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Programa de doctorado de BIOMEDICINA, INVESTIGACIÓN TRASLACIONAL Y NUEVAS TECNOLOGIAS EN SALUD, Facultad de Medicina, de la Universidad de Málaga, España

ON THE ROLE OF THE BALANCE OF G PROTEIN-COUPLED RECEPTOR (GPCR) HOMO- AND HETERORECEPTOR COMPLEXES AND THEIR INTEGRATION OF SIGNALS IN NEURONS AND ASTROGLIA. RELEVANCE FOR BRAIN DISORDERS

Ramón Forés Pons

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Directors

Dr. Dasiel Oscar Borroto-Escuela

Dr. Manuel Narváez Peláez

UNIVERSIDAD DE MÁLAGA

AUTOR: Ramón Forés Pons

https://orcid.org/0000-0002-0198-7260

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D. RAMÓN FORÉS PONS

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Fdo. Doctorando Ramón Forés Pons:

Fdo.Tutor Manuel Narváez Peláez:

Fdo.Director Dasiel Oscar Borroto Escuela:

Fdo.Director Manuel Narváez Peláez:

Edificio Pabellón de Gobierno. Campus El Ejido. 29071
Tel.: 952 13 10 28 / 952 13 14 61 / 952 13 71 10
E-mail: doctorado@uma.es

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ABSTRACT

There exists substantial evidence for the existence of G protein-coupled receptor (GPCR) homo and heteroreceptor complexes with allosteric receptor-receptor interactions in the Central Nervous System (CNS). GPCR heteroreceptor complexes can also involve ion channel receptors, receptor tyrosine kinases (RTKs), sets of G protein interacting proteins, ion channels and/or transmitter transporters increasing their integrative capability. The allosteric receptor–receptor interactions in GPCR heteroreceptor complexes gave a new dimension to brain integration and neuropsychopharmacology.

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. Usually there is a competition between different receptor complexes for the same receptor protomer since they are in balance with each other. This is the case of a heteromer and its corresponding homomers and different heteromers sharing one or two receptor protomers. However, there is a need to improve our understanding of the molecular organization of the receptor oligomers, their allosteric communication, and the features of the receptor interface.

The molecular basis of learning and memory was proposed to be based on the reorganization of the homo- and heteroreceptor complexes in the postjunctional membrane of synapses. Long-term memory may be created by the transformation of parts of the heteroreceptor complexes into unique transcription factors which can lead to the formation of specific adapter proteins. The observation of the GPCR heterodimer network (GPCR-HetNet) indicated that the allosteric receptor-receptor interactions dramatically increase GPCR diversity and biased recognition and signaling leading to enhanced specificity in signaling. Dysfunction of the GPCR heteroreceptor complexes can lead to brain disease. The findings of serotonin (5-HT) hetero and isoreceptor complexes in the brain over the last decade give new targets for drug development in major depression. In depression neuromodulation of neuronal networks in the raphe-hippocampal system and the cortical regions via 5-HT, galanin peptides and neuropeptide Y involve a number of GPCR heteroreceptor complexes in the raphe-hippocampal system: GalR1-5-HT1A, GalR2-NPYY1R, GalR1-GalR2, and putative GalR1-GalR2-5-HT1A heteroreceptor complexes or the 5-HT isoreceptor complexes such as 5-HT1A-5-HT7 and 5-HT1A-5-HT2A. The 5-

HT1A receptor protomer remains a receptor enhancing antidepressant actions through its participation in hetero and homoreceptor complexes listed above in balance with each other. Neuromodulation of neuronal networks in cocaine use disorder via dopamine and adenosine signals involve A2AR-D2R, A2AR-D2R-mGluR5 and A2AR-D2R-Sigma1R heteroreceptor complexes in the dorsal and ventral striatum. The excitatory modulation by A2AR agonists of the ventral striato-pallidal GABA anti-reward system via targeting the A2AR-D2R, A2AR-D2R-mGluR5 and A2AR-D2R-Sigma1R heteroreceptor complex holds high promise as a new way to treat cocaine use disorders. Neuromodulation of neuronal networks in schizophrenia via dopamine, adenosine, glutamate, 5-HT and neurotensin peptides and oxytocin, involving A2AR-D2R, D2R-NMDAR, A2AR-D2R-mGluR5, D2R-5-HT2A and D2R-oxytocinR heteroreceptor complexes opens up a new world of D2R protomer targets in the listed heterocomplexes for treatment of positive, negative and cognitive symptoms of schizophrenia.

Therefore, the overall aim of this thesis was to gain insight into molecular aspects of several GPCR 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 (D2R-5-HT2AR, GalR2-NPYY1R, A2AR-D2R, A2AR-D2R-mGluR5 and A2AR-D2R-Sigma1R heteroreceptor complex) using the proximity ligation assay (PLA) techniques and biophysical (BRET) and biochemical methods (Co-IP, radioligand binding assays). The understanding of the balance of GPCR homo- and heteroreceptor complexes and their integration of neuronal signal is of a high relevance in view of the involvement of these complexes on several mental and neurological diseases.

RESUMEN EN ESPAÑOL

Los mecanismos de señalización mediados por los receptores acoplados a la proteína G (GPCR) son procesos muchos más complejos e interconectados que los descritos hasta el presente. Habida cuenta que cada receptor o complejo de receptores requiere un conjunto de interacciones proteicas que faciliten de forma orquestada, en tiempo y espacio, la transmisión de la señal.

Los complejos de homo y heteroreceptores de los GPCR, no solo están constituidos por protómeros de GPCR, sino que también pueden incluir canales iónicos, receptores tirosina quinasas receptoras (RTK), conjuntos de proteínas que interactúan con proteínas G y/o transportadores de transmisores contribuyendo a una mayor integración. La formación de estos complejos de homo y heterorreceptores en un área sináptica o extrasináptica de la membrana plasmática están gobernadas por varios factores y especialmente por la densidad de los protómeros participantes y la afinidad de unión entre los diferentes protómeros. Por lo general, existe una competencia entre diferentes complejos de receptores por el mismo protómero y se establece por tanto un balance entre las diferentes poblaciones de homo- and heteroreceptores.

Los complejos de homo y heteroreceptores de GPCR a través de sus interacciones alostéricas entre los diversos componentes del complejo se han convertido en centros de integración molecular muy sofisticados a nivel molecular que facilitan el desarrollo de capacidades multifuncionales de sus protómeros. Estos se convierten así, en sí mismos, en excelente dianas o blancos para el desarrollo de nuevos neurofármacos o líneas neuroterapéuticas en enfermedades tales como el Parkinson, la esquizofrenia, la adicción a drogas, la ansiedad y la depresión. Por tanto, es de sumo interés comprender y estudiar la dinámica de estos procesos de interacción entre los receptores de membrana y las proteínas intracelulares que interactúan con los mismos, tanto en el tiempo como en el espacio, para poder tener una compresión global de los mecanismos de integración de la señal en el Sistema Nervioso Central (SNC) y su implicación en el desarrollo de las enfermedades mentales y neurodegenerativas.

Los hallazgos de complejos hetero e isorreceptores de serotonina (5-HT) en el cerebro durante la última década brindan nuevos objetivos para el desarrollo de fármacos en la depresión mayor. En la depresión, la neuromodulación de las redes neuronales en el sistema rafe-hipocampo y las regiones corticales a través de 5-HT, los péptidos de galanina y el neuropéptido Y involucran una serie de complejos

heterorreceptores GPCR en el sistema rafe-hipocampo: GalR1-5-HT1A, GalR2-NPYY1R, GalR1-GalR2, y complejos heterorreceptores posibles GalR1-GalR2-5-HT1A o los complejos de isorreceptores 5-HT tales como 5-HT1A-5-HT7 y 5-HT1A-5-HT2A. El protómero del receptor 5-HT1A sigue siendo un receptor que mejora las acciones antidepresivas a través de su participación en los complejos hetero y homorreceptores enumerados anteriormente en equilibrio entre sí. La neuromodulación de las redes neuronales en el trastorno por consumo de cocaína a través de señales de dopamina y adenosina involucra los complejos heterorreceptores A2AR-D2R, A2AR-D2R-mGluR5 y A2AR-D2R-Sigma1R en el estriado dorsal y ventral. La modulación excitatoria por los agonistas A2AR del sistema anti-recompensa GABA estriado palidal ventral a través de la orientación del complejo heterorreceptor A2AR-D2R, A2AR-D2R-mGluR5 y A2AR-D2R-Sigma1R es muy prometedora como una nueva forma de tratar los trastornos por consumo de cocaína. Se abre la neuromodulación de las redes neuronales en la esquizofrenia a través de dopamina, adenosina, glutamato, 5-HT y péptidos de neurotensina y oxitocina, que involucran los complejos heterorreceptores A2AR-D2R, D2R-NMDAR, A2AR-D2R-mGluR5, D2R-5-HT2A y D2R-oxitocinaR un nuevo mundo de objetivos de protómero D2R en los heterocomplejos enumerados para el tratamiento de síntomas positivos, negativos y cognitivos de la esquizofrenia.

Avances en los conocimientos de las interacciones más allá de los GPCR-GPCR. Interacciones GPCR-RTK.

Existe actualmente cierto consenso en las evidencias de que los receptores tirosina quinasa (RTK) pueden ser trans-activados vía la liberación por factores tróficos a través de la activación de los GPCR, pero también mediante mecanismos más directos en los que tienen lugar la formación de complejos de interacción en la membrana citoplasmática de estas dos familias de receptores de membranas. Los RTK son una familia de receptores transmembrana que operan como receptores de señal de factores neurotróficos como son: el factor de crecimiento epidérmico (EGF), el factor de crecimiento derivados de plaquetas (PDGF), el factor neurotrófico derivado del cerebro (BDNF), y el factor de crecimiento de fibroblastos (FGF).

La hipótesis de una interacción directa en la membrana citoplasmática entre receptores RTK y GPCR fue introducida en el año 2007 por Kjell Fuxe y sus colaboradores. Un año más tarde fue confirmada por el premio Nobel Paul Greengard y su equipo de investigación de la Universidad de Rockefeller. El grupo de Fuxe también propuso en el año 2007 la existencia de un complejo de heteroreceptores (5-HT1A-FGFR1) en el SNC y su posible implicación o relevancia en los mecanismos moleculares de las enfermedades depresivas. Se sugirió que el sistema neurotrófico FGF-2/FGFR1 puede considerarse como un buen candidato o diana para el desarrollo

de nuevos compuestos anti-depresivos si se consideraba desde el punto de vista de la interacción de estos receptores (FGFR1) con los receptores de serotonina, especialmente los receptores del subtipo 5-HT1A. La transactivación de los receptores RTK en respuesta a los tratamientos antidepresivos se postuló desde la base de los mecanismos alostéricos de interacción receptor-receptor que pueden operar entre los receptores FGFR1-5HT1A.

Cinco años más tarde, en el año 2012, Dasiel Borroto-Escuela descubrió la existencia de complejos de heteroreceptores entre el FGFR1-5HT1A en el cerebro de las ratas, especialmente en el hipocampo, las regiones del rafe medio dorsal y el córtex prefrontal. Se demostraron además una serie de mecanismos de señalización celular orquestados por la interacción de estos dos receptores, que contribuyen el incremento de la plasticidad neuronal y la formación de nuevos botones y dendritas en cultivos primarios de células del hipocampo. También, basado en la teoría del rompecabezas por triplete de aminoácidos (amino acid triplet puzzle theory) se demostró la existencia de una serie de tripletes que conforman la interfase de interacción entre el receptor FGFR1 y el 5-HT1A. Fidel Corrales y el equipo del Dr. Borroto-Escuela y Manuel Narváez aportaron también nuevas evidencias de que la co-estimulación de ambos protómeros en el heterocomplejo 5-HT1A-FGFR1 resulta en un significativo incremento de la formación de nuevos complejos en la membrana plasmática, así como un aumento en los procesos de homodimerización de los respectivos protómeros. Se observó, además, que el mecanismo alostérico como resultado de las interacciones receptor-receptor, resultan en un aumento significativo de la unión de ciertas proteínas intracelulares a ambos protómeros (por ejemplo, la β-arrestina), así como un aumento de la señalización vía las MAPK. Estos mecanismos de integración de la señal son recíprocos y las plataformas de señalización para los RTK pueden subordinarse a las de los GPCR y viceversa. La formación de los complejos RTK-GPCR son dinámicas y muy interrelacionadas, impactando en todos los niveles estructurales y funcionales de cada uno de estos receptores, desde el reconocimiento, hasta la señalización, la internalización y tráfico.

Importancia de los balances entre diferentes complejos de receptores en la membrana sináptica pre- y postsináptica.

El desarrollo de complejos de homo y heteroreceptores de GPCR en el área sináptica o extrasináptica de la membrana plasmática está mediado por varios factores, por ejemplo, por la densidad de los protómeros participantes en la formación de dichos complejos. Otro factor importante es la afinidad entre los protómero. Dicha afinidad viene determinada o mediada por la cantidad de puntos de interacción que se desarrollan en la interfaz de interacción de los receptores. La presencia o ausencia de algunas proteínas adaptadoras en el complejo heteroreceptor constituyen también otro

factor significativo para determinar la afinidad que se desarrolla entre dos o más protómeros. Por tanto, un aumento en la afinidad de unión entre los dos protómeros de receptores, conllevará a un aumento en el número de complejos de receptores. Debe destacarse también el impacto que tienen la estimulación mediada por diferentes ligandos, por ejemplo, los agonistas de los protómeros constituyentes partes del complejo heteroreceptor, que pueden modular los complejos de los receptores a través de cambios conformacionales como se estableció en los experimentos BRET.

Un punto muy interesante y del cúal ahora es que comenzamos a tener mejor comprensión y contamos con los medios técnicos para el análisis, es el rol de los balances entre las diferentes poblaciones de homo y heteroreceptores en una misma célula. Por lo general, existe una competencia entre diferentes complejos de receptores por el mismo protómero receptor, ya que están equilibrados entre sí. Este es el caso de un heterómero y sus correspondientes homómeros y diferentes heterómeros que comparten uno o dos protómeros receptores. Existe la necesidad de mejorar nuestra comprensión de la organización molecular de los oligómeros, su comunicación alostérica y las características de la interfaz del receptor.

Los complejos homo y heteroreceptores de la membrana plasmática proporcionan una base molecular para el aprendizaje y la memoria.

Junto con los hallazgos de las interacciones intramembrana entre receptores y la formación de complejos macromoleculares entre receptores de membrana, ha quedado claro que esta enorme área de la membrana plasmática juega un papel muy importante en la integración molecular de la señalización. Estos mecanismos de integración solo pueden tener lugar gracias al concurso de la formación de complejos de receptores, cuya estequiometría varía considerablemente, pasando de simples dímeros hasta oligómeros de orden superior. Los protómeros constituyentes de los complejos de receptores, así como otras proteínas, podrían comunicarse entre sí a través de interacciones directas receptor-receptor y receptor-proteína a través de mecanismos alostéricos. Con la integración de la señal continuada en las múltiples vías intracelulares, queda clara la importancia de este primer paso del manejo de la información en la membrana plasmática.

Fuxe y Borroto-Escuela propusieron la teoría alostérica del aprendizaje y la memoria basada en la existencia de muchos complejos receptores oligoméricos en las membranas postsináptica y extrasináptica y las interacciones receptor-receptor alostérico dentro de ellos. Establecieron que la base molecular del aprendizaje y la memoria puede ser representada por la reorganización de los complejos homo- y heteroreceptores en la membrana postsináptica guiando a los cambios en los complejos receptores presinápticos para facilitar el patrón de liberación del transmisor

a ser aprendido. Ante un cambio en el patrón de liberación de transmisores en la sinapsis, el aprendizaje de este nuevo patrón ocurrirá en la membrana postsináptica a través de una reorganización de los complejos homo y heteroreceptores. También ocurrirán cambios en los complejos receptores presinápticos para permitir que se reconozca el mantenimiento del patrón de liberación múltiple de transmisores. Considerando una especie de "código de barras basal" y por tanto a través de una reorganización transitoria de los complejos receptores se obtiene un nuevo código de barras que indica una memoria a corto plazo. La memoria a largo plazo se desarrolla a través de la transformación de partes intracelulares de los complejos heteroreceptores en moléculas solubles que pueden unirse a factores de transcripción y modular sus acciones transcripcionales a nivel del ADN. De esta manera, se pueden formar proteínas adaptadoras nuevas y específicas dispuestas a unirse a los complejos receptores que forman la memoria a corto plazo. Luego se consolida en una memoria a largo plazo (engrama molecular) con interacciones receptor-receptor conservadas. Las proteínas adaptadoras pueden actuar aumentando los enlaces entre los protómeros de receptores, los protómeros de receptores y las proteínas del citoesqueleto y los protómeros de receptores y las proteínas de andamiaje.

Por lo tanto, los complejos homo-heteroreceptores se consideran conjuntos altamente dinámicos formados o interrumpidos por señales de transmisión sináptica y de volumen integradas. Estos eventos son necesarios para el aprendizaje y pueden transformarse en un estado rígido consolidado con comunicación alostérica conservada. Esto representará engramas moleculares que darán como resultado una importante modulación a largo plazo de las redes neuronales. Este cambio de plasticidad molecular ya sea transitorio o a largo plazo, puede modificar los patrones de salida en los circuitos cerebrales y fomentar cambios transitorios y a largo plazo en los comportamientos y las funciones cognitivas. De acuerdo con esta hipótesis, el bloqueo de la eliminación sináptica de los receptores AMPA que contienen GluA2 previene el olvido natural de los recuerdos a largo plazo. Ha sido de especial relevancia para la plasticidad estructural, por ejemplo, el árbol dendrítico y sus espinas. Esto puede ser debido al reclutamiento del receptor tirosina quinasa a los complejos heteroreceptores formados, lo que puede resultar, por ejemplo, en aumentos sinérgicos en las densidades de neuritas y sus protuberancias en cultivos neuronales primarios.

De gran importancia es el trabajo de Everitt y colaboradores quienes plantearon la hipótesis de que la adicción a las drogas es causada por una memoria patológica, llamada memoria de la droga. También propusieron que comprender su base molecular podría conducir a la introducción de nuevas terapias contra las recaídas. La hipótesis de Borroto-Escuela y Fuxe sobre las bases moleculares del aprendizaje y la memoria está en línea con su visión. Implican que los recuerdos de

drogas se pueden producir a través de una reorganización de los complejos homo y heteroreceptores en las sinapsis y sus regiones extrasinápticas, entre otras cosas, en las sinapsis de glutamato en las neuronas anti-recompensa GABA estriado-palidales. Específicamente, el apoyo se basa en aclarar que la cocaína puede producir complejos patológicos A2AR-D2R-Sigma1R en tales sinapsis. Parece encarnar una memoria a largo plazo con una inhibición permanente y fuerte de la afinidad y la señalización de D2R, lo que puede conducir a la adicción a la cocaína. Por lo tanto, los complejos A2AR-D2R-Sigma1R pueden convertirse en un objetivo para el tratamiento de la adicción a la cocaína.

Complejos heterorreceptores GPCR como dianas para el tratamiento farmacológico en enfermedades cerebrales

Los complejos de heterorreceptores GPCR en el SNC se han convertido en nuevos y emocionantes objetivos para la neuroterapia en la enfermedad de Parkinson, la esquizofrenia, el trastorno por uso de sustancias, la ansiedad y la depresión, lo que abre un nuevo campo en la neuropsicofarmacología. Las posibles estrategias novedosas para dirigirse a los complejos heterorreceptores en la enfermedad del SNC son el tratamiento combinado con fármacos dirigidos a dos protómeros de receptores. Para mejorar el cumplimiento de los pacientes, se pueden desarrollar formulaciones fijas de los dos fármacos que proporcionen una farmacocinética óptima durante una ventana de tiempo de larga duración para las acciones terapéuticas combinadas. También se pueden desarrollar fármacos de acción dual dirigidos a dos protómeros en el complejo del receptor, así como profármacos de acción dual como un enfoque potencial y novedoso de objetivos múltiples para tratar la enfermedad del SNC. Los fármacos heterobivalentes son otras opciones para dirigirse selectivamente a los heterodímeros, como los antagonistas de D2R y los farmacóforos agonistas de A2AR que se dirigen a los heterodímeros A2AR-D2R en el trastorno por consumo de cocaína.

Todos estos aspectos novedosos sobre la comunicación cerebral y la integración en complejos heterorreceptores conducen a una mayor comprensión de las bases moleculares de las enfermedades del SNC y de sus tratamientos. Existe una mayor demanda de saber y comprender cómo funciona el cerebro a nivel de los receptores. El campo de los heteroreceptores es novedoso ya que generalmente se considera que los receptores existen como monómeros y esta revisión enfocada mostrará que el campo de los receptores se ha movido hacia los homo y heterodímeros y los complejos de homo y heteroreceptores de orden superior mediante el uso de metodologías novedosas. Por lo tanto, esta investigación hará avanzar sustancialmente el campo de los receptores. Introduce un principio biológico novedoso y una neuropsicofarmacología que se dirige a los complejos

heteroreceptores. Los complejos homo-heteroreceptores también están presentes, entre otros, en el sistema nervioso periférico, el sistema endocrino, el cardiovascular y el gastrointestinal. Representan nuevas dianas para fármacos en medicina molecular.

Alcance de este trabajo

En la presente tesis, las interacciones alostéricas entre los receptores componentes de los homos y heterocomplejos de los receptores D2R -5-HT2AR, GalR2-NPYY1R, A2AR-D2R, A2AR-D2R-mGluR5 y A2AR-D2R-Sigma1R complejos de heteroreceptores son exploradas y evidenciadas mediante el uso métodos bioquímicos, de biología molecular y neurofarmacología. Los métodos para el estudio de las interacciones receptores-receptores en modelos celulares utilizados a lo largo del presente trabajo incluyen las modernas técnicas biofísicas de FRET y BRET. Así mismo para el estudio y comprensión de las interacciones en tejidos animales, *ex vivo*, se han empleado y optimizado los ensayos *in situ* de ligación por proximidad (*in situ* PLA). El papel relevante de las interacciones entre los complejos heteroreceptores y la β-arrestina son también descritos y estudiados mediante ensayos de microscopía confocal y BRET. Sin embargo, su incidencia en los mecanismos alostéricos que se producen a través de las interacciones receptor-receptor en los heterocomplejos permanecen aún sin descifrar.

Las nuevas evidencias experimentales expuestas enfatizan y confirman la relevancia fisiológica y patofisiológica de las modulaciones alostéricas que operan a través de las interacciones receptor-receptor en los homos y heteroreceptores. Así como la necesidad de hacer énfasis en el desarrollo de nuevas moléculas o fármacos, que consideren esta nueva realidad molecular o principio biológico de integración de la señal, para el desarrollo de terapias farmacéuticas más eficaces en el tratamiento de enfermedades mentales y neurodegenerativas.

Se aportan, además, nuevas evidencias que apoyan de manera contundente el estado actual de los conocimientos en el campo de la integración molecular a nivel sináptico mediante la formación de homo y heterocomplejos dinámicos y sus respectivas modulaciones alostéricas a través de la interfase de interacciones de sus correspondientes protómeros. Estos complejos de heteroreceptores pueden considerarse parte de las bases moleculares de los mecanismos de aprendizaje y memoria a nivel neuronal. Alteraciones o cambios en su composición, el balance o equilibrio entre las diferentes poblaciones de homo- y heteroreceptores o sus mecanismos alostéricos por variaciones en su interfase de interacción; pueden representar las bases moleculares de muchas enfermedades neurológicas o neurodegenerativas en el SNC.

Se ha tomado como modelo de estudio el sistema serotoninérgico, adenosinérgico y dopaminérgico, que a través de su densa innervación, cortical-meso-límbico, de las regiones límbicos subcorticales y el estriado dorsal, está asociado fundamentalmente a dos patologías neuronales: adicciones, depresión, esquizofrenia y el Parkinson. A partir de esto y el hecho de la elevada complejidad a nivel molecular de las membranas sinápticas, donde un alto número de complejos homo y heteroreceptores para los receptores de la dopamina han sido recientemente descritos y estudiados (*www.gpcr-hetnet.com*), se hizo imprescindible considerar que perturbaciones en algunos de estos homo y heterocomplejos pueden estar en la base de los mecanismos moleculares de estas enfermedades del SNC. Se sabe que el receptor de la dopamina tipo D2R, serotonina tipo 5-HT1A y adenosina tipo A2A pueden ser considerados como un receptor "hubs", que se caracteriza por una gran plasticidad para las interacciones con diversos tipos de receptores, no solo dentro de la propia familia de los GPCR, sino también con otras proteínas y receptores de membranas como los canales iónicos, los transportadores, proteínas RAMPS, etc. Cambios en los balances de diversos homos y heterocomplejos del receptor de la adenosina A2A, especialmente en la vía estriado-palidal-gabaérgica, pueden acarrear cambios no solo en los mecanismos de transmisión de la señal sino también en el control de los impulsos glutamatérgicos.

La amplia distribución de complejos heteroreceptores con interacciones receptor-receptor alostérico en el SNC representa un nuevo mecanismo molecular integrador en la membrana plasmática de las neuronas y las células gliales. Se propuso que forman la base molecular para el aprendizaje y la memoria a corto y largo plazo. Esto también es cierto para los recuerdos de drogas formados durante el desarrollo de trastornos por consumo de sustancias como los trastornos por consumo de morfina y cocaína.

Por lo tanto, el objetivo general de esta tesis fue profundizar en los aspectos moleculares de varios complejos heteroreceptores GPCR y su interacción receptor-receptor alostérico en el Sistema Nervioso Central, con especial énfasis en el papel del equilibrio entre sus complejos homo y heteroreceptores. La comprensión del equilibrio de los complejos homo y heteroreceptores de GPCR y su integración de la señal neuronal es de gran relevancia en vista de la implicación de estos complejos en varias enfermedades mentales y neurológicas.

Se consideraron los siguientes objetivos específicos:

OBJETIVO-1: Para probar la hipótesis actual sobre un papel significativo de los complejos heterorreceptores D2R-5-HT2AR en las acciones de los agonistas alucinógenos de 5-HT2AR, hemos comparado las acciones agudas in vivo de DOI con las de un agonista estándar de 5-HT2R TCB2 . Ambos pertenecen al grupo de fenetilaminas con alta afinidad por el 5-HT2AR. Los efectos agudos in vivo de DOI y

TCB2 en dosis que producen efectos similares en la locomoción125,126 se estudiarán en los complejos heterorreceptores D2R-5-HT2AR en el núcleo accumbens en vista de sus neuronas de recompensa y anti-recompensa, entre otras cosas involucradas. en la interpretación de la relevancia de los estímulos sensoriales usando el ensayo de ligadura de proximidad (PLA) (Capítulo 1, Anexo 3).

