the plasma membrane of neuron-glia networks in the brain. Marco Bartolini, Verty Ochoa, Catalina Pérez, **Mariana Pita-Rodríguez**, Aracelis Hernández-García, Rasiel Beltran-Casanueva, Rafael franco, Kjell Fuxe and Dasiel O. Borroto-Escuela1. (MANUSCRIPT IN PREPARATION).

II. Dopamine D4R and adenosine A2AR heteroreceptor complexes and its allosteric receptor-receptor interactions. **Mariana Pita-Rodríguez**, Marco Bartolini, Malak Choucri, Aracelis Hernández-García, Rasiel Beltran-Casanueva, Francisco Ciruela, Kjell Fuxe and Dasiel O. Borroto-Escuela. (MANUSCRIPT IN PREPARATION).

III. Evidence for the existence of Alpha-Synuclein-GPCR and GPCR-GPCR Heterocomplex Alteration in the BSSG Rat Model of Parkinson's Disease, and In Vitro. Malak Choucri, **Mariana Pita-Rodríguez**, Sarah Beggiato, Aracelis Hernández-García, Rasiel Beltran-Casanueva, Luca Ferraro, Per Svegnisson, Kjell Fuxe1 and Dasiel O. Borroto-Escuela. (MANUSCRIPT IN PREPARATION).

IV. Manuel Narváez, Minerva Crespo-Rodríguez, Ramon Fores-Pons, **Mariana Pita-Rodríguez**, Francisco Ciruela, Malgorzata Filip, Sarah Beggiato, Luca Ferraro, Sergio Tanganelli, Patrizia Ambrogini, Miguel Perez de la Mora, Kjell Fuxe and Dasiel O. Borroto-Escuela**.** Study of GPCR homo and heteroreceptor complexes in specific neuronal cell populations using the in situ Proximity Ligation Assay. Chapter 9 (pp.299-315). *Ciruela and Lujan (Editors).* Receptor-Receptor Interactions in the Central Nervous System Neuromethods, Volume 144. Humana Press. Series Editor, W. Walz. 2021.

V. Borroto-Escuela DO, **Pita-Rodriguez M**, Fores-Pons R, Barbancho MA, Fuxe K, Narváez M. Galanin and neuropeptide Y interactions elicit antidepressant activity linked to neuronal precursor cells of the dentate gyrus in the ventral hippocampus. J Cell Physiol. 2021 May;236(5):3565-3578.

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dorsal hippocampus of the rat brain: Potential implications of A2AR and TrkB interplay upon ageing. Mech Ageing Dev. 2020 Sep; 190:111289. doi: 10.1016/j.mad.2020.111289.

VII. Dasiel O. Borroto-Escuela, Manuel Narvaez, Martina Zannoni, Chiara Contri, Minerva Crespo-Ramírez, Michael di Palma, Patrizia Ambrogini, Daily Y. Borroto-Escuela, Ismel Brito, **Mariana Pita-Rodríguez**, Ismael Valladolid-Acebes, Miguel Pérez de la Mora and Kjell Fuxe. Isolation and Detection of G Protein–Coupled receptor (GPCR) Heteroreceptor Complexes in Rat Brain Synaptosomal Preparation using a Combined Brain Subcellular Fractionation/Co-Immunoprecipitation (co-IP) Procedures. Chapter 10 (pp.299-315). Odagaki, Y. and Borroto-Escuela, D.O. (Editors). *Coimmunoprecipitation methods for brain tissue.* Neuromethods, Volume 144. Humana Press. Series Editor, W. Walz. 2018

VIII. Michael Di Palma, Manuel Narvaez, **Mariana Pita-Rodríguez**, Chiara Contri, Martina Zannoni, Riccardo Cuppini, Kjell Fuxe, Patrizia Ambrogini and Dasiel O. Borroto-Escuela. Co-Immunoprecipitation (co-IP) of G Protein-Coupled Receptors (GPCR)-(RTK) Complexes from the Dorsal Hippocampus of the Rat Brain. Chapter 13 (pp xx-xx). Odagaki, Y. and Borroto-Escuela, D.O. (Editors). *Coimmunoprecipitation methods for brain tissue.* Neuromethods, Volume 144. Humana Press. Series Editor, W. Walz. 2018.

RELATED WORK BY THE AUTHOR

IX. Ryan M, Zaldívar Vaillant T, McLaughlin RL, Doherty MA, Rooney J, Heverin M, Gutierrez J, Lara-Fernández GE, **Pita Rodríguez M**, Hackembruch J, Perna A, Vazquez MC, Musio M, Ketzoian CN, Logroscino G, Hardiman O. Comparison of the clinical and genetic features of amyotrophic lateral sclerosis across Cuban, Uruguayan and Irish clinic-based populations. J Neurol Neurosurg Psychiatry. 2019 Mar 7.

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INTRODUCTION

1. Introduction to the field of GPCR receptor-receptor interactions.

In 1980-1981, Fuxe and Agnati proposed the integration of peptide and monoamine signals in the Central Nervous System (CNS) through direct interactions between peptide receptors and monoamine receptors in the plasma membrane [1-3]. Their findings demonstrated that specific peptides could modulate the binding characteristics, particularly the affinity, of monoamine receptors in membrane preparations, exhibiting receptor subtype specificity [4]. Later on, in 1993, they introduced the hypothesis that allosteric receptor-receptor interactions could occur via the formation of GPCR heterodimers alongside corresponding homodimers [5, 6]. These receptor-receptor interactions were found to impact recognition, pharmacology, signaling, and trafficking of the partner receptor protomers [7-12]. The cloning of the Gamma-aminobutyric acid (GABA) B receptor 1 (GABABR1) gene was achieved in 1997 [13]. However, it was discovered that GABABR1 alone did not reach the plasma membrane but instead remained on intracellular membranes, exhibiting low affinity for GABAB receptor agonists. In 1998, Marshall et al. made a significant breakthrough by demonstrating that heterodimerization was essential for the formation of a functional GABA B receptor [14-16]. They identified a novel GABAB receptor subtype, GABABR2, which physically interacted with GABABR1 through their intracellular carboxy-terminal regions, facilitated by a coiled-coil domain. This interaction led to the transport of GABABR1 to the plasma membrane, resulting in a high affinity for GABA. The functional response of the GABAB heterodimer was mediated by the GABABR2 protomer coupled to Gi/o, as observed in *Xenopus oocytes* possessing G protein-coupled inwardly-rectifying potassium channels (GIRKs)[14].

In 1998, it was also discovered that receptor activity-modifying proteins (RAMPs) could regulate the family B GPCR calcitonin-receptor-like receptor (CLR) in terms of membrane transport and recognition specificity [17, 18]. RAMPs, including RAMP1, RAMP2, and RAMP3, played a crucial role in association with CLR. Their association transformed CLR into receptors for calcitonin gene-related peptide (CGRP), adrenomedullin1, adrenomedullin 2, and amylin, each with distinct recognition profiles [19, 20].

Furthermore, it was demonstrated that adaptor/chaperone proteins like Sigma1R could significantly modulate receptor-receptor interactions [21-23]. These allosteric receptor-receptor interactions occur through conformational changes induced in

partner receptor protomers upon ligand binding to orthosteric or allosteric sites, passing through the receptor interface. These interactions can lead to multiple allosteric changes, including alterations in affinity and signaling efficacy. Heterodimerization of somatostatin and opioid receptors was also found to allosterically modulate phosphorylation, trafficking, and desensitization of the receptor protomers [24].

In 2000 Fang Liu et al., were the first to demonstrate that GPCR-Ionotropic receptor heterocomplexes also exist [25]. Physical protein-protein coupling was observed between dopamine D5R and GABA A receptor. The second intracellular loop of GABA A gamma 2 (short) receptor subunit interacted with dopamine D5R carboxy terminal domain. A dynamic modulation of synaptic inhibition of the GABA A ion channels was found through inhibitory allosteric receptor -receptor interactions [25]. There exist indications from 1997 that activation of GABA A receptors in membrane preparations reduce the affinity of the high affinity of the D2R agonist binding sites [26] but these results should be validated. It opens the possibility of potential D2R-GABA (A)R interactions.

Overall, allosterism in GPCR have an impact at the intracellular signalling level [27, 28]. The allosteric receptor-receptor interaction can here modulate the strength of the receptor-G protein coupling or switch the G protein coupling from one type of G protein to another type of G protein as is the case in the D1R-D2R heteromer [29] and DOR-MOR[30, 31]. It is also known that GPCR kinases (GRK) can assist GPCRs in switching from G protein mediated signalling to beta-arrestin mediated signalling [32]. It seems likely that allosteric A2AR-D2R interactions can participate in such events since they were shown to inhibit D2R Gi/o mediated signalling and increase D2R mediated beta-arrestin signalling through recruitment of beta-arrestin to the intracellular surface of the D2R [33].

2. Homo and heteroreceptor complexes and their allosteric receptor-receptor interactions.

As already indicated, the work on neuropeptide-monoamine receptor-receptor interactions in the CNS indicated the existence not only of GPCR monomers [34] but also of GPCR homo and heteroreceptor complexes [35-40]. It can include receptor dimers, higher order receptor complexes and receptor interacting proteins like different types of adapter proteins and synaptic and/or non-synaptic proteins [41-43].

Early on there have been a large number of studies on the existence of allosteric receptor-receptor interactions in brain dopamine (DA) transmission belonging to the basal ganglia (**Figure 1**). It is based on biochemical binding studies [2, 44], especially with regard to A2AR-D2R interactions [44, 45] but also to A2AR-D3R

complexes [46] and the potential existence of A2AR-D4R complexes [47, 48]. The DA receptors are mainly located in extra-synaptic regions [49], which will then likely be true also for these heterocomplexes. In 1998 antagonistic A1R-D1R interactions were also observed in binding studies in the basal ganglia, likely reflecting allosteric receptor-receptor interactions in the extra-synaptic regions [9]. Later in 2003 and 2008[10, 50] came evidence from BRET and FRET studies on the existence of A2AR-D2R and A1R-D1R heterocomplexes with the A2AR-D2R complexes mainly modulating the striatal-pallidal GABA neurons, known to inhibit initiation of movements and the A1R-D1R complexes mainly modulating the direct GABA pathway, known to enhance movements. The DA receptor subtypes also interact with each other to form e.g., D2R-D4R[51] and D1R-D2R[29] heteroreceptor complexes, especially in the basal ganglia. The available evidence suggests that the allosteric receptor-receptor interactions can involve bi-directional modulation of both receptor recognition, signaling and trafficking[42, 47, 48, 52-60], and include high order heteroreceptor complexes.

Furthermore, the richness of 5-HT receptor subtypes in the brain is well-known [61] and they form a large number of 5-HT heteroreceptor complexes among themselves like 5-HT1AR-5-HT2AR heterocomplexes [62] and with other types of receptors like the DA receptor subtypes [63-65]. The DA and serotonin nerve terminal networks are also known to overlap with each other in multiple brain regions [66] (Fuxe et al.2010). It is therefore of substantial interest that D2R-5-HT2AR and D2R-5-HT1A heteroreceptor complexes have been identified in the brain [67-70].

It is of substantial interest that only the hallucinogenic 5-HT2AR agonists could enhance the Bmax values and the affinity of the high affinity component of the D2R protomers through allosteric receptor-receptor interactions in the dorsal and ventral striatum with the D2R signaling also becoming increased [68]. One molecular mechanism for the ability of atypical antipsychotic drugs to diminish psychosis can therefore be by blocking the allosteric enhancement of D2R protomer signaling through e. g., 5-HT2AR antagonism (**Figure 1**).

It should also be considered that the 5-HT2AR forms a heterocomplex with the oxytocin receptor but in this receptor complex, it exerts an allosteric antagonistic action on the oxytocin receptor signaling [71]. In view of the importance of oxytocin for social behavior and for reward, this action of the 5-HT2AR agonist will contribute to its known depressant actions[35, 63, 65]. The oxytocin receptor (OXTR) represents a key hub in the GPCR heteroreceptor network with significant relevance for brain and behavior [72] and even stronger antagonistic allosteric actions are exerted on the oxytocin receptor protomer by the 5-HT2CR protomer in OXTR-5-HT2CR heterocomplexes [73].

We should also consider the DA and serotonin heteroreceptor complexes as key hubs in the integration of DA and serotonin transmission [53]. It is of substantial interest that D2R-5-HT1AR heterocomplexes also have been discovered [69]. The method was based on the FRET principle and fluorescence lifetime imaging microscopy. These complexes were found to a high degree in the medial prefrontal cortex while found to a much lower extent in the striatum. In 2018 it was found that the atypical antipsychotic drug risperidone in a low dose which can reduce both D2R and 5-HT1AR protomer signaling, increased the D2R-5-HT1AR heteromerization, using in situ PLA, in the prefrontal cortex of the mouse [70]. It may reflect an enhancement in the affinity of the two receptor protomers for each other in the prefrontal cortex that may e.g., lead to enhanced inhibition of the D2R and /or 5-HT1AR protomer signaling which should be further investigated (**Figure 1**).

There also exist 5-HT1AR-5-HT2A heteroreceptor complexes in the anterior cingulate cortex and the hippocampus [62]. It should therefore be tested if also higher order D2R-5-HT1A-5-HT2A exist in a dynamic balance with D2R-5-HT1A and D2R-5-HT2AR heterocomplexes, including also the corresponding homomeric complexes and monomers [63, 64, 72, 74]. These results underline the fundamental role the various DAR-5-HTR heterocomplexes can have in the integration of the DA and 5-HT signaling in the dopamine and serotonin nerve terminal networks. [2, 7, 11, 38, 42, 75-78 *79-81*]

Plasma membrane homo and heteroreceptor complexes were mainly identified with coimmunoprecipitation[51, 82, 83], bioluminescence and fluorescence resonance energy transfer (BRET and FRET [84-87]. It should also be noted that in the period of 2010-2013 it became possible to demonstrate heteroreceptor complexes in the brain at endogenous expression levels of receptors based on a novel technique, proximity ligation assay (PLA), in combination with immunohistochemistry [41, 51, 82, 88-92].

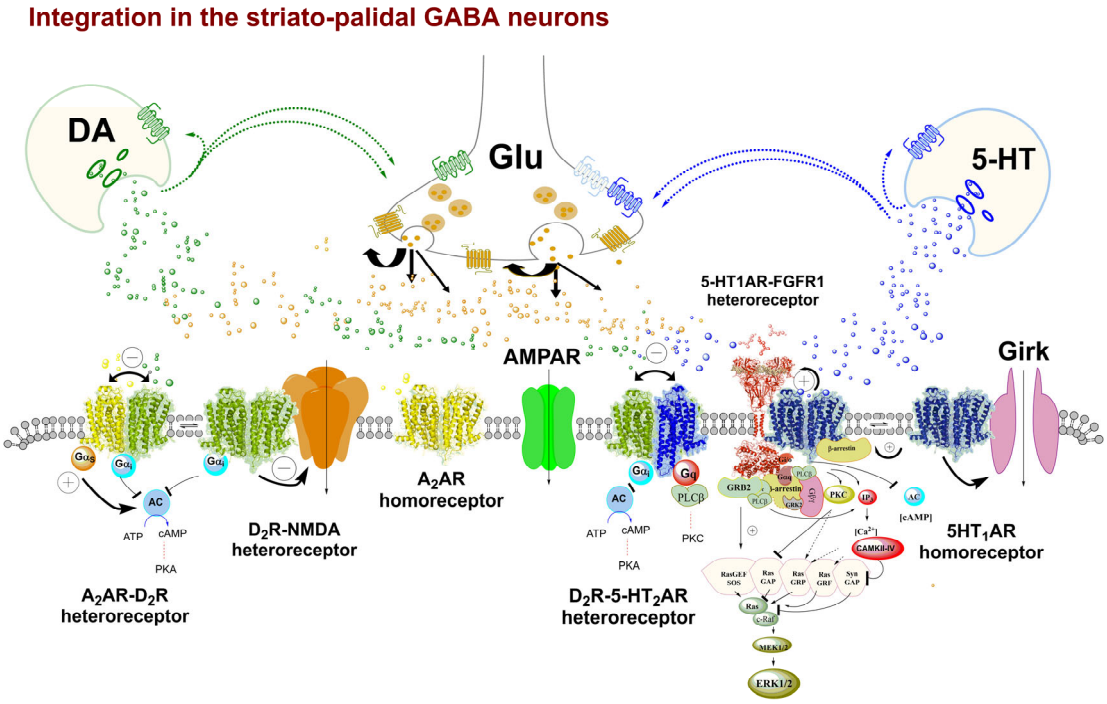

Figure 1. **GPCR heteroreceptor complexes as second order modulators. A novel integrative mechanism through allosteric receptor-receptor interactions at the plasma membrane which gives concepts like meta-modulation and protein modulation.** The discovery of GPCR receptor-receptor interactions in the plasma membrane was made already in the early 1980ies with the hypothesis in 1993 that these receptor-receptor interactions were caused by the formation of homo and hetero receptor dimers and higher order homo- and heteroreceptor complexes. The molecular integrative receptor mechanisms are in operation both in extra-and presynaptic and extra-and postsynaptic locations. They play a major role in modulating pre -and extra-synaptic release of neurotransmitters. In the extra-and postsynaptic places, the integration in and between multiple heteroreceptor complexes will lead to significant alterations in recognition, signaling and trafficking of the extra-and postsynaptic hetero-receptor complexes. Meta-modulation in 1999 stood for the fine tuning or modulation of nerve cell transmission through receptors of different types located in the same nerve cell and with functional interactions. Here we expand the concept of meta-modulation (second order modulation) and build it on the existence of physical receptor-receptor interactions in heteroreceptor complexes in synaptic and extra synaptic membranes (manuscript in preparation, Borroto-Escuela et al. 2023)

3. GPCR-RTK receptor-receptor interactions.

In 2007, Fuxe proposed the existence of GPCR-receptor tyrosine kinase (RTK) heteroreceptor complexes based on their potential direct receptor-receptor interactions [4]. Specifically, he suggested the presence of the FGFR1-5-HT1AR heteromer (**Figure 1**). The following year, Flajolet et al., [93] made the discovery that FGFR1can form heteroreceptor complexes with A2AR, a GPCR., based on the use of the yeast two hybrid method. Coactivation of the two protomers resulted in neurite extension of the cells and enhanced cortico-striatal plasticity. Twelve years later we found that A2AR also formed heteroreceptor complexes with TrkB receptors in the dorsal hippocampus using in situ PLA [94]. The

complexes were inter alia found in high densities in the pyramidal cell layers of the CA1-CA3 regions but lacked presence in the molecular and granular cell layers of the dentate gyrus. These A2AR-TrkB heteroreceptor complexes may have implications for hippocampal plasticity, which is impaired in aging [94].

The discovery of the FGFR1-5-HT1A heteroreceptor complexes in the dorsal hippocampus was made in 2012 [82] using PLA followed by observations of their presence also in the dorsal raphe [95, 96]. In the dorsal raphe the FGFR1 forms a complex with the 5-HT1A auto-receptor. It was found that combined 5-HT1AR agonist and FGF2 treatment increased the density of these heteroreceptor complexes in the hippocampus. Furthermore, the enhanced positive allosteric receptor-receptor interactions in these complexes led to improved FGFR1 signaling, linked to antidepressant actions [97]. Taken together, these results bring together the serotonin and neurotrophic hypothesis of major depression.

Disturbances have been observed in the FGFR1-5-HT1AR heterocomplexes in the raphe-hippocampal 5-HT neuronal system in a genetic rat model of depression rats (Flinders sensitive line rat) [98]. Such deficits may involve a failure of combined agonist treatment to uncouple the 5-HT1A auto receptor from the GIRK channels in the raphe 5-HT nerve cells which increases their hyperpolarization and may reduce their firing. This may be related to a reduced ability of the FGFR1 protomer to reduce the signaling of the 5-HT1A auto-receptor protomer via allosteric receptor-receptor interactions [63, 74, 98]. Through a neurochemical and electrophysiological analysis, it was demonstrated that astrocytic FGFR1-5-HT1AR heterocomplexes also exist in hippocampus [99]. Localization of hippocampal FGFR1-5-HT1AR heterocomplexes in astrocytes was found using in situ proximity ligation assay combined with immunohistochemistry using glial fibrillary acidic protein (GFAP) immunoreactivity as a marker for astroglia. Acute i.c.v. treatment with 8-OH-DPAT alone or together with basic fibroblast growth factor (FGF2) significantly increased FGFR1-5-HT1AR heterocomplexes in the GFAP positive cells, especially in the polymorphic layer of the dentate gyrus (PoDG) but also in the CA3 area upon combined treatment. Also, structural plasticity changes were observed in the astrocytes, especially in the PoDG region, upon these pharmacological treatment [99]. FGFR1-5-HT1AR heterocomplexes in astrocytes modulate the structure and function of astroglia in the hippocampus leading to possible changes in the gamma oscillations.

There also exist indications for the existence of muscarinic acetylcholine receptor, mAChR-FGFR1 heteroreceptor complexes which should be linked to the cholinergic neurons [100], which is of high interest. However, it is important to note that in some cases, the GPCR and RTK trophic interactions involve

functional crosstalk in intracellular pathways for receptor transactivation. One example is the interaction between GABAB and insulin growth factor 1.

In 2007 we introduced the hypothesis of the existence of GPCR- receptor tyrosine kinase (RTK) heteroreceptor complexes based on their potential direct receptor-receptor interactions [101]. More precisely the FGFR1-5-HT1AR heteromer was proposed to exist. The following year a direct physical interaction was demonstrated between FGFR1 and A2AR (Figure 2) [84, 93]. FGF was found to act as a cotransmitter to the A2AR protomer to enhance synaptic plasticity at the morphological and functional level. The FGFR1-5-HT1AR heteroreceptor complexes were found in 2012 [82, 97, 102] and produced an enhancement of hippocampal plasticity. This heteroreceptor complex was proposed to be a novel target for anti-depressant drugs at which neurotropic and antidepressant actions can be induced by restoring tropism and activity in the neuronal hippocampal circuits showing deficits in depression [97].

It was also found in 2017 that FGFR can form heteroreceptor complexes with muscarinic acetylcholine receptors (mAChR) associated with increased neurite outgrowth in neural hippocampal cultures [100]. However, it should be considered that sometimes the GPCR and RTK trophic interactions only involve a functional crosstalk in the intracellular pathways for receptor transactivation to develop as is the case for the GABAB and insulin growth factor 1 interaction [103].

4. Allosteric receptor-receptor interactions and their involvement in brain disease and its treatment. Pathophysiological relevance for Parkinson's disease and drug development.

The A2AR-D2R and A1-D1R heterocomplexes modulating the key indirect and direct pathways of the basal ganglia are strongly implicated in Parkinson's disease and its treatment [11, 50]. These homo- and heteroreceptor complexes, including their antagonistic allosteric receptor-receptor interactions play a key role in the basal ganglia [48, 104]. The A2AR-D2R complex modulates motor inhibition mediated by the indirect pathway and the A1R-D1R complex modulates the motor initiation of the direct pathway. Ionotropic and metabotropic glutamate receptor protomers in heterocomplexes also participate in the regulation of these pathways. It has led to potentially improved treatment of Parkinson's disease [105, 106].

It is also suggested that A2AR and their heteroreceptor complexes including also the A2AR-alpha-synuclein complex ([107] and Borroto-Escuela et al. unpublished data) can have a relevant role in potentially increasing the propagation of alpha synuclein monomers/dimers into oligomers and fibrils. Such events may also interfere with the function of various A2A heteroreceptor complexes including

A2AR-D2R, A2AR-mGluR5, A2AR-mGluR1and A2AR-NMDAR contributing to deficits in learning and memory and enhanced neurodegeneration [49, 78, 107], involving especially the mGluR1 protomer [108, 109]. It is proposed that the A2AR agonist through activation of the A2AR protomer of the alpha synuclein-A2AR homo and heterocomplex may increase the alpha synuclein propagation and produce disturbances in the A2A homo and heteroreceptor complexes interfering with learning and memory and increasing neurodegeneration through their dysfunction. In contrast, the A2AR antagonists like istradefylline can exert neuroprotective actions and improve cognition by e.g., reducing the formation of alpha synuclein-A2AR heterocomplexes and blocking the actions of A2AR agonists leading to reduced dysfunction [107] (**Figure 2**).

Aggregates of misfolded alpha synuclein is a hallmark of Parkinson's disease. The misfolding is caused by mutations of the alpha synuclein [110] and its degree likely enhances the misfolding. It should be investigated if the misfolding of the alpha synuclein due to mutations will increase the formation of alpha synclein-A2AR heterocomplexes which may enhance the disturbances they may induce in multiple A2A heteroreceptor complexes in the dorsal striato-pallidal GABA neurons which upon their activation inhibit movements [111, 112] (**Figure 2**).

The alpha synuclein complexes of various types may also be internalized into surrounding DA nerve terminals from e.g., their release from dorsal striatal-pallidal GABA neurons via the vesicle mode of volume transmission. These events may start the degeneration of the striatal DA nerve terminals of the nigro-striatal DA neurons in Parkinson's disease [58, 106, 107, 113].These neurons are highly vulnerable to Parkinson's disease.

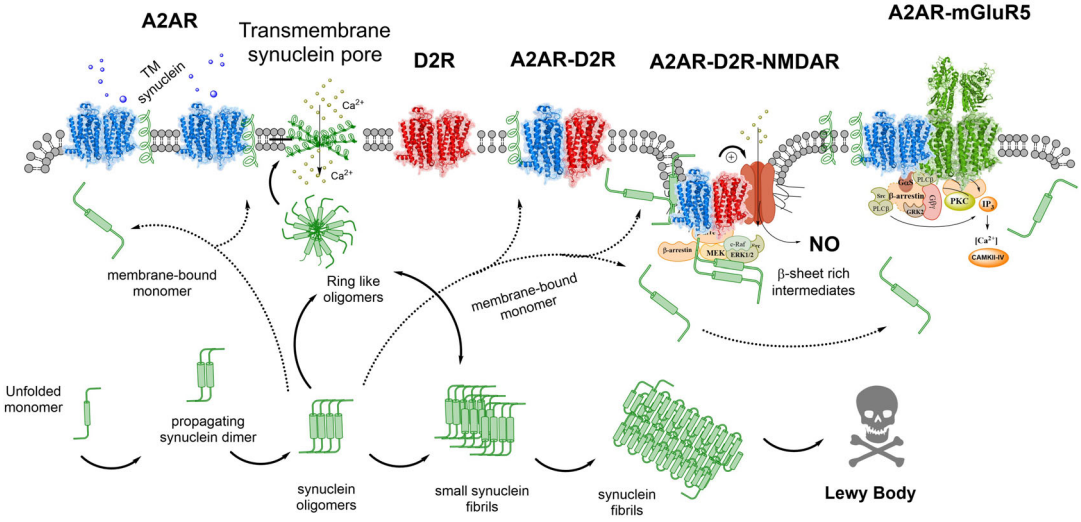

Figure 2. **Illustration of possible molecular mechanism by which misfolded alpha synuclein can interact through a receptor-protein interaction with the A2AR and modulate the adenosine A2AR homo-heteroreceptor complexes balance and panorama in the plasma membrane.** In the plasma membrane is indicated that monomeric alpha-synuclein transmembrane

(TM) peptide (in green) can become linked to A2AR homo- and heteroreceptor complex and modulate the A2AR function. From left to right they are indicated to interact with an A2AR-homodimer, an A2AR protomer in an A2AR-D2R heterodimer, and an A2AR protomer in an A2AR-mGluR5 heterodimer. In the alpha synuclein interaction with the A2AR-D2R-NMDAR heteroreceptor complex also involves the kinases like MEK and ERK1/2 as well as beta-arrestin in the cytoplasm, in which they can have another conformation, indicated as rectangular. Ring-like synuclein oligomers may also be formed which may enter the membrane and there produce beta sheet structures that associate and give rise to pores in the plasma membrane (shown between the A2AR homodimer and the D2R homodimer) through which calcium ions may pass. ((The text and the figure have been obtained from [106])

5. Novel methods and techniques to study GPCR homo and heteroreceptor complexes.

The first evidence of GPCR receptor-receptor interactions was obtained through recognition-level experiments using saturation and competition binding assays [1-3, 114]. These studies revealed modulation of binding through changes in Kd and Bmax values (saturation analysis) and KL, KH, and RH values (competition analysis), which allowed for the determination of modulation of the high- and low-affinity agonist states of the receptor. Additionally, the effect on G protein coupling and receptor efficacy could be assessed by studying how the modulator controlled the GTP-induced disappearance of the high-affinity state of the receptor (reduction of RH values) (**Figure 3**).

Fluorescence resonance energy transfer (FRET)[85, 115-118] and bioluminescence resonance energy transfer (BRET)[84, 116, 119] methods provided further evidence for heteromers among class A GPCRs. FRET involved genetically fused "donor" and "acceptor" fluorescent proteins in receptor constructs. If the donor and acceptor fluorophores were in close proximity (less than 10 nm), energy transfer between the two fluorophores occurred upon donor excitation, resulting in acceptor emission [115-117]. For intracellular protein interactions, cell surface FRET detection technologies were developed to study GPCR heteromerization in the plasma membrane. Total internal reflection fluorescence microscopy (TIRF) allowed for improved excitation field definition and restricted light propagation, enabling excitation of appropriate fluorophores within a limited distance of approximately 100 nm [85]. Bimolecular fluorescence complementation (BiFC) was another technique used to demonstrate protein dimerization [116]. By splitting the yellow fluorescent protein (YFP) into N-terminal and C-terminal fragments and fusing them to putative interacting proteins, a fluorescent fusion complex could be generated upon dimerization, visualized through YFP excitation.

The principle of BRET detection was similar to FRET, but it utilized bioluminescence instead of fluorescence (**Figure 3**). A donor, such as Renilla

luciferase (Rluc), oxidized a substrate (e.g., h-coelenterazine or coelenterazine-400) to produce bioluminescence, which could then transfer energy to an acceptor, such as yellow fluorescent protein (YFP) or green fluorescent protein 2 (GFP2), if they were within 10 nm of each other. This resulted in fluorescence emission from the acceptor (BRET signal)[84, 102, 120]. BRET assays were particularly useful for determining the oligomeric order of receptor complexes and the proportion of receptors engaged in dimers or oligomers. They also allowed for assessment of ligand-induced signals and affinity relationships between homo- and heterodimers [84]. Different generations of BRET have been developed, including BRET1, BRET2, eBRET2, BRET3, and QD-BRET, depending on the enzyme substrate and donor/acceptor pairs used. Rluc8, a mutated form of Renilla luciferase, was often used as the donor due to its higher quantum yield compared to non-mutated Rluc, leading to improved spectral separation and lower background[87, 116].

Higher-order heteroreceptor complexes could be detected through combined BRET/BiFC or BRET/FRET assays. The sequential BRET-FRET technique, or SRET, utilized blue bioluminescence from deep blueC to activate GFP2 (BRET2), followed by FRET transfer to a yellow or red acceptor fluorescent protein, resulting in emission in the respective spectral range [37, 87, 116]. These techniques have been employed to study complex heteroreceptor interactions, such as the A2A-D2-CB1 complex[87].

Despite the utility of FRET and BRET-based techniques in analyzing GPCR dimerization, certain challenges must be considered. Large fluorescent protein attachments may obstruct proper receptor function, and false FRET and BRET signals due to photophysic cross-talk can occur. Non-specific FRET and BRET can increase when FRET- or BRET-tagged receptors randomly collide, particularly when transiently overexpressed. Therefore, careful evaluation and interpretation of results are necessary. Nonetheless, when used appropriately, FRET and BRET-based methods clearly demonstrate the oligomerization of heterologously expressed GPCRs and cells from transgenic animals. It is widely accepted that FRET and BRET methods provide strong support for the existence of receptor heteromers in living cells.

In recent years, in situ Proximity Ligation Assay (in situ PLA) has been employed to establish the existence of native heteroreceptor complexes in the central nervous system (CNS)[51, 91] (**Figure 3**). This assay involves primary antibodies recognizing the dimer, followed by secondary antibodies linked to oligonucleotides. When the two antibodies recognize the dimer, the oligonucleotides join through enzymatic ligation, forming a DNA circle strand that serves as a template for amplification and detection through hybridization

with fluorescent oligonucleotides. In situ PLA enables the visualization of heteroreceptor complexes by fluorescence microscopy, allowing for the study of their number, localization, and modulation in formalin-fixed brain tissue [41, 88, 121]. The findings indicate that *in situ* PLA can be used to demonstrate heteroreceptor complexes *ex vivo* in brain tissue like the striatal A2A-D2 heteroreceptor complexes [88, 90] and the accumbal and dorsal striatal D2-5HT2A heteroreceptor complexes [53].

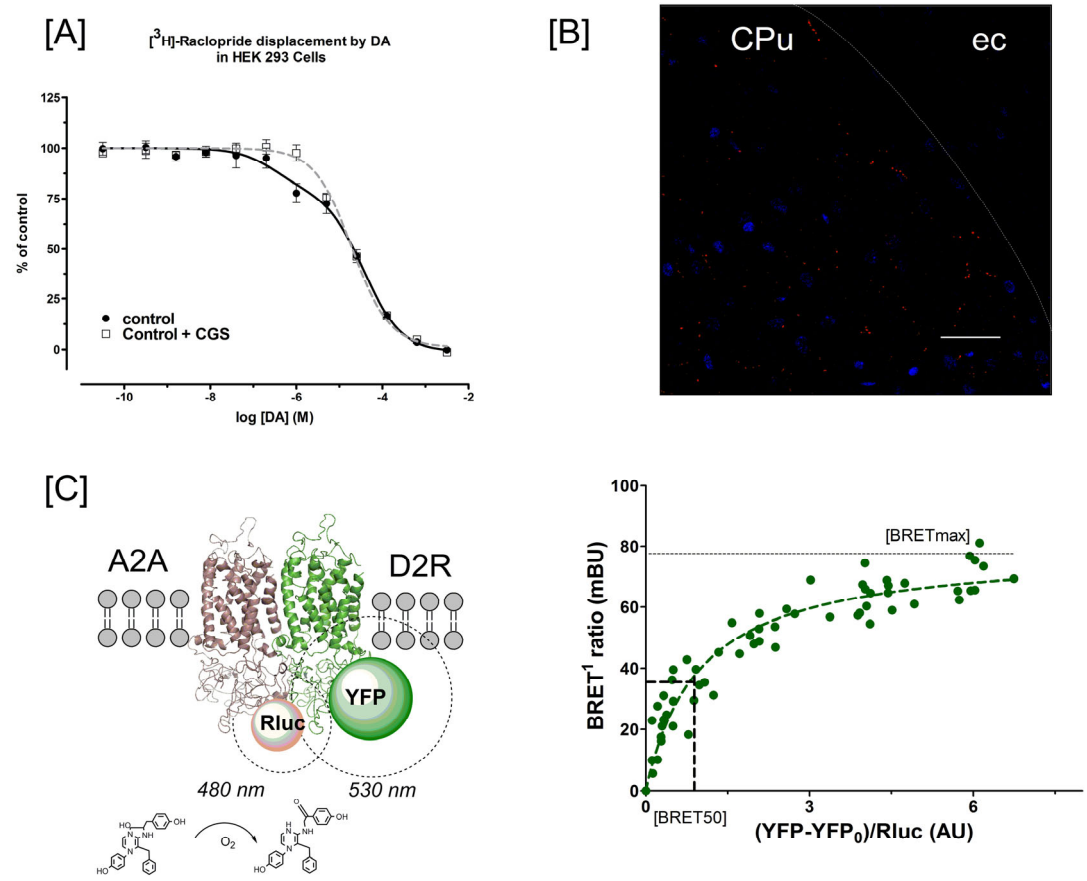

Figure 3. **Different methods have been used to study GPCR homo and heteroreceptor complexes. As examples are shown methods used to study the existence of A2A-D2 heteroreceptor complexes in heterologous expression systems and in brain ex vivo. (A)** Competition experiments using D_2-like receptor antagonist [^{3}H]-Raclopride (2 nM) versus increasing concentrations of dopamine in transiently cotransfected HEK293T cell membranes. Panel A illustrates the right shift of the competition curve after treatment with the A2A agonist CGS21680 in the high affinity range of the D2R agonist binding site demonstrating a reduction of D2R affinity in the high affinity state **(B)** Detection of A2A-D2 heteroreceptor complexes in ex vivo brain sections can be found by in situ proximity ligation assay (PLA). PLA-positive A2A-D2 heteroreceptor complexes in striatal sections have been visualized as red clusters (blobs, dots) within the neuropil of caudate putamen (CPu) which were almost absent within the external capsule (ec). **(C)** In C the principle of the BRET^1 method is illustrated giving strong support for the existence of A2AR-D2R heteromers. In the presence of h-coelenterazine, an energy transfer between Rluc and YFP occurs when the distance between them is less than 100 Å. To the right a BRET^1 saturation curve for the A2A-D2 hetero receptor complex (D2LR^{Rluc} + A2AR^{YFP}, filled circles) is shown giving BRET max and BRET EC50 values[122]. (The text and the figure have been obtained from [122]).

In conclusion, FRET and BRET-based techniques, including in situ PLA, have emerged as valuable tools for analyzing GPCR dimerization in living cells and ex vivo brain tissue. Although certain limitations and challenges exist, these methods provide substantial evidence for the existence and characterization of receptor heteromers, enhancing our understanding of their roles in cellular signaling and function.

6. In situ proximity ligation assays

The in situ proximity ligation assay (in situ PLA) is a method for visualizing the proximity between two or three interacting molecules within a biological sample. It is an advancement of the proximity ligation assay (PLA), which was originally developed to detect protein interactions in vitro. This important technique (PLA) was developed by Fredriksson, Gullberg, Söderberg and colleagues [91, 92]. In the PLA, pairs of DNA aptamers were used as affinity probes to detect homodimers of specific proteins. The PLA probes were extended with additional DNA sequences, and when the aptamers bound to their target proteins in close proximity, the additional DNA sequences could hybridize to a common connector oligonucleotide, allowing for covalent ligation. The ligation product was then quantified using real-time PCR. In in situ PLA, the method was adapted to visualize the proximity between endogenous proteins using pairs of antibodies as affinity probes. Each antibody is conjugated to an oligonucleotide, creating a PLA probe. When a pair of PLA probes binds to their respective targets in close proximity, they can template the hybridization of two additional oligonucleotides to form a circular DNA molecule. After ligation by T4 ligase, the circular DNA molecule can be amplified through rolling circle amplification (RCA) using phi29 DNA polymerase. This process generates a long single-stranded DNA molecule consisting of repeats complementary to the DNA circle. The RCA product remains attached to the protein complex via one of the PLA probes. The DNA circle includes a detection sequence that allows the RCA products to be visualized using fluorescently labeled oligonucleotides, which hybridize to the repeated detection sequence in the RCA product. Each RCA product appears as a bright dot of approximately 1 μm in size when visualized using fluorescence microscopy. The large number of fluorophores per RCA product reduces background fluorescence from the sample compared to other methods such as immunofluorescence (IF) and FRET/BRET. Each RCA product serves as a localized marker for the proximity of the two PLA probes. Secondary in situ PLA probes, consisting of oligonucleotide-coupled species-specific pairs of antibodies, can be introduced to enable the use of general in situ PLA probes with primary

antibodies from different species. Recombinant affinity binders like DARpins can also be utilized in in situ PLA.

In addition to protein-protein interactions, in situ PLA has been used to detect protein-RNA interactions, protein-DNA interactions, and post-translational modifications (PTMs). For recent work on the analysis and quantitation of the GPCR heterocomplexes with in situ PLA (see, [123]. In current procedures the receptor complexes appear as red blobs with a diameter of 0.5 to 1.5 um and their density in sampled fields is determined using confocal laser microscopy. The assay is performed in combination with a Neuro-ChromPan neuronal marker antibody-Alexa488 giving a green fluorescence to the neurons, enabling to study the link of the red blobs to the neurons. Based on different types of glial markers the link of the red blobs to glial cells can also be established.

Recently a new method for Boolean analysis at a molecular level has been introduced for protein interactions [92].The method is called MolBoolean and uses the Boolean operations AND and NOT at the molecular level. Like the PLA method, the pool of protein A and protein B is visualized when they are in sufficient proximity (AND). The difference with the MolBoolean method is that at the same time it also visualizes proteins A and B when they do not take part in an interaction with each other (NOT). Thus, at the same time the relative quantities of non-interacting protein A and protein B can be determined. It should be considered that the non-interacting components protein A and protein B, at least some of them, interact with other types of proteins or form homomers. This aspect is of high relevance for understanding the complexity and diversity of receptor-receptor interactions.

In situ PLA has advantages and drawbacks like any other detection method, and its success depends on its sensitivity and specificity. The limit of detection (LOD) is the lowest number of analytes that can be reliably detected, often defined as the number of molecules that produce a detection signal 2 or 3 standard deviations above the background. The sensitivity and LOD depend on factors such as background signal, signal variation among replicates, and detection efficiency. In situ PLA has a lower background compared to other methods due to the high local concentrations of fluorescence generated by the binding of detection oligonucleotides to RCA products. To produce a detection signal, two or sometimes three independent affinity binders are required, reducing the risk of false positive signals from nonspecific binding. The use of commercial antibodies makes the method versatile and easy to apply, but careful selection and validation of antibodies are crucial. In situ PLA detects proximity rather than interaction, similar to other methods like FRET, BRET, and IF. Although the distance criteria indicate interaction, there is always the possibility that the proteins are simply in

proximity without direct interaction. Detection efficiency, which determines the LOD and dynamic range of a method, is influenced by factors such as the dissociation constant (KD) of the affinity binders, enzymatic efficiency, sample composition and complexity, and the design of the oligonucleotide system. Experimental conditions such as buffers, temperatures, and reaction times can also affect detection efficiency. In in situ PLA, the formation of a circular ligation product may be limited by factors such as inefficient enzymatic reactions or the design of the oligonucleotide system used for the PLA probes. Additionally, the assembly of several independent oligonucleotides prior to ligation may limit the formation of a circular ligation product.

AIMS

There exists substantial evidence for the existence of GPCR homo and heteroreceptor complexes with allosteric receptor-receptor interactions in the Central Nervous System (CNS). Homo and heteroreceptor receptor complexes with allosteric receptor-receptor interactions give a new dimension to molecular neuroscience and brain integration and represent a new biological principle to integrate biological signals in all tissues. Through the receptor heteromerization the allosteric receptor-receptor interactions can develop and produce alterations in recognition including novel allosteric binding sites, pharmacology, signaling, and trafficking of the participating receptors (receptor protomers). This leads to biased and diverse signaling of the receptor heteromer signaling and to a specific integrated response at the molecular level.

The GPCR complexes can also contain ion channel receptors, receptor tyrosine kinases (RTKs), sets of G protein interacting proteins and/or transmitter transporters increasing their integrative capability. The formation of homo-and heteroreceptor complexes in a synaptic or extrasynaptic area of the plasma membrane is governed by several factors and especially by the density of the participating receptor protomers. Another factor is the affinity of one receptor protomer for another protomer, which is related to the number of hot spots that can develop in the receptor interface. The presence or absence of adapter proteins in the heteroreceptor complex can be a significant factor for determining the affinity that develops between two or more receptor protomers.

There is also lack of knowledge on the stoichiometry of the participating receptor protomers in GPCR heteroeceptor complexes. However, to-day super-resolution imaging methods, spatial intensity distribution analysis and in situ PLA methods have been developed which can be used to determine the stoichiometry in cellular models.

