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HETERORECEPTOR COMPLEXES AND THEIR ALLOSTERIC RECEPTOR-RECEPTOR INTERACTIONS IN THE CENTRAL NERVOUS SYSTEM. FOCUS ON EXAMPLES FROM DOPAMINE D2 AND SEROTONIN 5-HT1A RECEPTORS

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A mi madre y mi abuelo por su infinito cariño y esfuerzos

A mis amigos de laurea

A mi isla quebrada

... a todos ellos mi mas sinceros afectos, agradecimientos, y estima

TABLE OF CONTENTS

ABSTRACT

G-protein coupled receptors (GPCR)-mediated signalling is a more complicated process than described previously since every GPCR and GPCR heteromer requires a set of G protein interacting proteins (GIP) which interacts with the receptor in an orchestrated spatio-temporal fashion. Therefore, there is a high interest in understanding the dynamics of the receptor-receptor and receptor-protein interactions in space and time, and specially, their integration in GPCR heterocomplexes of the Central Nervous System (CNS). Also, pathological protein-protein interactions in homocomplexes and heterocomplexes of Aβ, Tau, and α-Syn are at the heart of the development of conformational protein disorders. Along this work, experimental evidences are given to illustrate that GPCR interactions have relevance for neurological and mental diseases and are targets for drug development. GPCR containing heteromers and higher order heteromers through allosteric receptor-receptor interactions have become major integrative centers at the molecular level and their receptor protomers act as moonlighting proteins. They have become exciting new targets for neurotherapeutics in e.g. Parkinson's disease, schizophrenia, drug addiction, anxiety and depression opening up a new field in neuropsychopharmacology.

Along this work, the allosteric receptor-receptor interactions over the interfaces in A2AR-D2R, D2R-NTS1R, D2R-Sigma1R and 5-HT1A-FGFR1 heteroreceptor complexes will be explored and their biochemical, pharmacological and functional integrative implications in the CNS described. Methodologies for studies on receptor-receptor interactions are discussed including the use of FRET and BRET-based techniques in the analysis of G protein coupled receptor (GPCR) dimerization in living cells. *In situ* proximity ligation assay is performed to establish the existence of native heteroreceptor complexes in the CNS. GPCR interacting proteins (specially β-arrestin) and their receptor-protein interactions are also covered but their interactions with the allosteric receptor-receptor interactions in heteroreceptor complexes remain to be elucidated. The physiological and pathological relevance of the allosteric receptor-receptor interactions in heteroreceptor complexes is emphasized and novel strategies for treatment of mental and neurological disease are introduced based on this new biological principle of integration.

This work gives further experimental evidences which strongly support the current view that allosteric receptor–receptor interactions in heteroreceptor complexes appear to represent a new principle in biology making possible integration of signals already at the level of the plasma membrane. These heteroreceptor complexes and their dynamics may be part of the molecular basis of learning and memory. The receptor

protomers and their allosteric receptor-receptor interactions can be disturbed in neurological and mental disorders, and in diseases of peripheral tissues like the endocrine, cardiovascular and immune systems.

The dopamine (DA) neuron system most relevant for schizophrenia and Parkinson´s diseases is the meso-limbic-cortical DA system inter alia densely innervating subcortical limbic regions as well as the dorsal striatum. The field of dopamine D2Rs changed significantly with the discovery of many types of D2R heteroreceptor complexes in the ventral and dorsal striatum. The results indicate that the D2R is a hub receptor (*www.gpcr-hetnet.com*) which interacts not only with many other GPCRs including DA isoreceptors but also with ion-channel receptors, receptor tyrosine kinases, scaffolding proteins and DA transporters. Disturbances in several of these D2R heteroreceptor complexes may contribute to the development of schizophrenia and Parkinson´s diseases through changes in the balance of diverse D2R homo- and heteroreceptor complexes mediating the DA signal, especially to the ventral striato-pallidal GABA pathway. In schizophrenia, this will have consequences for the control of this pathway of the glutamate drive to the prefrontal cortex via the mediodorsal thalamic nucleus which can contribute to psychotic processes.

Allosteric receptor-receptor interactions in GPCR heteromers appeared to introduce an intermolecular allosteric mechanism contributing to the diversity and bias in the GPCR protomers. In A2A-D2R heteroreceptor complexes allosteric A2A-D2R receptor-receptor interaction brings about a biased modulation of the D2R protomer signalling (Chapter 1). A conformational state of the D2R is induced which moves away from Gi/o signaling and instead favours b-arrestin2 mediated signalling which may be the main mechanism for its atypical antipsychotic properties especially linked to the limbic A2AR-D2R heteroreceptor complexes. Furthermore, D2R-NTS1R heterocomplexes also exist in the ventral and dorsal striatum (Chapter 2) and likely also in midbrain DA nerve cells as D2R-NTS1R autoreceptor complexes where neurotensin produces antipsychotic and propsychotic actions, respectively. D2R protomer appeared to bias the specificity of the NT orthosteric binding site towards neuromedin N vs neurotensin in the heteroreceptor complex.

There is a new awareness that Receptor tyrosine kinases (RTK) and transmitter activated GPCR possess the capacity for transactivation not only via GPCR induced release of neurotrophic factors, but also during signal initiation and propagation, using shared signaling pathways or using themselves as signaling platforms via direct allosteric

receptor–receptor interactions. RTK are a family of transmembrane-spanning receptors that mediate the signaling from ligands such as growth factors, like the platelet-derived growth factor (PDGF), epidermal growth factor (EGF), the brain derived neurotrophic factor (BDNF), and the fibroblast growth factor (FGF). This hypothesis on direct GPCR-RTK receptor-receptor interactions in heteroreceptor complexes was introduced by Fuxe et al 1983. They also proposed the existence of 5-HT1A-FGFR1 heteroreceptor complexes having a role in depression. The hypothesis was introduced that the neurotrophic system FGF-2/FGFR1 may be a good candidate to mediate antidepressant induced improvement in 5-HT neuronal communication and neurotrophism with regeneration of connections lost during depression. RTK transactivation in response to antidepressant drug treatment was postulated to take place via a new allosteric receptor–receptor between distinct serotonin receptor subtypes and FGFR1 in heteroreceptor complexes.

The discovery of brain FGFR1-5-HT1A heteroreceptor complexes and their enhancement of neuroplasticity offers an integration of the serotonin and the neurotrophic factor hypotheses of depression at the molecular level. These heteroreceptor complexes were found in the hippocampus and midbrain raphe 5-HT nerve cells, enriched in 5-HT1A autoreceptors. Based on the triplet puzzle theory several sets of triplet homologies were identified that may be part of the receptor interface. Combined FGF-2 and 5-HT1A agonist treatment increased the formation of these heterocomplexes and the facilitatory allosteric receptor-receptor interactions within them leading to an enhancement of FGFR1 signaling (Chapter 3). This integrative phenomenon is reciprocal and RTK signaling can be placed downstream of GPCRs. Formation of such heterocomplexes involving two major classes of membrane receptors can be involved in regulating all aspects of receptor protomer function including recognition, signaling, trafficking, desensitization, and downregulation (Chapter 3). These events were associated with development of rapid antidepressant effects. These heteroreceptor complexes are a novel target for antidepressant drugs.

These examples, based on solid experimental evidences, serve to illustrate that allosteric receptor-receptor interactions in GPCR heteroreceptor complexes play a significant role in receptor diversity and bias of the participating GPCR protomers.

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. 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. Por ejemplo, se sabe que ciertas interacciones disfuncionales entre proteínas, los péptidos Aβ, Tau, y α-Syn, son consideradas el centro de los mecanismos moleculares de muchas enfermedades neurodegenerativas. También, a lo largo del presente trabajo, y basado evidencias experimentales, se ilustra la importancia de diversos complejos heteroreceptores de la familia de los GPCR como futuras dianas o blancos para el tratamiento de enfermedades neurodegenerativas (A2AR-D2R, enfermedad del Parkinson) y mentales (5-HT1A-FGFR1, depresión).