OBJETIVO-2: Evaluar las acciones beneficiosas de la coadministración intranasal (i.n.) de agonistas GALR2 e Y1R sobre el rendimiento de la memoria espacial y sus efectos sobre la proliferación de células neuronales y las densidades de los complejos heteroreceptores GALR2/Y1R dentro de giro dentado del hipocampo dorsal (Anexo 4).

OBJETIVO-3: Explorar si el protómero del receptor de adenosina A2A (A2AR) juega un papel importante en la modulación de la densidad y la interacción receptor-receptor alostérico dentro del componente heteromérico D2R-mGluR5 del complejo A2AR-D2R-mGluR5 in vitro y en vivo. Además, para confirmar si los protómeros A2AR y mGluR5 interactúan y modulan el reconocimiento y la señalización del protómero D2R al formar un complejo trimérico a partir de estos receptores. Además, usar ratones knockout para A2AR para comprender el papel funcional de mGluR5 y A2AR en la mejora del bloqueo de D2R que da como resultado la catalepsia. (Capítulo 2, Anexos 1-2).

OBJETIVO-4: El trabajo anterior indicó que el tratamiento agudo con el estabilizador de monoamina OSU-6162 (5 mg/kg) a través de su alta afinidad por el Sigma1R, aumentó significativamente la densidad de los complejos heterorreceptores D2R-Sigma1R y A2AR-D2R de la capa accumbal después de la autoinyección de la cocaína. -administración. Las acciones ex vivo del agonista A2AR CGS21680 también sugirieron la existencia de interacciones alostéricas antagónicas A2AR-D2R mejoradas después del tratamiento con OSU-6162 en la autoadministración de cocaína. Sin embargo, el tratamiento de tres días con OSU-6162 (5 mg/kg) no logró alterar los efectos conductuales de la autoadministración de cocaína. Para probar si el tratamiento combinado con una dosis baja de OSU-6162 (2,5 mg/kg) y CGS21680 (0,05 mg/kg) no logra alterar la autoadministración de cocaína y aún produce cambios significativos en los complejos heterorreceptores A2AR-D2R y en sus interacciones antagónicas receptor-receptor alostérico. Tales resultados validarían el trabajo anterior y abrirían una nueva comprensión de las heterogeneidades en las neuronas GABA ventrales estriatal-palida en cuanto a las respuestas a la autoadministración de cocaína. (Capítulo 3, Anexo 1).

A modo de conclusiones

A modo de conclusiones de la presente tesis de doctorado, y tomando en cuenta todos los resultados experimentales de los últimos tres años, podemos concluir que:

I. De acuerdo con la hipótesis de la dopamina de la esquizofrenia, los receptores D2 accumbales son objetivos relevantes para los fármacos antipsicóticos. Por lo tanto, se propone que los heterocomplejos accumbales 5-HT2AR-D2R pueden ser un objetivo relevante para los agonistas alucinógenos de 5-HT2AR además de un objetivo para los fármacos antipsicóticos atípicos con alta afinidad, especialmente para el 5-HT2AR que conduce al bloqueo de su señalización. Por lo tanto, se compararon los efectos de un agonista estándar de 5-HT2AR TCB-2 y un agonista alucinógeno de 5-HT2AR DOI L en los heterocomplejos 5-HT2AR-D2R en el núcleo accumbens con el objetivo de descubrir acciones diferenciales utilizando el Ensayo de ligadura de proximidad. Los resultados revelan un aumento significativo por el tratamiento con DOI pero no con TC-2 en la densidad de los complejos heteroreceptores D2R-5-HT2AR en el núcleo accumbens. Esta acción fue bloqueada por un antagonista del receptor 5-HT2A, MDL-100907, conocido previamente por contrarrestar las acciones mediadas por DOI. Los hallazgos actuales indican que los heterocomplejos D2R-5-HT2AR en la capa del núcleo accumbens, pero no en el núcleo, pueden ser un objetivo para las acciones alucinógenas de DOI. Parece posible que los agonistas alucinógenos de 5-HT2AR puedan contribuir al desarrollo de la psicosis a través de la mejora de la señalización mediada por Gi/o del protómero D2R en los heterocomplejos D2R-5-HT2AR del núcleo accumbens. El bloqueo selectivo del sitio de unión del agonista del protómero 5-HT2AR alucinógeno puede ofrecer un tratamiento novedoso para la esquizofrenia.

II. La necesidad de nuevos enfoques terapéuticos es necesaria para las condiciones de demencia y los déficits de memoria de diferentes orígenes, como la enfermedad de Alzheimer. Hay mecanismos fisiopatológicos complejos involucrados que afectan la neurogénesis del hipocampo adulto, en los que parecen participar los neuropéptidos y su regulación de la neurogénesis. El receptor Y1 del neuropéptido Y(NPY) (Y1R) y el receptor 2 de galanina (GAL) (GALR2) interactúan en las regiones cerebrales responsables de los procesos de aprendizaje y memoria, con énfasis en el hipocampo. Además, un desafío significativo para los tratamientos que involucran fármacos peptídicos es eludir la barrera hematoencefálica. El estudio actual evalúa el rendimiento de la memoria sostenida inducida por la coadministración intranasal de los agonistas GALR2 y NPYY1R y sus correlatos hipocampales neuroquímicos. La recuperación de la memoria se llevó a cabo en la tarea de objeto en el lugar junto con el ensayo de ligadura de proximidad in situ (PLA) para manifestar la formación de complejos heterorreceptores GALR2/Y1R y su dinámica bajo los diferentes tratamientos. Evaluamos la proliferación celular a

través de un estudio de expresión de 5-Bromo-2'-desoxiuridina (BrdU) dentro del giro dentado del hipocampo dorsal. Se demostró que el agonista de GalR2 M1145 actúa con el agonista de Y1R para mejorar la recuperación de la memoria a las 24 horas en la tarea de objeto en el lugar. Nuestros datos muestran que la administración intranasal es una técnica factible para administrar directamente compuestos de galanina o neuropéptido Y en el SNC. Además, observamos la capacidad del tratamiento con coagonistas para mejorar la proliferación celular en el DG del hipocampo dorsal a través de 5- Bromo- Análisis de expresión de 2'-desoxiuridina (BrdU) a las 24 horas. La comprensión de los mecanismos celulares se logró analizando los complejos heterorreceptores GALR2/Y1R tras la coactivación agonista de sus dos tipos de protómeros receptores en neuroblastos que expresan doblecortina. Nuestros resultados pueden proporcionar la base para desarrollar farmacóforos agonistas heterobivalentes, dirigidos a los heterocomplejos GALR2-Y1R. Se trata especialmente de las células precursoras neuronales del giro dentado en el hipocampo dorsal para el tratamiento novedoso de patologías neurodegenerativas como la enfermedad de Alzheimer.

III. El receptor de adenosina A2A (A2AR), el receptor de dopamina D2 (D2R) y el receptor metabotrópico de glutamato tipo 5 (mGluR5) forman complejos heteroreceptores A2AR-D2R-mGluR5 en células vivas y en neuronas del estriado de rata. En el estudio actual, presentamos datos experimentales que respaldan la opinión de que el protómero A2AR juega un papel importante en la modulación inhibitoria de la densidad y la interacción receptor-receptor alostérico dentro del componente heteromérico D2R-mGluR5 del complejo A2AR-D2R-mGluR5 en vitro e in vivo. Los protómeros A2AR y mGluR5 interactúan y modulan el reconocimiento y la señalización del protómero D2R al formar un complejo trimérico a partir de estos receptores. La expresión de A2AR en células HEK293T que coexpresan D2R y mGluR5 resultó en un aumento significativo y marcado en la formación del componente heteromérico D2R-mGluR5 tanto en la transferencia de energía de resonancia bioluminiscente como en los ensayos de ligadura de proximidad. Se encontró un aumento muy significativo de los valores del componente de alta afinidad de D2R (D2RKi High) tras el cotratamiento con los agonistas mGluR5 y A2AR en las células que expresan A2AR, D2R y mGluR5 con un efecto significativo observado también con el agonista mGluR5 solo en comparación con células que expresan sólo D2R y mGluR5. En las células que coexpresan A2AR, D2R y mGluR5, la estimulación de las células con un agonista de mGluR5 o un antagonista de D2R contrarrestó por completo la inhibición inducida por el agonista de D2R de los niveles de AMPc, lo que no fue cierto en las células que solo expresaban mGluR5 y D2R. De acuerdo, el modulador alostérico negativo mGluR5 raseglurant redujo significativamente la catalepsia inducida por haloperidol en ratones y en ratones

knockout para A2AR la acción del haloperidol casi había desaparecido, lo que respalda un papel funcional para mGluR5 y A2AR en la mejora del bloqueo de D2R que da como resultado la catalepsia. Los resultados representan un ejemplo relevante de actividad integradora dentro de complejos heterorreceptores de orden superior.

IV. Trabajos previos indicaron que el tratamiento agudo con el estabilizador de monoamina OSU-6162 (5 mg/kg) a través de su alta afinidad por Sigma1R aumentó significativamente la densidad de los complejos heteroreceptores D2R-Sigma1R y A2AR-D2R de capa accumbal después de la autoadministración de cocaína. Las acciones ex vivo del agonista A2AR CGS21680 también sugirieron la existencia de interacciones alostéricas antagónicas A2AR-D2R mejoradas después del tratamiento con OSU-6162 en la autoadministración de cocaína. Sin embargo, el tratamiento de tres días con OSU-6162 (5 mg/kg) no logró alterar los efectos conductuales de la autoadministración de cocaína. Para probar estos resultados y la relevancia de las interacciones agonistas de OSU-6162 (2,5 mg/kg) y/o A2AR (0,05 mg/kg), el tratamiento con estas dosis bajas de los agonistas del receptor se realizó en la autoadministración de cocaína y neuroquímicos y conductuales. efectos estudiados. No se demostraron efectos en la autoadministración de cocaína, pero el cotratamiento indujo aumentos marcados y altamente significativos mediante el ensayo de ligadura de proximidad (PLA) en la densidad de los heterocomplejos A2AR-D2R en el núcleo accumbens. También se observaron disminuciones significativas en la afinidad de los sitios de unión del agonista de alta afinidad D2R. Por lo tanto, en dosis bajas, los efectos neuroquímicos altamente significativos observados en el cotratamiento con agonistas A2A y Sigma1R en los heterocomplejos A2AR-D2R y su potenciación de la inhibición alostérica de la unión de alta afinidad de D2R no están relacionados con la modulación de la autoadministración de cocaína. En cambio, estas dosis bajas de los agonistas Sigma1R y A2AR pueden haber modulado selectivamente los heterocomplejos A2AR-D2R de distintas poblaciones neuronales de las neuronas GABA del estriado palidal ventral que tienen una mayor afinidad por estos agonistas de los receptores. Sus funciones en las redes de capa accumbal aún no se han determinado, pero los heterocomplejos A2AR-D2R-Sigma1R pueden tener un papel único en la alteración de ciertas acciones de abuso de la cocaína, relacionadas con la modulación de distintas proyecciones de estas neuronas GABA ventrales estriatal-palidal.

Las nuevas evidencias experimentales expuestas enfatizan y confirman la relevancia fisiológica y patofisiológica de las modulaciones alostéricas que operan a través de las interacciones receptor-receptor y el balance o equilibrio entre poblaciones de homo y heterocomplejos. Así como la necesidad de hacer énfasis en el desarrollo de nuevas moléculas o fármacos, que consideren esta nueva realidad molecular o principio biológico de integración de la señal, para el desarrollo de

terapias farmacéuticas más eficaces en el tratamiento de enfermedades mentales y neurodegenerativas.

En resumen, el presente trabajo mediante el estudio de una seria de complejos de homo y heteroreceptores en el SNC, aporta las bases experimentales suficientes para demostrar e ilustrar que los mecanismos alostéricos mediados por interacciones "receptor-receptor" en complejos multiprotéicos desempeñan un importante papel en la integración de la señal y en los mecanismos de selectividad y diversidad de los protómeros involucrados. También se abre la puerta al desarrollo de nuevos fármacos y terapias en los que se tengan en cuenta estas particularidades.

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. The Balance of MU-Opioid, Dopamine D2 and Adenosine A2A Heteroreceptor Complexes in the Ventral Striatal-Pallidal GABA Antireward Neurons May Have a Significant Role in Morphine and Cocaine Use Disorders. Borroto-Escuela DO, Wydra K, **Fores-Pons R**, Vasudevan L, Romero-Fernandez W, Frankowska M, Ferraro L, Beggiato S, Crespo-Ramirez M, Rivera A, Rocha LL, Perez de la Mora M, Stove C, Filip M, Fuxe K. *Front Pharmacol*. 2021 Mar 15;12:627032. doi: 10.3389/fphar.2021.627032. eCollection 2021.

II. Serotonin Heteroreceptor Complexes and Their Integration of Signals in Neurons and Astroglia-Relevance for Mental Diseases. Borroto-Escuela DO, Ambrogini P, Narvaez M, Di Liberto V, Beggiato S, Ferraro L, **Fores-Pons R**, Alvarez-Contino JE, Lopez-Salas A, Mudò G, Díaz-Cabiale Z, Fuxe K. *Cells*. 2021 Jul 27;10(8):1902. d10.3390/cells10081902.

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

IV. Intranasal Delivery of Galanin 2 and Neuropeptide Y1 Agonists Enhanced Spatial Memory Performance and Neuronal Precursor Cells Proliferation in the Dorsal Hippocampus in Rats. Borroto-Escuela DO, **Fores R**, Pita M, Barbancho MA, Zamorano-Gonzalez P, Casares NG, Fuxe K, Narváez M. *Front Pharmacol*. 2022 Feb 14;13:820210. doi: 10.3389/fphar.2022.820210. eCollection 2022.

V. The mGlu5 receptor protomer mediated dopamine D2 receptor trans-inhibition is dependent on the adenosine A2A receptor protomer: implications for Parkinson's disease. Wilber Romero-Fernandez, Jaume J. Taura, René A. J Crans, Marc Lopez-Cano, **Ramon Fores-Pons**, Manuel Narváez, Jens

Carlsson, Francisco Ciruela Alferez, Kjell Fuxe and Dasiel O. Borroto-Escuela. (ACCEPTED FOR PUBLICATION May/10/2022. *Molecular Neurobiology*).

VI. Differential acute effects of a standard 5-HT2A receptor agonist TCB-2 and a hallucinogenic agonist DOI on 5-HT2AR-D2R heteroreceptor complexes in the nucleus accumbens of the rat forebrain. Michael Plach, **Fores-Pons, R**., Lopez-Salas, A., Manuel Narváez, Kristina Friedland, Kjell Fuxe and Dasiel O. Borroto-Escuela. (SUBMITTED FOR PUBLICATION IJMS, May/03/2022).

VII. Combined treatment with low doses of OSU-6162, a Sigma1R ligand, and an A2AR agonist fails to alter cocaine self-administration but increases accumbal A2AR-D2R heteroreceptor complexes and their antagonistic allosteric receptor-receptor interactions. Dasiel O. Borroto-Escuela, **Ramon Fores-Pons**, Wilber Romero-Fernandez, Karolina Wydra, Zilong Zhou, Malgorzata Frankowska**,** Agata Suder, Alexander Lopez-Salas, Malgorzata Filip and and Kjell Fuxe. (SUBMITTED FOR PUBLICATION PBB, May/06/2022).

INTRODUCTION

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

There exists substantial evidence for the existence of G protein-coupled receptor (GPCR) homo and heteroreceptor complexes with allosteric receptor-receptor interactions in the Central Nervous System (CNS) [1-11]. GPCR heteroreceptor complexes may also include ion channel receptors, receptor tyrosine kinases (RTKs), sets of G protein interacting proteins, ion channels and/or transmitter transporters [9,12-19] expanding their integrative capability [7,14,17,20,21]. They embody a new biological principle to integrate biological signals in all tissues [22,23].

Allosteric receptor–receptor interactions within GPCR heteroreceptor complexes lead to novel receptor dynamics during which the receptor protomers alter their recognition, pharmacology, signalling and trafficking so, novel allosteric binding sites could develop [13,24-27]. This leads to diverse heteroreceptor complexes signaling and to a specific integrated response at the molecular level [28]. Allosteric receptor-receptor interactions in heteroreceptor complexes are distintive for each heteroreceptor complexes. Either antagonistic or facilitatory receptor-receptor interactions may improve upon agonist coactivation of the receptor protomers [29-31] (**Figure 1**).

The allosteric receptor-receptor interactions are usually affected in a dynamic way by activation of their respective ligands, especially upon coactivation of the two receptor protomers [27,28,32]. They also markedly transform when a receptor complex moves from a dimer to a trimer and when the amount of adapter protein increases in the complex like cocaine recruitment of Sigma 1R into the A2AR-D2R heteroreceptor complex [33,34]. The allosteric interactions in such dynamic higher order receptor complexes take place in a coordinated spatio-temporal fashion, giving a new dimension to molecular neuroscience and brain integration (**Figure 1**).

There is some missing knowledge on the stoichiometry of the participating receptor protomers in GPCR heteroreceptor complexes. Nevertheless, to-day super-resolution imaging methods [35] and spatial intensity distribution analysis [36] have been developed. This may be used to understand the stoichiometry in cellular models. The structural determinants that make the desicion if a receptor pair forms a heteromer or not, exist in the receptor interface that mediates the allosteric receptor-receptor interaction [37]. A substantial amount of work has been devoted to distinguish the key residues involved, including the development of a model of the A2AR-D2R heterodimer [38]. It is recognized that the transmembrane helices in class A, B and C

dimers play a significant role (www.gpcr-hetnet.com)[4]. Interfaces usually acquire complementary pockets in the core of the interface. The remained amino acids that reconcile the binding between the two interfaces are called hot spots and may be isolated from the surrounding solvent by adjacent amino acids [39]. Based on bioinformatic methods and original software Borroto-Escuela and Fuxe stablished the protriplet puzzle theory, affirming that parts of triplet amino acid homologies may form hot spots and importantly contribute withing in receptor-receptor interactions in receptor heteromers and other types of protein-protein interactions [40-43].

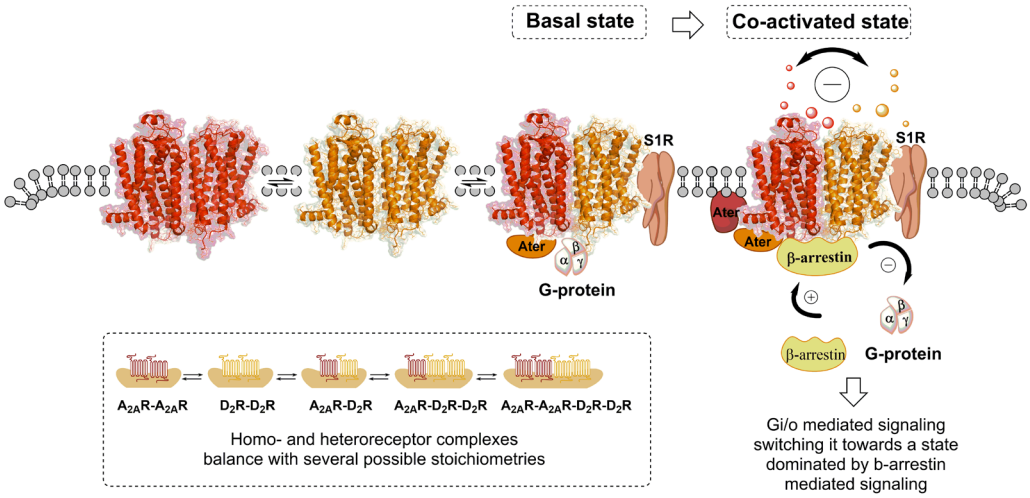


Figure 1. Illustration of the antagonistic allosteric receptor-receptor interactions in the $A_{2A}R$-D_2R heteroreceptor complexes with several possible stoichiometries from heterodimers to higher order heteromers of various types (heterotrimer and heterotetramer are shown) with or without the participation of adapter proteins (Ater), like for example the Sigma1R. These proteins may participate in the mediation of the allosteric interaction by e.g., guiding the receptors towards each other through a scaffolding function. Such actions may also regulate the time of the heteromerization from being transient to becoming more stable and long lasting. The major allosteric interaction appears to be an antagonistic $A_{2A}R$-D_2R interaction by which the $A_{2A}R$ protomer inhibits the D_2R protomer recognition (reduced affinity) and Gi/o mediated signaling switching it towards a state dominated by beta-arrestin mediated signaling. The heterocomplexes are in balance especially with the corresponding $A_{2A}R$ and D_2R homoreceptor complexes but also with other colocated D_2R heterocomplexes and $A_{2A}R$ heterocomplexes in the synapses and their extrasynaptic regions in the striato-pallidal GABA neurons. The text and the figure have been modified from [13].

The development of homo-and heteroreceptor complexes in a synaptic or extrasynaptic area of the plasma membrane is mediated by several factors, especially by the density of the participating receptor protomers [13]. Another factor is the affinity of one receptor protomer for another protomer, which is connected to the number of hot spots that can develop in the receptor interface [38]. The presence, or absence, of some adapter proteins in the heteroreceptor complex may lead to a significant factor to determine the affinity that develops between two or more receptor protomers [38].

With an increase in the binding affinity between the two receptor protomers an increase in the number of receptor complexes can develop. Receptor agonists can modulate receptor complexes through conformational changes as stablished in the BRET experiments [21,44,45].

Usually, there is a competition between different receptor complexes for the same receptor protomer since they are balanced with each other [5,27,46-48]. This is the case of a heteromer and its corresponding homomers and different heteromers sharing one or two receptor protomers [33,47]. There is a necessity to improve our understanding of the molecular organization of the receptor oligomers, their allosteric communication and the features of the receptor interface [4,40,49].

2. Homo-and heteroreceptor complexes allosteric mechanisms

Adaptor/chaperone proteins like Sigma1R, RAMP1 and Sigma2R that bind to the receptor protomers may modulate substantially the receptor-receptor interactions [50-52]. Allosteric mechanisms were suggested to mediate the reciprocal receptor-receptor interactions via the receptor interface of homo-heteroreceptor complexes over which the allosteric waves passed based on the early work on allosteric transitions in the 1960s [53,54], updated in 2009 [55]. The allosteric receptor-receptor interactions take place when the binding of a ligand to an orthosteric site or an allosteric site induces a conformational change in the partner receptor protomer after passing the receptor interface (see **Figure 1**). Several allosteric changes can be induced in the partner receptor with e.g., changes in affinity and/or signalling efficacy. In case of allosteric enhancement of orthosteric ligand binding in receptor heteromers the terms positive cooperativity or agonistic allosteric modulation are often used while in case of allosteric antagonism negative cooperativity or antagonistic allosteric modulation can be used as well (see [2,56-63]. Heterodimerization of somatostatin and opioid receptors were also found to allosterically modulate phosphorylation, trafficking and desensitization of the receptor protomers [64].

Therefore, allosterism in GPCR has an impact at the intracellular signalling level [65,66]. 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 [67]. It is also known that GPCR kinases (GRK) can assist GPCRs in switching from G protein mediated signalling to beta-arrestin mediated signalling [68]. It seems most 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 [69].

3. Homo-and heteroreceptor complexes in the plasma membrane provide a molecular basis of learning and memory.

Along with the findings of intramembrane receptor-receptor interactions in receptor complexes, it became clear that this huge plasma membrane area was used for molecular integration of receptor signalling by the formation of receptor complexes from dimers to higher order oligomers. The receptor protomers as well as other proteins could communicate with each other via direct receptor-receptor and receptor-protein interactions through allosteric mechanisms. With the signal integration continued in the multiple intracellular pathways, the importance of this first step of information handling in the plasma membrane became clear.

Fuxe and Borroto-Escuela proposed the allosteric theory of learning and memory based on the existence of many oligomeric receptor complexes in the postsynaptic and extra-synaptic membranes and the allosteric receptor-receptor interactions within them [3,22,23,70,71]. They stablished that the molecular basis of learning and memory can be represented by the reorganization of the homo- and heteroreceptor complexes in the postjunctional membrane of synapses guiding to the changes in the prejunctional receptor complexes to facilitate the pattern of transmitter release to be learned [22,23]. Upon a change in the release pattern of transmitters in the synapse, learning of this new pattern will take place in the postsynaptic membrane through a reorganization of the homo and heteroreceptor complexes [22,23]. Changes will also happen in the presynaptic receptor complexes to enable the maintenance of the pattern of multiple release of transmitters to be acknowledged. Considering a kind of "basal barcode" and therefore through a transient reorganization of the receptor complexes a new barcode is obtained indicating a short-term memory. A long-term memory is developed through transformation of intracellular parts of the heteroreceptor complexes into soluble molecules that can bind to transcription factors and modulate their transcriptional actions at the DNA level. In this way novel and specific adaptor proteins can be formed willing to bind to the receptor complexes forming the short-term memory. It then becomes consolidated into a long-term memory (molecular engram) with conserved receptor-receptor interactions. The adaptor proteins may act by increasing the links between receptor protomers, receptor protomers and cytoskeletal proteins and receptor protomers and scaffolding proteins [5,22,23,72].

Thus, the homo-heteroreceptor complexes are considered as highly dynamic assemblies formed or disrupted by integrated synaptic and volume transmission signals. These events are necessary for learning and can become transformed into a consolidated rigid state with conserved allosteric communication. This will represent molecular engrams resulting in a major long-term modulation of the neuronal networks. This molecular plasticity change, whether transient or long term, can then

modify the patterns of outflow in the brain circuits and encourage transient and long-term changes in behaviors and cognitive functions. In line with this hypothesis blocking synaptic removal of GluA2-containing AMPA receptors prevents the natural forgetting of long-term memories [73]. It has been of special relevance for structural plasticity, example, the dendritic tree and its spines. This may be the recruitment of receptor tyrosine kinase to the heteroreceptor complexes formed, which may result, for example in synergistic increases in neurite densities and their protrusions in primary neuronal cultures [14,17,74].

Of high significance is the work of Everitt and colleagues [75,76] who hypothesized that drug addiction is caused by a pathological memory, called drug memory. They also proposed that understanding its molecular basis could lead to introduction of novel anti relapse therapies. The Borroto-Escuela and Fuxe´s hypothesis on the molecular basis of learning and memory is in line with their view. They imply that drug memories can be produced through a reorganization of the homo and heteroreceptor complexes in synapses and their extra-synaptic regions inter alia in glutamate synapses on the striato-pallidal GABA anti-reward neurons [33,43]. Specifically, support is grained to make clear that cocaine can produce pathological A2AR-D2R-Sigma1R complexes in such synapses. It seems to embody a long-term memory with a permanent and strong inhibition of D2R affinity and signalling, which may lead to cocaine addiction. Therefore, A2AR-D2R-Sigma1R complexes can become a target for treatment of cocaine addiction [33].

4. The class A GPCR heteromers. The example of multiple adenosine A2AR, serotonin 5-HT2AR, galanin and neuropeptide Y heterocomplexes.