Therefore, **the overall aim of this thesis** was to gain insight into molecular aspects of several adenosine A2AR and dopamine D2R heteroreceptor complexes and their allosteric receptor-receptor interaction in the Central Nervous System, with special emphasis on the role of the balance between their homo and heteroreceptor complexes. As a proof of concept, we optimized and used the situ Proximity Ligation Assays methods to study A2AR and D2R iso, homo and heteroreceptor complexes. Also, to improve and expand the use of proximity ligation assays to the study of GPCR heteroreceptor complexes in the brain.

The following specific aims were considered:

AIM-1: The effects of adenosine are mediated via four adenosine receptor subtypes: A1R, A2AR, A2BR, A3R. affecting crucial processes such as neuronal signaling, astrocytic function, learning and memory, motor function and normal aging processes. Along with these normal physiological processes, adenosine is also involved in neuropathologies such as Schizophrenia, addiction and Parkinson's disease. Of the four adenosine receptors, the A1R and A2AR are both highly expressed in many brain regions and are of special interest since they are hub components of the global GPCR heterodimer network, each forming ten or more different types of heteroreceptor complexes. However, little is known in how this established integrative mechanism operating in the plasma membrane leads to formation of adenosine A2A isoreceptor complexes. Therefore, it became of interest to study the distribution pattern of the A2AR homoreceptor complexes and the A1R-A2AR isoreceptor complexes in the hippocampus and other areas of the forebrain using the proximity ligation assay (PLA).

AIM-2: The discovery of adenosine and dopamine receptor containing receptor mosaics (higher-order receptor heteromers) in the striatum opened a new understanding of adenosine-dopamine interactions in the CNS. Initial findings indicated the existence of $A_{2A}R$-D_2R heterodimers and A_1R-D_1R heterodimers in the striatum that were followed by indications for the existence of striatal $A_{2A}R$-D_3R and $A_{2A}R$-D_4R heterodimers. Adenosine A2AR and dopamine D_4R partially co-distribute in the dorsal striatum and appear to play a fundamental role in complex behaviours and motor function. I*n situ* Proximity ligation assays (*in situ* PLA) will be used to explore the existence of A2AR-D4R heteroreceptor complexes in several regions of the rat brain.

AIM-3: It is of substantial interest that A2ARs modulate alpha synuclein aggregation. Furthermore, A2AR antagonists can block the late stages of aggregation of the alpha synuclein leading to reduced toxicity. Thus, we aim to explore potential interactions between alpha-synuclein and several GPCRs, including A2AR, D2R, D4R. This will be done using in situ proximity ligation assay (PLA) in the BSSG animal model. DAT-alpha-synuclein interactions will be regarded as a positive control. And finally, to investigate how alpha-synuclein expression (wild type and A53T mutant) modulates A2AR-D2R and A2AR-D4R heteroreceptor complexes, using in situ PLA, in cell lines and in the BSSG model.

MATERIALES AND METHODS

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

On the balance and distribution of adenosine A2A isoreceptor complexes in hippocampus and other areas of cerebral cortex operating as an integrative mechanism in the plasma membrane of neuron-glia networks in the brain

Animals. All studies involving animals were performed in accordance with the European Communities Council Directive (Cons 123/2006/3) guidelines and the Institutional Animal Ethics Committee of the University of Málaga, the Spanish Directive (Real Decretory 53/2013) guidelines for accommodation and care of laboratory animals. Male Sprague-Dawley rats, 10 weeks old, weighing 310–350 g were obtained from Charles River Laboratories (Germany). The animals were housed one week before experiments under a 12-h light/dark cycle, with ambient temperature of 21 ± 2 °C, relative humidity of 50 ± 5%. Food and water available *ad libitum*. Rats (n = 5) were anaesthetized with sodium pentobarbital (40 mg/kg, i.p., Nembutal injection, Dainippon Pharmaceutical, Japan) and perfused intracardiacally with ice-cold 4% paraformaldehyde (PFA) in 0.1 M Na_2HPO_4/NaH_2PO_4, pH 7.4, buffer. Brains were removed and postfixed overnight in the same solution of PFA at 4°C and then incubated first for 24 h in 10% sucrose and then one week in 30% sucrose in 0.1 M Na_2HPO_4/NaH_2PO_4, pH 7.4, buffer. Coronal sections (30 µm) were generated using a cryostat (Leica), collected in a cryoprotective solution (30% glycerol, 30% ethylene glycol in 0.1 M Tris, pH 7.4), and kept at -20°C until *in situ* PLA processing.

In situ proximity ligation assay (Duolink, see also ANNEX 3-5). Rat hippocampal free-floating sections (30 µm) at Bregma – 3.36 and – 3.48 were employed for *in situ* PLA [88, 124], that was performed as described previously [41, 88]. Briefly, sections were quenched with 10 mM Glycine buffer, for 20 min and washed twice with phosphate buffer saline (PBS) at room temperature. Then, were incubated with the permeabilization buffer (0.1% Triton X-100 in PBS) for 30 min at room temperature followed by some wash steps and incubated with a blocking buffer (0.2% BSA in PBS) for 30 min at room temperature. The brain sections were incubated with the primary antibodies of different species, monoclonal anti-A2AR

produced in mouse (05-717, Millipore, Sweden), monoclonal anti-A2AR rabbit (AB1559F, Millipore, Sweden), monoclonal anti-A1R rabbit (AB1587P, Millipore, Sweden), diluted in a suitable concentration in the blocking solution at 4°C. After this step, the in situ PLA experiments were performed with the Duolink® In Situ Red Starter Kit Mouse/Rabbit (Sigma Aldrich) according to the manufacturer's instructions, with PLA plus or minus probes for rabbit or mouse antibodies. Sections were mounted on slides with fluorescent mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Sweden), staining the nuclei with blue color. The PLA signal was visualized, and images acquired, by using a confocal microscope (Leica TCS-SL, USA).

Quantification of In situ PLA signal. Quantification of positive PLA signal (observed as a red blobs/puncta) in different areas of the rat hippocampus was carried out using the BlobFinder and/or Duolink Software. The analyses were performed on images from at least two slices per animal, five images per hippocampal subfields. High-resolution (40x N.A. = 1.25) images from maximum projection obtained from eighteen optical section of 1µm of StepSize were acquired and analyzed. Mainly three important parameters were taken into account for a proper analysis and result interpretation: the number of DAPI nuclei in the sample field; 2) the number of positive PLA/dots per sample field; 3) the total number of positive PLA cells/nuclei per sample field. The quantitative results in the different regions of the hippocampus were expressed as mean number of PLA clusters/blobs per cell (identified by DAPI positive nuclei) in the sampled field (40 x 40 µm) from four rats. They are given in Figure 3 in percent of maximal signal of positive PLA per cell in the same sample field. Densities per cell above two/three standard deviations of the background of the negative controls (omission of one of the antibodies used) were regarded as specific PLA clusters/blobs. The values of the columns, each showing a different brain region, which are more than 25% above background, are described to represent moderate to high densities.

MolBoolean experimental procedure. All in MolBoolean experiments were performed with the MolBoolean® In Situ Starter Kit Mouse/Rabbit (Human Altas, Stokholm, Sweden) according to the manufacturer's instructions. Fixed brains free-floating slices were permeabilized with 1× TBS (Thermo Fisher Scientific) 0.2% v/v Triton X-100 (Sigma- Aldrich) for 10min, followed by 2 min wash with 1× TBS. Blocking was done either with Odyssey blocking buffer (LiCor), or homemade blocking buffer (2% BSA w/v (Jackson Immunoresearch) in 1× TBS 0.1% Tween (Sigma-Aldrich) 0.02% sodium azide (Sigma-Aldrich)), either one supplementedwith 2.5mg/mL salmon spermDNA (Thermo Fisher Scientific) for 1h at 37°Cin a humidified chamber. The slices were then incubated with pairs of mouse and rabbit primary antibodies against the proteins of interest, diluted in either Odyssey blocking

buffer, or homemade blocking buffer, and were incubated overnight at 4 °C in a humidified chamber, followed by 3 × 3min washes in 1× TBS. The primary antibodies used are described above. The brain slices were incubated with 3 µg/mL of each proximity probe (A and B), diluted in either Odyssey blocking buffer, or homemade blocking buffer for 1 h at 37 °C in a humidified chamber, followed by 1 × 3min wash in 1× TBS 1MNaCl (Thermo Fisher Scientific) 0.05% v/v Tween-20, followed by 2 × 3min wash in 1× TBS 0.05% Tween-20 (TBS-T). Subsequently, the slices were incubated in 1× T4 DNA ligase buffer, supplemented with 0.25mg/mL BSA (Sigma-Aldrich) with 0.05 µMcircle for 1 h at 37 °C in a humidified chamber, followed by 3 × 3min wash with 1× TBS-T. Afterwards a mix of 0.125 U/µl Nt.BsmAI in 1× NEBuffer CutSmart (NewEngland Biolabs), and 0.25mg/mL BSA was added for 30 min at 37 °C in a humidified chamber, followed by 3 × 3min wash with 1× TBS-T. For the hybridization of the tag oligonucleotides, the cells were incubated in 1× TBS, 0.25mg/mL BSA, and 0.5µM tag oligonucleotides A and B for 30 min at 37 °C in a humidified chamber and ligated in 1× T4DNA ligase buffer, 0.25 mg/mL BSA, 0.05 U/µl T4 ligase for 30min at 37 °C, followed by 1 × 3min wash with 1× TBS 1MNaCl 0.05% v/v Tween-20, and 1 × 3min wash with 1× TBS-T. For the RCA, the slices were incubated in 1x phi29 polymerase buffer (Monserate), 0.25mg/mL BSA, 1.25mM dNTPs (Thermo Fisher Scientific), and 1 U/µl phi29 polymerase (Monserate) for 90min at 37 °C in a humidified chamber, followed by 2 × 10 min wash with 1× TBS-T and then incubated in 1× TBS 1M NaCl 0.05% v/v Tween-20, 0.25mg/mL UltraPure Salmon Sperm DNA Solution, Hoechst33342 (1:250) (Thermo Fisher Scientific), 0.025 µM detection oligonucleotides A and B (Table 1) for 30min at 37°C in a dark humidified chamber, followed by 1 × 10min wash with 1× TBS 1M NaCl, 1 × 10min wash with 1x TBS, and 1 × 5min wash with 0.2× TBS in the dark. Slides were mounted with SlowFade Gold antifade reagent (Thermo Fisher Scientific) according to manufacturer's instructions and sealed with Menzel Gläser coverglass #1.5 (VWR). During brain tissue section imaging, at least three images per sample tissue section were taken in a single focal plane according to the Nyquist criteria. The microscope images were acquired using either a Zeiss AxioImager M2 or a Leica TCS SP8 X microscope (all other images). Adjustments of brightness and contrast were then made on figure images for visualization purposes only. Pseudo-coloring was applied to all images; Hoechst33342, Texas Red, and Atto647N are depicted in blue, magenta, and green respectively. Quantification and colocalization analyses were performed with the Duolink software on the deconvolved but otherwise unaltered images.

Naveni™TriFlex Cell Experimental Procedure. A significant portion of this study involved the optimization and standardization of a novel technique known as Naveni™TriFlex Cell, which is a commercial kit developed by Navinci (Uppsala,

Stockholm). This technique is based on proximity ligation assay technology, enabling the detection of both the close proximity and the total protein levels of two proteins in a sample. In our study, we aimed to quantify the formation of heteromeric complexes between the adenosine receptor 1 (A1R) and the adenosine receptor 2A (A2AR) using the Naveni™TriFlex Cell kit.The interaction between A1R and A2AR has been previously reported; however, our study focused on analyzing the stoichiometry of A1R-A2AR heteromers. To simulate conditions relevant to ischemic/reperfusion scenarios, we used varying concentrations of adenosine in C6 cells, a stable rat glioblastoma cell line expressing endogenous A1R and A2AR, namely 10 μM. Intermediate concentrations of adenosine mimicking intense physiological conditions associated with heavy exercise (1 μM) and low or physiological concentrations in a healthy brain (100 nM) were also employed. The chosen concentrations were based on relevant literature.To evaluate the impact of caffeine, a well-known non-selective antagonist of adenosine receptors, on the stoichiometry of A1R-A2AR heteromers, cells were treated with different concentrations of caffeine. These concentrations ranged from innocuous amounts (1 μM, 10 μM) to concentrations previously described as toxic (100 μM). C6 cells, were used for optimizing the Naveni™TriFlex Cell kit's ability to detect A1R-A2AR heteromer complexes. The cells were treated with the indicated concentrations of adenosine or caffeine for 48 hours, with treatments applied every 24 hours. After fixing the samples with 3.75% formalin and quenching with a 10 mM glycine solution, the samples were permeabilized using a 0.5% Triton X-100 solution in PBS. Subsequently, the samples were incubated overnight at 4 ºC with a mixture of primary antibodies: anti-A1R antibody (ADORA1 antibody, ref.A268, Sigma Aldrich, MO, USA) (1:500) and anti-A2AR antibody (ADORA2A antibody 7F6-G5-A2, ref.05-717, Sigma Aldrich, MO, USA) (1:500) in blocking buffer. The following day, the manufacturer's instructions were followed. Briefly, the samples were incubated with Navenibodies (secondary antibodies) for 2 hours. Polymerization and amplification steps were performed according to the distributor's guidelines. Finally, the samples were mounted using Duolink in situ mounting medium with DAPI (Sigma-Aldrich, MA, USA) for subsequent observation under a confocal Leica SP5 microscope (Leica, Wetzlar, Germany). For each field of view, a stack of four channels (one per staining) and 10 Z stacks with a step size of 5 μm were acquired. Dot quantification and cell analysis were conducted using Duolink ImageTool.

Statistical analysis. All data were analysed using the commercial program GraphPad PRISM 6.0 (GraphPad Software, USA). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-hoc test. The *P* value 0.05 and lower was considered significant.

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

Dopamine D4R and adenosine A2AR heteroreceptor complexes and its allosteric receptor-receptor interactions

Animals. Male Sprague–Dawley (derived from the licensed animal breeder Charles River), weighing between 260-310 g at the beginning of the experiment were used. The animals were housed individually in standard plastic rodent cages (39 cm x 28 cm x 28 cm) in a colony room maintained at 21±1ºC and 45-65% humidity under a 12-hr light-dark cycle (lights on at 6:00 am). Rodent food (VRF1 pellets, UK) and water were available *ad libitum*. All protocols were conducted during the light phase of the light-dark cycle between 9:00 and 13:00 hour. All animals used in the study were experimentally naive. The experiments were carried out in accordance with the European Directive 2010/63/EU and were approved by the Ethical Committee at Karolinska Institutet.

Plasmid constructs. The constructs presented herein ($D_{4.x}R$ and A2AR tagged receptor) were made using standard molecular biology techniques employing PCR and fragment replacement strategies. The other constructs used are described previously [125].

Cell culture and transfection. HEK293T cells (American Type Culture Collection, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 10% (v/v) fetal bovine serum (FBS) at 37 °C and in an atmosphere of 5% CO_2. Cells were transiently transfected using linear PolyEthyleneImine reagent (PEI) (Polysciences Inc., USA).

Co-immunoprecipitation. Co-immunoprecipitation studies were performed as previously described (for more details see [125] and ANNEX 1-2).

Immunohistochemistry and in situ PLA experiments (see also ANNEX 4). For immunohistochemical staining the animals were immediately after the experimental sessions injected with pentobarbital (133.3 mg/kg, i.p) and perfused intracardially with saline followed by 4% paraformaldehyde solution (VWR). Each brain was immersed in the same fixative for 12 hrs. The brain was left at 4-8ºC in 10% w/v sucrose up to 7 days followed by 30% w/v sucrose for two weeks. The *in situ* PLA was performed as described previously [41, 82, 84]. Free-floating formalin fixed brain sections (30 µm-thick, cut using a cryostat) at Bregma level (1.0 mm) (see Online Resource 1) from rats after cocaine-self administration were employed using the following primary antibodies: rabbit monoclonal anti-D4R (1 µg/ml, Biorbyt, Stockholm, Sweden) and rabbit monoclonal anti-A2AR (AB1559F, 1:250; Millipore, Sweden). Control experiments employed only one primary antibody or cells transfected with cDNAs encoding only one type of receptor. The PLA signal was visualized and quantified by using a Leica TCS-SL SP5 confocal microscope (Leica,

USA) and the Duolink Image Tool software. Briefly, fixed free-floating rat brain sections (storage at −20°C in Hoffman solution) were washed four times with PBS and quenched with 10 mM glycine buffer, for 20 min at room temperature. Then, after three PBS washes, incubation took place with a permeabilization buffer (10% fetal bovine serum (FBS) and 0.5% Triton X-100 or Tween 20 in Tris buffer saline (TBS), pH 7.4) for 30 min at room temperature. Again the sections were washed twice, 5 min each, with PBS at room temperature and incubated with the blocking buffer (0.2% BSA in PBS) for 30 min at room temperature. The brain sections were then incubated with the primary antibodies diluted in a suitable concentration in the blocking solution for 1-2 hrs at 37°C or at 4°C overnight. The day after, the sections were washed twice, and the proximity probe mixture (minus and plus probes, for details see: Duolink instructions) was applied to the sample and incubated for 1 hr at 37°C in a humidity chamber. The unbound proximity probes were removed by washing the slides twice, 5 min each time, with blocking solution at room temperature under gentle agitation. The sections were incubated with the hybridization-ligation solution (BSA (250 g/ml), T4 DNA ligase (final concentration of 0.05 U/µl), Tween-20 (0.05%), NaCl 250 mM, ATP 1 mM and the circularization or connector oligonucleotides (125-250 nM)) and incubated in a humidity chamber at 37°C for 30 min. The excess of connector oligonucleotides was removed by washing twice, for 5 min each, with the washing buffer A (Sigma-Aldrich, Duolink Buffer A (8.8 g NaCl, 1.2 g Tris Base, 0.5 ml Tween 20. Dissolved in 800 ml high purity water, pH to 7.4) at room temperature under gentle agitation and the rolling circle amplification buffer was added to the sections and incubated in a humidity chamber for 100 min at 37°C. Then, the sections were incubated with the detection solution through hybridization (fluorescent oligonucleotide probes) in a humidity chamber at 37°C for 30 min. In a last step, the sections were washed twice in the dark, for 10 min each, with the washing buffer B (Sigma-Aldrich, Duolink Buffer B (5.84 g NaCl, 4.24 g Tris Base, 26.0 g Tris-HCl. Dissolved in 500 ml high purity water, pH 7.5) at room temperature under gentle agitation. The free-floating sections were put on a microscope slide and a drop of appropriate mounting medium containing DAPI giving a blue staining of the nuclei (e.g., VectaShield or Dako) was applied. The cover slip was placed on the section and sealed with nail polish. The sections were protected against light and stored for several days at −20°C before confocal microscope analysis.

BRET^1 saturation assay. Forty-eight hours after transfection, HEK293T cells transiently transfected with constant (1 µg) or increasing amounts (0.12-7 µg) of plasmids encoding for $D_{4.x}R^{Rluc}$ and $A2AR^{YFP}$ respectively, were rapidly washed twice in PBS, detached, and resuspended in the same buffer. Cell suspensions (20 µg protein) were distributed in duplicate into the 96-well microplate black plates with a

transparent bottom (Corning 3651) (Corning, Stockholm, Sweden) for fluorescence measurement or white plates with a white bottom (Corning 3600) for BRET determination. For $BRET^1$ measurement, h-coelenterazine substrate (Molecular Probes, Eugene, OR, USA) was added at a final concentration of 5 μM, and readings were performed 1 min after using the POLARstar Optima plate-reader that allows the sequential integration of the signals detected with two filter settings [485 nm (440-500 nm) and 530 nm (510-560 nm)]. The $BRET^1$ ratio is defined as previously described [10]. The specificities of $D_{4.x}R$-A2AR interactions were assessed by comparison with co-expression of $D_{4.x}R^{Rluc}$ and $T2R38^{YFP}$.

Luciferase reporter gene assay. We used a dual luciferase reporter assay to indirectly detect variations of cAMP levels in transiently transfected cell lines treated with different compounds in a range of concentrations (typically 10 nM to 1 μM) . For luciferase assays, 24 h before transfection, cells were seeded at a density of 1×10^6 cells/well in 6-well dishes and transfected with TransFectin. Cells were co-transfected with plasmids corresponding to three constructs as follows (per 6-well): 1 μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p; Promega, Stockholm, Sweden), 1 μg of $D_{4.x}R$ plus A2AR expression vectors and 50 ng *R*luc-encoding internal control plasmid (phRG-B; Promega). Approximately 36 h post transfection, after the cells were incubated with appropriate ligands and harvested with passive lysis buffer (Promega), the luciferase activity of cell extracts was determined using a luciferase assay system according to the manufacturer's protocol in a POLARstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany) using a 535 nm filter with a 30-nm bandwidth. Firefly luciferase was measured as firefly luciferase luminescence over a 15 s reaction period. The luciferase values were normalized against *R*luc luminescence values.

Statistical analysis. The number of samples (*n*) in each experimental condition is indicated in Figure legends. For statistical evaluation of the biochemical data, unless otherwise specified, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The *P* value 0.05 and lower was considered significant.

III- RELATED TO SPECIFIC AIM 3 (see, Chapter 3)

Evidence for the existence of Alpha-Synuclein-GPCR and GPCR-GPCR Heterocomplex Alteration in the BSSG Rat Model of Parkinson's Disease, and In Vitro

Drugs and chemicals. To investigate the effect that receptor ligands (agonists and antagonists) had on the given receptor's potential interaction with

alpha-synuclein, and on heteroreceptor complexes, ligands were used in BRET and in situ PLA. The D2R agonists utilised included (4a*R*-trans)-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1*H*-pyrazolo[3,4-*g*]quinoline hydrochloride (Quinpirole, 1061), and (*R*)-5,6,6a,7-Tetrahydro-6-methyl-4*H*-dibenzo[*de*,*g*]quinoline-10,11-diol hydrochloride (Apomorphine, 2073). Antagonists for D2R included (6*S*)-5,6,7,8-Tetrahydro-6-[propyl[2-(2-thienyl)ethyl]amino]-1-naphthalenol hydrochloride (Rotigotine, 3896) and (1*R*,6*R*,8*R*)-6-*tert*-butyl-3-azapentacyclo[11.8.1.03,8.09,22.016,21]docosa-9,11,13(22),16,18,20-hexaen-6-ol;hydrochloride (Butaclamol, D033). Furthermore, the D4R ligands included the high affinity, selective agonist *N*-[[4-(2-Cyanophenyl)-1-piperazinyl]methyl]-3-methylbenzamide maleate (PD 168077 maleate, 1065), and the antagonists 3-(4-[4-Chlorophenyl]piperazin-1-yl)- methyl-1*H*-pyrrolo[2,3-*b*]pyridine trihydrochloride (L-745,870 trihydrochloride, 1002) and 8-Chloro-11-(4-methyl-1-piperazinyl)-5*H*-dibenzo[*b*,*e*][1,4]diazepine (clozapine, 0444). In addition, the DAT agonist methyl (1R,2R,3S,5S)-3- (benzoyloxy)-8-methyl-8-azabicyclo[3.2.1] octane-2-carboxylate (cocaine, C-008) was used. Additionally, 3,4-Dihydroxyphenethylamine hydrochloride (Dopamine, 3548) was utilised as DR agonist. The A2AR agonist 4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS-21680, 1063), and antagonists 8-[(1*E*)-2-(2-(3,4-Dimethoxyphenyl)ethenyl]-1,3-diethyl-3,7-dihydro-7-methyl-1*H*-purine-2,6-dione (Istradefylline, 5147), and 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]-ethyl)phenol (ZM- 241385, 1036) were used. All the drugs used were purchased from Tocris, except butaclamol and cocaine (Sigma-Aldrich). The concentration selected for the antagonists were 1 μM, and the agonists were 500 nM. These concentrations were selected on the basis that, for the agonists, the concentrations were at least 3x the Kd values. For the antagonists, at least 10x the Kd values were used, to ensure complete saturation of the receptors.

Animals and brain sectioning. In order to generate the BSSG rat model of PD, Sprague Dawley rats were treated with BSSG for 25 weeks. The progressive PD model was produced by Dr. Per Svenningsson's laboratory at Karolinska Institutet, and our laboratory later acquired the brains. In addition, the WT brains were also obtained from Dr. Per Svenningsson laboratory; the rats were kept in the same conditions, with their dietary pellet lacking BSSG. The brains were then perfused with phosphate buffered saline (PBS) and paraformaldehyde (PFA), left in sucrose 30%, frozen, and then sectioned. Using a cryostat, the brains were sliced to obtain coronal sections of the hippocampus and striatum, each with a thickness of 30 μm. A total of 6 brains were cut, with n = 3 per group. After the brains were sectioned, they were stored in Hoffman solution, at -20 °C, until use for in situ PLA. All animal

procedures were approved by the Ethical committee of Karolinska Institutet and were performed in accordance with the European Directive (2010/63/EU).

Cell culture. Human embryonic kidney 293T cells (HEK293T cells) were purchased from ATCC (American Type Culture Collection). The HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich), supplemented with 10% (v/v) foetal bovine serum (FBS, gibco) and 1% Penicillin/Streptomycin (PS, Sigma-Aldrich). SH-SY5Y and SK-N-MC human neuroblastoma cell lines, and SN56 fusion of mouse septal neurons and mouse neuroblastoma cells were purchased from also purchased from ATCC (American Type Culture Collection), then cultured in DMEM / Ham's F12 (1:1) (Sigma-Aldrich), supplemented with 10% FBS and 1% PS. All cell lines were incubated at 37 °C, 5% CO2 in a humidified incubator. Once reaching 90% confluency in a T75 flask (Avantor), the cells were regularly washed with Phosphate-Buffered Saline (PBS, Sigma-Aldrich), detached using Trypsin-EDTA (0.25%) phenol red (gibco), and split. The cells were maintained for a maximum of ten passages and then discarded to avoid the effect of genetic drift.

Plasmid amplification. In order to transfect cells with adequate quantity and high purity cDNA, plasmid isolation was carried out using the QIAGEN Plasmid Maxi Endotoxin-Free kit. Transformed bacteria was spread onto two agar plates; one containing ampicillin (1µg/ml) and the other kanamycin (1µg/ml), and the plates were incubated at 37°C overnight. The cDNA library has different antibiotic resistant genes, so both kanamycin and ampicillin were used to allow for appropriate selection of the colonies that have the desired plasmid. After the incubation, colonies were collected and transferred to separate ampicillin and kanamycin containing LB media (1µg/ml) and were incubated under vigorous agitation overnight. The cells were then pelleted through centrifugation for 15 minutes at 6000 × g, and the experiment was carried out according to the manufacturer's protocol. Briefly, the plasmid DNA was isolated from the bacteria by alkaline lysis, and then this lysate was centrifuged, and the supernatant cDNA was collected. The cDNA was then eluted using anion-exchange resin columns, and then precipitated using isopropanol. Following the precipitation, the cDNA was washed with ethanol, centrifuged, and the pellet was collected and stored in DNase-free water. To determine the concentration and purity of the plasmid, a NanoDrop spectrophotometer was used. The ratio of absorbance at 260nm and 280nm is indicative of the cDNA purity; absorbance at 260 nm is that of the cDNA, and absorbance at 280 nm is of contaminants such as proteins. Values obtained were 1.9-2, indicative of high purity cDNA. Regarding the concentration of the cDNA, this was measured at 1µg/µl.

Transient transfection of cells. SH-SY5Y, SK-N-MC, HEK-293T and SN-56 cells were seeded in 75cm3 and 25cm3 flasks, and 6 well plates, and cultured

overnight prior to transfection. To investigate how alpha-synuclein alters heteroreceptor complexes using in situ-PLA, WT and A53T alpha-synuclein were transfected (separately) to allow for their overexpression in the cell line. For the BRET experiments, the pair of alpha-synuclein and given receptor which were investigating whether these two interact, are co-transfected in equal amounts. Linear PEI (Polyethylenimine hydrochloride, Sigma-Aldrich) was utilised to enable the transient transfection of the cDNA into cells, and was prepared by dissolving 100 mg of PEI in 100 ml of H2O, resulting in a concentration of 40 µM. It was then adjusted for pH by adding NaOH. The ratio of cDNA:PEI used was 1:3. For the BRET experiments, for the 75cm3 flasks, 625 µl of non-supplemented DMEM (without FBS) was added to a 15ml conical tube; non supplemented DMEM is used so that the cells enter a quiescent state. Next, 5 µl of each cDNA was added (cDNA is 1 µg, so a total of 5 µg per µl of each cDNA), and then 30 µl of PEI was added. The conical tube was then topped up to 7 ml with non-supplemented DMEM. This was then added to the cells, and incubated for 4 h at 37 °C. After 4h, the medium was replaced with 11 ml of DMEM supplemented DMEM. This then incubated for 2 days at 37 °C prior to the BRET experiment. The 1:3 cDNA:PEI ratio was maintained, and volumes were adjusted for the 15cm3 flask, 6 well plate, and for the experiments where only one cDNA (WT or A53T alpha-synuclein) was transfected.

In situ PLA. In situ PLA was carried out in cell culture and tissue to explore protein-protein interactions. All cell lines were seeded in 24 well plates (Avantor), 8 or 4 chamber tissue culture-treated glass slides (Corning). After reaching a confluency of about 50%, cells were then treated as shown in figure 1 for 30 minutes. The culture medium was removed, and cells were washed with 1X PBS (Sigma-Aldrich) at room temperature (RT), and fixed with 4% Formaldehyde solution (Sigma-Aldrich) at RT for 15 mins, then washed 3 times for 10 min with cold 1X PBS. Quenching with 10mM Glycine (Sigma-Aldrich) in PBS was carried out for 20 minutes at RT. Following this, permeabilization was performed at RT using 1X PBS containing 0.1% Triton X-100 (Sigma-Aldrich) for 30 minutes at RT. The cells were washed 2 times for 5 mins with 1X PBS at RT and incubated in a blocking buffer in PBS (ThermoFisher) for 30 mins at 37 °C in a pre-heated humidity chamber. Primary antibodies were incubated at 4 °C overnight in the humidity chamber. The following antibodies were used: mouse monoclonal anti-Adenosine Receptor A2a 05-717 Sigma-Aldrich (1:250), mouse monoclonal anti-Dopamine D2 receptor (DRD2) MABN53 Sigma-Aldrich (1:500), mouse monoclonal anti-Dopamine Transporter MA524796 Invitrogen (1:500), rabbit monoclonal, anti-α-synuclein ZRB1134 Sigma-Aldrich (1:250), mouse monoclonal antibody anti-α-synuclein MA5-45837 Invitrogen (1:100), rabbit polyclonal anti-D4DR orb643932 Biorbyt (1:100). The antibody dilutions were used based on manufacturer's instructions, and

supplementary papers. PLA was performed using two different kits, according to the manufacturer's protocols: the Duolink® in situ Detection Reagents Red (Sigma-Aldrich DUO92008) using anti-rabbit plus (Sigma-Aldrich DUO92002) and anti-mouse minus (Sigma-Aldrich DUO92004) probes, and the NaveniFlex Tissue MR kit (NTB1MR002). Fluoroshield with DAPI histology mounting medium (Sigma-Aldrich) was used as a nuclear stain. Image acquisition was performed using a TCS-SL confocal microscope (Leica Laser technik GmbH, Heidelberg, Germany). A total number of three animals per group (n=3) were used. In parallel, control experiments in SH-SY5Y, SN-56, and SK-N-MC cells were performed. Images were acquired in the dorsal medial component of the striatum (caudate putamen), nucleus accumbens core and shell, and corpus callosum. All image stacks were acquired with comparable settings, at a resolution of 300 x 300 (40X objective) and 120 x 120 pixels for a zoom in, and a z-stack size of 20 µm, each scan being 1µm.

BRET[1] assay. The bioluminescence resonance energy transfer (BRET) assay was used to investigate protein-protein interactions between the DA receptors D2, D4.2 and D4.7 and alpha-synuclein (WT and A53T). HEK293T cells were transiently co-transfected with constant (1 µg) amounts of plasmids encoding the receptors fused with Renilla Luciferase (RLuc), and alpha-synuclein tagged with enhanced green fluorescent protein (EGFP), as described in section 2.5, to allow for their overexpression. If the excited state luciferin donor is in close proximity with the acceptor, the luciferin molecule transfers its energy to the fluorescent acceptor molecule. Preparation of the cells for BRET assay began with cell detachment using trypsin Trypsin-EDTA (0.25%), phenol red (gibco). In order to separate the cells, they were centrifuged at 1300 rpm for 3 minutes. The trypsin supernatant was then aspirated, and the cells were washed and resuspended thoroughly in HEPES-buffered DMEM without phenol red. To determine the effect of D2R-like ligands, that function as receptor agonists and antagonists, on their potential interactions with a-syn, transfected HEK293T cells (90 µl/well) were plated on a 96 well OptiPlate (PerkinElmer) plate and incubated with the drugs (10 µl/well) mentioned in section 2.1 for 15 minutes. Next, 10 µl/well of coelenterazine (Molecular Probes, Eugene), which is the substrate for the luciferase enzyme, was added at a final concentration of 5 µM, and readings were performed 1 minute after. Then, the plate was placed in a luminometer, specifically the POLARstar OPTIMA microplate reader (BMG Labtech). This records both the BRET signal, which is the emission signals from both the donor and acceptor, and the BRET ratio, which is the acceptor signal divided by the donor signal, and this is indicative of the proximity of the two proteins. The BRET[1] ratio = [(EGFP emission at 530 ± 10 nm)/(Rluc emission 485 ± 10 nm)]– correction factor (cf.) The correction factor, cf. = (emission at 530 ± 10 nm)/(emission at 485 ± 10 nm) of the receptor-Rluc construct expressed

alone, in the same experiment. The ligand-induced BRET^1 ratio is indicative of the change in BRET^1 ratio induced by the ligand and is calculated as follows: BRET^1 ratio of treated cells-BRET^1 ratio of cells lacking treatment. A total number of three independent experiments (8 replicates per group) were performed.

Data analysis. Quantification of the fluorescent in situ PLA signals was carried out using the Duolink ImageTool software. In situ PLA and BRET data was analysed using GraphPad Prism 5.0. Statistical analysis was done using one-way and two-way ANOVA, and student's t-test, with α values set at $\leq$ 0.01, 0.05 and 0.001 significance.

CHPATER 1
Results and Discussion

SPECIFIC AIM 1. *On the balance and distribution of adenosine A2A isoreceptor complexes in hippocampus and other areas of cerebral cortex operating as an integrative mechanism in the plasma membrane of neuron-glia networks in the brain*

1.1. Background

G protein-coupled receptors (GPCR) homo and heteromerization is an established integrative mechanism operating in the plasma membrane leading to formation of homoreceptor (identical receptors) and heteroreceptor (different receptors) complexes [126, 127]. Adenosine A2AR and A1R receptors are of special interest since they are hub components of the global GPCR heterodimer network (GPCR-HetNET [53] each forming ten or more different types of heteroreceptor complexes [106, 128-130]. The ligand is the neuromodulator adenosine, an endogenous nucleoside [131] which operates as a volume transmission molecule in the extracellular fluid of the CNS with origin both in glia and neurons [49, 132]. Homomerization of adenosine A2ARs and A1Rs was also demonstrated in cell lines using fluorescence and bioluminescence energy transfer (FRET and BRET) techniques [133]. A further development took place when adenosine A1-A2A isoreceptor complexes were found in the plasma membrane of A1-A2A cotransfected HEK cells using BRET, time-resolved FRET and coimmunoprecipitation [134]. With immunogold and coimmunoprecipitation their colocation was indicated on striatal glutamate nerve terminals and on postjunctional membranes of these glutamate synapses. The existence of striatal A1R-A2AR isoreceptor complexes was suggested based on the observations that the A2A agonist CGS 21680 reduced the affinity of the agonist binding sites of the striatal A1 receptors as studied in membrane preparations [134]. Later on Gi protein coupling of the A1R protomer of the A1R-A2AR isoreceptor complex was found to be impaired by the A2A agonist in the human caudate nucleus [135].

Based on this work it became of interest to study the distribution pattern of the A2AR homoreceptor complexes and the A1R-A2AR isoreceptor complexes in the hippocampus and other areas of the forebrain using the proximity ligation assay [41, 62,

[88, 94]. It may serve to illustrate if they show a correlated distribution in different network components of certain regions while in others no correlation exists. A correlation may indicate a balance between the A2A homoreceptor and A1R-A2AR isoreceptor complexes present in the same cell, which may have an impact on the decoding of the adenosine signaling which operates via volume transmission. The hippocampus is of special interest since optogenetic activation of hippocampal A2A receptor signaling elicited CREB phosphorylation and impaired memory.

1. 2. Results

A2A-A2A homoreceptor complexes. Studies with Duolink in situ PLA. The quantitative results in the different regions of the hippocampus, the dorsolateral cerebral cortex and the piriform cortex are expressed as mean number of PLA blobs per cell (identified by DAPI positive nuclei) in the sampled field (20 x 20 um) from five rats. They are given in **Figure 4** in percent of blobs over negative controls (omission of one of the antibodies used). Densities per cell above two standard deviations of the background of the negative controls were regarded as specific PLA blobs. The values of the columns in red, each showing a different brain region, are more than 25 % above background and are described to represent moderate to high densities.

The highest density was observed in the pyramidal cell layers of CA1-CA3 and the polymorphic layer of dentate gyrus. CA4 appeared to have similar densities but quantitation was not performed. Image from CA2 exemplifies the high density of PLA positive blobs (size 0.5-1 μm) in the pyramidal cell layer, while **Figure 4**, right panels show an image of the high density in the polymorphic layer. The molecular layer and the sub granular layer of the dentate gyrus had low and moderate densities of PLA blobs per cell, respectively (**Figure 4**). The molecular and radiatum layers of the CA regions lacked or had a low density of PLA blobs. ‘

High densities were also found in layers III-V of the dorsolateral cerebral cortex and layer II of the piriform cortex (**Figure 4**). The density of blobs in the corpus callosum was not significantly different from the background of negative controls (**Figure 4**). The overall A2A-A2A PLA blob distribution is illustrated in Figure 1, regarding the hippocampus and the cerebral cortex. The density of red circles indicates the relative PLA blob distribution over the hippocampus, the dorsolateral cerebral cortex and the piriform cortex.

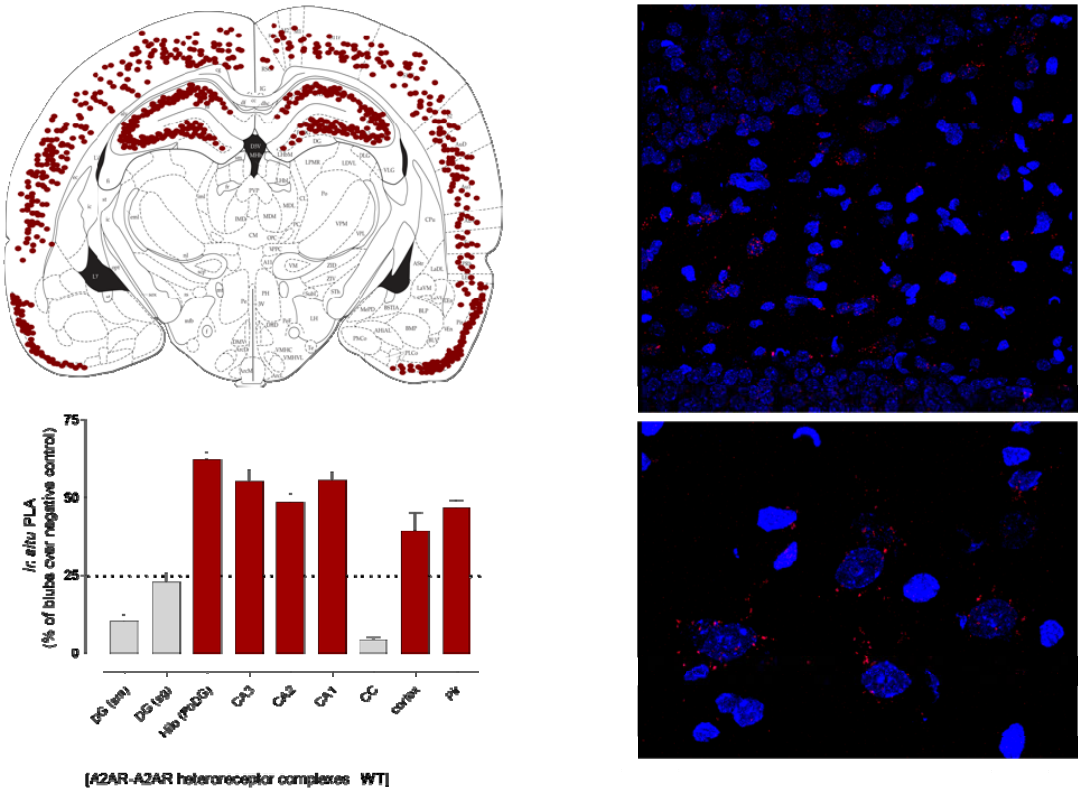

Figure 4. **Detection of A2AR-A2AR homoreceptor complexes in the different subfields of dorsal rat hippocampus by in situ proximity ligation assay (in situ PLA).** The nuclei are shown in blue (DAPI) and the positive PLA puncta/blobs (A2AR-TrkB heteroreceptor complexes) in red. **(Right panels)** Representative confocal images of in situ PLA at the polymorphic layers of the dentate gyrus (PoDG). Some positive PLA clusters/blobs are indicated by an arrow. In the cc, the PLA values obtained were similar to thus obtained in the negative control (in situ PLA experiment performed without one of the adenosine A2A primary antibody). The scale bar for these three panels is 35 µm. **(Left panel, bottom)** Number of positive in situ PLA blobs per nuclei per field as a percent of the maximal signal. The analyses (Means ± S.E.M.) were performed on images from at least two slices per animal (four rats in total), five images per hippocampal subfields.

A1-A2A isoreceptor complexes. Studies with Duolink in situ PLA and MolBoolean analysis. The quantitative results in the different regions of the hippocampus, the dorsolateral cerebral cortex and the piriform cortex are again expressed as mean number of PLA blobs per cell in the sampled fields as shown in **Figure 5**. As observed for A2A-A2A PLA blobs, high densities of A1R-A2AR blobs were observed in the pyramidal cell layers of CA1-CA3 regions and in the polymorphic layer of the dentate gyrus with low-moderate densities in the subgranular layer (**Figure 5**).

In panels of **Figure 5** PLA images are presented from the polyform and subgranular layer of the dentate gyrus and pyramidal cell layer of CA3. The PLA blob density in the molecular layer of the dentate gyrus and in the corpus callosum did not significantly differ from the PLA blob density found in negative controls. A

moderate density of A1R-A2AR PLA blobs was found in layers III-V of the dorsolateral cerebral cortex and a high density in layer II of the piriform cortex (**Figure 5**). The density of red circles in Figure 2 indicates the relative overall PLA blob distribution within the hippocampus, the dorsolateral cerebral cortex and the piriform cortex. It is like the overall distribution found of the A2A-A2A PLA blobs in these regions.

