



Receptores de dopamina y heterómeros de receptores de dopamina en la modulación de la neurotransmisión

Sergio González González

ADVERTIMENT. La consulta d'aquesta tesi queda condicionada a l'acceptació de les següents condicions d'ús: La difusió d'aquesta tesi per mitjà del servei TDX (www.tdx.cat) ha estat autoritzada pels titulars dels drets de propietat intel·lectual únicament per a usos privats emmarcats en activitats d'investigació i docència. No s'autoritza la seva reproducció amb finalitats de lucre ni la seva difusió i posada a disposició des d'un lloc aliè al servei TDX. No s'autoritza la presentació del seu contingut en una finestra o marc aliè a TDX (framing). Aquesta reserva de drets afecta tant al resum de presentació de la tesi com als seus continguts. En la utilització o cita de parts de la tesi és obligat indicar el nom de la persona autora.

ADVERTENCIA. La consulta de esta tesis queda condicionada a la aceptación de las siguientes condiciones de uso: La difusión de esta tesis por medio del servicio TDR (www.tdx.cat) ha sido autorizada por los titulares de los derechos de propiedad intelectual únicamente para usos privados enmarcados en actividades de investigación y docencia. No se autoriza su reproducción con finalidades de lucro ni su difusión y puesta a disposición desde un sitio ajeno al servicio TDR. No se autoriza la presentación de su contenido en una ventana o marco ajeno a TDR (framing). Esta reserva de derechos afecta tanto al resumen de presentación de la tesis como a sus contenidos. En la utilización o cita de partes de la tesis es obligado indicar el nombre de la persona autora.

WARNING. On having consulted this thesis you're accepting the following use conditions: Spreading this thesis by the TDX (www.tdx.cat) service has been authorized by the titular of the intellectual property rights only for private uses placed in investigation and teaching activities. Reproduction with lucrative aims is not authorized neither its spreading and availability from a site foreign to the TDX service. Introducing its content in a window or frame foreign to the TDX service is not authorized (framing). This rights affect to the presentation summary of the thesis as well as to its contents. In the using or citation of parts of the thesis it's obliged to indicate the name of the author.



UNIVERSIDAD DE BARCELONA

FACULTAD DE BIOLOGÍA

DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR

RECEPTORES DE DOPAMINA Y HETERÓMEROS DE RECEPTORES DE DOPAMINA EN LA MODULACIÓN DE LA NEUROTRANSMISIÓN

Memoria presentada por el licenciado en Bioquímica

SERGIO GONZÁLEZ GONZÁLEZ

para optar al grado de Doctor por la Universidad de Barcelona

Esta Tesis se ha inscrito dentro del programa de doctorado de Fisiología del Departamento de Fisiología e Inmunología de la Universidad de Barcelona, bienio 2007-2009.

El trabajo experimental y la redacción de la presente memoria han sido realizados por Sergio González González bajo la dirección del Dr. Rafael Franco Fernández y la Dra. Josefa Mallol Montero.

Barcelona, Abril de 2012

Dr. Rafael Franco Fernández

Dra. Josefa Mallol Montero

Sergio González González

*« Et il revint vers le renard : - Adieu, dit-il...
- Adieu, dit le renard. Voici mon secret. Il est très simple: on ne voit bien qu'avec le cœur. L'essentiel est invisible pour les yeux.
- L'essentiel est invisible pour les yeux, répéta le petit prince, afin de se souvenir.
- C'est le temps que tu as perdu pour ta rose qui fait ta rose si importante.
- C'est le temps que j'ai perdu pour ma rose... fit le petit prince, afin de se souvenir. »*

Le Petit Prince. Antoine de Saint-Exupéry

**À mon renard apprivoisé
et mes roses.**

Y llegó el momento de cerrar otra etapa del camino. Un camino que empezó exactamente la fría mañana del 25 de diciembre de 1997. Recuerdo que me levanté nervioso e ilusionado cuando apenas empezaban a salir los primeros rayos de sol para ver que me había traído Papa Noel y... ahí estaba!!! Bajo el árbol que había decorado con mi hermana unos días antes... Mi Quimifeca!!! Mi primer laboratorio!!! Todavía recuerdo el primer experimento de todos: leí más de diez veces el protocolo y mezclé cuidadosamente la disolución saturada de sulfato de cobre con una disolución básica de hidróxido sódico formándose así lo que sería el primer precipitado de mi vida. Fue en aquel momento cuando me giré a mi madre y le dije: - Mama, yo quiero ser científico. - Y sin duda, decidí el camino que seguiría desde entonces y que todavía sigo. Es por ello que quiero dar las gracias, no únicamente a la gente que ha estado a mi lado durante el periodo de la tesis, sino a todos aquellos que han compartido o comparten parte de este camino.

En primer lugar, quiero agradecer a mis jefes toda la ayuda durante estos cuatro años. A Rafa por darme la oportunidad de realizar la tesis en el grupo y convertirme en el 'pseudo-profe' de BAC. A Enric, por su ayuda con los papeles y la eterna burocracia. A Vicent, por los cortos pero intensos momentos de binding y por no perder la esperanza de que aprendiésemos a pipetear bien. A 'papá' Antoni, por llegar cada mañana al laboratorio con una gran sonrisa, pero sobretodo, por las increíbles clases de enzimología. No te preocupes, que no me olvidaré nunca de la ecuación de Michaelis Mentel! A Pepi, por hacernos el día a día más fácil y pensar siempre en lo mejor para el grupo. A Peter, por todas las nuevas ideas en los proyectos y enseñarme lo que hay detrás del misterioso mundo de las publicaciones. A Sergi, por estar cerca a pesar de estar a más de 1000 Km. Y a 'mamá' Carme, por su criterio científico, y por saber regañarnos cuando hacíamos mal las cosas y felicitarnos cuando las hacíamos bien.

Quiero también dar las gracias a todos mis compañeros de laboratorio, por los buenos y malos momentos vividos. Especialmente quiero dar las gracias a los viejos; a la capitana Aroa por preguntarme continuamente si era feliz, a Daniel, por ser la primera persona con quien hablé al entrar al grupo y a Chemita, por ser el hermano mayor. Y sobretodo a los nuevos, a Edu, por ser un ejemplo de paciencia y templanza. A Milena, por las cientos de recetas con extra de frankfurts. A Estefanía, mi compañera de batallas con los slices, por cada mirada de desesperación y por cada sonrisa de complicidad compartida, sin las cuales este camino hubiera sido imposible. A Marta, mi hombro de apoyo, por los cientos de 'tuppers' compartidos y las largas conversaciones con el oráculo. To Benni, always my German friend! For each shared adventure: tourism in Barcelona, the party in Kamil' home, the Purines Congress and the ski day in 'La Molina'. - Vamos Benni! The last blue pista!-. A Kamil, por nuestras horas compartidas sacando pineales y por enseñarme que el dialogo es el mejor camino para solucionar los problemas. A David, por nuestros indispensables coffee-break acompañados de

bromas sobre los experimentos: -Si cargo 5 veces un gel, tengo una $n=5$? jejeje- . A Lucía, por ser la mañica de Zárágózá! y por sus increíbles obras de teatro. Be espora my friend!!! Y por supuesto a todos los miembros del lab con los que he compartido el difícil día a día entre estas cuatro paredes: a Newton, a Victor, a Sandra, a Dani, a Marta, a Julia, a Mireia, etc... Gracias también a los grupos de Lipos, Integrativa y RST, a Ingrid, a Itziar, a Paula, a Santi, a Úrsula, a Ana... y a Roser, Manolo, Raquel, Ana, David y todos los técnicos del departamento que han hecho este camino un poquito más fácil. Gracias también a Cata y Patri por largas horas de cultivos juntos y por nuestras conversaciones que me ayudaban a desconectar un poquito del mundo. Gracias a Carmen Benito por toda la ayuda y consejos con los experimentos de radioactividad, y a Manel por las horas compartidas buscando células en esa nevera llamada sala de microscopia confocal.

Dicen que los buenos amigos se pueden contar con los dedos de la una mano. Yo debo ser muy afortunado, pues a mi me faltan dedos para contar a las increíbles personas que han estado a mi lado durante este camino. Gracias a Natàlia e Isa por tantas clases compartidas, tantos exámenes y tantas risas. -Como era? Kinase!-. A mi vasca favorita, Andrea la Txaku, I love u darling! Por ser una de las personas más importantes de mi vida, por cada paseo hasta el lab por las mañanas, por nuestros viajes por el País Vasco, por conducir más de 50km bajo la nieve para encontrar la masía de las cuajadas y por la futura casa que nos compraremos en Cadaqués. Gracias a Gabriel, por las cenas que acababan con conversaciones trascendentales hasta las 3 de la madrugada. A Minerva, mi amiga 'Yanki', por las largas tardes de invierno en el Starbucks tomando un café y hablando de ciencia. A Raquel, por explicarme sus aventuras en Suecia en formato telenovela (Continuará...?). Y a Cris, mi mafioso siciliano, por convertirme en su matarratas personal. Gracias a Elisa, mi madriña escarallada, por ser un ejemplo de alegría y optimismo, por las risas en clase de francés, por nuestro paseo en las Islas Cíes y por aquel helado en Playa América cuando estábamos a 2°C bajo cero. A Olaf y Andrea, por nuestros picnics en el Parc de la Ciutadella y por convertirnos en los tíos oficiales de 'la petite puce'. A Uge y Aida por tantos años juntos, por nuestras peligrosísimas vacaciones en Sicilia comiendo 'aranchinis', por los chapuzones en las aguas 'termales' de la piscina de Prades y por cada vaso de cava que nos hemos tirado encima. Gracias a Mireia, por tantos años de felicidad compartidos, por subir sin aliento hasta el Templo de Delfos y por compartir conmigo el más impresionante de los amaneceres sobre las falucas del río Nilo. Mil gracias también a todos mis amigos del Gym: a Juan el cachitas, a Dimpel la periodista, a Nuria -viva nuestro barrio Sandin!-, a Marc el informático, a Luz por sus consejos de enfermera, etc... pero especialmente gracias a xiketa Marta y xiket Kike por nuestro viaje a Lisboa acompañado de pastelitos de crema y café de 50 céntimos, a Conxita, por ser la 'mami panxi' del grupo y estar dispuesta a darte un abrazo para demostrarte que el mundo no es tan malo como parece. Y a Maria, mi tata,

por hacer que cada pedaleada, cada café, cada excursión, cada cena, cada cotilleo, cada sonrisa y cada mirada de complicidad sea especial.

Je tiens aussi à remercier ma famille et amis de France pour m'avoir accueilli et protégé en fuyant mes problèmes, et me permettant de me déconnecter un peu du monde. Merci à Florence pour me traiter comme ses enfants et me faire grossir avec la tartiflette. À Francis pour me faire découvrir le monde envoutant du vin français et le fromage. À mes oncles Roland et Sylvie, et à ma cousine Perrine pour nos séjours à Oléron, Font Romeu et Tossa de Mar. À papi Dominique et mamie Christiane pour les après-midi en jouant à la belote. À Lauren et à Mark pour la belle ballade dans le Marais Poitevin, pour nos vacances à Llançà et pour notre formidable aventure en bicyclette sur l'Île d'Oléron. -Allez Lauren, plus qu'un km!!!- Merci à Claire la femme à lunettes, à Alexis mon blanc ami, à Flo la bretonne, à Ludivine Madame Cracotte, à Manue la parisienne, à Romain le père Beauval, à Kevin le prof et à Lucie ma gitane! Merci pour les fêtes dans l'appart'bourgeois d'Alexis, pour manger la truffade sous un soleil d'août, pour les crevettes à la vanille, pour les mojito-fêtes et pour notre escapade sur l'île de Noirmoutier.

Dicen que más esenciales son las raíces del árbol que sus propios frutos, y por ello, quiero agradecer especialmente a todas esas raíces que me han hecho crecer y me han mantenido firme y mirando hacia arriba cada día. Gracias a mis titos Antonio y Toni por las barbacoas llenas de ánimos y risas. A mi tita Angustia y tito Enrique por todos los consejos acompañados de rosquillas con azúcar. A mi tío Paquito por haber sido mi padre en los peores momentos. A mis primos Jordi, Eric y Raúl por cada chapuzón en la piscina, por cada sábado por la mañana con las bicis y por nuestros findes en el 'Continente'. Gracias especialmente a mis padres por su confianza, su coraje y por enseñarme que la posibilidad de realizar un sueño es lo que hace que la vida sea interesante, y que solo el miedo a fracasar puede volver este sueño imposible. Gracias también a mi hermana, por todos los incontables momentos vividos, por ser la primera mirada que recuerdo y por cada sonrisa de complicidad que recordaré siempre. A Xabi y mis sobrinos, por enseñarme que la simplicidad de la vida es lo que hace que valga la pena vivirla. Et spécialement merci à mon renard apprivoisé, pour le plus romantique pique-nique au bord de la rivière de Parthenay, pour la silencieuse promenade dans le château de Chambord et pour faire de ma vie un rêve duquel je ne veux pas me réveiller.

Gracias a todos por enseñarme que dans le grand jeu de la vie, la vraie règle c'est d'être heureux. Sin vosotros esta tesis nos hubiera sido posible.

ABREVIATURAS	1
I. INTRODUCCIÓN	5
1.1 CARACTERÍSTICAS GENERALES DE LOS RECEPTORES ACOPLADOS A PROTEÍNA G	7
1.1.1 Estructura de los receptores acoplados a proteína G	8
1.2.1 Nomenclatura de los receptores acoplados a proteína G	10
1.3.1 Vías de señalización de los receptores acoplados a proteína G	13
1.4.1 Actividad constitutiva de los receptores acoplados a proteína G	18
1.5.1 Desensibilización de los receptores acoplados a proteína G	19
1.2 LOS RECEPTORES ACOPLADOS A PROTEÍNA G COMO DIANAS TERAPÉUTICAS	22
1.2.1 Ligandos Múltiples	23
1.2.2 Ligandos Alostéricos	24
1.3 HOMÓMEROS Y HETERÓMEROS DE RECEPTORES ACOPLADOS A PROTEÍNA G	28
1.3.1 Estructura cuaternaria de los homómeros y heterómeros de receptores acoplados a proteína G	30
1.3.2 Consecuencias funcionales de la formación de homómeros y heterómeros entre receptores acoplados a proteína G	33
1.3.3 Técnicas para el estudio de la oligomerización de receptores acoplados a proteína G	37
1.4 RECEPTORES DE DOPAMINA Y VÍAS DOPAMINÉRGICAS EN EL SNC	46
1.4.1 La dopamina como neurotransmisor	46
1.4.2 Receptores de dopamina D ₁ y D ₂	47
1.4.3 El receptor de dopamina D ₄ y su relación con el Trastorno	

de Hiperactividad y Déficit de Atención (ADHD)	54
1.4.4 Los ganglios basales y las vías dopaminérgicas en el SNC	60
1.5 RECEPTORES ADRENÉRGICOS Y VÍAS ADRENÉRGICAS EN EL SNC	67
1.5.1 La adrenalina como neurotransmisor	67
1.5.2 Clasificación, estructura y farmacología de los receptores adrenérgicos	69
1.5.3 Los receptores α_1 y β_1 adrenérgicos	72
1.5.4 Vías noradrenérgicas en el SNC	74
1.6 RECEPTORES ADRENÉRGICOS Y DOPAMINÉRGICOS EN LA GLÁNDULA PINEAL	77
II. OBJETIVOS	83
III. RESULTADOS	89
Informe del Director	91
3.1 A Hybrid Indoloquinolizidine Peptide as Allosteric Modulator of Dopamine D ₁ Receptors.	95
3.2 Dopamine D ₄ receptor, but not the ADHD-associated D _{4.7} variant, forms functional heteromers with the dopamine D ₂ receptor in the brain.	111
3.3 Circadian-related heteromerization of adrenergic and dopamine D ₄ receptors modulates melatonin synthesis and release in the pineal gland.	131
3.4 Biotin Ergopeptide Probes for Dopamine Receptors	199
IV. RESUMEN DE RESULTADOS Y DISCUSIÓN	227
V. CONCLUSIONES	241
VI. BIBLIOGRAFÍA	245

ABREVIATURAS

5-HT	Receptor de Serotonina
7TMD	Siete dominios de transmembrana
α_1	Receptor alfa 1 adrenérgico
β_1	Receptor beta 1 adrenérgico
A ₁ R	Receptor A1 de adenosina
A _{2A}	Receptor A2A de adenosina
AA-NAT	AminoÁcido N-AcetylTransferasa
AC	Adenilato ciclasa
Ach	Acetilcolina
ADHD	Attention-Deficit and Hyperactivity Disorder
AMPC	Adenosina Monofosfato Cíclico
ATP	Adenosina Trifosfato
BLAST	Basic Local Alignment Search Tool
BRET	Bioluminescence resonante energy transfer
BSA	Albúmina de Suero Bovino
C	Citosina
CaCl ₂	Cloruro de calcio
CaMKII	Calmodulin-dependent Protein Kinase II
CK1/2	Casein Kinase 1/2
CREB	cAMP reponse element-binding proteína
D	Aminoácido Aspártico
D1 o DRD1	Receptor D1 de dopamina
D2l	Receptor D2 long de dopamina
D2s	Receptor D2 short de dopamina
D3	Receptor D3 de dopamina
D4	Receptor D4 de dopamina
D4.2	Receptor D4.2 de dopamina (variante polimórfica 2)
D4.4	Receptor D4.4 de dopamina (variante polimórfica 4)
D4.7	Receptor D4.7 de dopamina (variante polimórfica 7)
D5 o DRD5	Receptor D5 de dopamina
DARPP-32	Dopamine and cyclic adenosine 3', 5'- monophosphate Regulated Phospho Protein
DAG	Diacilglicerol
DAT	Transportador de Dopamina

DMEM	Dulbecco's Modified Eagle Medium
α -MEM	Alfa Medified Eagle Medium
DNA	Ácido Desoxiribonucleico
dNTPs	Deoxynucleoside Triphosphates
E	Aminoácido Glutámico
EDTA	Ácido Tetraacético Etilendiamina
ERK	Extracelular Regulated Kinases
FBS	Suero Fetal Bovino
FRET	Fluorescence Resonance Energy Transfer
G	Guanosina
GABA	Ácido γ -aminobutírico
GDP	Guanosina Difosfato
GFP	Proteína Verde Fluorescente
GMP	Guanosina Monofosfato
GPCR	Receptores Acoplados a Proteína G
GRK	G protein-coupled receptor kinase
GTP	Guanosina Trifosfato
H3	Receptor h3 de histamina
HBSS	Hank's Balance-Salt Solution
HCl	Ácido Clorhídrico
HEK	Human Embryonic Kidney
IC	Intracelular
IP3	Inositol 1,4,5-Trifosfato
JNK	Jun N-terminal Kinase
K	Aminoácido Lisina
KD	Constante de Disociación
KDa	KiloDalton
KO	Knockout
L	Ligando
λ	Longitud de onda
LC	Locus Coeruleus
L-type VDCC	Canales de Calcio Dependientes de Voltaje Tipo L
LB	Medio de Luria-Bertani
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen Activated Protein Kinase Kinase
mRNA	Ácido Ribonucleico Mensajero
NaCl	Cloruro de Sodio

NAD ⁺	Nicotinamide Adenine Dinucleotide
PBS	Tampón de Fosfato Salino
PCR	Reacción en Cadena de la Polimerasa
PEI	PolyEthylenImine
PIP2	Fosfatidilinositol 4,5-bifosfato
PDZ	Post-synaptic-Density-95/ZOI domain
PKA	Proteína Kinasa A
PKC	Proteína Kinasa C
PLA	Fosfolipasa A
PLC	Fosfolipasa C
PP1	Proteína Fosfatasa 1
PP-2A	Serina/Treonina Fosfatasa Dependiente de Calcio
R	Aminoácido Arginina
Raf	Proto-oncogene serine/threonine-protein kinase Raf
RAS	RAt Sarcosoma Protein
RE	Retículo Endoplasmático
REC	1-(4-amino-6,7-dimethoxy-2-quinazoliny1)-4-((2-methoxy-6-(1-methylethyl)phenoxy)acetyl)piperazine dihydrochloride
RET	Transferencia de Energía por Resonancia
RTK	Receptor Tirosina Kinasa
RLuc	Renilla Luciferasa
RNA	Ácido Ribonucleico
SDS	Sodium Dodecyl Sulfate
SH2	Scr-Homology 2 domain
SNC	Sistema Nervioso Central
SOS	Son of Sevenless Protein
TEMED	TetraMetilEtilenDiamina
TM	Transmembrana
TPH	Triptófano Hidroxilasa
TRIS	2-amino-2-hydroxymethyl-1,3-propanodiol
VNTR	Número Variable de Repeticiones en Tándem
VTA	Área Tegmental Ventral
YFP	Proteína Amarilla Fluorescente

« Je ne pouvais entendre sa voix ni sentir son contact, mais sa lumière et sa chaleur rayonnaient dans chaque recoin de notre logis, et moi, avec la confiance d'un enfant qui peut encore compter ses années sur les doigts, je croyais qu'il me suffisait de fermer les yeux et de lui parler pour qu'elle m'écoute, d'où qu'elle fût. »

L'Ombre du vent. Carlos Ruiz Zafón

INTRODUCCIÓN

AGTCATCGTCGTACGTACGATCGTACTTACAGTAAAATCTGTAGCAGCTAGCATGTTTTCTTTGGGTGTACGATCGACTGATCGTGCATACTACGATCACTACTACGGCGA

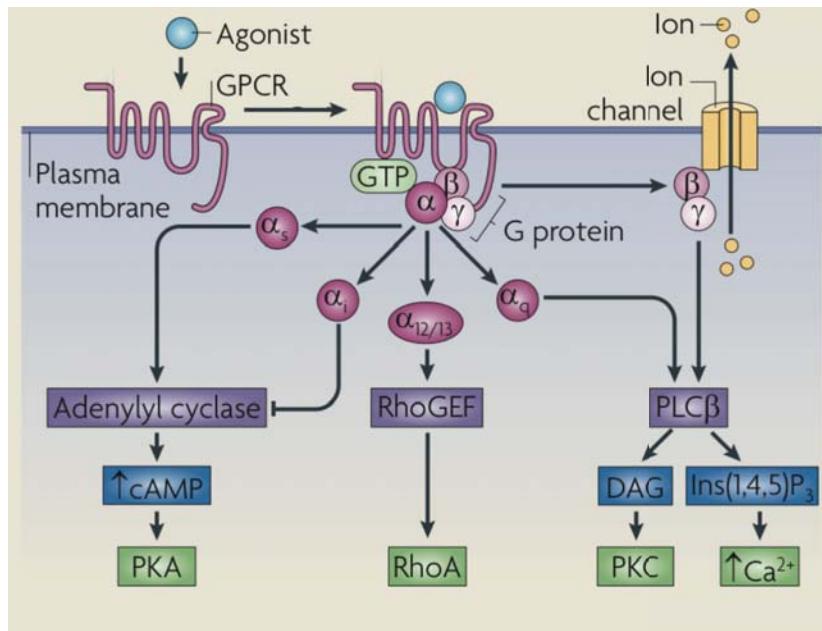
1.1 Características generales de los receptores acoplados a proteína G

La comunicación celular es un requisito necesario para el mantenimiento y regulación de la homeostasis de los seres vivos. Para ello, las células del organismo tienen la capacidad de reconocer moléculas liberadas al medio extracelular, con la finalidad de procesar gran cantidad de información procedente de otras células. Debido a que muchas de estas moléculas liberadas no entran dentro de la célula a causa de su naturaleza polar, es necesario la presencia de receptores en la membrana citoplasmática con los que interactuar para ejercer su función. Los receptores acoplados a proteína G (GPCR) o también llamados receptores de siete dominios de transmembrana (7TMD) constituyen una importante familia de receptores de la membrana celular (Marinissen y Gutkind, 2001; Gudermann et al., 1997). Estos receptores están codificados por una gran familia de genes; en el caso del genoma humano, más del 1% codifica para más de 1000 proteínas con esta estructura, de las que más del 90% se expresan en el Sistema Nervioso Central (SNC) (George et al., 2002).

Los GPCRs son activados por una gran variedad de ligandos tanto endógenos como exógenos, entre los que se incluyen hormonas, péptidos, aminoácidos, iones y fotones de luz; y traducen la señal a través de un gran número de efectores como la adenilato ciclasa (AC), las fosfolipasas o los canales iónicos, entre otros. Muchas de estas vías de señalización, aunque no todas, están mediadas por el acoplamiento del receptor a una proteína G y, en su conjunto, desempeñan un papel clave en la fisiología celular controlando procesos del organismo como la secreción, la diferenciación o la neurotransmisión, entre otros. Más del 50% de los agentes terapéuticos actualmente comercializados actúan sobre estas proteínas activando (agonistas) o antagonizando (antagonistas) estos receptores (Marinissen y Gutkind, 2001; Flower, 1999). En la actualidad los GPCRs son considerados como dianas terapéuticas para el desarrollo de nuevos fármacos en amplios campos de la medicina.

En la figura 1 se ilustra como un agonista actúan a través de un GPCR. La unión del agonista a este receptor activa una cascada de señalización intracelular en la que intervienen dianas citoplasmáticas a través de un mecanismo dependiente de proteína heterotrimérica G. Estas vías de señalización regulan funciones biológicas clave como

la proliferación, apertura de canales iónicos, diferenciación y supervivencia celular, entre muchas otras.



Extraído de Ritter y Hall, 2009

Figura 1. Unión de un ligando agonista a un GPCR y activación de mecanismos de señalización celular responsables de las funciones biológicas.

1.1.1 Estructura de los receptores acoplados a proteína G

Los GPCRs se caracterizan por estar constituidos por una única cadena proteica que atraviesa siete veces la membrana celular gracias a que en su estructura existen 7 secuencias de unos 25-35 residuos aminoacídicos, principalmente hidrofóbicos, que se disponen en una estructura de hélices α y se internan en la membrana plasmática. Así, estos receptores están parcialmente inmersos en un ambiente no-polar de la bicapa lipídica formando una estructura compacta de hélices transmembrana conectadas por bucles extracelulares e intracelulares (Figura 2). Otra característica de estos receptores es que son capaces de interactuar estructuralmente con la proteína heterotrimérica G mediante sus dominios o bucles intracelulares.

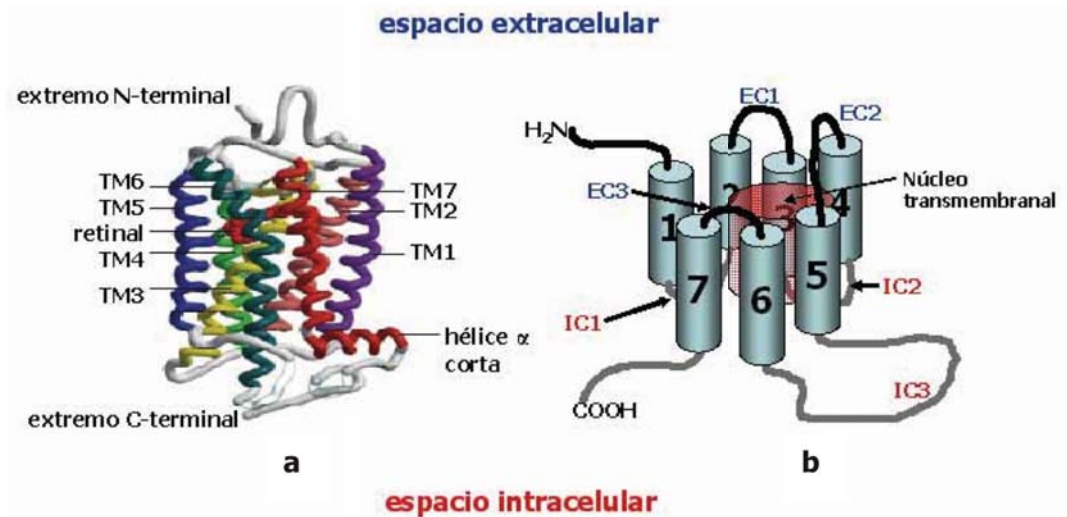
Un aparato complejo de translocación presente en el retículo endoplasmáticos (RE) es el encargado de la correcta orientación de la cadena polipeptídica. Se distinguen dos estados de plegamiento diferentes que se producen tras la translocación inicial del

receptor a través del extremo N-terminal dentro del lumen del RE. En el primer plegamiento, las hélices α se disponen a través de la bicapa lipídica, y el plegamiento de la proteína está dirigido principalmente por los efectos hidrofóbicos. Los dominios transmembrana adoptan una estructura para minimizar la superficie polar expuesta dentro del ambiente lipídico y, como resultado, los aminoácidos hidrofóbicos se encaran a la bicapa lipídica y los aminoácidos más hidrofílicos quedan orientados a través de la hendidura generada por el empaquetamiento de los dominios transmembrana (Scarselli et al., 2000). Finalmente, en el segundo plegamiento se forma una estructura terciaria por interacciones específicas hélice-hélice y por interacciones entre los dominios extracelulares y citoplasmáticos, permitiendo un fuerte empaquetamiento. En una gran parte de los receptores acoplados a proteína G, podemos encontrar dos residuos de cisteína en los bucles extracelulares 1 y 2 que forman un puente disulfuro, que posiblemente se encarga del empaquetamiento y la estabilización de un número restrictivo de conformaciones de los 7 dominios transmembrana (Kniazeff et al., 2011; Peeters et al., 2011; Agnati et al., 2003; Scarselli et al., 2000)

La región N-terminal puede estar glicosilada y la región C-terminal está expuesta a la interacción con otras moléculas de señalización, como kinasas y proteínas β -arrestinas, responsables de procesos de sensibilización, desensibilización e internalización (DeFea, 2011; Schulte et al., 2010; Pflieger et al., 2007; Lefkowitz, 1998). Además, la región carboxi terminal y los bucles intracelulares IC2 e IC3 son críticos para la transducción de la señal hacia el interior de la célula ya que son los dominios de unión a la proteína G responsable, en la mayoría de los casos, de iniciar la señalización intracelular.

La primera estructura cristalina de un GPCR fue descrita en el año 2000, cuando se estudió, con una alta resolución, la estructura cristalina del receptor de rodopsina bovino (Palczewski et al., 2000) (Figura 2a). Pese a la alta resolución queda pendiente información estructural detallada de algunas partes de los bucles y del extremo carboxi terminal; así como de la conformación en estado activo del receptor ya que la estructura cristalizada se estabilizó con un antagonista del receptor. Más recientemente se ha obtenido la estructura cristalina del receptor de opsina acoplado a la proteína G, lo que ha permitido una mejor interpretación de los cambios estructurales asociados a la transducción de señal (Park et al., 2008; Scheerer et al., 2008). Desde entonces, se han cristalizado otros GPCR incluyendo el receptor D_3 de dopamina (Chien et al., 2010;

Rosenbaum et al., 2009; Weis y Kobilka, 2008) el receptor A_{2A} de adenosina (Jaakola y Izerman, 2010; Jaakola et al., 2008) y el receptor β_2 -adrenérgico (Rasmussen et al., 2011; Tebben y Schnur, 2011; Cherezov et al., 2007).

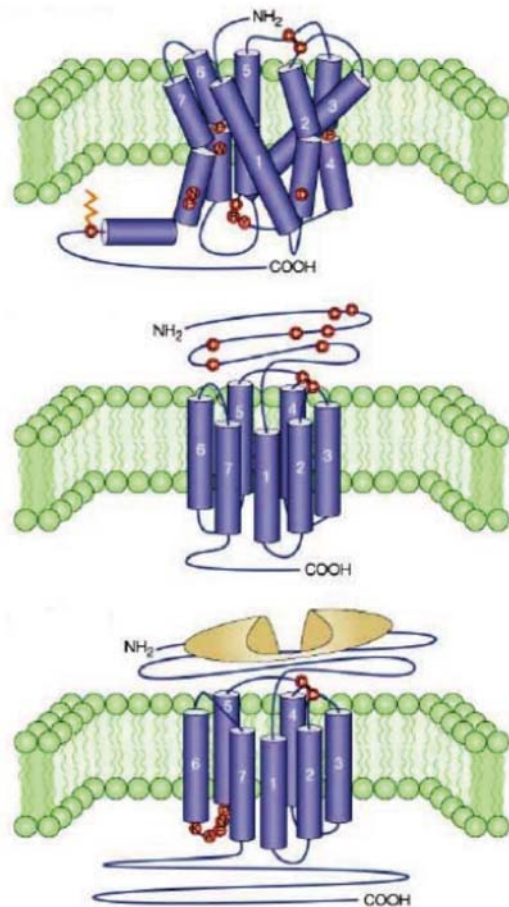


Extraído y modificado de Palczewski, 2000

Figura 2. Representación gráfica de la estructura cristalina de la rodopsina y esquema de la estructura típica de un GPCR.

1.1.2 Nomenclatura de los receptores acoplados a proteína G

Existen diversos sistemas de clasificación de los GPCRs. El más clásico es el sistema Kolakowski (Kolakowski, 1994) basado en la agrupación de familias en función de su estructura y características genéticas (Kolakowski, 1994; Probst et al., 1992). Como se observa en la figura 3, las diferentes familias se diferencian en el tamaño y en la función del dominio N-terminal, C-terminal y los bucles intracelulares. Pese a que todos los GPCRs comparten una estructura común, las distintas familias no tienen secuencias homólogas, indicando que probablemente no están relacionadas filogenéticamente y que la similitud de su estructura transmembrana se debe sólo a tener requerimientos funcionales comunes.



Familia 1 Receptores de aminas biogénicas (adrenérgico, serotonina, dopamina, muscarínico, histamina)
 Receptores de neurotensina y TRH
 Receptores de adenosina y cannabinoides
 Receptores de nucleótidos, oxitocina y somatostatina
 Receptores de melatonina

Familia 2 Receptores de calcitonina
 Receptores de PTH
 Receptores de glucagón, VIP y secretina
 Receptores de latrotoxina

Familia 3 Receptores metabotrópicos de glutamato, Receptores GABA
 Receptores de calcio
 Receptores de feromonas vomeronasales
 Receptores gustativos

Extraído y modificado de George et al., 2002

Figura 3. Representación de las tres principales familias de receptores acoplados a proteínas G.

Aproximadamente el 90% de todos los GPCRs pertenecen a la familia 1, también llamada A o rodopsin-like. Esta familia contiene receptores para odorantes, neurotransmisores y hormonas glicoproteicas. La homología entre los receptores de esta familia es baja y restringida a un número limitado pero con un alto grado de conservación de residuos, lo que sugiere que estos tienen un papel esencial en la estructura y/o funcionalidad de estos receptores (Probst et al., 1992). Los receptores de esta familia se caracterizan también por tener un puente disulfuro que conecta el primer y segundo bucle extracelular y muchos de estos tienen también una cisteína palmitoilada en la cola C-terminal que le sirve para anclarse a la membrana. En esta familia, el ligando se une en una cavidad formada por los dominios transmembrana aunque para alguna subfamilia, activada por pequeños péptidos, el reconocimiento se produce a nivel de los bucles extracelulares y del dominio N-terminal (Pin et al., 2003; Ulric et al., 1998).

La familia B, o también llamada familia 2, incluye aproximadamente 20 receptores diferentes para una variedad de hormonas peptídicas y neuropéptidos, como el péptido intestinal vasoactivo, la calcitonina y el glucagón. Esta familia se caracteriza por poseer un extremo N-terminal de cerca de unos 100 residuos, el cual contiene diversas cisteínas que forman una red de puentes disulfuro (Ulrich et al., 1998). La morfología de estos receptores es similar a la de los de la familia 1, pero no parecen palmitoilarse y los residuos y motivos conservados son diferentes. Se sabe poco de la orientación de los dominios de transmembrana (TM), pero teniendo en cuenta la divergencia de la secuencia aminoacídica, probablemente son diferentes de la familia 1.

La familia 3 o C se caracterizan por un largo extremo carboxi y amino terminal (500-600 aminoácidos). A esta familia pertenece el receptor metabotrópico de glutamato y el receptor GABA (ácido γ -aminobutírico). La estructura del lugar de unión (representado en la figura 3 en amarillo) se ha deducido mediante estudios de cristalografía del extremo N-terminal del receptor metabotrópico de glutamato solubilizado y unido a glutamato (He et al., 2002). Se ha visto que forma un dímero unido por puente disulfuro que puede abrirse y cerrarse en el proceso de unión del ligando (He et al., 2002). Esta familia no tiene ningún rasgo común con la familia 1 y 2 excepto por la presencia de cisteínas en el bucle extracelular que forman puentes disulfuro. Una característica única de estos receptores es que el tercer bucle intracelular es corto y altamente conservado, y al igual que en la familia 2 tampoco se conoce la orientación de los dominios de transmembrana (TM) (Jacoby et al., 2006; George et al., 2002).

Los receptores de feromonas de levadura configuran dos familias menores no relacionadas, familias D y E. Finalmente, 4 receptores diferentes de AMPc constituyen otra familia menor, pero única, la familia F (Kolakowski, 1994).

Aunque la clasificación A-F esta ampliamente aceptada, Fredriksson y colaboradores (Fredriksson et al., 2003) efectuaron el primer estudio filogénico de toda la superfamilia de GPCRs en el genoma de mamífero proponiendo una clasificación más detallada. Sus análisis demuestran que hay 5 familias principales para los GPCRs humanos: Glutamate, Rodopsin, Adhesion, Frizzled/Tasted2 y Secretin; y que dentro de cada familia los receptores comparten un origen evolutivo común. Tres de estas

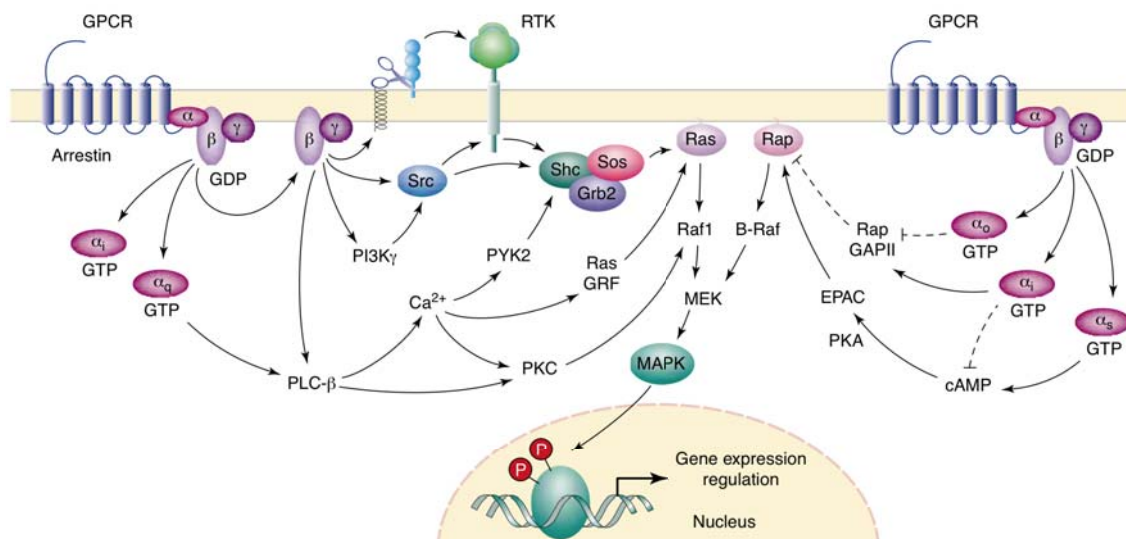
familias, rodopsina (A), secretina (B) y glutamato (C) se corresponden con la clasificación A-F, mientras que las otras dos familias, adhesión y frizzled, no están incluidas. En esta clasificación la superfamilia de la rodopsina sigue siendo la mayor, y se ha dividido en 4 grupos principales con 13 ramas distintas. Los autores de este nuevo sistema de clasificación defienden la teoría de que los receptores acoplados a proteína G surgieron a partir de un único predecesor común, que evolucionó a través de duplicaciones génicas, evolucionando desde la mayor simplicidad en cuanto a sus orígenes a la enorme complejidad que muestra la superfamilia de estos receptores en la actualidad.

1.1.3 Vías de señalización de receptores acoplados a proteína G

Cuando un receptor es activado por un ligando, se inicia una serie de eventos intracelulares que modulan la función celular. Estos eventos dependen de la proteína G a la que se encuentran acoplados y de la maquinaria molecular intracelular. Las proteínas G están presentes en todos los organismos eucariotas y tienen un papel esencial en la transducción de señales debido a su asociación al receptor y a otras proteínas citoplasmáticas efectoras. Cada proteína G tiene una estructura heterotrimérica constituida por la subunidades α (39-46 KDa), β (37 KDa) y γ (8KDa). La interacción del ligando con el receptor produce una serie de cambios conformacionales que modifican la estructura de la proteína G, los cuales repercuten en la afinidad de la subunidad G_α por $G_{\beta\gamma}$ los nucleótidos de guanina, haciéndola más afín por GTP que por GDP (Bourne et al., 1991) Al intercambiar GDP por GTP, la subunidad G_α se activa y se desensambla tanto del receptor como del complejo estable $G_{\beta\gamma}$ (Marinissen y Gutkind, 2001). Tanto la subunidad G_α como el complejo $G_{\beta\gamma}$ son moléculas señalizadoras de forma que activan o inhiben a moléculas efectoras, como las adenilato y guanilato ciclasas, fosfodiesterasas, las fosfolipasas A2 y C, la fosfoinositol 3-kinasa entre otras; dando lugar a una activación o inhibición de una gran variedad de segundos mensajeros como el AMPc, GMPc, diacilglicerol (DAG), inositol (1,4,5)-trifosfato (IP3), fosfatidil inositol (3,4,5)-trifosfato, ácidos araquidónico y fosfatídico, por citar algunos (Marinissen y Gutkind, 2001).

Dos ejemplos típicos de cascadas de señalización iniciadas por receptores

acoplados a proteína G son las que conducen a la formación de inositol- 1,4,5-trifosfato (IP3)/DAG y AMPc como segundos mensajeros. Para la subunidad $G_{\alpha q}$, la proteína efectora diana es la PLC, enzima que hidroliza fosfoinosítoles de membrana generando IP3 y DAG como segundos mensajeros. El IP3 aumenta la concentración de calcio intracelular vaciando los depósitos intracelulares, mientras que el DAG activa a la PKC. En el caso de las subunidades $G_{\alpha s}$ o $G_{\alpha i}$, la proteína efectora es la adenilato ciclasa (AC), enzima que cataliza la conversión de ATP a AMPc, mientras $G_{\alpha s}$ estimula esta enzima, la subunidad $G_{\alpha i}$ la inhibe. El AMPc activa la PKA que igual que la PKC fosforila a múltiples y diversas proteínas (receptores, canales iónicos, enzimas o factores de transcripción) regulando así el funcionamiento celular (Berridge, 2009; Puzianowska-Kuznicka y Kuznicki, 2009; Faure et al., 2004; Marinissen y Gutkind, 2001)



Extraído de Marinissen y Gutkind, 2001

Figura 4. Representación de algunas de las vías que enlazan los GPCR con la vía de las MAPK.

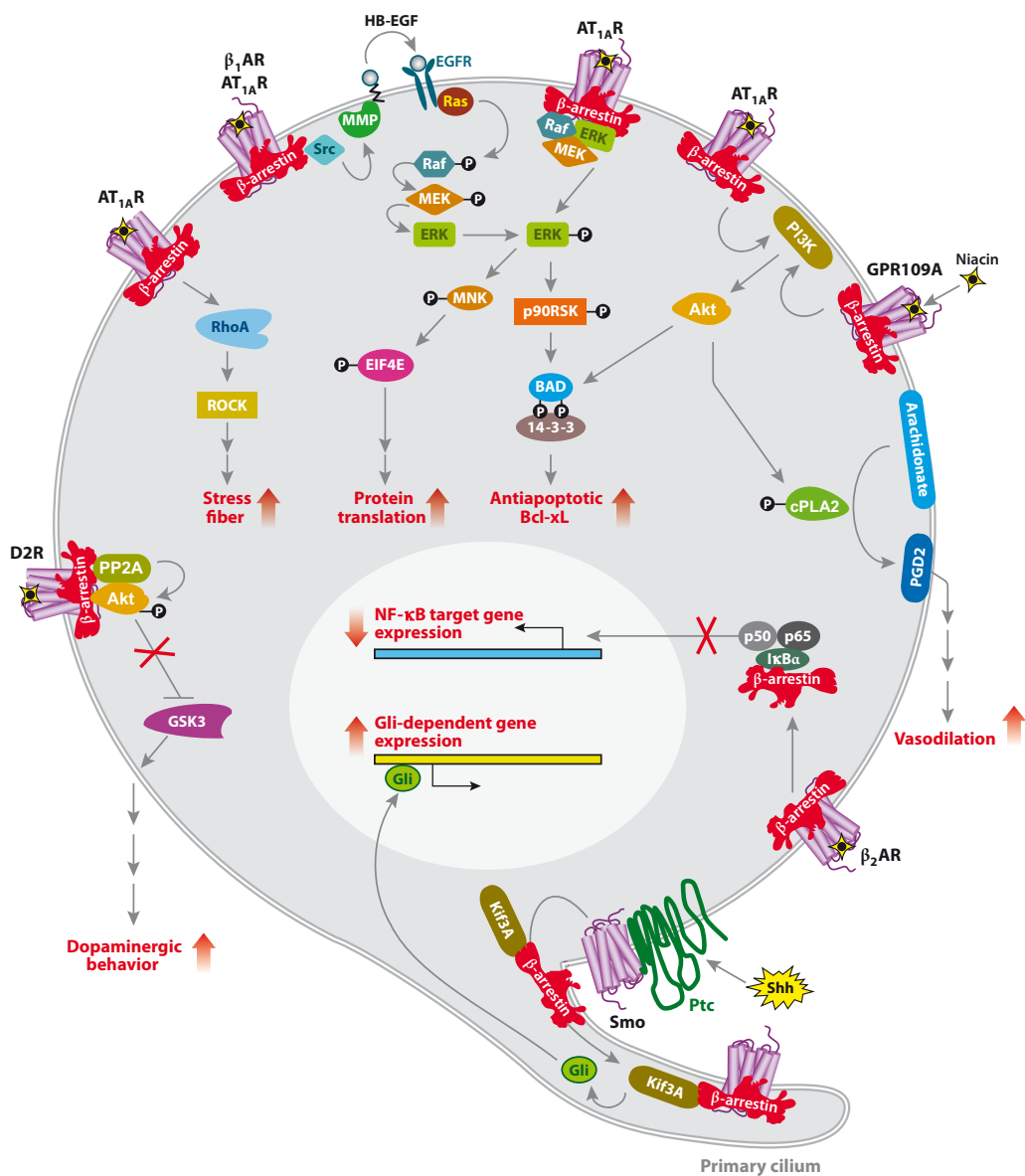
Muchas de las respuestas mediadas por estos receptores no consisten únicamente en la estimulación de segundos mensajeros convencionales, si no que son el resultado de la integración de diferentes redes de señalización, entre las que se incluyen la vía de las MAPKs y las JNKs. Al principio se creía que la activación de la ruta de las MAPK por GPCR involucraba proteína G sensible a la toxina de la *Bordetella pertussis* ($G_{\alpha i}$) y que dependía fuertemente del complejo $G_{\beta\gamma}$ de la proteína G y de tirosina quinasas no identificadas (Faure et al., 1994; Koch et al., 1994; Van Corven et al., 1993). Se postuló

que, en ausencia de ligandos para receptores con actividad tirosina quinasas (RTK), la activación de receptores acoplados a proteína G podía inducir la estimulación de un RTK generando señales mitogénicas. Este fenómeno se denominó transactivación. Una vez transactivado, el RTK inicia una cascada de señalización idéntica a la generada por su propio ligando; es decir, la activación de las MAPK es a través de la vía Ras, Raf, MEK y ERK (Figura 4). El proceso es iniciado con las subunidades $G_{\beta\gamma}$ dando lugar a que se reclute Sos hacia la membrana. Ello activa el intercambio de GDP por GTP en la proteína Ras, siendo esta proteína el intermediario que conecta la cascada de señalización generada por la transactivación de un RTK con la fosforilación de ERK (Marinissen y Gutkind, 2001).

Sin embargo hay evidencias que indican que la señalización de los receptores de siete dominios transmembrana es mucho más compleja puesto que pueden activar la vía de las MAPKs a través de vías de señalización dependientes (Figura 4) e independientes de proteínas G (Beaulieu y Gainetdinov, 2011; Luttrell et al., 1999; Daaka et al., 1998; Lefkowitz, 1998). Un ejemplo paradigmático es la señalización mediada por la fosforilación del receptor por GRKs (G protein-coupled Receptor Kinases) la unión de β -arrestinas y el subsiguiente secuestro del receptor de la superficie celular (Krupnick y Benovic, 1998), que no sólo es importante para la finalización de la señal, sino que también juega un papel importante en el intercambio entre las vías de señalización dependientes de proteína G e independientes como las utilizadas normalmente por receptores de factores de crecimiento (Luttrell et al., 1999).

Estudios relativamente recientes muestran que las β -arrestinas desempeñan un papel en la señalización celular que va más allá del simple desacoplamiento entre receptor y la proteína G. El hecho de que las β -arrestinas puedan interaccionar directamente con tirosina kinasas de la familia de las Src y con componentes de la cascada de MAP kinasas (Reiter et al., 2011; Perry y Lefkowitz, 2002), sugiere que las β -arrestinas pueden funcionar como adaptadores o scaffolds reclutando proteínas involucradas en la señalización de un determinado receptor. De esta manera, se ha demostrado la capacidad de diferentes tipos de receptores acoplados a proteína G de reclutar componentes de las cascadas de las JNKs o las ERKs, incluyendo las kinasas más relevantes de la cascada, como pueden ser JNK3, Raf-1, MEK1 o ERK1/2. Estos

complejos pueden permanecer unidos incluso durante la internalización del receptor, presentando diferentes localizaciones subcelulares, presumiblemente en los endosomas hacia donde el receptor es conducido en su proceso de internalización y por lo tanto aproximando las quinasas a sus posibles substratos citosólicos. En este sentido, tal y como se muestra en la figura 5, las β -arrestinas actúan como scaffolds permitiendo al receptor regular la actividad y la distribución de dichas quinasas en el interior celular, lo que puede tener unas implicaciones funcionales muy importantes (Reiter et al., 2011; Kelly et al., 2008; Viloin y Lefkowitz, 2007).



Extraído de Reiter et al., 2011

Figura 5. Representación de las β -arrestinas regulando la función y localización subcelular de otras proteínas.

Además de las interacciones receptor-proteína intracelulares implicadas en la transducción de señal, se han descrito un gran número de interacciones receptor-proteína que son importantes para la formación de complejos macromoleculares responsables de la localización de estos receptores en determinados dominios celulares. Las proteínas andamio o scaffolding proteins o scaffolds, actualmente son consideradas como organizadoras de complejos multiproteicos en diversos compartimentos celulares como por ejemplo las densidades post-sinápticas neuronales y son las responsables de mantener estos receptores en esta localización (Bruneau et al., 2009; Beresewicz, 2007; Triller et al., 2005). En un mismo scaffold se agrupan varios dominios de interacción proteína-proteína, proporcionando un soporte específico que permite el ensamblaje de complejos multiméricos concretos para cada necesidad estructural o funcional (Huber, 2001). Los GPCRs interactúan con proteínas andamio que los conectan con el citoesqueleto celular (Hering y Sheng, 2001). Las proteínas scaffolds son ricas en dominios tales como los SH2 (Src-Homology 2), SH3 o PDZ (Post-synaptic-Density-95/Discs-large/ZO1), que se han conservado a lo largo de la evolución. En los últimos años se ha descrito un gran número de interacciones entre los receptores acoplados a proteína G y proteínas que contienen el dominio PDZ (Feng y Zhang, 2009; Pawson y Scott, 1997). Estas proteínas tienen un papel importante en la modulación de la señal, ya que definen la composición molecular de los complejos de señalización en microcompartimentos y, en algunos casos, la localización precisa de estos complejos en la célula. Por ejemplo, el factor regulador del intercambio Na⁺/H⁺ (NHERF: Na⁺/H⁺ Exchange Regulatory Factor) que interactúa con el receptor α_2 -adrenérgico, se ha visto que promueve la clusterización y la endocitosis del receptor (Hall et al., 1998). También la proteína Homer-1b que interactúa con el receptor metabotrópico de glutamato tipo 1 (mGluR₁) se ha demostrado que, por acción del Ca²⁺, induce movilización hacia la membrana de estos receptores (Roche et al., 1999). Por otra parte, proteínas con dominios PDZ, como la espinofilina, se ha visto que pueden interactuar con receptores D₂ de dopamina y α_2 -adrenérgicos vía un nuevo dominio no-PDZ, actuando como una proteína andamio que liga estos GPCRs con proteínas señalizadoras como PP-1 (Richman et al., 2001; Smith et al., 1999). Otros ejemplos de proteínas andamio que interactúan con receptores de siete dominios transmembrana son la α -filamina y el receptor de dopamina D₂ (Lin et al., 2001), la α -actinina y el receptor mGluR₅ de glutamato (Cabello et al., 2007), la α -actinina y el receptor A_{2A} de

adenosina (Burgueño et al., 2003a) y la familia de proteínas Shank y varios GPCRs como el receptor metabotrópico de glutamato 1 (mGluR₁) o el receptor de somatostatina tipo 2 (SSTR₂) (Sheng y Kim, 2000).

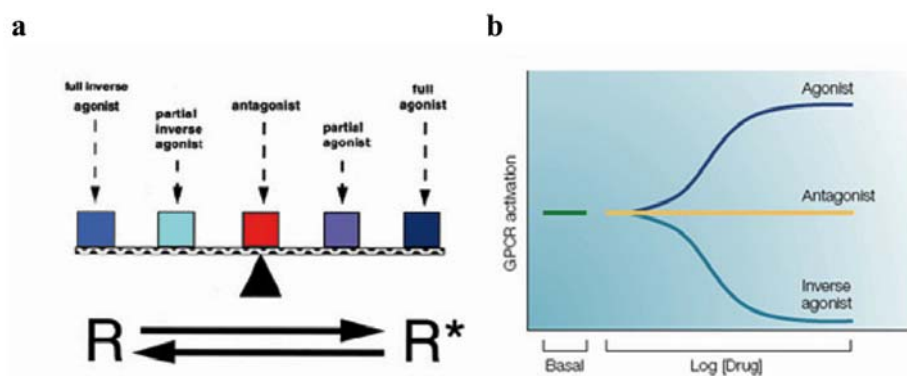
Además de las interacciones receptor-proteínas intracelulares, existen crecientes evidencias de que las interacciones receptor-proteína extracelulares pueden jugar un papel importante en la farmacología de los GPCRs. Un ejemplo es el caso del enzima adenosina desaminasa (ADA), proteína multifuncional que puede estar presente en la superficie de la célula anclada a diferentes proteínas como los receptores de adenosina A₁ y A_{2A} (Gracia et al., 2011; Herrera et al., 2001; Saura et al., 1996). Estas interacciones parecen ser esenciales para que estos receptores muestren el estado de alta afinidad por su ligando.

1.1.4 Actividad constitutiva de los receptores acoplados a proteína

La activación de un receptor acoplado a proteína G se basa en un cambio conformacional de la estructura terciaria debido a la unión al receptor de un ligando agonista. El receptor pasa de una conformación inactiva a una activa, existiendo una constante de equilibrio entre los dos estados del receptor. La actividad constitutiva que presentan estos receptores representa una isomerización del receptor a la conformación activa en ausencia de ligando (Seifert, 2002). Como consecuencia se promueve el intercambio GDP-GTP en la proteína G acoplada, aumentando así la actividad basal de dicha proteína G y de los siguientes sistemas efectoros (Costa y Herz, 1989)

Esta actividad constitutiva es inhibida por los compuestos denominados agonistas inversos, los cuales actúan sobre el receptor de manera que estabilizan la conformación inactiva y por lo tanto minimizan el intercambio GDP-GTP. Estos compuestos actúan de forma opuesta a los agonistas, cuya función es estabilizar al receptor en la conformación activa y, por lo tanto, inducir su señalización intracelular. Se ha propuesto la existencia de múltiples conformaciones de los receptores con distintas funciones biológicas (Seifert y Wenzel-Seifert, 2002). Estas conformaciones estarían estabilizadas por diferentes tipos de compuestos, siendo la más favorable para la señalización aquella

conformación del receptor estabilizada por el agonista; seguidas por los agonistas parciales, que serían compuestos con una menor eficiencia para estabilizar el receptor en la conformación más activa y por lo tanto promueven un menor intercambio GDP-GTP. A continuación vendrían los antagonistas neutros o simplemente antagonistas que no alterarían el equilibrio entre las conformaciones activa e inactiva, pero con la capacidad de bloquear el efecto de los agonistas y de los agonistas inversos. Por último, estarían los agonistas inversos parciales y los agonistas inversos, que serían capaces de estabilizar al receptor en su estado inactivo, en un menor y mayor grado respectivamente, reduciendo la actividad basal o constitutiva del receptor (Figura 6).



Extraído y modificado de Seifert y Wenzel-Seifert, 2002

Figura 6. Activación de los receptores acoplados a proteína G según el modelo de dos estados.

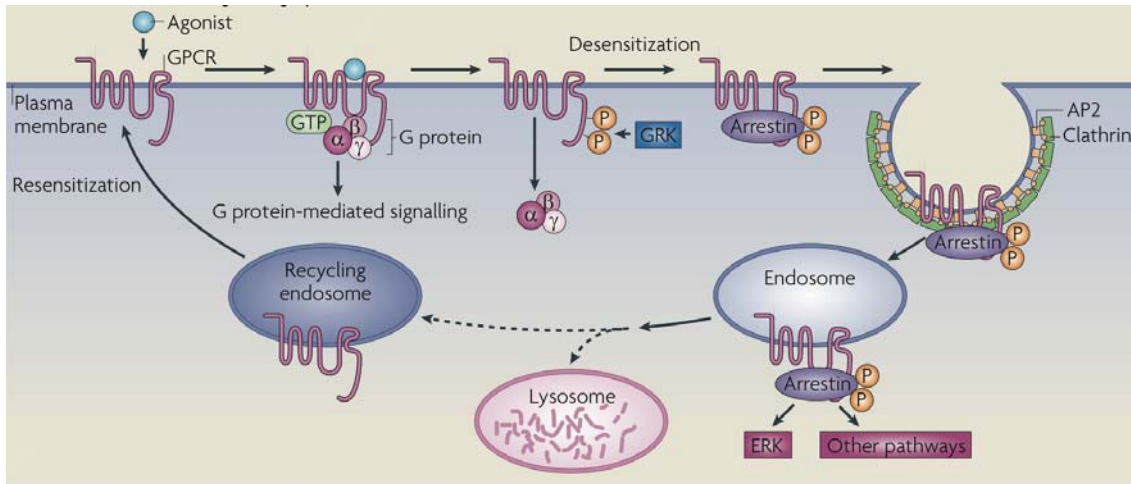
a) El modelo de dos estados asume que el receptor isomeriza desde un estado inactivo R a uno activo R*. Los diferentes tipos de ligando de un receptor se clasifican desde los que consiguen una mayor actividad del receptor, los agonistas totales, hasta los que consiguen inhibir completamente la funcionalidad del receptor, los agonistas inversos totales. b) La actividad constitutiva de los receptores acoplados a proteína G da lugar a una cierta actividad basal de la proteína G y del sistema efector asociado a dicho receptor. Los agonistas incrementan esta actividad, los antagonistas no tienen ningún efecto sobre la actividad basal y los agonistas inversos consiguen disminuirla.

1.1.5 Desensibilización de los receptores acoplados a proteína G

La rápida atenuación de la respuesta del receptor tras su activación mediante unión de un agonista recibe el nombre de desensibilización (Moser et al., 2010; Golan et al., 2009). Este fenómeno puede manifestarse mediante diferentes mecanismos como el desacoplamiento del receptor de su proteína G en respuesta a la fosforilación del receptor (Golan et al., 2009; Ferguson, 2001; Lohse et al., 1990; Hausdorff et al.,

1989), la internalización de los receptores de la superficie celular a compartimientos intracelulares (Ferguson, 2001; Trejo et al., 1998; Hermans et al., 1997) y la disminución del número de receptores debido a la disminución del RNA mensajero y a la síntesis proteica, así como la degradación de los receptores preexistentes (Jockers et al., 1999; Pak et al., 1999). En el caso de las fosforilaciones, estos fenómenos tienen lugar en segundos, minutos en el caso de las endocitosis y horas cuando es regulada la expresión. La desensibilización del receptor puede ser completa, como ocurre en el sistema olfativo y visual o atenuada, disminuyendo la respuesta máxima, como ocurre con el receptor β_2 - adrenérgico (Sakmar, 1998). La manera más rápida por la cual un GPCR se desacopla de la proteína G es a través de modificaciones covalentes en el receptor como consecuencia de su fosforilación por quinasas intracelulares, siendo de especial importancia las quinasas específicas para GPCR o GRK (Golan et al., 2009; Kelly et al., 2008).

La internalización de GPCR es un fenómeno común tras la estimulación por agonista. El tráfico de receptores a compartimientos endosomales permite la desfosforilación y reciclaje del receptor a la superficie celular (Boulay y Rabet, 2005; Gainetdinov et al., 2004; Ferguson, 2001; Pierce y Lefkowitz, 2001; Krueger et al., 1997). Parte de los receptores internalizados pueden degradarse tras la exposición prolongada al agonista, lo que implica que el receptor sea marcado para entrar en la vía de degradación (Böhm et al., 1997). El mecanismo de internalización de GPCR mejor caracterizado es a través de la fosforilación del receptor mediada por las proteínas quinasas específicas de GPCR (GRK) y β -arrestinas (Kelly et al., 2008). Una vez el receptor es fosforilado por GRK, la β -arrestina actúa como molécula reguladora que interactúa con componentes de la vía endocítica mediada por vesículas de clatrina. En respuesta a la activación de los GPCR, la β -arrestina citosólica transloca a la membrana plasmática uniéndose a los receptores a la vez que se inicia el proceso de endocitosis mediado por clatrina (Ritter y Hall, 2009) (Figura 7). Como se ha mencionado anteriormente, las β -arrestinas no solo funcionan en el secuestro de GPCR para la desensibilización e internalización, sino como proteínas para transducir y compartimentar las señales alternativas (Golan et al., 2009). Ya que estas proteínas tienen la habilidad de interactuar con una gran variedad de proteínas endocíticas y de señalización como las c-Src (Luttrell et al., 1999), MAPK y Raf (DeFea et al., 2000).



Extraído de Ritter y Hall, 2009

Figura 7. Ejemplo de un modelo de desensibilización, internalización y degradación de los GPCRs.

Algunos GPCR se han encontrado en estructuras de membrana ricas en colesterol llamadas caveolas (Jin et al., 2011; Burgueño et al., 2003b; Chun et al., 1994). Estos dominios son otro mecanismo para mediar la internalización de un receptor inducida por ligando (Wu et al., 2008; Kong et al., 2007; Escriche et al., 2003; Ginés et al., 2001). Las caveolas también son conocidas como dominios de señalización donde los GPCR pueden localizarse e interactuar específicamente con proteínas de señalización (Ostrom y Insel, 2004).

Una vez internalizados, los receptores son marcados para entrar en vías de reciclaje o degradativas. Algunos GPCRs, entre los que se incluye el receptor β_2 -adrenérgico, pueden ser reciclados a la membrana plasmática, como receptores totalmente competentes después de unos minutos tras ser internalizados (Ritter y Hall, 2009; Pipping et al., 1995). Otros como el receptor de vasopresina tipo 2 es retenido en la célula durante un cierto periodo de tiempo antes de ser reciclado a la membrana (Innamorati et al., 2001), mientras que otros como los receptores de δ -opioides o trombina son mayoritariamente degradados (Tsao y Von Zastrow, 2000). Para la mayoría de GPCRs parte es reciclada y parte es degradada, como ocurre con los receptores de adenosina (Escriche et al., 2003).

1.2 Los receptores acoplados a proteína G como dianas terapéuticas

Los GPCRs han sido el centro de interés de fisiólogos y farmacólogos mucho antes de que se supiera que estaban acoplados a proteína G. Estos receptores representan la familia de proteínas de mayor impacto social, terapéutico y económico (Fredholm et al., 2007; Lefkowitz, 2007). Hoy en día, más del 50% de los fármacos, con unas ventas anuales en el mundo que superan los 50 billones de dólares, regulan la función de los GPCR, y un 30% de estos fármacos está directamente dirigidos a los GPCRs (Jacoby et al., 2006; Lundstrom, 2006). Los GPCRs están involucrados en una amplia diversidad de enfermedades como son; alergias, disfunción cardiovascular, depresión, obesidad, cáncer, dolor, diabetes, y una variedad de trastornos del sistema nervioso central. Dado que los GPCRs representan alrededor del 2% del genoma humano, sólo una proporción muy pequeña de todos los GPCRs son actualmente diana de fármacos. Por lo tanto, hay mucho interés en la identificación de nuevos receptores que puedan ser utilizados para el desarrollo de fármacos (Fredholm et al., 2007; Lin y Civelli, 2004). En la tabla 1 se muestra un pequeño ejemplo de los fármacos más vendidos dirigidos a GPCRs, dónde se observa el amplio rango de indicaciones terapéuticas que cubren.

Acción	Nombre Comercial	Entidad Molecular	Compañía	Indicación terapéutica	Ventas mundiales (Millones de US\$)
Antagonista de H ₁	Allegra/Telfast®	fexofenadine	Sanofi-Aventis	Alergias	1792
Antagonista de AT ₁	Diovan®	valsartan	Novartis	Hipertensión	2214
Antagonista de H ₂	Gaster®	famotidine	Yamanouchi	Úlcera gástrica	656
Agonista de 5HT _{1D}	Imigran®	sumatriptan	GlaxoSmithKline	Migraña	1454
Agonista de LH-RH	Leuplin/Lupron®	leuprorelin	Takeda/Abbot	Cáncer	904
Agonista de GABA _B	Neurontin®	GABApentin	Pfizer	Dolor neurológico	2480
Antagonista de P2Y ₁₂	Plavix®	clopidogrel	Bristol-Myers Squibb	Ictus	5277
Antagonista de 5HT _{2/D2}	Risperdal®	risperidone	Johnson&Johnson	Esquizofrenia	371

Extraído y modificado de Jacoby et al., 2006

Tabla 1. Algunos de los fármacos más vendidos de GPCR.

La mayoría de estrategias terapéuticas dirigidas a receptores acoplados a proteína G involucrados en algún desorden se basan en el uso de agonistas o antagonistas específicos del receptor. En los últimos años se han propuesto nuevas estrategias que consideran el complejo funcionamiento de los GPCR. Así, se considera la complejidad de los receptores a diferentes niveles moleculares por lo que se buscan fármacos específicos para un subtipo de GPCR, fármacos que reconocen un centro de unión alostérico, fármacos que consideran la oligomerización de GPCR, fármacos que neutralizan la actividad constitutiva y fármacos que tienen como diana otros elementos moleculares que regulan las diferentes vías de señalización (Liebmann, 2004).

1.2.1 Ligandos Múltiples

Se han descrito evidencias que indican que la aplicación de tratamientos con ligandos altamente específicos es insuficiente en la modulación de algunos sistemas complejos *in vivo* (Roth et al., 2004). Por otro lado, también existen evidencias que señalan la interacción con más de un receptor como una estrategia más efectiva en algunos desórdenes, en contra de la establecida filosofía ‘una diana para una enfermedad’. Los primeros fármacos con acción múltiple fueron descubiertos de manera fortuita y su modo de acción se ha ido descubriendo retrospectivamente. Sin embargo, en la actualidad, el diseño deliberado y racional de ligandos múltiples, capaces de interactuar con más de un receptor simultáneamente, se ha establecido como una nueva tendencia en el desarrollo de agentes terapéuticos y moduladores de receptores. El uso de ligandos múltiples presenta ciertas ventajas respecto a otras aproximaciones para la interacción simultánea con múltiples dianas, como son los cócteles de fármacos (un principio activo por preparado) o los fármacos multicomponente (varios principios activos en un único preparado). Si la aplicación de una única diana da lugar a resultados insuficientes (Roth et al., 2004; Law et al., 2003), la modulación equilibrada de un número pequeño de dianas es más eficaz y da lugar a menos efectos secundarios que los tratamientos con fármacos altamente selectivos (Morphy et al., 2004). El diseño de este tipo de ligandos consiste en una primera etapa basada en el conocimiento adquirido y focalizada en la interpretación de datos biológicos de fármacos o ligandos antiguos; y en una segunda etapa centrada en la evaluación biológica de estos compuestos, que puede

ser masiva (extensas colecciones de biomoléculas) o dirigida (compuestos de los que se conoce su actividad en una de las dianas).

La aplicación de ligandos múltiples para la regulación de la neurotransmisión en desórdenes del SNC es de especial relevancia, puesto que en numerosos casos, los tratamientos dirigidos a la interacción con una sola diana han dado lugar a resultados insuficientes (Buccafusco y Terry, 2000). Un ejemplo de esta situación son los desórdenes poligénicos complejos como el ADHD y la esquizofrenia (Gray y Roth, 2006) o las enfermedades neurodegenerativas como Alzheimer y Parkinson (Cavalli et al., 2008; Youdim y Buccafusco, 2005). Vendrell y colaboradores (Vendrell et al., 2007) derivatizaron la estructura privilegiada del ergoleno con distintos tripéptidos lineales consiguiendo ligandos duales para los receptores de dopamina y adenosina. Se identificaron ergopéptidos (ergoleno-péptidos) con actividad agonista de receptores de dopamina y antagonista de receptores de adenosina cuyo perfil farmacológico indica que podrían ser útiles en el estudio del cross-talk dopamina-adenosina en el SNC y para probar el potencial terapéutico de fármacos múltiples en la enfermedad de Parkinson.

Utilizando las similitudes estructurales entre la fluoxetina (inhibidor selectivo de la recaptación de serotonina) y la rivastigmina (inhibidor de la acetilcolinesterasa) se ha diseñado un ligando múltiple que mantiene la alta afinidad por ambas dianas y puede ser un tratamiento útil en la enfermedad de Alzheimer (Toda et al., 2003).

En el campo de la depresión, actualmente se están evaluando agentes múltiples de doble y triple acción para su tratamiento. Entre ellos están los triple inhibidores de la recaptación de dopamina, serotonina y noradrenalina o los fármacos como la agomelatina, que actúa como agonista de los receptores de melatonina y antagonista de los receptores 5-HT_{2C} (Millan, 2009).

1.2.2 Ligandos Alostéricos

Los ligandos clásicos de los GPCR modulan la señalización del receptor estimulando directamente la respuesta del receptor (agonismo), bloqueando la unión de los agonistas endógenos (antagonismo competitivo), o bloqueando la actividad constitutiva de los receptores (agonismo inverso). Estos ligandos clásicos ejercen sus efectos interaccionando con el centro de unión ortostérico del receptor (por ejemplo, el

centro que reconoce el agonista endógeno) y esta interacción ha sido caracterizada clásicamente utilizando métodos de unión de radioligandos ortostéricos (Oldham y Hamm, 2006). En el caso del descubrimiento de fármacos, la mayor parte de la atención se focaliza en la identificación y estudio de moléculas que actúan como ligandos ortostéricos de determinados receptores diana para obtener un efecto farmacológico (la activación o inhibición de la señal de transducción). Estos compuestos compiten con los ligandos endógenos impidiendo la ocupación simultánea del receptor por ambas moléculas.

Además de los sitios ortostéricos, se ha encontrado que muchos GPCR poseen sitios de unión alostéricos, los cuales son topográficamente distintos de los sitios ortostéricos (Keov et al., 2011; Mohr et al., 2010; Bridges y Lindsley, 2008; Gilchrist, 2007; May et al., 2007). La presencia de sitios de unión alostéricos permite numerosas interacciones ligando-receptor, más allá de las asociadas con el sitio ortostérico. Además, los sitios alostéricos pueden estar menos conservados entre subtipos de receptores que los sitios ortostéricos, proporcionando un medio para conseguir una verdadera selectividad de la acción farmacológica (Keov et al., 2011; Bridges y Lindsley, 2008; May et al., 2007). Un rasgo característico de la interacción alostérica es que un ligando ortostérico y un ligando alostérico pueden co-unirse al receptor. De este modo, los ligandos alostéricos pueden introducir complejidad a las respuestas farmacológicas modificando la afinidad y/o la señal impartida por el receptor por la unión concomitante del ligando ortostérico (Keov et al., 2011; May et al., 2007). La habilidad de los moduladores alostéricos de afinar de manera precisa las respuestas farmacológicas ha suscitado interés en sus posibles aplicaciones tanto en investigación básica como en clínica (Keov et al., 2011; Conn et al., 2009; Bridges y Lindsley, 2008; Springael et al., 2007; Ballesteros y Ramsom, 2006). Este interés es más relevante en el caso de los receptores de neurotransmisores, dado que la transmisión sináptica tiene lugar en circuitos extremadamente complejos y redes implicadas en numerosas funciones neurológicas. Un modulador alostérico preservaría la relevancia fisiológica de la señalización del receptor mientras modula la eficiencia del neurotransmisor endógeno (Conn et al., 2009). Hasta la fecha, se ha descrito un gran número de moduladores alostéricos de receptores acoplados a proteína G, muchos de ellos dirigidos a receptores del SNC, entre los que cabe destacar tanto moduladores exógenos como endógenos (Conn et al., 2009). Entre las moléculas endógenas que actúan como moduladores

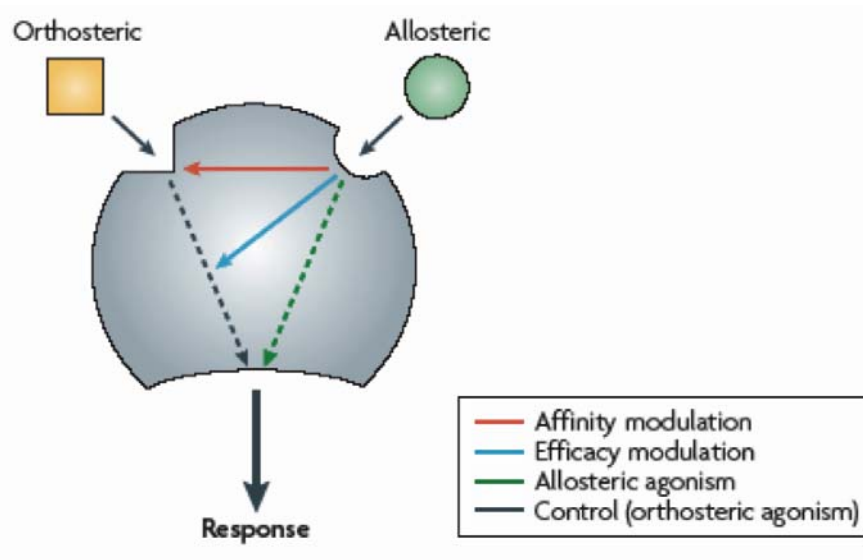
alostéricos encontramos, iones como Zn^{2+} , Na^+ y Ca^{2+} , lípidos y péptidos (May et al., 2007)

Los moduladores alostéricos de los GPCR acostumbran a presentar una o varias de las siguientes propiedades farmacológicas, representadas en la figura 8: modulación de la afinidad, en general se asume que la interacción del ligando alostérico con su sitio de unión causa un cambio conformacional en el receptor que es transmitido al sitio ortostérico, y viceversa. La cualidad del efecto alostérico se define como modulación positiva si el modulador facilita la interacción o modulación negativa si el modulador inhibe la interacción del ligando con el sitio de unión ortostérico (Conn et al., 2009; May, 2007). Modulación de la eficacia, el efecto alostérico puede provocar el cambio de las respuestas intracelulares, lo que conduce a un cambio en la capacidad de señalización (o eficacia intrínseca) de un ligando ortostérico; agonismo/agonismo inverso, el modulador alostérico puede alterar la señalización del receptor en un sentido positivo (agonismo) o negativo (agonismo inverso), independientemente de la presencia o ausencia de un ligando ortostérico.

Se han propuesto, al menos, tres ventajas terapéuticas que pueden proporcionar los moduladores alostéricos respecto a los ligandos ortostéricos. En primer lugar, el efecto de los moduladores alostéricos es saturable, lo que significa que incluso a altas dosis no sobreestimularía o sobreinhibiría al sistema entero y no se produciría toxicidad. Esta saturabilidad depende del grado de cooperatividad que existe entre el ligando alostérico y ortostérico. En segundo lugar, al menos que el fármaco presente actividades adicionales no-alostéricas o agonismos por ellos mismos, el fármaco alostérico es activo únicamente en los sitios del tejido donde se produce la liberación fisiológica normal del ligando endógeno, y con la misma pauta temporal que el ligando endógeno. Y por último, como se ha mencionado anteriormente, los moduladores alostéricos pueden ser selectivos de un determinado subtipo de receptor mediante su unión a regiones no conservadas y/o a la cooperatividad con ligandos ortostéricos de un determinado subtipo de receptor (Conn et al., 2009; Ballesteros y Ransom, 2006).

Desde un punto de vista estructural, los centros alostéricos y ortostéricos pueden estar cercanos o distantes entre sí. Diversos experimentos de mutagénesis han dado soporte a esta idea. Por ejemplo, en receptores muscarínicos se ha encontrado al menos un sitio alostérico en el que intervienen epítomos de los bucles extracelulares y de la

parte superior de los dominios de transmembrana (May et al., 2007; Prilla et al., 2006; Matsui et al., 1995). Por lo contrario, en GPCR que unen péptidos, como los de chemokinas o CRF1, se han localizado sitios alostéricos en las regiones transmembrana lejos de los dominios extracelulares utilizados por los péptidos ortostéricos (Tsamis et al., 2003; Dragic et al., 2000). Finalmente, para la clase C de GPCR se ha descrito la separación más sorprendente entre sitios de unión alostéricos y ortostéricos, la región transmembrana de estos GPCR presenta más de un posible sitio de unión alostéricos para pequeñas moléculas moduladoras mientras que la unión de ligandos ortostéricos está situada en los dominios extracelulares (Schaffhauser et al., 2003; Knoflach et al., 2001).



Extraído de Conn et al., 2009

Figura 8. Modos de acción de los moduladores alostéricos.

Los ligandos alostéricos se unen a un sitio topográficamente distinto en el receptor para modular la afinidad del ligando ortostérico (rojo) y/o la eficacia (azul). Algunos ligandos alostéricos pueden por ellos mismos alterar directamente la señalización del receptor (verde).

1.3 Homómeros y heterómeros de receptores acoplados a proteína G

Como consecuencia de las características estructurales de los GPCR y de su localización subcelular, éstos pueden interactuar con otras proteínas tanto en el lado intracelular como extracelular de la membrana plasmática, así como también pueden exhibir interacciones proteína-proteína con otros receptores o canales iónicos a nivel de membrana plasmática (Rozenfeld y Devi, 2011; Franco et al., 2003). Este último aspecto ha suscitado un elevado interés. Desde mediados de los años 90, diversos estudios han demostrado la oligomerización de numerosos GPCR (George et al., 2002), hoy en día se acepta que la oligomerización es un hecho común en la biología de estos receptores y que pueden formar homodímeros, heterodímeros y/o homo o heterooligómeros de orden superior (Birdsall, 2010; Ferré et al., 2010; Ferré et al., 2009; Prinster et al., 2005; Agnati et al., 2003; Franco et al., 2003; Pin et al., 2003; Bouvier, 2001). La homomerización de receptores se define como la asociación física entre receptores idénticos, mientras que la heteromerización es la asociación entre receptores distintos. Debido que hasta la fecha, las técnicas disponibles no permitían la distinción entre dímeros u oligómeros de orden superior, el término dímero es a menudo usado entendiendo que es la forma más simple de una unidad funcional oligomérica; a pesar de ello, en la actualidad se empiezan a desarrollar técnicas que permiten discernir entre dímeros, trímeros y tetrámeros (Vidi y Watts; 2009; Carriba et al., 2008). Los dímeros/oligómeros presentan características funcionales diferentes a las de los receptores que los constituyen, así la oligomerización confiere nuevas propiedades a los GPCR, lo que establece un posible mecanismo para generar nuevas funciones en estos receptores. Este fenómeno ha dado lugar a un nuevo nivel de complejidad que gobierna la señalización y regulación de estas proteínas (Ferré et al., 2009).

A mediados de los años 70, ciertas evidencias farmacológicas indirectas llevaron a pensar a los investigadores en la posibilidad de que los receptores acoplados a proteína G pudieran actuar como dímeros. Las complejas curvas de unión de agonistas y antagonistas de estos receptores se interpretaron como evidencias de una cooperatividad que se podía explicar mediante interacciones entre lugares de unión de los receptores en complejos diméricos o multiméricos (Franco et al., 1996; Wreggett y Wells, 1995;

Limbird et al., 1975). De igual manera, otros experimentos de tipo bioquímico apoyaron también la idea de la oligomerización de los receptores acoplados a proteína G (Maggio et al., 1993). Cuando los receptores α_2 -adrenérgicos marcados con los epítomos Myc y HA se co-expresaban y se inmunoprecipitaban con un anticuerpo contra el epítomo Myc, se detectaba inmunoreactividad para el epítomo HA en los inmunoprecipitados, lo que evidenciaba una interacción intermolecular entre los dos tipos de receptores diferencialmente marcados (Hebert et al., 1996).

Se ha demostrado la formación de dímeros para una gran variedad de receptores. En la tabla 2 se enumeran algunos ejemplos de homodímeros y heterodímeros, cuya existencia permite comprender la diversidad funcional de estos receptores. Las interacciones entre GPCRs son cruciales para entender el variado cross-talk que se observa, sobre todo entre receptores de neurotransmisores. La oligomerización de receptores neuronales permite hipotetizar sobre el alto grado de diversidad y plasticidad que es característico de una estructura altamente organizada y compleja como es el cerebro.

Homodímeros	Heterodímeros
Adenosina A ₁	Adenosina A ₁ -Dopamina D ₁
Adenosina A _{2A}	Adenosina A ₁ - mGlu ₁
Dopamina D ₁	Adenosina A ₁ - Purinérgico P2Y ₁
Dopamina D ₂	Adenosina A _{2A} - Dopamina D ₂
Dopamina D ₃	Adenosina A _{2A} - mGlu ₅
Histamina H ₂	Angiotensina AT ₁ -AT ₂
Histamina H ₄	Dopamina D ₁ - Dopamina D ₃
Melatonina MT ₁	Melatonina MT ₁ - MT ₂
Citoquina CCR ₂	Muscarinico M ₂ - M ₃
Serotonina 5-HT _{1B}	Citoquina CCR ₂ - CCR ₅
Vasopresina V ₂	Serotonina 5-HT _{1B} - 5-HT _{1D}
GABA _{BR1}	TIR ₁ - TIR ₃

Tabla 2. Algunos ejemplos de homodímeros y heterodímeros de GPCR.

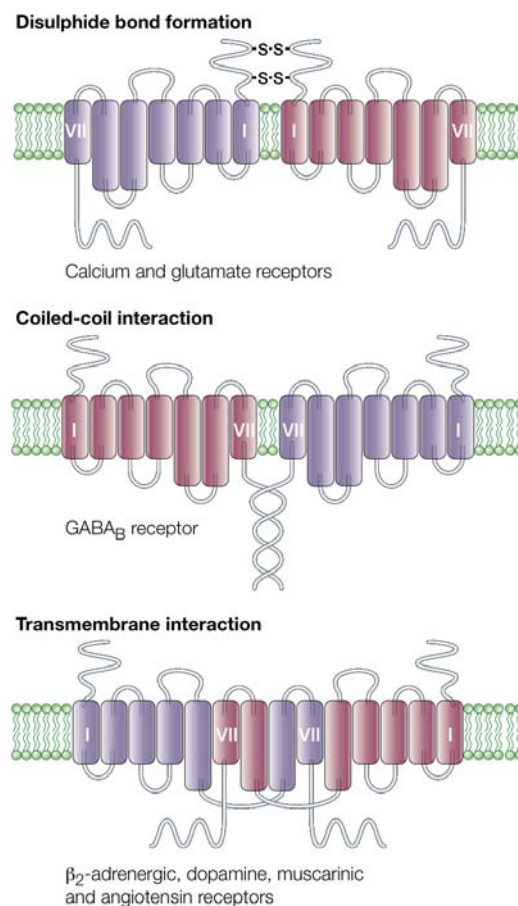
1.3.1 Estructura cuaternaria de los homómeros y heterómeros de receptores acoplados a proteína G

Para explicar el fenómeno de la dimerización de los GPCRs se consideran dos posibilidades de interacción: interacciones indirectas o interacciones directas. En el caso de las interacciones indirectas entre GPCRs es necesario la mediación de terceras proteínas que hagan de puente, como por ejemplo las proteínas citoesqueléticas. Muchas de estas proteínas son proteínas andamio o *scaffolding proteins*, que proporcionan una estructura compleja en la cual diversos receptores pueden interactuar entre ellos y con otras proteínas involucradas en la transducción de señal, controlando la velocidad y la especificidad de dicha señalización (Ciruela et al., 2010).

Las interacciones directas entre miembros de la familia de GPCRs no precisan de otras proteínas. Se cree que para muchos receptores los oligómeros se forman en el retículo endoplasmático (RE), por lo que los ligandos no modulan la interacción. La gran complejidad estructural que existe en esta superfamilia no permite pensar en un único mecanismo de interacción directa. De este modo, las interacciones directas pueden tener lugar mediante enlaces covalentes como puentes disulfuro y/o no covalentes como fuerzas hidrofóbicas y/o electrostáticas entre los dominios de transmembrana y/o los dominios intracelulares de los receptores (Bouvier, 2001) (Figura 9). En la familia C de receptores acoplados a proteína G, el gran dominio N-terminal extracelular tiene varios residuos de cisteína que pueden contribuir en la dimerización mediante puentes disulfuro (Romano et al., 2001). Este es el caso de los receptores sensibles a calcio y los metabotrópicos de glutamato, así como de algunos receptores de la familia A como los receptores κ - y δ -opioides o los receptores D_1 de dopamina (Jordan y Devi, 1999)

Se ha descrito que la interacción directa *coiled-coil* de la cola C-terminal de los receptores $GABA_{B1}$ y $GABA_{B2}$ está implicada en la formación del heterómero (Margeta-Mitrovic et al., 2000). Así como también en la homodimerización del receptor δ -opioide, en el que el dominio C-terminal tiene un papel fundamental en la formación del dímero ya que al deletarse los últimos 15 aminoácidos el receptor pierde la capacidad de heteromerizar. Finalmente, la dimerización directa entre GPCR puede estar mediada por interacciones iónicas o hidrofóbicas entre los dominios extracelulares,

intracelulares o transmembrana del receptor. Se ha demostrado la existencia de interacciones iónicas entre péptidos presentes en los dominios intracelulares que contienen respectivamente dos o más cargas positivas adyacentes (RR, KK o RKR) y dos o más cargas negativas (DD o EE) o residuos aminoacídicos fosforilados. Un ejemplo de estas interacciones sería la participación de residuos cargados y/o fosforilados en la heteromerización de los receptores A_{2A} de adenosina y D_2 de dopamina (Ciruela et al., 2004) o en la heterotrimerización de receptores A_{2A} de adenosina, CB_1 de cannabinoides y D_2 de dopamina (Navarro et al., 2010).



Extraído de Bouvier, 2001

Figura 9. Determinantes moleculares de la dimerización de GPCRs.

La idea de que las interacciones hidrofóbicas podrían tener un papel relevante en la formación de los dímeros se propuso por primera vez para el receptor β₂-adrenérgico. Mediante el uso de péptidos sintéticos y mutagénesis dirigida se propuso que residuos concretos de glicina y leucina situados en el sexto dominio transmembrana

del receptor estaban involucrados en su dimerización (Hebert et al., 1996). Sin embargo, todos estos mecanismos de interacción propuestos, más que reflejar diferentes estrategias utilizadas por diferentes clases de receptores, indican que múltiples sitios de interacción están implicados en el ensamblaje y la estabilización de los dímeros.

Mediante estudios computacionales (Gouldson et al., 2000) se propuso dos modelos alternativos tridimensionales que explican la dimerización de los receptores acoplados a proteína G (Figura 10). En ambos modelos se propone que los dominios de transmembrana cinco y seis están involucrados en el contacto o interfase entre los receptores, así como un papel importante del tercer bucle intracelular. El primer modelo, denominado *domain swapping model* o modelo de intercambio de dominio, considera que cada unidad funcional en el dímero está compuesta por los cinco primeros dominios de transmembrana de un receptor y los dos últimos del otro. Este modelo explica la complementación funcional en las quimeras de los receptores α_2 -adrenérgicos y M_3 muscarínico observadas por Maggio y colaboradores (Maggio et al., 1993). El segundo modelo, denominado de contacto, considera que los dímeros se forman por empaquetamiento lateral de monómeros individuales, donde los dominios cinco y seis forman la interfase de interacción. Éste sería el caso para el receptor V_2 de vasopresina (Schulz et al., 2000).

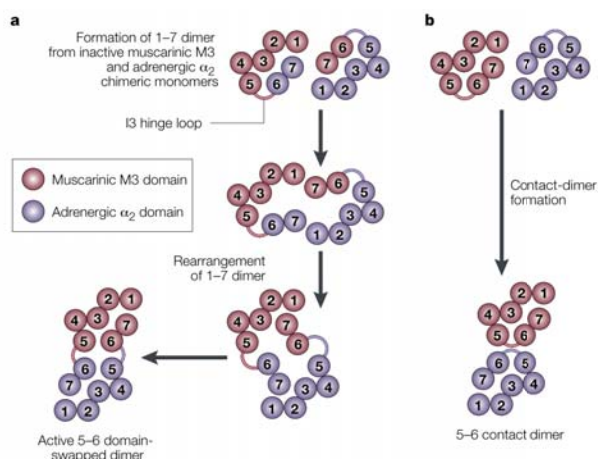


Figura 10. Modelos tridimensionales alternativos de la dimerización de GPCRs.

Extraído de Bouvier, 2001

El estudio de la estructura cuaternaria de los heterómeros de receptores A_{2A} de adenosina, CB_1 de cannabinoides y D_2 de dopamina ha supuesto un avance en el conocimiento de la estructura cuaternaria de heterómeros (Navarro et al., 2010). Se ha

podido establecer que la disposición de los receptores en el heterómero es triangular. Al analizar los dominios de interacción entre los receptores que cumplieran con las observaciones experimentales, los resultados fueron compatibles con los modelos propuestos para dímeros de otros GPCRs de la familia A, en los que la heteromerización involucra principalmente la interacción entre las hélices TM4 y TM5. Una disposición triangular de los receptores en la que se forman interacciones entre estas hélices es compatible con la existencia de homómeros de cada uno de los protómeros en los que la hélice TM1 constituye la interfase entre los homodímeros, lo que es un fenómeno descrito para GPCRs (Guo et al., 2008; Guo et al., 2005; Klco et al., 2003).

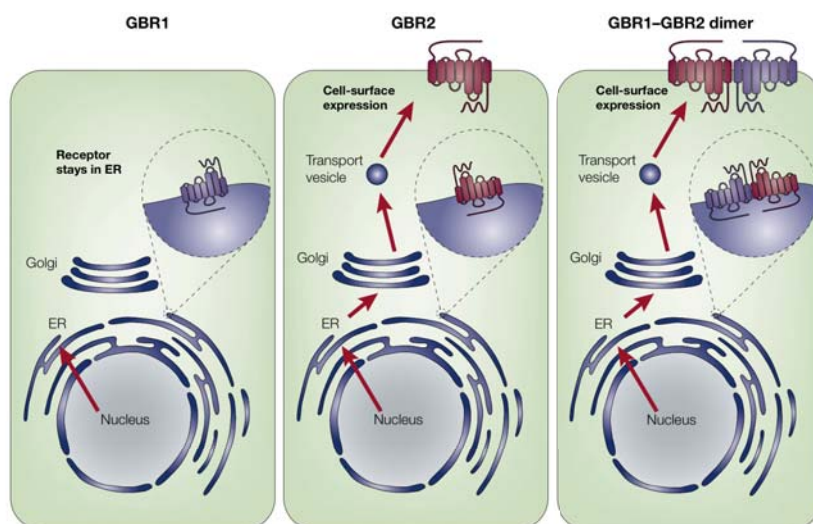
1.3.2 Consecuencias funcionales de la formación de homómeros y heterómeros entre receptores acoplados a proteína G

La formación de homómeros y heterómeros tiene un papel importante en la regulación de la función de los receptores implicados (Ferré et al., 2009). Esta regulación tiene lugar a diferentes niveles, desde la modulación de la expresión del receptor en la superficie celular hasta el hecho de conferirles nuevas propiedades farmacológicas a los oligómeros, lo que tiene que tenerse muy en cuenta en el diseño racional de drogas que actúan a través de estos receptores.

Un ejemplo claro del papel de la heteromerización en la modulación de la expresión del receptor en la superficie celular lo constituyen los receptores metabotrópicos GABA_B (Figura 11). La heteromerización de los receptores GABA_{BR1} y GABA_{BR2} es necesaria para el correcto plegamiento del receptor GABA_{BR1} y su transporte a la membrana plasmática, además de para su señalización (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). Cuando se expresa individualmente la isoforma GABA_{BR1} del receptor, ésta queda retenida intracelularmente en el retículo endoplasmático como glicoproteína inmadura. Por el contrario, cuando es la isoforma GABA_{BR2} la que se expresa, ésta sí que llega a la membrana plasmática pero no puede unir GABA ni iniciar la transducción de la señal. Cuando ambos receptores se coexpresan, las dos proteínas alcanzan la superficie celular y forman el receptor funcional (White et al., 1998). Posteriormente se demostró que GABA_{BR2} sirve como una chaperona que es esencial para el correcto plegamiento y el transporte a la

membrana de GABA_{BR1}. La dimerización a través de interacciones *coiled-coil* de las colas C-terminales enmascara la señal de retención en el retículo endoplasmático; por lo tanto, permite el transporte del receptor desde éste hasta la membrana plasmática como dímero (Margeta-Mitrovic et al., 2000).