4.1 Adenosine heteroreceptor complexes in the brain, especially in the dorsal and ventral striatum (see Anexo I and Anexo II)

A large number of A2AR heteromers exist and exemplify a hub component in the GPCR heterodimer network (GPCR-HetNet www.gpcr-hetnet.com [5]. The A2AR heteromers can be listed as follows: A1R-A2AR, A2AR-A2BR, A2AR-D2R, A2AR-D3R, A2AR-D4R4. They are usually named A2AR homo, iso and heteroreceptor complexes in view of the involvement of adaptor/chaperone proteins directly binding to the GPCR protomers [77]. The A2AR-D2R heteroreceptor complex is of high benefit with tremendous relevance for Parkinson disease, Schizophrenia and cocaine addiction [33,67,78,79]. This receptor complex is distinguished by a dynamic antagonistic allosteric receptor-receptor interaction by which the A2AR agonist can inhibit the D2R protomer recognition, and the Gi/o mediated signalling of the D2R protomer which instead becomes coupled to beta-arrestin 2. The dorsal striatum the A2AR-D2R heteroreceptor complexes are in balance mainly with A2A homoreceptor complexes and A2AR monomers are mainly located on the dorsal striato-pallidal

GABA neurons mediating motor inhibition. The inhibitory D2R signalling is therefore necessary to reduce activity in this neuron system and allow movements to develop. In agreement the A2AR antagonists produce antiparkinsonian actions which can involve blockade of A2AR protomer in the A2AR-D2R complex, especially in view of the reduction of the extracellular DA levels in Parkinson's disease [80].

In cocaine use disorder it was found that irreversible A2AR-D2R-Sigma1R complexes with an allosteric brake on D2R recognition and signaling are formed in higher densities in the ventral enkephalin positive striatal-pallidal GABA antireward neurons. Increased opioid heteroreceptor complexes, containing MOR-DOR, MOR-MOR and MOR-D2R, and their balance with each other and A2AR-D2R complexes in the striatal-pallidal enkephalin positive GABA antireward neurons, are proposed to be markers for development of morphine and cocaine use disorders. We suggest that increased formation of MOR-DOR complexes takes place in the striatal-pallidal enkephalin positive GABA antireward neurons after chronic morphine treatment in part through recruitment of MOR from the MOR-D2R complexes due to the possibility that MOR upon morphine treatment can develop a higher affinity for DOR. As a result, increased numbers of D2R monomers/homomers in these neurons become free to interact with the A2A receptors found in high densities within such neurons. Higher numbers of A2AR-D2R heteroreceptor complexes are formed and aid to enhanced firing of these antireward neurons due to loss of inhibitory D2R protomer signaling which finally leads to the development of morphine use disorder. Development of cocaine use disorder may instead be reduced through enkephalin induced activation of the MOR-DOR complex inhibiting the activity of the enkephalin positive GABA antireward neurons.

Confirming the role of the balance of D2R-MOR, MOR-DOR and A2AR-D2R heteroreceptor complexes, including their corresponding homoreceptor complexes, in the GABA antireward neurons appears to be of high significance for understanding the molecular basis of morphine and cocaine use disorder. Therefore, we should consider these receptor protomers as new targets for novel treatments of these brain diseases. Patients suffering from morphine dependence can become more dependent on morphine actions at MOR protomers in MOR-DOR and MOR-MOR complexes in the antireward GABA neurons in view of increased expression of A2AR-D2R complexes antagonizing inhibitory D2R signaling. Therefore, morphine may produce inhibition of these neurons most likely through the activation of the Gi/o and Galphaz mediated inhibitory signaling of MOR in a receptor complex with DOR. The integration of signaling in D2R-MOR complex remains to be stablished but is proposed to be reduced in density in morphine withdrawal due to a postulated increase in hypersensitive MOR-MOR homoreceptor complexes. Overall, the activation of the A2AR-D2R complex in the striatal-pallidal GABA antireward

neurons by favoring antireward and aversion may reduce morphine induced reward produced via activation of the MOR homo- and heteroreceptor complexes, inhibiting activity in these antireward neurons.

4.2. Serotonin 5-HT2AR heteroreceptor complexes in the brain (see Anexo III)

The heteroreceptor complexes present a novel biological principle for signal integration. These complexes and their allosteric receptor-receptor interactions are bidirectional and novel targets for treatment of CNS diseases including mental diseases. In 2010 two groups [81,82], by means of FRET and BRET methods, respectively, provided the first evidence for the existence of the DA D2R-5-HT2AR heteromer in cellular models. Electrostatic interactions between the third intracellular loop of the D2R protomer and the C-tail of the 5-HT2AR protomer [81] as observed in A2AR-D2R heterocomplexes [83] and triplet amino acid homologies in transmembrane regions [82], appeared to be involved in forming the receptor interface. They also assembled into functionally interacting heteromers. Their existence in the brain was proposed based *inter alia* on their colocalization in the medial prefrontal cortex [81].

It is of interest that hallucinogenic drugs, like d-Lysergic acid diethylamide (LSD) and those of the indole-alkyl-amine type like psilocybin, proposed to act as postsynaptic 5-HTR agonists [84,85] turned out to be 5-HTR agonists that mainly target the 5-HT2AR subtype [86]. The effects of the standard 5-HT2AR agonist TCB2 were therefore compared with those of the hallucinogenic 5-HT2AR agonists LSD and 2,5-Dimethoxy-4-iodoamphetamine (DOI) on the biochemical binding and signaling of the D2R protomer in the D2R-5-HT2A heteroreceptor complexes. Only the hallucinogenic 5-HT2AR agonists were able to increase the Bmax values of the D2R antagonist binding sites and to increase the affinity of the high affinity D2R protomer agonist binding sites in the ventral and dorsal striatum and HEK293 cells [87]. Based on these findings the hypothesis was introduced that hallucinogenic 5-HT2AR agonists at least in part induce their psychotic-like actions by being biased 5-HT2AR agonists at the orthosteric binding site of the 5-HT2AR protomer of the D2R-5-HT2AR heterocomplex, especially located in the ventral striatum. This biased property of the hallucinogenic 5-HT2AR agonists leads to the activation of a facilitatory allosteric mechanism that increases the density and the affinity of the high affinity component of the D2R protomer, associated with an enhancement of D2R protomer signaling mediated via Gi/o [87,88].

Evidence for the existence of 5-HT1AR-5-HT2AR heterocomplexes in brain has been gathered within dorsal hippocampus and the anterior cingulate cortex of the rat [89], which are well known for their important participation in the biology of MDD [90]. The existence of this heterocomplex was validated by BRET in HEK293T cells [89]. In turn, functional studies demonstrated antagonistic allosteric receptor-receptor interactions between the two 5-HT protomers, whereby agonist activated 5-HT-2AR

reduced the affinity of the 5-HT1A protomer for ipsapirone, a 5-HT1AR partial agonist [89] (**Figure 2**). In view of these results it is of considerable interest that in mice previously exposed to a post-traumatic stress model treatment with WAY100635, a 5-HT1A receptor antagonist, enhances the expression of 5-HT2AR and at the same time reduces the 5-HT1AR expression within the hippocampus. These results support the existence of antagonistic interactions between the 5-HT1A and 5-HT2A receptor protomers in the hippocampus and indicate that the 5-HT2A receptor mediated inhibition of the 5-HT1A receptor protomer can be involved in producing posttraumatic stress.

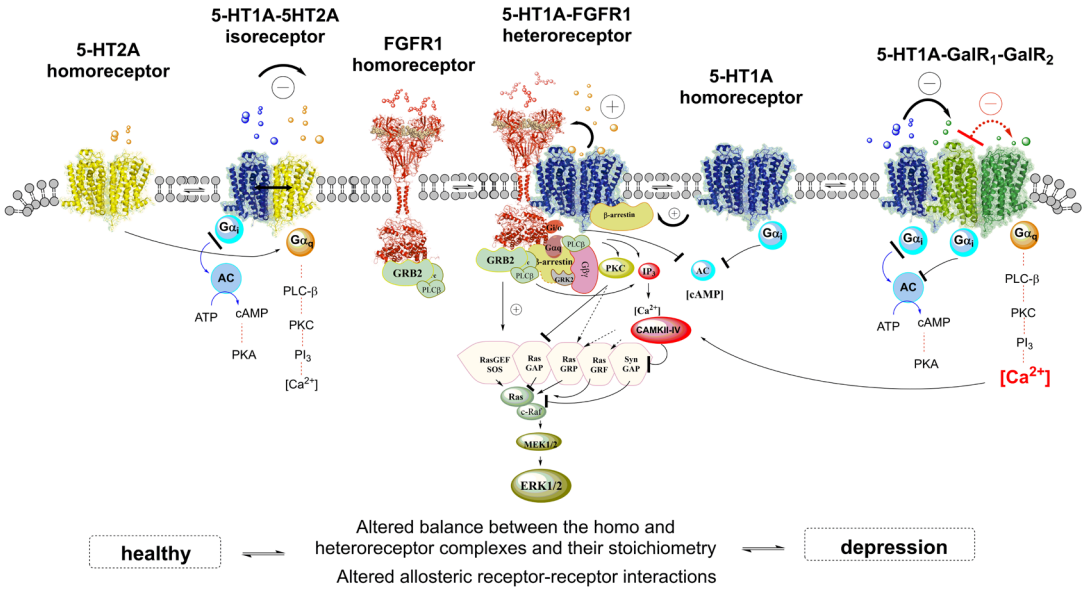


Figure 2. Panorama of serotonin homo and heteroreceptor complexes of relevance for Major Depressive Disorders (MDD) with a location in the plasma membrane. Recent work has strongly indicated the impact of 5-HT1A-5-HT2A isoreceptor complexes and the 5-HT1A-FGFR1, 5-HT1A-GalR1-GalR2 heteroreceptor complexes and their balance between each other in MDD. Significant molecular mechanism is represented by allosteric receptor-receptor interactions within the heteroreceptor complexes. Inhibitory and enhancing allosteric receptor-receptor interactions develop in the 5-HT1A-5HT2A and 5-HT1A-FGFR1 heteroreceptor complexes, respectively. In the trimeric 5-HT1A-GalR1-GalR2 heteroreceptor complexes, a cascade of inhibitory interactions take place. The 5-HT1A protomer produced a negative allosteric modulation of the GalR1 protomer, inducing a removal of the inhibitory allosteric interactions between the GalR1 and GalR2 protomers (indicated as dash red lines), leading to antidepressant events. Another molecular mechanism of strong interest is the potential disbalance that may develop between the various types of homo and heteroreceptor complexes. These molecular mechanisms involve both changes in receptor protomers recognition, signaling (including the panorama of interacting proteins and the intracellular cascades) and internalization. These events are taking place in key neuronal pathways of the limbic system, having an important role in emotional events.

Additionally, it is rather interesting that a reduction in the density of PLA positive clusters was also found in the CA1 and CA2 regions of the hippocampus 2

hours after the exposure of the rats to the Forced Swim Test [89] pointing to a likely rapid participation of the 5-HT1AR-5-HT2AR heterocomplex in the pathophysiology of MDD. Thus, considering that an elevated 5-HT1AR activity is associated with antidepressant effects and that a depressant-like behavior is linked to an enhancement of 5-HT2AR activity it was proposed by Borroto-Escuela et al. that heterobivalent drugs having both 5-HT1a agonistic and 5-HT2a antagonistic pharmacophores may be useful for the treatment of MDD [5].

4.3. GalR1 heteroreceptor complexes in the brain (see Anexo III).

Accumulating evidence has shown that Galanin, referred here to Gal (1-29)[91], binds with high affinity to several receptor subtypes designated as GalR1, GalR2 and GALR3. Gal (1-29) has also a critical role in the mesencephalic serotonergic neurotransmission since it has the ability to reduce firing in ascending 5-HT neurons [91-93]. Gal (1-29) receptors are widely distributed in the brain including some regions having a special involvement in MDD such as the dorsal raphe, where the coexistence of Gal (1-29) and 5-HT was demonstrated [5,94,95]. GALR1 and GALR3 signal through the inhibitory G-protein Gi/o, whereas GALR2 activates excitatory G protein Gq/11.

The GalR1-GalR2 heterodimer and the putative GalR1-GalR2-5-HT1 heteroreceptor complexes are targets for Galanin N-terminal fragment Gal (1-15), a major modulator of emotional networks in models of mental disease. Gal (1-15) has a high affinity for and preferentially activates the GalR1-GalR2 heterodimer located especially in the raphe-limbic-cortical systems and has a significant role in producing anxiety and depression related behaviors [93,96] (**Figure 2**). GalR1 activation produces depressive actions while GalR2 stimulation induces antidepressant effects [97]. The reason for the stronger depressive effects of the Gal (1-15) fragment compared with parent galanin peptide [97] may be its ability to preferentially activate with higher affinity and efficacy the GalR1 protomer *vs* the GalR2 protomer of the GalR1-GalR2 complex [94] (Figure 1). This should also lead to increased allosteric inhibition of the GalR2 protomer signaling with its antidepressant activity.

It should be noted that high affinity galanin fragment binding sites were observed also in brain regions with few galanin high affinity binding sites like dorsal hippocampus, striatum and cerebral cortex [93]. In regions with both galanin fragment and galanin binding sites, the galanin fragment was more potent than galanin in reducing the affinity of the high affinity 5-HT1AR agonist binding sites [98,99]. In line with these results, only the galanin fragment but not galanin was able to reduce the high affinity 5-HT1AR agonist binding sites in the hippocampus [92].

The mechanism for the ability of the Gal (1-15) fragment to enhance the antidepressant effects of the 5-HT1AR agonist [100] is unknown but it may be that the combined treatment enhances GalR2 signaling via an allosteric mechanism in the putative GalR1-GalR2-5-HT1AR complex. The 5-HT1AR agonist may preferentially

enhance the GalR2 protomer signaling which in turn can allosterically inhibit the GalR1 protomer signaling coupled to Gi/o, causing inhibition of the cAMP-PKA signaling pathway and leading to depressive effects.

4.4. NPY heteroreceptor complexes in the brain (see Anexo III).

NPY is a 36 amino acid neuropeptide, which was originally extracted from the brain by Tatemoto et al. (1982) [101,102]. It has been encountered in many brain regions, particularly in the hippocampus and the raphe nuclei [93,103]. In the hippocampus it has been found in a large population of neurons within the polymorphic layer of the dentate gyrus. Furthermore, in line with these findings, NPYY1 receptors are also expressed in this anatomical location. Behaviorally, high NPY receptor signaling seems to be associated in rat and mouse with decreased immobility in the Forced Swim Test suggesting that it plays an important role in the modulation of mood [3,98,99,104].

Recent work by Narváez et al. [105] using receptor autoradiography, in situ hybridization and proximity ligation assay has unveiled the likely existence of a physical interaction between NPYY1R and GalR2R in the hippocampus giving rise to the formation of putative GalR2-NPYY1R heterocomplexes within the polymorphic and subgranular subregions of the dentate gyrus. In support, it was found that the i.c.v. injection of galanin increased both NPYY1R agonist binding and NPYY1R mRNA expression in the dentate gyrus. In addition, evidence was obtained by the same group suggesting that signaling through such heterocomplexes may be important for modulation of mood. It was demonstrated that despite the observations that galanin itself, as previously discussed, elicits anxiogenic effects, it can induce a strong enhancement of the NPYY1R antidepressant activity in the Forced Swim Test when co-administered together with a NPYY1R agonist.

5. Dysfunction of the GPCR heteroreceptor complexes can lead to brain diseases

A dysfunction or a disruption of the GPCR heteroreceptor complexes can be a molecular basis for a pathological change in brain circuits. For example, increase in D2R function leads to alterations in the activity of glutamate prefrontal afferents [106], followed by development of schizophrenic symptoms. Understanding GPCR heteroreceptor complexes and their dysfunction in brain disorders can lead to new strategies for its treatment and for avoiding side-effects of e.g., antipsychotics and antidepressants[107], including a way to optimize combined treatment or single use of heterobivalent drugs targeting GPCR heteroreceptor complexes in mental and neurodegenerative disorders.

With the discovery also of many 5-HT1A iso and heteroreceptor complexes, like the 5-HT1A-5-HT7 [108], the FGFR1-5-HT1A [15-18,109] and the putative trimer complex GalR1-GalR2-5-HT1A [110,111], an increased understanding of the molecular

basis of major depression was obtained based on the 5-HT hypothesis of depression [112] (**Figure 2**). Postjunctional 5-HT1A receptors can strongly contribute to the mediation of the antidepressive effects of 5-HT [113]. It is of high interest that in these heteroreceptor complexes the receptor interacting proteins, like the scaffolding protein p11 [114-116], appear to play a substantial role [13]. In human suicide victims reductions of p11 mRNA were established in hippocampus and amygdala and antidepressants enhance the expression of p11 in limbic regions of rodents [117].

6. GPCR heteroreceptor complexes as targets for drug treatment in brain diseases

The GPCR heteroreceptor complexes in the CNS have become exciting new targets for neurotherapeutics in Parkinson's disease, schizophrenia, substance use disorder, anxiety and depression opening a new field in neuropsychopharmacology [78,79,118-121]. Possible novel strategies for targeting heteroreceptor complexes in CNS disease are combined treatment with drugs targeting two receptor protomers. To enhance compliance of patients, fixed formulations of the two drugs can be developed which give optimal pharmacokinetics for a time window of long duration for the combined therapeutic actions. Dual acting drugs targeting two protomers in the receptor complex can also be developed as well as dual acting pro-drugs as a potential and novel multi-target approach to treat CNS disease [79,122,123]. Heterobivalent drugs are other options to selectively target heterodimers [118,119,124] such as D2R antagonist and A2AR agonist pharmacophors targeting A2AR-D2R heterodimers in cocaine use disorder [13].

All these novel aspects on brain communication and integration in heteroreceptor complexes lead to increased understanding of the molecular basis of diseases in the CNS and of their treatments. There is increased demand to know and understand how the brain operates at the receptor level. The heteroreceptor field is novel since receptors are usually regarded to exist as monomers and this focused review will show that the receptor field has moved into homo and heterodimers and higher order homo and heteroreceptor complexes through use of novel methodologies [47,70]. This research will therefore substantially advance the receptor field. It introduces a novel biological principle and neuropsychopharmacology which targets the heteroreceptor complexes. The homo-heteroreceptor complexes are also present inter alia in the peripheral nervous system, the endocrine, the cardiovascular and gastrointestinal systems. They represent new targets for drugs in molecular medicine.

AIMS

The widespread distribution of heteroreceptor complexes with allosteric receptor-receptor interactions in the CNS represents a novel integrative molecular mechanism in the plasma membrane of neurons and glial cells. It was proposed that they form the molecular basis for learning and short-and long-term memories. This is also true for drug memories formed during the development of substance use disorders like morphine and cocaine use disorders.

Therefore, **the overall aim of this thesis** was to gain insight into molecular aspects of several GPCR 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. The understanding of the balance of GPCR homo- and heteroreceptor complexes and their integration of neuronal signal is of a high relevance in view of the involvement of these complexes on several mental and neurological diseases.

The following specific aims were considered:

AIM-1: To test the current hypothesis on a significant role of D2R-5-HT2AR heteroreceptor complexes in the actions of hallucinogenic 5-HT2AR agonists we have compared the acute *in vivo* actions of DOI with those of a standard 5-HT2R agonist TCB2. They both belong to the group of phenethylamines with high affinity for the 5-HT2AR. The acute in vivo effects of DOI and TCB2 in doses producing similar effects on locomotion[125,126] will be studied on the D2R-5-HT2AR heteroreceptor complexes in the nucleus accumbens core and shell in view of their reward and anti-reward neurons inter alia involved in interpreting the relevance of sensory stimuli using the proximity ligation assay (PLA) (**Chapter 1, Annex 3**).

AIM-2: To assesses the beneficial actions of intranasal (i.n.) coadministration of GALR2 and Y1R agonists on sustained memory performance and their effects on neuronal cell proliferation and the densities of GALR2/Y1R heteroreceptor complexes within the dentate gyrus of the dorsal hippocampus (**Annex 4**).

AIM-3: To explore if the adenosine A_{2A} receptor ($A_{2A}R$) protomer plays a major role in the modulation of the density and the allosteric receptor-receptor interaction within the D_2R-$mGluR_5$ heteromeric component of the $A_{2A}R$-D_2R-$mGluR_5$ complex *in vitro* and *in vivo*. Also, to confirm if $A_{2A}R$ and $mGluR_5$ protomers interact and modulate D_2R protomer recognition and signalling upon forming a trimeric complex from these receptors. Furthermore, using a $A_{2A}R$ knockout mice to understand the functional role for $mGluR_5$ and $A_{2A}R$ in enhancing D_2R blockade resulting in catalepsy. (**Chapter 2, Annexes 1-2**).

AIM-4: Previous work indicated that acute treatment with the monoamine stabilizer OSU-6162 (5 mg/kg) via its high affinity for the Sigma1R, significantly increased the density of accumbal shell D2R-Sigma1R and A2AR-D2R heteroreceptor complexes following cocaine self-administration. Ex vivo actions of the A2AR agonist CGS21680 also suggested the existence of enhanced antagonistic accumbal A2AR-D2R allosteric interactions after treatment with OSU-6162in cocaine self-administration. However, the three days treatment with OSU-6162 (5 mg/kg) failed to alter the behavioral effects of cocaine self-administration. To test if combined treatment with a low dose of OSU-6162, (2.5 mg/kg), and CGS21680 (0.05 mg/kg) fails to alter cocaine self-administration while still producing significant changes in the A2AR-D2R heteroreceptor complexes and in their antagonistic allosteric receptor-receptor interactions. Such results would validate the previous work and open a new understanding of heterogeneities in the ventral striatal-pallidal GABA neurons as to responses to cocaine self-administration. (**Chapter 3, Annex 1**).

MATERIALES AND METHODS

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

Differential acute effects of a standard 5-HT2A receptor agonist TCB-2 and a hallucinogenic agonist DOI on 5-HT2AR-D2R heteroreceptor complexes in the nucleus accumbens of the rat forebrain.

Animals. Male Sprague–Dawley (derived from the licensed animal breeder Charles River, Spain), 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* except for the period of the initial training sessions when rats were maintained on limited water. 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 guideline of the Institutional Animal Ethics Committee of the University of Málaga, the Spanish Directive (Real Decretory 53/2013) and the European Communities Council Directive (Cons 123/2006/3) guidelines for accommodation and care of laboratory animals.

Drugs. The 5-HT2AR agonist (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide (TCB-2) and the selective 5-HT2AR antagonist MDL-100907 were obtained from Tocris Bioscience (UK), and the hallucinogenic 5-HT2A/2C agonist (T)-2,5-dimethoxy-4-iodophenyl-2- aminopropane (DOI) was obtained from Sigma Aldrich (Sweden). TCB-2 was dissolved in distilled water, MDL-100907 was dissolved in a few drops of acetic acid and prepared in distilled water (pH of ~6.5), and DOI was dissolved in saline. All drugs and their vehicles were administered via intraperitoneal injection at 0.5 mg/kg.

Behavioral test and surgery. Briefly, rats were intraperitoneally (i.p.) injected with an acute dose of TCB-2 or DOI (0.5 mg/kg). 10 min earlier, either vehicle (saline) or a 5-HT2A receptor antagonist (MDL-100907) had been given at a dose of 0.5 mg/kg, i.p. 20 min after TCB-2/DOI treatment, locomotor activity was assessed on the open field test. No locomotor differences were found between groups (One way ANOVA $F(4,15)=0,2$; Distance travelled in cm (Mean±SEM); Vehicle

(3205±163 cm); TCB-2 (3099±227 cm); DOI (3133±197 cm); MDL-100907 (3219±330 cm); MDL-100907+DOI (2957±204 cm). All experiments were performed according to a Latin square design, whereby every animal received each dose of the drug in a pseudo-randomized order. Immediately after, rats were anesthetized with pentobarbital (60 mg/ml, 0.1 ml/0.1 kg, i.p.) and perfused with 0.1 M PBS (pH 7.4, room temperature) using an intracardiac blood pump. After this procedure, the animals were perfused with 4% PFA in PBS at 4 °C to fix the brain tissue. The rats were then decapitated, the brains dissected and post-fixed in PFA overnight. Cryoprotection was facilitated by incubating the tissue at 4 °C in 10% sucrose in PBS (0.1 M, pH 7.4), which was replaced by a 30% sucrose solution after 24 h. Several changes of the 30% sucrose solution within the following 3 - 4 days prepared the brains for sectioning.

Tissue preparation. At first, each rat brain was placed in a cutting matrix (1 mm thick coronal slices). Then, the samples were frozen in isopentane at -45 °C for 30 s and kept at -20 °C overnight. The next day, the rat brains were cut using a Leica CM1950 cryostat at -21 °C. 30 - 40 coronal sections of cortical-striatal regions (from bregma 2.70 mm to -1.40 mm). Sections were prepared with a thickness of 35 µm each and stored in Hoffman solution at -20 °C.

In situ Proximity Ligation Assay (in situ PLA). To study the effects of the different drugs on the D2-5-HT2A heteroreceptor complex density changes, the *in situ* PLA was performed as described previously [31,47,109,127,128]. Free-floating formalin fixed brain sections (35 µm-thick, cut using a cryostat, see above) at Bregma levels given above were employed using the following primary antibodies: mouse monoclonal anti-D2R (1:250 - 1:600, Stockholm, Sweden) and rabbit anti-5-HT2AR (1:100). 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 hours 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 h at 37°C in a humidity chamber. The unbound proximity probes were removed by washing the slides twice, 5 min each

time, with blocking solution at room temperature under gentle agitation. 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., Vecta Shield 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.

Confocal imaging acquisition and analysis. Confocal imaging experiments were performed with an inverted Leica DM IRE2 confocal microscope at 20 °C. The microscope was equipped with a 40x oil immersion objective, a 405 nm UV-laser (DAPI excitation) and a 543 nm argon-laser (Texas Red excitation). Fluorescent PLA signals were visualized by a Texas Red filter (fluorophore emission at 624 nm) and DAPI stained nuclei by a shorter wavelength filter (emission at 475 nm). In each investigated brain region, first a z-stack of a 400 x 400 µm field of vision was recorded (10 images were taken over a thickness of 20 µm, 40x magnification). Within this area, 20 µm z-scan images of two representative zoomed regions (120 x 120 µm) were generated. Data was presented as PLA signals per cell per sample field. Thus, z-stack images were processed with Fiji ImageJ 1.52i (separate background correction of blue and red channels and subsequent merge to RGB). Using the Duolink Image Tool software (Olink) nuclei and red fluorescent signals were quantified adjusting individual threshold values to preclude unspecific signals. Minimum detectable signal size was set to 1 x 1 pixel. Ratios of total PLA signals to number of cells were calculated and plotted with GraphPad Prism 6.07.

Statistical analysis. Data were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). All the data are shown as means ± SEM. Data from *in situ* PLA experiments showing cluster density (clusters per nucleus per

sampled field) were analyzed using a one-way ANOVA followed by post-hoc Tukey's test. The number of rats (*n*) in each experimental condition is indicated in figure legends. The *P* value 0.05 and lower was considered significant.