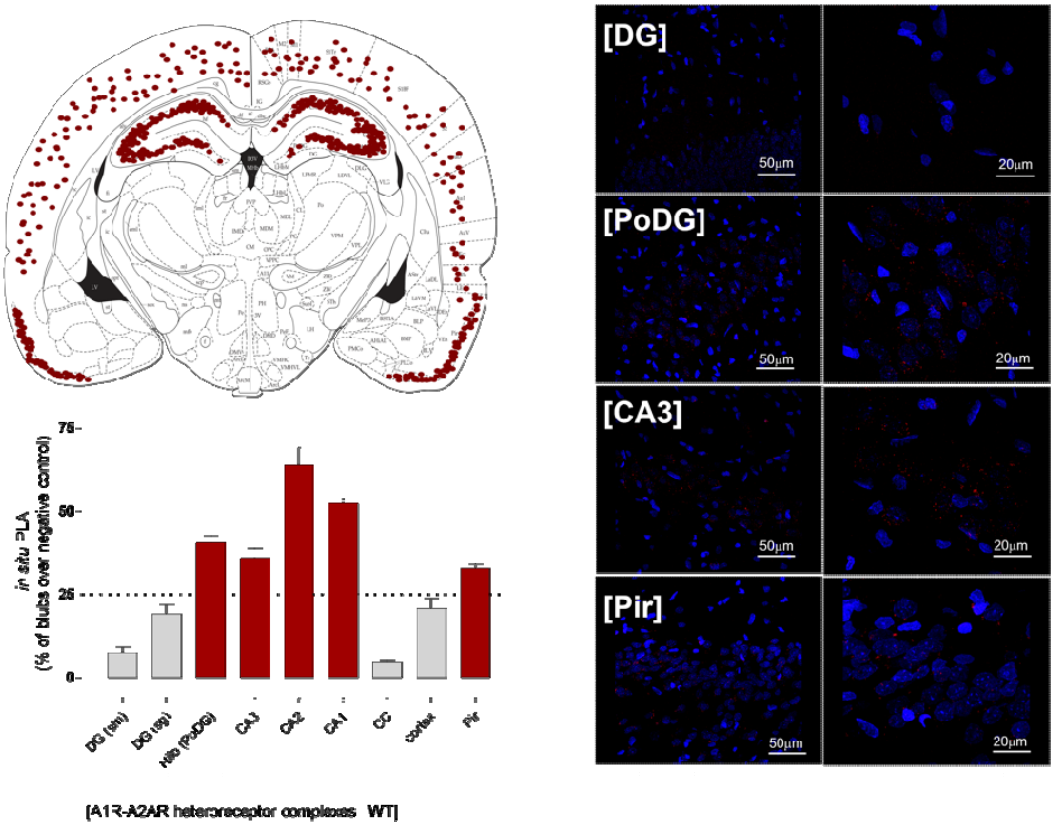

Figure 5. **Detection of A1R-A2AR isoreceptor complexes in the different subfields of dorsal rat hippocampus by in situ proximity ligation assay (in situ PLA).** The nuclei are shown in blue (DAPI) and the positive PLA puncta/blobs (A2AR-TrkB heteroreceptor complexes) in red. **(Right panels)** Representative confocal images of in situ PLA at the pyramidal cell layer of the CA1, the granular (grDG) and polymorphic layers of the dentate gyrus (PoDG), and the corpus callosum (cc). Some positive PLA clusters/blobs are indicated by an arrow. In the cc, the PLA values obtained were similar to thus obtained in the negative control (in situ PLA experiment performed without one of the adenosine A2A primary antibody). The scale bar for these three panels is 35 µm. **(Left panel, bottom)** Number of positive in situ PLA blobs per nuclei per field as a percent of the maximal signal. The analyses (Means ± S.E.M.) were performed on images from at least two slices per animal (four rats in total), five images per hippocampal subfields.

These results were also confirmed by means of MolBoolean analysis (**Figure 6**). Similar densities were found in the CA4 regions but not quantitated. Again, the molecular and radiatum layers of the CA regions had no specific PLA blobs or a low

density of specific PLA blobs. This distribution pattern is demonstrated in **Figure 7** in the CA2 region.

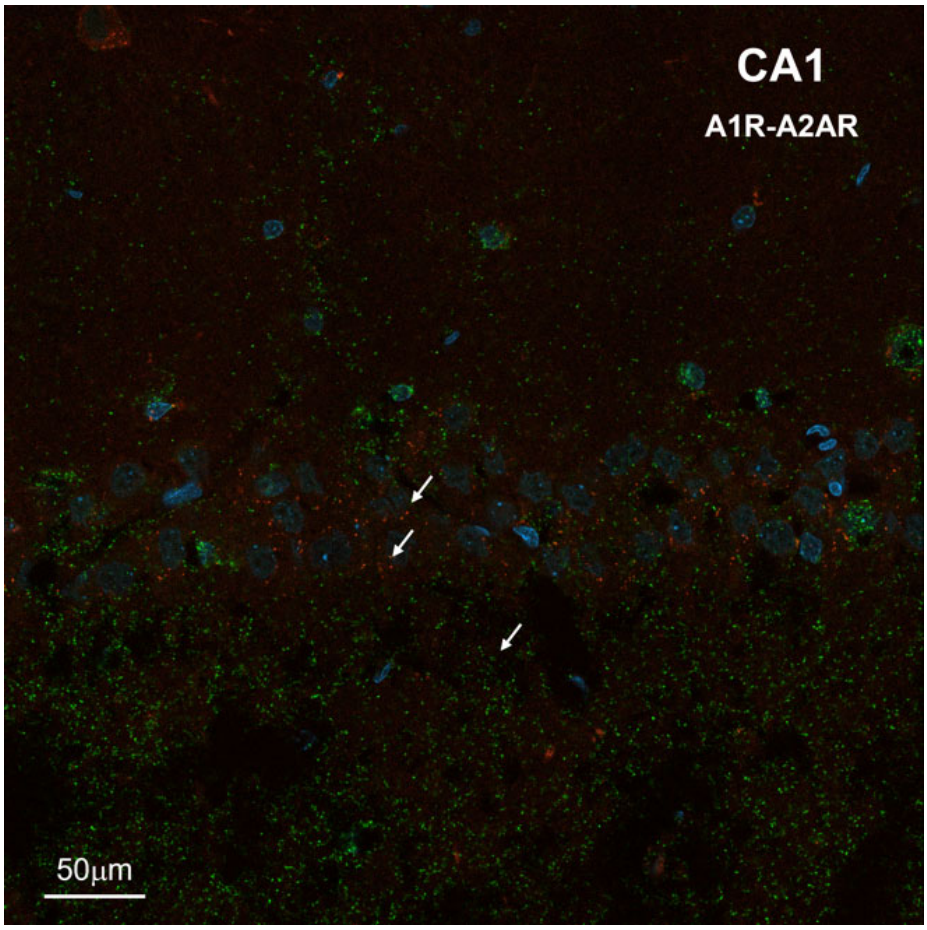

Figure 6. **Detection of A1R-A2AR isoreceptor complexes in the different subfields of dorsal rat hippocampus by MolBoolean assay.** The nuclei are shown in blue (DAPI) and the positive PLA puncta/blobs (A1R-A2AR isoreceptor complexes) in red.

Analysis of A1R-A2AR isoreceptor complexes upon agonist and antagonist treatments. Naveni™TriFlex Cell experiments. C6 cells were stained with three different fluorophores to detect the endogenous levels of total receptor A1R, total receptor A2AR, and the formation of interacting A1R-A2AR heteromers.
Quantification of dots per cell, containing dots, was performed using the individual masks. We observed that under conditions simulating ischemia with adenosine concentrations of 10 µM, the total levels of A1R and A2AR receptors were reduced compared to other adenosine treatments and the vehicle control. Similarly, the signal for heteromer formation was also decreased in cells treated with adenosine at 10 µM. In cells treated with caffeine, we observed a significant increase in the total amount of A1R and A2AR receptors with the toxic concentration of 100 µM. However, no differences were observed in the amount of heteromer signal between caffeine treatments and the vehicle control. Although statistical comparisons between

adenosine and caffeine-treated cells were not performed, there is a tendency towards increased levels of both total receptors and interacting A1R-A2AR in cells treated with caffeine.

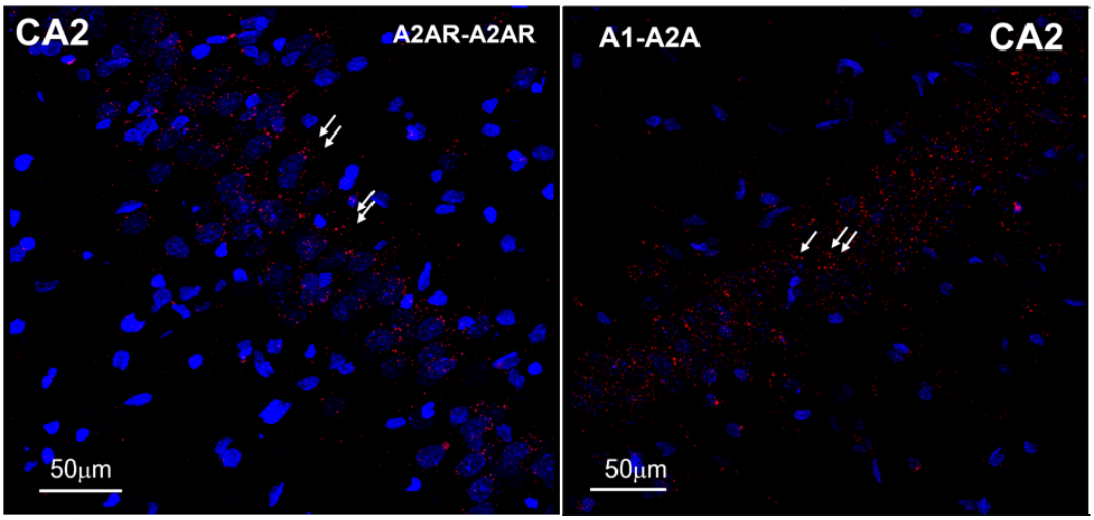

Figure 7. **A representative confocal image of the in situ PLA at the level of the pyramidal cell layer of the CA2 for both, the A2AR-A2AR homoreceptor complexes and the A1R-A2AR isoreceptor complexes.** Positive PLA signal (red clusters/blobs) is mainly detected on the cells of the pyramidal cell layer (pcl) and within a lower density on the layer of the stratum radiatum (sr). Some positive PLA puncta/blobs are indicated by an arrow and higher order of heteroreceptor compelxes (clusters) are indicated by a dash circle. The scale bar for this panel is indicated on the right bottom of the panel.

Specificity of the adenosine A1R-A2AR heteroreceptor interface. Bioinformatic analysis using the triplet puzzle theory. Based on a mathematical approach it was deduced, based on 48 pairs of receptors that form or not form heterodimers, a set of triplet amino acid homologies that may be critically involved in receptor–receptor interactions [105, 136]. This theory was called the triplet puzzle. Such triplets of amino acid residues and their 'teams' was utilized to construct a kind of code that help determine which receptors should or should not form heterodimers. A 'guide-and clasp' manner for receptor–receptor interactions was proposed where 'adhesive guides' may be the triplet aminoacid homologies.

To understand the specificity of the A1R-A2AR receptor interface the triplet amino acid homologies involved in the A1R-A2AR interface were identified in a bioinformatic analysis (**Figure 8-9**). In **Figure 8** protriplets AKS and FSL are shown to exist in the border zone between intracellular loop3 (IC3)and transmembrane 6 (TM6) of A1R and A2AR receptors. In the case of AKS it may also participate in the extracellular part of the interface between the integrin ITGA3 and ITGB1 but not in any other human receptor heteromer like the A1-D1 or A2A-D2 heteromers.

The KSL protriplet homology of the A1-A2A heteromer can instead participate also in the intracellular part of the interface of A1-mGluR1 and A1-P2Y1 as well as in the

extracellular part (LRR domain) of the interface between the TLR1-TLR2. However, it is not involved in the interface of the A2AR-D2R heteromer. These results support a substantial degree of specificity in the A1R-A2AR interface through the AKS and KSL protriplet homologies.

Specificity is also underlined by finding an EDL protriplet homology in the C-terminal tail of A1, D1, mGluR1 and ADRB2 which can participate in the interface of A1-mGluR1 and A1-D1 heteromers and a potential A1-ADRB2 heteromer as well as in an A1-P2Y1 heteromer. In contrast, it does not participate in the A2A-D2 heteromer. Specificity is also supported by the demonstration of a PNG protriplet in the C-terminal tail of A2A and D5 and in the IC3 of D2. This protriplet homology does not exist in the A1-A2A heteromer but can participate in the interface of the A2A-D2 and the D2-D5 heteromer.

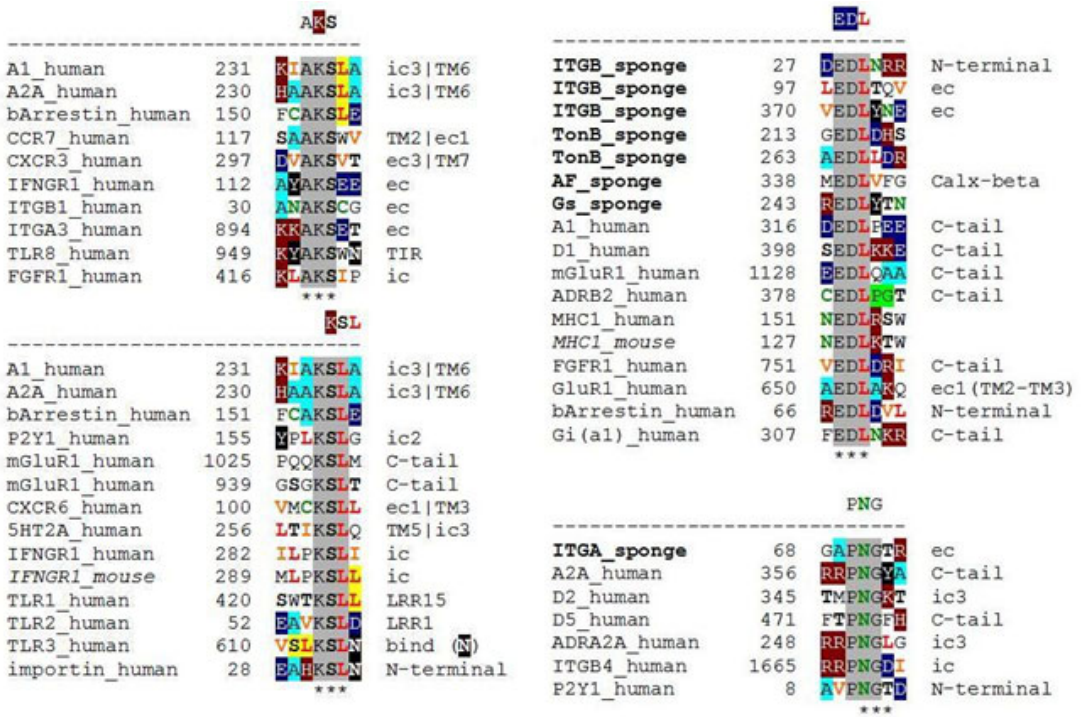

Figure 8. **Example of protriplets AKS, KSL, EDL and P NG in the protomers of human receptor heteromers and other proteins and receptors.**

Figure 9. **Example of protriplets in the protomers of human receptor heteromers**

Receptor heteromer	Reference	AKS	KSL	EDL	PNG
ITGA3-ITGB1 Laminin receptor	Barczyk et al. (2010)	#	–	–	–
A1-A2A	Ciruela et al. (2006)	#	#	–	–
A1-mGluR1	Ciruela et al. (2001)	–	#	#	–
TLR1-TLR2	Jim et al. (2007)	–	#	–	–
A1-D1	Gines et al. (2000)	–	–	#	–
A1-ADRB2		–	–	#	–
A2A-D2	Canals et al. (2003)	–	–	–	#
D2-D5	So et al. (2009)	–	–	–	#
A1-P2Y1	Yoshioka et al. (2001)	–	#	–	–

yes in both receptors and may mediate their interaction, – no in any receptor.

1. 3. DISCUSSION

The current study reports the existence of A2A-A2A homoreceptor and A1-A2A heteroreceptor complexes in the hippocampus, the dorsolateral cerebral cortex and the piriform cortex with similar distribution patterns using the PLA technique [88]. These results may open up an understanding of which brain circuits such adenosine receptor complexes can modulate and their relevance for learning and memory and psychiatric disease.

The current bioinformatics analysis of the A1-A2A heteromers indicated that their receptor interface is different from the one in A1-D1 and A2A-D2 receptor heteromers [137, 138]. Thus, the AKS and KSL amino acid homologies found in the A1-A2A heteromer were not present in the A1-D1 and A2A-D2 heteromers which contained other types of homologies. These results support the specificity in the heteromerzation process and indicate that the interface of adenosine receptor heteromers built up of adenosine isoreceptors can differ from those formed from adenosine receptors by interacting with other receptors using different transmitter/modulator ligands.

In the hippocampus the highest densities were found in the pyramidal cell layers of CA1-CA3 and the polymorphic cell layer in the hilus of the dentate gyrus. Previous work demonstrated that adenosine A1 and A2A receptors can be coexpressed in many hippocampal glutamatergic pyramidal nerve cells and in glutamate nerve terminals of the hippocampus [134]. Furthermore, in the striatum indications were obtained using coimmunoprecipitation and biochemical binding experiments that A1-A2A heteroreceptor complexes may be present on striatal glutamate nerve terminals. Antagonistic A2A-A1 receptor-receptor interactions can take place in these

complexes with A2A receptor activation reducing the affinity of the A1 agonist binding sites. Based on these observations it is proposed that the A2A-A2A and A1-A2A PLA blobs in the pyramidal cell layer of the hippocampus may in part be located in the soma-dendrites of the pyramidal cells. They may also be located in local glutamate collaterals of the CA3 and CA2 regions and in extrinsic glutamate inputs to CA1 and CA3 from the CA2 pyramidal cells. The local CA1 collaterals mainly reach into the stratum oriens and alveus. The Schaffer collaterals from CA3 are likely not linked to these adenosine receptor complexes since they project to the stratum radiatum and oriens where only a low density of these complexes was found. The same is also true for the glutamate inputs from the entorhinal cortex which project mainly into the outer two thirds of the molecular layer but also into other regions such as the stratum lacunosum-moleculare which appear to lack specific PLA blobs.

Based on these observations it is proposed that appropriate regulation of A1-Gi/o and A2A-Gs/olf mediated receptor signaling in the hippocampus which have opposing actions on the AC-PKA-CREB signaling pathway to the nucleus, glutamate release [139] and brain function pathology such as seizures is made possible made by a balance of adenosine receptor signaling in A2A-A2A, A1-A1 homoreceptor complexes and A1-A2A heteroreceptor complexes. This can take place within individual pyramidal cells and between pyramidal cells with different ratios between these major complexes mediating adenosine signaling. The importance of this balance is demonstrated by the ability of optogentic activation of hippocampal A2A receptor signaling through phosphorylation of CREB to impair memory [140]. In line with these results A2A inactivation also enhances working memory and reversal learning. Recently a reorganization of homo and heteroreceptor complexes in the postsynaptic membrane was proposed as a possible molecular basis for learning and memory [49, 126]. In learning, a new temporal pattern of release of transmitters is learned through a transient reorganization of sets of homo and heteroreceptor complexes in postsynaptic and associated extrasynaptic membranes. This receptor reorganization leads to a novel bar code forming a short term memory of the novel pattern of transmitter release to be learned, which can be converted into long-term memory via newly formed adapter proteins stabilizing the receptor complexes and linking them to the cytoskeleton [35, 126]. It seems possible that disruption of the balance of the signaling of adenosine homo and heteroreceptor complexes can lead to errors in the molecular engrams formed of the hippocampal glutamate synapses and impairment of spatial memory performance develops.

Tamminga and colleagues (2012) introduced an interesting hypothesis that glutamate dysfunction in dentate gyrus and CA3 signaling participate in psychosis development including appearance of deficient declarative memory [141]. The model proposes a

reduced glutamate signaling in the mossy fibres to the CA3 due to a molecular dysfunction of the granular nerve cells of the dentate gyrus, which therefore cannot be driven properly by their entorhinal afferents. Through a reduction in the activation of the inhibitory CA3 GABA interneurons by the Mossy fibers this dysfunction may produce an increased activity in the excitatory output of CA3 to the CA1 subfield. This may generate false associations which leads to memories of psychotic thoughts and signs of schizophrenia as the output of this brain circuit reaches subiculum and then the cerebral cortex [141].

It seems possible that a disbalance of the signaling of the adenosine homo and heteroreceptor complexes in the CA3-CA1 regions can contribute to this pathological output with psychosis consequences through their modulation of the GABA-glutamate interactions. It is known that A2A receptor agonists are atypical antipsychotic drugs in animal models of schizophrenia [12, 48]. It may be that part of the mechanism for this action could be to increase A2A signaling and restore the balance of adenosine isoreceptor signaling in some glutamate synapses in the CA regions. It is speculated that this action may block the formation of molecular engrams of these false memories in the postsynaptic membrane and its extrasynaptic components of these hippocampal glutamate synapses present in the CA3 and CA1 regions.

The mossy fibers of the granular nerve cells also project into the polymorphic layer of the dentate gyrus where they also contact mossy cells and different types of inhibitory interneurons [142]. Also, these synaptic interactions may be modulated by adenosine mediated signaling in view of the existence of high densities of A2A-A2A and A1-A2A PLA positive blobs in the polymorphic layer. The low to moderate densities of these receptor complexes in the subgranular layer opens up the possibility that these integrative A1 and A2A homo and heteroreceptor mechanisms can modulate the activity also of the dentate pyramidal basket cells mainly found in this layer [142]. Thus, the current study indicates that the entire hippocampal circuit from the dentate gyrus via CA3 and CA1 to the subiculum can be modulated by these integrative adenosine receptor mechanisms fine-tuning the glutamate and GABA synapses and their interactions of these hippocampal regions.

It should be noted that the A2A homo-and isoreceptor complexes may control also neuroinflammation in the hippocampus and the associated dysfunction of its neuron-glia networks [143]. It was found that the A2A receptor antagonist SCH58261 given icv diminishes the LPS induced inflammation in the hippocampus produced by microglia activation. Thus, the phenotype of the hippocampal microglia in inflammation likely express increased densities of A2A receptors which may enhance inter alia the release of chemokines and cytokines and other inflammatory agents from the microglia. This action appears to dominate when the invasion of peripheral immune cell is restricted. These findings can help explain the neuroprotective effects of A2A

receptor antagonists in neurodegenerative disorders where the neuroinflammatory processes play a role.

In the piriform cortex also a codistribution of A2A-A2A and A1-A2A specific PLA blobs of high densities were observed in layer 2 but not in the other layers. They may have a role in regulating the balance of activity in excitatory pyramidal cells and inhibitory interneurons [144]. The piriform cortex also belongs to the limbic system and is known to have the ability to induce seizures [145]. In fact, it was found that A1 but not A2A receptor agonists exerted anticonvulsive effects when microinjected into the piriform cortex of amygdala kindled rats [146].

It seems possible that such effects can be exerted in part in layer 2 in A1-A2A heteroreceptor complexes and in postulated A1-A1 homoreceptor complexes. Furthermore, microinjections of A1 and A2A agonists in the CA1 region had reducing and increasing effects on piriform cortex kindled seizures [147]. Together with the current findings it seems possible that the balance of adenosine signaling in homoreceptor and isoreceptor complexes of A1 and A2A receptors in limbic regions have an impact on limbic seizure development.

High densities of A2A-A2A and A1-A2A specific PLA blobs were observed in layers III-V of the dorsolateral cerebral cortex. In view of the fact that A2A agonists centrally administered produce a marked increase in sleep it seems likely these A2A receptor complexes with the granular and pyramidal layers of the cerebral cortex studied at Bregma level 3.6 may participate in this action. It is also known that in A2A but not A1 knockout mice the arousal action of caffeine I counteracted. The role of the A1 receptor protomers in the cortical A1-A2A isoreceptor complexes, however, is not clear with regard to sleep induction. In fact, A2A protomer agonist activation reduces the affinity of the A1 receptors [134]. This indicates reduced A1 receptor signaling in these complexes when A2A receptor protomer becomes activated and induces sleep.

The evidence suggests that the brain site determines if sleep is induced by A1 receptor activation. A1 activation e.g. in the tuberomamillary nucleus and in the lateral hypothalamus elicits sleep (Oishi et al. 2008, Thakkar et al.2002) while in the lateral preoptic area increased wakefulness develops (Methippara et al.2005). Thus, the role of the high densities of A1 receptors in the cerebral cortex in sleep remains to be determined but the current findings open up the possibility that they can modulate sleep by allosterically interacting with A2A protomer signaling in the isoreceptor complexes in the cerebral cortex and balance the actions of cortical A2A-A2A homoreceptor complexes through potentially codistributed A1-A1 homoreceptor complexes in layers III-V.

Future work may clarify the complexities of the integration of adenosine VT signaling in the brain through the adenosine isoreceptor signaling and their balance

with the corresponding A2A and A1 homreceptor signaling in the neuron-glia networks.

CHPATER 2
Results and Discussion

SPECIFIC AIM 2. *Dopamine D4R and adenosine A2AR heteroreceptor complexes and its allosteric receptor-receptor interactions*

1. 1. Background

There exists substantial evidence for the existence of GPCR homo and heteromers with allosteric receptor-receptor interactions in the CNS [41, 43, 148]. Through the receptor heteromerization the allosteric receptor-receptor interactions can develop and produce alterations in the recognition, pharmacology, signaling and trafficking of the participating receptors (receptor protomers) [122, 149]. This leads to biased and diverse signaling of the receptor heteromer signaling and to a specific integrated response at the molecular level [9, 38]. In tissues e.g., the brain, the terms homo and heteroreceptor complexes are instead used since the receptors also bind to a substantial number of adapter proteins many of which have not been identified. Also, their stoichiometry has not been determined**.**

Adenosine is a significant neuromodulator in the CNS that originates from ATP located intracellularly and extracellularly in the neuronal and glial cells. Adenosine located intracellularly can reach the extracellular space via transporters present in the plasma membrane. It uses volume transmission for communication taking place in the extracellular space of the brain through diffusion and flow [49, 81]. Adenosine receptors play a crucial role in mediating adenosine communication. There are four different subtypes namely A1R, A2AR, A2BR and A3R [150-152]. It has been reported that several adenosine receptor subtypes exist in the brain as homo and heteroreceptor complexes [53, 134]. For instance, the A2AR enhances the activity of dorsal striato-pallidal GABA neurons mainly through allosteric inhibition of the Gi/o mediated D2R protomer signaling in an A2AR-D2R heteroreceptor complex located in the soma-dendritic level of these GABA neurons [33, 153-155]. This activation of the dorsal striato-pallidal GABA pathway by A2AR agonists leads to motor inhibition.

On the other hand, co-immunoprecipitation experiments suggest the existence of D_2R-D_3R heteromers in D_2R and D_3R cotransfected HEK293 cells [156]. Antiparkinsonian D_2 agonists like ropinirole and pramipexole appear to have a more potent effect at the D_2R-D_3R heteromer binding pockets thus the heteromer allows the D_3R to become more strongly coupled to the adenylate cyclase resulting in

increased inhibition of its activity. The D_2R has also been found to form a phospholipase C-coupled heteromer with the dopamine D_1R in D_1R-D_2R cotransfected cells and both receptors co-immunoprecipitate in rat striatal membrane preparation [157]. There is evidence that the D_1R-D_2R heteromer possesses a specific coupling to the Gq/11–coupled pathway giving it a unique pharmacology with high PLC activation and low AC activation [157]. Upon coactivation of the two receptors in the D_1R-D_2R heteromer the allosteric receptor-receptor interactions may only allow the Gq/11 to couple to the D_1R-D_2R heteromer.

Dopamine D_4 receptors appear to play a fundamental role in complex behaviors mediated by the limbic system including novelty seeking behaviors and in the control and coordination of movements involving the dorsal striatum and has been postulated to form heteromers with adenosine A_{2A} receptors [158-160]. Furthermore, the D_2 and D_4 receptors partially codistribute in the dorsal striatum, especially in the matrix compartment and both of them are associated with post-synaptic elements mainly involving dendrite shafts and dendritic spines [161].

In this paper we show, for the first time, that A2A receptors can also form heteroreceptor complexes with three more common human dopamine $D_{4.x}R$ polymorphism variants ($D_{4.2}R$, $D_{4.4}R$ or $D_{4.7}R$) to different degrees in HEK293T cells cotransfected with human A2A receptors and with any of these $D_{4.x}R$ isoforms by means of BRET[1]. Furthermore, by means of *in situ* Proximity ligation assays (*in situ* PLA) we show evidence for the existance of A2AR-D4R hteroreceptor complexes in several regions of the rat brain.

1. 2. Results

A2AR and $D_{4.x}R$ form constitutive heteromers in HEK293T cells. It has been demonstrated that adenosine A2AR form homo and heterodimers, especially with several types of rhodopsin like receptors, even in the absence of agonist [53]. In order to analyse if adenosine A2AR and the most common polymorphic variants of dopamine $D_{4.x}R$ in the human population, namely $D_{4.2}R$, $D_{4.4}R$ and $D_{4.7}R$, can interact and exists as an heteroreceptor complex, we used three different approaches, co-immunoprecipitation, *in situ* PLA and the BRET technology. Classic co-immunoprecipitation performed on extracts of transiently cotransfected HEK293T cells was conducted with different combinations of the three polymorphic variants of 3xHA-$D_{4.X}R$ and with FLAG-A2AR. The immunoblot obtained with anti-HA antibodies revealing the presence and position of the HA tagged $D_{4.2}R$, $D_{4.4}R$ and $D_{4.7}R$ immunoreactive bands after immunoprecipitation of the FLAG tagged A2AR by means of antibodies against the FLAG. The differential position of the $D_{4.2}R$,

$D_{4.4}R$ and $D_{4.7}R$ immunoreactive bands is clearly seen, demonstrating co-immunoprecipitation of the $D_{4.2}R$, $D_{4.4}R$ and $D_{4.7}R$ with the A2AR (data not shown).
Next, to further characterize the heterodimeric interactions of dopamine $D_{4.x}R$ polymorphic variants and adensoineA2AR we examined the possibility of direct receptor-receptor interactions by BRET saturation curves. BRET assay was performed on HEK293T in cells transiently co-transfected with a constant amount of $D_{4.2}R$-Rluc, $D_{4.4}$-Rluc or $D_{4.7}$-Rluc construct and increasing concentrations of the adenosine A2A-YFP plasmids. As a positive control, we used the cell expressing a GFP^2-YFP tandem fusion protein (0.5 µg cDNA). Upon co-expression of the $D_{4.2}R$-Rluc, $D_{4.4}$-Rluc or $D_{4.7}$-Rluc and $A_{2A}R^{YFP}$ cDNA, a significantly higher BRET signal was observed compared to the BRET signal obtained from cells individually expressing each of the two receptors (**Figure 10**).

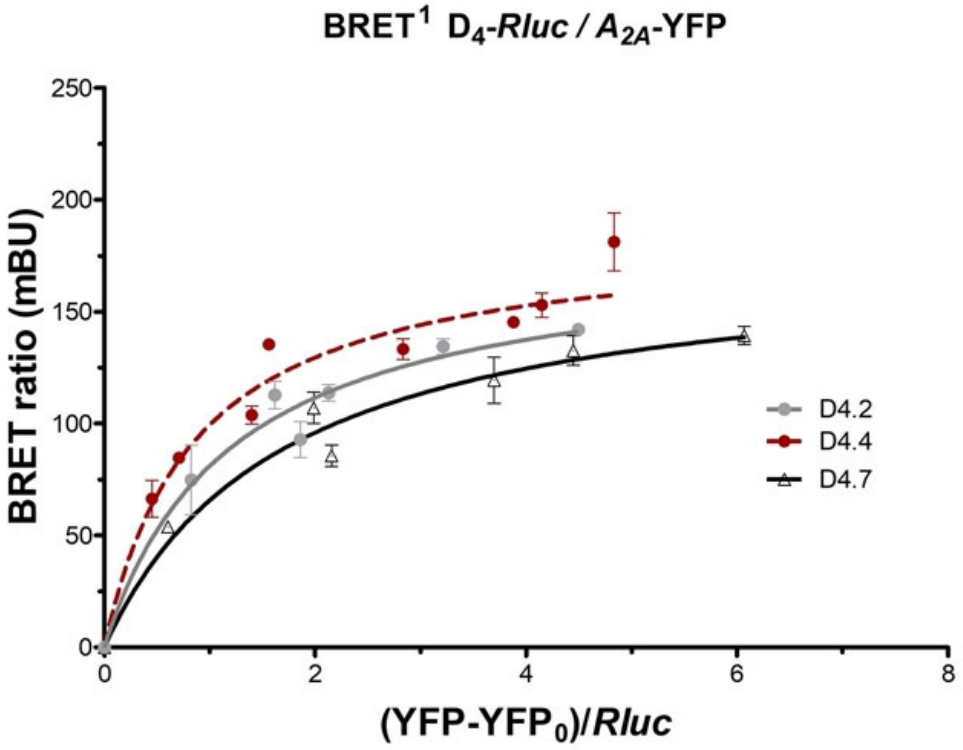

Figure 10. **Quantitative analysis of A2AR and D4.xR heterodimerization.** BRET donor saturation curves were performed by transfecting HEK293T cells with a constant DNA concentration of acceptor receptor-Rluc and increasing concentrations of donor receptor-YFP constructs. BRET ratio, total fluorescence and total luminescence were determined as described under material and methods. The curves represent 8 saturation curves that were fitted using a non-linear regression equation assuming a single binding site.

As shown in **Figure 10**, significant quantitative BRET signals were found for the A2AR-$D_{4.2}R$ and A2AR-$D_{4.4}R$ pairs, giving also the highest maximum BRET values ($BRET_{max}$). A lower $BRET_{max}$ signal was obtained with the A2AR-$D_{4.7}R$ pair which

was also shown to possess a significantly higher $BRET_{50}$ value. In all cases, BRET increased as a hyperbolic function of the increasing concentration of the YFP fusion construct, reaching an asymptote at the highest concentrations used. To test that the BRET signal was indeed a result of a specific protein-protein interaction, we performed two essential control experiments. First, we co-expressed the $D_{4.4}$R-Rluc with soluble YFP which indeed led to marginal signals that increased linearly with increasing amounts of YFP expressed. Secondly, we over-expressed increasing concentrations of WT receptor in combination with the protomers of the BRET pair (constant ratio 1:1) and investigated whether the WT receptor could reduce the BRET signal. Over-expression of the WT receptor significantly reduced the BRET ratio.

In Situ PLA Detection of A2AR and D_4R heteroreceptor complexes in parts of the rat forebrain. An overall high density of PLA positive clusters was found in the dorsal striatum (**Figure 11**) based on the average number clusters per nucleus (in blue) per sample field. It was highly significant increased vs values in negative controls and the myelinated bundles of the crus cerebri (CC) and the anterior limb of the anterior commissure (aca). In the nucleus accumbens shell a medium density of PLA positive A2AR-D4R heteroreceptor complexes was found and, in the nucleus accumbens core a low density (**Figure 11**). They were both significantly different from the number of PLA positive clusters in aca and negative controls.

The quantitative results in the different regions of the hippocampus, the dorsolateral cerebral cortex and the piriform cortex are expressed as mean number of PLA blobs per cell (identified by DAPI positive nuclei) in the sampled field (25 x 25 um) from five rats (**Figure 12** and **Figure 13**). Densities per cell above two standard deviations of the background of the negative controls were regarded as specific PLA blobs. The values of the columns in red, each showing a different brain region, are more than 25 % above background and are described to represent moderate to high densities.

The highest density was observed in the pyramidal cell layers of CA1-CA3 and the polymorphic layer of dentate gyrus. Image from CA1 exemplifies the high density of PLA positive blobs (**Figure 12**) in the pyramidal cell layer. The molecular layer and the subgranular layer of the dentate gyrus had low densities of PLA blobs per cell, respectively. The molecular and radiatum layers of the CA regions had a low density of PLA blobs too.

High densities were also found in layers III-V of the dorsolateral cerebral cortex and layer II of the piriform cortex (**Figure 13**). The density of blobs in the corpus callosum was not significantly different from the background of negative controls.

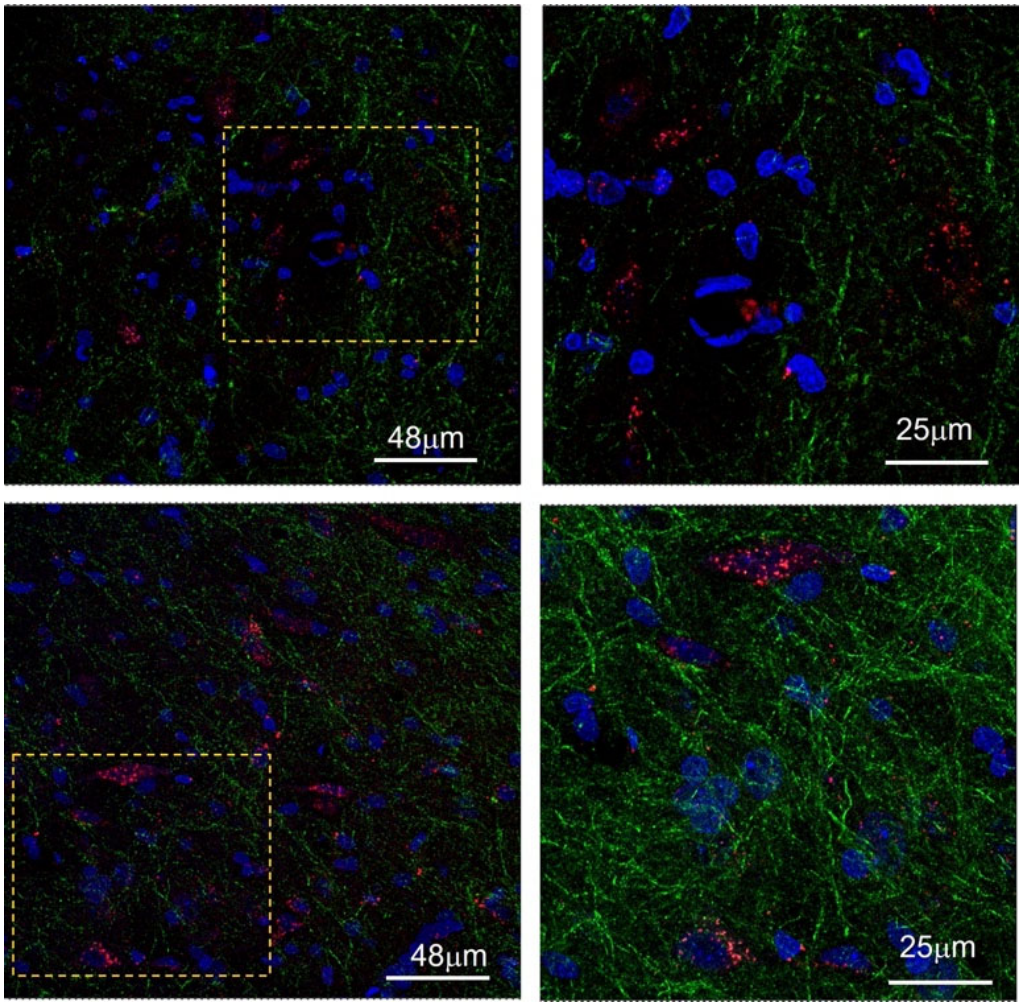

Figure 11. **Examples of combined in situ PLA assay and neuronal labelling, using the Milli-Neuronal marker antibody AlexaFluor488 conjugate, in the ventral striatum.** A high density of PLA positives profiles (red) is shown in the dorsal (top panels) and ventral (bottom panels) striatum regions (green), representing the adenosine A2AR-dopamine D4R (A2AR-D4R) heteroreceptor complex. Microphotograph from transverse sections of the rat striatum showing the distribution of the A2AR-D4R heteroreceptor complexes using the in situ proximity ligation assay (in situ PLA) technique combined with the Neuro-Chrom™ Pan neuronal marker antibody-Alexa488 conjugate immunostaining. The combined use of in situ PLA and neuronal labelling indicate the expression of the A2AR-D4R mainly at the somatic membrane level. The nuclei are shown in blue by DAPI.

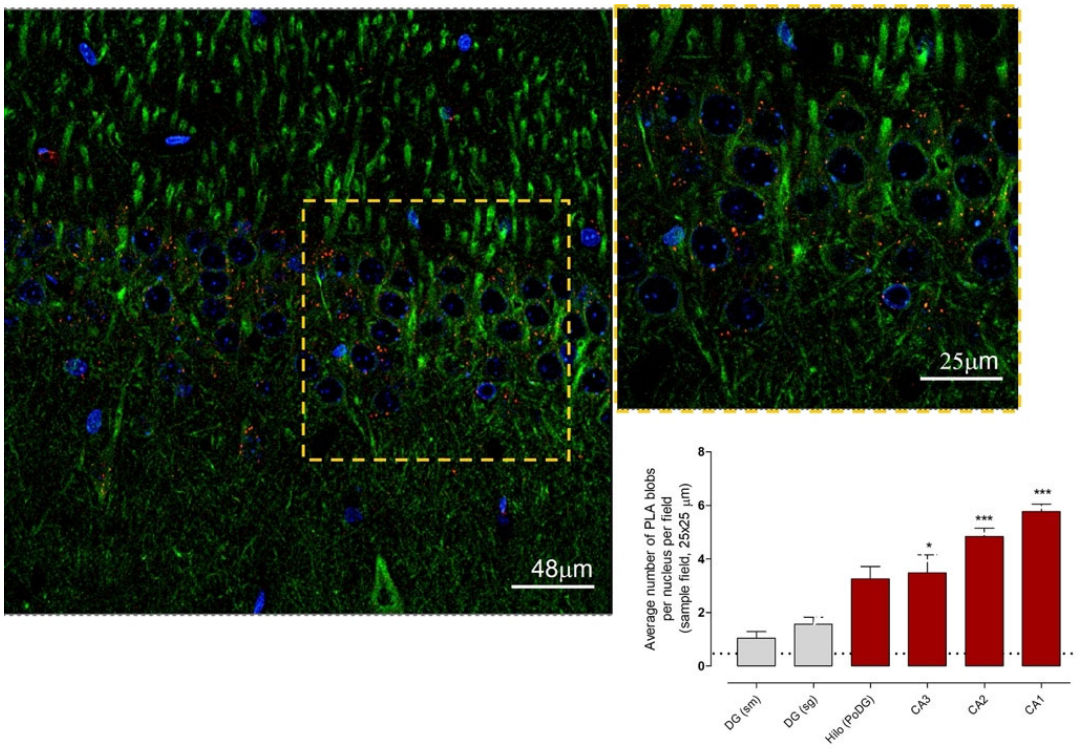

Figure 12. **Illustration of the A2AR-D4R heteroreceptor complexes in the dorsal hippocampus of the rat brain. (Left panel)** Microphotograph from transverse sections of the rat dorsal hippocampus (Bregma level: -3.6 mm) showing the distribution of the A2AR-D4R heteroreceptor complexes in CA1 using the in situ proximity ligation assay (in situ PLA) technique [27,31,32]. They are shown as red PLA blobs (clusters) found in high densities per cell in a large number of nerves cells in the pyramidal cell layer using confocal laser microscopy. The nuclei are shown in blue by DAPI. The neurons are seen in green by the use of the Neuro-ChromTM Pan neuronal marker antibody-Alexa488 conjugate. **(Right panel)** In the right inset the PLA blobs in the pyramidal cell layer are shown in higher magnification. The square outlines the CA1 area from which the picture was taken. **(Bottom right panel)** Quantitative analysis of the A2AR-D4R heteroreceptor complexes expression in the dorsal hippocampus of the rat brain detected by means of the in situ PLA. PLA was quantified as PLA (red puncta/blobs) per nuclei per field by an experimenter blind to the experiment conditions. The analyses (Means ± S.E.M.) were performed on images from at least two slices per animal (four rats in total), five images per hippocampal subfields. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The P value 0.05 and lower was considered significant: *$P < 0.05$, ***$P < 0.001$. Significant changes in CA1-CA3 areas versus DG (molecular and granular layers) area of the dorsal hippocampus.