Los complejos de homo y heteroreceptores 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 si mismos, en excelente dianas o blancos para el desarrollo de nuevos neurofármacos o líneas neuroterapéuticas en enfermedades tales como el Parkinson, la esquizofrenia, la adición a drogas, la ansiedad y la depresión.

En la presente tesis, las interacciones alostéricas entre los receptores componentes de los homo y heterocomplejos de los receptores A2AR-D2R, D2R-NTS1R, D2R-Sigma1R y 5-HT1A-FGFR1 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 en el presente estudio 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 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 tratamientos farmacológicos 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 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: la 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 puede ser considerados como un receptor "hubs", que se caracteriza por una gran plasticidad para la 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 homo y heterocomplejos del receptores de la dopamina D2R, 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.

Las modulaciones alostéricas que operan a través de interacciones receptor-receptor introducen un nuevo mecanismo intermolecular que facilitan y contribuyen al desarrollo de procesos de diversificación y especificidad de la señal de los respectivos protómeros. En los complejos de heteroreceptores para la adenosina A2AR- y la

dopamina D2R (A2AR-D2R), las modulaciones alostéricas que operan y tienen lugar entre la interacción del receptor A2AR y D2R resultan en una compensación antagónica y un cambio en la selectividad por la señal de cada uno de los respectivos protómeros (Capítulo 1). El cambio conformacional inducido en el receptor de la dopamina D2R resulta en una perdida por la afinidad a la proteína G i/0 y un incremento significativo en la interacción del receptor D2R por la proteína citoplasmática β-arrestina, cambio que puede estar en la base de las propiedades antipsicóticas atípicas relacionadas con el receptor D2R. Estas modulaciones alostéricas operan a través de una interfase de interacción receptor-receptor muy bien definida y caracterizada, en la que solo ciertos amino ácidos actúan como puntos calientes o de encuentros, en lugar de amplios segmentos de la secuencia polipeptídica (Capítulo 1). En línea con estos resultados, también se ha demostrado la existencia de un complejo heteroreceptor entre el receptor de dopamina D2R y el receptor de neurotensina 1 (NTS1R) en el estriado dorsal y ventral del cerebro de rata (Capítulo 2). Se ha demostrado que el protómero (D2R) produce un cambio en la selectividad y afinidad del sitio ortostérico del receptor NTSR1, resultando en un aumento de la interacción y afinidad de la neuromedina N respecto a la neurotensina, así como cambios en los patrones de internalización.

Por otra parte, 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 el Dr. sueco 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, se 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 amino ácidos (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. En el presente trabajo (Capítulo 3) se aportan 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 (Capítulo 3).

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

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I. Borroto-Escuela DO, Romero-Fernandez W, Tarakanov AO, Gómez-Soler M, **Corrales F**, Marcellino D, Narvaez M, Frankowska M, Flajolet M, Heintz N, Agnati LF, Ciruela F, Fuxe K. Characterization of the A2AR-D2R interface: focus on the role of the C-terminal tail and the transmembrane helices. Biochem Biophys Res Commun. 2010 Nov 26;402(4):801-7. doi: 10.1016/j.bbrc.2010.10.122.

II. Borroto-Escuela DO, Ravani A, Tarakanov AO, Brito I, Narvaez M, Romero-Fernandez W, **Corrales F**, Agnati LF, Tanganelli S, Ferraro L, Fuxe K. Dopamine D2 receptor signaling dynamics of dopamine D2-neurotensin 1 receptor heteromers. Biochem Biophys Res Commun. 2013 May 24;435(1):140-6. doi: 10.1016/j.bbrc.2013.04.058.

III. Borroto-Escuela DO, **Corrales F**, Narvaez M, Oflijan J, Agnati LF, Palkovits M, Fuxe K. Dynamic modulation of FGFR1-5-HT1A heteroreceptor complexes. Agonist treatment enhances participation of FGFR1 and 5-HT1A homodimers and recruitment of β-arrestin2.Biochem Biophys Res Commun. 2013 Nov 15;441(2):387-92.

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INTRODUCTION

1.1 History of receptor-receptor interactions

In the early 1980ies it was observed that neuropeptides can alter the affinity and density of the monoamine agonist and antagonist binding sites in different regions of the Central Nervous System (CNS), in a receptor subtype specific way [1-4]. The molecular mechanisms for these intramembrane events between neuropeptide and monoamine receptor subtypes were unknown but direct interactions between the two receptors were postulated**.** Thus, it indicated the presence of neuropeptide-monoamine receptor-receptor interactions in the plasma membrane and the introduction of the heteroreceptor complexes hypothesis [2, 5].

The demonstration of neuropeptide-monoamine receptor–receptor interactions in membrane preparations from different regions of the CNS indicated e.g. the existence of DA receptor subtype–specific interactions with neurotensin (NT) and cholecystokinin (CCK) receptors in putative brain heteroreceptor complexes [3, 6-9]. The stronger allosteric NTR-D2R and CCKR-D2R interactions found in sections than in membrane preparations indicated the requirement of intracellular mechanisms and/or a more intact membrane structure for optimal receptor-receptor interactions [3, 4, 10].

In line with these results on neuropeptide induced changes in the affinity of the monoamine receptor subtypes [11, 12], Lefkowitz, Limbird and colleagues had earlier discovered negative cooperativity in beta adrenergic receptors [13, 14]. This can be explained on the basis of the existence of beta adrenergic homoreceptor complexes which leads to site–site interactions. The first symposium on receptor–receptor interactions among GPCRs was held in Stockholm in 1986 [5]. In the preface to the symposium book the receptor-receptor interaction field was proposed to become wider and include also interactions between different classes of biologically active macromolecules such as receptors, ion channels and ion pumps.

Heteroreceptor complexes was postulated again in 1993 to be the molecular basis for the receptor–receptor interaction [15]. The first observations indicating the existence of homodimerization of GPCR were made in 1982 [16, 17]. In 1987 homodimerization was found to take place upon epidermal growth factor (EGF) induced stimulation of the epidermal growth hormone receptor [18]. Ten years later the demonstration of the GABA B receptor heterodimer (see [19-21]) validated Fuxe group early findings indicating receptor-receptor interactions in putative GPCR heteroreceptor complexes [2, 15]. Thus, in words of the eminent Swedish neuroscientist Kjell Fuxe, the entire decoding process becomes a branched process already at the receptor level in the plasma membrane. For a review of the early work on receptor-receptor interactions, see [3, 4, 6-8, 22-29].

The allosteric receptor-receptor interactions over the interfaces in homo and heteroreceptor complexes and their biochemical, pharmacological and functional integrative implications in the CNS will be dealt with in this thesis work. The resemblance of the receptor protomers to moonlighting proteins will be also underlined [27, 30, 31]. Heteroreceptor complexes appear to be a fundamental principle for molecular integration in biology [23, 28]. The involvement of GPCR interacting proteins in these heteroreceptor complexes will also be covered [6, 27, 32]. We will also reveal how the discovery of different types of receptor–receptor interactions in such complexes in the brain led to novel strategies for treatment of Parkinson's disease (e.g. A2A and mGluR5 receptor antagonists) [33], schizophrenia (e.g.A2A and mGluR5 agonists) [4, 34], depression (e.g. 5-HT1A agonists enhancing FGFR1 function) [35] and cocaine addiction (e.g.A2A agonists) [36]. It contributed to the introduction of A2A receptor antagonists in the treatment of Parkinson's disease [33, 37, 38].

1.2. Heteroreceptor complexes and their allosteric receptor-receptor interactions

It was proposed that the receptor-receptor interactions in the plasma membrane took place in postulated heteroreceptor complexes of GPCRs which could involve the participation of adapter proteins; see [3, 7, 9, 27]. Now the receptor field in the CNS has changed and include not only the monomers but also homo and heteroreceptor complexes of unknown stoichiometry and geometry as novel targets for treatment of neurological and mental diseases (Figure 1)[23, 28].