II- RELATED TO SPECIFIC AIM 1 (see, Annex 4)

Intranasal Delivery of Galanin 2 and Neuropeptide Y1 Agonists Enhanced Spatial Memory Performance and Neuronal Precursor Cells Proliferation in the Dorsal Hippocampus in Rats

Materials and methods related to the experimental work performed on the analysis of the intranasal delivery of Galanin 2 and Neuropeptide Y1 agonists in the spatial memory performance and neuronal precursors cells proliferation in the dorsal hippocampus of the rat brain, as well as, the in situ proximity ligation assays for the study of GalR2-NPYR1 interactions, are described in details in Annex 4.

III- RELATED TO SPECIFIC AIM 1 (see, Chapter 2)

The mGlu5 receptor protomer mediated dopamine D2 receptor trans-inhibition is dependent on the adenosine A2A receptor protomer: implications for Parkinson's disease

Plasmid constructs. The cDNA encoding the rat $mGluR_5$ was cloned (without stop codon) in $pGFP^2$-N1 vector (PerkinElmer, Waltham, MA, United States) using standard molecular biology techniques. The D_2R^{Rluc} construct used have been described previously in Borroto-Escuela *et al.* 2010 [129].

Drugs and chemicals. The $A_{2A}R$ agonist 4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS-21680), the selective $A_{2A}R$ antagonist 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM-241385), the $mGluR_5$ agonist (*RS*)-2-Chloro-5-hydroxyphenylglycine sodium salt (CHPG), the $mGluR_5$ antagonist 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) and the D_2R antagonist 4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone hydrochloride (haloperidol) were purchased from Tocris Bioscience (UK), and the $mGluR_5$ negative allosteric modulator 2-[(3-Fluorophenyl)ethynyl]-4,6-dimethyl-3-pyridinamine hydrochloride (raseglurant) was purchased from HelloBio (Republic of Ireland). The concentrations of CGS-21680 (100 nM) and ZM-241385 (1 μM) were chosen in agreement with our previous studies [130,131]. The concentrations of CHPG (500 nM) and MPEP (300 nM) have been selected on the basis of previous studies suggesting that, in this concentration range, the compounds selectively act as agonist or antagonist of $mGluR_5$, respectively [132-

[135]. Finally, the dose of haloperidol (1mg/kg) and raseglurant (1mg/kg) used in mouse behavioural experiments were previously described [136,137]. Also, isobutyl-1-methylxanthine (IBMX) and 4-(3-butoxy-4-methoxybenzyl) imidazolidone (Ro 20-1724) were purchased from Tocris Bioscience (Bristol, UK).

Cell culture and transfection. Human embryonic kidney 293T (HEK293T cells (American Type Culture Collection, Manassas, VA, USA) cells 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 at 37 °C in an atmosphere of 5% CO_2. Cells were plated in 6-well plates (1×10^6cells/well), 96-well plates (1×10^4cells/well) or in 75 cm^2 flasks and cultured overnight prior to transfection or experimental procedures. Cells were transiently transfected using linear polyethyleneimines (Polysciences Inc., Warrington, PA, USA) according to the manufacturer's instructions.

Animals. $A_{2A}R^{-/-}$ and $D_2R^{-/-}$ mice generated on a CD-1 genetic background [136,138] and the corresponding wild type littermates weighing 20-25 g were used. The animal protocol (#7085) was approved by the University of Barcelona Committee on Animal Use and Care. Animals were housed and tested in compliance with the guidelines provided by the Guide for the Care and Use of Laboratory Animals [139] and following the European Union directives (2010/63/EU) the ARRIVE guidelines [140]. Mice were housed in groups of five in standard cages with access to food and water *ad libitum, while* maintained under a 12 h dark/light cycle (starting at 7:30 AM), 22°C temperature, and 66% humidity (standard conditions). All animal experimentation was carried out in a period comprehended between 9:00 AM to 6:00 PM by a researcher blind to drug treatments.

Locomotor activity tests. Mice spontaneous or drug-induced locomotor activity was assessed by the open field test. In brief, animals were administered intraperitoneal (i.p.) with raseglurant (1 mg/kg) or vehicle-saline with 5% DMSO and 5% Tween-20 30 min before the testing session. Non-habituated mice were placed in the center of an activity field arena (30 x 30 cm, surrounded by four 50 cm-high black painted walls) equipped with a camera above to record activity and connected to the light source. The total distance travelled was analyzed using Spot tracker function from Image J (NIH, Bethesda, MD, USA), as previously described [136].

Catalepsy test. Mouse catalepsy was induced by the administration (i.p.) of haloperidol (1 mg/kg) [136]. After one h, haloperidol-induced catalepsy was measured as the duration in seconds of an abnormal upright posture in which the forepaws of the mouse were placed on a horizontal wooden bar (0.6 cm of diameter) that was located 4.5 cm above the floor. Subsequently, mice were administered (i.p.) with either vehicle (i.e., saline with 5% DMSO and 5% Tween) or raseglurant (1 mg/kg). After 20 min a second haloperidol-induced catalepsy measurement was performed.

The rational for the use of raseglurant (a mGluR5 negative allosteric modulator) instead of a full antagonist was based on the theoretical advantages that allosteric modulators offer compared with their competitive counterparts. mGluR5 allosteric modulators (negative allosteric modulators (NAM) and positive allosteric modulators (PAM)) have the potential for greater subtype selectivity when compared to orthosteric ligands. Also, mGluR5 NAM and PAM do not possess intrinsic activity and are assumed to be quiescent in the absence of an endogenous agonist and only modulate receptor function when the endogenous agonist is present. In this manner, NAM and PAM have the potential to retain spatial and temporal aspects of endogenous receptor signaling. This is of particular interest for CNS targets where optimal neurotransmission is likely to have an improved therapeutic outcome as opposed to sustained receptor blockade or activation.

Haloperidol-induced catalepsy. Mice (n=10) were randomly assigned to treatment groups and behavioral testing was performed blind to treatment. The dopamine D_2 receptor (D_2R) antagonist, haloperidol (1 mg/kg, s.c.) was administered to induce catalepsy. Thirty minutes after the haloperidol administration, mice experienced a full cataleptic response. At this time point, for each mouse the state of catalepsy was tested by gently placing their front limbs over an 8-cm high horizontal bar. The intensity of catalepsy was assessed by measuring the time the mice remain in this position being completely immobile for a maximum of 120 s. Only mice that remained cataleptic for the entire 120 s were used for subsequent drug testing. After 30 minutes of the baseline measurement vehicle (0.5% methylcellulose and 2% DMSO) or PBF509 was administered orally via gavage (3, 10 or 30 mg/kg, p.o.) and the catalepsy was then determined at 15, 30 and 60 minutes PBF509 administration. For each time point the number of responding mice and the total cataleptic time for each animal was determined.

Membrane preparation. HEK293T cells or mouse striata were homogenized in ice-cold 10 mM Tris HCl, pH 7.4, 1 mM EDTA, 300 mM KCl buffer containing a protease inhibitor cocktail (Roche, Penzberg, Germany) using a Polytron for three periods of 10 s each. The homogenate was centrifuged for 10 min at 1000 x *g*. The resulting supernatant was centrifuged for 30 min at 12000 x *g*. The membranes were dispersed in 50 mM Tris HCl (pH 7.4) and 10 mM $MgCl_2$, washed, and resuspended in the same medium. Protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

Bioluminescence resonance energy transfer saturation assay. The bioluminescence resonance energy transfer ($BRET^2$) saturation assays were carried out using plasmids encoding for D_2R^{Rluc} and $mGluR_5^{GFP2}$ according to previously published methods [60,130,141,142]. The $netBRET^2$ ratio was defined as the BRET ratio for co-expressed Rluc and GFP^2 constructs normalized against the BRET ratio for the

Rluc expression construct alone: netBRET^2 ratio = [(GFP^2 emission at 515 ± 30 nm)/(Rluc emission 410 ± 80 nm)]-cf. The correction factor, cf, corresponds to (emission at 515 ± 30 nm)/(emission at 410 ± 80 nm) found with the receptor-Rluc construct expressed alone in the same experiment. The maximal value of BRET (netBRET^{2}max) corresponds to the situation when all available donor molecules are paired up with acceptor molecules [2]. The specificity of D_2R^{Rluc}-$mGluR_5^{GFP2}$ interactions was assessed by comparison with co-expression of A_1R^{GFP2} and D_2R^{Rluc}.

***In situ* PLA in cultured cells.** *In situ* proximity ligation assay (PLA) in cultured cells was performed using the Duolink *in situ* PLA detection kit (Sigma-Aldrich, St. Louis, MO, USA), following the protocol described previously [47,61,143] using mouse monoclonal anti-D_2R (X μg/ml, MABN53; Millipore, Billerica, MA, USA) and rabbit polyclonal anti-$mGluR_5$ (X μg/ml, AB5675; Millipore) primary antibodies. PLA control experiments employed only one primary antibody. The PLA signal was visualized and quantified by using a TCS-SL confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) and the Duolink Image Tool software. High magnifications of the microphotograph were taken and visualized using multiple z-scan projections.

Immunohistofluorescence and *in situ* PLA in mouse brain. Mice were anesthetized and intracardially perfused with 50-200 ml of ice-cold 4% formaldehyde solution (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS; 1.47 mM KH_2PO_4, 8.07 mM Na_2HPO_4, 137 mM NaCl, 0.27 mM KCl with pH 7.2). The brains were post-fixed overnight in the same 4% formaldehyde solution at 4°C. The vibratome (Leica Lasertechnik GmbH, Heidelberg, Germany) was used to make coronal section (50 μm). Slices were collected and kept in Walter's antifreezing solution (30% glycerol, 30% ethylene glycol in PBS with pH 7.2) at -20°C until further processing [144].

For immunohistofluorescence (IHF) experiments coronal brain slices were washed three times with PBS for 10 min at 22°C, then permeabilized with 0.3% Triton X-100 in PBS (2 hours at 22°C) and rinsed (3 x) with washing solution (PBS containing 0.05% Triton X-100, 10 min, at 22°C). Blocking of the slices was performed with washing solution containing 10% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 hours at 22°C. To avoid unspecific binding, the slices were incubated with secondary anti-mouse IgG (#715-005-150; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in washing solution (2 h at 22°C). Then, the slices were incubated with mouse anti-$mGluR_5$ monoclonal (20 μg/ml, MABN540; Millipore) and rabbit anti-D_2R polyclonal (1 μg/ml, D_2R-Rb-Af960; Frontier Institute Co. Ltd, Shinko-nishi, Ishikari, Hokkaido, Japan) in washing solution with 5% NDS overnight at 4°C. Subsequently, the slices were washed twice with a washing solution containing 1%

NDS (10 min at 22°C). Next, the slices were incubated with anti-Cy2 donkey anti-rabbit (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and anti-Cy3 donkey anti-mouse (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in washing solution with 1% NDS for 2 hours at 22°C. Finally, slices were washed two times with washing solution containing 1% NDS (10 min at 22°C), two times with PBS (10 min at 22°C) and then mounted with Duolink® *in situ* mounting medium with DAPI (Sigma-Aldrich). The Leica TCS 4D confocal scanning laser microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) was used to capture the fluorescence striatal images.

For *in situ* PLA in mouse brain **t**he Duolink *in situ* PLA detection kit (Sigma-Aldrich) was used as previously described [47,109,144]. Thus, the experimental procedure until the secondary antibody incubation step was the same as the IHF (*see* above). Subsequently, the following steps were performed according to the manufacturer's protocol. Images were acquired and analyzed as previously described [144].

Radioligand competition binding experiments. For the binding experiments, membrane preparations (60 µg protein/ml) were obtained from HEK293T cells expressing either D_2R and $mGluR_5$ or $A_{2A}R$, D_2R, and $mGluR_5$ and [^{3}H]-raclopride (Novandi Chemistry AB, Södertälje, Sweden) competition assays with minor modifications were performed according to previously published methods [130,131,145]. [^{3}H]-raclopride (75 Ci/mmol), a D_2-like receptor antagonist competing [146] with quinpirole for binding to D_2-like receptors in HEK293T membrane preparations, was used to determine the D_2R high-affinity ($K_{i,\ High}$) and D_2R low-affinity ($K_{i,\ Low}$) values. (+)-Butaclamol (100 µM, Sigma-Aldrich) was used to determine the non-specific binding. The amount of bound [^{3}H]-raclopride was determined by liquid scintillation spectrometry.

cAMP functional assay. Intracellular cAMP levels were determined using a cAMP-Glo™ assay detection kit (Promega, Madison, WI, USA). HEK293T cells expressing either D_2R and $mGluR_5$ or $A_{2A}R$, D_2R, and $mGluR_5$ were plated at a density of 10,000 cells/well in 96-well microtiter plates coated with poly-L-lysine (Sigma-Aldrich) and incubated overnight. Culture medium was then removed, cells were washed with 1 x PBS before the induction buffer (red phenol/serum-free DMEM containing 500 µM IBMX and 100 µM Ro 20-1724) was added. The cells were incubated for 1 h prior to drug incubation. To examine the G_i protein-mediated inhibition of adenylyl cyclase, the levels of cAMP were first raised with 5 µM forskolin for 10 min. Drug dilutions were prepared in the induction buffer and the temperature- and carbon dioxide-equilibrated drug dilutions (37°C cell culture incubator for 30 min) were added as indicated, and cells were then incubated at 37 °C for 30 min. The assay was performed accordingly to the manufacturer's specifications (Promega, Sweden). Readings of luminescence intensity were

performed using the POLARstar Optima plate reader (BMG Lab Technologies, Offenburg, Germany). cAMP levels in non-transfected, non-treated cells and non-transfected cells treated only with forskolin were defined as basal and control, respectively.

Gel electrophoresis and immunoblotting. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) was performed using 7% polyacrylamide gels. Proteins were transferred to Hybond-LFP polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Chicago, IL, USA) using the Trans-Blot TurboTM transfer system (Bio-Rad, Hercules, CA, USA) at 200 mA/membrane for 30 min. PVDF membranes were blocked with 5% (wt/vol) dry non-fat milk in phosphate-buffered saline (PBS; 8.07 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 0.27 mM KCl, pH 7.2) containing 0.05% Tween-20 (PBS-T) during 1h at 20 °C before being immunoblotted with the indicated antibody in blocking solution overnight at 4°C. PVDF membranes were washed with PBS-T three times (5 min each) before incubation with either a HRP-conjugated rabbit anti-mouse IgG (1/10,000) or HRP-conjugated goat anti-rabbit IgG (1/30,000) in blocking solution at 20°C during 2 h. After washing the PVDF membranes with PBS-T three times (5 min each), the immunoreactive bands were developed using a chemiluminescent detection kit (Thermo Fisher Scientific) and detected with an Amersham Imager 600 (GE Healthcare Europe, Barcelona, Spain).

Statistical Analysis. The number of independent experiments (*n*) in each group is indicated in figure legends. Data are represented as mean ± standard error of mean (SEM). Outliers were assessed by the ROUT method [147], thus, subjects were excluded assuming a Q value of 1% in GraphPad Prism 9 (San Diego, CA, USA). Data normality was assessed by the Shapiro-Wilk normality test ($p < 0.05$). When two groups were evaluated unpaired Student's *t*-test or Mann-Whitney *U* test was used. Comparisons among more than two experimental groups were performed by one-, two- or three-way factor analysis of variance (ANOVA) followed by either Dunnett's, Šídák's or Tukey post-hoc test using GraphPad Prism 9, as indicated in the figure legends. A p value ≤ 0.05 was considered significant.

IV- RELATED TO SPECIFIC AIM 1 (see, Chapter 3)

Combined treatment with low doses of OSU-6162, a Sigma1R ligand, and an A2AR agonist fails to alter cocaine self-administration but increases accumbal A2AR-D2R heteroreceptor complexes and their antagonistic allosteric receptor-receptor interactions.

Animals. Male Sprague–Dawley (derived from the licensed animal breeder Charles River, Sulzfeld, Germany), weighing between 250 and 270 g at the

beginning of the experiment, were used. All animals used in the study were experimentally tested. The animals were housed individually in standard plastic rodent cages in a colony room maintained at 22 ± 1°C and 55 ± 10% humidity under a 12-h light-dark cycle (lights on at 6:00 am). Rodent food (VRF1 pellets, UK) and water were available ad libitum except for the period of the initial lever pressing training when rats were maintained on limited food. All protocols were conducted during the light phase of the light-dark cycle between 9:00 and 15:00 hours. The experiments were carried out in accordance with the European Directive 2010/63/EU and were approved by the Ethical Committee (76/2019, 2020/2019) at the Institute of Pharmacology, Polish Academy of Sciences, Krakow.

Drugs. Cocaine hydrochloride ((1R, 2R, 3S, 5S)-3-(benzoyloxy-8-methyl-8-azabicyclo [3.2.1] octane-2-carboxylic acid methyl ester hydrochloride; Toronto Research Chemicals (TRC), Canada) was dissolved in sterile 0.9% Na Cl. It was administered *i. v.* in a volume of 0.1 ml per infusion. OSU-6162 hydrochloride ((3*S*)-3-[3-(methyl sulfonyl)phenyl]-1-propylpiperidine hydrochloride; (Tocris, UK, 2.5 mg/kg, *s .c.*) and CGS 21680 (Tocris; UK, 0.05 mg/kg, *i. p*) were dissolved in 0.9% NaCl and administrated 60 and 10 min respectively, before a 2-h self-administration (SA) session in a volume of 0.1 ml/kg.

Surgery. Animals were anesthetized with ketamine HCl (Biowet, Poland; 75 mg/kg, *i.m.*;) and xylazine (Biowet, Poland; 5 mg/kg, *i.m.*;) given as a cocktail and chronically implanted with a silastic catheter in the external jugular vein, as described previously [148]. During 3 days after surgery, meloxicam (*Metacam*, Boehringer Iingelheim; 5 mg/kg, *s. c.*) was used to reduce post-operative pain. Rats were allowed 7-9 days to recover from surgery before the start of the experiments. Catheters were flushed daily with 0.2 ml of saline solution containing heparin (Biochemie, Austria;100 U/ml) and 0.1 ml of a cephazolin solution (Biochemie GmbH, Austria; 100 mg/ml) to prevent catheter non-patency. No problems with catheter patency were reported in the tested rats.

Initial training (lever presses). Before self-administration training, rats were trained to press the lever for food pellets (VRF1 pellets, UK) under a fixed ratio (FR) from 1 to 5 schedule of reinforcement of sweetened milk. Food training and SA sessions were performed in a sound attenuated, standard operant conditioning chambers (Med-Associates, St. Albans, VT, USA) from 2 to 3 days for 2-h daily. Starting first day prior to the food training session, rats received rations of ~25 g of chow daily sessions. Each chamber was equipped with a reward feeder; presses on the "active" lever resulted in the delivery of 0.1 mL of sweetened milk.

Cocaine Self-Administration. After the recovery period, all animals began lever pressing for cocaine reinforcement during 2-h daily sessions performed 6 days per week (Monday-Saturday). The house light was illuminated throughout each

session. Each press on the "active" lever (FR-5 schedule of reinforcement) resulted in a 5-s infusion of cocaine (0.5 mg/kg per 0.1 ml) and a 5-s presentation of a stimulus complex (activation of the white stimulus light directly above the "active" lever and the tone generator). Following each injection, there was a 20-s time-out period during which responding was recorded but had no programmed consequences. Presses on the "inactive" lever were recorded, but not reinforced. After the 7 days of acquisition, rats were used to complete a cocaine (0.25–0.5 mg/kg/infusion) dose-response curve. Cocaine SA was conducted daily for 17 sessions. Following stabilization of responding rates with cocaine (0.25 mg/kg/infusion) SA, the animals were divided into separate groups (n = 8) to undergo test procedures. Vehicle or OSU-6162 was administered daily for 3 last cocaine SA sessions, while CGS 21680 was administered only before the last cocaine SA session. Immediately after the last cocaine SA sessions, animals were either sacrificed (for biochemical analysis) or injected with pentobarbital and perfused intracardially (for IHC and in situ PLA analysis).

Yoked "procedure". In this procedure, each rat actively self-administering cocaine has been assigned for rats that were passively receiving intravenous saline in the same amount and manner as the active animal. Lever pressing by the "yoked" rats was recorded but had no programmed consequences. The yoked saline group were sacrificed at the same time as the corresponding self-administered cocaine groups of rats after a 2-h experimental session of SA.

Biochemical binding experiments. Membrane preparation. Frozen tissue was homogenized in ice-cold preparation buffer using a sonicator (Soniprep 150). The buffer contained 50 mM Tris-HCl, pH 7.4, 7 mM $MgCl_2$, 1 mM EDTA, a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany) and 0.3 IU/ml adenosine deaminase (EC 3.5.4.4, Sigma-Aldrich). The membranes were precipitated by centrifugation at 4°C for 40 min at 40,000 × g (Thermo scientific, Sorvall Lynx 6000, Stockholm, Sweden) and washed through re-homogenization in the same buffer once more. The protein concentration was determined for the membrane homogenates by means of BCA Protein Assay (Pierce, Sweden) using as a standard bovine serum albumin. Pelleted membranes were resuspended to a concentration of 0.15 mg/ml, immediately used or stored at -80°C until required.

[^{3}H]-raclopride competition binding experiments. [3H]-raclopride binding was displaced by quinpirole to determine the proportion of receptors in the high-affinity state (RH), the high-affinity (Ki, High), and low-affinity (Ki, Low) values. Ventral striatum membrane preparations (60 µg protein/ml) were incubated with increasing concentrations of quinpirole (0.01 nM to 1 mM) and 2 nM [3H]-raclopride (75 Ci/mmol, Novandi Chemistry AB, Sweden) in 250 µl of incubation buffer (50 mM Tris-HCl, 100 mM NaCl, 7 mM MgCl2, 1 mM EDTA, 0.05% BSA, 1 mM

DTT) and 0.3 IU/ml adenosine deaminase (EC 3.5.4.4, Sigma-Aldrich) for 90 min at 30°C in the presence or absence of 100 nM of the A2AR agonist CGS-21680. Non-specific binding was defined by radioligand binding in the presence of 100 µM (+)-butaclamol (Sigma-Aldrich, Sweden). The incubation was terminated by rapid filtration using Whatman GF/B filters (Millipore Corp, Sweden) and a MultiScreenTM Vacuum Manifold 96-well followed by five washes (250 µl per wash) with ice-cold washing buffer (50 mM Tris-HCl pH 7.4). The filters were dried, 5 ml of scintillation cocktail was added, and the amount of bound ligand was determined after 12 h by liquid scintillation spectrometry.

***In situ* Proximity Ligation Assay (*in situ* PLA).** To study the effects of OSU-6162, a Sigma1R ligand, in low doses, on the D2R-A2AR and D2R-Sigma1R heteroreceptor complexes, density changes after cocaine self-administration was performed with *in situ* PLA as described previously [17,47,141]. Free-floating formalin fixed brain sections (30 µm-thick, cut using a cryostat) at Bregma level (1.0 mm) from rats after cocaine-self administration were employed using the following primary antibodies: rabbit monoclonal anti-A2AR (AB1559F, 1:250; Millipore, Sweden), mouse monoclonal anti-D2R (MABN53, 1:600, Millipore, Sweden) and rabbit monoclonal anti-sigma1R (ab53852, 1:500, Abcam, Sweden). Primary antibodies were validated previously by means of immunohistochemistry in both rat brain tissue and HEK293 cell line ([34,52,149]). To localize the heteroreceptor complexes in relation with neurons and astrocytes in the brains, traditional immunohistochemistry (IHC) was employed parallelly to in situ PLA. For this purpose, a glial marker (GFAP) and a nucleic marker (DAPI) was used.

Control experiments for in situ PLA procedures were performed in free-floating formalin fixed rat brain sections employing only one primary antibody (mouse monoclonal anti-D2R (MABN53, 1:600, Millipore, Sweden). The *insitu* PLA analysis of this negative control showed 15.6% false positive clusters compared to the positive control group value (100%). This false positive signal was reduced even further (less than 4%) when the brain sections were kept in citric acid Buffer for 45-60 min at 65°C prior to the primary antibody incubation. Control experiments with similar results were also performed in 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 Duo link 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.

Image acquisition and analysis. Images of the samples were taken with a confocal microscope LEICA TCS-SL SP5 confocal microscope. Three different areas of the NAc shell were selected from which two randomly chosen magnified sample fields (150 x 150µm) were used for image acquisition yielding a total of six pictures per animal. Images were inspected before analyzed to exclude unrepresentable pictures, e.g., pictures containing blood vessels (which naturally attract astrocytes). Nuclei and PLA signal quantification was performed with DuoLink Image Tool software. Astrocyte quantification including branch analysis was performed with the open-source Fiji ImageJ software using the Ridge detection plugin.

Statistical Analysis. Data were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). All the data are shown as means ± SEM.

In behavioral experiments, the number of responses on the „active" and „inactive" lever or the number of cocaine infusions were analyzed using factorial analysis of variance (ANOVA) for factors (groups and levers). Data from competition experiments were analyzed by nonlinear regression analysis. The absolute values and the percent changes induced by A2A agonist CGS-21680 in the dopamine D2R high-affinity, low-affinity and proportion of receptors in the high-affinity state were evaluated with paired Student's *t*-test and nonparametric Mann-Whitney *U*-test respectively. Data from *in situ* PLA experiments showing cluster density (clusters per nucleus per sampled field) were analyzed using a one-way ANOVA followed by post-hoc Tukey's test. The number of rats (*n*) in each experimental condition is indicated in figure legends. The *P* value 0.05 and lower was considered significant.

CHPATER 1
Results and Discussion

SPECIFIC AIM 1. *Differential acute effects of a standard 5-HT2A receptor agonist TCB-2 and a hallucinogenic agonist DOI on 5-HT2AR-D2R heteroreceptor complexes in the nucleus accumbens of the rat forebrain.*

1. 1. Background

Several decades ago it was proposed based on changes in 5-HT turnover and behavioral experiments that hallucinogenic compounds like d-LSD and other drugs of the indolamine type like psilocybin produced their hallucinogenic actions via activation of post-junctional 5-HT receptors in the brain [84,85,150,151]. In this period the hypothesis was also advanced that hallucinogenic indolamines like d-LSD preferentially acted at presynaptic serotonin receptors based on neurophysiological work [152,153]. Subsequently implications were obtained that d-LSD and other hallucinogens targeted the 5-HT2 receptor (5-HT2AR) [154,155]. It was later classified as the 5-HT2AR [156].

It was shown that LSD and phenethylamine hallucinogen DOI acted as partial agonists at post-synaptic 5-HT2ARs of piriform neurons with high potency [157] in line with the work of the Swedish group [84,151,158]. In 1998 it was suggested that post-synaptic 5-HT2ARs located apical dendrites of cortical pyramidal neurons could be a relevant target for hallucinogenic drugs [159].