En muchos casos la oligomerización es un evento temprano en la maduración del receptor y su transporte y, en este mismo sentido, cabe mencionar que los receptores que forman heterómeros pueden tener diferentes características de internalización, es decir, la oligomerización puede también modular las propiedades de tráfico de GPCRs mediadas por agonista. Este es el caso de los heterodímeros de los receptores de somatostatina SSTR₁ y SSTR₅, en el cual la internalización del heterodímero ocurre a pesar de la resistencia a la internalización que presenta el monómero SSTR₁ (Rocheville et al., 2000).



Extraído de Bouvier, 2001

Figura 11. Papel de la heteromerización de los receptores GABA_{1B} y GABA_{2B}.

Los estudios de unión de ligando han dado algunas pistas de la relevancia fisiológica de la formación de oligómeros de GPCRs, ya que la formación de estos complejos puede resultar en la generación de centros con nuevas propiedades para la unión de ligando. La formación de homodímeros de GPCRs puede conferir cooperatividad a la unión de ligandos ya que se ha visto que la unión de un ligando específico sobre uno de los protómeros del homómero puede incrementar o disminuir la afinidad del ligando para el otro protómero. En un escenario donde se asumía que los

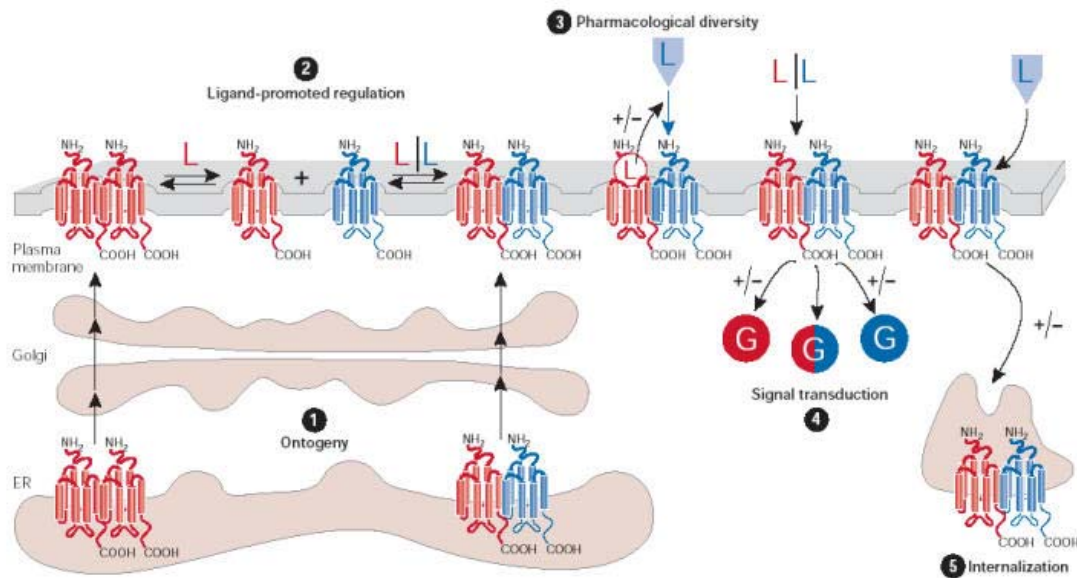
GPCRs actuaban como monómeros, esta cooperatividad era difícil de explicar. Una posible explicación era que el receptor podía estar en dos estados conformacionales diferentes con diferentes afinidades por los agonistas: un estado de alta afinidad en el cual el receptor estaba acoplado a proteína G y otro de baja afinidad en el que no estaba acoplado. En cambio la existencia de oligómeros permite un nuevo modelo en el cual la interacción receptor-receptor es la base de la cooperatividad entre receptores. Un ejemplo de ello lo constituye la cooperatividad negativa en el homodímero de receptores de glutamato mGluR₁ (Suzuki et al., 2004) o de receptores A₁ de adenosina o D₁ de dopamina (Casadó et al., 2009; Franco et al., 2008a). Considerando todos estos aspectos, se han formulado modelos que tienen en cuenta la formación de homodímeros (Franco et al., 2008) y recientemente se han desarrollado las ecuaciones para ajustar los datos de unión de ligandos a partir de uno de estos modelos, el *two-state dimer receptor model* (Casadó et al., 2009; Casadó et al., 2007).

La formación de heterómeros entre dos receptores distintos implica que se pueda establecer una interacción alostérica entre ellos, de manera que la unión de un ligando a uno de los receptores en el heterómero modifique la afinidad del otro ligando por el otro receptor en el heterómero. El primer heterómero descrito con distintas propiedades respecto de los receptores constituyentes fue el heterómero formado por los receptores κ -opioides y δ -opioides (Jordan y Devi, 1999). Este heterodímero no presenta alta afinidad por la unión de sus ligandos selectivos, en cambio si presenta alta afinidad por ligandos selectivos parciales. Un caso especialmente interesante es el de los receptores de dopamina y adenosina, entre los cuales se ha descrito un cross-talk negativo. Los agonistas del receptor de adenosina A₁ inducen la desaparición del lugar de alta afinidad en preparaciones de membrana que contienen el receptor de dopamina D₁ (Gines et al., 2000) y los ligandos del receptor de dopamina D₂ consiguen la desensibilización heteróloga del receptor de adenosina A_{2A} (Hillion et al., 2002). Por otro lado, algunos antagonistas del receptor de adenosina A_{2A} muestran una mayor selectividad para el heterómero de receptores de adenosina A₁-A_{2A} que para el heterómero adenosina A_{2A}-dopamina D₂ (Orru et al., 2011)

La homomerización y la heteromerización pueden afectar diferencialmente la señal inducida por diversos agonistas. Una de las primeras evidencias de que los dímeros forman una unidad compleja de señalización proviene de la demostración de

que la disrupción del homodímero del receptor β_2 -adrenérgico con un péptido derivado del sexto dominio transmembrana, implicado en la dimerización, inhibía la producción de AMPc inducida por el agonista (Hebert et al., 1996). Estos resultados indican que el dímero es la especie activa del receptor, aunque tampoco se puede descartar la posibilidad de que el péptido esté modificando interacciones intramoleculares dentro del monómero que provocarían la falta de funcionalidad, siendo la pérdida de la unidad dimerica más una consecuencia que no una causa de la no señalización por parte del receptor. La heteromerización entre los receptores de angiotensina AT₁ y B₂ de bradikina mejora la señal del receptor AT₁ mientras que inhibe la del B₂ de bradikina, mostrando que la heteromerización entre receptores diferentes puede ser un nuevo modelo para la modulación de la respuesta de GPCRs por sus respectivos ligandos (AbdAlla et al., 2000). El acoplamiento del receptor de histamina H₃ a la vía de las MAPKs solo se produce si el receptor H₃ forma heterómeros con el receptor de dopamina D₁ (Moreno et al., 2011; Ferrada et al., 2009). De todo lo comentado se deduce que la oligomerización puede también tener relevancia en la funcionalidad de los receptores que conforman el oligómero. De hecho existen evidencias que indican que es el dímero el que interacciona con una única proteína G y que la dimerización es un prerrequisito para la activación de la proteína G, como en el caso del receptor de leucotrieno B₄ (Banères, 2003).

En la figura 12 se resumen las posibles implicaciones funcionales de la oligomerización. La oligomerización puede estar implicada en la ontogénesis de GPCRs, es decir en el control de calidad del plegamiento y de la destinación a la membrana de receptores sintetizados de novo (**12.1**). Así mismo, confiere diversidad farmacológica ya que la unión de un ligando específico de uno de los receptores puede verse afectada por la unión de un ligando al otro receptor (**12.3**). La oligomerización también puede modificar las propiedades de señalización de un determinado ligando afectando la selectividad de interacción entre el receptor correspondiente y su proteína G, resultando en una potenciación, atenuación o acoplamiento con otra proteína G (**12.4**). Finalmente, también se ha visto que la oligomerización puede alterar el patrón endocítico para un determinado receptor (**12.5**).



Extraído de Terrillon y Bouvier, 2004

Figura 12. Posibles papeles funcionales de la oligomerización de GPCRs.

1.3.3 Técnicas para el estudio de la oligomerización de receptores acoplados a proteína G

Las técnicas utilizadas para el establecimiento de la formación de oligómeros de GPCRs son de índole muy variada, como técnicas farmacológicas, utilización de quimeras, aproximaciones bioquímicas y técnicas de biofísica. A menudo la demostración de la oligomerización de GPCRs requiere la utilización de algunas o incluso todas ellas.

Los estudios farmacológicos, como hemos comentado anteriormente, pueden constituir la primera evidencia de la existencia de homodímeros entre GPCRs en aquellos casos en los que se detecte tanto cooperatividad positiva como negativa en la unión de ligando. El fenómeno de la cooperatividad no puede ser explicado considerando la existencia de distintos estados de activación de los receptores monoméricos en equilibrio. La manera más simple de explicar la cooperatividad requiere la formulación de un modelo que considera la forma dimérica del receptor y explica la cooperatividad de manera natural por analogía con los enzimas (Franco et al.,

2008b) (véase apartado 1.3.2.). Por otro lado, una evidencia farmacológica contundente de la existencia de heterómeros la constituyen los cambios cinéticos en la unión de ligandos a un receptor provocados por la unión de ligandos al otro receptor en el heterómero, en preparados de membrana de células o de tejido que expresen los receptores. En preparaciones de membrana aisladas no existe ninguna maquinaria celular que pueda producir un cross-talk indirecto (por ejemplo, un cross-talk a nivel de segundos mensajeros) y la existencia de una modulación a nivel de unión de ligandos sólo puede ser explicada mediante una interacción molecular entre ambos receptores. En estos casos la unión de un ligando a un receptor induce cambios conformacionales en el otro receptor que modulan su capacidad de unir ligandos. Estos cambios conformacionales sólo se pueden producir si ambas proteínas interactúan molecularmente.

La utilización de receptores quimera y mutantes es otra herramienta válida para detectar oligómeros. Un estudio pionero que demostraba que los GPCRs pueden funcionar como dímeros fue el estudio llevado a cabo por Maggio y colaboradores (Maggio et al., 1993), usando quimeras de los receptores α_2 -adrenérgico/ M_3 muscarínico compuestas por los 5 primeros dominios transmembrana de uno de los receptores y los dos últimos dominios transmembrana del otro. En la misma línea se ha observado que diversos receptores mutantes actúan de dominantes negativos cuando son expresados con su receptor en la forma nativa (*wild type*) (Bai et al., 1998; Zhu y Wess, 1998; Benkirane et al., 1997). En estos casos, la dimerización entre el *wild type* y el receptor inactivo es la única explicación de este fenómeno.

En los últimos años, una de las aproximaciones bioquímicas más usadas para el estudio de la dimerización de GPCRs ha sido la coimmunoprecipitación de receptores diferencialmente marcados. El primer estudio que se llevo a cabo utilizando esta aproximación fue realizado por Hebert y colaboradores (Hebert et al., 1996) en el cual demostraban la existencia de interacciones específicas entre los receptores β_2 -adrenérgicos. Desde entonces, se han utilizado estrategias similares para documentar la homodimerización de receptores D_2 de dopamina (Ng et al., 1996), receptores metabotrópicos de glutamato tipo 5 (mGluR₅) (Romano et al., 1996) y otros. Más recientemente, se han efectuado experimentos de coimmunoprecipitación para demostrar la existencia de heterodímeros entre receptores relacionados, como los subtipos GABA_{BR1} y GABA_{BR2} (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998) y

menos relacionados como los receptores de adenosina A₁ y D₁ de dopamina (Gines et al., 2000), los receptores de angiotensina AT₁ y bradikina B₂ (AbdAlla et al., 2000), el de δ -opioides y β_2 - adrenérgico (Jordan et al., 2001) o los de histamina H₃ y dopamina D₁ (Moreno et al., 2011). Aunque las coimmunoprecipitaciones y los análisis por western-blot son bastante convincentes requieren de la solubilización del receptor de la membrana, lo que no permite descartar que los dímeros observados puedan ser artefactos debidos al tratamiento con detergentes, considerando la naturaleza hidrofóbica de estas proteínas. A pesar de todos los controles usados para descartar esta posibilidad, la aceptación generalizada de la dimerización de GPCRs permanecía pendiente de una demostración directa de que estos complejos existen en células vivas. Esto fue posible con el desarrollo y la utilización de métodos biofísicos basados en la transferencia de energía por resonancia.

Estas aproximaciones están basadas en la transferencia no radioactiva de energía de excitación entre un dador energético y un aceptor. En el caso de la transferencia de energía de resonancia fluorescente (FRET: Fluorescence Resonance Energy Transfer), tanto el dador como el aceptor son moléculas fluorescentes, mientras que en la transferencia de energía de resonancia bioluminiscente (BRET: Bioluminescence Resonance Energy Transfer) el dador es bioluminiscente y el aceptor fluorescente (De A, 2011; Schäferling y Nagl, 2011; Ciruela et al., 2010; Ferré et al., 2010; Gandía et al., 2008; Bouvier et al., 2007). Para que este fenómeno tenga lugar es necesario que se cumplan dos requisitos. El primero, que el espectro de emisión del dador y el espectro de excitación del aceptor se solapen, de forma que el dador no emite completamente la energía que debiera, si no que transfiere parte de su energía al fluoróforo aceptor, el cual emite como si hubiera sido excitado directamente. Y el segundo, que la distancia entre el emisor y el aceptor sea menor o igual a 100 Å. Esta dependencia crítica de la distancia entre dador y aceptor para la transferencia de energía hace que los sistemas de BRET/FRET sean los elegidos para monitorizar las interacciones proteína-proteína en cultivos celulares.

Para la técnica de FRET se utilizan las diferentes variantes de la proteína verde fluorescente (GFP: Green Fluorescence Protein) obtenidas por mutación. Estas mutaciones confieren diferentes propiedades espectrales, de forma que utilizando dos formas diferentes de mutantes con las características espectrales adecuadas, fusionadas a las proteínas en estudio, permite determinar si estas están lo suficientemente cercanas

como para transferirse energía (Schäferling y Nagl, 2011; Ferré et al., 2010; Pflieger y Eidne, 2005). La pareja más ampliamente utilizada para los experimentos de FRET son las variantes YFP (Yellow Fluorescence Protein) y GFP². Esta última variante de la GFP ha sido optimizada para ser usada como pareja de FRET con la YFP. La GFP² se excita a 400 nm y emite a 510 nm, mientras que la YFP se excita a 485 nm y emite a 530 nm. De esta forma, tal y como se muestra en la figura 13, la excitación de la células que expresan la proteína de fusión receptor-GFP² con un láser de una longitud de onda de 393-403nm, produce la emisión de energía a 510nm, que es capaz de excitar a la proteína de fusión receptor-YFP, cuya emisión a 530nm es cuantificable. Debido a que hay un cierto solapamiento entre los espectros de emisión de ambas proteínas de fusión, es necesario separar los dos espectros de emisión para cuantificar la señal de FRET (Zimmermann et al., 2002).

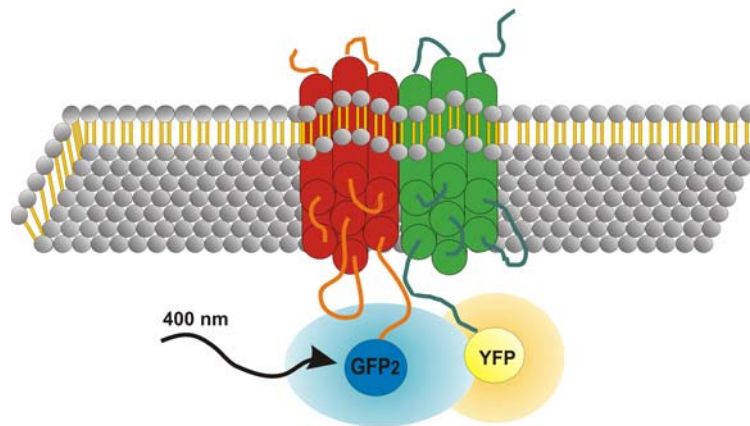


Figura 13. Representación esquemática del fenómeno de FRET.

En la técnica de BRET se utiliza el enzima *Renilla luciferasa* (Rluc) fusionado a uno de los receptores. Se produce la degradación catalítica del substrato coelenterazina H por la luciferasa en presencia de oxígeno, de forma que se genera luz que al ser transferida a una variante de la proteína GFP fusionada al otro receptor, ésta emite fluorescencia a su longitud de onda característica si ambas proteínas están lo suficientemente cercanas (De A, 2011; Ciruela et al., 2010; Ferré et al., 2010; Bouvier et al., 2007). En el estudio de la dimerización de GPCRs, se generan proteínas de fusión que consisten en la unión de la proteína fluorescente GFP o sus variantes (por ejemplo

YFP) en el extremo carboxi terminal de un receptor y la proteína luminiscente RLuc en el extremo carboxi terminal del otro receptor y, al igual que en la técnica de FRET, se co-expresan ambas proteínas de fusión en células vivas. Como se muestra en la figura 14a, en ausencia de dimerización la adición del sustrato coelenterazina H genera una señal bioluminiscente característica, mientras que, como se muestra en la figura 14b, si se produce dimerización entre ambos receptores, la energía es transferida de la RLuc a la proteína fluorescente, dando lugar a la aparición de una señal adicional fluorescente con un pico de emisión característico de la variante usada.

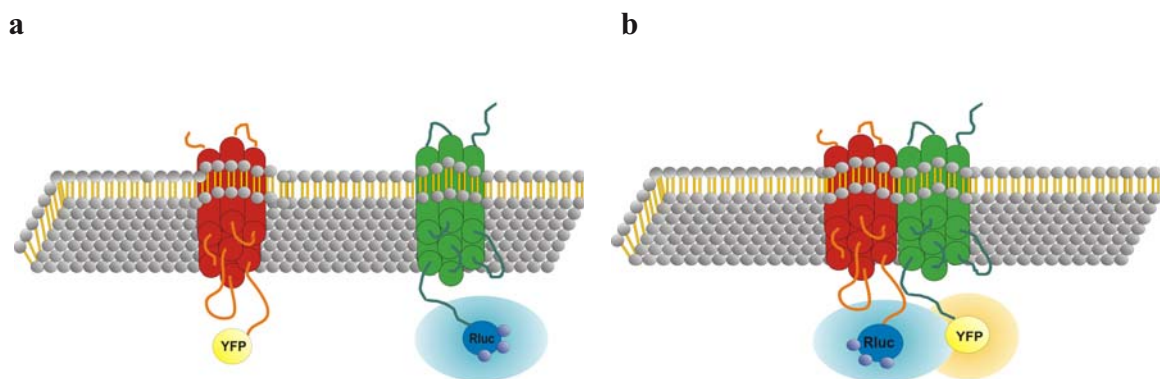


Figura 14. Representación esquemática del fenómeno de BRET.

Hasta la fecha se han descrito dos variantes principales de esta técnica, la que se conoce como BRET o BRET¹ y la denominada BRET². Aunque el principio biofísico de ambas técnicas es el mismo, difieren por el sustrato que cataliza la RLuc y por la proteína aceptora. En el BRET el sustrato que se usa es la coelenterazina H, que al ser metabolizado por la RLuc genera luz con un pico de emisión a 480 nm; emisión que permite excitar a la YFP (ya que se solapa con su pico de excitación), de forma que ésta emite a 530 nm. En el BRET² el sustrato es DeepBlueC que al ser oxidado por la RLuc emite una luz a 400 nm de forma que puede excitar a la GFP²; en este caso la longitud de onda a la que emite esta variante de la GFP es 510 nm.

Mediante técnicas de transferencia de energía se ha demostrado la existencia de homodímeros de los receptor β_2 -adrenérgico (Angers et al., 2000), δ -opioides (McVey et al., 2001) y A_{2A} de adenosina (Canals et al., 2004) entre muchos otros. También se ha

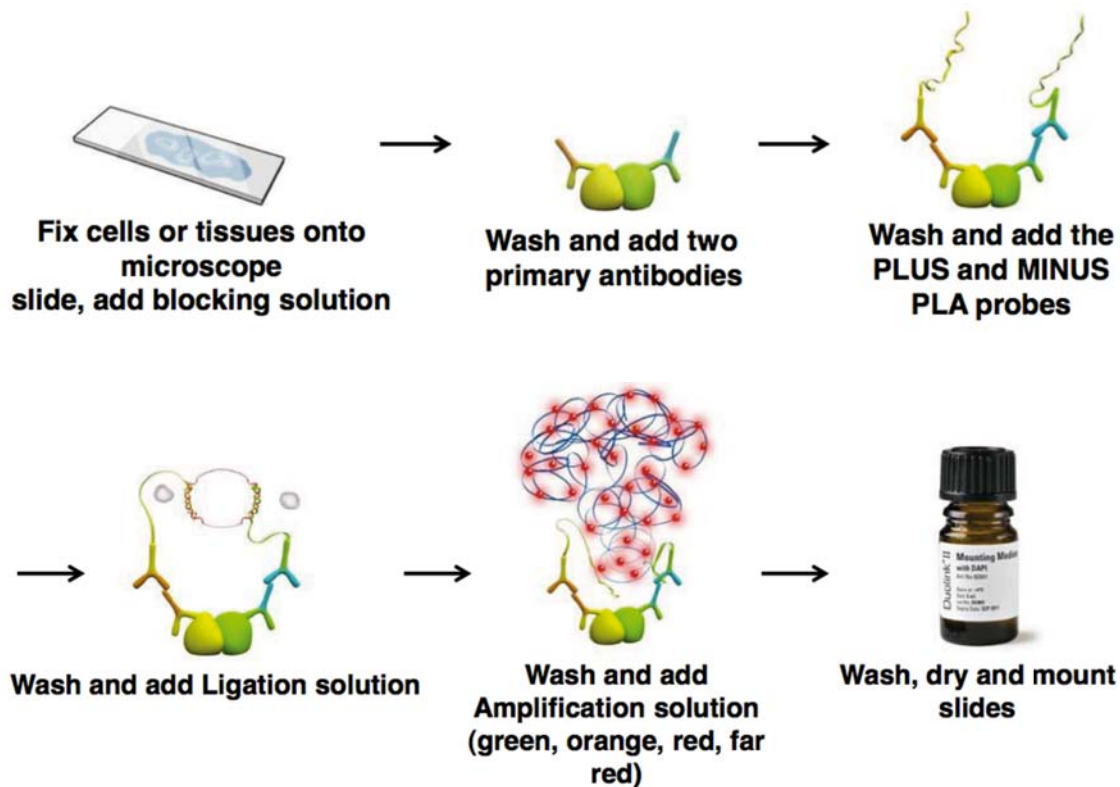
realizado una aproximación similar para el estudio de heterómeros de receptores acoplados a proteína G, como por ejemplo entre los receptores de somatostatina SSTR_{2A} y SSTR_{1B} (Rocheville et al., 2000), los receptores de somatostatina SSTR_{1B} y los D₂ de dopamina (Rocheville et al., 2000), los receptores A_{2A} de adenosina y D₂ de dopamina (Canals et al., 2003), los receptores de dopamina D₁ y D₃ (Marcellino et al., 2008), los receptores A_{2A} de adenosina y CB₁ de cannabinoides (Carriba et al., 2007) y los receptores D₁ de dopamina y H₃ de histamina (Ferrada et al., 2009) entre otros. En los últimos años se han desarrollado multitud de variantes de estas técnicas (véase Ciruela, 2008 como revision) entre las que cabe destacar la técnica de SRET (sequential resonante energy transfer) basada en las técnicas de BRET y FRET (Carriba et al., 2008) y la técnica de BRET y complementación bimolecular (Vidi y Watts, 2009) que permiten la detección de heterómeros de más de dos receptores.

Las técnicas de transferencia de energía ponen de manifiesto que dos proteínas tienen la capacidad de interactuar molecularmente en cultivos celulares, y esta es evidentemente la primera condición que se debe cumplir para que las proteínas en estudio estén formando heterómeros in vivo. Sin embargo, una señal positiva en células transfectadas con proteínas de fusión no significa necesariamente que en un tejido que exprese endógenamente estas proteínas, éstas formen heterómeros. Para detectar heterómeros en tejidos nativos deben utilizarse otro tipo de estrategias.

Existen técnicas directas para detectar oligómeros en tejidos nativos. Una de ellas utiliza la microscopía de fuerza atómica. Palczewski y colaboradores (Fotiadis et al., 2003) usando microscopia de fuerza atómica demostraron, por primera vez, oligómeros de rodopsina en la retina con un determinado patrón de distribución. Esta técnica es factible cuando la concentración de receptores en el tejido es muy elevada como ocurre con la rodopsina en la retina, pero es de difícil aplicación para la mayoría de receptores del sistema nervioso central cuya expresión es moderada.

La técnica de *In Situ Proximity Ligation Assay* (PLA) es una técnica directa muy útil para el estudio de heterómeros de receptores si se dispone de anticuerpos específicos para ello (Bonanomi et al., 2012; Carmena et al., 2012; Hervouet et al., 2011; Renfrow et al., 2011; Vuoriluoto et al., 2010; Weibrecht et al., 2010). Como se muestra en la figura 15, la técnica se basa en la unión de los anticuerpos primarios a los receptores a estudiar y la posterior unión de anticuerpos secundarios, previamente

conjugados a las sondas PLA, a estos anticuerpos primarios. A continuación, se produce la hibridación de unos oligonucleótidos a las dos sondas PLA conjugadas utilizando una solución de ligación. Esta hibridación da lugar a un DNA circular que es posteriormente amplificado utilizando polimerasas y, a continuación, detectado utilizando sondas específicas previamente marcadas con un fluoróforo.

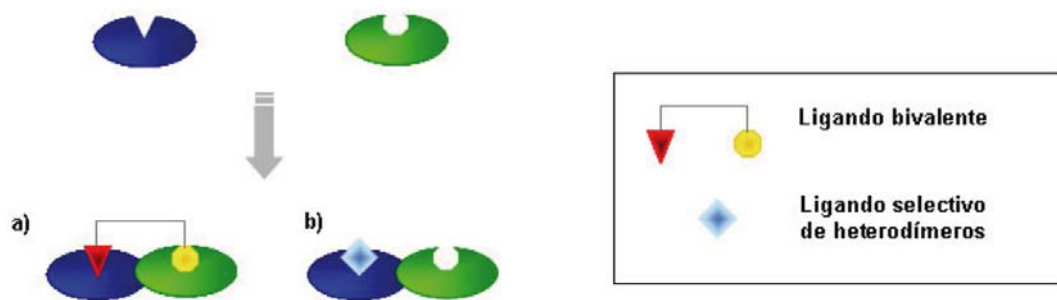


Extraído de Olink Bioscience, 2011

Figura 15. Representación de la técnica ‘In Situ Proximity Ligation Assay’ (PLA) utilizada para la detección de interacciones entre receptores.

La utilización de ligandos para heterómeros constituye otra técnica directa para su detección. Como se muestra en la figura 16, se pueden seguir varias estrategias dependiendo de las propiedades del heterodímero (Rozenfeld et al., 2006). Uno de los enfoques consiste en el diseño y síntesis de ligandos bivalentes que interaccionen con los dos receptores del dímero (Figura 16a). Estos ligandos pueden tener mayor afinidad y selectividad si se compara con los ligandos clásicos de los receptores. Esta estrategia se ha utilizado para determinar la presencia de heterómeros de receptores de adenosina A_{2A} y de dopamina D_2 en el estriado de cerebro de cordero (Soriano et al., 2009). Otra aproximación es el desarrollo de ligandos selectivos de un determinado heterodímero

(Figura 16b). Estos ligandos interactúan con el centro de unión únicamente cuando forma parte del heterodímero. Waldhoer y colaboradores (Waldhoer et al., 2005) demostraron que el compuesto 6'-guanidinonaltrindole (6'-GNTI) no podía unirse a receptores opioides individuales pero sí era capaz de unirse y activar al heterodímero de receptores opioides κ - δ . Experimentos *in vivo* demostraron que el compuesto 6'-GNTI producía analgesia cuando se administraba directamente en la médula espinal, pero prácticamente no tenía efecto cuando se administraba directamente en el cerebro. Además, este efecto analgésico selectivo de la médula espinal se bloqueaba por un antagonista bivalente selectivo del heterómero κ - δ confirmando a éste como diana funcional para la analgesia *in vivo*.



Extraído de Rozenfeld et al., 2006

Figura 16. Ligandos que interactúan específicamente con heterodímeros.

Un ejemplo interesante de ligando que no se une al heterómero pero sí selectivamente a los receptores individuales es el antagonista del receptor adrenérgico α_{1D} denominado BMY 7378 (Hague et al., 2006). Este resultado sugiere que es posible generar fármacos o ligandos que interactúen selectivamente con homodímeros, y no heterodímeros, de receptores o vice-versa. Tanto la estrategia de los ligandos bivalentes como la de los ligandos selectivos de un heterodímero tiene sus pros y sus contras. En la tabla 4 se indican las ventajas e inconvenientes de cada estrategia (Rozenfeld et al., 2006). Actualmente existen varias empresas, como CARA Therapeutics, Dimerix Bioscience o PatoBIOS Incorporated, que están desarrollando estrategias adecuadas para la identificación de heterodímeros o de moléculas que interactúan selectivamente con heterodímeros (Milligan, 2006).

	Ventajas	Inconvenientes
Ligandos Bivalentes	Alta selectividad. Pocos efectos secundarios. Diseño racional del compuesto.	Alto peso molecular de los compuestos. No poseer las propiedades fisicoquímicas de un fármaco (i.e. no satisfacer las reglas de Lipinski; (Lipinski, et al., 2001)).
Ligandos selectivos de heterodímero	Alta selectividad. Pocos efectos secundarios. Bajo peso molecular de los compuestos. Pueden satisfacer las reglas de Lipinski (Lipinski, et al., 2001).	Se basa en el supuesto de que la heterodimerización altera drásticamente el sitio de unión. Difícil diseño racional de los compuestos. Necesita una extensa exploración para la identificación del ligando.

Extraído y modificado de Rozenfeld et al., 2006

Tabla 4. Ventajas e inconvenientes del desarrollo de ligandos bivalentes y selectivos de heterodímeros.

Existen técnicas indirectas para detectar oligómeros en tejidos nativos como por ejemplo la coimmunoprecipitación de las proteínas implicadas a partir de tejido, pero como hemos comentado anteriormente, la coimmunoprecipitación requiere de la solubilización de los receptores de la membrana, lo que no permite descartar que los oligómeros observados puedan ser artefactos debidos al tratamiento con detergentes. Una manera bastante eficaz de detectar oligómeros en tejidos nativos es determinar alguna característica específica de los heterómeros en células donde se haya demostrado la heteromerización y utilizar esta propiedad como huella dactilar para detectar el heterómero en tejidos nativos. La determinación de cross-talk entre cascadas de señalización intracelular, el antagonismo cruzado en el que un antagonista específico de un receptor inhibe la señalización mediada por un agonista del otro receptor o bien el estudio de cambios en la unión de ligandos en uno de los receptores en presencia de un ligando para el otro receptor en preparaciones de membranas obtenidas de tejidos vivos pueden constituir una huella dactilar si se ha demostrado previamente que es una característica del heterómero. Estas estrategias se han utilizado para detectar heterómeros entre receptores de dopamina D₁ y histamina H₃ o entre receptores de dopamina D₁ y receptores sigma 1 en el tejido estriatal de cerebro (Moreno et al., 2011, Navarro et al., 2010) entre otros.

1.4 Receptores de dopamina y vías dopaminérgicas en el SNC

En el sistema nervioso central (SNC) se expresan un elevado número de receptores acoplados a proteína G, más del 90% de los receptores de siete dominios transmembrana se expresan en el cerebro y para algunos de ellos su expresión está restringida a este tejido. La combinación de técnicas de inmunohistoquímica, RT-PCR e hibridación in situ en diferentes regiones del cerebro ha permitido descubrir que la expresión de estos receptores presenta patrones diferenciales, lo que sugiere que la expresión de un grupo de receptores concretos y no otros, es clave en la regulación de diferentes procesos neurofisiológicos. Un ejemplo clásico lo constituyen los receptores de dopamina.

1.4.1 La dopamina como neurotransmisor

La dopamina es la principal catecolamina que actúa como neurotransmisor en el sistema nervioso central (representa el 80% del contenido total de catecolaminas del cerebro) y controla una gran variedad de funciones como la modulación de la actividad sensorial, la actividad motora, la actividad endocrina, el aprendizaje, la memoria, la emotividad, la afectividad y la motivación (Missale et al., 1998). Como otros neurotransmisores, la dopamina no es capaz de cruzar la barrera hematoencefálica, pero sí sus precursores fenilalanina y tirosina. Así pues, a partir de sus precursores, la biosíntesis de dopamina tiene lugar en el citosol de las terminales nerviosas dopaminérgicas (Cooper et al., 1996; Fuxe et al., 1965; Levitt et al., 1965). La liberación de dopamina en la hendidura sináptica tiene lugar mediante un mecanismo clásico de liberación de neurotransmisores: la entrada de calcio a través de canales de calcio dependientes de voltaje promueve la fusión de vesículas con la membrana pre-sináptica, dando lugar a la exocitosis de la dopamina, de forma que ésta difunde a través de la hendidura sináptica hasta unirse a sus receptores tanto pre- como post-sinápticos.

La señal dopaminérgica finaliza con la eliminación de la dopamina del espacio inter-sináptico, implicando mecanismos de recaptación específicos en el terminal pre-sináptico donde se vuelve a almacenar o es metabolizada. Aunque existen enzimas

extraneuronales que catabolizan la dopamina liberada, la terminación del efecto se debe principalmente a la captura del neurotransmisor por los propios terminales nerviosos que la liberaron. Esto tiene lugar mediante transportadores específicos (DAT: DopAmine Transporters) (Feldman et al., 1997; Cooper et al., 1996; Amara y Kuhar, 1993; McGeer et al., 1987) que juegan un papel esencial en la función, inactivación y reciclaje de la dopamina liberada.

1.4.2 Receptores de dopamina D_1 y D_2

Los receptores de dopamina se clasifican en dos subfamilias en función de sus propiedades bioquímicas y farmacológicas: la subfamilia D_1 -like, que comprende a los receptores D_1 y D_5 , y la subfamilia D_2 -like que incluye a los receptores D_2 , D_3 y D_4 . Los receptores D_1 -like producen incrementos del AMPc intracelular a través de proteínas $G_{s/olf}$ que estimulan a la AC y se localizan principalmente en los terminales postsinápticos (Neve et al., 2004; Nieoullon y Almaric, 2002; Missale et al., 1998; Civelli et al., 1993). Los receptores D_2 -like, en cambio, inhiben la AC por acoplamiento a proteínas $G_{i/o}$, además de activar canales de K^+ y disminuir la entrada de Ca^{+2} a través de canales dependientes de voltaje (Gershon et al., 2007; Neve et al., 2004; Nicola et al., 2000; Missale et al., 1998). Los receptores D_2 -like pueden localizarse en terminales presinápticas y postsinápticas (Dal Toso et al., 1989). Los dos subgrupos presentan peculiaridades estructurales diferentes como se muestra en la figura 17: los receptores D_1 -like tienen un dominio carboxiterminal unas siete veces más largo que los receptores D_2 -like, mientras que estos últimos tienen un tercer bucle intracelular mucho más largo, característica común en muchos receptores acoplados a proteína G_i (Missale et al., 1998).

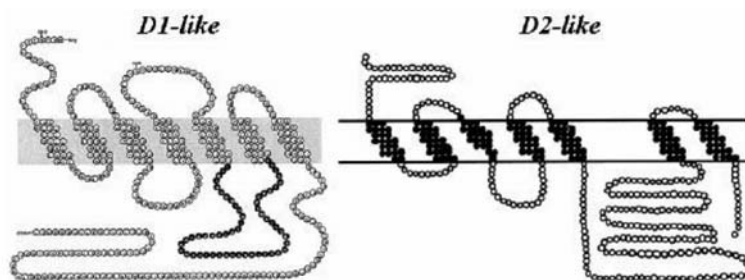


Figura 17. Representación esquemática de las dos subfamilias de receptores de dopamina.

Existe una homología del 80% en la secuencia entre los dos miembros de la familia de receptores *D₁-like*. En cambio, la homología es del 75% entre los miembros *D₂* y *D₃* de la familia *D₂-like* y un 53% entre los *D₂* y *D₄* de dicha familia. Por contraste, la homología entre los receptores *D₁-like* y *D₂-like* es solo del 42-46%. La región con homología más elevada es aquella que se encuentra en los dominios de transmembrana y en aquellos residuos que son clave para la unión de catecolaminas. El extremo carboxiterminal, en ambas familias, contiene lugares de fosforilación y palmitoilación que se cree juegan un papel esencial en la desensibilización del receptor y en la formación de un cuarto bucle intracelular, respectivamente. Por el contrario, los receptores de dopamina presentan diferencias en las modificaciones post-traduccionales, como diferentes lugares consenso de N-glicosilación.

Para el estudio de las propiedades farmacológicas de los receptores de dopamina se dispone de ligandos que discriminan fácilmente entre las subfamilias *D₁-like* y *D₂-like*, sin embargo, es mucho más difícil encontrar ligandos selectivos para los miembros de cada subfamilia. Cada uno de los receptores *D₁-like* muestra alta afinidad por benzazepinas (agonistas) y baja afinidad por butiroferonas y benzanidas sustituidas (antagonistas). Se ha detectado una diferencia remarcable de su afinidad por la dopamina, presentado el receptor *D₅* una afinidad 10 veces superior a la que presenta el receptor *D₁* (Missale et al., 1998). Dentro de los receptores *D₂-like* las afinidades por muchos antagonistas y agonistas pueden variar en uno o dos órdenes de magnitud. Cada uno de estos receptores se caracteriza por tener alta afinidad por butiroferonas, como las espiperonas y el haloperidol y baja afinidad por benzazepinas como el SKF 38393.

De los tres receptores que componen la familia *D₂-like*, el *D₂* tiene las propiedades farmacológicas más distintivas, pues en general, presenta baja afinidad por la mayoría de los antagonistas dopaminérgicos de esta subfamilia, por ejemplo, el raclopride, exhibiendo a su vez una relativa afinidad por el neuroléptico atípico clozapina (Missale et al., 1998). En cambio, el receptor *D₃* presenta una afinidad 20 veces mayor por la dopamina que el receptor *D₂*, existiendo algunos ligandos comerciales selectivos como el antagonista GSK 789472. Por último, el receptor *D₄* es el que presenta menor afinidad por la dopamina de entre los miembros de la subfamilia *D₂-like*, existiendo también algunos ligandos sintéticos selectivos para este receptor como el agonista RO 10-5824 o el antagonista L-745,870.

La diferencia de afinidad que presentan los receptores de dopamina por su ligando endógeno puede permitir la activación de unos receptores u otros en función de la cantidad de dopamina liberada. Teniendo en cuenta los diferentes mecanismos de transducción de señal de cada subtipo de receptor, se puede generar una gran variedad de respuestas a una misma sustancia. Esta diversidad dentro de los receptores de dopamina es un reflejo de la diversidad funcional que ejerce este neurotransmisor, sobretodo si se considera la expresión diferencial de estos receptores dentro del SNC. En la tabla 3 se resumen la información más relevante de los diferentes subtipos de receptores de dopamina.

Familia	D₁R-like		D₂R-like		
Subtipo	D₁R	D₅R	D₂R	D₃R	D₄R
Proteína G	G_s/olf	G_s/olf	G_{i/o}	G_{i/o}	G_{i/o}
Mecanismo de transducción de señal	+ AC + PLC	+ AC	- AC + PLC - canales Ca²⁺ + canales K⁺	- AC + PLC - canales Ca²⁺ + canales K⁺	- AC + PLC
Moléculas efectoras	↑ AMPc ↑ PKA ↑ IP₃	↑ AMPc	↓ AMPc ↑ IP₃ ↓ Ca²⁺ ↑ K⁺	↓ AMPc ↑ IP₃ ↓ Ca²⁺ ↑ K⁺ ↑ NKE*	↓ AMPc ↑ ác. araquid. ↑ NKE*
Afinidad por la dopamina K _D en nM	2340	261	2,8-474	4-27	28-450
Agonista K _D en nM	SKF-38393 1-150	NPA 187	Quinpirole 4,8-474	Bromocriptina 5-7,4	(-) Apomorfina 4
Antagonista K _D en nM	SCH-23390 0,11-0,35	SCH-23390 0,11-0,54	Raclopride 1-5	UH 232 2,9-9,2	Clozapina 9-42

*NKE: Na⁺/K⁺ exchange: intercambiador Na⁺/K⁺.

Tabla 3. Resumen de las principales características de los receptores de dopamina.

La expresión de los distintos subtipos de receptores de dopamina en el cerebro ha sido determinada mediante la combinación de técnicas de unión de radioligandos y de hibridación in situ. Así se ha demostrado, por ejemplo, que el receptor D₁ es el más abundante y su distribución es la más amplia de todos los receptores dopaminérgicos (Beaulieu et al., 2011; Barishpolets et al., 2009; Dearry et al., 1990); en cambio, el receptor D₅ presenta una distribución más restringida a regiones tales como el hipocampo o el tálamo (Centonze et al., 2003). Estudios de RT-PCR y proteómica han demostrado la expresión del receptor D₁ en el estriado (tanto dorsal como ventral), en el

núcleo accumbens, el tubérculo olfatorio, y en menor medida en el sistema límbico, el hipotálamo y el tálamo. La expresión mayoritaria del receptor D₁ se localiza postsinápticamente en las neuronas GABAérgicas estriatales, junto con la expresión de la sustancia P (Gerfen et al., 1990). El receptor D₃ se localiza específicamente en regiones límbicas del núcleo accumbens con una localización post-sináptica en neuronas que expresan sustancia P y neurotensina. El receptor D₄ se expresa en interneuronas GABAérgicas tanto piramidales como no-piramidales de la corteza prefrontal e hipocampo, en el bulbo olfatorio, la amígdala, el mesencéfalo (Missale et al., 1998), en la glándula pineal (Klein et al., 2010) y en menor medida en el núcleo accumbens y estriado (Almeida y Mengod, 2010; Gasca-Martinez et al., 2010).

El receptor D₂ de dopamina ha sido ampliamente estudiado, demostrándose su participación en numerosas e importantes funciones fisiológicas como el control de la actividad motora. Este receptor se distribuye ampliamente en el cerebro y se localiza a nivel postsináptico y presináptico en función de su splice alternativo (short o long) (Beaulieu y Gainetdinov, 2011; De Mei et al., 2009; Usiello et al., 2000; Giros et al., 1989). El receptor D₂ presináptico actúa como un autoreceptor e inhibe la liberación de dopamina. Esto hace que los receptores D₂ representen la principal diana de drogas antipsicóticas, además de estar implicados en varias neuropatologías como el Parkinson, el síndrome de Tourette y la adicción a drogas (Vallone et al., 2000). El receptor de dopamina D₂ también se localiza en los lóbulos anteriores y neurointermedios de la glándula pituitaria, siendo uno de los principales receptores dopaminérgicos que regulan la liberación de hormonas (Vallone et al., 2000).

En el SNC se detecta una elevada densidad de este receptor en el caudatoputamen o estriado dorsal, región del estriado responsable del control motor; en esta región se detecta en neuronas GABAérgicas estriatopallidales que coexpresan encefalina. Su expresión es alta en el tubérculo olfatorio y el núcleo accumbens o estriado ventral, región del estriado responsable de los fenómenos de recompensa asociados a la adicción a drogas de abuso. De forma moderada se detecta en neuronas de la sustancia nigra, tanto reticulata como compacta; en esta última la expresión del receptor D₂ tiene lugar en neuronas dopaminérgicas y actúa como autoreceptor. Además, también se ha encontrado una distribución moderada de este receptor en la corteza cerebral, el globus pallidus, el núcleo subtalámico, la amígdala, el tálamo y el hipotálamo (Jackson et al., 1994). La distribución del mRNA es prácticamente paralela

a la descrita para la proteína. Así, se ha descrito un elevado nivel de mRNA para este receptor en el área ventral tegmental, núcleo que da lugar a la mayor vía dopaminérgica del cerebro, lo que indica que el receptor D₂ es uno de los principales receptores dopaminérgicos que controla directamente la actividad de neuronas que contienen dopamina. El estriado recibe la mayor densidad de inervaciones dopaminérgicas y contiene, como se ha comentado anteriormente, la mayor concentración de receptores de dopamina del cerebro (Seeman, 2006; Gerfen, 2000; Vallone et al., 2000; Björklund et al., 1984).

El gen del receptor D₂ está compuesto por 8 exones, 7 de los cuales se transcriben. En el sexto exón tienen lugar un splicing alternativo, el cual codifica para 29 aminoácidos adicionales en el tercer bucle intracelular (IC3), generando las dos isoformas que se encuentran tanto en rata como humano. El primer cDNA de los receptores de dopamina aislado fue el del receptor D₂ (Bunzow et al., 1988) que se clonó a partir de una librería de cDNAs de pituitaria de rata. La región codificadora de esta proteína se encuentra en el cromosoma 11q23. El cDNA aislado por Bunzow y colaboradores contenía una secuencia de 1245 nucleótidos que codificaban para una proteína de 415 residuos, que posteriormente se llamó D_{2S}R (receptor de dopamina D₂ short), con un perfil farmacológico típico de los receptores *D₂-like*. Más tarde, varios grupos clonaron una variante por splice de este receptor, el D_{2L}R (receptor de dopamina D₂ long) de diferentes especies (rata, ratón, bovino, humano) y tejidos (cerebro, pituitaria, retina), que contenía 444 aminoácidos.

En la figura 18 se representa la estructura prototipo del receptor de dopamina D_{2L}. Los D₂R tienen un extremo C-terminal corto y un IC3 largo, el cual parece estar implicado en el acoplamiento a la proteína G (Ilani et al., 2002; Filteau et al., 1999; Lachowicz et al., 1997; Malek et al., 1993), además de permitirle interactuar con otras proteínas, como el receptor de adenosina A_{2a} (Canals et al., 2003).

Ambas isoformas del receptor D₂, D_{2L} y D_{2S}, tienen la misma capacidad de unir ligando pero difieren tanto en la expresión como en la capacidad de acoplarse a la proteína G. La isoforma larga se expresa unas 10 veces más que la corta y tiene una capacidad de acoplarse a la proteína G_i mucho menor, lo que da lugar a una diversidad de señal. De hecho existen evidencias que indican que los receptores D_{2L} y el D_{2S} se acoplan a distintas proteínas G, G_i y G_o respectivamente, debido principalmente a sus

diferencias estructurales (Beaulieu y Gainetdinov, 2011; De Keyser et al., 1989; Ohara et al., 1988).

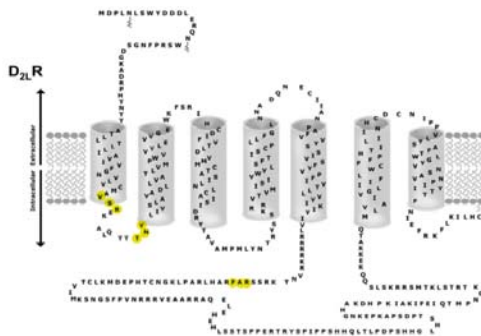


Figura 18. Representación esquemática de la estructura del receptor D_{2L} de dopamina.

Extraído de Borroto-Escuela et al., 2011

Las proteínas G_i y G_o son sensibles a la toxina pertusis, por tanto, inhiben a la adenilato ciclasa (AC), lo que parece ser la vía de señalización predominante utilizada por los receptores D₂, al menos en el estriado (Missale et al., 1998). La inactivación genética tanto de la AC₅, la principal isoforma de la AC en el estriado (Lee et al., 2002; Mons et al., 1995), como de la PKA provoca un daño importante en la función de estos receptores, como la pérdida de los efectos bioquímicos y del comportamiento de los antagonistas de los receptores D₂ (Lee et al., 2002; Adams et al., 1997). La inhibición sobre la AC provocada por la activación de los receptores D₂ se ha observado en varias células y parece ser dependiente del acoplamiento del receptor a la proteína G_{i/o} (Banihashemi y Albert, 2002; Ghahremani et al., 1999).

Además de inhibir la AC, la activación de los receptores D₂ da lugar a cambios en la actividad de canales de Ca²⁺ (Hernández-López et al., 2000; Taraskevich y Douglas, 1978) y de K⁺ (Castelletti et al., 1989; Missale et al., 1998) provocando una hiperpolarización celular. Los agonistas del receptor activan a la fosfolipasa C (PLC) e incrementan la concentración de Ca²⁺ intracelular (Beaulieu y Gainetdinov, 2011; Martemyanov y Arshavsky, 2009; Beaudry et al., 1986; Enjalbert et al., 1986) dependiente de IP₃ y la activación de la calcineurina, una serina-treonina fosfatasa dependiente de Ca²⁺ (PP-2A) (Hernández-López et al., 2000). Esta vía parece implicar a las subunidades G_{βγ} de la proteína G_o. La calcineurina no solo reduce las corrientes de Ca²⁺ a través de los canales de Ca²⁺ dependientes de voltaje tipo L (L-type VDCC: L-type Voltage Dependent Ca²⁺ Channel) (Banihashemi y Albert, 2002; Hernández-López et al., 2000; Ghahremani et al., 1999), sino que además, la calcineurina parece ser la principal fosfatasa implicada en la desfosforilación de DARPP-32 (Dopamine and

cyclic adenosine 3', 5'- monophosphate Regulated Phospho Protein, 32 kDa). Por lo tanto, la activación del receptor D₂ de dopamina produce la desfosforilación de DARPP-32 debida tanto a la inhibición de la actividad de la AC como a la calcineurina dependiente de Ca²⁺ e independiente de AC (Nishi et al., 1997).

La estimulación de los receptores D₂ activa también la vía de las MAPKs y la fosforilación de CREB (cAMP Response Element-Binding protein) en cortes cerebrales (Yan et al., 1999), en cultivos estriatales primarios (Brami-Cherrier et al., 2002) y en diferentes líneas celulares (Banihashemi y Albert, 2002; Oak et al., 2001; Faure et al., 1994). Tanto la PKC como DARPP-32 y la calmodulina kinasa, junto con los incrementos en los niveles de Ca²⁺, parecen ser importantes en la activación de estas vías (Sahu et al., 2009; Lee et al., 2004; Yan et al., 1999). La activación de la vía de las MAPKs, en las interneuronas estriatales, se cree juega un papel importante en la regulación de la expresión génica inducida por dopamina y la adaptación neuronal a largo plazo en el estriado. La activación de la vía de las MAPKs puede estar implicada en la sensibilización locomotora en respuesta a la estimulación del receptor D₂ en ratas lesionadas unilateralmente con 6-hidroxi dopamina (Santini et al., 2009; Cai et al., 2000). Curiosamente existen datos contradictorios, los agonistas del receptor D₂ se ha descrito que inhiben específicamente la activación de la vía de las MAPK en neuronas de proyección estriatopalidales activadas por estimulación aferente corticoestriatal (Yoon et al., 2011; Chen et al., 2009; Gerfen et al., 2002). Para la activación de la vía de las MAPKs es necesario la formación de un complejo entre la calmodulina y el VDCC tipo L, que juega un papel importante en la conversión de la información de la membrana (activación neuronal) hacia el núcleo (plasticidad neuronal) (Dolmetsch et al., 2001). En las neuronas de proyección estriatopalidales el receptor D₂ media la inhibición de los VDCC tipo L lo que parece ser el principal mecanismo implicado en la inhibición de la activación de las MAPKs.

Una de las diferencias más significativas entre las dos isoformas del receptor D₂ se encuentra en su tráfico intracelular. El proceso de maduración del receptor difiere entre las dos isoformas; así, los receptores D_{2S} se procesan más rápido que los D_{2L} y alcanzan en mayor proporción la membrana. Un porcentaje significativo de receptores D_{2L} permanece en una forma inmadura en compartimentos intracelulares sin alcanzar la membrana plasmática (De Mei et al., 2009; Fishburn et al., 1995). Más relevante desde un punto de vista terapéutico y patofisiológico es la respuesta diferencial que presentan

ambas isoformas a la exposición prolongada a agonistas. En algunas células se ha visto que ambas isoformas experimentan una internalización tras exposición a agonista, proceso que implica a GRKs y β -arrestinas (Kim et al., 2001; Ito et al., 1999). Sin embargo, el grado de internalización del receptor D_{2S} es mayor que el del D_{2L} (Ito et al., 1999) de acuerdo con el hecho de que ambas isoformas pueden ser fosforiladas diferencialmente por GRKs y β -arrestina (Guiramand et al., 1995; Senogles, 1994; Liu et al., 1992) La resistencia a la internalización inducida por ligando del receptor D_{2L} se hace muy patente en algunas células en las que el receptor D_{2L} se up-regula en respuesta al pretratamiento con agonistas (Ng et al., 1997; Starr et al., 1995; Zhang et al., 1994; Filtz et al., 1993) lo que es debido a la translocación a la membrana de los receptores intracelulares ya existentes y a la síntesis de novo de receptores (Thibault et al., 2011; Ng et al., 1997). Se ha descrito que esta up- regulación puede ser la causa de la resistencia que presentan los receptores D_{2L} a la desensibilización (Hillion et al., 2002; Ng et al., 1997; Starr et al., 1995; Zhang et al., 1994; Filtz et al., 1993).

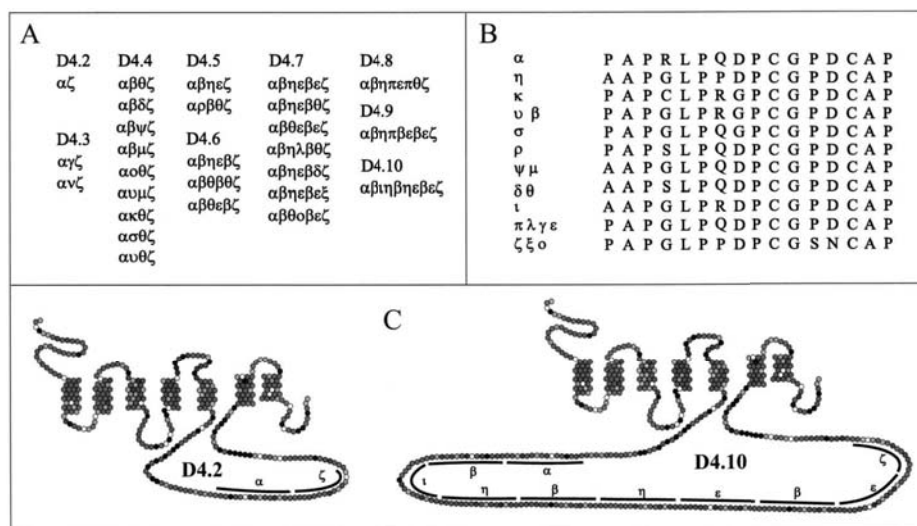
Ambas isoformas presentan también diferencias en su distribución. En general, parece que hay pocos tejidos específicos para la expresión de las isoformas larga y corta del receptor D₂, aunque sí que existe una distribución diferencial en el neostriado y la hipófisis, donde se observa una mayor expresión de la forma larga (Jackson et al., 1994; O'Dowd, 1993). Los receptores D_{2S} son presinápticos y se encuentran principalmente en neuronas dopaminérgicas del cerebro medio (Centonze et al., 2002; Usiello et al., 2000; Mercuri et al., 1997) donde actúa como autoreceptor inhibitorio (Lindgren et al., 2003). En las neuronas del estriado, los receptores D_{2L} se localizan preferencialmente de forma post-sináptica (Rouge-Pont et al., 2002), y principalmente en neuronas estriatopallidales GABAérgicas, donde colocalizan con diferentes receptores de dopamina y adenosina, entre otros.

1.4.3 El receptor de dopamina D₄ y su relación con el Trastorno de Hiperactividad y Déficit de Atención (ADHD)

El gen correspondiente al receptor D₄ de dopamina se clonó por primera vez en 1991 por Van Tol y colaboradores; localizado en el extremo distal del brazo corto de cromosoma 11 en la posición 11p15.5 y próximo al oncogen Harvey-RAS y al

gen de la tirosina hidrolasa (Oak et al., 2000), el gen del receptor D₄ contiene cuatro exones y un número de polimorfismos variable en la secuencia de codificación. El polimorfismo más extendido es el que se encuentra en el tercer axón, cuya región codifica para el tercer loop citoplasmático. Este polimorfismo consiste en un número variable de repeticiones en tándem (VNTR), es decir, unas secuencias de 48bp que codifican para diferentes polipéptidos formados por 16 aminoácidos. Éstos pueden formar combinaciones distintas que pueden presentar de 2 a 11 repeticiones dando así el subnombre al receptor de D_{4.2} a D_{4.11} y proporcionando un tamaño variable a cada polimorfismo (Figura 19).

La frecuencia de la presencia de los diferentes polimorfismos varía de forma considerable en función de la población y etnia (Yilmaz et al., 2012; McGeary, 2009; Swanson et al., 2001), a pesar de ello, estudios genéticos de población sobre la diversidad alélicas demostraron que los polimorfismos D_{4.2}, D_{4.4} y D_{4.7} son los más prevalentes, presentándose así en el 90% de la población. Como se muestra en la figura 20, dentro de los polimorfismos más abundantes, el D_{4.4} ocurre con más frecuencia, en aproximadamente el 65% de la población, seguido del D_{4.7} en aproximadamente el 19% y D_{4.2} en aproximadamente el 8% (Floet et al., 2010; Ding et al., 2002).



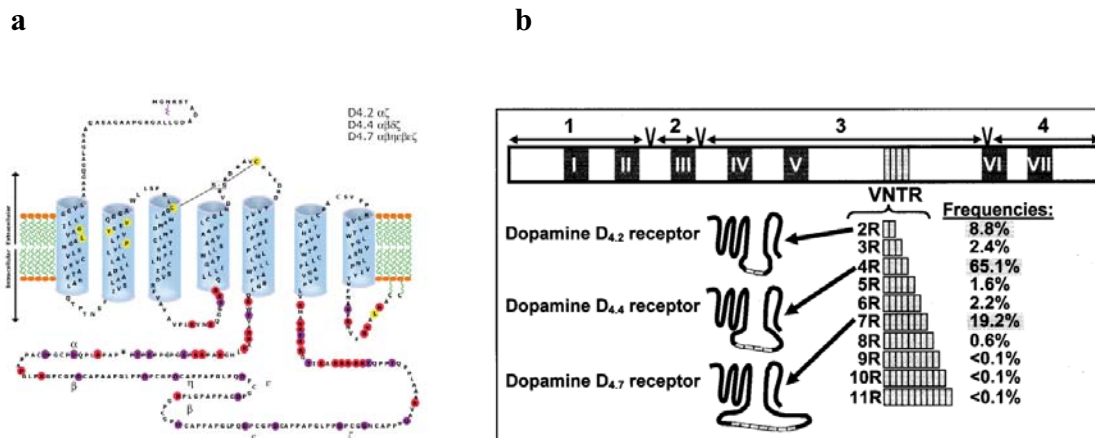
Extraído de Oak et al., 2000

Figura 19. Representación de las diferentes VNTR presentes en el tercer loop intracelular de los receptores D₄ de dopamina.

a) Representación de los diferentes haplotipos presentes en la población humana y las combinaciones de secuencias de 48bp que los forman . b) Los péptidos formados por 16 aminoácidos correspondientes a los diferentes haplotipos. c) Representación esquemática del largo loop intracelular como consecuencia de las diferentes VNTR.

Como se ha explicado anteriormente, el receptor D₄ pertenece a la subfamilia de receptores *D₂-like* y, por lo tanto, presenta gran homología con los otros miembros de esta subfamilia, especialmente en los siete dominios de transmembrana que son altamente conservados. Muestra una serie de modificaciones post-transcripcionales como N-glicosilaciones en la cola amino-terminal extracelular, y la presencia de regiones de fosforilación de proteína quinasa A (PKA), proteína quinasa C (PKC) y casein quinasa II (Rondou et al., 2010; Neve et al., 2004; Jovanovic et al., 1999; Asghari et al., 1995).

El tercer loop citoplasmático del receptor D₄ es característicamente largo si lo comparamos con cualquier otro miembro de su subfamilia *D₂-like*. Posee regiones ricas en prolina además de la región hipervariable VNTR anteriormente descrita y contiene una secuencia SH3 que le permite interactuar con proteínas como Src, Grb2 y Nck (Rondou et al., 2010; Oak et al., 2001, Oldenhof et al., 1998). Aunque se conocen las diferentes regiones de unión a proteínas de señalización intracelular todavía se desconocen sus funciones y el porqué de la existencia de los diferentes polimorfismos.



Extraído y modificado de Ding et al., 2002

Figura 20. Representación esquemática de la estructura del receptor D₄ de dopamina y la frecuencia de los diferentes polimorfismos.

El receptor D₄ se expresa mayoritariamente en córtex prefrontal, hipocampo, amígdala, hipotálamo (Missale et al., 1998) y en las neuronas piramidales glutamatérgicas y no piramidales del córtex cerebral y en las terminaciones de sus proyecciones estriatales (Lauzon et al., 2010; Svingos et al., 2000; Tarazi et al., 1998),

en las que se incluyen el núcleo talámico, globus pallidus y sustancia nigra pars reticulata, donde también se encuentran las interneuronas GABAérgicas. Estudios de northern-blot, hibridación *in situ*, inmunohistoquímica y RT-PCR han demostrado que el receptor D₄ presenta una gran diversidad de expresión en diferentes tejidos como la glándula pineal, linfocitos o retina (Beaulieu y Gainetdinov, 2011; Burgueño et al., 2007)

Debido a la tardía clonación de este receptor y a la limitación en la disponibilidad de agonistas selectivos y/o antagonistas, la farmacología y los estudios de señalización intracelular del receptor D₄ son todavía muy reducidos. A pesar de ello ya se había descrito que en células mesencefálicas MN9D de rata y en otras líneas celulares éste receptor se acopla mayoritariamente a proteína G_i (Kazmi et al., 2000; Watts et al., 1999; Chio et al., 1994) pero también puede acoplarse a G_{0A}, G_{0B} y G_{1r2r3} dependiendo del tejido en el que se encuentra (O'Hara et al., 1996). Es el primer receptor no-opsina capaz de unirse a la proteína mutante G_{t2} resistente a toxina pertusis (Yamaguchi et al., 1997). La activación de GIRK1 en oocitos de *Xenopus* mediada por D₄ sugiere también que éstos están implicados en la apertura de canales de K⁺ por vía G_{βγ} (Pillai et al., 1998; Werner et al., 1996).

Recientemente, tras la síntesis de nuevos ligandos para D₄R como los agonistas parciales RO 10-5824 (K_D 5,2nM), PD 168077 (K_D 8,7nM) o CP-226,269 (K_D 6nM) y antagonistas como L-741,742 (K_D 3,5nM) o IPMPP (K_D 0,39nM), se ha comenzado a entender y definir la vías de señalización mediante las cuales el receptor es capaz de abrir canales iónicos, fosforilar segundos mensajeros como las proteínas CaMKII y PP1 o activar factores de transcripción (Lauzon et al., 2011; Newman-Tancredi et al., 2007; Powell et al., 2003; Clifford y Waddington, 2000; Pillai et al., 1998).

Una de las características más interesantes del receptor D₄ de dopamina humano es que ha sido relacionado con trastorno de hiperactividad y déficit de atención (ADHD: *Attention-Deficit Hyperactivity Disorder*). El ADHD es un desorden del desarrollo caracterizado por un patrón persistente de inatención e hiperactividad, así como falta de memoria y elevada impulsividad, agitación y distracción. Este desorden afecta del 1 al 5% de la población infantil mundial, dependiendo del método de evaluación (American Academy of Pediatrics, 2001) con una prevalencia de 2 a 6 niños por cada niña; entre los cuales, el 60% de los casos persiste en adultos (American Academy of Pediatrics,

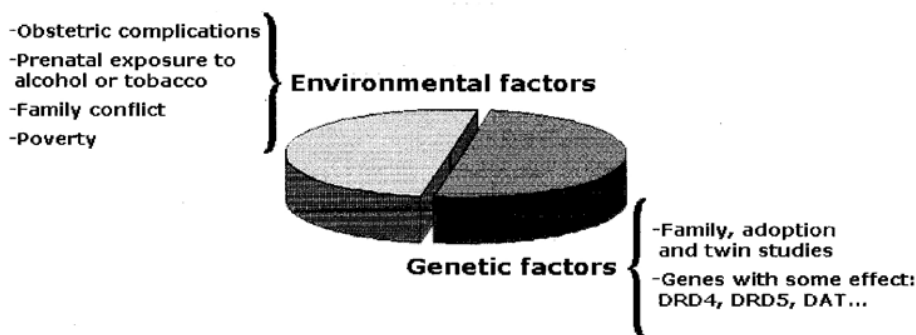
2001; Scahill y Schwab-Stone, 2000). Dicho trastorno se clasifica clínicamente en tres subtipos en función de sus características psicomotrices (Antshel et al., 2011; Faraone et al., 2005; Mediavilla-García, 2003; Adler y Chua, 2002; Carte et al., 1996):

a) Subtipo de hiperactividad-impulsividad: se caracteriza por movimientos de manos y pies, gran dificultad para relajarse, habla excesiva y un patrón de agresividad en las relaciones sociales.

b) Subtipo de déficit de atención: caracterizado por una inatención en los detalles, no sigue instrucciones ni escucha, presenta dificultad para organizar tareas, fácil pérdida de objetos y gran facilidad para la distracción.

c) Subtipo combinado: es el más perjudicial de los subtipos y el menos frecuente en la población. Presenta desordenes bipolares, ansiedad y gran dificultad en la integración social.

Diversos estudios clasifican las causas de ADHD en niños con edad escolar en dos subgrupos (Figura 21): factores socioambientales y factores genético-moleculares.



DRD4, dopamine D4 receptor; DRD5, dopamine D5 receptor; DAT, dopamine transporter.

Extraído de Burgueño et al., Neurobiological Aspects of ADHD. Antidepressants, Antipsychotics, Anxiolytics. Editado por Buschmann et al., Wiley-VCH. 2007

Figura 21. Gráfico representativo de los factores causantes del trastorno de hiperactividad y déficit de atención (ADHD).

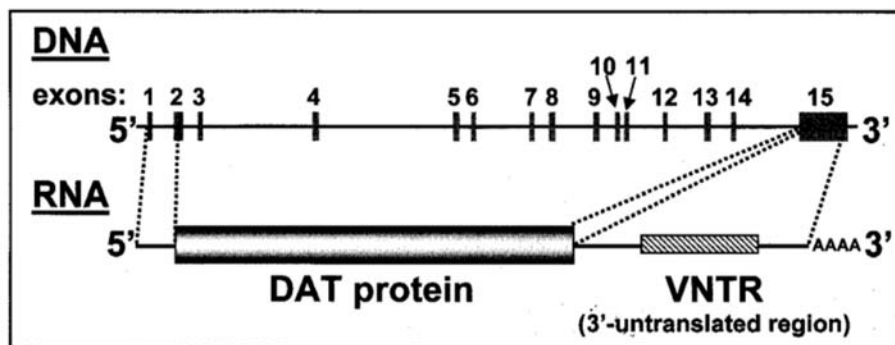
Factores socioambientales como complicaciones en el nacimiento, exposición prenatal a alcohol y/o tabaco, conflictos familiares y pobreza, entre otros, son causantes de un trastorno de hiperactividad y falta de atención que no tiene una base molecular,

pero son igualmente perjudiciales y dificultan la capacidad del individuo para concentrarse e integrarse socialmente (Burgueño et al., 2007). Por otro lado, los factores genéticos tienen un carácter hereditario ya que están relacionados con la presencia del polimorfismo de un gen o el mal funcionamiento de una proteína, lo que implica un patrón de ADHD debido a una anomalía en el funcionamiento de la comunicación neuronal. En este tipo de casos, se observa, entre otras alteraciones, una disminución de un 8,1% en el metabolismo de la glucosa en el cerebro, así como una disminución de la actividad en córtex prefrontal, ganglio basal y cerebelo.

Desde hace ya varios años, se ha relacionado el receptor D₄ de dopamina, concretamente el polimorfismo D_{4.7}, y el transportador de dopamina DAT, como responsables en parte de ADHD, pero a pesar de ello, hoy en día se desconocen las bases moleculares mediante las cuales estas proteínas pueden causar dichas anomalías. Estudios de meta-análisis realizados con individuos ADHD e individuos control (sanos) han descrito una relación estadística significativa entre la presencia del polimorfismo D_{4.7} y el trastorno ADHD (Faraone et al., 2005; Grady et al., 2003; Holmes et al., 2002; Roman et al., 2001). Estos estudios definen un *odds ratio* (OR) de 1.9 (OR superior a 1.0 implica un incremento de riesgo significativo) de asociación entre D_{4.7} – ADHD con un 95% de intervalo de confianza [CI] 1.4 – 2.2 (Faraone et al., 2005; Faraone et al., 2001)

El receptor de dopamina D_{4.7} presente en los niños con ADHD presenta una mayor expresión en ciertas áreas del cerebro como estriado y córtex prefrontal, y a su vez, estas áreas presentan una morfología variable comparadas con un individuo sano (Yang et al., 2008; Eisenberg et al., 2000). Filbey y colaboradores, han descrito que el córtex prefrontal y el estriado de individuos con ADHD presentaban una activación menor respecto a individuos control tras la administración de diferentes fármacos dirigidos al receptor D₄ de dopamina, pero actualmente se desconocen las causas de estas diferencias en la actividad cerebral de estos individuos (Filbey et al., 2008). A pesar de no existir diferencias significativas respecto al perfil farmacológico de los diferentes polimorfismos de D₄, la activación de D_{4.7} presenta una menor inhibición de la adenilato ciclasa y, por lo tanto, una mayor concentración de AMP cíclico intracelular comparada con los otros polimorfismos (Burgueño et al., 2007; Wang et al., 2004; Oak et al., 2000).

El transportador de dopamina DAT, que tiene la función de recaptar la dopamina liberada en el espacio sináptico, tiene una mayor actividad en niños que padecen este trastorno, con lo que la concentración de dopamina presente en la comunicación sináptica es menor y, por consiguiente, también lo es la activación de los receptores dopaminérgicos presentes en la sinapsis (Ciruela et al., 2007, Madras et al., 2005). Una de las teorías más aceptadas es la presencia de 10 repeticiones en tándem (480-bp) presentes en el extremo a 3' del gen que codifica para el transportador DAT que da lugar a un mRNA cuya conformación le proporciona una gran estabilidad y resistencia frente a la actividad de la RNAsas celulares. De este modo, se produce una gran densidad presináptica de transportadores DAT, dando lugar a una disminución de la concentración de dopamina en el espacio sináptico (Madras et al., 2005; Thapar et al., 2005; Curran et al., 2001).



Extraído de Ciruela et al., *Pharmacology: Targets for Drug Action. Antidepressants, Antipsychotics, Anxiolytics*. Editado por Buschmann et al., Wiley-VCH, 2007

Figura 22. Estructura del gen del transportador de dopamina DAT

1.4.4 Los ganglios basales y las vías dopaminérgicas en el SNC

En roedores, los ganglios basales están constituidos por cinco núcleos principales: el estriado, la sustancia nigra, el globus pallidus, el núcleo subtalámico y el núcleo entopeduncular. El estriado es la principal estructura de entrada de los ganglios basales y está funcionalmente subdividido en estriado dorsal y ventral. El estriado dorsal (núcleo caudato y putamen) está implicado en la ejecución y aprendizaje de actos motores complejos. El estriado ventral (núcleo accumbens) forma parte de los circuitos

cerebrales implicados en la conversión de la motivación en acción. En el estriado, más del 90% de las neuronas son GABAérgicas de proyección o *médium-size spiny neurons* y reciben dos vías de entrada que convergen en sus espinas dendríticas: por un lado las neuronas dopaminérgicas del mesencéfalo, localizadas en la sustancia nigra pars compacta y el área ventral tegmental y por otro lado las neuronas glutamatérgicas procedentes de áreas corticales, límbicas y talámicas (hipocampo y amígdala) (Gerfen, 2004).

Hay dos subtipos de neuronas GABAérgicas eferentes en el estriado, que proyectan al tálamo a través de dos vías (Figura 23): las neuronas estriatopalidales (vía indirecta) y las neuronas estriatonigroentopedunculares (vía directa). Los dos tipos de neuronas GABAérgicas estriatales se pueden distinguir neuroanatómicamente. Las neuronas estriatopalidales contienen el péptido encefalina, receptores de dopamina, (predominantemente del subtipo D₂) y receptores A₁ y A_{2A} de adenosina, entre otros. Las neuronas estriatonigroentopedunculares contienen dinorfina, sustancia P, receptores de dopamina (predominantemente de subtipo D₁) (Alexander y Crutcher, 1990) y receptores A₁ de adenosina, pero no receptores A_{2A} (Ferré et al., 2007; Schiffmann et al., 2007;).

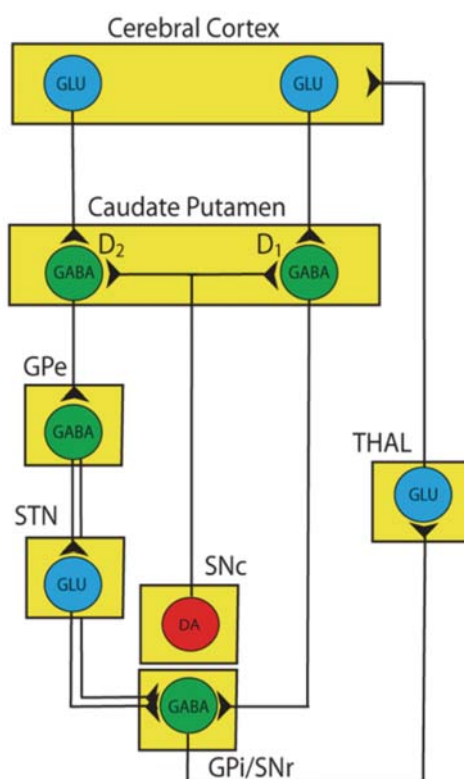


Figura 23. Funcionamiento de los ganglios basales.

Existen dos vías de salida del estriado: la vía directa, que conecta el estriado al núcleo entopeduncular (GPi en humanos)/sustancia nigra pars reticulata (GPi/SNr) y la vía indirecta, que conecta el estriado con el globus pallidus (GP), el núcleo subtalámico (STN) y la sustancia nigra pars reticulata (GPi en humanos)/núcleo entopeduncular (GPi/SNr).

Cedido por Dr. Sergi Ferré

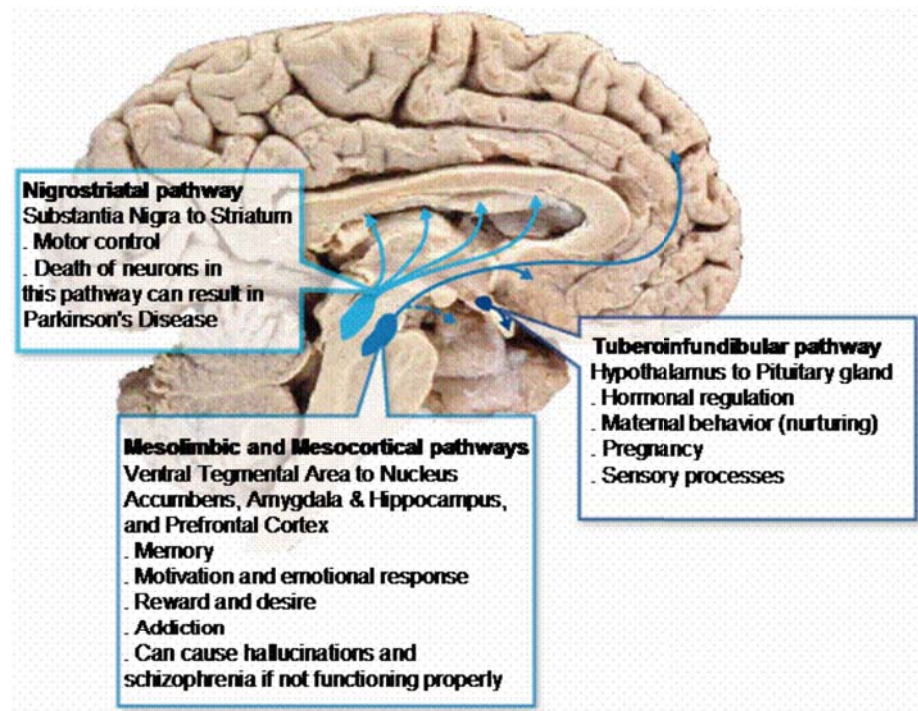
La estimulación de la vía directa produce activación motora, mientras que la de la vía indirecta produce inactivación motora. La vía directa tiende a activar los movimientos voluntarios y la vía indirecta a inhibir la aparición de componentes involuntarios en el movimiento. Un adecuado equilibrio entre las dos produce los movimientos normales. La dopamina induce la activación de la actividad motora mediante los receptores D₁ de las neuronas estriatonigroentopedunculares, mientras que deprime la actividad de las neuronas estriatopalidales actuando sobre los receptores D₂, produciendo indirectamente una actividad motora (Alexander y Crutcher, 1990). La dopamina por tanto, estimula el movimiento a través de las dos vías, porque estimula la vía estimuladora e inhibe a la vía inhibidora (Figura 23).

La enfermedad de Parkinson está producida por la degeneración progresiva de las neuronas dopaminérgicas nigroestriatales que proyectan de la sustancia nigra, al caudado-putamen. Esto da lugar a una disminución de la liberación de dopamina en el estriado, lo que provoca una hipoactividad de las neuronas GABAérgicas estriatonigroentopedunculares (vía directa) y una hiperactividad de las neuronas GABAérgicas estriatopalidales (vía indirecta) debido a la liberación de los efectos inhibitorios de la dopamina endógena (Obeso et al., 2008), con el consiguiente descontrol de la actividad de los ganglios basales. Los síntomas clínicos más relevantes incluyen bradiquinesia (lentitud en los movimientos), rigidez, temblor en reposo y alteraciones en el equilibrio. El tratamiento paliativo de esta enfermedad es suministrar un precursor de dopamina L-DOPA, que aunque efectivo en los primeros estadios de la enfermedad, acaba por perder la efectividad y provoca la aparición de complicaciones motoras como la discinesia (Nutt, 1990). Actualmente, existen avances importantes en el desarrollo de nuevos fármacos dopaminérgicos y no dopaminérgicos para la enfermedad de Parkinson, así como para las complicaciones motoras de las terapias en uso (Schapira et al., 2006).

Los ganglios basales constituyen solo una parte de los circuitos dopaminérgicos del SNC. A pesar del reducido número de neuronas presentes en el cerebro que utilizan dopamina como neurotransmisor, este sistema de transmisión juega un papel esencial en la regulación del movimiento, conducta y liberación de hormonas (Dale, 2000). Los circuitos dopaminérgicos del SNC se pueden dividir en tres tipos: nigroestriado, mesolímbico-mesocortical y tuberohipofisario (Figura 24). Cuya alteración en

cualquiera de estas vías de transmisión esta asociada con una o más patologías. Así, por ejemplo, una alteración en la vía nigroestriada esta relacionada con la enfermedad de Parkinson, una alteración en la vía mesolímbica-mesocortical está relacionada con esquizofrenia y una gran variedad de alteraciones hormonales está relacionada con anomalías en la vía tuberohipofisaria (Dale, 2000).

El sistema nigroestriado se origina en la sustancia nigra, que es un núcleo de neuronas localizado en el mesencéfalo. Éste se divide en dos partes: la compacta, formada por neuronas dopaminérgicas, y la reticulata, formada principalmente por neuronas GABAérgicas. Las neuronas dopaminérgicas con origen en la sustancia nigra constituyen el principal tracto dopaminérgicos en el cerebro, y proyectan axones que proporcionan una densa inervación al núcleo caudado y al putamen del estriado; aproximadamente un 80% de toda la dopamina que se encuentra en el cerebro se halla en el estriado. Este sistema es el implicado en la regulación motora y la ejecución de tareas, permitiendo que el movimiento se realice de forma armoniosa y obedezca a las órdenes voluntarias del individuo de acuerdo con patrones motores bien establecidos (Flóres y Pazos, 2003).

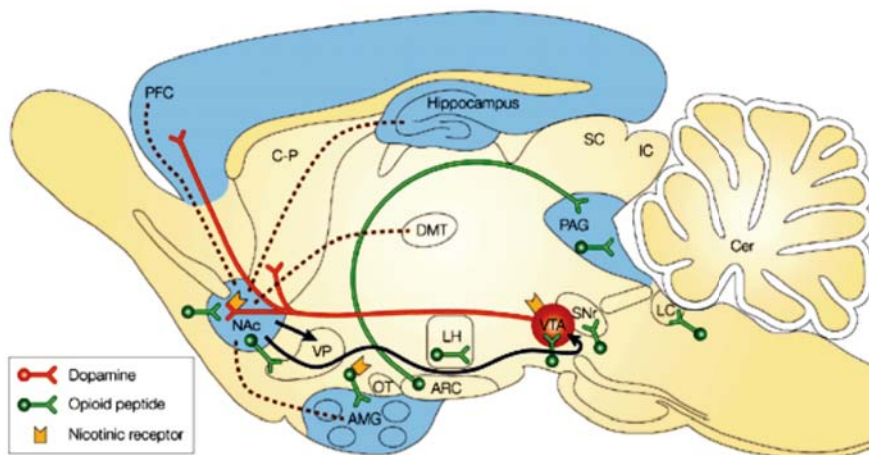


Cedido por Dr. Sergi Ferré

Figura 24. Representación de los circuitos dopaminérgicos.

Como hemos comentado anteriormente, lo que ocurre en los pacientes de Parkinson es que hay una pérdida de neuronas dopaminérgicas de la vía nigroestriada, dando lugar a claras anomalías motoras. La innervación dopaminérgica hacia regiones límbicas y corticales también está alterada, aunque en menor medida y al parecer, la enfermedad se manifiesta cuando la pérdida neuronal en el estriado representa el 80% (Elsworth y Roth, 1997).

El sistema mesolímbico-mesocortical tiene su origen en el área tegmental ventral, también localizada en el mesencefalo. Dicho núcleo contiene células dopaminérgicas que envían proyecciones a la corteza frontal y el lóbulo límbico, conformando los circuitos mesocortical y límbico respectivamente (Figura 25). El sistema mesolímbico se distribuye por el sistema límbico con excepción del hipocampo; principalmente se proyecta hacia el núcleo accumbens, tubérculo olfatorio, núcleo central de la amígdala, septum lateral y núcleo intersticial de la estria terminal (Flóres y Pazos, 2003).



Cedido por Dr. Sergi Ferré

Figura 25. Representación de las vías dopaminérgicas que se proyectan desde el área tegmental ventral (VTA) hasta el córtex prefrontal (PFC) y el núcleo accumbens (NAc), responsables de la conducta esencial del individuo.

El sistema mesocortical se proyecta desde la sustancia nigra y el área tegmental ventral hacia las cortezas motoras, promotoras y suplementarias y a las cortezas parietal, temporal y singular posterior, es decir, hasta las principales áreas sensorimotoras y de

asociación. Ambos sistemas contribuyen a mantener la atención, la ideación, la evaluación correcta de la realidad, la motivación y el control del pensamiento (Flóres y Pazos, 2003), es decir, están implicados en todos aquellos procesos en los que la motivación forma parte esencial de la conducta, ya sea fisiológica para atender necesidades elementales del individuo, o patológica, creada por hiperestimulación del sistema, que es lo que ocurre en procesos de adicción a sustancias de abuso. Los mecanismos implicados en estos últimos procesos se denominan sistemas de premio o recompensa, ya que son circuitos que al activarse producen un efecto placentero (Wise, 1996). La mayoría de sustancias que provocan adicción, interaccionan directa o indirectamente con proteínas presentes en las neuronas dopaminérgicas a nivel de vía mesolímbica-mesocortical, provocando un incremento de la liberación de dopamina, consiguiéndose sensaciones positivas y perdiéndose la sensibilidad a estímulos habituales. Cuando se interrumpe la administración aparecen sensaciones desagradables, depresión o falta de motivación (Noble et al., 1994).

El sistema mesolímbico-mesocortical parece jugar un papel importante en el desarrollo de la esquizofrenia. Las conexiones con el núcleo accumbens tienen una especial relevancia, ya que la falta de regulación de las vías dopaminérgicas mesolímbicas provocaría una descoordinación en el núcleo accumbens, que a su vez sobre-estimularía ciertas regiones implicadas en el procesamiento de la información de los sentidos, contribuyendo a los síntomas positivos de la esquizofrenia (alucinaciones, delirios, pensamientos incoherentes...). Por otro lado, dado que las vías dopaminérgicas mesocorticales juegan un papel fundamental en el buen funcionamiento cognitivo de la corteza prefrontal, alteraciones en este sistema estarían relacionadas con los síntomas negativos de la esquizofrenia (aislamiento social, retraimiento social, falta de iniciativa) (Abi-Dargham, 2004; Pani, 2002).

En las situaciones patológicas que se acaban de comentar se ha observado la existencia de diferencias cuantitativas en cuanto a la expresión de los receptores de dopamina o bien en su señalización. Por ejemplo, los receptores D₁ se ven incrementados en la esquizofrenia y su señalización varía en la enfermedad de Parkinson. La densidad de los receptores D₂ localizados post-sinápticamente incrementa en la esquizofrenia y también en los enfermos de Parkinson no tratados con L-DOPA (precursor de la dopamina capaz de traspasar la barrera hematoencefálica). Es por ello que el estudio de los receptores de dopamina es altamente importante, tanto para poder

entender una gran cantidad de anomalías funcionales tales como Parkinson, Alzheimer, esquizofrenia e hiperactividad, como para crear nuevas dianas terapéuticas para dichas anomalías.

Por último, el sistema tuberohipofisario se origina en el hipotálamo y se proyecta hacia la hipófisis. Las neuronas del sistema tuberohipofisario desempeñan un papel importante en la regulación de la liberación de hormonas pituitarias, como por ejemplo la prolactina, en la que la dopamina juega un papel inhibitorio en la liberación de esta hormona (Dale, 2000).

1. 5 Receptores adrenérgicos y vías adrenérgicas en el SNC

1.5.1 La adrenalina como neurotransmisor

A pesar de que fue en 1913 cuando se aisló por primera vez la adrenalina y se probaron sus efectos sobre la vasodilatación y la vasoconstricción, no fue hasta la década de 1950 que se estableció la función neurotransmisora de las catecolaminas (noradrenalina y adrenalina) en el encéfalo. Fue en 1988 cuando se definió que las neuronas noradrenérgicas de la periferia son neuronas simpáticas postganglionares, cuyos cuerpos celulares se encuentran en los ganglios simpáticos (Marshall et al., 1991; Fillenz, 1990; Weiner et al., 1967). Generalmente poseen largos axones que terminan en una serie de varicosidades dispersas a lo largo de la red terminal ramificada. Estas varicosidades contienen numerosas vesículas sinápticas que constituyen el lugar de síntesis y liberación de la adrenalina y noradrenalina, junto a otros mediadores tales como ATP y neuropéptido Y.

El precursor metabólico de la adrenalina y noradrenalina es la L-tirosina, un aminoácido aromático presente en el plasma y los fluidos intersticiales, que es captado por las neuronas adrenérgicas. La tirosina hidroxilasa, una enzima citosólica que cataliza la conversión de tirosina a dihidroxifenilalanina, se encuentra sólo en las células que contienen catecolaminas (Figura 26). Es una enzima bastante selectiva, ya que a diferencia de otras enzimas implicadas en el metabolismo de las catecolaminas, no acepta derivados indólicos como sustratos y, por tanto, no interviene en el metabolismo de la 5-hidroxitriptamina. Este primer paso de hidroxilación es el principal punto de control para la síntesis de noradrenalina. La tirosina hidroxilasa es inhibida por el producto final de la vía biosintética, la adrenalina; lo que proporciona un mecanismo de regulación de la velocidad de síntesis (Kanagy, 2005; Wurtman, 2002; Sneader, 2001; Fuller y Wong, 1977).

El siguiente paso, la conversión de DOPA en dopamina, está catalizado por la dopa descarboxilasa, una enzima citosólica que no se limita a las células que sintetizan catecolaminas. Es una enzima relativamente inespecífica y cataliza la descarboxilación de otros aminoácidos L-aromáticos, como L-histidina y L-triptófano, que son los

precursores en la síntesis de histamina, y 5-hidroxitriptamina, respectivamente. La actividad de la dopa descarboxilasa no limita la velocidad de síntesis de adrenalina, con lo que, a pesar de que existen varios fármacos que actúan sobre esta enzima, no es un medio eficaz de regulación de la síntesis de noradrenalina y adrenalina (Daubner, 2011; Kanagy, 2005; Elsworth, 1997; Axelrod, 1972; Axelrod y Weinshilboum, 1972)

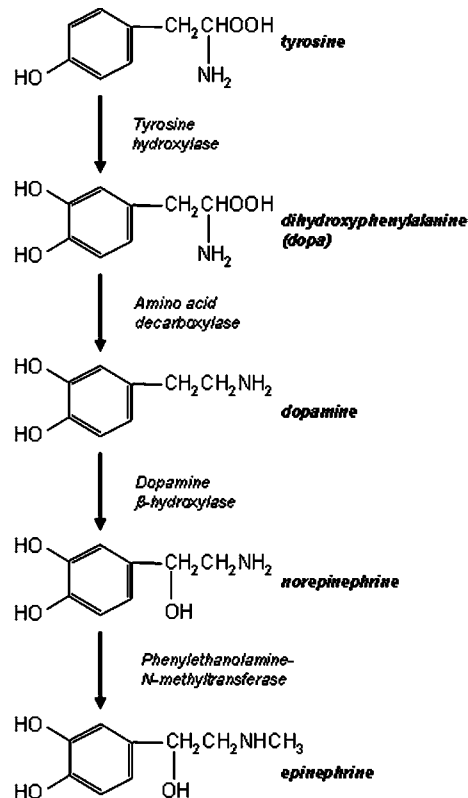


Figura 26. Esquema de la vía de biosíntesis de la noradrenalina y adrenalina.

Extraído de Kanagy, 2005

La dopamina-β-hidroxilasa también es una enzima relativamente inespecífica, pero se encuentra restringida a las células que sintetizan catecolaminas y se localiza en las vesículas sinápticas, con lo que una pequeña cantidad se libera en las terminaciones nerviosas adrenérgicas. Muchos fármacos que inhiben la dopamina-β-hidroxilasa, como los quelantes de cobre y disulfiram, pueden causar depleción parcial de los depósitos de noradrenalina y una interferencia en la transmisión simpática (Daubner, 2011; Liu y Edwards, 1997).

La feniletanolamina N-metiltransferasa cataliza la N-metilación de noradrenalina a adrenalina. La principal localización de este enzima es la médula suprarrenal, pero

también se encuentra en ciertas zonas del encéfalo donde la adrenalina puede funcionar como neurotransmisor.

La mayor parte de noradrenalina y adrenalina de las terminaciones nerviosas se encuentra en las vesículas y sólo una pequeña parte está libre en el citoplasma en circunstancias normales. La concentración en las vesículas es muy elevada (0,3-1mol/l) y se conserva por un mecanismo de transporte similar al del transportador de aminas responsable de la recaptación de noradrenalina en la terminación nerviosa. En estas vesículas, y junto a la noradrenalina, hay otros constituyentes como ATP y cromogranina A que se liberan en el momento de la sinapsis (Sugita, 2008; Esler et al., 2003; Lundberg, 1996), y tienen funciones diversas tal como la producción del potencial sináptico excitador rápido.

Los procesos ligados a la llegada de un impulso nervioso a una terminación nerviosa noradrenérgica para la liberación de noradrenalina son básicamente los mismos que los de otras sinapsis de transmisión química. La despolarización de la membrana de la terminación nerviosa abre sus canales de calcio y la entrada resultante induce la fusión y descarga de las vesículas sinápticas. Se produce un mecanismo de efecto inhibitor inducido por la adrenalina liberada al espacio sináptico denominado retroalimentación autoinhibidora y mediado por receptores α_2 -adrenérgicos presentes en la membrana de la neurona presináptica (Kanagy, 2005; Starke et al., 1989).

1.5.2 Clasificación, estructura y farmacología de los receptores adrenérgicos

En la primera clasificación de los receptores adrenérgicos, realizada por Ahlquist y colaboradores, se definió que el orden de la potencia de diversas catecolaminas, como adrenalina, noradrenalina e isoprenalina, tenía dos patrones diferentes dependiendo de la respuesta que se determinara. Así, se postuló por primera vez la existencia de dos tipos de receptores, definidos como α y β , en función de la potencia del agonista. Los α tenían más afinidad por la noradrenalina y la adrenalina que por la isoprenalina (una catecolamina sintética) y los β mostraban más afinidad por isoprenalina que por noradrenalina. Experimentos posteriores con antagonistas específicos para receptores α

y β adrenérgicos hipotetizaron la existencia de subdivisiones adicionales dentro de estas subfamilias (Rang et al., 2008). Esta hipótesis se confirmó al analizar la farmacología de los receptores y permitió determinar la existencia de dos subfamilias dentro de los receptores α adrenérgicos (α_1 y α_2) y tres subfamilias dentro de los receptores β adrenérgicos (β_1 , β_2 y β_3) (Figura 27). Todos los receptores adrenérgicos son receptores acoplados a proteína G típicos y su clonación ha revelado que cada uno de los receptores α_1 y α_2 comprende tres subclases adicionales que se expresan en diferentes localizaciones (Sugita, 2008; Liggett, 2003; Bylund et al., 1994).

Cada una de estas clases de receptor se asocia a un sistema de segundo mensajero específico. Por ejemplo, los receptores α_1 adrenérgicos están acoplados a la fosfolipasa C y ejercen sus efectos principalmente mediante la liberación de calcio intracelular. Los receptores α_2 adrenérgicos están acoplados a G_i , con lo que su activación hace disminuir los niveles de cAMP intracelular e inhibe canales de calcio. Y por último, los tres subtipo de receptores β actúan estimulando la adenilato ciclasa y activando canales de calcio (Figura 27).