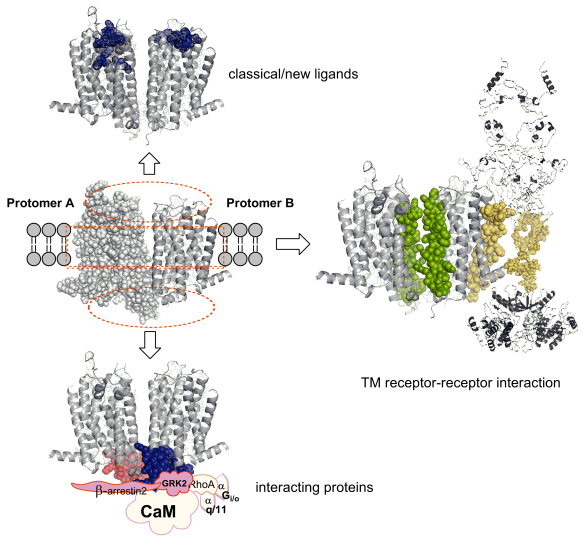

Figure 1. GPCR protomers forming heteroreceptor complexes results in increases in the diversity of recognition and signalling. Theses results underline the view of GPCRs as moonlighting proteins. Changes in the allosteric receptor-receptor interaction through the formation of different types of heteroreceptor complexes and receptor/protein complexes can change the function of an individual receptor present as homomer or monomer (This figure and text has been obtained from [27]).

It is of highly relevant that dopamine D2Rs form higher order homoreceptors at physiological expression levels in living cells as was demonstrated using protein complementation assays combined with BRET [39]. Also, it was observed that allosteric receptor-receptor interactions are in operation between protomers of D2R homoreceptor complexes which modulate their activation [40]. Using a functional complementation assay it became possible to evaluate the D2R homoreceptor functional unit and directly study their receptor-G protein interactions. The evidence suggests an asymmetrical activated D2R homoreceptor complexe where the second D2R protomer inhibits signalling.

The allosteric receptor-receptor interactions in heteroreceptor complexes give diversity, specificity and bias to the receptor protomers due to conformational changes in discrete domains leading to changes in receptor protomer function and their pharmacology [3, 7, 9, 27, 28]. The discovery of the adenosine A2AR- dopamine D2R heteroreceptor complexes in the dorsal striato-pallidal GABA neurons with antagonistic A2AR-D2R interactions reducing D2R signalling led to the development of A2AR antagonists for treatment of Parkinson's disease [7, 27] and A2AR agonist for the treatment of cocaine used disorders [36] (Figure 2). The motor complications found with levodopa like dyskinesias and wearing off can involve a reorganization of these heteroreceptor complexes involving also a disbalance with A2AR and D2R homoreceptor complexes [7, 8, 37].

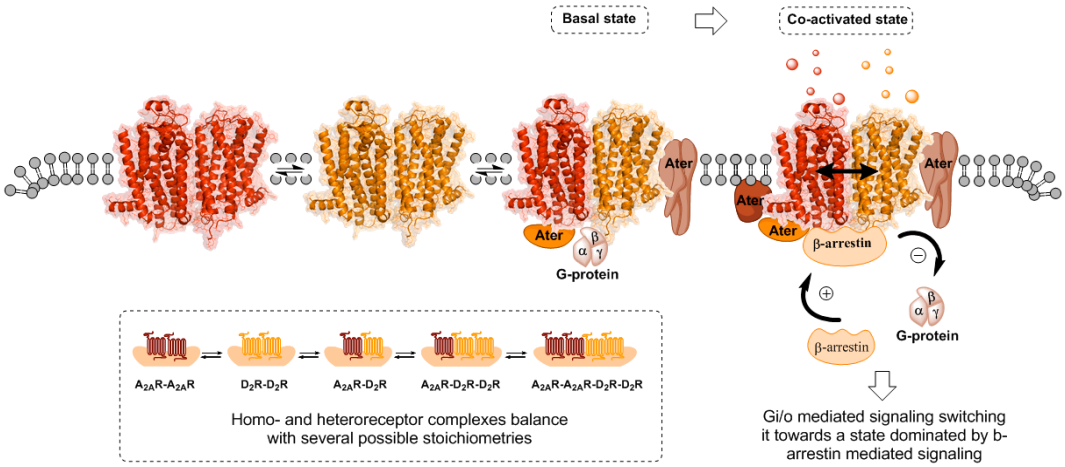

Figure 2. Illustration of the antagonistic allosteric receptor-receptor interactions in the $A_{2A}R$-D_2R heteroreceptor complexes with several possible stoichiometries from heterodimers to higher order heteromers of various types (heterotrimer and heterotetramer are shown) with or without the participation of adapter proteins (Ater). 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 has been obtained from [23])

Neurotrophic and antidepressant effects of 5-HT in brain may, in part, be mediated by activation of the 5-HT1A receptor protomer in the hippocampal FGFR1–5-HT1A heteroreceptor complex enhancing the FGFR1 signalling [24, 35, 41, 42]. The FGFR1-5-HT1A heteroreceptor complex likely represents a novel target for antidepressant drugs and offers a novel strategy for treatment of depression [24].

FGFR1–5-HT1A heteroreceptor complexes are also found in the majority of the 5-HT nerve cells in the dorsal and median raphe nuclei [41]. 5-HT1A autoreceptors by being part of this heteroreceptor complex may have also a trophic role in the 5-HT neuron systems. The majority of the ascending midbrain 5-HT neurons may be dysregulated in depression and have a reduced trophic support due to disruption or dysfunction of these receptor complexes in the 5-HT nerve cells [3, 24].

Furthermore, a single GPCR through conformational changes due to its participation in an altered receptor complex may undergo changes in co-trafficking with increased internalization and can become possible transcription factors through their altered conformation and possible receptor fragments formed. As possible transcription factors the GPCRs would have a novel function by directly modulating gene expression and likely trophism, possible actions of relevance for neurodegeneration. Allosteric receptor-receptor interactions in GPCR-RTK heteromers likely will lead to moonlighting of the participating GPCR and RTK protomers with high relevance for neurodegeneration [43-45].

Taken together, GPCR heteroreceptor complexes and their receptor-receptor interactions represent a new fundamental principle in molecular medicine for integration of transmitter signals in the plasma membrane. A novel understanding of the molecular basis of CNS diseases is given together with new strategies for their treatment by targeting heteroreceptor complexes based on a new pharmacology with combined treatment, multi-targeted drugs and heterobivalent drugs [23, 28].

1.3 D2R-NTR1 heteroreceptor complexes

Neurotensin (NT) was found to reduce the affinity of D2R agonist binding sites which correlated with its ability to counteract the DA agonist induced inhibition of striatal DA and GABA release and to induce neuroleptic actions with relevance for its postulated anti-psychotic actions [2, 3, 6]. Thus, in the striatum a prejunctional allosteric antagonistic D2R-NTR1 autoreceptor interaction in striatal DA terminals increasing DA release and a

postjunctional allosteric antagonistic D2R-NTR1 interaction mainly located on the cortico-striatal glutamate nerve terminals but also on the striato-pallidal GABA neurons increasing their activity and GABA release.

It was of substantial interest that the C-terminal NT(8-13) fragment also potently and antagonistically modulated rat neostriatal D2Rs and that neuromedin N (NN) also was a potent inhibitory modulator of D2R agonist binding in rat neostriatal membranes [1, 2, 5]. In view of the higher potency of NN versus NT to modulate the affinity of neostriatal D2Rs, in contrast to the higher potency of NT vs NN to bind to the cloned NTRs, the NN-activated neostriatal NT receptors involved in the affinity regulation of the D2Rs, may have developed a bias towards NN vs NT in terms of affinity and/or potentially efficacy. This may have been accomplished through a reciprocal allosteric D2R-NTR interaction from the D2R protomer biasing the specificity of the NT orthosteric binding site towards NN vs NT binding in the heteroreceptor complex [3, 7].