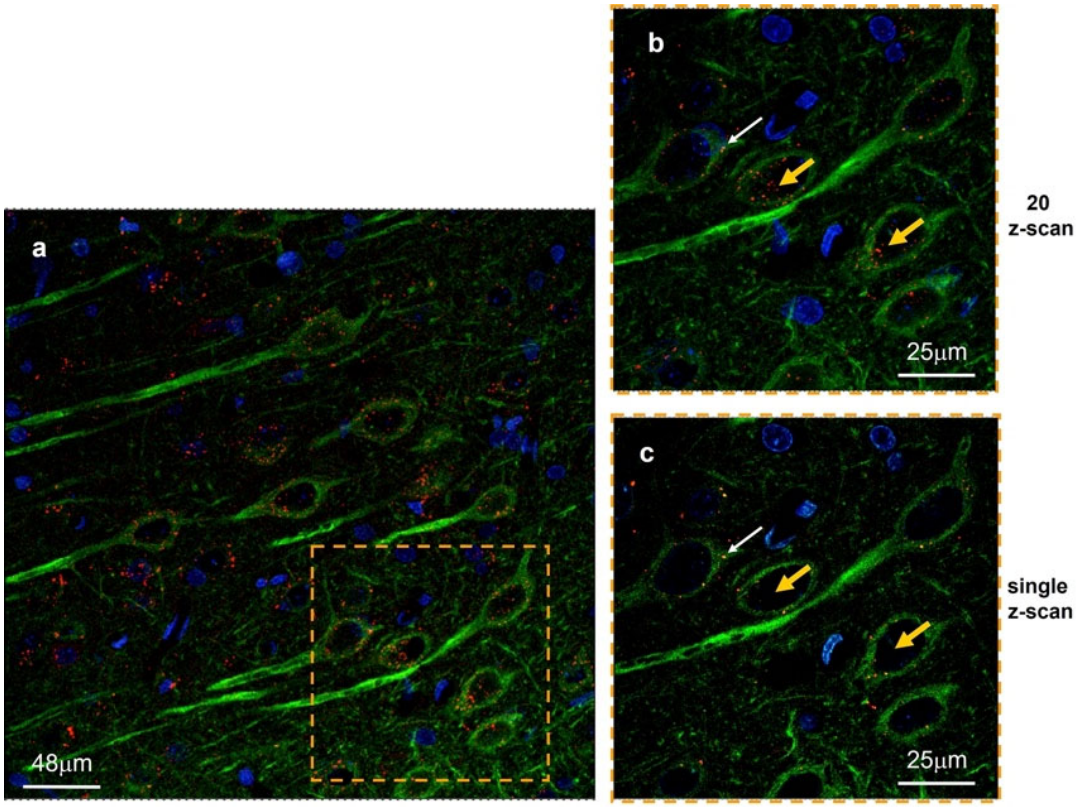

Figure 13. **Examples of combined in situ PLA assay and neuronal labelling, using the Milli-Neuronal marker antibody AlexaFluor488 conjugate, in the cerebral cortex.** A high density of PLA positives profiles (red) is shown in the internal pyramidal nerve cell layer V (green) of the prefrontal cortex, representing the adenosine A2AR-dopamine D4R (A2AR-D4R) heteroreceptor complex. **(a)** Microphotograph from transverse sections of the rat anterior cingulate cortex (Bregma level: 1.2 mm) showing the distribution of the A2AR-D4R heteroreceptor complexes using the in situ proximity ligation assay (in situ PLA) technique [27,31,32] combined with the Neuro-ChromTM Pan neuronal marker antibody-Alexa488 conjugate immunostaining. The combined use of in situ PLA and neuronal labelling indicate the expression of the A2AR-D4R mainly at the somatic membrane level. White arrows point to red PLA clusters. The nuclei are shown in blue by DAPI. **(b** and **c)** High magnifications of this microphotograph are shown in the right panels and the pictures are taken and visualized using multiple z-scan (20 z-scan) and single s-can projection respectively. Positive PLA blobs/clusters are located on the cytoplasmic membrane (see yellow arrows in panel **b** and **c**). The scale bars are shown in the lower-right panels

Functional consequences of A2AR-$D_{4.x}$R heteromerization on CREB phosphorylation. It has been well documented that dopamine D2 like receptor activation via the $G_{i/o}$-AC-PKA cascade, can decrease the phosphorylation of the CREB transcription factor leading to a reduction of the activity of cAMP response element (or CRE) (in our experimental approach represented as a CRE-luciferase reporter gene).The receptor-receptor interactions within the A2AR-$D_{4.x}$R heteroreceptor complexes were studied by analysis of CREB phosphorylation after selective A2A (CGS21680) and D_4R (PD168077) agonist treatment and their combination in A2AR-$D_{4.4}$R cotransfected cells. In A2AR-$D_{4.4}$R transfected cells, the forskolin-induced increase of luciferase activity by a direct activation of AC is

significantly reduced by PD168077, an action which is fully counteracted by 50 nM of CGS21680 and by the D_4R like antagonist l-741742 (1 μM) (**Figure 14, left panel**). CGS21680 (50 nM) alone does not affect the forskolin-induced increase of luciferase activity. In A2AR-D4R cotransfected cells (**Figure 14, right panel**) the concentration response curve obtained with the D_4R agonist PD168077 to inhibit forskolin-mediated CRE-Luc induction was shifted to the right by the A2AR receptor agonist CGS21680 (100Nm), showing a reduced potency of the D4R agonist to inhibit the CRE signal.

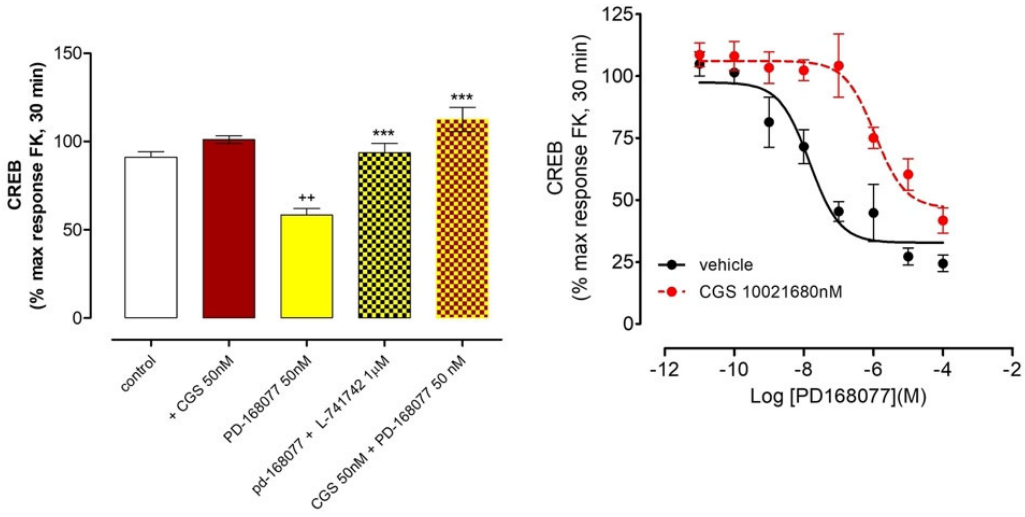

Figure 14. **Functional consequences of A2AR-D4.4R heteromerization on CREB phosphorylation**. **(Left panel)** HEK293T cells were transiently co-transfected with 1 μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p), 1 μg of both (D4.4R and A2AR) and the expression vectors and 50 ng Renilla luciferase-encoding internal control plasmid (phRG-B). Thirty-six hours after transfection, cells were treated 4 hours with agonist or antagonist (in presence of 1 μM of submaximal value of forskolin). The fairly to see an action of the A2A agonist may be related to an endogenous adenosine tone. Light emission is expressed as a percentage of the control forskolin-induced value. The data represent the means ± S.E.M. of three independent experiments performed in triplicate. +++: Significantly different compared to CGS 50nM ($P<0.001$) and control; ***: Significantly different compared to PD-168077 50nM ($P<0.001$). **(Right panel)** Dose-response curves with PD168077 in A2AR-D4R co-transfected HEK293T cells. HEK293T cells were transiently co-transfected with 1 μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p), 1 μg of both (D4.4R and A2AR) and the expression vectors and 50 ng Renilla luciferase-encoding internal control plasmid (phRG-B). Thirty-six hours after transfection, cells were treated 4 hours with 2μM forskolin (sub-maximal concentration), D4R receptor agonist PD168077 in presence or absence of the A2AR agonist CGS21680 (100nM) and the luciferase activity was measured. Light emission is expressed as a percentage of the control forskolin-induced value. The data represent the mean ± S.E.M. of three independent experiments performed in quadruplicate. (EC50 ~ 14.6 nM in control group and EC50 ~ 1.1 μM in the A2AR agonist CGS21680 groups at 100nM, respectively).

Overall, $A_{2A}R$ activation can, via the G_s-AC-PKA cascade (AC: adenylate cyclase) increase the phosphorylation of CREB leading to an increase of the CRE

transcription. $A_{2A}R$ activation can also lead to an increased transcriptional activity by antagonizing the $D_{4.4}R$-$G_{o/i}$-AC inhibition through a receptor-receptor interaction.

1. 3. DISCUSSION

The existence of A2A-D4R heteroreceptor complexes after cotransfection of the two receptors in cell lines is demonstrated by means of co-immunoprecipitation and BRET assay experiments. In addition. An allosteric A2AR-D4R receptor-receptor interaction also appears to exist in the control of D4R protomer signaling. In the analysis of the Gi/o-AC-PKA-CREB pathway using the CREB luciferase reporter gene assay the presence of a A2AR-D4R heteroreceptor complex led to a reduced potency of the D4R specific agonist PD-168077 to inhibit the CREB signal. Their antagonistic allosteric receptor-receptor interactions led to a significant inhibition of the Gi/o mediated D4R protomer signaling. The results indicate the possibility that the A2AR-D2R heterorecepor complexes can play a significant role in neuroplasticity.

By means of proximity ligation assay these receptor complexes were identified in high densities mainly in the ventral striatum, the prefrontal cortex and the pyramidal cell layer of the hippocampus. They were observed as red clusters with a size range of 0.5 to 2 um located in the soma outside the nuclei. They are constitutive and were also demonstrated in cellular models with BRET.

Indications that A2AR can heteromerize also with D3 receptors were obtained in cellular models in 2005 using FRET [46]. Antagonistic A2AR-D3R interactions were also demonstrated leading to reduction of the affinity of the high affinity D3 agonist binding site. The receptor interface involved also for this receptor heteromer electrostatic forces between positively charged arginins (D3R intracellular loop 3) and negatively charged aspartates (A2AR C-terminus) [160]. The D3 receptors in brain are mainly found in the ventral striatum unlike the D2R which is located in high densities in both ventral and dorsal striatum. These results strengthen the view that A2AR agonists should counteract psychosis development in PD patients upon dopaminergic treatment by targeting A2AR-D2R and A2AR-D3R heteroreceptor complexes in the ventral striatum [48].

It was early on indicated that also A2AR can D4R can form heteroreceptor complexes based their interface which involved electrostatic interactions as outlined above for the A2AR-D3R [160]. It was demonstrated by Rivera et al. in 2002 that the D4R was enriched in the striosomes but also found in the matrix of the dorsal striatum [161]. It is therefore of high interest that we observed A2A-D4 heteroreceptor complexes in the dorsal and ventral striatum using the proximity ligation assay.

In the dorsal striatum, the location of the A2AR-D4R appear mainly into the striosomes. The striosomes using GABA as transmitter can project directly to the nigral DA neurons and inhibit their activity. It is therefore of high interest that A2AR-D4R heteroreceptor complexes can in part be located to the striosomal GABA neurons. It seems likely that also in this receptor complex an antagonistic allosteric receptor-receptor interaction can develop reducing the Gi/o mediated inhibitory D4R signaling. This may result in an enhanced activity of the striosomal GABA neurons directly projecting to nigral DA cells. As a result, a reduced activity with reduced DA turnover should develop in the DA nerve cells projecting to the striosomes.

It should be noticed that also D4R-MOR heterorecepor complexes appear to exist in the striosomes [161]. Facilitatory allosteric D4R-MOR interactions were observed in striosome nerve cells enhancing the affinity of MOR, which should enhance inhibition by MOR of the striosome GABA projection neurons. These results serve to illustrate that multiple heteroreceptor complexes perform molecular integration also exist in the striosome projection neurons involving in this case A2AR-D4R complexes. The D4R seems to have taken over the role of the D2R in the striosome system and interacts both with A2AR protomers and MOR protomers.

CHPATER 3
Results and Discussion

SPECIFIC AIM 3. *Evidence for the existence of Alpha-Synuclein-GPCR and GPCR-GPCR Heterocomplex Alteration in the BSSG Rat Model of Parkinson's Disease, and In Vitro*

1. 1. Background

Parkinson's Disease (PD) is a prevalent neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the accumulation of alpha-synuclein, forming Lewy bodies [162]. It affects approximately ten million people worldwide and leads to both motor and non-motor symptoms that significantly impact patients' quality of life [163, 164]. Among other protein and gene, alpha-synuclein plays a pivotal role in PD pathology, and its abnormal accumulation and aggregation contribute to the formation of Lewy bodies [165, 166]. While previous research has primarily focused on intracellular alpha-synuclein, recent studies have shed light on the actions of extracellular alpha-synuclein [106]. This form of alpha-synuclein propagates between cells, binds to cell surface receptors, and triggers signaling cascades, resulting in increased synaptic damage, cell death, and Lewy body formation.

G protein-coupled receptors (GPCRs) are a diverse group of transmembrane proteins involved in various physiological processes and implicated in diseases such as PD [12, 54]. GPCRs undergo conformational changes upon agonist binding, leading to the activation of specific G proteins and subsequent modulation of intracellular signaling pathways. GPCR heteroreceptor complexes, discovered in the early 1980s, exhibit allosteric interactions that modify the function of the involved receptors, making them valuable targets for research and therapeutic interventions in brain diseases like PD[106, 107, 167-171]. Dopamine receptors (DR) are a subset of GPCRs that play a crucial role in PD [172-178]. They are divided into two subclasses: D1-like family receptors (D1 and D5) and D2-like family receptors (D2, D3, and D4). The imbalance between the direct and indirect pathways, regulated by D1-like and D2-like receptors, respectively, contributes to the motor symptoms of PD. Modulating these receptors presents a potential therapeutic approach for PD treatment. The adenosine A2A receptor (A2AR), another GPCR, is highly expressed in the striatum and primarily located in striato-pallidal GABA neurons. A2AR forms heteroreceptor complexes

with D2 receptors, leading to a reduction in D2 receptor signaling [164, 171, 179-181]. Targeting A2AR has emerged as a therapeutic strategy to alleviate the brake on the indirect pathway, potentially improving motor symptoms in PD.

Alpha-synuclein interacts with various cell surface receptors, including dopamine receptors and the dopamine transporter (DAT) [182-184]. These interactions can impact the configuration and function of the associated GPCRs, leading to downstream signaling pathway modifications and physiological responses relevant to PD pathophysiology. Investigating the interactions between alpha-synuclein and DA receptors, particularly D2 and D4, is of interest due to their involvement in the indirect pathway of basal ganglia motor control.

To study these interactions and their implications, a recent BSSG rat model of PD has been developed [185]. This model replicates many pathologies observed in PD patients, including the progressive loss of dopaminergic neurons, alpha-synuclein accumulation, and motor and non-motor symptoms. By utilizing this model, the present research aims to explore potential GPCR-alpha-synuclein interactions, investigate the modulation of alpha-synuclein-dopamine receptor interactions, and examine the impact of alpha-synuclein expression on A2AR-D2R and A2AR-D4R heteroreceptor complexes. The proposed studies have the potential to uncover new alpha-synuclein receptor interactions, shed light on the modulation of GPCR signaling pathways, and identify therapeutic targets for PD. Additionally, investigating the differences between wild-type and A53T mutant alpha-synuclein may provide valuable insights into the disease mechanisms and facilitate the development of innovative therapeutic approaches.

In summary, this research chapter we aim to elucidate GPCR-alpha-synuclein interactions and their implications in PD using the BSSG rat model. By investigating these interactions and their modulation, this study aims to contribute to our understanding of PD pathophysiology and pave the way for the development of novel therapeutic strategies and the validation of existing PD therapies.

1. 2. Results

Characterization of GPCR-alpha-synuclein interactions by in situ PLA. To investigate the interactions between alpha-synuclein and the receptors D2, D4, A2A, and DAT, in situ proximity ligation assay (PLA) was performed in the BSSG rat model of PD and in vehicle-treated WT rats as a control. The analysed brain regions are depicted in **Figure 15-A**. In the SD control brain, PLA signals (red puncta) representing D2R-alpha-synuclein interactions were observed in the caudate putamen, accumbens core and shell of the ventral striatum, while very few positive

signals were detected in the corpus callosum, serving as an internal negative control (**Figure 15-B**).

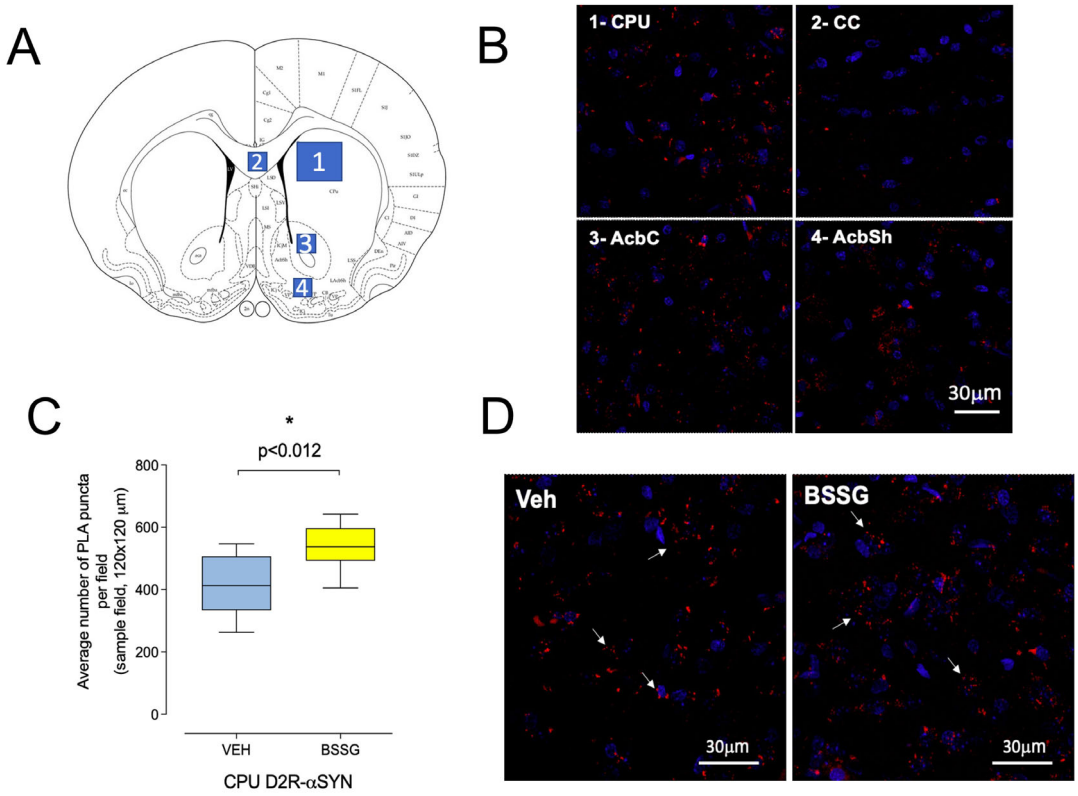

Figure 15. **Characterization of D2R-alpha-synuclein interactions. A.** Diagram of Bregma (-1.0 mm) striatal section, indicating the regions of the brain where analysis was performed; 1) caudate putamen, 2) corpus callosum, 3) accumbens core, 4) accumbens shell. **B.** Distribution of PLA puncta (red signals) in the areas of interest in the SD control brain. **C.** Comparison of average no. of PLA puncta per sample field in vehicle and BSSG treated brains ($p<0.05$, student's t-test, indicated by (*)). **D.** Images of vehicle and BSSG treated dorsal striatal samples, with arrows indicating PLA signals.

Quantification of PLA signals in the caudate putamen revealed a significant increase in D2R-alpha-synuclein interactions in the BSSG-treated brains compared to the vehicle-treated brains ($p<0.05$, student's t-test), as shown in **Figure 15-C**. D4R-alpha-synuclein interactions were also observed in both vehicle and BSSG-treated brains in the caudate putamen and ventral striatum, with fewer PLA signals detected in the corpus callosum. However, no significant difference was found between the vehicle and BSSG-treated groups (**Figure 16-A and 16-C**).

As a positive control, the interaction between DAT and alpha-synuclein was examined, showing a significant increase in DAT-alpha-synuclein interactions in the BSSG model compared to the WT model ($p<0.05$, student's t-test) (**Figure 17-A and 17-B**). Additionally, the analysis of A2AR-alpha-synuclein complexes in the SD

control and BSSG animal model brains revealed no significant changes in the number of PLA signals between the vehicle and BSSG-treated caudate putamen (**Figure 18**).

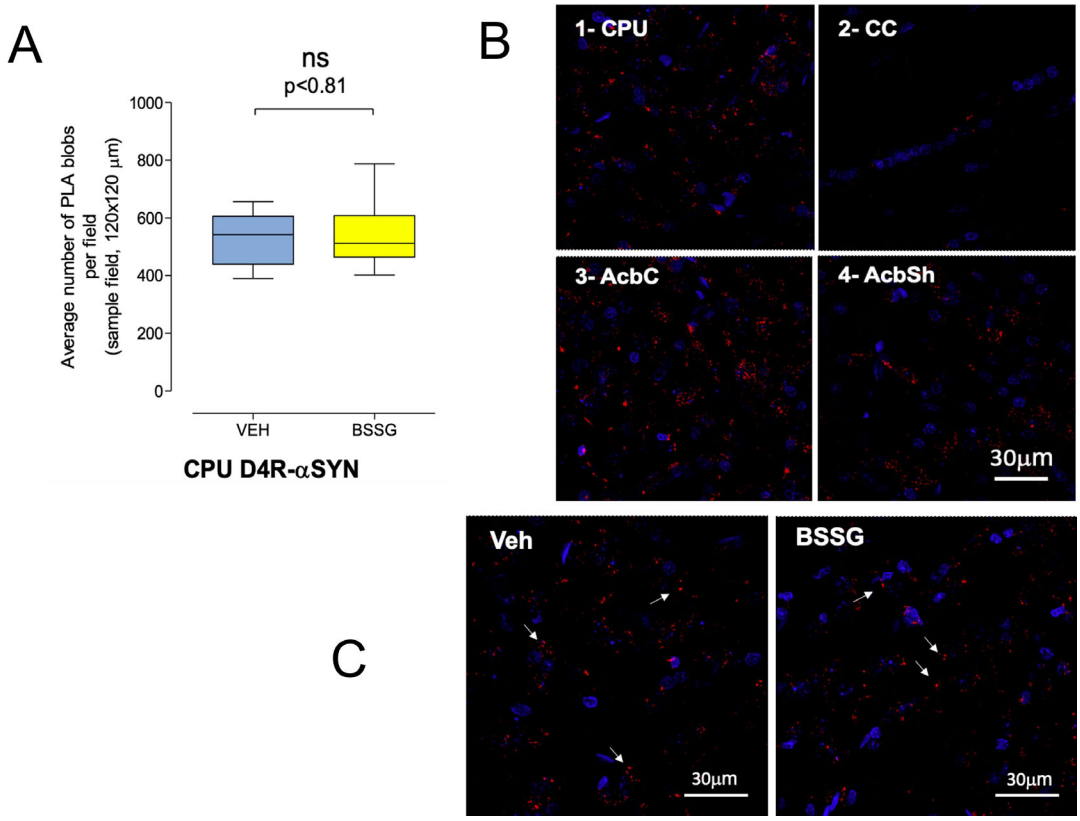

Figure 16. **Identification of D4R-alpha-synuclein interactions. A.** PLA signal visualisation in the 1) caudate putamen, 2) corpus callosum, 3) accumbens core, 4) accumbens shell. **B.** Comparison between the PLA signals in the vehicle vs BSSG treated animals (p>0.05, student's t-test). **C.** Images indicating PLA puncta in the caudate putamen of vehicle and BSSG treated brains.

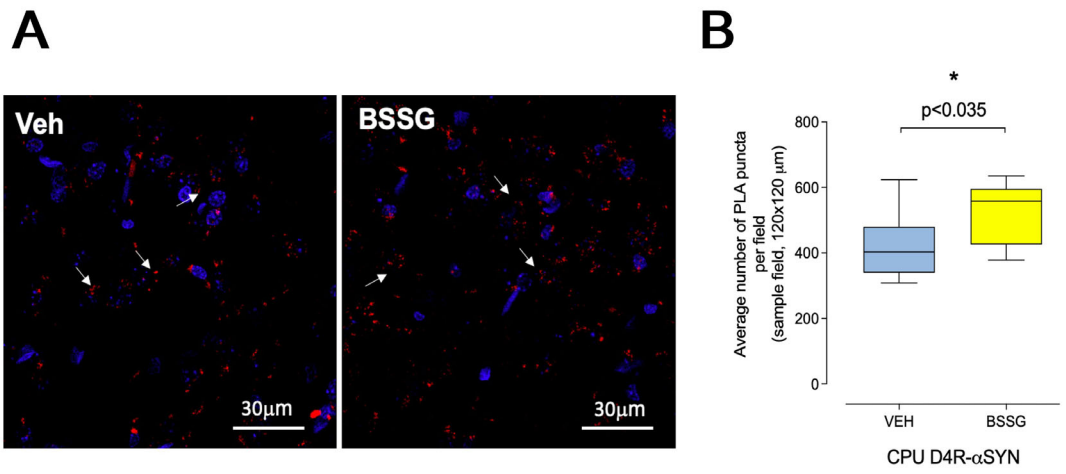

Figure 17. **Characterization of DAT-alpha-synuclein interactions. A.** Images of the caudate putamen, displaying PLA signals in both vehicle and BSSG treated animals. **B.** Comparison between

the Caudate Putamen of the vehicle and BSSG treated brains, indicating a significant increase in average no. of PLA puncta of the BSSG animal (*p<0.05, student's t-test).

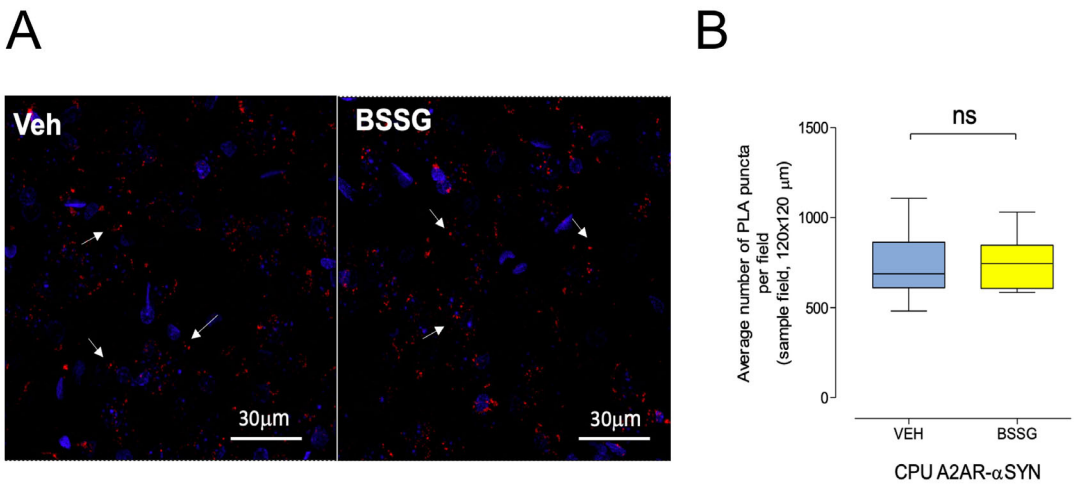

Figure 18. **Characterization of A2AR-alpha-synuclein interactions. A.** Images of the caudate putamen, indicating PLA signals in the SD control and BSSG models. **B.** Comparison between the caudate putamen of both groups, displaying a non-significant change in the number of PLA puncta (p>0.05, student's t-test).

BRET1 analysis. BRET1 assays were performed in live HEK293T cells to investigate ligand-induced modulations of D2R-alpha-synuclein, D4.2R-alpha-synuclein, and D4.7R-alpha-synuclein complexes. In the D2R-alpha-synuclein (WT) complexes, all D2R ligands induced positive BRET ratios, except for Apomorphine, which significantly increased the BRET ratio compared to all the agonists (p<0.05), antagonists (p<0.01), and the vehicle (H2O) (p<0.001) (**Figure 19-A**). No significant change was observed in the BRET ratio between D2R-alpha-synuclein complexes and A53T mutant alpha-synuclein (**Figure 19-B**). Two-way ANOVA analysis indicated a significant interaction between treatment and alpha-synuclein variants (p<0.01), with both factors being individually significant (p<0.001 for alpha-synuclein variants and p<0.01 for drug treatment) (**Figure 19-C**).

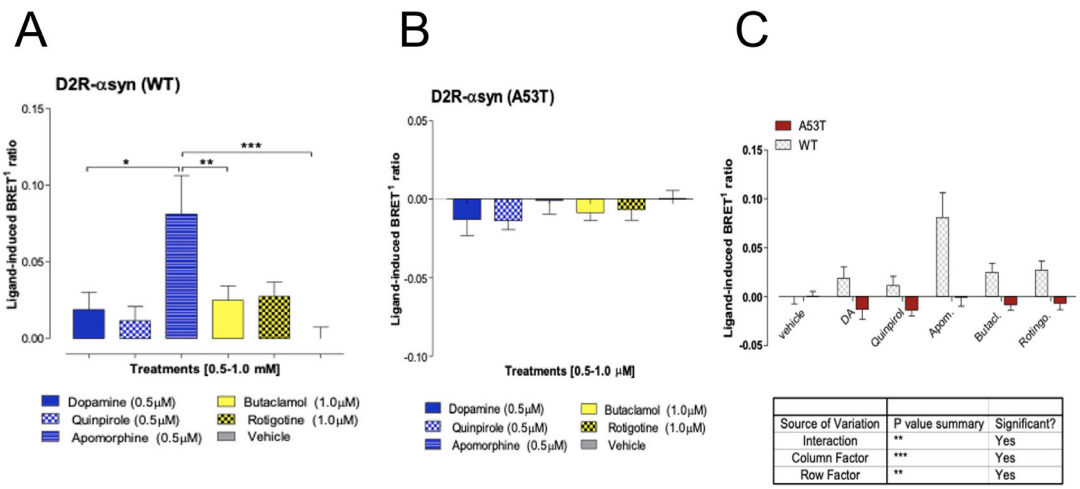

Figure 19. **D2R agonists and antagonists' effects on the interaction between D2R and alpha-synuclein in HEK293T cells using BRET[1].** Three independent experiments (8 replicates per group) were performed. **A.** WT alpha-synuclein, **B.** A53T mutant alpha-synuclein. Significant differences (*p<0.05, **p<0.01, ***p<0.001, one-way ANOVA) are indicated. **C.** Ligand-induced BRET ratios of alpha-synuclein variants were compared by drug. Significant differences (**p<0.01, ***p<0.001, two-way ANOVA) are indicated. Receptor agonists are coloured in blue, and antagonists in yellow.

For D4.2R-alpha-synuclein interactions, all D4R ligands positively modulated the BRET ratio, but statistical analysis showed no significant differences compared to each other or the vehicle (**Figure 20-A**). The ligands and L-745,870 trihydrochloride negatively affected the BRET ratio between D4.2R and the mutant alpha-synuclein, but these results were not significant (**Figure 20-B**). Two-way ANOVA analysis indicated no interaction between drug effects and alpha-synuclein variants but revealed a significant difference between the variants (WT vs. A53T) (p<0.01) (**Figure 20-C**).

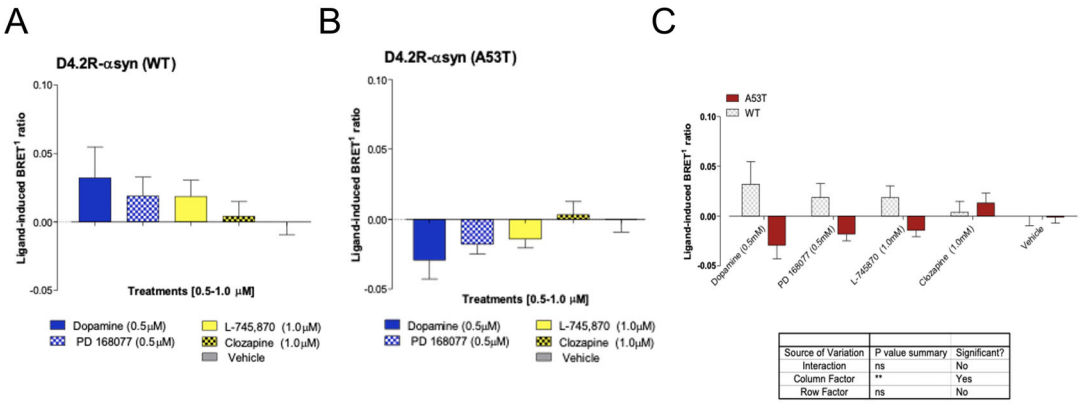

Figure 20. **The effects of D4R ligands on the interaction between D4.2R and alpha-synuclein. A.** D4.2R and WT alpha-synuclein, **B.** D4.2R and A53T mutant alpha-synuclein. Three independent experiments (8 replicates per group) were performed. In A-B, non-significant changes were observed (p>0.05, one way ANOVA, Bonferroni post-test). **C.** Comparison between the effects that drugs have on the modulation of the D4.2R-WT alpha-synuclein and D4.2R-A53T alpha-synuclein interactions, using two-way ANOVA analysis.

In the case of D4.7R-alpha-synuclein interactions, D4R antagonists significantly increased the BRET ratio compared to the agonist dopamine, which showed a negative modulation of the interaction ($p<0.05$) (**Figure 21-A**). No significant effects of the drugs on the interaction between D4.7R and A53T alpha-synuclein were observed (**Figure 21-B**).

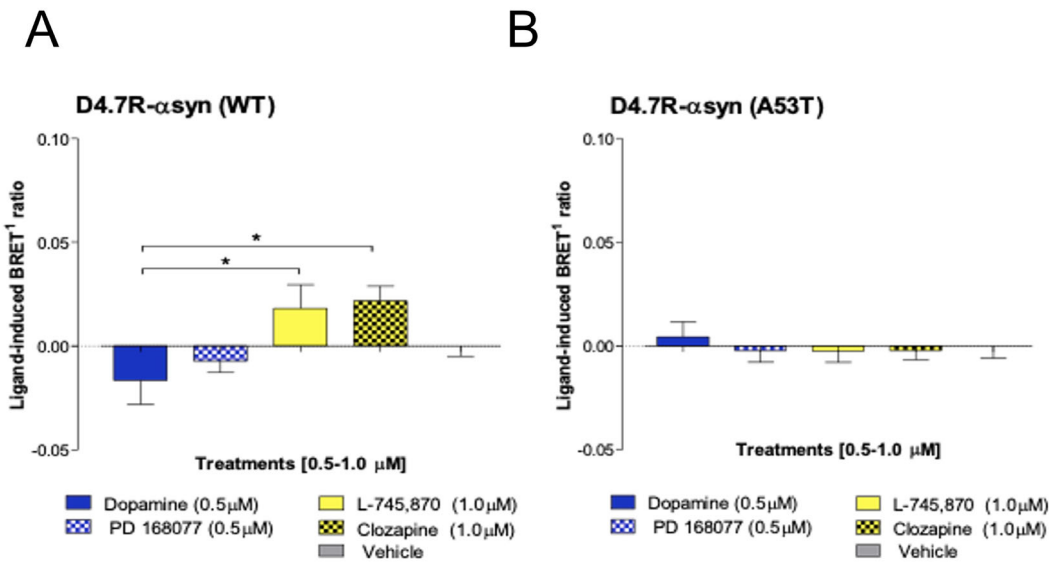

Figure 21. The effects of D4R ligands on the interaction between D4.7R and alpha-synuclein A. WT alpha-synuclein, **B.** A53T mutant alpha-synuclein.

Overall, the results obtained from in situ PLA and BRET1 analysis provide insights into GPCR-alpha-synuclein interactions in the BSSG rat model of PD. The increased D2R-alpha-synuclein interactions and enhanced DAT-alpha-synuclein interactions suggest their involvement in PD pathophysiology. However, no significant changes were observed in D4R and A2AR interactions with alpha-synuclein. Furthermore, ligand-induced modulations in the BRET ratios of D2R-alpha-synuclein and D4.2R-alpha-synuclein complexes were observed, suggesting potential regulatory mechanisms in these interactions.

Figure 1: Characterization of D2R-alpha-synuclein interactions. A. Schematic representation of a striatal section at Bregma (-1.0 mm), indicating the specific regions analyzed: 1) caudate putamen, 2) corpus callosum, 3) accumbens core, 4) accumbens shell. B. In situ PLA signals (red puncta) representing D2R-alpha-synuclein interactions in the areas of interest in the SD control brain. C. Quantification of the average number of PLA puncta per sample field in vehicle and BSSG-treated brains, showing a significant difference between the two treatments ($p<0.05$, student's t-test, indicated by (*)). D. Representative images of vehicle and BSSG-treated dorsal striatal samples, with arrows indicating PLA signals.

Figure 2: Identification of D4R-alpha-synuclein interactions. A. Visualization of in situ PLA signals (red puncta) in the specific regions: 1) caudate putamen, 2) corpus callosum, 3) accumbens core, 4) accumbens shell. B. Comparison of PLA signals between the vehicle and BSSG-treated animals, indicating no significant difference ($p>0.05$, student's t-test). C. Representative images showing PLA puncta in the caudate putamen of vehicle and BSSG-treated brains.

Figure 3: Characterization of DAT-alpha-synuclein interactions. A. Representative images of the caudate putamen displaying in situ PLA signals (red puncta) in both vehicle and BSSG-treated animals. B. Comparison of the average number of PLA puncta in the caudate putamen between the vehicle and BSSG-treated brains, indicating a significant increase in the BSSG animal (*$p<0.05$, student's t-test).

Figure 4: Characterization of A2AR-alpha-synuclein interactions. A. Representative images of the caudate putamen showing in situ PLA signals (red puncta) in the SD control and BSSG models. B. Comparison of the number of PLA puncta between the caudate putamen of both groups, indicating no significant change ($p>0.05$, student's t-test).

Figure 5: Effects of D2R agonists and antagonists on the interaction between D2R and alpha-synuclein in HEK293T cells using BRET1. A. BRET ratios of WT alpha-synuclein with different D2R ligands. Significant differences (*$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA) are indicated. B. BRET ratios of A53T mutant alpha-synuclein with different D2R ligands. C. Comparison of ligand-induced BRET ratios of alpha-synuclein variants by drug treatment. Significant differences (**$p<0.01$, ***$p<0.001$, two-way ANOVA) are indicated. Receptor agonists are coloured in blue, and antagonists in yellow.

Figure 6: Effects of D4R ligands on the interaction between D4.2R and alpha-synuclein. A. BRET ratios of D4.2R with WT alpha-synuclein. B. BRET ratios of D4.2R with A53T mutant alpha-synuclein. C. Comparison of the effects of drugs on the modulation of D4.2R-WT alpha-synuclein and D4.2R-A53T alpha-synuclein interactions using two-way ANOVA analysis.

1. 3. DISCUSSION

This study provides evidence, for the first time, of the existence of D2R-alpha-synuclein and D4R-alpha-synuclein interactions in the striatum of the rat brain. In

addition, A2AR-alpha-synuclein and DAT-alpha-synuclein interactions are confirmed by in situ PLA. The complexes are demonstrated in SD control rat brains and in the BSSG rat model of PD and are enriched in the dorsal and ventral striatum. These results are in line with previous observations that alpha-synuclein interacts with several membrane receptors, e.g NMDAR 2B, B2-adrenergic, mGluR5, M5R and EGFR. So far, it has been demonstrated by means of mass spectrometry, co-immunoprecipitation, affinity-capture-western and Two-hybrid that alpha-synuclein can interact with more than 500 proteins (both, intracellular and membrane located proteins). These facts highlight the complexity of the synucleinopathies. Our in-situ PLA experiments in the rat brain have also demonstrated the capacity of alpha-synuclein to interact with several proteins involved in the dopamine signalling and neurotransmission process (D2R, D4R and DAT).

A significant increase in receptor-alpha-synuclein interactions, in the BSSG model compared to SD control group, is seen for receptors D2 and DAT, supporting the hypothesis. Alternatively, there is no significant difference in the alpha-synuclein-D4R interactions between the BSSG model of PD and the SD control groups. The significant increase in the densities of the D2R-alpha-synuclein and DAT-alpha-synuclein complexes may be due to a preferential affinity of the D2R and DAT by a particular conformational state of alpha-synuclein protein. Certain types of oligomeric forms of alpha-synuclein are found in the BSSG rat model (replicating human PD alpha-synuclein forms), and not in the SD control group. The changes observed in the densities of each complex in the BSSG animal model may also be due to cell type vulnerability e.g., presynaptic dopamine terminals (varicosities) versus postsynaptic striatopallidal GABA neurons and/or astrocytes, or a difference in the striatal compartments (matrix versus striosomes). The identification of such complexes is a tremendous step towards a better understanding of the molecular pathophysiology behind PD and has great therapeutic and diagnostic potential.

Further studies should explore how the signal transduction pathways of the involved GPCRs are modified in response to such complex formations. This will provide insight into how the GPCRs functions are altered in the presence of alpha-synuclein (GPCR-alpha-synuclein complex), and shed light on the resulting cellular effects, and pathology of PD. This could be done by, for example, conducting a cAMP-Glo assay, which measures the levels of cAMP upon GPCR activation (Gs/olf and Gi/o). Therefore, the extent to which allosteric receptor-alpha-synuclein interactions contribute to this can be explored. A plausible hypothesis could be that alpha-synuclein binding to the D2R and DAT antagonises the receptors, therefore reducing D2R signalling and DAT activity, leading to reduced dopamine activity. This could therefore exacerbate the dopamine depleted conditions that essentially cause PD. Also, it could be exciting to test how GPCR protomer activation (agonist) or

blockade (antagonist), within the GPCR-alpha-synuclein complexes, have an impact on alpha-synuclein distribution, function and/or posttranslational modification (e.g., oligomerization). This could guide potential novel therapeutic approaches and reveal the molecular mechanisms of such drugs in new contexts.

Drugs targeting the GPCRs-alpha-synuclein complexes that are altered in PD, such as the D2R, may be of therapeutic relevance, potentially relieving symptoms. Moreover, alpha-synuclein and GPCRs are expressed in blood cells (34, 35). This is of great potential from a diagnostic standpoint, as blood tests could be designed to measure the levels of alpha-synuclein-GPCR interactions, providing insight to whether an individual is susceptible to developing PD. It can also be used to monitor how well PD therapies are working, through consistently measuring relevant alpha-synuclein-GPCR interactions throughout the course of treatment.