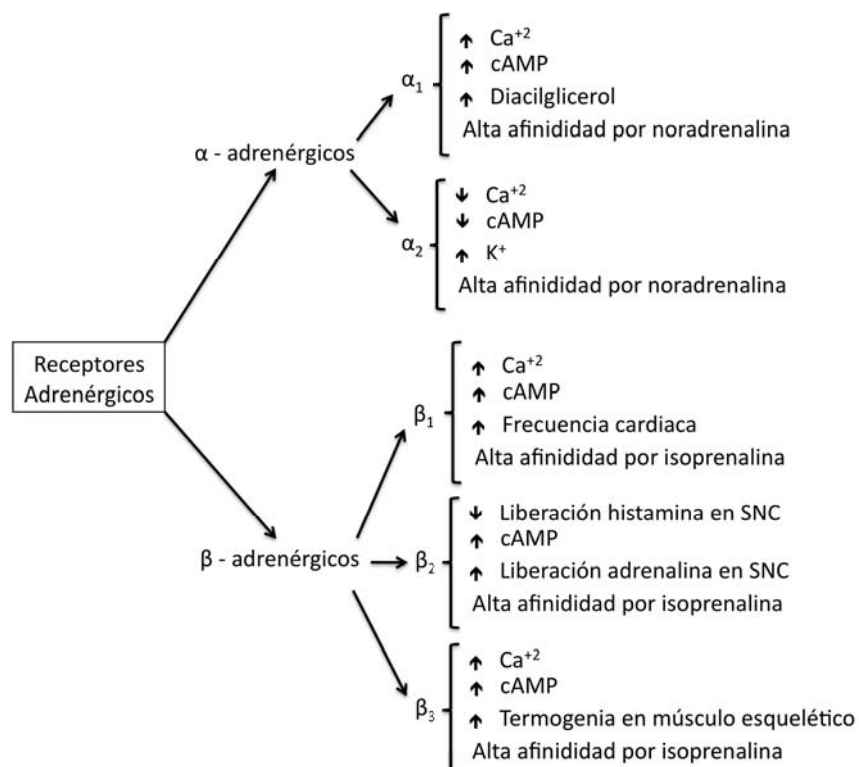
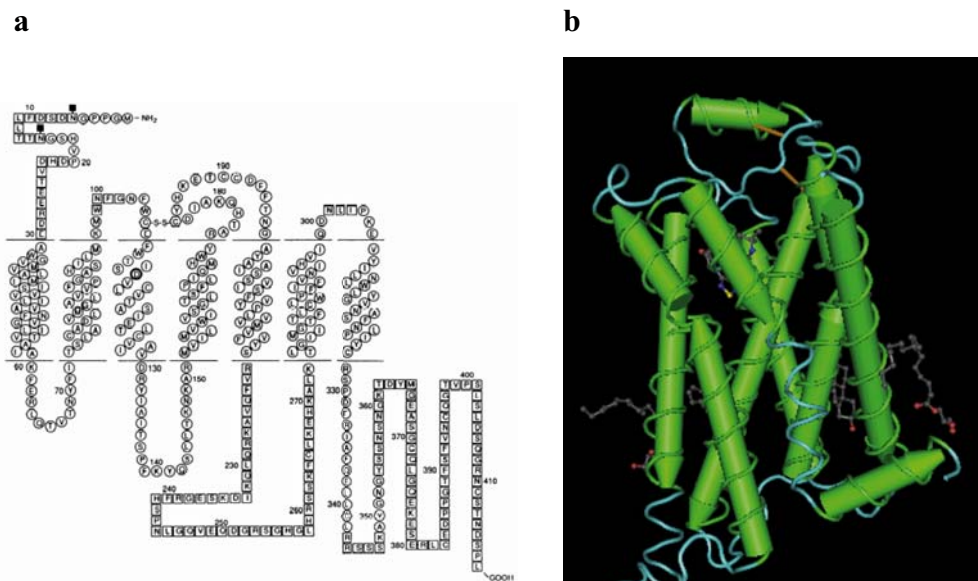


Figura 27. Esquema de la clasificación de los receptores adrenérgicos y sus funciones principales.

Los receptores adrenérgicos, como otros GPCRs, poseen un dominio N-terminal extracelular y un dominio C-terminal intracelular y diferentes bucles intracelulares y extracelulares producidos por los loops que conectan unas hélices con otras (Figura 28). Los bucles intracelulares permiten a los receptores adrenérgicos interactuar con proteínas de la cascada de señalización tales como β -arrestina y dinamina (Cotecchia, 2010; Tan et al., 2009; Small et al., 2006; Volovyk et al., 2006), mientras que los bucles extracelulares forman un ‘bolsillo’ estructural que permite la unión del ligando al receptor. A pesar de que la homología en la secuencia aminoacídica entre la familia α y β es baja, no hay diferencias significativas en la estructura y tamaño entre ambas familias. Cabe destacar que dentro de las distintas subfamilias se encuentran secuencias muy homólogas, indicando que probablemente están relacionadas filogenéticamente y que la similitud de su estructura transmembrana no se deba únicamente a requerimientos funcionales comunes, sino a un antecesor funcional común (Rang et al., 2008; Garland y Biaggioni, 2001).



Extraído de Rang et al., *Farmacología*. Editado por ELSEVIER. 2008 y Warne et al., 2011

Figura 28. Estructura de un receptor adrenérgico.

(a) Modelo de distribución de siete hélices de transmembrana de un receptor β -adrenérgico. (b) Modelo tridimensional del receptor β_1 adrenérgico.

A pesar de que en el organismo, los ligandos endógenos principales son la adrenalina y noradrenalina (con diferente afinidad para cada receptor adrenérgico), actualmente existe una gran variedad de agonistas y antagonistas selectivos y

específicos para cada familia y cada miembro de las subfamilias que se utilizan como fármacos para diferentes patologías. Estos fármacos, dirigidos directa o indirectamente a los receptores adrenérgicos, se clasifican en cinco subclases dependiendo de su funcionalidad (Kobilka, 2011; Rang et al., 2008; de Boer et al., 1999; Pfeffer y Stevenson, 1996):

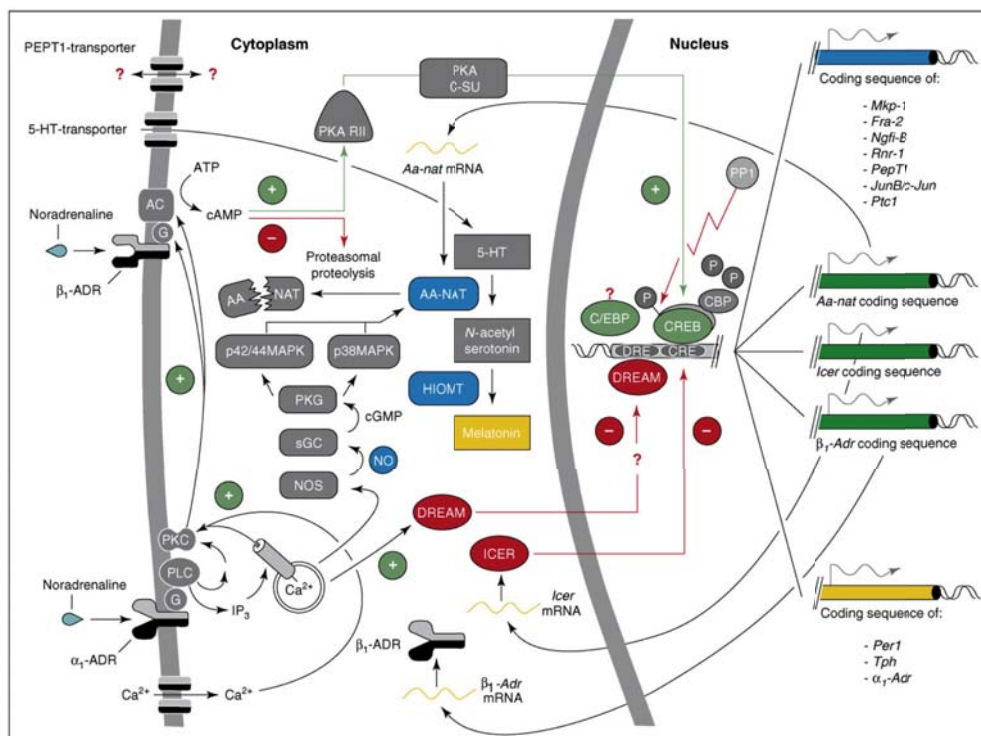
- 1- Simpaticomiméticos: son fármacos selectivos para receptores α o β adrenérgicos cuya acción varía dependiendo de la subfamilia del receptor y el tejido de acción. Por ejemplo, el salmeterol o la terbutalina son agonistas β_2 selectivos que permite la vasodilatación en situaciones de asma o asfíxia.
- 2- Antagonistas: son ligandos selectivos que se unen a los receptores α o β adrenérgicos que antagonizan la función de éstos y bloquean la unión del ligando endógeno. Su efecto también varía en función del receptor y el tejido. Por ejemplo, el propranolol o el alprenolol que actúan sobre toda la familia de receptores β adrenérgicos regulando la hipertensión mediante relajación de la musculatura lisa.
- 3- Inhibidores de síntesis: tales como la α -metil-p-tirosina o la metildopa, son fármacos que inhiben la síntesis de noradrenalina endógena alterando la funcionalidad de los enzimas o la concentración de precursores.
- 4- Inhibidores de liberación: son fármacos que inhiben la liberación de noradrenalina endógena causando depleción de adrenalina y daño irreversible en neuronas noradrenérgicas. El más conocido es la guanetidina.
- 5- Alteradores de la recaptación: tales como la imipramina, son aquellos fármacos que alteran la recaptación de noradrenalina del espacio sináptico aumentando o disminuyendo la funcionalidad de los transportadores de catecolaminas presentes en la membrana de la neurona pre-sináptica.

1.5.3 Los receptores α_1 y β_1 adrenérgicos

El receptor α_1 adrenérgico es el primer miembro de la subfamilia de α -receptores. Existen tres subtipos de receptores denominados α_{1A} , α_{1B} y α_{1D} que se diferencian entre ellos por sus propiedad farmacológicas y localización en el organismo. En general, los receptores α_1 adrenérgicos se acoplan a proteína G_q , cuya activación

inducida por el intercambio de GDP por GTP produce la activación de la fosfolipasa C (PLC) y la consiguiente fosforilación de la proteína quinasa C (PKC). Esta fosfolipasa hidroliza al fosfatidilinositol 4,5-bifosfato (PIP₂) dando lugar a inositol trifosfato (IP₃) y a diacilglicerol (DAG). Estos metabolitos secundarios permiten la apertura de los canales de calcio de los retículos endoplasmático y sarcoplasmático aumentando así los niveles de calcio intracelular, con la activación de una cascada de fosforilación que conlleva la inducción de varios factores de transcripción (Johnson y Liggett, 2011; Cotecchia, 2010; Maronde y Stehle, 2007; Schmitz et al., 1981) (Figura 29).

El receptor β_1 adrenérgico es el primer miembro de la subfamilia de β -receptores, y actualmente no se han descrito submiembros dentro de esta subfamilia. Se acopla a proteína G_s, con lo que su activación produce un aumento de AMPc catalizado por la adenilato ciclasa. Este incremento de AMPc intracelular induce la activación de CREB mediante fosforilación y la inhibición de la degradación del enzima AA-NAT (Grimm y Brown, 2010; Schiattarella et al., 2010; Maronde y Stehle, 2007), enzima responsable de la síntesis de melatonina.



Extraído de Maronde y Stehle, 2007

Figura 29. Cascada de activación intracelular inducida por la unión de noradrenalina a los receptores α_{1B} y β_1 adrenérgicos en la glándula pineal.

La acción fisiológica inducida por la activación de estos receptores depende directamente de su localización en el organismo. El receptor α_1 adrenérgico se localiza principalmente en la musculatura lisa, cuya activación produce la vasoconstricción de los vasos sanguíneos (Elliott, 1997; Stiles et al., 1983a); y el receptor β_1 adrenérgico se localiza principalmente en glándulas salivales, cardiomiocitos y córtex cerebral, cuya activación produce la secreción de amilasa y un aumento de la frecuencia cardíaca (Grimm y Brown, 2010; Ranade et al., 2002; Moore et al., 1999; Stiles et al., 1983b). Cabe destacar que la presencia de receptores adrenérgicos en el sistema nervioso central (SNC) es minoritaria en comparación con otros tejidos, ya que el número de neuronas noradrenérgicas en el encéfalo es reducido y estos receptores ejercen una gran variedad de funciones en diferentes tejidos del organismo.

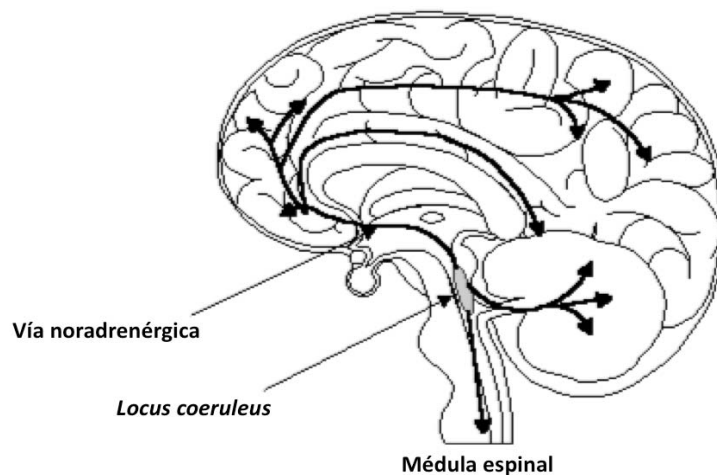
Entre los ligandos más utilizados para los receptores α_1 encontramos la fenilefrina ($K_D = 4,7$ nM) que actúa como agonista selectivo de la familia α_1 con mayor afinidad por α_{1A} y α_{1B} , (Morton et al., 2007; Minneman et al., 1994) y REC 15/2615 ($K_D = 0,3$ nM) que actúa como antagonista selectivo de la familia α_1 con mayor afinidad por α_{1B} (Morston et al., 2007). Y para los receptores β_1 adrenérgicos se utiliza principalmente el isoproterenol, que actúa como agonista de receptores β adrenérgicos sin ser selectivo para β_1 (Akimoto et al., 2002; Schmitt y Stork, 2000) y CGP 20712 ($K_D = 0,3$ nM) que actúa como antagonista selectivo para receptores β_1 adrenérgicos con mil veces más afinidad por β_1 que por β_2 (Hieble et al., 1995).

1.5.4 Vías noradrenérgicas en el SNC

Los primeros mapas detallados de las vías noradrenérgicas en el encéfalo se realizaron a partir de estudios de fluorescencia basada en la formación de un derivado fluorescente de las catecolaminas cuando los cortes histológicos se exponían al formaldehído. Los cuerpos celulares de las neuronas noradrenérgicas se agrupan en pequeños núcleos situados en la protuberancia y el bulbo raquídeo, desde donde envían axones con extensas ramificaciones hasta otras muchas partes del encéfalo y la médula espinal (Figura 30). El núcleo más importante es el *locus coeruleus* (LC), localizado en la sustancia gris de la protuberancia. Aunque en el ser humano sólo contiene unas

10.000 neuronas noradrenérgicas, los axones acaban en muchos millones de terminaciones nerviosas noradrenérgicas distribuidas por toda la corteza, el hipocampo, el área tegmental ventral (VTA) y el cerebelo (Meitzen et al., 2011; Mandela y Ordway, 2006; Aston-Jones, 2005a; Aston-Jones y Cohen, 2005b). Estas terminaciones nerviosas no establecen contactos sinápticos separados, sino que parecen liberar el transmisor de una manera difusa.

El *locus coeruleus* (LC) es el origen de la mayor parte de la noradrenalina liberada en el encéfalo y, por lo tanto, es el núcleo de neuronas noradrenérgicas que ha recibido más atención, ya que en él se puede medir la actividad neuronal mediante electrodos implantados. En general, las neuronas del LC permanecen silentes durante el sueño y su actividad aumenta con la activación conductual. Los estímulos amenazantes excitan a estas neuronas con mucha mayor eficacia que los estímulos familiares y, debido a ello, se cree que la depresión se debe, en parte, a una diferencia funcional de noradrenalina en determinadas regiones encefálicas (Garland et al., 2002; Delgado y Moreno, 2000), a pesar de que la 5-hidroxitriptamina (5-HT) también podría tener un papel importante en las mismas áreas del SNC en esta patología.



Extraído y modificado de *Brain Vascular Disorder. Canada*

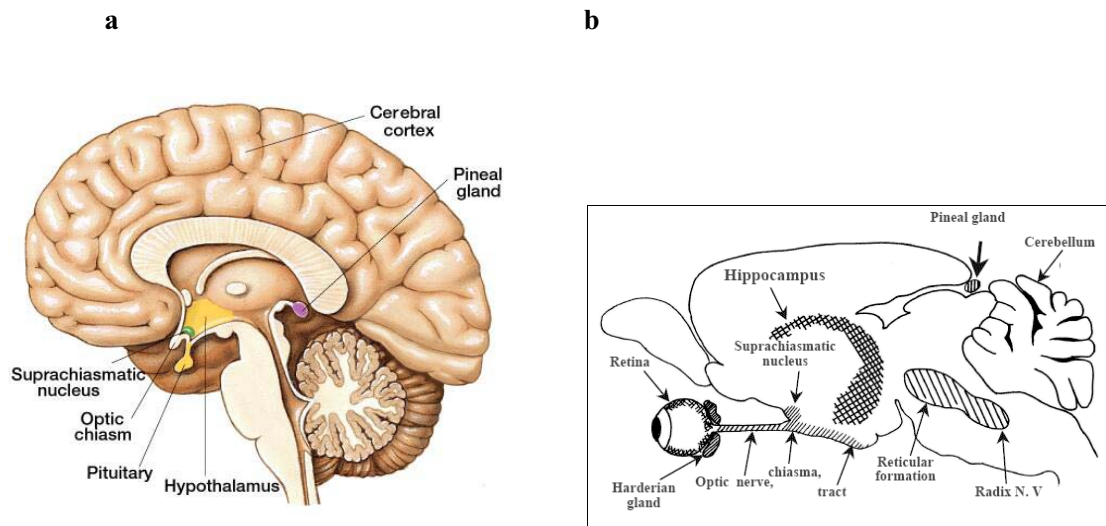
Figura 30. Vías noradrenérgicas en el SNC que parten del *locus coeruleus*.

En las proximidades del *locus coeruleus* en la protuberancia y el bulbo raquídeo existen otras neuronas noradrenérgicas cuyos axones inervan el hipotálamo y el

hipocampo, entre otras partes, además de proyectarse hacia el cerebelo y la médula espinal (Gargaglioni et al., 2010; Sara, 2009; Fung et al., 1994; Sasa y Yoshimura, 1994). Existe también otro grupo más pequeño de neuronas adrenérgicas cuyos cuerpos celulares se encuentran en una situación más ventral del tronco del encéfalo. Sus fibras se dirigen sobre todo a la protuberancia, el bulbo y el hipotálamo, y liberan adrenalina en lugar de noradrenalina. Los conocimientos sobre estas últimas neuronas son muy escasos, pero se cree que son importantes para el control cardiovascular (Keys y Koch, 2004; Ma y Huang, 2002)

1.6 Receptores adrenérgicos y dopaminérgicos en la Glándula pineal

La glándula pineal, también llamada cuerpo pineal o epífisis, es una pequeña glándula endocrina de secreción interna en el sistema nervioso central presente en el cerebro de los vertebrados. En los humanos, tiene un tamaño de cinco milímetros de diámetro y presenta una formación ovoidea y aplanada, que descansa sobre la lámina cuadrigémina, en el tercer ventrículo cerebral en el techo del diencefalo (Figura 31a). En los roedores, su tamaño varía en función de la especie, pero se sitúa siempre en la parte exterior del encéfalo, entre el córtex posterior y el cerebelo (Figura 31b).



Extraído y modificado de Humanity Healing Network, 2010 y Konturek et al., 2006

Figura 31. Localización de la glándula pineal en el SNC de humanos y roedores.

a) Representación esquemática de la localización de la glándula pineal en el encéfalo de humanos. Situada en el techo del diencefalo. b) Representación de la localización de la glándula pineal en el encéfalo de roedores. Situada en la parte posterior frente al cerebelo.

Muchas de las funciones de la glándula pineal son actualmente desconocidas, así como su relación con el sueño y las visiones y experiencias extra-corpóreas. A pesar de ello, se sabe que es la glándula responsable de la síntesis y secreción de melatonina (N-acetil-5-metoxitriptamina), una hormona que deriva de la serotonina y es responsable de la regulación de una gran variedad de procesos celulares neuroendocrinos y neurofisiológicos; entre los cuales, cabe destacar el ritmo circadiano

de noche y día y de las funciones estacionales (Arendt, 2005; Macchi et al., 2004; Lerner et al., 1960).

A pesar de que la glándula pineal está formada en un 90% por pinealocitos, también posee otros tipos de células con funciones diversas:

- Pinealocitos: es la población mayoritaria de células de la glándula pineal que son las encargadas de la producción y liberación de melatonina. Poseen un orgánulo llamado cinta sináptica en el que se encuentran las enzimas serotonina N-acetiltransferasa y acetilserotonina O-metiltransferasa capaces de transformar la serotonina en melatonina. Los pinealocitos poseen prolongaciones citoplasmáticas largas que les permiten establecer contacto con los capilares y liberar la melatonina al torrente sanguíneo (Maronde y Stehle, 2007; Borjigin y Deng, 2000).

- Células intersticiales: son células que se localizan entre los pinealocitos y tienen como función dar soporte estructural a la glándula pineal y aportar nutrientes y metabolitos para los pinealocitos (Kaissling et al., 1996).

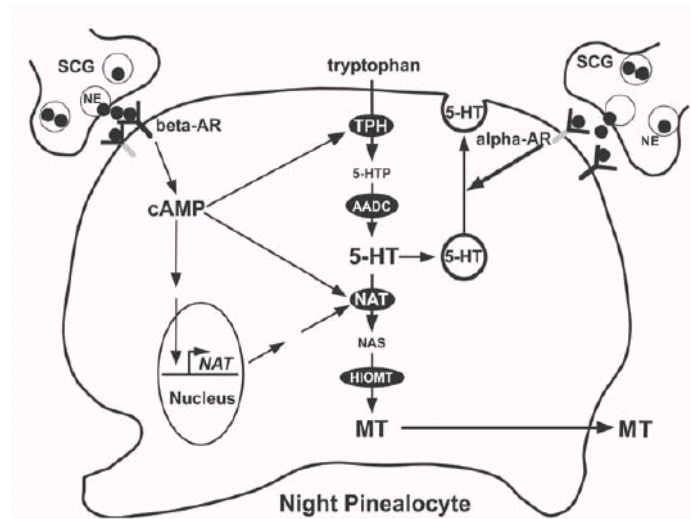
- Fagocitos perivascuales: localizados en los microcapilares que penetran en la glándula pineal, tienen como función la defensa inmunológica y la presentación de antígenos (Møller et al., 2006; Møller y Baeres, 2002).

- Neuronas pineales: su población es muy reducida (<1%) y están solo presentes en los vertebrados superiores, pero no en roedores. Actualmente se desconoce su función (Kriegebaum et al., 2010; López-Muñoz et al., 2010).

- Neuronas peptidérgicas: solo presentes en algunas especies de los mamíferos superiores, la población de estas neuronas es extremadamente reducida (<0,1%) y su función es la liberación de polipéptidos con acción paracrina (Nowicki et al., 2007; Møller y Baeres, 2002; Matsushima et al., 1999).

La glándula pineal recibe inervaciones simpáticas procedentes del ganglio cervical superior e inervaciones parasimpáticas procedentes de la esfenoopalatina y el ganglio ótico. Algunas de estas innervaciones penetran en la glándula, pero la mayor parte de ellas la irrigan de forma superficial permitiendo la entrada del metabolito por difusión. A su vez, neuronas procedentes del núcleo supraquiasmático (Maronde y Stehle, 2007), el ganglio cervical superior y del ganglio trigeminal inervan a la

glándula pineal, liberando de forma superficial neuropeptina, PACAP (polipéptido activador de la adenilato ciclasa pituitaria), dopamina y noradrenalina, que penetran en ella por difusión pasiva (Tapp et al., 1972; Axelrod, 1970). Desde hace ya varias décadas, se conoce que en la glándula pineal se expresan los receptores α_{1B} y β_1 adrenérgicos, a los cuales, se une la noradrenalina liberada permitiendo así la activación o inhibición de muchas funciones celulares como la regulación de la síntesis de melatonina y su liberación. En los mamíferos superiores existe una inervación entre el nervio ocular y la glándula pineal, lo que demuestra una relación directa de las funciones de esta glándula con los ciclos de luz y oscuridad y, por consiguiente, una relación directa con la regulación del ritmo circadiano.



Extraído de Borjigin y Deng. Madame Curie

Bioscience Database. Landes Bioscience. 2000

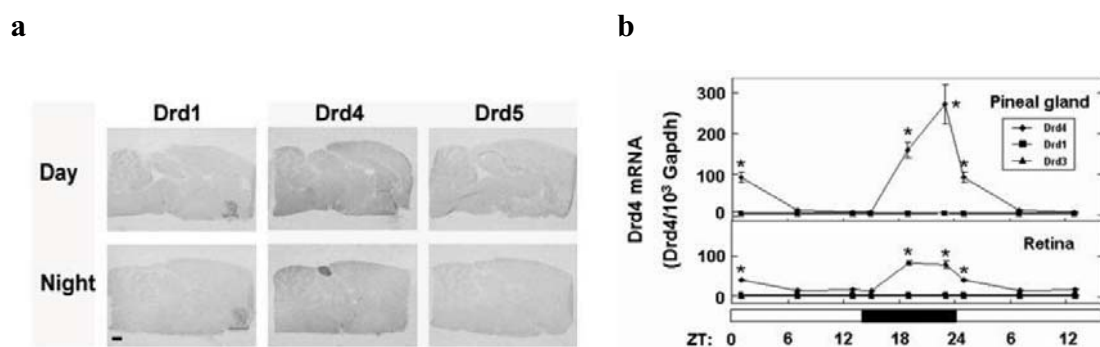
Figura 32. Localización de la glándula pineal en el SNC de humanos y roedores

Se ha demostrado que los receptores adrenérgicos presentes en la membrana de los pinealocitos son los responsables directos de la síntesis y liberación de melatonina en la glándula pineal (Hardeland et al., 2011; Abbas et al., 2010; Mitchell et al., 2010; Rios et al., 2010; Borjigin y Deng, 2000). La noradrenalina liberada en la glándula pineal activa receptores β -adrenérgicos que inducen un incremento de los niveles de AMPc, responsable de la activación de los enzimas Triptófano Hidroxilasa (TPH) y Serotonina-N-Acetiltransferasa (NAT) induciendo la activación de la síntesis de

melatonina. A su vez, la noradrenalina activa receptores α -adrenérgicos que inducen la liberación de serotonina y melatonina mediante difusión vesicular probablemente mediada por incrementos en la concentración de Ca^{+2} intracelular.

A pesar de que la expresión de los receptores adrenérgicos no está en función del ritmo circadiano, se conoce que durante el periodo de oscuridad se produce un aumento significativo de la producción de melatonina durante las primeras horas del sueño, mientras que durante el periodo de luz, esta producción está altamente inhibida (Sun et al., 2002; Borjigin et al., 1999)

Ha sido recientemente cuando se ha descrito, por primera vez, que en la glándula pineal se expresa también el receptor D_4 de dopamina y que presenta un patrón de expresión característico regulado por el ritmo circadiano (Figura 33), siendo éste el único receptor dopaminérgico presente en la glándula pineal de rata (Kim et al., 2010; Baylei et col., 2008).



Extraído de Kim et al., 2010

Figura 33. Variación del mRNA de D_4 en la glándula pineal en periodos de luz/oscuridad.

a) Hibridación in situ del mRNA del receptor de D_4 de dopamina en la glándula pineal de rata durante el día (luz) o la noche (oscuridad). b) qRT-PCR del mRNA de D_4 de glándulas pineales de ratas extraídas a diferentes horas del día y la noche para determinar el aumento de expresión en periodos de oscuridad.

Mediante técnicas de hibridación *in situ* se observó que la expresión del mRNA de D_4 era prácticamente nula durante el periodo de luz, en cambio, la expresión de este receptor aumentaba drásticamente durante el periodo de oscuridad (Figura 33a). Mediante experimentos de qRT-PCR con glándulas pineales de ratas extraídas a diferentes horas del día y la noche, se demostró que el mRNA del receptor D_4 de dopamina aumentaba hasta 300 veces su expresión en el periodo de oscuridad (Figura

33b) (Kim et al., 2010), lo que indica que este receptor juega un papel importante en la glándula pineal en los periodos de oscuridad y, por lo tanto, posiblemente tiene relación con la regulación del ritmo circadiano.

Se ha descrito la existencia de receptores D_1 y D_2 de dopamina en la glándula pineal de pollo y buey (Santanavanich et al., 2005; Zawilska et al., 2004; Simonneaux et al., 1990). Se ha demostrado que la glándula pineal está inervada por terminaciones nerviosas que liberan dopamina. Esta catecolamina se une a los receptores D_1 y D_2 de dopamina presentes en la membrana de los pinealocitos, produciendo una activación y una inhibición de la síntesis de melatonina, respectivamente. Zawilska y colaboradores demostraron que el tratamiento de glándulas pineales con el agonista SKF 38393 (agonista selectivo de D_1) producía la activación del enzima AA-NAT, cuya función es la síntesis directa de melatonina (Zawilska et al., 2004). Un año después, otro estudio demostró que el tratamiento de glándulas pineales con el mismo agonista SKF 38393 producía un aumento del AMPc intracelular que inducía la fosforilación de la proteína CREB, responsable de la activación del enzima AA-NAT. A su vez, se demostró que el tratamiento de las glándulas pineales con quinpirole (agonista selectivo de D_2 -like) producía una disminución del AMPc intracelular, disminuyendo la fosforilación de la proteína CREB y la consecuente inhibición del enzima AA-NAT (Santanavanich et al., 2005). Estos trabajos demostraron que existe algún tipo de interacción (por ejemplo: cross-talk intracelular) entre receptores de dopamina y adrenérgicos que permite una fina regulación de la síntesis de melatonina y un mantenimiento de los ritmos circadianos en la glándula pineal.

II. OBJETIVOS

La dopamina desempeña un papel muy importante en la neurotransmisión. Ejerce sus acciones a través de la interacción con receptores acoplados a proteína G que se subdividen en dos grandes subtipos, los receptores de la familia D₁ (D₁ y D₅) y los receptores de la familia D₂ (D₂, D₃ y D₄). Todos ellos están expresados de manera diferencial en distintas áreas del cerebro por lo que son las dianas de un elevado porcentaje de los agentes terapéuticos de acción en sistema nervioso central y continúan siendo el principal foco de atención de muchas investigaciones biomédicas. La mayoría de estrategias terapéuticas dirigidas a GPCR neuronales se basan en el uso de agonistas o de antagonistas específicos para un determinado tipo de receptor. Sin embargo, en la actualidad, los ligandos alostéricos que discriminan entre distintos subtipos de receptores se están revelando como fármacos alternativos a agonistas o antagonistas ortostéricos. Teniendo en cuenta este hecho, *el primer Objetivo General de esta Tesis ha sido caracterizar ligandos alostéricos para receptores de dopamina de la familia D₁-like*. El grupo de investigación, en el cual se ha desarrollado esta Tesis Doctoral, dispone de una quimioteca de híbridos indoloquinolicidina-péptido como ligandos múltiples de receptores D₁ y D₂ de dopamina que podrían ser potenciales moduladores alostéricos de estos receptores, por lo que el primer objetivo concreto de esta Tesis ha sido:

- **Objetivo 1.1. Caracterizar un ligando indoloquinolicidinapéptido como modulador alostérico de los receptores de dopamina D₁ y su posible importancia en alteraciones psicóticas.**

La modulación de los receptores acoplados a proteína G no solo ocurre a través de ligandos. La visión de estos receptores ha evolucionado en los últimos años. Clásicamente se consideraban unidades individuales capaces de producir una señal intracelular pero actualmente está aceptado que estas proteínas de membrana pueden interaccionar con otros receptores acoplados a proteína G para formar heterómeros y que estas interacciones pueden dar lugar a nuevas propiedades farmacológicas y funcionales de los receptores involucrados. Los heterómeros son, por tanto, nuevas entidades funcionales en las cuales un receptor puede modular la actividad del otro

receptor en el heterómero. Teniendo en cuenta este hecho, *un segundo Objetivo General de esta Tesis ha sido investigar si distintos receptores de dopamina de la familia D₂-like pueden modular su función mediante un proceso de heteromerización*. Dado que los receptores de dopamina D₂ y D₄ pueden colocalizar en varias zonas del cerebro, incluyendo el estriado y que existe una clara relación entre la forma polimórfica D_{4,7} del receptor D₄ humano con el trastorno de hiperactividad y déficit de atención, se plantearon los siguientes objetivos concretos:

- **Objetivo 2.1. Determinar si los receptores D₂ y D₄ de dopamina pueden formar heterómeros en células vivas y, en tal caso, estudiar su funcionalidad y la existencia de diferencias en la heteromerización entre las distintas formas polimórficas del receptor D₄ humano.**
- **Objetivo 2.2. Investigar la presencia de heterómeros de receptores de dopamina D₂-D₄ funcionales en el tejido estriatal y estudiar su papel en la liberación de glutamato en el estriado**

Recientemente, se ha descrito la presencia del receptor D₄ de dopamina como único receptor dopaminérgico en la glándula pineal de rata. Este receptor presenta un patrón de ritmo circadiano, ya que su expresión aumenta durante la noche (oscuridad) y disminuye durante el día (luz) sin que se conozca cual es su función en la glándula pineal. Ello sugiere que quizás su función principal sea la modulación de otros receptores. Se conoce que la funcionalidad de la glándula pineal está bajo el control de los receptores α_{1B} y β_1 adrenérgicos, cuya activación está altamente relacionadas con la regulación del ritmo circadiano y la síntesis y liberación de serotonina y melatonina. Dentro de este marco, *un tercer Objetivo General de esta Tesis ha sido investigar si los receptores de dopamina D₄ pueden modular la función de los receptores adrenérgicos de la glándula pineal mediante un proceso de heteromerización*. Para ello se han propuesto los siguientes objetivos concretos:

• **Objetivo 3.1. Determinar si los receptores D₄ de dopamina pueden formar heterómeros con los receptores α_{1B} y β_1 adrenérgicos en células vivas y, en tal caso, estudiar su funcionalidad.**

• **Objetivo 3.2. Investigar la presencia de heterómeros funcionales entre receptores de dopamina D₄ y receptores α_{1B} y β_1 adrenérgicos en la glándula pineal de rata y estudiar su función en la síntesis y liberación de serotonina y melatonina.**

Si bien técnicas biofísicas de transferencia de energía como BRET, FRET o sus derivados han permitido detectar la formación de heterómeros entre receptores acoplados a proteína G en células vivas, es difícil la aplicación de estas técnicas para determinar la expresión de heterómeros en tejidos nativos. Por ello la búsqueda de ligandos específicos para receptores que puedan acoplarse a un sistema de transferencia de energía es de gran interés. A este respecto *un último Objetivo General de esta Tesis ha sido contribuir al desarrollo de ligandos para receptores de dopamina útiles para detectar heterómeros que contengan estos receptores*. Para ello se ha propuesto el siguiente objetivo concreto:

• **Objetivo 4.1. Desarrollar y caracterizar ergopéptidos biotinilados como herramientas para el estudio de heterómeros que contengan receptores de dopamina**

CGTAAATCTGTAAGCAGCTAAGCATGCTACGGGGAAAGCTATGCAATTATATAATTACGTGGCCATGATGCTACGATCTAAACACAGCTGGATGATGACGATCGATGATGAGCCTAAGCTAAGCACTTATAATAATAGCTAAGATGGGGCTCGTCTGTAGTCGTGTGCTAGCTAGATTATCGATGCTGCTAAGGATCGATGCT

« Si la question de la priorité de l'œuf sur la poule ou de la poule sur l'œuf vous embarrasse, c'est que vous supposez que les animaux ont été originaires ce qu'ils sont à présent.

Quelle folie! »

Denis Diderot

RESULTADOS

AGTCATCGTCGTAAGTACGATCGTACTTACAGTAAAATCTGTAGCAGCTAGCATGTTTTCTTTGGGTGTACGATCGACTGATCGTGCATACTACGATCACTACTACGGCGA

Rafael Franco Fernández y Josefa Mallol Montero
Grupo de Neurobiología Molecular
Dep. de Bioquímica y Biología Molecular
Av. Diagonal, 645
Edificio Nuevo, Planta-2
08028 Barcelona

La tesis doctoral de Sergio González González “Receptores de Dopamina y Heterómeros de Receptores de Dopamina en la Modulación de la Neurotransmisión” se presenta como un compendio de publicaciones.

El manuscrito “**A Hybrid Indoloquinolizidine Peptide as Allosteric Modulator of Dopamine D₁ Receptors**” ha sido publicado en *The Journal of Pharmacology and Experimental Therapeutics* con un factor de impacto de 4,090. El manuscrito “**Dopamine D₄ receptor, but not the ADHD-associated D_{4.7} variant, forms functional heteromers with the dopamine D₂ receptor in the brain**” ha sido publicado en *Molecular Psychiatry* con un factor de impacto de 15.049. El manuscrito “**Circadian-related heteromerization of adrenergic and dopamine D₄ receptors modulates melatonin synthesis and release in the pineal gland**” se encuentra en vías de publicación. El manuscrito “**Biotin Ergopeptide Probes for Dopamine Receptors**” ha sido publicado en *Journal of Medical Chemistry* con un factor de impacto de 5.207.

En el trabajo “**A Hybrid Indoloquinolizidine Peptide as Allosteric Modulator of Dopamine D₁ Receptors**” el doctorando Sergio González ha realizado los experimentos de disociación en presencia o ausencia del ligando IP28 con todos los miembros de las subfamilias de receptores D1-like y D2-like. En el trabajo “**Dopamine D₄ receptor, but not the ADHD-associated D_{4.7} variant, forms functional heteromers with the dopamine D₂ receptor in the brain**”, exceptuando la obtención de los ratones transgénicos y los experimentos de microdiálisis y liberación de neurotransmisores, el doctorando Sergio González ha realizado la totalidad del trabajo experimental. En el trabajo “**Circadian-related heteromerization of adrenergic and dopamine D₄ receptors modulates melatonin synthesis and release in the pineal gland**” exceptuando la determinación de serotonina por HPLC y los experimentos de

PLA, el doctorando Sergio González ha realizado la totalidad del trabajo experimental. Finalmente, en el trabajo **“Biotin Ergopeptide Probes for Dopamine Receptors”** el doctorando Sergio González ha realizado los experimentos de competición del compuesto 13 por los receptores D₁, D₂ y D₃ y los experimentos de funcionalidad y señalización intracelular.

Barcelona, a 16 de Abril de 2012

Dra. Josefa Mallol Montero

Dr. Rafael Franco Fernández

Los resultados de la presente tesis están reflejados en los siguientes manuscritos:

3.1 Aroa Soriano, Marc Vendrell, **Sergio González**, Josefa Mallol, Fernando Albericio, Miriam Royo, Carmen Lluís, Enric I. Canela, Rafael Franco, Antoni Cortés y Vicent Casadó. **A Hybrid Indoloquinolizidine Peptide as Allosteric Modulator of Dopamine D₁ Receptors.**

Manuscrito publicado en *The Journal of Pharmacology and Experimental Therapeutics*. 332(3):876-85, Mar. 2010

3.2 **Sergio González**, Claudia Rangel-Barajas, Marcela Peper, Ramiro Lorenzo, Estefanía Moreno, Francisco Ciruela, Janusz Borycz, Carme Lluís, Rafael Franco, Peter J. McCormick, Nora Volkow, Marcelo Rubinstein, Benjamin Floran y Sergi Ferré. **Dopamine D₄ receptor, but not the ADHD-associated D_{4.7} variant, forms functional heteromers with the dopamine D₂ receptor in the brain.**

Manuscrito publicado en *Molecular Psychiatry*. 2010. August 16. doi: 10.1038/mp.2011.93. [Epub ahead of print]

3.3 **Sergio González**, David Moreno-Delgado, Estefanía Moreno, Kamil Pérez-Capote, Josefa Mallol, Antoni Cortés, Vicent Casadó, Carme Lluís, Jordi Ortiz, Sergi Ferré, Enric Canela y Peter J. McCormick. **Circadian-related heteromerization of adrenergic and dopamine D₄ receptors modulates melatonin synthesis and release in the pineal gland.**

Manuscrito en vías de publicación.

3.4 Marc Vendrell, Anabel Molero, **Sergio González**, Kamil Pérez-Capote, Carme Lluís, Peter J. McCormick, Rafael Franco, Antoni Cortés, Vicent Casadó, Fernando Albericio y Miriam Royo. **Biotin Ergopeptide Probes for Dopamine Receptors.**

Manuscrito publicado en *Journal of Medical Chemistry*. 24;54(4):1080-90, Feb. 2011

3.1 A Hybrid Indoloquinolizidine Peptide as Allosteric Modulator of Dopamine D₁ Receptors.

Aroa Soriano, Marc Vendrell, **Sergio González**, Josefa Mallol, Fernando Albericio, Miriam Royo, Carmen Lluís, Enric I. Canela, Rafael Franco, Antoni Cortés y Vicent Casadó

Manuscrito publicado en *The Journal of Pharmacology and Experimental Therapeutics*. 332(3):876-85, Mar. 2010

El ligando indoloquinolizidina-péptido 28, un híbrido trans-indoloquinolizidina-péptido que se obtuvo mediante una aproximación combinatoria, se comportó como un modulador alostérico negativo de la unión tanto de agonistas como de antagonistas a receptores D₁ de dopamina estriatales. En presencia de concentraciones crecientes de indoloquinolizidina-péptido 28 se observó que la constante de disociación aparente para el antagonista incrementaba hiperbólicamente. Este compuesto también alteró la cinética de disociación del antagonista del receptor de dopamina D₁. Esta modulación alostérica negativa también se encontró cuando se investigó la unión de un agonista. El ligando indoloquinolizidina-péptido 28 se comportó como un modulador ago-alostérico débil que provoca una considerable disminución de la potencia sin reducir el efecto máximo mediado por el agonista en los niveles de AMPc. Compuestos capaces de disminuir la potencia mientras preservan la eficacia de los agonistas del receptor D₁ son prometedores para su exploración en patologías psicóticas.

A Hybrid Indoloquinolizidine Peptide as Allosteric Modulator of Dopamine D₁ Receptors^[S]

Aroa Soriano, Marc Vendrell,¹ Sergio Gonzalez, Josefa Mallol, Fernando Albericio, Miriam Royo, Carmen Lluís, Enric I. Canela, Rafael Franco, Antoni Cortés, and Vicent Casadó

Institut d'Investigacions Biomèdiques August Pi i Sunyer, Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas, and Department of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain (A.S., S.G., J.M., C.L., E.I.C., A.C., V.C.); Combinatorial Chemistry Unit, Barcelona Science Park-University of Barcelona, Barcelona, Spain (M.V., M.R.); Institute of Research in Biomedicine, Barcelona Science Park-University of Barcelona, Barcelona, Spain and Department of Organic Chemistry, University of Barcelona, Barcelona, Spain (F.A.); Centro de Investigación Biomédica en Red en Bioingeniería, Biomateriales, y Nanomedicina, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Barcelona, Spain (F.A., M.R.); and Department of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain, Molecular Neurobiology, Neurosciences Division, Centro de Investigación Médica Aplicada, University of Navarra, Pamplona, Spain, and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas, Barcelona, Spain (R.F.)

Received July 13, 2009; accepted December 17, 2009

ABSTRACT

The indoloquinolizidine-peptide 28 [(3*S*,12*bR*)-*N*-((*S*)-1-((*S*)-1-((*S*)-2-carbamoylpyrrolidin-1-yl)-3-(4-fluorophenyl)-1-oxopropan-2-ylamino)-4-cyclohexyl-1-oxobutan-2-yl)-1,2,3,4,6,7,12,12*b*-octahydroindolo[2,3-*a*]quinolizine-3-carboxamide], a *trans*-indoloquinolizidine-peptide hybrid obtained by a combinatorial approach, behaved as an orthosteric ligand of all dopamine D₂-like receptors (D₂, D₃, and D₄) and dopamine D₅ receptors, but as a negative allosteric modulator of agonist and antagonist binding to striatal dopamine D₁ receptors. Indoloquinolizidine-peptide 28 induced a concentration-dependent hyperbolic increase in the

antagonist apparent equilibrium dissociation constant values and altered the dissociation kinetics of dopamine D₁ receptor antagonists. The negative allosteric modulation was also found when agonist binding to D₁ receptors was assayed. Indoloquinolizidine-peptide 28 was a weak ago-allosteric modulator but markedly led to a decreased potency without decreasing the maximum partial/full agonist-mediated effect on cAMP levels. Compounds able to decrease the potency while preserving the efficacy of D₁ receptor agonists are promising for exploration in psychotic pathologies.

G-protein-coupled receptors (GPCRs) represent a high percentage of the current market for therapeutic agents and remain a primary focus of many biomedical research and

pharmaceutical drug discovery programs. Much attention is focused in the identification and study of molecules that act as orthosteric ligands at a given GPCR to elicit a pharmacological effect. These compounds compete with the endogenous ligand(s) and thus preclude simultaneous occupation of the receptor by the two molecules. In addition to orthosteric sites, many GPCRs have been found to possess allosteric binding sites that are structurally distinct from the orthosteric sites (Christopoulos, 2002; May et al., 2007; Bridges and Lindsley, 2008). Allosteric sites may be less conserved across subtypes than orthosteric sites, providing a means for true selectivity (Bridges and Lindsley, 2008). One characteristic feature of

This work was supported by the Spanish Ministerio de Ciencia y Tecnología [Grants SAF2008-00146, SAF2009-07276] (to E.I.C. and R.F., respectively); and Fundació La Marató de TV3 [Grant 060110] (to E.I.C.).

A.S. and M.V. contributed equally to this work.

¹ Current affiliation: Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium, Helios, Singapore.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

doi:10.1124/jpet.109.158824.

[S] The online version of this article (available at <http://jpet.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: GPCR, G-protein-coupled receptor; IP28, indoloquinolizidine-peptide 28, (3*S*,12*bR*)-*N*-((*S*)-1-((*S*)-1-((*S*)-2-carbamoylpyrrolidin-1-yl)-3-(4-fluorophenyl)-1-oxopropan-2-ylamino)-4-cyclohexyl-1-oxobutan-2-yl)-1,2,3,4,6,7,12,12*b*-octahydroindolo[2,3-*a*]quinolizine-3-carboxamide (C₄₀H₅₁FN₆O₄); SCH 23390, *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF 38393, 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1*H*-3-benzazepine; SKF 81297, (±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide; A77636, (1*R*,3*S*)-3-(1'-adamantyl)-1-aminomethyl-3,4-dihydro-5,6-dihydroxy-1*H*-2-benzopyran; SKF 83566, 8-bromo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepine-7-ol hydrobromide; YM-09151-2, *cis*-5-chloro-2-methoxy-4(methylamino)-*N*-[2-methyl-1-(phenylmethyl)-3-pyrrolidinyl]-benzamide; HBSS, Hank's balanced salt solution; FRT, Flp recombination target; HEK, human embryonic kidney.

the allosteric interaction is that the receptor is able to simultaneously bind an orthosteric and an allosteric ligand, introducing complexity into pharmacological responses by modifying the affinity or the signal imparted by orthosteric ligand (May et al., 2007). The ability of allosteric modulators to fine tune pharmacological responses has sparked interest in their potential applications in both clinical and basic science settings (Bridges and Lindsley, 2008; Conn et al., 2009). This interest is more relevant in the case of neurotransmitter receptor targets, where synaptic neurotransmission occurs in extremely complex circuits implicated in many neurological functions. An allosteric modulator will preserve the physiological relevance of receptor signaling while modulating the potency of the endogenous neurotransmitter (Conn et al., 2009).

Allosteric sites have been described for dopamine receptors (Schetz, 2005). Dopamine receptors are grouped into two classes: D₁-like receptors, which include D₁ and D₅ receptors, and D₂-like receptors, which include D₂, D₃, and D₄ receptors (Neve et al., 2004). As for many GPCRs (see Ferré et al., 2009 for review), there is evidence that dimerization/oligomerization of D₁ receptors (Ng et al., 1994; George et al., 1998) is important for membrane expression (Kong et al., 2006). In addition, dopamine D₂ receptors form higher-order oligomers at physiological expression levels with dimers, the minimal repetitive structural unit (Guo et al., 2008; Han et al., 2009). An allosteric site associated with the D₂ dopamine receptor is recognized by amiloride and analogs of this diuretic drug, such as benzamil and methylisobutylamiloride (Hoare et al., 2000). These compounds also decrease antagonist binding to D₁, D₃, and D₄ dopamine receptors (Hoare et al., 2000). L-Proline-L-leucine-L-glycine, an endogenous hypothalamic factor that inhibits the release of melanocyte-stimulating hormone from the anterior pituitary, is another dopamine receptor allosteric modulator enhancing agonist binding to D₂ and D₄ receptors (Verma et al., 2005). The allosteric modulation of dopamine receptors by ions has been described extensively (Schetz, 2005). At millimolar concentrations, sodium decreases agonist binding but increases antagonist binding to D₂ dopamine receptors (Erickson et al., 2009). Zinc ion allosterically modulates dopamine receptors (Schetz and Sibley, 2001), and it may be therapeutically relevant in antipsychotic drug treatments (Schetz, 2005).

Because dopamine is an important neurotransmitter involved in the regulation of several biological functions, including locomotor activity, emotion, cognition, and neuroendocrine secretion, and because striatum receives the densest dopamine innervations and contains the highest density of dopamine receptors in the brain (Gerfen, 2004), dopamine receptors are targets in the pathophysiology of Parkinson's disease and schizophrenia (Andersen and Nielsen, 1991; Wu et al., 2005). We have recently developed a library of indoloquinolizidine-peptide hybrids as multiple ligands for different dopamine receptor subtypes by a combinatorial approach that combines the solution-phase synthesis of two indolo[2,3-*a*]quinolizidine scaffolds with solid-phase peptide chemistry (Vendrell et al., 2009). Some *trans*-indoloquinolizidine-peptide hybrids were selected showing an affinity (K_D) in the low-micromolar range for both families of dopamine receptors (Vendrell et al., 2009). Here, the functional characterization of one of these compounds, the indoloquinolizidine-peptide 28 (IP28), on binding to dopamine D₁-like and D₂-like

receptors is described. By means of kinetic assays and competition experiments in radioligand binding, it has been demonstrated that the IP28 behaved as an orthosteric ligand of dopamine D₂, D₃, D₄, and D₅ receptors but as an allosteric modulator of the D₁ dopamine receptors. IP28 decreased the affinity of both agonist and antagonist binding to the receptor and, at the same time, behaved as a D₁ receptor partial agonist. IP28, which was then the first described ago-allosteric modulator of D₁ dopamine receptors, decreased receptor potency, whereas it preserved agonist-induced maximal cAMP production. This type of compound may be relevant to treat some psychotic pathologies.

Materials and Methods

Cell Transfection and Generation of an Inducible Cell Line Expressing the Human Dopamine D₁ Receptor. Human embryonic kidney 293 (HEK-293) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 2 mM L-glutamine, and 100 µg ml⁻¹ sodium pyruvate (all from Invitrogen, Paisley, UK), at 37°C in a humidified atmosphere of 5% CO₂. HEK-293 cells were grown to 60% confluence and transfected by the polyethylenimine (Sigma-Aldrich Chemie, Steinheim, Germany) method with 5 µg of cDNA corresponding to human dopamine D₃, D_{4.4}, or D₅ receptors. Cells were incubated (4 h) with the corresponding cDNA together with polyethylenimine (5.47 mM in nitrogen residues) and 150 mM NaCl in a serum-starved medium. After 4 h, the medium was changed to a fresh complete culture medium. Forty-eight hours after transfection, cells were washed twice in quick succession in Hank's balanced salt solution (HBSS) with 10 mM glucose, detached, and resuspended in the same buffer containing 1 mM EDTA. Human dopamine receptor expression was tested by binding to membranes from these cells.

The Flp-In T-Rex System (Invitrogen) was used to generate a stable mammalian cell line exhibiting tetracycline-inducible expression of human dopamine D₁ receptor from a specific genomic cDNA location. The Flp-In T-REx-293 cells used in this system contain a single integrated Flp recombination target (FRT) site, stably express the Tet repressor, and allow research to proceed directly to the generation of a stable cell line. The human dopamine D₁ receptor cDNA was amplified by use of sense and antisense primers and the iProof kit (Bio-Rad, Hercules, CA) as indicated by the manufacturer. To the amplified fragment, an adenine nucleotide was added in its 3' end, with use of the iTag kit (Bio-Rad), to be cloned into pcDNA5/FRT/TO-TOPO vector (Invitrogen), as indicated by the manufacturer, to generate the pcDNA5/FRT/TO-TOPO-D₁R construct. Flp-In T-REx-293 cells were cotransfected with the pcDNA5/FRT/TO-TOPO-D₁R construct and the pOG44 plasmid (Invitrogen) to allow the integration of the expression vector pcDNA5/FRT/TO-TOPO-D₁R under the control of a tetracycline-inducible promoter into the genome via Flp recombinase-mediated DNA recombination at the FRT site. A polyclonal selection of isogenic cell lines was performed by use of 15 µg/ml blasticidin and 200 µg/ml hygromycin B, as recommended by manufacturer. Flp-In T-REx-293-D₁R cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 4.5 mg/ml glucose and 0.11 mg/ml sodium pyruvate, 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 15 µg/ml blasticidin, and 200 µg/ml hygromycin B (all supplements were from Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂. Expression of human dopamine D₁ receptor was induced by overnight incubation of Flp-In T-REx-293-D₁R cells with different concentrations of tetracycline (Invitrogen) and tested by binding experiments.

Membrane Preparation and Protein Determination. Membrane suspensions from sheep brain striatum or from cells expressing particular subtypes of dopamine receptors were processed as

described previously (Casadó et al., 1990; Sarrió et al., 2000). Tissue or cells were disrupted with a Polytron homogenizer (PTA 20 TS rotor, setting 3; Kinematica, Basel, Switzerland) for three 5-s periods in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4, containing a proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Cell debris was removed by centrifugation at 1500g for 10 min at 4°C, and membranes were obtained by centrifugation at 105,000g (40 min, 4°C). Membranes were resuspended and recentrifuged under the same conditions. The pellet was stored at -80°C, washed once more as described earlier, and resuspended in 50 mM Tris-HCl buffer for immediate use. Protein was quantified by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL) by use of bovine serum albumin dilutions as standard.

Radioligand Binding Experiments. For competition experiments, membrane suspensions (0.5 mg of protein/ml) were incubated for 2 h at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ with the indicated free concentration of the dopamine D₁-like receptor antagonist [³H]SCH 23390 (PerkinElmer Life and Analytical Sciences, Wellesley, MA) or the D₂-like receptor antagonist [³H]YM-09151-2 (PerkinElmer Life and Analytical Sciences) and increasing concentrations of IP28 (11 different concentrations from 10 nM to 50 μM), increasing concentrations of YM-09151-2 (10 different concentrations from 0.01 nM to 10 μM; Tocris, Aronmouth, UK), increasing concentrations of SCH 23390 (13 different concentrations from 0.01 nM to 50 μM; Sigma-Aldrich) in the absence or in the presence of the indicated concentration of IP28 (preincubated 60 min with membranes) or increasing concentrations of dopamine D₁-like receptor agonist SKF 38393 (13 different concentrations from 0.1 nM to 50 μM; Tocris) in the absence or in the presence of the indicated concentration of IP28 (preincubated 60 min with membranes). Nonspecific binding was determined in the presence of 50 μM SKF 83566 (the same values were obtained in the presence of 50 μM SCH 23390 or SKF 38393) or 50 μM YM-09151-2 (the same nonspecific binding was obtained in the presence of 50 μM quinpirole). Free and membrane-bound ligand were separated by rapid filtration of 500-μl aliquots in a cell harvester (Brandel, Gaithersburg, MD) through Whatman GF/C filters embedded in 0.3% polyethylenimine that were subsequently washed for 5 s with 5 ml of ice-cold Tris-HCl buffer. The filters were incubated with 10 ml of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA) overnight at room temperature, and radioactivity counts were determined by use of a Tri-Carb 1600 scintillation counter (PerkinElmer Life and Analytical Sciences) with an efficiency of 62% (Sarrió et al., 2000).

For dissociation kinetic assays, sheep brain striatum membranes (0.5 mg of protein/ml) or membranes from receptor-expressing cells (0.2 mg of protein/ml) were incubated 60 min at 25°C with medium, the indicated concentrations of SKF 83566 (Tocris) or IP28 in Tris-HCl buffer (50 mM, pH 7.4) containing 10 mM MgCl₂ before adding the indicated concentrations of [³H]SCH 23390 or [³H]YM-09151-2. After 2 h the dissociation was initiated by the addition of 10 μM SCH 23390 or YM-09151-2. At the indicated time interval total binding was measured by rapid filtration and determination of radioactivity counts as indicated above. Nonspecific binding was measured after a 90-min incubation in the presence of 50 μM SKF 83566 or 50 μM quinpirole.

All displacers were dissolved in dimethyl sulfoxide and diluted in the binding medium. The dimethyl sulfoxide concentration in the binding incubates was less than 0.5% and, at this concentration, it did not affect agonist or antagonist affinity for dopamine receptors.

Binding Data Analysis. Because dopamine D₁ and D₂ receptors are expressed as dimers or higher-order oligomers (see the introduction), radioligand competition curves were analyzed by nonlinear regression with use of the commercial Graft curve-fitting software (Erithacus Software, Surrey, UK), by fitting the specific binding data to the mechanistic two-state dimer receptor model (Franco et al., 2005, 2006). This model considers a homodimer as the minimal structural unit of the receptor. To calculate the macroscopic equilib-

rium dissociation constants, the following equation for a competition binding experiment deduced by Casadó et al. (2007) was considered:

$$A_{\text{total bound}} = (K_{\text{DA}2} A + 2A^2 + K_{\text{DA}2} A B/K_{\text{DAB}})R_{\text{T}}/(K_{\text{DA}1} K_{\text{DA}2} + K_{\text{DA}2} A + A^2 + K_{\text{DA}2} A B/K_{\text{DAB}} + K_{\text{DA}1} K_{\text{DA}2} B/K_{\text{DB1}} + K_{\text{DA}1} K_{\text{DA}2} B^2/(K_{\text{DB1}} K_{\text{DB2}})) + A_{\text{nonspecific bound}} \quad (1)$$

where A represents free radioligand (the D₁-like receptor antagonist [³H]SCH 23390 or the D₂-like receptor antagonist [³H]YM-09151-2) concentration, R_{T} is the total amount of receptor dimers, and $K_{\text{DA}1}$ and $K_{\text{DA}2}$ are the macroscopic equilibrium dissociation constants describing the binding of the first and the second radioligand molecule (A) to the dimeric receptor; B represents the assayed competing compound (the dopamine D₁-like receptor antagonist SCH 23390, the dopamine D₁-like receptor agonist SKF 38393, or the dopamine D₂-like receptor antagonist YM-09151-2) concentration, and K_{DB1} and K_{DB2} are, respectively, the macroscopic equilibrium dissociation constants of the first and second binding of B ; K_{DAB} is the hybrid equilibrium radioligand/competitor dissociation constant, which is the dissociation constant of B binding to a receptor dimer semioccupied by A .

Because the radioligand A (A being the antagonist [³H]SCH 23390 or the antagonist [³H]YM-09151-2) showed noncooperative behavior (Franco et al., 2006; Casadó et al., 2007), eq. 1 was simplified to eq. 2 because $K_{\text{DA}2} = 4K_{\text{DA}1}$ (Casadó et al., 2007):

$$A_{\text{total bound}} = (4K_{\text{DA}1} A + 2A^2 + 4K_{\text{DA}1} A B/K_{\text{DAB}})R_{\text{T}}/(4K_{\text{DA}1}^2 + 4K_{\text{DA}1} A + A^2 + 4K_{\text{DA}1} A B/K_{\text{DAB}} + 4K_{\text{DA}1}^2 B/K_{\text{DB1}} + 4K_{\text{DA}1}^2 B^2/(K_{\text{DB1}} K_{\text{DB2}})) + A_{\text{nonspecific bound}} \quad (2)$$

The dimer cooperativity index for the competing ligand B (the dopamine D₁ receptor agonist SKF 38393) was calculated as (Casadó et al., 2007):

$$D_{\text{CB}} = \log(4K_{\text{DB1}}/K_{\text{DB2}}) \quad (3)$$

When $K_{\text{DAB}} = 2K_{\text{DB1}}$, the binding of the radioligand to one protomer in the dimer does not modify the binding of the competing ligand to the other empty protomer in the dimer. In contrast, values of $K_{\text{DAB}} < 2K_{\text{DB1}}$ or $K_{\text{DAB}} > 2K_{\text{DB1}}$ indicate, respectively, a positive or negative effect (see radioligand/competitor modulation in Supplemental Data). According to this, a new parameter, "the dimer radioligand/competitor modulation index" (D_{AB}), ("SCH 23390/SKF 38393 modulation index" for the D₁ receptor ligands reported here) is introduced, which is defined as $D_{\text{AB}} = \log(2K_{\text{DB1}}/K_{\text{DAB}})$. The index is defined in such a way that its value is "0" when the presence of radioligand does not affect the competitor binding to the empty protomer in the dimer. Positive or negative values of D_{AB} indicate that the presence of radioligand increases or decreases, respectively, the competitor affinity for binding to the empty protomer in the dimer.

A direct calculation of the concentration of B providing half-saturation (B_{50}) was obtained according to (Casadó et al., 2007):

$$B_{50} = (K_{\text{DB1}} K_{\text{DB2}})^{1/2} \quad (4)$$

In the experimental conditions, when both radioligand A (the antagonist [³H]SCH 23390) and competitor B (the dopamine D₁ receptor agonist SKF 38393) show noncooperativity, $K_{\text{DA}2} = 4K_{\text{DA}1}$ and $K_{\text{DB2}} = 4K_{\text{DB1}}$, and eq. 1 is simplified to:

$$A_{\text{total bound}} = (4K_{\text{DA}1} A + 2A^2 + 4K_{\text{DA}1} A B/K_{\text{DAB}})R_{\text{T}}/(4K_{\text{DA}1}^2 + 4K_{\text{DA}1} A + A^2 + 4K_{\text{DA}1} A B/K_{\text{DAB}} + 4K_{\text{DA}1}^2 B/K_{\text{DB1}} + K_{\text{DA}1}^2 B^2/K_{\text{DB1}}^2) + A_{\text{nonspecific bound}} \quad (5)$$

When both radioligand A (A being the antagonist [³H]SCH 23390 or the antagonist [³H]YM-09151-2) and competitor B (SCH 23390 or

YM-09151-2) are the same compound and the binding is noncooperative, eq. 5 is simplified to:

$$A_{\text{total bound}} = (4K_{\text{DA1}} A + 2A^2 + A B)R_{\text{T}} / (4K_{\text{DA1}}^2 + 4K_{\text{DA1}} A + A^2 + A B + 4K_{\text{DA1}} B + B^2) + A_{\text{nonspecific bound}} \quad (6)$$

Dissociation kinetic data were fitted to the following empirical equation:

$$A_{\text{total bound}} = \sum_{i=1}^n A_{ei} e^{-tk_i} + A_{\text{nonspecific bound}} \quad (7)$$

where A_{ei} represents the initial radioligand (the D₁ receptor antagonist [³H]SCH 23390 or the D₂ receptor antagonist [³H]YM-09151-2) bound at equilibrium for each molecular specie i , t is time, and k_i is the dissociation rate constants for the n different molecular species. For monophasic curves (or simple dissociation kinetics), $n = 1$, and for biphasic curves (or complex dissociation kinetics), $n = 2$.

Goodness of fit was tested according to reduced χ^2 value given by the nonlinear regression program. The test of significance for two different population variances was based on the F distribution (see Casadó et al., 1990 for details). By use of this F test, a probability greater than 95% ($p < 0.05$) was considered the criterion to select a more complex equation to fit binding data over the simplest one. In all cases, a probability of less than 70% ($p > 0.30$) resulted when one equation to fit binding data was not significantly better than the other. Results are given as parameter values \pm S.E.M. of three to four independent experiments.

cAMP Determination. The Flp-In T-REx-293-D₁R cells were grown in six-well plates to 80% confluence and incubated with 15 ng/ml tetracycline for 16 h in serum-free medium before the experiment [in these conditions, the expression level of dopamine D₁ receptors was similar to the expression in brain striatum (0.4–0.5 pmol/mg protein)]. Cells (equivalent to 0.4 mg of protein) were harvested, washed twice in HBSS containing 10 mM glucose, and suspended in HBSS supplemented with 10 mM glucose and 10 mM MgCl₂ to a final volume of 1 ml in plastic tubes. Cells were preincubated with 50 μ M zardaverine (Tocris) as phosphodiesterase inhibitor for 10 min at 37°C and treated or not with the indicated ligands. After 5 min, cells were placed on ice and centrifuged at 2500g for 5 min at 4°C. The pellet was washed with ice-cold HBSS with 10 mM glucose and resuspended with 200 μ l of ice-cold HClO₄ (4%) for 30 min; 1.5 M KOH was added to reach neutral pH. Samples were centrifuged at 15,000g for 30 min at 4°C, and the supernatant was frozen at –20°C. The accumulation of cAMP in the samples was measured by a [³H]cAMP assay system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as instructed by the manufacturer. Student's t test for unpaired samples was used for statistics.

Results

The IP28 Binds to Dopamine D₁ Receptors but Is Unable to Completely Displace Agonist Binding. The IP28 (Fig. 1a) is a *trans*-indoloquinolizidine-peptide hybrid obtained as described previously by applying a combinatorial approach that combines the solution-phase synthesis of an indolo[2,3-*a*]quinolizidine scaffold with solid-phase peptide chemistry (Vendrell et al., 2009). As described in Vendrell et al. (2009), IP28 binds to dopamine D₁ and D₂ receptors. We have tested whether IP28 has some degree of selectivity for dopamine receptors because its affinity constants for other GPCRs, namely adenosine A₁ or histamine H₃ receptors, were 70 or 50 μ M, respectively (results not shown). These values are more than 30-fold higher than the affinity for the D₂ receptors (1.5 μ M; Vendrell et al., 2009). To characterize the IP28 binding to dopamine D₁ receptors, IP28 was com-

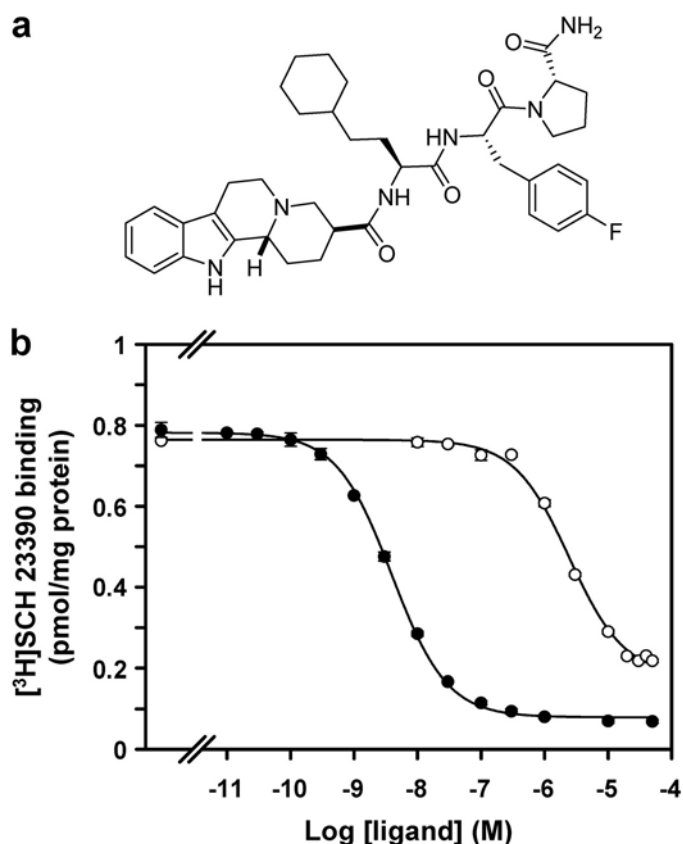


Fig. 1. Competition curves of dopamine D₁ receptor antagonist [³H]SCH 23390 binding versus increasing concentrations of SCH 23390 or IP28. a, structure of IP28, Smiles formula: O=C(N[C@@H](CC1=CC=C(F)C=C1)C(N2CCC[C@H]2C(N)=O)=O)[C@H](CCC3CCCC3)NC([C@H](C4)CC[C@@]5([H])N4CCC6=C5NC7=CC=CC=C76)=O. b, competition experiments of the antagonist [³H]SCH 23390 (2.5 nM) versus increasing concentrations of SCH 23390 (0.01 nM to 50 μ M) (●) or IP28 (10 nM to 50 μ M) (○) were performed as indicated under *Materials and Methods* by use of brain striatal membranes. Data are mean \pm S.E.M. from a representative experiment ($n = 3$) performed in triplicate. For some data points, error is smaller than symbol.

pared with the D₁ dopamine receptors antagonist SCH 23390 as displacer of [³H]SCH 23390 binding. Competition experiments were performed with a constant concentration of [³H]SCH 23390 (2.5 nM) and increasing concentrations of unlabeled SCH 23390 (0.01 nM to 50 μ M) or IP28 (10 nM to 50 μ M) by using brain striatal membranes as described under *Materials and Methods*. As expected (see Fig. 1b), SCH 23390 fully displaced the specific binding of the [³H]SCH 23390, whereas IP28 decreased the radioligand binding with a EC₅₀ of 2.5 ± 0.2 μ M, but it was unable to completely inhibit the specific binding of [³H]SCH 23390 (Fig. 1b). Very high IP28 concentrations led to 78% reduction of radioligand binding, and this cannot be explained by binding to the orthosteric site. Therefore, the results suggested that IP28 might behave as an allosteric effector of D₁ receptors.

IP28 Is a Negative Allosteric Modulator of Antagonist SCH 23390 Binding to Dopamine D₁ Receptors. To test whether the IP28 was an allosteric modulator of the antagonist binding to dopamine D₁ receptors, its effect on the antagonist SCH 23390 binding affinity was carefully evaluated. Competition experiments were performed with a constant amount of [³H]SCH 23390 (2.8 nM) and increasing concentrations of unlabeled antagonist (0.01 nM to 50 μ M) in

the absence or presence of different IP28 concentrations (1–50 μM) using brain striatal membranes. From Fig. 2a, two related effects can be seen. One is that total amount of [^3H]SCH 23390 bound is decreased by increasing IP28 concentration in agreement with the results shown in Fig. 1b. The other is that IP28 concentration-dependently increased the SCH 23390 concentration needed to displace 50% the radioligand binding, indicating that antagonist affinity decreases in the presence of IP28. Because it has been described that D_1 receptors are dimers or higher-order oligomers (Ng et al., 1994; George et al., 1998; Kong et al., 2006), binding data were fitted to a dimeric receptor model as described under *Materials and Methods*, i.e., to eq. 6, and the equilibrium dissociation constant K_{DA1} values, which are a measure of affinity for the antagonist, were calculated by nonlinear regression (K_{DA2} was calculated as $4K_{\text{DA1}}$ for this noncooperative antagonist; see *Materials and Methods*). IP28 concentration-dependently increased the apparent antagonist equilibrium

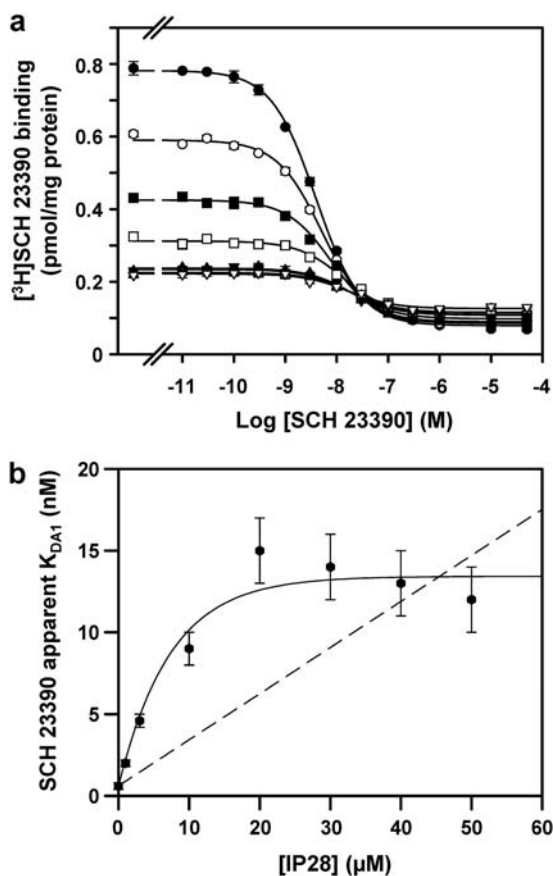


Fig. 2. Competition curves of dopamine D_1 receptor antagonist [^3H]SCH 23390 binding versus increasing concentrations of SCH 23390 in the presence or in the absence of IP28. a, competition experiments of the antagonist [^3H]SCH 23390 (2.8 nM) versus increasing concentrations of SCH 23390 were performed with brain striatal membranes as indicated under *Materials and Methods* in the presence of increasing concentrations of IP28 (top to bottom: 0 (\bullet), 1 (\circ), 3 (\blacksquare), 10 (\square), 20 (\blacktriangle), 30 (\triangle), 40 (\blacktriangledown), 50 (\triangledown) μM). Binding data were fitted to eq. 6 (see *Materials and Methods*) to obtain the equilibrium binding parameters. b, the calculated values for the apparent equilibrium dissociation constant (apparent K_{DA1}) versus the IP28 concentrations are plotted. The dotted straight line represents the plot of the apparent equilibrium dissociation constants, assuming that IP28 binds to the orthosteric site and competes for the [^3H]SCH 23390 binding (see Supplemental Data). Data are mean \pm S.E.M. from a representative experiment ($n = 3$) performed in triplicate. In a, for some data points, error is smaller than symbol.

dissociation constant (K_{DA1}) values until a seemingly constant K_{DA1} value was reached (Fig. 2b). To better understand the results, an analysis of the variation of dissociation constants assuming the binding of IP28 to the orthosteric site was performed (see Supplemental Data). The results of this analysis (dotted line in Fig. 2b) indicate that, in such circumstances, when the [^3H]SCH 23390 binding is noncooperative and there is not radioligand-competitor modulation ($K_{\text{DAB}} = 2K_{\text{DB1}}$; see Supplemental Data), a linear relationship between apparent K_{DA1} and the IP28 concentration would happen. Therefore, the apparently hyperbolic relationship depicted in Fig. 2b (solid line connecting real data points) indicates that IP28 is not an orthosteric ligand and suggest that it is a negative allosteric modulator of the antagonist binding to dopamine D_1 receptors.

To further prove the allosteric behavior of IP28 on dopamine D_1 receptors binding, dissociation kinetic experiments were performed. First, 2.5 nM [^3H]SCH 23390 was incubated with brain striatal membranes in the absence or in the presence of 3 nM dopamine D_1 receptor antagonist SKF 83566 or 3 μM IP28, as indicated under *Materials and Methods*; dissociation was then initiated by the addition of 10 μM SCH 23390. The time course of the different dissociations is displayed in Fig. 3. In the absence of SKF 83566 or IP28, the dissociation of [^3H]SCH 23390 is biphasic, and dissociation rate constants are listed in Table 1. As expected, the presence of the orthosteric competitor, SKF 83566, did not affect the values of the dissociation rate constants for [^3H]SCH 23390 (Table 1). In contrast, IP28 led to a different dissociation curve in such a way that the presence of the indoloquinolizidine-peptide led to simple [^3H]SCH 23390 dissociation kinetics and to the disappearance of the slow radioligand dissociation rate constant (Fig. 3, Table 1). This suggests that IP28 modifies the receptor conformation increasing orthosteric ligand dissociation. This finding is consistent with a negative allosteric modulation exerted by IP28 on the antagonist SCH 23390 binding to dopamine D_1 receptors. Thus, IP28 binds to an allosteric site on D_1 receptors.

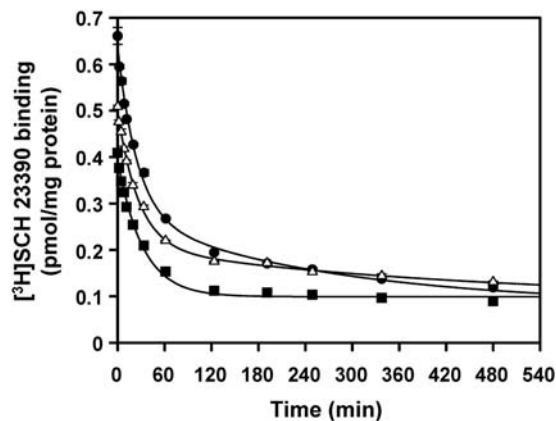


Fig. 3. Dissociation kinetic curves for [^3H]SCH 23390 binding to dopamine D_1 receptors in the absence or in the presence of the antagonist SKF 83566 or IP28. Brain striatal membranes were incubated with 2.5 nM [^3H]SCH 23390 in the absence (\bullet), or in the presence of 3 nM SKF 83566 (Δ), or in the presence of 3 μM IP28 (\blacksquare). In all cases, the dissociation was initiated by the addition of 10 μM unlabeled SCH 23390 (see *Materials and Methods*). Data are mean \pm S.E.M. from a representative experiment ($n = 3$) performed in triplicate. For some data points, error is smaller than symbol.

TABLE 1

Dissociation rate constants for [³H]SCH 23390 binding to dopamine D₁ receptors determined in the absence or in the presence of either the antagonist SKF 83566 (3 nM) or IP28 (3 μM)

Data points in Fig. 3 were fitted assuming monophasic ($n = 1$ in eq. 7) and biphasic ($n = 2$ in eq. 7) dissociation curves. Parameter values represent the mean ± S.E.M. of three experiments.

	$k_{i \text{ slow}}$	$k_{i \text{ fast}}$
	min^{-1}	
Control	0.004 ± 0.001	0.041 ± 0.004
SKF 83566	0.002 ± 0.001	0.037 ± 0.002
IP28 ^a	—	0.031 ± 0.001

^a Using the *F* test, no significantly better fit was obtained by considering $n = 2$ (see *Materials and Methods*).

Because the D₁ receptor expression in striatal membranes is very high with respect to D₅ receptor expression (Araki et al., 2007), the above-described results are representative of the IP28 binding to D₁ receptors. There is no selective radiolabeled ligand available for the different subtypes of D₁-like receptors (D₁ and D₅ receptors); therefore, the IP28 binding to D₅ receptors was analyzed in membranes from HEK-293 cells expressing D₅ receptors. Cells were generated, and membranes were obtained as described under *Materials and Methods*. Dissociation kinetic experiments were performed by incubating cell membranes (0.2 mg of protein/ml) with 2.5 nM [³H]SCH23390 in the absence or in the presence of 3 μM IP28, and dissociation was then initiated by the addition of 10 μM SCH23390. In the presence of IP28, the [³H]SCH23390 binding decreased by 40%. In the absence of IP28, the dissociation of [³H]SCH 23390 was monophasic, and the dissociation rate constant was 0.12 ± 0.02 min⁻¹. It is noteworthy that the presence of IP28 did not change the [³H]SCH 23390 dissociation rate for D₅ receptor, which was 0.11 ± 0.02 min⁻¹ for the calculated constant. Thus, IP28 is not an allosteric modulator of dopamine D₅ receptors, which suggests that IP28 behaves as an orthosteric ligand.

IP28 Is a Negative Allosteric Modulator of Agonist SKF 38393 Binding to Dopamine D₁ Receptors. To test whether IP28 was an allosteric modulator of agonist binding to dopamine D₁ receptors, competition experiments were performed with a constant amount of [³H]SCH 23390 (2.3 nM) and increasing concentrations of the D₁ receptor agonist SKF 38393 (0.1 nM to 50 μM), in the absence or presence of different IP28 concentrations (1 μM to 30 μM) using brain striatal membranes. Binding data (Fig. 4) were fitted assuming that D₁ receptors are dimers and statistically (*F* test) testing whether the agonist SKF 38393 binding was cooperative (fitting to eq. 2) or noncooperative (fitting to eq. 5). In the absence of IP28 or at low IP28 concentrations (1 and 3 μM), binding data were well fitted to eq. 2. In contrast, at high concentrations (10–30 μM), binding data fitted to eq. 5. Therefore, the presence of IP28 led to a progressive disappearance of agonist binding cooperativity (see Table 2). One advantage provided by the two-state dimer receptor model is the quantification of cooperativity by calculation of a cooperativity index (D_{CB} ; Table 2). D_{CB} measures the affinity modifications occurring when a protomer senses the binding of the same ligand molecule to the partner protomer in a dimer (see *Materials and Methods*). The D_{CB} value of -0.7 indicates negative cooperativity for SKF 38393 binding. The D_{CB} values (Table 2) changed in the presence of IP28 until cooperativity completely disappeared ($D_{CB} = 0$), which indicates

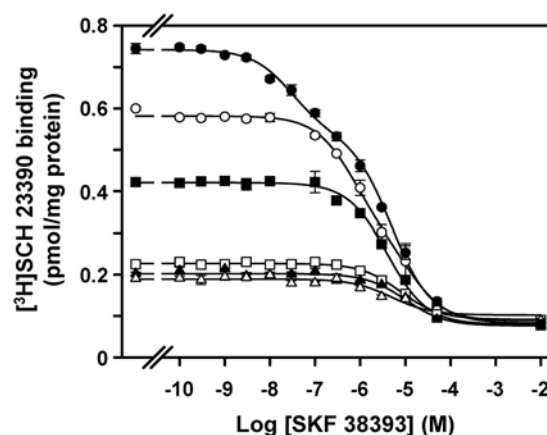


Fig. 4. Competition curves of dopamine D₁ receptor antagonist [³H]SCH 23390 binding versus increasing concentrations of the receptor agonist SKF 38393 in the presence or in the absence of IP28. Competition experiments of [³H]SCH 23390 (2.3 nM) versus increasing concentrations of SKF 38393 (0.1 nM to 50 μM) were performed with brain striatal membranes, as indicated under *Materials and Methods*, in the presence of increasing concentrations of IP28 (top to bottom: 0 (●), 1 (○), 3 (■), 10 (□), 20 (▲), 30 (△) μM). Data are mean ± S.E.M. from a representative experiment ($n = 3$) performed in triplicate. For some data points, error is smaller than symbol.

that IP28 binding to its allosteric site prevents the homotropic cooperativity in agonist binding. Furthermore, apparent dissociation constants for the agonist/competitor (K_{DB1} and K_{DB2}) increased ($p < 0.01$) in the presence of increasing IP28 concentrations until a constant value was attained. These results indicate that IP28 is also a negative allosteric modulator of the agonist binding.

The two-state dimer receptor model can even provide an hybrid equilibrium dissociation constant (K_{DAB} ; Table 2) that corresponds to the equilibrium dissociation constant of the agonist SKF 38393 binding to a receptor dimer semioccupied by another compound, such as an antagonist (SCH 23390; see Casadó et al., 2009a,b). Accordingly, a “dimer radioligand/competitor modulation index” (D_{AB}) can be calculated as indicated under *Materials and Methods*. D_{AB} is a measure of competitor affinity modifications occurring when a protomer senses the binding of another molecule to the partner protomer (in a dimer). As shown in Table 2, in the absence of IP28, the antagonist [³H]SCH 23390 binding to an empty receptor dimer positively modulates the agonist SKF 38393 binding to the other subunit in the dimer ($D_{AB} = 0.43$), whereas the modulation is completely disrupted by IP28 ($D_{AB} = 0$).

IP28 Is a Weak Ago-Allosteric Effector but a Strong Negative Modulator of D₁ Receptor Potency. Dopamine D₁ receptors couple to Gs/olf proteins and its main signaling pathway is the stimulation of the adenylyl cyclase-protein kinase A cascade (Neve et al., 2004). Thus, to investigate the IP28-mediated consequences on dopamine D₁ receptor signaling, cAMP assays were performed. Flp-In T-REx-293-D₁R cells were treated with increasing concentrations of IP28 or with the dopamine D₁ receptor partial or full agonists in the absence or the presence of 10 μM IP28. IP28 was able by itself to increase concentration-dependently the cAMP concentration (Fig. 5) in cells induced with tetracycline, but not in noninduced cells, which lack D₁ receptor expression. The maximum effect was approximately 22% of that exerted by the partial agonist (Fig. 6a) and approximately

TABLE 2

Parameter values obtained by fitting data of antagonist [3 H]SCH 23390 binding to dopamine D₁ receptors in competition experiments with the agonist SKF 38393 and different concentrations of IP28

Data points in Fig. 4 were fitted assuming radioligand binding to D₁ receptor dimers. Values were calculated by fitting data to eq. 2 or 5, introducing the K_{DA1} value for [3 H]SCH 23390 binding to D₁ receptor obtained from Fig. 2 to calculate the macroscopic dissociation constants in the absence or in the presence of IP28. Data are mean \pm S.E.M. values of three experiments.

Parameters ^a	IP28					
	0	1	3	10	20	30
	μM					
Apparent K_{DB1} , μM	0.020 \pm 0.002	0.35 \pm 0.04	1.2 \pm 0.2	1.7 \pm 0.3	3 \pm 1	2.2 \pm 0.5
Apparent K_{DB2} , μM	0.4 \pm 0.1	7 \pm 1	9 \pm 1	7 \pm 1	12 \pm 4	9 \pm 2
Apparent K_{DAB} , μM	0.015 \pm 0.009	0.70 \pm 0.08	2.4 \pm 0.4	3.4 \pm 0.6	6 \pm 3	4 \pm 1
D_{CB}	-0.7 \pm 0.1	-0.7 \pm 0.1	-0.3 \pm 0.1	0	0	0
D_{AB}	0.5 \pm 0.3	0	0	0	0	0
B_{50} , μM	0.09 \pm 0.01	1.5 \pm 0.2	3.3 \pm 0.4	3.4 \pm 0.6	6 \pm 2	4.4 \pm 0.9

^a K_{DB1} and K_{DB2} , respectively, are the equilibrium dissociation constants of the first and second binding of *B* (SKF 38393) to the dimer. K_{DAB} is the hybrid equilibrium dissociation constant of *B* binding to a receptor dimer semioccupied by *A* ([3 H]SCH 23390). D_{CB} is the dimer cooperativity index for the binding of ligand *B*. D_{AB} is the dimer radioligand/competitor modulation index (SCH 23390/SKF 38393 modulation index). B_{50} is the concentration providing half-saturation for *B*.

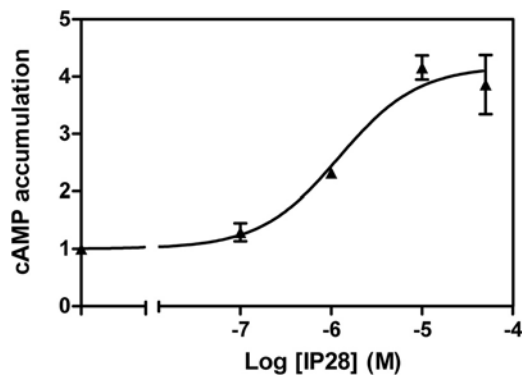


Fig. 5. cAMP production induced by IP28. cAMP concentration was measured in Flp-In T-REx-293-D₁R cells after human dopamine D₁ receptor induction with tetracycline. Cells were treated with increasing concentrations of the IP28 (100 nM to 50 μM) and cAMP was determined as described under *Materials and Methods*. Values are represented as infolds over the basal (nontreated cells). Data are mean \pm S.E.M. of a representative experiment ($n = 3$) performed in duplicate.

17% of that exerted by the full agonist (Fig. 6b); therefore, IP28 behaved as a weak ago-allosteric agonist. It is noteworthy that the presence of IP28 led to a marked shift to the right of the dose-response curves of any orthosteric agonists (Fig. 6). The EC_{50} values for the partial agonist SKF 38393 or the full agonist SKF 81297 in the absence of IP28 (116 \pm 15 nM or 41 \pm 8 nM, respectively) were lower ($p < 0.001$) than the values obtained in the presence of IP28 (4.4 \pm 0.5 μM or 1.2 \pm 0.2 μM , respectively), indicating that IP28 strongly decreased the receptor potency. IP28 did not decrease the efficacy of full/partial agonists (Fig. 6). All of these results indicate that IP28 is an ago-allosteric modulator of dopamine D₁ receptors that decreases the agonist potency while preserving the receptor efficacy.

IP28 Is Not an Allosteric Modulator of Dopamine D₂ Receptors. IP28 not only binds to dopamine D₁ receptors, but also to dopamine D₂ receptors (Vendrell et al., 2009). To characterize IP28 binding to dopamine D₂ receptors, IP28 was compared with the D₂ dopamine receptors antagonist YM-09151-2 as displacer of [3 H]YM-09151-2 binding. Competition experiments were performed with a constant concentration of [3 H]YM-09151-2 (1 nM) and increasing concentrations of unlabeled YM-09151-2 (0.01 nM to 50 μM) or IP28 (10 nM to 100 μM) using brain striatal membranes as de-

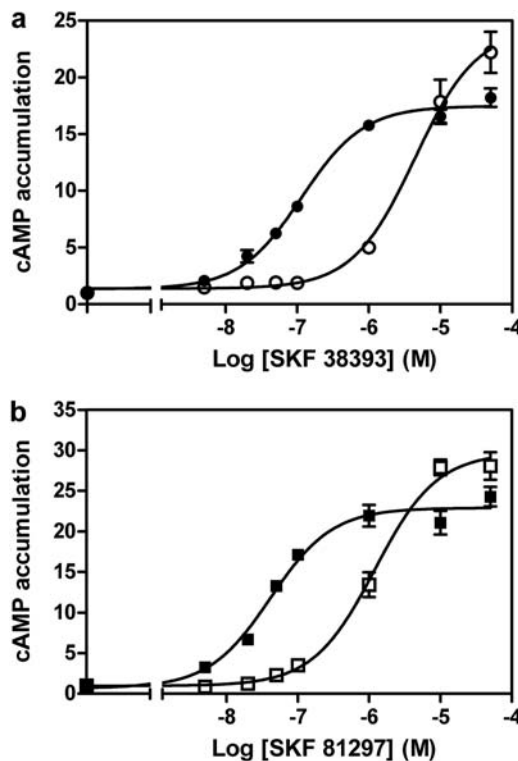


Fig. 6. Modulation of agonist-induced cAMP production by IP28. Cyclic AMP concentration was measured in Flp-In T-REx-293-D₁R cells after human dopamine D₁ receptor induction with tetracycline as described under *Materials and Methods*. Cells were treated with increasing concentrations of the partial agonist SKF 38393 (5 nM to 50 μM) in the absence (●) or presence (○) of 10 μM IP28 (a); the full agonist SKF 81297 (5 nM to 50 μM) in the absence (■) or presence (□) of 10 μM IP28 (b). Values are represented as infolds over the basal (nontreated cells). Data are mean \pm S.E.M. of a representative experiment ($n = 3$) performed in duplicate. For some data points, error is smaller than symbol.

scribed under *Materials and Methods*. Both YM-09151-2 and IP28 fully displaced the specific binding of [3 H]YM-09151-2 (Fig. 7a). For IP28 a EC_{50} of 2.6 \pm 0.4 μM was calculated. These results are compatible with IP28 binding to the orthosteric site of D₂ receptors. To further prove this hypothesis, dissociation kinetic experiments were performed. First of all, 0.5 nM [3 H]YM-09151-2 was incubated with brain striatal membranes in the absence or in the presence of 3 μM IP28 as indicated under *Materials and Methods* and dissociation was then initiated by the addition of 10 μM YM-09151-2. The

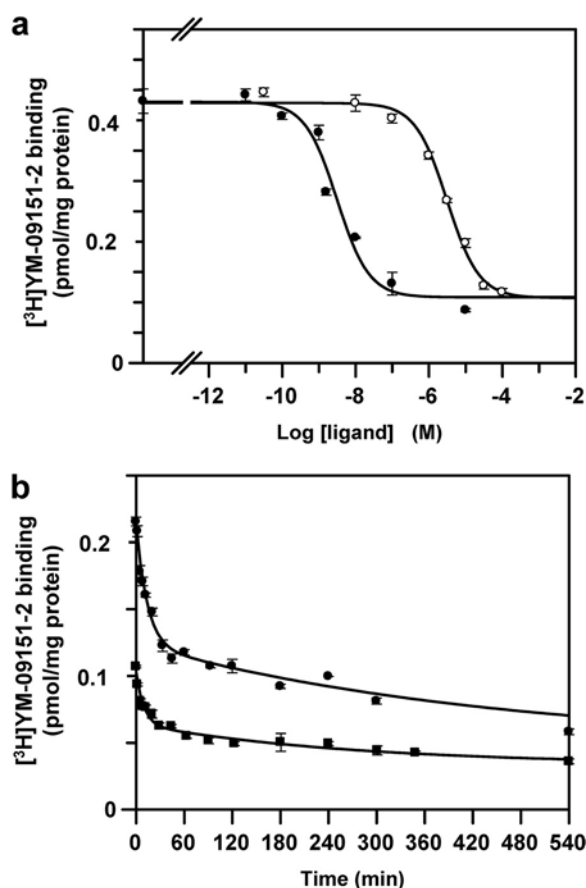


Fig. 7. Effect of IP28 on ligand binding to dopamine D₂ receptors. a, competition experiments of the dopamine D₂ receptor antagonist [³H]YM-09151-2 (0.65 nM) versus increasing concentrations of YM-09151-2 (0.01 nM to 10 μM) (●) or IP28 (0.1 nM to 100 μM) (○) were performed with brain striatal membranes as indicated under *Materials and Methods*. b, dissociation kinetic experiments were performed with brain striatal membranes incubated with 0.45 nM [³H]YM-09151-2 in the absence (●), or in the presence of 3 μM IP28 (■). In all cases, the dissociation was initiated by the addition of 10 μM unlabeled YM-09151-2 (see *Materials and Methods*). Data are mean ± S.E.M. from a representative experiment (*n* = 3) performed in triplicate. For some data points, error is smaller than symbol.

time course of dissociation is displayed in Fig. 7b. In the presence of IP28 the [³H]YM-09151-2 binding decreased (55%). In the absence of IP28, the dissociation curve was biphasic, and dissociation rate constants were 0.08 ± 0.01 and $0.002 \pm 0.001 \text{ min}^{-1}$. The presence of IP28 did not affect the dissociation rate, and the values of the dissociation rate constants were 0.09 ± 0.01 and $0.003 \pm 0.001 \text{ min}^{-1}$. This indicated that IP28 did not modify the dissociation of the orthosteric ligand. This finding indicates that IP28 is not an allosteric modulator of D₂ receptors and is consistent with a orthosteric binding of IP28 to dopamine D₂ receptors.