It should be noticed that the antagonistic presynaptic but not the postsynaptic D2R-NTR1 receptor-receptor interaction was missing in the nucleus accumbens in contrast to dorsal striatum. This led to increases in ventral striatal GABA release as in the dorsal striatum but without increases in DA release. Instead reductions of accumbens DA release were observed likely at least in part related to activation of GABA A receptors on the DA terminals. Thus, regional differences in the microanatomy of the NTR-D2R heteroreceptor complexes can have important regional functional consequences at the local circuit levels of the ventral and dorsal striatum leading to a differential regulation of the striato-pallidal GABA outflow from these two regions by NT. The NT induced reduction of D2R signaling in the ventral striatum therefore become stronger in the ventral striatum vs the dorsal striatum which will favor antipsychotic actions vs development of motor side-effects [3, 4, 6, 7].

1.4 Dynamics and bias through antagonistic reciprocal receptor-receptor interactions in A2AR-D2R heteroreceptor complexes

Adenosine A2A receptor (A2AR)–dopamine D2 receptor heteroreceptor complexes was demonstrated by means of biochemical and biophysical methods, namely co-immunoprecipitation, bioluminescence resonance energy transfer (BRET), and fluorescence resonance energy transfer analyses, upon transient cotransfection of the two receptors in cell lines [46-48]. A2AR-D2R heteroreceptor complexes were later on also found in the striatum using the PLA technique [49, 50].

Antagonistic A2AR-D2R receptor-receptor interactions in striatal membrane preparations were early on demonstrated after incubation with A2AR agonist CGS21680 as seen from demonstrated by the reduction of the affinity of the high affinity D2R agonist binding site [33]. With receptor autoradiograpy strong reductions in affinity were observed after A2AR agonist treatment in the nucleus accumbens core and shell of the

rat [37]. Significant increases in the EC50 values were also observed in the human caudate after incubation of the sections with CGS21680. Furthermore, the antagonistic A2AR–D2R interaction in A2AR–D2R heteroreceptor complexes diminished the Gi/o mediated signaling of the D2R [51]. In D2R-A2AR cotransfected neuroblastoma cells, coactivation of A2AR and D2R resulted in the coaggregation, cointernalization, and codesensitization of the A2AR and D2R [52]. Recently it was shown that that the activation of the A2AR-D2R interaction also favors β-arrestin2 recruitment to the D2LR protomer with subsequent increase in cointernalization [53]. Thus, this allosteric A2AR-D2R receptor-receptor interaction brings about a biased modulation of the D2 protomer signaling (Figure 2). A conformational state of the D2R protomer is induced which moves away from Gi/o signaling and instead favours binding of β-arrestin2 and β-arrestin2 mediated signaling [52, 53]. This change in functional selectivity can in part explain the reduced time onset of Akt phosphorylation after the A2AR-D2R coactivation followed by a rapid dephosphorylation [3, 6-9].

The existence of an electrostatic interaction between the C-terminal tail of the A2AR and the third intracellular loop (IL3) of the D2R was demonstrated to be importantly involved in the A2AR protomer-mediated allosteric effects on the D2R protomer recognition, signaling and trafficking [52]. Electrostatic bonds of covalent-like strength are formed in this interaction and a detailed mutational analysis was made in the A2AR C-terminal tail [7, 52] (Figure 3).

Recently evidence was obtained for the existence of a reciprocal allosteric communication from the D2R to the A2AR protomer of the A2AR-D2R heteromer [51]. This allosteric receptor–receptor interaction was found to be mostly mediated by two regions rich in arginins known to give with positive charges, located in IL3 of the D2R. The negative allosteric modulation by the D2R on A2AR agonist binding was shown in a real-time mode. It was possible to determine that D2R activation in part inhibited and also slowed the binding a of the fluorescent A2AR agonist to the A2AR [3, 51, 54]. The interaction was abolished by mutating the IL3 of the D2R. The Arg residues (217–222 and 267–269) in IL3 of the D2R were as demonstrated to play a major role in the antagonistic allosteric D2R-A2AR receptor-receptor interaction (Figure 3). This allosteric receptor-receptor interactions makes possible also an inhibitory modulation by the D2R protomer of the A2AR protomer binding and function.

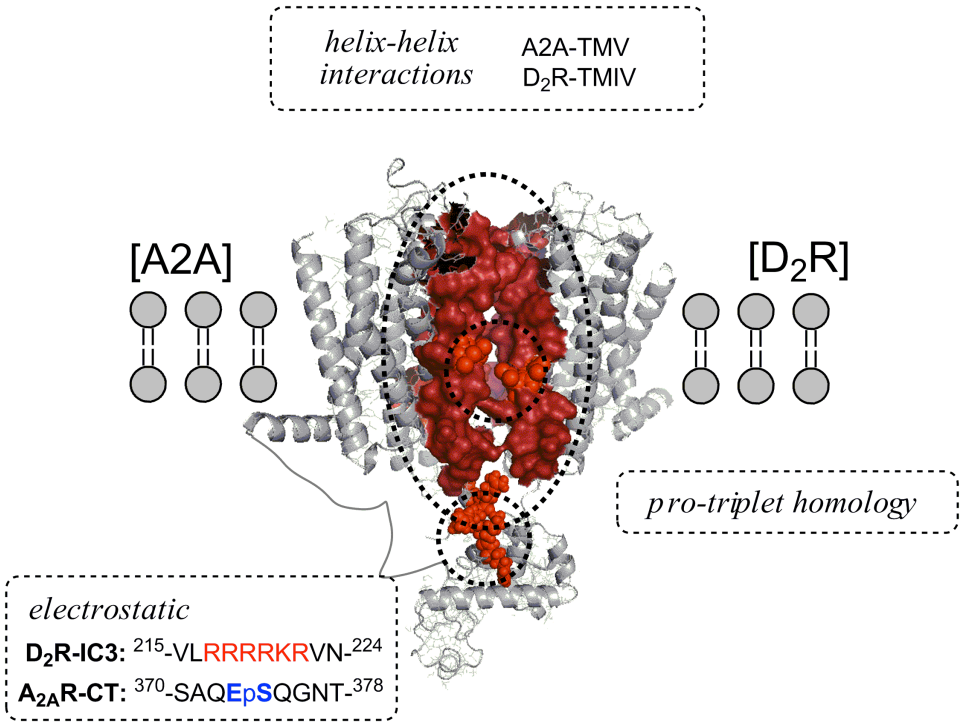

Figure 3. GPCR heteromer receptor interface ilustrated in the $A_{2A}R$-D_2R heteromers . (*Helix-helix interaction*) Representations are shown from a lateral view of the D_2R (TM-IV/V)-$A_{2A}R$(TM-IV/V) model of A2AR-D2R heterodimer. (*Electrostatic interactions*) Illustration of positively charged arginine-rich epitope ^{215}VL**RRRRKR**VN^{224} in the N-terminal part of the third intracellular loop of D2R electrostatically interacting with the negatively charged C-terminal epitopes of the $A_{2A}R$ (^{370}SAQE**pS**QGNT^{378}**,** ^{388}H**E**LKGVCP**E**PPGL**DD**PLAQ**D**GAGVS^{412}**)**. (*Pro-triplet homology interactions*) *In silico* analysis and sequence alignment of A2AR and D2R receptor homologies containing the triplet homologies AAR, AQE, VLS, and VYI (in red) in the receptor interface. The triplet homology VYI is located in the N-terminal of A2AR and in the fifth transmembrane α-helix (TM-V) of D2R, the triplet homology VLS is located in the TM-IV of both A2AR and D2R, the triplet homology AAR is located in the third intracellular loop (IL3, between TM-V and TM-VI) of both A2AR and D2R and the triplet homology AQE is located in the IL3 of D2R and in the C-tail of A2AR. Note that the triplet AQE is adjacent to S374 which seems to play a key role in the A2AR-D2R receptor interfaces. Also note that the triplet VYI cannot play a role since the homology is located in the N-terminal part of the A2AR and the fifth TM region of the D2R. (the text and figure have been obtained from [27])

1.5 GPCR-RTK receptor-receptor interactions

It has been observed that the dopamine D2R stimulation of MAPK is mediated by a cell type-dependent transactivation of RTK [43]. There may also exist RTK ligand independent mechanisms for RTK transactivation with participation of both GPCRs and the RTK in a multi-receptor signaling complex [55, 56]. In fact, D2R agonist enhance the coimmunoprecipitation of D2R and EGFRs in neuroblastoma cells, thereby suggesting that D2R activation induces the formation of a D2R-EGFR heteroreceptor signalling

complex [43] as a D2/RTK heteromer.