BRET[1] assays were carried out to investigate whether dopamine receptor ligands (agonists and antagonists) induced a change in the interactions between alpha-synuclein (WT and A53T variants) and dopamine receptors (D2R and D4R). These complexes were already validated in this study by in situ PLA. The results indicate that the agonists Dopamine, Quinpirole, and antagonists Butaclamol and Rotigotine, did not induce significant modulations of the D2R-alpha-synuclein (WT and A53T) complexes. However, preliminary indications highlight the importance of Apomorphine; the drug positively modulates the D2R-alpha-synuclein (WT) interaction. Indeed, Apomorphine was the first available dopaminergic drug for PD, and is the only drug with comparable efficacy to that of levodopa, the gold standard for treating PD (36). A possible explanation for the difference in the BRET signals observed upon apomorphine treatment in the D2R-alpha synuclein (WT) vs the D2R-alpha synuclein (A53T) could be that the conformational changes induced by apomorphine favour the interaction with the WT and not to the A53T mutant. Further studies should focus on the role of Apomorphine in altering GPCR-alpha synuclein complexes that are implicated in PD. This could provide valuable insights into the D2R-alpha-synuclein interface interaction and their selectivity and specificity and guide the development of novel therapeutics treatments targeting these complexes.

For instance, the design of novel small interface interacting peptides. Similarly, no significant changes in the ligand-induced BRET signal were obtained for D4.2R-alpha-synuclein interactions with both variants (WT vs A53T). However, Two WAY ANOVA analysis indicated that there is a significant difference between the column factor (variants: WT vs A53T), meaning that a differential interface interaction is in operation between the D4.2R-alpha-Synuclein (WT) and the D4.2R-alpha-Synuclein (A53T).

Furthermore, receptor antagonists L-745 and clozapine induced significant positive modulations of D4.7-alpha-synuclein (WT) complex when compared to the agonist

dopamine, which negatively modulated the complex. This could indicate that agonists induced a particular interface interaction or conformational state between the D4.7R and alpha-synuclein WT protein which is significantly different from the one induced by the antagonists. In contrast, no change was seen in the ligand-induced BRET ratios of the D4.7-alpha-synuclein (A53T) interactions as similar to the effects observed for the D2R. Further BRET assays should be performed, to clarify the effects of the ligands on the interactions between alpha-synuclein and D2R, D4.2R and D4.7R interactions. For instance, a BRET saturation assay should be carried out. This will indicate the affinity ($BRET_{50}$ value) and number of the interactions ($BRET_{max}$ value). Additionally, BRET assays with ligand treatment should be conducted to investigate the interactions of the A2AR and DAT with alpha-synuclein.

CONCLUSIONS

I. While the integrative mechanism of adenosine receptor complexes in the plasma membrane has been extensively studied, the formation and distribution patterns of adenosine A2AR isoreceptor complexes remain poorly understood. Our study aimed to investigate the distribution of A2AR homoreceptor complexes and A1R-A2AR isoreceptor complexes in the hippocampus and other forebrain areas using proximity ligation assay (PLA) and immunohistochemistry techniques. The findings from our in situ PLA analysis revealed the highest densities of A2AR-A1R isoreceptor complexes and A2AR-A2AR homoreceptor complexes in the pyramidal cell layers of CA1-CA3 and the polymorphic cell layer in the hilus of the dentate gyrus in the hippocampus. Additionally, in the piriform cortex, a co-distribution of A2AR-A2AR and A1R-specific PLA signals with high densities was observed in layer II, while other layers showed different patterns. Based on these observations, we propose that the regulation of adenosine A2AR signaling in the hippocampus relies on a balance between A2AR-A2AR homoreceptor complexes and A1R-A2AR isoreceptor complexes. Furthermore, the A2AR homo- and isoreceptor complexes may play a role in controlling neuroinflammation within the hippocampus and the associated dysfunction of its neuron-glia networks. Dysregulation of adenosine signaling within these complexes, particularly in the CA3-CA1 regions, may contribute to pathological outcomes such as psychosis or depression by modulating the interactions of the GABA-glutamate network. Additionally, adenosine isoreceptor complexes in limbic regions could impact the development of limbic seizures. Moving forward, further research is needed to unravel the complexities of adenosine volume transmission signaling in the brain, with a focus on adenosine isoreceptor signaling and its balance with corresponding A2A and A1 homoreceptor signaling in neuron-glia networks. Exploring these intricate interactions will deepen our understanding of adenosine-mediated processes and potentially unveil new therapeutic avenues for neurological disorders associated with adenosine dysregulation.

II. Our findings provide valuable insights into the complex interactions between adenosine and dopamine receptors in the central nervous system (CNS). The discovery of receptor mosaics, specifically higher-order heteromers, comprising adenosine A2AR and dopamine D4R in the striatum, has revolutionized our understanding of these interactions. We have demonstrated the existence of A2AR-D2R and A1R-D1R heterodimers in the striatum, followed by indications

of A2AR-D3R and A2AR-D4R heterodimers. Particularly, our study focuses on the A2AR-D4R heteroreceptor complexes, which we have detected in cellular models and various regions of the rat brain using in situ Proximity Ligation Assays (PLA). Additionally, we have confirmed the formation of these complexes through BRET1 techniques in HEK cells, highlighting the lower effectiveness of the A2AR and D4.7R splice variant receptors in heteromer formation. Interestingly, our research expands on the heteroreceptor complexity by demonstrating, for the first time, that A2A receptors can form heteroreceptor complexes with the common human dopamine D4.xR polymorphism variants (D4.2R, D4.4R, and D4.7R) to varying degrees in HEK293T cells co-transfected with both receptor types. Moreover, through in situ PLA, we provide evidence for the existence of A2AR-D4R heteroreceptor complexes in several regions of the rat brain. Our results also shed light on the allosteric receptor-receptor interactions within A2AR-D4R heteromers, as observed through CREB gene reports assays. These findings suggest the presence of antagonistic allosteric receptor-receptor interactions between the orthosteric binding sites of the corresponding heteromers. Overall, our study significantly contributes to the understanding of adenosine-dopamine interactions in the CNS, highlighting the formation of heteroreceptor complexes involving A2AR and D4R. These findings have important implications for comprehending the role of these complexes in complex behaviors and motor function. Further investigations into the functional implications of A2AR-D4R heteroreceptor complexes and their modulation in various CNS disorders hold promise for developing targeted therapeutic interventions in the future.

III. Chapter 3 new findings sheds light on the molecular mechanisms underlying Parkinson's disease (PD) by investigating the interactions between alpha-synuclein and D2-like G protein-coupled receptors (GPCRs), namely D2R and D4R. By utilizing advanced techniques such as in situ proximity ligation assay (PLA) and Bioluminescence Resonance Energy Transfer (BRET), we have provided evidence for the existence of D2R and D4R complexes with alpha-synuclein in the rat brain, specifically in the dorsal and ventral striatum. Moreover, we have validated the presence of alpha-synuclein complexes with A2AR and DAT, further implicating these interactions in the pathophysiology of PD. Notably, our findings demonstrate an increase in D2R-alpha-synuclein and DAT-alpha-synuclein interactions in the BSSG animal model of PD, highlighting the potential role of these interactions in disease progression. Furthermore, our investigations into the modulatory effects of receptor agonists and antagonists revealed the significant impact of Apomorphine on D2R-alpha-synuclein interactions, providing insights into the therapeutic potential of

dopaminergic drugs. The identification and characterization of these GPCR-alpha-synuclein complexes contribute to our understanding of the molecular basis of PD neurodegeneration. They provide a foundation for future research exploring how alpha-synuclein influences the structure and function of A2AR, D2R, and D4R, particularly in the context of GPCR-GPCR heteroreceptor complexes relevant to PD, such as A2AR-D2R and A2AR-D4R. Overall, this study advances our knowledge of the intricate interactions between alpha-synuclein and GPCRs in PD, paving the way for further investigations into the therapeutic targeting of these complexes for the development of novel treatments and interventions for this debilitating neurodegenerative disorder.

IV. The interplay between adenosine A2A receptors (A2AR) and tropomyosin-related kinase B receptor (TrkB) holds a pivotal role in modulating BDNF-induced synaptic transmission in the aging rat hippocampus. Our findings, demonstrated through in situ proximity ligation assay (PLA), confirm the direct physical interaction between A2AR and TrkB, forming heterogeneous heteroreceptor complexes within the hippocampus. Particularly, high densities of these complexes were observed in the pyramidal cell layers of CA1-CA3 regions and the polymorphic layer of the dentate gyrus (DG). These results shed light on the mechanisms underlying this powerful crosstalk and underscore the importance of understanding A2AR-TrkB interactions for enhancing hippocampal plasticity.

V. Our study has also elucidated the functional interaction between Neuropeptide Y (NPY) Y1 receptor (NPYY1R) and galanin receptor 2 (GALR2) in the hippocampus, particularly in the ventral dentate gyrus (vDG). The in-situ proximity ligation assay revealed a significant increase in GALR2/NPYY1R heteroreceptor complexes, particularly on doublecortin-expressing neuroblasts. Notably, coactivation of GALR2 and NPYY1R in cell cultures led to the recruitment of GALR2 expression to the plasma membrane, indicating a potential mechanism for antidepressant behavior through enhanced ERK pathway. These findings offer valuable insights into the basis for developing heterobivalent agonist pharmacophores targeting GALR2/NPYY1R heteromers, especially in neuronal precursor cells of the dentate gyrus within the ventral hippocampus, paving the way for novel depression treatments.

Overall, our proximity ligation assays studies provide compelling evidence for the significance of GPCR iso- homo- and heteroreceptor complexes in modulating synaptic transmission and neuroplasticity in the hippocampus and other important areas of the brain. These findings could potentially lead to the development of innovative therapeutic strategies for neurological conditions associated with synaptic dysfunction and neurodegeneration. Further research in this area holds promise for advancing our understanding of brain function and/ or neuronal signal integration and contributing to future treatments for various neuropsychiatric and neurodegenerative disorders.

CONCLUSIONES EN ESPAÑOL

I. Con anterioridad, otros estudios demostraron que la adenosina, actuando como neuromodulador, ejerce sus efectos a través de la difusión y el flujo en el espacio extracelular, influenciando la señalización neuronal, la función astrocítica, el aprendizaje y/o la memoria, la función motora y los procesos normales de envejecimiento. En el presente estudio, basado en los complejos iso-receptores de adenosina (A1R-A2AR, A2AR-A2AR, A2AR-A2BR y A2AR-A3R), se arroja luz sobre el papel de la adenosina y sus cuatro subtipos de receptores en la modulación de diversos procesos fisiológicos, así como su participación en diversas neuropatologías. Entre los receptores de adenosina los subtipos A1R y A2AR son de particular interés, debido a su amplia expresión en el cerebro y su participación en múltiples complejos hetero-receptores dentro de la familia de los GPCRs, pero también en su interacción y formación de complejos con otras proteínas de membranas o receptores, como por ejemplo los RTK. Aunque el mecanismo integrador de los complejos de receptores de adenosina en la membrana plasmática de las neuronas ha sido ampliamente estudiado, no se tenía una comprensión adecuada de los patrones de formación y distribución de los complejos iso-receptores de A2AR. Por lo tanto, partiendo de estas limitaciones en los conocimientos actuales o el estado-del-arte, nuestro estudio tuvo como objetivo investigar la distribución de los complejos homo-receptores de A2AR y los complejos iso-receptores de A1R-A2AR en el hipocampo y otras áreas del cerebro mediante la técnica de ensayo de ligadura de proximidad (PLA) e inmunohistoquímica. Los hallazgos de nuestro análisis mediante in situ PLA muestran que las mayores densidades de complejos iso-receptores A2AR-A1R y complejos homo-receptores A2AR-A2AR tienen lugar en las capas de células piramidales de CA1-CA3 y la capa de células polimórficas en el hilio del giro dentado en el hipocampo en el cerebro de ratas. Además, en la corteza piriforme, se observó una co-distribución de señales PLA específicas de A2AR-A2AR y A2AR-A1R con altas densidades en la capa II, mientras que otras capas mostraron menores densidades. Por tanto, tomando en cuenta todas estas observaciones y/o elementos propusimos que la regulación de la señalización de A2AR en el hipocampo depende de un equilibrio entre los complejos homo-receptores A2AR-A2AR y los complejos iso-receptores A1R-A2AR. Además, los complejos homo- e iso-receptores de A2AR podrían desempeñar un papel en el control de la neuroinflamación dentro del hipocampo y la disfunción asociada

de sus redes neuronales y gliales. La desregulación de la señalización de adenosina dentro de estos complejos, especialmente en las regiones CA3-CA1, podría contribuir a neuropatologías como la psicosis o la depresión al modular las interacciones de la red GABA-glutamato. Además, los complejos iso-receptores de adenosina en las regiones límbicas podrían afectar el desarrollo de convulsiones límbicas. Podemos concluir, además, que se requiere profundizar en el estudio de estos complejos para desentrañar las complejidades de la señalización de adenosina en el cerebro en términos de los mecanismos de reconocimiento ortostérico-activación-señal. Dichos estudios deben abordarse desde la perspectiva o consideración de los balances de los complejos iso-receptores y su equilibrio con la señalización de los homo-receptores correspondiente de A2A y A1 en las redes neuronales y gliales. Explorar estas interacciones profundizará nuestra comprensión de los procesos mediados por la adenosina y potencialmente revelará nuevas vías terapéuticas para los trastornos neurológicos asociados con la desregulación de la adenosina.

II. En el capítulo 2 se resumen una serie de evidencias experimentales sobre las complejas interacciones entre los receptores de adenosina y dopamina en el sistema nervioso central (SNC), particularmente las interacciones entre el receptor de dopamina D4R y el receptor de adenosina A2A. El descubrimiento de complejos heterodímeros de los receptores de dopamina y adenosina, específicamente los complejos que comprenden los receptores de adenosina A2AR y dopamina D4R en el estriado, ha revolucionado nuestra comprensión sobre la integración de la señal entre la adenosina y la dopamina. En particular, nuestro estudio se enfocó a los complejos de A2AR-D4R en modelos celulares y diversas regiones del cerebro de la rata utilizando ensayos de ligadura de proximidad in situ (PLA). Además, se confirmó la formación de estos complejos mediante técnicas BRET1 en células HEK. Por primera vez, en el presente estudio, se demuestra que los receptores A2A pueden formar complejos de hetero-receptores con las variantes polimórficas más comunes del receptor de dopamina D4.xR (D4.2R, D4.4R y D4.7R) en diferentes grados en células HEK293T co-transfectadas con ambos tipos de receptores. Se observa, además, una baja afinidad o eficacia en la formación del complejo A2AR-D4.7R. Además, a través de los ensayos de in situ PLA, proporcionamos evidencia de la existencia de complejos de hetero-receptores A2AR-D4R en varias regiones del cerebro de la rata. Nuestros resultados también arrojan luz sobre las interacciones alostéricas receptor-receptor dentro de los heterómeros A2AR-D4R, como se observa a través de ensayos de informes del gen CREB. Estos hallazgos sugieren la presencia de interacciones alostéricas antagónicas entre los sitios de unión ortostáticos de los heterómeros correspondientes. Por otra parte,

se validó la existencia de heterodímeros A2AR-D2R y A1R-D1R en el estriado, y se demostró la existencia también de los heterodímeros A2AR-D3R tanto en cerebros de ratas como en cultivos primarios estriatales. En general, nuestro estudio contribuye significativamente a la comprensión de las interacciones entre adenosina y dopamina en el SNC, destacando la formación de complejos de hetero-receptores que involucran a A2AR y D4R. Estos hallazgos tienen implicaciones importantes para comprender el papel de estos complejos en la integración de la señal y la función motora. Aun así, se requieren otros estudios funcionales y el análisis de los balances y cambios en las densidades de este complejo en otros modelos animales.

III. En el Capítulo 3 se presentan nuevos hallazgos que arrojan luz sobre los mecanismos moleculares subyacentes a la enfermedad de Parkinson mediante la investigación de las interacciones entre la proteína intracelular alfa-sinucleína y los receptores acoplados a proteínas G tipo D2, específicamente los receptores D2R y D4R, y los receptores de adenosina A2AR en el modelo animal BSSG de la enfermedad de Parkinson. Utilizando técnicas avanzadas como el ensayo de ligadura de proximidad in situ (PLA) y la transferencia de energía de resonancia de bioluminiscencia (BRET), hemos proporcionado evidencia de la existencia de complejos de D2R y D4R con la alfa-sinucleína en el cerebro de la rata, específicamente en el estriado dorsal y ventral. Además, se validó la presencia de complejos de alfa-sinucleína con A2AR y DAT, demostrándose que estas interacciones pueden ser relevantes o subyacer a los mecanismos moleculares relacionados con la fisiopatología de la enfermedad de Parkinson. Es importante destacar que nuestros hallazgos demuestran un aumento en las interacciones de D2R-alfa-sinucleína y DAT-alfa-sinucleína en el modelo animal BSSG de la enfermedad de Parkinson, lo que resalta el papel potencial de estas interacciones en la progresión de la enfermedad. Además, nuestras investigaciones sobre los efectos moduladores de los agonistas y antagonistas de los receptores revelan el impacto significativo de la apomorfina en las interacciones de D2R-alfa-sinucleína, proporcionando nuevas perspectivas sobre el potencial terapéutico de los fármacos dopaminérgicos. Estos hallazgos podrían sentar las bases para futuras investigaciones que exploren cómo la alfa-sinucleína influye en la estructura y función de los receptores de A2AR, D2R y D4R, especialmente en el contexto de los complejos hetero-receptores GPCR-GPCR relevantes para la enfermedad de Parkinson, como son los complejos de A2AR-D2R y A2AR-D4R. En general, este estudio avanza en nuestro conocimiento de las complejas interacciones entre la alfa-sinucleína y los GPCRs en la enfermedad de Parkinson, allanando el camino para futuras investigaciones sobre el objetivo

terapéutico de estos complejos para el desarrollo de tratamientos e intervenciones novedosas para este debilitante trastorno neurodegenerativo.

IV. La interacción entre los receptores de adenosina A2A (A2AR) y el receptor del factor neurotrófico derivado del cerebro (TrkB) desempeña un papel crucial en la modulación de la transmisión sináptica inducida por BDNF en el hipocampo de ratas envejecidas. Nuestros hallazgos, demostrados mediante el ensayo de proximidad in situ (PLA), confirman la interacción física directa entre A2AR y TrkB, formando complejos heterorreceptoras heterogéneos dentro del hipocampo. En particular, se observaron altas densidades de estos complejos en las capas de células piramidales de las regiones CA1-CA3 y la capa polimórfica del giro dentado (DG). Estos resultados arrojan luz sobre los mecanismos que subyacen a esta poderosa interacción y subrayan la importancia de comprender las interacciones A2AR-TrkB para mejorar la plasticidad del hipocampo.

V. Estudios previos demostraron la interacción funcional entre el receptor Y1 de neuropeptido Y (NPYY1R) y el receptor 2 de galanina (GALR2) en el hipocampo, especialmente en el giro dentado ventral (vDG). En el presente trabajo se demostró mediante ensayo de proximidad en situ no sólo la existencia de complejos heteroreceptores GALR2/NPYY1R, sino también un aumento significativo de los mismos en los neuroblastos del vDG que expresan doublecortin. Es importante destacar que la coactivación de GALR2 y NPYY1R en cultivos celulares condujo al reclutamiento de la expresión de GALR2 en la membrana plasmática, indicando un posible role facilitatorio de NPYY1R en el reclutamiento de GalR2. Estos hallazgos ofrecen conocimientos valiosos sobre la base para el desarrollo de agonistas heterobivalentes dirigidos a los heterómeros GALR2/NPYY1R, especialmente en las células precursoras neuronales del giro dentado en el hipocampo ventral, abriendo camino para nuevos tratamientos contra la depresión.

En general, nuestros estudios mediante técnicas de ligación por proximidad (in situ PLA) proporcionan evidencia convincente sobre la importancia de la interacción entre los complejos homo-, iso- y hetero-receptores en la modulación de la transmisión sináptica y la neuro-plasticidad en el hipocampo y otras áreas importantes del cerebro. También validan la importancia y utilidad de las técnicas por proximidad para el estudio y análisis de interacciones protéicas en tejido. Nuestros resultados experimentales pueden de cierto modo conducir al desarrollo de nuevas estrategias terapéuticas para el tratamiento de afecciones neurológicas asociadas con la disfunción sináptica y la neurodegeneración, por ejemplo, la enfermedad de Parkinson.

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Annexes

Annex 1

Chapter 10

Isolation and Detection of G Protein-Coupled Receptor (GPCR) Heteroreceptor Complexes in Rat Brain Synaptosomal Preparation Using a Combined Brain Subcellular Fractionation/Co-immunoprecipitation (Co-IP) Procedures

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Abstract

The isolation and characterization of GPCR heteroreceptor complexes, specially those present at the central nervous system, are of crucial relevance for the understanding of the molecular mechanisms behind several mental and neurodegenerative disorders. The existence of homo- and heteroreceptor complexes with allosteric receptor-receptor interactions increases the diversity of receptor function including recognition, trafficking, and signaling. This phenomenon increases our understanding of how brain function is altered through molecular integration of receptor signals. An alteration in specific heteroreceptor complexes or their neuronal localization is considered to have a role in the pathogenic mechanisms that lead to mental and neurological diseases, including drug addiction, depression, Parkinson's disease, and schizophrenia. Therefore, it is fundamental to understand the appropriate localization and synaptic clustering of these GPCR heteroreceptor complexes. This chapter represents a workflow for the analysis of GPCR heteroreceptor complexes by means of combined use of differential centrifugation/co-immunoprecipitation in rat brain tissue. The combination of differential centrifugation/co-immunoprecipitation allows the separation and detection of GPCR heteroreceptor complexes present at synaptic sites from those found in intracellular compartments and vesicular pools. It is a reproducible protocol and produces reliable quantitative data.

Key words G protein-coupled receptors (GPCRs), GPCR heteroreceptor complexes, Receptor-receptor interactions, Heterodimerization, Co-immunoprecipitation, Subcellular, Synaptic membrane protein, Ultracentrifugation

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1 Introduction

Mental and neurological diseases, including drug addiction, depression, Parkinson's disease, and schizophrenia, are highly complex in their etiology. It is not surprising that the underlying pathological processes are poorly understood, and treatment possibilities are inadequate. One emerging concept is that direct physical interactions of different receptors named homo−/heteroreceptor complexes may be involved with disease onset and progression [1–3]. Among all membrane receptor complexes, the G protein-coupled receptors (GPCR) heteroreceptor complexes are of special relevance [4–8].

In the last decades, GPCR heteroreceptor complexes were demonstrated through diverse biophysical, biochemical, or microscopy-based procedures (e.g., co-immunolocalization, co-immunoprecipitation, radioligand binding, co-internalization analysis, fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) methods) [9–11]. Each methodology used has provided precise and valuable information which was considered with caution in view of the limitations of the techniques. Some controversy regarding some approaches also emerged [12, 13].

In this chapter, we will describe how the combined use of brain tissue subcellular fractionation procedure and co-immunoprecipitation method may make it possible in a highly selective and sensitive assay to study and characterize also the GPCR homo- and heteroreceptor complexes, specially their existence and distribution within a subcellular compartment. Although the combined procedures of brain tissue subcellular fractionation by differential centrifugation and co-immunoprecipitation require a great deal of optimization and troubleshooting, in most cases the method allows the study of the synaptic clustering of GPCR heteroreceptor complexes in a fine-tuning manner.

2 Materials

2.1 Animals

3- to 4-week-old male Sprague Dawley rats (Scanbur, Sweden). All studies involving animals are performed in accordance with the Stockholm North Committee on Ethics of Animal Experimentation, the Swedish National Board for Laboratory Animal, and the European Communities Council Directive (Cons 123/2006/3) guidelines for accommodation and care of laboratory animals. Thus, animals are housed in standard cages with ad libitum access to food and water and maintained under controlled standard conditions (12 h dark/light cycle, 22 °C temperature, and 66% humidity).

2.2 Reagents

Protein A-immobilized agarose fast flow, 50% (v/v) (Sigma-Aldrich); TrueBlot™ anti-mouse IgG IP resin (eBioscience); Protease Inhibitor Cocktail Set III (Millipore), phosphatase inhibitors (aprotinin, β-glycerophosphate, pefabloc, leupeptin); acrylamide/bis-acrylamide (30%/0.8% w/v) (Bio-Rad); ammonium persulfate (APS) (Sigma-Aldrich); tetramethylethylenediamine (TEMED) (Sigma-Aldrich), sodium dodecyl sulfate (SDS) (Sigma-Aldrich); methanol 99.8% (Pancreac Química SL, Barcelona, Spain); Precision Plus Protein Standards (Bio-Rad); Tween 20 (Sigma-Aldrich); nonfat dry milk or bovine serum albumin (BSA) or commercial blocking buffer (e.g., Odyssey blocking buffer from LI-COR Biosciences, cat. nr. 927–40,000); SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.); BCA Protein Assay Kit (Pierce Biotechnology). *Antibodies specific for subcellular fractions*: e.g., anti-synaptophysin (Chemicon) or anti-VAMP (Stressgen) for synaptic vesicles, anti-Na^+/K^+-ATPase (Transduction Laboratories) for plasma membranes, and anti-Lactate Dehydrogenase (anti-LDH, Abcam) for cytosol. *Primary antibodies*: rabbit monoclonal anti-5HT1A (vtg544, 1 μg/mL, VTG Biosciences), goat monoclonal anti-5HT1A (ab101914, 1:500, Abcam, Sweden), rabbit monoclonal anti-5HT2A (HPA014011, 1 μg/mL; Human Atlas Project, Stockholm, Sweden), and goat monoclonal anti-5HT2A (ab140824, 1:500, Abcam, Sweden). *Secondary antibodies*: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) and HRP-conjugated anti-mouse IgG TrueBlot™ (eBioscience).

2.3 Buffers

1. *Phosphate-buffered saline (PBS)*: 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 17 mM Na_2HPO_4, pH 7.2.
2. *Percoll gradients*: To create a discontinuous gradient, carefully layer 2 mL of each Percoll solution in an 11 mL (16 × 100 mm) polycarbonate high-speed centrifuge tube, starting with 23% at the bottom. Prepare gradients on the day of or the day before the experiment, and keep at 4 °C until use. *23% Percoll*: 12 mL 100% Percoll and 14 mL H_2O in 26 mL 2× sucrose buffer. Adjust solutions to pH 7.4 and store up to 5 days at 4 °C. *15% Percoll*. 30 mL 23% Percoll in 16 mL 1× sucrose buffer. Adjust solutions to pH 7.4 and store up to 5 days at 4 °C. *10% Percoll*: 20 mL 15% Percoll in 10 mL 1× sucrose buffer. Adjust solutions to pH 7.4 and store up to 5 days at 4 °C. *3% Percoll*: 6 mL 10% Percoll in 14 mL 1× sucrose buffer. Adjust solutions to pH 7.4 and store up to 5 days at 4 °C.
3. *Sucrose gradients* in thin-walled ultracentrifuge tubes (ultra-clear tubes, 38.5 mL capacity, 25 × 89 mm). 7 mL 1.2 M sucrose. 6 mL 1.0 M sucrose. 6 mL 0.8 M sucrose. 6 mL 0.6 M sucrose. 6 mL 0.4 M sucrose. Prepare sucrose solutions

individually in Medium L, and store up to 5 days at 4 °C. On the day of experiment, layer solutions on top of each other in a thin-walled 38.5 mL, 25 × 89 mm ultracentrifuge tube, starting with 1.2 M at the bottom. Keep at 4 °C until use.

4. *Wash buffer*: 6.142 mL 2 M NaCl (122 mM final). 500 μL 1 M KCl (5 mM final). 460 μL 0.25 M NaH_2PO_4 (1.15 mM final). 4 mL 0.5 M PIPES (20 mM final). 0.1 g D-(+)-glucose (1 mg/mL final). 100 mL H_2O. Adjust to pH 6.8. Prepare fresh on the day of use and keep at 4 °C.
5. *Hypoosmotic Medium L*. 0.174 g K_2HPO_4 (1 mM final). 2 mL 0.5 M EDTA (0.1 mM final). 1 L H_2O. Adjust to pH 8.0. Store up to several months at 4 °C.
6. *Radioimmunoprecipitation assay (RIPA) lysis buffer*: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, and 1 mM EDTA.
7. *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer*: 125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 0.004% bromophenol blue, and freshly prepared 50 mM dithiothreitol (DTT).
8. *Urea sample buffer*: 375 mM Tris–HCl, pH 6.8, 8 M Urea, 50 mM DTT, 2% SDS, 0.004% bromophenol blue.
9. *Glycine buffer*: 0.1–0.2 M glycine, pH 2.0–3.0.
10. *Electrophoresis buffer*: 25 mM Tris base, pH 8.0, 380 mM Glycine, 0.1% SDS.
11. *Anode buffer*: 300 mM Tris base, 20% methanol.
12. *Cathode buffer*: 40 mM 6-Aminocaproic acid, 20% methanol.
13. *IB blocking solution*: PBS containing 0.05% Tween 20 and 5% (w/v). You can also use the commercial blocking buffer (e.g., Odyssey blocking buffer from LI-COR Biosciences, cat. nr. 927-40000).
14. *Immunoblot (IB) washing solution*: PBS containing 0.05% Tween 20.

2.3.1 Instrumentation, Equipment, and Software

1. Rat guillotine.
2. Surgical tools for isolation of the cortex.
3. 25 and 55 mL Potter-Elvehjem homogenizers (Teflon glass).
4. High-speed centrifuge (e.g., Sorvall RC-5C with SS-34 rotor and adaptors for eight 16 × 100 mm tubes).
5. 50 mL (29 × 102 mm) polycarbonate high-speed centrifuge tubes.
6. Ultracentrifuge capable of 100,000 × *g* with swinging-bucket rotor (e.g., Beckman SW 28) and 31 mL, 25 × 89 mm, thick-walled polycarbonate ultracentrifuge tubes.

7. Fixed angle rotor (e.g., Beckman 70.1 Ti or 50 Ti) with 10 mL, 16 × 76 mm thick-walled polycarbonate ultracentrifuge tubes.
8. 10,000 MWCO dialysis membranes.
9. Centriprep 10 centrifugal concentrations (Millipore).
10. Mini-PROTEAN casting frame (Bio-Rad).
11. Immobilon-P transfer membrane (pore 0.45 μm, Millipore).
12. Extra thick blot paper, filter paper (Bio-Rad).
13. Western blot equipment (Bio-Rad).
14. Proper detection equipment, for instance, for Kodak Image Station 440 CF from Kodak for HRP-coupled antibodies and Odyssey from LI-COR Biosciences for infrared dye-coupled antibodies.

3 Methods

3.1 Synaptosomes Preparation (Fig. 1)

1. Sacrifice 4–8 rats weighting 200–250 g under ether anesthesia by decapitation with a guillotine. Remove carefully the brains and place in ice-cold 1× sucrose buffer.
2. Dissect quickly the brain area of interest (e.g., hippocampus, raphe nucleus, striatum (dorsal and ventral components)). Pools 4–8 are of interest together, and place in 9 mL ice-cold 1× sucrose buffer containing the Protease Inhibitor Cocktail.
3. Homogenize with the polytron for four periods of 20 s each.
4. Centrifuge the homogenate for 10 min at 1000 × *g* (3000 rpm in an SS-34 rotor) at 4 °C to remove the nucleus and tissue debris.
5. Recover the supernatant containing the membrane extracts, and carefully load 2 mL supernatant onto each of four Percoll gradients. Centrifuge gradients for exactly 5 min at 32,500 × *g* (17,250 rpm in an SS-34 rotor), 4 °C. Alternatively, the supernatant may be loaded onto a discontinuous sucrose gradient consisting of 1.2/1.0/0.8 M sucrose layers and centrifuged for 20 min at 48,200 × *g*.
6. Collect synaptosomes from the interface at 15%/23% using a Pasteur pipet. In this case, the synaptosome fraction will be collected from a discontinuous sucrose gradient consisting of 1.2/1.0/0.8 M sucrose; the synaptosomes will be collected from the interface between the 1.2 and 1.0 M sucrose layers. Synaptosomes are visible as a cloud of material concentrated at the bottom interface (15%/23% for Percoll or 1.2/1.0 M for sucrose). Use a separate Pasteur pipet to remove upper layers before collecting the synaptosome layer.

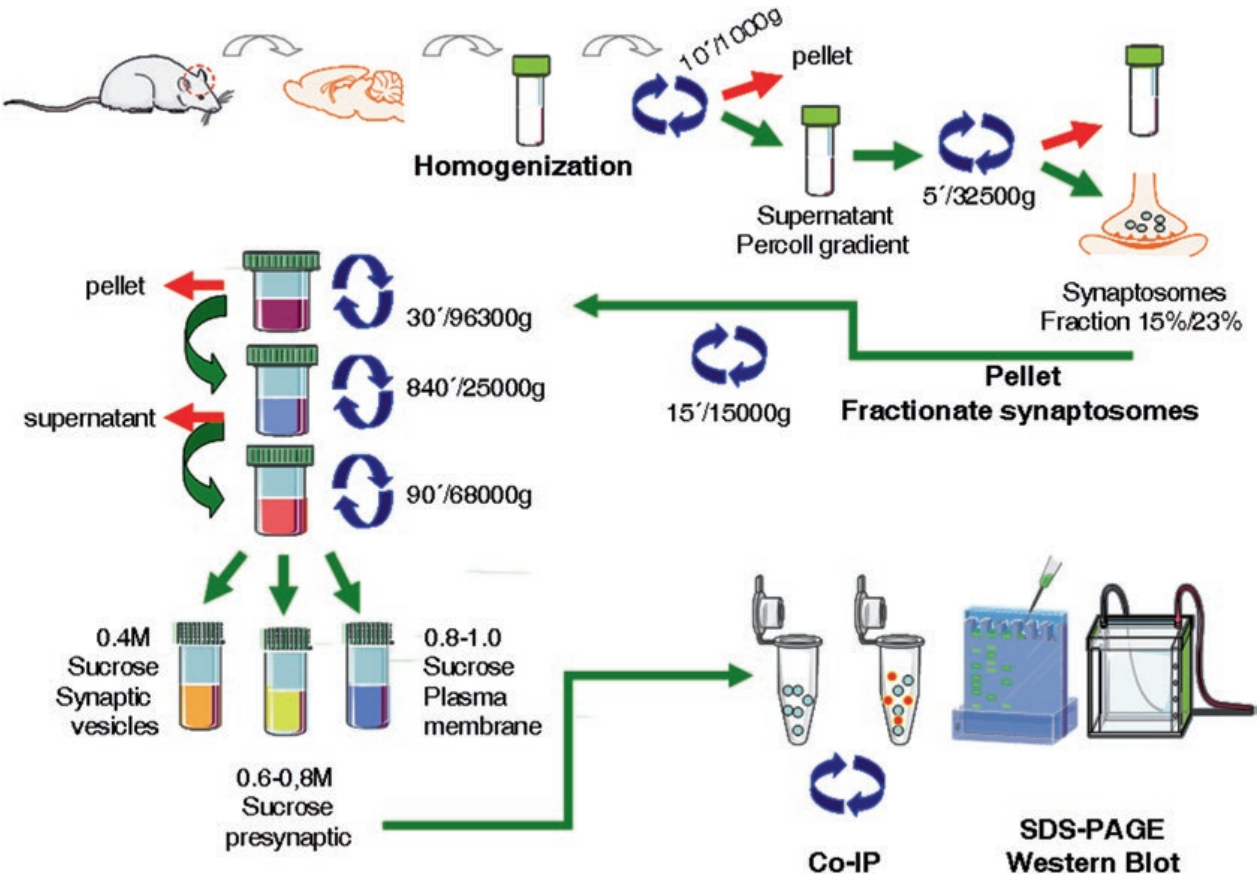

Fig. 1 Illustration of the flow chart for synaptosomes preparation and co-immunoprecipiation (Co-IP) experiment which is done using these fractions. The method is represented step by step. It includes the brain region dissection, the synaptosomes isolation, and the subcellular fraction of the synaptosomes samples by differential centrifugation and the co-immunoprecipitation, electrophoresis, and Western blot. Each of these steps is described in more details in the corresponding method section

7. Transfer the synaptosomes to a 50 mL polycarbonate centrifuge tube, and add 30 mL chilled wash buffer. Centrifuge 15 min at 15,000 × *g* (11,000 rpm in an SS-34 rotor), 4 °C.
8. Carefully discard the supernatant, and use immediately the synaptosomes pellet for fractionation (see below) or alternatively frozen down in liquid nitrogen and stored at −80 °C until use.
9. Determine the total protein content of each pellet (synaptosomes) by means of the BCA Protein Assay Kit (Pierce, USA). Adjust all membrane extracts to the same final concentration, normally between 1 and 2 mg/mL in wash buffer containing the Protease Inhibitor Cocktail.

3.2 Fractionate Synaptosomes (Fig. 1)

1. Resuspend the synaptosomes pellet (Step 8) in 12–15 mL ice-cold hypoosmotic Medium L. Save a 0.5 mL aliquot of this synaptosomal suspension for validation of the fractionation procedure.

2. Homogenize with the polytron for three periods of 10 s each, and then incubate suspension on ice for 15 min.
3. Repeat homogenization once more, then adjust pH to 8.0 with 1 M KOH, and incubate homogenized synaptosomal suspension on ice for another 45 min.
4. Layer, as much as you need, 5 mL synaptosomal suspension onto 5 mL of 1.0 M sucrose in Medium L in a 31 mL thick-walled polycarbonate ultracentrifuge tube.
5. Centrifuge 30 min at 96,300 × *g* (27,000 rpm in an SW 28 rotor), 0° to 4 °C. Collect the supernatant in fresh 31 mL ultracentrifuge tubes. Discard the pellet, which contain the crude synaptosomal mitochondrial fraction.
6. Cover the supernatant tubes with Parafilm and gently mix by inverting. Centrifuge 14 h at 25,000 × *g* (14,000 rpm in an SW 28 rotor), 4 °C. Collect the pellet on ice and discard the supernatant (cytosolic fraction).
7. Resuspend the pellet from Step 6 in Medium L (1.0–4.0 mL). Layer 5 mL of suspension onto separate sucrose gradients prepared in 38.5 mL thin-walled ultracentrifuge tubes. Mark phase interfaces on the tubes to facilitate recognition of the desired fractions after centrifugation.
8. Centrifuge sucrose gradients 90 min at 68,000 × *g* (23,000 rpm in an SW 28 rotor), 0° to 4 °C. Collect each fraction carefully with a Pasteur pipet. The synaptic vesicle fraction is located above the 0.4 M sucrose layer. The fraction enriched with the presynaptic plasma membrane proteins is located at the phase interfaces between 0.6/0.8, 0.8/1.0, and 1.0/1.2 M sucrose. The 0.8/1.0 M interface usually contains the purest plasma membrane fraction.
9. Transfer each fraction to a thick-walled 10 mL ultracentrifuge tube. Add Medium L to fill 3/4 of each tube. Centrifuge 45 min at 106,500 × *g* (40,000 rpm in 50 Ti rotor), 0° to 4 °C.
10. Collect each pellet, and resuspend in 0.5–1 mL of 20 mM Tris·Cl, pH 7.4, containing protease inhibitors. Store in 50–100 µL aliquots for up to several years at −20 °C.
11. Determine the total protein content of each synaptosomes fraction by means of the BCA Protein Assay Kit (Pierce, USA). Analyze aliquots containing equal amounts of protein from each fraction by SDS-PAGE. Aliquots containing 10 µg total protein should be sufficient to detect most synaptic vesicle proteins. Detect proteins of interest by immunoblotting with the appropriate specific antibodies.

3.3 Detection and Visualization of Synaptic Vesicle GPCR Heteroreceptor Complexes by Co-immuno-precipitation (A General Scheme for the Co-immuno-precipitation Procedure Is Provided in Fig. 1 (Note 1))

1. Take 0.5–1 mL membrane fraction (e.g., synaptosomes or the fraction enriched with the presynaptic plasma membrane proteins) for each antibody to be tested in the experiment.
2. Centrifuge during 30 min at 12,000 × *g* at 4 °C.
3. Resuspend pellet in 1 mL of RIPA buffer containing the Protease Inhibitor Cocktail, and homogenize using the polytron (**Note 2**).
4. Incubate during 60–120 min in constant rotation at 4 °C.
5. Centrifuge for 15 min at 10,000 × *g* at 4 °C. The membrane receptors will mainly be in the supernatant fraction.
6. Collect each supernatant in a new microcentrifuge tubes, and add 1–4 μg of the indicated primary antibody (**Note 3**).
7. Incubate the sample with the antibody overnight at 4 °C, upon constant rotation. The length of the incubation period depends on the amount of protein and affinity properties of the antibody.
8. Meanwhile prepare the Sepharose beads (**Note 4**) or use ready-to-use protein A beads (delivered as a 50% slurry). These beads are first washed three times (centrifugation after each washing step: 110 × *g*, 4 °C, 1 min) with RIPA buffer supplemented with protease and phosphatase inhibitors.
9. The washed beads are added to the incubate samples (Step 7) and incubated 2 h with rotation at 4 °C.
10. The beads are again washed. Wash the resin three times in 1 mL of ice-cold RIPA buffer (supplemented with protease and phosphatase inhibitors) by centrifuging at 10,000 × *g* for 1 min at 4 °C. In the last wash, aspirate the supernatant to dryness with a 28-gauge needle. The protein of interest should now be specifically bound to the antibody coating the beads.
11. Resuspend the resin and elute the beads. There are several possibilities: *glycine buffer elution (go to Step 5), SDS buffer elution (go to Step 6), or urea buffer elution (go to Step 7)*. In the glycine buffer, the complex is eluted from the beads by acidification using a buffer containing 0.1–0.2 M glycine, pH 2.0–3.0. The low pH of glycine weakens the interaction between the antibody and the beads. This method is advantageous as beads can be reused after removal of the glycine buffer. However, the eluted sample should be immediately neutralized with Tris, pH 8.0–8.5. On the other hand, the SDS buffer is the harshest, which will also elute non-covalently bound antibodies and antibody fragments along with the protein of interest. The Ag-Ab complex is eluted from the beads by heating or boiling samples in loading buffer with denaturant SDS. This method is advantageous because the extraction method is highly efficient and the resulting sample is more concentrated. The urea elu-

tion is advantageous for mass spectrometry because the sample can be digested by proteolytic enzymes.

12. Elute the beads (50 μL) with 3 × 50 μL 0.2 M glycine pH 2.6 (1:1) by incubating the sample for 10 min with frequent agitation before gentle centrifugation. Pool the eluate and neutralize by adding equal volume of Tris pH 8.0. Neutralize the beads by washing 2× with 150 μL lysis buffer (without detergent), and pool with eluate. Run the samples on SDS-PAGE (see below Step 8).
13. Elute 50 μL of beads by heating in 50 μL of 2× SDS loading buffer without DTT for 10 min at 50 °C. Pellet beads, transfer supernatant to a new tube, and add DTT at 100 mM (elution 1). Add 50 μL 2× SDS buffer with DTT to pelleted beads (elution 2). Boil the eluted samples for 5 min, and analyze content of the sample by SDS-PAGE and Western blot (see below Step 8).
14. Wash beads with pre-urea wash buffer (50 mM Tris pH 8.5, 1 mM EGTA, 75 mM KCl). Remove all residual supernatant. Add 2–5 volumes urea elution buffer (6–8 M Urea, 20 mM Tris pH 7.5, and 100 mM NaCl), and rotate for 30 min at room temperature with frequent agitation before gentle centrifugation. Repeat this process at least twice more to ensure that the entire captured complex has been released from the beads. Pellet beads and remove urea to a new tube. Run the samples on SDS-PAGE (see below Step 8).
15. Finally, when the receptors are eluted from the beads, standard SDS-PAGE can be performed to separate the receptors according to their molecular weight. Prepare samples for SDS-PAGE. Load the mini-PROTEAN tetra cell with electrophoresis buffer. Load protein marker and samples into the gel wells.
16. Run the gels at 75 V until samples reach the separating gel, and then increase voltage until 150 V. Stop SDS-PAGE running when the downmost band of the protein marker reaches the foot line of the glass plate.
17. Following electrophoresis, separate glass plates, recover the gel, and equilibrate it in anode buffer for 5 min.
18. Cut two immunoblot thick filter papers in 6.25 × 9.25 cm sheet. Soak one of them in anode buffer and the other in cathode buffer.
19. Cut Immobilon-P transfer membranes in 9 cm × 6 cm sheets. Completely soak membranes in methanol for 3 s to activate them and finally equilibrate in anode buffer.
20. Prepare the Western blot sandwich by placing (a) the anode buffer pre-soaked sheet of filter paper onto the anode cassette; (b) the anode buffer pre-wetted transfer membrane on top of

the filter paper; (c) the anode buffer equilibrated gel on top of the transfer membrane, aligning the gel on the center of the membrane; and finally (d) the other cathode buffer pre-soaked extra thick filter paper on top of the gel.