Because the D₂ receptor expression in striatal membranes is very high with respect to the expression of other D₂-like receptors, namely D₃ and D₄, (Lidow et al., 1998; Araki et al., 2007), the above-described results are representative of the IP28 binding to D₂ receptors. Because there are no selective radiolabeled ligands for the different subtypes of D₂-like receptors, the IP28 binding to D₃ and D₄ receptors was analyzed in cells expressing D₃ or D₄ receptors. Cells were generated and membranes were obtained as described under

Materials and Methods. Dissociation kinetic experiments were performed by incubating cell membranes (0.2 mg of protein/ml) from HEK-293 expressing D₃ or D₄ receptors with 0.5 nM [³H]YM-09151-2 in the absence or in the presence of 3 μM IP28, as indicated under *Materials and Methods*, and dissociation was then initiated by the addition of 10 μM YM-09151-2. In both cases, the [³H]YM-09151-2 binding decreased in the presence of IP28 (33% for the D₃ receptor and 36% for the D₄ receptor), and the dissociation of [³H]YM-09151-2 was biphasic. It is noteworthy that the presence of IP28 did not affect the values of the dissociation rate constants (Table 3). This indicates that IP28 did not modify the orthosteric ligand dissociation from D₃ or D₄ receptors. This finding is consistent with orthosteric binding of IP28 to all dopamine D₂-like receptors.

Discussion

The indoloquinolizidine scaffold constitutes a novel and synthetically accessible structure, which can potentially interact at transmembrane binding sites and exhibits good solubility in ethanol-aqueous media. The combinatorial exploration of indoloquinolizidine-peptide hybrids and their behavior at dopamine receptors has been studied recently (Vendrell et al., 2009). Here, we reported that one of the indoloquinolizidine peptide hybrids, IP28, is able to decrease the antagonist affinity for D₁ dopamine receptors. The increase in the *K_D* values for the antagonist in the presence of increasing concentrations of IP28 was nonlinear, thus indicating that IP28 binds to an allosteric site. To detect and quantify such allosteric interaction, the allosteric modulator effect on the rates of dissociation of orthosteric ligands has been one of the methods of election (May et al., 2007). Affinity modulators induce a conformational change that may alter one or both association or dissociation rates of binding to the orthosteric site. The most common method is to assay dissociation kinetics, because the only way dissociation of a pre-bound GPCR orthosteric ligand complex can be modified is by the concomitant binding of a modulator to a topographically distinct site (May et al., 2007). For this purpose, dissociation experiments (of a radiolabeled orthosteric ligand) can be performed in the presence of the allosteric modulator. These assays demonstrated that IP28 is an allosteric modulator that increased the dissociation rate of the radiolabeled neutral antagonist bound to D₁ dopamine receptors. This effect is specific for D₁ dopamine receptors because the dissociation rate of the radiolabeled antagonist bound to membranes from cells expressing D₅ dopamine receptors did not change in the presence of IP28. In addition, IP28 did not change the disso-

TABLE 3

Dissociation rate constants for [³H]YM-09151-2 binding to dopamine D₃ and D₄ receptors determined in the absence or in the presence of IP28 (3 μM)

Binding data from dissociation kinetic experiments were fitted assuming monophasic (*n* = 1 in eq. 7) and biphasic (*n* = 2 in eq. 7) dissociation curves. By the *F* test, a significant better fit was obtained by considering *n* = 2 (see *Materials and Methods*). Parameter values represent the mean ± S.E.M. of three experiments.

Receptor Subtype	Experimental Conditions	<i>k_i</i> <i>min</i> ⁻¹	
		<i>k_i</i> <i>slow</i>	<i>k_i</i> <i>fast</i>
D ₃	Control	0.002 ± 0.001	0.13 ± 0.08
D ₃	IP28	0.003 ± 0.002	0.13 ± 0.04
D ₄	Control	0.0033 ± 0.0003	0.04 ± 0.02
D ₄	IP28	0.004 ± 0.001	0.05 ± 0.02

ciation rate of the radiolabeled antagonist bound to D₂, D₃, or D₄ dopamine receptors, indicating that IP28 is not an allosteric modulator of D₂-like receptors and suggesting that it behaves as an orthosteric ligand of D₂-like dopamine receptors. Although IP28 binds to all subtypes of dopamine receptors, it is a specific allosteric modulator for dopamine D₁ receptors. This is interesting from a pharmacological point of view, because the study of IP28 could open new avenues for the design of more affinity and selective drugs acting as allosteric modulators.

An allosteric effect results in a positive modulation if the modulator facilitates an interaction, or in a negative modulation if it inhibits the interaction of the ligand with the orthosteric binding site (May et al., 2007; Schwartz and Holst, 2007; Conn et al., 2009). According to these concepts, IP28 is an allosteric ligand of dopamine D₁ receptors that negatively modulates the agonist and antagonist binding to the orthosteric site of the receptor. Moreover, IP28 was able to disrupt the homotropic cooperativity in agonist binding to dopamine D₁ receptors and also the positive cross talk observed between the antagonist and the agonist binding in competition experiments. In summary, the binding of IP28 to its allosteric site reduces the orthosteric-mediated ligand-induced molecular cross-talk between the two protomers in the D₁ receptor dimer. In terms of signaling, it is interesting that IP28 was able to induce cAMP increases in cells expressing D₁ dopamine receptors (and not in parental cells). This result suggested that IP28 would be an ago-allosteric modulator, which recently has been defined as a ligand that functions both as an agonist on its own and as an allosteric modulator of the effect of the agonists (Schwartz and Holst, 2006, 2007; Bridges and Lindsley, 2008). This distinguishes ago-allosteric from allosteric modulators, i.e., modulators that, as defined by International Union of Pharmacology (IUPHAR: <http://www.iuphar.org/>) (Neubig et al., 2003), enhance or inhibit the affinity and/or the effect of the orthosteric agonist but have no effect on their own. The effect of the ago-allosteric modulator can be positive with regard to both efficacy and potency but might also be negative or inhibitory in terms of, for example, potency while being positive in terms of efficacy (Schwartz and Holst, 2007). As demonstrated in this article, IP28 increased the EC₅₀ values obtained from curves of cAMP response versus increasing D₁ dopamine receptor agonist concentrations. This indicates that IP28 is an ago-allosteric negative modulator of agonist potency without decreasing the agonist-mediated maximum effect, i.e., without affecting efficacy of the full/partial agonists used.

Diverse evidence suggests that D₁ dopamine antagonists may have neuroleptic properties (Andersen and Nielsen, 1991; Wu et al., 2005). An important role for D₁ dopamine receptors in the pathophysiology of schizophrenia has been described (Goldman-Rakic, 1999; Sedvall et al., 1995). It was demonstrated that selective D₁ antagonists had antipsychotic activity in preclinical studies, but a clinical trial of selective D₁ antagonists demonstrated no antipsychotic activity, and instead may have aggravated psychoses in some patients (Miyamoto et al., 2005). In contrast to the ineffectiveness of D₁ antagonists in the treatment of schizophrenia, low doses of selective full D₁ receptor agonists, such as dihydrexidine, A77636 and SKF81297, have been reported to have cognitive-enhancing actions in non-human primates

(Cai and Arnsten, 1997). Cognitive impairment has been found across all subtypes of schizophrenia. It is postulated that either insufficient or excessive D₁ receptor stimulation is deleterious to cognitive function of the prefrontal cortex; thus, an "optimal" level of D₁ receptor activation is necessary for normal cognitive function (Goldman-Rakic et al., 2000), and overactivation of D₁ dopamine receptors may exacerbate psychotic symptoms in patients with schizophrenia (Bubenikova-Valesova et al., 2009). It may be speculated that compounds able to decrease the potency while preserving the efficacy of D₁ receptor agonists are promising targets of exploration as modulators of dopamine compounds in psychotic pathologies. Because IP28 binding to an allosteric site on D₁ dopamine receptors decreases the ligand affinity and the receptor potency while preserving the receptor efficacy, a D₁-like receptor agonist stimulation in the presence of this type of compound, showing weak agonist properties by its own, is promising in terms of therapeutic potential. Mapping the interacting zone in the D₁ receptor combined with strategies to improve selectivity and the affinity of the interaction between the allosteric compound and the receptor are required to meet this desirable objective.

Acknowledgments

We thank Jasmina Jiménez (Molecular Neurobiology Laboratory, Barcelona University) for technical assistance.

References

- Andersen PH and Nielsen EB (1991) Novel approaches to development of antipsychotics. *Drug News Perspect* **4**:150–157.
- Araki KY, Sims JR, and Bhide PG (2007) Dopamine receptors mRNA and protein expression in the mouse corpus striatum and cerebral cortex during pre- and post-natal development. *Brain Res* **1156**:31–45.
- Bridges TM and Lindsley CW (2008) G-protein-coupled receptors: from classical modes of modulation to allosteric mechanisms. *ACS Chem Biol* **3**:530–541.
- Bubenikova-Valesova V, Svoboda J, Horacek J and Vales K (2009) The effect of a full agonist/antagonist of the D1 receptor on locomotor activity, sensorimotor gating and cognitive function in dizocilpine-treated rats. *Int J Neuropsychopharmacol* **12**:873–883.
- Cai JX and Arnsten AF (1997) Dose-dependent effects of the dopamine D1 receptor agonists A77636 or SKF81297 on spatial working memory in aged monkeys. *J Pharmacol Exp Ther* **283**:183–189.
- Casadó V, Cantí C, Mallol J, Canela EI, Lluís C, and Franco R (1990) Solubilization of A1 adenosine receptor from pig brain: characterization and evidence of the role of the cell membrane on the coexistence of high- and low-affinity states. *J Neurosci Res* **26**:461–473.
- Casadó V, Cortés A, Ciruela F, Mallol J, Ferré S, Lluís C, Canela EI, and Franco R (2007) Old and new ways to calculate the affinity of agonists and antagonists interacting with G-protein-coupled monomeric and dimeric receptors: the receptor-dimer cooperativity index. *Pharmacol Ther* **116**:343–354.
- Casadó V, Cortés A, Mallol J, Pérez-Capote K, Ferré S, Lluís C, Franco R, and Canela EI (2009a) GPCR homomers and heteromers: a better choice as targets for drug development than GPCR monomers?. *Pharmacol Ther* **124**:248–257.
- Casadó V, Ferrada C, Bonaventura J, Gracia E, Mallol J, Canela EI, Lluís C, Cortés A, and Franco R (2009b) Useful pharmacological parameters for G-protein-coupled receptor homodimers obtained from competition experiments. Agonist-antagonist binding modulation. *Biochem Pharmacol* **78**:1456–1463.
- Christopoulos A (2002) Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat Rev Drug Discov* **1**:198–210.
- Conn PJ, Christopoulos A, and Lindsley CW (2009) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat Rev Drug Discov* **8**:41–54.
- Erickson SS, Cummings DF, Weinstein H, and Schetz JA (2009) Ligand selectivity of D2 dopamine receptors is modulated by changes in local dynamics produced by sodium binding. *J Pharmacol Exp Ther* **328**:40–54.
- Ferré S, Baler R, Bouvier M, Caron MG, Devi LA, Durroux T, Fuxe K, George SR, Javitch JA, Lohse MJ, et al. (2009). Building a new conceptual framework for receptor heteromers. *Nat Chem Biol* **5**:131–134.
- Franco R, Casadó V, Mallol J, Ferré S, Fuxe K, Cortés A, Ciruela F, Lluís C, and Canela EI (2005) Dimer-based model for heptaspanning membrane receptors. *Trends Biochem Sci* **30**:360–366.
- Franco R, Casadó V, Mallol J, Ferrada C, Ferré S, Fuxe K, Cortés A, Ciruela F, Lluís C, and Canela EI (2006) The two-state dimer receptor model: a general model for receptor dimers. *Mol Pharmacol* **69**:1905–1912.
- George SR, Lee SP, Varghese G, Zeman PR, Seeman P, Ng GY, and O'Dowd BF (1998) A transmembrane domain-derived peptide inhibits D1 dopamine receptor function without affecting receptor oligomerization. *J Biol Chem* **273**:30244–30248.

- Gerfen CR (2004) *The Rat Nervous System* (Paxinos G ed) pp 445–508, Elsevier Academic Press, Amsterdam.
- Goldman-Rakic PS (1999) The physiological approach: functional architecture of working memory and disordered cognition in schizophrenia. *Biol Psychiatry* **46**: 650–661.
- Goldman-Rakic PS, Muly EC III, and Williams GV (2000) D(1) receptors in prefrontal cells and circuits. *Brain Res Brain Res Rev* **31**:295–301.
- Guo W, Urizar E, Kralikova M, Mobarec JC, Shi L, Filizola M, and Javitch JA (2008) Dopamine D2 receptors form higher order oligomers at physiological expression levels. *EMBO J* **27**:2293–2304.
- Han Y, Moreira IS, Urizar E, Weinstein H, and Javitch JA (2009) Allosteric communication between protomers of dopamine class A GPCR dimers modulates activation. *Nat Chem Biol* **9**:688–695.
- Hoare SR, Coldwell MC, Armstrong D, and Strange PG (2000) Regulation of human D(1), d(2(long)), d(2(short)), D(3) and D(4) dopamine receptors by amiloride and amiloride analogues. *Br J Pharmacol* **130**:1045–1059.
- Kong MM, Fan T, Varghese G, O'Dowd BF, and George SR (2006) Agonist-induced cell surface trafficking of an intracellularly sequestered D1 dopamine receptor homo-oligomer. *Mol Pharmacol* **70**:78–89.
- Lidow MS, Wang F, Cao Y, and Goldman-Rakic PS (1998) Layer V neurons bear the majority of mRNA encoding the five distinct dopamine receptor subtypes in the primate prefrontal cortex. *Synapse* **28**:10–20.
- May LT, Leach K, Sexton PM, and Christopoulos A (2007) Allosteric modulation of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **47**:1–51.
- Miyamoto S, Duncan GE, Marx CE, and Lieberman JA (2005) Treatments for schizophrenia: a critical review of pharmacology and mechanisms of action of antipsychotic drugs. *Mol Psychiatry* **10**:79–104.
- Neubig RR, Spedding M, Kenakin T, Christopoulos A, and International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (2003) International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol Rev* **55**:597–606.
- Neve KA, Seamans JK, and Trantham-Davidson H (2004) Dopamine receptor signaling. *J Recept Signal Transduct Res* **24**:165–205.
- Ng GY, Mouillac B, George SR, Caron M, Dennis M, Bouvier M, and O'Dowd BF (1994) Desensitization, phosphorylation and palmitoylation of the human dopamine D1 receptor. *Eur J Pharmacol* **267**:7–19.
- Sarrió S, Casadó V, Escriche M, Ciruela F, Mallol J, Canela EI, Lluís C, and Franco R (2000) The heat shock cognate protein hsc73 assembles with A(1) adenosine receptors to form functional modules in the cell membrane. *Mol Cell Biol* **20**:5164–5174.
- Schetz JA (2005) Allosteric modulation of dopamine receptors. *Mini Rev Med Chem* **5**:555–561.
- Schetz JA and Sibley DR (2001) The binding-site crevice of the D4 dopamine receptor is coupled to three distinct sites of allosteric modulation. *J Pharmacol Exp Ther* **296**:359–363.
- Schwartz TW and Holst B (2006) Ago-allosteric modulation and other types of allostery in dimeric 7TM receptors. *J Recept Signal Transduct Res* **26**:107–128.
- Schwartz TW and Holst B (2007) Allosteric enhancers, allosteric agonists and ago-allosteric modulators: where do they bind and how do they act? *Trends Pharmacol Sci* **28**:366–373.
- Sedvall G, Farde L, Hall H, Halldin C, Karlsson P, Nordström AL, Nyberg S, and Pauli S (1995) Utilization of radioligands in schizophrenia research. *Clin Neurosci* **3**:112–121.
- Vendrell M, Soriano A, Casadó V, Díaz JL, Lavilla R, Canela EI, Lluís C, Franco R, Albericio F and Royo M (2009) Indoloquinolizidine-Peptide Hybrids as Multiple Agonists for D₁ and D₂ Dopamine Receptors. *ChemMedChem* **4**:1514–1522.
- Verma V, Mann A, Costain W, Pontoriero G, Castellano JM, Skoblenick K, Gupta SK, Pristupa Z, Niznik HB, Johnson RL, et al. (2005) Modulation of agonist binding to human dopamine receptor subtypes by L-prolyl-L-leucyl-glycinamide and a peptidomimetic analog. *J Pharmacol Exp Ther* **315**:1228–1236.
- Wu WL, Burnett DA, Spring R, Greenlee WJ, Smith M, Favreau L, Fawzi A, Zhang H, and Lachowicz JE (2005) Dopamine D1/D5 receptor antagonists with improved pharmacokinetics: design, synthesis, and biological evaluation of phenol bioisosteric analogues of benzazepine D1/D5 antagonists. *J Med Chem* **48**:680–693.

Address correspondence to: Dr. Enric I Canela, Department of Biochemistry and Molecular Biology, Faculty of Biology, Avda. Diagonal 645, 08028 Barcelona, Spain. E-mail: ecanela@ub.edu

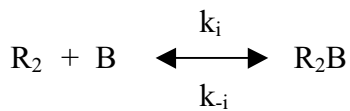
SUPPLEMENTAL DATA

A HYBRID INDOLOQUINOLIZIDINE/ PEPTIDE AS ALLOSTERIC MODULATOR OF DOPAMINE D₁ RECEPTORS

Aroa Soriano, Marc Vendrell, Sergio González, Josefa Mallol, Fernando Albericio, Miriam Royo Carmen Lluís, Enric I. Canela, Rafael Franco, Antoni Cortés, Vicent Casadó.

J Pharmacol Exp Ther

A) RADIOLIGAND/COMPETITOR MODULATION

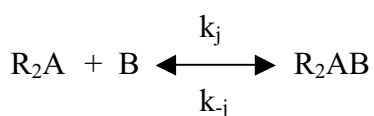


$$v_{fR_2B} = k_i [B] [\text{free sites in } R_2] = k_i [B]^2 [R_2]$$

$$v_{dR_2B} = k_{-i} [\text{occupied sites in } R_2B] = k_{-i} [R_2B]$$

in the equilibrium: $k_i [B]^2 [R_2] = k_{-i} [R_2B]$

$$k_{-i}/k_i = 2 [B] [R_2] / [R_2B] = 2 K_{DB1}$$



$$V_{fR_{2AB}} = k_j[B] [\text{free sites in } R_2A] = k_j [B] [R_2A]$$

$$V_{dR_{2AB}} = k_{-j} [\text{occupied sites in } R_2AB] = k_{-j}[R_2AB]$$

in the equilibrium: $k_j [B] [R_2A] = k_{-j} [R_2AB]$

$$k_{-j}/k_j = [B] [R_2A] / [R_2AB] = K_{DAB}$$

when:

$$k_{-i}/k_i = k_{-j}/k_j \rightarrow K_{DAB} = 2 K_{DB1} \quad (\text{non A / B modulation})$$

$$k_{-i}/k_i > k_{-j}/k_j \rightarrow K_{DAB} > 2 K_{DB1} \quad (\text{negative A / B modulation})$$

$$k_{-i}/k_i < k_{-j}/k_j \rightarrow K_{DAB} < 2 K_{DB1} \quad (\text{positive A / B modulation})$$

B) APPARENT EQUILIBRIUM DISSOCIATION CONSTANTS AS FUNCTION OF COMPETITOR CONCENTRATION

According to the two-state dimer receptor model, in competition experiments between two orthosteric ligands, the radioligand A and the competitor B, the saturation function of the receptor by A (\bar{Y}_A) can be easily deduced by rearranging eq. 1 in Materials and Methods:

$$\bar{Y}_A = \frac{A_{bound}}{R_T} = \frac{2A^2 + AK_{DA2} \left(1 + \frac{B}{K_{DAB}}\right)}{A^2 + AK_{DA2} \left(1 + \frac{B}{K_{DAB}}\right) + K_{DA1}K_{DA2} \left(1 + \frac{B}{K_{DB1}} + \frac{B^2}{K_{DB1}K_{DB2}}\right)}$$

From this expression, the apparent macroscopic equilibrium dissociation constants for the radioligand A (K_{DA1_app} and K_{DA2_app}) are deduced as function of the competitor (B) concentration as follows:

$$K_{DA2_app} = K_{DA2} \left(1 + \frac{B}{K_{DAB}} \right)$$

$$K_{DA1_app} = K_{DA1} \frac{1 + \frac{B}{K_{DB1}} + \frac{B^2}{K_{DB1}K_{DB2}}}{1 + \frac{B}{K_{DAB}}}$$

When the competitor B is non-cooperative, K_{DB2} is $4 \cdot K_{DB1}$, and the apparent K_{DA1} expression simplifies to:

$$K_{DA1_app} = K_{DA1} \frac{\left(1 + \frac{B}{2K_{DB1}} \right)^2}{1 + \frac{B}{K_{DAB}}}$$

The graphical representation of this function is a parabolic, non-hyperbolic, curve.

When there is not radioligand-competitor modulation K_{DAB} is $2 \cdot K_{DB1}$ (see A in supplemental data) and the relationship between the K_{DA1_app} and the competitor B concentration is a straight line:

$$K_{DA1_app} = K_{DA1} \left(1 + \frac{B}{2K_{DB1}} \right)$$

Assuming that IP28 (B) competes with [³H]SCH 23390 (A) for the binding to dopamine D₁ receptor orthosteric site, the competition curves in Figure 1 (see Results) were fitted to the equation derived from the dimer receptor model (equations 2 and 6, see Materials and Methods), and the parameter values obtained were: $K_{DA1} = 0.62$ nM, $K_{DB1} = 1.1$ μM, $K_{DB2} = 4.4$ μM and $K_{DAB} = 2.2$ μM. Taking into account these values and considering the above last equation, it was simulated the linear dependence between the apparent equilibrium dissociation constants and the concentration of IP28 depicted as a dotted line in Figure 2b (see Results).

3.2 Dopamine D₄ receptor, but not the ADHD-associated D_{4.7} variant, forms functional heteromers with the dopamine D₂ receptor in the brain.

Sergio González, Claudia Rangel-Barajas, Marcela Peper, Ramiro Lorenzo, Estefanía Moreno, Francisco Ciruela, Janusz Borycz, Carme Lluís, Rafael Franco, Peter J. McCormick, Nora Volkow, Marcelo Rubinstein, Benjamin Floran y Sergi Ferré

Manuscrito publicado en *Molecular Psychiatry*. 2010. August 16. doi: 10.1038/mp.2011.93. [Epub ahead of print]

Las variantes polimórficas del receptor de la dopamina D₄ se han asociado al desorden de hiperactividad y déficit de atención (ADHD). Sin embargo, la significación funcional de los diferentes polimorfismos (número variable de repeticiones en tándem en el exón 3) se desconoce todavía. En este trabajo se describe que las variantes polimórficas más frecuentes que contienen 4 (D_{4.4}) y 2 (D_{4.2}) repeticiones, forman heterómeros funcionales con los receptores de dopamina D₂, mientras que la forma polimórfica asociada a ADHD, que contiene 7 repeticiones en tándem (D_{4.7}), no es capaz de heteromerizar. La activación del receptor D₂ en el heterómero D₂-D₄ potencia la señalización de las MAPK mediada por la activación del receptor D₄ en células transfectadas y en estriado de ratón, pero no ocurre lo mismo en células que expresan el receptor D_{4.7} o en ratones knock-in que expresan el receptor D_{4.7} humano. En el estriado, el heterómero D₂-D₄ modula la liberación de glutamato. Se concluye que la disrupción funcional del heterómero D₂-D₄ puede alterar el control dopaminérgico presináptico de la neurotransmisión glutamatérgica cortico-estriatal lo que podría estar asociado al déficit funcional del trastorno de ADHD.

ORIGINAL ARTICLE

Dopamine D₄ receptor, but not the ADHD-associated D_{4.7} variant, forms functional heteromers with the dopamine D_{2S} receptor in the brain

S González¹, C Rangel-Barajas², M Peper³, R Lorenzo³, E Moreno¹, F Ciruela⁴, J Borycz⁵, J Ortiz⁶, C Lluís¹, R Franco⁷, PJ McCormick¹, ND Volkow⁸, M Rubinstein³, B Floran² and S Ferré⁵

¹Department of Biochemistry and Molecular Biology, Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas, University of Barcelona, Barcelona, Spain; ²Departamento de Fisiología, Biofísica y Neurociencias, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México D.F., México; ³Departamento de Fisiología y Biología Molecular y Celular, Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina; ⁴Unitat de Farmacologia, Departament Patologia i Terapèutica Experimental, Universitat de Barcelona, L'Hospitalet de Llobregat, Barcelona, Spain; ⁵CNS Receptor-Receptor Interactions Unit, National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, Baltimore, MD, USA; ⁶Department of Biochemistry and Molecular Biology, Neuroscience Institute, Universitat Autònoma de Barcelona, Bellaterra, Spain; ⁷Departamento de Neurociencias, Centro de Investigación Médica Aplicada, Universidad de Navarra, Pamplona, Spain and ⁸National Institute on Drug Abuse, National Institutes of Health, Bethesda MD, USA

Polymorphic variants of the dopamine D₄ receptor have been consistently associated with attention-deficit hyperactivity disorder (ADHD). However, the functional significance of the risk polymorphism (variable number of tandem repeats in exon 3) is still unclear. Here, we show that whereas the most frequent 4-repeat (D_{4.4}) and the 2-repeat (D_{4.2}) variants form functional heteromers with the short isoform of the dopamine D₂ receptor (D_{2S}), the 7-repeat risk allele (D_{4.7}) does not. D₂ receptor activation in the D_{2S}–D₄ receptor heteromer potentiates D₄ receptor-mediated MAPK signaling in transfected cells and in the striatum, which did not occur in cells expressing D_{4.7} or in the striatum of knockin mutant mice carrying the 7 repeats of the human D_{4.7} in the third intracellular loop of the D₄ receptor. In the striatum, D₄ receptors are localized in corticostriatal glutamatergic terminals, where they selectively modulate glutamatergic neurotransmission by interacting with D_{2S} receptors. This interaction shows the same qualitative characteristics than the D_{2S}–D₄ receptor heteromer-mediated mitogen-activated protein kinase (MAPK) signaling and D_{2S} receptor activation potentiates D₄ receptor-mediated inhibition of striatal glutamate release. It is therefore postulated that dysfunctional D_{2S}–D_{4.7} heteromers may impair presynaptic dopaminergic control of corticostriatal glutamatergic neurotransmission and explain functional deficits associated with ADHD.

Molecular Psychiatry advance online publication, 16 August 2011; doi:10.1038/mp.2011.93

Keywords: dopamine receptors; receptor heteromers; ADHD; striatum; glutamate

Introduction

Dopamine D₄ receptors are expressed in the prefrontal cortex, in GABAergic interneurons and in glutamatergic pyramidal neurons, including their striatal projections.^{1–3} D₄ receptors have been implicated in attention-deficit hyperactivity disorder (ADHD).^{1,4–6} In fact, the prefrontal cortex and associated frontostriatal circuits are critical for executive function and are involved in ADHD.⁵ The gene encoding the

human D₄ receptor contains a large number of polymorphisms in its coding sequence.⁴ The most extensive polymorphism is found in exon 3, a region that codes for the third intracellular loop (3IL) of the receptor. This polymorphism consists of a variable number of tandem repeats in which a 48-bp sequence exists as 2- to 11-fold repeats.⁷ The three most common variants contain 2, 4 and 7 repeats (D_{4.2}, D_{4.4} and D_{4.7}, respectively). D_{4.4} constitutes the most frequent variant, with a global frequency of 64%, followed by D_{4.7} (21%) and D_{4.2} (8%).⁸ Importantly, a high prevalence of the D_{4.7} variant has been demonstrated in children diagnosed with ADHD.⁵ Though stimulation of the D_{4.7} variant has been reported to be less potent at inhibiting cAMP than D_{4.2} or D_{4.4},⁹ the functional significance of these variants are poorly understood.

Correspondence: Dr S Ferré, National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, 251 Bayview Boulevard, Baltimore, MD 21224, USA.

E-mail: sferre@intra.nida.nih.gov

Received 25 March 2011; revised 27 June 2011; accepted 7 July 2011

Receptor heteromers are becoming the focus of extensive research in the field of G-protein-coupled receptors.¹⁰ A receptor heteromer is currently defined as a macromolecular complex composed of at least two (functional) receptor units with biochemical properties that are demonstrably different from those of its individual components.¹⁰ In some cases, receptor heteromers provide a framework in which to understand the role of receptors with no clear functional significance, and example being the D₃ receptor, which forms heteromers with the D₁ receptor and modifies its function.¹¹ A recent study showed that in mammalian transfected cells, the long isoform of the D₂ receptor (D_{2L}) heteromerizes with the three main D₄ receptor variants, D_{4.2}, D_{4.4} and D_{4.7}.¹² Interestingly, results from the same study suggested that D_{4.7} was less effective in forming heteromers with D_{2L} receptors.¹² In view of the reported evidence of predominant co-localization of D₄ receptors with the short isoform of the D₂ receptor (D_{2S}) in corticostriatal glutamatergic terminals,^{2,3,13} we first investigated if any of the three main human variants of the D₄ receptor could interact both physically and functionally with D_{2S}. By using the Bioluminescence Resonance Energy Transfer (BRET) technique, here we show evidence for the formation of heteromers between D_{2S} and D_{4.2} and D_{4.4} variants of the D₄ receptor. In contrast, the D_{4.7} variant failed to form heteromers with the D_{2S} receptor. In transfected cells, we found a biochemical property of the D_{2S}-D₄ receptor heteromer, which consists of the ability of D_{2S} receptor activation to potentiate D₄ receptor-mediated mitogen-activated protein kinase (MAPK) signaling. A similar result was observed in striata from wild-type (WT) mice, a species that expresses D₄ receptors with a short 3IL comparable to human D_{4.2}. In contrast, potentiation of D₄ receptor-mediated MAPK signaling was not observed in transfected cells expressing D_{4.7} or in striata taken from *knockin* mice carrying a humanized 7-repeat intracellular loop identical to that found in human D_{4.7}. Finally, analyzing neurotransmitter release in striatal slices and with *in vivo* microdialysis in rats, evidence was obtained for a key role of D₂-D₄ receptor interaction in the modulation of striatal glutamatergic neurotransmission.

Materials and methods

Fusion proteins and expression vectors

The synthetic cDNAs for the human D_{4.2}, D_{4.4} and D_{4.7} receptor gene (kindly provided by TP Sakmar, Rockefeller University, USA) were amplified using sense oligonucleotide primer (5'-TCAACGGGACTTTCCA AAATGT-3') and antisense primer (5'-CTCCGAGAT CAACTTCTGCTCGCTTCGGTTACCC-3'), resulting in a cDNA fragment of 200bp. A second product was generated using the sense oligonucleotide primer (5'-AAGTTGATCTCGGAGGAAGATACAGCAGATGC AG-3') and antisense primer (5'-GCGAATTCGCAGC AAGCAGGTAGAGCCTTACG-3'), resulting in a cDNA

fragment of 1500bp. Equimolar quantities of both fragments were used to produce a third product corresponding to the myc-D_{4.2}, myc-D_{4.4} or myc-D_{4.7}-tagged gene using the sense primer (5'-GTGCTCGAG CACCATGGGTAACCGAAGCACAG-3') and antisense primer without its stop codon (5'-GCGAATTCTCAG CAGCAAGCACGTAGAGCCTTACG-3'), harboring unique *Xho*I and *Eco*RI restriction sites, respectively. The fragments were then subcloned in-frame into *Xho*I/*Eco*RI sites of the pcDNA3.1 vector (Invitrogen, Paisley, Scotland, UK). Next, the human cDNAs for the adenosine A₁ receptor and dopamine D_{4.2}, D_{4.4}, D_{4.7} and D_{2S} receptors, cloned in *pcDNA3.1* were amplified without their stop codons using sense and antisense primers harboring unique *Xho*I and *Eco*RI sites to clone A₁, D_{4.2}, D_{4.4} and D_{4.7} receptors in the RLuc and the yellow fluorescent protein (YFP) corresponding vectors, and *Hind*III and *Bam*HI to clone D_{2S} in the RLuc and the YFP corresponding vectors. The mouse cDNAs for the D₄ and D_{2S} receptors, cloned in pCMV-SPORT6 (American Type Culture Collection, Manassas, USA) and pReceiver-M16 vectors, respectively (GeneCopeia, Rockville, MD, USA), were amplified without their stop codons using sense and antisense primers harboring unique *Xho*I and *Eco*RV sites to clone D₄ receptor in the RLuc corresponding vector, and *Xho*I and *Kpn*I to clone D_{2S} receptor in the RLuc and the YFP corresponding vectors. The amplified fragments were subcloned to be in-frame into restriction sites of the multiple cloning sites of EYFP-N3 vector (enhanced yellow variant of YFP; Clontech, Heidelberg, Germany) or the mammalian humanized pRLuc-N1 vectors (Perkin-Elmer, Waltham, MA, USA) to give the plasmids that express the receptors fused to either RLuc or YFP on the C-terminal end of the receptor (D_{4.2}-RLuc, D_{4.4}-RLuc, D_{4.7}-RLuc, D_{2S}-RLuc and A₁-RLuc or D_{2S}-YFP, D_{4.7}-YFP and D₁-YFP, respectively). All constructs were verified by nucleotide sequencing and the fusion proteins are functional and expressed at the membrane level (see Results).

Cell culture and transient transfection

HEK (human embryonic kidney)-293T cells were grown in DMEM (Dulbecco's modified Eagle's medium) (Gibco Paisley, Scotland, UK) supplemented with 2 mM L-glutamine, 100 U ml⁻¹ penicillin/streptomycin and 5% (v/v) heat-inactivated fetal bovine serum (all supplements were from Invitrogen). CHO cell lines were maintained in α -MEM medium without nucleosides, containing 10% fetal calf serum, 50 μ g ml⁻¹ penicillin, 50 μ g ml⁻¹ streptomycin and 2 mM L-glutamine (300 μ g ml⁻¹). Cells were maintained at 37°C in an atmosphere of 5% CO₂, and were passaged when they were 80–90% confluent, twice a week. HEK-293T or CHO cells growing in six-well dishes or in 25 cm² flasks were transiently transfected with the corresponding fusion protein cDNA by the PEI (PolyEthylenImine; Sigma, Steinheim, Germany) method as previously described.¹⁴

Immunostaining

For immunocytochemistry, HEK-293T cells were grown on glass coverslips and transiently transfected with 1 µg of cDNA corresponding to human D_{4.2}-RLuc, D_{4.4}-RLuc or D_{4.7}-RLuc and 0.5 µg of cDNA corresponding to human D_{2S}-YFP or 0.8 µg of cDNA corresponding to mouse D₄-RLuc and 0.5 µg of cDNA corresponding to mouse D_{2S}-YFP. After 48 h of transfection, cells were fixed in 4% paraformaldehyde for 15 min and washed with phosphate-buffered saline containing 20 mM glycine to quench the aldehyde groups. After permeabilization with phosphate-buffered saline containing 0.05% Triton X-100 for 15 min, cells were treated with phosphate-buffered saline containing 1% bovine serum albumin. After 1 h at room temperature, cells were labeled with the primary rabbit monoclonal anti-human D₄ receptor (1/10 000; Abcam, Cambridge, UK) or with the primary goat polyclonal anti-D₄ receptor (1/500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, washed and stained with the secondary antibody Cy3 anti-rabbit (1/200; Jackson ImmunoResearch, Baltimore, PA, USA) or with the secondary antibody Cy3 anti-goat (1/200; Jackson ImmunoResearch). The D_{2S}-YFP construct was detected by its fluorescence properties. Samples were rinsed and observed in an Olympus confocal microscope.

BRET assay

HEK-293T cells were co-transfected with a constant amount of cDNA encoding for the receptor fused to RLuc and with increasingly amounts of cDNA encoding to the receptor fused to YFP to measure BRET as previously described.¹⁴ Both fluorescence and luminescence for each sample were measured before every experiment to confirm similar donor expressions (~100 000 bioluminescence units) while monitoring the increase in acceptor expression (2000–20 000 fluorescence units). The relative amounts of BRET acceptor are expressed as the ratio between the net fluorescence of the acceptor and the luciferase activity of the donor being the net fluorescence the fluorescence of the acceptor minus the fluorescence detected in cells only expressing the donor. The BRET ratio is defined as [(emission at 510–590)/(emission at 440–500)]–Cf, where Cf corresponds to (emission at 510–590)/(emission at 440–500) for the D₄-RLuc or D_{2S}-RLuc constructs expressed alone in the same experimental conditions. Curves were fitted by using a non-linear regression equation, assuming a single phase with GraphPad Prism software (San Diego, CA, USA).

Generation of knockin mutant mice carrying human expansions in the 3IL of the D₄ receptor

A targeting vector was designed such that coding sequences of the 3IL of mouse *Drd4* were replaced by human ortholog sequences corresponding to the most frequent 7-variable number of tandem repeat human variant allele (see Figure 4). The vector included a selectable PGK-*neo* cassette, flanked by two loxP

sites, placed just downstream of *Drd4* polyadenylation site and an herpes simplex virus-thymidine kinase cassette placed at one of the extremes of the targeting vector to select for the absence of random integrations. A long and short arm of *Drd4* homology were inserted flanking the swapped sequence and the selectable marker, respectively. The linearized vector was used to electroporate hybrid 129svev/C57BL/6 ES cells (inGenious Targeting Laboratory, Stony Brook, NY, USA) and homologous recombinant clones were selected in the presence of G418 and gancyclovir. Two selected clones carrying the human 7-variable number of tandem repeat were used to microinject C57BL/6J blastocysts and one high percentage chimeric male mouse was used to produce heterozygote *Drd4*^{+ /7repeat.neo} mice. The neo cassette was excised from the recombinant allele by crossing mutant mice with transgenic mice expressing Cre recombinase from an EIIa promoter (Jackson Laboratories; Cat. No. 003724). The resulting heterozygote *Drd4*^{+ /7repeat} (D_{4.7} knockin) mice were successively bred to C57BL/6J mice to obtain a congenic heterozygote strain (*n* = 10) that was used to establish a breeding colony. Homozygous D_{4.7} knockin mice and their WT littermates were used for the experiments. Knockin animals were characterized as indicated in Figure 4.

Mouse striatal slices preparation

Mice were housed five per cage in a temperature (21 ± 1 °C) and humidity-controlled (55 ± 10%) room with a 12:12-h light/dark cycle (light between 0800 and 2000 hours) with food and water *ad libitum*. All animal procedures were conducted according to the standard ethical guidelines (National Institutes of Health Animal care guidelines and European Communities Council Directive 86/609/EEC) and approved by the Local Ethical and Animal Care Committees. Transgenic mice and littermates were decapitated with a guillotine and the brains were rapidly removed and placed in ice-cold oxygenated (O₂/CO₂:95%/5%) Krebs-HCO₃⁻ buffer (124 mM NaCl, 4 mM KCl, 1.25 mM NaH₂PO₄, 1.5 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose and 26 mM NaHCO₃, pH 7.4). The brains were sliced at 4 °C in a brain matrix (Zivic Instruments, Pittsburgh, PA, USA) into 0.5 mm coronal slices. Slices were kept at 4 °C in Krebs-HCO₃⁻ buffer during the dissection of the striatum. Each slice was transferred into an incubation tube containing 1 ml of ice-cold Krebs-HCO₃⁻ buffer. The temperature was raised to 23 °C and after 30 min, the media was replaced by 2 ml Krebs-HCO₃⁻ buffer (23 °C).

ERK phosphorylation assay

Striatal slices from transgenic mice and littermates were incubated under constant oxygenation (O₂/CO₂:95%/5%) at 30 °C for 4–5 h in an Eppendorf Thermomixer (5 Prime, Boulder, CO, USA) with Krebs-HCO₃⁻ buffer. The media was replaced by 200 µl of fresh Krebs-HCO₃⁻ buffer and incubated for 30 min before the addition of ligands. Transfected CHO cells were cultured in serum-free medium for

16 h before the addition of the indicated concentration of ligands for the indicated time. Both, cells and slices were lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaF, 150 mM NaCl, 45 mM β -glycerophosphate, 1% Triton X-100, 20 μ M phenylarsine oxide, 0.4 mM NaVO₄ and protease inhibitor cocktail). Cellular debris was removed by centrifugation at 13 000 g for 5 min at 4 °C and protein was quantified by the bicinchoninic acid method using bovine serum albumin dilutions as standard. To determine the level of extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation, equivalent amounts of protein (10 μ g) were separated by electrophoresis on a denaturing 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene fluoride for fluorescence membranes. Odyssey blocking buffer (LICOR Biosciences, Lincoln, NE, USA) was then added and membranes were blocked for 90 min. Membranes were then probed with a mixture of a mouse anti-phospho-ERK1/2 antibody (1:2500; Sigma) and rabbit anti-ERK1/2 antibody (1:40 000; Sigma) for 2–3 h. Bands were visualized by the addition of a mixture of IRDye 800 (anti-mouse) antibody (1:10 000; Sigma) and IRDye 680 (anti-rabbit) antibody (1:10 000; Sigma) for 1 h and scanned by the Odyssey infrared scanner (LICOR Biosciences). Bands densities were quantified using the scanner software and exported to Excel (Microsoft, Redmond, WA, USA). The level of phosphorylated ERK1/2 isoforms was normalized for differences in loading using the total ERK protein band intensities.

In vivo microdialysis in rat striatum

Male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA, USA), weighing 300–350 g were used. Concentric microdialysis probes with 2 mm long dialysis membranes were prepared as described previously.¹⁵ Animals were anesthetized with Equithesin (NIDA Pharmacy, Baltimore, MD, USA) and microdialysis probes were implanted in the ventral striatum (core of the nucleus accumbens); coordinates with respect to bregma: A 1.7, L + 1.2 and V – 7.6 mm. The experiments were performed on freely moving rats 24 h after the probe implantation. A Ringer solution (in mmol l⁻¹) of 147 NaCl, 4 KCl and 2.2 CaCl₂ was pumped through the dialysis probe at a constant rate of 1 μ l per minute. After a washout period of 90 min, samples were collected at 20 min intervals and split into two fractions of 10 μ l, to separately measure glutamate and dopamine contents. Each animal was used to study the effect of one treatment by local administration (perfusion by reverse dialysis) of the D₄ receptor agonist RO-10-5824 or the D₄ receptor antagonist L-745870. At the end of the experiment, rats were killed with an overdose of Equithesin and methylene blue was perfused through the probe. The brain was removed and placed in a 10% formaldehyde solution, and coronal sections were cut to verify the probe location. Dopamine content was measured by reverse high-performance liquid chromatography coupled to an electrochemical detector, as described in

detail previously. Glutamate content was measured by high-performance liquid chromatography coupled to a fluorimetric detector, as described before.¹⁶ The limit of detection (which represents three times baseline noise levels) for dopamine and glutamate was 0.5 and 50 nM, respectively. Dopamine and glutamate values were transformed as percentage of the mean of the three values before the stimulation and transformed values were statistically analyzed with one-way repeated measures analysis of variance followed by Newman-Keuls tests, to compare glutamate and dopamine values of the samples obtained after drug perfusion with those obtained just before drug perfusion.

Neurotransmitter release in rat striatal slices

Rat brain slices were obtained from male Wistar rats weighing 180–220 g. After rapid killing of the rat, the brain was immersed in oxygenated ice-cold artificial cerebrospinal fluid (ACSF) solution, and coronal brain slices (300 μ m thick) were obtained with a vibratome. The striatum (caudate-putamen and nucleus accumbens) was microdissected under a stereoscopic microscope and the slices were incubated for 30 min at 37 °C in ACSF (in mM: NaCl 118.25, KCl 1.75, MgSO₄ 1, KH₂PO₄ 1.25, NaHCO₃ 25, CaCl₂ 2 and D-glucose 10), gassed continuously with O₂/CO₂ (95:5, v/v). For γ -aminobutyric acid (GABA) release, the slices were then incubated for 30 min with 8 nM [³H]GABA in 2 ml solution containing 10 μ M aminooxyacetic acid (to inhibit GABA transaminase, thus preventing degradation of the labeled GABA). At the end of this period, excess radiolabeled compound was removed by washing twice with ACSF containing, in addition to aminooxyacetic acid and 10 μ M nipecotic acid (to prevent the reuptake of the released [³H]GABA). Both compounds were present in the perfusion solution for the rest of the experiment. For dopamine release, the slices were labeled with 77 nM [³H]dopamine in Krebs-Henseleit solution containing 10 μ M pargyline, 0.57 mM ascorbic acid and 0.03 mM EDTA, which were present in the solutions for the rest of the experiment. For glutamate release, the tissues were incubated for 30 min with 100 nM [³H]glutamate in 2 ml of ACSF containing 200 μ M aminooxyacetic acid (to inhibit glutamate decarboxylase and prevent the conversion of glutamate to GABA) and 200 μ M dihydrokainic acid (to prevent the uptake of [³H]glutamate by astrocytes). Dihydrokainic acid was present in the medium only during the incubation period. At the end of this period, the excess radiolabeled compound was removed by washing twice with ACSF. Methods for measuring [³H]neurotransmitter release and data analysis used in the present work were the same as those described previously.^{17,18} The slices were apportioned randomly between the chambers (usually three slices per chamber) of a superfusion system (volume of each chamber 80 μ l; 20 chambers in parallel) and perfused with the ACSF at a flow rate of 0.5 ml per minute for 1 h. Basal release of

[³H]neurotransmitter was measured by collecting four fractions of the superfusate (total volume 2 ml) before depolarizing the slices with a solution in which the [K⁺] was raised to 25 mM. The composition of the high K⁺ solution was (in mM): NaCl 101.25, KCl 23.75, MgSO₄ 1, KH₂PO₄ 1.25, NaHCO₃ 25, CaCl₂ 2 and D-glucose 10. Six more fractions were collected in the high K⁺ medium. All drugs were added to the medium at fraction 2, before changing the superfusion to the high K⁺ medium, to explore effects on basal release. To determine the total amount of tritium remaining in the tissue, the slices were collected, treated with 1 ml of 1 M HCl and allowed to stand for 1 h before adding the scintillator. The [³H]neurotransmitter release was expressed initially as a fraction of the total amount of tritium remaining in the tissue. The effect of drugs on the basal release of [³H]neurotransmitter was assessed by comparing the fractional release in fraction 2 (immediately before exposure of the tissue to the drug) and fraction four (immediately before exposure to 25 mM of K⁺), using Student's paired *t*-test. Changes in depolarization-induced [³H]GABA release by drugs and treatments were assessed by comparing the area under the appropriate release curves between the first and last fractions collected after the change to high K⁺. The significance of drug effects was assessed by one-way analysis of variance and Tukey-Kramer test, using Prism Graph Pad Software 4.0 (Graph Pad Software). To obtain an unbiased estimate of IC₅₀ values, concentration-response data were fitted by non-linear regression using the same software.

Statistical analysis

Statistical analyses were performed with Prism Graph Pad Software 4.0 (Graph Pad Software). See above and figure legends (Figure 1 to Figure 7) for details.

Results

D_{2S} and D₄ receptors form heteromers in transfected cells

BRET experiments were performed where one of the receptor is fused to the bioluminescent protein *Renilla Luciferase* (RLuc) and the other receptor is fused to a YFP. The fusion proteins were functional (Supplementary Figure 1) and expressed at the membrane level (Figure 1c). Clear BRET saturation curves were obtained in cells expressing D_{4.2}-RLuc or D_{4.4}-RLuc receptors and increasing amounts of D_{2S}-YFP (Figure 1a), but not in cells expressing D_{4.2}-RLuc or D_{4.4}-RLuc receptors and increasing amounts of D₁-YFP (Figure 1a), indicating that the D_{4.2} and the D_{4.4} form heteromers with D_{2S} but not with D₁ receptors. Interestingly, in cells expressing the D_{4.7}-RLuc variant and D₁-YFP or D_{2S}-YFP (Figure 1a) low linear BRET was detected, which was qualitatively similar to the results obtained with the negative control, with adenosine A₁-RLuc and D_{2S}-YFP receptors (Figure 1a). This result was not due to the particular BRET

donor and acceptor chosen, as low and linear BRET were obtained when we swapped the fused proteins, that is, in cells co-expressing D_{2S}-RLuc and D_{4.7}-YFP (Figure 1a). These results strongly suggest that the human D_{4.7} polymorphic variant does not form heteromers with the human D_{2S} receptor or if heteromers are formed, the fusion proteins are not properly oriented or are not within proximity to allow energy transfer (<10 nm). One way to test if the receptors are indeed forming heteromers in such a way that impedes energy transfer is to titrate one receptor in the presence of the heteromer and look for changes in the BRET signal. In BRET displacement experiments, D_{4.2}, but not D_{4.7} receptors were able to compete with D_{4.4}-RLuc and alter heteromer formation with D_{2S}-YFP (Figure 1b), meaning that D_{4.2} and D_{4.4}, but not D_{4.7} receptors use the same molecular determinants to establish intermolecular interactions with D_{2S} receptor and strongly suggesting that D_{4.7} receptors are unable to form heteromers with D_{2S}.

D_{2S}-D₄ receptor heteromer signals through MAPK

To investigate the function of the D_{2S}-D₄ receptor heteromer, MAPK signaling (ERK1/2 phosphorylation) was determined. RO-10-5824 and quinolorane, selective D₄ and D_{2/3} receptor agonists respectively,^{19,20} selectively stimulated MAPK in cells transfected with D₄ or D_{2S} receptors, respectively (Supplementary Figure 2). Dose-response experiments with RO-10-5824 showed no significant differences between cells transfected with D_{4.2}, D_{4.4} or D_{4.7} receptors (Supplementary Figure 2). However, in co-transfected cells, stimulation of D_{2S} receptors potentiated D₄ receptor-mediated MAPK activation, but not the other way around. Importantly, this functional interaction only occurred in cells transfected with D_{2S} and D_{4.2} or D_{4.4}, but not in cells expressing D_{4.7} receptors (Figure 2). Since disruption of D_{2S}-D₄ receptor heteromers (by substituting D_{4.2} or D_{4.4} with the D_{4.7} variant) is associated with the loss of the D_{2S}-D₄ receptor interaction at the MAPK level, this interaction constitutes a specific biochemical property of the D_{2S}-D₄ receptor heteromer and can be used as a biochemical fingerprint to detect the heteromer in native tissues.¹⁰

D_{2S}-D₄ receptor heteromers in the mouse brain

D₄ receptors are preferentially expressed in limbic areas and the prefrontal cortex, where they can be found in interneurons and also projecting neurons.¹ In corticostriatal neurons, D₄ receptors have also been localized at their nerve terminals,^{2,3} where they can co-localize with D_{2S} receptors.¹³ We therefore investigated the existence of D_{2S}-D₄ receptor heteromers in the striatum. Biophysical techniques cannot be easily applied in native tissues, but indirect methods can be used, such as the identification of a biochemical property of the heteromer (biochemical fingerprint).¹⁰ In this case, the biochemical fingerprint would be the potentiation by D_{2S} receptor activation of D₄ receptor-mediated MAPK activation, which should not occur

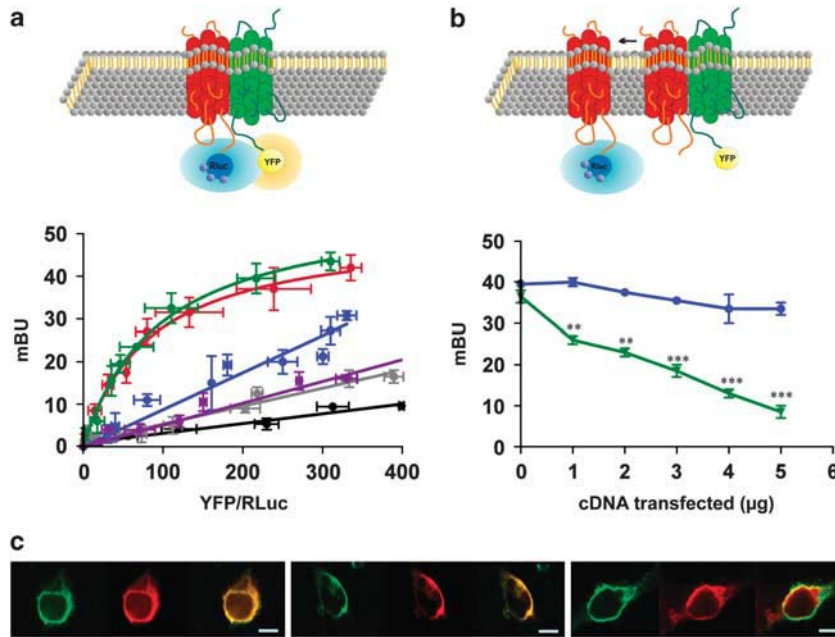


Figure 1 Human D_{2S} and D₄ receptors form heteromers in transfected cells. **(a)** Bioluminescence Resonance Energy Transfer (BRET) saturation curves were obtained from experiments with cells co-expressing, top to bottom, D_{2S}-YFP (yellow fluorescent protein) and D_{4.2}-RLuc (red), D_{4.4}-RLuc (green) or D_{4.7}-RLuc (blue), D_{2S}-RLuc and D_{4.7}-YFP (purple), A₁-RLuc and D_{2S}-YFP (black) or D_{4.4}-RLuc and D₁-YFP (gray). Co-transfections were performed with a constant amount of cDNA corresponding to the receptor-RLuc construct (2 μg of cDNA for D₄-RLuc or 1 μg of cDNA for A₁-RLuc) and increasing amounts of cDNA corresponding to the receptor-YFP construct (0.2–6 μg of cDNA for D_{2S}-YFP or 1–4 μg of cDNA for D₁-YFP). Both fluorescence and luminescence of each sample were measured before every experiment to confirm equal expression of RLuc (about 100 000 luminescence units) while monitoring the increase of YFP expression (2000–20 000 fluorescence units). BRET data are expressed as mean values ± s.d. of four to nine different experiments grouped as a function of the amount of BRET acceptor. **(b)** BRET displacement experiments were performed in cells expressing constant amounts of D_{4.4}-RLuc (2 μg cDNA transfected) and D_{2S}-YFP (2 μg cDNA transfected) and increasing amounts (1–5 μg of cDNA transfected) of D_{4.7} (blue) or D_{4.2} (green). Both fluorescence and luminescence of each sample were measured before every experiment to confirm no changes in the expression of D_{4.4}-RLuc and D_{2S}-YFP. BRET data are expressed as mean values ± s.d. of five different experiments grouped as a function of the amount of BRET acceptor. Significant differences with respect to the samples without D_{4.2} or D_{4.7} were calculated by one-way analysis of variance (ANOVA) and Bonferroni's test (***P* < 0.01 and ****P* < 0.001). In **(a, b)**, the relative amounts of BRET acceptor are expressed as the ratio between the fluorescence of the acceptor minus the fluorescence detected in cells only expressing the donor, and the luciferase activity of the donor. In the top, schematic representations of BRET **(a)** or BRET displacement **(b)** are shown. **(c)** Confocal microscopy images of cells transfected with 1 μg of cDNA corresponding to, left to right, D_{4.2}-RLuc, D_{4.4}-RLuc or D_{4.7}-RLuc and 0.5 μg cDNA corresponding to D_{2S}-YFP. Proteins were identified by fluorescence or by immunocytochemistry. D₄-RLuc receptors are shown in red, D_{2S}-YFP is shown in green and co-localization is shown in yellow. Scale bar: 5 μm.

with the human D_{4.7} variant. Before these experiments with mouse brain, we demonstrated by BRET saturation experiments in transfected cells that the mouse D_{2S} receptor forms heteromers with the mouse D₄ receptor (which has an amino-acid sequence in the 3IL similar to that from the human D_{4.2}). Mouse fusion proteins were expressed in the plasma membrane of transfected cells (Figure 3a) and shown to be functional (Supplementary Figure 3). Like the human receptors, mouse D_{2S} receptors were found to form heteromers with mouse D₄ receptors and also with human D_{4.4} receptors, but not with human D_{4.7} receptors (Figure 3b). Furthermore, it was also shown that, in co-transfected cells, stimulation of the mouse D_{2S} receptor potentiates the effect of the mouse D₄, but not the human D_{4.7}, on MAPK signaling (Figures 3c and d). This result was not reciprocal (Supplementary

Figure 4) and mirrors the results obtained with human D₄ and D_{2S} receptors (Figure 2). We next analyzed the effects of D₂ and D₄ receptor agonists on MAPK signaling on striatal slices taken from knockin mice carrying the 7 repeats of the human D_{4.7} in replacement of the mouse region and from WT littermates (Figure 4). Neither quinlorane nor RO-10-5824 induced a significant ERK1/2 phosphorylation in striatal slices of WT mice when administered alone, but co-administration of both agonists produced a significant dose-dependent effect with an increase of up to fourfold (Figure 3e). This synergistic interaction between D₂ and D₄ receptors, which constitutes the biochemical fingerprint of the D_{2S}-D₄ receptor heteromer, was completely absent in the D_{4.7} mutant mouse (Figure 3e), confirming both the existence of D_{2S}-D₄ receptor heteromers and the

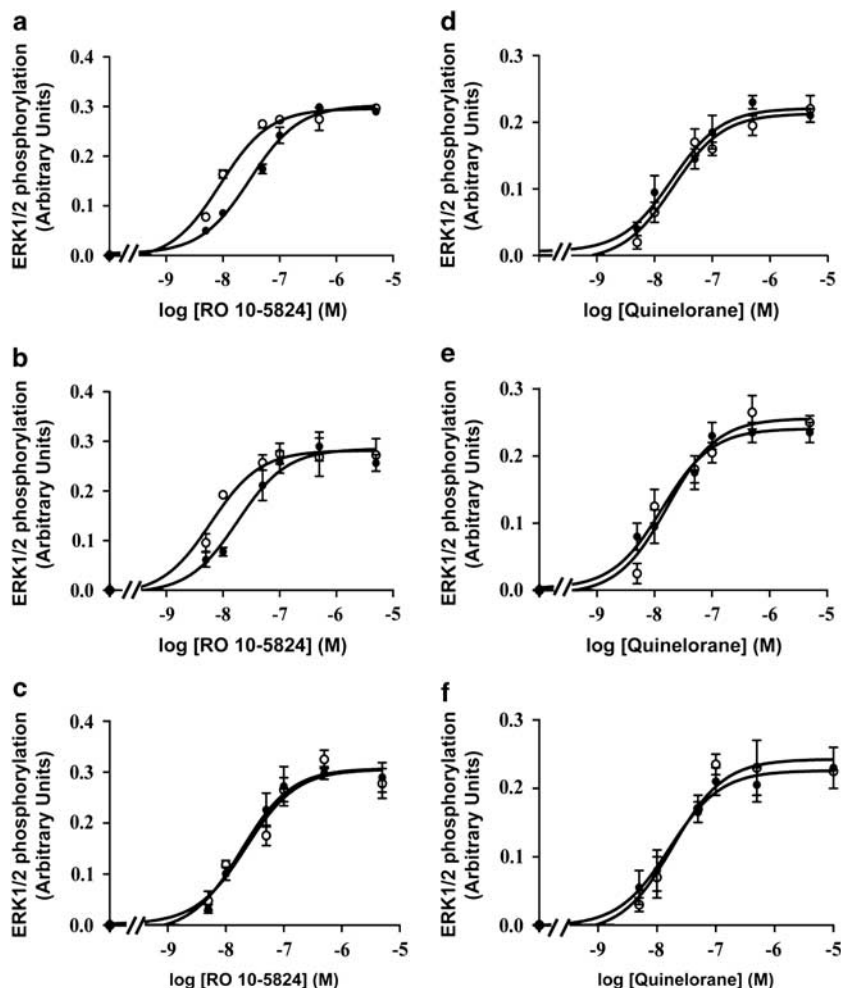


Figure 2 Crosstalk between human D₄ and D_{2S} receptors in ERK1/2 phosphorylation. Cells were transiently co-transfected with 2.5 μ g of cDNA corresponding to D_{2S} and 2.5 μ g of cDNA corresponding to D_{4.2} (a, d), D_{4.4} (b, e) or D_{4.7} (c, f). In (a–c), cells were treated for 10 min with increasing concentrations of RO-10-5824 in the presence (○) or in the absence (●) of quinelorane (50 nM). In (d–f), cells were treated for 10 min with increasing concentrations of quinelorane in the presence (○) or in the absence (●) of RO-10-5824 (50 nM). The immunoreactive bands, corresponding to ERK1/2 phosphorylation, of three to six experiments were quantified and expressed as arbitrary units. For each curve, EC₅₀ values were calculated as mean \pm s.e.m. and statistical differences between curves obtained in the presence or in the absence of quinelorane (a–c) or RO-10-5824 (d–f) were determined by Student's *t*-test. EC₅₀ with and without quinelorane: (a) 9 \pm 1 and 26 \pm 1 nM (P < 0.01), (b) 7 \pm 1 and 23 \pm 1 nM (P < 0.01), (c) 18 \pm 1 and 22 \pm 1 nM (N.S.). EC₅₀ with and without RO-10-5824: (d) 22 \pm 1 and 20 \pm 1 nM (N.S.), (e) 20 \pm 1 and 17 \pm 1 nM (N.S.), (f) 18 \pm 1 and 13 \pm 1 nM (N.S.). N.S., non-statistical differences.

absence of functional interactions between D₂ and D_{4.7} receptors in the brain.

D₂-D₄ receptor interactions modulate striatal glutamate release

To investigate the functional significance of D₄ receptor activation, we determined D₄ receptor-mediated modulation of striatal glutamate release by *in vivo* microdialysis in freely moving rats. The local perfusion of the D₄ receptor agonist RO-10-5824 in the ventral striatum (in the nucleus accumbens) produced a dose-dependent decrease in the striatal extracellular concentration of glutamate and a concomitant increase in the extracellular concentration of dopamine (Figures 5a and 5b), which were counteracted by co-perfusion with the selective D₄ receptor

antagonist L-745 870 (which was inactive when perfused alone) (Figures 5a–c). These results suggest that inhibitory D₄ receptors are located in glutamatergic terminals, whose activation decreases basal striatal glutamate release. The increase in dopamine concentration can best be explained by a decreased activation of striatal GABAergic efferent neurons that tonically inhibit dopaminergic mesencephalic neurons. This interpretation could be confirmed in experiments with striatal slices, where dopamine should not be modified due to the interruption of the striatal-mesencephalic loop. In fact, in slices of dorsal or ventral rat striatum, the D₄ receptor agonist RO-10-5824 decreased K⁺-induced glutamate release, an effect that was counteracted by the selective D₄ receptor antagonist L-745 870, but did not change

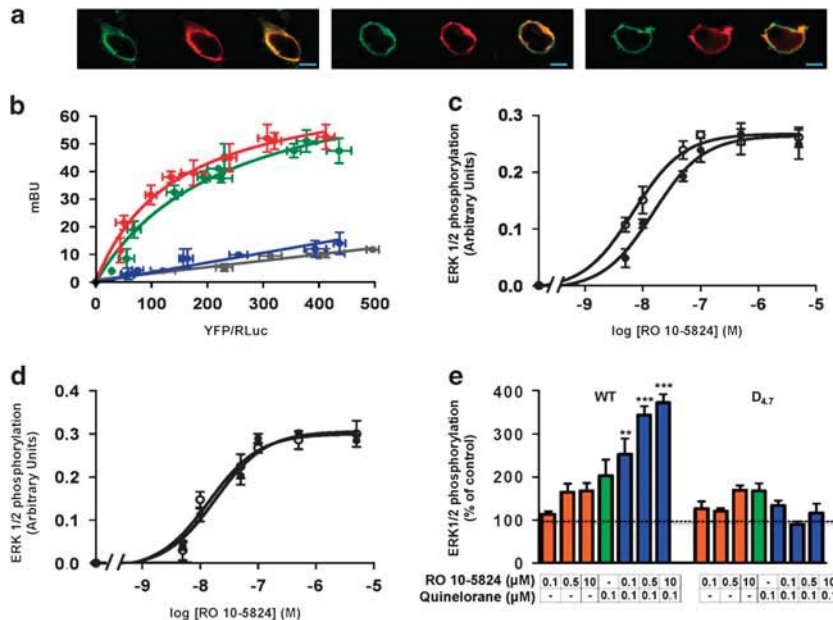


Figure 3 D_{2s}-D₄ receptor heteromers in the mouse brain. **(a)** Confocal microscopy images of cells transfected with 1 μ g of cDNA corresponding to, left to right, mouse D₄-RLuc, human D_{4.4}-RLuc and human D_{4.7}-RLuc and 0.5 μ g of cDNA corresponding to D_{2s}- yellow fluorescent protein (YFP). Proteins were identified by fluorescence or by immunocytochemistry. D₄-RLuc receptors are shown in red, D_{2s}-YFP is shown in green and co-localization is shown in yellow. Scale bar: 5 μ m. **(b)** Mouse D_{2s} receptor heteromerization with mouse and human D₄ receptors. Bioluminescence Resonance Energy Transfer (BRET) saturation curves were obtained from cells co-expressing mouse D₄-RLuc (green), human D_{4.4}-RLuc (red), human D_{4.7}-RLuc (blue) or human A₁-RLuc (gray) and mouse D_{2s}-YFP receptors. Co-transfections were performed with a constant amount of cDNA corresponding to the receptor-RLuc construct (2 μ g of cDNA for mouse D₄-RLuc, 2.5 μ g of cDNA for human D_{4.4}-RLuc or 1 μ g of cDNA for A₁-RLuc) and increasing amounts of cDNA corresponding to the receptor-YFP construct (0.2–6 μ g cDNA). Both fluorescence and luminescence of each sample were measured before every experiment to confirm equal expression of RLuc (about 100 000 luminescence units) while monitoring the increase of YFP expression (2000–20 000 fluorescence units). The relative amounts of BRET acceptor are expressed as the ratio between the fluorescence of the acceptor minus the fluorescence detected in cells only expressing the donor, and the luciferase activity of the donor. BRET data are expressed as mean values \pm s.d. of three to six different experiments grouped as a function of the amount of BRET acceptor. **(c, d)** Crosstalk between mouse D_{2s} receptors and mouse or human D₄ receptors in ERK1/2 phosphorylation. Cells transiently co-expressing mouse D_{2s} receptors and mouse D₄ receptors **(c)** or human D_{4.7} receptors **(d)** were treated for 10 min with increasing RO-10-5824 concentrations in the presence (\circ) or in the absence (\bullet) of quinelorane (50 nM) before the ERK1/2 phosphorylation determination. The immunoreactive bands of three experiments (mean \pm s.e.m.; $n=3$) were quantified and expressed as arbitrary units. EC₅₀ values with or without quinelorane were: **(c)** 7 ± 0.1 and 15 ± 0.1 nM (Student's *t*-test: $P < 0.01$) or **(d)** 18 ± 0.1 and 15 ± 0.1 nM (Student's *t*-test: N.S.). **(e)** Striatal slices from wild-type (WT) or D_{4.7} mutant mice were treated for 10 min with the indicated concentrations of RO-10-5824 (orange) or quinelorane (green) or with RO-10-5824 plus quinelorane (blue) and ERK1/2 phosphorylation was determined. For each treatment, the immunoreactive bands from four to six slices from a total 10 WT and 10 D_{4.7} mutant animals were quantified and values represent the mean \pm s.e.m. of the percentage of phosphorylation relative to basal levels found in untreated slices (100%). No significant differences were obtained between the basal levels of the WT and the D_{4.7} mutant mice. Significant treatment and genotype effects were shown by a bifactorial analysis of variance (ANOVA) followed by *post hoc* Bonferroni's tests (** $P < 0.01$ and *** $P < 0.001$, as compared with the lowest concentration of RO-10-5824).

dopamine or GABA release (Figure 6), indicating that striatal D₄ receptors selectively and locally modulate glutamate release. This role of D₄ receptors in the striatum can also explain previous results obtained with D₄ receptor KO mice, which show an increase and decrease in the striatal extracellular concentration of glutamate and dopamine, respectively.^{21,22}

As mentioned before, there is evidence for co-localization of both D₂ and D₄ receptors in corticostriatal glutamatergic terminals^{2,3,13} and previous studies have demonstrated that presynaptic D₂-like receptors have an inhibitory role in the modulation of striatal glutamate release.^{13,23} However,

since those studies did not use selective compounds, they could not distinguish between effects due to D₂ or D₄ receptor stimulation. Therefore, in this study we tested the effect of quinelorane alone and in combination with RO-10-5824 on glutamate release in rat striatal slices. To eliminate endogenous dopamine, rats were treated with reserpine, and the experiments performed in the presence of the D₁-like receptor antagonist SCH-23390. Quinelorane significantly decreased K⁺-induced glutamate release, whereas the co-application of quinelorane with RO-10-5824 showed a more significant effect (Figure 7a). Dopamine strongly decreased K⁺-induced glutamate

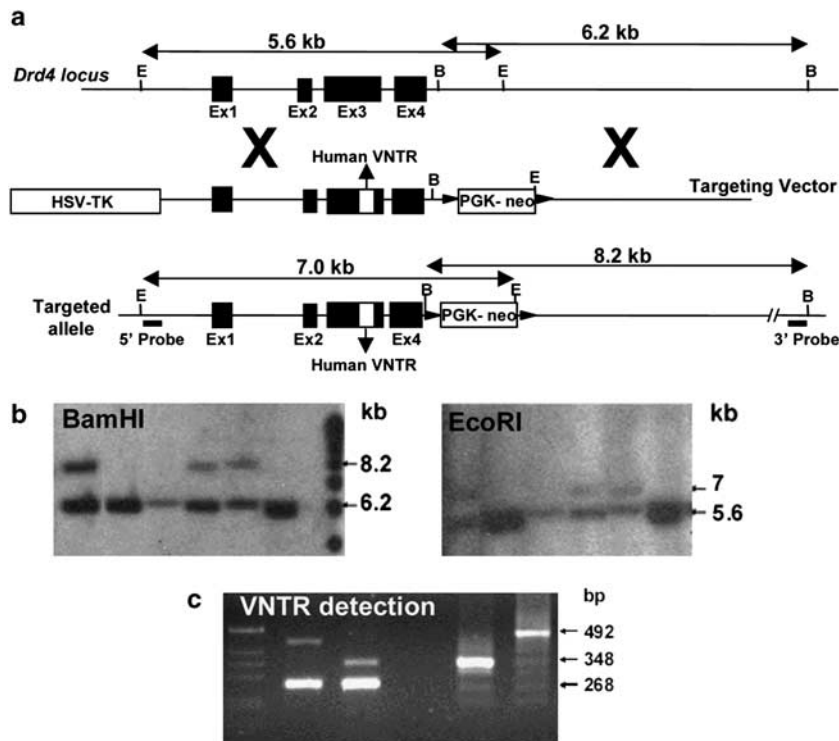


Figure 4 Targeted insertion of human variable number of tandem repeats (VNTRs) carrying 7 repeats into the mouse *Drd4* exon 3 by homologous recombination in ES cells. **(a)** Structure of the *Drd4* locus, targeting vector and targeted allele. **(b)** Southern blot analysis detected double homologous recombination events at the 5' and 3' ends using external probes after digestion with *Bam*HI or *Eco*RI. **(c)** The presence of inserted human VNTR was verified by PCR using mouse primers flanking the expansion.

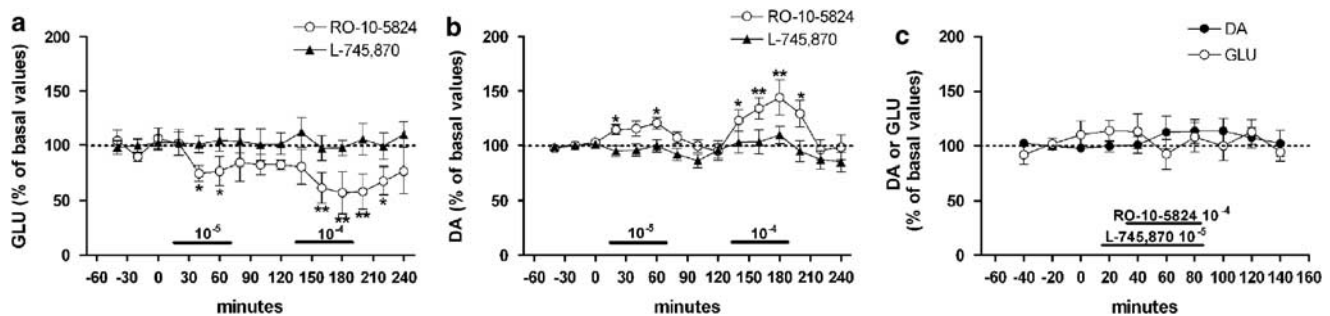


Figure 5 *In vivo* D₄ receptor-mediated modulation of basal extracellular levels of glutamate in the rat ventral striatum. Effects of the local perfusion with the D₄ receptor agonist RO-10-5824 and the D₄ receptor antagonist L-745 870 on the basal extracellular concentrations of glutamate (GLU) and dopamine (DA) in the ventral striatum (core of the nucleus accumbens). Horizontal bars show the periods of drug perfusion (concentrations are indicated in M). Data represent mean values \pm s.e.m. of the percentage of the mean of the three basal values before the first drug perfusion ($n=6-8$ per group): * $P<0.05$ and ** $P<0.01$, compared with the values previous in time '0' (repeated measures analysis of variance (ANOVA) followed by Newman-Keuls tests).

release, an effect partially counteracted by the D₂ receptor antagonist L-741 626 or by the D₄ receptor antagonist L-745 870, but completely counteracted by the simultaneous application of both antagonists (Figure 7b). In agreement with the reported higher *in vitro* affinity of D₄ versus D₂ receptor for dopamine,²⁴ the IC₅₀ of dopamine-mediated inhibition of K⁺-induced glutamate release was significantly higher in the presence of the D₄ receptor antagonist

(D₂-mediated effect) than in the presence of the D₂ receptor antagonist (D₄-mediated effect) (Figure 7b). Finally, and more importantly, the D₂ receptor agonist quinolorane synergistically potentiated the inhibitory effect of the D₄ receptor agonist RO-10-5824 on K⁺-induced glutamate release (significant decrease in IC₅₀ value) (Figure 7c), but not the other way around (Figure 7d). These results therefore show the same kind of D₂-D₄ receptor interaction demonstrated by

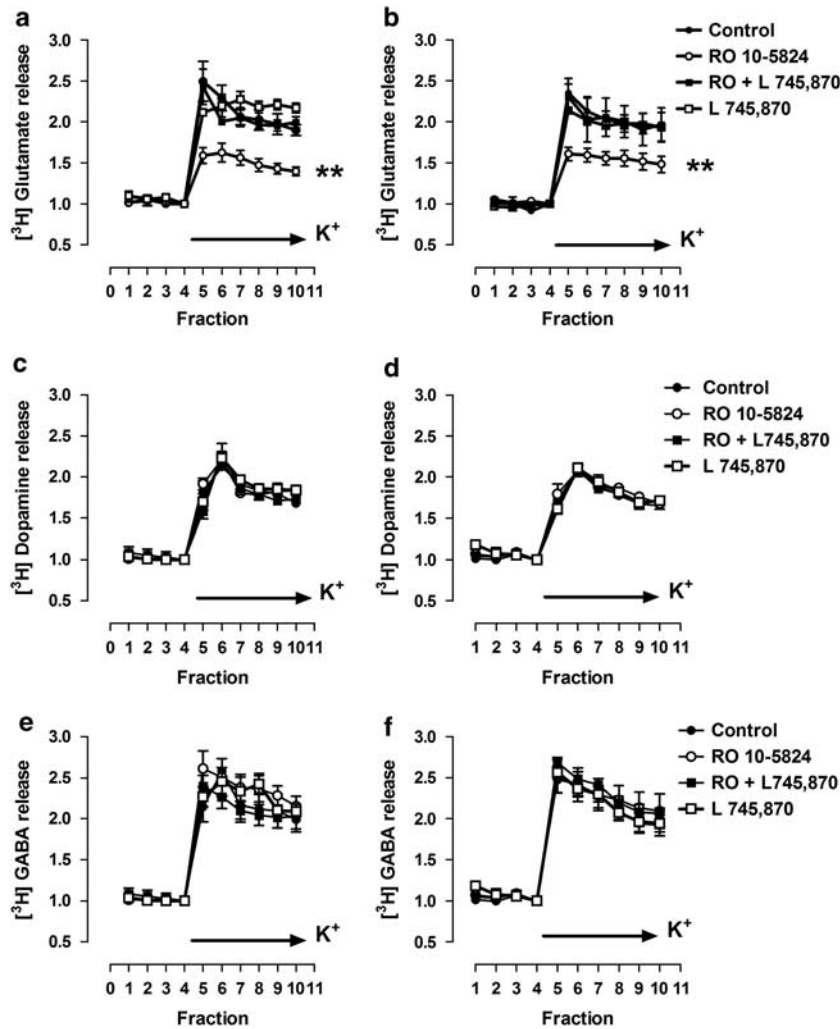


Figure 6 D₄ receptor-mediated modulation of [³H]glutamate, but not [³H]dopamine or [³H]GABA release from slices of dorsal and ventral striatum. Slices from the dorsal striatum (caudate-putamen; **a**, **c**, **e**) or the ventral striatum (nucleus accumbens; **b**, **d**, **f**) of reserpine-treated rats were treated with the D₄ receptor agonist RO-10-5824 (100 nM) or with the D₄ receptor antagonist L-745 870 (10 nM) alone or in combination and the time course of K⁺-stimulated [³H]glutamate (**a**, **b**), [³H]dopamine (**c**, **d**) or [³H]GABA (**e**, **f**) release was determined. The RO-10-5824-induced effect (open circles) was prevented by the antagonist L-745 870 (dark squares), which itself had no effect (open squares). Values are mean ± s.e.m. of samples from three different animals performed in four replicates. Drug effect was assessed by comparing the relative area under the curve for each condition. ***P* < 0.01 with respect to the control (analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison *post hoc* test).

D_{2S}-D₄ receptor heteromers in transfected cells with MAPK signaling. Our combined *in vitro* and *in vivo* data strongly suggest that D_{2S}-D₄ receptor heteromers are likely to have a key role in dopamine-mediated modulation of striatal glutamate release.

Discussion

The present study shows that dopamine D_{2S} and D_{4.2} or D_{4.4} receptors, but not the ADHD-associated human D_{4.7} variant, form functional heteromers in transfected cells and in the rodent brain. Co-stimulation of D_{2S} and D₄ receptors in the D_{2S}-D₄ receptor heteromer has a synergistic effect on MAPK signaling, which could be demonstrated in transfected cells and in the mouse

striatum, but not in cells expressing D_{4.7} or in the striatum of a mutant mouse carrying the 7 repeats of the human D_{4.7} in the 3IL of the D₄ receptor. These results provide a significant functional difference of one of the human receptor variants, D_{4.7}, compared with the D_{4.2} and D_{4.4} variants, which can have important implications for the understanding of the pathogenesis of ADHD. Importantly, we also demonstrated, for the first time, that D_{2S}-D₄ receptor interactions modulate striatal glutamate release, suggesting that the D_{2S}-D₄ receptor heteromer allows dopamine to fine-tune glutamate neurotransmission.

The molecular mechanism involved in preventing heteromer formation between D_{2S} and D_{4.7} receptors is not yet known. Indeed, the control of heteromer

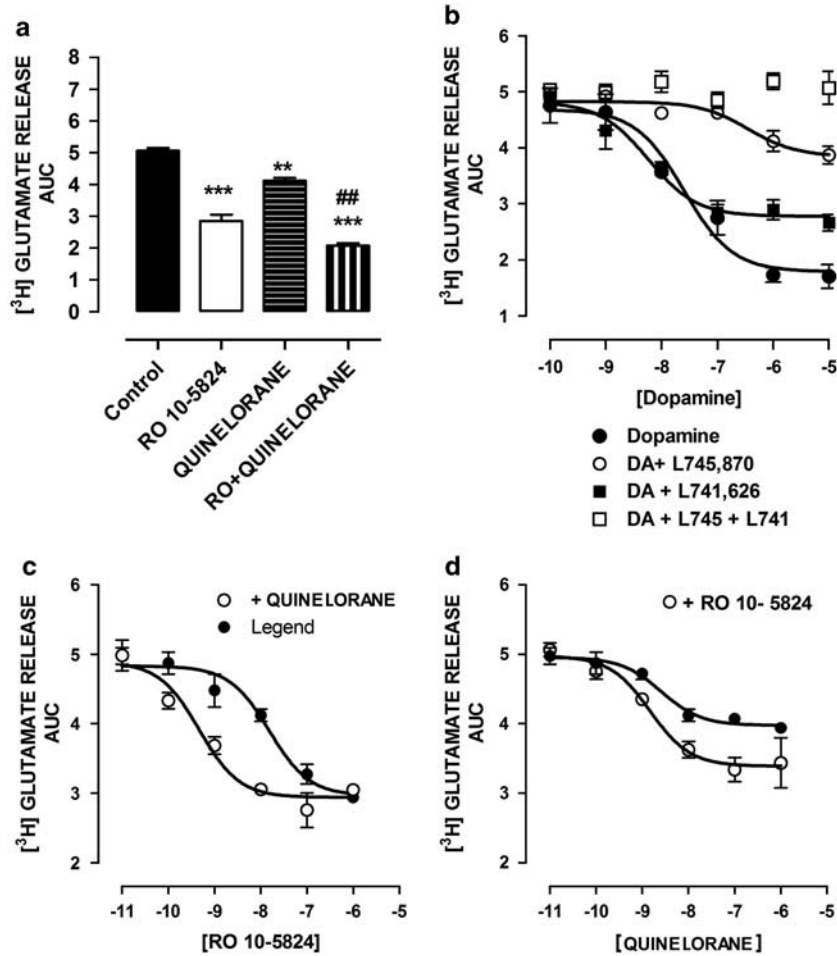


Figure 7 D₂ and D₄ receptor interactions in the modulation of striatal [³H]glutamate release. Striatal slices (dorsal striatum) from reserpine-treated rats were incubated with SCH-23390 (100 nM) to block D₁ receptor activation. In (a), slices were treated for 32 min (fraction 2 to fraction 10) with medium (control), with the D₄ receptor agonist RO-10-5824 (100 nM), with the D_{2/3} receptor agonist quinolorane (100 nM) or with both and K⁺-stimulated [³H]glutamate release was determined. Values are mean ± s.e.m. of samples from three different animals performed in four replicates. Drug effects were assessed by comparing the relative area under the curve for each condition. ***P* < 0.01 and ****P* < 0.001 with respect to the control and ##*P* < 0.01 with respect to slices treated with RO-10-5824 or quinolorane alone (analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison *post hoc* test). In (b), slices were treated for 32 min with increasing dopamine concentrations in the absence (dark circles) or in the presence of the D₄ receptor antagonist L-45 870 (10 nM, dark squares), the D₂ receptor antagonist L-741 626 (10 nM, open circles) or both (open squares) and K⁺-stimulated [³H]glutamate release was determined. Values are mean ± s.e.m. of samples from three different animals performed in four replicates. Drug effects were assessed by comparing the relative area under the curve for each condition. The IC₅₀ values were: 25.25 nM (C.I.: 9.63–66.20 nM) for dopamine alone, 5.75 nM (2.12–15 nM) for dopamine in the presence of L-741 626 and 357.27 nM (C.I.: 73.40–1739 nM) for dopamine in the presence of L-745 870. In (c), slices were treated for 32 min with increasing concentrations of RO-10-5824 in the absence (black circles) or in the presence (open circles) of quinolorane (10 nM) and K⁺-stimulated [³H]glutamate release was determined. In (d), slices were treated for 32 min with increasing concentrations of quinolorane in the absence (black circles) or in the presence (open circles) of RO-10-5824 (10 nM) and K⁺-stimulated [³H]glutamate release was determined. In (c, d), values are mean ± s.e.m. of samples from three different animals performed in four replicates. The IC₅₀ values were (c) 15 nM (35.15–6.55 nM) for RO-10-5824 alone and 0.05 nM (1.21–0.02 nM) for RO-10-5824 in the presence of quinolorane (Student's *t*-test; *P* < 0.01) and (d) 2.55 nM (7.31–0.89 nM) for quinolorane alone and 1.48 nM (4.5–0.45 nM) for quinolorane in the presence of RO-10-5824 (Student's *t*-test; N.S.).

formation between G-protein-coupled receptors is still a large question in the field. Since the D_{4.7} receptor variant has the longest 3IL and is the only polymorphic form not forming heteromers with the D_{2S} receptor, steric hindrance of the 3IL of D_{4.7} receptor is a probable mechanism responsible for this lack of heteromerization, but other mechanisms cannot be

ruled out. Using two-hybrid methodologies as well as proteomic studies, interactions between dopamine receptors and a cohort of DRIPs (dopamine receptor interacting proteins) have been demonstrated, forming signaling complexes or signalplexes.^{25,26} Some of these DRIPs show selectivity for some dopamine receptor subtypes. For example, filamin or protein 4.1N

interact with D₂ and D₃ receptors but not with D₁, D₅ or D₄ receptors,^{27,28} the PDZ domain-containing protein, GIPC (GAIP interacting protein, C terminus) interacts with D₂ and D₃ receptor but not with the D₄ receptor subtype²⁹ and paralemmin interacts exclusively with D₃, but not with D₂ or D₄ receptors.³⁰ All of these interactions modulate receptor targeting, trafficking and signaling. Proline-rich sequences of the D₄ receptor, mainly located in the polymorphic region of the 3IL, constitute putative SH3 binding domains, which can potentially interact with adapter proteins like Grb2 and Nck, which do not have any known catalytic activity but are capable of recruiting multi-protein complexes to the receptor.²⁴ It can be hypothesized that differences in DRIPs recruitment by D_{4.7} and the other D₄ polymorphic forms can influence the D_{4.7} ability to form heteromers, but future studies will be required.

Previous experiments indicated that locally in the striatum, dopamine inhibits glutamate release by activating D₂ receptors (predominantly D_{2S}) localized in glutamatergic terminals.^{13,15} Other studies also indicate that striatal postsynaptic D₂ receptors (predominantly D_{2L}) indirectly modulate glutamate release by retrograde endocannabinoids signaling.³¹ The present results indicate that D₄ receptors also have a key role in the modulation of striatal glutamate release, likely through its ability to form heteromers with presynaptic D_{2S} receptors. In the striatal D_{2S}-D₄ receptor heteromer, low concentrations of dopamine should bind to the D₄ receptor, which has more affinity for dopamine than the D_{2S} receptor,²⁴ causing a certain degree of inhibition of glutamate release. However, at higher concentrations, dopamine should also bind to the D_{2S} receptor and under these conditions, the synergistic interaction in the D_{2S}-D₄ receptor heteromer will produce an even stronger inhibition of glutamate release. Therefore, the D_{2S}-D₄ receptor heteromer seems to act as a concentration-dependent device that establishes two different degrees of presynaptic dopaminergic control over striatal glutamatergic neurotransmission. Since the strong modulation observed with higher concentrations of dopamine depends on D_{2S}-D₄ receptor heteromerization, the existence of a D_{4.7} variant implies a weaker control of glutamatergic neurotransmission, which could be a main mechanism involved in the pathogenesis of ADHD. This could also explain at least part of the so far not understood successful effect of psychostimulants in ADHD, which amplify dopaminergic signaling and these medications appear to be more effective in ADHD patients with the D_{4.4} than with the D_{4.7} variants.^{32,33} We have to take into account that the existence of a D_{4.7} variant does not imply ADHD is the result of this variant, but rather that it is one factor that contributes to its development. In fact, the D_{4.7} variant might constitute a successful evolutionary trait under the appropriate environmental exposure.^{7,34} The present study provides a new element of interest in the field of receptor heteromers, which now become new targets to be

studied when dealing with functional differences associated with polymorphisms of G-protein-coupled receptor genes.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank the technical help from Jasmina Jiménez (University of Barcelona). The study was supported by the NIDA IRP funds and from Grants from Spanish Ministerio de Ciencia y Tecnología (SAF2008-03229-E, SAF2009-07276, SAF2010-18472, SAF2008-01462 and Consolider-Ingenio CSD2008-00005) and from Consejo Nacional de Ciencia y Tecnología de México (50428-M). PJM is a Ramón y Cajal Fellow.

References

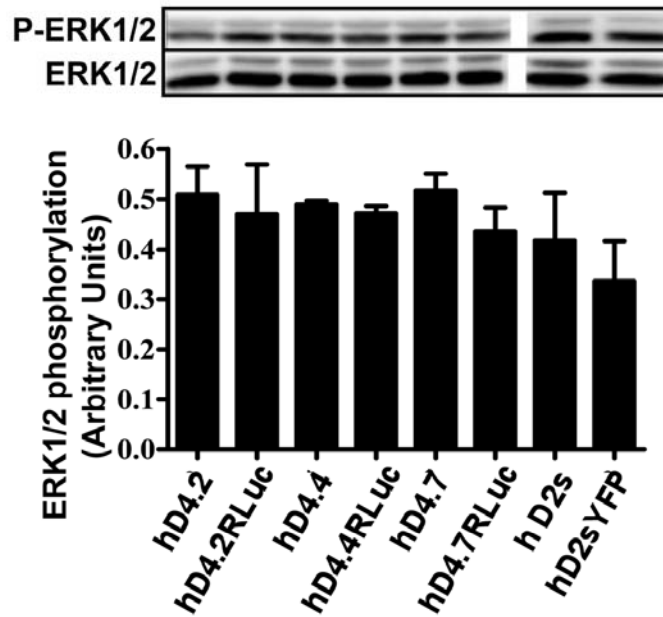
- 1 Lauzon NM, Laviolette SR. Dopamine D4-receptor modulation of cortical neuronal network activity and emotional processing: implications for neuropsychiatric disorders. *Behav Brain Res* 2010; **208**: 12–22.
- 2 Tarazi FI, Campbell A, Yeghiayan SK, Baldessarini RJ. Localization of dopamine receptor subtypes in corpus striatum and nucleus accumbens septi of rat brain: comparison of D1-, D2-, and D4-like receptors. *Neuroscience* 1998; **83**: 169–176.
- 3 Svingos AL, Periasamy S, Pickel VM. Presynaptic dopamine D(4) receptor localization in the rat nucleus accumbens shell. *Synapse* 2000; **36**: 222–232.
- 4 LaHoste GJ, Swanson JM, Wigal SB, Glabe C, Wigal T, King N *et al*. Dopamine D4 receptor gene polymorphism is associated with attention deficit hyperactivity disorder. *Mol Psychiatry* 1996; **1**: 121–124.
- 5 Swanson JM, Kinsbourne M, Nigg J, Lanphear B, Stefanos GA, Volkow N *et al*. Etiologic subtypes of attention-deficit/hyperactivity disorder: brain imaging, molecular genetics and environmental factors and the dopamine hypothesis. *Neuropsychol Rev* 2007; **17**: 39–59.
- 6 Casey BJ, Nigg JT, Durston S. New potential leads in the biology and treatment of attention deficit-hyperactivity disorder. *Curr Opin Neurol* 2007; **20**: 119–124.
- 7 Wang E, Ding YC, Flodman P, Kidd JR, Kidd KK, Grady DL *et al*. The genetic architecture of selection at the human dopamine receptor D4 (DRD4) gene locus. *Am J Hum Genet* 2004; **74**: 931–944.
- 8 Chang FM, Kidd JR, Livak KJ, Pakstis AJ, Kidd KK. The worldwide distribution of allele frequencies at the human dopamine D4 receptor locus. *Hum Genet* 1996; **98**: 91–101.
- 9 Asghari V, Sanyal S, Buchwaldt S, Paterson A, Jovanovic V, Van Tol HH. Modulation of intracellular cyclic AMP levels by different human dopamine D4 receptor variants. *J Neurochem* 1995; **65**: 1157–1165.
- 10 Ferré S, Baler R, Bouvier M, Caron MG, Devi LA, Durrux T *et al*. Building a new conceptual framework for receptor heteromers. *Nat Chem Biol* 2009; **5**: 131–134.
- 11 Marcellino D, Ferré S, Casadó V, Cortés A, Le Foll B, Mazzola C *et al*. Identification of dopamine D1-D3 receptor heteromers. Indications for a role of synergistic D1-D3 receptor interactions in the striatum. *J Biol Chem* 2008; **283**: 26016–26025.
- 12 Borroto-Escuela DO, Van Craenenbroeck K, Romero-Fernandez W, Guidolin D, Woods AS, Rivera A *et al*. Dopamine D2 and D4 receptor heteromerization and its allosteric receptor-receptor interactions. *Biochem Biophys Res Commun* 2011; **404**: 928–934.
- 13 De Mei C, Ramos M, Iitaka C, Borrelli E. Getting specialized: presynaptic and postsynaptic dopamine D2 receptors. *Curr Opin Pharmacol* 2009; **9**: 53–58.