In addition, a physical interaction between the A2AR and the FGFR1 has been demonstrated [45]. Coactivation of these two classes of receptors caused a substantially increased activation of the MAPK/ERK pathway associated with increases in synaptic plasticity with spine morphogenesis. Novel research in this field has increased our understanding of the mechanisms of antidepressant drug action and led to the development of new strategies for treatment of depression. For the first time it has been possible to identify in the mesencephalic 5-HT neurons and their telencephalic targets a 5-HT1A-FGFR1 heteromer the activation of which may contribute to a relief from depression (Borroto-Escuela et al., unpublished data). Ongoing work based on the present hypothesis seems to identify that the activation of the 5-HT1A-FGFR1 heteromer can mediate improvement in 5-HT neuronal communication and neurotrophism. Moonlighting in the FGFR1 of the GPCR-FGFR1 heteromers may develop as a result of the allosteric receptor-receptor interaction in the heteromer achieved inter alia by coactivation of its protomers. This allosteric change in the catalytic region of the FGFR1 may change its recruitment of adaptor proteins which may alter the pattern of activation of its five major signaling pathways: the RAS/RAF/MAPK pathway, the PI3K/AKT/mTOR pathway, the PLC gamma pathway, the JAK/STAT pathway, and the IKK/NF-KB pathway. It may be that the receptor-receptor interaction may favor the selection of one of its pathways, the RAS/RAF/MAPK pathway, according to the results obtained (Borroto-Escuela et al., unpublished data). The results open up the exciting possibility that moonlighting in the GPCR-RTK heteromers may inter alia lead to altered functions of the RTK protomer with relevance both for trophism and neurodegeneration.

1.5.1 Brain FGFR1-5-HT1A heteroreceptor complexes: Implications for major depression and its treatment

The coming together of the serotonin hypothesis and the trophic factor hypothesis of depression.

Coppen introduced the serotonin theory of depression[57] in 1967 based inter alia on studies on free and total tryptophan, and on brains of depressed suicides. The same year Fuxe and Ungerstedt[58] demonstrated the 5-HT uptake mechanism of the central serotonin neurons, discovered three years earlier[59]. In 1968 Carlsson, Fuxe and Ungerstedt[60] reported that imipramine can block the 5-HT uptake mechanism which led to the search for serotonin selective reuptake inhibitors SSRIs in the treatment of major depression. Today, neurobiological basic research as well as clinical studies on SSRIs have further established that the ascending serotonin neuron systems to the forebrain are involved in the etiology and therapy of major depression[61, 62]. The therapeutic action of antidepressant drugs is of proven effectiveness, but the

mechanisms underlying their effect are still unclear. It is known that, although biochemical changes produced by SSRIs occur within hours of administration, therapeutic effects become evident only after a latency of about 2–3 weeks, suggesting that adaptive processes induced via activation of signaling patterns of different 5-HT receptor subtypes, including the regulation of specific genes, are necessary for the long-term effects of these drugs.

According to a current view, antidepressants induce processes of neuroplasticity that lead to a reorganization of central neural networks thus and thereby generating their therapeutic effects. Increasing evidence suggests that antidepressant drugs via actions on the 5-HT neurons may exert their effects on neuroplasticity at least in part, through the enhancement of neurotrophic factor expression and function leading to therapeutic activity[63]. A role for fibroblast growth factors (FGFs) has inter alia been proposed in mood disorders[64]. Antidepressant drugs and chronic electroconvulsive shock treatment may increase the expression of fibroblast growth factor-2 (FGF-2) in frontal cortices and hippocampus.

Serotonin receptor subtypes and the possible transactivation of the FGF-2/FGFR1 neurotrophic system

It seems possible that a certain pattern of activity at different 5-HT receptor subtypes of the seven 5-HT receptor families identified is necessary to be induced by SSRIs to counteract the depression since some like postjunctional 5-HT1A receptors mediate antidepressant activity while others like 5-HT2A receptors mediate depressive actions[25, 62, 65-67]. In contrast, a down-regulation of the inhibitory 5-HT1A autoreceptors in the midbrain raphe regions is of importance[67, 68].

The hypothesis was introduced that the neurotrophic system FGF-2/FGFR1 may be a good candidate to mediate antidepressant induced improvement in 5-HT neuronal communication and neurotrophism with regeneration of connections lost during depression[63]. RTK transactivation in response to antidepressant drug treatment was postulated to take place via a new allosteric receptor–receptor interactions between distinct serotonin receptor subtypes and FGFR1 heteroreceptor complexes.

Discovery of hippocampal FGFR1-5-HT1A heteroreceptor complexes and their enhancement of hippocampal plasticity

By means of several biochemical, biophysical and behavioral assays indications were obtained that neurotrophic and antidepressant effects of 5-HT in brain may, in part, be mediated by activation of the 5-HT1A receptor protomer in the hippocampal FGFR1–5-HT1A receptor complex enhancing the FGFR1 signaling[35]. Dynamic modulation of FGFR1–5-HT1A heteroreceptor complexes was found to exist in cellular models[42]. Agonist treatment enhanced participation of FGFR1 and 5-HT1A homodimers in and recruitment of b-arrestin2 to the FGFR1-5-HT 1A heteroreceptor complexes[42].

Discovery of the FGFR1–5-HT1A heteroreceptor complexes in the midbrain raphe 5-HT nerve cells and their enhancement of the FGFR1 signaling and plasticity

With *in situ* PLA and supported by co-location of the FGFR1 and 5-HT1A immunoreactivities in midbrain raphe 5-HT cells, evidence for the existence of FGFR1–5-HT1A heteroreceptor complexes were obtained in the dorsal and median raphe nuclei of the rat[69] (Figure 5). The heteroreceptor complex formation was dependent on TM5 of the 5-HT1A receptor since it was blocked by incubation with TM5 but not with TM2.