21. Roll a plastic pipet over the surface of the filter paper in both directions to eliminate air bubbles and to ensure complete contact between polyacrylamide gel and the transfer membrane.
22. Carefully place the cathode top onto the cassette.
23. Turn on the power and transfer mini gels for 30 min at 25 V.
24. Following transfer, discard filter papers and polyacrylamide gel.
25. Block transfer membrane in IB blocking solution for 30 min at room temperature with continuous shacking.
26. Incubate the membrane overnight at 4 °C with the indicated primary antibody diluted in IB blocking solution with continuous shaking. You can also use the commercial blocking buffer (e.g., Odyssey blocking buffer from LI-COR Biosciences, cat. nr. 927–40,000).
27. Make three washes of 10 min with IB washing solution to eliminate unbound primary antibody.
28. Incubate with the indicated secondary antibody for 90 min at room temperature in continuous shaking. For instance, the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific), HRP-conjugated anti-mouse IgG TrueBlot™ (eBioscience), or Odyssey for infrared dye-coupled antibodies from LI-COR.
29. Make three washes of 10 min with IB washing solution to eliminate unbound secondary antibody.
30. If using the horseradish peroxidase (HRP)-conjugated antibodies, incubate membrane with SuperSignal West Pico Chemiluminescent Substrate (prepare the mix following the proportion 1:1 of solution A and B provided by the manufacturer in dark conditions).
31. Develop membrane in, for instance, Kodak Image Station 440 CF from Kodak for HRP-coupled antibodies and Odyssey from LI-COR Biosciences for infrared dye-coupled antibodies.

4 Notes

1. There are a few different methods to co-immunoprecipitate GPCRs. One approach consists in the pre-mixture of the pri-

mary antibody with your membrane fraction sample, followed by addition of protein A/G support. This method yields high purity of receptor; however, the antibodies are also co-eluted with receptor of interest which sometimes creates difficulties in Western blot detection. The other approach is to bind the primary antibody that recognizes one of the protomers of interest to the protein A/G beads and then mix with the antigen. This method gives lesser yield than the first one but avoids the problem of co-elution of antibodies. Herein we will describe the first approach.

2. There are a lot of possibilities concerning detergent choice for the solubilization of membrane receptors. For instance, CHAPS buffer (30 mM Tris–HCl pH 7.5; 150 mM NaCl; 1% CHAPS), TNT buffer (20 mM Tris/HCl pH 7.5; 200 mM NaCl; 0.1% Tween 20), NP-40 buffer (0.5% NP-40; 140 mM NaCl; 1.5 mM $MgCl_2$; 10 mM Tris HCl pH 7.5), and Triton X-100 buffer (10% glycerol; 1% Triton X-100; 160 mM NaCl; 50 mM Tris HCl pH 7.5; 1 mM EDTA; 1 mM EGTA). So far, for GPCR the RIPA buffer (150 mM NaCl; 50 mM Tris HCl pH 7.5; 1% NP-40; 0.1% SDS; 0.5% deoxycholic acid) seemed to be the optimal choice, because it gave the best signal. The RIPA lyses buffer is based on moderate concentrations of the nonionic detergent NP-40 that will disrupt the integrity of the cell membrane, causing the extraction of membrane proteins in their native form. The buffer also contains a low concentration of the harsh denaturing anionic detergent SDS and ionic detergent sodium deoxycholate. SDS and sodium deoxycholate will help to disrupt the membrane, resulting in a higher yield of extracted membrane proteins. The concentration of these two is however quite low and will not be able to disrupt the protein–protein interactions. In addition, the detergent digitonin (0.1%) can also be added to optimize the solubilization of the membrane receptors.
3. Typically in a pilot experiment, a fixed amount of protein is precipitated by increasing amounts of antibody. Check the antibody datasheet for recommended antibody concentration. As a guideline use 1–5 μL polyclonal antiserum, 1 μg affinity purified polyclonal antibody, 0.2–1 μL ascites fluid (monoclonal antibody), or 20–100 μL culture supernatant (monoclonal antibody).
4. If using a monoclonal antibody choose protein G-coupled Sepharose beads. If using a polyclonal antibody, protein A-coupled Sepharose beads are usually a better option. If the beads come as a powder, incubate 100 mg of beads in 1 mL 0.1 M PBS, wash for 1 h so they swell up, and then centrifuge, remove the supernatant, and discard. Add 1 mL PBS 0.1% BSA, mix for 1 h using an Eppendorf rotator and rinse twice in

PBS. Remove the supernatant and add 400 μL of buffer made with protease inhibitors. The slurry is now ready for use. It can be stored at 4 °C for a few days. You can also buy pre-swollen beads as slurry ready for use. If use IgM antibodies, do not use protein A- or protein G-conjugated beads. Use, instead, anti-IgM coupled protein A or protein G beads. The IgM will bind to the beads by binding to the anti-IgM antibody.

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Annex 2

Chapter 13

Co-immunoprecipitation (Co-IP) of G Protein-Coupled Receptor (GPCR)-Receptor Tyrosine Kinase (RTK) Complexes from the Dorsal Hippocampus of the Rat Brain

Michael Di Palma, Manuel Narvaez, Mariana Pita-Rodríguez, Chiara Contri, Martina Zannoni, Riccardo Cuppini, Kjell Fuxe, Patrizia Ambrogini, and Dasiel O. Borroto-Escuela

Abstract

A large body of evidence indicates that G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) can form heteroreceptor complexes. In these complexes, the signaling from each interacting protomer is modulated to produce an integrated and therefore novel response upon agonist(s) activation. In the GPCR-RTK heteroreceptor complexes, GPCRs can activate RTK in the absence of added growth factor through the use of RTK signaling molecules. This integrative phenomenon is reciprocal and can place also RTK signaling downstream of GPCR. The existence of either stable or transient GPCR-RTK homo- and heteroreceptor complexes with allosteric receptor-receptor interactions increases the diversity of receptor function including recognition, trafficking, and signaling. The isolation and characterization of GPCR-RTK heteroreceptor complexes are therefore important to understand these processes. Co-immunoprecipitation (Co-IP) is a straightforward technique to study in vivo GPCR-RTK interactions and can identify interacting proteins or heteroreceptor complexes present in cell extracts. Here, we present detailed protocol for Co-IP of GPCR-RTK heteroreceptor complexes from brain membrane preparations using as an example the study of A2AR-TrkB heteroreceptor complexes in the rat dorsal hippocampus.

Key words G protein-coupled receptors (GPCRs), Receptor tyrosine kinases (RTKs), Heteroreceptor complexes, GPCR-RTK heteroreceptor complexes, Receptor-receptor interactions, Homodimerization, Heterodimerization, Co-immunoprecipitation

1 Introduction

In the beginning of the 1980s, the idea of the existence of direct interactions between two different G protein-coupled receptors (GPCRs) at the level of plasma membrane was introduced in Sweden [1, 2]. Nowadays, the ability of GPCRs to form homo- and heteroreceptor complexes is a well-documented phenomenon of high relevance, since these receptor complexes represent centers for molecular integration in the central nervous system (CNS) [3, 4].

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The integration of signals in the heteroreceptor complexes takes place via allosteric receptor-receptor interactions at the plasma membrane level, which makes possible a marked increase of the repertoire of GPCR signaling [5, 6]. This results in biochemical and/or pharmacological activities of the receptor protomers different from those of the corresponding monomers and homomers [7, 8]. However, over the past decade, the concept of GPCR-GPCR interactions has expanded, considering the ability of different GPCRs to interact not only with themselves but also with receptor tyrosine kinases (RTKs), ion channel receptors, and receptor activity-modifying proteins, forming large homo- and heteroreceptor complexes [9–14].

The growth-promoting activity of many GPCR ligands in fact involves the activation of RTKs and their downstream signaling pathways [15]. This evidence led to the emergence of the "transactivation" concept, which refers to the activation of RTK signaling pathways by GPCR ligands via GPCR-induced release of neurotrophic factors and/or also crosstalk in the GPCR-RTK downstream signaling pathways [16]. In 2007, GPCRs were proposed to directly interact with RTKs themselves to alter the RTK signaling through allosteric mechanisms via direct receptor-receptor interactions (GPCR-RTK heteroreceptor complexes) [16], and evidence for this view was obtained [9–12] (Fig. 1). Among all GPCRs able to trigger RTK activation, the adenosine receptor A2AR is of particular interest. This GPCR is able to activate, in the absence of neurotrophins, the tropomyosin-related kinase B (TrkB) receptor, which is the high-affinity receptor of brain-derived neurotrophic factor (BDNF) [17]. As to the CNS, especially the hippocampus is of interest since the two receptors are coexpressed in this region [18–20] and are known to be involved in learning and memory processes [21–23]. The activation of A2AR was found to be crucial for facilitating synaptic transmission modulation by BDNF at hippocampal synapses leading to the subsequent long-term potentiation (LTP) development [24, 25]. Taking into account that A2AR and TrkB receptors are colocalized on rat motor neurons [26], it is plausible to propose the existence of A2AR-TrkB heteroreceptor complexes in the CNS. The proof of the presence of this heteroreceptor complex in the hippocampus may be crucial to further characterize the action of these receptors in hippocampal functions. Therefore, in the current work, we have used *co-immunoprecipitation* (*Co-IP*) assay to determine the existence of A2AR-TrkB heteroreceptor complexes in the rat dorsal hippocampus and to map their distribution within this brain region.

1.1 Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) is a protein extraction method that specifically targets protein-protein or receptor-receptor interactions. Although such an analysis supports the coexistence of receptors in a complex, a direct receptor-receptor interaction

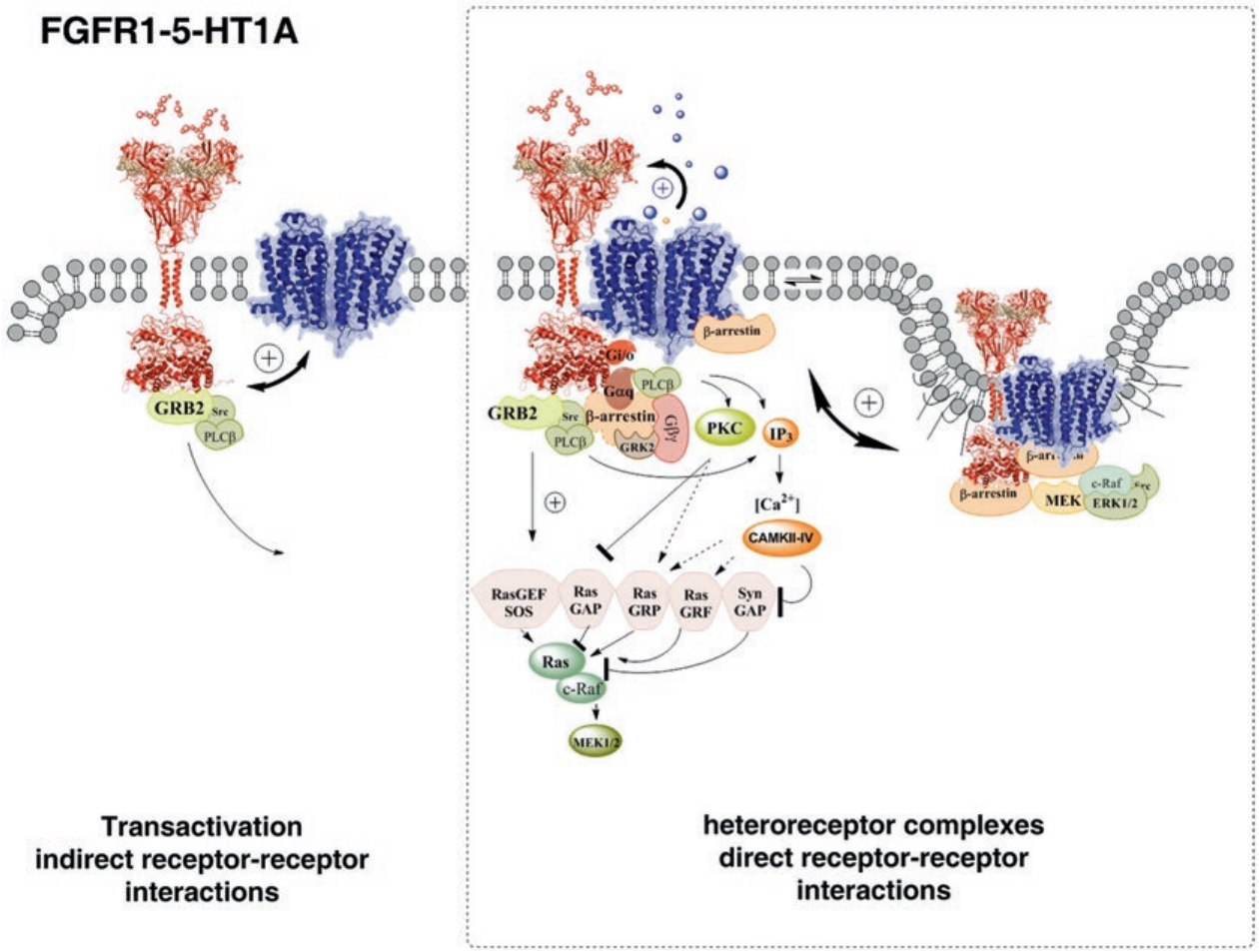

Fig. 1 There is a new awareness that receptor tyrosine kinase (RTK) and G protein-coupled receptor (GPCR) possess the capacity for transactivation not only via GPCR-induced release of neurotrophic factors but also using shared signaling pathways or for GPCRs using RTKs themselves as signaling platforms via direct receptor-receptor interactions. Thus, over the past decade, many examples of transactivation of mitogenic growth factor receptors in response to GPCR signaling have been reported indicating that there are alternative modes of activating receptor tyrosine kinases even in the absence of neurotrophic factor binding (left panel). The receptors for epidermal growth factor, platelet-derived growth factor, insulin-like growth factor-1, FGFs, and neurotrophins can be transactivated in response to GPCR activation in the absence of neurotrophic factor binding via direct (heteromeric receptor complexes) (right panel) and/or indirect GPCR-RTK receptor interaction (left panel). This RTK transactivation via GPCR-RTK receptor interactions can lead to effects on cell proliferation, differentiation, and synaptic plasticity and may play crucial roles in the induction of enduring change in the nervous system. Thus, there appears to exist a general mechanism of receptor-receptor interactions between these two receptor systems, but the molecular mechanisms by which a GPCR may directly and indirectly interact with and activate receptor tyrosine kinases in the absence of neurotrophic factor binding are not fully understood. Recently FGFR1-5-HT1A receptor complexes have been demonstrated in the raphe-hippocampal 5-HT system and shown to enhance hippocampal and dorsal raphe plasticity [9, 10]. A dynamic modulation of FGFR1-5-HT1A heteroreceptor complexes was demonstrated by which agonist treatment enhances participation of FGFR1 and 5-HT1A homodimers and recruitment of β-arrestin2

cannot be concluded unless further in vitro data are available. Co-IP is slightly different from immunoprecipitation since it takes the concept of immunoprecipitation one step further by using antibodies to target not the direct antigen that binds to the antibody

but any protein that binds to the antigen and is pulled out with it. This makes Co-IP an ideal assay for studying homo- and heteroreceptor complexes. Co-IP can provide physiologically relevant information about receptor interactions, but several parameters like the type of detergents and antibodies to be used must be considered in order to have a reliable assay.

2 Material

2.1 Animals

Sprague Dawley rats (Charles River Laboratories, Stockholm, Sweden).

2.2 Buffers and Reagents

1. *Tris 50 mM buffer*: 50 mM Tris Base, pH 7.4.
2. *Radioimmunoprecipitation assay (RIPA) buffer*: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, and 1 mM EDTA.
3. *Urea sample buffer:* 375 mM Tris–HCl, pH 6.8, 8 M urea, 50 mM DTT, 2% SDS, and 0.004% bromophenol blue.
4. *Electrophoresis buffer:* 25 mM Tris Base, pH 8.0, 180 mM glycine, and 0.1% SDS.
5. *Anode buffer:* 300 mM Tris Base and 20% methanol.
6. *Cathode buffer:* 40 mM 6-aminocaproic acid and 20% methanol.
7. *Immunoblot (IB) washing solution*: PBS containing 0.05% Tween 20.
8. *IB blocking solution*: PBS containing 0.05% Tween 20 and 5% (w/v) of nonfat dry milk.

2.3 Reagents

Protein A-immobilized agarose fast flow, 50% (v/v) (Sigma-Aldrich, Stockholm, Sweden); Protease Inhibitor Cocktail; SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Stockholm, Sweden); BCA Protein Assay Kit (Pierce, Stockholm, Sweden). *Primary antibodies*: rabbit anti-A2A receptor (Abcam) and mouse anti-TrkB (Abcam). *Secondary antibodies*: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) and HRP-conjugated anti-mouse IgG TrueBlot™ (eBioscience).

3 Methods

3.1 Preparation of Total Brain Membranes

1. Sacrifice rats weighting 200–300 g by decapitation and after anesthesia, and carefully dissect the brain area of interest (e.g., the hippocampus). Keep the sample at −20 °C.

2. To each brain sample, add 1 mL of ice-cold Tris 50 mM buffer containing the Protease Inhibitor Cocktail (Roche, Switzerland).
3. Homogenize with the Polytron for three periods of 10 seconds each.
4. Spin the homogenate for 15 min at 1000 × *g* at 4 °C to remove the nucleus and tissue debris.
5. Recover the supernatant containing the membrane extracts, and centrifuge for 40 min at 12,000 × *g* at 4 °C.
6. Discard the supernatant containing the cytosolic proteins, and resuspend the pellet containing the membrane extracts in 1 mL of Tris 50 mM with Protease Inhibitor Cocktail.
7. Determine the total protein concentration of each membrane extract by means of the BCA Protein Assay Kit. Adjust all membrane extracts to the same final concentration, normally between 1 and 2 mg/mL in Tris 50 mM containing the Protease Inhibitor Cocktail.
8. Use the fresh obtained membrane extracts immediately. Alternately, they can be frozen down and stored at −20 °C until use.

3.2 Co-immunoprecipitation

1. Take 1 mL of the brain membrane extract for each antibody to be tested in the experiment.
2. Centrifuge during 40 min at 12,000 × *g* at 4 °C.
3. Resuspend pellet in 1 mL of RIPA buffer containing the Protease Inhibitor Cocktail, and homogenize using the Polytron.
4. Incubate during 40 min in constant rotation at 4 °C.
5. Centrifuge for 40 min at 12,000 × *g* at 4 °C.
6. Collect each supernatant in a new Eppendorf, add 1–5 μg of the indicated primary antibody, and incubate overnight in constant rotation at 4 °C to allow the formation of the immune complexes.
7. Add 50 μL of a suspension of protein A-immobilized agarose.
8. Incubate it for 4 h at 4 °C to allow the antibody to bind to the protein complexes.
9. Wash the resin three times by centrifuging at 10,000 × *g* for 1 min at 4 °C. Discard the supernatant. Add again to the pellet 1 mL of ice-cold RIPA buffer. In the last wash, aspirate the supernatant to dry with a needle.
10. Resuspend the resin and the acetone precipitated extract with SDS-PAGE sample buffer.

3.3 SDS-PAGE

1. Load the Mini-PROTEAN Tetra cell with electrophoresis buffer.
2. Load protein marker and samples (see above) into the gel wells.
3. Run the gels at 75 V until samples reach the separating gel, and then increase voltage until 150 V. Stop SDS-PAGE running when the lower protein marker reaches the foot line of the glass plate.

3.4 Immunobloting

1. Cut Immobilon-P transfer membranes or similar. Soak the transfer membranes in methanol for 3 s to activate them, and finally equilibrate in anode buffer. To avoid membrane contamination, always use tweezers with no teeth or gloves when handling them.
2. Cut two immunoblot thick filter papers. Completely soak each of them, one of them in anode buffer and the other in cathode buffer.
3. After electrophoresis, separate glass plates, recover the gel, and equilibrate it in anode buffer for 5 min.
4. Place the anode buffer pre-soaked sheet of filter paper onto the anode cassette.
5. Place the anode buffer pre-wetted transfer membrane on top of the filter paper.
6. Place the anode buffer equilibrated gel on top of the transfer membrane, aligning the gel on the center of the membrane.
7. Place the other cathode buffer pre-soaked extra-thick filter paper on top of the gel.
8. Roll a plastic pipet over the surface of the filter paper in both directions to eliminate air bubbles and to ensure complete contact between polyacrylamide gel and the transfer membrane.
9. Place the cathode top onto the cassette.
10. Turn on the power and transfer mini gels for 30 min at 25 V.
11. After transfer, turn off the power supply, and discard filter papers and polyacrylamide gel.
12. Block transfer membrane in IB blocking solution or similar blocking buffers for 60 min at room temperature with continuous shaking.
13. Incubate the membrane overnight at 4 °C with the indicated primary antibody diluted in IB blocking solution with continuous shaking.
14. Make three washes of 10 min with IB washing solution to eliminate unbound primary antibody.

15. Incubate with the indicated secondary antibody for 120 min at room temperature in continuous shaking.
16. Make three washes of 10 min with IB washing solution to eliminate unbound secondary antibody.
17. Incubate membrane with SuperSignal West Pico Chemiluminescent Substrate (prepare the mix following the proportion 1:1 of solution A and B provided by the manufacturer in dark conditions).
18. Develop membrane in a ChemiDoc.

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Annex 3

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Evidence for the existence of A2AR-TrkB heteroreceptor complexes in the dorsal hippocampus of the rat brain: Potential implications of A2AR and TrkB interplay upon ageing

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ABSTRACT

Adenosine A2A receptors (A2AR) are crucial in facilitating the BDNF action on synaptic transmission in the rat hippocampus primarily upon ageing. Furthermore, it has been suggested that A2AR-Tropomyosin related kinase B receptor (TrkB) crosstalk has a pivotal role in adenosine A2AR-mediated modulation of the BDNF action on hippocampal plasticity. Considering the impact of the above receptors interplay on what concerns BDNF-induced enhancement of synaptic transmission, gaining a better insight into the mechanisms behind this powerful crosstalk becomes of primary interest. Using *in situ* proximity ligation assay (PLA), the existence of a direct physical interaction between adenosine A2AR and TrkB is demonstrated. The A2AR-TrkB heteroreceptor complexes show a heterogeneous distribution within the rat dorsal hippocampus. High densities of the heteroreceptor complexes were observed in the pyramidal cell layers of CA1-CA3 regions and in the polymorphic layer of the dentate gyrus (DG). The stratum radiatum of the CA1-3 regions showed positive PLA signal in contrast to the oriens region. The molecular and granular layers of the DG also lacked significant densities of PLA positive heteroreceptor complexes, but subgranular zone showed some PLA positive cells. Their allosteric receptor-receptor interactions may significantly modulate BDNF signaling impacting on hippocampal plasticity which is impaired upon ageing.

1. Introduction

The idea of the existence of direct interactions between two different G protein-coupled receptors (GPCRs) at the level of the plasma membrane was introduced in Sweden at the beginning of the 1980s (Agnati et al., 1980; Fuxe et al., 1981). Currently, a large body of evidence has highlighted the relevance of GPCRs homo-heteroreceptor complexes for molecular integration in the central nervous system (CNS) (Borroto-Escuela et al., 2015a; Fuxe et al., 2014a), which mainly takes place *via* allosteric receptor-receptor interactions at the plasma membrane level. This integration process makes possible a marked increase of the repertoire of GPCR signalling (Borroto-Escuela et al., 2014; Fuxe et al., 2014b), resulting in biochemical and/or pharmacological activities of the receptor protomers different from those of the corresponding monomers and homomers (Borroto-Escuela et al., 2015b, a). Over the past decade, an increasing amount of evidence has pointed out the ability of different GPCRs to interact not only with themselves but also with receptor tyrosine kinases (RTKs), ion channel receptors and receptor activity-modifying proteins, forming large homo-heteroreceptor complexes (Borroto-Escuela et al., 2017, 2015c; Borroto-Escuela et al., 2015d, 2012; Flajolet et al., 2008; Pei et al., 2004; Wang et al., 2012). In this scenario, the growth-promoting activity of many GPCR ligands implicates the activation of RTKs and their downstream signalling pathways (Luttrell et al., 1999) by the GPCR-

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induced release of neurotrophic factors and/or crosstalk in the GPCR-RTK downstream signalling pathways (Fuxe et al., 2007), thus paving the way to the emergence of the 'transactivation' concept.

Beyond the classical 'transactivation process', where the GPCR is not in direct interaction with the RTK, nowadays, there is a new awareness that RTKs and GPCRs possess the capacity for transactivation using RTKs themselves as signalling platforms *via* direct receptor–receptor interactions (Borroto-Escuela et al., 2015c, d; Borroto-Escuela et al., 2012; Di Palma et al., 2019; Flajolet et al., 2008; Fuxe et al., 2007). In this line of reasoning, among all GPCRs able to trigger RTKs activation, the adenosine receptor A2AR is particularly intriguing. This GPCR can transactivate, in the absence of neurotrophins, the high-affinity receptor of brain-derived neurotrophic factor (BDNF), namely, the tropomyosin related kinase B receptor (TrkB) (Lee and Chao, 2001). Interestingly, these two receptors are coexpressed in different brain areas, including the hippocampus, which is a region noteworthy for its relevance on cognition (Rosin et al., 1998; Spencer-Segal et al., 2011) since it is involved in learning, memory processes (Ambrogini et al., 2013; Gimenez-Llort et al., 2007; Lee et al., 2012; Zeng et al., 2012), and adult neurogenesis (Ambrogini et al., 2004). In addition, much work has been focused on the hippocampus as a proxy to study the ageing process, considering that age-related decline in memory and learning performance dependent on this brain area is consistently found across species and tasks (Barnes, 1979; Colombo et al., 1997; Erickson and Barnes, 2003; Mabry et al., 1996; Oler and Markus, 1998; Tanila et al., 1997; Temido-Ferreira et al., 2019). In this regard, the transactivation of TrkB receptors by A2AR was found to have a pivotal role in gating the BDNF action at hippocampal synapses inducing long-term potentiation (LTP) development (Diogenes et al., 2004; Fontinha et al., 2008) from young to aged animals (Costenla et al., 2011; Diogenes et al., 2007; Temido-Ferreira et al., 2019). Notably, the effect of this neurotrophin on rat hippocampal plasticity is affected by ageing, and this seems to be related to the antithetical differential density of TrkB and A2A receptors induced by age (Costenla et al., 2011; Diogenes et al., 2007; Lopes et al., 1999; Rebola et al., 2003; Silhol et al., 2005; Temido-Ferreira et al., 2019; Webster et al., 2006). Indeed, both receptors expression and signalling are profoundly altered in the hippocampus during the ageing with a concomitant age-related down-regulation of TrkB receptors (Diogenes et al., 2007; Silhol et al., 2005) and an up-regulation of A2A ones (Costenla et al., 2011; Diogenes et al., 2007; Lopes et al., 1999; Rebola et al., 2003). This relationship between age-related reorganisation in the density of these receptors with a consequent increase of A2A-receptor-mediated adenosinergic tonus was found to be crucial in triggering the BDNF action upon hippocampal synaptic transmission in aged rats despite the marked loss of TrkB receptors (Costenla et al., 2011; Diogenes et al., 2007; Temido-Ferreira et al., 2019). Thus, considering the impact of the above receptors interplay on what concerns BDNF-induced enhancement of synaptic transmission in the hippocampus primarily upon ageing, gaining a better insight into the mechanisms behind this powerful adenosine/A2AR and BDNF/TrkB crosstalk becomes of primary interest (Diogenes et al., 2007). In this context, in the light of the capacity of RTKs and GPCRs to physically interact (Fuxe et al., 2007), and that TrkB and A2A receptors were found to associate into complexes in lipid raft and nonlipid raft membranes on rat motor neurons (Mojsilovic-Petrovic et al., 2006), it is plausible to propose the existence of A2AR-TrkB heteroreceptor complexes in the hippocampus as a further interplay tool beyond the classical 'transactivation process' (Lee and Chao, 2001; Sebastiao et al., 2011). Therefore, the current work aims to take advantage of a cutting-edge technique as the *in situ* proximity ligation assay (*in situ* PLA) (Borroto-Escuela et al., 2016, 2018; Borroto-Escuela et al., 2013, 2012) to determine the existence of A2AR-TrkB heteroreceptor complexes and their distribution in the rat dorsal hippocampus.

2. Materials and methods

2.1. Animals

All studies involving animals were performed in accordance with the European Communities Council Directive (Cons 123/2006/3) guidelines. Male Sprague-Dawley rats, ten weeks old, weighing 310–350 g were obtained from Charles River Laboratories (Germany). The animals were housed one week before experiments under a 12-h light/dark cycle, with an ambient temperature of 21 ± 2 °C and a relative humidity of 50 ± 5 %. Food and water available *ad libitum*.

Rats (n = 4) were anaesthetised with sodium pentobarbital (40 mg/kg, i.p., Nembutal injection, Dainippon Pharmaceutical, Japan) and perfused intracardially with ice-cold 4 % paraformaldehyde (PFA) in 0.1 M Na_2HPO_4/NaH_2PO_4, pH 7.4, buffer. Brains were removed and postfixed overnight in the same solution of PFA at 4 °C and then incubated first for 24 h in 10 % sucrose and then one week in 30 % sucrose in 0.1 M Na_2HPO_4/NaH_2PO_4, pH 7.4, buffer. Coronal sections (30 μm) were generated using a cryostat (Leica), collected in a cryoprotective solution (30 % glycerol, 30 % ethylene glycol in 0.1 M Tris, pH 7.4), and kept at −20 °C until *in situ* PLA processing.

2.2. In situ proximity ligation assay

Rat hippocampal free-floating sections (30 μm) at Bregma – 3.36 and – 3.48 were employed for *in situ* PLA (Borroto-Escuela et al., 2013, 2011b), that was performed as described previously (Borroto-Escuela et al., 2013). Briefly, sections were quenched with 10 mM Glycine buffer, for 20 min and washed twice with phosphate buffer saline (PBS) at room temperature. Then, were incubated with the permeabilisation buffer (0.1 % Triton X-100 in PBS) for 30 min at room temperature followed by some wash steps and incubated with a blocking buffer (0.2 % BSA in PBS) for 30 min at room temperature. The brain sections were incubated with the primary antibodies of different species, polyclonal anti-TrkB produced in rabbit (ab18987, Abcam, Sweden) and monoclonal anti-A2AR produced in mouse (05–717, Millipore, Sweden) (for details see Supplementary Information), diluted in a suitable concentration in the blocking solution at 4 °C. After this step, the experiment was performed according to the manufacturer's instructions (Duolink *in situ* PLA detection kit, Olink, Sweden) with PLA plus or minus probes for rabbit or mouse antibodies. Sections were mounted on slides with fluorescent mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Sweden), staining the nuclei with blue colour. The PLA signal was visualized, and images acquired by using a confocal microscope (Leica TCS-SL, USA).

2.3. Quantification of in situ PLA signal

Quantification of positive PLA signal (observed as a red blobs/puncta) in different areas of the rat hippocampus was carried out using the Duolink Image Tool Software. The analyses were performed on images from at least two slices per animal, five images per hippocampal subfields. High-resolution (40x

N.A. = 1.25) images from maximum projection obtained from eighteen optical sections of 1 μm of StepSize were acquired and analysed. Mainly three important parameters were taken into account for proper analysis and result interpretation: 1) the number of DAPI nuclei in the sample field; 2) the number of positive PLA/dots per sample field; 3) the total number of positive PLA cells/nuclei per sample field (for details see Supplementary Information; S-Tables 1 and 2). The quantitative results in the different regions of the hippocampus were expressed as the average number of PLA clusters/blobs per cell (identified by DAPI positive nuclei) in the sampled field (40 × 40 μm) from four rats. They are given in Fig. 4 as per cent of the maximal signal of positive PLA per cell in the same sample field. Densities per cell above three standard deviations of the background of the negative controls

(omission of one of the antibodies used) were regarded as specific PLA clusters/blobs. The values of the columns, each showing a different brain region, which are more than 25 % above background, are described to represent moderate to high densities.

2.4. Statistical analysis

To properly apply parametric or non-parametric statistics, data distributions were explored using D'agostino-Pearson normality test, while the homoscedasticity/homogeneity of variance means was checked using the Levene's test (for details see Supplementary Information; S-Tables 3 and 4).

All data were analysed using the commercial program GraphPad PRISM 6.0 (GraphPad Software, USA). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-hoc test. The *P*-value .05 and lower was considered significant.

3. Results

PLA positive clusters/puncta representing A2AR-TrkB heteroreceptor complexes were observed in the dorsal hippocampus, including both the CA regions and the dentate gyrus. However, a heterogeneous distribution of these PLA positive heteroreceptor complexes was detected (Figs. 1–3).

3.1. CA regions of Ammon´s horn

The highest density of PLA positive clusters was observed in the pyramidal cell layers of CA3-CA2 (Figs. 1, 2 and 4), with a high to medium densities in the pyramidal cell layers of CA1 and CA4 (Figs. 3 and 4). The Stratum Lucidum (sl) displayed, on average, a low PLA signal similar to the one gathered in the negative control, despite some high-density PLA positive cells were observed at the border close to the pyramidal cell layer of CA3 (Figs. 1 and 4). Furthermore, a higher number of A2AR-TrkB heteroreceptor complexes clusters (higher receptor orders) were detected in the pyramidal cell layers of CA2. This clusterisation phenomenon is indicated as dashed circles in yellow colour in Fig. 2.

Moderate densities of A2AR-TrkB puncta/blobs were obtained in the Stratum Radiatum (sr) of the CA regions (Figs. 2 and 4). The Stratum Oriens (so) layers of the CA regions lacked or had a low density of PLA puncta/blobs and were not significantly different from the values obtained for the negative controls (see Figs. 1 and 4). When we compared the number of positive PLA cells per field (as a per cent of the positive PLA cells over the total number cells in the field, as observed from the DAPI staining), a significant increase in the Stratum Radiatum values in comparison to the Stratum Lucidum and Oriens was observed (Fig. 4b).

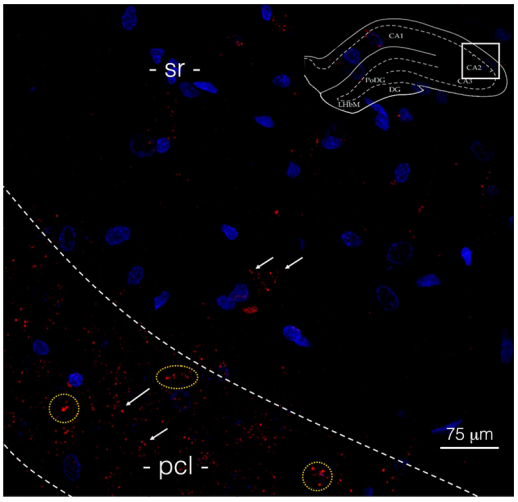

Fig. 2. A representative confocal image of the *in situ* PLA at the level of the pyramidal cell layer of the CA2, in which the positive PLA signal (red clusters/blobs) is mainly detected on the cells of the pyramidal cell layer (pcl) and within a lower densities on the layer of the stratum radiatum (sr). An arrow indicates some positive PLA clusters/blobs and a dash circle marks higher order of heteroreceptor complexes (clusters). The scale bar for this panel is indicated on the right bottom of the panel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2. Dentate gyrus (DG)

As observed for A2AR-TrkB positive PLA densities in the pyramidal cell layers of CA regions, a high density of these heteroreceptor complexes were also detected in the polymorphic layer of the dentate gyrus (PoDG) (Fig. 3). Conversely, both the molecular (molDG; data not shown) and the granular layer (grDG) displayed, on average, a low density of PLA blobs per cell in a similar fashion to the PLA signal gathered in the negative control, as well as, in the corpus callosum (cc) (Fig. 3 and 4). However, it is worth noting that some PLA positive cells in the PoDG were observed at the border close to granular layer (arrows in Fig. 3), an interface defined subgranular zone in which are located neural precursors/neuronal progenitors able to generate new granule neurons in adulthood (Ambrogini et al., 2000, 2004).

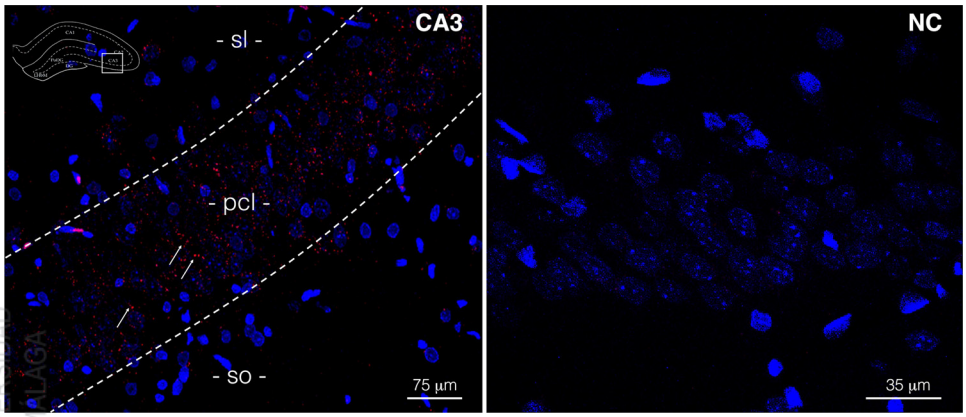

Fig. 1. Detection of A2AR-TrkB heteroreceptor complexes in the different subfields of dorsal rat hippocampus by *in situ* proximity ligation assay (*in situ* PLA). The nuclei are shown in blue (DAPI) and the positive PLA puncta/blobs (A2AR-TrkB heteroreceptor complexes) in red. On the left panel, a representative confocal image of the *in situ* PLA at the level of the pyramidal layer of the CA3, in which the positive PLA signal (red clusters/blobs) are mainly detected on the cells of the pyramidal cell layer (pcl). An arrow indicates some positive PLA clusters/blobs, and the scale bar for this panel is indicated on the right bottom of the panel. On the right panel, a representative confocal image of a negative control of the pyramidal layer (omission of one of the antibodies used). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

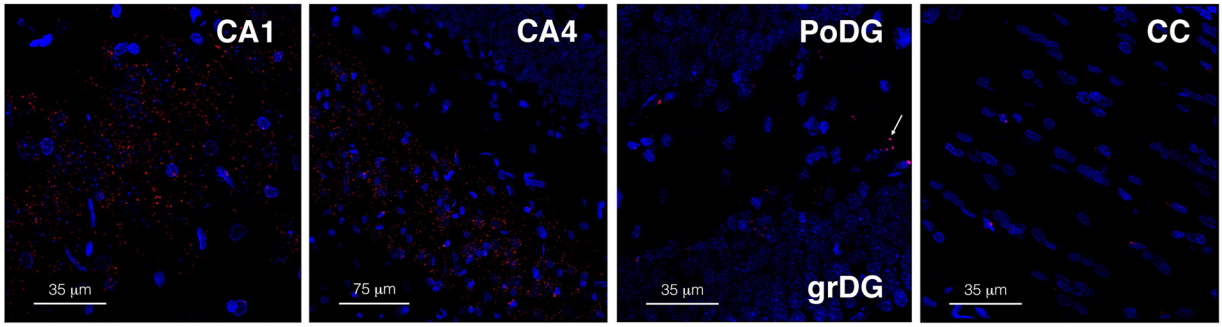

Fig. 3. Representative confocal images of *in situ* PLA at the pyramidal cell layer of the CA1 and CA4, the granular (grDG) and polymorphic layers of the dentate gyrus (PoDG), and the corpus callosum (cc). An arrow indicates some positive PLA clusters/blobs. In the cc, the PLA values obtained were similar to those gathered in the negative control (*in situ* PLA experiment performed without one of the adenosine A2A primary antibody). The scale bar is indicated at the bottom of each panel.

4. Discussion

In the current paper, using a cutting-edge technique to study receptor-receptor physical interactions as the *in situ* PLA, we provided evidence for the existence of A2AR-TrkB heteroreceptor complexes in the rat dorsal hippocampus, showing a specific distribution pattern of the density of these complexes within this brain region. In particular, our data are in line with previous immunoprecipitation findings by Mojsilovic-Petrovic et al. (2006) gathered from lipid raft and nonlipid raft membranes of rat motor neurons, and from a mechanistic point of view, provide a novel interplay tool available for the powerful adenosine/A2A and BDNF/TrkB crosstalk in the hippocampus beyond the classical 'transactivation process' (Lee and Chao, 2001; Sebastiao et al., 2011). Regarding the distribution pattern, in the present work, we observed a high-density of PLA positive clusters of A2AR-TrkB heterocomplexes throughout the pyramidal cell layer of the CA1 to CA3 with lower densities in the stratum radiatum and lack of significant densities in the stratum oriens. This specific pattern of expression, circumscribed to CA areas, is particularly intriguing, considering the integrative properties of CA1-CA3 pyramidal cells and their critical role in modulating hippocampal synaptic plasticity (Langston et al., 2010). In this respect, the A2AR-mediated-facilitatory modulation of the BDNF action on synaptic transmission in CA areas was found to be necessary for the LTP development (Diogenes et al., 2004; Fontinha et al., 2008), notably upon ageing characterised by an age-related downregulation of TrkB receptors (Diogenes et al., 2007; Silhol et al., 2005) and an up-regulation of A2A ones (Diogenes et al., 2007; Lopes et al., 1999; Rebola et al., 2003). Thus, the discovered A2AR-TrkB heteroreceptor complexes and their receptor-receptor interactions, mainly located in the pyramidal cell layers, may provide a novel tool at the service of the adenosine/A2AR and BDNF/TrkB interplay underpinning learning processes and neural plasticity, even if it is worth noting that further studies are needed to precisely map, using different techniques as the post-embedding immunogold labelling (Ciruela et al., 2006), the cellular distribution of these complexes.