- 14 Carriba P, Navarro G, Ciruela F, Ferré S, Casadó V, Agnati L *et al*. Detection of heteromerization of more than two proteins by sequential BRET-FRET. *Nat Methods* 2008; **5**: 727–733.
- 15 Pontieri FE, Tanda G, Di Chiara G. Intravenous cocaine, morphine, and amphetamine preferentially increase extracellular dopamine in the ‘shell’ as compared with the ‘core’ of the rat nucleus accumbens. *Proc Natl Acad Sci USA* 1995; **92**: 12304–12308.
- 16 Quarta D, Ferré S, Solinas M, You ZB, Hockemeyer J, Popoli P *et al*. Opposite modulatory roles for adenosine A1 and A2A receptors on glutamate and dopamine release in the shell of the nucleus accumbens. Effects of chronic caffeine exposure. *J Neurochem* 2004; **88**: 1151–1158.
- 17 García M, Floran B, Arias-Montaño JA, Young JM, Aceves J. Histamine H3 receptor activation selectively inhibits dopamine D1 receptor-dependent [3H]GABA release from depolarization-stimulated slices of rat substantia nigra pars reticulata. *Neuroscience* 1997; **80**: 241–249.
- 18 Cortés H, Paz F, Erlij D, Aceves J, Florán B. GABA(B) receptors modulate depolarization-stimulated [H]glutamate release in slices of the pars reticulata of the rat substantia nigra. *Eur J Pharmacol* 2010; **649**: 161–167.
- 19 Powell SB, Paulus MP, Hartman DS, Godel T, Geyer MA. RO-10-5824 is a selective dopamine D4 receptor agonist that increases novel object exploration in C57 mice. *Neuropharmacology* 2003; **44**: 473–481.
- 20 Gackenheim SL, Schaus JM, Gehlert DR. [3H]-quinelorane binds to D2 and D3 dopamine receptors in the rat brain. *J Pharmacol Exp Ther* 1995; **274**: 1558–1565.
- 21 Thomas TC, Kruzich PJ, Joyce BM, Gash CR, Suchland K, Surgener SP *et al*. Dopamine D4 receptor knockout mice exhibit neurochemical changes consistent with decreased dopamine release. *J Neurosci Meth* 2007; **166**: 306–314.
- 22 Thomas TC, Grandy DK, Gerhardt GA, Glaser PE. Decreased dopamine D4 receptor expression increases extracellular glutamate and alters its regulation in mouse striatum. *Neuropsychopharmacology* 2008; **34**: 436–445.
- 23 Bamford NS, Zhang H, Schmitz Y, Wu NP, Cepeda C, Levine SM *et al*. Heterosynaptic dopamine neurotransmission selects sets of corticostriatal terminals. *Neuron* 2004; **42**: 653–663.
- 24 Rondou P, Haegeman G, Van Craenenbroeck K. The dopamine D4 receptor: biochemical and signalling properties. *Cell Mol Life Sci* 2010; **67**: 1971–1986.
- 25 Kabbani N, Levenson R. A proteomic approach to receptor signaling: molecular mechanisms and therapeutic implications derived from discovery of the dopamine D2 receptor signalplex. *Eur J Pharmacol* 2007; **572**: 83–93.
- 26 Yao WD, Spealman RD, Zhang J. Dopaminergic signaling in dendritic spines. *Biochem Pharmacol* 2008; **75**: 2055–2069.
- 27 Lin R, Karpa K, Kabbani N, Goldman-Rakic P, Levenson R. Dopamine D2 and D3 receptors are linked to the actin cytoskeleton via interaction with filamin A. *Proc Natl Acad Sci USA* 2001; **98**: 5258–5263.
- 28 Binda AV, Kabbani N, Lin R, Levenson R. D2 and D3 dopamine receptor cell surface localization mediated by interaction with protein 4.1N. *Mol Pharmacol* 2002; **62**: 507–513.
- 29 Jeanneteau F, Diaz J, Sokoloff P, Griffon N. Interactions of GIPC with dopamine D2, D3 but not D4 receptors define a novel mode of regulation of G protein-coupled receptors. *Mol Biol Cell* 2004; **15**: 696–705.
- 30 Basile M, Lin R, Kabbani N, Karpa K, Kilimann M, Simpson I *et al*. Paralemmin interacts with D3 dopamine receptors: implications for membrane localization and cAMP signaling. *Arch Biochem Biophys* 2006; **446**: 60–68.
- 31 Yin HH, Lovinger DM. Frequency-specific and D2 receptor-mediated inhibition of glutamate release by retrograde endocannabinoid signaling. *Proc Natl Acad Sci USA* 2006; **103**: 8251–8256.
- 32 Cheon KA, Kim BN, Cho SC. Association of 4-repeat allele of the dopamine D4 receptor gene exon III polymorphism and response to methylphenidate treatment in Korean ADHD children. *Neuropsychopharmacology* 2007; **32**: 1377–1383.
- 33 Hamarman S, Fossella J, Ulger C, Brimacombe M, Dermody J. Dopamine receptor 4 (DRD4) 7-repeat allele predicts methylphenidate dose response in children with attention-deficit/hyperactivity disorder: a pharmacogenetic study. *J Child Adolesc Psychopharmacol* 2004; **14**: 564–574.
- 34 Ding YC, Chi HC, Grady DL, Morishima A, Kidd JR, Kidd KK *et al*. Evidence of positive selection acting at the human dopamine receptor D4 gene locus. *Proc Natl Acad Sci USA* 2002; **99**: 309–314.

Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)

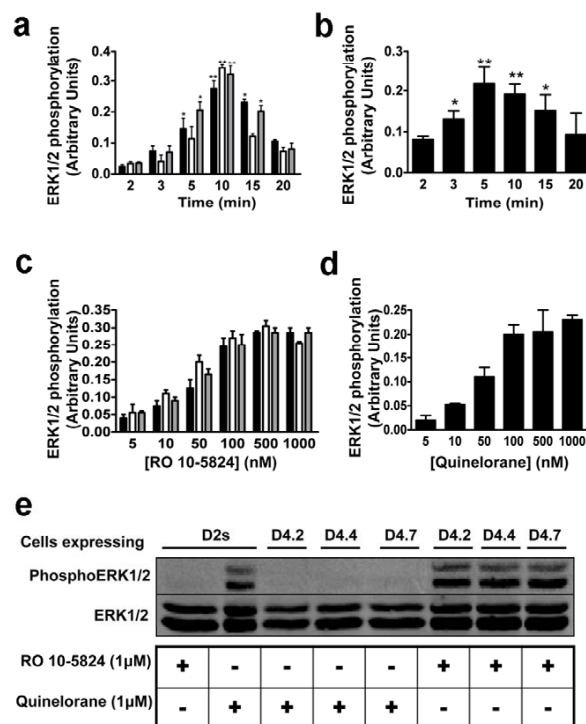
SUPPLEMENTARY INFORMATION

Supp Figure 1



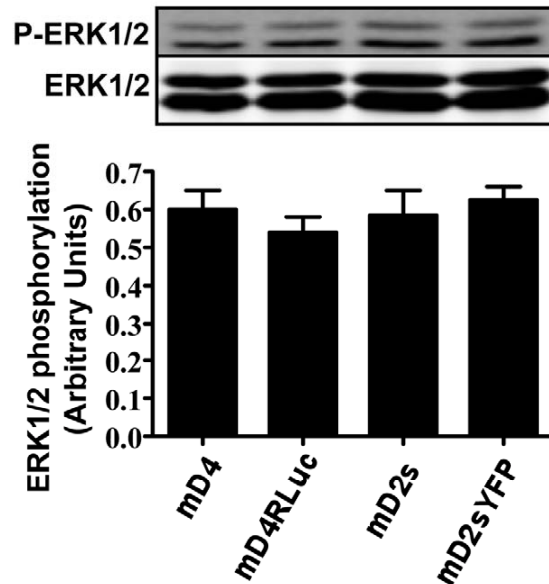
Suppl. Fig. 1. Functionality of the human fusion proteins. Cells were transfected with 2.5 μ g of cDNA corresponding to the human D4.2, D4.4, D4.7 or D2S dopamine receptors or to the corresponding fusion proteins D4.2-RLuc, D4.4-RLuc, D4.7-RLuc or D2S-YFP. 48 h post-transfection, cells expressing D4 or D4-RLuc receptors were treated for 10 minutes with RO 10-5824 (100 nM) and cells expressing D2S or D2S-YFP receptors were treated for 10 min with quinolorane (300 nM) and ERK1/2 phosphorylation was determined. The immunoreactive bands of three to four experiments were quantified and expressed as arbitrary units. A representative Western blot is shown at the top.

Supp Figure 2



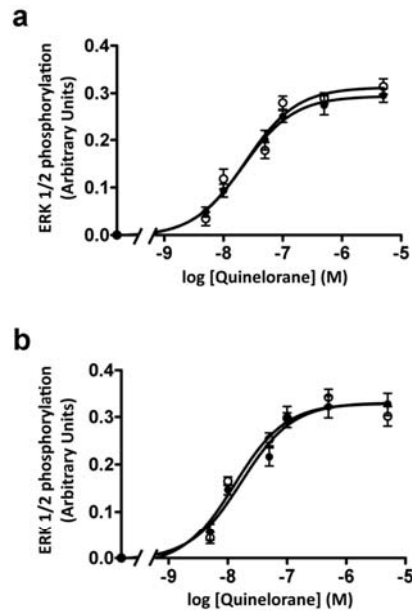
Suppl. Fig. 2. Agonist selectivity and time-response of ERK1/2 phosphorylation in cells transfected with D4 or D2 receptors. In **(a)** and **(c)** cells were transfected with 2.5 µg of cDNA corresponding to the human D4.2 (black), D4.4 (white) or D4.7 (gray) dopamine receptors. 48 h post-transfection, cells were treated for increasing time **(a)** or for 10 min **(c)** with 500 nM **(a)** or increasing concentrations **(c)** of RO 10-5824. In **(b)** and **(d)** cells were transfected with 2 µg of cDNA corresponding to the human D2S receptor and 48 h post-transfection, cells were treated for increasing time **(b)** or for 10 min **(d)** with 300 nM **(a)** or increasing concentrations **(d)** of quinelorane. In all cases the immunoreactive bands of three to four experiments were quantified and expressed as arbitrary units. Statistical differences with respect to non-treated cells were determined by Student's *t* test (**p*<0.05 and ***p*<0.01). In **(e)** the selectivity of RO-10-5824 and quinelorane was proved by the lack of ERK1/2 phosphorylation observed in cells not expressing D4 receptors or D2S receptors, respectively, when stimulated by high concentrations (1 µM) of these agonists.

Supp Figure 3



Suppl. Fig. 3. Functionality of the mouse fusion proteins. Cells were transfected with 2.5 μ g of cDNA corresponding to mouse D4 or D2S dopamine receptors or to the corresponding fusion proteins D4-RLuc or D2S-YFP. 48 h post-transfection, cells expressing D4 or D4-RLuc receptors were treated for 10 minutes with RO 10-5824 (100 nM) and cells expressing D2S or D2S-YFP receptors were treated for 10 min with quinolorane (300 nM) and ERK1/2 phosphorylation was determined. The immunoreactive bands of three to four experiments were quantified and expressed as arbitrary units. A representative Western blot is shown at the top.

Supp Figure 4



Suppl. Fig. 4. Crosstalk between mouse D2 receptors and mouse or human D4 receptors. Cells transiently co-expressing mouse D2S receptors and mouse D4 receptors **(a)** or human D4.7 receptors **(b)** were treated for 10 minutes with increasing quinolerane concentrations in the presence (○) or in the absence (●) of RO 10-5824 (50 nM) prior to the ERK1/2 phosphorylation determination. The immunoreactive bands of three experiments (mean \pm SEM; $n = 3$) were quantified and expressed as arbitrary units. EC50 values with or without RO 10-5824 were: **(a)** 23.8 ± 0.1 and 21.4 ± 0.1 nM (Student's t test: N.S.) or **(b)** 15.2 ± 0.1 and 18.2 ± 0.1 nM (Student's t test: NS).

3.3 Circadian-related heteromerization of adrenergic and dopamine D₄ receptors modulates melatonin synthesis and release in the pineal gland.

Sergio González, David Moreno-Delgado, Estefanía Moreno, Kamil Pérez-Capote, Josefa Mallol, Antoni Cortés, Vicent Casadó, Carme Lluís, Jordi Ortiz, Sergi Ferré, Enric Canela y Peter J. McCormick

Manuscrito en vías de publicación

El papel de la glándula pineal es traducir los ciclos rítmicos de noche y día controlados por la retina en señales hormonales que son transmitidas al resto del sistema neural en forma de síntesis y liberación de serotonina y melatonina. En este trabajo se describe que la producción y secreción de melatonina y serotonina de la glándula pineal está regulada por la formación de heterómeros entre receptores adrenérgicos α_{1B} y β_1 y de dopamina D₄ que sigue un patrón de ritmo circadiano. Por unión a los heterómeros α_{1B} -D₄ y β_1 -D₄, la dopamina es capaz de inhibir la señalización de los receptores adrenérgicos y bloquear la síntesis y liberación de serotonina inducida por activación con agonistas de los receptores adrenérgicos. Estos resultados proporcionan una nueva perspectiva en la función de la dopamina y constituyen el primer ejemplo de heterómeros de receptores controlados por el ritmo circadiano. La formación de heterómeros entre receptores adrenérgicos y de dopamina D₄ permite un mecanismo de feedback en el sistema neuro-hormonal, en el cual, la dopamina controla los inputs circadianos.

Circadian-related heteromerization of adrenergic and dopamine D₄ receptors modulates melatonin synthesis and release in the pineal gland

Sergio González¹, David Moreno-Delgado¹, Estefanía Moreno¹, Kamil Pérez-Capote¹, Rafael Franco¹§, Josefa Mallol¹, Antoni Cortés¹, Vicent Casadó¹, Carme Lluís¹, Jordi Ortiz², Sergi Ferré³, Enric Canela¹*, Peter J. McCormick¹**

¹Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), and Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Avda. Diagonal 645, 08028 Barcelona, Spain. ²Neuroscience Institute and Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; ³National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, Department of Health and Human Services, Baltimore, MD 21224

Corresponding author: Peter J. McCormick, Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Avda. Diagonal 645, 08028 Barcelona, Spain. Tel +(93)4021208; Fax +(93)4021559 pmccormick@ub.edu

Additional Footnotes:

- * These authors contributed equally to the manuscript.
- § Present address: Centro de Investigación Médica Aplicada (CIMA), University of Navarra, Pamplona, Spain

Running Title: Pineal gland α_{1B} -D₄ and β_1 -D₄ receptor heteromers

Abstract

The role of the pineal gland is to translate the rhythmic cycles of night and day encoded by the retina into hormonal signals that are transmitted to the rest of the neuronal system in the form of serotonin and melatonin synthesis and release. Here we describe that the production of both melatonin and serotonin by the pineal gland is regulated by a circadian-related heteromerization of adrenergic and dopamine D4 receptors. Through $\alpha 1B$ -D4 and $\beta 1$ -D4 receptor heteromers dopamine inhibits adrenergic receptor signaling and blocks the synthesis of melatonin induced by adrenergic receptor ligands. This inhibition was not observed at hours of the day when D4 was not expressed. These data provide a new perspective on dopamine function and constitutes the first example of a circadian-controlled receptor heteromer. The unanticipated heteromerization between adrenergic and dopamine D4 receptors provides a feedback mechanism for the neuronal hormone system in the form of dopamine to control circadian inputs.

Introduction

Dopamine receptors are G protein-coupled receptors (GPCRs) that consist of two major families, the D₁-like and D₂-like receptors. D₁-like receptors include D₁ and D₅ subtypes that are known to stimulate adenylate cyclase activity via a G_s mechanism and D₂-like receptors include D₂, D₃ and D₄ subtypes that inhibit adenylate cyclase activity via a G_i mechanism [1]. Of these subtypes, D₁ and D₂ and their heteromers constitute the most abundant in the brain [2–4]. The function of the other dopamine receptor subtypes has been more difficult to determine. The dopamine D₄ receptor was discovered twenty years ago and initially drew a lot of attention in view of its significantly higher affinity for the atypical antipsychotic clozapine compared to the previously discovered D₂ and D₃ receptors [5,6]. In the retina, D₄ receptors modulate phototransduction through a mechanism that requires cAMP [7]. It has been described that *Drd4* is the dominant dopamine receptor gene expressed in the rat pineal gland and that it is expressed in pinealocytes and retina at levels which are greater than in other tissues [8]. Rat pineal *Drd4* mRNA expression was found to be circadian in nature and under photoneural control [8,9]. In the pineal gland, mRNA expression for D₄ receptors has been shown to be tightly regulated and stimulated by norepinephrine through a mechanism involving thyroid hormone [8]. Nevertheless, the amount of D₄ receptor protein expression or function in the pineal gland is currently not known. In this study, the primary issue under consideration is whether or not dopamine D₄ receptor is active within the pineal gland and what is the physiological role of agonist binding to D₄ receptors with respect to pineal gland function.

The role of the pineal gland is to translate light inputs from the retina into chemical signals for the rest of the body. This is achieved via production and secretion of melatonin by the pineal gland. Melatonin production occurs on a night / day cycle and is heavily dependent on the concentration of serotonin (5-HT)[10–14]. The β_1 and α_{1B} adrenergic receptors are the main receptors that control melatonin production by different mechanisms. One of them is to control the availability of 5-HT, the melatonin precursor, by increasing both the activity of tryptophan hydroxylase (TPH) and the release of 5-HT. Another is via a strict regulation of the enzyme that converts 5-HT to melatonin, the arylalkylamine N-acetyltransferase (AANAT)[15–18]. Despite tight regulation by the adrenergic receptors it is unclear what limits the nighttime and daytime rates of melatonin and 5-HT production. We hypothesized that one important role of dopamine D_4 receptors in the pineal gland can be the modulation of β_1 and α_{1B} adrenergic receptor function. One possibility for such a modulation could be through a concept becoming widely accepted for GPCRs, the modulation of function through receptor heteromer formation [19–29]. A receptor heteromer is a macromolecular complex composed of at least two functional receptor units with biochemical properties that are demonstrably different from those of its individual receptors [30]. Here, using a combination of approaches including biophysical, molecular and cellular biology, and metabolic assays from cultured cells to whole, intact, pineal gland, we explored the possibility that D_4 receptor might modify adrenergic receptor function through direct receptor-receptor interaction. We report, to our knowledge, the first heteromer between dopamine and adrenergic receptors, provide new data that adrenergic receptor control of 5-HT levels can be modulated

via the D₄ receptor, and show that D₄-adrenergic receptor regulation can alter melatonin production from the pineal gland.

Results

D₄ receptors are functional in the pineal gland.

The expression of D₄ receptor mRNA in the pineal gland during the dark period has been described but the functional role of the protein is unknown [8,31]. Thus we first assessed whether the receptor was active in the pineal gland. Pineal gland dissected from rats 1 h from the start of the light period were stimulated with increasing concentrations of dopamine or with the D₄ receptor agonist RO 10-5824 and the levels of p-ERK 1/2 and p-Akt/PKB were determined. Dopamine increased both p-ERK 1/2 and p-Akt/PKB to a similar extent as RO 10-5824 (Figure 1A and B). Moreover, primary cultures of pinealocytes stimulated with RO 10-5824, the adrenergic α_1 receptor agonist phenylephrine or the adrenergic β receptor agonist isoproterenol showed signaling via p-ERK 1/2 (Figure 1C, red staining). The subcellular distribution of the pinealocyte marker S-arrestin (green staining) in the absence of ligands was diffuse, suggesting cytosolic localization, and in the presence of ligands, was found in punctate structures, indicating recruitment to membrane structures. In addition, these punctate structures co-localized with the p-ERK 1/2, confirming receptor activation, since endosomes containing receptor-arrestin complexes are known to serve as a signaling platform for p-ERK 1/2 [32] (Figure 1C). Thus, in both intact pineal gland and isolated pinealocytes D₄ receptors are functional.

D₄ receptors form heteromers with α_{1B} and β_1 receptors in transfected cells.

Having shown that D₄ receptors are functional in the pineal gland, we sought to test whether D₄ receptors might form heteromers with the adrenergic receptors α_{1B} and β_1 . We first examined this possibility using transfected cells. The best assay for detecting an interaction between two membrane receptors in transfected cells is through biophysical means using Bioluminescence Resonance Energy Transfer (BRET) assays. BRET experiments were performed by fusing one of the receptors to the bioluminescent protein *Renilla Luciferase* (RLuc) and the other to a yellow fluorescent protein (YFP) (Experimental Procedures). Prior to BRET experiments, preliminary experiments showed that fusion proteins were able to bind their respective ligands with similar affinities (data not shown). Next, we confirmed that the fusion proteins were able to activate p-ERK 1/2 in the same manner as the native protein (Figure S1) and that all receptors were properly trafficked to the cell membrane as observed by confocal microscopy (Figure 2D). Clear BRET saturation curves were obtained in cells expressing D₄-RLuc receptors and increasing amounts of α_{1B} -YFP or β_1 -YFP receptors (Figure 2A) with BRET_{max} values of 74 ± 4 mBU and 120 ± 10 mBU respectively and BRET₅₀ values of 37 ± 2 and 61 ± 4 respectively, indicating that the two receptors are indeed forming a higher order structure that allows energy transfer. In contrast, a low and linear BRET was detected in cells expressing α_{1B} -RLuc and increasing amounts of β_1 -YFP (Figure 2A, gray line); this was qualitatively similar to the results obtained with the negative control, cells expressing D₄-RLuc receptors and increasing amounts of D₁-YFP (Figure 2A, green line). Taken together, these results strongly suggest that the D₄ receptor forms heteromers with both, α_{1B} and β_1 receptors, but heteromers are not formed between α_{1B} and β_1 receptors.

Although these results show that α_{1B} and β_1 do not form heteromers in cells not expressing D_4 receptors, they do not discount the possibility that there are heterotrimers between D_4 , α_{1B} and β_1 receptors in cells expressing all three, as has been previously reported for other GPCRs [33]. If α_{1B} - β_1 - D_4 heterotrimers are formed, the molecular determinants on the D_4 receptor which interact with α_{1B} receptor must be different from those required to interact with β_1 receptors. On the other hand, if α_{1B} and β_1 receptors interact with the same molecular determinants on the D_4 receptor, α_{1B} - β_1 - D_4 receptor heterotrimers will not form due to the steric hindrance of two receptors competing for the same region. To test this we performed two parallel experiments. In the first one we titrated α_{1B} receptors in cells expressing a constant amount of D_4 -RLuc and α_{1B} -YFP (Figure 2B). As more unlabeled α_{1B} was expressed (red line) energy transfer was decreased due to the receptor competing with itself. We observed a nearly identical decrease in energy transfer when we titrated β_1 receptor. We obtained similar results in the second experiment, when we titrated α_{1B} or β_1 receptors in cells expressing a constant amount of D_4 -RLuc and β_1 -YFP (Figure 2C). One important observation is that the BRET approached zero as more competing receptor was added, arguing against the possibility that the unlabeled receptor is forming a complex with an existing BRET complex. In the latter scenario, the BRET is likely to remain relatively constant over a range of concentrations of the competing receptor.

The advantage of BRET experiments is that they are performed on live cells in native membranes. However, we sought to confirm these interactions using the classical method of co-immunoprecipitation. We first confirmed that α_{1B} and β_1

receptors could be co-precipitated with D₄ receptor. In cells expressing D₄-YFP, α_{1B} and β_1 receptors (Figure 2E) immunoprecipitating with anti-D₄ receptor antibodies led to co-precipitation of both α_{1B} and β_1 receptors (Figure 2F lanes 7 and 14). We also performed the reverse, immunoprecipitating with antibodies to α_{1B} or β_1 receptors and looking for co-precipitation of D₄ receptor. However, the D₄ receptor antibodies do not function by Western Blot, so we blotted the membrane with an anti-YFP antibody. Immunoprecipitating with either α_{1B} and β_1 receptor antibodies led to co-precipitation of D₄ receptors (Figure 2E lanes 1 and 8). As controls we performed the immunoprecipitation with an unrelated antibody and no α_{1B} and β_1 receptors were precipitated (Figure 2F lanes 6 and 13). Next, to confirm the BRET competition experiments detailed above (Figures 2B and C) we examined the ability of α_{1B} and β_1 receptors to co-precipitate each other. As can be seen in Figure 2F lanes 2 and 9, α_{1B} and β_1 receptors did not co-precipitate. Taken together, these results confirm the BRET experiments and prompted us to discard the possibility of α_{1B} - β_1 -D₄ receptor heterotrimers. Control experiments using cells expressing a single receptor or two receptors were also performed (Figure S2) confirming the above described results.

Functional consequences of α_{1B} -D₄ and β_1 -D₄ receptor heteromer formation in transfected cells. A common and often essential attribute of receptor heteromers is the ability to modify downstream signaling versus the single constituent receptors. This type of receptor-receptor interaction has been observed for several receptor heteromers [34–37]. To understand the function of α_{1B} -D₄ and β_1 -D₄ receptor heteromers, we investigated whether there were changes in MAPK (ERK 1/2 phosphorylation) and Akt/PKB (Ser-473 Akt phosphorylation) signaling when

heteromers were co-stimulated with both agonists or blocked with antagonists. In terms of pineal function, phosphorylation of ERK 1/2 is the last step in a cascade of signaling that modulates the enzyme that converts 5-HT to N-acetyl serotonin, thus we felt it pertinent to study changes in this signaling pathway. First, the selectivity of receptor agonists, RO 10-5824, phenylephrine and isoproterenol was tested in cells expressing D_4 , α_{1B} or β_1 receptors (Figure 3A). Using selective agonist in time-response assays we found an increase in ERK 1/2 and Akt/PKB phosphorylation in cells only expressing D_4 , α_{1B} or β_1 receptors (Figure S3). We next explored whether any cross-talk between the receptors could be detected in cells co-expressing the receptors. In α_{1B} - D_4 and β_1 - D_4 receptor co-expressing cells, stimulation of D_4 receptors for 7 min with the D_4 specific ligand RO 10-5824 inhibited α_{1B} and β_1 receptor-mediated ERK 1/2 and Akt/PKB activation induced by increasing amounts of phenylephrine and isoproterenol (Figure 3 B to E). We observed an almost complete block in the amount of p-ERK 1/2 induced by adrenergic agonists in the presence of RO 10-5824 (Figure 3B and D), indicating that D_4 activation inhibited the α_{1B} and β_1 receptor-mediated ERK 1/2 phosphorylation. In addition, a complete block of p-Akt production was observed in the presence of both adrenergic receptor agonist and D_4 receptor agonist (Figure 3C and E), demonstrating that D_4 receptor activation inhibited the α_{1B} and β_1 receptor-mediated Akt/PKB phosphorylation and vice-versa. These results are not due to a change in the time in which the signaling peaks, since differences were not observed in time-response curves when co-transfected cells were activated with one or both agonists (Figure S4). In addition, as a negative control, we confirmed that RO 10-5824 did not modify ERK 1/2 or Akt/PKB phosphorylation induced by

phenylephrine or isoproterenol in cells transfected with α_{1B} or β_1 receptors alone (Figure S5).

In addition to cross-talk at the level of receptor signaling, some GPCR heterodimers act at the level of ligand binding [35,38–40]. To explore whether D_4 receptor ligands can modify the binding of α_{1B} or β_1 receptor ligands we performed radioligand competition assays in transfected cells in the presence or absence of the D_4 receptor specific ligand RO 10-5824. As can be seen in figure 3F, the addition of RO 10-5824 led to a decrease in the ability of phenylephrine, the α_{1B} receptor agonist, to displace the radiolabeled α_{1B} receptor antagonist [3H]-prazosin. The monophasic competition curve giving an affinity constant (K_{D1}) of 10 ± 1 nM changed to a biphasic curve giving a K_{D1} of 27 ± 7 nM and K_{D2} of 1600 ± 400 nM in the presence of RO 10-5824, showing negative cooperativity (cooperativity index of -1.17). These results point out that agonist binding to D_4 receptor in the heteromer decreases the affinity of agonist binding to α_{1B} receptor. Interestingly, when similar experiments were performed testing agonist binding to β_1 receptors, there were no differences observed in the displacement curve or the affinity in the presence or absence of RO 10-5824 (K_{D1} of 300 ± 50 nM and 460 ± 80 nM respectively)(Figure 3G). Taken together, these results imply differences between α_{1B} - D_4 and β_1 - D_4 receptor heteromers in their allosteric interactions.

We next looked for a heteromer specific biochemical property. Antagonists, by definition, do not signal; thus, cross-antagonism, any change in α_{1B} or β_1 mediated signaling caused by an antagonist of D_4 receptors, could only be due to protein-protein contact between the receptors, and would constitute a specific biochemical characteristic of the heteromer. Prior to looking for cross-antagonism, we

investigated the selectivity of D_4 , α_{1B} and β_1 receptor antagonists by measuring MAPK and Akt/PKB signaling in cells transfected with only D_4 , α_{1B} or β_1 receptors and stimulated or not with agonist and treated with the selective D_4 , α_{1B} and β_1 receptor antagonists L-745,870, REC 15/2615 and CGP 20712, respectively. All antagonists behaved as classical antagonists, since none demonstrated any signaling properties in transfected cells (Figure S6). Importantly, all antagonists were selective, as expected and were able to attenuate agonist-induced signaling in only their respective receptors (Figure S6). Next, cells co-expressing α_{1B} - D_4 and β_1 - D_4 receptors were treated with antagonists prior to activation with agonist. We obtained a striking cross-antagonism in MAPK and Akt/PKB activation (Figure 4). In both cases, the D_4 receptor antagonist, L-745,870 was able to completely block signaling caused by isoproterenol or phenylephrine. Moreover, signaling induced by the D_4 receptor agonist was blocked by the adrenergic receptor antagonist REC 15/2615 and CGP 20712. These results demonstrate that the dopamine D_4 receptor is able to modify α_{1B} and β_1 function via receptor heteromers and vice-versa. In addition, this cross-antagonism constitutes a specific biochemical property of the α_{1B} - D_4 and β_1 - D_4 receptor heteromers and can be used as a biochemical fingerprint to detect the heteromers in native tissues.

Functional α_{1B} - D_4 and β_1 - D_4 receptor heteromers in the pineal gland.

We next sought to detect α_{1B} - D_4 and β_1 - D_4 receptor heteromers in the pineal gland. We looked for the heteromer biochemical property identified above, the cross-antagonism, as an initial demonstration of the existence of α_{1B} - D_4 and β_1 - D_4 receptor heteromers in the pineal gland. Therefore, whole pineal glands were isolated one hour after starting the light period and stimulated with the respective

D₄, α_{1B} and β_1 agonists RO 10-5824, phenylephrine and isoproterenol and p-ERK 1/2 (Figure 4E) and p-Akt (Figure 4F) signaling were measured with respect to basal levels. As can be seen in Figure 4E and F, all three receptors showed robust signaling that could be attenuated with the respective antagonist (L-745,870, REC 15/2615, and CGP 20712). We also detected a cross-antagonism in MAPK and Akt/PKB activation. In both cases, the D₄ receptor antagonist, L-745,870, was able to block completely the signaling caused by isoproterenol or phenylephrine and the signaling induced by the D₄ receptor agonist was blocked by the adrenergic receptor antagonist REC 15/2615 and CGP 20712 (Figure 4E and F). These results matched the cross-antagonism observed in transfected cells, thus strongly indicating that D₄ receptors form functional heteromers with α_{1B} and β_1 receptors in the pineal gland.

Direct detection of α_{1B} -D₄ and β_1 -D₄ receptor heteromers in the pineal gland.

Biophysical techniques to detect heteromers directly cannot be easily applied in native tissue, but other direct methods can be used. One example is the application of the newly developed proximity ligation assay (PLA). This technique has been successfully employed to detect protein dimers in cells and in tissue [41]. Prior to performing PLA, we first confirmed the antibody specificity. The antibody against D₄, α_{1B} or β_1 receptor, only stained cells expressing the corresponding receptor but not non-transfected cells and cells expressing D₄ receptors were not stained by antibodies against adrenergic receptors and cells expressing α_{1B} or β_1 receptors are not stained with anti-D₄ receptors antibody (Figure S7). The selectivity for anti-D₄ antibody was also demonstrated by taking advantage of the fact that rat pineal *Drd4* mRNA expression was found to be circadian in nature being high at the

last part of the dark period and very low during the light period [8,9]. Thus, without the need of genetically manipulated animals, we observed that the anti-D₄ antibody was able to stain pinealocytes from pineal glands extracted just after the darkness period but not pinealocytes from glands extracted at the end of the light period (Figure 5A). The expression of both adrenergic receptors was similar in both periods (Figure 5B and C). After testing the expression of the individual receptors using immunofluorescence in pinealocytes, we next looked for evidence of expression of α_{1B} -D₄ and β_1 -D₄ receptor heteromers in pineal gland using the proximity ligation assay. This direct method requires that both receptors be close enough to allow the two different antibody-probes to be able to ligate (<17nm) [41,42]. If the receptors are within sufficient proximity, a punctate fluorescent signal can be detected by confocal microscopy (see Experimental Procedures). We found that the endogenously expressed D₄ receptors do indeed form heteromers with the endogenous expressed α_{1B} and β_1 receptors in a primary culture of pinealocytes obtained from a pineal gland dissected 1 h after the start of the light period (Figure 5D and E, punctate pattern of fluorescence in the upper images) but we did not observe receptor interaction, in the form of a fluorescent signal, for negative controls tested in the absence of primary antibodies (Figure S8) or for α_{1B} - β_1 receptors (Figure 5F). These results were consistent with the BRET experiments and demonstrated α_{1B} -D₄ and β_1 -D₄ receptor heteromers expression in pinealocytes. As we observed a severe depletion of D₄ receptor expression in pinealocytes from glands isolated at the end of the light period, we performed the PLA experiments also with glands isolated at the end of the light period. As expected, no α_{1B} -D₄ and β_1 -D₄ receptor heteromers were detected (Figure 5D and E, lower images). These results not only confirm the specificity of the results in

Figure 5D and E (top images), but also demonstrate the circadian nature of heteromer formation. To confirm the circadian nature of heteromer formation we performed co-immunoprecipitation experiments using glands dissected 1 h after the start of the light period (sunrise) or glands isolated at the end of the light period (sunset). Although adrenergic receptors are expressed in sunrise and sunset periods (Figure 5G), immunoprecipitating with anti-D₄ receptor antibodies led to co-precipitation of both α_{1B} and β_1 receptors only from glands extracted at the sunrise period and not from glands extracted at the sunset period (Figure 5H) indicating the heteromer expression in the pineal gland and the circadian nature of the heteromerization. The lack of heteromer formation between α_{1B} and β_1 receptors seen earlier by BRET and immunoprecipitation in transfected cells was confirmed in pineal gland by co-immunoprecipitation experiments (Figure 5 H).

Functional consequences of α_{1B} -D₄ and β_1 -D₄ receptor heteromer formation in the pineal gland.

To test the effect of receptor co-activation in α_{1B} -D₄ and β_1 -D₄ receptor heteromers in the p-ERK 1/2 and p-Akt/PKB production, pineal glands, isolated at 9:00h, one hour after the start of the light period (at sunrise), were stimulated with RO 10-5824, phenylephrine or isoproterenol alone or in combination. Co-activation with RO 10-5824 and phenylephrine or with RO 10-5824 and isoproterenol induced a significant decrease of p-ERK 1/2 production compared with stimulation with one agonist alone (Figure 6A). Co-activation completely blocked the formation of p-Akt/PKB in cells stimulated with RO 10-5824, phenylephrine or isoproterenol (Figure 6B). These results indicate that there is a negative cross-talk between D₄ and α_{1B} or β_1 receptors not only in transfected cells but also in the pineal gland. To

be sure that the data reflected a true negative cross-talk between D_4 and α_{1B} or β_1 receptors, and not a time displacement of the signaling, we performed time-response experiments with pineal glands (Figure S9). The effect of co-activation with RO 10-5824 and phenylephrine or with RO 10-5824 and isoproterenol on α_{1B} and β_1 signaling was not due to a change in timing of the signal with maximal signal obtained at 10 min. In addition, at all times examined no p-Akt/PKB signal was detected in the presence of both adrenergic agonists and RO 10-5824. These data support the conclusion that the result observed in Figure 6A and B were indeed due to a true negative cross-talk.

As the expression of D_4 receptor in the pineal gland is regulated by a cycle of light/dark, we reasoned that if we isolated pineal gland after 12 hours of light (at sunset) when the levels of D_4 receptor are low, then we should now lose the negative cross-talk seen in Figure 6 A and B. To test this, we stimulated pineal gland extracted at 20:00 h and compared signaling after stimulation with RO 10-5824 in the presence or absence of phenylephrine and isoproterenol. As shown in Figure 6C and D, there was no inhibition of α_{1B} and β_1 receptor-mediated MAPK and Akt/PKB activation by the D_4 receptor agonist RO 10-5824 in glands isolated at the end of the light period (sunset), a time of low D_4 receptors expression. This was in contrast to signaling in glands extracted at 9:00h, just after the dark period (sunrise) where D_4 receptors are expressed and negative cross-talk in agonist-induced signaling was observed (Figure 6 A and B).

The metabolic consequences of α_{1B} - D_4 and β_1 - D_4 receptor heteromers activation in the pineal gland.

Finally, we sought to understand how α_{1B} -D₄ and β_1 -D₄ receptor heteromers might modulate pineal gland function. A major role of the pineal gland is controlling the levels of melatonin and its precursor 5-HT via synthesis and release. The α_{1B} receptor controls 5-HT and melatonin release via potentiation of the calcium-induced exocytosis, while the β_1 receptors can modify the synthesis of both 5-HT and melatonin [15–18]. With this in mind, we tested the role of the α_{1B} -D₄ and β_1 -D₄ receptor heteromers in 5-HT and melatonin synthesis and release. Ideally, to test the physiological importance of heteromers, one would like to create a targeted knockout animal lacking one of the partner receptors in the tissue of interest to be compared with wild type animals. However, in the case of D₄ receptor expression in the pineal gland nature provided a suitable alternative. We decided to take advantage of the fact that D₄ receptor expression is altered by the cycle of light and dark and compare results obtained with pineal gland extracted at the end of the light period (sunset) when D₄ receptors are not expressed with those obtained with glands extracted at the end of the dark period (sunrise) when D₄ receptors are expressed.

We treated pineal glands, isolated at 20:00h, when α_{1B} -D₄ and β_1 -D₄ receptor heteromers are not expressed, with specific agonists or/and antagonists and measured the amount of 5-HT synthesized (Figure 7A and C) or released (Figure 7B and D) and the amount of melatonin synthesized (Figure 7E and G) or released (Figure 7F and H). As can be seen in Figure 7A to H, treatment with the D₄ specific agonist, RO 10-5824, showed no increase in either 5-HT or melatonin synthesis or release compared to basal levels. In contrast, we observed a large increase in melatonin synthesis and release when the glands were treated with the β_1 receptor

agonist isoproterenol or the α_{1B} agonist phenylephrine respectively (Figure 7E to H) and a significant increase in 5-HT synthesis and release when the glands were treated with isoproterenol or phenylephrine (Figure 7A to D). The increases in 5-HT and melatonin synthesis and release could be blocked only by the corresponding specific antagonists of adrenergic receptors but not by the D_4 receptor antagonist L-745,870 (Figure 7A, B, E and F) demonstrating a lack of cross-antagonism due to the absence of the heteromers. In addition, when we treated the glands with either phenylephrine or isoproterenol in the presence of the dopamine D_4 receptor agonist RO 10-5824 (Figure 7C, D, G and H) no negative cross-talk between dopamine D_4 and adrenergic receptors could be detected. The role of adrenergic receptors is represented in Figure 7I. In contrast and very interestingly, when pineal glands were isolated at 9:00h, at sunrise, (when D_4 receptor expression increases and α_{1B} - D_4 and β_1 - D_4 receptor heteromers are expressed) and were stimulated as before with agonists of both α_{1B} and β_1 receptors in the presence of either the pertinent antagonist or the D_4 antagonist, we observed that 5-HT and melatonin synthesis and release could be blocked not only by the corresponding specific antagonists of adrenergic receptors but also by the D_4 receptor antagonist L-745,870 (Figure 7J, K, N and O). This demonstrates a clear cross-antagonism. In addition, when we treated the glands with either phenylephrine or isoproterenol in the presence of the dopamine D_4 receptor agonist RO 10-5824, a complete block in the ability of either ligand to increase 5-HT or melatonin synthesis or release was observed (Figure 7L, M, P and Q). This shows that, in these conditions, a negative cross-talk between dopamine D_4 and adrenergic receptors exists in the pineal gland. A schematic representing the influence of dopamine on 5-HT and melatonin synthesis and release in these

conditions is represented in Figure 7R. These data provide strong evidence that the role of the dopamine D₄ receptor via either α_{1B} -D₄ and β_1 -D₄ receptor heteromers is to modify the melatonin metabolic pathway in the pineal gland.

Discussion

In the present study we identified a previously unknown mechanism for how dopamine can regulate adrenergic receptor function in a circadian fashion. By applying a number of different experimental approaches, we were able to identify: 1) That functional dopamine D₄ receptors form heteromers with both α_{1B} - and β_1 adrenergic receptors in transfected cells and in the pineal gland; 2) that the α_{1B} -D₄ and β_1 -D₄ receptor heteromers allow direct modulation of the adrenergic agonist-induced MAPK and Akt signaling by the D₄ receptor agonist and antagonist in transfected cells and in the pineal gland; 3) that the synthesis of melatonin and its precursor 5-HT, promoted by adrenergic receptor stimulation in the pineal gland, can be controlled by D₄ receptor activation via α_{1B} -D₄ and β_1 -D₄ receptor heteromers and 4) that this D₄ receptor heteromer-mediated modulation is dependent on the circadian light/dark cycle. This is the first example, to our knowledge, of a circadian-dependent modulation of receptor heteromerization. Together these findings point to a new role for the D₄ receptor in the pineal gland where D₄ receptor activation modifies α_{1B} - and β_1 adrenergic receptor function by a direct receptor-receptor interaction which can limit the levels of melatonin secreted by the pineal gland.

The adrenergic receptors are the mainstay receptors of pineal gland function. They form the bridge between the circadian controlled release of norepinephrine by the surrounding sympathetic nerve terminals and the melatonin production of the pineal gland. The adrenergic receptors are thought to control the production of melatonin through a variety of mechanisms, including control of the levels of the melatonin precursor 5-HT [15,16]. Dopamine is also present in the afferent sympathetic nerves in the pineal gland, not only as a precursor of norepinephrine, but is also proposed to be co-released to a lesser extent with norepinephrine [8].

The 'receptor heteromer' concept, in which receptors of the same and different gene families can combine among themselves to generate new and unique biochemical and functional characteristics, is becoming widely accepted for GPCRs and constitutes an emerging area in the field of GPCR signaling and function regulation [30]. To date there had been no demonstration of heteromers involving dopamine and the adrenergic receptors. Here, by means of BRET experiments in transfected cells and by proximity ligation assays in pinealocytes, we present direct evidence that the D₄ receptor forms heteromers with both the α_{1B} and β_1 adrenergic receptors. The formation of α_{1B} -D₄ and β_1 -D₄ receptor heteromers in the pineal gland manifests itself in the form of cross-antagonism. We observed that a D₄ receptor specific antagonist was able to block the signaling through both α_{1B} - and β_1 adrenergic receptors. The specific antagonists of α_{1B} - and β_1 adrenergic receptors were also able to block signaling through D₄ receptors. This is a clear example of cross-antagonism in a receptor heteromer [43–45], since, by definition an antagonist is not able to induce intracellular signaling. This statement is *a propos* in our case since none of the antagonists used here demonstrated any

signaling activity. Thus the simplest way to explain the effect of a D₄ receptor antagonist on α_{1B} and β_1 receptor activation and vice-versa, is through a direct protein-protein interaction between both receptors.

The functional consequences of this protein-protein interaction is a negative cross-talk between both receptors in the α_{1B} -D₄ and β_1 -D₄ receptor heteromers, i.e. the block in the amount of p-ERK 1/2 induced by adrenergic agonists in the presence of D₄ receptor agonist and the complete block of p-Akt production when both receptors in the heteromer were co-stimulated. In the pineal gland, D₄ receptor mRNA expression is tightly regulated so that it is highest during the last part of the dark period [8]. Accordingly, we show that the D₄ receptor is expressed and is functional in pineal glands isolated at sunrise and we saw no activity and no expression when pineal glands were isolated at sunset, the end of the light period. Our finding that the D₄ receptor can modify the downstream signaling of the α_{1B} and β_1 adrenergic receptors is particularly interesting as D₄ receptor expression was found to be modified by an increase in norepinephrine levels [8]. Norepinephrine levels are also known to increase at night and it is through its binding to the adrenergic receptors that the level of D₄ receptor mRNA is thought to reach the maximum at the end of the dark period [8]. Thus, the mechanism we describe may represent a feedback inhibition, where increased expression of D₄ receptor via adrenergic signaling leads to an increase of α_{1B} -D₄ and β_1 -D₄ receptor heteromers which then inhibit adrenergic-induced signaling through the above described cross-talk. The detailed molecular mechanism for how this feedback occurs is less clear. It is known that heteromers can function in a variety of different mechanisms, including allosterically, asymmetrically, and / or through

cooperativity [23,29,46]. The binding experiments in transfected cells suggest, at least for the α_{1B} -D₄ receptor heteromer, there is inhibition at the level of ligand binding. Why is this not seen for β_1 -D₄ receptor heteromer? Does this reflect differences in heteromer plasticity?, e.g. protomer-protomer molecular interactions promoted by ligand binding to one protomer inducing structural changes in the other protomer that are sensed at both ligand binding and signaling levels in one case and only at the signaling level in the other case. Or are these results due to something experimentally related eg. the differences of ligands used? More experiments will be required to identify how exactly these particular heteromers function. An interesting corollary to heteromer function and the data presented here is that a recent proposal arguing against the existence of heteromers and heteromer function suggested that GPCRs were competing for available G-proteins and that any cross-signaling effects observed were due to this competition [47]. Our results argue against this possibility, at least in the case of α_{1B} -D₄ and β_1 -D₄ receptor heteromers, as none of these receptors use the same G-proteins to signal. We have observed cross-talk at the level of p-ERK 1/2 and p-Akt, two steps along the production and release of melatonin. Separately all three receptors studied can activate both signaling pathways, thus heteromer formation by a protein-protein interaction clearly alters the ability of these receptors to signal using these pathways. Cross-talk has been observed for other heteromers [24,25,37,48], the mechanisms have varied from changes in β -arrestin recruitment, changes in receptor trafficking, changes in G-protein coupling, and / or changes in ligand binding. More experiments will be required to understand the molecular mechanism at play in the pineal gland. Another possible interpretation of our signaling results is that some downstream effect after D₄ receptor activation might

cause adrenergic receptor signaling to be inhibited. This does not seem to be the mechanism of action for the α_{1B} -D₄ heteromers based on the fact that the inhibition occurs at the binding level as well. However, although we cannot completely rule out this possibility for β_1 -D₄, the fact that there is cross-antagonism and that the receptors are in a complex suggests an indirect mechanism of inhibition is less likely.

We have also studied the metabolic consequences of α_{1B} -D₄ and β_1 -D₄ receptor heteromer activation at the level of melatonin synthesis and release, as well as the precursor of melatonin, 5-HT. Melatonin levels are increased at night while 5-HT levels fluctuate in the opposite manner, with production and secretion increasing during the day with the levels of AANAT, the enzyme in the last step to melatonin synthesis. Through mass action, large changes in AANAT activity at night can rapidly decrease the levels of 5-HT, yielding large increases in melatonin [49]. It is important to point out that 5-HT synthesis is thought to occur both during the day and at night and nocturnal synthesis and release of 5-HT is required for maximal adrenergic stimulation of melatonin synthesis [50,51]. Extracellular 5-HT is either taken up by surrounding sympathetic nerves or binds 5HT_{2C} receptors on the pineal gland, which in turn can lead to increased melatonin synthesis and release [50,52]. To date it has not been entirely clear what limits the maximum nighttime and minimum daytime rates of melatonin production. Our data suggest that α_{1B} -D₄ and β_1 -D₄ receptor heteromers may play an important role in this process. In pineal glands, isolated at the end of the light period (sunset) when the expression of D₄ receptor is negligible, treated with adrenergic ligands, we have seen a large increase in melatonin and a moderate increase in 5-HT synthesis mediated by β_1

receptors and release mediated by α_{1B} receptors (Figure 7I). In this case neither synthesis nor release of 5-HT or melatonin were blocked by treating the gland simultaneously with a D_4 receptor agonist or were modified in the presence of D_4 receptor antagonist. In these conditions the pineal gland is starting the melatonin production during the dark period. In pineal glands, isolated at the end of the dark period (sunrise) when the D_4 receptor is expressed, treated with adrenergic ligands, we have also seen a large increase in melatonin and 5-HT synthesis mediated by β_1 receptors and release mediated by α_{1B} receptors. Interestingly, both synthesis and release were blocked by treating the gland simultaneously with a D_4 receptor agonist (Figure 7). Thus, dopamine appears to be able to regulate the melatonin and 5-HT levels as seen in Figure 7R. This suggests that dopamine, via α_{1B} - D_4 and β_1 - D_4 receptor heteromers, may serve both as a buffer to control the amount of 5-HT that can be made and released during the light period, limiting total melatonin production, and be partially responsible for the block of melatonin production after the dark period. During the day, D_4 receptors would begin to be down-regulated, less α_{1B} - D_4 and β_1 - D_4 receptor heteromers would be formed, AANAT would be also down regulated, maintaining a reduced melatonin production, 5-HT levels would gradually increase, and the cycle could repeat. These findings provide the first report of a role for the D_4 receptor in the pineal gland and suggest a new area of research on how dopamine receptors, by means of a circadian-related heteromerization with adrenergic receptors, may help maintain the circadian rhythm signals emulating from the pineal gland.

Experimental Procedures

Fusion proteins and expression vectors. The cDNA for human dopamine D₄, and adrenergic α_{1B} and β_1 receptor genes expressed in the *pcDNA3.1* vector were amplified without its stop codon using sense and antisense primers to be cloned in the mammalian humanized pRluc-N1 or in the EYFP-N3 vectors (*See Supplementary Experimental Procedures*).

Cell culture and transient transfection. CHO or HEK-293T cells were grown in supplemented α -MEM or Dulbecco's modified Eagle's medium (DMEM) medium, respectively, and they were transfected by the polyethylenimine (PEI) method. (*See Supplementary Experimental Procedures*).

Immunostaining. HEK-293T cells were grown on glass coverslips and transiently transfected. After 48h of transfection, cells were fixed and labeled with the corresponding antibodies. (*See Supplementary Experimental Procedures*).

BRET assay. HEK-293T cells were co-transfected with a constant amount of cDNA encoding for the receptor fused to Rluc and with increasing amounts of cDNA encoding to the receptor fused to YFP to measure BRET. BRET was expressed as milli BRET Units (mBU) and is the BRET ratio x 1000. (*See Supplementary Experimental Procedures*).

Pineal glands dissection and culture. Male Sprague Dawley rats (3 month old, \approx 350g), receiving water and food *ad libitum*, were obtained from the animal facility of the Faculty of Biology (University of Barcelona). Rats were housed in light:dark 12:12 lighting cycles starting light at 9:00 h and dark at 21:00 h 4% Isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) anesthetized animals were killed by decapitation at 9:00h (just after the dark period) or at 20:00h (after light period) and pineal glands were immediately dissected. All procedures were approved by the Catalan Ethical Committee for Animal Use (CEAA/ DMAH 4049 and 5664). Rat pineal glands were cultured in defined culture medium (BGJb, Invitrogen, Carlsbad, CA) containing 10% (v/v) fetal bovine serum (heat-inactivated) for 24-36h and cultured in serum-free medium for 16 h before the addition of agonists and/or antagonist for signaling experiments or were overnight cultured in HBSS medium (137 mM NaCl, 5 mM KCl, 0.34 mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.44 mM KH_2PO_4 , 1.26 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.4 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 mM MgCl_2 , 10 mM HEPES, pH 7.4), supplemented with 0,1% glucose, 100 U/ml penicillin/streptomycin and 1mg/ml bovine serum albumin, containing agonist and/or antagonist for serotonin synthesis and release determination .

Coimmunoprecipitation. Transfected cells or pineal glands were solubilized by homogenization in ice-cold immunoprecipitation buffer and were processed for immunoprecipitation as described in immunoprecipitation protocol using a Dynabeads Protein G kit (Invitrogen) (*See Supplementary Experimental Procedures*)

Detection of MAPK and Akt/PKB phosphorylation. Transfected cells or pineal glands were cultured in serum-free medium before the addition of the indicated

concentration of ligands for the indicated time. Both, cells and pineal glands were washed and lysed. Proteins were separated by electrophoresis and ERK 1/2 (Thr¹⁸³-Tyr¹⁸⁵) and Akt (Ser⁴⁷³) phosphorylation was determined by western blot and band densities were quantified (*See Supplementary Experimental Procedures*).

Radioligand Binding Experiments. Competition experiments were performed using membranes from cells expressing D₄ and α_{1B} or β_1 receptors. Membranes were incubated with the indicated concentration of the α_{1B} receptor antagonist [³H]prazosin or β_1 receptor antagonist [³H]CGP-12177 (PerkinElmer Life and Analytical Sciences) and increasing concentrations of phenylephrine or isoproterenol respectively, in the absence or in the presence of the indicated concentration of the D₄ receptor agonist RO 10-5824 (Tocris, Aronmouth, UK) (*See Supplementary Experimental Procedures*).

Pinealocyte culture, signaling and immunocytochemistry. Pinealocytes were prepared from rat pineal glands as previously described by Silveira Cruz-Machado et al. [53] and maintained in culture no more than 48h. For signaling experiments, pinealocytes were treated with specific agonist, fixed with paraformaldehyde and treated with the corresponding antibodies (*See Supplementary Experimental Procedures*).

In situ Proximity Ligation Assay (PLA). The primary cultures of pinealocytes were fixed and permeabilized as described above. The receptor-receptor molecular interaction was detected using the Duolink II in situ PLA detection Kit (*See Supplementary Experimental Procedures*).

Serotonin synthesis and release determination. After 36 h of culture in BGJb medium, pineal glands were incubated in supplemented HBSS medium for 12 h with specific agonist and/or antagonist and radioactive [^{14}C]-Tryptophan (10 μM). Medium and pineal glands were collected separately and [^{14}C]-serotonin in medium or in homogenized glands, was separated by HPLC chromatography coupled to detection by fluorescence and counted in a liquid scintillation counter (*See Supplementary Experimental Procedures*).

Melatonin synthesis and release determination. After 36 h of culture in BGJb medium, the pineal glands were incubated for 12 h with specific agonist and/or antagonist in supplemented HBSS medium. After incubation, media were collected into eppendorf tubes and pineal glands were homogenized by sonication in a Dynatech/Sonic Dismembrator (Dynatech Labs, Chantilly, VA) for 15 seconds. An aliquot was reserved for protein quantification by the Lowry method and cellular debris was removed by centrifugation at 10,000 g for 10 min at 4°C. Melatonin was quantified using a radioimmunoassay kit with [^{125}I]-melatonin (DiaSource, Belgium) following the instructions of the supplier.

Acknowledgments

We would like to thank D.C. Klein for helpful suggestions. We thank Jasmina Jiménez for technical help (University of Barcelona). We would like to thank S. Lefrancois (Univerite de Montreal) for critical reading of the manuscript. This study was supported by grants from the Spanish Ministerio de Ciencia y Tecnología (SAF2010-18472, SAF2008-01462), funds from the Generalitat of Catalonia (2009SGR12) and NIDA intramural funds to S.F. PJM is a Ramón y Cajal Fellow.

References

1. Missale C, Nash SR, Robinson SW, Jaber M, Caron MG (1998) Dopamine receptors: from structure to function. *Physiol Rev* 78: 189–225.
2. Rashid AJ, O'Dowd BF, Verma V, George SR (2007) Neuronal Gq/11-coupled dopamine receptors: an uncharted role for dopamine. *Trends Pharmacol Sci* 28: 551–555. doi:10.1016/j.tips.2007.10.001.
3. Hasbi A, O'Dowd BF, George SR (2010) Heteromerization of dopamine D2 receptors with dopamine D1 or D5 receptors generates intracellular calcium signaling by different mechanisms. *Curr Opin Pharmacol* 10: 93–99. doi:10.1016/j.coph.2009.09.011.
4. Perreault ML, O'Dowd BF, George SR (2011) Dopamine receptor homooligomers and heterooligomers in schizophrenia. *CNS Neurosci Ther* 17: 52–57. doi:10.1111/j.1755-5949.2010.00228.x.
5. Van Tol HH, Bunzow JR, Guan HC, Sunahara RK, Seeman P, et al. (1991) Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. *Nature* 350: 610–614. doi:10.1038/350610a0.
6. Rondou P, Haegeman G, Van Craenenbroeck K (2010) The dopamine D4 receptor: biochemical and signalling properties. *Cell Mol Life Sci* 67: 1971–1986. doi:10.1007/s00018-010-0293-y.
7. Ivanova TN, Alonso-Gomez AL, Iuvone PM (2008) Dopamine D4 receptors regulate intracellular calcium concentration in cultured chicken cone photoreceptor cells: relationship to dopamine receptor-mediated inhibition of cAMP formation. *Brain Res* 1207: 111–119. doi:10.1016/j.brainres.2008.02.025.

8. Kim J-S, Bailey MJ, Weller JL, Sugden D, Rath MF, et al. (2010) Thyroid hormone and adrenergic signaling interact to control pineal expression of the dopamine receptor D4 gene (*Drd4*). *Mol Cell Endocrinol* 314: 128–135. doi:10.1016/j.mce.2009.05.013.
9. Bai L, Zimmer S, Rickes O, Rohleder N, Holthues H, et al. (2008) Daily oscillation of gene expression in the retina is phase-advanced with respect to the pineal gland. *Brain Research* 1203: 89–96. doi:16/j.brainres.2008.01.073.
10. Delgado MJ, Vivien-Roels B (1989) Effect of environmental temperature and photoperiod on the melatonin levels in the pineal, lateral eye, and plasma of the frog, *Rana perezi*: importance of ocular melatonin. *Gen Comp Endocrinol* 75: 46–53.
11. Alonso-Gómez AL, Tejera M, Alonso-Bedate M, Delgado MJ (1990) Response to pinealectomy and blinding in vitellogenic female frogs (*Rana perezi*) subjected to high temperature in autumn. *Can J Physiol Pharmacol* 68: 94–98.
12. Ganguly S, Coon SL, Klein DC (2002) Control of melatonin synthesis in the mammalian pineal gland: the critical role of serotonin acetylation. *Cell Tissue Res* 309: 127–137. doi:10.1007/s00441-002-0579-y.
13. Velarde E, Cerdá-Reverter JM, Alonso-Gómez AL, Sánchez E, Isorna E, et al. (2010) Melatonin-synthesizing enzymes in pineal, retina, liver, and gut of the goldfish (*Carassius*): mRNA expression pattern and regulation of daily rhythms by lighting conditions. *Chronobiol Int* 27: 1178–1201. doi:10.3109/07420528.2010.496911.
14. Klein DC, Bailey MJ, Carter DA, Kim J, Shi Q, et al. (2010) Pineal function: impact of microarray analysis. *Mol Cell Endocrinol* 314: 170–183. doi:10.1016/j.mce.2009.07.010.
15. Gonzalez-Brito A, Troiani ME, Menendez-Pelaez A, Delgado MJ, Reiter RJ (1990) mRNA transcription determines the lag period for the induction of pineal melatonin synthesis in the Syrian hamster pineal gland. *J Cell Biochem* 44: 55–60. doi:10.1002/jcb.240440105.
16. Zheng W, Cole PA (2002) Serotonin N-acetyltransferase: mechanism and inhibition. *Curr Med Chem* 9: 1187–1199.
17. Klein DC (2007) Arylalkylamine N-acetyltransferase: “the Timezyme.” *J Biol Chem* 282: 4233–4237. doi:10.1074/jbc.R600036200.
18. Ho AK, Chik CL (2010) Modulation of Aanat gene transcription in the rat pineal gland. *J Neurochem* 112: 321–331. doi:10.1111/j.1471-4159.2009.06457.x.
19. Ferré S, Ciruela F, Woods AS, Lluís C, Franco R (2007) Functional relevance of neurotransmitter receptor heteromers in the central nervous system. *Trends Neurosci* 30: 440–446. doi:10.1016/j.tins.2007.07.001.
20. Terrillon S, Bouvier M (2004) Roles of G-protein-coupled receptor dimerization. *EMBO Rep* 5: 30–34. doi:10.1038/sj.embor.7400052.

21. Bulenger S, Marullo S, Bouvier M (2005) Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. *Trends Pharmacol Sci* 26: 131–137. doi:10.1016/j.tips.2005.01.004.
22. Waldhoer M, Fong J, Jones RM, Lunzer MM, Sharma SK, et al. (2005) A heterodimer-selective agonist shows in vivo relevance of G protein-coupled receptor dimers. *Proc Natl Acad Sci USA* 102: 9050–9055. doi:10.1073/pnas.050112102.
23. Smith NJ, Milligan G (2010) Allostery at G protein-coupled receptor homo- and heteromers: uncharted pharmacological landscapes. *Pharmacol Rev* 62: 701–725. doi:10.1124/pr.110.002667.
24. Kern A, Albarran-Zeckler R, Walsh HE, Smith RG (2012) Apo-Ghrelin Receptor Forms Heteromers with DRD2 in Hypothalamic Neurons and Is Essential for Anorexigenic Effects of DRD2 Agonism. *Neuron* 73: 317–332. doi:10.1016/j.neuron.2011.10.038.
25. Decaillot FM, Kazmi MA, Lin Y, Ray-Saha S, Sakmar TP, et al. (2011) CXCR7/CXCR4 heterodimer constitutively recruits β -arrestin to enhance cell migration. *J Biol Chem*. Available: <http://www.ncbi.nlm.nih.gov/pubmed/21730065>. Accessed 18 July 2011.
26. Moreno E, Vaz SH, Cai N-S, Ferrada C, Quiroz C, et al. (2011) Dopamine-Galanin Receptor Heteromers Modulate Cholinergic Neurotransmission in the Rat Ventral Hippocampus. *J Neurosci* 31: 7412–7423. doi:10.1523/JNEUROSCI.0191-11.2011.
27. He S-Q, Zhang Z-N, Guan J-S, Liu H-R, Zhao B, et al. (2011) Facilitation of μ -Opioid Receptor Activity by Preventing δ -Opioid Receptor-Mediated Codegradation. *Neuron* 69: 120–131. doi:10.1016/j.neuron.2010.12.001.
28. Pei L, Li S, Wang M, Diwan M, Anisman H, et al. (2010) Uncoupling the dopamine D1-D2 receptor complex exerts antidepressant-like effects. *Nat Med* 16: 1393–1395. doi:10.1038/nm.2263.
29. Maurice P, Kamal M, Jockers R (2011) Asymmetry of GPCR oligomers supports their functional relevance. *Trends Pharmacol Sci* 32: 514–520. doi:10.1016/j.tips.2011.05.006.
30. Ferré S, Baler R, Bouvier M, Caron MG, Devi LA, et al. (2009) Building a new conceptual framework for receptor heteromers. *Nat Chem Biol* 5: 131–134. doi:10.1038/nchembio0309-131.
31. Klitten LL, Rath MF, Coon SL, Kim J-S, Klein DC, et al. (2008) Localization and regulation of dopamine receptor D4 expression in the adult and developing rat retina. *Exp Eye Res* 87: 471–477. doi:10.1016/j.exer.2008.08.004.
32. DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK (2007) β -Arrestins and Cell Signaling. *Annu Rev Physiol* 69: 483–510. doi:10.1146/annurev.physiol.69.022405.154749.

33. Navarro G, Carriba P, Gandía J, Ciruela F, Casadó V, et al. (2008) Detection of heteromers formed by cannabinoid CB1, dopamine D2, and adenosine A2A G-protein-coupled receptors by combining bimolecular fluorescence complementation and bioluminescence energy transfer. *ScientificWorldJournal* 8: 1088–1097. doi:10.1100/tsw.2008.136.
34. Jiang H, Betancourt L, Smith RG (2006) Ghrelin Amplifies Dopamine Signaling by Cross Talk Involving Formation of Growth Hormone Secretagogue Receptor/Dopamine Receptor Subtype 1 Heterodimers. *Mol Endocrinol* 20: 1772–1785. doi:10.1210/me.2005-0084.
35. Sohy D, Parmentier M, Springael J-Y (2007) Allosteric Transinhibition by Specific Antagonists in CCR2/CXCR4 Heterodimers. *Journal of Biological Chemistry* 282: 30062–30069. doi:10.1074/jbc.M705302200.
36. Levoye A, Balabanian K, Baleux F, Bachelier F, Lagane B (2009) CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signalling. *Blood*. Available: <http://www.ncbi.nlm.nih.gov/pubmed/19380869>. Accessed 1 May 2009.
37. Moreno E, Hoffmann H, Gonzalez-Sepúlveda M, Navarro G, Casadó V, et al. (2011) Dopamine D1-histamine H3 receptor heteromers provide a selective link to MAPK signaling in GABAergic neurons of the direct striatal pathway. *J Biol Chem* 286: 5846–5854. doi:10.1074/jbc.M110.161489.
38. George SR, Fan T, Xie Z, Tse R, Tam V, et al. (2000) Oligomerization of μ - and δ -Opioid Receptors. *J Biol Chem* 275: 26128–26135. doi:10.1074/jbc.M000345200.
39. Rashid AJ, So CH, Kong MMC, Furtak T, El-Ghundi M, et al. (2007) D1–D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of Gq/11 in the striatum. *PNAS* 104: 654–659. doi:10.1073/pnas.0604049104.
40. Levoye A, Dam J, Ayoub MA, Guillaume J-L, Couturier C, et al. (2006) The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *The EMBO Journal* 25: 3012–3023. doi:10.1038/sj.emboj.7601193.
41. Trifilieff P, Rives M-L, Urizar E, Piskorowski RA, Vishwasrao HD, et al. (2011) Detection of antigen interactions ex vivo by proximity ligation assay: endogenous dopamine D2-adenosine A2A receptor complexes in the striatum. *BioTechniques* 51: 111–118. doi:10.2144/000113719.
42. Söderberg O, Leuchowius K-J, Gullberg M, Jarvius M, Weibrecht I, et al. (2008) Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay. *Methods* 45: 227–232. doi:10.1016/j.ymeth.2008.06.014.
43. Carriba P, Navarro G, Ciruela F, Ferré S, Casadó V, et al. (2008) Detection of heteromerization of more than two proteins by sequential BRET-FRET. *Nat Methods* 5: 727–733. doi:10.1038/nmeth.1229.

44. Ferrada C, Moreno E, Casadó V, Bongers G, Cortés A, et al. (2009) Marked changes in signal transduction upon heteromerization of dopamine D1 and histamine H3 receptors. *Br J Pharmacol* 157: 64–75. doi:10.1111/j.1476-5381.2009.00152.x.
45. Navarro G, Moreno E, Aymerich M, Marcellino D, McCormick PJ, et al. (2010) Direct involvement of sigma-1 receptors in the dopamine D1 receptor-mediated effects of cocaine. *Proc Natl Acad Sci USA* 107: 18676–18681. doi:10.1073/pnas.1008911107.
46. Comps-Agrar L, Kniazeff J, Norskov-Lauritsen L, Maurel D, Gassmann M, et al. (2011) The oligomeric state sets GABAB receptor signalling efficacy. *EMBO J* 30: 2336–2349. doi:10.1038/emboj.2011.143.
47. Chabre M, Deterre P, Antony B (2009) The apparent cooperativity of some GPCRs does not necessarily imply dimerization. *Trends in Pharmacological Sciences* 30: 182–187. doi:10.1016/j.tips.2009.01.003.
48. Milan-Lobo L, Whistler JL (2011) Heteromerization of the {micro}- and {delta}-Opioid Receptors Produces Ligand-Biased Antagonism and Alters {micro}-Receptor Trafficking. *J Pharmacol Exp Ther* 337: 868–875. doi:10.1124/jpet.111.179093.
49. Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, et al. (1997) The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. *Recent Prog Horm Res* 52: 307–357; discussion 357–358.
50. Miguez JM, Simonneaux V, Pevet P (1997) The role of the intracellular and extracellular serotonin in the regulation of melatonin production in rat pinealocytes. *J Pineal Res* 23: 63–71.
51. Simonneaux V, Ribelayga C (2003) Generation of the Melatonin Endocrine Message in Mammals: A Review of the Complex Regulation of Melatonin Synthesis by Norepinephrine, Peptides, and Other Pineal Transmitters. *Pharmacological Reviews* 55: 325–395. doi:10.1124/pr.55.2.2.
52. Sugden D (1990) 5-Hydroxytryptamine amplifies beta-adrenergic stimulation of N-acetyltransferase activity in rat pinealocytes. *J Neurochem* 55: 1655–1658.
53. Da Silveira Cruz-Machado S, Carvalho-Sousa CE, Tamura EK, Pinato L, Cecon E, et al. (2010) TLR4 and CD14 receptors expressed in rat pineal gland trigger NFkB pathway. *Journal of Pineal Research* 49: 183–192. doi:10.1111/j.1600-079X.2010.00785.x.

Figures

Figure 1

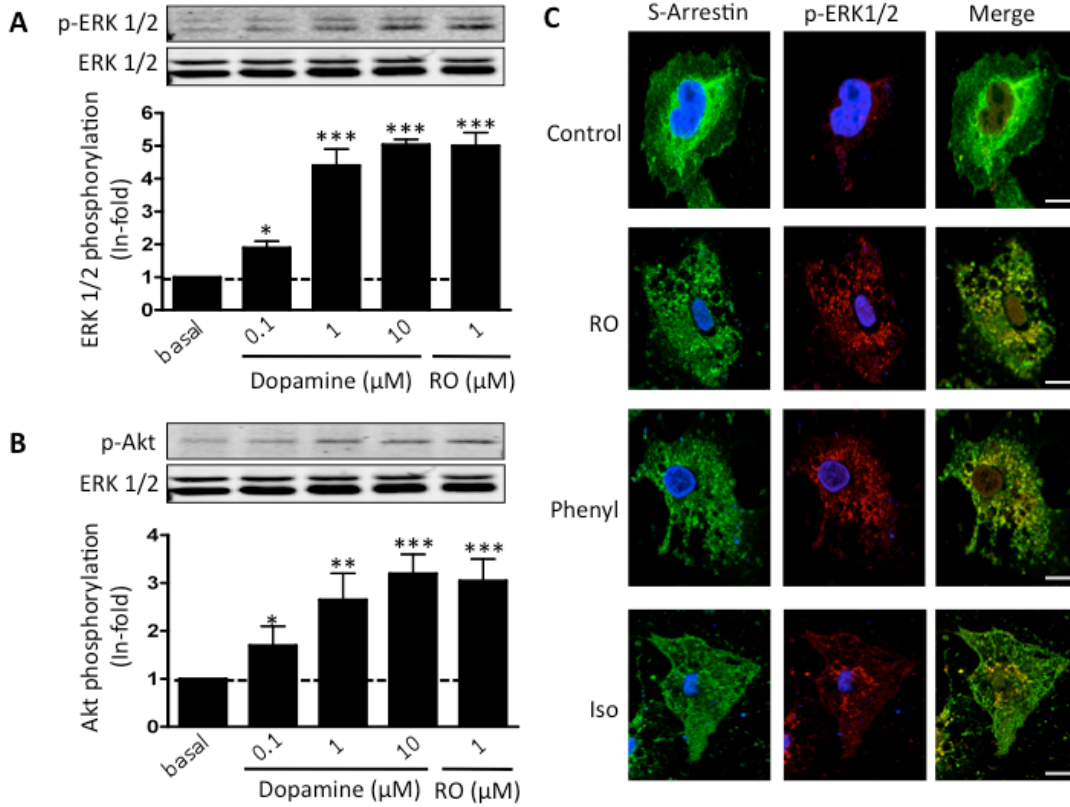


Figure 1. Functionality of dopamine D₄ receptors in pineal gland and pinealocytes. Pineal glands extracted at 9:00 h were treated for 10 min with increasing amounts of dopamine or with 1 μ M of RO 10-5824 (RO). The immunoreactive bands, corresponding to ERK 1/2 (Thr¹⁸³-Tyr¹⁸⁵) phosphorylation **(A)** and Akt (Ser⁴⁷³) phosphorylation **(B)**, of two separate experiments performed in duplicate were quantified and values represent the mean \pm S.D. of the fold increase relative to basal levels found in untreated cells. Significant differences with respect to basal levels were determined by one way ANOVA followed by a Dunnett's multiple comparison post hoc test (* $p < 0.05$, ** $p < 0.01$ and *** $p <$

0.001). A representative Western blot is shown at the top (see Experimental Procedures). **(C)** Pinealocytes were isolated from pineal glands extracted at 9:00h and were treated with medium (Control), 1 μ M of RO 10-5824 (RO), 1 μ M phenylephrine (Phenyl) or 1 μ M isoproterenol (Iso) for 10 min before labeling with anti-S-arrestin (green) and anti-phospho- ERK1/2 (red) as indicated in Experimental Procedures. Cell nuclei were stained with DAPI (blue). Scale bar: 5 μ m.

Figure 2

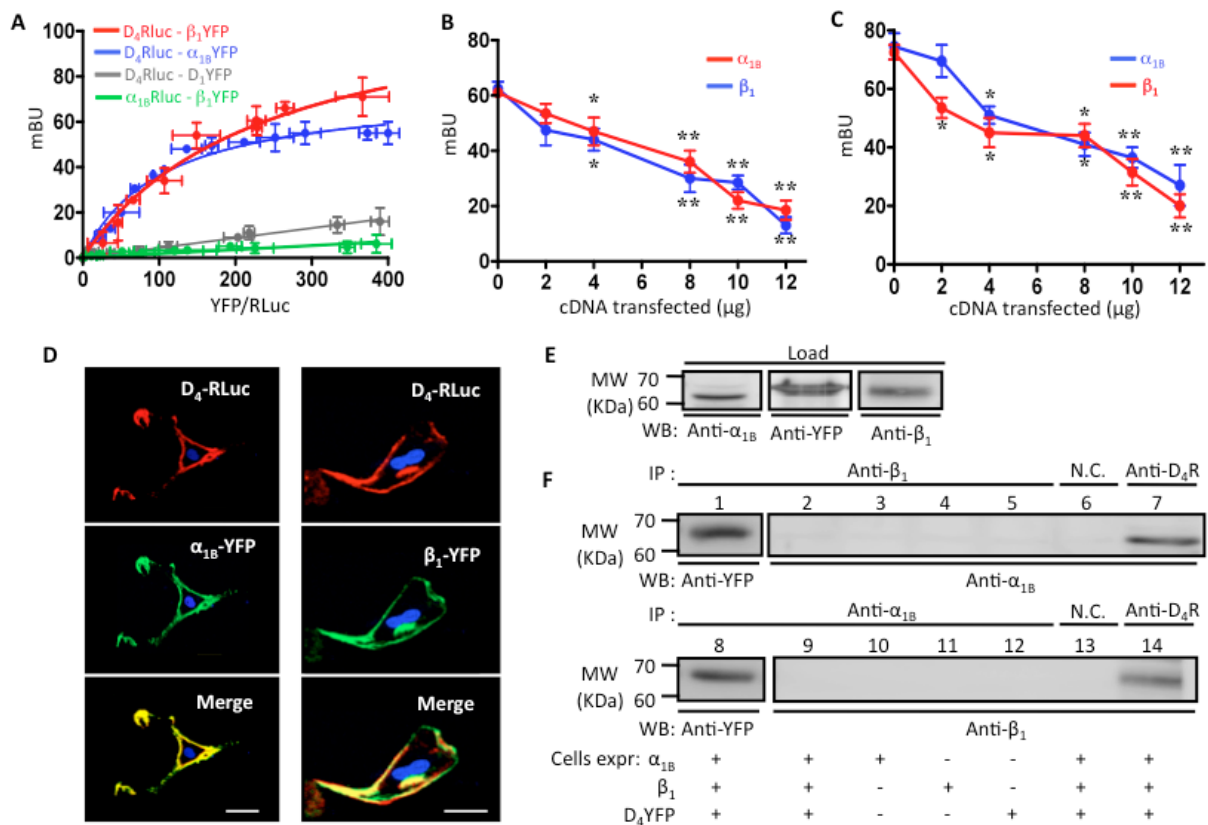


Figure 2. D_4 receptors form heteromers with α_{1B} and β_1 receptors in transfected cells. **(A)** BRET saturation curves were performed in HEK-293T cells co-expressing a constant amount D_4 -RLuc construct (2 μ g of plasmid transfected)

and increasing amounts of β_1 -YFP construct (0.4-5 μg plasmid transfected, red), α_{1B} -YFP construct (0.4-5 μg of plasmid transfected, blue) or D_1 -YFP construct (1-4 μg of plasmid transfected, gray) or with cells co-expressing a constant amount of α_{1B} -RLuc construct (3 μg of plasmid transfected) and increasing amounts of β_1 -YFP construct (0.4-5 μg of plasmid transfected, green). Both fluorescence and luminescence of each sample were measured prior to every experiment to confirm equal expression of RLuc construct ($\sim 100,000$ luminescence units) while monitoring the increase of YFP construct expression (2000 to 40,000 fluorescence units). Milli BRET Units (mBU) are BRET ratio (see Experimental Procedures) $\times 1000$ and are expressed as means \pm S.D. of five different experiments grouped as a function of the amount of BRET acceptor normalized with respect to the BRET donor (YFP/RLuc). **(B and C)** BRET was determined in HEK-293T cells expressing a constant amount of D_4 -RLuc construct (2 μg of plasmid transfected) and **(B)** α_{1B} -YFP construct (4 μg of plasmid transfected) or **(C)** β_1 -YFP construct (4 μg of plasmid transfected) and increasing amounts (2-12 μg of plasmid transfected) of **(B)** α_{1B} receptor (red) or β_1 receptor (blue) or **(C)** β_1 receptor (red) or α_{1B} receptor (blue). Both fluorescence and luminescence of each sample were measured prior to every experiment to confirm that there were no changes in the expression of D_4 -RLuc, α_{1B} -YFP or β_1 -YFP constructs. BRET data (see above) are expressed as means \pm S.D. of three different experiments. Significant differences with respect to cells not expressing α_{1B} or β_1 receptors were calculated by one way ANOVA followed by a Dunnett's multiple comparison post hoc test (* $p < 0.05$ and ** $p < 0.01$). **(D)** Confocal microscopy images of HEK-293T cells transfected with 1 μg of plasmid coding for D_4 -RLuc and 0.5 μg of plasmid coding for α_{1B} -YFP or β_1 -YFP. Proteins were identified by fluorescence or by immunocytochemistry. D_4 -RLuc receptor is

shown in red, α_{1B} -YFP and β_1 -YFP receptors are shown in green and co-localization is shown in yellow. Scale bar: 5 μ m. **(E and F)** Co-immunoprecipitation of D_4 and α_{1B} or D_4 and β_1 receptors expressed in HEK-293T cells. Membranes from cells transfected with the indicated receptors were solubilized and processed for immunoprecipitation as described under Experimental Procedures using goat anti- D_4 R, rabbit anti- α_1 or goat anti- β_1 receptor antibodies or, as negative controls (NC), goat anti-adenosine A_{2B} receptor antibody (top in F) or rabbit anti-adenosine A_1 receptor antibody (bottom in F). Solubilized membranes **(E)** and immunoprecipitates **(F)** were analyzed by SDS-PAGE and immunoblotted using rabbit anti-YFP, rabbit anti- α_1 or goat anti- β_1 antibody. IP: immunoprecipitation; WB: western blotting (numbers are included to delineate the different lanes on the SDS-PAGE); MW, molecular mass.

Figure 3

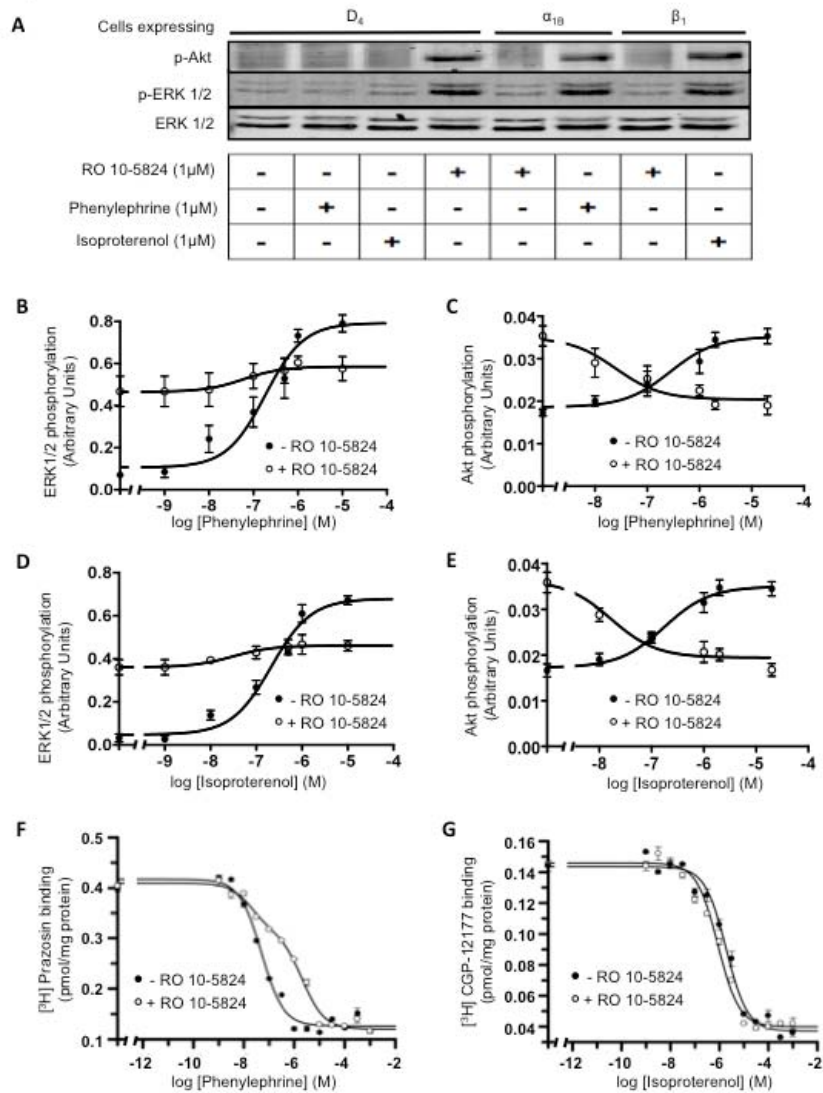


Figure 3. Functional characteristics of α_{1B} -D₄ and β_1 -D₄ receptor heteromers in transfected cells. CHO cells were transfected with 2 μ g of plasmid coding for D₄ receptors or with 3 μ g of plasmid coding for α_{1B} receptors or β_1 receptors alone (**A**) or in combination (**B to G**). In (**A**), the selectivity of ligands was tested by measuring ERK 1/2 (Thr¹⁸³-Tyr¹⁸⁵) and Akt (Ser⁴⁷³) phosphorylation in cells expressing D₄, α_{1B} or β_1 receptors, treated for 7 min with 1 μ M RO 10-5824, phenylephrine or isoproterenol. In (**B to E**), cells expressing D₄ and α_{1B} receptors

(B and C) or D_4 and β_1 receptors **(D and E)** were treated for 7 min with increasing concentrations of phenylephrine **(B and C)** or isoproterenol **(D and E)** in the presence (\circ) or in the absence (\bullet) of 500 nM RO 10-5824. The immunoreactive bands, corresponding to ERK 1/2 **(B and D)** and Akt **(C and E)** phosphorylation of four experiments were quantified and expressed as mean \pm S.E.M. of arbitrary units. In **(F and G)** membranes of cells expressing D_4 and α_{1B} receptors **(F)** or D_4 and β_1 receptors **(G)** were used to perform competition binding experiments of α_1 receptor antagonist [3 H]prazosin (1 nM) versus increasing concentrations of phenylephrine (1 nM to 1 mM) **(F)** or β_1 receptor antagonist [3 H]CGP-12177 (1 nM) versus increasing concentrations of isoproterenol (1 nM to 1 mM) **(F)** in the presence (\circ) or in the absence (\bullet) of 500 nM RO 10-5824.

Figure 4

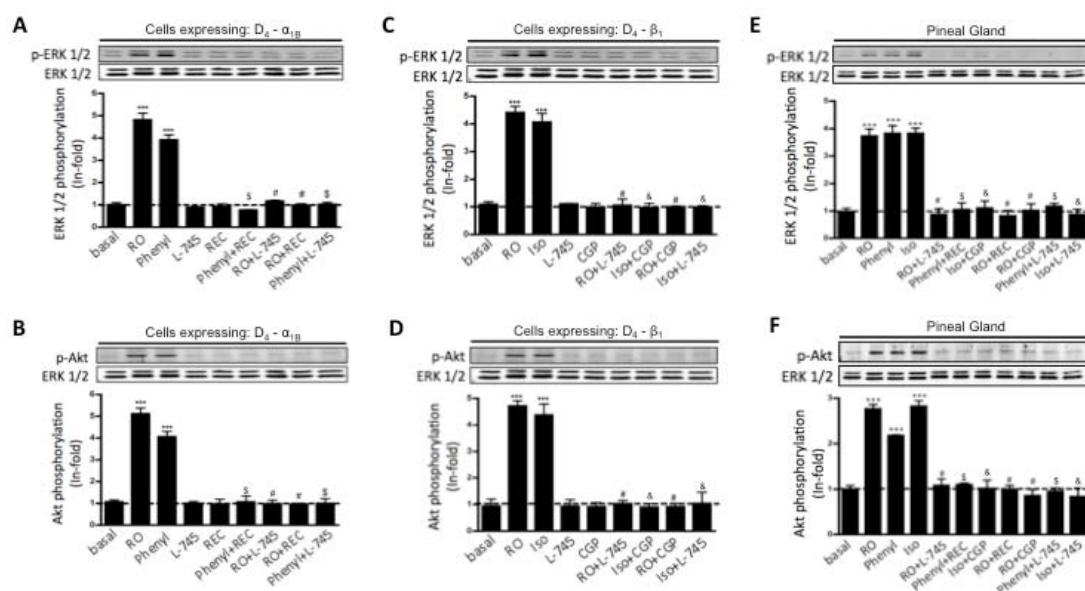


Figure 4. Cross-antagonism between D_4 and α_{1B} or β_1 receptors in transfected cells and in pineal gland. In **(A to D)** CHO cells were transiently co-

transfected with 2 μ g of plasmid coding for D₄ receptors and with 3 μ g of plasmid coding for α_{1B} receptors **(A and B)** or β_1 receptors **(C and D)**. In **(E and F)** rat pineal glands were extracted at 9:00 h and processed as indicated in Experimental Procedures. Cells were treated for 7 min and pineal glands were treated for 10 min with 500 nM of RO 10-5824 (RO), phenylephrine (Phenyl) or isoproterenol (Iso) or with 1 μ M of L-745,870 (L-745), REC 15/2615 (REC) or CGP 20712 (CGP), alone or in combination. The immunoreactive bands, corresponding to ERK 1/2 (Thr¹⁸³-Tyr¹⁸⁵) phosphorylation **(A, C and E)** and Akt (Ser⁴⁷³) phosphorylation **(B, D and F)** of four experiments were quantified and values represent the mean \pm S.E.M. of the fold increase with respect to basal levels found in untreated cells. Significant differences were calculated by an one way ANOVA followed by post-hoc Bonferroni's tests (**p < 0.001, as compared to the basal level. #p < 0.001, as compared to the sample treated with RO 10-5824. \$p < 0.001, as compared to the sample treated with phenylephrine. &p < 0.001, as compared to the sample treated with isoproterenol). A representative Western blot is shown at the top of each panel.

Figure 5

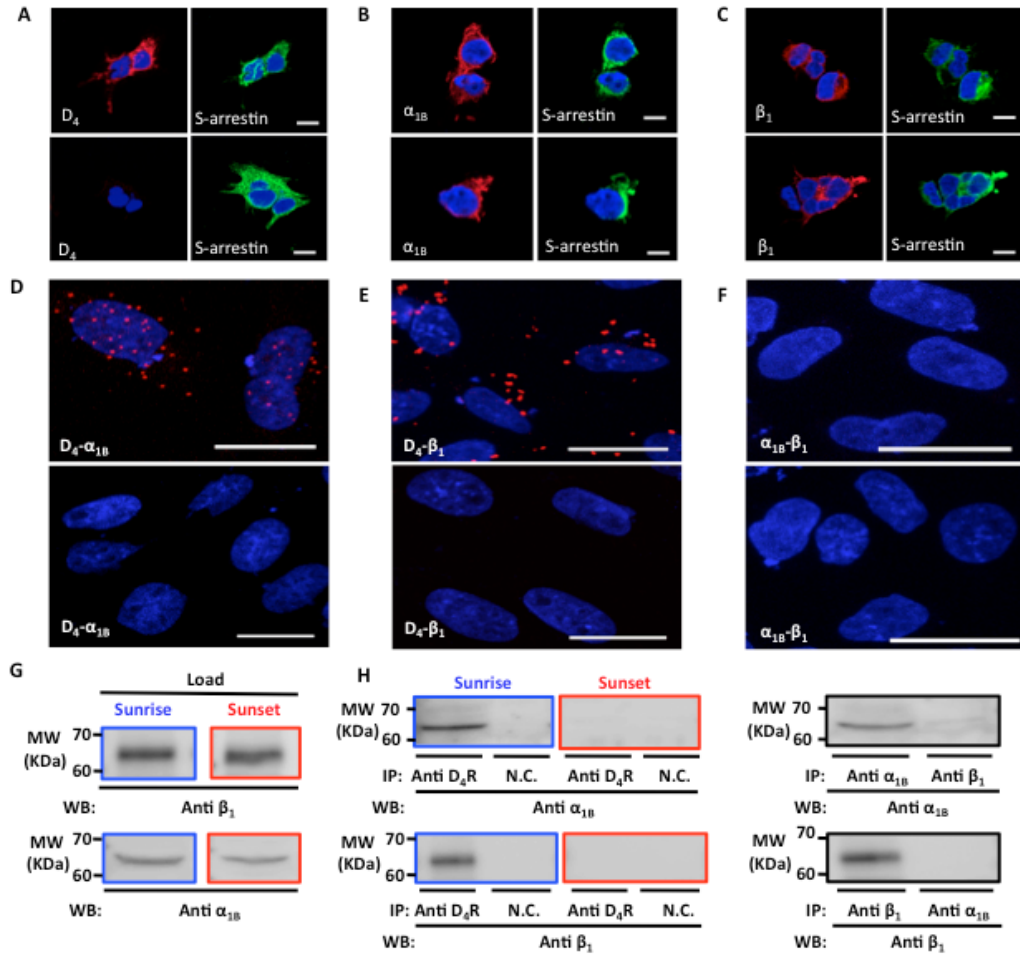


Figure 5. D_4 receptors form heteromers with α_{1B} and β_1 receptors in the pineal gland. In **(A to C)**, pinealocytes were isolated from pineal glands extracted at 9:00 h (top) or at 20:00 h (bottom) and stained using anti-S-arrestin antibody (green) and anti- D_4 **(A)**, anti- α_{1B} **(B)** or anti- β_1 **(C)** antibodies (red) as indicated in Experimental Procedures. Scale bar: 5 μ m. In **(D to F)**, pinealocytes were isolated from pineal glands extracted at 9:00 h (top) or at 20:00 h (bottom) and the expression of α_{1B} - D_4 **(D)** and β_1 - D_4 **(E)** receptor heteromers was visualized as punctate red fluorescent spots detected by confocal microscopy using the proximity ligation assay (see Experimental procedures). Any expression of α_{1B} - β_1

receptor heteromers was seen **(F)**. Scale bar: 20 μm . In **(G and H)**, co-immunoprecipitation of D_4 and α_{1B} or D_4 and β_1 receptors from pineal gland extracted at 9:00 h (sunrise) or at 20:00 h (sunset) was performed. Glands were solubilized and processed for immunoprecipitation as described under Experimental Procedures using goat anti- D_4 , rabbit anti- α_1 or goat anti- β_1 receptor antibodies or goat anti-adenosine A_{2B} receptor antibody as a negative control (N.C.). Solubilized gland membranes **(G)** and immunoprecipitates **(H)** were analyzed by SDS-PAGE and immunoblotted using rabbit anti- α_1 , rabbit anti- β_1 receptor antibodies or goat anti- β_1 receptor antibody. Immunoprecipitation experiments with anti- α_1 or anti- β_1 receptor antibodies (right image in H) were performed with pineal glands extracted at 9:00 h. IP: immunoprecipitation; WB: western blotting; MW, molecular mass.