The discovery of the D2R-5-HT2AR heteroreceptor complex in living cells using BRET2 and FRET techniques was of high interest [81,82], since disturbances in these receptors are likely involved in contributing to development of schizophrenia [78,87,88,106,160]. A major finding was the observation that these heteroreceptor complexes exist in the nucleus accumbens and the dorsal striatum using *in situ* proximity ligation assay (*in situ* PLA)[109]. Thus, the D2R-5-HT2AR heteroreceptor complex likely represents a new important target for hallucinogenic 5-HT2AR agonists, in addition to being a target for atypical antipsychotic drugs.

As to their allosteric receptor-receptor interactions, the 5-HT transmitter counteracted the D2R agonist induced activation of the D2R protomer determined in a cAMP response element-luciferase reporter gene assay while the standard 5-HT2AR agonist TCB2 lacked effects [82,87]. Of significant interest was the finding that the hallucinogenic 5-HT2AR agonists d-LSD and DOI potently enhanced the D2R

agonist action on the D2R protomer recognition and Gi/o mediated signaling in contrast to the case with 5-HT and TCB2 [47,87]. It was therefore proposed that these hallucinogenic 5-HT2AR agonists can have this property due to their ability to produce a pathological conformational change in the 5-HT2A receptor protomer upon binding to its orthosteric site. This change led to the development of a facilitatory allosteric receptor-receptor interaction causing an enhanced D2R protomer signaling. Such events can contribute to development of hallucinations and psychosis based on the DA hypothesis of schizophrenia. This hypothesis includes giving evidence for a key role of enhanced and disturbed D2R function in schizophrenia [107,161]. This molecular mechanism can help explain the advantage of atypical antipsychotic drugs with a higher potency to block the 5-HT2A receptor vs the D2 receptors compared to first-generation antipsychotics in the treatment of schizophrenia. It also leads to reduced side-effects [162,163].

To test the current hypothesis on a significant role of D2R-5-HT2AR heteroreceptor complexes in the actions of hallucinogenic 5-HT2AR agonists we have compared the acute *in vivo* actions of DOI with those of a standard 5-HT2R agonist TCB2. They both belong to the group of phenethylamines with high affinity for the 5-HT2AR [164,165]. The acute in vivo effects of DOI and TCB2 in doses producing similar effects on locomotion [125,126] were studied on the D2R-5-HT2AR heteroreceptor complexes in the nucleus accumbens core and shell in view of their reward and anti-reward neurons inter alia involved in interpreting the relevance of sensory stimuli [78,161] using the proximity ligation assay (PLA). Colocation of the D2R and 5HT2AR IR was found in nucleus accumbens as well as PLA-positive blobs in discrete regions of nucleus accumbens, indicating the existence of accumbal D2R–5-HT2AR heteroreceptor complexes. It was of special interest to compare the effects of DOI and TCB-2 on the nucleus accumbens core and shell since there exist in the dorsomedial part of the shell large numbers of patches of strong D1R immunoreactivity (IR) with low D2R, dopamine transporter and tyrosine hydroxylase IRs [166]. They form a tubular rostro-caudal neuronal system, in which the D1R is reached by DA through volume transmission after a diffusion distance 30-50 um. In contrast, in the accumbens core you have only a moderate number of patches rich in D1R IR and in this case also in D2R IR having a low number of DA nerve terminals [166].

The results reveal a unique increase by DOI treatment on the density of the D2R-5-HT2AR heteroreceptor complexes in the nucleus accumbens shell which can be blocked by a 5-HT2A receptor antagonist MDL-100907. This compound is a highly selective 5-HT2AR antagonist [167,168] and has been demonstrated to unfold its activity in the nucleus accumbens and the cerebral cortex [160] and has shown efficacy to counteract DOI-mediated action. The current findings support the hypothesis of a

role of accumbal shell but not core D2R-5-HT2AR heteroreceptor complexes as a putative target for the hallucinogenic actions of DOI. They which may contribute to the development of psychosis through enhancement of the Gi/o mediated signaling of the D2R protomer in the nucleus accumbens shell.

1. 2. Results

Distribution pattern of the density of 5-HT2AR-D2R heteroreceptor complexes in the nucleus accumbens with or without acute treatment with DOI or TCB-2 using PLA. Five areas within the nucleus accumbens were analyzed: the anterior part of the anterior commissure (aca) and the core and the shell region of the nucleus accumbens (NAc) including the medial and ventral part of the core and shell (Fig. 1). Due to the large number of brain regions studied, few significant differences between regions could be observed with the limited number of rats used per group. Only possible small significant changes in the density of PLA positive 5-HT2AR-D2R complexes among regions are reported with or without treatment with DOI.

Vehicle treated group (rats). There was a low density of 5-HT2AR-D2R complexes as shown with PLA in the aca and all the regions studied of the nucleus accumbens, except the nucleus accumbens shell ventral region (**Fig. 1.1 A**). The nucleus accumbens shell ventral regions instead showed medium densities around 4 PLA clusters/cell/sampled field.

TCB-2 (standard 5-HT2AR agonist) acutely treated group (rats). There was a low density of 5-HT2AR-D2R complexes as shown with PLA in the aca and all the regions studied of the nucleus accumbens (**Fig. 1.1. B**).

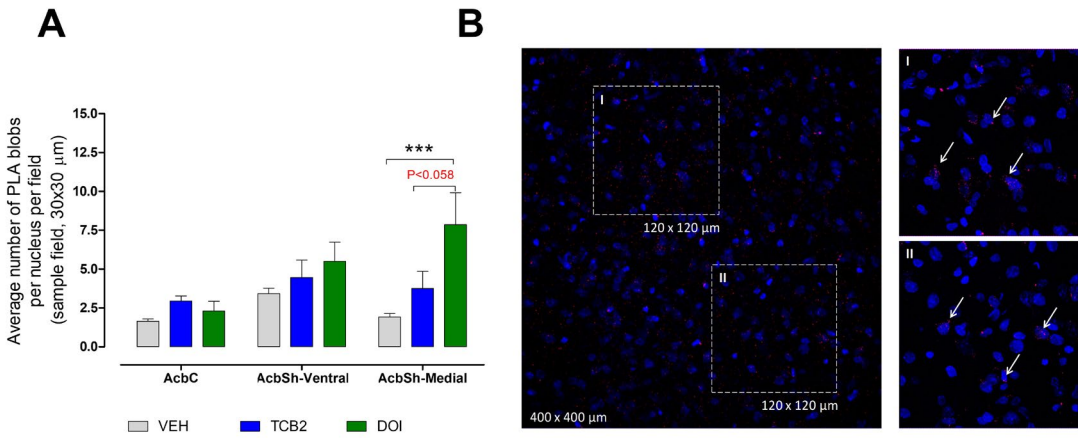
DOI (hallucinogenic 5-HT2AR agonist) acutely treated group (rats). An increased density of PLA positive 5-HT2AR-D2R complexes was found in the accumbens shell, especially in its medial part (7.9 ± 2.1, means s.e.m.) (Figure 1C).

Comparison between the effects of DOI and TCB-2 vs the vehicle group in the medial part of the nucleus accumbens shell.

Significant and selective increases ($p < 0.05$) in the PLA positive 5-HT2AR-D2R complexes were demonstrated with acute DOI but not TCB-2 treatment (**Fig. 1.1. B**).

Figure 1.1. Quantification of PLA clusters in striatal areas. Plotting of the number of PLA-positive signals per cell per sample field over different brain areas visualizes the density of 5-HT2AR-D2R heteroreceptor complexes following acute treatments of rats with vehicle, hallucinogenic DOI, and (non-hallucinogenic TCB-2). **(A)** Effects of DOI and TCB-2 on the average number of PLA clusters per cell per sample field in the nucleus accumbens shell of the rat brain. Compared to the vehicle group, within the medial part of the nucleus accumbens shell application of the hallucinogenic 5-HT2AR agonist DOI increased the number of detectable PLA-positive 5-HT2AR-D2R clusters approximately by the factor of four, whereas TCB-2 was less potent. Four animals per treatment group and two sample fields per animal were measured in each brain area. Data were analyzed with one-way ANOVA followed by Dunnett's multiple comparisons test compared to vehicle and are presented as

mean ± SEM. * p < 0.05. **(B)** Illustration panels of positive PLA (5-HT2AR-D2R heteroreceptor complexes) following acute treatments of rats with non-hallucinogenic TCB-2.



Effects of the 5-HT2A receptor antagonist MDL-100907 on the DOI induced increases in the density of the 5-HT2AR-D2R complexes in the nucleus accumbens shell. In a next step, it was evaluated whether the increases in the density of 5-HT2AR-D2R complexes induced by DOI were a direct result of the 5-HT2A agonist activation of the 5-HT2A receptor. Therefore, rats were treated with the highly selective 5-HT2AR antagonist MDL-100907 to see if the DOI-induced increase in the density of the PLA positive 5-HT2AR-D2R complexes could be reversed. We investigated the potential of MDL-100907 to influence the density of PLA positive 5-HT2AR-D2R complexes in the nucleus accumbens shell, a brain area where DOI produced a significant increase. A comparison was also made with the effects of DOI in the Claustrum and the nucleus accumbens core. In none of the analyzed regions, the antagonistic MDL-100907 alone changed the number of PLA signals (Fig. 3a - c). The DOI-induced elevated density of the PLA positive 5-HT2AR-D2R complexes in the medial accumbens shell (7.2 ± 1.1, Fig. 3b) and in the claustrum (11.0 ± 1.1, Fig. 3c) were significantly decreased by the co-administration of MDL-100907 to 3.0 ± 0.3 (NAc shell, **Fig. 1.2.**) and 6.1 ± 0.3 (claustrum, data not shown), respectively. Moreover, MDL-100907 could as expected not significantly affect the 5-HT2AR-D2R complexes in the nucleus accumbens core where DOI could not significantly increase the 5-HT2AR-D2R complexes (**Fig. 1.2.**). Thus, the specific 5-HT2AR antagonist MDL-100907 can significantly inhibit DOI-induced increases in 5-HT2AR-D2R heteroreceptor complexes in the claustrum and the medial shell of the nucleus accumbens. These data further support the finding that DOI exerts its influence on the number of 5-HT2AR-D2R heteroreceptor complexes in the nucleus accumbens selectively in the shell rather than the core region.

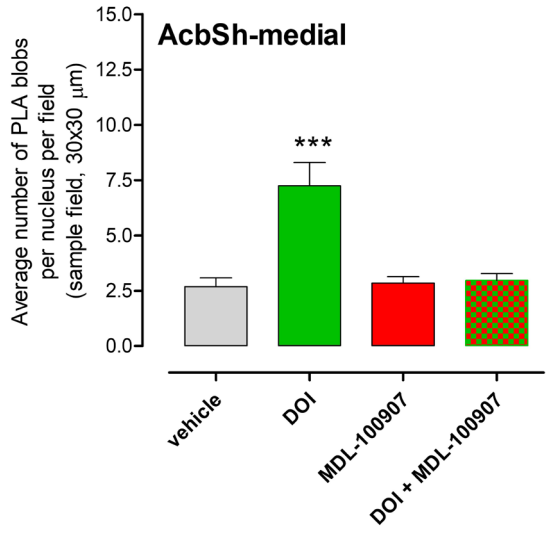


Figure 1.2. Reduction of DOI-induced increases in the density of 5-HT2AR-D2R heteroreceptor complexes by MDL-100907. (MDL-100907 marginally decreased the density of PLA clusters in the nucleus accumbens core, like the number of the vehicle control. MDL-100907 successfully reduced the effect of DOI in the NAc shell and the claustrum with high significance (**** $p < 0.0001$). Four animals per treatment group and two sample fields per animal in each brain area were measured. Data were analyzed with two-way ANOVA followed by Bonferroni multiple comparisons test and are presented as mean ± SEM. **** $p < 0.0001$.

1. 3. Discussion

The major result obtained in the current experiments was the demonstration that acute treatment with the hallucinogenic 5-HT2AR agonist DOI significantly and specifically increased the density of the 5-HT2AR-D2R complexes in the nucleus accumbens shell (medial part) versus the standard 5-HT2A receptor TCB-2. In contrast, both these types of 5-HT2AR agonists lacked effects in the nucleus accumbens core. These results are of substantial interest since the hallucinogenic 5-HT2AR agonists d-LSD and DOI potently enhance the D2R agonist action on the D2R protomer recognition and Gi/o mediated signaling in contrast to the case with 5-HT and TCB-2 (Borroto-Esccuela et al.2014,2016). The current findings with DOI therefore indicate that the increased density of 5-HT2AR-D2R complexes induced by DOI in the nucleus accumbens shell can lead to a further increase in the enhancement of D2R signaling. It strengthens the hypothesis (Borroto-Escuela et al.2014,2016) that the hallucinogenic 5-HT2AR agonists may produce hallucinations and psychotic effects in part through increased density of 5-HT2AR-D2R complexes involving also a switch towards allosteric facilitatory receptor-receptor interactions with enhancement of D2R protomer signaling. These 5-HT2AR-D2R complexes are likely mainly located in the ventral D2R rich striatal-pallidal GABA antireward neurons (Borroto-Escuela et al. 2010, 2016, Fuxe et al.2014). By enhancing the D2R protomer mediated inhibition of these antireward neurons, antireward becomes pathologically reduced leading to brain overflow of irrelevant sensory stimuli due to inadequate filtration of such stimuli. As a result, hallucinations become facilitated.

The specificity of the DOI action at the 5-HT2A receptor was demonstrated by the ability of the 5-HT2AR antagonist MDL-100907 to block the DOI-induced increase in the density of the 5-HT2AR-D2R complexes in the nucleus accumbens shell

(medial part). No significant effects of DOI and MDL-100907 were observed in the nucleus accumbens core (medial part) indicating that the major action of DOI is in the accumbens shell. These results indicate that only the 5-HT2AR-D2R complexes on the ventral striatal-pallidal GABA antireward neurons from the shell may be targets for hallucinogenic 5-HT2AR agonists. Furthermore, the increase in the 5-HT2AR-D2R complexes induced by DOI in the claustrum present in the cerebral cortex could also be counteracted by the 5-HT2AR antagonist. However, in the claustrum also the standard 5-HT2A receptor agonist produced trends for similar increases in the PLA positive 5-HT2AR-D2R complexes as found with DOI. Therefore, the increase produced by DOI in this region may not be of relevance for its ability to cause hallucinations. Blockade of the 5-HT2AR protomer alone with this antagonist failed to induce changes in the density of the 5-HT2AR-D2R complexes in the brain areas discussed above.

Furthermore, without 5-HT2AR agonist treatment the distribution pattern of the PLA positive 5-HT2AR-D2R complexes in the forebrain was characterized by higher densities of these complexes in the cortical regions studied. Treatment with DOI or TCB-2 did not appear to produce any overall change in this pattern of distribution of the 5-HT2AR-D2R complexes in the forebrain. The unique change in the action of DOI vs TCB-2 on the density of the 5-HT2AR-D2R complexes was only identified in the nucleus accumbens shell.

Taken together, the current findings indicate that the hallucinogenic 5-HT2AR agonist DOI vs the standard 5-HT2AR agonist TCB2 can produce a unique increase in the 5-HT2AR-D2R heteroreceptor complexes in the nucleus accumbens shell which may lead to enhanced D2R protomer inhibitory signaling. This site of action is proposed to be one mechanism for the hallucinations produced by hallucinogenic 5-HT2AR agonists like DOI, psilocybin and d-LSD which can contribute to psychosis development.

CHPATER 2
Results and Discussion

SPECIFIC AIM 2. *The mGlu5 receptor protomer mediated dopamine D2 receptor trans-inhibition is dependent on the adenosine A2A receptor protomer: implications for Parkinson's disease*

2. 1. Background

The first pieces of evidence for antagonistic glutamate receptor with dopamine D_2 receptor (D_2R) interactions were found in 1983-1984 through the ability of glutamate to reduce the affinity of the high-affinity D_2R agonist binding sites in striatal membrane preparations. Subsequently, it was observed that $mGluR_5$ agonists alone or combined with an $A_{2A}R$ agonist (CGS-21680) can reduce the affinity of the high-affinity state of D_2R for agonist binding sites in the rat striatum [169]. Co-immunoprecipitation experiments also indicated the existence of $A_{2A}R$-$mGluR_5$ heteroreceptor complexes in HEK293 cells and rat striatal membrane preparations [170]. The colocation of the receptors in striatal neurons was demonstrated [52,171] as well as their synergistic interactions as studied with *in vivo* micro-dialysis and intracellular signalling in striatal preparations [170,172,173].

In 1974, the discovery that the methylxanthines caffeine and theophylline could enhance the contralateral turning behaviour induced by levodopa and dopamine receptor agonists in the hemi-Parkinsonian rat model was one early finding leading to the hypothesis that antagonistic adenosine-dopamine interactions existed [2,174]. Today, a considerable amount of molecular and functional experimental data supports the view that $A_{2A}R$ and D_2R form heteroreceptor complexes with antagonistic receptor-receptor interactions on the plasma membrane [34,38,49,60,127,143,175,176].

The existence of $A_{2A}R$-D_2R-$mGluR_5$ higher-order oligomers was postulated and it was proposed that the receptor-receptor interactions within this high order complex are important to modulate the dorsal and ventral striatal-pallidal GABA neurons [2,170,171]. Years later, it was proposed that combined treatment with $A_{2A}R$ and $mGluR_5$ agonists targeting $A_{2A}R$-D_2R-$mGluR_5$ heteroreceptor complexes in the ventral striatal-pallidal GABA pathway can represent a new strategy for the treatment of schizophrenia[106]. Also, the combine treatment with selective A_{2A} and $mGluR_5$ receptors antagonists represent an alternative therapeutic approach to Parkinson's disease [132,177,178].

A combination of bimolecular fluorescence complementation assays and bioluminescence resonance energy transfer assays as well as the sequential resonance energy transfer technique was used to show that $A_{2A}R$-D_2R-$mGluR_5$ heteroreceptor complexes exist in living cells [179]. In addition, high-resolution immunoelectron microscopy was also used to further demonstrate their existence in striatal glutamate synapses [179]. An integrative role of these receptor complexes in adenosine, dopamine and glutamate transmission was also proposed [2,180,181]. Recently, $A_{2A}R$, D_2R, and $mGluR_5$ receptor-receptor interactions were also found to modulate the activity of the striatal-pallidal GABA neurons based on *in vivo* dual-probe microdialysis [134].

Herein, new findings that further expand the understanding of $A_{2A}R$-D_2R-$mGluR_5$ heteroreceptor complexes are presented. Results in cellular models first demonstrated that $A_{2A}R$ promotes the D_2R and $mGluR_5$ receptor-receptor interactions and its participation increases the density of the D_2R-$mGluR_5$ heterocomplexes. Binding and functional experiments indicated that $A_{2A}R$ and $mGluR_5$ upon agonist activation play a significant role in modulating the composition, density, and signalling of $A_{2A}R$-D_2R-$mGluR_5$ heteroreceptor complexes. This was also observed in $A_{2A}R$ or D_2R knockout mice when studying the effects of the $mGluR_5$ negative allosteric modulator raseglurant on locomotor activity.

2. 2. Results

BRET^2 experiments. HEK293T cells were transiently transfected with constant amounts of D_2R^{Rluc} and increasing amounts of plasmids encoding for $mGluR_5^{GFP2}$ with/without transient co-expression of $A_{2A}R$. The transient co-expression of $A_{2A}R$ with D_2R^{Rluc} and $mGluR_5^{GFP2}$ had a significant impact on D_2R^{Rluc}-$mGluR_5^{GFP2}$ heteroreceptor complex formation (**Fig. 2.1.A**). Transient co-expression of $A_{2A}R$ promoted a significant increase of netBRET^{2}max ratio value (0.084 ± 0.003 AU) compared to that found in cells without transient co-expression of $A_{2A}R$ (0.043 ± 0.002 AU) (**Fig. 2.1.B**).

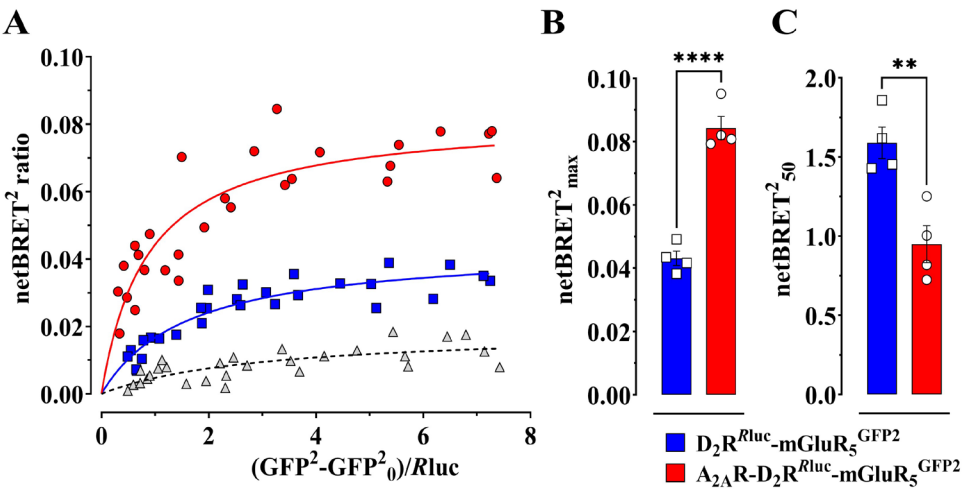


Figure 2.1. Effect of $A_{2A}R$ expression in D_2R-$mGluR_5$ heteromer formation assessed by $BRET^2$ assay in HEK293T cells. Cells were transiently transfected with plasmids encoding the D_2R tagged with *R*luc (i.e., D_2R^{Rluc}) and $mGluR_5$ with GFP2 (i.e., $mGluR_5^{GFP2}$) in the absence (blue squares) or presence (red circles) of $A_{2A}R$ expression. The A_1R^{GFP2}-D_2R^{Rluc} pair was used as a control (grey triangles). $BRET^2$ saturation curves **(A)** were constructed by co-transfecting a constant amount of the plasmid for D_2R^{Rluc} and increasing amounts of the $mGluR_5^{GFP2}$ plasmid or A_1R^{GFP2} plasmid. Curves are based on mean values of four independent experiments performed in quadruplicates. The $netBRET^2_{max}$ **(B)** and $netBRET^2_{50}$ **(C)** values from the $BRET^2$ saturation curves shown in (A) are represented. BRET ratio is calculated from florescence and bioluminescence values expressed as arbitrary units. Results are expressed as mean ± SEM ($n = 4$, each determination performed in quadruplicates). ****$p < 0.0001$ and **$p < 0.01$, Student's *t*-test.

When the $A_{2A}R$ was co-expressed with D_2R^{Rluc} and $mGluR_5^{GFP2}$, these receptors hence showed an increased ability to heteromerize. In addition, the $netBRET^250$ ratio value for D_2R^{Rluc}-$mGluR_5^{GFP2}$ heteromerization was significantly reduced by transient co-expression of $A_{2A}R$ from (1.58 ± 0.09 AU) to (0.94 ± 0.11 AU) (**Fig. 2.1.C**) indicating increased affinity of the two receptor protomers for each other.

Proximity ligation assay experiments. The role of $A_{2A}R$ in the dynamics of the D_2R-$mGluR_5$ heteromers was also evaluated by *in-situ* proximity ligation assays (PLA) in transiently co-transfected HEK293T cells. The PLA results were *in line* with the results from the $BRET^2$ assays. The *in-situ* PLA demonstrated the existence of D_2R-$mGluR_5$ heteroreceptor complexes in cells to a low degree without transient co-expression of $A_{2A}R$ (**Fig. 2.2.A**). Furthermore, the transient co-expression of $A_{2A}R$ highly significantly promoted the formation D_2R-$mGluR_5$ heteroreceptor complexes as shown by the marked increase in the number of PLA-positive D_2R-$mGluR_5$ complexes, while this was significantly reduced in HEK293T cells without co-expressing $A_{2A}R$ (**Fig. 2.2. B-D**). Few and weak PLA clusters were detected in the PLA negative controls (lack of D_2R antibodies) representing background labelling (**Fig. 2.2. C**).

The specificity of the PLA positive D_2R-$mGluR_5$ complexes, shown as red blobs in the mouse dorsal striatum (**Fig. 2.3 A**), was demonstrated using $D_2R^{-/-}$ mice (**Fig. 2.3 C**). In the sections from the mouse striatum the appearance of the red PLA positive D_2R-$GluR_5$ complexes, shown as mean number of red blobs/Nucleus, was markedly and highly significantly reduced (**Fig. 2.3 D**). Furthermore, the loss of the red D_2R-$mGluR_5$ blobs to the same high degree in the $A_{2A}R^{-/-}$ mice (**Fig. 2.3 D**) likely reflects the requirement of D_2R-$mGluR_5$ heterocomplexes to be part of an $A_{2A}R$-D_2R-$mGluR_5$ to be expressed in the mouse striatum, probably by dorsal striatal-pallidal GABAergic neurons. In this way it forms D_2R-$mGluR_5$ complexes that are close enough to be visualized by PLA.