Besides CA areas, a distinct distribution pattern of the A2AR-TrkB heteroreceptor complexes was detected in the dentate gyrus. Specifically, among all the layers of this hippocampal area, the PoDG showed a significant and high-density PLA positive puncta made up of A2AR-TrkB heteroreceptor complexes. On the other hand, the molecular and granular layers showed no significant density of PLA positive complexes that were, on average, similar to those detected in the corpus callosum and the negative controls. These latter findings are in line with the low expression level of adenosine A2A receptors previously observed in the granular layer of the rat hippocampus (Rosin et al., 1998). However, it is worth noting that the detected high-density of

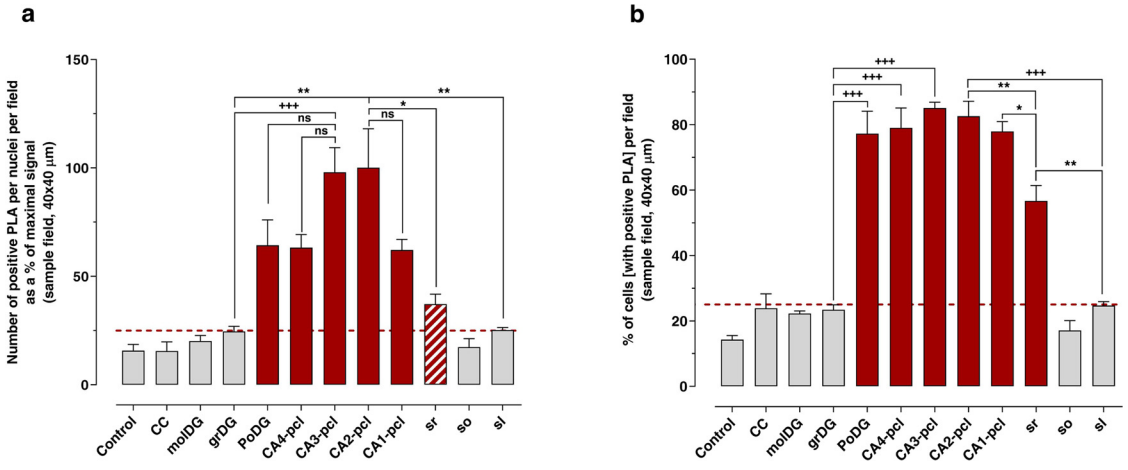

Fig. 4. Quantitative analysis of the A2AR-TrkB heteroreceptor complexes expression in the dorsal hippocampus of the rat brain detected by means of the *in situ* PLA. The PLA was blind quantified as PLA (red puncta/blobs) per nuclei per field. (**a**) The number of positive *in situ* PLA blobs per nuclei per field as a per cent of the maximal signal. The analyses (Mean ± S.E.M.) were performed on images from at least two slices per animal (four rats in total), five images per hippocampal subfields. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The *P*-value .05 and lower was considered significant: $^{*}P < .05$, $^{**}P < .01$ and $^{+++}P < .001$. (**b**) The percent number of PLA positive cells in per cent of total number of nuclei per sampled field are represented. The analyses (Mean ± S.E.M.) were performed on images from at least two slices per animal (four rats in total), five images per hippocampal subfields. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The *P*-value .05 and lower was considered significant. $^{*}P < .05$, $^{**}P < .01$ and $^{+++}P < .001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

A2AR-TrkB heteroreceptor complexes in the PoDG, close to granular layer (subgranular zone), suggests a possible role of these heteroreceptor complexes in the adult hippocampal neurogenesis (Ambrogini et al., 2013) which is known to be reduced upon ageing contributing to age-related cognitive impairment (Drapeau et al., 2003; Lazarov et al., 2010; Okamoto et al., 2011; Takei, 2019). In this line of reasoning, further studies will be required to functional characterise the impact of these heteroreceptor complexes on hippocampal synaptic plasticity in young and aged animals, especially taking into account that usually the GPCR-RTK physical interaction entails the establishment of different integration process that makes possible a marked increase of the downstream signalling (Borroto-Escuela et al., 2014; Fuxe et al., 2014b), resulting in biochemical and/or pharmacological activities of the receptor protomers different from those of the corresponding monomers and homomers (Borroto-Escuela et al., 2015b, 2017; Borroto-Escuela et al., 2011a; Flajolet et al., 2008) (for details see Supplementary Information; S-Fig. 1).

Taken together, the existence of hippocampal A2AR-TrkB heteroreceptor complexes with distinct distribution patterns in the dorsal hippocampus helps to gain a better insight into the mechanisms behind the powerful adenosine/A2AR and BDNF/TrkB crosstalk on the hippocampal plasticity (Diogenes et al., 2007) beyond the classical 'transactivation process' (Lee and Chao, 2001). Finally, considering the therapeutic potential of BDNF-based strategies to counteract the age-related cognitive impairments (Lu et al., 2013), this novel interplay tool should be taken into consideration since it may turn out to be relevant in the design of BDNF-based therapeutic strategies.

Declaration of Competing Interest

The authors have no affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mad.2020.111289.

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Annex 4

Chapter 9

Study of GPCR Homo- and Heteroreceptor Complexes in Specific Neuronal Cell Populations Using the In Situ Proximity Ligation Assay

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Abstract

Membrane receptor, for example, G-protein-coupled receptors **(GPCRs)**, operates via coordinated changes between the receptor expression, their modifications**,** and interactions between each other. Perturbation in specific heteroreceptor complexes and/or their balance/equilibrium with other heteroreceptor complexes and corresponding homoreceptor complexes is considered to have a role in pathogenic mechanisms, including drug addiction, depression, Parkinson's disease**,** and schizophrenia. To understand the associations of GPCRs and to unravel the global picture of their receptor–receptor interactions in the brain, different experimental detection techniques for receptor–receptor interactions have been established (e.g., co-immunoprecipitation-based approach). However, they have been criticized for not reflecting the cellular situation or the dynamic nature of receptor–receptor interactions. Therefore, the detection and visualization of GPCR homo- and heteroreceptor complexes in the brain remained largely unknown until recent years, when a well-characterized in situ proximity ligation assay (in situ PLA) was adapted to validate the receptor complexes in their native environment. The in situ PLA protocol presented here can be used to visualize GPCR receptor–receptor interactions in cells and tissues in a highly sensitive and specific manner. We have developed a combined method using immunohistochemistry and PLA, particularly aimed to monitor interactions between GPCRs in specific neuronal cell populations. This allows the analysis of homo- and heteroreceptor complexes at a cellular and subcellular level. The method has the advantage that it can be used in clinical specimens, providing localized, quantifiable homo and heteroreceptor complexes detected in single cells. We compare the advantages and limitations of the methods, underlining recent progress and the growing importance of these techniques in basic research. We discuss also their potential as tools for drug development and diagnostics.

Key words Protein–protein interaction, Immunoprecipitation, Antibody, Co-immunoprecipitation

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1 Introduction

Mental and neurological diseases, including drug addiction, depression, Parkinson's disease, and schizophrenia, are highly complex in their etiology [1–7]. It is not surprising that the underlying multiple molecular mechanisms are poorly understood and treatment possibilities are inadequate. One emerging hypothesis is that direct physical interactions of different receptors named homo-/heteroreceptor complexes may be involved with disease onset and progression [8–16]. Thus, these homo and heteroreceptor complexes could serve as a biomarker and/or drug target of the disease [17–24].

Experimental evidence confirmed that G-protein-coupled receptors (GPCRs) not only exist as monomers but also as homo- and heteroreceptor complexes in the cell membrane in which allosteric receptor–receptor interactions take place modulating the functions of the participating GPCR protomers. GPCRs can also form heteroreceptor complexes with receptor tyrosine kinases [17, 25, 26] and ionotropic receptors [27] leading to an integrated activation of intracellular signaling cascades generating inter alia changes in gene expression [21, 28, 29]. Furthermore, adaptor proteins interact with receptor protomers and modulate the interactions. The state of the art is that the allosteric receptor–receptor interactions are reciprocal, highly dynamic, and substantially alter the signaling, trafficking, recognition, and pharmacology of the participating protomers. The pattern of changes appears to be unique for each heteromer and can favor antagonistic or facilitatory interactions or switch the G protein coupling from Gi/o to Gq or to beta-arrestin signaling, for example. It gives a new dimension to molecular integration in the nervous system. Future direction should be to determine the receptor interface involving building models of selected heterodimers. This will make it possible to design interface interfering peptides that specifically disrupt the heterodimer. This will help to determine the functional role of the allosteric receptor–receptor interaction versus integration of the intracellular signaling pathways in the function of the receptor heterodimer. Integration of signals already at the plasma membrane seems crucial also in view of the hypothesis that learning and memory at molecular level takes place by reorganization of homo- and heteroreceptor complexes in the postsynaptic membrane. Homo- and heteroreceptor complexes are in balance with each other, and their disbalance is linked to diseases. Targeting heteroreceptor complexes represents a novel strategy for treatment of brain disorders.

The majority of identified GPCR homo- and heteroreceptor complexes have been found using the yeast two-hybrid screen [26], co-immunoprecipitation [25, 30, 31], fluorescence (Förster), or

bioluminescence resonance energy transfer (FRET) and BRET [32–38]. Each approach used has provided precise and valuable information, which was considered with caution in view of their varying number of false-positive results and technical limitations. Some controversy regarding some approaches also emerged [38, 39]. However, when these methods are properly assessed, it is possible to safely demonstrate the direct interactions between membrane receptors [40, 41]. Improvement of the previous methods and introduction of novel techniques have also been developed, such as real-time FRET experiments in living cells [42] and dual-color fluorescence cross-correlation spectroscopy [43]. However, despite the extensive experimental results obtained with these biophysical/biochemical techniques, supporting the formation of GPCR homo- and heteroreceptor complexes in overexpressing systems (cell lines), the existence of GPCR complexes at endogenous expression levels (their native environment) was not demonstrated until 2010–2013 [17, 21–23, 25, 31, 44–48].

In this chapter, we present an approach that combines the well-established immunohistochemical (IHC) technique for immunofluorescence labeling of cells and the recently introduced in situ proximity ligation assay (in situ PLA). Combining IHC with PLA analysis allows a simultaneous visualization of a homo- and heteroreceptor complexes of interest in specific neuronal and nonneuronal cell populations (cellular level) or cellular components like, for instance, the terminal, the soma or the dendritic spines (subcellular level). The introduction of a neuronal or glial cell marker during the in situ PLA technique simultaneously allows an additional readout of GPCR homo- and heteroreceptor complexes by combining the advantages of the two methods. At the end of the chapter, we will discuss the advantages and disadvantages of this method compared to other available techniques.

1.1 The Value of Proximity Ligation Assay in Understanding the Heteroreceptor Complexes and Their Allosteric Receptor–Receptor Interactions

In tissues, in situ PLA is of highest importance since it allows the detection and quantitation of GPCR homo-and heteroreceptor complexes in specific neuronal cell populations and in different types of glial cells like astroglia and microglia [11, 25, 46–51]. This became possible through the use of antibodies against neuronal and glial markers based on the immunofluorescence technique in situ PLA procedure. The in situ PLA has been fundamental in understanding how the homo- and heteroreceptor complexes change in discrete brain circuits in models of cocaine use disorder [18–20, 52, 53], Parkinson's disease, schizophrenia, and depression [11, 25, 47, 48, 50, 54, 55]. In adjacent sections, it is also possible to determine with PLA if the corresponding homoreceptor complexes to the heteroreceptor complex studied are altered and show a disbalance with the heteroreceptor complex [10, 51]. Such a comparison can also be made between different heteroreceptor complexes that share one or two receptor protomers

[18, 56]. These experiments will expand our understanding of brain integration and function in health and disease and if a disbalance of the receptor complexes in the same part of the neuronal network may contribute to disease development. In this integrative process, a set of proteins often called adapter proteins play a special role by inter alia markedly modulating the allosteric receptor–receptor interactions [57–61]. In tissues and also in cellular models, the correct name for the homo- and heteromers is therefore homo- and heteroreceptor complexes built up of two or more receptor protomers and different types of adaptor proteins and GPCR kinases.

Each heteroreceptor complex has its own unique and dynamic allosteric receptor–receptor interactions that modulate the recognition, pharmacology, signaling, and/or trafficking of the receptor protomers. The allosteric receptor–receptor interactions can be influenced by multiple signals like orthosteric agonists/antagonists and positive and negative allosteric modulators acting at allosteric binding sites and by participating adaptor proteins and GRKs that can enhance the recruitment of beta-arrestin to the phosphorylated receptor protomer [30, 62, 63].

1.2 The In Situ PLA: Principle of the Assay

In situ proximity ligation assay (in situ PLA) was first described by Fredriksson and colleagues in 2002 [64] and further developed for protein–protein interactions by Gullberg et al. and Soderberg et al. [65–70]. It was optimized for GPCR receptor–receptor interaction detection by the Fuxe laboratory [31, 44, 47, 48, 51] and the Javitch group [45]. It became commercialized by Olink Bioscience (http://www.olink.com/) and nowadays its reagents are sold by Sigma-Aldrich, Biomol, among other companies becoming an established and generally applicable immunohistochemical tool for advanced and precise protein–protein interactions and protein modification analysis.

The in situ PLA technique combines the dual recognition of a probe-targeted assay with a split-reporter approach, creating a selective and sensitive method for specific detection of two receptors in proximity forming either a homo- or a heteroreceptor complex [25, 31, 47, 48]. This innovative method utilizes one pair of oligonucleotide-labeled antibodies binding in proximity (20–30 nm apart) to different epitopes of the same receptor or two receptors in a homo- or heteroreceptor complex, respectively. The assay is used for localization detection, visualization, and quantification of a single receptor (useful to detect receptor post-translational modification, e.g., phosphorylation, palmitoylation, etc.), receptor–scaffold or chaperon interactions or receptor–receptor interactions in adherent cell lines, cytospin preparations or tissues, including frozen or paraffin-embedded patient samples. The cells or tissue need to be fixed with a fixative appropriate for antibodies used in the in situ PLA protocol and if necessary antigen retrieval and antibody-specific blocking must be performed.

There are two different approaches [67, 69, 70]: one method uses direct primary antibody conjugation and the other uses detection with secondary conjugates. In the detection of PLA with secondary conjugates, the unmodified primary antibodies are detected with two secondary antibodies that are equipped or conjugated to short DNA oligonucleotides. Two additional DNA strands, called connector oligonucleotides, are then introduced. The two DNA strands on the secondary antibodies are ligated to the two additionally introduced connector oligonucleotides, leading to the formation of a circular, single-stranded DNA molecule. In the created DNA circle, one of the secondary antibody conjugated DNA probes serves as a primer for the rolling circle amplification, the so-called positive probe (+). As a result, when adding a DNA polymerase, a long DNA product is formed and it remains covalently attached to the PLA probes (+). After finalizing the rolling circle amplification, the concatameric repetitions of the same sequence enable hybridization of multiple detection fluorescent-labeled oligonucleotides that can be visualized under a fluorescent microscope and quantified. The principle is the same for the detection of PLA with direct primary antibody conjugation; however, in this method, the secondary antibodies that are equipped with DNA strands are not required.

2 Materials and Buffers

1. *Hoffman solution* (cryoprotection for free-floating brain sections): 250 ml 0.4 M PBS, ethylene glycol 300 ml, 300 g sucrose, 10 g polyvinylpyrrolidone, 9 g NaCl. Add high-purity water to 1000 ml. Keep the resulting solution in a freezer (cold storage: −20 °C.).
2. *Brain tissue samples and their preparation*. For the study of GPCR homo- and heteroreceptor complexes in the rat brain, we highly recommend the use of formaldehyde-fixed frozen free-floating sections. First, animals should be deeply anesthetized by an intraperitoneal (i.p.) injection of a high dose of pentobarbital (60 mg/ml, [0.1 ml/100 g]) and then perfused intracardially with 30–50 ml ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS, pH 7.4) solution. After perfusion, brains are collected and transferred into well-labeled glass vials filled with 4% PFA fixative solution for 6–12 h. Then, the brains are successively placed in 10% and 30% sucrose in 0.1 M PBS, pH 7.4 in such a way that they are incubated for 1 day in 10% sucrose and for a number of days in 30% sucrose both at 4 °C with several sucrose buffer changes until freezing. Brains are frozen (in a bowl containing a beaker with isopentane surrounded by dry ice. Molds with tissue are

introduced into the beaker, when the isopentane reaches −45 °C. Once frozen store the brains at −80 °C. Proceed to generate the tissue sections (10–30 μm thick) using a cryostat (stored tissue at −20 °C on the day before cutting). After cutting, store slices in Hoffman solution (e.g., in a 24-well plate). Alternatively, to the use of fixed free-floating sections, you can use fixed frozen sections attached to microscopy slides. The mounted sections on slides must be kept at −20 °C until use.

3. *Phosphate-buffered saline* (PBS). PBS is prepared by mixing 0.23 g NaH_2PO_4 (anhydrous; 1.90 mM), 1.15 g Na_2HPO_4 (anhydrous; 8.10 mM), 9.00 g NaCl (154 mM). Then, add H_2O to 900 ml and if needed, adjust to desired pH (7.2–7.4) with 1 M NaOH or 1 M HCl. Finally, add H_2O to 1 L, filter, sterilize and store indefinitely at 4 °C. PBS could also be prepared at a 10× concentration (commercially available at Sigma-Aldrich (Cat. No.: P5493-1L)) and stored until dilution into the working solution.
4. *Glycine buffer* (10 mM). Dissolve 0.75 g glycine in 100 ml PBS. Store at 4 °C.
5. *Citric acid buffer*. 1 mM Citric acid buffer in water, pH 7.4. Store at 4 °C.
6. *Permeabilization buffer*. 0.1% Triton X-100 in PBS (e.g., 0.1 ml Triton X-100 in 100 ml PBS). Store at 4 °C.
7. *Blocking solution*. Prepare the blocking solution by preparing 0.2% BSA in PBS (e.g., 0.2 g in 100 ml PBS, stored at 4 °C). Adjust the amount of reagents accordingly so that the total volume is kept at 400 μl × well (12-well plate). Prepare this solution fresh. This blocking solution can be replaced by, for example, the Duolink Blocking solution (Sigma-Aldrich) or the Odyssey Blocking buffer (Licor Biosciences). However, choose the blocking agent best suited for the antibodies used. If animal serum is used to replace the BSA, like, for example, 5% sterile-filtered goat serum, make sure that it is sterile filtered, as unfiltered serum may increase the amount of background signals.
8. *Primary antibodies and proximity probes (oligonucleotide-labeled secondary antibodies) incubation buffer*. We strongly recommend to dilute the antibodies in the blocking solution to be used (*see* above 7).
   - Proximity probes are created through the attachment of oligonucleotides to antibodies. The antibodies are functionalized by either direct covalent coupling of an oligonucleotide [70] or noncovalent coupling by incubating biotinylated antibodies with a streptavidin-modified oligonucleotide [66]. The oligonucleotide component of the

proximity probes can be functionalized to either primary antibodies or secondary antibodies. The latter approach avoids the need to conjugate the oligonucleotide components to each primary antibody protomer pair, saving time and costs. Conjugation reactions can severely affect the antibodies specificities and functionality. For this reason, we strongly recommend to use validated proximity probes from specialized companies like Sigma-Aldrich, which can be bought directly.

- *Primary antibodies validation* [71]**.** GPCR antibodies are among the most frequently used tools in basic neurochemistry research and in clinical assays. However, the quality and consistency of data generated through the use of GPCR antibodies may widely vary. This poses a serious drawback to the rigor and reproducibility that are the cornerstones of GPCR research. Therefore, a validation of each antibody used in our in situ PLA experiments for its specificity and reproducibility is strongly recommended. How did we determine the primary antibody specificity? As a general principle, a highly specific primary antibody recognizes its target with minimal cross-reactivity (off-target binding) within a given application and experimental context. Therefore, the evaluation of potential cross-reactivity under the conditions tested is needed. (1) Cross reactivity can be assessed by measuring the relevant signal in control cells or tissues in which the target receptor has been either knocked out or knocked down using techniques such as CRISPR–Cas9 or RNA interference (RNAi). In this way, the expression of the target receptor is either eliminated or reduced; any signal observed after substantial reduction of receptor levels indicates cross-reactivity. This approach is powerful and particularly useful for examining antibody specificity for GPCR receptors, which originate from a related multigene family. However, this strategy cannot be used for some applications and certain types of samples like the human tissue samples and body fluids. (2) Also two (or more) independent antibodies (they must be able to bind to different epitopes/regions of the receptor) that recognize the same receptor target can be used to assess antibody specificity in a range of assays. This approach requires that the expression pattern generated by the two antibodies correlate within a given application environment. (3) Primary antibodies may be validated by expressing a receptor containing an affinity tag (such as 3XHA, myc, flag, etc.) or a fluorescent protein (such as GFP2, YFP, sRed). This will allow for parallel detection via additional well-validated immunoreagents or direct observation through the fluorescent protein.

The detection pattern of the antibody being validated must correlate with the pattern demonstrated by the anti-affinity tag antibody or the fluorescent signal. If substantial discord between these patterns comes out, it will suggest cross-reactivity.

9. *Ligation buffer*: 10 mM Tris–acetate, 10 mM magnesium acetate, 50 mM potassium acetate, pH 7.5 adjusted with HCl. Stored at −20 °C [70].
10. *Hybridization-ligation solution*: BSA (250 g/ml), T4 DNA ligase (final concentration of 0.05 U/μl), Tween-20 (0.05%), NaCl 250 mM, ATP 1 mM and the circularization or connector oligonucleotides (125–250 nM). Circularization or connector oligonucleotides can be designed and synthesized as described previously [70]. Before usage, vortex briefly to mix the ligase with the solution. Alternatively, the ligation buffer and the hybridization-ligation solution can be ordered from Olink Bioscience or Sigma-Aldrich (Cat No. DUO92008).
11. *Washing buffer A*: 8.8 g NaCl, 1.2 g Tris Base, 0.5 ml Tween 20. Dissolve in 800 ml high-purity water and adjust pH to 7.4 using HCl. Adjust with high-purity water to 1000 ml and filter through a 0.22 μm filter. Store at 4 °C.
12. *Amplification solution*: Instead of preparing the solutions described below (11–13) one amplification solution can be purchased (Olink Bioscience or Sigma-Aldrich (Cat. No. DUO92008)) and used.
13. *Rolling circle amplification (RCA) buffer*: 50 mM Tris–HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, pH 7.5 adjusted with HCl. Stored at −20 °C.
14. *RCA solution* (final concentration: phi-29 polymerase 0.125–0.200 U/μl, BSA (250 g/ml), 1× RCA buffer, Tween-20 (0.05%), and dNTP (250 mM for each)).
15. *Detection solution* (final concentration: BSA (250 g/ml), 2 μl Sodium citrate 20× (A 20× stock solution consists of 3 M sodium chloride and 300 mM trisodium citrate (adjusted to pH 7.0 with HCl)), 4 μl of Tween-20 (0.05%), and the fluorescence detection by fluorophors (e.g. Texas Red or Alexa 555)-oligonucleotide strand (5 μM) *see* [70].
16. *Washing buffer B*: 5.84 g NaCl, 4.24 g Tris Base, 26.0 g Tris–HCl. Dissolve in 500 ml high-purity water and adjust pH to 7.5 using HCl. Then add again high-purity water to 1000 ml. Filter through a 0.22 μm filter. Store at 4 °C.
17. *Mounting medium* (e.g., VectaShield, Vector Labs).

3 Assay Protocol

1. Wash the fixed free-floating sections (storage at −20 °C in Hoffman solution) four times with PBS.
2. Enhance the exposition of the receptor epitope by keeping your brain slices in citric acid buffer, for 45–60 min at 65 °C.
3. Wash twice, for 5 min each, with PBS at room temperature.
4. Quench your brain slices with 10 mM glycine buffer for 20 min at room temperature.
5. Wash twice, for 5 min each, with PBS at room temperature.
6. Incubate with the permeabilization buffer (10% fetal bovine serum (FBS) and 0.5% Triton X-100 or Tween 20 in Tris buffer saline (TBS), pH 7.4) for 30 min at room temperature.
7. Wash twice, for 5 min each, with PBS at room temperature.
8. Incubate with the blocking buffer (0.2% BSA in PBS) for 30 min at room temperature.
9. Turn on the incubator and preheat the humidity chamber until usage.
10. Incubate the brain sections with a mixture of the primary antibodies diluted in a suitable concentration in the blocking solution (or at 1:100 in the Duolink II Antibody Diluent (1×)) for 1–2 h at 37 °C or at 4 °C overnight. Prepare primary antibodies alone at the same concentration to be used for the control groups. In one well, add only antibody diluent as an additional negative control.
11. Tap off the primary antibody solution from the slides. Wash twice, for 5 min each, with the blocking solution at room temperature under gentle agitation to remove the excess of primary antibodies.
12. In the meantime, dilute the secondary antibodies proximity probes to a suitable concentration in the blocking solution. Alternatively, dilute the two commercial available PLA probes (e.g., Duolink II anti-Mouse MINUS and Duolink II anti-Rabbit PLUS) 1:5 in antibody diluent. It is important to use the same buffer as those for the primary antibody to avoid background staining. Apply the proximity probe mixture to the sample and incubate for 1 h at 37 °C in a humidity chamber. It should be checked regularly that the reaction, in each plate well, does not dry out since this will cause a high background signal.
13. To remove the unbound proximity probes, wash the slides twice, 5 min each time, with blocking solution at room temperature under gentle agitation.

14. Prepare the hybridization-ligation solution. To ensure optimal conditions for the enzymatic reactions, the sections should be soaked for 1 min in ligation buffer (10 mM Tris–acetate, 10 mM magnesium acetate, 50 mM potassium acetate, pH 7.5 [70]), prior to addition of the hybridization-ligation solution (final concentration: BSA (250 g/ml), T4 DNA ligase buffer, Tween-20 (0.05%), NaCl 250 mM, ATP 1 mM, and the circularization or connector oligonucleotides 125–250 nM). Remove the soaking solution (ligation buffer), and add T4 DNA ligase at a final concentration of 0.05 U/μl to the hybridization-ligation solution. Vortex briefly to mix the ligase with the solution. Apply the mixture immediately to the sections and incubate slides in a humidity chamber at 37 °C for 30 min. Alternatively, the ligation buffer and the hybridization-ligation solution can be ordered from Olink Bioscience or Sigma-Aldrich (Cat No. DUO92008). Vortex the Duolink II Ligation stock (5×) and dilute 1:5 in high-purity water and mix. Take out the Ligase from the freezer using a freezing block (−20 °C). Add the ligase to the ligation solution at a 1:40 dilution immediately before addition to brain samples, vortex and incubate the brain slides with this solution in a preheated humidity chamber for 30 min at 37 °C.
15. To remove the excess of connector oligonucleotides, wash twice, for 5 min each, with the washing buffer A at room temperature under gentle agitation. Tap off all excess washing solution.
16. Add to the brain slices the neuronal and/or cell process marker (e.g., astrocyte marker) antibody conjugated with AlexaFluor (e.g., Neuro-Chrom™ Pan neuronal marker antibody-Alexa488 conjugate or the anti-GFAP Alexa Fluor 488 monoclonal antibody, Merck Millipore, MAB 3402X (*see* Fig. 1)) and diluted in the rolling circle amplification mixture (*see* below). Directly conjugated antibodies already have the Alexa Fluor® dye, so you can skip that secondary antibody staining step. *A selection of direct conjugates against neural markers can be found in* http://www.abcam.com/neuroscience/directly-conjugated-neural-markers *or buy it from Millipore*. The use of antibodies to neuronal proteins have become critical tools for identifying neurons and discerning morphological characteristics in culture and complex tissue. While the labeling from GFP constructs yield excellent cytoarchitectural detail, this approach is technically challenging and impractical for many neuroscience research needs. Neuron-specific antibodies are convenient precision tools useful in revealing cytoarchitecture. They can be used for labeling a single protein target within the neuron or to achieve a morphological staining across all parts of neurons (e.g., the Millipore Neuro-Chrom™ Pan neuronal marker

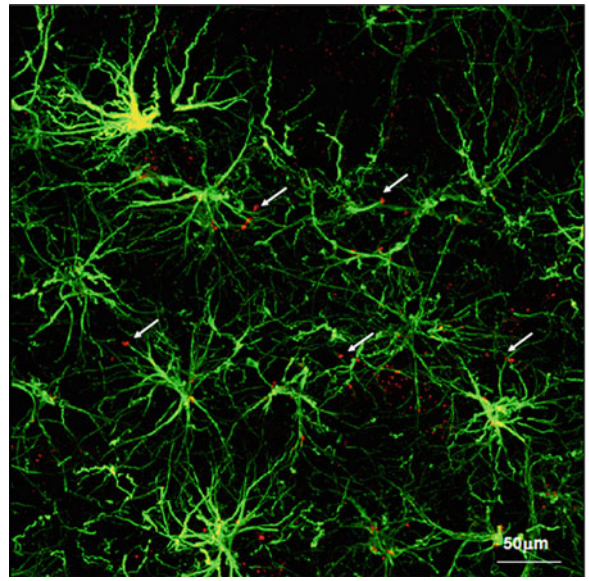

Fig. 1 Demonstration of the expression of the FGFR1-5-HT1AR heteroreceptor complexes in astrocytes of the PoDG region in the dorsal hippocampus of rat brain. Microphotographs from transverse sections of the rat dorsal hippocampus (Bregma level: −3.6 mm) show the distribution of the FGFR1-5-HT1AR heteroreceptor complexes expressed in astrocytes in the PoDG region using the combined IHC with in situ PLA techniques. Scale bar = 50 μm. Receptor complexes are shown as red PLA blobs (clusters, indicated by arrows) found in high densities per astrocyte using confocal laser microscopy. The astrocyte GFAP marker are shown in green (anti-GFAP Alexa Fluor 488 monoclonal antibody, Merck Millipore, MAB 3402X). The pictures are taken and visualized using multiple *z*-scan (20 *z*-scan)

antibody, a polyclonal antibody that reacts against key somatic, nuclear, dendritic, and axonal proteins distributed across the pan-neuronal architecture that can then be detected by a single secondary antibody or used directly conjugated with AlexaFluor488).

17. Prepare the rolling circle amplification mixture. Soak the sections in RCA buffer for 1 min. Remove the soaking solution, add the RCA solution (final concentration: phi-29 polymerase 0.125–0.200 U/μl, BSA (250 g/ml), RCA buffer, Tween-20 (0.05%), and dNTP (250 M for each)). Vortex briefly the RCA solution and incubate in a humidity chamber for 100 min at 37 °C. Prepare the detection solution and incubate the sections in a humidity chamber for at 37 °C for 30 min. Keep the detection solution in the dark to prevent fluorophore bleaching. From now on, all reactions and washing steps should be performed in the dark. Alternatively, to prepare these buffers and solutions by yourself, the amplification mixture and the detection solution can be ordered from Olink Bioscience or

Sigma-Aldrich (Cat. No. DUO92008). Dilute the Duolink II Amplification stock (5×) 1:5 in high purity water and mix. Take out the Polymerase from the freezer using a freezing block (−20 °C). Add the Polymerase to the amplification solution at a 1:80 dilution and vortex. Add the amplification-polymerase solution to each well. Incubate the brain slides in a preheated humidity chamber for 100 min at 37 °C.

18. Wash the sections twice in the dark, for 10 min each, with the washing buffer B at room temperature under gentle agitation. Light-sensitive reagents. Keep the slides protected from light.
19. Dip the sections in a washing buffer B dilution of 1:10 and let sections dry at room temperature in the dark. Light-sensitive reagents. Keep the slides protected from light.
20. The free-floating sections are put on a microscope slide and a drop of appropriate mounting medium (e.g., VectaShield or Dako) is applied. The cover slip is placed on the section and sealed with nail polish. The sections should be protected against light and can be stored for several days at 4 °C or for several months/years at −20 °C. Light-sensitive reagents. Keep the slides protected from light.
21. Visualize the samples with a confocal or fluorescent microscope equipped with excitation/emission filters compatible with the fluorophores used. The in situ PLA signals have a very characteristic bright sub-micrometer-sized punctate appearance that is easily recognized and distinguishable from potential background fluorescence. While moving the focus through the sample tissue, appearance and disappearance of PLA signals should be noticeable (Fig. 1). Up to a certain density of PLA signals, they appear as discrete dots (puncta, blobs) that can be easily counted/quantified using image analysis software. Obtain digital images.
22. Analyze the captured images using image techniques to quantify the number of dots (Fig. 1). Many commercial image analysis software packages can be used (e.g., Duolink ImageTool (Sigma-Aldrich)) but also free software packages are available (e.g., Blob Finder, Cell profiles). Usually four important parameters should be kept in mind for a proper analysis and result interpretation: (1) the number of DAPI nuclei in the sample field, (2) the number of positive PLA/dots per sample field, (3) the total number of positive PLA cells/nuclei per sample field, and (4) the diameter sizes of the individual PLA blobs (the diameter may indicate if aggregates (higher order) of receptor complexes exist). Within these four values, it will be possible to get an overall view of the expression/enrichment of GPCR heteroreceptor complexes in the different brain areas analyzed and extract relevant conclusions from the comparisons between brain areas.

23. Compare the measurements and make a graph. We have proposed the molecular phenomenon of receptor–receptor interactions as a fruitful way to understand how brain function can increase through molecular integration of signals. An alteration in specific receptor–receptor interactions or their balance/equilibrium (with the corresponding monomers–homomers) are indeed considered to have a role in the pathogenic mechanisms that lead to various brain diseases. Therefore, targeting protomer–protomer interactions in heteroreceptor complexes or changing the balance with their corresponding homoreceptor complexes in discrete brain regions may become an important field for developing novel drugs, including hetero-bivalent drugs and optimal types of combined treatments. The analysis of animal or human brain material with in situ PLA can reveal if the relative abundance of specific homo- and heteroreceptor complexes in discrete brain regions is altered in brain diseases or under certain drug treatments, for instance, chronic L-dopa treatment in Parkinson's disease [72]. In this analysis, it is important also to determine the ratio between heteroreceptor complex populations versus total number of the two participating protomer populations, using in addition to Western blots, receptor autoradiography, and biochemical binding methods. The two latter methods show the densities and affinities of the two functional receptor populations. The relationship between these parameters will help to normalize the heteroreceptor complexes values for comparison between groups in addition to evaluating the potential changes in the total number of the two protomer populations [44]. Certainly, we cannot compare or determine directly a balance between the homo- and heteroreceptor complexes populations in the same tissue using the in situ PLA approach, because of a technical limitation of the procedure itself. But the method could help us determine each population independently and compare their relative expression levels after an appropriate numerical analysis. Furthermore, of increasing importance will be to determine the agonist/antagonist regulation of these homo-/heteroreceptor complexes in order to understand their potential roles as drug targets or as markers of brain disease progression.

4 Advantages and Disadvantages of the PLA Method

In situ PLA can offer advantages by:

1. Giving the opportunity to study the existence of any potential homo–heteroreceptor complexes, for which a pair of suitable antibodies are available without the need of employing a genetic constructs.

2. The method could be performed in both cells and tissue samples, including human specimens collected from biobanks.
3. The in situ PLA could be useful to monitor the effects of different compounds like agonists and antagonists or their combined treatment on the balance of homo- and heteroreceptor complexes in cells and tissue.
4. The information obtained by the in situ PLA is at the resolution of individual cells or even of subcellular compartments providing profound insights into cellular heterogeneity in tissues.
5. The method also provides an enhanced sensitivity and selectivity compared to many other methods since powerful rolling circle amplification and dual target recognition are used.

As with any method, there are limitations, for instance:

1. The in situ PLA cannot be used in live cells, as fixation it is a prerequisite for the cell/tissue material employed.
2. When studying receptor–receptor interactions, it is important to remember that the method, like many other methods for studying protein–protein interactions, can show that two proteins are in proximity and therefore, likely directly interact. Proteins can also interact indirectly through an adapter protein. The maximal distance between two epitopes to give rise to a signal with in situ PLA is 10–30 nm with direct-conjugated proximity probes, and slightly longer when secondary proximity probes are used. This distance will be dependent on the size of the receptors/antibodies and the respective length of the attached oligonucleotides. By changing the length of the oligonucleotides, the maximal distance limits can be reduced or increased. However, in general, the functional distance is usually close to the one detected in a FRET-assay [73].
3. Another critical parameters for achieving good results is the use of excellent antibodies. Importantly, the antibodies have to be used under optimal conditions taking into consideration parameters such as antibody concentration, epitopes targeted by the antibodies, fixation, antigen-retrieval, blocking conditions, and so on.
4. A range of controls, both positive and negative ones, should be used to guarantee the specificity of the PLA signal. Positive controls can include cells where the protein is known to be expressed, such as in certain cells or tissues or in cells transfected to express the protein. Negative controls include cells or tissues that do not express the protein or where the protein has been knocked out or downregulated by, for example, siRNA.

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Annex 5

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ORIGINAL RESEARCH ARTICLE

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Galanin and neuropeptide Y interactions elicit antidepressant activity linked to neuronal precursor cells of the dentate gyrus in the ventral hippocampus

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Abstract

A need for new antidepressants is necessary since traditional antidepressants have several flaws. Neuropeptide Y(NPY) Y1 receptor (NPYY1R) and galanin (GAL) receptor 2 (GALR2) interact in several regions of the limbic system, including the hippocampus. The current study assesses the antidepressant effects induced by GALR2 and NPYY1R coactivation, together with the evaluation of cell proliferation through 5-Bromo-2'-deoxyuridine expression within the dentate gyrus of the ventral hippocampus (vDG). We employed in situ proximity ligation assay to manifest GALR2/NPYY1R heteroreceptor complexes. Additionally, the expression pattern of GALR2 and the activation of the extracellular-regulated kinases (ERK) pathway after GALR2 and NPYY1R costimulation in cell cultures were examined. GALR2 and NPYY1R coactivation resulted in sustained antidepressant behaviors in the FST after 24 h, linked to increased cell proliferation in the vDG. Moreover, an increased density of GALR2/NPYY1R heteroreceptor complexes was observed in vDG, on doublecortin-expressing neuroblasts. Recruitment of the GALR2 expression to the plasma membrane was observed upon the coactivation of GALR2 and NPYY1R in cell cultures, presumably associated to the enhanced effects on the activation of ERK pathway. GALR2 may promote the GALR2/NPYY1R heteroreceptor complexes formation in the ventral hippocampus. It may induce a transformation of cell proliferation toward a neuronal lineage by enhancement of ERK pathway. Thus, it may give the mechanism for the antidepressant behavior observed. These results may provide the basis for the development of heterobivalent agonist pharmacophores, targeting GALR2/NPYY1R heteromers, especially in the neuronal precursor cells of the dentate gyrus in the ventral hippocampus for the novel treatment of depression.

KEYWORDS

Neuropeptides, Galanin, Neuropeptide Y, Receptor interaction, Depression

1 | INTRODUCTION

According to the World Health Organization (WHO), major depressive disorder (MDD) is the most prevalent mood disorder and the leading cause of disability worldwide, with more than 300 million patients affected (WHO, 2017). MDD is defined by a constellation of behavioral, emotional, and cognitive symptoms, and confer a challenge for the medical community. Traditional antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) or tricyclic antidepressants, have several flaws, as their therapeutic efficacy is often limited due to side effects and delayed onset of effect, from weeks to months (Harmer et al., 2017). Furthermore, those who failed standard antidepressant treatment are regarded as having treatment-resistant depression (TRD), as studies determine that ~50% of such patients fail to respond to such therapy, and about 65% of them fail to achieve remission (Chen, 2019). Presently, only electroconvulsive treatment and infusion of the N-methyl-D-aspartate receptor antagonist ketamine (DiazGranados et al., 2010) are available for TRD treatment.

MDD, as a heterogeneous disease, may have a contribution to its etiology from multiple neural regions, and the hippocampus has been one of the most broadly considered (Miller & Hen, 2015). The ventral hippocampus in rodents plays a significant role in stress and modulation of emotional behavior, where immature adult-born neurons might actively participate in limbic functions and influence MDD pathophysiology (Baptista & Andrade, 2018; Fanselow & Dong, 2010; Tanti & Belzung, 2013). The precursor cells reside in a narrow band of tissue between the granule cell and the polymorphic layers, called the subgranular zone (SGZ). Critical precursor cell proliferation is modulated and being subject to "control" and "regulation" by multiple factors (Kempermann et al., 2015). The existence of neurogenesis in humans was first demonstrated by Eriksson et al. (1998). Their findings have been supported (Boldrini et al., 2018; Moreno-Jimenez et al., 2019; Spalding et al., 2013) but also questioned (Cipriani et al., 2018; Sorrells et al., 2018) by other studies. However, the limited availability of accurately preserved human brain tissue samples, together with the heterogeneity of tissue processing methodologies, is considered to have contributed to this disagreement (Kempermann et al., 2018).

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

The NPY is a 36 amino acid polypeptide neurotransmitter broadly distributed in the mammalian central nervous system, with high expression in several limbic regions. In the hippocampus, a subset of GABAergic interneurons in the polymorphic layer of the DG coreleases NPY, which innervates the granule cell layer mainly via volume transmission (Fuxe et al., 1991), close to the SGZ (Sperk et al., 2007). NPY has a physiological role in modulating the granule cell excitability and enhances the proliferation of neuronal precursor cells both in vitro (Howell et al., 2003, 2007) and in vivo (Decressac et al., 2011). In vitro evidence suggests a proliferative effect, explicitly involving the NPY Y1 receptor (NPYY1R), which is mediated by the extracellular-regulated kinases (ERK) 1/2 signaling (Cheung et al., 2012). In vivo administration of NPY validated its proliferative role and a preferential differentiation of newly generated cells toward a neuronal lineage trough NPYY1R (Decressac et al., 2011; Geloso et al., 2015). Furthermore, increased NPY and NPYY1R expression in DG is related to decreased immobility in the forced swimming test (FST) in rats, implying the participation of the NPY-NPYY1R system in the pathophysiology of MDD (Catena-Dell'Osso et al., 2013). Conversely, genetic, and experimental rat models of depression show decreased levels of NPY and NPYY1R in the DG (Jimenez-Vasquez et al., 2007; Mathe et al., 1998).

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

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

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

2 | MATERIALS AND METHODS

2.1 | Animals

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

2.2 | Drugs used

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

2.3 | Behavioral analysis

2.3.1 | Forced swimming test

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

Behavioral experiments were performed between 09:00 and 14:00 h. Animals were adapted to handling and were taken into the experimental room (80–90 lux) for at least 1 h to reach habituation and assigned randomly to the experimental groups. Peptides were freshly prepared and injected icv into the right lateral ventricle (5 μl total volume). Doses for GAL (3 nmol), the NPYY1R agonist [Leu^{31},-Pro^{34}]NPY (3 nmol), and for GALR2 antagonist M871 (3 nmol) were chosen based on previous dose–response curves (Narvaez et al., 2016, 2018, 2015).

Swimming sessions were conducted by placing individually the rats in cylinders containing water (25 ± 0.5°C), 30-cm deep. Two sessions were conducted: an initial 15-min pretest followed 48 h later by a 5-min test. Drugs were administered 24 h before the test. The water in the cylinders was changed after every trial. The FST was performed according to a previously reported method (Koike & Chaki, 2014). The total duration of floating (immobility), swimming, and climbing periods were scored during the 5-min test and analyzed using the Raton Time 1.0 software (Fixma S.L.). Floating in the water without struggling and only making movements necessary to keep its head above the water was regarded as immobility. Swimming was scored when they actively swam around the cylinder, while climbing was defined as vigorous forepaw movements directed toward the walls of the cylinder. Following swimming sessions, the rats were removed from the tank, carefully dried in heated cages, and then returned to their home cages. Behavioral experiments were carried out by observers blinded to all experimental conditions.

2.3.2 | Evaluation of hippocampal cell proliferation

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

Animals were distributed randomly into five experimental groups: (1) aCSF: control group; (2) GAL-treated group (3 nmol); (3) Y1-treated group receiving an NPYY1R agonist [Leu^{31},Pro^{34}]NPY (3 nmol); (4) GAL+Y1: group administered with both substances; (5) GAL + Y1 + M871: group injected with GAL, [Leu^{31},Pro^{34}]NPY and the GALR2 antagonist (M871; 3 nmol; $N = 4$ in each group). Doses indicated above (Narvaez et al., 2016, 2018, 2015) and the BrdU procedure (Abrial et al., 2014; Pilar-Cuellar et al., 2012) are based on previously published protocols.