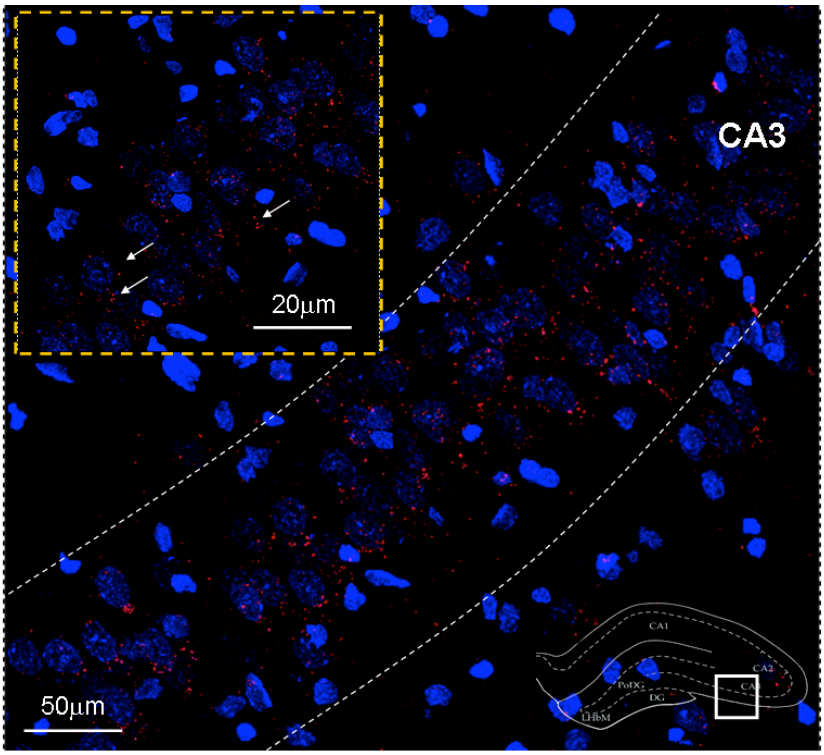

Figure 5. Illustration of the FGFR1-5-HT1A heteroreceptor complex in the dorsal hippocampus of rat brain. Microphotographs from transverse sections of the rat dorsal hippocampus (Bregma level: -3.6 mm) showing the distribution of the 5-HT1A-FGFR1 heteroreceptor complexes in CA3 using the *in situ* proximity ligation assay (*in situ* PLA) technique. They are shown as red PLA blobs (clusters) found in high densities per cell in a large number of cells in the pyramidal cell layer using confocal laser microscopy. The nuclei are shown in blue by DAPI. In the left inset the PLA blobs in the pyramidal cell layer are shown in higher magnification. In the lower right part of the figure the different parts of the dorsal hippocampus are given in a transverse section. The square outlines the CA3 area from which the picture was taken (for further details see [35]) Abbreviations: CA3: region III of hippocampus proper is a portion of the hippocampal formation. CA stands for the latin *cornus ammonis*. (the text and figure have been taken from [24])

This can be explained based on the triplet puzzle theory[70]. Namely, the triplet homology (protriplet) Gly-Ala-Phe may mediate interaction between TM5 of 5-

HT1A and TM of FGFR1. Another protriplet Ala-Ala-Arg may also mediate interaction 5-HT1A-FGFR1 in the cytoplasmic domain between TM5 and TM6 of 5-HT1A and substrate binding site of FGFR1. Third protriplet Thr-Leu-Gly in the border of this cytoplasmic domain and TM6 of 5-HT1A may also mediate interaction 5-HT1A-FGFR1.

The combined i.c.v. treatment with FGF-2 and the 5-HT1A agonist 8-OHDPAT synergistically increased FGFR1 and ERK1/2 phosphorylation in the raphe midline area of the midbrain and in the raphe RN33B cells. Cotreatment with FGF2 and the 5-HT1A agonist induced RN33B cell differentiation as seen from development of an increased number and length of extensions per cell and their increased 5-HT immunoreactivity[71].

1.6 Methods for studying receptor-receptor interactions

The first evidence of GPCRs receptor-receptor interactions was obtained at the recognition level of the receptors, using saturation and competition binding experiments (Figure 6). The modulation of binding was shown as changes in Kd and Bmax values (saturation analysis) and in K_L, K_H, and RH values (competition analysis) allowing a determination of modulation of the high- versus the low-affinity agonist states of the receptor. An indication of an effect on the G protein-coupling and thus on the efficacy of the modulated receptor could be obtained by studying how, e.g., the modulator could control the GTP-induced disappearance of the high-affinity state of the receptor (reduction of the RH values).

FRET and BRET methods gave the evidence needed to demonstrate heteromers among class A GPCRs (Figure 6). Schematic representation of the basic principle of FRET are displayed here. Two putative proteins (A and B) bearing a ‘donor’ (CFP) or an ‘acceptor’ (YFP) fluorophore are depicted. If these two proteins interact then the donor and acceptor fluorophores are likely in proximity (10 nm or less) and energy transfer between donor and acceptor can occur after donor excitation by demonstration of YFP emission. Two putative proteins (A and B) bearing a ‘donor’ (Rluciferase, Rluc) or an ‘acceptor’ fluorophore (YFP) are depicted. If these proteins are in proximity, e.g. do interact, then a donor-acceptor energy transfer can occur after Rluc substrate (h-coelenterazine) oxidation. The bioluminescence formed can activate YFP and YFP emission develops.

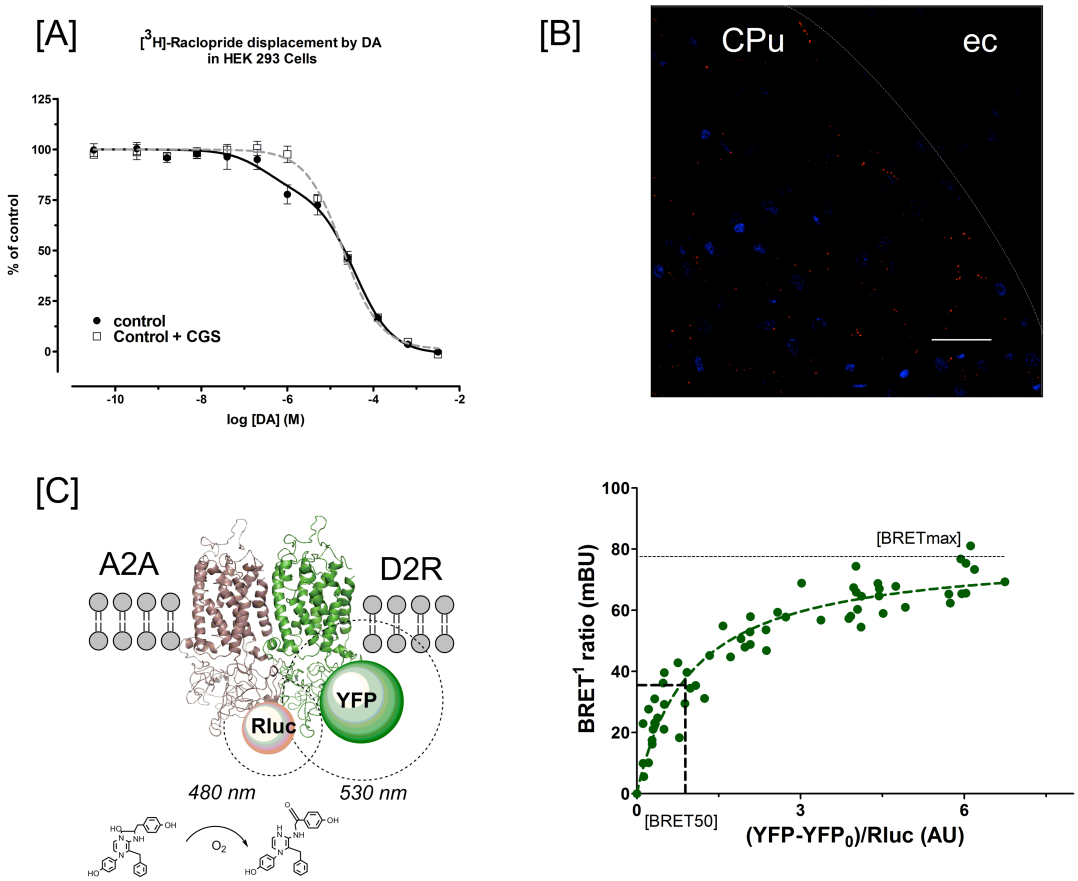

Figure 6. Different methods have been used to study GPCR homo and heteroreceptor complexes. As examples are shown methods used to study the existence of A2A-D2 heteroreceptor complexes in heterologous expression systems and in brain ex vivo. **(A)** Competition experiments using D_2-like receptor antagonist [^{3}H]-Raclopride (2 nM) versus increasing concentrations of dopamine in transiently cotransfected HEK293T cell membranes. Panel A illustrates the right shift of the competition curve after treatment with the A2A agonist CGS21680 in the high affinity range of the D2R agonist binding site demonstrating a reduction of D2R affinity in the high affinity state **(B)** Detection of A2A-D2 heteroreceptor complexes in ex vivo brain sections can be found by in situ proximity ligation assay (PLA). PLA-positive A2A-D2 heteroreceptor complexes in striatal sections have been visualized as red clusters (blobs, dots) within the neuropil of caudate putamen (CPu) which were almost absent within the external capsule (ec). **(C)** In C the principle of the BRET^1 method is illustrated giving strong support for the existence of A2AR-D2R heteromers. In the presence of h-coelenterazine, an energy transfer between Rluc and YFP occurs when the distance between them is less than 100 Å. To the right a BRET^1 saturation curve for the A2A-D2 hetero receptor complex (D2LR^{Rluc} + A2AR^{YFP}, filled circles) is shown giving BRET max and BRET EC50 values[27]. (The text and the figure have been obtained from [27])

Fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) methods were introduced which could be used to study homo and heteromerization of proteins including receptors in living cells. It involved the preparations of receptor constructs having genetically fused ‘donor’ and ‘acceptor’ fluorescent proteins.