Figure 6

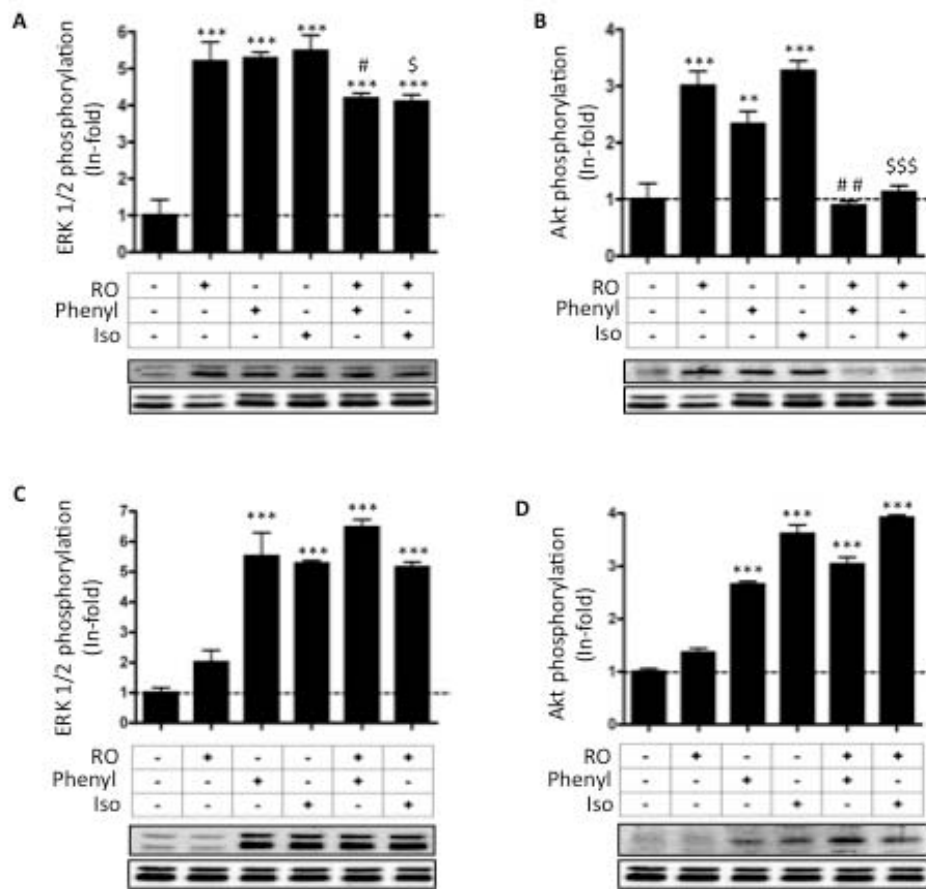


Figure 6. Functional characteristics of α_{1B} -D₄ and β_{1} -D₄ receptor heteromers in pineal gland. Pineal glands extracted at 9:00 h (**A and B**) or at 20:00 h (**C and D**) were treated for 10 min with RO 10-5824 (RO), phenylephrine (Phenyl) or isoproterenol (Iso) at 1 μ M concentration alone or in combination. The immunoreactive bands, corresponding to ERK 1/2 (Thr¹⁸³-Tyr¹⁸⁵) (**A and C**) or Akt (Ser⁴⁷³) (**B and D**) phosphorylation, of three experiments performed in duplicates were quantified and values represent the mean \pm S.E.M. of the fold increase with respect to basal levels found in untreated pineal glands. Significant differences were calculated by an one way ANOVA followed by post-hoc Bonferroni's tests (**p

< 0.01 and ***p < 0.001, as compared to the basal level. #p < 0.05 and ##p < 0.01, as compared to the sample treated with phenylephrine. \$p < 0.05 and \$\$\$p < 0.001, as compared to the sample treated with isoproterenol). A representative Western blot is shown at the bottom of each panel.

Figure 7

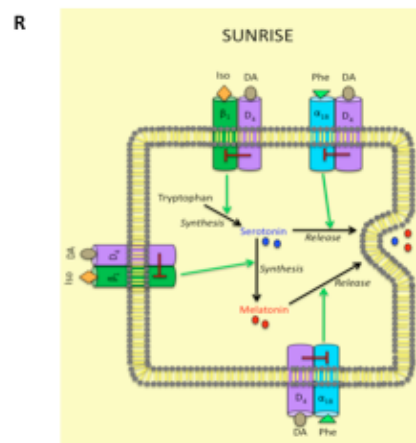
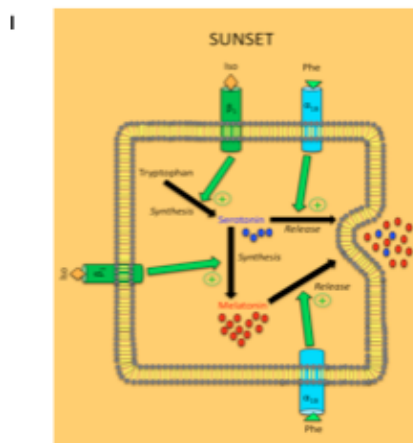
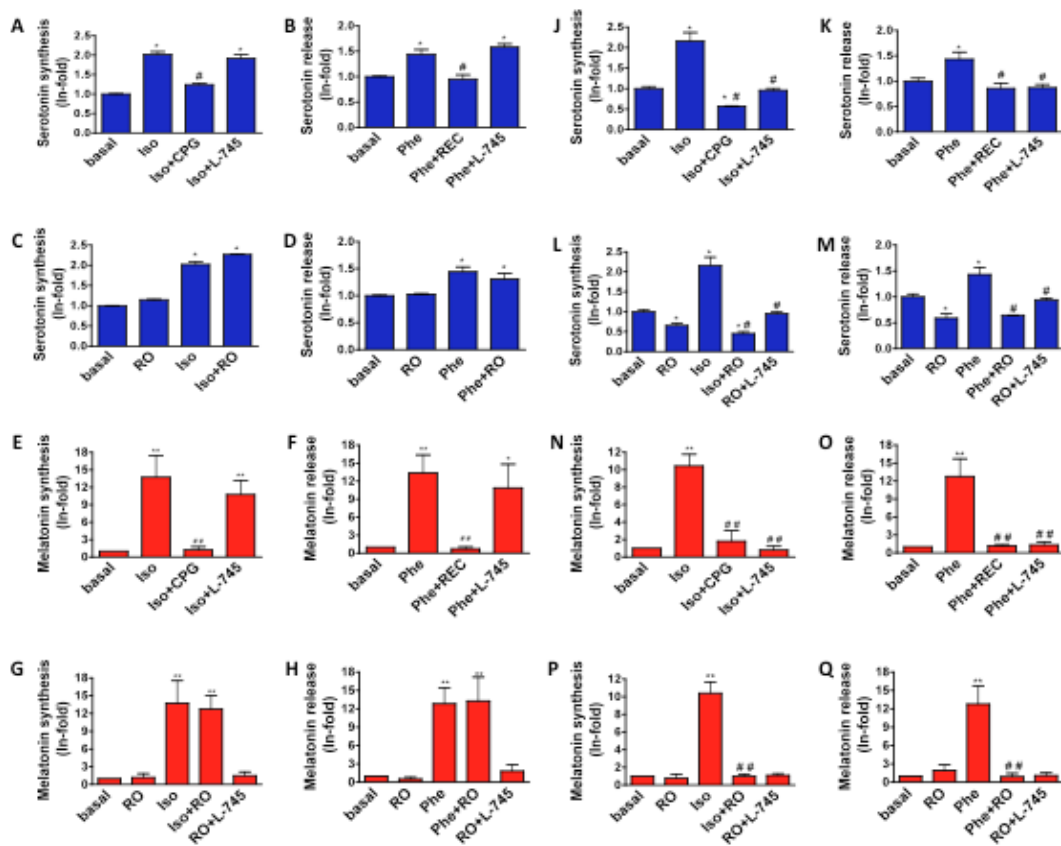


Figure 7. Metabolic consequences of α_{1B} -D₄ and β_1 -D₄ receptor heteromers activation. 5-HT synthesis (**A, C, J, L**) and release (**B, D, K, M**) and melatonin synthesis (**E,G, N, P**) and release (**F, H,O, Q**) were measured as described in Experimental Procedures in pineal gland extracted at 20:00 h (**A to H**) or at 9:00 h (**J to Q**). Pineal glands were not treated (basal) or treated with 500 nM RO 10-5824 (RO), 500 nM phenylephrine (Phe), 500 nM isoproterenol (Iso), 1 μ M L-745,870 (L-745), 1 μ M REC 15/2615 (REC) or 1 μ M CGP 20712 (CGP), alone or in combination. Three experiments were quantified and values represent the mean \pm S.E.M. of the fold increase with respect to basal levels found in untreated pineal glands. Significant differences were calculated by an one way ANOVA followed by post-hoc Bonferroni's tests (*p < 0.01 as compared to the basal level. #p < 0.005 as compared to the sample treated with isoproterenol or with phenylephrine). In (**I and R**) the overall results are presented as a scheme.

SUPPLEMENTARY INFORMATION

Supplementary Experimental Procedures

Fusion proteins and expression vectors. The cDNA for the human dopamine D₄ receptor expressed in the *pcDNA3.1* vector (Invitrogen, Paisley, Scotland, UK) was amplified without its stop codon using sense and antisense primers harboring unique XhoI and EcoRI sites to be cloned it in the mammalian humanized pRluc-N1 vectors (Perkin-Elmer, Waltham, MA, USA). The cDNA for the human adrenergic α_{1B} receptor gene, cloned in pOmicsLink ORF Expression Clone (GeneCopoeia, Maryland, USA) was amplified without its stop codon using sense and antisense primers harbouring unique KpnI and ApaI restriction sites, to be subcloned into KpnI/ApaI sites of the *pcDNA3.1* vector, the pRluc-N1 vector or the EYFP-N3 vector (enhanced yellow variant of YFP; Clontech, Heidelberg, Germany). Finally, the cDNA for the human adrenergic β_1 receptor gene, (kindly provided by Dr. S. Dorsch, University of Wuerzburg, Germany) and D₁ receptor cloned in *pcDNA3.1* vector were amplified without their stop codon using sense and antisense primers harboring unique BamHI and HindIII sites or EcoRI and KpnI, respectively to be cloned in the EYFP-N3 vector. The resulting plasmids express the receptors fused to either Rluc or YFP on the C-terminal end of the receptor (D₄-RLuc, α_{1B} -RLuc, α_{1B} -YFP, β_1 -YFP, and D₁-YFP, respectively). All constructs were verified by nucleotide sequencing and the fusion proteins were functional and expressed at the membrane level (see Results).

Cell culture and transient transfection. CHO cell were maintained in α -MEM medium without nucleosides (Invitrogen), containing 10% fetal calf serum, 50

$\mu\text{g/ml}$ penicillin, 50 $\mu\text{g/ml}$ streptomycin and 2 mM L-glutamine. Human embryonic kidney (HEK)-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% (v/v) heat inactivated Foetal Bovine Serum (FBS) (all from Invitrogen). Cells were maintained at 37°C in an atmosphere of 5% CO₂, and were passaged every 3 or 4 days when they were 80-90% confluent. HEK-293T or CHO cells growing in 6-well dishes or in 25 cm² flasks were transiently transfected with the corresponding fusion protein cDNA by the polyethylenimine (PEI) (PolyEthylenImine, Sigma, Steinheim, Germany) method. Cells were incubated (4 h) with the corresponding cDNA together with ramified PEI (5 ml of 10 mM PEI for each mg cDNA) and 150 mM NaCl in a serum-starved medium. After 4 h, the medium was changed to a fresh complete culture medium. Forty-eight hours after transfection, cells were washed twice in quick succession in Hanks' balanced salt solution HBSS (137 mM NaCl, 5 mM KCl, 0.34 mM Na₂HPO₄·12H₂O, 0.44 mM KH₂PO₄, 1.26 mM CaCl₂·2H₂O, 0.4 mM MgSO₄·7H₂O, 0.5 mM MgCl₂, 10 mM HEPES, pH 7.4) supplemented with 0.1% glucose (w/v), detached, and resuspended in the same buffer. To control the cell number, sample protein concentration was determined using the Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin dilutions as standards.

Immunostaining. For immunocytochemistry, HEK-293T cells were grown on glass coverslips and transiently transfected as indicated in the Figure legends. After 48h of transfection cells were fixed in 4% paraformaldehyde for 15 min and washed with phosphate-buffered saline (PBS) containing 20 mM glycine to quench the aldehyde groups. After permeabilization with PBS containing 0.05% Triton X-100 for 15 min, cells were incubated 1 h at room temperature with PBS containing

1% bovine serum albumin and were labeled with the primary goat polyclonal anti-D₄ receptor antibody (1/500, Santa Cruz Biotechnology), rabbit anti- α_1 receptor antibody (1:100, Abcam, Cambridge, UK) or rabbit anti- β_1 receptor antibody (1:100, Santa Cruz Biotechnology) for 1 h, washed and stained with the secondary antibody Cy3 labeled anti-goat (1/200, Jackson ImmunoResearch, Baltimore, PA) or Cy3 labeled anti-rabbit (1/200, Jackson ImmunoResearch, Baltimore, PA). The D₄-YFP, α_{1B} -YFP and β_1 -YFP constructs were detected by monitoring fluorescence emission at 530 nm. Samples were rinsed and observed using an Olympus FV1000 confocal microscope.

BRET assay. HEK-293T cells were co-transfected with a constant amount of cDNA encoding for the receptor fused to Rluc and with increasing amounts of cDNA encoding the receptor fused to YFP and used after 48 h of transfection. With aliquots of transfected cells (20 μ g of total protein), three different determinations were performed in parallel: i) To quantify fluorescence protein expression, cells were distributed in 96-well microplates (black plates with a transparent bottom, Corning, King's Lynn, UK,) and fluorescence was read in a Fluostar Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter at 400 nm reading. Receptor-fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing receptor-Rluc alone. ii) For BRET measurements, the equivalent of 20 μ g of cell suspension were distributed in 96-well microplates (Corning 3600, white plates with white bottom, Sigma) and 5 μ M coelenterazine H (Molecular Probes, Eugene, OR) was added. After 1 minute of

adding coelenterazine H, the readings were collected using a Mithras LB 940 that allows the integration of the signals detected by a filter at 485 nm and a filter at 530 nm. iii) To quantify receptor-Rluc expression luminescence readings were also performed after 10 minutes of adding 5 μ M coelenterazine H. Both fluorescence and luminescence for each sample were measured before every experiment to confirm similar donor expressions (approximately 100,000 bioluminescence units) while monitoring the increase in acceptor expression (2000 to 40,000 fluorescence units). The relative amounts of BRET acceptor are expressed as the ratio between the net fluorescence of the acceptor and the luciferase activity of the donor being the net fluorescence the fluorescence of the acceptor minus the fluorescence detected in cells only expressing the donor. The BRET ratio is defined as $[(\text{emission at } 510\text{-}590)/(\text{emission at } 440\text{-}500)] - C_f$, where C_f corresponds to $(\text{emission at } 510\text{-}590)/(\text{emission at } 440\text{-}500)$ for the D₄-RLuc construct expressed alone in the same experimental conditions. BRET was expressed as mili BRET Units (mBU) and is the BRET ratio x 1000. Curves were fitted by using a non-linear regression equation, assuming a single phase with GraphPad Prism software (San Diego, CA, USA).

Coimmunoprecipitation. Transfected cells or pineal glands were disrupted with a Polytron homogenizer in 50 mM Tris-HCl buffer, pH 7.4, containing a protease inhibitor mixture (1/ 1000, Sigma). The cellular debris was removed by centrifugation at 13,000 g for 5 min at 4 °C, and membranes were obtained by centrifugation at 105,000 g for 1 h at 4 °C. Membranes were washed two more times at the same conditions and were solubilized by homogenization in ice-cold immunoprecipitation buffer (phosphate-buffered saline, pH 7.4, containing 1% (v/v) Nonidet P-40) and incubated for 30 min on ice before centrifugation at

105,000 g for 1 h at 4 °C. The supernatant (1 mg/ml of protein) was processed for immunoprecipitation as described in the immunoprecipitation protocol using a Dynabeads Protein G kit (Invitrogen) using goat anti-D₄ receptor antibody (1:1000, Santa Cruz Biotechnology), rabbit anti- α_1 receptor antibody (1:1000, Abcam), rabbit anti- β_1 receptor antibody (1:1000, Santa Cruz Biotechnology) or goat anti- β_1 receptor antibody (1:1000, Abcam). As negative control goat anti-adenosine A_{2B} receptor antibody (1:1000, Santa Cruz Biotechnology) or rabbit anti-adenosine A₁ receptor antibody (1:1000, Affinity BioReagents, Rockford USA) was used. Protein was quantified by the bicinchoninic acid method (Pierce) using bovine serum albumin dilutions as standard. Immunoprecipitates were separated on a denaturing 10% SDS-polyacrylamide gel and transferred onto PVDF membranes. Membranes were blocked for 90 min in 5% milk and PBS-Tween 20 (0.05% V/V). The following primary antibodies were incubated overnight at 4°C in 5% milk and PBS-Tween 20 (0.05% V/V): rabbit anti-YFP antibody (1:800, Santa Cruz Biotechnology), rabbit anti- α_1 receptor antibody (1:800, Abcam), rabbit anti- β_1 receptor antibody (1:500, Santa Cruz Biotechnology) or goat anti- β_1 receptor antibody (1:800, Abcam). The following day the membranes were washed 3 times for 10 minutes in PBS Tween-20 (0.05% V/V) and the secondary antibodies: mouse anti-rabbit-peroxidase (1:20,000, Sigma) or donkey anti-goat-peroxidase (1:20,000, Jackson ImmunoResearch Laboratories, West Grove, PA) incubated for 1 hr at room temperature in 5% milk and PBS-Tween 20 (0.05% V/V). Following an additional three washes for 10 min each with PBS Tween-20 (0.05% V/V) and a final wash with PBS, bands were detected with the addition of SuperSignal West Pico Chemiluminescent Substrate (Pierce) and visualized with a LAS-3000

(Fujifilm). Analysis of detected bands was performed by Image Gauge software (version 4.0) and Multi Gauge software (version 3.0).

MAPK and Akt/PKB determination. Transfected CHO cells or pineal glands were cultured in serum-free medium for 16h before the addition of the indicated concentration of ligands for the indicated time. Both, cells and pineal glands were rinsed with ice-cold PBS and lysed by the addition of 300 μ l of ice-cold lysis buffer (50mM Tris-HCl pH 7.4, 50mM NaF, 150 mM NaCl, 45 mM β -glycerophosphate, 1% Triton X-100, 20 mM phenylarsine oxide, 0.4 mM NaVO₄ and protease inhibitor cocktail) and by shaking (cells) or sonicating (pineal glands, Branson Digital Sonifier S-250 from Branson Ultrasonic Corporation, Dambury, USA with an amplitude of 10% for 10 seconds). Cellular debris was removed by centrifugation at 13,000 g for 5 min at 4°C and protein was quantified by the bicinchoninic acid method using bovine serum albumin as a standard. To determine the level of MAPK (ERK1/2) and Akt/PKB phosphorylation, equivalent amounts of protein (10 μ g) were separated by electrophoresis on a denaturing 10% SDS-polyacrylamide gel and transferred onto PVDF-FL membranes. Odyssey blocking buffer (LICOR Biosciences, Lincoln, Nebraska, USA) was then added and membranes were blocked for 90 min. Membranes were then probed for 2-3 h with a mixture of a mouse anti-phospho-Thr¹⁸³-Tyr¹⁸⁵-ERK 1/2 antibody (1:2500, Sigma, Steinheim, Germany), a rabbit anti-phospho-Ser⁴⁷³-Akt antibody (1/2500, SAB Signalway Antibody, Pearland, USA) and a rabbit anti-ERK 1/2 antibody (1:40000, Sigma, Steinheim, Germany) to control differences in loading. Bands were visualized by the addition of a mixture of IRDye 800 (anti-mouse) antibody (1:10000, Sigma) and/or IRDye 680 (anti-rabbit) antibody (1:10000, Sigma) for 1 h and scanned by the Odyssey infrared scanner. Bands densities were quantified

using the scanner software and exported to Excel (Microsoft, Redmond, WA, USA). The level of phosphorylated ERK 1/2 isoforms or phosphorylated Akt in the same membrane were normalized for differences in loading using the total ERK protein band intensities.

Radioligand Binding Experiments. Competition experiments were performed. Membrane suspensions (2 mg of protein/ml) from CHO cells expressing D₄ and α_{1B} or β_1 receptors, were incubated for 2 h at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ with the indicated concentration of the α_{1B} receptor antagonist [³H]prazosin or β_1 receptor antagonist [³H]CGP-12177 (PerkinElmer Life and Analytical Sciences) and increasing concentrations of phenylephrine or isoproterenol respectively, in the absence or in the presence of the indicated concentration of the D₄ receptor agonist RO 10-5824 (Tocris, Aronmouth, UK). Free and membrane-bound ligand were separated by rapid filtration of 500 μ l aliquots in a cell harvester (Brandel, Gaithers- burg, MD) through Whatman GF/C filters embedded in 0.3% polyethylenimine that were subsequently washed for 5 s with 5 ml of ice-cold Tris-HCl buffer. The filters were incubated with 10 ml of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA) overnight at room temperature, and radioactivity counts were determined by use of a Tri-Carb 1600 scintillation counter (PerkinElmer Life and Analytical Sciences) with an efficiency of 62%.

Competition curves were analyzed by non-linear regression, using the commercial Grafit software (Erithacus Software), by fitting the binding data to the two-state dimer receptor model [1,2]. To calculate the macroscopic equilibrium

dissociation constants the equations deduced by Casadó et al. were used [3]. Goodness of fit was tested according to reduced chi-squared value given by the non-linear regression program.

Pinealocyte culture, signaling and immunocytochemistry. Pinealocytes were prepared from rat pineal glands as previously described by Silveira Cruz-Machado et al. [4]. Briefly, pinealocytes were obtained by trypsinization (0.25%, 37°C, 15 min) followed by mechanical dispersion in the presence of fetal bovine serum. Cells were pelleted and resuspended in BGJb medium supplemented with 10% v/v fetal bovine serum (heat-inactivated), 100 U/mL penicillin/streptomycin (pH 7.4). The total number of cells and fractional survival was estimated by Trypan blue exclusion. Cells (200,000 x well) were plated on polylysine coated 6-well chamber plate and maintained at 37°C, 5% CO₂ for 48 h prior to use. For signalling experiments, pinealocytes were treated with specific agonist for 10 min, fixed in 4% paraformaldehyde for 15 min and washed with PBS containing 20 mM glycine. After permeabilization with PBS containing 0.05% Triton X-100 for 15 min, pinealocytes were treated 1 h at room temperature with PBS containing 1% bovine serum albumin and were labeled with the mouse monoclonal anti-S-arrestin 2 (1/100, Thermo Scientific, Rockford, USA) and the rabbit polyclonal anti-phospho-Thr²⁰²/Tyr²⁰⁴ ERK1/2 (1/300, Cell Signaling Technology, Danvers, MA) for 1 h, washed and stained with the secondary chicken anti-rabbit (1/200, Alexa Fluor 594, Invitrogen) and goat anti-mouse (1/200, Alexa Fluor 488, Invitrogen). For D₄, α_{1B} or β₁ receptor staining pinealocytes were labeled with the goat anti D₄ receptor antibody (1:100, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-α₁ receptor antibody (1:100, Abcam, Cambridge, UK) or rabbit anti-β₁ receptor antibody (1:100, Santa Cruz Biotechnology) and mouse

monoclonal anti-S-arrestin 2 (1/100, Thermo Scientific) and the secondary antibody Cy3 labeled anti-goat (1/200, Jackson ImmunoResearch, Baltimore, PA) or Cy3 labeled anti-rabbit (1/200, Jackson ImmunoResearch) and Cy3 labeled anti-mouse (1/200, Jackson ImmunoResearch). Coverslips were rinsed with PBS three times at room temperature and fluorescence was observed in a Leica SP2 confocal microscope equipped with an Argon UV lamp with excitation at 351nm or 364nm, an Argon laser at 488nm and a DPSS laser at 561nm. A 63X oil objective was used.

In Situ Proximity Ligation Assay (PLA). The primary cultures of pinealocytes were fixed and permeabilized as described above. The receptor-receptor molecular interaction was detected using the Duolink II in situ PLA detection Kit (OLink; Bioscience, Uppsala, Sweden). After 1 h incubation at 37°C with the blocking solution in a pre-heated humidity chamber, pinealocytes were incubated overnight with the primary antibodies: goat anti-D₄ antibody (1:100, Santa Cruz Biotechnology, Heidelberg, Germany) and rabbit anti- α_1 antibody (1:100, Abcam, Cambridge, UK) to detect α_{1B} -D₄ receptor heteromers, goat anti-D₄ antibody and rabbit anti β_1 antibody (1:100, Santa Cruz Biotechnology) to detect β_1 -D₄ receptor heteromers or rabbit anti- α_1 antibody and goat anti- β_1 antibody (1:100, Abcam) to detect α_{1B} - β_1 receptor heteromers, in the antibody diluent medium. The pinealocytes were washed with buffer A (10 mM Tris, 150mM NaCl, and 0.05% (v/v) Tween-20,) at room temperature and incubated for 2 h in a pre-heated humidity chamber at 37°C with PLA probes detecting rabbit or goat antibodies (Duolink II PLA probe anti-Rabbit plus and Duolink II PLA probe anti-Goat minus diluted in the antibody diluent to a concentration of 1:5). After washing with wash buffer A at room temperature, pinealocytes were incubated in a pre-heated

humidity chamber for 30 min at 37°C, with the ligation solution (Duolink II Ligation stock 1:5 and Duolink II Ligase 1:40). Detection of the amplified probe was done with the Duolink II Detection Reagents Red Kit. After exhaustively washing at room temperature with wash buffer B (200 mM Tris and 100 mM NaCl), the pinealocytes were mounted using the mounting medium with DAPI. The samples were observed in a Leica SP2 confocal microscope. As negative controls for the technique, the same procedure was done but omitting the primary antibodies. As negative control for heteromerization, heteromers between α_{1B} and β_1 receptors were tested.

Serotonin synthesis and release determination. After 36 h of culture in BGJb medium (Invitrogen, Carlsbad, CA), the pineal glands were incubated in HBSS medium supplemented with 0,1% glucose, 100 U/ml penicillin/streptomycin and 1mg/ml bovine serum albumin for 12 h with specific agonist and/or antagonist and radioactive [14 C]-Tryptophan (10 μ M). After incubation, medium and pineal glands were collected separately into eppendorf tubes with 35 μ l of trichloroacetic acid (TCA 1%) and were kept at 4°C. Pineal glands were homogenized in a Dynatech/Sonic Dismembrator (Dynatech Labs, Chantilly, VA) for 15 seconds. An aliquot was reserved for protein quantification by the Lowry method and cellular debris were removed by centrifugation at 10,000 g for 10 min at 4°C. [14 C]-Serotonin present in the supernatant was separated from [14 C]-Tryptophan by HPLC coupled to detection by fluorescence (excitation: 252nm; emission:382). The chromatography system consisted of a reverse-phase C18 column (2.5 μ m particle Fortis C18, 100 x 4.6, Sugelabor, Spain) and an ion-pair mobile phase, made up of 500mM

sodium acetate, 500mM citric acid, 1mM EDTA, 5 mM octanesulfonic acid plus 20% methanol (v/v) , pH 3.8. The flow rate was 1ml/min. Serotonin fractions were recovered in scintillation vials, mixed with Optiphase HiSafe III cocktail, and [¹⁴C]-serotonin was quantified in a liquid scintillation counter.

References

1. Franco R, Casadó V, Mallol J, Ferré S, Fuxe K, et al. (2005) Dimer-based model for heptaspanning membrane receptors. *Trends Biochem Sci* 30: 360–366. doi:10.1016/j.tibs.2005.05.010.
2. Franco R, Casadó V, Mallol J, Ferrada C, Ferré S, et al. (2006) The two-state dimer receptor model: a general model for receptor dimers. *Mol Pharmacol* 69: 1905–1912. doi:10.1124/mol.105.020685.
3. Casadó V, Ferrada C, Bonaventura J, Gracia E, Mallol J, et al. (2009) Useful pharmacological parameters for G-protein-coupled receptor homodimers obtained from competition experiments. *Agonist-antagonist binding modulation. Biochem Pharmacol* 78: 1456–1463. doi:10.1016/j.bcp.2009.07.012.
4. Da Silveira Cruz-Machado S, Carvalho-Sousa CE, Tamura EK, Pinato L, Cecon E, et al. (2010) TLR4 and CD14 receptors expressed in rat pineal gland trigger NFκB pathway. *Journal of Pineal Research* 49: 183–192. doi:10.1111/j.1600-079X.2010.00785.x.

Supplementary Figures

Figure S1

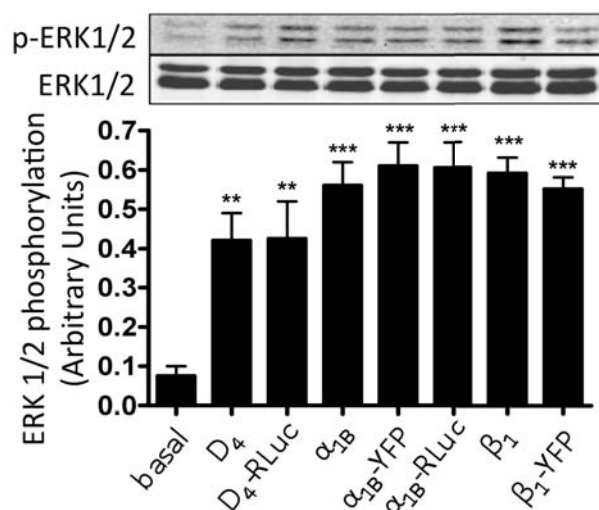


Figure S1. Functionality of the fusion proteins. HEK 293T cells were transfected with 2 μ g of plasmid coding for the D₄ receptor or with 3 μ g of plasmid coding for the adrenergic α_{1B} or β_1 receptors or to the corresponding fusion proteins D₄-RLuc, α_{1B} -YFP, α_{1B} -RLuc or β_1 -YFP. 48 h post-transfection, cells expressing D₄ or D₄-RLuc receptors were treated with 500 nM RO 10-5824, cells expressing α_{1B} , α_{1B} -YFP or α_{1B} -RLuc receptors were treated with 1 μ M phenylephrine or cells expressing β_1 or β_1 -YFP were treated with 1 μ M isoproterenol for 7 min and ERK 1/2 (Thr¹⁸³-Tyr¹⁸⁵) phosphorylation was determined. The immunoreactive bands of three experiments performed in duplicates were quantified and expressed as mean \pm S.E.M. of arbitrary units. A representative Western blot is shown at the top. Significant differences with respect to basal levels were calculated one way ANOVA followed by a Dunnett's multiple comparison post hoc test (**p < 0.01 and ***p < 0.001).

Figure S2

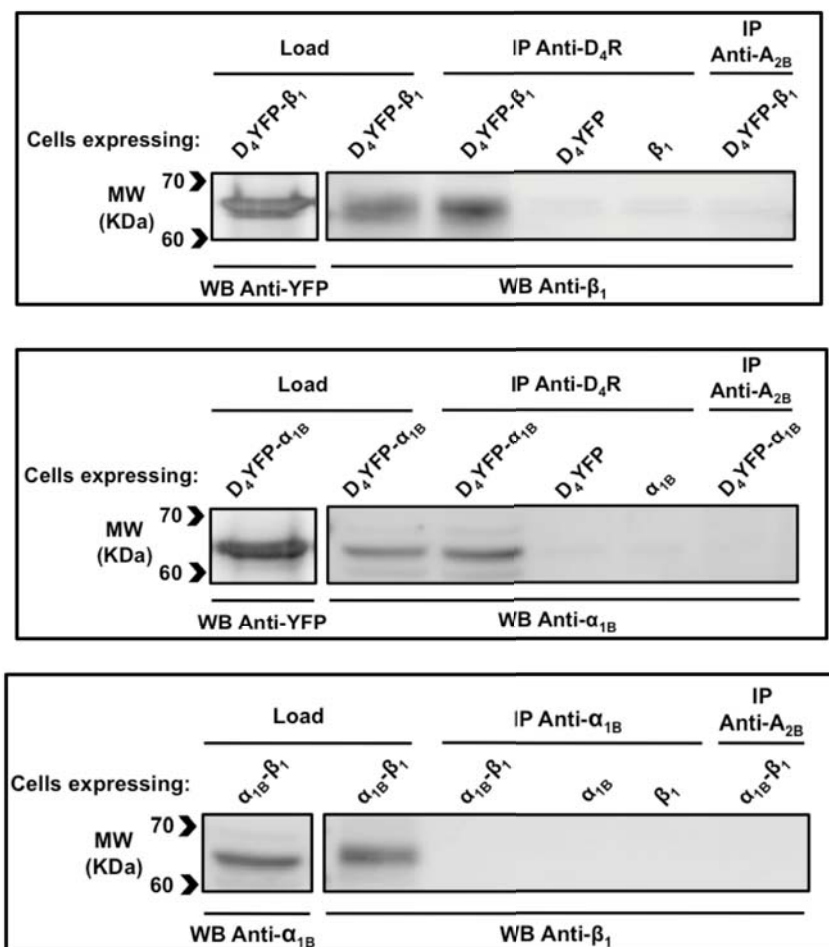


Figure S2. Specificity of the antibodies used for co-immunoprecipitation experiments. Membranes from cells expressing the indicated receptors were solubilized and processed for immunoprecipitation as described under Experimental Procedures using goat anti-D₄ or rabbit anti-α₁ receptor antibodies or goat anti-adenosine A_{2B} or rabbit anti-adenosine A₁ receptor antibodies as negative controls. Solubilized membranes (Load) and immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using rabbit anti-YFP, rabbit anti-α₁, rabbit anti-β₁ or goat anti-β₁ receptor antibodies. IP: immunoprecipitation; WB: western blotting; MW, molecular mass.

Figure S3

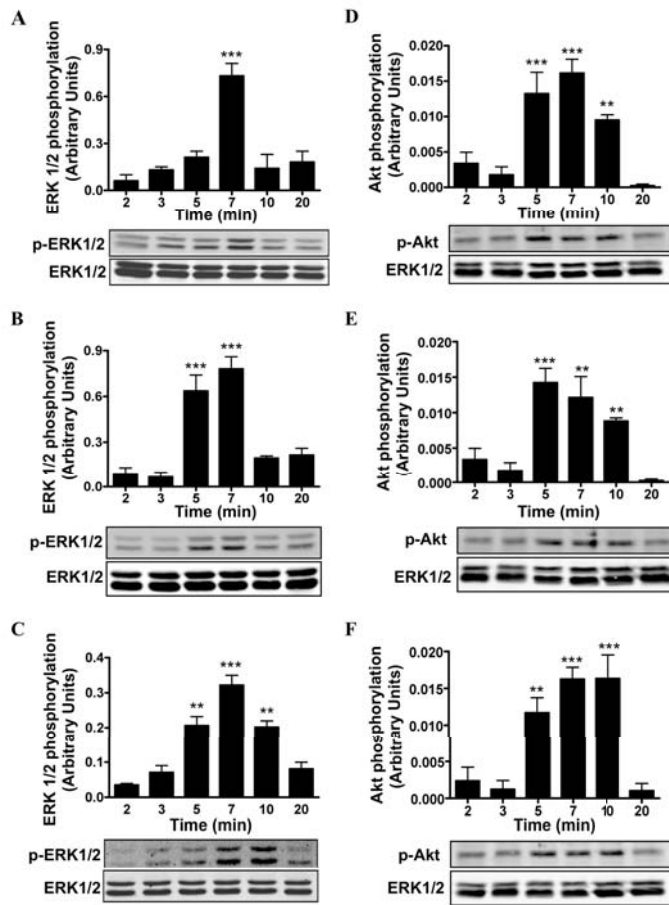


Figure S3. ERK 1/2 and Akt phosphorylation in cells transfected with D₄, α_{1B} or β_1 receptors. CHO cells were transfected with 2 μ g of plasmid coding for the D₄ receptor (A, D), 3 μ g of plasmid coding for the α_{1B} receptor (B, E) or 3 μ g of plasmid coding for the β_1 receptor (C, F). 48 h post-transfection, cells were treated for increasing time with 500 nM RO 10-5824 (A, D), 1 μ M phenylephrine (B, E) or 1 μ M isoproterenol (C, F). The immunoreactive bands, corresponding to ERK 1/2 (Thr¹⁸³-Tyr¹⁸⁵) (A to C) and Akt (Ser⁴⁷³) (D to F) phosphorylation of three experiments were quantified and expressed as mean \pm S.E.M of arbitrary units. Statistical differences over non-treated cells were determined one way ANOVA followed by a Dunnett's multiple comparison post hoc test (*p < 0.05, **p < 0.01 and ***p < 0.001).

Figure S4

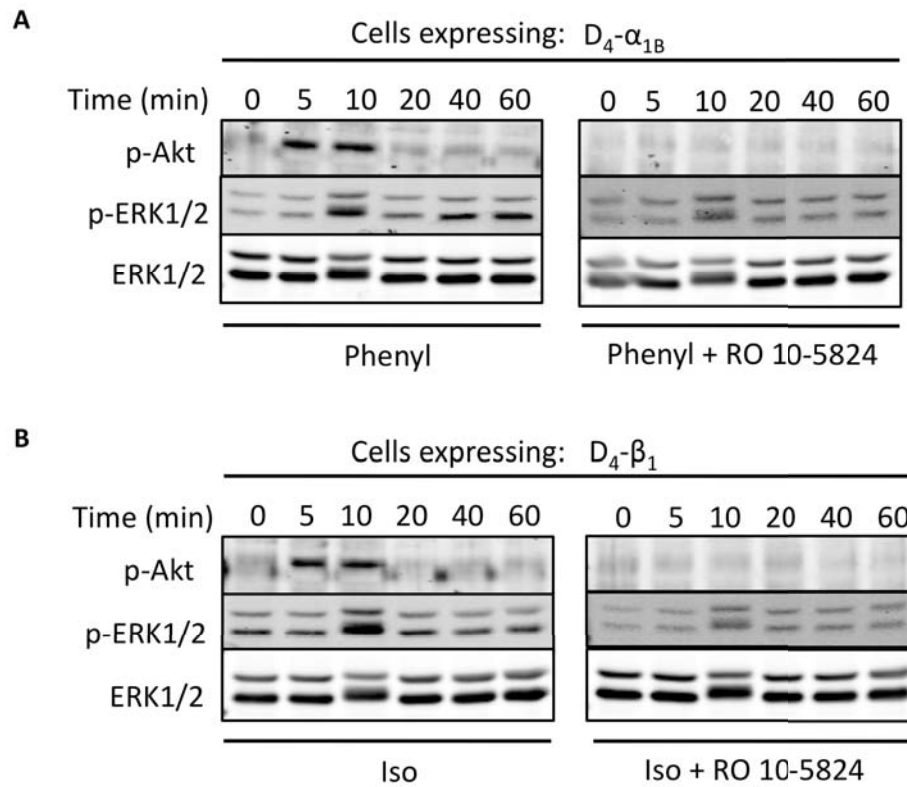


Figure S4. Time-response on ERK 1/2 and Akt phosphorylation by co-activation of $\alpha_{1B}\text{-D}_4$ and $\beta_1\text{-D}_4$ receptor heteromers in cell cultures. CHO cells were transfected with 2 μg of plasmid coding for the D_4 receptor and 3 μg of plasmid coding for the α_{1B} receptor (**A**) or the β_1 receptor (**B**). 48 h post-transfection, cells were treated with 1 μM phenylephrine (Phenyl, **A**) or 1 μM isoproterenol (Iso, **B**) alone or in the presence of 1 μM RO 10-5824 for different times. A representative Western blot is shown.

Figure S5

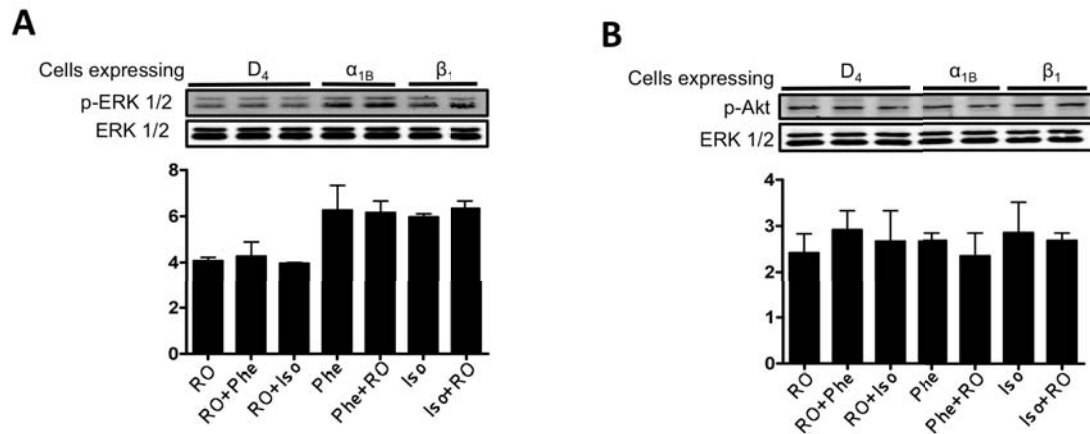


Figure S5. Selectivity of D_4 , α_{1B} or β_1 receptor agonists. The selectivity of ligands was tested by measuring ERK 1/2 (Thr¹⁸³-Tyr¹⁸⁵) **(A)** and Akt (Ser⁴⁷³) **(B)** phosphorylation in cells expressing D_4 , α_{1B} or β_1 receptors, treated for 7 min with 1 μ M RO 10-5824 (RO), phenylephrine (Phe) or isoproterenol (Iso) alone or in combination as indicated.

Figure S6

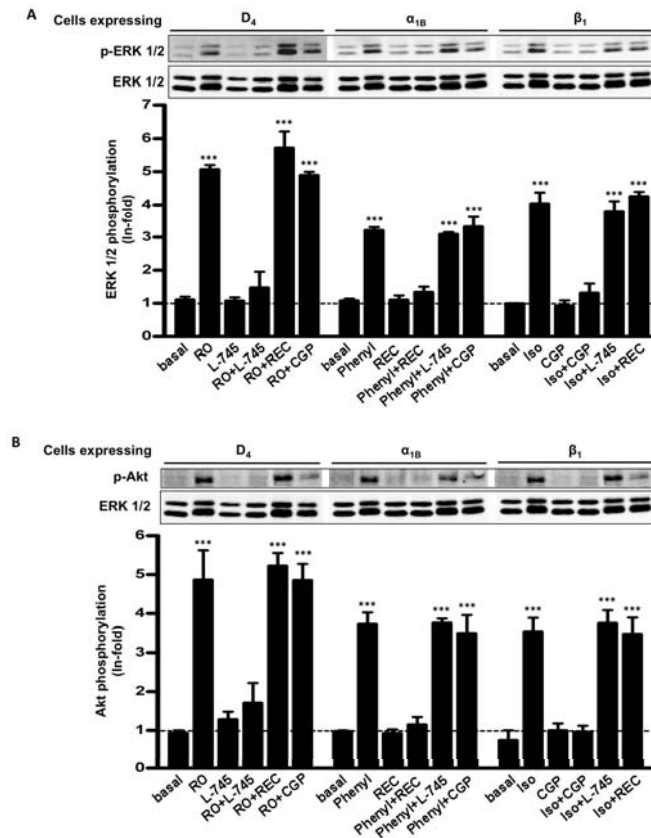


Figure S6. Selectivity of D₄, α_{1B} or β₁ receptor antagonists. CHO cells were transfected with 2 μg of plasmid coding for the D₄ receptor or with 3 μg of plasmid coding for α_{1B} or β₁ receptors. 48 h post-transfection, cells were treated for 7 min with 500 nM RO 10-5824 (RO), 500 nM phenylephrine (Phenyl), 500 nM isoproterenol (Iso), 1 μM L-745,870 (L-745), 1 μM REC 15/2615 (REC) or 1 μM CGP 20712 (CGP) alone or in combination. The immunoreactive bands, corresponding to ERK 1/2 (Thr¹⁸³-Tyr¹⁸⁵) **(A)** and Akt (Ser⁴⁷³) **(B)** phosphorylation, of three experiments were quantified and values represent the mean ± S.E.M. of the fold increase over basal levels found in untreated cells (basal). Significant differences over basal levels were determined by one way ANOVA followed by a Dunnett's multiple comparison post hoc test (**p < 0.01, ***p < 0.001). A representative Western blot is shown at the top.

Figure S7

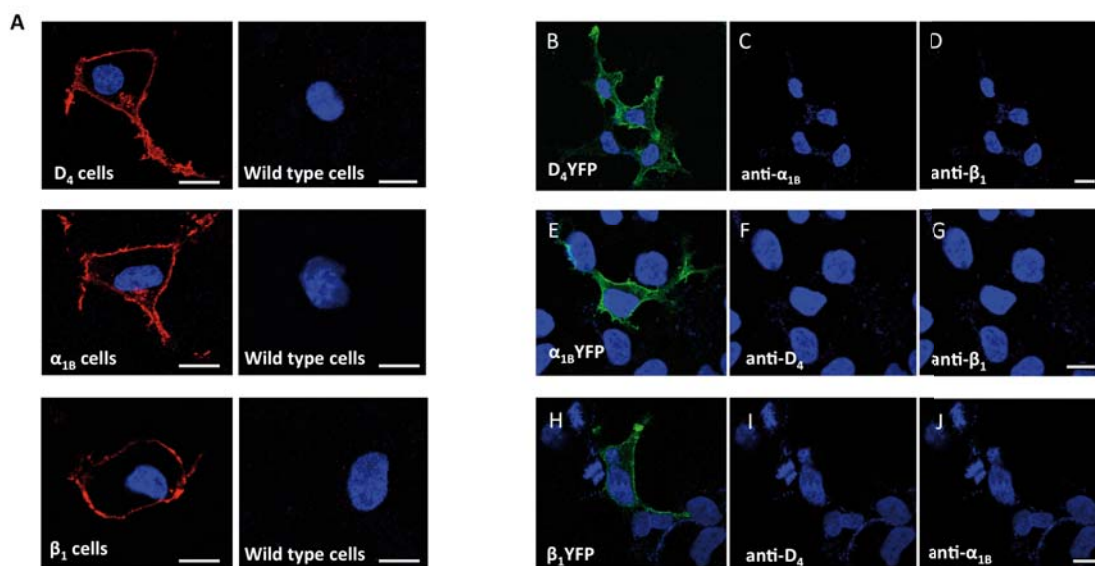


Figure S7. Specificity of the antibodies tested by immunocytochemistry. In **(A)** non transfected HEK-293T cells (right panels) and cells transfected with, top to bottom, 1 μg of plasmid coding for D₄ receptor, 0.5 μg cDNA coding for α_{1B} receptor or 0.5 μg cDNA coding for β₁ receptor (left panels) were stained using, top to bottom, anti-D₄, anti-α₁ or anti-β₁ antibodies as indicated in Experimental Procedures. Scale bar: 5 μm. In **(B to J)**, cells were transfected with 1 μg of plasmid coding for D₄-YFP receptor **(B to D)**, 0.5 μg cDNA coding for α_{1B}-YFP receptor **(E to G)** or 0.5 μg cDNA coding for β₁-YFP receptor **(H to J)**. The expression of the receptors was detected by its own YFP fluorescence **(B, E and H)** or by using anti-α₁ **(C and J)**, anti-β₁ **(D and G)** or anti-D₄ **(F and I)** receptor antibodies. Scale bar: 5 μm.

Figure S8

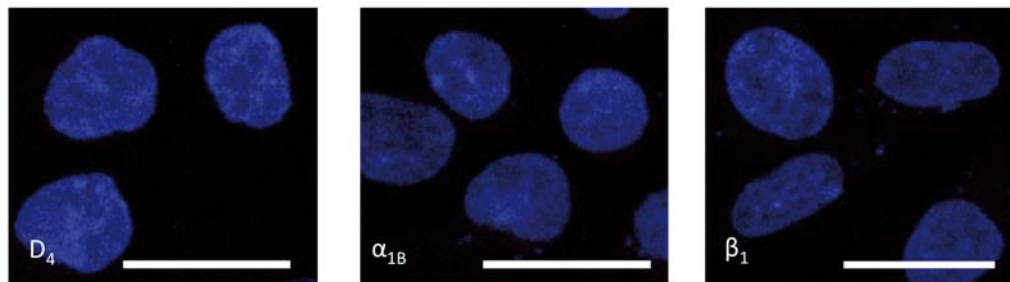


Figure S8. Negative controls for in situ proximity ligation assays. Negative controls for in situ proximity ligation assays (PLA, see Experimental Procedures) are shown demonstrating a lack of punctate red fluorescence staining in pinealocytes in the absence of primary antibodies, left to right, anti-D₄, anti-α₁ or anti-β₁ antibodies. Scale bar represents 20 μm.

Figure S9

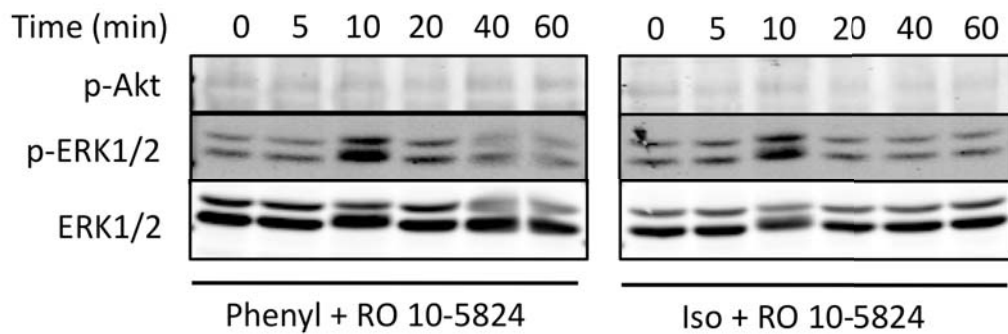
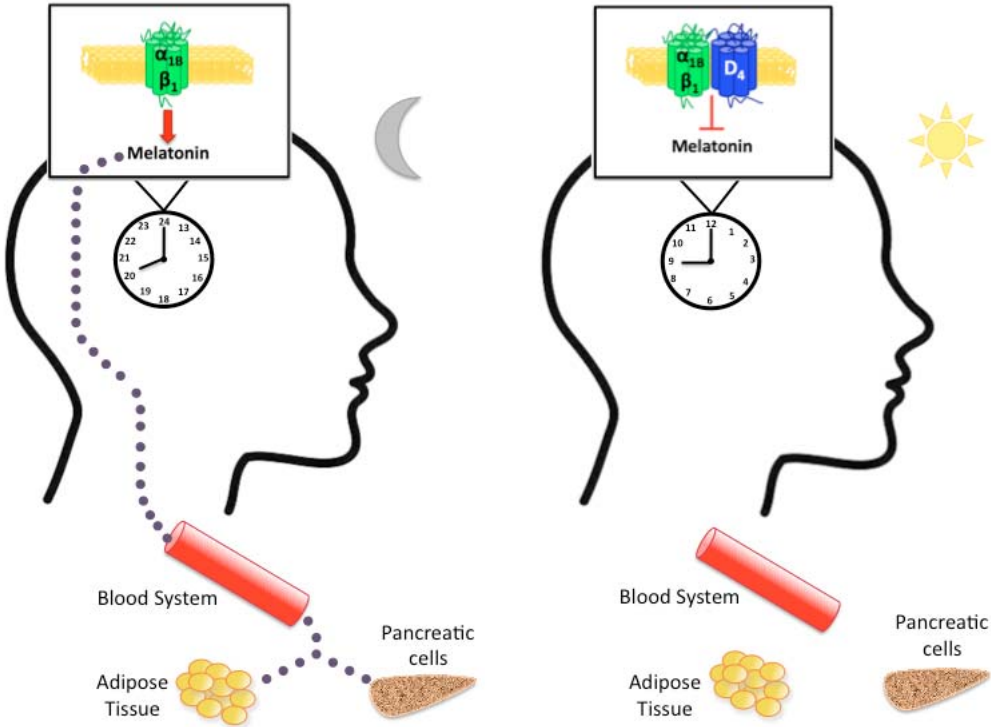


Figure S9. Time-response on ERK 1/2 and Akt phosphorylation by co-activation of α_{1B} -D₄ and β_1 -D₄ receptor heteromers in pineal gland. Pineal glands extracted at 9:00 h were treated with 1 μ M phenylephrine (Phenyl) or 1 μ M isoproterenol (Iso) in the presence of 1 μ M RO 10-5824 for the times indicated. A representative Western blot is shown.

Graphical Abstract



3.4 Biotin Ergopeptide Probes for Dopamine Receptors

Marc Vendrell, Anabel Molero, **Sergio González**, Kamil Pérez-Capote, Carme Lluís, Peter J. McCormick, Rafael Franco, Antoni Cortés, Vicent Casadó, Fernando Albericio y Miriam Royo

Manuscrito publicado en *Journal of Medical Chemistry*. 24;54(4):1080-90, Feb. 2011

La incorporación de modificaciones químicas dentro de la estructura de compuestos bioactivos es a menudo difícil debido a que las propiedades biológicas de las nuevas moléculas deben conservarse respecto a las del ligando nativo. Los ergopéptidos, con gran afinidad por los receptores D₁ y D₂ de dopamina, son ejemplos particularmente complejos. En este trabajo se estudia la derivación sistemática de dos ergopéptidos con diferentes espaciadores de base peptídica y su evaluación usando técnicas de unión de radioligando. Se seleccionaron ergopéptidos que contenían espaciadores selectivos que producían mínimas alteraciones biológicas y se derivatizaron usando un compuesto biotinilado. Se identificó el compuesto 13 como el ergopéptido que mantenía una buena afinidad y un comportamiento como agonista. Este compuesto es una herramienta útil para el estudio de heterómeros que contienen receptores D₁, D₂ o D₃ de dopamina.

Biotin Ergopeptide Probes for Dopamine Receptors

Marc Vendrell,^{†,‡} Anabel Molero,^{†,§} Sergio González,[‡] Kamil Pérez-Capote,[‡] Carme Lluís,[‡] Peter J. McCormick,[‡] Rafael Franco,[‡] Antoni Cortés,[‡] Vicent Casadó,[‡] Fernando Albericio,^{||,§} and Miriam Royo^{*,†}

[†]Combinatorial Chemistry Unit, Barcelona Science Park, University of Barcelona, 08028 Barcelona, Spain, [‡]Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), and Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, 08028 Barcelona, Spain, [§]CIBER-BBN, Networking Centre on Bioengineering, Biomaterials, and Nanomedicine, Barcelona Science Park, 08028 Barcelona, Spain, and ^{||}Institute for Research in Biomedicine, 08028 Barcelona, Spain. [‡]Current address: Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium, 138667 Singapore

Received December 8, 2010

The incorporation of chemical modifications into the structure of bioactive compounds is often difficult because the biological properties of the new molecules must be retained with respect to the native ligand. Ergopeptides, with their high affinities at D₁ and D₂ dopamine receptors, are particularly complex examples. Here, we report the systematic derivatization of two ergopeptides with different peptide-based spacers and their evaluation by radioligand binding assays. Selected spacer-containing ergopeptides with minimal biological alteration and a proper anchoring point were further derivatized with a biotin reporter. Detailed characterization studies identified **13** as a biotin ergopeptide maintaining high affinity and agonist behavior at dopamine receptors, being a useful tool for the study of heteromers involving D₁R, D₂R, or D₃R.

Introduction

Ergopeptides, with their high affinity at D₁ and D₂ dopamine receptors (D₁Rs and D₂Rs^α), are valuable molecules to study dopamine receptors.¹ The therapeutic significance of dopamine receptors containing heteromers has been extensively

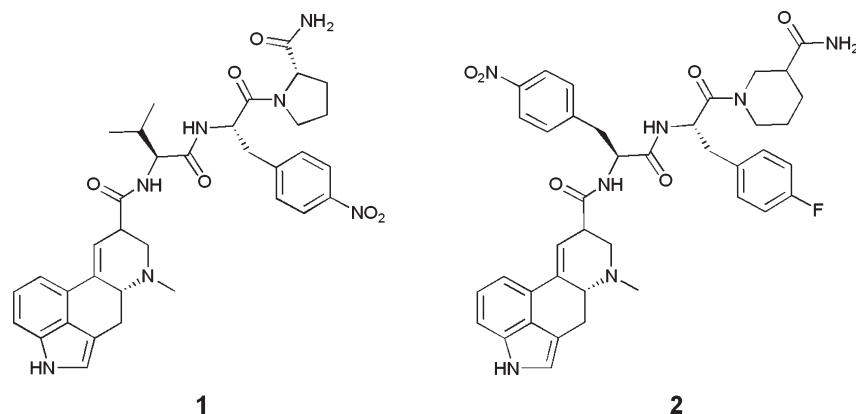
reported.^{2–4} Dual ligands have been successfully applied to the study of G-protein coupled receptors (GPCRs) oligomerization.^{5,6} While ergopeptides may provide insights into the D₁ or D₂ receptors containing heteromers, they require a proper reporter tag prior to their use in protein localization and profiling studies.^{7–10} The modification of hit compounds is a critical step as the biological properties of the labeled molecules can be significantly altered with respect to the native ligand.¹¹ This step is particularly important when applied to small molecule ligands, such as ergopeptides, because the ligand affinity and efficacy of the pharmacophore is likely to be compromised.¹² In the present work, we report a systematic study to optimize the length and chemical nature of different spacer moieties attached to two ergopeptides with high affinity at D₁R and D₂R (**1** and **2**, Chart 1) and identify those linkers that retain their binding profile. Trifunctional amino acids, such as lysine and glutamic acid, are excellent scaffolds for the synthesis of peptide-based spacers. In addition to their low toxicity, they can be easily adapted to a solid-phase synthesis approach to allow the incorporation of a range of functional groups (amines, anilides, and carboxamides) within the spacer structure. A number of peptide-based spacers were incorporated to **1** and **2** to render a 40-member library of new ergopeptides, and the evaluation of their affinities at D₁R and D₂R identified two linker moieties with minimal biological interference. The subsequent incorporation of a biotin reporter led to identification of **13** as a biotin ergopeptide for dopamine receptors with nanomolar binding affinities and agonist behavior.

Results

Design of the Library. The incorporation of a spacer moiety into a bioactive molecule requires the selection of a suitable attachment point, which must be well separated

*To whom correspondence should be addressed: Phone: 0034 934037122. Fax: 0034 934037126. E-mail: mroyo@pcb.ub.cat.

^αAbbreviations: A_{2A}R, adenosine A_{2A} receptor; A₁R, adenosine A₁ receptor; Ac₂O, acetic anhydride; ACN, acetonitrile; Alloc, allyloxy-carbonyl; All, allyl ester; Ahx, aminohexanoic acid; Akt, protein kinase B; Boc, *t*-butoxycarbonyl; CDI, 1,1'-carbonyldiimidazole; CHO, Chinese hamster ovary; DCM, dichloromethane; cDNA, cDNA; [³H]-CP55940, tritium labeled 2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl)-cyclohexyl]-5-(2-methyloctan-2-yl)phenol; DIPCPI, *N,N'*-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; D₁R, dopamine D₁ receptor; D₂R, dopamine D₂ receptor; D₃R, dopamine D₃ receptor; ERK, extracellular-signal-regulated kinases; Fmoc, fluorenylmethoxycarbonyl; GPCR, G-protein coupled receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOBt, hydroxybenzotriazole; HOAt, 1-hydroxy-7-azabenzotriazole; HRMS, high resolution mass spectrometry; MAPK, mitogen-activated protein kinases; PEG, polyethylene glycol; PEI, polyethylenimine; Rink-MBHA-PS, 4-(2',4'-dimethoxyphenyl)-Fmoc-aminomethyl)-phenoxyacetamido *p*-methylbenzhydriylamine resin; RP-HPLC, reversed phase-high performance liquid chromatography; RP-HPLC-MS, reversed phase-high performance liquid chromatography–mass spectrometry; SKF81297, (±)-6-chloro-2,3,4,5-tetrahydro-1-phenyl-1*H*-3-benzazepine hydrobromide; TFA, trifluoroacetic acid; TMUCl Cl, *N*-[chloro(dimethylamino)methylene]-*N*-methylmethanaminium chloride; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloric acid; [³H]-RAMH, tritium labeled *R*-methyl histamine; [³H]-*R*-PIA, tritium labeled *R*-phenylisopropyladenosine; [³H]-SCH23390, tritium labeled [*R*-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepine-7-ol]; [³H]-YM09151-2, tritium labeled nemonapride (*N*-(1-benzyl-2-methylpyrrolidin-3-yl)-5-chloro-2-methoxy-4-(methylamino)benzamide); [³H]-ZM241358, tritium labeled 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol. Abbreviations used for amino acids follow the IUPAC-IUB Commission of Biochemical Nomenclature in Jones, J. H. *J. Pept. Sci.* 2003, 9, 1–8.

Chart 1. Ergopeptides with High Affinity at D₁ and D₂ Dopamine Receptors

from the pharmacophore in order to prevent any interference on the binding between biomolecule and receptor. The binding mode analysis of **1** and **2** revealed the interaction of the ergolene scaffold at the transmembrane binding sites while the peptide moieties interacted with adjacent amino acids.¹ Bearing this in mind, the attachment of distinct spacer moieties into **1** and **2** was designed by means of the C-terminal carboxamide group modification, which would presumably produce a minor change to the biological properties of the final compounds.

Several spacer moieties were designed on the basis of two trifunctional amino acids, lysine and glutamic acid, which are key scaffolds for the derivatization of biomolecules when a suitable protecting group scheme is used.¹³ The introduction of a number of building blocks onto the lysine and glutamic acid side chains afforded 19 spacer moieties with various length and chemical functionalities (Table 1), including aromatic and saturated rings, primary amines, carboxamides, anilides, and polyethyleneglycol (PEG) units. Moreover, a common *N*^α-group at the C-terminus was acetylated to mimic the further incorporation of a reporter molecule.

Synthesis of the Spacer-Containing Ergopeptides. The synthesis of the peptide-based spacers was entirely performed on solid-phase, using Rink-MBHA-PS as a polymeric support. Coupling Fmoc-Lys(Alloc)-OH or Fmoc-Glu(OAll)-OH onto the resin, Fmoc elimination, acetylation of the *N*^α-group, and removal of the side chain protecting groups led to resins **3** and **4**, which were used for the construction of the 19 peptide-based spacers (Scheme S1 in Supporting Information (SI)).

The introduction of distinct units (amino acids, diamines, anhydrides, and dicarboxylic acids) onto resins **3** and **4** was carried out using standard solid-phase peptide synthesis (SPPS) protocols and 1,1'-carbonyldiimidazole (CDI) for the activation of supported carboxylic acids. The poor nucleophilicity of some anilines required the use of a previously described procedure, based on *N*-[chloro(dimethylamino)methylene]-*N*-methylmethanaminium chloride (TMUCI Cl) as the activating reagent, for the solid-phase synthesis of anilides¹⁴ (compounds **1p**, **2p**, **1r**, and **2r**). The incorporation of the 19 spacer moieties into the ergopeptides structure was carried out in two steps: (1) *N*^α-Boc-tripeptides **5** and **6** were attached to the supported spacers in a convergent protocol instead of a stepwise procedure to favor the parallelization synthetic process; (2) the crude mixtures released after cleavage (TFA-H₂O, 95:5) were further coupled to the D-lysergic acid in solution using DIPCDI and HOAt (Scheme 1). Although this method involved an extra step compared to

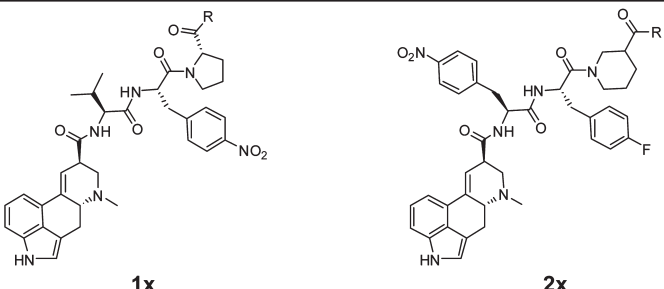
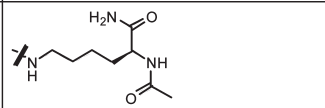
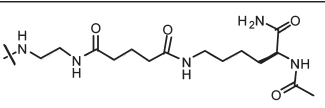
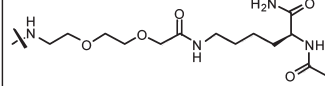
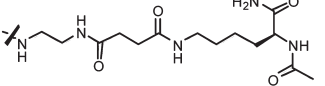
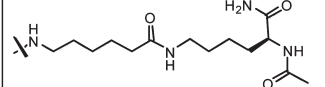
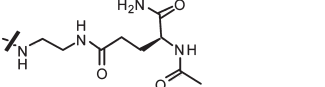
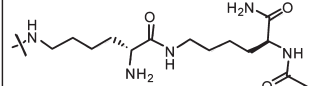
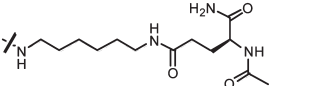
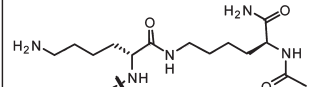
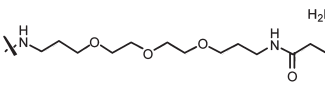
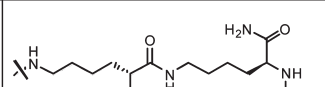
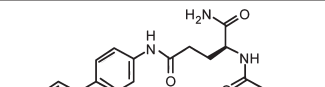
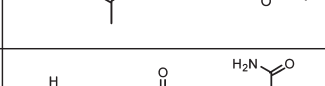
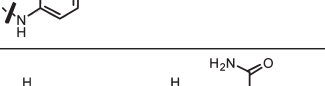
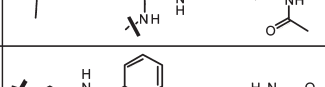
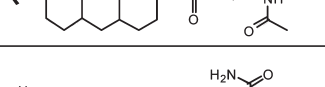
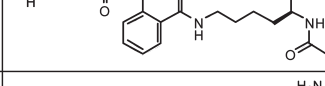
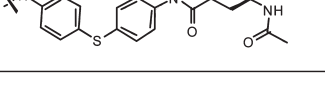
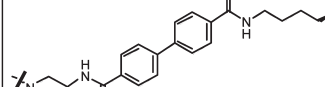
the direct coupling of ergopeptides onto the spacer moieties, the amount of the costly D-lysergic acid could be minimized. The purification of the whole library by semipreparative RP-HPLC afforded the 38 final products with excellent purities (**1a–s** and **2a–s**, Table S1 in SI).

Biological Assays: Binding Properties of Spacer-Containing Ergopeptides. The binding properties of **1a–s** and **2a–s** were assayed by displacement experiments of D₁R or D₂R radiolabeled ligands (concentration indicated in the legends of Figures 1 and 2) by 25 μM **1a–s** or **2a–s**. The binding screening at D₁R and D₂R (Figures 1 and 2) indicated a significant decrease of binding affinity when aromatic rings were included within the spacer moieties (**1p** (mainly at D₂R), **2i**, **2j**, **2p**, and **2r**). In contrast, the incorporation of linear aliphatic spacers resulted in compounds showing a more favorable binding, with slightly stronger interactions in the presence of medium-length spacers (**1c**, **1f**, **1n** at D₁R; **1i**, **1n**, **2f**, and **2k** at D₂R) and some shorter ones (**1a**, **1m** at D₁R; **1a**, **1m**, **2a** at D₂R). Regarding the inclusion of primary amines within the spacer structure, the results were both ergopeptide and receptor-dependent; **1d** did not show a significantly different binding at D₁R, but **1d** and **1e** binding affinities were remarkably improved at D₂R. Whereas determining the exact nature of this enhancement would require further studies, the incorporation of medium-sized aliphatic spacers (**c** and **f**, Table 1) at the C-terminus of ergopeptides **1** and **2** proved to be successful in preserving their binding affinities at both D₁R and D₂R.

We further investigated the behavior of the modified ergopeptides (**1c**, **1f**, **2c**, and **2f**) at A₁ and A_{2A} adenosine receptors because heteromers containing adenosine-dopamine receptors have been very well described.^{15–17} The binding affinities of **1c**, **1f**, **2c**, and **2f** were assayed by displacement experiments of A₁R or A_{2A}R radiolabeled ligands (concentration indicated in the Figure S1 and S2 legends in SI) by 25 μM ergopeptides. The binding of **1c**, **1f**, **2c**, and **2f** at adenosine receptors (Figures S1 and S2 in SI) proved to be much lower than at D₁R and D₂R and confirmed their specificity for dopamine receptors.

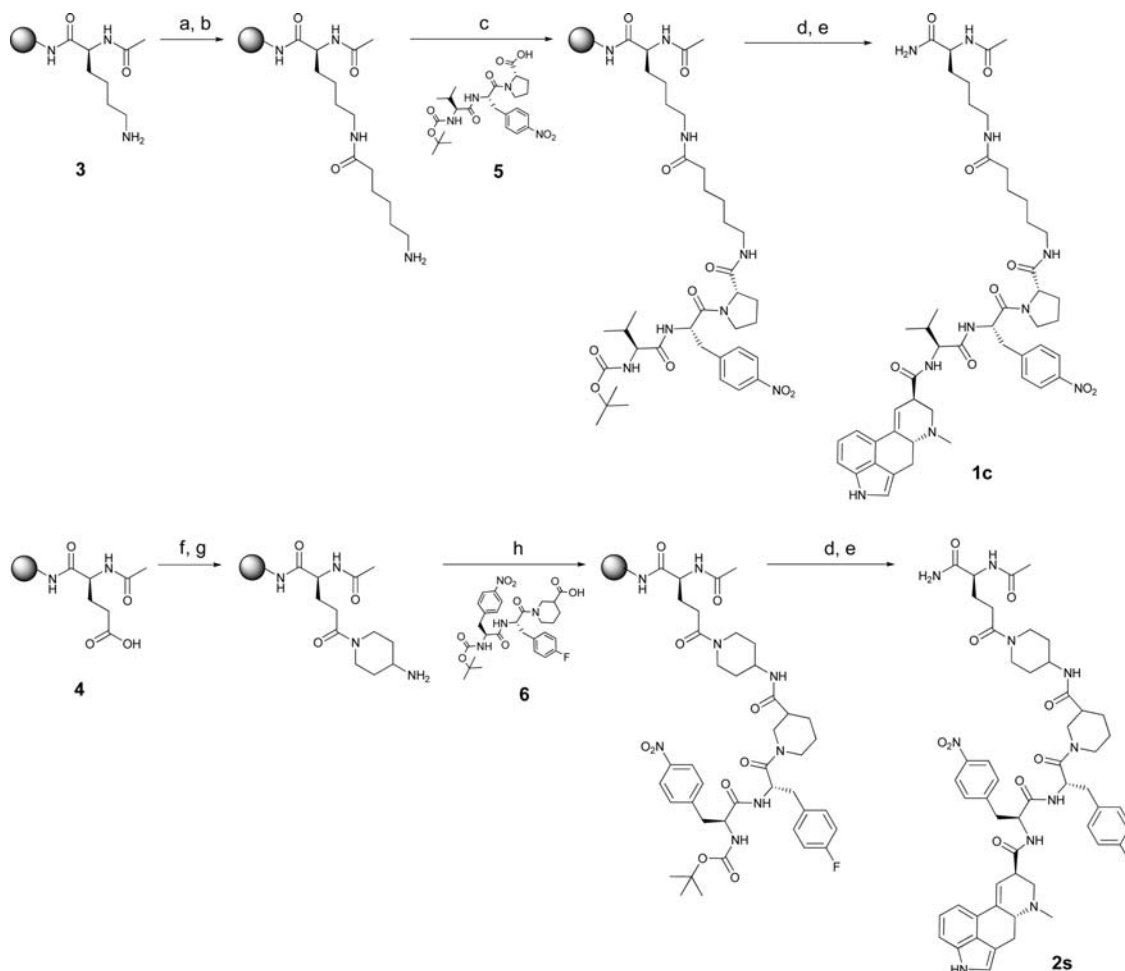
Synthesis and Pharmacological Characterization of Biotin Ergopeptides (9–16). Biotin has been extensively used for protein profiling studies because its tight interaction with avidin can facilitate the isolation of protein complexes.^{18–20} Moreover, the availability of numerous biotin/streptavidin-labeled antibodies and reagents makes biotin one of the most versatile reporters for protein characterization. To construct the corresponding biotin ergopeptide probes, a biotin molecule

Table 1. Chemical Structures of Ergopeptides after the Incorporation of Peptide-Based Spacers

			
compd	R	compd	R
1a, 2a		1k, 2k	
1b, 2b		1l, 2l	
1c, 2c		1m, 2m	
1d, 2d		1n, 2n	
1e, 2e		1o, 2o	
1f, 2f		1p, 2p	
1g, 2g		1q, 2q	
1h, 2h		1r, 2r	
1i, 2i		1s, 2s	
1j, 2j			

was incorporated to the C-terminus of **1c**, **2c**, **1f**, and **2f**. Biotin ergopeptides were synthesized using a slightly modified procedure from the previously described (Scheme 2), and the biotin reporter was coupled to the N^α -group of the C-terminus of ergopeptides using TBTU and HOBt.

The larger size of the biotin ergopeptides facilitated the isolation of the two ergolene diastereomers derived from the epimerization of D-lysergic acid under the coupling conditions,²¹ and eight biotin ergopeptides (**9–16**, Chart 2) were subjected to primary radioligand binding assays at D₁R and

Scheme 1. Synthesis of Representative Compounds **1c** and **2s**

^a Conditions: (a) Fmoc-Ahx-OH, DIPCDI/HOBt; (b) piperidine-DMF (2:8); (c) **5**, DIPCDI/HOBt; (d) TFA-H₂O (95:5); (e) D-lysergic acid, DIPCDI/HOAt, DIEA; (f) (Fmoc-4-amino) piperidine·HCl, DIEA, CDI; (g) piperidine-DMF (2:8); (h) **6**, DIPCDI/HOBt.

D₂R. The binding properties of **9–16** were initially evaluated using displacement experiments of a fixed concentration of D₁R, D₂R, A₁R or A_{2A}R radiolabeled ligands (indicated in the legends of Figures S3–S6 in SI) by 25 μ M **9–16** (Figures S3–S6 in SI). The incorporation of the biotin molecule retained the binding properties of the parent compounds **1** and **2** and confirmed that both selected spacers (**c** and **f**) separate well the pharmacophore from the reporter tag. We selected compounds **9**, **12**, **13**, and **14** for their pharmacological characterization, and their affinity constants at D₁R and D₂R were calculated by competition experiments: brain membranes (0.5 mg/mL) were incubated with a fixed concentration of D₁R or D₂R radiolabeled antagonists in the absence or in the presence of increasing concentrations of compounds **9**, **12**, **13**, or **14**, as described in the Experimental Section. From the resulting competition curves (Figure 3 for compound **13**, and Figures S7–S9 in SI for compounds **9**, **12**, and **14**), their corresponding K_D values were determined (Table 2). The biotin ergopeptide **13** proved to be the compound that best maintained the nanomolar range affinities at both dopamine receptor subtypes.

To test whether the compound **13** behaved as an agonist, we examined two different signal transduction pathways (e.g., MAPK and Akt (PKB)) in cells that were separately transfected with D₁R and D₂R. As shown in Figure 4a,b, **13** increased the ERK1/2 phosphorylation in cells expressing

D₁R or D₂R in a dose-dependent manner and to a similar extent than a D₁R full agonist (e.g., SKF 81297)²² or a D₂R agonist (e.g., quinpirole).²³ The **13**-mediated effect (at 0.1 or 1 μ M) was also reverted when cells were preincubated with 5 or 50 μ M of D₁R (e.g., SCH 23390) or D₂R antagonists (e.g., raclopride), further demonstrating the agonist behavior of **13** at both dopamine receptor subtypes. Regarding the Akt (PKB) pathway, both SKF 81297 and quinpirole (D₁R and D₂R agonists, respectively) induced a decrease in the Akt Ser⁴⁷³ phosphorylation, a signaling that has been also observed in mouse brain.^{24,25} Similarly, **13** decreased the Akt phosphorylation in a dose-dependent manner in cells expressing D₁R or D₂R, and its effect was also reverted upon preincubation with D₁R (e.g., SCH 23390) or D₂R antagonists (e.g., raclopride), confirming the agonist behavior of **13** at both D₁R and D₂R (Figure 4c,d).

Since it has been described that D₁R can also heteromerize with dopamine D₃R,²⁶ we analyzed the binding affinity of compound **13** at D₃R. Because of the low expression level of D₃R in the striatum when compared to D₂R, we transiently transfected CHO cells with D₃R to study the binding affinity of compound **13** to this receptor. Membranes (0.5 mg/mL) from transfected cells were incubated with 4 nM [³H]-raclopride, a D₃R antagonist, in the absence or in the presence of increasing concentrations of compound **13**. The competition curve was used to determine the corresponding

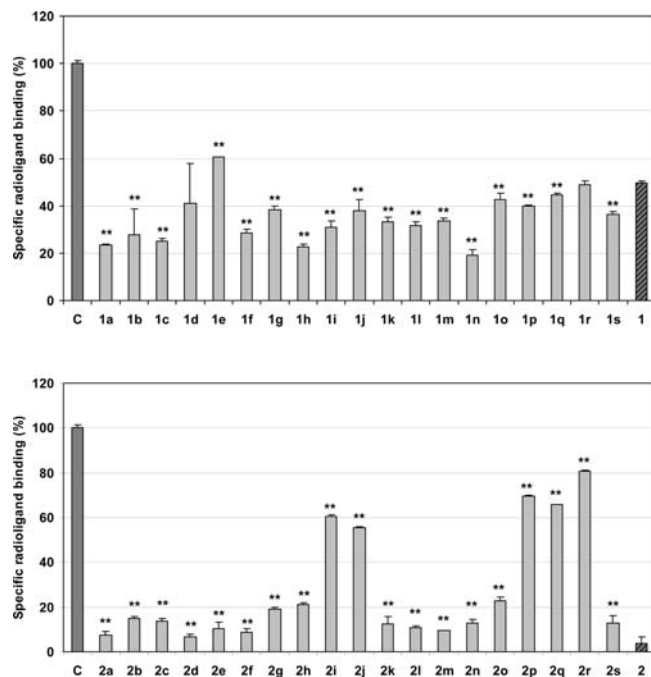


Figure 1. Displacement experiments at D₁R. Specific binding of 0.9 nM D₁R antagonist [³H]-SCH23390 in the absence or in the presence of competing ligands was measured as indicated in the Experimental Section. C, control of radioligand binding without competing ligand; **1a–2s**, radioligand specific binding in the presence of 25 μM ergopeptides; **1–2**, radioligand specific binding in the presence of 25 μM ergopeptides **1** and **2**, respectively. Values are represented as means ± SD (*n* = 3). Student's *t*-test for unpaired samples showed significant differences (***p* < 0.01) compared to the ergopeptides **1** or **2**.

K_D values ($K_{DB1} = 23 \pm 9$ nM and $K_{DB2} = 93 \pm 36$ nM), proving that **13** can be a useful tool for the study of heteromers involving D₁, D₂, or D₃ dopamine receptors (Figure 5). The binding properties of **13** were also examined at other GPCRs that can form heteromers with D₁Rs or D₂Rs (e.g., histamine H₃, metabotropic glutamate 5, somatostatin SST5, and cannabinoids CB₁ receptors).^{27–31} Displacement experiments (Figure S10 in SI) showed that high concentrations of **13** did not significantly decrease the radioligand binding at the studied receptors and proved that the binding of **13** at other GPCRs was very low when compared to dopamine receptors.

Conclusions

The derivatization of two ergopeptides showing high affinity at dopamine receptors has been optimized using a combinatorial chemistry approach to develop of a novel biotin ergopeptide that maintained both nanomolar binding affinities and an agonist behavior at dopamine receptors. The systematic modification of the two parent ergopeptides using a solid-phase synthesis approach afforded a 40-member library including different peptide-based spacers at the C-terminus of the ergopeptides. The binding analysis of the library identified two modified ergopeptides incorporating medium-length aliphatic spacers as the compounds that best retained the affinity profile at D₁R and D₂R. Subsequent derivatization of the spacer-containing ergopeptides with a biotin molecule rendered a set of biotin ergopeptides that bound at D₁R and D₂R with K_D values in the nanomolar range. Further characterization studies identified **13** as a biotin ergopeptide

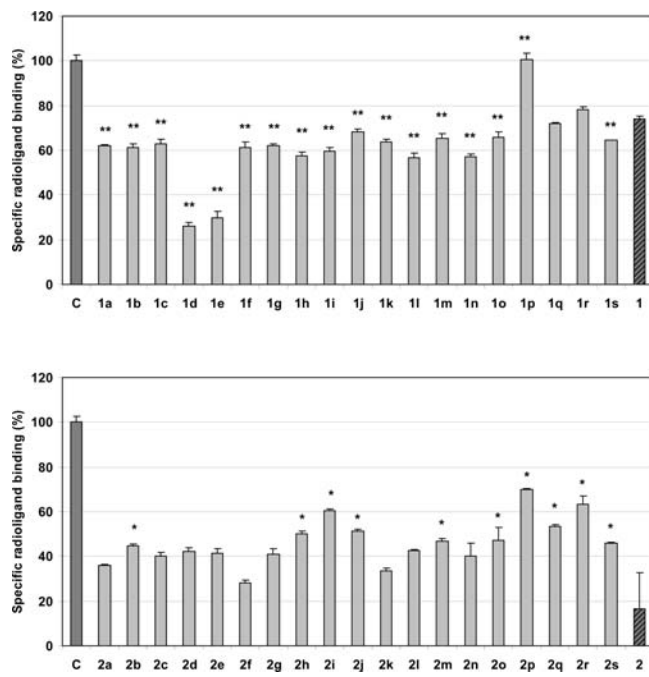


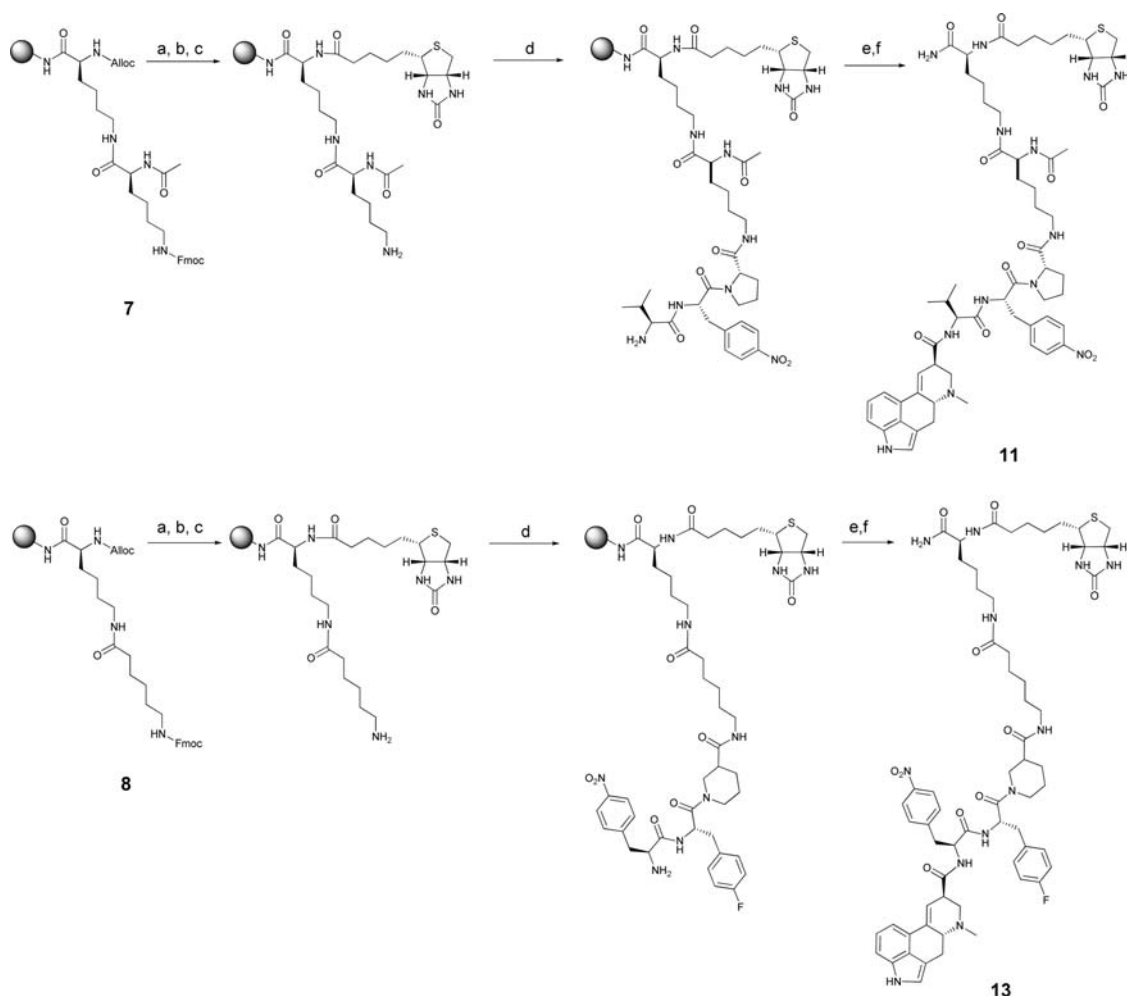
Figure 2. Displacement experiments at D₂R. Specific binding of 0.7 nM D₂R antagonist [³H]-YM 09151-2 in the absence or in the presence of competing ligands was measured as indicated in the Experimental Section. C, control of radioligand binding without competing ligand; **1a–2s**, radioligand specific binding in the presence of 25 μM ergopeptides; **1–2**, radioligand specific binding in the presence of 25 μM ergopeptides **1** and **2**, respectively. Values are represented as means ± SD (*n* = 3). Student's *t*-test for unpaired samples showed significant differences (**p* < 0.05; ***p* < 0.01) compared to the ergopeptides **1** or **2**.

with agonist behavior and significantly higher binding affinities at D₁R, D₂R, and D₃R when compared to adenosine (A₁/A_{2A}), histamine H₃, metabotropic glutamate 1/5, somatostatin SST, and cannabinoid CB₁ receptors. These results attest the potential application of **13** to study heteromer complexes involving dopamine receptors. Experiments with biotin ergopeptides to study dopamine receptors heteromers are currently ongoing and will be reported in due course.

Experimental Section

Materials and Equipment. All Fmoc-amino acids were purchased from Neosystem (Strasbourg, France), and Fmoc-Rink-PS and 2-chlorotriyl resins were supplied by Calbiochem-Novabiochem AG. DIPCDI was obtained from Fluka Chemika (Buchs, Switzerland) and HOBt from Albatross Chem, Inc. (Montreal, Canada). Solvents for peptide synthesis and RP-HPLC equipment were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid was supplied by KaliChemie (Bad Wimpfen, Germany). Other chemicals of the highest commercially available purity were purchased from Aldrich (Milwaukee, WI). All commercial reagents and solvents were used as received.

Adenosine deaminase (EC 3.5.4.4) was purchased from Roche (Basel, Switzerland), and [³H]-*R*-PIA was supplied by Amersham Biosciences (Buckinghamshire, UK). Raclopride, polyethylenimine (PEI), MgCl₂, DPCPX, mouse antiphospho-ERK1/2 antibody, rabbit anti-ERK1/2 antibody, IRDye 800 antimouse antibody, and IRDye 680 antirabbit antibodies were purchased from Sigma (St Louis, MO). Rabbit anti-P-Ser⁴⁷³ Akt antibody was purchased from SAB Signalway (Pearland, USA). ZM241385, SCH23390, RAMH, quisqualic acid, somatostatin, and CP55940 were supplied by Tocris Biosciences (Avonmouth, UK). [³H]-SCH23390, [³H]-YM09151-2, [³H]-quisqualic acid,

Scheme 2. Synthesis of Representative Biotin Ergopeptides **11** and **13**.^a

^a Conditions: (a) Pd(PPh₃)₄-PhSiH₃ in DCM_{anh}; (b) biotin, TBTU/DIEA, HOBT; (c) piperidine-DMF (2:8); (d) sequential peptide synthesis: couplings in DIPCDI/HOBT, Fmoc removal in piperidine-DMF (2:8); (e) TFA-H₂O (95:5); (f) D-lysergic acid, DIPCDI/HOAt.

[³H]-CP55940, [¹²⁵I]-Tyr¹¹-somatostatin 14, and [³H]-ZM241385 were supplied by Perkin-Elmer (Boston, MA). [³H]-RAMH was purchased from GE Healthcare (Buckinghamshire, U.K.). Ecoscint H scintillation cocktail was purchased from National Diagnostics (Atlanta, GA). Bradford assay kit was purchased from Bio-Rad (Munich, Germany). All other supplements were purchased from Invitrogen (Paisley, UK).

Analytical RP-HPLC-MS was performed using 2795 Waters (Milford, MA) Alliance with a Micromass ZQ mass spectrometer and a 996 PDA detector. Semipreparative RP-HPLC was performed on a 2767 Waters chromatography system with a Micromass ZQ mass spectrometer. Multiple sample evaporation was carried out in a Discovery SpeedVac ThermoSavant (Waltham, MA). Radioligand binding experiments were performed using a Brandel (Gaithersburg, MD) cell harvester and a Packard 1600 TRI-CARB scintillation counter. Fitting data binding program GRAFIT was obtained from Erithacus Software (Surrey, UK). For ERK1/2 or P-Ser⁴⁷³Akt phosphorylation determination, the Odyssey infrared scanner (LI-COR Biosciences, Lincoln, Nebraska, USA) was used. Band densities were quantified using the scanner software and exported to Excel (Microsoft, Redmond, WA, USA).

Synthesis. Solid-Phase General Procedure. Peptide syntheses were performed manually in a polypropylene syringes, each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by filtration. Washings between deprotection, coupling, and subsequent deprotection steps were carried

out with DMF (5 × 1 min) and DCM (5 × 1 min) using 10 mL of solvent/g of resin each time.

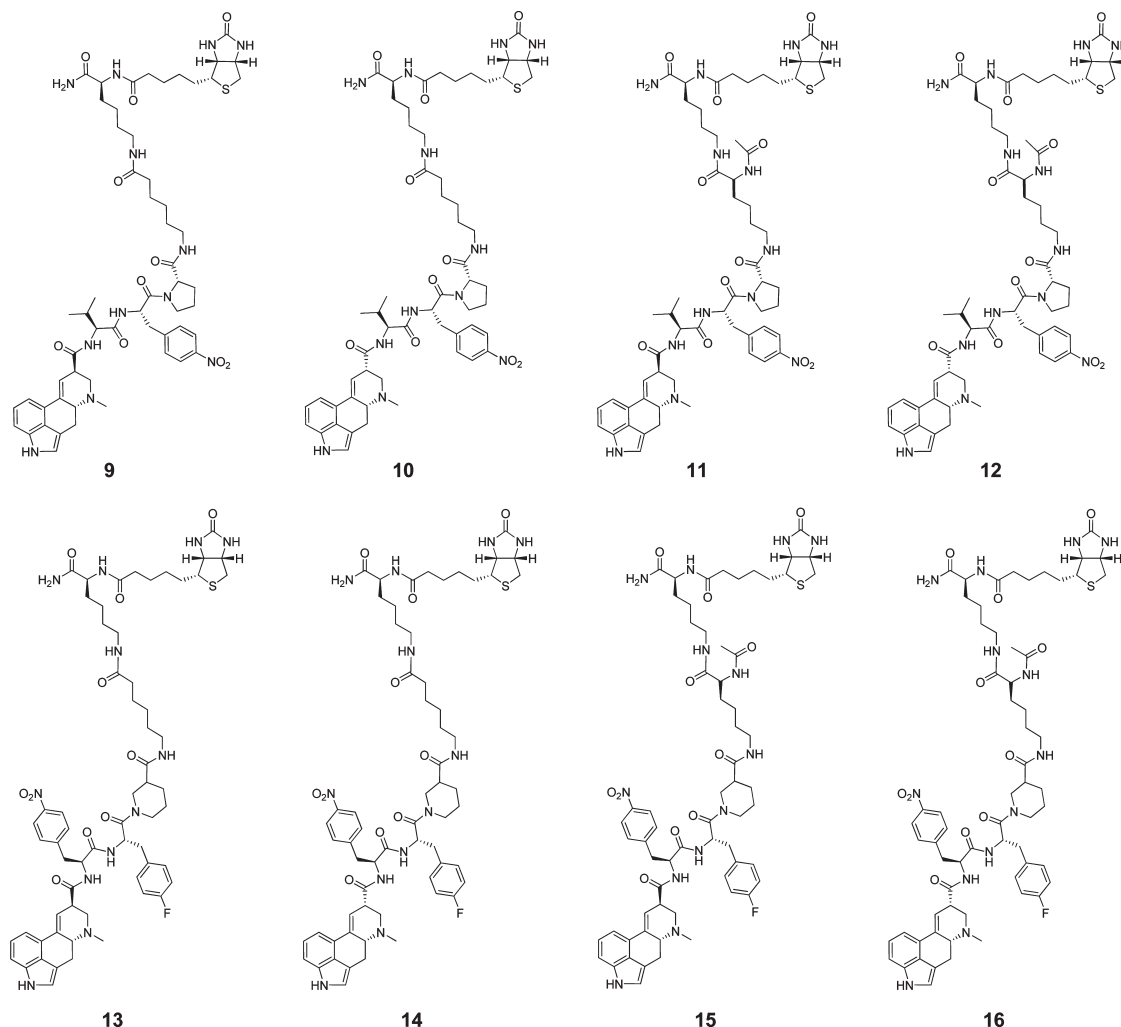
Coupling using DIPCDI and HOBT-HOAt. Fmoc-AA-OH, carboxylic acids or Boc-peptides (3 equiv) were coupled using DIPCDI (3 equiv) as coupling reagent and HOBT (3 equiv) or HOAt (3 equiv) as additives in DCM-DMF (1:1) for 2–4 h at rt. After each coupling, the resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min). Reaction completion was checked by means of the Kaiser or chloranil tests.³²

Anhydrides Coupling. Anhydrides (10 equiv) were coupled using DIEA (10 equiv) in DCM for 2–4 h at rt. After each coupling, the resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min). Reaction completion was checked by means of the Kaiser or chloranil tests.

Coupling using CDI.³³ Solid-supported carboxylic acids were washed with DCM (5 × 1 min) and DMF (5 × 1 min) and treated with CDI (25 equiv) in DMF (30 min). After filtering the resin and washing with DMF, amines (5 equiv) were added in DCM-DMF (1:1) and kept under orbital agitation for 2–4 h at rt. After each coupling, the resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min). Reaction completion was checked by means of the malachite green test.³⁴

Coupling using TMUCl.¹⁴ Solid-supported carboxylic acids were washed with DCM (5 × 1 min) and treated with TMUCl (10 equiv) and DIEA (10 equiv) in DCM (10 min). After filtering the resin and washing with DCM and DMF, anilines (5 equiv) were added in DCM-DMF (1:1) and left

Chart 2. Structures of Biotin Ergopeptides 9–16



under orbital agitation for 2 h at rt. After each coupling, the resin was washed with DMF (5×1 min) and DCM (5×1 min).