2.3.3 | Immunohistochemistry

Free-floating sections were incubated in saline sodium citrate buffer (pH 6; 10 nM sodium citrate) for 90 min at 65°C, followed by 30 min with 0,6% H_2O_2 to eliminate endogenous peroxidases. After 30 min in 2M hydrochloric acid (HCl) to denature DNA, sections were incubated for neutralization with 0.1M sodium borate (pH 8). Then, sections were incubated at 4°C overnight with a primary antibody against BrdU (1:1,000; Abcam) in 2.5% donkey serum. Sections were then washed with phosphate-buffered saline (PBS) and incubated with a secondary antibody for 90 min (biotinylated anti-rabbit Immunoglobulin G, 1/200; Vector Laboratories), followed by amplification with ExtrAvidin peroxidase (Sigma-Aldrich) diluted 1:1,000 in darkness at room temperature for 1 h. Immunolabeling was revealed

with 0.05% diaminobenzidine (Sigma-Aldrich) and 0.03% H_2O_2 in PBS. After several rinses, sections were mounted on gelatin-coated slides, dehydrated in graded alcohols, and cover-slipped in DePeX mounting medium (VWR).

2.3.4 | Counting procedure

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

2.3.5 | In situ proximity ligation assay

To study the GALR2-NPYY1R heteroreceptor complexes, the in situ proximity ligation assay (in situ PLA) was performed as described previously (Borroto-Escuela et al., 2016; Fuxe & Borroto-Escuela, 2018). Treated rats were divided into experimental groups: (1) aCSF: control group; (2) GAL-treated group (3 nmol); (3) Y1- treated group receiving an NPYY1R agonist [Leu^{31},Pro^{34}]NPY (3 nmol); (4) GAL + Y1: group administered with both substances; (5) GAL + Y1 + M871: group injected with GAL, [Leu^{31},Pro^{34}]NPY and the GALR2 antagonist (M871; 3 nmol; $n = 4$ in each group). Animals were perfused with 4% paraformaldehyde 24 h after icv injections, brains were removed, and sections were obtained.

Free-floating sections were washed four times with PBS and quenched with 10 mM Glycine buffer for 20 min at room temperature. Then, after three PBS washes, slices were permeabilized with a permeabilization buffer (10% fetal bovine serum [FBS] and 0.5% Triton X-100 in Tris buffer saline), pH 7.4) for 30 min at room temperature. Again, the sections were washed twice, 5 min each, with PBS at room temperature and incubated with the blocking buffer (0.2% bovine serum albumin [BSA] in PBS) for 30 min at room temperature. The brain sections were then incubated with the primary antibodies (GALR2 rabbit, Alomone Lab, 1:100; NPYY1R goat, sc-21992 Santa Cruz Biotechnology Inc., 1:200) diluted in a suitable concentration in the blocking solution at 4°C overnight. Then, the sections were washed twice, and the proximity probe mixture (Duolink PLA probe anti-mouse MINUS and Duolink PLA probe anti-rabbit PLUS, Sigma-Aldrich) was applied to the sample and incubated for one hour at 37°C in a humidity chamber. The unbound proximity probes were removed by washing the slides twice, 5 min each time, with blocking solution at room temperature under gentle agitation, and the sections were incubated with the hybridization-ligation solution (BSA, 250 g/ml), T4 DNA ligase (final concentration of 0.05 U/μl), 0.05% Tween-20, 250 mM NaCl, 1 mM ATP, and the circularization or connector oligonucleotides [125–250 nM]) and incubated in a humidity chamber at 37°C for 30 min. The excess of connector oligonucleotides was removed by washing twice, for 5 min each, with the washing buffer (Sigma-Aldrich, Duolink Buffer A (8.8 g NaCl, 1.2 g Tris Base, 0.5 ml Tween 20 dissolved in 800 ml high purity water, pH 7.4) at room temperature under gentle agitation and the rolling circle amplification mixture (Duolink amplification red, DUO82011, Sigma-Aldrich) was added to the slices and incubated in a humidity chamber at 37°C for 100 min. Then, the sections were incubated with the detection solution in a humidity chamber at 37°C for 30 min. In the last step, the sections were washed twice in the dark, for 10 min each, with the washing buffer (Sigma-Aldrich, Duolink Buffer B (5.84 g NaCl, 4.24 g Tris Base, 26.0 g Tris-HCl.) dissolved in 500 ml high purity water, pH 7.5) at room temperature under gentle agitation. The free-floating sections were put on a microscope slide, and a drop of appropriate mounting medium (Duolink Mounting Medium) was applied. The coverslip was placed on the section and sealed with nail polish. The sections were protected against light and stored for several days at −20°C before confocal microscope analysis. The mounting medium contained 4′,6-diamidino-2-phenylindole (DAPI). DAPI is a blue-fluorescent DNA stain that exhibits ~20-fold enhancement of fluorescence upon binding to AT regions of double-stranded DNA. It is excited by the violet (405 nm) laser line and is commonly used as a nuclear counterstain in fluorescence microscopy.

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

2.3.6 | Cloning of GALR2-GFP^2, cell culture, and transfection

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

Human embryonic kidney 293T (HEK293T) cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium supplemented with L-glutamine 2 mM, penicillin/streptomycin 100 units/ml, and FBS 10% (vol/vol) at 37°C and 5% CO_2. For transfection, cells were plated in six-well dishes at a concentration of 1×10^6 cells/well and cultured overnight before transfection. Cells were transiently transfected (cDNA molar ratio 1:1) using Fugene HD Transfection Reagent (Promega) and empty pcDNA3.1 vector DNA to maintain a constant total amount of DNA per well. Cells were transfected with 1 μg of GALR2-GFP^2 and NPYY1R.

2.3.7 | GALR2-GFP^2 internalization using confocal microscopy

Twenty-four hours before imaging, the FBS growth media was removed and replaced with serum-free media (Cellgro-Free, Mediatech). GALR2-GFP^2/NPYY1R HEK293T coexpressing cells were incubated with GAL 1 μM and/or NPY 1 μM, at different times. Antagonist studies were performed 15 min before the addition of agonist with NPYY1R antagonist BIBP3226 10 μM. Cells were fixed in 4% paraformaldehyde for 10 min, washed with Glycine 10 mM in PBS, and mounted in a Vectashield immunofluorescence medium (Vector Laboratories) as previously described (Narvaez et al., 2015).

GALR2-GFP^2 endosomes, seen as green fluorescent molecules, were excited with a krypton/argon laser at 488 nm and are shown as a single z-scan image. Timed-interval images of different cell groups were acquired (×63, Leica TCS-SL confocal microscope) following agonist addition. The percentage of internalization was determined by Leica software analysis of total membrane fluorescence compared to total internal compartment fluorescence at the various time points (Gehlert et al., 2007; Narvaez et al., 2015).

2.3.8 | Luciferase reporter gene assay

Dual-luciferase gene reporter assay was used to indirectly detect activation of ERK pathway in transiently transfected HEK293T cells treated with different compounds in a range of concentrations (typically 25 nM–1 μM; Borroto-Escuela et al., 2010; Narvaez et al., 2015).

For luciferase assays, 24 h before transfection, cells were seeded at a density of 1×10^6 cells/well in six-well dishes and transfected with Fugene HD. Cells were cotransfected with plasmids corresponding to three constructs as follows (per six-well): 1 μg firefly luciferase-encoding experimental plasmid (pGL4-SRE-luc2p; Promega), 1 μg of NPYY1R and/or GALR2, and 50 ng Renilla luciferase-encoding internal control plasmid (phRG-B; Promega). Approximately 36 h post-transfection, the cells were treated for 4 h with appropriate ligands (GAL 50 nM; GAL 100 nM; NPY 50 nM; BIBP3226 1 μM; M871 1 μM) and harvested with passive lysis buffer (Promega). The luciferase activity of cell extracts was determined using a luciferase assay system according to the manufacturer's protocol in a POLARstar Optima plate reader (BMG Labtech) using a 30-nm bandwidth excitation filter at 535 nm. Luciferase was measured as firefly luciferase luminescence over a 15-s reaction period. The values were normalized against Renilla luciferase luminescence values. Transfection experiments were performed in quadruplicate and repeated at least three times.

2.4 | Statistical analysis

Data are presented as mean ± *SEM*, and sample number (*n*) is indicated in figure legends. All data were analyzed using GraphPad PRISM 8.0 (GraphPad Software).

For comparing two experimental conditions, Student's unpaired *t* test statistical analysis was performed. Otherwise, a one-way analysis of variance (ANOVA) followed by the Newman–Keuls comparison posttest was performed. Differences were considered significant at $p < .05$ ($^*p < .05$ $^{**}p < .01$ $^{***}p < .001$).

3 | RESULTS

3.1 | Antidepressant-like behavior profile induced in the FST by GALR2/NPYY1R interaction

In the FST, rats were pre-exposed to water for 15 min. Twenty-four hours after the icv administration, the immobility, swimming, and climbing parameters were measured during a second 5-min exposure to water to find signs of depression-like behavior.

The icv administration of the NPYY1R agonist at 3 nmol decreased the time of immobility (one-way ANOVA, $F4, 30 = 53.62$, $p < .001$, Newman–Keuls post hoc test: $p < .001$; Figure 1a) and the time of climbing behavior (one-way ANOVA, $F4, 30 = 13.97$, $p < .001$, Newman–Keuls post hoc test: $p < .01$; Figure 1c) compared with control animals. Increased time of swimming (one-way ANOVA, $F4, 30 = 16.89$, $p < .001$, Newman–Keuls post hoc test: $p < .05$; Figure 1b) was observed after NPYY1R agonist injections.

In contrast, a significant increase in the immobility time (Newman–Keuls post hoc test: $p < .05$; Figure 1a) was observed after the icv GAL administration at 3 nmol compared with control animals. A decreased time in climbing behavior (Newman–Keuls post hoc test: $p < .001$; Figure 1c) was observed after GAL injection alone.

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

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

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

To investigate the neurophysiological mechanisms associated with the behavioral effects, we evaluated the impact of GAL and NPYY1R agonist coinjection on adult ventral hippocampal cell proliferation by using the thymidine analog BrdU.

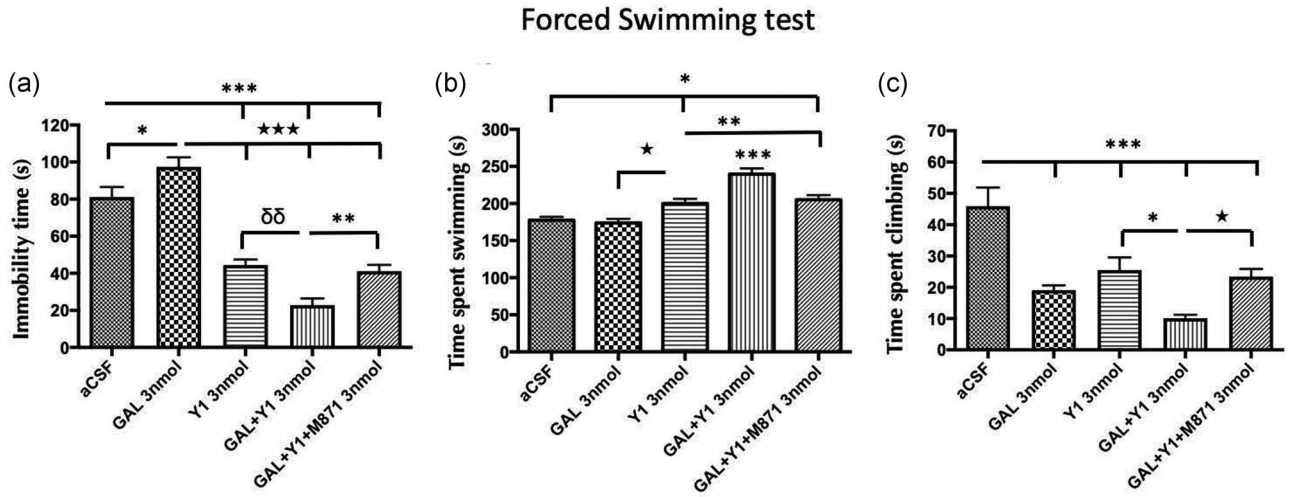

FIGURE 1 Behavioral effects produced by galanin (GAL) and the neuropeptide Y Y1 receptor (NPYY1R) agonist alone and in combination in the forced swimming test (FST). The response induced by GAL on the NPYY1-produced behaviors is blocked by the GAL 2 receptor (GALR2) antagonist M871. Cumulative behavioral duration of Immobility (a), swimming (b), and climbing (c) time in the FST. Data represent mean ± *SEM*. *N* = 6–8 animals in each group. For a: $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; ★★★$p < .001$; δδ$p < .01$. For b: $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; ★$p < .05$. For c: $^{*}p < .05$; $^{***}p < .001$; ★$p < .05$ according to one-way ANOVA (immobility: F4, 30 = 53.62, $p < .001$; swimming: F4, 30 = 16.89, $p < .001$; climbing: F4, 30 = 13.97, $p < .001$) followed by Newman–Keuls post hoc test. Intergroup comparisons are indicated by the vertical lines from the horizontal line above bars. aCSF, control (artificial cerebrospinal fluid); GAL 3 nmol, galanin 3 nmol; Y1 3 nmol, NPY Y1 receptor agonist [Leu31-Pro34]NPY 3 nmol; GAL + Y1 3 nmol, coadministration of GAL and Y1; GAL + Y1 + M871 3 nmol, coadministration of GAL, Y1, and GALR2 antagonist M871 3nmol

The icv injection of the NPYY1R agonist alone induced an increase in the number of BrdU-IR profiles in the SGZ of the ventral hippocampus (one-way ANOVA, F4, 15 = 75.37, $p < .001$, Newman–Keuls post hoc test: $p < .001$; Figure 2b) compared with the control group. The injection of GAL alone lacked effects on the BrdU-IR profiles (Figure 2b) compared with the aCSF control group (Figure 2b,c).

However, GAL and NPYY1R agonist coinjection significantly increased the number of BrdU-IR profiles, specifically in the SGZ of the ventral hippocampus compared with NPYY1R agonist alone (Newman–Keuls post hoc test: $p < .001$; Figures 2b and 2d). The cotreatment with the GALR2 antagonist M871 completely reversed the GAL contribution to the NPYY1R agonist response in the ventral hippocampus (Newman–Keuls post hoc test: $p < .001$; Figure 2b), demonstrating the involvement of GALR2 in the GAL/NPYY1R agonist interaction to promote cell proliferation.

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

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

PLA-positive red clusters were found specifically in the subgranular zone and the polymorphic layer of the ventral DG (Figure 3a). Quantification of PLA demonstrated an increase in the density of the PLA-positive red clusters after NPYY1R agonist injection compared to control (one-way ANOVA, F4, 15 = 11.76, $p < .001$, Newman–Keuls post hoc test: $p < .05$) or GAL group (Newman–Keuls post hoc test: $p < .05$; Figure 3b–d). Moreover, the coinjection of GAL and NPYYR1 agonist significantly increased (Newman–Keuls post hoc test: $p < .05$) the number of PLA-positive red clusters in the ventral hippocampal DG (Figures 3b and 3d,e) compared with NPYYR1 agonist alone. Similarly to the BrdU-IR profiles response described above, the presence of the GALR2 antagonist M871 completely blocked this increase (Newman–Keuls post hoc test: $p < .05$; Figure 3b), demonstrating the involvement of GALR2 in this interaction. The signal specificity was validated with no PLA clusters observed in the molecular layer of the DG, an area that seems to lack GALR2 (O'Donnell et al., 1999).

Furthermore, to study the specific cell population potentially involved in the results described above, we analyzed the neuroblast-expressing marker Doublecortin (Figure S1). We found a particular colocalization of GALR2 (Figure S1a) and NPYY1R (Figure S1b) with doublecortin-expressing cells. Unexpectedly, GALR1 did not colocalize with doublecortin-expressing cells, suggesting a specific role for GALR2/NPYY1R interaction on the neuroblasts located in the DG of the ventral hippocampus.

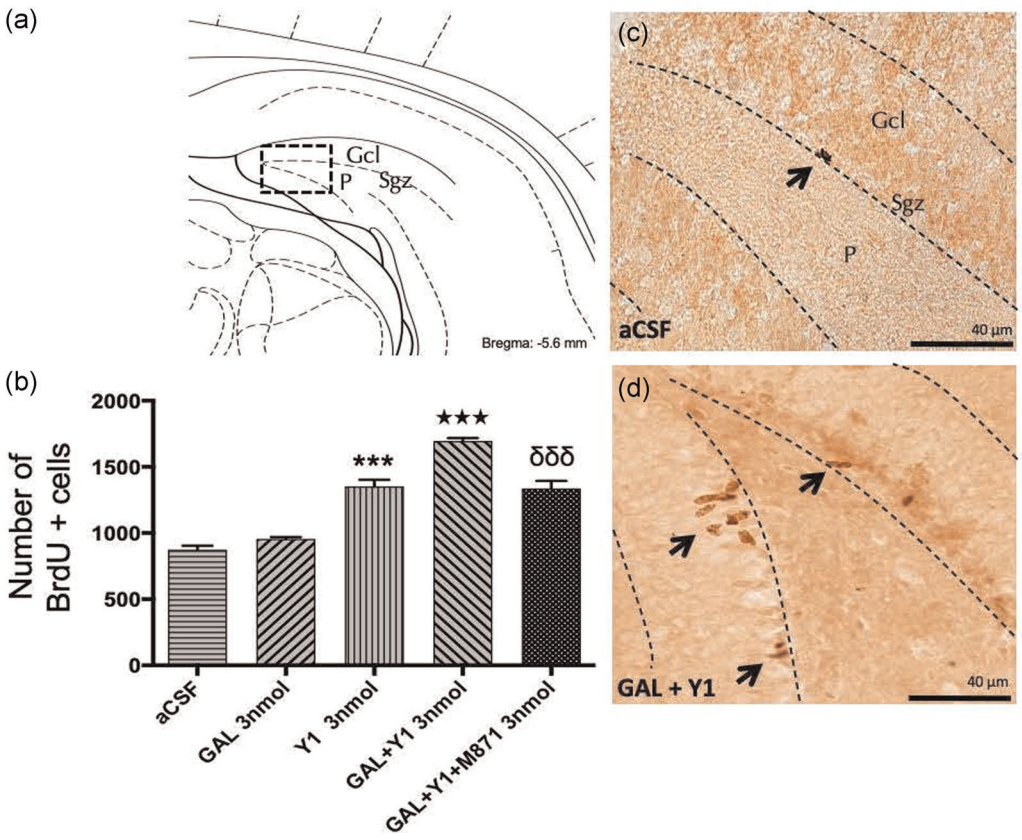

FIGURE 2 5'-Bromo-2'-deoxyuridine (BrdU) immunolabelling (BrdU+) in the dentate gyrus of the hippocampal ventral or anterior portion, after the intracerebroventricular (icv) administration of galanin (GAL) and neuropeptide Y Y1 receptor (NPYY1R) agonist, either alone or in combination with or without the GAL 2 receptor (GALR2) antagonist M871. (a and d) The majority of the BrdU-labeled cells were located in the subgranular zone (SGZ) of the dentate gyrus at the border between the granular cell layer (Gcl) and the polymorphic layer (P) of the dentate gyrus in the ventral hippocampus. They appeared to be nerve cells forming clusters of 3–4 cells. (Bregma: −5.6 mm; according to the Paxinos and Watson stereotaxic atlas (1998)). (b) Quantification of total BrdU IR cells at this Bregma level. Data represent mean ± *SEM*, to show the differences between groups after icv injection of aCSF, GAL, NPYY1 agonist [Leu^{31}-Pro^{34}]NPY, or the coadministration of both peptides with or without M871. GAL and the NPYY1 agonist coinjection increased the number of cells with BrdU expression in the ventral hippocampus compared with the effects of the two peptides given alone and the aCSF group. Furthermore, this effect was blocked by GALR2 antagonist M871. ***$p < .001$; ★★★$p < .001$; δδδ $p < .001$ according to one-way ANOVA ($F4, 15 = 75.37$, $p < .001$) followed by Newman–Keuls post hoc test. Intergroup comparisons are indicated by the vertical lines from the horizontal line above bars. $N = 4$ in each group. GAL and NPYY1R agonist coinjection (d) increased the BrdU immunolabelling in SGZ in the ventral hippocampus compared with the control group (c). Arrows indicate examples of clusters of BrdU positive nerve cells. Dashed lines outline the Gcl of the dentate gyrus. Treatments were performed 24 h before brain processing; for further details, see Section 2. aCSF, control (artificial cerebrospinal fluid); GAL 3 nmol, galanin 3 nmol; GAL + Y1 3 nmol, coadministration of GAL and Y1; GAL + Y1 + M871 3 nmol, coadministration of GAL, Y1, and GALR2 antagonist M871 3 nmol; Y1 3 nmol, NPY Y1 receptor agonist [Leu31-Pro34]NPY 3 nmol

3.4 | GALR2 and NPYY1R coactivation increased GALR2 availability on the plasma membrane and enhanced serum response element/mitogen-activated protein kinase pathway signaling in cell cultures

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

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

The presence of GALR2-GFP^2 expression in the plasma membrane but also in the intracellular compartment indicated constitutive GALR2 internalization in the basal state without agonist addition (Figure S2). The addition of GAL induced a rapid internalization of GALR2-GFP^2 expression, which slowly recovered during the 36-h measuring period (Figures 4a and 4d). However, combined treatment with GAL and NPY induced GALR2-GFP^2 recruitment, moving up toward the plasma membrane. We observed a maximal

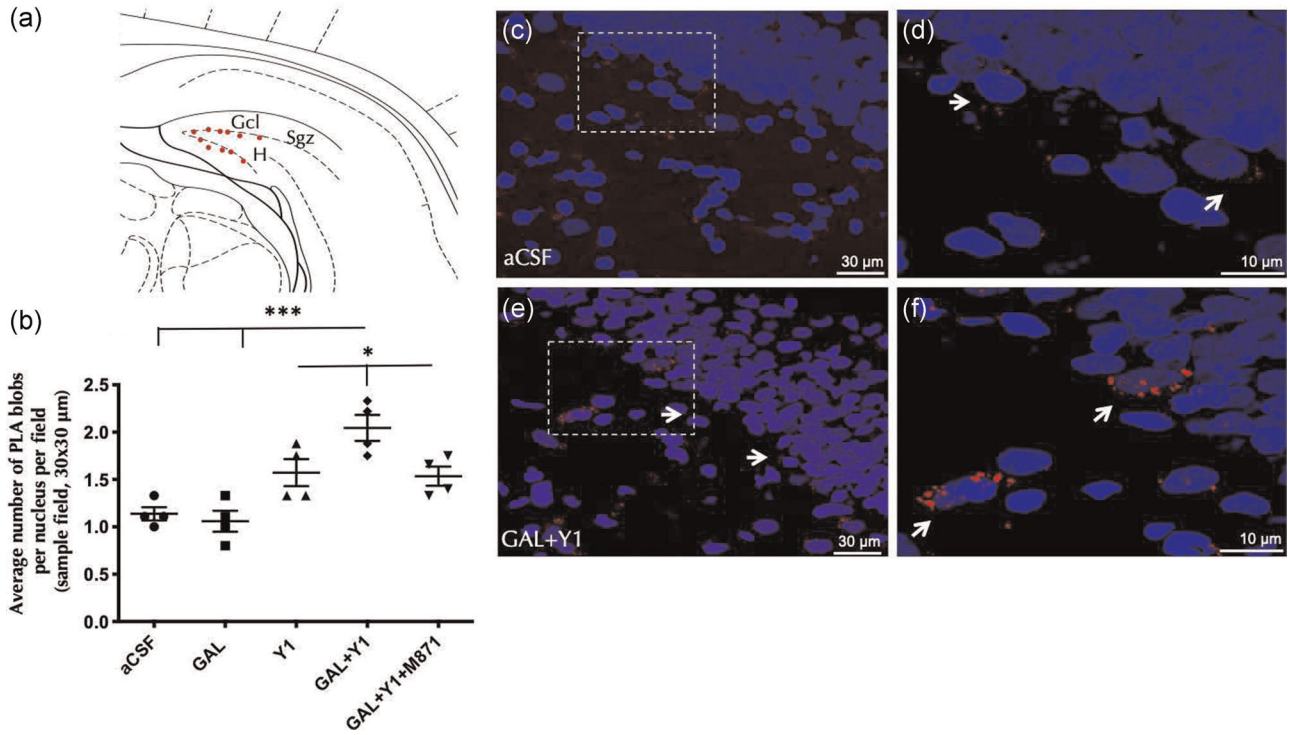

FIGURE 3 Detection of GALR2/NPYY1R heteroreceptor complexes by in situ PLA in the ventral hippocampal dentate gyrus (DG). (a) The diagram shows the presence of positive PLA signals (red circles) mainly in the subgranular zone (SGZ) of the dentate gyrus at the border between the granular cell layer (Gcl) and polymorphic layer (P) of the dentate gyrus in the ventral hippocampus. PLA-positive signals were also observed in the polymorphic layer. Blue filled circles indicate a negative PLA signal in the molecular layer (M). (Bregma: −5.6 mm; according to the Paxinos and Watson (1998) stereotaxic atlas]). (b) Quantification of PLA signals in SGZ was performed by measuring red PLA-positive blobs per nucleus per sampled field by an experimenter blind to treatment conditions. Sprague–Dawley rats showed a significant increase in GALR2/NPYY1R heteroreceptor complexes (PLA blobs) in the SGZ after GAL and NPYY1R agonist coinjection. This effect was blocked by treatment with the GALR2 antagonist M871. $^{*}p < .05$; $^{***}p < .001$; according to one-way ANOVA ($F4, 15 = 11.76$, $p < .001$) followed by Newman–Keuls post hoc test. Intergroup comparisons are indicated by the vertical lines from the horizontal line above bars. Data are expressed as mean ± *SEM*, four rats per group, duplicates). (c–f) Representative microphotographs of the significant increase in the density of GALR2/NPYY1R positive red PLA blobs in the Sgz subregion after GAL and NPYY1R agonist coinjection (e) compared with the control group (c). Magnified views from dashed boxes in (c) and (e) are shown in (d) and (f), respectively. GALR2/NPYY1R heteroreceptor complexes are shown as red PLA blobs (clusters) found in high densities per cell in a large number of nerve cells using confocal laser microscopy. White arrows point to PLA-positive clusters. Dashed lines outline the Gcl of the dentate gyrus. The nuclei are shown in blue by DAPI. Treatments were performed 24 hours before brain processing; for further details, see Section 2. aCSF, control (artificial cerebrospinal fluid); DAPI, 4′,6-diamidino-2-phenylindole; GAL 3 nmol, galanin 3 nmol; GAL + Y1 3 nmol, coadministration of GAL and Y1; GAL + Y1 + M871 3 nmol, coadministration of GAL, Y1, and GALR2 antagonist M871 3 nmol; Y1 3 nmol, NPY Y1 receptor agonist [Leu31-Pro34]NPY 3 nmol

effect at 30 min, compared to internalized GALR2-GFP^2 after GAL alone ($t = 7.842$, $p < .001$, $df = 21$; Figures 4a,b and 4d). This effect was sustained for 24 h ($t = 2.240$, $p < .05$, $df = 20$; Figure 4d). At this time, GALR-GFP^2 expression was greater in the plasma membrane after GAL and NPY costimulation, compared to GAL alone ($t = 4.213$, $p < .001$, $df = 21$; Figures 4a,b and 4d). The specific NPYY1R antagonist BIBP3226 abolished this recruitment of GALR2-GFP^2 expression (Figure 4c,d), demonstrating that this effect was mediated through the coactivation of GALR2 and NPYY1R.

The extent of phosphorylation of ERK1/2 was studied upon the coactivation of GALR2 and NPYY1R using the principle of the serum response element (SRE)-luciferase gene reporter assay, in transiently transfected HEK293T cells coexpressing both receptors.

Treatment with NPY (50 nM) or GAL (100 nM) leads to a significant and similar increase in the MAPK pathway that subsequently leads to SRE activation in cells coexpressing GALR2 and NPYY1R receptors (Figure 5a; one-way ANOVA, $F7, 56 = 89.25$, $p < .001$, Newman–Keuls post hoc test: $p < .001$). The corresponding selective antagonists fully counteract these effects, M871 (1 μM) for GALR2 and BIBP3226 (1 μM) for NPYY1R (Newman–Keuls post hoc test: $p < .001$; Figure 5a). GAL at 50 nM lacks effects on the SRE response.

Combined treatment with a threshold dose of GAL (50 nM) and NPY (50 nM) leads to a significant increment of SRE activity compared with NPY alone (Newman–Keuls post hoc test: $p < .001$). Furthermore, the effective dose of GAL (100 nM) in combination with NPY (50 nM) induce a significant increment of SRE activity compared with the observed when stimulated by GAL or NPY alone (Newman–Keuls post hoc test: $p < .001$; Figure 5a).

With these results, we can propose a schematic diagram with possible direct and indirect interactions between GALR2-NPYY1R

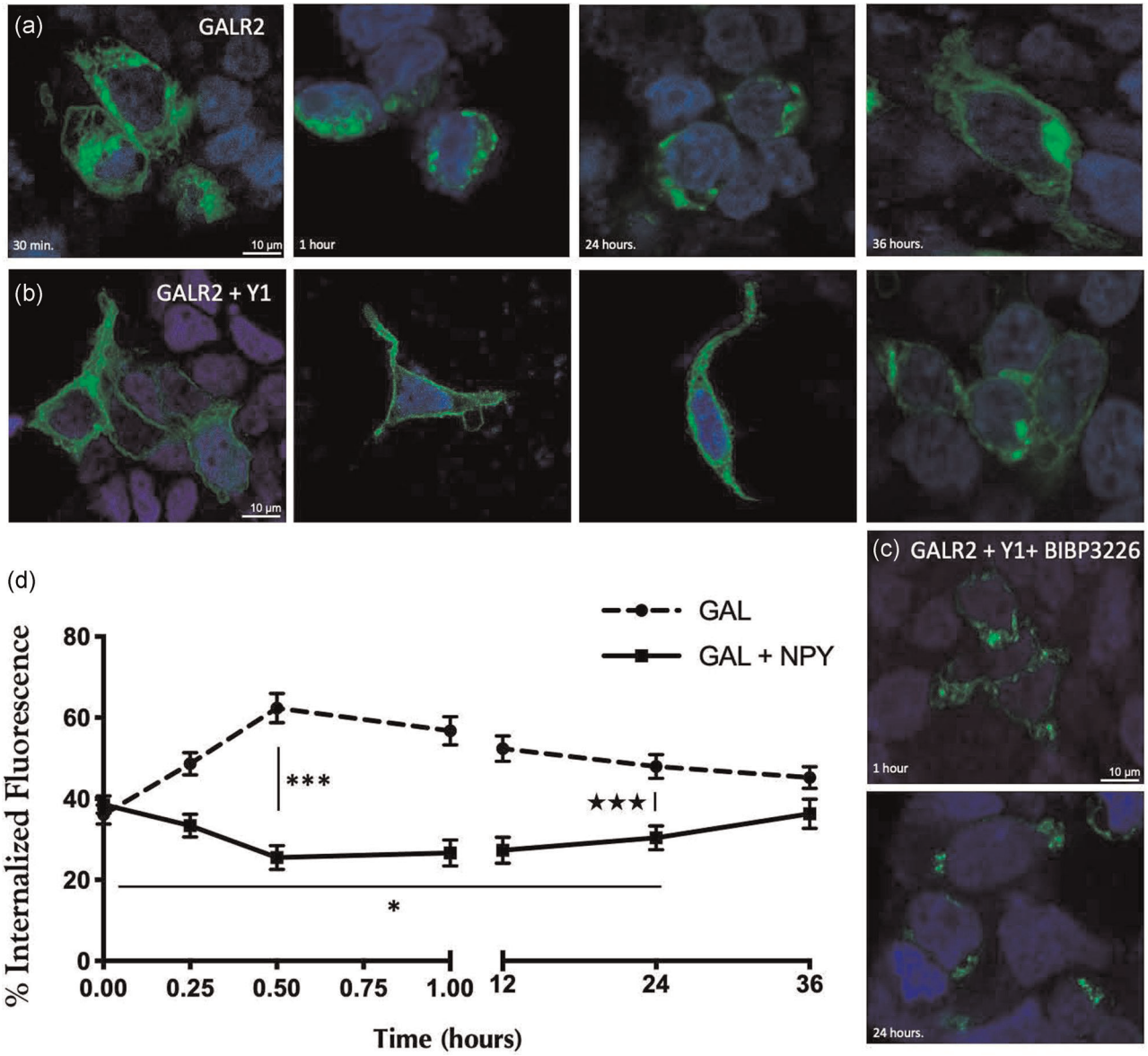

FIGURE 4 Analysis of the peak internalization values of galanin receptor 2 (GALR2)-GFP^2 with GAL alone in the1st-h period versus the combined treatment with NPY, and the coagonist-induced disappearance of the internalization at the 24-h time interval. (a–c) Representative confocal micrographs show the distribution of GALR2-GFP^2 in GALR2-GFP^2/NPYY1R HEK293T coexpressing cells. Timed-interval images of different cell groups were acquired with the confocal microscope after GAL 1 μM and/or NPY 1 μM addition and washed with ice-cold PBS to stop the internalization; for further details, see Section 2. Antagonist studies were performed 15 min before the addition of agonist with NPYY1R antagonist BIBP3226 10 μM. Following costimulation with 1 μM GAL and NPY (b), there was an increased GALR2-GFP^2 presence in the plasma membrane at different time points, compared with GAL alone (a). This effect was counteracted by the NPYY1R antagonist BIBP3226 (10 μM). Representative confocal micrographs are shown at 1 and 24 h (c). (d) Representation of time course of quantitation of GALR2-GFP^2 internalization. Data, as mean ± *SEM*, showed maximal internalization after GAL stimulation alone at 30 min. However, after GAL and NPY costimulation, GALR2-GFP^2 recruitment was observed toward the plasma membrane. Internalization took place only after 60 min until 36 h. $^*p < .05$ ($t = 2.240$, $df = 20$; 24 h after GAL and NPY costimulation vs. basal state); $^{***}p < .001$ ($t = 7.842$, $df = 21$; 30 min after GAL and NPY costimulation vs. GAL alone); ★★★$p < .001$ ($t = 4.213$, $df = 21$; 24 h after GAL and NPY costimulation vs. GAL alone) according to Student's unpaired *t* test statistical analysis. The lines indicate group comparisons. Micrographs are representative images of multiple cells imaged in three independent experiments. NPYY1R, neuropeptide Y Y1 receptor; PBS, phosphate-buffered saline

heteromer and receptor tyrosine kinases, known to be interalia linked to the MAPK pathway (Cheung et al., 2012; Luo et al., 2019; McKay & Morrison, 2007). It can involve the Gβγ subunits modulating the activity of the Ras–Raf–MEK1–MAPK pathway to the SRE (Figure 5b). The presence of an enhanced interaction in SRE activity upon coactivation of the two receptors suggests the existence of allosteric excitatory communication in the interface between the two receptors of the GALR2–NPYY1R heteroreceptor complex.

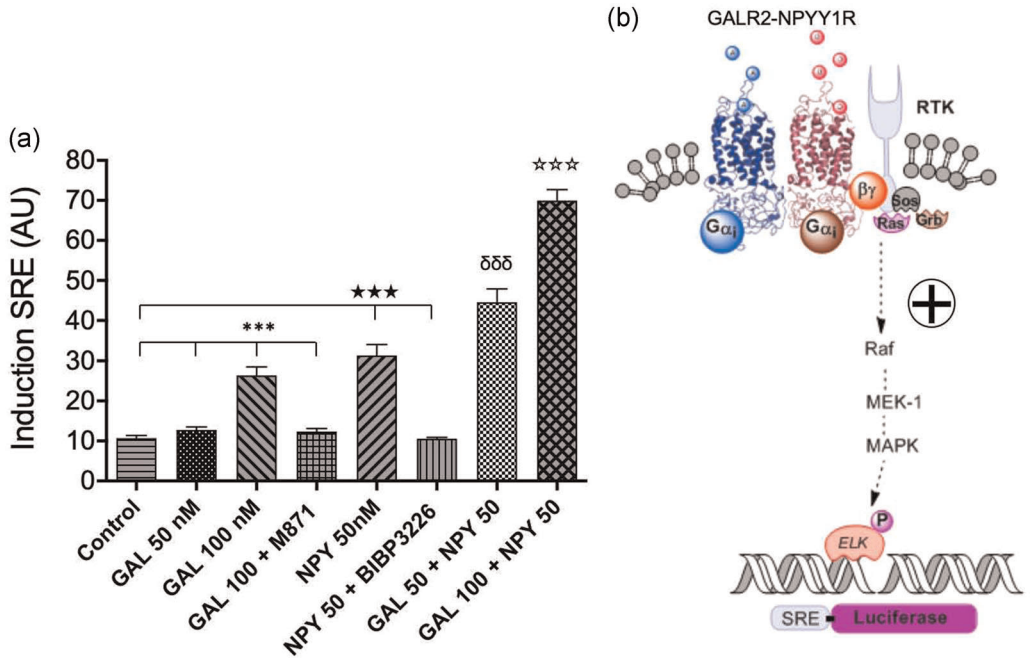

FIGURE 5 Agonist-induced galanin 2 receptor (GALR2) and neuropeptide Y Y1 receptor (NPYY1R) activation in the serum response element (SRE)-luciferase reporter gene assay. HEK293T cells were transiently cotransfected with 1 μg firefly luciferase-encoding experimental plasmid (pGL4-SRE-luc2p), 1 μg of (GALR2 and/or NPYY1R) expression vectors, and 50 ng Renilla luciferase-encoding internal control plasmid (phRG-B). Thirty-six hours after transfection, cells were treated for 6 h with GAL and/or NPY or their specific antagonists (in the presence of agonist). (a) The costimulation with GAL (50 and 100 nM) and NPY (50 nM) substantially increased the induction of the SRE-Reporter expression compared with the increase found with GAL and NPY alone. The specific antagonists for GALR2 (M871) and NPYY1R (BIBP3226) counteracted the increase of SRE-expression found with effective doses of GAL and NPY, respectively. Data represent the mean ± *SEM* of three independent experiments performed in triplicate. ***$p < .001$; ★★★$p < .001$; δδδ$p < .001$; ☆☆☆$p < .001$ according to one-way ANOVA one-way ANOVA, ($F7, 56 = 89.25$, $p < .001$) followed by the Newman–Keuls post hoc test. Intergroup comparisons are indicated by the vertical lines from the long horizontal line above bars. (b) Schematic cross-talk signaling pathway of GALR2-NPYY1R heteroreceptor complex with a positive modulation on SRE pathway signaling. There can be a putative involvement also of RTK in the GALR2-NPYY1R heteroreceptor complex contributing to the enhancement of the SRE signaling. ANOVA, analysis of variance; BIBP3226, NPYY1R antagonist 1 μM; control, basal medium; GAL 50 nM, galanin 50 nM; GAL 100 nM, galanin 100 nM; M871, GALR2 antagonist 1 μM; NPY 50 nM, neuropeptide Y 50 nM; SRE, serum response element

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

4 | DISCUSSION

The current study demonstrates, for the first time, the existence of an interaction between GALR2 and NPYY1R in the DG of the ventral hippocampus upon combined receptor agonist treatment. GAL was demonstrated to act via GALR2 to enhance NPYY1R-sustained effects at 24 h in the FST, reducing immobility and climbing time while increasing swimming time, which strongly supports antidepressant-like actions. This effect seems to be linked to the ability of the coagonist treatment to enhance the NPYY1R-mediated increase of cell proliferation in the DG of the ventral hippocampus through BrdU expression analysis at 24 h. This event may be determined by the increased presence of GALR2/NPYY1R heteroreceptor complexes, on doublecortin-expressing neuroblasts, upon agonist coactivation of their two types of receptor protomers. Thus, it is proposed that the mechanism of how the GAL and NPY Y1 agonist coinjection underlies the antidepressant-like actions is mediated by the increase of GALR2/NPYY1R heteroreceptor complexes leading to an enhancement of the neuronal proliferation in the ventral DG upon their receptor protomer coactivation. Accordingly, it was validated that boosting neural cell proliferation in the hippocampal DG leads to antidepressants effects in rodents (Bauman et al., 2018; Walker et al., 2015).

The understanding of the cellular mechanisms was achieved by analyzing the GALR2 expression and the SRE–luciferase gene reporter response in transiently GALR2 and NPYY1R cotransfected HEK cells. Recruitment in the GALR2 expression to the plasma membrane was observed upon the coactivation of GALR2 and NPYY1R in the cotransfected HEK cells, presumably linked to the increase in GALR2/NPYY1R heteroreceptor complexes observed. These heteroreceptor complexes may allow enhanced effects on the

activation of the ERK pathway, as observed in the SRE-luciferase assay after the agonist coactivation of GALR2 and NPYY1R.

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

Furthermore, the effects achieved after the administration of GAL or NPYY1 agonist alone agree with the previous studies and support the prodepressant-like role of GAL and the antidepressant-like actions of the NPYY1 agonist in the FST (Kotagale et al., 2013; Kuteeva et al., 2008; Redrobe et al., 2002). These behavioral effects observed in the FST were independent of the motor activity since neither GAL, and NPYY1 agonist nor their coadministration has shown locomotor modifications (Narvaez et al., 2016, 2018, 2015).

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

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

Our data support the hypothesis that GAL and the NPPYY1R agonist can act on the doublecortin (DCX)-cells expressing GALR2 and NPY1R in the ventral hippocampus with the formation of GALR2/NPYY1R heteroreceptor complexes. DCX is expressed at the proliferative stage, first appearing at the Type 2b stage of stem cell, widely used as a neuronal lineage marker (Kempermann et al., 2015). According to the results obtained, Decressac et al. (2011) confirmed that NPY-sensitive cells are the neuroblast expressing DCX, which selectively express the Y1R.

Furthermore, we observed a reduction of the GALR2 internalization with this receptor, mainly localized to the plasma membrane upon the coactivation of GALR2 and NPYY1R, for 24 h. This effect may promote the formation of GALR2-NPYY1R heteroreceptor complexes formation since NPYY1R was also shown to mainly remain at the plasma membrane upon the coactivation of the two receptors (Narvaez et al., 2015). Moreover, we recognized a constitutive internalization for GALR2, in line with previous results (Xia et al., 2005). This increased GALR2-NPYY1R interaction could lead to increase integration in the intracellular signaling, as we observed in the ERK pathway, through the SRE reporter assay. ERK kinases, as a group of MAPK, are implicated in the control of cellular proliferation, including neural precursors in the hippocampus (Li et al., 2001). In agreement, both receptors GALR2 and NPYY1R were shown to provoke activation of the MAPK ERK1/2 pathway in the hippocampus (Cheung et al., 2012; Luo et al., 2019).

Taken together, changes in the formation, activation, and trafficking of the GALR2 may promote the GALR2/NPYY1R heteroreceptor complexes formation in the ventral hippocampus. It may induce a transformation of cell proliferation toward a neuronal lineage by enhancement of the MAP ERK 1/2 pathway. This effect may be linked with the antidepressant-like response observed upon cotreatment with GAL and the NPYY1R agonist. Therefore, our results may provide the basis for the development of heterobivalent agonist pharmacophores, targeting GALR2/NPYY1R heteromers, especially in the neuronal precursor cells of the dentate gyrus in the ventral hippocampus for the novel treatment of depression.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in: Narváez, Manuel (2020), "Raw data-Statistical analysis," Mendeley Data, V1, https://doi.org/10.17632/pcf28dx3z5.1

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SUPPORTING INFORMATION

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