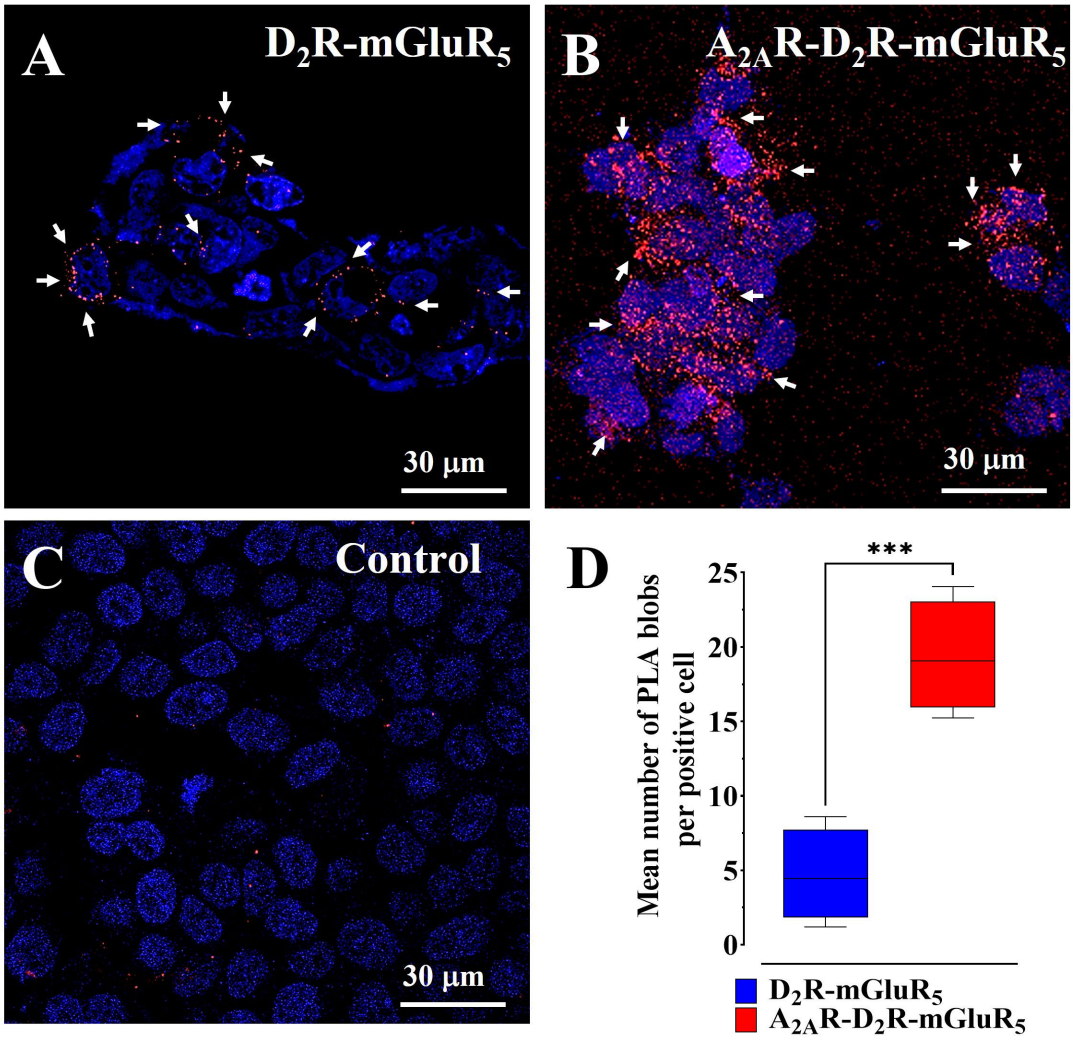
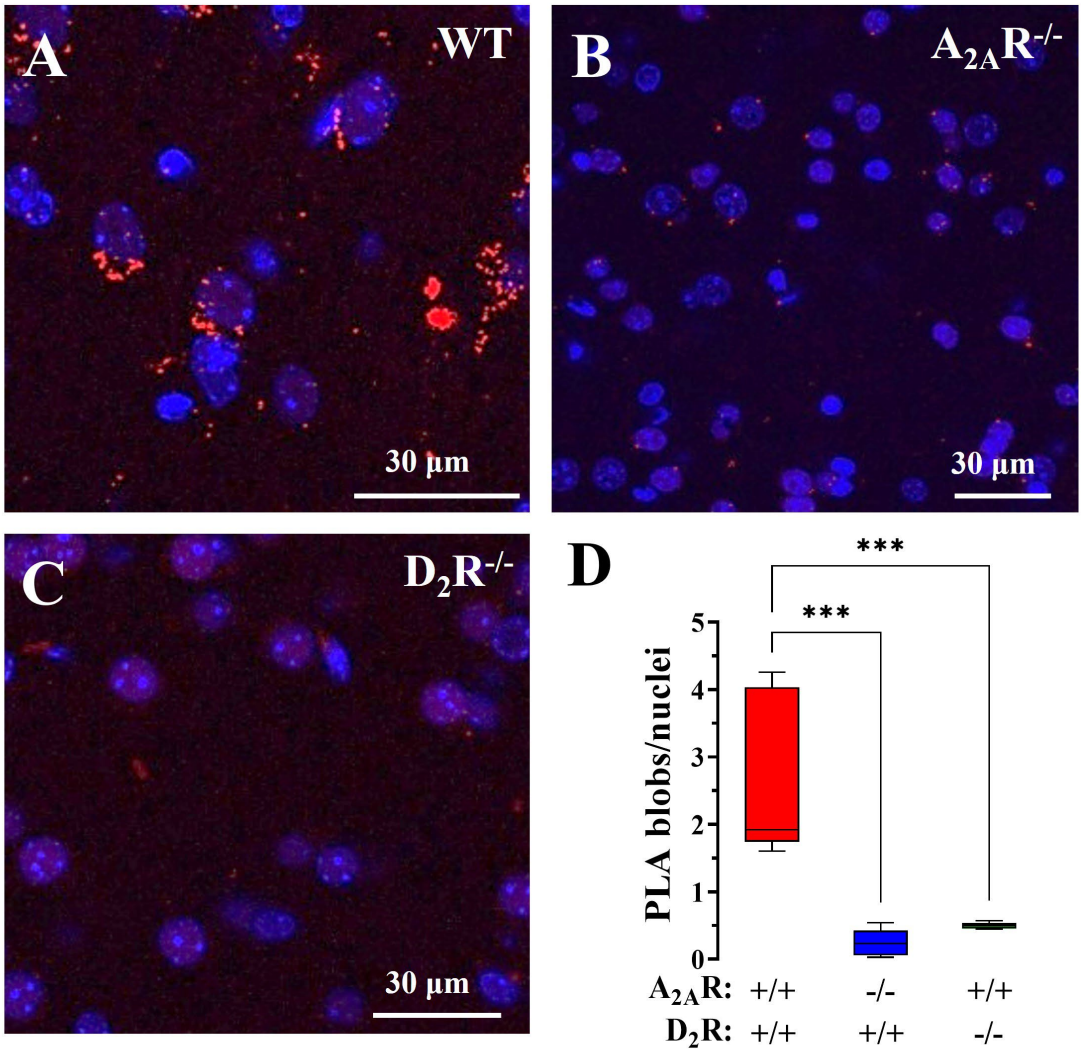


Figure 2.2. *In-situ* PLA assessment of D_2R-$mGluR_5$ heteromer formation in the absence **(A)** or presence **(B)** of $A_{2A}R$ (*see* Methods). The *in-situ* PLA positive D_2R-$mGluR_5$ heteroreceptor complexes were shown as red blobs (arrows) and nuclei in blue (DAPI staining). A negative *in-situ* PLA control **(C)** was included by incubating the cells in the absence of the primary anti-D_2R antibody. **(D)** Quantification of D_2R-$mGluR_5$ complexes. The number of PLA blobs (red clusters) per positive cell (n = 4x50 cells) was assessed as described in Methods. Results were expressed as mean ± SEM (n = 4 independent experiments). ****$p < 0.0001$ and **$p < 0.01$, Student's t-test.

Figure 2.3. Assessment of D_2R-$mGluR_5$ heteromer formation in mouse dorsal striatum by *in-situ* PLA. Photomicrographs showing PLA recognition of D_2R-$mGluR_5$ heteromers in the dorsal striatum of wild-type (**A**), $A_{2A}R^{-/-}$ (**B**) and $D_2R^{-/-}$ (**C**) mice. The *in-situ* PLA positive D_2R-$mGluR_5$ heteroreceptor complexes are shown as red blobs (arrows) and nuclei in blue (DAPI staining). (**D**) Quantification of D_2R-$mGluR_5$ complexes showing a highly significant reduction of D_2R-$mGluR_5$ positive red blobs in the absence of $A_{2A}R^{-/-}$ or $D_2R^{-/-}$. The number of PLA blobs (red clusters) per nucleus was assessed as described in Methods. Results were expressed as mean ± SEM (n = 5 animals). ***$p < 0.001$, one-way ANOVA followed by Dunnett's post-hoc test when compared with wild-type animals.

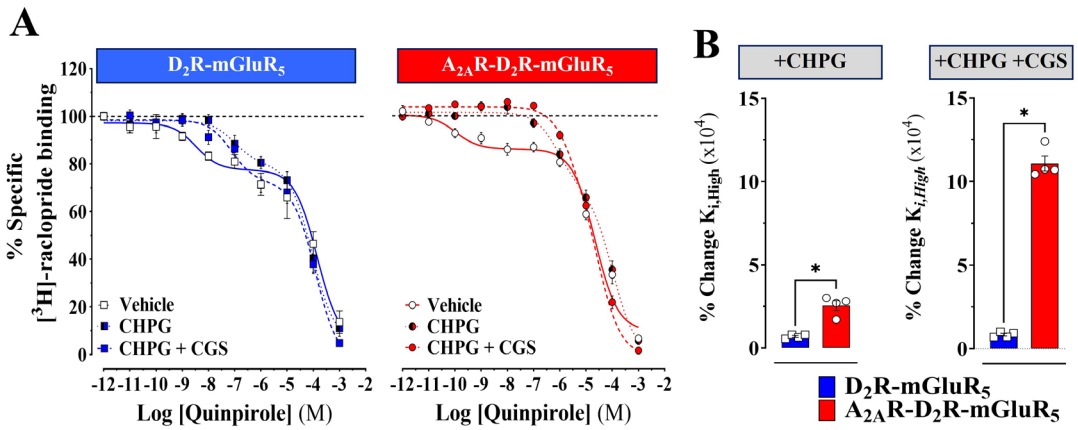


[^{3}H]-raclopride/quinpirole competition experiments. In HEK293T cells expressing D_2R and $mGluR_5$, the $mGluR_5$ agonist CHPG (500 nM) reduced the affinity of the high-affinity state ($K_{i,\ High}$) of the D_2R for the agonist quinpirole with no effects on its low-affinity state ($K_{i,\ Low}$). Co-treatment with $A_{2A}R$ agonist CGS-21680 (100 nM) did not significantly alter the D_2R $K_{i,\ High}$, and $K_{i,\ Low}$ values obtained when the cells were treated only with CHPG (500 nM) (**Fig. 2.4** and Table 1). In HEK293T cells expressing $A_{2A}R$, D_2R, and $mGluR_5$, $mGluR_5$ agonist stimulation also reduced the affinity of the high-affinity state ($K_{i,\ High}$) of the D_2R for the agonist quinpirole with no statistically significant effects on its low-affinity state ($K_{i,\ Low}$) (Figure 4A and Table 2). However, the transient co-expression of $A_{2A}R$ by itself (without agonist stimulation) potentiate $mGluR_5$ agonist effects on the high-affinity D_2R agonist binding sites (**Fig. 2.4 B**, Tables 1 and 2). Finally, the co-stimulation of $A_{2A}R$ and $mGluR_5$ synergistically increased in the $K_{i,\ High}$ values of the D_2R protomer upon co-expression of the $A_{2A}R$ (Table 2). Nevertheless, in cells expressing $A_{2A}R$, D_2R, and $mGluR_5$, further analysis should be performed to test the effect of combine treatment of $A_{2A}R$ (ZM-241385) and

mGluR$_5$ (CHPG) to figure out if the expression of $A_{2A}R$, without agonist stimulation and its corresponding constitutive activity, is responsible for increased in the $K_{i,\ High}$ values of the D_2R protomer upon co-expression of the $A_{2A}R$.

In both HEK293T cells expressing D_2R and mGluR$_5$ or $A_{2A}R$, D_2R, and mGluR$_5$, the incubation with $A_{2A}R$ antagonist ZM-241385 (1 μ M) and mGluR$_5$ antagonist MPEP (300 μ M) alone or in combination resulted in an almost complete blockade of the mGluR$_5$ increase of the D_2R $K_{i,\ High}$ values and $A_{2A}R$ agonist-induced increase of mGluR$_5$ agonist effects on the high-affinity D_2R agonist binding sites (Tables 1 and 2).

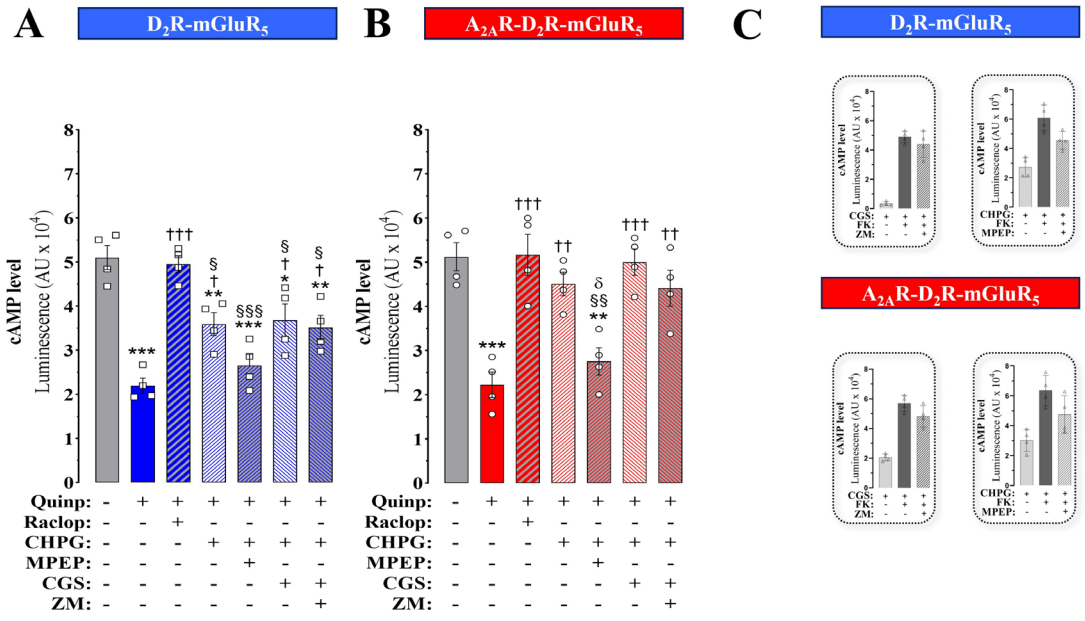
Figure 2.4 Assessing $A_{2A}R$ dependent allosteric modulation of D_2R-GluR$_5$ heteromer by [^{3}H]-raclopride/quinpirole competition binding experiments. (**A**) Competition assays were performed in HEK293T cells transiently expressing D_2R and mGluR$_5$ (blue squeares) or $A_{2A}R$, D_2R, and mGluR$_5$ (red circles) with/without agonist(s)/antagonist(s) for adenosine $A_{2A}R$ or mGluR$_5$ either alone or in combination as indicated. (+)-Butaclamol (100 μM) was used to determine the non-specific binding and the specific binding at the lowest concentration of the [^{3}H]-raclopride employed was defined as one hundred percent. Results are expressed as percentage of specific binding (mean ± SEM; n = 4 independent experiments performed in triplicate). (**B**) Percentage of change comparing CHPG alone or CHPG plus CGS-21680 induced changes in the D_2R high affinity values (($K_{i,\ High}$ (nM)) with/without transient co-expression of $A_{2A}R$. Results are expressed as means ± S.E.M.; n = 4, each determination performed in triplicate. *$p < 0.05$, Mann-Whitney U-test.



cAMP functional experiments. In cells expressing D_2R and mGluR$_5$ forming D_2R-mGluR$_5$ heterocomplexes, the D_2R agonist activation with quinpirole (100 nM) induced a G_i protein-mediated inhibition of adenylyl cyclase that first was raised with 5 μM forskolin (**Fig. 2.5**). This effect was highly significantly blocked by the D_2R antagonist raclopride (1 μM). In these cells, the mGluR$_5$ agonist CHPG stimulation significantly counteracted the D_2R agonist-induced reduction of cAMP accumulation (**Fig. 2.5 A**). The significant effect of CHPG (500 μ M) was significantly reduced by the mGluR$_5$ antagonist MPEP (300 μM). We should consider also that mGluR$_5$ might simply activate Gs, inducing cAMP accumulation, independently of D2-Gi-induced

inhibition of adenylate cyclase. The co-treatment with the $A_{2A}R$ agonist did not enhance the counteraction of the inhibitory D_2R signalling by CHPG (**Fig. 2.5 A**).

Figure 2.5 Functional evaluation of $A_{2A}R$-mediated modulation of D_2R-$GluR_5$ heteromer. HEK293T cells transiently expressing D_2R and $mGluR_5$ (**A**) or $A_{2A}R$, D_2R, and $mGluR_5$ (**B**) were treated with forskolin before incubation with quinpirole (Quinp, X nM), raclopride (Raclop, X nM), CHPG (X nM), MPEP (X nM), CGS-21680 (CGS, X nM) and ZM-241385 (ZM, X nM). The cAMP levels were determined using a cAMP-Glo™ Assay detection kit (*see* Methods). Intracellular cAMP levels are given in luminescence intensity (AU, arbitrary units) after subtracting background basal luminescence (cAMP levels measured in non-transfected, non-treated cells). Results are expressed as means ± S.E.M.; $n = 4$ independent experiments, each determination performed in quadruplicates. $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$, one-way ANOVA followed by Tukey's post-hoc test compared with cells treated only with forskolin (control). $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger}p < 0.01$ and $^{\dagger}p < 0.05$, one-way ANOVA followed by Tukey's post-hoc test when compared with cells treated only with quinpirole; $^{\S\S\S}p < 0.001$, $^{\S\S}p < 0.01$ and $^{\S}p < 0.05$, when compared with cells treated only with quinpirole plus raclopride; $^{\delta}p < 0.05$, when compared to cells treated with quinpirole and CHPG. (**C**) CHPG and CGS21680-induced cAMP levels. HEK293T cells transiently expressing D_2R and $mGluR_5$ (Top inset box) or $A_{2A}R$, D_2R, and $mGluR_5$ (Bottom inset box) were treated with and without forskolin before incubation with ligands. Results are expressed as means ± S.E.M.; $n = 4$ independent experiments, each determination performed in quadruplicates. Statistical analysis performed using one-way ANOVA followed by Tukey's post-hoc test. (Top inset box) CHPG versus CHPG + forskolin ($p < 0.001$), CHPG versus CHPG + forskolin + MPEP ($p < 0.05$); CGS-21680 versus CGS-21680 + forskolin ($p < 0.001$), CGS-21680 versus CGS-21680 + forskolin + ZM-241385 ($p < 0.001$). (Bottom inset box) CHPG versus CHPG + forskolin ($p < 0.01$), CHPG versus CHPG + forskolin + MPEP (*ns*); CGS-21680 versus CGS-21680 + forskolin ($p < 0.001$), CGS-21680 versus CGS-21680 + forskolin + ZM-241385 ($p < 0.001$).



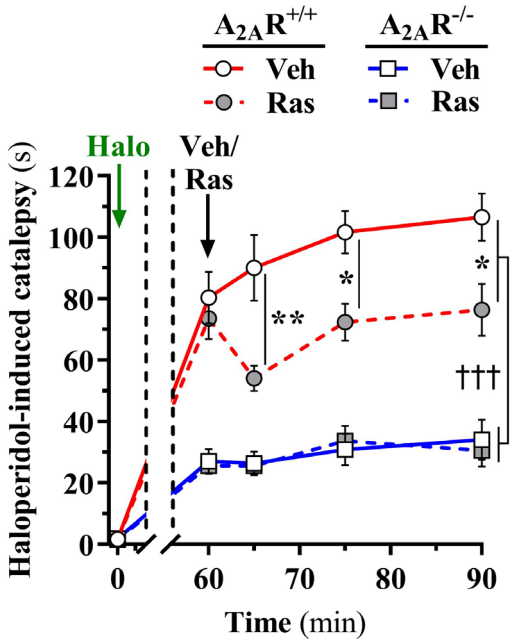
Likewise, quinpirole significantly reduced the cAMP level in cells expressing $A_{2A}R$, D_2R, and $mGluR_5$ (**Fig. 2.5 B**). The $mGluR_5$ agonist CHPG had an improved ability to counteract the adenylyl cyclase inhibition produced by the D_2R agonist in these cells,

yielding cAMP levels similar to those obtained after blocking D_2R signalling with raclopride (**Fig. 2.5 B**). Upon $A_{2A}R$ and $mGluR_5$ agonist co-activation, a larger counteraction of the D_2R agonist action was found compared to that obtained with such a co-treatment performed in cells expressing only D_2R and $mGluR_5$. These results suggest a synergistic and significant counteraction by $A_{2A}R$ and $mGluR_5$ agonists of the D_2R agonist-induced decrease of cAMP accumulation (**Fig. 2.5 B**). Such effects of the combined agonist treatment were only weakly reduced by the $A_{2A}R$ antagonist (ZM-241385). In cells not expressing the $A_{2A}R$, the $A_{2A}R$ antagonist failed to produce any changes in the cAMP accumulation under such co-agonist treatments.

Experiments on haloperidol-induced catalepsy. The catalepsy is mainly produced by haloperidol induced blockade of D2R complexes in the dorsal striatal-pallidal GABA neurons within the dorsal striatum. These GABA neurons mediate motor inhibition, counteracted by D2R agonist induced activation of the D2R homo and heterocomplexes like the D2R-A2AR or the D2R-mGluR5 heterocomplexes. The D2R activation of the dorsal striatal-pallidal GABA neurons is also essential for maintenance of normal locomotor activity.

The catalepsy induced by the D_2R antagonist haloperidol was evaluated in 10-minute time intervals from 60 min to 90 min after the injection of haloperidol (Figure 6). In wild type mice the $mGluR_5$ negative allosteric modulator raseglurant produced in this time-period a significant reduction of the catalepsy time which was in the order of 25% (**Fig. 2. 6**). In contrast, such a reduction of catalepsy was not observed by raseglurant treatment of $A_{2A}R^{-/-}$ mice. Furthermore, in vehicle treated $A_{2A}R^{-/-}$ animals the haloperidol induced catalepsy was markedly reduced compared to that obtained in vehicle treated wild-type mice (**Fig. 2. 6**).

Figure 2.6 $A_{2A}R$ expression is needed for $mGluR_5$ modulation of D_2R-dependent behaviour in mice. Raseglurant reverses haloperidol-induced catalepsy. Haloperidol-induced cataleptic behaviour was measured as the time spent with both front paws resting on the bar (*see* Methods). Wild type (circles), $A_{2A}R^{-/-}$ (squares) animals were pre-treated with haloperidol (i.p., 1 mg/kg, i.p.) at time 0 (green arrow). Subsequently, 1h later, animals were administered (i.p.; black arrow) with either vehicle (20% DMSO in saline) or raseglurant (Ras, 1 mg/kg). The time spent in a cataleptic position was measured after 5, 15 and 30 min after raseglurant (or vehicle) administration. Results are expressed as the mean time spent cataleptic ± SEM over a period of 120 s measurement (n=6 animals per group). The cataleptic behaviour was calculated and compared within groups by a multiple t-test statistical analysis. *$p < 0.05$ and **$p < 0.01$, two-way ANOVA followed by Šídák's post-hoc test. ††††$p < 0.0001$, three-way ANOVA (Phenotype, $F_{(1, 100)} = 279.6$).



2. 3. Discussion

The existence of the D_2R-$mGluR_5$ heterodimers in the bio-membranes of living cells was first demonstrated by bimolecular fluorescence complementation experiments in cellular models [179]. Our new findings are that transient co-expression of $A_{2A}R$ in HEK293T cells together with D_2R^{Rluc} and $mGluR_5^{GFP2}$ resulted in a significant and marked increase in the formation of the D_2R-$mGluR_5$ heterodimer, a component of the $A_{2A}R$-D_2R-$mGluR_5$ heterocomplex, based on the increase in the $BRET^2$ max values. Such an increase could be related to the development of an increased affinity of the two D_2R and $mGluR_5$ protomers for each other due to allosteric changes related to the formation of the $A_{2A}R$-D_2R-$mGluR_5$ complex. In line with this hypothesis, the $BRET^2$50 values were significantly reduced for the D_2R-$mGluR_5$ heteromeric component of this trimeric heteroreceptor complex.

These results are also supported by the demonstration with PLA that an increased density of PLA positive D_2R-$mGluR_5$ clusters was observed when $A_{2A}R$ expression had been added to the cells compared to cells only expressing D_2R and $mGluR_5$. In agreement, in the mouse dorsal striatum the D_2R-$mGluR_5$ complexes were significantly reduced in the $A_{2A}R^{-/-}$ mice. Thus, it becomes clear that the expression of the A2AR in the mouse dorsal striatum is necessary to facilitate that the D_2R and $mGluR_5$ form a complex. It underlines that the multiple receptor protomers in the high order heteroreceptor complexes are dependent on each other to improve or facilitate the formation of such complexes in the dorsal striatum.

The different results obtained on haloperidol induced catalepsy in wild type mice *vs* $A_{2A}R^{-/-}$ mice are of substantial interest since they can indicate a functional role of the $A_{2A}R$-D_2R-$mGluR_5$ heteroreceptor complexes in the dorsal striatum as previously

discussed [2,106]. There was a marked reduction in the haloperidol induced catalepsy in the $A_2AR^{-/-}$ mice compared to wild-type mice. Thus, in the absence of the $A_{2A}R$, the D_2R antagonist haloperidol appears to have a substantially reduced potency to block the D_2R which can be caused by the loss of the antagonistic $A_{2A}R$-D_2R interaction [60,182]. According to the current findings in cell lines the D_2R-$mGluR_5$ heterocomplexes should be also formed to a much lower degree in the absence of $A_{2A}R$ in view of their dependency of $A_{2A}R$ according to the PLA experiments performed. The counteraction of the D2R mediated inhibitory actions on cAMP signalling by CHPG, a $mGluR_5$ agonist, was in our cell line also more effective in cells co-expressing beside D_2R and $mGluR_5$ also $A_{2A}R$.

It seems likely that the formation of the $A_{2A}R$-D_2R-$mGluR_5$ complex enhances the affinity of the D_2R and $mGluR_5$ protomers for each other in this complex. It is of high interest that the biochemical binding experiments reveal that the $mGluR_5$ CHPG agonist-induced increase in D_2R $K_{i,\ High}$ values becomes significantly higher in the $A_{2A}R$-D_2R-$mGluR_5$ complex compared to the D_2R-$mGluR_5$ complex despite the absence of $A_{2A}R$ agonist exposure. Thus, although agonist activation of the $A_{2A}R$ seems necessary to exert negative allosteric modulation of the D_2R protomer agonist binding via heteroreceptor complexes, an increased constitutive activity of the $A_{2A}R$ protomer could explain the above results.

As expected, the combined incubation with CHPG and CGS-21680 led to an even stronger increase in the D_2R $K_{i,\ High}$ values of the $A_{2A}R$-D_2R-$mGluR_5$ complex, demonstrating the impact of the $A_{2A}R$ protomer on the D_2R-$mGluR_5$ allosteric interactions, which can involve both constitutive and $A_{2A}R$ agonist induced inhibition of D_2R agonist binding. Our findings represent one of the first examples of integrative activity within a higher-order heteroreceptor complex and show how one receptor ($A_{2A}R$) can substantially modulate the structure and recognition of a participating receptor heterodimer (D_2R-$mGluR_5$) in such a trimeric receptor complex.

The pharmacological analysis of the $A_{2A}R$-D_2R-$mGluR_5$ complex and its impact on cAMP levels indicated that the $A_{2A}R$ can modulate the effects of the D_2R-$mGluR_5$ interactions on cAMP signalling. It was found that when the $A_{2A}R$-D_2R-$mGluR_5$ complex was likely to be formed through the expression also of the $A_{2A}R$, the $mGluR_5$ agonist had an increased ability to counteract the D_2 agonist-induced $G_{i/o}$ mediated inhibition of the cAMP levels in comparison to the counteraction observed in the absence of $A_{2A}R$ expression. The same was also true for the combined treatment with the $mGluR_5$ agonist CHPG and the $A_{2A}R$ agonist CGS-21680 when the $A_{2A}R$ was co-expressed. A stronger counteraction of the D_2R induced inhibition of the cAMP levels was observed when $A_{2A}R$ expression was present.