In FRET if the donor and acceptor fluorophores are in close proximity (less than 10nm) energy transfer between the two fluorophores can occur after donor excitation and acceptor emission develops [83, 84]. Energy transfer is inversely proportional to the sixth power of the distance (r) between donor and acceptor fluorophors and the distance leading to 50% of energy transfer from the donor to the acceptor is around 5nm. There are drawbacks of using classical FRET when the protein is located in intracellular stores, making measurements of protein interactions at the plasma membrane difficult.

However, there exist cell surface FRET detection technologies to study GPCR heteromerization in the plasma membrane [51]. Using the total internal reflection fluorescence microscopy (TIRF) approach it is possible to narrow and better define the field of excitation. The light decays in an exponential way and is restricted in terms of distance propagation, for instance, appropriate fluorophores can be excite only within a distance of 100nm [83]. With cell surface TIRF-FRET detection technology a collimated light at an angle of projection exceeding a critical angle generates at the cover slip surface an evanescent field, the light of which decays exponentially, penetrating into the cell only approximately 100nm.

There is also the technique of bimolecular fluorescence complementation (BiFC) to demonstrate protein dimerization. Two putative proteins (A and B) can carry N-YFP and C-YFP halves, respectively. If a dimerization/interaction phenomenon between A and B takes place, then a fluorescent fusion complex of the two complemented fragments of the YFP protein (N and C) will be generated. And thereby, allowing a direct visualization of dimerization of A and B after the excitation of the YFP reconstructed protein [46, 85].

The principle of the detection of GPCR heterodimerization using the BRET method is similar to FRET. In the presence of the substrate h-coelenterazine or coelenterazine-400 on which *Renilla luciferase* fused to the donor acts to produce through their oxidation a compound showing bioluminence. An energy transfer between the generated luminescence and YFP or GFP2 occurs when the distance between these proteins is less than 10nm. This leads to a fluorescence emission from YFP ($BRET^1$) or GFP2 ($BRET^2$) representing the BRET signal. BRET gives strong support for the existence of receptor heteromers in artificial cell systems.

In titration or saturation $BRET^2$ experiments cells are transfected with a constant amount of $BRET^2$-donor in presence or absence of increasing amounts of the acceptor. Theoretically, for any specific interaction between the Receptor-donor and Receptor-acceptor fusions, the $BRET^2$ ratio signal is developed as an hyperbole function of GFP/Rluc values, reaching an asymptote (saturation) when all donor molecules are associated with acceptors (BRETmax) [48, 86, 87].

The BRETmax values are influenced by several parameters or factors, e.g., on the energy transfer distance between receptor protomers, their relative orientation and the dimer numbers. Therefore, these values cannot be used as a quantitative measure of the relative number of receptor complex formed. $BRET^2$ saturation curves have been particularly used with the aim to establish the oligomeric order of receptor complexes, as well as the proportion of receptors engaged in dimers or oligomers. They are also used to determine whether ligand-induced $BRET^2$ signals depend on conformational changes or association/dissociation of interacting receptors. Also saturation assays have been used to compare the relative affinity of receptors for each other and their probability to form a complex, the so called $BRET_{50}$, which represents the acceptor/donor ratio giving 50% of the maximal signal. This value could also be used to compare the homo vs heterodimer affinity relationships [87]. Many GPCR-GPCR heteromers show no difference in the relative affinity between their receptor homomers and their specific heteromers. Furthermore, neither $BRET_{max}$ nor $BRET_{50}$ values may be modified following the agonist activation of the heteromer, consistent with the general consensus that GPCR homo- and heteromerization is often constitutive. In contrast, GPCR-RTK heteroreceptor complexes show more dynamic features where agonist treatment markedly affect $BRET_{max}$, $BRET_{50}$ or both these values [35, 42, 48].

Over the last years different generations of BRET ($BRET^1$, $BRET^2$, $eBRET^2$, $BRET^3$ and QD-BRET) have been developed, depending on the type of enzyme substrate and the nature of donor/acceptor pairs. As a result, the nomenclature for describing each of the BRET forms has not followed a unique rigorous pattern.

In our work we often used the $BRET^2$ variant assay with $Rluc^8$ as a donor and GFP^2 as an acceptor with improved spectral separation of the donor bioluminescence and acceptor emission peaks. This implies less bleed-through at the acceptor emission maximum and lower background. Also, the rational for the use of mutated Rluc (Rluc8, Genbank: EF446136) instead of the non-mutated Rluc is mainly based on the fact that $Rluc^8$ gives a higher quantum yield compared to the non-mutated Rluc. Therefore, we do not need to overexpress the luciferase fusion receptor which is detrimental for interaction specificity.

Detection of higher order heteroreceptor complexes can be obtained by combined BRET/BiFC assays in which BiFC is followed by BRET. It may be achieved by combining the bimolecular fluorescence complementation with BRET [47, 72]. The trimeric heteromers of GPCRs can also be demonstrated with a combination of BRET and FRET. It is called the sequential BRET-FRET technique or SRET technique [73]. In $SRET^2$ you use the blue bioluminenscence from deep blueC which activates the GFP^2 ($BRET^2$) and its emission activates the yellow fluorescent protein which is the FRET component. In $SRET^1$ you use instead $BRET^1$which involves

addition of the Rluc substrate coelenterazine h and energy transfer to YFP. This is followed by FRET transfer to the DsRed acceptor fluorescent protein and elicitation of emission in the red range. Also, the sequential BRET–FRET methods is used for the demonstration of the higher-order heteroreceptor complex in like the A2A-D2-CB1 complex.

Taken together, the use of FRET and BRET-based techniques emerged as important tools in the analysis of GPCR dimerization in living cells. Nevertheless there are inherent problems linked to the use of these technologies. For instance, the attachment of such large fluorescent proteins may block proper receptor function, which makes it difficult to interpret of results. In addition, the existence of false FRET and BRET signals produced by photophysic cross-talks should be considered. An increase of non-specific FRET and BRET can take place as a result of the random collision of intracellularly accumulated FRET or BRET-tagged receptors when transiently overexpressed. In view of this some controversy surrounds these approaches [77, 78]. Nevertheless, when FRET and BRET-based results are properly evaluated, we can demonstrate clearly an oligomerization of heterologously expressed GPCRs and of cells from transgenic animals [48, 78-81]. Consensus exists that FRET and BRET methods powerfully support the existence of receptor heteromers in living cells [82].