Fmoc Group Removal involved the following sequence: (i) DMF (5×1 min); (ii) piperidine-DMF (2:8) (1×1 min + 2×15 min); (iii) DMF (5×1 min).

Alloc/All Group Removal involved the following sequence: (i) DCM (5×1 min); (ii) Pd(PPh₃)₄ (0.1 equiv) and PhSiH₃ (10 equiv) in anhydrous DCM (3×15 min); (iii) anhydrous DCM (5×1 min); (iv) DCM (5×1 min); (v) DMF (5×1 min); (vi) 0.02 M solution of sodium diethyldithiocarbamate in DMF (3×15 min), DMF (5×1 min), DCM (5×1 min), and DMF (5×1 min).

N^α-Terminus Acetylation. The resins were treated with Ac₂O (10 equiv) and DIEA (10 equiv) in DCM (2×15 min). Reaction completion was checked by the Kaiser test.

Cleavage Conditions. A. 2-Chlorotrityl-Based Resins. The resins were treated with a solution of TFA-DCM (5:95) (5×1 min). Filtrates were collected, washed with DCM (3×1 min), and evaporated under vacuum.

B. Rink-Based Resins. The resins were treated with a solution of TFA-H₂O (95:5) and orbitally shaken for 2 h at rt. Filtrates were collected, washed with TFA (2×1 min) and DCM (3×1 min), and evaporated under vacuum.

Synthesis of Boc-Val-pNO₂Phe-Pro-OH (5). Starting from 2-chlorotrityl resin (6.0 g, loading: 1.3 mmol/g), the peptide was synthesized as described above. Cleavage and lyophilization in H₂O-ACN (2:1) rendered a white solid powder; 1.75 g (yield: 39%); M_{exp} 507.1 (M_{calc} 506.3), 96%.

Synthesis of Boc-pNO₂Phe-pFpHe-Nip-OH (6). Starting from 2-chlorotrityl resin (6.0 g, loading: 1.3 mmol/g), the peptide was synthesized as described above. Cleavage and lyophilization in H₂O-ACN (2:1) yielded a white solid powder; 3.58 g (yield: 68%); M_{exp} 587.0 (M_{calc} 586.2), 95%.

Coupling of D-Lysergic Acid. Evaporated crude cleavages for the different peptide moieties (23.7–79.0 mg, 0.03–0.09 mmol) were dissolved in individual vials using 0.5 mL of DMF, treated with DIEA (1 equiv, 5–15 μ L, 0.03–0.09 mmol), and stirred for 5 min at rt. Second, D-lysergic acid (1.2 equiv, 11–29 mg, 0.04–0.11 mmol) and HOAt (1.5 equiv, 6–18 mg, 0.05–0.14 mmol) were dissolved in 1 mL of DMF (each reaction) and added to every vial. Finally, DIPCDI was added (2 equiv, 9–27 μ L, 0.06–0.18 mmol) and the set of reactions was stirred in a parallel synthesizer for 16 h. Multiple sample evaporation rendered the crude mixtures further purified by semipreparative RP-HPLC.

Library Characterization. The purified compounds **1a–1s** and **2a–2s** were characterized by analytical RP-HPLC-MS, using a reverse-phase Symmetry C₁₈ (5 μ m, 3.9 mm \times 150 mm) column and 1 mL/min flow. Elution system A: H₂O-TFA, 99.9:0.1; B: ACN-TFA, 99.9:0.1; gradient 0% B to 100% B in 25 min. The purities of all library compounds (average $\geq 95\%$) were determined by UV absorption at 220 nm (HPLC-MS data, quantities, and purities of all library compounds are included in Table S1 in SI).

Synthesis of Biotin Ergopeptides (9–16). Rink-MBHA PS (1 g, loading: 0.56 mmol/g) was swollen with DCM (1×1 min, 2×10 min) and DMF (5×1 min, 1×15 min) before

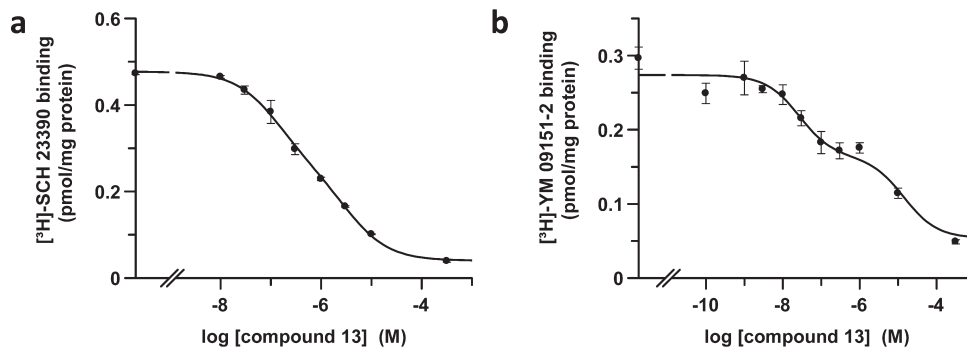


Figure 3. Competition curves of dopamine receptors antagonists binding versus increasing concentrations of compound **13**. Competition experiments of 0.9 nM D₁R antagonist [³H]-SCH23390 (a) or 0.7 nM D₂R antagonist [³H]-YM 09151-2 (b) versus increasing concentrations of compound **13** were performed with brain striatal membranes (0.5 mg prot/mL) as indicated in the Experimental Section. Data are represented as means ± SD from a representative experiment (*n* = 3) performed in triplicate.

Table 2. *K_D* Values of Selected Biotin Ergopeptides at D₁R and D₂R

	D ₁ R		D ₂ R	
	<i>K_{DB1}</i> (nM)	<i>K_{DB2}</i> (μM)	<i>K_{DB1}</i> (nM)	<i>K_{DB2}</i> (μM)
9	107 ± 9	3.2 ± 0.3	260 ± 60	7 ± 2
12	2100 ± 300	> 50	2000 ± 400	23 ± 8
13	56 ± 6	1.5 ± 0.2	17 ± 5	9 ± 3
14	490 ± 80	> 50	160 ± 50	10 ± 4

^a *K_{DB1}* and *K_{DB2}* are respectively the equilibrium dissociation constants of the high and low binding affinities of ergopeptides to the dimeric D₁R and D₂R.

use. After washing, Alloc-L-Lys(Fmoc)-OH (3 equiv) was coupled to the resin, using DIPCI (3 equiv) and HOBT (3 equiv) as a coupling system in DMF. The resin was washed and the Fmoc group removed, yielding Alloc-L-Lys(NH₂)-AM-MBHA. At this point, the resin was split into two equal aliquots. Ac-Lys(Fmoc)-OH (3 equiv) and the Fmoc-Ahx-OH (3 equiv) were respectively coupled on each one as previously described to render the resins **7** and **8**. After that, Alloc group was eliminated in both resins and biotin (3 equiv) was introduced using TBTU (3 equiv), DIEA (6 equiv), and HOBT (3 equiv) in DMF for 1 h at rt. After the Fmoc group removal, the resins were again divided in two parts, obtaining at that point four different resins. From this point onward, the attachment of the three different amino acids (corresponding to the tripeptides of **1** and **2**) was performed according to the procedure described previously. The biotin peptide moieties were cleaved following the B cleavage conditions, and the coupling of D-lysergic acid was carried out as described above. Evaporated crude cleavages for the different biotin peptide moieties (95–127 mg, 0.10–0.13 mmol) were dissolved in individual vials using 0.5 mL of DMF, treated with DIEA (1 equiv), and stirred for 5 min at rt. Second, D-lysergic acid (1.2 equiv) and HOAt (1.5 equiv) were dissolved in 1 mL of DMF (each reaction) and added to every vial. Finally, DIPCDI was added (2 equiv) and the set of reactions were stirred for 16 h at rt. Evaporation rendered the crude mixtures further purified by semipreparative RP-HPLC-MS under basic conditions (A: 20 mM NH₄COOCH₃, pH 9; B: ACN) using a reverse-phase X-Bridge C₁₈ column (5 μm, 19 mm × 100 mm²) to yield the biotin ergopeptides **9–16**.

Biotin Ergopeptides Characterization (9–16). Biotin ergopeptides **9–16** were characterized by analytical RP-HPLC-MS and RP-HPLC using a reverse-phase XBridge C₁₈ column (3.5 μm, 4.6 mm × 50 mm²) at 2 mL/min (two separate elution solvent systems: (a) A, H₂O–HCOOH (99.9:0.1); B, ACN–HCOOH (99.93:0.07). (b) A, H₂O–TFA (99.9:0.1); B, ACN–TFA (99.9:0.1). The purities of **9–16** were determined by RP-HPLC at 220 nm UV absorption using as elution system b and a gradient 5% B to 50% B in 4.5 min. All purities were confirmed to be ≥95%. HRMS spectra for **9–16** were recorded confirming the identity of each biotin ergopeptides (Table 3).

Identity of compound **13** was confirmed by ¹H NMR (400 MHz, DMSO-*d*₆): 10.7 (s, 1H), 8.44 (s, 1H), 8.37 (s, 1H), 8.29 (s, 2H), 8.17 (d, 2H), 7.90 (s, 1H), 7.54 (d, 2H), 7.3–6.9 (m, 7H), 7.30 (s, 1H), 7.09 (s, 1H), 7.02 (s, 1H), 6.42 and 6.36 (s, 2H), 6.26 (s, 1H), 4.98 (m, 1H), 4.64 (m, 1H), 4.4–3.8 (m, 7H), 3.00 (dd, 1H), 2.78 (m, 1H), 2.59 (m, 2H), 2.51 (s, 3H), 2.3–1.24 (m, 4H).

Biological Assays. Radioligand Binding Experiments. General Procedure. Membrane suspensions from lamb brain striatum or D₃R transiently transfected CHO cells were obtained by following the method previously described.³⁵ Radioligand binding assays using membrane suspensions (0.5 mg prot/mL) determined with bicinchoninic acid kits) were carried out at 22 °C in 50 mM Tris-HCl buffer, pH 7.4 (see conditions below used for each receptor). After radioligand incubation, free and membrane-bound ligand were separated by rapid filtration of 500 μL aliquots in a cell harvester through Whatman GF/C filters embedded in 0.3% polyethylenimine (PEI) that were subsequently washed for 5 s with 5 mL of ice-cold Tris-HCl buffer. The filters were incubated with 10 mL of Ecocint H scintillation cocktail overnight at room temperature, and radioactivity counts were determined using a scintillation counter with an efficiency of 62% for tritium labeled compounds and 99% for the ¹²⁵I-labeled compound.

Screening of the Library. Binding experiments of the whole library were performed at a concentration of 25 μM for compounds **1**, **2**, **1a–1s**, **2a–2s** and **9–16**.

Dopamine D₁, D₂, and D₃, Histamine H₃, and Metabotropic Glutamate 1/5 Receptors. Membranes were incubated with 0.9 nM [³H]-SCH23390 (85 Ci/mmol), 0.7 nM [³H]-YM09151-2 (85.5 Ci/mmol), 4 nM [³H]-raclopride (82.8 Ci/mmol), 2.5 nM [³H]-RAMH (34 Ci/mmol), or 27 nM [³H]-quisqualic acid (30.9 Ci/mmol) in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl₂ for 2 h in the absence or in the presence of tested compounds. Nonspecific binding was measured in the presence of 10 μM SCH23390, raclopride, RAMH, or quisqualic acid, respectively.

Somatostatin Receptors. Membranes were incubated with 0.1 nM [¹²⁵I]-Tyr¹¹-somatostatin **14** (2,200 Ci/mmol) in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl₂ for 3 h in the absence or in the presence of tested compound. Nonspecific binding was measured in the presence of 100 nM somatostatin.

Adenosine A₁ and A_{2A} Receptors. Membranes were incubated with 1.0 nM [³H]-R-PIA (30.5 Ci/mmol) or 1.6 nM [³H]-ZM241385 (27.4 Ci/mmol) in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl₂ and 0.2 U/mL ADA for 2 h in the absence or in the presence of tested compounds. Nonspecific binding was measured in the presence of 10 μM DPCPX or ZM 241385, respectively.

Cannabinoid CB₁ Receptor. Membranes were incubated in siliconated tubes with 0.5 nM [³H]-CP 55940 (144 Ci/mmol) in

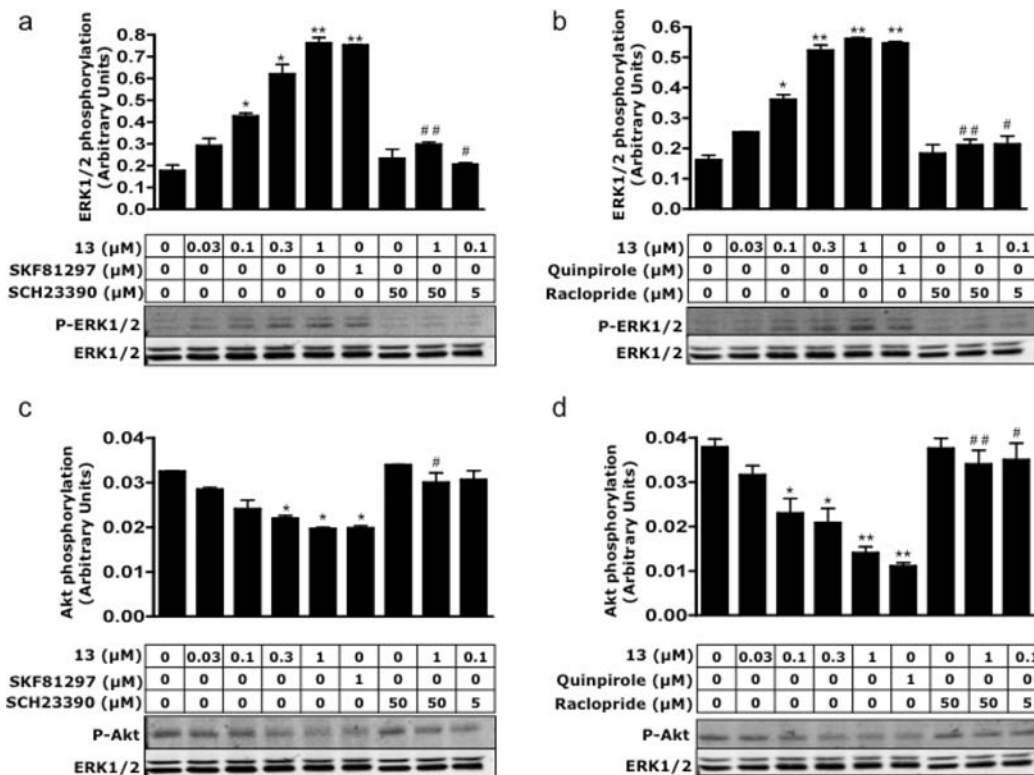


Figure 4. Functional characterization of the biotin ergopeptide **13**. CHO cells expressing D₁R (a and c) or D₂R (b and d) were cultured in serum-free medium for 16 h prior to the addition of any ligand. Cells were treated (or not) with 50 μM of the D₁R antagonist SCH23390 (a and c) or the D₂R antagonist raclopride (b and d). After 5 min, increasing concentrations of compound **13**, D₁R agonist SKF 81297, or the D₂R agonist quinpirole were added for further 5 min of incubation, and ERK1/2 phosphorylation (a and b) or P-Ser⁴⁷³Akt (c and d) were determined as indicated in the Experimental Section. Results are expressed as means ± SEM of four independent experiments. Student's *t*-test for unpaired samples showed significant increases (a and b) or decreases (c and d) over basal (not treated cells, **p* < 0.05, ***p* < 0.01) or significant decreases (a and b) or increases (c and d) respect to cells stimulated with the same concentration of compound **13** in the absence of antagonist (#*p* < 0.05, ##*p* < 0.01).

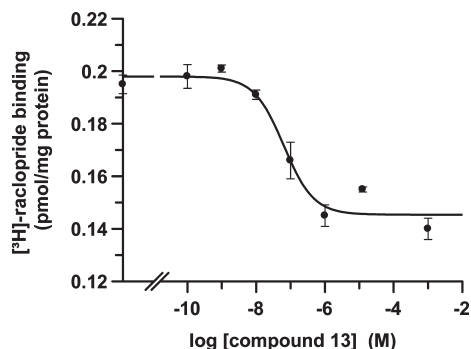


Figure 5. Competition curve of a D₃R antagonist binding versus increasing concentrations of compound **13**. Competition experiments of 4 nM D₃R antagonist [³H]-raclopride versus increasing concentrations of compound **13** were performed with membranes (0.5 mg prot/mL) from CHO cells transiently transfected with 2 μg of the cDNA corresponding to D₃R, as indicated in the Experimental Section. Data are represented as means ± SD from a representative experiment (*n* = 3) performed in triplicate.

50 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl₂ and 1 mg/mL fatty acid-free bovine serum albumin (BSA) for 2 h in the absence or in the presence of tested compounds. In this case, filters were presoaked with buffer containing BSA, and BSA was maintained in the washing medium. Nonspecific binding was measured in the presence of 10 μM CP55940.

K_D Determination. Competition experiments of 0.9 nM [³H]-SCH23390, 0.7 nM [³H]-YM09151-2, or 4 nM [³H]-raclopride binding versus increasing concentrations of compounds **9**, **12**,

13, or **14** were performed by incubating membranes under the same conditions as described above for D₁R, D₂R, or D₃R binding. Nonspecific binding was determined as previously outlined. Radioligand displacement curves were analyzed by nonlinear regression using the commercial program GRAFIT (Erithacus Software, Surrey, UK) by fitting the specific binding data to the mechanistic two-state dimer receptor model.^{35,36} To calculate the macroscopic equilibrium dissociation constants, the equations used for a competition binding experiment were deduced by Casadó.³⁵ Goodness-of-fit was tested following the reduced χ^2 value given by the nonlinear regression program GRAFIT. A modified *F* test was used to analyze whether the fit to cooperativity model significantly improved upon the fit to noncooperative model, and *p* < 0.05 was taken as a criterion of significance; when no significant improvement over the noncooperative model was detected, the *p* values were > 0.30.

Cell Culture, Transient Transfection, and Protein Determination. Chinese hamster ovary (CHO) cells were cultured in MEM α medium without nucleosides supplemented with 100 U/mL penicillin/streptomycin and 10% (v/v) heat inactivated fetal bovine serum (FBS). CHO cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and were passaged when they were 80–90% confluent, i.e., approximately twice a week. CHO cells were transiently transfected with 2 μg cDNA corresponding to human D₁R, D₂R, or D₃R by the ramified PEI method. Cells were incubated for 4 h with the corresponding cDNA together with ramified PEI (5 mL/mg cDNA of 10 mM PEI) and 150 mM NaCl in a serum-starved medium. After 4 h, the medium was changed to a fresh complete culture medium. Forty-eight h after transfection, cells were washed twice in quick succession in HBSS (Hanks' balanced salt solution: 137 mM

Table 3

compd	t_R (min)	molecular formula	M_{calc}	M_{exp} (HRMS)	purity (220 nm) (%)	mg
9	3.33	C ₅₇ H ₇₈ N ₁₂ O ₁₀ S	1122.5685	1123.5747	95	12.6
10	3.68	C ₅₇ H ₇₈ N ₁₂ O ₁₀ S	1122.5685	1123.5747	99	14.3
11	3.20	C ₅₉ H ₈₁ N ₁₃ O ₁₁ S	1179.5899	1180.5961	99	14.5
12	3.58	C ₅₉ H ₈₁ N ₁₃ O ₁₁ S	1179.5899	1180.5963	98	13.6
13	3.88	C ₆₂ H ₇₉ FN ₁₂ O ₁₀ S	1202.5747	1203.5815	99	14.7
14	3.85	C ₆₂ H ₇₉ FN ₁₂ O ₁₀ S	1202.5747	1203.5804	94	15.5
15	3.81	C ₆₄ H ₈₂ FN ₁₃ O ₁₁ S	1259.5961	1260.6015	95	12.9
16	3.77	C ₆₄ H ₈₂ FN ₁₃ O ₁₁ S	1259.5961	1260.6016	96	25.1

NaCl, 5 mM KCl, 0.34 mM Na₂HPO₄·12H₂O, 0.44 mM KH₂PO₄, 1.26 mM CaCl₂·2H₂O, 0.4 mM MgSO₄·7H₂O, 0.5 mM MgCl₂, 10 mM HEPES, pH 7.4) supplemented with 0.1% glucose (w/v), detached by gently pipetting and resuspended in the same buffer. To control the cell number, sample protein concentration was determined using a Bradford assay kit using BSA dilutions as standards.

ERK and Akt Phosphorylation Assay. Transfected CHO cells were cultured in serum-free medium for 16 h before the addition of any agent. Cells were treated or not with 5 or 50 μM of the D₁R antagonist SCH 23390 or the D₂R antagonist raclopride. After 5 min, increasing concentrations of compound **13**, 1 μM D₁R agonist SKF 81297, or 1 μM D₂R agonist quinpirole were added for further 5 min incubation. Cells were rinsed with ice-cold phosphate-buffered saline and lysed by the addition of 500 μL of ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaF, 150 mM NaCl, 45 mM β-glycerophosphate, 1% Triton X-100, 20 μM phenyl-arsine oxide, 0.4 mM NaVO₄, and protease inhibitor cocktail). The cellular debris was removed by centrifugation at 13000g for 5 min at 4 °C, and the protein was quantified by the bicinchoninic acid method using bovine serum albumin dilutions as standard. To determine the level of ERK1/2 or Akt-phosphorylation, equivalent amounts of protein (10 μg) were separated by electrophoresis on a denaturing 7.5% SDS-polyacrylamide gel and transferred onto PVDF-FL membranes. Odyssey blocking buffer was then added, and the membrane was rocked for 90 min. The membranes were then probed with a mixture of antiphospho-ERK1/2 antibody (1:2500) or anti-P-Ser⁴⁷³Akt antibody (1:2500) and anti-ERK1/2 antibody that recognizes both phosphorylated and nonphosphorylated ERK1/2 (1:40000) for 2–3 h. Bands were visualized by the addition of a mixture of IRDye 800 (antimouse) antibody (1:10000) and IRDye 680 (antirabbit) antibody (1:10000) for 1 h and scanned by the Odyssey infrared scanner. Bands densities were quantified using the scanner software, exported to Excel. The level of phosphorylated ERK1/2 isoforms or P-Ser⁴⁷³Akt was normalized for differences in loading using the total ERK protein band intensities.

Acknowledgment. We acknowledge the technical help obtained from Jasmina Jiménez (Molecular Neurobiology Laboratory, University of Barcelona), from Serveis Científic-Tècnics of University of Barcelona for their support in the HRMS, and María Macías (Institute for Research in Biomedicine) for their support in NMR analysis. This work was partially supported by grants from Spanish Ministerio de Ciencia e Innovación MICINN (SAF2008-00146, SAF2008-03229-E, SAF2009-07276, BQU2006-03794, CTQ2005-00315/BQU, CTQ2008-00177, SAF2005-00170, and SAF2006-05481), grant 060110 from Fundació La Marató de TV3, Generalitat de Catalunya, CIBER-BBN, Networking Centre on Bioengineering, Biomaterials, and Nanomedicine Institute for Research in Biomedicine, and the Barcelona Science Park. P.J.M. is a Ramon y Cajal Fellow.

Supporting Information Available: Additional synthetic schemes, tables with chemical structures, and complete

characterization data for the ergopeptide library, radioligand binding assays of **1c**, **1f**, **2c**, and **2f** (A₁R and A_{2A}R) and **9–16** (D₁R, D₂R, A₁R, and A_{2A}R), competition curves of **9**, **12**, and **14** at D₁R and D₂R and binding experiments of **13** at different GPCRs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- Vendrell, M.; Angulo, E.; Casadó, V.; Lluis, C.; Franco, R.; Albericio, F.; Royo, M. Novel ergopeptides as dual ligands for adenosine and dopamine receptors. *J. Med. Chem.* **2007**, *50*, 3062–3069.
- Maggio, R.; Aloisi, G.; Silvano, E.; Rossi, M.; Millan, M. J. Heterodimerization of dopamine receptors: new insights into functional and therapeutic significance. *Parkinsonism Relat. Disord.* **2009**, *15* (S4), S2–S7.
- So, C. H.; Verma, V.; O'Dowd, B. F.; George, S. R. Desensitization of the dopamine D1 and D2 receptor hetero-oligomer mediated calcium signal by agonist occupancy of either receptor. *Mol. Pharmacol.* **2007**, *72*, 450–462.
- So, C. H.; Varghese, G.; Curley, K. J.; Kong, M. M.; Alijaniam, M.; Ji, X.; Nguyen, T.; O'Dowd, B. F.; George, S. R. D₁ and D₂ dopamine receptors form heterooligomers and cointernalize after selective activation of either receptor. *Mol. Pharmacol.* **2005**, *68*, 568–578.
- Bhushan, R. G.; Sharma, S. K.; Xie, Z.; Daniels, D. J.; Portoghese, P. S. A bivalent ligand (KDN-21) reveals spinal delta and kappa opioid receptors are organized as heterodimers that give rise to delta(1) and kappa(2) phenotypes. Selective targeting of delta-kappa heterodimers. *J. Med. Chem.* **2004**, *47*, 2969–2972.
- Waldhoer, M.; Fong, J.; Jones, R. M.; Lunzer, M. M.; Sharma, S. K.; Kostenis, E.; Portoghese, P. S.; Whistler, J. L. A heterodimer-selective agonist shows in vivo relevance of G protein-coupled receptor dimers. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9050–9055.
- Adam, G. C.; Sorensen, E. J.; Cravatt, B. F. Proteomic profiling of mechanistically distinct enzyme classes using a common chemotype. *Nature Biotechnol.* **2002**, *20*, 805–809.
- Speers, A. E.; Adam, G. C.; Cravatt, B. F. Activity-based protein profiling in vivo using a copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J. Am. Chem. Soc.* **2003**, *125*, 4686–4687.
- Walsh, D. P.; Chang, Y. T. Chemical genetics. *Chem. Rev.* **2006**, *106*, 2476–2530.
- Middleton, R. J.; Bridton, S. J.; Cordeaux, Y.; Yates, A. S.; Dale, C. L.; George, M. W.; Baker, J. G.; Hill, S. J.; Kellam, B. New fluorescent adenosine A₁-receptor agonists that allow quantification of ligand–receptor interactions in microdomains of single living cells. *J. Med. Chem.* **2007**, *50*, 782–793.
- Lokey, R. S. Forward chemical genetics: progress and obstacles on the path to a new pharmacopoeia. *Curr. Opin. Chem. Biol.* **2003**, *7*, 91–96.
- Middleton, R. J.; Kellam, B. Fluorophore-tagged GPCR ligands. *Curr. Opin. Chem. Biol.* **2005**, *9*, 517–525.
- Gellerman, G.; Elgavi, A.; Salitra, A.; Kramer, M. Novel Gly building units for backbone cyclization: synthesis and incorporation into model peptides. *J. Pept. Res.* **2001**, *57*, 277–291.
- Vendrell, M.; Ventura, R.; Ewenson, A.; Royo, M.; Albericio, F. N-[Chloro(dimethylamino)methylene]-N-methylmethanaminium chloride (TMUCl·Cl), the reagent of choice for the solid-phase synthesis of anilides. *Tetrahedron Lett.* **2005**, *46*, 5383–5386.
- Baraldi, P. G.; Tabrizi, M. A.; Gessi, S.; Borea, P. A. Adenosine receptor antagonists: translating medicinal chemistry and pharmacology into clinical utility. *Chem. Rev.* **2008**, *108*, 238–263.
- Agnati, L. F.; Ferré, S.; Lluis, C.; Franco, R.; Fuxe, K. Molecular mechanisms and therapeutic implications of intramembrane receptor/receptor interactions among heptahelical receptors with

- examples from the striatopallidal GABA neurons. *Pharmacol. Rev.* **2003**, *55*, 509–550.
- (17) Franco, R.; Casadó, V.; Mallol, J.; Ferré, S.; Fuxe, K.; Cortés, A.; Ciruela, F.; Lluís, C.; Canela, E. I. Dimer-based model for heptaspanning membrane receptors. *Trends Biochem. Sci.* **2005**, *300*, 360–366.
- (18) Bayer, E. A.; Wilchek, M. Application of avidin–biotin technology to affinity-based separations. *J. Chromatogr.* **1990**, *510*, 3–11.
- (19) Elia, G. Biotinylation reagents for the study of cell surface proteins. *Proteomics* **2008**, *8*, 4012–4024.
- (20) Bratthauer, G. L. The avidin–biotin complex (ABC) and other avidin-biotin binding methods. *Methods Mol. Biol.* **1999**, *115*, 203–214.
- (21) Smith, D. J.; Shappell, N. W. Technical note: epimerization of ergopeptine alkaloids in organic and aqueous solvents. *J. Anim. Sci.* **2002**, *80*, 1616–1622.
- (22) Chausmer, A. L.; Katz, J. L. Comparison of interactions of D1-like agonists, SKF 81297, SKF 82958, and A-77636, with cocaine: locomotor activity and drug discrimination studies in rodents. *Psychopharmacology* **2002**, *159*, 145–153.
- (23) Levant, B.; Grigoriadis, D. E.; De Souza, E. B. Characterization of [³H]-quinpirole binding to D₂-like dopamine receptors in rat brain. *J. Pharmacol. Exp. Ther.* **1992**, *262*, 929–935.
- (24) Beaulieu, J.; Gainetdinov, R. R.; Caron, M. G. The Akt/GSK-3 signaling cascade in the actions of dopamine. *Trends Pharmacol. Sci.* **2007**, *28*, 166–172.
- (25) Beaulieu, J.; Gainetdinov, R. R.; Caron, M. G. Akt/GSK3 signaling in the action of psychotropic drugs. *Annu. Rev. Pharmacol. Toxicol.* **2009**, *49*, 327–347.
- (26) Marcellino, D.; Ferré, S.; Casadó, V.; Cortés, A.; Le Foll, B.; Mazzola, C.; Drago, F.; Saur, O.; Stark, H.; Soriano, A.; Barnes, C.; Goldberg, S. R.; Lluís, C.; Fuxe, K.; Franco, R. Identification of dopamine D₁–D₃ receptor heteromers. Indications for a role of synergistic D₁–D₃ receptor interactions in the striatum. *J. Biol. Chem.* **2008**, *283*, 26016–26025.
- (27) Ferrada, C.; Moreno, E.; Casadó, V.; Bongers, G.; Cortés, A.; Mallol, J.; Canela, E. I.; Leurs, R.; Ferré, S.; Lluís, C.; Franco, R. Marked changes in signal transduction upon heteromerization of dopamine D₁ and histamine H₃ receptors. *Br. J. Pharmacol.* **2009**, *157*, 64–75.
- (28) Ferrada, C.; Ferré, S.; Casadó, V.; Cortés, A.; Justinova, Z.; Barnes, C.; Canela, E. I.; Goldberg, S. R.; Leurs, R.; Lluís, C.; Franco, R. Interactions between histamine H₃ and dopamine D₂ receptors and the implications for striatal function. *Neuropharmacology* **2008**, *55*, 190–197.
- (29) Cabello, N.; Gandía, J.; Bertarelli, D. C.; Watanabe, M.; Lluís, C.; Franco, R.; Ferré, S.; Luján, R.; Ciruela, F. Metabotropic glutamate type 5, dopamine D₂ and adenosine A_{2A} receptors form higher-order oligomers in living cells. *J. Neurochem.* **2009**, *109*, 1497–1507.
- (30) Kearn, C. S.; Blake-Palmer, K.; Daniel, E.; Mackie, K.; Glass, M. Concurrent stimulation of cannabinoid CB₁ and dopamine D₂ receptors enhances heterodimer formation: a mechanism for receptor cross-talk? *Mol. Pharmacol.* **2005**, *67*, 1697–1704.
- (31) Rocheville, M.; Lange, D. C.; Kumar, U.; Patel, S. C.; Patel, R. C.; Patel, Y. C. Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity. *Science* **2000**, *288*, 154–157.
- (32) Vazquez, J.; Qushair, G.; Albericio, F. Qualitative colorimetric tests for solid phase synthesis. *Methods Enzymol.* **2003**, *369*, 21–35.
- (33) Strohmeier, G. A.; Haas, W.; Kappe, C. O. Synthesis of functionalized 1,3-thiazine libraries combining solid-phase synthesis and post-cleavage modification methods. *Chem.—Eur. J.* **2004**, *10*, 2919–2926.
- (34) Attardi, M. E.; Porcu, G.; Taddei, M. Malachite green, a valuable reagent to monitor the presence of free COOH on the solid-phase. *Tetrahedron. Lett.* **2000**, *41*, 7391–7394.
- (35) Casadó, V.; Ferrada, C.; Bonaventura, J.; Gracia, E.; Mallol, J.; Canela, E. I.; Lluís, C.; Cortés, A.; Franco, R. Useful pharmacological parameters for G-protein-coupled receptor homodimers obtained from competition experiments. Agonist–antagonist binding modulation. *Biochem. Pharmacol.* **2009**, *78*, 1456–1463.
- (36) Casadó, V.; Cortés, A.; Ciruela, F.; Mallol, J.; Ferré, S.; Lluís, C.; Canela, E. I.; Franco, R. Old and new ways to calculate the affinity of agonists and antagonists interacting with G-protein-coupled monomeric and dimeric receptors: the receptor–dimer cooperativity index. *Pharmacol. Ther.* **2007**, *116*, 343–354.

Biotin Ergopeptide Probes for Dopamine Receptors

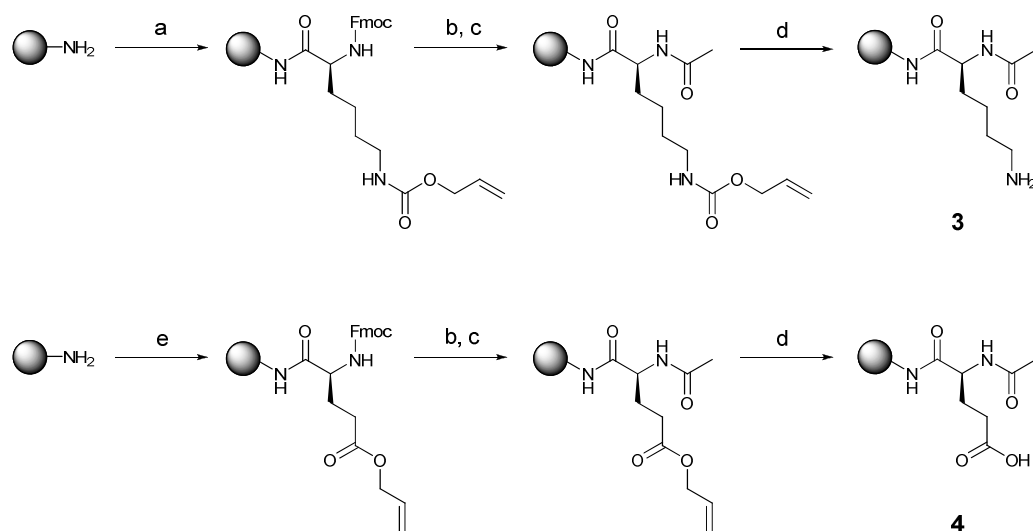
Marc Vendrell, Anabel Molero, Sergio Gonzalez, Kamil Pérez-Capote, Carme Lluís, Peter J. McCormick, Rafael Franco, Antoni Cortés, Vicent Casadó, Fernando Albericio and Miriam Royo

Table of Contents

1. Synthetic schemes for the preparation of the resins **3** and **4**.
2. Table with chemical structures and complete HPLC data for the whole library of ergopeptides containing peptide-based spacers.
3. Radioligand binding assays of **1c**, **1f**, **2c**, and **2f** at A₁ and A_{2A} adenosine receptors.
4. Radioligand binding assays of **9-16** D₁R, D₂R, A₁R and A_{2A}R.
5. Binding curves of D₁R and D₂R antagonists in competition with increasing concentrations of ergopeptides **9**, **12** and **14**.
6. Affinity of compound **13** at histamine, glutamate, somatostatin and cannabinoid receptors.

1. Synthetic schemes for the preparation of the resins 3 and 4.

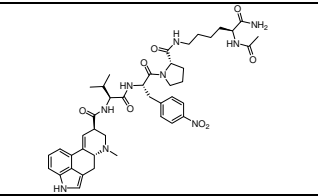
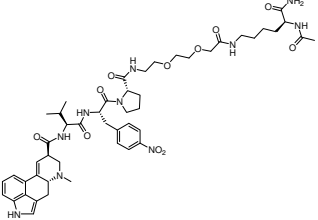
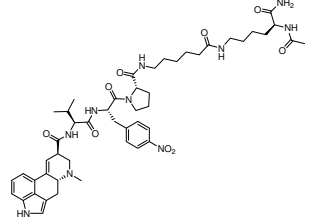
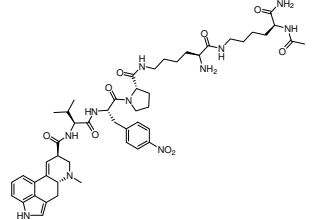
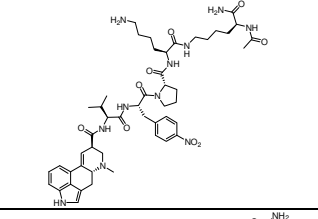
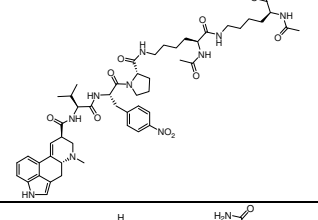
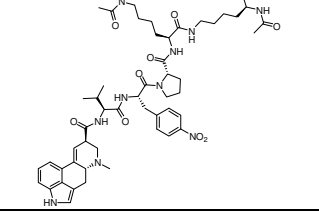
Scheme S1. Preparation of resins 3 and 4.



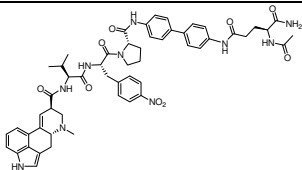
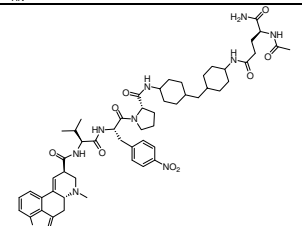
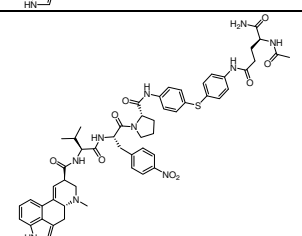
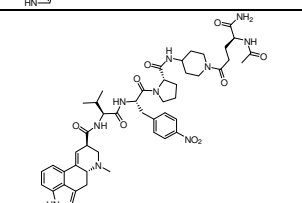
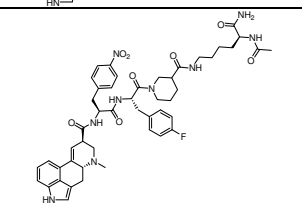
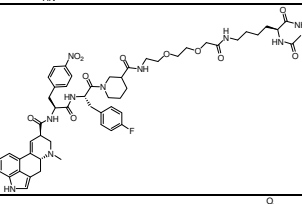
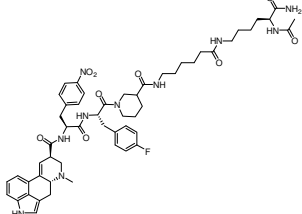
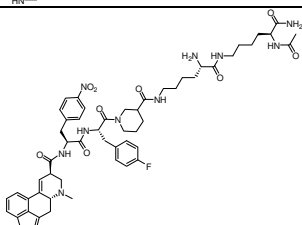
Reaction conditions: a) Fmoc-Lys(Alloc)-OH, DIC/HOBt, b) piperidine-DMF (2:8), c) Ac₂O-DIEA, d) Pd(PPh₃)₄-PhSiH₃ in DCM, e) Fmoc-Glu(OAll)-OH, DIC/HOBt.

2. Table with chemical structures and complete HPLC data for the whole library of ergopeptides containing peptide-based spacers.

Table S1. Chemical structures and HPLC data for the whole library of ergopeptides containing peptide-based spacers.

Compd.	Chemical structure	Mcalc.	Mexp. *	%purity (HPLC 1)	%purity (HPLC 2)	mg
1a		825.4	414.1 826.6	100%	100%	6.4
1b		970.5	486.6 971.6	97%	95%	8.0
1c		938.5	470.6 939.6	99%	99%	7.0
1d		953.5	478.0 954.6	93%	88%	0.4
1e		953.5	478.1 954.6	95%	90%	0.3
1f		995.5	499.1 996.7	98%	95%	2.6
1g		995.5	499.1 996.6	98%	98%	9.8

Compd.	Chemical structure	Mcalc.	Mexp.	%purity (HPLC 1)	%purity (HPLC 2)	mg
1h		1091.5	547.1 1092.6	98%	96%	7.3
1i		1091.5	547.1 1092.6	100%	98%	4.1
1j		1119.5	561.1 1120.6	95%	95%	8.5
1k		981.5	492.1 982.7	98%	97%	2.7
1l		967.5	485.1 968.5	96%	94%	4.1
1m		868.4	435.3 869.5	98%	96%	9.4
1n		924.5	463.5 925.6	97%	95%	7.0
1o		1028.5	515.6 1029.6	98%	94%	9.7

Compd.	Chemical structure	Mcalc.	Mexp.	%purity (HPLC 1)	%purity (HPLC 2)	mg
1p		992.5	497.7 993.7	100%	100%	2.4
1q		1018.6	510.6 1019.7	93%	95%	1.0
1r		1024.4	513.6 1025.6	100%	100%	1.3
1s		908.5	455.5 909.5	97%	94%	3.0
2a		905.4	445.6 906.6	99%	95%	0.9
2b		1050.5	526.6 1051.6	100%	100%	5.8
2c		1018.5	510.6 1019.6	95%	94%	2.4
2d		1033.5	518.2 1034.7	92%	93%	0.4

Compd.	Chemical structure	Mcalc.	Mexp.	%purity (HPLC 1)	%purity (HPLC 2)	mg
2e		1033.5	518.2 1034.8	85%	89%	1.0
2f		1075.5	539.2 1076.7	97%	99%	0.4
2g		1075.5	539.3 1076.6	100%	96%	1.4
2h		1171.5	587.1 1172.7	99%	96%	2.0
2i		1171.5	587.1 1172.7	96%	93%	2.9
2j		1199.5	601.2 1200.7	97%	98%	2.0
2k		1061.5	532.2 1062.7	93%	94%	0.9
2l		1047.5	525.2 1048.6	95%	95%	1.1

Compd.	Chemical structure	Mcalc.	Mexp.	%purity (HPLC 1)	%purity (HPLC 2)	mg
2m		948.4	475.5 949.5	99%	94%	1.2
2n		1004.5	503.6 1005.5	97%	93%	2.1
2o		1108.6	555.6 1109.8	97%	99%	1.8
2p		1072.5	537.7 1073.7	100%	99%	2.2
2q		1098.6	550.6 1099.7	90%	93%	0.4
2r		1104.4	553.7 1105.7	100%	100%	2.6
2s		988.5	495.5 989.5	98%	95%	1.1

HPLC system 1: A: H₂O-TFA: 99.9:0.1. B: ACN-TFA: 99.9:0.1; gradient 0% B to 100% B in 25 min. HPLC system 2: A: H₂O-HCOOH: 99.9:0.1. B: ACN-HCOOH: 99.9:0.1; gradient 0% B to 100% B in 25 min. The purities of all compounds were determined by UV absorption at 220 nm. * *m/z* corresponding to [M+2H]²⁺ and [M+H]⁺ respectively.

3. Radioligand binding assays of 1c, 1f, 2c, and 2f at A₁ and A_{2A} adenosine receptors.

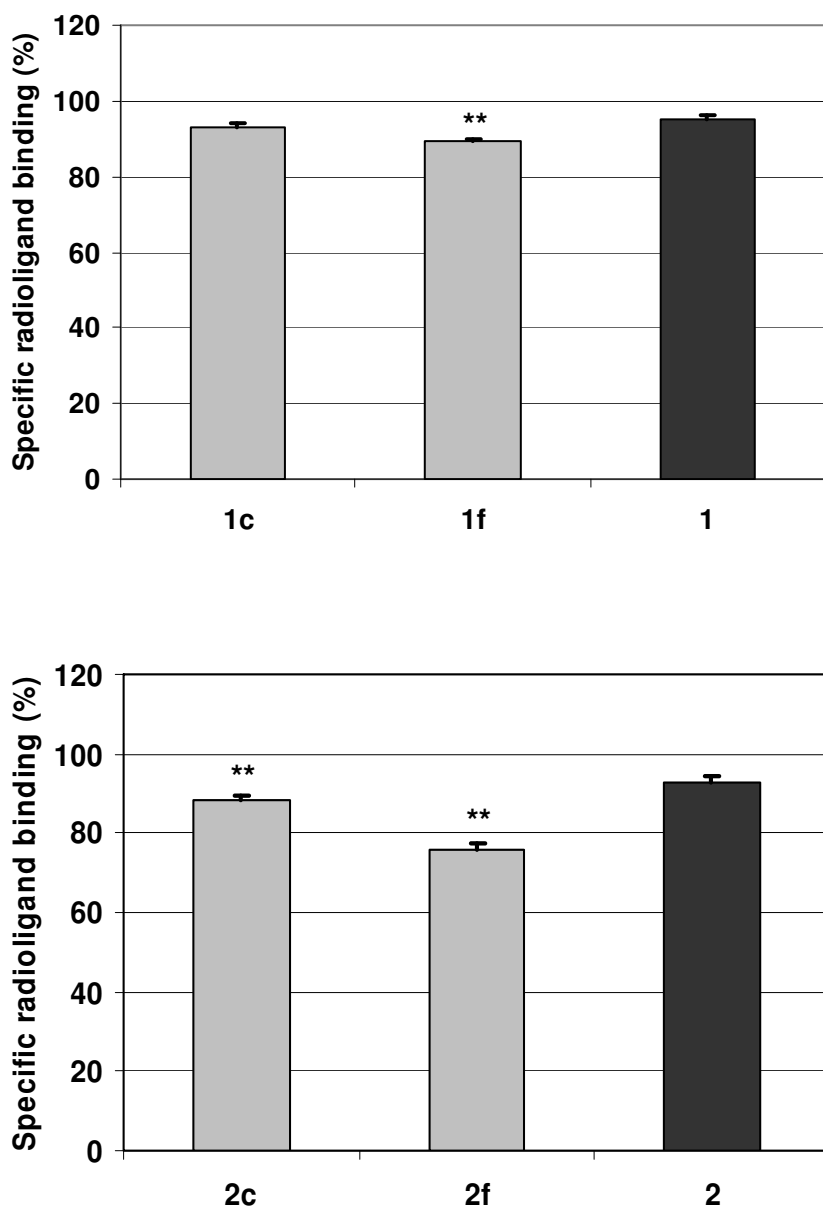


Figure S1. Displacement experiments at A₁R. Specific binding of 10 nM A₁R agonist [³H]-R-PIA in the absence or in the presence of competing ligands was measured as indicated in the Experimental Section. C: control of radioligand binding without competing ligand; **1c**, **1f**, **2c**, **2f**: radioligand specific binding in the presence of 25 μM ergopeptides; **1-2**: radioligand specific binding in the presence of 25 μM ergopeptides **1** and **2** respectively. Values are represented as means ± SD (n=3). Student's *t*-test for unpaired samples showed significant differences (***p* < 0.01) compared to the ergopeptides **1** or **2**.

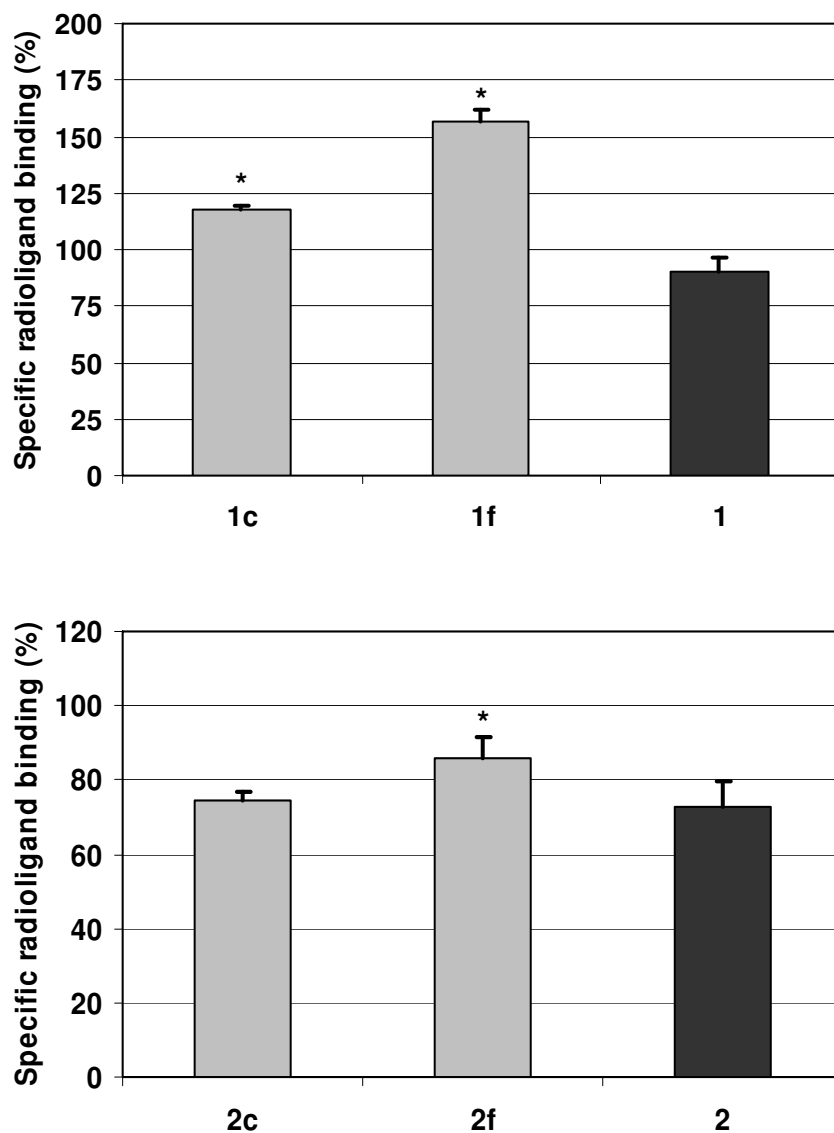


Figure S2. Displacement experiments at $A_{2A}R$. Specific binding of 1.6 nM $A_{2A}R$ antagonist [3H]-ZM 241385 in the absence or in the presence of competing ligands was measured as indicated in the Experimental Section. C: control of radioligand binding without competing ligand; **1c**, **1f**, **2c**, **2f**: radioligand specific binding in the presence of 25 μ M ergopeptides; **1-2**: radioligand specific binding in the presence of 25 μ M ergopeptides **1** and **2** respectively. Values are represented as means \pm SD (n=3). Student's *t*-test for unpaired samples showed significant differences (**p* < 0.05) compared to the ergopeptides **1** or **2**.

4. Radioligand binding assays of 9-16 at D₁R, D₂R, A₁R and A_{2A}R.

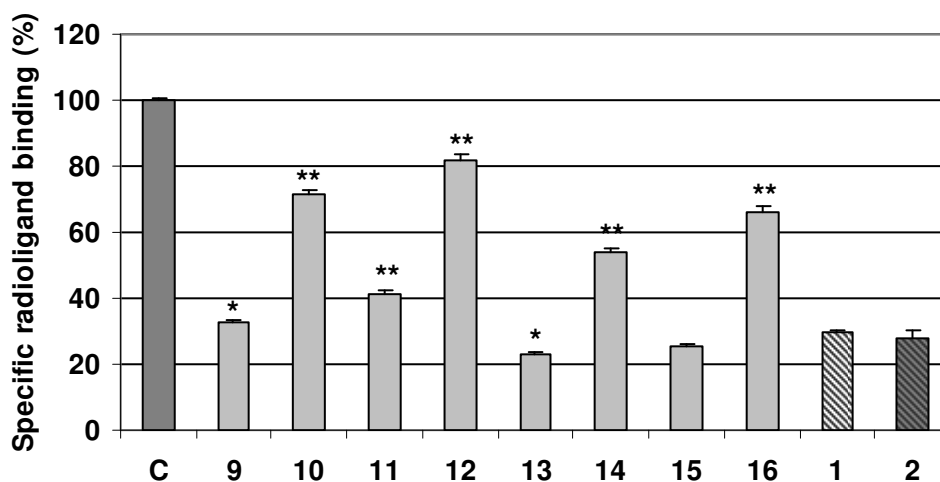


Figure S3. Displacement experiments at D₁R. Specific binding of 0.9 nM D₁R antagonist [³H]-SCH 23390 in the absence or in the presence of competing ligands was measured as indicated in the Experimental Section. C: control of radioligand binding without competing ligand; 9-16: radioligand specific binding in the presence of 25 μM biotin ergopeptides; 1-2: radioligand specific binding in the presence of 25 μM ergopeptides 1 and 2. Values are represented as means ± SD (n=3). Student's *t*-test for unpaired samples showed significant differences (**p* < 0.05; ***p* < 0.01) compared to the ergopeptides 1 (9 to 12) or 2 (13 to 16).

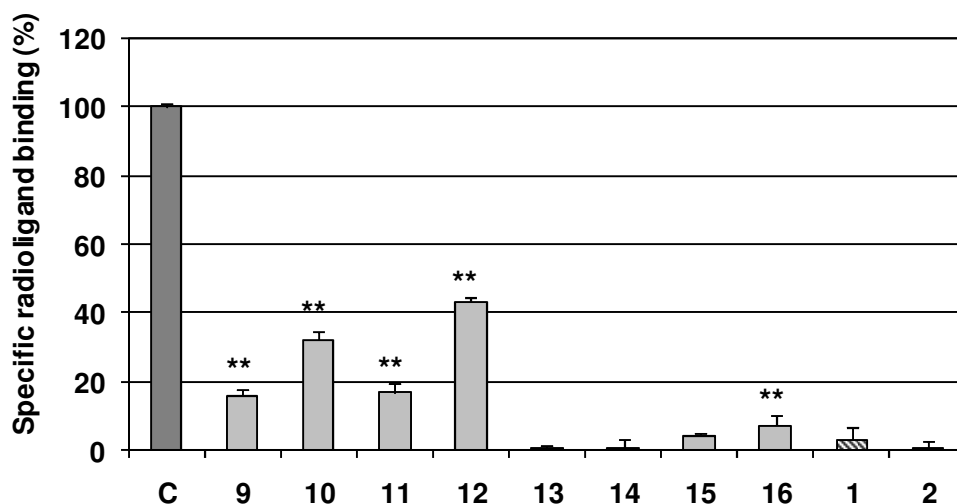


Figure S4. Competition experiments at D₂R. Specific binding of 0.7 nM D₂R antagonist [³H]-YM 09151-2 in the absence or in the presence of competing ligands was measured as indicated in the Experimental Section. C: control of radioligand binding without competing ligand; 9-16: radioligand specific binding in the presence of 25 μM biotin ergopeptides; 1-2: radioligand specific binding in the presence of 25 μM ergopeptides 1 and 2. Values are represented as means ± SD (n=3). Student's *t*-test for unpaired samples showed significant differences (***p* < 0.01) compared to the ergopeptides 1 (9 to 12) or 2 (13 to 16).

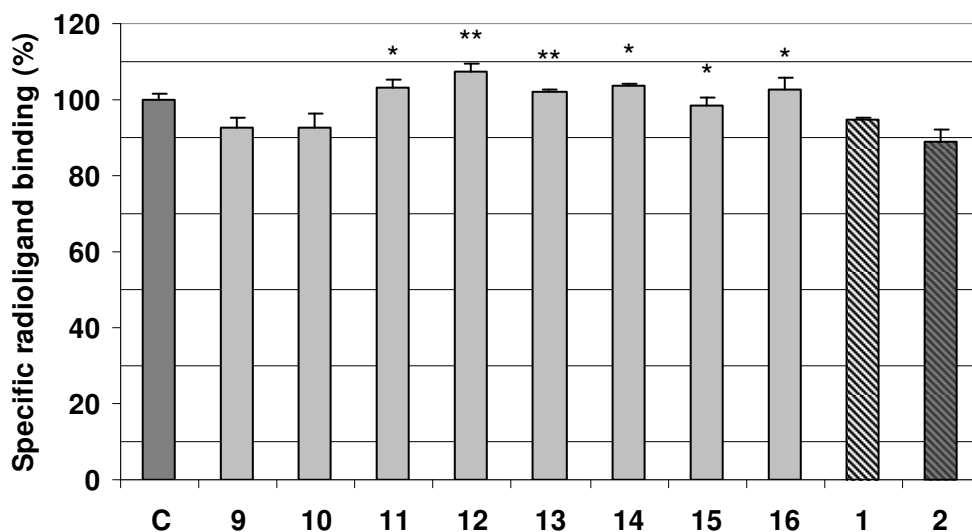


Figure S5. Competition experiments at A₁R. Specific binding of 1.0 nM [³H]-R-PIA in the absence or in the presence of competing ligands was measured as indicated in the Experimental Section. C: control of radioligand binding without competing ligand; **9-16**: radioligand specific binding in the presence of 25 μM biotin ergopeptides; **1-2**: radioligand specific binding in the presence of 25 μM ergopeptides **1** and **2**. Values are represented as means ± SD (n=3). Student's *t*-test for unpaired samples showed significant differences (**p* < 0.05; ***p* < 0.01) compared to the ergopeptides **1** (**9** to **12**) or **2** (**13** to **16**).

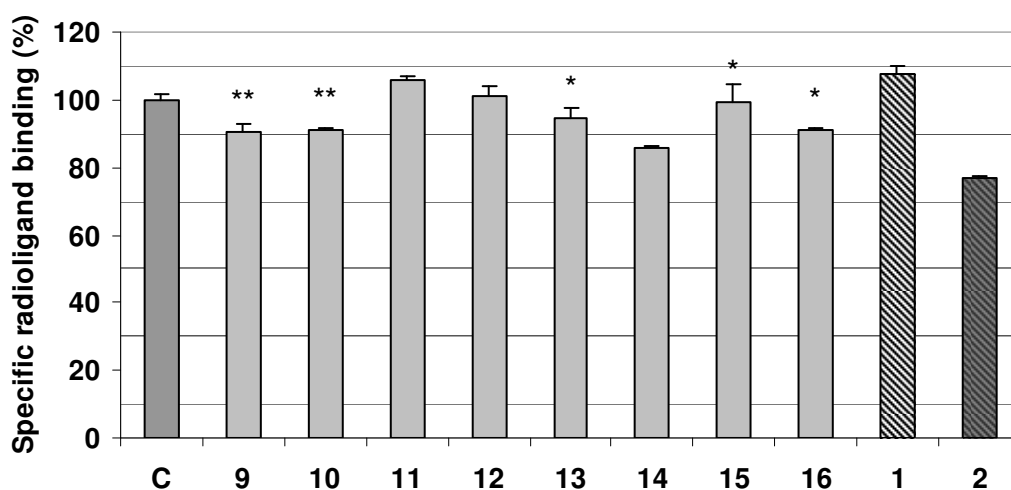


Figure S6. Competition experiments at A_{2A}R. Specific binding of 1.6 nM [³H]-ZM241385 in the absence or in the presence of competing ligands was measured as indicated in the Experimental Section. C: control of radioligand binding without competing ligand; **9-16**: radioligand specific binding in the presence of 25 μM biotin ergopeptides; **1-2**: radioligand specific binding in the presence of 25 μM ergopeptides **1** and **2**. Values are represented as means ± SD (n=3). Student's *t*-test for unpaired samples showed significant differences (**p* < 0.05; ***p* < 0.01) compared to the ergopeptides **1** (**9** to **12**) or **2** (**13** to **16**).

5. Binding curves of D₁R and D₂R antagonists in competition with increasing concentrations of compounds 9, 12 and 14.

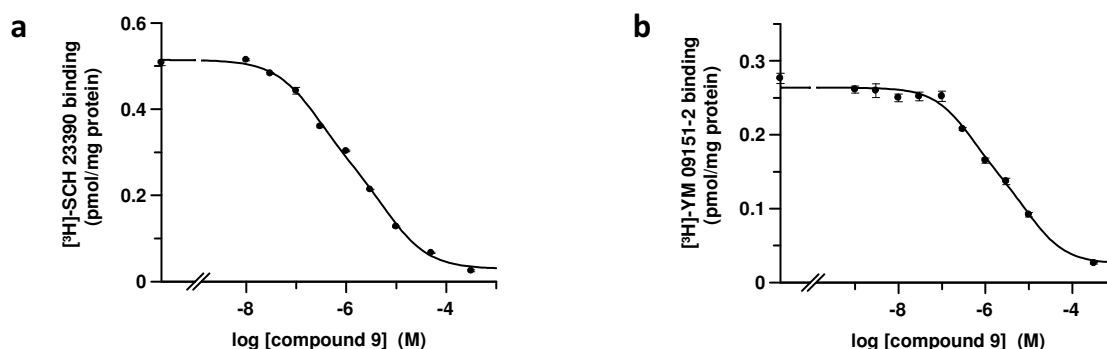


Figure S7. Competition curves of dopamine receptors antagonists binding vs increasing concentrations of compound **9**. Competition experiments of: a) 0.9 nM [³H]-SCH23390 (D₁R antagonist), b) 0.7 nM [³H]-YM09151-2 (D₂R antagonist) with increasing concentrations of ergopeptide **9** were performed in brain striatal membranes (0.5 mg prot/mL) as indicated in the Experimental Section. Data plotted as means ± SEM from a representative experiment (n = 3) performed in triplicate.

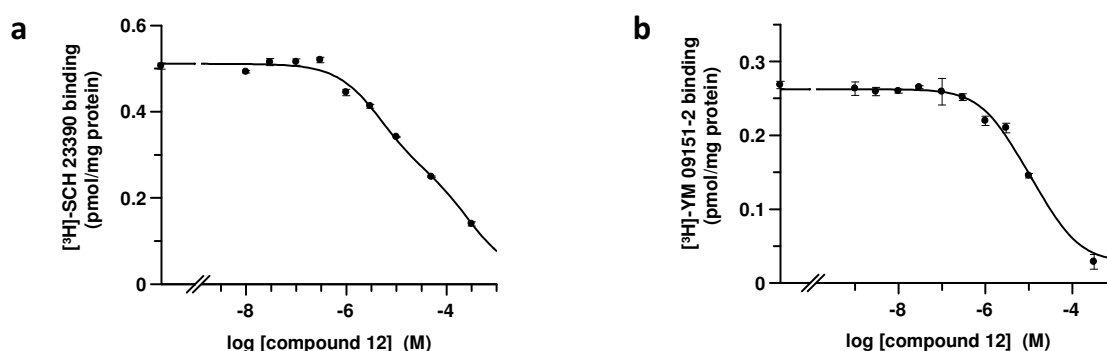


Figure S8. Competition curves of dopamine receptors antagonists binding vs increasing concentrations of compound **12**. Competition experiments of: a) 0.9 nM [³H]-SCH23390 (D₁R antagonist), b) 0.7 nM [³H]-YM09151-2 (D₂R antagonist) with increasing concentrations of ergopeptide **12** were performed in brain striatal membranes (0.5 mg prot/mL) as indicated in the Experimental Section. Data plotted as means ± SEM from a representative experiment (n = 3) performed in triplicate.

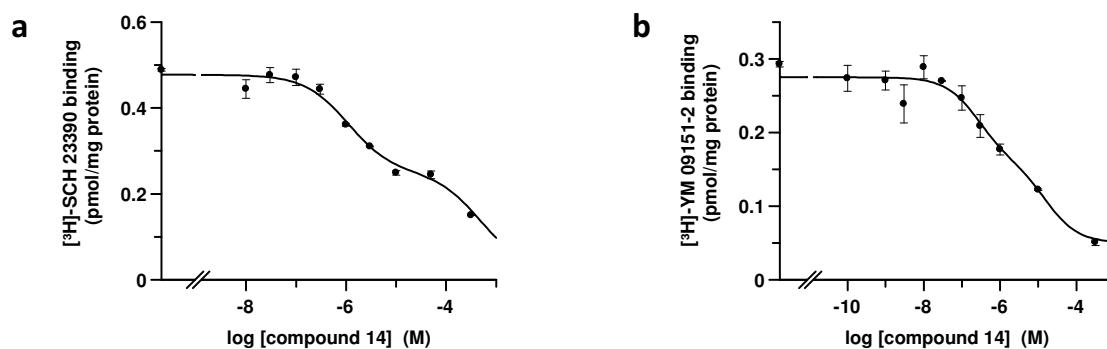


Figure S9. Competition curves of dopamine receptors antagonists binding vs increasing concentrations of compound **14**. Competition experiments of: a) 0.9 nM [³H]-SCH23390 (D₁R antagonist), b) 0.7 nM [³H]-YM09151-2 (D₂R antagonist) with increasing concentrations of ergopeptide **14** were performed in brain striatal membranes (0.5 mg prot/mL) as indicated in the Experimental Section. Data plotted as means ± SEM from a representative experiment (n = 3) performed in triplicate.

6. Affinity of compound 13 at histamine, glutamate, somatostatin and cannabinoid receptors.

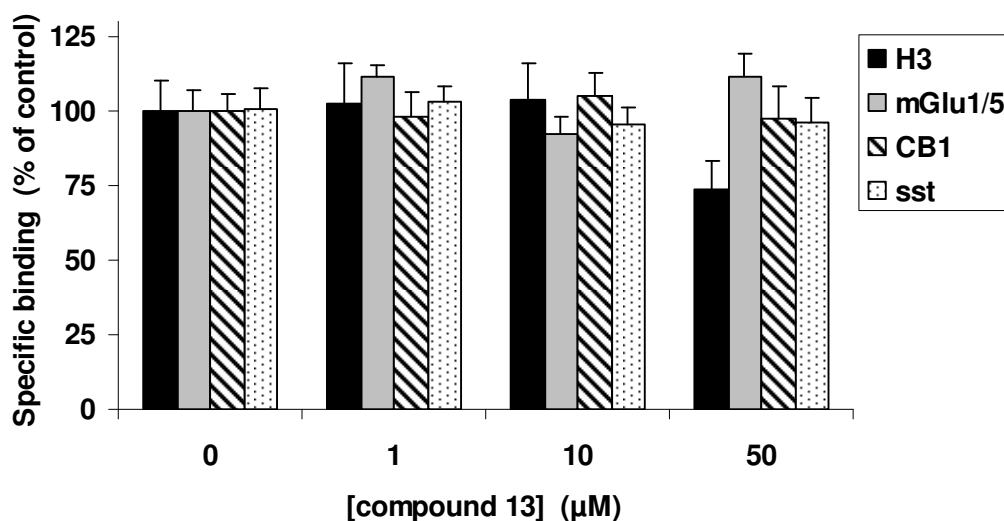


Figure S10. Affinity of compound **13** for different GPCRs. The ability of compound **13** (at different concentrations) to decrease the binding of 2.5 nM [³H]-RAMH (histamine H₃ receptors agonist), 27 nM [³H]-quisqualic acid (metabotropic glutamate 1/5 receptors agonist), 0.1 nM [¹²⁵I]-Tyr¹¹-somatostatin 14 (somatostatin receptors agonist) and 0.5 nM [³H]-CP55940 (cannabinoid CB₁ receptors agonist) was measured as indicated in the Experimental Section. Specific binding as percentage of radioligand binding in the absence of compound **13** was shown. Values are represented by means ± SD (n=3).

La mayoría de los ligandos de GPCR que actúan como agonistas, antagonistas o agonistas inversos, se unen al mismo dominio del receptor reconocido por los agonistas endógenos, es decir, el lugar de unión ortostérico (Neubig et al., 2003). Muchos GPCR poseen sitios alostéricos topográficamente distintos a los ortostéricos. Esto ha llevado a la identificación y estudio de ligandos que actúan como moduladores alostéricos, que pueden regular indirectamente la actividad de los ligandos ortostéricos y/o mediar directamente efectos agonista/antagonista (Christopoulos, 2002). Este es el caso del híbrido *trans*-indoloquinolicidina-péptido 28 (IP28) estudiado en la primera parte de esta Tesis Doctoral en el trabajo titulado *A híbrid indoloquinolizidine peptide as allosteric modulator of dopamine D1 receptor*. Este compuesto es un modulador alostérico de los receptores de dopamina D₁, actuando como ligando ortostérico para los receptores D₂, D₃, D₄ y D₅ de dopamina.

La estructura de los grupos sintéticos indoloquinolicidina les permite interactuar con dominios de transmembrana y presentan una buena solubilidad en medios etanol-acuosos. En este estudio, hemos demostrado que uno de los híbridos indoloquinolicidina-péptido, el IP28, es útil para disminuir la afinidad de los antagonistas del receptor D₁ de dopamina. Se observó que el aumento del valor de K_D para el antagonista en presencia de concentraciones crecientes de IP28 no era lineal, indicando que IP28 se une a un sitio alostérico del receptor. Se efectuaron experimentos de disociación para detectar y cuantificar esta interacción alostérica (May et al., 2007). El método más común para caracterizar este tipo de ligandos son los ensayos de disociación. Se realizaron experimentos de asociación con un ligando ortostérico marcado radioactivamente, en presencia o ausencia del modulador alostérico IP28 y se efectuaron a continuación experimentos de disociación. Estos ensayos demostraron que IP28 es un modulador alostérico que aumenta la velocidad de disociación de un antagonista neutro del receptor D₁ de dopamina y que este efecto era específico para D₁, ya que no se observaron cambios significativos en la velocidad de disociación de un antagonista unido a D₅. A su vez, IP28 no producía cambios en la velocidad de disociación de los antagonistas de los receptores D₂, D₃ o D₄, indicando que este ligando no es un modulador alostérico de la familia *D₂-like*, sino que los resultados sugieren que IP28 actúa como ligando ortostérico de esta familia.

Se define como efecto alostérico positivo aquel cuyo modulador facilita la interacción del ligando ortostérico, y efecto alostérico negativo aquel que inhibe la interacción con el ligando ortostérico (Conn et al., 2009; May et al., 2007; Schwartz y Holst, 2007). Basado en este concepto, el ligando IP28 es un ligando alostérico negativo de D₁, ya que modula negativamente la unión del agonista y del antagonista al sitio ortostérico del receptor. En términos de señalización, es interesante destacar que IP28 induce un aumento del AMPc en células que expresan el receptor D₁ de dopamina. Este resultado sugiere que IP28 podría ser un modulador ago-alostérico, que recientemente han sido definidos como ligandos moduladores alostéricos que inducen un efecto agonista en el receptor (Bridges y Lindsley, 2008; Schwartz y Holst, 2006). Cabe destacar que el efecto del modulador alostérico puede ser positivo con respecto a la eficacia y potencia, pero también puede ser negativo o inhibitorio en términos de, por ejemplo, la potencia, mientras que ser positivo en términos de eficacia (Schwartz y Holst, 2007). Como demostramos en este trabajo, IP28 aumenta el valor de EC₅₀ obtenido de curvas dosis-respuestas de AMPc versus concentraciones crecientes del agonista del receptor D₁. Esto indica que IP28 es un modulador ago-alostérico negativo de la potencia del agonista sin disminuir el efecto máximo mediado por el agonista.

Diversas evidencias sugieren que los antagonistas del receptor D₁ de dopamina juegan un papel esencial por sus las propiedades neurolépticas (Wu et al., 2005; Nielsen y Andersen, 1992) y que este receptor se relaciona directamente con la fisiopatología de la esquizofrenia (Goldman-Rakic, 1999; Sedvall et al., 1999). Estudios pre-clínicos demostraron que los antagonistas selectivos de D₁ presentaban propiedades antipsicóticas, pero estudios clínicos posteriores no demostraron ninguna actividad antipsicótica en incluso, en algunos casos, se agravaban los estados temporales de psicosis (Miyamoto et al., 2005). En contra de estos resultados, la administración de bajas dosis del agonista total del receptor D₁ a primates no-humanos con esquizofrenia demostraron un aumento de las acciones cognitivas (Cai y Arnsten, 1997). El deterioro cognitivo se ha encontrado en todos los subtipos de esquizofrenia. Se cree que la estimulación insuficiente o excesiva de los receptores D₁ es perjudicial para la función cognitiva de la corteza prefrontal, por lo que un nivel óptimo de la activación de D₁ permitiría una función cognitiva normal (Goldman-Rakic et al., 2000). De este modo, la sobreactivación de los receptores D₁ de dopamina pueden aumentar los síntomas

psicóticos en pacientes con esquizofrenia. Se puede especular que los compuestos que disminuyen la potencia y preservan la eficacia de los agonistas del receptor D_1 son dianas prometedoras para la exploración de compuesto moduladores de las patologías psicóticas relacionadas con la dopamina. Es el caso de IP28, que disminuye la afinidad del ligando y la potencia de los receptores, preservando al mismo tiempo la eficacia de los mismos y actuando como un agonista débil por si mismo. A pesar de ello, es necesario diseñar estrategias para mejorar la selectividad y afinidad de la interacción entre el ligando y el receptor, con el fin de poder emplear este tipo de moduladores como fármacos para trastornos psicóticos.

A pesar de una cierta resistencia inicial por parte de la comunidad científica, la existencia de heterómeros entre diversos receptores de neurotransmisores y neuromoduladores es, hoy por hoy, un hecho aceptado. La heteromerización implica cambios en la forma de entender la neurotransmisión. Así, los receptores no pueden considerarse como una única unidad funcional, sino como agregados multimoleculares localizados en el plano de la membrana plasmática (Franco et al., 2003). La heteromerización confiere a los receptores propiedades bioquímicas distintas de los componentes individuales, como cambios en la funcionalidad y en las propiedades farmacológicas (Terrillon y Bouvier, 2004). Los ensayos de doble híbrido, pull-down o coimmunoprecipitación han hecho posible la construcción de mapas de las redes moleculares formadas por interacciones proteína-proteína entre las proteínas citosólicas; sin embargo, estas técnicas se ven limitadas cuando se analizan proteínas de membrana. Recientemente, el desarrollo de las técnicas biofísicas basadas en la transferencia de energía de resonancia, como BRET y FRET han facilitado la demostración de la homodimerización y heterodimerización de proteínas de membrana y especialmente GPCR, en cultivos celulares (Pfleger et al., 2006a; Pfleger et al., 2006b; Milligan et al., 2004; Agnati et al., 2003; Franco et al., 2003; Bouvier et al., 2001). Al iniciarse esta Tesis se sabía que los miembros de una misma familia de receptores de dopamina (*D1-like* y *D2-like*) eran capaces de formar heterómeros entre si, como es el caso del heterómero D_1 - D_3 (Marcellino et al., 2008) pero se desconocía si el receptor D_2 de dopamina podían formar heterómeros con el receptor D_4 y si existían diferencias en la heteromerización de los diferentes polimorfismos de este receptor. A su vez, se desconocía también la implicación y funcionalidad del receptor D_4 de dopamina en el

estriado, así como el motivo de la existencia de los diferentes polimorfismos y su relación con el trastorno de hiperactividad y déficit de atención. Así pues, en el trabajo titulado *Dopamine D₄ receptor, but not the ADHD-associated D_{4.7} variant, forms functional heteromers with the dopamine D₂ receptor in the brain*, se demostró que los receptores de dopamina D_{2S}, D_{4.2} y D_{4.4}, pero no la variante D_{4.7} asociada con el trastorno por déficit de atención y hiperactividad (ADHD), forman heterómeros funcionales en células transfectadas y en cerebro de ratón. La coestimulación de los receptores D_{2S} y D₄ en el heterómero D_{2S}-D₄ tiene un efecto sinérgico en la señalización, hecho que no ocurre en células que expresaban la variante D_{4.7} o en el estriado de ratones mutados (*knock-in*) portadores de la variante de 7 repeticiones (D_{4.7}) en el tercer bucle intracelular del receptor D₄ de dopamina. Estos resultados indican una diferencia funcional de la variante D_{4.7} del receptor D₄ respecto a las variantes D_{4.2} y D_{4.4}, la cual puede tener implicaciones importantes para la comprensión de la patogénesis de ADHD. Se ha demostrado, por primera vez, que las interacciones entre los receptores D_{2S}-D₄ modulan la secreción de glutamato en el estriado, hecho que sugiere que los heterómeros de receptores D_{2S}-D₄ permiten a la dopamina ejercer una modulación más precisa en la neurotransmisión glutamatérgica.

Puesto que la variante D_{4.7} del receptor D₄ de dopamina posee el tercer bucle intracelular (IL3) más largo y es la única variante polimórfica que no forma heterómeros con el receptor D_{2S}, el impedimento estérico de este bucle del receptor D₄ podría ser el mecanismo responsable de obstaculizar la heteromerización, aunque no se pueden descartar otros mecanismos como la intervención de otras proteínas intracelulares en la formación de los heterómeros. Utilizando metodologías de proteómica, se han demostrado interacciones entre receptores de dopamina y DRIPS (*dopamine receptor interacting proteins*), formando complejos de señalización o “*signalplexes*” (Yao et al., 2008; Kabbani y Levenson, 2007). Algunas de las proteínas DRIPS muestran selectividad hacia algunos subtipos de receptores de dopamina. Por ejemplo, la filamina o proteína 4.1N interacciona con los subtipos D₂ y D₃, pero no lo hace con los subtipos D₁, D₅ o D₄ (Binda et al., 2002; Lin et al.; 2001), las proteínas con dominios PDZ, como GIPC (*GAIP interacting protein, C terminus*) interaccionan con los subtipos D₂ y D₃, pero no lo hacen con los subtipos D₄ (Jeanneteau et al., 2004) y la proteína *paralemmin* interacciona exclusivamente con el receptor D₃, pero no con los

receptores D₂ y D₄ (Basile et al., 2006). Todas estas interacciones modulan la especificidad del receptor, el tráfico y la señalización. Las secuencias ricas en prolina del receptor D₄, principalmente localizadas en la región polimórfica del IL3, constituyen los supuestos dominios de unión que, potencialmente, pueden interactuar con proteínas “adaptadoras” como Grb2 y Nck, que no tienen ninguna actividad catalítica conocida pero son capaces de reclutar complejos multiproteicos con el receptor (Rondou et al., 2010). Se podría hipotetizar que las diferencias en el reclutamiento de DRIP's por el receptor D_{4.7} y otras variantes polimórficas podrían influenciar la habilidad del receptor D_{4.7} para formar heterómeros, pero serían necesarios más estudios para corroborar esta hipótesis.

Experimentos previos indicaban que la dopamina en el estriado inhibe la secreción de glutamato activando los receptores D₂, predominantemente el receptor D_{2S} localizado en terminales glutamatérgicas (De Mei et al., 2009; Pontieri et al., 1995). Otros estudios también indicaban que los receptores estriatales postsinápticos, predominantemente el receptor D_{2L}, modulan de forma indirecta la liberación de glutamato por un mecanismo de señalización retrogrado mediado por endocannabinoides (Yin y Lovinger, 2006). Los resultados de esta Tesis indican que los receptores D₄ tienen un papel muy importante en la modulación de la secreción de glutamato en el estriado, muy posiblemente a través de su capacidad para formar heterómeros con receptores D_{2S} presinápticos. Los resultados sugieren que a través de los heterómeros D_{2S}-D₄, la dopamina, a concentraciones bajas, se unirían al receptor D₄, el cual tiene mayor afinidad para la dopamina que el receptor D_{2S} (Rondou et al., 2010), causando un cierto nivel de inhibición en la secreción de glutamato. Sin embargo, a altas concentraciones, la dopamina debería unirse también al receptor D_{2S} y, en estas condiciones, el efecto sinérgico de la interacción de los receptores D_{2S}-D₄ en el heterómero produciría una mayor inhibición de la liberación de glutamato. Por lo tanto, el heterómero de los receptores D_{2S}-D₄ podría actuar a través de un mecanismo dependiente de la concentración de dopamina para establecer dos niveles de control dopaminérgico presináptico sobre la neurotransmisión estriatal glutamatérgica. Dado que la potente modulación sinérgica observada dependen de la heteromerización de los receptores D_{2S}-D₄, la existencia de la variante D_{4.7} implicaría un control más débil de la neurotransmisión glutamatérgica, lo cual podría constituir un mecanismo involucrado en la patogénesis de ADHD. Este hecho también podría explicar en parte los efectos,

hasta estos momentos poco claros, de los psicoestimulantes en ADHD, los cuales amplifican la señalización dopaminérgica y la efectividad de este tipo de tratamientos en pacientes ADHD con la variante D_{4.4} y no así con la variante D_{4.7} (Cheon et al., 2007; Hamarman et al., 2004). Hay que tener en cuenta que la existencia de la variante D_{4.7} no implica que ésta sea la causante de ADHD, sino que puede ser un factor que contribuye a su desarrollo. De hecho, la variante D_{4.7} podría constituir una característica evolutiva exitosa bajo la exposición adecuada al medio (Wang et al., 2004; Ding et al., 2002). El presente estudio aporta un nuevo elemento de interés en el campo de los heterómeros, los cuales son nuevas dianas para el estudio de diferencias funcionales asociadas a polimorfismos de los genes de los receptores acoplados a proteína G.

Otra particularidad del receptor de dopamina D₄ es que es el único receptor dopaminérgico en la glándula pineal de rata (Kim et al., 2010; Bailey et al., 2009; Bai et al., 2008) sin que se conozca cual es su función a pesar de que se expresa de manera circadiana. Por tanto, la glándula pineal de rata es una región del cerebro con gran interés para investigar las características del receptor D₄. Dado que la glándula pineal está bajo el control de los receptores α_{1B} y β_1 adrenérgicos, de cuya activación depende la regulación del ritmo circadiano y la síntesis y liberación de serotonina y melatonina, una posibilidad es que los receptores de dopamina D₄, que en animales no presenta formas polimórficas, puedan modular la función de los receptores adrenérgicos de la glándula pineal mediante un proceso de heteromerización. Esta posibilidad se ha estudiado en esta Tesis y los resultados aparecen en el trabajo “*Circadian-related heteromerization of adrenergic and dopamine D₄ receptors modulates melatonin synthesis and release in the pineal gland*” En este trabajo se ha identificado un nuevo mecanismo que describe como la dopamina regula la función de los receptores adrenérgicos durante el ritmo circadiano. Utilizando diversas técnicas experimentales se ha puesto de manifiesto que: 1) los receptores D₄ de dopamina forman heterómeros con receptores adrenérgicos α_{1B} y β_1 en células transfectadas y en la glándula pineal 2) los heterómeros α_{1B} -D₄ y β_1 -D₄ permiten la modulación inducida por agonistas y antagonistas del receptor D₄ de la activación de MAPK y Akt mediada por agonistas de los receptores adrenérgicos en células transfectadas y en la glándula pineal 3) la síntesis y la liberación de serotonina y melanina, promovida por la estimulación de los receptores adrenérgicos en la glándula pineal, está controlada por el receptor D₄ a través

de la activación de los heterómeros α_{1B} -D₄ y β_1 -D₄, y 4) la modulación de los heterómeros a través de los receptores D₄ depende de los ciclos circadianos de día/noche. Este es el primer ejemplo de la modulación de la heteromerización de receptores dependiente de ritmos circadianos. Todos estos resultados apuntan un nuevo papel del receptor D₄ en la glándula pineal que lleva a la reducción de la función de los receptores α_{1B} y β_1 adrenérgicos a través de una interacción directa receptor-receptor.

Los receptores adrenérgicos son el sostén principal de la función en la glándula pineal. Forman el puente entre la secreción de noradrenalina por las terminaciones nerviosas del sistema nervioso simpático controlado por ritmos circadianos y la producción de melatonina en la glándula pineal. Los receptores adrenérgicos son los responsables de la producción de melatonina a través de varios mecanismos, incluyendo el control de los niveles del precursor serotonina (5-HT) (Zheng y Cole, 2002; Gonzalez-Brito et al., 1990). La dopamina también está presente en los nervios simpáticos aferentes en la glándula pineal, no solo como un precursor de la noradrenalina, sino que también es co-secretada junto con la noradrenalina (Kim et al., 2010). En la glándula pineal de rata la noradrenalina ejerce sus funciones por interacción con los receptores α_{1B} y β_1 y la dopamina actúa activando a los receptores D₄. Hasta este momento no existía ningún indicio de interacción entre los receptores de dopamina y los receptores adrenérgicos. En este trabajo a través de experimentos de BRET en células transfectadas y ensayos de ligación por proximidad (PLA, *proximity ligation assays*) en pinealocitos, se muestran evidencias directas de la formación de heterómeros de los receptores D₄ y receptores α_{1B} y β_1 . La formación de heterómeros α_{1B} -D₄ y β_1 -D₄ en la glándula pineal se ha demostrado, también, por determinación de antagonismo cruzado. Se observó que un antagonista específico del receptor D₄ era capaz de bloquear la señalización mediada por los receptores α_{1B} y β_1 y que los antagonistas de los receptores α_{1B} y β_1 eran también capaces de bloquear la señalización mediada por el receptor D₄. Este es un claro ejemplo del antagonismo cruzado en un heterómero de receptores que ya había sido detectado para otros heterómeros (Carriba et al., 2008). Ya que por definición un antagonista es incapaz de inducir la señalización intracelular, la forma más sencilla de explicar el efecto de un antagonista del receptor D₄ sobre los receptores α_{1B} y β_1 y viceversa, es a través de una interacción proteína-proteína directa entre ambos receptores.

Las consecuencias funcionales de la heteromerización es que se establece una interacción negativa cuando los dos receptores del heterómero se co-activan. En los heterómeros α_{1B} -D₄ y β_1 -D₄, se produce la inhibición de fosforilación de ERK 1/2 y el bloqueo total de la fosforilación de Akt inducidas por los agonistas adrenérgicos en presencia agonistas del receptor D₄. El hecho de que el receptor D₄ pueda modificar la señalización de los receptores α_{1B} y β_1 adrenérgicos es particularmente interesante ya que la expresión del receptor D₄ está regulada por el incremento de los niveles de norepinefrina (Kim et al., 2010). El mecanismo que describimos puede representar una inhibición por retroceso (*feedback* negativo) donde el incremento de la expresión del receptor D₄ a través de la señalización adrenérgica da lugar a un incremento de la formación de heterómeros α_{1B} -D₄ y β_1 -D₄, los cuales inhiben la señalización mediada por los receptores adrenérgicos a través de la interacción negativa descrita anteriormente.

La expresión de ARNm del receptor D₄ en la glándula pineal está estrictamente regulada y alcanza sus máximos niveles en la última parte del período oscuro y no se expresa en medio del periodo de luz (Kim et al., 2010). Se ha observado que el receptor D₄ se expresa y es funcional en la glándula pineal cuando ésta se disecciona en las primeras horas del período de luz, y no se observó actividad ni expresión cuando las glándulas pineales fueron aisladas al final del período de luz. Ello significa que la modulación ejercida por el receptor D₄ está bajo control circadiano.

Se han estudiado las consecuencias metabólicas de la activación de los heterómeros α_{1B} -D₄ y β_1 -D₄ en la síntesis y secreción de 5-HT y de melatonina. Los niveles de 5-HT incrementan durante el día, mientras que los niveles de melatonina fluctúan de manera opuesta, incrementando su producción y secreción por la noche a través de la activación del enzima AANAT, enzima involucrada en las últimas etapas de su síntesis. A través de la acción de masas, cambios significativos en la actividad de AANAT por la noche pueden reducir rápidamente los niveles de 5-HT (Klein et al., 1997). Es importante destacar que la síntesis de 5-HT parece ocurrir tanto durante el día como durante la noche, y la síntesis y secreción nocturna de 5-HT es necesaria para la síntesis de melatonina a través de la estimulación adrenérgica (Simonneaux y Ribelayga, 2003; Miguez et al., 1997). La 5-HT extracelular es absorbida por terminaciones nerviosas simpáticas o se une a los receptores 5HT_{2C} de la glándula

pineal, que a su vez puede llevar a un incremento de la síntesis y secreción de melatonina (Miguez et al., 1997; Sugden, 1990). Hasta este momento no está completamente claro como se limitan los niveles de producción de 5-HT y melatonina durante los ciclos día/noche. Nuestros datos sugieren que los heterómeros α_{1B} -D₄ y β_1 -D₄ pueden jugar un papel importante en este proceso.

Cuando las glándulas pineales aisladas al final del período de luz, cuando la expresión del receptor D₄ es inapreciable, se trataron con ligandos adrenérgicos, se produjo un incremento en la síntesis de 5-HT y un gran incremento en la síntesis de melatonina mediadas por el receptor β_1 y se detectó, también, un incremento en la secreción de 5-HT y un gran incremento en la secreción de melatonina mediadas por el receptor α_{1B} . En este caso cabe destacar que ni la síntesis ni la secreción fueron bloqueadas por el tratamiento simultáneo con el agonista del receptor D₄ cuya expresión en estas condiciones es muy baja. Por el contrario, se observó que el incremento en la síntesis de 5-HT y de melatonina mediado por los receptores β_1 y la secreción mediada por los receptores α_{1B} al tratar con ligandos adrenérgicos glándulas pineales, aisladas al principio del período de luz, cuando el receptor D₄ se expresa, se inhibían drásticamente al tratar las glándulas pineales simultáneamente con un agonista del receptor D₄. Este bloqueo podría ser debido a una interacción negativa a nivel de señalización. Sin embargo, se ha visto que un antagonista del receptor D₄ también produce inhibición tanto de la síntesis como de la secreción de 5-HT y melatonina mediada por los receptores adrenérgicos. Puesto que los antagonistas no pueden por si solos señalar, este bloqueo debe ser causado por una interacción proteína-proteína a través de los heterómeros α_{1B} -D₄ y β_1 -D₄.

El conjunto de resultados muestra que la dopamina parece que es capaz de regular los niveles de 5-HT y de melatonina. Esto sugiere que la dopamina, a través de los heterómeros α_{1B} -D₄ y β_1 -D₄, puede servir como molécula reguladora para reducir la cantidad de melatonina sintetizada y secretada al principio del período de luz. Al anochecer, la ausencia de receptores D₄ favorecería la síntesis de 5-HT y una rápida transformación de serotonina en melatonina inducida por la noradrenalina. La progresiva aparición de mRNA durante la noche y el incremento de la expresión del receptor D₄ al amanecer facilitarían el bloqueo de la síntesis y liberación de la melatonina. Durante el día, los receptores D₄ “desaparecerían” de la membrana, por lo

que los heterómeros α_{1B} -D₄ y β_1 -D₄ tampoco se formarían. La actividad AANAT también disminuiría, y los niveles de 5-HT incrementarían gradualmente y el ciclo se repetiría. Estos resultados ponen de manifiesto el papel del receptor D₄ en la glándula pineal y abren una nueva área de investigación sobre el papel de los heterómeros entre receptores de dopamina y noradrenalina para mantener las señales de los ritmos circadianos de la glándula pineal.

Tal como se ha discutido anteriormente, a lo largo de esta Tesis se ha puesto de manifiesto la importancia funcional de la formación de heterómeros en los que intervienen receptores de dopamina. Una evidencia destacada en estos estudios es la necesidad de investigar la presencia de estos heterómeros en tejidos nativos. Si bien técnicas biofísicas de transferencia de energía como BRET, FRET o sus derivados han permitido detectar la formación de heterómeros entre receptores acoplados a proteína G en cultivos celulares, es difícil la aplicación de estas técnicas para determinar la expresión de heterómeros en tejidos nativos. Por ello la búsqueda de ligandos específicos para receptores que puedan acoplarse a un sistema de transferencia de energía es de gran interés. En la parte final de esta Tesis y concretamente en el trabajo titulado *Biotin Ergopeptide Probes for Dopamine Receptors* se abordó el desarrollo y caracterización de ergopéptidos biotinilados como herramientas para el estudio de heterómeros que contengan receptores de dopamina.

En trabajos previos nuestro grupo de investigación había caracterizado una familia de ergopéptidos como moléculas con elevada afinidad para los receptores D₁ y D₂ de dopamina (Vendrell et al., 2007). Se utilizaron estos compuestos como base para obtener los derivados biotinilados. Ello requirió la incorporación de modificaciones químicas en la estructura que actuaran como brazo espaciador entre el ergopéptido y la biotina. La incorporación de modificaciones químicas en estructuras con actividad biológica siempre conlleva la dificultad de preservar la afinidad del ligando nativo. En este trabajo, basándonos en aproximaciones de química combinatoria, se sintetizó un conjunto de brazos espaciadores mediante síntesis en fase sólida, los que se incorporaron al extremo C-terminal del ergopéptido sintético por un lado y a una molécula de biotina en el otro extremo. Entre los diferentes compuestos sintetizados, se seleccionó el compuesto 13 por sus características farmacológicas y su afinidad por los

receptores D₁ y D₂ de dopamina, la cual fue examinada mediante experimentos de competición con ligandos radiomarcados de los receptores presentes membranas estriatales y concentraciones crecientes de los diferentes compuestos.

El compuesto 13 mantuvo la afinidad en el rango de nanomolar por ambos receptores. A su vez, se determinó la afinidad del compuesto 13 por los receptores de histamina H₃, metabotrópico de glutamato 5, somatostatina SST y canabinoide CB₁, observándose que este compuesto no se producía un decremento de la unión del ligando radioactivo a altas concentraciones, indicando de esta forma que el compuesto 13 tiene muy baja afinidad por estos receptores comparado con los receptores de dopamina y mostrando, por lo tanto, su especificidad.