Taken together, our work on cell lines gives strong indications that, in the $A_{2A}R$-D_2R-$mGluR_5$ complex, the $A_{2A}R$ protomer enhances the formation of the D_2R-$mGluR_5$

component of the complex with enhanced inhibition of D_2R agonist binding recognition and its $G_{i/o}$ mediated cAMP signalling. The inhibitory effects by $A_{2A}R$ and $mGluR_5$ on D_2R recognition and signalling reveal a significant molecular integration in $A_{2A}R$-D_2R-$mGluR_5$ complexes, likely formed also in the dorsal striatum. The $A_{2A}R$ and $mGluR_5$ antagonists targeting the $A_{2A}R$-D_2R-$mGluR_5$ complexes in dorsal striatum may reduce the haloperidol-induced catalepsy by removal of the $A_{2A}R$ and $mGluR_5$ protomer mediated allosteric inhibition of the D2R protomer. Understanding of the trimeric complexes formed by these GPCRs could provide novel strategies for development of drugs against neuropsychiatric and neurodegenerative diseases by targeting their antagonistic receptor-receptor interactions.

CHPATER 3
Results and Discussion

SPECIFIC AIM 4. *Combined treatment with low doses of OSU-6162, a Sigma1R ligand, and an A2AR agonist fails to alter cocaine self-administration but increases accumbal A2AR-D2R heteroreceptor complexes and their antagonistic allosteric receptor-receptor interactions.*

3. 1. Background

It was previously demonstrated that cocaine can target the Sigma1R which can counteract actions of cocaine [183]. However, the Sigma1R agonist OSU-6162 which in low doses (5 mg/kg) selectively binds to the Sigma1R [184] failed to alter the behavior found in cocaine self-administration in rats [185]. Nevertheless, based on the proximity ligation assay (PLA) the low dose of OSU-6162 could still produce in cocaine self-administration significant increases in the density of the A2AR-D2R and D2R-Sigma1R heterocomplexes in the nucleus accumbens shell compared to its effects in yoked saline treated rats [185]. Furthermore, the effects of the A2AR agonist CGS 21680 on its inhibition of D2R affinity, given *ex vivo*, were significantly enhanced by the OSU-6162 treatment versus vehicle treatment. Thus, neurochemical effects on the heteroreceptor complexes were observed in the absence of changes in cocaine infusion and active lever pressing.

It therefore became of interest to test if combined treatment with a low dose of OSU-6162, (2.5 mg/kg), and CGS21680 (0.05 mg/kg) also fails to alter cocaine self-administration while still producing significant changes in the A2AR-D2R heteroreceptor complexes and in their antagonistic allosteric receptor-receptor interactions. Such results would validate the previous work [185] and open up a new understanding of heterogeneities in the ventral striatal-pallidal GABA neurons as to responses to cocaine self-administration. It is based on the use of low doses of A2AR and Sigma1R agonists that by themselves do not alter cocaine self-administration.

3. 2. Results

Behavioral pharmacological analysis in cocaine self-administration

Following 17 sessions, rats acquired cocaine SA (i.e., they received > 25 infusion/2-h under 0.25 mg/kg/infusion of cocaine) and displayed < 10 % variation in the number of cocaine infusions (**Fig. 3.1**).

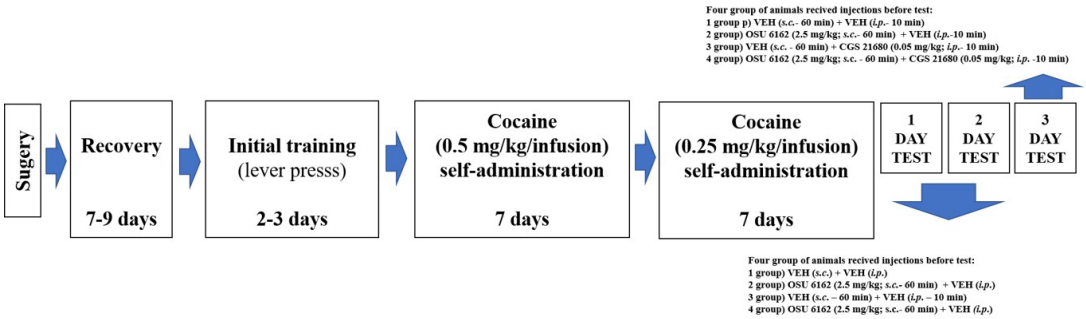


Figure 3.1. Experimental design of the study. Schematic diagram illustrating the experimental procedure.

The mean number of cocaine infusions per day during the last 3 self-administration days varied from 23 to 28. The total cocaine intake during 17 days (means ± SEM of 8 rats) was 103 ± 10 mg/rat for vehicle group, 148 ± 33 mg/rat for OSU-6162 group, 123± 18 mg/rat for CGS 21680 group (see **Fig. 3.2** for more details), and 134 ± 15 mg/rat for OSU-6162 + CGS 21680 groups (**Fig. 3.3**).

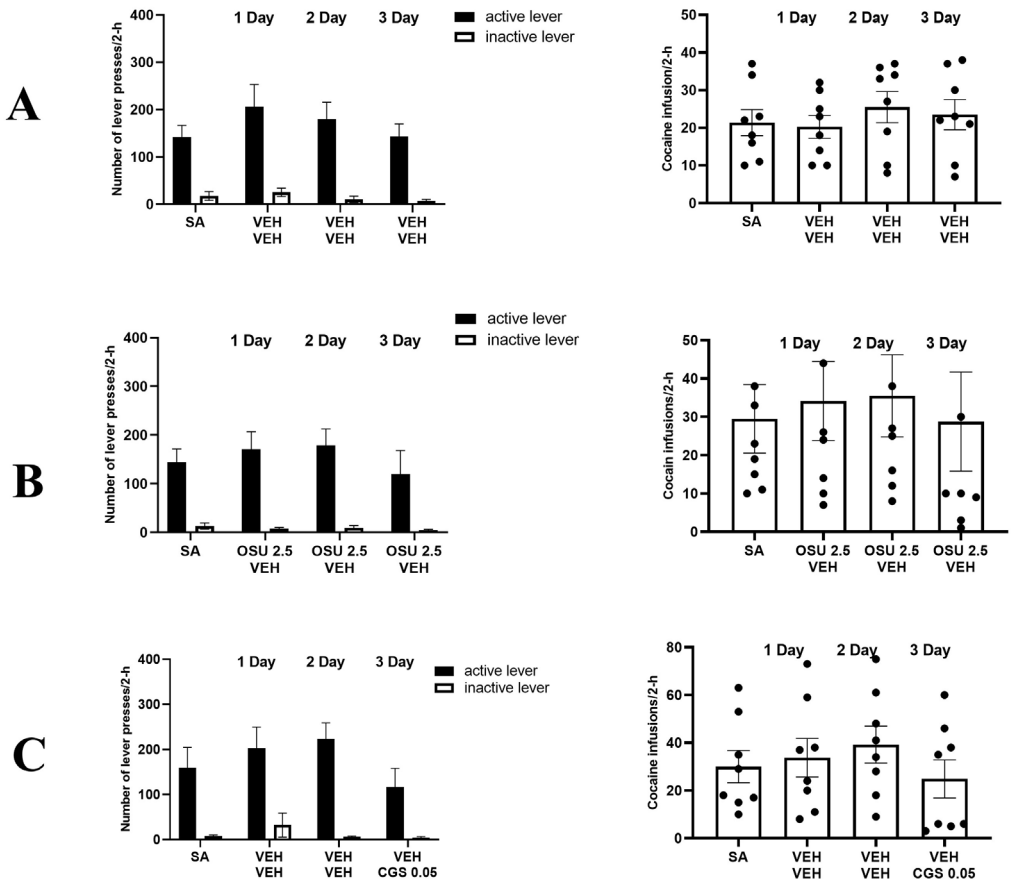


Figure 3.2. (A) Effects of repeated administration of vehicle (VEH; 0.9% saline, *s.c.*) **(B)** OSU-6162 (OSU; 2.5 mg/kg, *s.c.*) and **(C)** CGS 21680 (CGS; 0.05 mg/kg, *i.p*) treatments on the maintenance of cocaine (0.25 mg/kg/infusion) self-administration under the FR 5 schedule of reinforcement in the rats. The numbers of "active" and "inactive" lever presses (left panels) as well as cocaine infusions (right panels) after 2-h sessions are expressed as the means (± SEM) of the data from 8 rats/group. SA – mean of last three cocaine self-administration sessions.

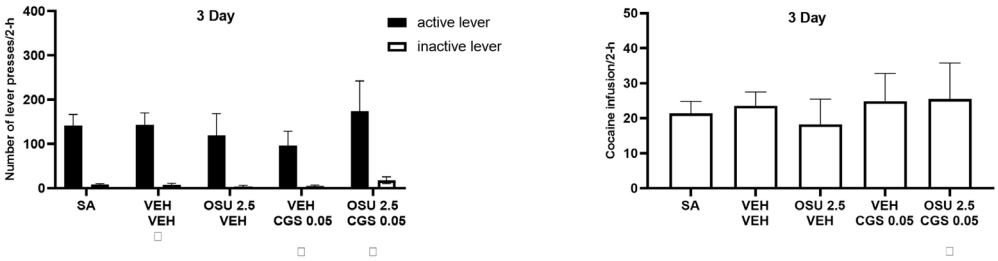


Figure 3.3. Effects of repeated administration of OSU-6162 (OSU; 2.5 mg/kg, *s.c.*) with combination with CGS 21680 (CGS; 0.05 mg/kg, *i.p*.) treatments on the maintenance of cocaine (0.25 mg/kg/infusion) self-administration under the FR 5 schedule of reinforcement in the rats. The numbers of „active" and „inactive" lever presses (left panels) as well as cocaine infusions (right panels) after 2-h sessions are expressed as the means (± SEM) of the data from 8 rats/group. SA – mean of last three cocaine self-administration sessions.

No difference between the groups assigned as vehicle, OSU-6162 or CGS 21680 groups in context of „active: and „inactive" lever presses and cocaine infusions was observed (**Fig. 3.2**).

OSU-6162 in a dose of 2.5 mg/kg given in combination with CGS 21680, 0.05 mg/kg, did not change the number of „active" and „inactive" lever presses [group x lever $F(3, 56) = 0.174$, $p = 0.91$; group $F(3, 56) = 0.045$, $p = 0.71$; lever $F(1, 56) = 28.58$; $p = 0.000$] and cocaine infusions [$F(3,28)$]=0.14; p=0.093 (**Fig. 3.3**).

Effects of combined systemic OSU-6162 and CGS 21680 treatment on the antagonistic allosteric A2AR-D2R receptor-receptor interactions in the ventral striatum in cocaine self-administration

Co-treatment with OSU-6162 (2.5 mg/kg) and CGS 21680 (0.05 mg/kg) with the acute A2AR agonist given on the last day 180 min (because 60 min pretreatment + 120 min session SA) before decapitation, resulted in a marked and significant increase in the D2R $K_{i,\ High}$ values in the ventral striatum compared to vehicle controls (**Fig. 3.4**)

Treatment with OSU-6162 alone in this dose led to a smaller but still significant increase in these values vs vehicle controls reflecting reductions in the affinity of the high affinity component of the D2R, while CGS 21680 alone in 0.05 mg/kg failed to have a significant change *vs*. vehicle treated controls.

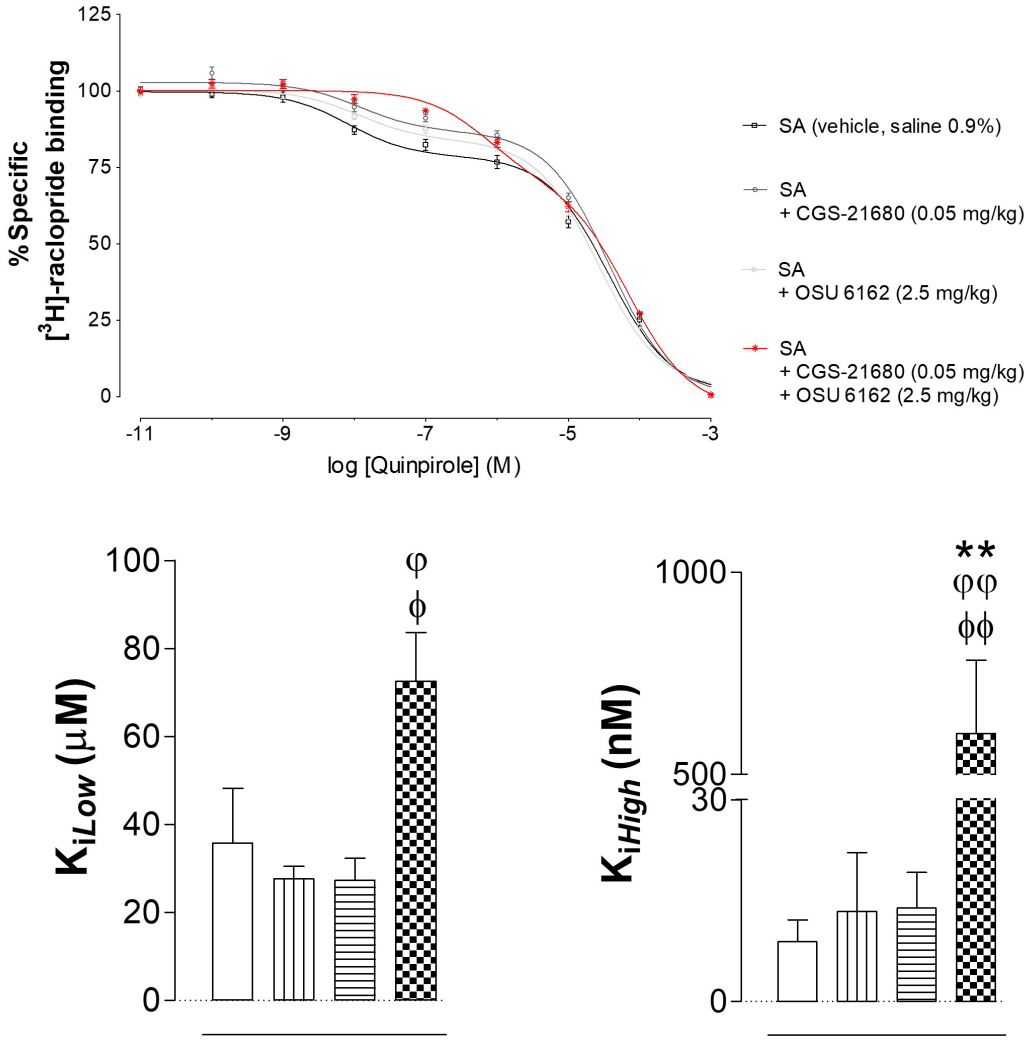


Figure 3.4. [^{3}H]-raclopride/quinpirole competition experiments to determine changes in D2R affinities induced by adenosine A2AR agonist CGS-21680 in repeated administration of OSU-6162 (OSU; 2.5 mg/kg, *s.c.*) with combination with CGS 21680 (CGS; 0.05 mg/kg, *i.p.*) treatments on the maintenance of cocaine (0.25 mg/kg/infusion) self-administration. (Top) Competition experiments involving dopamine D2-likeR antagonist [^{3}H]-raclopride binding versus increasing concentrations of quinpirole were performed in ventral striatal membrane preparations from cocaine self-administration rat group (60 µg/ml) in the presence or absence of the adenosine A2A agonist CGS-21680 (100 nM) as indicated. Nonspecific binding was defined as the binding in the presence of 100 µM (+)-butaclamol. (A) [^{3}H]-raclopride/quinpirole displacement curve based on the values of four rats with each experiment performed in duplicate. The binding values are given in percent of specific binding at the lowest concentration of quinpirole employed. (Bottom panels) Analysis and presentation are given of the A2AR agonist CGS-21680 (100 nM) induced changes in the high-affinity value ($K_{i,\ High}$) and low-affinity value ($K_{i,\ Low}$),. Means ± SEM. are given from four independent

experiments performed in duplicate. Statistical analysis was performed by paired Student's t-test. Changes in the high-affinity value ($K_{i,\ High}$) **($p < 0.01$): the group of rats co-treated with CGS-21680 (0.05 mg/kg, *i.p.*)) + OSU-6162 (OSU; 2.5 mg/kg, *s.c.*) is significantly different compared to the group receiving saline-solution or treated with CGS-21680 (0.05 mg/kg, *i.p.*) alone or OSU-6162 (OSU; 2.5 mg/kg, *s.c.*).

Studies within situ PLA on the effects of low doses of OSU-6162 and CGS 21680 in cocaine self-administration on A2AR-D2R and D2R-Sigma1R heteroreceptor complexes of nucleus accumbens shell

A2AR-D2R heteroreceptor complexes

OSU-6162 treatment by itself in the low dose of 2.5 mg/kg given once daily over three days produced a significant increase in the density of red PLA blobs (complexes) per nucleus in the sampled field in the nucleus accumbens shell (**Fig. 3.5**). In combination with a low dose (0.05 mg/kg) of the A2AR agonist CGS 21680, which by itself did not change the density of the PLA, significantly enhanced the density of the PLA complexes in the nucleus accumbens shell (**Fig. 3.5**).

D2R-Sigma1R heteroreceptor complexes

Unlike the case with the A2AR-D2R heterocomplexes OSU-6162 alone in a daily dose of 2.5 mg/kg failed to significantly change the density of these heteroreceptor complexes in the nucleus accumbens shell (**Fig. 3.5**).

Evidence for the existence of A2AR/D2R and D2R/Sigma1R heteroreceptor complexes in astrocytes and effects of OSU6162 (2.5 mg/kg) and cocaine self-administration on astroglia in the nucleus accumbens shell

Density of astrocytes

Cocaine self-administration versus yoked saline controls produced by itself a significant increase in the density of astroglial cells in the nucleus accumbens shell. This increase was not further altered by the current 3 days treatment with OSU-6162 in a low dose 2.5 mg/kg (**Fig. 3.6**).

Astrocytic branches

Again, cocaine self-administration alone caused a highly significant and substantial increase in the number of branches per astrocyte in the nucleus accumbens shell versus yoked saline controls. Furthermore, the current OSU treatment significantly increased the number of branches per astrocyte in the vehicle treated yoked saline rats but not in the cocaine self-administration group (**Fig. 3.6**).

Figure 3.5. Effects of OSU-6162 (OSU; 2.5 mg/kg, *s.c.*) treatment **in cocaine self-administration** on the density of PLA positive **A2AR-D2R and D2R-Sigma1R** heteroreceptor complexes in the nucleus accumbens shell (AcbSh). Means +- SEM is shown.

Representative images of the red PLA positive complexes (**D2R-Sigma1R** heteroreceptor complexes) are presented in the nucleus accumbens shell (AcbSh) in vehicle and OSU-6162 (2.5 mg/kg) treatment.

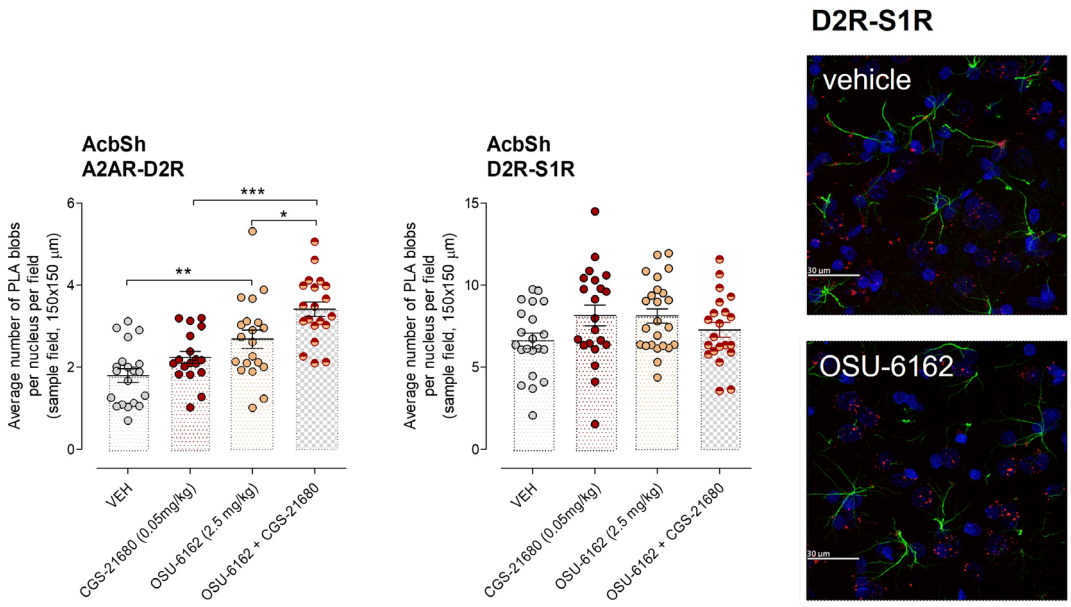
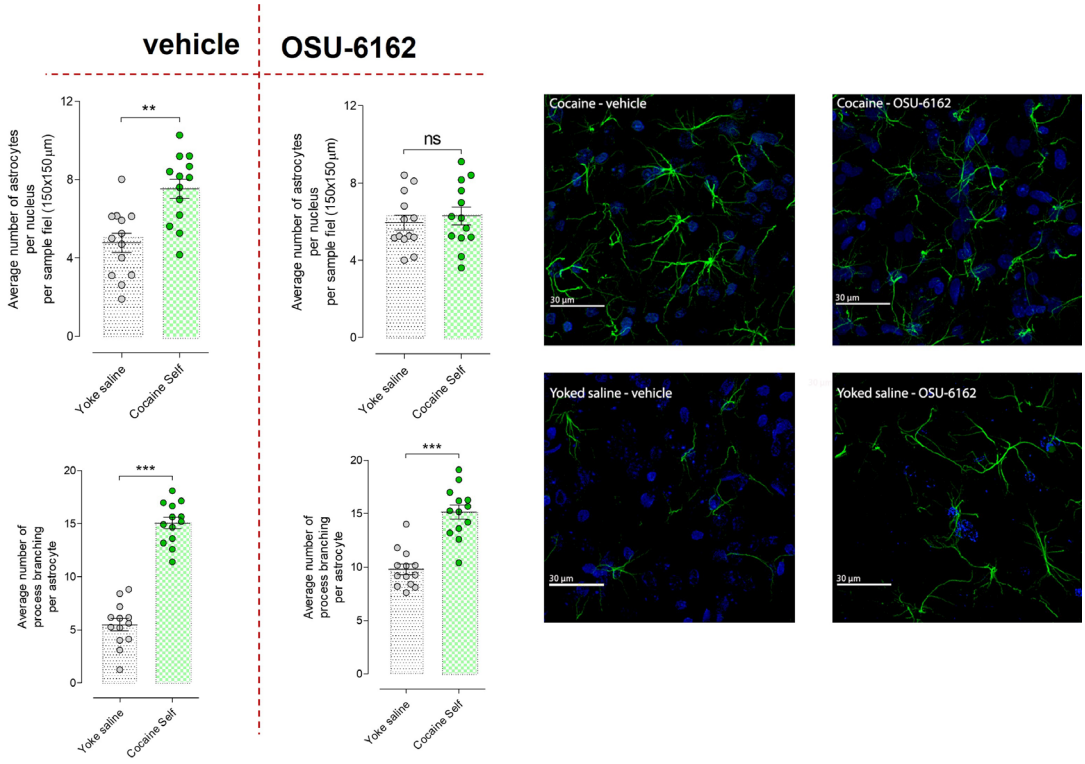


Figure 3.6. Effect of cocaine self-administration compared with yoked saline control on the average number of branches per astrocyte in rats treated with vehicle and OSU-6162, respectively. Means +- SEM are shown. Differences between groups were tested for significance with Welch's t-test. (Left panels) Representative image samples from rats in cocaine self-administration model (top) and yoked saline (bottom). Astrocytes in green (GFAP) and nuclei in blue (DAPI).



3. 3. Discussion

Previous work failed to demonstrate that the monoamine stabilizer OSU-6162 [186] in a low dose (5 mg/kg)which mainly activates the Sigma1R [184] altered cocaine self-administration in terms of active lever pressing and total cocaine intake even if a small trend for a reduction was found [185]. However, in the experiments involving three-day treatments with OSU-6162 significant increases in the density of the A2AR-D2R and D2R-Sigma1R heterocomplexes of the nucleus accumbens shell did develop in cocaine self-administration vs vehicle treated rats. This may involve also an increased formation of putative trimeric A2AR-D2R-Sigma1R heterocomplexes [33,50,187]. It was also found that when adding the A2AR agonist *ex vivo* to the membrane preparations from OSU-6162 treated rats undergoing cocaine self-administration a substantial and significant enhancement of the A2AR mediated allosteric inhibition of the D2R protomer affinity was observed versus vehicle treated rats [185]. The mechanism was proposed to involve a cocaine induced increase in the expression of Sigma1R in the ventral striatum [50,185,187,188]. It was proposed to produce increased formation of A2AR-D2R-Sigma1R in the ventral striatal-pallidal GABA neurons in which the Sigma1R protomer can enhance the allosteric A2AR protomer mediated inhibition of the D2R protomer function through a conformational change in the D2R protomer induced by the sigma1R activation [33].

In the current experiments the previous work on cocaine self-administration has been further explored by use of even a lower dose of OSU-6162, namely 2.5 mg/kg, given daily for three days and by use of systemic treatment with a low threshold dose of the A2AR agonist CGS 21680 (0.05 mg/kg) with or without combined systemic treatment with OSU-6162. No significant effects of these treatments were observed regarding active lever pressing for cocaine and total cocaine intake. Not even a trend for a reduction of this behavior was found. Despite these negative results on cocaine self-administration it was possible to demonstrate that combined treatment with the OSU-6162 and CGS 21680 in low doses caused highly significant increases in the A2AR-D2Rand D2R-Sigma1R heterocomplexes in the nucleus accumbens shell compared to vehicle treated animals. Treatment with a low dose of OSU-6162 alone but not with CCS21680 alone produced significant but reduced increases of the density of these two heteroreceptor complexes compared to vehicle treated animals in the nucleus accumbens shell. These results enhance the impact of the previous work. It is of considerable interest that these results are matched by corresponding degrees of reductions in the high affinity of the D2R protomers in the A2AR-D2R heterocomplexes with combined treatment of the Sigma1R and A2AR agonists producing the strongest reductions of D2R protomer affinity.