In situ Proximity Ligation Assay (*in situ* PLA) has been performed to establish the existence of native heteroreceptor complexes in the CNS [35, 49, 89, 90]. *In situ* PLA is based on a pair of primary antibodies followed by the use of secondary antibodies to which oligonucleotides have been linked. When the two antibodies recognize a dimer, the oligonucleotides of the secondary antibodies are in a sufficiently close proximity (16nm or less) to allow them to recognize each other and join followed by an enzymatic ligation reaction. Then, the DNA circle strand formed can act as a template for an amplification reaction of the rolling circle. It is linked to one of the proximity probes and can be detected and quantified by hybridizing fluorescent oligonucleotides. This sequential reaction process (antibody recognition, joining of oligonucleotides, ligation, amplification and hybridization) rendered the heteroreceptor complexes observable by fluorescence microscopy. In this way you can study the number, localization and modulation of CNS heteroreceptor complexes since formalin fixed tissue is used [10, 35, 49, 74, 75, 89-91]. The main drawback of *in situ* PLA lies in the quality of fixed tissue, the specificity and bivalent character of the primary antibodies and the use of proper controls.

Taken together, the findings indicate that *in situ* PLA can be used to demonstrate heteroreceptor complexes *ex vivo* in brain tissue like the striatal A2A-D2 heteroreceptor complexes [49, 89] and the accumbal and dorsal striatal D2-5HT2A heteroreceptor complexes [91].

AIMS

The overall aim of this thesis was to gain insight into molecular aspects of the dopamine D2 and serotonin 5-HT1A heteroreceptor complexes and their allosteric receptor-receptor interaction in the Central Nervous System in view of the relevant role of both receptor on several mental and neurological diseases. The following specific aims were considered:

- To explore the role of negatively charged double aspartates (D401A-D402A) and single serine (S374A) in the $A_{2A}R$-D_2R interface and their impact on the allosteric mechanisms operating on the $A_{2A}R$-D_2R heteroreceptor complexes . Also to confirm the role of the TM helix interactions of the $A_{2A}R$-D_2R interface using TM synthetic peptides and BRET[1] saturation and competition assays. Furthermore, based on the experimental results, and using a novel bioinformatics approach to predict the receptor-receptor interface, to perform a molecular dynamic simulation and a rigid-body protein-protein docking of the $A_{2A}R$-D_2R heteromers (Chapter 1 and 4).
- To validate that D2R form heteroreceptor complex with NTS1R and determine their ratios to total D2R or NTS1R populations in accumbens and dorsal striatal sections and primary striatal cultures through molecular assessment using *in situ* proximity ligation assay supported with coimmunoprecipitation data and bioluminescence and fluorescence resonance energy transfer methods (BRET/FRET). To further validate the allosteric mechanisms and the dynamic changes in D_2R signalling in D_2R-NTS1R heteroreceptor complexes upon agonist activation of NTS1R using gene reporter assays to follow the adenylyl cyclase (AC) and MAPK activities in line with the previous findings of an NTS1R mediated antagonistic regulation of D_2R recognition (Chapter 2).
- To study the dynamic modulation of 5-HT1A-FGFR1 heteroreceptor complexes using the BRET[2] assay in HEK293T cells. Also, to understand the role and participation of 5-HT1A and FGFR1 homoreceptor complexes and recruitment of β-rrestin2 upon agonist treatment (Chapter 3 and 5).

CHAPTERS 1-5

CONCLUSIONS

I. A single serine point mutation (S374A) in the adenosine A_{2A} receptor ($A_{2A}R$) C-terminal tail reduces $A_{2A}R$-D_2R heteromerization and prevents its allosteric modulation of the dopamine D_2 receptor (D_2R). We found evidence that the TM domains IV and V of the D_2R play a major role in the $A_{2A}R$-D_2R heteromer interface since the incubation with peptides corresponding to these domains significantly reduced the ability of $A_{2A}R$ and D_2R to form a heteroreceptor complexe. In addition, the incubation with TM-IV or TM-V blocked the allosteric modulation normally found in $A_{2A}R$-D_2R heteromers. The mutation of two negatively charged aspartates in the $A_{2A}R$ C-terminal tail (D401A/D402A) in combination with the S374A mutation drastically reduced the physical $A_{2A}R$-D_2R interaction and lost the ability of antagonistic allosteric modulation over the $A_{2A}R$-D_2R interface, suggesting further evidence for the existence of an electrostatic interaction between the C-terminal tail of $A_{2A}R$ and the intracellular loop 3 (IL3) of D_2R. On the other hand, molecular dynamic model and bioinformatic analysis propose that specific AAR, AQE, and VLS protriplets as an important motive in the $A_{2A}R$-$D_{2L}R$ heteromer interface together with $D_{2L}R$ TM segments IV/V interacting with $A_{2A}R$ TM-IV/V or TM-I/VII.

II. Biochemical, histochemical and coimmunoprecipitation experiments have indicated the existence of antagonistic dopamine D2 (D_2R) and neurotensin 1 (NTS1R) receptor-receptor interactions in the dorsal and ventral striatum suggesting a potential role of these receptor-receptor interactions in Parkinson's disease and schizophrenia. By means of Bioluminiscence Resonance energy transfer ($BRET^2$) evidence has for the first time been obtained in the current study for the existence of both $D_{2L}R$/NTS1R and $D_{2S}R$/NTS1R heteroreceptor complexes in living HEK293T cells. Through confocal laser microscopy the $NTS1R^{GFP2}$ and $D2R^{YFP}$ were also shown to be colocated in the plasma membrane of these cells. A bioinformatic analysis suggests the existence of a basic set of three homology protriplets (TVM, DLL and/or LRA) in the two participating receptors which may contribute to the formation of the D_2R/NTS1R heteromers by participating in guide-clasp interactions in the receptor interface. The CREB reporter gene assay indicated that the neurotensin receptor agonist JMV 449 markedly reduced the potency of the D_2R like agonist quinpirole to inhibit the forskolin induced increase of the CREB signal. In contrast, the neurotensin agonist was found to markedly increase the quinpirole potency to

activate the MAPK pathway as also studied with luciferase reporter gene assay measuring the degree of SRE activity as well as with ERK1/2 phosphorylation assays. These dynamic changes in D_2R signaling produced by the neurotensin receptor agonist may involve antagonistic allosteric receptor-receptor interactions in the $D_{2L}R$-NTS1R heteromers at the plasma membrane level (CREB pathway) and synergistic interactions in PKC activation at the cytoplasmatic level (MAPK pathway).

III. New findings show that neurotrophic and antidepressant effects of 5-HT in brain can, in part, be mediated by activation of the 5-HT1A receptor protomer in the hippocampal and raphe FGFR1-5-HT1A heteroreceptor complexes enhancing the FGFR1 signaling. The dynamic agonist modulation of the FGFR1-5-HT1A heteroreceptor complexes and their recruitment of β-arrestin is now determined in cellular models with focus on its impact on 5-HT1AR and FGFR1 homodimerization in the heteroreceptor complexes based on $BRET^2$ assays. The findings show that coagonist treatment with 8-OH-DPAT and FGF2 but not treatment with the 5-HT1A agonist alone markedly increases the BRETmax values and significantly reduces the BRET50 values of 5HT1A homodimerization. The effects of FGF2 or FGF20 with or without the 5-HT1A agonist were also studied on the FGFR1 homodimerization of the heteroreceptor complexes. FGF2 produced a marked and rapid increase in FGFR1 homodimerization which partially declined over a 10 min period. Cotreatment with FGF2 and 5-HT1A agonist blocked this decline in FGFR1 homodimerization. Furthermore, FGF2 alone produced a small increase in the $BRET^2$ signal from the 5-HT1A-β-arrestin2 receptor-protein complex which was additive to the marked effect of 8-OH-DPAT alone. Taken together, the results indicate a dynamic agonist regulation of the FGFR1-5-HT1A heteroreceptor complexes, which via allosteric receptor-receptor interactions leads to a structural change in this heteroreceptor complex. This change results in an expanded FGFR-5-HT1A heteroreceptor complex in which an increased presence of FGFR1 and 5-HT1A homodimers develops. Thus, the agonist regulation of these heteroreceptor complexes produces a reorganization in their structure to include an increased participation of FGFR1 and 5-HT1A homodimers and recruitment of β-arrestin2 to the 5-HT1A and also to the FGFR1 protomers.

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