Heterogeneities of striatal D2R neurons have been previously described[189]. It is proposed that there exist discrete nerve cell population of ventral striatal-pallidal GABA neurons in the nucleus accumbens shell in which A2AR, D2R and Sigma 1R can form A2AR-D2R, D2R-Sigma1R and putative A2AR-D2R-Sigma1R heterocomplexes in a dynamic way with changes in their allosteric receptor-receptor interactions. They seem to have high affinity for low doses of A2AR and Sigma1R agonists under the impact of cocaine that appear to enhance their impact. However, their role in mediating effects of cocaine remains to be determined, since these low doses of A2AR and Sigma1R agonists do not alter the behavior of cocaine self-administration. It seems likely that one vulnerable molecular target can be the putative A2AR-D2R-Sigma1R heterocomplex containing A2AR and Sigma1R protomers, located in the ventral striatal-pallidal GABA antireward neurons [33,190].

It should be noted that cocaine self-administration also produced a substantial increase in the density of the astrocytes and in the number of branches per astrocyte in the nucleus accumbens shell compared to yoked saline animals. The cocaine induced changes in the mean number of astrocytes were not clearly altered by the low dose treatment with OSU-6162 but the average number of astrocytes was no longer significantly different in the yoked saline group versus the cocaine self-administration group. However, the increase in the number of branches per astrocyte in the yoked saline treated rats remained highly significantly reduced after OSU-6162 treatment compared with the OSU-6162 treated cocaine self-administration group. The results indicate that structural plasticity can develop in astrocytes upon cocaine self-administration and support the possibility that astrocytes can modulate cocaine actions on neuronal function in the nucleus accumbens shell. It may develop via alteration of astrocytic-neuronal crosstalk through changes in release of astrocytic signals, especially adenosine, operating via volume transmission (18), to reach and activate especially neuronal A1R protomers with a high affinity for adenosine. They can form presynaptic A1R-A2AR heterocomplexes (Ciruela et al.2006, Franco et al. 2008) that can inhibit the A2AR protomer signaling in the cortical-striatal glutamate terminals forming synapses on the ventral striatal-pallidal GABA anti-reward neurons (Borroto-Escuela et al.2018). It is proposed that also A1R-A2AR-D2R higher order complexes can exist on these glutamate terminals. Therefore, the reduced inhibition of the presynaptic D2R protomer signaling by the A2AR protomer leads to increased inhibition of glutamate release onto the ventral striatal-pallidal GABA antireward neurons. As a result, astrocytic release of adenosine can contribute to the reduction of activity in the GABA anti-reward neurons.

Such a mechanism may help explain why cocaine self-administration was not reduced in the current experiments in spite of increased formation of post-junctional A2AR-D2R complexes. In this complex located in the GABA anti-reward neurons

the A2AR protomer is known to reduce the inhibitory D2R protomer signaling that should cause increased firing in these anti-reward neurons and a reduction of cocaine self-administration as found previously but using a higher dose of the Sigma1R antagonist OSU6162 (Borroto-Escuela et al. 2021).

The explanation may be that the low dose of the Sigma 1R antagonist used in the current work (2.5 mg/kg) is not sufficient to produce a significant enhancement of the ability of the A1R-A2AR-D2R heterocomplex, located on the glutamate terminals, to reduce D2R protomer function and increase glutamate release. Thus, astrocytic induced adenosine release leading to A1R protomer activation followed by its inhibition of the A2AR protomer signaling is not sufficiently strong. The failure to produce a sufficient counteraction of the inhibitory D2R signaling on synaptic glutamate release onto the GABA anti-reward neurons can be one significant mechanism involved in causing the failure to produce a reduction of cocaine self-administrationö It occurred in spite of the findings of increased densities of A2AR-D2R heterocomplexes in the nucleus accumbens shell associated with A2AR agonist induced affinity reduction of the high affinity component of the D2R binding in the nucleus accumbens. However, the A1R-A2AR-D2R heterocomplex induced inhibition of glutamate release from other cortical glutamate terminal networks innervating other parts of the GABA anti-reward pathways, can be less strong in inhibition of glutamate releaase and lead to a reduction of cocaine self-administration

In conclusion, combined treatment with very low doses of Sigma1R antagonist and low dose of the A2AR agonist produced significant and clear-cut increases in the density of A2AR-D2R but not in D2R-Sigma1R heterocomplexes associated of the nucleus accumbens shell. A significant allosteric inhibition of the high and low affinity D2R agonist binding sites was found upon combined treatment with CGS21680 and OSU6162 in the nucleus accumbens. These observations were made in spite of failure of these actions to reduce the behavior of cocaine self-administration which was unexpected in view of the substantial increases in A2AR-D2R complexes and their antagonistic A2AR-D2R interactions in the nucleus accumbens. It is proposed that with the low doses used of A2AR and OSU6162, the cortical glutamate synapses on the GABA anti-reward neurons remain relatively intact with regard to its regulation of glutamate release by A1R-A2AR-D2R heterocomplex and cocaine (Shen et al.2008, Borroto-Escuela and Fuxe.2019). The astrocytic activation by cocaine can also contribute by release of adenosine stimulating the neuronal A1R which allosterically inhibits A2AR which leads to reduced inhibition of the D2R followed by its reduction of the glutamate release. Through these dynamic mechanisms involving the regulation of glutamate release from cortical glutamate terminals the GABA anti-reward neurons should become properly modulated and in balance with the A2AR-D2R-mGluR5 hetercomplexes in

the GABA anti-reward neurons. The result can be a maintenance of cocaine self - administration in spite of enhanced formation of antagonistic A2AR-D2R heterocomplexes in the ventral-striatal-pallidal GABA anti-reward neurons.

CONCLUSIONS

I. According to the dopamine hypothesis of schizophrenia the accumbal D2 receptors are relevant targets for antipsychotic drugs. It is therefore proposed that accumbal 5-HT2AR-D2R heterocomplexes can be a relevant target for hallucinogenic 5-HT2AR agonists besides a target for atypical antipsychotic drugs with high affinity, especially for the 5-HT2AR leading to blockade of its signaling. The effects of a standard 5-HT2AR agonist TCB-2 and a hallucinogenic 5-HT2AR agonist DOI L were therefore compared on the 5-HT2AR-D2R heterocomplexes in the shell and core of the nucleus accumbens with the aim to discover differential actions using the proximity ligation assay. The results reveal a significant increase by DOI but not TC-2 treatment in the density of the D2R-5-HT2AR heteroreceptor complexes in the nucleus accumbens shell but in the core. This action was blocked by a 5-HT2A receptor antagonist MDL-100907 previously known to counteract DOI-mediated actions. The current findings indicate that D2R-5-HT2AR heterocomplexes in the nucleus accumbens shell but not core may be a putative target for the hallucinogenic actions of DOI. It seems possible that hallucinogenic 5-HT2AR agonists may contribute to the development of psychosis through enhancement of the Gi/o mediated signaling of the D2R protomer in the D2R-5-HT2AR heterocomplexes of the nucleus accumbens shell. Selective blockade of the hallucinogenic 5-HT2AR protomer agonist binding site may offer a novel treatment of schizophrenia.

II. A need for new therapeutic approaches is necessary for dementia conditions and memory deficits of different origins, such as Alzheimer's disease. There are complex pathophysiological mechanisms involved, affecting adult hippocampal neurogenesis, in which neuropeptides and its neurogenesis regulation seem to participate. Neuropeptide Y(NPY) Y1 receptor (Y1R) and galanin (GAL) receptor 2 (GALR2) interact in brain regions responsible for learning and memory processes, emphasizing the hippocampus. Moreover, a significant challenge for treatments involving peptide drugs is bypassing the blood-brain barrier. The current study assesses the sustained memory performance induced by GALR2 and NPYY1R agonists intranasal coadministration and their neurochemical hippocampal correlates. Memory retrieval was conducted in the object-in-place task together with in situ proximity ligation assay (PLA) to

manifest the formation of GALR2/Y1R heteroreceptor complexes and their dynamics under the different treatments. We evaluated cell proliferation through a 5-Bromo-2'-deoxyuridine (BrdU) expression study within the dentate gyrus of the dorsal hippocampus. The GalR2 agonist M1145 was demonstrated to act with the Y1R agonist to improve memory retrieval at 24 hours in the object-in-place task. Our data show that the intranasal administration is a feasible technique for directly deliveringGalanin or Neuropeptide Y compounds into CNS.Moreover, we observed the ability of the co-agonist treatment to enhance the cell proliferation in the DG of the dorsal hippocampus through 5- Bromo-2'-deoxyuridine (BrdU) expression analysis at 24 hours. The understanding of the cellular mechanisms was achieved by analyzing the GALR2/Y1R heteroreceptor complexes upon agonist coactivation of their two types of receptor protomers in Doublecortin-expressing neuroblasts. Our results may provide the basis for developing heterobivalent agonist pharmacophores, targeting GALR2-Y1R heterocomplexes. It involves especially the neuronal precursor cells of the dentate gyrus in the dorsal hippocampus for the novel treatment of neurodegenerative pathologies as in the Alzheimer's disease.

III. The adenosine A_{2A} receptor ($A_{2A}R$), dopamine D_2 receptor (D_2R), and metabotropic glutamate receptor type 5 ($mGluR_5$) form $A_{2A}R$-D_2R-$mGluR_5$ heteroreceptor complexes in living cells and in rat striatal neurons. In the current study, we present experimental data supporting the view that the $A_{2A}R$ protomer plays a major role in the inhibitory modulation of the density and the allosteric receptor-receptor interaction within the D_2R-$mGluR_5$ heteromeric component of the $A_{2A}R$-D_2R-$mGluR_5$ complex *in vitro* and *in vivo*. The $A_{2A}R$ and $mGluR_5$ protomers interact and modulate D_2R protomer recognition and signalling upon forming a trimeric complex from these receptors. Expression of $A_{2A}R$ in HEK293T cells co-expressing D_2R and $mGluR_5$ resulted in a significant and marked increase in the formation of the D_2R-$mGluR_5$ heteromeric component in both bioluminescence resonance energy transfer and proximity ligation assays. A highly significant increase of the the high affinity component of D_2R ($D2R_{Ki\ High}$) values was found upon cotreatment with the $mGluR_5$ and $A_{2A}R$ agonists in the cells expressing $A_{2A}R$, D_2R, and $mGluR_5$ with a significant effect observed also with the $mGluR_5$ agonist alone compared to cells expressing only D_2R and $mGluR_5$. In cells co-expressing $A_{2A}R$, D_2R, and $mGluR_5$, stimulation of the cells with an $mGluR_5$ agonist like or D_2R antagonist fully counteracted the D_2R agonist induced inhibition of the cAMP levels which was not true in cells only expressing $mGluR_5$ and D_2R. In agreement, the $mGluR_5$ negative allosteric modulator raseglurant significantly reduced the haloperidol induced catalepsy in mice and in

$A_{2A}R$ knockout mice the haloperidol action had almost disappeared, supporting a functional role for $mGluR_5$ and $A_{2A}R$ in enhancing D_2R blockade resulting in catalepsy. The results represent a relevant example of integrative activity within higher order heteroreceptor complexes.

IV. Previous work indicated that acute treatment with the monoamine stabilizer OSU-6162 (5 mg/kg) via its high affinity for the Sigma1R, significantly increased the density of accumbal shell D2R-Sigma1R and A2AR-D2R heteroreceptor complexes following cocaine self-administration. Ex vivo actions of the A2AR agonist CGS21680 also suggested the existence of enhanced antagonistic accumbal A2AR-D2R allosteric interactions after treatment with OSU-6162in cocaine self-administration. However, the three days treatment with OSU-6162 (5 mg/kg) failed to alter the behavioral effects of cocaine self-administration. To test these results and the relevance of OSU-6162 (2.5 mg/kg) and/or A2AR (0.05 mg/kg) agonist interactions, treatment with these low doses of the receptor agonists were performed in cocaine self-administration and neurochemical and behavioral effects studied. No effects were demonstrated on cocaine self-administration but marked and highly significant increases using proximity ligation assay (PLA) were induced by the co treatment on the density of the A2AR-D2R heterocomplexes in nucleus accumbens shell. Significant decreases in the affinity of the D2R high affinity agonist binding sites were also observed. Thus, in low doses the highly significant neurochemical effects observed upon cotreatment with A2A and Sigma1R agonists on the A2AR-D2R heterocomplexes and their enhancement of allosteric inhibition of D2R high affinity binding are not linked to modulation of cocaine self-administration. Instead, these low doses of the Sigma1R and A2AR agonists can have selectively modulated the A2AR-D2R heterocomplexes of distinct neuronal populations of the ventral striatal-pallidal GABA neurons having an enhanced affinity for these receptor agonists. Their functions in the accumbal shell networks remain to be determined but putative A2AR-D2R-Sigma1R heterocomplexes may have a unique role in altering certain abuse actions of cocaine, related to modulation of distinct projections of these ventral striatal-pallidal GABA neurons.

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Annex 1

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SPECIAL ISSUE: REVIEW

The coming together of allosteric and phosphorylation mechanisms in the molecular integration of A2A heteroreceptor complexes in the dorsal and ventral striatal-pallidal GABA neurons

Dasiel O. Borroto-Escuela[1,4] · Luca Ferraro[2] · Sarah Beggiato[3] · Manuel Narváez[4] · Ramon Fores-Pons[4] · Jose E. Alvarez-Contino[4,5] · Karolina Wydra[6] · Małgorzata Frankowska[6] · Michael Bader[7] · Małgorzata Filip[6] · Kjell Fuxe[1]

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Abstract

The role of adenosine A2A receptor (A2AR) and striatal-enriched protein tyrosine phosphatase (STEP) interactions in the striatal-pallidal GABA neurons was recently discussed in relation to A2AR overexpression and cocaine-induced increases of brain adenosine levels. As to phosphorylation, combined activation of A2AR and metabotropic glutamate receptor 5 (mGluR5) in the striatal-pallidal GABA neurons appears necessary for phosphorylation of the GluA1 unit of the AMPA receptor to take place. Robert Yasuda (J Neurochem 152: 270–272, 2020) focused on finding a general mechanism by which STEP activation is enhanced by increased A2AR transmission in striatal-pallidal GABA neurons expressing A2AR and dopamine D2 receptor. In his Editorial, he summarized in a clear way the significant effects of A2AR activation on STEP in the dorsal striatal-pallidal GABA neurons which involves a rise of intracellular levels of calcium causing STEP activation through its dephosphorylation. However, the presence of the A2AR in an A2AR-fibroblast growth factor receptor 1 (FGFR1) heteroreceptor complex can be required in the dorsal striatal-pallidal GABA neurons for the STEP activation. Furthermore, Won et al. (Proc Natl Acad Sci USA 116: 8028–8037, 2019) found in mass spectrometry experiments that the STEP splice variant $STEP_{61}$ can bind to mGluR5 and inactivate it. In addition, A2AR overexpression can lead to increased formation of A2AR-mGluR5 heterocomplexes in ventral striatal-pallidal GABA neurons. It involves enhanced facilitatory allosteric interactions leading to increased Gq-mediated mGluR5 signaling activating STEP. The involvement of both A2AR and STEP in the actions of cocaine on synaptic downregulation was also demonstrated. The enhancement of mGluR5 protomer activity by the A2AR protomer in A2AR-mGluR5 heterocomplexes in the nucleus accumbens shell appears to have a novel significant role in STEP mechanisms by both enhancing the activation of STEP and being a target for $STEP_{61}$.

Keywords Allosteric receptor–receptor interactions · Adenosine A2A receptor · Cocaine · Striatal-enriched protein tyrosine phosphatase · Oligomerization · Phosphorylation

Introduction

In a recent Editorial by Robert Yasuda [1] on striatal-enriched protein tyrosine phosphatase (STEP) activity in central nervous system (CNS), the role of adenosine and STEP in modulating synaptic glutamate receptor function has been discussed, especially in relation to how STEP might be modulated by the adenosine A2A receptor (A2AR) activation [2]. Changes induced in striatal synaptic glutamate transmission were modulated by cocaine (10 μM) by an increase in adenosine levels and the consequent A2AR activation, a mechanism involving the participation of STEP [3] and mediating the A2AR agonist-induced reduction in cocaine self-administration. The possible involvement of A2AR-dopamine (DA) D2R heteroreceptor complex in STEP-mediated A2AR agonist-induced reduction in cocaine self-administration has also be proposed by R. Yasuda in its Editorial [1]. In this heteroreceptor complex, the A2AR protomer allosterically inhibits the DA D2R protomer

✉ Dasiel O. Borroto-Escuela
dasiel.borroto.escuela@ki.se

✉ Kjell Fuxe
kjell.fuxe@ki.se

Extended author information available on the last page of the article

Annex 2

frontiers in Pharmacology

PERSPECTIVE
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The Balance of MU-Opioid, Dopamine D2 and Adenosine A2A Heteroreceptor Complexes in the Ventral Striatal-Pallidal GABA Antireward Neurons May Have a Significant Role in Morphine and Cocaine Use Disorders

Dasiel O. Borroto-Escuela[1], Karolina Wydra[2], Ramon Fores-Pons[1], Lakshmi Vasudevan[3], Wilber Romero-Fernandez[1], Małgorzata Frankowska[2], Luca Ferraro[4], Sarah Beggiato[5], Minerva Crespo-Ramirez[6], Alicia Rivera[7], Luisa L. Rocha[8], Miguel Perez de la Mora[6], Christophe Stove[3], Małgorzata Filip[2] and Kjell Fuxe[1]**

[1]Department of Neuroscience, Karolinska Institutet, Biomedicum, Stockholm, Sweden, [2]Department of Drug Addiction Pharmacology, Maj Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland, [3]Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium, [4]Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy, [5]Department of Medical, Oral and Biotechnological Sciences, University of Chieti-Pescara, Chieti, Italy, [6]Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, Mexico, [7]Department of Cell Biology, University of Malaga, Instituto de Investigación Biomédica (IBIMA), Malaga, Spain, [8]Pharmacobiology Department, Center for Research and Advanced Studies, Mexico City, Mexico

Edited by:
Vsevolod V. Gurevich, Vanderbilt University, Nashville, TN, United States

Reviewed by:
Diego Guidolin, University of Padua, Italy
Amaia Maite Erdozain, University of the Basque Country, Spain

***Correspondence:**
Dasiel O. Borroto-Escuela
dasiel.borroto.escuela@ki.se
Kjell Fuxe
kjell.fuxe@ki.se

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The widespread distribution of heteroreceptor complexes with allosteric receptor-receptor interactions in the CNS represents a novel integrative molecular mechanism in the plasma membrane of neurons and glial cells. It was proposed that they form the molecular basis for learning and short-and long-term memories. This is also true for drug memories formed during the development of substance use disorders like morphine and cocaine use disorders. In cocaine use disorder it was found that irreversible A2AR-D2R complexes with an allosteric brake on D2R recognition and signaling are formed in increased densities in the ventral enkephalin positive striatal-pallidal GABA antireward neurons. In this perspective article we discuss and propose how an increase in opioid heteroreceptor complexes, containing MOR-DOR, MOR-MOR and MOR-D2R, and their balance with each other and A2AR-D2R complexes in the striatal-pallidal enkephalin positive GABA antireward neurons, may represent markers for development of morphine use disorders. We suggest that increased formation of MOR-DOR complexes takes place in the striatal-pallidal enkephalin positive GABA antireward neurons after chronic morphine treatment in part through recruitment of MOR from the MOR-D2R complexes due to the possibility that MOR upon morphine treatment can develop a higher affinity for DOR. As a result, increased numbers of D2R monomers/homomers in these neurons become free to interact with the A2A receptors found in high densities within such neurons. Increased numbers of A2AR-D2R heteroreceptor complexes are formed and contribute to enhanced

Annex 3

cells

MDPI

Review

Serotonin Heteroreceptor Complexes and Their Integration of Signals in Neurons and Astroglia—Relevance for Mental Diseases

Dasiel O. Borroto-Escuela [1,2,3,*], Patrizia Ambrogini [2], Manuel Narvaez [3], Valentina Di Liberto [4], Sarah Beggiato [5], Luca Ferraro [6], Ramon Fores-Pons [1,3], Jose E. Alvarez-Contino [1,7], Alexander Lopez-Salas [1,3], Giuseppa Mudò [4], Zaida Díaz-Cabiale [3] and Kjell Fuxe [1,*]

1 Department of Neuroscience, Karolinska Institutet, 171 77 Stockholm, Sweden; rforespons@gmail.com (R.F.-P.); erickjose@infomed.sld.cu (J.E.A.-C.); alexanderlopezsalas@gmail.com (A.L.-S.)
2 Department of Biomolecular Science Section of Morphology, Physiology and Environmental Biology, Campus Scientifico Enrico Mattei, via Ca' le Suore 2, 61029 Urbino, Italy; patrizia.ambrogini@uniurb.it
3 Facultad de Medicina, Instituto de Investigacion de Málaga, Universidad de Malaga, Campus de Teatinos s/n, 29071 Málaga, Spain; mnarvaez@uma.es (M.N.); zaida@uma.es (Z.D.-C.)
4 Department of Biomedicine, Neuroscience and Advanced Diagnostic (BIND), University of Palermo, 90134 Palermo, Italy; valentina.diliberto@unipa.it (V.D.L.); giuseppa.mudo@unipa.it (G.M.)
5 Department of Medical, Oral and Biotechnological Sciences, University of Chieti-Pescara, 66100 Chieti, Italy; sara.beggiato@unich.it
6 Department of Life Sciences and Biotechnology and LTTA Center, University of Ferrara, 44121 Ferrara, Italy; frl@unife.it
7 Department of Education, School of Medicine, Villa Clara University of Medical Sciences, Polyclinic Juan Bruno Zayas, 52900 Cifuentes, Cuba
* Correspondence: Dasiel.Borroto.Escuela@ki.se (D.O.B.-E.); kjell.fuxe@ki.se (K.F.); Tel.: +46-760396319 (D.O.B.-E.)

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Abstract: The heteroreceptor complexes present a novel biological principle for signal integration. These complexes and their allosteric receptor–receptor interactions are bidirectional and novel targets for treatment of CNS diseases including mental diseases. The existence of D2R-5-HT2AR heterocomplexes can help explain the anti-schizophrenic effects of atypical antipsychotic drugs not only based on blockade of 5-HT2AR and of D2R in higher doses but also based on blocking the allosteric enhancement of D2R protomer signaling by 5-HT2AR protomer activation. This research opens a new understanding of the integration of DA and 5-HT signals released from DA and 5-HT nerve terminal networks. The biological principle of forming 5-HT and other heteroreceptor complexes in the brain also help understand the mechanism of action for especially the 5-HT hallucinogens, including putative positive effects of e.g., psilocybin and the indicated prosocial and anti-stress actions of MDMA (ecstasy). The GalR1-GalR2 heterodimer and the putative GalR1-GalR2-5-HT1 heteroreceptor complexes are targets for Galanin N-terminal fragment Gal (1–15), a major modulator of emotional networks in models of mental disease. GPCR-receptor tyrosine kinase (RTK) heteroreceptor complexes can operate through transactivation of FGFR1 via allosteric mechanisms and indirect interactions over GPCR intracellular pathways involving protein kinase Src which produces tyrosine phosphorylation of the RTK. The exciting discovery was made that several antidepressant drugs such as TCAs and SSRIs as well as the fast-acting antidepressant drug ketamine can directly bind to the TrkB receptor and provide a novel mechanism for their antidepressant actions. Understanding the role of astrocytes and their allosteric receptor–receptor interactions in modulating forebrain glutamate synapses with impact on dorsal raphe-forebrain serotonin neurons is also of high relevance for research on major depressive disorder.

Keywords: serotonin receptors; heteroreceptor complexes; depression; astroglia; receptor tyrosine kinase; rapid antidepressant drugs; G protein-coupled receptors

Annex 4

frontiers in Pharmacology

ORIGINAL RESEARCH
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Intranasal Delivery of Galanin 2 and Neuropeptide Y1 Agonists Enhanced Spatial Memory Performance and Neuronal Precursor Cells Proliferation in the Dorsal Hippocampus in Rats

Dasiel O. Borroto-Escuela[1,2,3], *Ramón Fores*[1,4], *Mariana Pita*[1,4,5], *Miguel A. Barbancho*[4], *Pablo Zamorano-Gonzalez*[4], *Natalia García Casares*[4], *Kjell Fuxe*[1] *and Manuel Narváez*[1,4]*

[1]Department of Neuroscience, Karolinska Institute, Stockholm, Sweden, [2]Department of Biomolecular Science, Section of Physiology, University of Urbino, Urbino, Italy, [3]Grupo Bohío-Estudio, Observatorio Cubano de Neurociencias, Yaguajay, Cuba, [4]Instituto de Investigación Biomédica de Málaga, Facultad de Medicina, Universidad de Málaga, Málaga, Spain, [5]Departamento de Neurogenética, Instituto de Neurología y Neurocirugía, La Habana, Cuba

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Arjan Blokland,
Maastricht University, Netherlands

Reviewed by:
Gavin Scott,
University of Calgary, Canada
Claudia Bregonzio,
CCT CONICET Córdoba, Argentina

***Correspondence:**
Manuel Narváez
mnarvaez@uma.es

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A need for new therapeutic approaches are necessary for dementia conditions and memory deficits of different origins, such as Alzheimer's disease. There is complex pathophysiological mechanisms involved, affecting adult hippocampal neurogenesis, in which neuropeptides and its neurogenesis regulation seem to participate. Neuropeptide Y(NPY) Y1 receptor (Y1R) and galanin (GAL) receptor 2 (GALR2) interact in brain regions responsible for learning and memory processes, emphasizing the hippocampus. Moreover, a significant challenge for treatments involving peptide drugs is bypassing the blood-brain barrier. The current study assesses the sustained memory performance induced by GALR2 and NPYY1R agonists intranasal coadministration and their neurochemical hippocampal correlates. Memory retrieval was conducted in the object-in-place task together with in situ proximity ligation assay (PLA) to manifest the formation of GALR2/Y1R heteroreceptor complexes and their dynamics under the different treatments. We evaluated cell proliferation through a 5-Bromo-2'-deoxyuridine (BrdU) expression study within the dentate gyrus of the dorsal hippocampus. The GalR2 agonist M1145 was demonstrated to act with the Y1R agonist to improve memory retrieval at 24 hours in the object-in-place task. Our data show that the intranasal administration is a feasible technique for directly delivering Galanin or Neuropeptide Y compounds into CNS. Moreover, we observed the ability of the co-agonist treatment to enhance the cell proliferation in the DG of the dorsal hippocampus through 5- Bromo-2'-deoxyuridine (BrdU) expression analysis at 24 hours. The understanding of the cellular mechanisms was achieved by analyzing the GALR2/Y1R heteroreceptor complexes upon agonist coactivation of their two types of receptor protomers in Doublecortin-expressing neuroblasts. Our results may provide the basis for developing heterobivalent agonist pharmacophores, targeting GALR2-Y1R heterocomplexes. It involves especially the neuronal precursor cells of the dentate gyrus in the dorsal hippocampus for the novel treatment of neurodegenerative pathologies as in the Alzheimer's disease.

Keywords: galanin receptor 2, neuropeptide Y receptor 1, spatial memory, neurogenesis, receptor-receptor heterodimers

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