El compuesto 13 conservó la naturaleza de agonista del ergopéptido inicial. En células transfectadas separadamente con los receptores D₁ y D₂, se observó un incremento de la fosforilación de ERK1/2 de forma dosis-dependiente, que era similar a la activación por el agonista total de D₁ (SKF 81297) o de D₂ (Quinpirole). Este efecto mediado por el compuesto 13 era revertido cuando las células se preincubaban con el antagonista selectivo de D₁ (SCH 23390) o de D₂ (Raclopride), demostrando así que este compuesto actúa como agonista para los receptores D₁ y D₂. Al analizar la señalización estudiando la vía de la Akt (PKB) se observó que el compuesto 13 producía un decremento de la fosforilación de Akt dosis-dependiente similar a los agonistas totales SKF 81297 y quinpirole, efecto ya observado en neuronas de cerebro de ratón (Beaulieu et al., 2007; Levant et al., 1992). Este efecto también era revertido mediante la preincubación con los antagonistas SCH 23390 y raclopride, confirmando la naturaleza agonista del compuesto 13.

Considerando la abundancia relativa de los receptores de dopamina en el cerebro, se analizó también la afinidad del compuesto 13 para el receptor D₃ de dopamina mediante experimentos de competición en membranas de células CHO transfectadas con receptor D₃. Se obtuvo un desplazamiento del ligando marcado radioactivamente que indicaba que este compuesto se unía con alta afinidad a D₃. Dado que la biotina puede acoplarse a diversos compuestos que pueden actuar como dadores o aceptores para la transferencia de energía, el compuesto 13 puede ser útil para el estudio de homómeros de receptores de dopamina o heterómeros que contengan los

receptores D₁, D₂ o D₃ de dopamina si se dispone del ligando para el otro receptor del heterómero que pueda actuar como dador o aceptor de transferencia de energía.

*« Comprendiendo su mala voluntad, les dijo Jesús:
-¡Hipócritas!, ¿por qué me tentáis? Enseñadme la
moneda del impuesto.*

Le presentaron un denario. Él les preguntó:

-¿De quién son esta cara y esta inscripción?

Le respondieron:

-Del César.

Entonces les replicó:

*-Pues devolvedle al César lo que es del César y a
Dios lo que es de Dios»*

Mateo 22, 15-21

CONCLUSIONES

AGTCATCGTCGTACGTACGATCGTACTTACAGTAAAATCTGTAGCAGCTAGCATGTTTTCTTTGGGTGTACGATCGACTGATCGTGCATACTACGATCACTACTACGGCGA

V. CONCLUSIONES

Conclusiones que hacen referencia al primer Objetivo General de esta Tesis (*Caracterizar ligandos alostéricos para receptores de dopamina de la familia D₁-like*):

- **El ligando indoloquinolicidina-péptido 28 es un modulador alostérico negativo de la unión de agonistas y antagonistas a receptores de dopamina D₁ estriatales. Este compuesto es un modulador ago-alostérico ya que se comporta como un agonista parcial del receptor D₁ a la vez que disminuye la potencia, mientras que preserva la eficacia, de la señalización inducida por agonistas ortostéricos. Estas características le confieren cierto potencial terapéutico en enfermedades psicóticas.**

Conclusiones que hacen referencia al segundo Objetivo General de esta Tesis (*Investigar si distintos receptores de dopamina de la familia D₂-like pueden modular su función mediante un proceso de heteromerización*):

- **La formas polimórficas D_{4.2} y D_{4.4} del receptor D₄ de dopamina humano, pero no la forma polimérica D_{4.7} asociada a ADHD, forman heterómeros con el receptor D_{2s} de dopamina en células transfectadas y en el tejido estriatal.**

- **La co-activación de los receptores D_{2s} y D₄ en los heterómeros tiene un efecto sinérgico en la activación de la vía de las MAPK que puede ser detectado en células transfectadas y en el tejido estriatal, pero no se detecta en células que expresan receptores D_{2s} y la forma polimórfica D_{4.7} ni tampoco en el estriado de ratones transgénicos que expresan un receptor D₄ con el 3IL correspondiente al receptor D_{4.7} humano**

- **La interacción entre receptores D_{2s} y D₄ modula la liberación de glutamato en el estriado. La activación de los receptores D_{2s} potencia la inhibición de la liberación de glutamato inducida por la activación de los receptores D₄, lo que sugiere que la dopamina, a través de los heterómeros D_{2s}-D₄ ejerce una modulación muy precisa de la neurotransmisión glutamatérgica estriatal.**

Conclusiones que hacen referencia al tercer Objetivo General de esta Tesis (*Investigar si los receptores de dopamina D₄ pueden modular la función de los receptores adrenérgicos de la glándula pineal mediante un proceso de heteromerización*):

- **Los receptores D₄ de dopamina forman heterómeros con los receptores α_{1B} y β_1 adrenérgicos en células transfectadas y en la glándula pineal de rata. En la glándula pineal, la formación de estos heterómeros está regulada por el ritmo circadiano**
- **La unión de agonistas o antagonistas del receptor de dopamina D₄ a los heterómeros α_{1B} -D₄ y β_1 -D₄ inhibe la fosforilación de ERK 1/2 y Akt/PKB inducida por los agonistas de los receptores adrenérgicos. En el caso de los agonistas del receptor D₄ es un cross-talk negativo y en el caso de los antagonistas del receptor D₄ es un proceso de antagonismo cruzado.**
- **La activación de los receptores D₄ en los heterómeros α_{1B} -D₄ y β_1 -D₄ inhibe la síntesis y liberación de serotonina y melatonina mediada por la activación de los receptores adrenérgicos, por lo que los receptores D₄ en la glándula pineal modulan negativamente y de manera circadiana la funcionalidad de los receptores adrenérgicos en la vía metabólica de la síntesis de melatonina.**

Conclusiones que hacen referencia al cuarto Objetivo General de esta Tesis (*Contribuir al desarrollo de ligandos para receptores de dopamina útiles para detectar heterómeros que contengan estos receptores*):

- **Se ha obtenido una quimioteca de ergopéptidos unidos a biotina mediante un espaciador apropiado que conservan sus propiedades como ligandos de receptores de dopamina. Se ha identificado el compuesto 13 como un agonista de elevada afinidad para los receptores D₁, D₂ y D₃ de dopamina siendo una herramienta útil para estudiar heterómeros que contengan estos receptores.**

*« Sans la liberté de blâmer, il n'est point
d'éloge flatteur. »*

Le mariage de Figaro. Beaumarchais. 1778

BIBLIOGRAFÍA

AGTCATCGTCGTACGTACGATCGTACTTACAGTAAAATCTGTAGCAGCTAGCATGTTTTCTTTGGGTGTACGATCGACTGATCGTGCATACTACGATCACTACTACGGCGA

VI. BIBLIOGRAFÍA

Abbas A, Raju J, Milles J, Ramachandran S. (2010). A circadian rhythm sleep disorder: melatonin resets the biological clock. *J. R. Coll Physicians Edinb.* 40(4):311-3.

AbdAlla S, Lothar H and Quitterer U. (2000). AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature* 407:94-98.

Abi-Dargham A. (2004). Do we still believe in the dopamine hypothesis? New data bring new evidence. *Int J Neuropsychopharmacol.* 7 Suppl 1:S1-5.

Adams M. R., Brandon E. P., Chartoff E. H., Idzerda R. L., Dorsa D. M. and McKnight G. S. (1997). Loss of haloperidol induced gene expression and catalepsy in protein kinase A-deficiente mice. *Proc. Natl. Acad. Sci. USA.* 94:12157-12161.

Adler LA, Chua HC. (2002). Management of ADHD in adults. *J Clin Psychiatry.* 63 Suppl 12:29-35.

Agnati LF, Ferre S., Lluís C., Franco R. and Fuxe K. (2003). Molecular mechanisms and therapeutical implications of intramembrane receptor/receptor interactions among heptahelical receptors with examples from the striatopallidal GABA neurons, *Pharmacol Rev.* 55(3):509-50.

Akimoto Y, Horinouchi T, Shibano M, Matsushita M, Yamashita Y, Okamoto T, Yamaki F, Tanaka Y, Koike K. (2002). Nitric oxide (NO) primarily accounts for endothelium-dependent component of beta-adrenoceptor-activated smooth muscle relaxation of mouse aorta in response to isoprenaline. *J Smooth Muscle Res.* 38(4-5):87-99.

Alexander GE and Crutcher MD. (1990). Functional architecture of basal ganglia circuits: neural substrates of parallel processing. *Trends Neurosci* 13:266-271.

Almeida J, Mengod G. (2010). D2 and D4 dopamine receptor mRNA distribution in pyramidal neurons and GABAergic subpopulations in monkey prefrontal cortex: implications for schizophrenia treatment. *Neuroscience.* 170(4):1133-9.

Amara S. G. and Kuhar M. J. (1993). Neurotransmitter transporters: recent progress. *Annu. Rev. Neurosci.* 16:73-93.

AMERICAN ACADEMY OF PEDIATRICS. Clinical Practice Guideline: Treatment of the School-Aged Child With Attention-Deficit/Hyperactivity Disorder. *PEDIATRICS* Vol. 108 No. 4 October 2001.

Angers S, Salahpour A, Joly E, Hilaiet S, Chelsky D, Dennis M and Bouvier M. (2000). Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc Natl Acad Sci U S A* 97:3684-3689.

Antshel KM, Hargrave TM, Simonescu M, Kaul P, Hendricks K, Faraone SV. (2011). Advances in understanding and treating ADHD. *BMC Med.* 9:72.

Arendt J. (2005). Melatonin: characteristics, concerns, and prospects. *J Biol Rhythms.* 20(4):291-303.

Asghari V, Sanyal S, Buchwaldt S, Paterson A, Jovanovic V, Van Tol HH. (1995). Modulation of intracellular cyclic AMP levels by different human dopamine D4 receptor variants. *J Neurochem.* 65(3):1157-65.

Aston-Jones G. (2005a) Brain structures and receptors involved in alertness. *Sleep Med.* 6 Suppl 1:S3-7.

Aston-Jones G, Cohen JD. (2005b). Adaptive gain and the role of the locus coeruleus-norepinephrine system in optimal performance. *J Comp Neurol.* 493(1):99-110.

Axelrod J. (1970). The pineal gland. *Endeavour.* 29(108):144-8.

Axelrod J. (1972). Dopamine-*-*hydroxylase: regulation of its synthesis and release from nerve terminals. *Pharmacol Rev.* 24(2):233-43.

Axelrod J, Weinshilboum R. (1972). Catecholamines. *N Engl J Med.* 287(5):237-42.

Bai L, Zimmer S, Rickes O, Rohleder N, Holthues H, Engel L, Leube R, Spessert R. (2008). Daily oscillation of gene expression in the retina is phase-advanced with respect to the pineal gland. *Brain Res.* 1203:89-96.

Bai M, Trivedi S and Brown EM. (1998). Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. *J Biol Chem* 273:23605-23610.

Bailey MJ, Coon SL, Carter DA, Humphries A, Kim JS, Shi Q, Gaildrat P, Morin F, Ganguly S, Hogenesch JB, Weller JL, Rath MF, Møller M, Baler R, Sugden D, Rangel ZG, Munson PJ, Klein DC. (2009). Night/day changes in pineal expression of >600 genes: central role of adrenergic/cAMP signaling. *J Biol Chem.* 284(12):7606-22.

Ballesteros J and Ransom J. (2006). In-Target versus Off-Target allosteric modulators of GPCRs. *Drug Discovery Today.* 3:445-450.

- Baneres JL and Parello J. (2003). Structure-based analysis of GPCR function: evidence for a novel pentameric assembly between the dimeric leukotriene B4 receptor BLT1 and the G-protein. *J Mol Biol* 329:815-829.
- Banihashemi B. and Albert P. R. (2002). Dopamine-D2S receptor inhibition of calcium influx, adenylyl cyclase, and mitogen-activated protein kinase in pituitary cells: distinct Galpha and Gbetagamma requirements. *Mol. Endocrinol.* 16:2393-2404.
- Barishpolets VV, Fedotova IuO, Saprionov NS. (2009). Structural and functional organization of the cerebral dopaminergic system. *Eksp Klin Farmakol.* 72(3):44-9.
- Basile M, Lin R, Kabbani N, Karpa K, Kilimann M, Simpson I. (2006). Paralemmin interacts with D3 dopamine receptors: implications for membrane localization and cAMP signaling. *Arch Biochem Biophys.* 446: 60–68.
- Beaudry P., Fontaine R., Chouinard G. and Annable L. (1986). Clonazepam in the treatment of patients with recurrent panic attacks. *J. Clin. Psychiatry.* 47(2):83-85.
- Beaulieu JM, Gainetdinov RR. (2011). The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol Rev.* 63(1):182-217.
- Beaulieu JM, Tirotta E, Sotnikova TD, Masri B, Salahpour A, Gainetdinov RR, Borrelli E, Caron MG. (2007). Regulation of Akt signaling by D2 and D3 dopamine receptors in vivo. *J Neurosci.* 27(4):881-5.
- Benkirane M, Jin DY, Chun RF, Koup RA and Jeang KT. (1997). Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. *J Biol Chem* 272:30603-30606.
- Beresewicz M. (2007). Scaffold proteins (MAGUK, Shank and Homer) in postsynaptic density in the central nervous system. *Postepy Biochem.* 53(2):188-97.
- Berridge MJ. (2009). Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta.* 1793(6):933-40.
- Binda AV, Kabbani N, Lin R, Levenson R. (2002). D2 and D3 dopamine receptor cell surface localization mediated by interaction with protein 4.1N. *Mol Pharmacol.* 62: 507–513.
- Birdsall NJ. (2010). Class A GPCR heterodimers: evidence from binding studies. *Trends Pharmacol Sci.* 31(11):499-508.
- Björklund A. and Lindvall O. (1984). In handbook of chemical Neuroanatomy. Björklund, A. and Hökfelt

T. Eds.; Elsevier, Amsterdam. Vol. 2:55-122.

Bohm SK, Grady EF and Bunnett NW. (1997). Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *Biochem J.* 322:1- 18.

Bonanomi D, Chivatakarn O, Bai G, Abdesselem H, Lettieri K, Marquardt T, Pierchala BA, Pfaff SL. (2012). Ret Is a Multifunctional Coreceptor that Integrates Diffusible- and Contact- Axon Guidance Signals. *Cell.* 148(3):568-82.

Borjigin J, Deng J. (2000). Madame Curie Bioscience Database. Editado por Landes Bioscience. Austin Texas. EEUU.

Borjigin J, Li X, Snyder SH. (1999). The pineal gland and melatonin: molecular and pharmacologic regulation. *Annu Rev Pharmacol Toxicol.* 39:53-65.

Borroto-Escuela DO, Romero-Fernandez W, Tarakanov AO, Ciruela F, Agnati LF, Fuxe K. (2011). On the existence of a possible A2A-D2- β -Arrestin2 complex: A2A agonist modulation of D2 agonist-induced β -arrestin2 recruitment. *J Mol Biol.* 406(5):687-99.

Boulay F and Rabiet MJ (2005) The chemoattractant receptors FPR and C5aR: same functions--different fates. *Traffic* 6:83-86.

Bourne HR, Sanders DA and McCormick F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature* 349:117-127.

Bouvier M (2001) Oligomerization of G-protein-coupled transmitter receptors. *Nat Rev Neurosci* 2:274-286.

Bouvier M, Heveker N, Jockers R, Marullo S, Milligan G. (2007). BRET analysis of GPCR oligomerization: newer does not mean better. *Nat Methods.* 4(1):3-4.

Brami-Cherrier K., Valjent E., Garcia M., Pages C., Hipskind R. A. and Caboche J. (2002). Dopamine induces a PI3-kinase-independent activation of Akt in striatal neurons: a new route to cAMP response element-binding protein phosphorylation. *J. Neurosci.* 22:8911-8921.

Bridges TM, Lindsley CW. (2008). G-protein-coupled receptors: from classical modes of modulation to allosteric mechanisms. *ACS Chem Biol.* 3(9):530-41.

Bruneau EG, Esteban JA, Akaaboune M. (2009). Receptor-associated proteins and synaptic plasticity. *FASEB J.* 23(3):679-88.

Buccafusco J.J. and Terry A.V. (2000). Multiple central nervous system targets for eliciting beneficial effects on memory and cognition. *J. Pharmacol Exp. Ther.* 295:438-446.

Bunzow J. R., Van Tol H. H., Grnady D. K., Albert P., Salon J., Christie M., Machida C. A., Neve K. A. and Civelli O. (1988). Cloning and expression of a rat D2 dopamine receptor cDNA. *Nature* 336:783-787.

Burgueño J., Blake D. J., Benson M. A., Tinsley C. L., Esapa C. T., Canela E. I., Penela P., Mallol J., Mayor F. Jr., Lluís C., Franco R. and Ciruela F. (2003a). The adenosine A2A receptor interacts with the actin-binding protein alpha-actinin. *J. Biol. Chem.* 278:37545- 37552.

Burgueño J, Enrich C, Canela EI, Mallol J, Lluís C, Franco R, Ciruela F. (2003b). Metabotropic glutamate type 1alpha receptor localizes in low-density caveolin-rich plasma membrane fractions. *J Neurochem.* 86(4):785-91.

Burgueño J, Franco R and Ciruela F. (2007). Antidepressants, Antipsychotics and Anxiolytics. *Neurobiological Aspects of Attention Deficit and Hyperactivity Disorders.* Volum. 2. Edited by H. Buschmann, J.L. Díaz, J. Holenz, A. Párraga, A. Torrens and J.M. Vela. Wiley-VCF.

Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, Molinoff PB, Ruffolo RR Jr, Trendelenburg U. (1994). International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol Rev.* 46(2):121-36.

Cabello N, Remelli R, Canela L, Soriguera A, Mallol J, Canela EI, Robbins MJ, Lluís C, Franco R, McIlhinney RA, Ciruela F. (2007). Actin-binding protein alpha-actinin-1 interacts with the metabotropic glutamate receptor type 5b and modulates the cell surface expression and function of the receptor. *J Biol Chem.* 20;282(16):12143-53.

Cai G., Zhen X., Uryu K. and Friedman E. (2000). Activation of extracellular signal-regulated protein kinases is associated with a sensitized locomotor response to D(2) dopamine receptor stimulation in unilateral 6-hydroxydopamine-lesioned rats. *J. Neurosci.* 20:1849-1857.

Cai JX, Arnsten AF. (1997). Dose-dependent effects of the dopamine D1 receptor agonists A77636 or SKF81297 on spatial working memory in aged monkeys. *J Pharmacol Exp Ther.* 283(1):183-9.

Canals M, Burgueno J, Marcellino D, Cabello N, Canela EI, Mallol J, Agnati L, Ferre S, Bouvier M, Fuxe K, Ciruela F, Lluís C and Franco R. (2004). Homodimerization of adenosine A2A receptors: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J*

Neurochem 88:726-734.

Canals M, Marcellino D, Fanelli F, Ciruela F, de Benedetti P, Goldberg SR, Neve K, Fuxe K, Agnati LF, Woods AS, Ferre S, Lluís C, Bouvier M, Franco R. (2003). Adenosine A2A-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J Biol Chem* 278:46741-46749.

Carmena M, Pinson X, Platani M, Salloum Z, Xu Z, Clark A, Macisaac F, Ogawa H, Eggert U, Glover DM, Archambault V, Earnshaw WC. (2012). The chromosomal passenger complex activates polo kinase at centromeres. *PLoS Biol.* 10(1):e1001250.

Carriba P, Navarro G, Ciruela F, Ferré S, Casadó V, Agnati L, Cortés A, Mallol J, Fuxe K, Canela EI, Lluís C, Franco R. (2008) Detection of heteromerization of more than two proteins by sequential BRET-FRET. *Nat Methods* 5: 727–733.

Carriba P, Ortiz O, Patkar K, Justinova Z, Stroik J, Themann A, Muller C, Woods AS, Hope BT, Ciruela F, Casado V, Canela EI, Lluís C, Goldberg SR, Moratalla R, Franco R and Ferre S. (2007). Striatal adenosine A2A and cannabinoid CB1 receptors form functional heteromeric complexes that mediate the motor effects of cannabinoids. *Neuropsychopharmacology* 32:2249- 2259.

Carte ET, Nigg JT, Hinshaw SP. (1996). Neuropsychological functioning, motor speed, and language processing in boys with and without ADHD. *J Abnorm Child Psychol.* 24(4):481-98.

Casadó V, Cortés A, Ciruela F, Mallol J, Ferré S, Lluís C, Canela EI, Franco R. (2007). Old and new ways to calculate the affinity of agonists and antagonists interacting with G-protein-coupled monomeric and dimeric receptors: the receptor-dimer cooperativity index. *Pharmacol Ther.* 116(3):343-54.

Casadó V, Cortés A, Mallol J, Pérez-Capote K, Ferré S, Lluís C, Franco R, Canela EI. (2009). GPCR homomers and heteromers: a better choice as targets for drug development than GPCR monomers? *Pharmacol Ther.* 124(2):248-57.

Castelleti L., Memo M., Missale C., Spano P. F and Valerio A. (1989). Potassium channels in the transduction mechanism of dopamine D2 receptors in rat lactotrophs. *J. Physiol.* 410:256-265.

Cavalli A, Bolognesi ML, Minarini A, Rosini M, Tumiatti V, Recanatini M, Melchiorre C. (2008) (Multi-target-directed ligands to combat neurodegenerative diseases. *J Med Chem.* 14;51(3):347-72.

Centonze D, Grande C, Saulle E, Martin AB, Gubellini P, Pavon N, Pisani A, Bernardi G, Moratalla R and Calabresi P. (2003). Distinct roles of D1 and D5 dopamine receptors in motor activity and striatal synaptic plasticity. *J Neurosci* 23:8506-8512.

- Centonze D., Usiello A., Gubellini P., Pisani A., Borrelli E., Bernardi G. and Calabresi P. (2002). Dopamine D2 receptor-mediated inhibition of dopaminergic neurons in mice lacking D2L receptors. *Neuropsychopharmacol.* 27:723-726.
- Chen JC, Chen PC and Chiang YC. (2009). Molecular mechanisms of psychostimulant addiction. *Chang Gung Med J* 32:148-154.
- Cheon KA, Kim BN, Cho SC. (2007). Association of 4-repeat allele of the dopamine D4 receptor gene exon III polymorphism and response to methylphenidate treatment in Korean ADHD children. *Neuropsychopharmacology.* 32: 1377–1383.
- Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK and Stevens RC. (2007). High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* 318:1258-1265.
- Chien EY, Liu W, Zhao Q, Katritch V, Han GW, Hanson MA, Shi L, Newman AH, Javitch JA, Cherezov V, Stevens RC. (2010) Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. *Science.* 330(6007):1091-5.
- Chio CL, Drong RF, Riley DT, Gill GS, Slightom JL, Huff RM. (1994). D4 dopamine receptor-mediated signaling events determined in transfected Chinese hamster ovary cells. *J Biol Chem.* 269(16):11813-9.
- Christopoulos A. (2002). Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat Rev Drug Discov.* 1(3):198-210.
- Chun M, Liyanage UK, Lisanti MP and Lodish HF. (1994). Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc Natl Acad Sci U S A* 91:11728-11732.
- Ciruela F, Albergaria C, Soriano A, Cuffi L, Carbonell L, Sánchez S, Gandía J, Fernández-Dueñas V. (2010). Adenosine receptors interacting proteins (ARIPs): Behind the biology of adenosine signaling. *Biochim Biophys Acta.* 1798(1):9-20.
- Ciruela F, Burgueno J, Casado V, Canals M, Marcellino D, Goldberg SR, Bader M, Fuxe K, Agnati LF, Lluís C, Franco R, Ferre S and Woods AS. (2004). Combining mass spectrometry and pull-down techniques for the study of receptor heteromerization. Direct epitope-epitope electrostatic interactions between adenosine A2A and dopamine D2 receptors. *Anal Chem* 76:5354-5363.
- Ciruela F., Franco R and Burgueño J. (2007). Antidepressants, Antipsychotics and Anxiolytics.

Pharmacology: Targets of Drug Actions. Attention Deficit and Hyper-activity Disorders. Volum. 2. Edited by H. Buschmann, J.L. Díaz, J. Holenz, A. Párraga, A. Torrens and J.M. Vela. Wiley-VCF.

Civelli O, Bunzow JR and Grandy DK. (1993). Molecular diversity of the dopamine receptors. *Annu Rev Pharmacol Toxicol* 33:281-307.

Clifford JJ, Waddington JL. (2000). Topographically based search for an "Ethogram" among a series of novel D(4) dopamine receptor agonists and antagonists. *Neuropsychopharmacology*. 22(5):538-44.

Conn PJ, Christopoulos A and Lindsley CW. (2009). Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat. Rev. Drug. Discov.* 8:41-54.

Cooper J. R., Bloom F. E. and Roth R. H. (1996). *The biochemical basis of neuropharmacology*. 7th Ed. New York/Oxford, Oxford University Press 293-351.

Costa T and Herz A (1989) Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci U S A* 86:7321-7325.

Cotecchia S. (2010). The α 1-adrenergic receptors: diversity of signaling networks and regulation. *J Recept Signal Transduct Res*. 30(6):410-9.

Curran S, Mill J, Tahir E, Kent L, Richards S, Gould A, Hockett L, Sharp J, Batten C, Fernando S, Ozbay F, Yazgan Y, Simonoff E, Thompson M, Taylor E, Asherson P. (2001). Association study of a dopamine transporter polymorphism and attention deficit hyperactivity disorder in UK and Turkish samples. *Mol Psychiatry*. 6(4):425-8.

Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, Caron MG and Lefkowitz RJ. (1998). Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J Biol Chem* 273:685- 688

Dal Toso R, Sommer B, Ewert M, Herb A, Pritchett DB, Bach A, Shivers BD and Seeburg PH (1989) The dopamine D2 receptor: two molecular forms generated by alternative splicing. *EMBO J* 8:4025-4034.

Dale M. (2000). *Sistema nervioso central: Otros neurotransmisores y neuromoduladores: Dopamina*. Farmacología. Editado por Harcourt, 4º edición. Barcelona. 517-535.

Daubner SC, Le T, Wang S. (2011). Tyrosine hydroxylase and regulation of dopamine synthesis. *Arch Biochem Biophys*. 508(1):1-12.

De A. (2011). The new era of bioluminescence resonance energy transfer technology. *Curr Pharm*

Biotechnol. 12(4):558-68.

Dearry A, Gingrich JA, Falardeau P, Fremeau RT, Jr., Bates MD and Caron MG. (1990). Molecular cloning and expression of the gene for a human D1 dopamine receptor. *Nature* 347:72-76.

De Boer SF, Lesourd M, Mocaer E, Koolhaas JM. (1999). Selective antiaggressive effects of alnespirone in resident-intruder test are mediated via 5-hydroxytryptamine_{1A} receptors: A comparative pharmacological study with 8-hydroxy-2-dipropylamino-tetralin, ipsapirone, buspirone, eltoprazine, and WAY-100635. *J Pharmacol Exp Ther.* 288(3):1125-33.

DeFea KA. (2011). Beta-arrestins as regulators of signal termination and transduction: how do they determine what to scaffold? *Cell Signal.* 23(4):621-9.

DeFea KA, Zalevsky J, Thoma MS, Dery O, Mullins RD and Bunnett NW (2000) beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* 148:1267-1281.

De Keyser J., Walraevens H., De Backer J. M. P., Ebinger G. and Vauquelin G. (1989). D2 dopamine receptors in the human brain: heterogeneity based on difference in guanine nucleotide effect on agonist binding and their presence on corticostriatal nerve terminals. *Brain Res.* 484:36-42.

Delgado PL, Moreno FA. (2000). Role of norepinephrine in depression. *J Clin Psychiatry.* 61 Suppl 1:5-12.

De Mei C, Ramos M, Itaka C and Borrelli E. (2009). Getting specialized: presynaptic and postsynaptic dopamine D2 receptors. *Curr Opin Pharmacol* 9:53-58.

Ding YC, Chi HC, Grady DL, Morishima A, Kidd JR, Kidd KK, Flodman P, Spence MA, Schuck S, Swanson JM, Zhang YP, Moyzis RK. (2002). Evidence of positive selection acting at the human dopamine receptor D4 gene locus. *Proc Natl Acad Sci U S A.* 99(1):309-14.

Dolmetsch RE, Pajvani U, Fife K, Spotts JM, Greenberg ME. (2001). Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. *Science.* 294(5541):333-9.

Dragic T, Trkola A, Thompson DA, Cormier EG, Kajumo FA, Maxwell E, Lin SW, Ying W, Smith SO, Sakmar TP, Moore JP. (2000). A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proc Natl Acad Sci U S A.* 97(10):5639-44.

Eisenberg J, Zohar A, Mei-Tal G, Steinberg A, Tartakovsky E, Gritsenko I, Nemanov L, Ebstein RP. (2000). A haplotype relative risk study of the dopamine D4 receptor (DRD4) exon III repeat

polymorphism and attention deficit hyperactivity disorder (ADHD). *Am J Med Genet.* 96(3):258-61.

Elliott ME, Goodfriend TL, Ball DL, Jefcoate CR. (2007). Angiotensin-responsive adrenal glomerulosa cell proteins: characterization by protease mapping, species comparison, and specific angiotensin receptor antagonists. *Endocrinology.* 138(6):2530-6.

Elsworth JD, Roth RH. (1997). Dopamine synthesis, uptake, metabolism, and receptors: relevance to gene therapy of Parkinson's disease. *Exp Neurol.* 144(1):4-9.

Enjalbert A., Sladczek F., Guillon G., Bertrand P., Shu C., Epelbaum J., Garcia-Sainz A., Jard S., Lombard C., Kordon C. and Bockaert J. (1986). Angiotensin II and dopamine modulate both cAMP and inositol phosphate productions in anterior pituitary cells. Involvement in prolactin secretion. *J. Biol. Chem.* 261:4071-4075.

Escrive M, Burgueno J, Ciruela F, Canela EI, Mallol J, Enrich C, Lluís C and Franco R. (2003). Ligand-induced caveolae-mediated internalization of A1 adenosine receptors: morphological evidence of endosomal sorting and receptor recycling. *Exp Cell Res* 285:72-90.

Esler M, Lambert G, Brunner-La Rocca HP, Vaddadi G, Kaye D. (2003). Sympathetic nerve activity and neurotransmitter release in humans: translation from pathophysiology into clinical practice. *Acta Physiol Scand.* 177(3):275-84.

Faraone SV, Doyle AE. (2001). The nature and heritability of attention-deficit/hyperactivity disorder. *Child Adolesc Psychiatr Clin N Am.* 10(2):299-316, viii-ix.

Faraone SV, Perlis RH, Doyle AE, Smoller JW, Goralnick JJ, Holmgren MA, Sklar P. (2005). Molecular genetics of attention-deficit/hyperactivity disorder. *Biol Psychiatry.* 57(11):1313-23.

Faure M, Voyno-Yasenetskaya TA and Bourne HR. (1994). cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J Biol Chem* 269:7851-7854.

Faure S, Salazar-Fontana LI, Semichon M, Tybulewicz VL, Bismuth G, Trautmann A, Germain RN, Delon J. (2004). ERM proteins regulate cytoskeleton relaxation promoting T cell-APC conjugation. *Nat Immunol.* 5(3):272-9

Feldman R. S., Meyer J. S. and Quenzer L. F. (1997). Principles of neuropsychopharmacology. Sunderland, Sinauer, 277-344.

Feng W, Zhang M. (2009). Organization and dynamics of PDZ-domain-related supramodules in the

postsynaptic density. *Nat Rev Neurosci.* 10(2):87-99.

Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* 53:1-24.

Ferrada C, Moreno E, Casadó V, Bongers G, Cortés A, Mallol J, Canela EI, Leurs R, Ferré S, Lluís C, Franco R. (2009). Marked changes in signal transduction upon heteromerization of dopamine D1 and histamine H3 receptors. *Br J Pharmacol.* 157(1):64-75.

Ferré S, Baler R, Bouvier M, Caron MG, Devi LA, Durroux T, Fuxe K, George SR, Javitch JA, Lohse MJ, Mackie K, Milligan G, Pflieger KD, Pin JP, Volkow ND, Waldhoer M, Woods AS and Franco R. (2009). Building a new conceptual framework for receptor heteromers. *Nat Chem Biol* 5:131-134.

Ferré S, Ciruela F, Woods AS, Lluís C and Franco R. (2007). Functional relevance of neurotransmitter receptor heteromers in the central nervous system. *Trends Neurosci* 30:440-446.

Ferré S, Navarro G, Casadó V, Cortés A, Mallol J, Canela EI, Lluís C, Franco R. (2010). G protein-coupled receptor heteromers as new targets for drug development. *Prog Mol Biol Transl Sci.* 91:41-52.

Filbey FM, Ray L, Smolen A, Claus ED, Audette A, Hutchison KE. (2008). Differential neural response to alcohol priming and alcohol taste cues is associated with DRD4 VNTR and OPRM1 genotypes. *Alcohol Clin Exp Res.* 32(7):1113-23.

Fillenz M. (1990). Regulation of catecholamine synthesis: Multiple mechanisms and their significance. *Neurochem Int.* 17(2):303-20.

Filteau F., Veilleux F. and Levesque D. (1999). Effects of reciprocal chimeras between the C- terminal portion of third intracellular loops of the human dopamine D2 and D3 receptors. *FEBS Lett.* 447:251-256.

Filtz T. M., Artymyshyn R. P., Guan W. and Molinoff P. B. (1993). Paradoxical regulation of dopamine receptors in transfected 293 cells. *Mol. Pharmacol.* 44:371-379.

Fishburn C. S., Elazar Z. and Fuchs S. (1995). Differential glycosylation and intracellular trafficking for the long and short isoforms of the D2 dopamine receptor. *J. Biol. Chem.* 270:29819-29824.

Floet AM, Scheiner C, Grossman L. (2010). Attention-deficit/hyperactivity disorder. *Pediatr Rev.* 31(2):56-69.

Flóres J y Pazos A. (2003). Neurotransmisión en el sistema nervioso central. *Farmacología Humana.* Flóres J, Armijo JA, Mediavilla A. Editado por Masson. 4º edición. Barcelona. 435-460.

Flower D. R. (1999). Modelling G-protein-coupled receptors for drug design. *Biochem. Biophys. Acta* 1422:207–234

Fotiadis D, Liang Y, Filipek S, Saperstein DA, Engel A and Palczewski K. (2003). Atomic-force microscopy: Rhodopsin dimers in native disc membranes. *Nature* 421:127-128.

Franco R, Canals M, Marcellino D, Ferre S, Agnati L, Mallol J, Casado V, Ciruela F, Fuxe K, Lluís C and Canela EI (2003) Regulation of heptaspanning- membrane-receptor function by dimerization and clustering. *Trends Biochem Sci* 28:238-243.

Franco R, Casado V, Ciruela F, Mallol J, Lluís C and Canela EI (1996) The cluster-arranged cooperative model: a model that accounts for the kinetics of binding to A1 adenosine receptors. *Biochemistry* 35:3007-3015.

Franco R, Casado V, Cortes A, Mallol J, Ciruela F, Ferre S, Lluís C and Canela EI (2008a) G-protein-coupled receptor heteromers: function and ligand pharmacology. *Br J Pharmacol* 153 Suppl 1:S90-98.

Franco R, Casado V, Cortes A, Perez-Capote K, Mallol J, Canela E, Ferre S and Lluís C (2008b) Novel pharmacological targets based on receptor heteromers. *Brain Res Rev* 58:475-482.

Franco R, Ciruela F, Casadó V, Cortes A, Canela EI, Mallol J, Agnati LF, Ferré S, Fuxe K, Lluís C. (2005). Partners for adenosine A1 receptors. *J Mol Neurosci.* (2-3):221-32.

Fredholm BB, Hökfelt T, Milligan G. (2007). G-protein-coupled receptors: an update. *Acta Physiol.* 190(1):3-7.

Fredriksson R, Lagerstrom MC, Lundin LG and Schiöth HB (2003) The G- protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* 63:1256-1272.

Fuller RW, Wong DT. (1977). Inhibition of serotonin reuptake. *Fed Proc.* 36(8):2154-8.

Fung SI, Chan JY, Manzoni D, White SR, Lai YY, Strahlendorf HK, Zhuo H, Liu RH, Reddy VK, Barnes CD. (1994). Cotransmitter-mediated locus coeruleus action on motoneurons. *Brain Res Bull.* 35(5-6):423-32.

Fuxe K. and Agnati L. F. (1965). Receptor-receptor interactions in the central nervous system. A new integrative mechanism in synapses. *Med. Res. Rev.* 5:441-482.

Gainetdinov RR and Caron MG (2003) Monoamine transporters: from genes to behavior. *Annu Rev Pharmacol Toxicol* 43:261-284.

Gandía J, Lluís C, Ferré S, Franco R, Ciruela F. (2008). Light resonance energy transfer-based methods in the study of G protein-coupled receptor oligomerization. *Bioessays*. 30(1):82-9.

Gargaglioni LH, Hartzler LK, Putnam RW. (2010). The locus coeruleus and central chemosensitivity. *Respir Physiol Neurobiol*. 173(3):264-73.

Garland EM, Biaggioni I. (2001). Genetic polymorphisms of adrenergic receptors. *Clin Auton Res*. 11(2):67-78.

Garland EM, Hahn MK, Ketch TP, Keller NR, Kim CH, Kim KS, Biaggioni I, Shannon JR, Blakely RD, Robertson D. (2002). Genetic basis of clinical catecholamine disorders. *Ann N Y Acad Sci*. 971:506-14.

Gasca-Martinez D, Hernandez A, Sierra A, Valdiosera R, Anaya-Martinez V, Floran B, Erlij D, Aceves J. (2010). Dopamine inhibits GABA transmission from the globus pallidus to the thalamic reticular nucleus via presynaptic D4 receptors. *Neuroscience*. 169(4):1672-81.

George SR, O'Dowd BF and Lee SP. (2002) G-protein-coupled receptor oligomerization and its potential for drug discovery. *Nat Rev Drug Discov* 1:808-820.

Gerfen CR. (2000). Dopamine-mediated gene regulation in models of Parkinson's disease. *Ann Neurol*. 47(4 Suppl 1):S42-50; discussion S50-2.

Gerfen CR. (2004). *The Rat Nervous System*. pp 445-508, Elsevier Academic Press, Amsterdam.

Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma FJ Jr, Sibley DR. (1990). D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science*. 250(4986):1429-32.

Gerfen CR, Miyachi S., Paletzki R. and Brown P. (2002). D1 dopamine receptor supersensitivity in the dopamine-depleted striatum results from a switch in the regulation of ERK1/2/MAP kinase. *J. Neurosci*. 22:5042-5054.

Gershon AA, Vishne T, Grunhaus L. (2007). Dopamine D2-like receptors and the antidepressant response. *Biol Psychiatry*. 61(2):145-53.

Ghahremani M. H., Cheng P., Lembo P. M. and Albert P. R. (1999). Distinct roles for Galphai2, Galphai3 and Gbetagamma in modulation of forskolin- or Gsmediated cAMP accumulation and calcium

mobilization by dopamine D2S receptor. *J. Biol. Chem.* 274:9238.

Gilchrist A. (2007). Modulating G-protein-coupled receptors: from tradicional pharmacology to allosterics. *Trends Pharmacol Sci.* 28:431-437.

Gines S, Ciruela F, Burgueno J, Casado V, Canela EI, Mallol J, Lluís C and Franco R. (2001). Involvement of caveolin in ligand-induced recruitment and internalization of A(1) adenosine receptor and adenosine deaminase in an epithelial cell line. *Mol Pharmacol.* 59:1314-1323

Gines S, Hillion J, Torvinen M, Le Crom S, Casado V, Canela EI, Rondin S, Lew JY, Watson S, Zoli M, Agnati LF, Verniera P, Lluís C, Ferre S, Fuxe K and Franco R. (2000). Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. *Proc Natl Acad Sci U S A* 97:8606-8611.

Giros B., Sokoloff P., Martres M. P., Riou J. F., Emorine L. J., Schwartz J. C. (1989). Alternative splicing directs the expression of two D2 dopamine receptor isoforms. *Nature* 342:923-926.

Golan M, Schreiber G and Avissar S (2009) Antidepressants, beta-arrestins and GRKs: from regulation of signal desensitization to intracellular multifunctional adaptor functions. *Curr Pharm Des* 15:1699-1708.

Goldman-Rakic PS. (1999). The relevance of the dopamine –D1 receptor in the cognitive symptoms of schizophrenia. *Neuropsychopharmacology.* 21:170-180.

Goldman-Rakic PS, Muly EC and Williams GC. (2000). D(1) receptors in prefrontal cells and circuits. *Brain Res Brain Res Rev.* 31:295-301.

Gonzalez-Brito A, Troiani ME, Menendez-Pelaez A, Delgado MJ, Reiter RJ. (1990). mRNA transcription determines the lag period for the induction of pineal melatonin synthesis in the Syrian hamster pineal gland. *J Cell Biochem.* 44(1):55-60.

Gouldson PR, Higgs C, Smith RE, Dean MK, Gkoutos GV and Reynolds CA (2000) Dimerization and domain swapping in G-protein-coupled receptors: a computational study. *Neuropsychopharmacology* 23:S60-77.

Gracia E, Pérez-Capote K, Moreno E, Barkešová J, Mallol J, Lluís C, Franco R, Cortés A, Casadó V, Canela EI. (2011). A2A adenosine receptor ligand binding and signalling is allosterically modulated by adenosine deaminase. *Biochem J.* 1;435(3):701-9.

Grady DL, Chi HC, Ding YC, Smith M, Wang E, Schuck S, Flodman P, Spence MA, Swanson JM, Moyzis RK. (2003). High prevalence of rare dopamine receptor D4 alleles in children diagnosed with attention-deficit hyperactivity disorder. *Mol Psychiatry.* 8(5):536-45.

- Gray J.A. and Roth B.L. (2006). Developing selectively nonselective drugs for treating CNS disorders. *Drug Discovery Today: Therapeutic Strategies* 3:413-419.
- Grimm M, Brown JH. (2010). Beta-adrenergic receptor signaling in the heart: role of CaMKII. *J Mol Cell Cardiol.* 48(2):322-30.
- Gudermann T, Schoneberg T and Schultz G (1997) Functional and structural complexity of signal transduction via G-protein-coupled receptors. *Annu Rev Neurosci* 20:399-427
- Guiramand J., Montmayeur J. P., Ceraline J., Bhatia M. and Borrelli E. (1995). Alternative splicing of the dopamine D2 receptor directs specificity of coupling to G-proteins. *J. Biol. Chem.* 270:7354-7358.
- Guo W, Shea JE, Berry RS. (2005). The physics of the interactions governing folding and association of proteins. *Ann N Y Acad Sci.* 1066:34-53.
- Guo S, Wu J, Ding M, Feng J. (2008). Uncovering interactions in the frequency domain. *PLoS Comput Biol.* 4(5):e1000087.
- Hague C, Lee SE, Chen Z, Prinster SC, Hall RA, Minneman KP. (2006). Heterodimers of alpha1B- and alpha1D-adrenergic receptors form a single functional entity. *Mol Pharmacol.* 69(1):45-55.
- Hall R. A., Premont R. T., Chow C. W., Blitzer J. T., Pitcher J. A., Claing A., Stoffel R. H., Barak L. S., Shenolikar S., Weinman E. J., Grinstein S. and Lefkowitz R. J. (1998). The beta2- adrenergic receptor interacts with the Na⁺/H⁺-exchanger regulatory factor to control Na⁺/H⁺ exchange. *Nature* 392:626-630.
- Hamarman S, Fossella J, Ulger C, Brimacombe M, Dermody J. (2004). Dopamine receptor 4 (DRD4) 7-repeat allele predicts methylphenidate dose response in children with attention-deficit/hyperactivity disorder: a pharmacogenetic study. *J Child Adolesc Psychopharmacol.* 14: 564–574.
- Hardeland R, Madrid JA, Tan DX, Reiter RJ. (2011). Melatonin, the circadian multioscillator system and health: the need for detailed analyses of peripheral melatonin signaling. *J Pineal Res.*
- Hausdorff WP, Bouvier M, O'Dowd BF, Irons GP, Caron MG and Lefkowitz RJ (1989) Phosphorylation sites on two domains of the beta 2-adrenergic receptor are involved in distinct pathways of receptor desensitization. *J Biol Chem* 264:12657-12665.
- He L., Fong J., Von Zastrow M. and Whistler J. L. (2002). Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell* 108:271–282.

Hebert TE, Moffett S, Morello JP, Loisel TP, Bichet DG, Barret C and Bouvier M (1996) A peptide derived from a beta2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J Biol Chem* 271:16384-16392.

Hering H, Sheng M. (2001). Dendritic spines: structure, dynamics and regulation. *Nat Rev Neurosci.* 2(12):880-8.

Hermans E, Vanisberg MA, Geurts M and Maloteaux JM (1997) Down- regulation of neurotensin receptors after ligand-induced internalization in rat primary cultured neurons. *Neurochem Int* 31:291-299.

Hernandez-Lopez S., Tkatch T., Perez-Garci E., Galarraga E., Bargas J., Hamm H. and Surmeier D. J. (2000). D2 dopamine receptors in striatal medium spiny neurons reduce L-type Ca²⁺ currents and excitability via a novel PLC[beta]1-IP3-calcineurin-signaling cascade. *J. Neurosci.* 20:8987-8995.

Herrera C, Casado V, Ciruela F, Schofield P, Mallol J, Lluís C and Franco R (2001) Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. *Mol Pharmacol* 59:127-134.

Hervouet E, Hulin P, Vallette FM, Cartron PF. (2011). Proximity ligation in situ assay for monitoring the global DNA methylation in cells. *BMC Biotechnol.* 11:31.

Hieble JP, Bondinell WE, Ruffolo RR Jr. (1995). Alpha- and beta-adrenoceptors: from the gene to the clinic. 1. Molecular biology and adrenoceptor subclassification. *J Med Chem.* 38(18):3415-44.

Hillion J, Canals M, Torvinen M, Casado V, Scott R, Terasmaa A, Hansson A, Watson S, Olah ME, Mallol J, Canela EI, Zoli M, Agnati LF, Ibanez CF, Lluís C, Franco R, Ferre S and Fuxe K. (2002). Coaggregation, cointernalization, and codesensitization of adenosine A2A receptors and dopamine D2 receptors. *J Biol Chem* 277:18091-18097.

Holmes J, Hever T, Hewitt L, Ball C, Taylor E, Rubia K, Thapar A. (2002). A pilot twin study of psychological measures of attention deficit hyperactivity disorder. *Behav Genet.* 32(6):389-95.

Huber A. (2001). Scaffolding proteins organize multimolecular protein complexes for sensory signal transduction. *Eur. J. Neurosci.* 14:769-776.

Ilani T., Fishburn C. S., Levavi-Sivan B., Carmon S., Raveh L. and Fuchs S. (2002). Coupling of dopamine receptors to G proteins: studies with chimeric D2/D3 dopamine receptors. *Cell. Mol. Neurobiol.* 22:47-56.

Innamorati G., Le Gouill C., Balamotis M. and Birnbaumer M. (2001). The long and the short cycle. Alternative intracellular routes for trafficking of G-proteincoupled receptors. *J. Biol. Chem.* 276:13096-13103.

Ito K., Haga T., Lamah J. and Sadee W. (1999). Sequestration of dopamine D2 receptors depends on coexpression of G-protein-coupled receptor kinases 2 or 5. *Eur. J. Biochem.* 260:112-119.

Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, Ijzerman AP and Stevens RC (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* 322:1211- 1217.

Jaakola VP, Ijzerman AP. (2010). The crystallographic structure of the human adenosine A2A receptor in a high-affinity antagonist-bound state: implications for GPCR drug screening and design. *Curr Opin Struct Biol.* (4):401-14.

Jackson D. M. and Westlind-Danielsson A. (1994). Dopamine receptors: molecular biology, biochemistry and behavioral aspects. *Pharmacol. Ther.* 64:291-369.

Jacoby E, Bouhelal R, Gerspacher M and Seuwen K. (2006). The 7 TM G- protein-coupled receptor target family. *ChemMedChem* 1:761-782.

Jeanneteau F, Diaz J, Sokoloff P, Griffon N. (2004). Interactions of GIPC with dopamine D2, D3 but not D4 receptors define a novel mode of regulation of G protein-coupled receptors. *Mol Biol Cell.* 15: 696–705.

Jin Y, Lee SJ, Minshall RD, Choi AM. (2011). Caveolin-1: a critical regulator of lung injury. *Am J Physiol Lung Cell Mol Physiol.* 300(2):L151-60.

Jockers R, Angers S, Da Silva A, Benaroch P, Strosberg AD, Bouvier M and Marullo S (1999) Beta(2)-adrenergic receptor down-regulation. Evidence for a pathway that does not require endocytosis. *J Biol Chem* 274:28900-28908.

Johnson JA, Liggett SB. (2011). Cardiovascular pharmacogenomics of adrenergic receptor signaling: clinical implications and future directions. *Clin Pharmacol Ther.* 89(3):366-78.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA and Gerald C. (1998). GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. *Nature* 396:674-679.

Jordan BA and Devi LA. (1999). G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* 399:697-700.

Jordan BA, Trapaidze N., Gomes I., Nivarthi R. and Devi LA. (2001). Oligomerization of opioid receptors with beta2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. *Proc. Natl. Acad. Sci. USA.* 98:343-348.

Jovanovic V, Guan HC, Van Tol HH. (1999). Comparative pharmacological and functional analysis of the human dopamine D4.2 and D4.10 receptor variants. *Pharmacogenetics.* 9(5):561-8.

Kabbani N, Levenson R. (2007). A proteomic approach to receptor signaling: molecular mechanisms and therapeutic implications derived from discovery of the dopamine D2 receptor signalplex. *Eur J Pharmacol.* 572: 83–93.

Kaissling B, Hegyi I, Loffing J, Le Hir M. (1996). Morphology of interstitial cells in the healthy kidney. *Anat Embryol.* 193(4):303-18.

Kanagy N. (2005). α 2-Adrenergic receptor signalling in hypertension. *Clinical Science.* 109:431-437.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A and Bettler B. (1998). GABA(B)-receptor subtypes assemble into functional heteromeric complexes. *Nature* 396:683-687.

Kazmi MA, Snyder LA, Cypess AM, Graber SG, Sakmar TP. (2000). Selective reconstitution of human D4 dopamine receptor variants with Gi alpha subtypes. *Biochemistry.* 39(13):3734-44.

Kelly E, Bailey CP and Henderson G (2008) Agonist-selective mechanisms of GPCR desensitization. *Br J Pharmacol* 153 Suppl 1:S379-388.

Keov P, Sexton PM, Christopoulos A. (2011). Allosteric modulation of G protein-coupled receptors: a pharmacological perspective. *Neuropharmacology.* 60(1):24-35.

Keys JR, Koch WJ. (2004). The adrenergic pathway and heart failure. *Recent Prog Horm Res.* 59:13-30.

Kim JS, Bailey MJ, Weller JL, Sugden D, Rath MF, Møller M, Klein DC. (2010). Thyroid hormone and adrenergic signaling interact to control pineal expression of the dopamine receptor D4 gene (*Drd4*). *Mol Cell Endocrinol.* 314(1):128-35.

Kim K. M., Valenzano K. J., Robinson S. R., Yao W. D., Barak L. S. and Caron M. G. (2001). Differential regulation of the dopamine D2 and D3 receptors by G protein-coupled receptor kinases and

beta-arrestins. *J. Biol. Chem.* 40:37409-37414.

Klco JM, Lassere TB, Baranski TJ. (2003). C5a receptor oligomerization. I. Disulfide trapping reveals oligomers and potential contact surfaces in a G protein-coupled receptor. *J Biol Chem.* 278(37):35345-53.

Klein DC, Bailey MJ, Carter DA, Kim JS, Shi Q, Ho AK, Chik CL, Gaildrat P, Morin F, Ganguly S, Rath MF, Møller M, Sugden D, Rangel ZG, Munson PJ, Weller JL, Coon SL. (2010). Pineal function: impact of microarray analysis. *Mol Cell Endocrinol.* 314(2):170-83.

Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone PM, Rodriguez IR, Bégay V, Falcón J, Cahill GM, Cassone VM, Baler R. (1997). The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. *Recent Prog Horm Res.* 52:307-57;

Kniazeff J, Prézeau L, Rondard P, Pin JP, Goudet C. (2011) Dimers and beyond: The functional puzzles of class C GPCRs. *Pharmacol Ther.* 130(1):9-25

Knoflach F, Mutel V, Jolidon S, Kew JN, Malherbe P, Vieira E, Wichmann J, Kemp JA. (2001). Positive allosteric modulators of metabotropic glutamate 1 receptor: characterization, mechanism of action, and binding site. *Proc Natl Acad Sci U S A.* 98(23):13402-7.

Kobilka BK. (2011). Structural insights into adrenergic receptor function and pharmacology. *Trends Pharmacol Sci.* 32(4):213-8.

Koch WJ, Hawes BE, Allen LF and Lefkowitz RJ. (1994). Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by G beta gamma activation of p21ras. *Proc Natl Acad Sci U S A* 91:12706-12710.

Kolakowski LF, Jr. (1994) GCRDb: a G-protein-coupled receptor database. *Receptors Channels* 2:1-7.

Kong MM, Hasbi A, Mattocks M, Fan T, O'Dowd BF and George SR (2007) Regulation of D1 dopamine receptor trafficking and signaling by caveolin-1. *Mol Pharmacol* 72:1157-1170.

Konturek SJ, Konturek PC, Brzozowski T. (2006). Melatonin in gastroprotection against stress-induced acute gastric lesions and in healing of chronic gastric ulcers. *J Physiol Pharmacol.* 57 Suppl 5:51-66.

Kriegebaum C, Gutknecht L, Schmitt A, Lesch KP, Reif A. (2010). Serotonin now: Part 1. Neurobiology and developmental genetics. *Fortschr Neurol Psychiatr.* 78(6):319-31.

Krueger KM, Daaka Y, Pitcher JA and Lefkowitz RJ. (1997). The role of sequestration in G protein-coupled receptor resensitization. Regulation of beta2-adrenergic receptor dephosphorylation by vesicular acidification. *J Biol Chem* 272:5-8.

Krupnick J. G. and Benovic J. L. (1998). The role of receptor kinases and arrestins in G-protein-coupled receptor regulation. *Annu. Rev. Pharmacol. Toxicol.* 38:289-319.

Lachowicz J. E. and Sibley D. R. (1997). Chimeric D2/D3 dopamine receptor coupling to adenylyl cyclase. *Biochem. Biophys. Res. Commun.* 237:394-399.

Lauzon NM, Ahmad T, Laviolette SR. (2011). Dopamine D4 Receptor Transmission in the Prefrontal Cortex Controls the Salience of Emotional Memory via Modulation of Calcium Calmodulin-Dependent Kinase II. *Cereb Cortex*.

Lauzon NM, Laviolette SR. (2010). Dopamine D4-receptor modulation of cortical neuronal network activity and emotional processing: Implications for neuropsychiatric disorders. *Behav Brain Res.* 208(1):12-22.

Law MR, Wald NJ, Morris JK, Jordan RE. (2003). Value of low dose combination treatment with blood pressure lowering drugs: analysis of 354 randomised trials. *BMJ.* 28;326(7404):1427.

Lee K. W., Hong J. H., Choi I. Y., Che Y., Lee J. K., Yang S. D., Song C. W., Kang H. S., Lee J. H., Noh J. S., Shin H. S. and Han P. L. (2002). Impaired D2 dopamine receptor function in mice lacking type 5 adenylyl cyclase. *J. Neurosci.* 22:7931-7940.

Lefkowitz R. J. (1993). G protein-coupled receptor kinases. *Cell.* 74:409-412.

Lefkowitz R. J. (1998). G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J. Biol. Chem.* 273:18677-18680.

Lefkowitz R. J. (2007). Seven transmembrane receptors: something old, something new. *Acta Physiol.* 190(1):9-19.

Lerner AB, Case JD, Takahashi Y. (1960). Isolation of melatonin and 5-methoxyindole-3-acetic acid from bovine pineal glands. *J Biol Chem.* 1960 Jul;235:1992-7.

Levant B, Grigoriadis D E, De Souza EB. (1992). Characterization of [3H]-quinpirole binding to D2-like dopamine receptors in rat brain. *J. Pharmacol. Exp. Ther.* 1992, 262, 929-935.

Levitt M., Spctor S., Sjoerdsma A. and Udenfriend S. (1965). Elucidation of the rate-limiting step in

norepinephrine biosynthesis in the perfused guinea-pig heart. *J. Pharmacol. Exp. Ther.* 23:1493-1501.

Liebmann C. (2004). G protein-coupled receptors and their signaling pathways: classical therapeutical targets susceptible to novel therapeutic concepts. *Curr Pharm Des.* 10(16):1937-58.

Liggett SB. (2003). Polymorphisms of adrenergic receptors: variations on a theme. *Assay Drug Dev Technol.* 1(2):317-26.

Limbird LE, Meyts PD and Lefkowitz RJ (1975) Beta-adrenergic receptors: evidence for negative cooperativity. *Biochem Biophys Res Commun* 64:1160- 1168.

Lin R., Karpa K., Kabbani N., Goldman-Rakic P. and Levenson R. (2001). Dopamine D2 and D3 receptors are linked to the actin cytoskeleton via interaction with filamin A. *Proc. Natl. Acad. Sci. USA.* 98:5258-5263.

Lin SH, Civelli O. (2004). Orphan G protein-coupled receptors: targets for new therapeutic interventions. *Ann Med.* 36(3):204-14.

Lindgren N., Usiello A., Goigny M., Haycock J., Erbs E., Greengard P., Hokfelt T., Borrelli E. and Fisone G. (2003). Distinct roles of dopamine D2L and D2S receptor isoforms in the regulation of protein phosphorylation at presynaptic and postsynaptic sites. *Proc. Natl. Acad. Sci. USA.* 100:4305-4309.

Liu YF, Civelli O., Grandy D. K. and Albert P. R. (1992). Differential sensitivity of the short and long human dopamine D2 receptor subtypes to protein kinase C. *J. Neurochem.* 59:2311-2317.

Liu YF, Edwards RH. (1997). Differential localization of vesicular acetylcholine and monoamine transporters in PC12 cells but not CHO cells. *J Cell Biol.* 139(4):907-16.

Lohse MJ, Benovic JL, Caron MG and Lefkowitz RJ (1990) Multiple pathways of rapid beta 2-adrenergic receptor desensitization. Delineation with specific inhibitors. *J Biol Chem* 265:3202-3211.

López-Muñoz F, Marín F, Alamo C. (2010). The historical background of the pineal gland: II. From the seat of the soul to a neuroendocrine organ. *Rev Neurol.* 50(2):117-25.

Lundberg JM. (1996). Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. *Pharmacol Rev.* 48(1):113-78.

Lundstrom K. (2006). Latest development in drug Discovery on G proteina-coupled receptors. *Curr. Protein Pept. Sci.* 7:465-470.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG and Lefkowitz RJ. (1999). Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* 283:655-661.

Ma YC, Huang XY. (2002). Novel signaling pathway through the beta-adrenergic receptor. *Trends Cardiovasc Med.*12(1):46-9.

Macchi MM, Bruce JN. (2004). Human pineal physiology and functional significance of melatonin. *Front Neuroendocrinol.* 25(3-4):177-95.

Madras BK, Miller GM, Fischman AJ. (2005). The dopamine transporter and attention-deficit/hyperactivity disorder. *Biol Psychiatry.* 57(11):1397-409.

Maggio R, Vogel Z and Wess J (1993) Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein-linked receptors. *Proc Natl Acad Sci U S A* 90:3103- 3107.

Malek D., Munch G. and Palm D. (1993). Two sites in the third inner loop of the dopamine D2 receptor are involved in functional G protein-mediated coupling to adenylate cyclase. *FEBS Lett.* 325:215-219.

Mandela P, Ordway GA. (2006). The norepinephrine transporter and its regulation. *J Neurochem.* 97(2):310-33.

Marcellino D, Ferre S, Casado V, Cortes A, Le Foll B, Mazzola C, Drago F, Saur O, Stark H, Soriano A, Barnes C, Goldberg SR, Lluís C, Fuxe K and Franco R. (2008). Identification of dopamine D1-D3 receptor heteromers. Indications for a role of synergistic D1-D3 receptor interactions in the striatum. *J Biol Chem* 283:26016-26025.

Margeta-Mitrovic M, Jan YN and Jan LY (2000) A trafficking checkpoint controls GABA(B) receptor heterodimerization. *Neuron* 27:97-106.

Marinissen M.J. and Gutkind, J.S. (2001), G-protein-coupled receptors and signaling networks: emerging paradigms, *Trends Pharmacol Sci.* 22(7): 368-76.

Maronde E, Stehle JH. (2007). The mammalian pineal gland: known facts, unknown facets. *Trends Endocrinol Metab.* 18(4):142-9.

Marshall KC, Christie MJ, Finlayson PG, Williams JT. (1991). Developmental aspects of the locus coeruleus-noradrenaline system. *Prog Brain Res.* 88:173-85.

- Martemyanov KA, Arshavsky VY. (2009). Biology and functions of the RGS9 isoforms. *Prog Mol Biol Transl Sci.* 86:205-27.
- Matsui H, Lazareno S, Birdsall NJ. (1995). Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis. *Mol Pharmacol.* 47(1):88-98.
- Matsushima S, Sakai Y, Hira Y. (1999). Peptidergic peripheral nervous systems in the mammalian pineal gland. *Microsc Res Tech.* 46(4-5):265-80.
- May LT, Leach K, Sexton PM and Christopoulos A. (2007). Allosteric modulation of G protein-coupled receptors. *Annu Rev. Pharmacol. Toxicol.* 47:1-51.
- McGeary J. (2009). The DRD4 exon 3 VNTR polymorphism and addiction-related phenotypes: a review. *Pharmacol Biochem Behav.* 93(3):222-9. Epub 2009 Mar 29.
- McGeer P. L., Eccles J. C. and McGeer E. G. (1987). *Molecular neurobiology of the mammalian brain.* 2nd. New York. Plenum Press. 265-317.
- McVey M, Ramsay D, Kellett E, Rees S, Wilson S, Pope AJ and Milligan G. (2001). Monitoring receptor oligomerization using time-resolved fluorescence resonance energy transfer and bioluminescence resonance energy transfer. The human delta -opioid receptor displays constitutive oligomerization at the cell surface, which is not regulated by receptor occupancy. *J Biol Chem* 276:14092- 14099.
- Mediavilla-García C. (2003). Neurobiology of hyperactivity disorder. *Rev Neurol.* 16-31;36(6):555-65.
- Meitzen J, Luoma JI, Stern CM, Mermelstein PG. (2011). β 1-Adrenergic receptors activate two distinct signaling pathways in striatal neurons. *J Neurochem.* 116(6):984-95.
- Mercuri N. B., Saiardi A., Bonci A., Picetti R., Calabresi P., Bernardi G. and Borrelli E. (1997). Loss of autoreceptor function in dopaminergic neurons from dopamine D2 receptor deficient mice. *Neurosci.* 79:323-327.
- Miguez JM, Simonneaux V, Pevet P. (1997). The role of the intracellular and extracellular serotonin in the regulation of melatonin production in rat pinealocytes. *J Pineal Res* 23: 63–71.
- Millan MJ. (2009). Dual- and triple-acting agents for treating core and co-morbid symptoms of major depression: novel concepts, new drugs. *Neurotherapeutics.* 6(1):53-77.
- Milligan G. (2006). G-protein-coupled receptor heterodimers: pharmacology, function and relevance to

drug discovery. *Drug Discov Today*. 11(11-12):541-9.

Milligan G, Murdoch H, Kellett E, White JH and Feng GJ (2004) Interactions between G-protein-coupled receptors and periplakin: a selective means to regulate G-protein activation. *Biochem Soc Trans* 32:878-880.

Minneman KP, Theroux TL, Hollinger S, Han C, Esbenshade TA. (1994). Selectivity of agonists for cloned alpha 1-adrenergic receptor subtypes. *Mol Pharmacol*. 46(5):929-36.

Missale C, Nash SR, Robinson SW, Jaber M and Caron MG (1998) Dopamine receptors: from structure to function. *Physiol Rev* 78:189-225.

Mitchell HA, Weinschenker D. (2010). Good night and good luck: norepinephrine in sleep pharmacology. *Biochem Pharmacol*. 79(6):801-9.

Miyamoto S, Duncan GE, Marx CE, Lieberman JA. (2005). Treatments for schizophrenia: a critical review of pharmacology and mechanisms of action of antipsychotic drugs. *Mol Psychiatry*. 10(1):79-104.

Mohr K, Tränkle C, Kostenis E, Barocelli E, De Amici M, Holzgrabe U. (2010). Rational design of dualsteric GPCR ligands: quests and promise. *Br J Pharmacol*. 159(5):997-1008

Møller M, Baeres FM. (2002). The anatomy and innervation of the mammalian pineal gland. *Cell Tissue Res*. 309(1):139-50.

Møller M, Rath MF, Klein DC. (2006). The perivascular phagocyte of the mouse pineal gland: an antigen-presenting cell. *Chronobiol Int*. 23(1-2):393-401.

Mons N., Harry A., Dubourg P., Premont R. T., Iyengar R. and Cooper D. M. (1995). Immunohistochemical localization of adenylyl cyclase in rat brain indicates a highly selective concentration at synapses. *Proc. Natl. Acad. Sci. USA* 92:8473-8477.

Moore JD, Mason DA, Green SA, Hsu J, Liggett SB. (1999). Racial differences in the frequencies of cardiac beta(1)-adrenergic receptor polymorphisms: analysis of c145A>G and c1165G>C. *Hum Mutat*. 14(3):271.

Moreno E, Hoffmann H, Gonzalez-Sepúlveda M, Navarro G, Casadó V, Cortés A, Mallol J, Vignes M, McCormick PJ, Canela EI, Lluís C, Moratalla R, Ferré S, Ortiz J, Franco R. (2011). Dopamine D1-histamine H3 receptor heteromers provide a selective link to MAPK signaling in GABAergic neurons of the direct striatal pathway. *J Biol Chem*. 286(7):5846-54.

Morphy R, Kay C and Rankovic Z. (2004). From magic bullets to designed multiple ligands. *Drug Discov. Today*. 9:641-651.

Morton JS, Daly CJ, Jackson VM, McGrath JC. (2007). Alpha(1A)-adrenoceptors mediate contractions to phenylephrine in rabbit penile arteries. *Br J Pharmacol*. 150(1):112-20.

Moser E, Kargl J, Whistler JL, Waldhoer M, Tschische P. (2010). G protein-coupled receptor-associated sorting protein 1 regulates the postendocytic sorting of seven-transmembrane-spanning G protein-coupled receptors. *Pharmacology*. 86(1):22-9.

Navarro G, Carriba P, Gandía J, Ciruela F, Casadó V, Cortés A, Mallol J, Canela EI, Lluís C, Franco R. (2008). Detection of heteromers formed by cannabinoid CB1, dopamine D2, and adenosine A2A G-protein-coupled receptors by combining bimolecular fluorescence complementation and bioluminescence energy transfer. *ScientificWorldJournal*. 8:1088-97.

Navarro G, Ferré S, Cordomi A, Moreno E, Mallol J, Casadó V, Cortés A, Hoffmann H, Ortiz J, Canela EI, Lluís C, Pardo L, Franco R, Woods AS. (2010). Interactions between intracellular domains as key determinants of the quaternary structure and function of receptor heteromers. *J Biol Chem*. 285(35):27346-59.

Neubig RR, Spedding M, Kenakin T, Christopoulos A. (2003). International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. *Pharmacol Rev*. 55(4):597-606.

Neve KA, Seamans JK, Trantham-Davidson H. (2004). Dopamine receptor signaling. *J Recept Signal Transduct Res*. 24(3):165-205.

Newman-Tancredi A, Cussac D, Depoortere R. (2007). Neuropharmacological profile of bifeprunox: merits and limitations in comparison with other third-generation antipsychotics. *Curr Opin Investig Drugs*. 8(7):539-54.

Ng GY, O'Dowd BF, Lee SP, Chung HT, Brann MR, Seeman P and George SR. (1996). Dopamine D2 receptor dimers and receptor-blocking peptides. *Biochem Biophys Res Commun* 227:200-204.

Ng G. Y., Varghese G., Chung H. T., Trogadis J., Seeman P., O'Dowd B. F. and George S. R. (1997). Resistance of the dopamine D2L receptor to desensitization accompanies the up-regulation of receptors on to the surface of Sf9 cells. *Endocrinol*. 138:4199-4206.

Nicola S. M., Surmeier J. and Malenka R. C. (2000). Dopaminergic modulation of neuronal excitability

in the striatum and nucleus accumbens. *Annu. Rev. Neurosci.* 23:185-215.

Nielsen EB, Andersen PH. (1992). Dopamine receptor occupancy in vivo: behavioral correlates using NNC-112, NNC-687 and NNC-756, new selective dopamine D1 receptor antagonists. *Eur J Pharmacol.* 219(1):35-44.

Nieoullon A, Amalric M. (2002). Dopaminergic receptors: structural features and functional implications. *Rev Neurol.* 158,1:S59-68.

Nishi A., Snyder G. L. and Greengard P. (1997). Bidirectional regulation of DARPP-32 phosphorylation by dopamine. *J. Neurosci.* 17:8147-8155.

Noble EP, St Jeor ST, Ritchie T, Syndulko K, St Jeor SC, Fitch RJ, Brunner RL, Sparkes RS. (1994). D2 dopamine receptor gene and cigarette smoking: a reward gene? *Med Hypotheses.* 42(4):257-60.

Nowicki M, Wojtkiewicz J, Lewczuk B, Kosacka J, Majewski M, Przybylska-Gornowicz B. (2007). Peptidergic and nitrergic innervation of the pineal gland in the domestic pig: an immunohistochemical study. *Anat Histol Embryol.* 36(4):311-20.

Nutt JG (1990) Levodopa-induced dyskinesia: review, observations, and speculations. *Neurology* 40:340-345.

O'Dowd F.B. (1993). Structure of dopamine receptors. *J. Neurochem.* 60:804-816.

O'Hara CM, Tang L, Taussig R, Todd RD, O'Malley KL. (1996). Dopamine D2L receptor couples to G alpha i2 and G alpha i3 but not G alpha i1, leading to the inhibition of adenylate cyclase in transfected cell lines. *J Pharmacol Exp Ther.* 278(1):354-60.

Oak J. N., Lavine N. and Van Tol H. H. (2001). Dopamine D(4) and D(2L) Receptor Stimulation of the Mitogen-Activated Protein Kinase Pathway Is Dependent on trans-Activation of the Platelet-Derived Growth Factor Receptor. *Mol. Pharmacol.* 60:92-103.

Oak J.N., Oldenhof J, Van Tol H.H. (2000). The dopamine D(4) receptor: one decade of research. *Eur J Pharmacol.* 405(1-3):303-27.

Obeso JA, Rodriguez-Oroz MC, Benitez-Temino B, Blesa FJ, Guridi J, Marin C and Rodriguez M. (2008). Functional organization of the basal ganglia: therapeutic implications for Parkinson's disease. *Mov Disord* 23 Suppl 3:S548- 559.

Ohara K., Haga K., Berstein G., Haga T., Ichiyama A. and Ohara K. (1988). The interaction between D2-

dopamine receptors and GTP-binding sites. *Mol. Pharmacol.* 33:290-296.

Oldenhof J, Vickery R, Anafi M, Oak J, Ray A, Schoots O, Pawson T, von Zastrow M, Van Tol HH. (1998). SH3 binding domains in the dopamine D4 receptor. *Biochemistry.* 37(45):15726-36.

Oldham WM, Hamm HE. (2006) Structural basis of function in heterotrimeric G proteins. *Q Rev Biophys.* 39(2):117-66.

Orru M, Bakešová J, Brugarolas M, Quiroz C, Beaumont V, Goldberg SR, Lluís C, Cortés A, Franco R, Casadó V, Canela EI, Ferré S. (2011). Striatal pre- and postsynaptic profile of adenosine A(2A) receptor antagonists. *PLoS One.* 6(1):e16088.

Ostrom RS and Insel PA. (2004). The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. *Br J Pharmacol.* 143:235-245.

Pak Y., O'Dowd B. F., Wang J. B. and George S. R. (1999). Agonist-induced, G protein-dependent and -independent down-regulation of the mu opioid receptor. The receptor is a direct substrate for protein-tyrosine kinase. *J. Biol. Chem.* 274:27610- 27616.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289:739-745

Pani L. (2002). Clinical implications of dopamine research in schizophrenia. *Curr Med Res Opin.* 18 Suppl 3:s3-7.

Park PS, Lodowski DT and Palczewski K (2008) Activation of G protein- coupled receptors: beyond two-state models and tertiary conformational changes. *Annu Rev Pharmacol Toxicol* 48:107-141.

Pawson T, Scott JD. (1997). Signaling through scaffold, anchoring, and adaptor proteins. *Science.* 19;278(5346):2075-80.

Peeters MC, van Westen GJ, Li Q, IJzerman AP. (2011) Importance of the extracellular loops in G protein-coupled receptors for ligand recognition and receptor activation. *Trends Pharmacol Sci.* 32(1):35-42

Perry SJ, Lefkowitz RJ. (2002). Arresting developments in heptahelical receptor signaling and regulation. *Trends Cell Biol.* 12(3):130-8.

Pfleffer MA, Stevenson LW. (1996). Beta-adrenergic blockers and survival in heart failure. *N Engl J*

Med. 334(21):1396-7.

Pfleger KD and Eidne KA. (2006a). Illuminating insights into protein-protein interactions using bioluminescence resonance energy transfer (BRET). *Nat. Meth.* 3:165-174.

Pfleger KD, Dalrymple MB, Dromey JR, Eidne KA. (2007). Monitoring interactions between G-protein-coupled receptors and beta-arrestins. *Biochem Soc Trans.* 35(Pt 4):764-6.

Pfleger KD, Dromey JR, Dalrymple MB, Lim EM, Thomas WG and Eidne KA. (2006b). Extended bioluminescence resonance energy transfer (eBRET) for monitoring prolonged protein-protein interactions in live cells. *Cell Signal.* 18:1664-1670.

Pfleger KD and Eidne KA. (2005). Monitoring the formation of dynamic G-protein-coupled receptor-protein complexes in living cells. *Biochem. J.* 385:625-637.

Pierce KL and Lefkowitz RJ. (2001). Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat Rev Neurosci* 2:727-733.

Pillai G, Brown NA, McAllister G, Milligan G, Seabrook GR. (1998). Human D2 and D4 dopamine receptors couple through betagamma G-protein subunits to inwardly rectifying K⁺ channels (GIRK1) in a *Xenopus* oocyte expression system: selective antagonism by L-741,626 and L-745,870 respectively. *Neuropharmacology.* 37(8):983-7.

Pin JP, Galvez T and Prezeau L (2003) Evolution, structure, and activation mechanism of family 3/C G-protein-coupled receptors. *Pharmacol Ther* 98:325- 354.

Pippig S., Andexinger S. and Lohse M. J. (1995). Sequestration and recycling of beta2- adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* 47:666-676.

Pontieri FE, Tanda G, Di Chiara G. (1995). Intravenous cocaine, morphine, and amphetamine preferentially increase extracellular dopamine in the "shell" as compared with the "core" of the rat nucleus accumbens. *Proc Natl Acad Sci U S A.* 92(26):12304-8.

Powell SB, Paulus MP, Hartman DS, Godel T, Geyer MA. (2003). RO-10-5824 is a selective dopamine D4 receptor agonist that increases novel object exploration in C57 mice. *Neuropharmacology.* 44(4):473-81.

Prilla S, Schrobang J, Ellis J, Höltje HD, Mohr K. (2006). Allosteric interactions with muscarinic acetylcholine receptors: complex role of the conserved tryptophan M2422Trp in a critical cluster of amino acids for baseline affinity, subtype selectivity, and cooperativity. *Mol Pharmacol.* 70(1):181-93.

Prinster SC, Hague C and Hall RA (2005) Heterodimerization of G protein-coupled receptors: specificity and functional significance. *Pharmacol Rev* 57:289-298.

Probst WC, Snyder LA, Schuster DI, Brosius J and Sealfon SC (1992) Sequence alignment of the G-protein coupled receptor superfamily. *DNA Cell Biol* 11:1-20.

Puzianowska-Kuznicka M, Kuznicki J. (2009). The ER and ageing II: calcium homeostasis. *Ageing Res Rev.* 8(3):160-72.

Ranade K, Jorgenson E, Sheu WH, Pei D, Hsiung CA, Chiang FT, Chen YD, Pratt R, Olshen RA, Curb D, Cox DR, Botstein D, Risch N. (2002). A polymorphism in the beta1 adrenergic receptor is associated with resting heart rate. *Am J Hum Genet.* 70(4):935-42.

Rang HP, Dale MM, Ritter JM and Flower RJ. (2008). *Farmacología*. Editado por ELSEVIER. 6ª edición. Madrid.

Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, Devree BT, Rosenbaum DM, Thian FS, Kobilka TS, Schnapp A, Konetcki I, Sunahara RK, Gellman SH, Pautsch A, Steyaert J, Weis WI, Kobilka BK. (2011). Structure of a nanobody-stabilized active state of the $\beta(2)$ adrenoceptor. *Nature.* 469(7329):175-80.

Reiter E, Ahn S, Shukla AK, Lefkowitz RJ. (2011). Molecular Mechanism of β -Arrestin-Biased Agonism at Seven-Transmembrane Receptors. *Annu Rev Pharmacol Toxicol.*

Renfrow JJ, Scheck AC, Dhawan NS, Lukac PJ, Vogel H, Chandler JP, Raizer JJ, Harsh GR, Chakravarti A, Bredel M. (2011). Gene-protein correlation in single cells. *Neuro Oncol.* 13(8):880-5.

Richman J. G., Brady A. E., Wang Q., Hensel J. L., Colbran R. J. and Limbird L. E. (2001). Agonist-regulated interaction between alpha2-adrenergic receptors and spinophilin. *J. Biol. Chem.* 276:15003-15008.

Rios ER, Venâncio ET, Rocha NF, Woods DJ, Vasconcelos S, Macedo D, Sousa FC, Fonteles MM. (2010). Melatonin: pharmacological aspects and clinical trends. *Int J Neurosci.* 120(9):583-90.

Ritter SL and Hall RA (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat Rev Mol Cell Biol* 10:819-830

Roche K. W., Tu J. C., Petralia R. S., Xiao B., Wenthold R. J. and Worley P. F. (1999). Homer 1b regulates the trafficking of group I metabotropic glutamate receptors. *J. Biol.*

Chem. 274:25953-25957.

Rocheville M, Lange DC, Kumar U, Sasi R, Patel RC and Patel YC. (2000). Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. *J Biol Chem* 275:7862-7869.

Roman T, Schmitz M, Polanczyk G, Eizirik M, Rohde LA, Hutz MH. (2001). Attention-deficit hyperactivity disorder: a study of association with both the dopamine transporter gene and the dopamine D4 receptor gene. *Am J Med Genet.* 105(5):471-8.

Romano C, Miller JK, Hyrc K, Dikranian S, Mennerick S, Takeuchi Y, Goldberg MP and O'Malley KL (2001) Covalent and noncovalent interactions mediate metabotropic glutamate receptor mGlu5 dimerization. *Mol Pharmacol* 59:46-53.

Romano C, Yang WL and O'Malley KL. (1996). Metabotropic glutamate receptor 5 is a disulfide-linked dimer. *J Biol Chem* 271:28612-28616.

Rondou P, Haegeman G, Van Craenenbroeck K. (2010). The dopamine D4 receptor: biochemical and signalling properties. *Cell Mol Life Sci.* 67(12):1971-86.

Rosenbaum DM, Rasmussen SG and Kobilka BK (2009) The structure and function of G-protein-coupled receptors. *Nature* 459:356-363.

Roth BL, Sheffler DJ and Kroeze WK. (2004). Magic shotguns versus magic bullets: selectively non-selective drugs for mood disorders and schizophrenia. *Nat. Rev. Drug Discov.* 3:437-443.

Rouge-Pont F., Usiello A., Benoit-Marand M., Gonon F., Piazza P. V. and Borrelli E. (2002). Changes in extracellular dopamine induced by morphine and cocaine: crucial control by D2 receptors. *J. Neurosci.* 22:3293-3301.

Rozenfeld R, Décaillot FM, IJzerman AP and Devi LA. (2006). Heterodimers of G protein-coupled receptors as novel and distinct drug targets. *Drug Discovery Today: Therapeutic Strategies.* 3:437-443.

Rozenfeld R, Devi LA. (2011). Exploring a role for heteromerization in GPCR signalling specificity. *Biochem J.* 433(1):11-8.

Sahu N, August A. (2009). ITK inhibitors in inflammation and immune-mediated disorders. *Curr Top Med Chem.* 9(8):690-703.

Sakmar TP (1998) Rhodopsin: a prototypical G protein-coupled receptor. *Prog Nucleic Acid Res Mol Biol.* 59:1-34.

- Santanavanich C, Ebadi M, Govitrapong P. (2005). Dopamine receptor activation in bovine pinealocyte via a cAMP-dependent transcription pathway. *J Pineal Res.* 38(3):170-5.
- Santini E, Alcacer C, Cacciatore S, Heiman M, Hervé D, Greengard P, Girault JA, Valjent E, Fisone G. (2009). L-DOPA activates ERK signaling and phosphorylates histone H3 in the striatonigral medium spiny neurons of hemiparkinsonian mice. *J Neurochem.* 108(3):621-33.
- Sara SJ. (2009). The locus coeruleus and noradrenergic modulation of cognition. *Nat Rev Neurosci.* 10(3):211-23.
- Sasa M, Yoshimura N. (1994). Locus coeruleus noradrenergic neurons as a micturition center. *Microsc Res Tech.* 29(3):226-30.
- Saura C, Ciruela F, Casado V, Canela EI, Mallol J, Lluís C and Franco R (1996) Adenosine deaminase interacts with A1 adenosine receptors in pig brain cortical membranes. *J Neurochem* 66:1675-1682.
- Scahill L, Schwab-Stone M. (2000). Epidemiology of ADHD in school-age children. *Child Adolesc Psychiatr Clin N Am.* 9(3):541-55, vii.
- Scarselli M., Armogida M., Chiacchio S., DeMontis M. G., Colzi A., Corsini G. U. and Maggio R. (2000). Reconstitution of functional dopamine D(2s) receptor by coexpression of amino and carboxyl-terminal receptor fragments. *Eur. J. Pharmacol.* 397:291-296.
- Schäferling M, Nagl S. (2011). Förster resonance energy transfer methods for quantification of protein-protein interactions on microarrays. *Methods Mol Biol.* 723:303-20.
- Schaffhauser H, Rowe BA, Morales S, Chavez-Noriega LE, Yin R, Jachec C, Rao SP, Bain G, Pinkerton AB, Vernier JM, Bristow LJ, Varney MA, Daggett LP. (2003). Pharmacological characterization and identification of amino acids involved in the positive modulation of metabotropic glutamate receptor subtype 2. *Mol Pharmacol.* 64(4):798-810.
- Schapira AH, Bezard E, Brotchie J, Calon F, Collingridge GL, Ferger B, Hengerer B, Hirsch E, Jenner P, Le Novere N, Obeso JA, Schwarzschild MA, Spampinato U and Davidai G. (2006). Novel pharmacological targets for the treatment of Parkinson's disease. *Nat Rev Drug Discov* 5:845-854.
- Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP and Ernst OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* 455:497-502.
- Schiattarella GG, Perrino C, Gargiulo G, Sorrentino S, Franzone A, Capretti G, Esposito G, Chiariello M. (2010). Novel concepts in beta-adrenergic receptor signaling: therapeutic options for heart failure. *G Ital*

Cardiol.11(3):221-8.

Schiffmann SN, Fisone G, Moresco R, Cunha RA and Ferre S. (2007). Adenosine A2A receptors and basal ganglia physiology. *Prog Neurobiol* 83:277-292.

Schmitt JM, Stork PJ. (2000). beta 2-adrenergic receptor activates extracellular signal-regulated kinases (ERKs) via the small G protein rap1 and the serine/threonine kinase B-Raf. *J Biol Chem*. 275(33):25342-50.

Schmitz JM, Graham RM, Sagalowsky A, Pettinger WA. (1981). Renal alpha-1 and alpha-2 adrenergic receptors: biochemical and pharmacological correlations. *J Pharmacol Exp Ther*. 219(2):400-6.

Schulte G, Schambony A, Bryja V. (2010). beta-Arrestins - scaffolds and signalling elements essential for WNT/Frizzled signalling pathways? *Br J Pharmacol*.159(5):1051-8.

Schulz A, Grosse R, Schultz G, Gudermann T and Schoneberg T. (2000). Structural implication for receptor oligomerization from functional reconstitution studies of mutant V2 vasopressin receptors. *J Biol Chem* 275:2381-2389.

Schwartz TW, Holst B. (2006). Ago-allosteric modulation and other types of allostery in dimeric 7TM receptors. *J Recept Signal Transduct Res*. 26(1-2):107-28.

Schwartz TW, Holst B. (2007). Allosteric enhancers, allosteric agonists and ago-allosteric modulators: where do they bind and how do they act? *Trends Pharmacol Sci*. 28(8):366-73

Sedvall GC and Karlsson P. (1999). Pharmacological manipulation of D1-dopamine receptor function in schizophrenia. *Neuropharmacology*. 22:181-188.

Seeman P. (2006). Targeting the dopamine D2 receptor in schizophrenia. *Expert Opin Ther Targets*. 10(4):515-31.

Seifert R and Wenzel-Seifert K (2002) Constitutive activity of G-protein- coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn Schmiedebergs Arch Pharmacol* 366:381-416.

Senogles S. E. (1994). The D2 dopamine receptor isoforms signal through distinct Gi alpha proteins to inhibit adenylyl cyclase. A study with site-directed mutant Gi alpha proteins. *J. Biol. Chem*. 269:23120-23127.

Sheng M. and Kim E. (2000). The Shank family of scaffold proteins. *J. Cell Sci*. 113(Pt11):1851-1856.

Simonneaux V, Murrin LC, Ebadi M. (1990). Characterization of D1 dopamine receptors in the bovine pineal gland with [3H]SCH 23390. *J Pharmacol Exp Ther.* 253(1):214-20.

Simonneaux V, Ribelayga C. (2003). Generation of the Melatonin Endocrine Message in Mammals: A Review of the Complex Regulation of Melatonin Synthesis by Norepinephrine, Peptides, and Other Pineal Transmitters. *Pharmacological Reviews* 55: 325–395. doi:10.1124/pr.55.2.2.

Small KM, Schwarb MR, Glinka C, Theiss CT, Brown KM, Seman CA, Liggett SB. (2006). Alpha2A- and alpha2C-adrenergic receptors form homo- and heterodimers: the heterodimeric state impairs agonist-promoted GRK phosphorylation and beta-arrestin recruitment. *Biochemistry.* 45(15):4760-7.

Smith F. D., Oxford G. S. and Milgram S. L. (1999). Association of the D2 dopamine receptor third cytoplasmic loop with spinophilin, a protein phosphatase-1-interacting protein. *J. Biol. Chem.* 274:19894-19900.

Sneider W. (2001). The discovery and synthesis of epinephrine. *Drug News Perspect.* 14(8):491-4.

Soriano A, Ventura R, Molero A, Hoen R, Casadó V, Cortés A, Fanelli F, Albericio F, Lluís C, Franco R, Royo M. (2009). Adenosine A2A receptor-antagonist/dopamine D2 receptor-agonist bivalent ligands as pharmacological tools to detect A2A-D2 receptor heteromers. *J Med Chem.* 52(18):5590-602.

Springael JY, Urizar E, Costagliola S, Vassart G and Parmentier M. (2007). Allosteric properties of G proteina-coupled receptor oligomers. *Pharmacol. Ther.* 115:410-418.

Starke K, Göthert M, Kilbinger H. (1989). Modulation of neurotransmitter release by presynaptic autoreceptors. *Physiol Rev.* 69(3):864-989.

Starr S., Kozell L. B. and Neve K. A. (1995). Drug-induced up-regulation of dopamine D2 receptors on culture cells. *J. Neurochem.* 65:569-577.

Stiles GL, Hoffman BB, Hubbard M, Caron MG, Lefkowitz RJ. (1983a). Guanine nucleotides and alpha 1 adrenergic receptors in the heart. *Biochem Pharmacol.* 32(1):69-71.

Stiles GL, Taylor S, Lefkowitz RJ. (1983b). Human cardiac beta-adrenergic receptors: subtype heterogeneity delineated by direct radioligand binding. *Life Sci.* 33(5):467-73.

Sugden D. (1990). 5-Hydroxytryptamine amplifies beta-adrenergic stimulation of N-acetyltransferase activity in rat pinealocytes. *J Neurochem* 55: 1655–1658.

- Sugita S. (2008). Mechanisms of exocytosis. *Acta Physiol (Oxf)*. 192(2):185-93.
- Sun X, Deng J, Liu T, Borjigin J. (2002). Circadian 5-HT production regulated by adrenergic signaling. *Proc Natl Acad Sci U S A*. 99(7):4686-91.
- Suzuki Y., Moriyoshi E., Tsuchiya D. and Jingami H. (2004). Negative cooperativity in glutamate binding in the dimeric metabotropic receptor subtype 1. *J. Biol. Chem.* 279:35526-35534.
- Svingos AL, Periasamy S, Pickel VM. (2000). Presynaptic dopamine D(4) receptor localization in the rat nucleus accumbens shell. *Synapse*. 36(3):222-32.
- Swanson J, Posner M, Fusella J, Wasdell M, Sommer T, Fan J. (2001). Genes and attention deficit hyperactivity disorder. *Curr Psychiatry Rep*. 3(2):92-100.
- Tan M, Walwyn WM, Evans CJ, Xie CW. (2009). p38 MAPK and beta-arrestin 2 mediate functional interactions between endogenous micro-opioid and alpha2A-adrenergic receptors in neurons. *J Biol Chem*. 284(10):6270-81.
- Tapp E, Huxley M. (1972). The histological appearance of the human pineal gland from puberty to old age. *J Pathol*. 108(2):137-44.
- Taraskevich P. S. and Douglas W. W. (1978). Catecholamines of supposed inhibitory hypophysiotrophic function suppress action potentials in prolactin cells. *Nat*. 276:832-834.
- Tarazi FI, Tomasini EC, Baldessarini RJ. (1998). Postnatal development of dopamine D4-like receptors in rat forebrain regions: comparison with D2-like receptors. *Brain Res Dev Brain Res*. 110(2):227-33.
- Tebben AJ, Schnur DM. (2011). Beyond rhodopsin: G protein-coupled receptor structure and modeling incorporating the beta2-adrenergic and adenosine A(2A) crystal structures. *Methods Mol Biol*. 672:359-86.
- Terrillon S and Bouvier M. (2004). Roles of G-protein-coupled receptor dimerization. *EMBO Rep* 5:30-34.
- Thapar A, O'Donovan M, Owen MJ. (2005). The genetics of attention deficit hyperactivity disorder. *Hum Mol Genet*. 14 Spec No. 2:R275-82.
- Thibault D, Albert PR, Pineyro G, Trudeau LÉ. (2011). Neurotensin triggers dopamine D2 receptor desensitization through a protein kinase C and beta-arrestin1-dependent mechanism. *J Biol Chem*. 286(11):9174-84.

Toda N, Tago K, Marumoto S, Takami K, Ori M, Yamada N, Koyama K, Naruto S, Abe K, Yamazaki R, Hara T, Aoyagi A, Abe Y, Kaneko T, Kogen H. (2003). A conformational restriction approach to the development of dual inhibitors of acetylcholinesterase and serotonin transporter as potential agents for Alzheimer's disease. *Bioorg Med Chem.* 11(20):4389-415.

Trejo J, Hammes SR and Coughlin SR (1998) Termination of signaling by protease-activated receptor-1 is linked to lysosomal sorting. *Proc Natl Acad Sci U S A* 95:13698-13702.

Triller A, Choquet D. (2005). Surface trafficking of receptors between synaptic and extrasynaptic membranes: and yet they do move! *Trends Neurosci.* 28(3):133-9.

Tsamis F, Gavrilov S, Kajumo F, Seibert C, Kuhmann S, Ketas T, Trkola A, Palani A, Clader JW, Tagat JR, McCombie S, Baroudy B, Moore JP, Sakmar TP, Dragic T. (2003). Analysis of the mechanism by which the small-molecule CCR5 antagonists SCH-351125 and SCH-350581 inhibit human immunodeficiency virus type 1 entry. *J Virol.* 77(9):5201-8.

Tsao P. and von Zastrow M. (2000). Downregulation of G protein-coupled receptors. *Curr. Opin. Neurobiol.* 10:365-369.

Ulrich CD, Holtmann M and Miller LJ. (1998). Secretin and vasoactive intestinal peptide receptors: members of a unique family of G protein-coupled receptors. *Gastroenterology* 114:382-397.

Usiello A, Baik JH, Rouge-Pont F, Picetti R, Dierich A, LeMeur M, Piazza PV and Borrelli E. (2000). Distinct functions of the two isoforms of dopamine D2 receptors. *Nature* 408:199-203.

Vallone D, Picetti R and Borrelli E. (2000). Structure and function of dopamine receptors. *Neurosci Biobehav Rev* 24:125-132.

Van Corven EJ, Hordijk PL, Medema RH, Bos JL and Moolenaar WH. (1993). Pertussis toxin-sensitive activation of p21ras by G protein-coupled receptor agonists in fibroblasts. *Proc Natl Acad Sci U S A* 90:1257-1261.

Vendrell M, Angulo E, Casadó V, Lluís C, Franco R, Albericio F, Royo M. (2007). Novel ergopeptides as dual ligands for adenosine and dopamine receptors. *J Med Chem.* 28;50(13):3062-9.

Vidi PA and Watts VJ. (2009). Fluorescent and Bioluminescent Protein-Fragment Complementation Assays in the Study of G Protein-Coupled Receptor Oligomerization and Signaling. *Mol Pharmacol* 75:733–739.

- Violin JD, Lefkowitz RJ. (2007). Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol Sci.* 28(8):416-22.
- Volovyk ZM, Wolf MJ, Prasad SV, Rockman HA. (2006). Agonist-stimulated beta-adrenergic receptor internalization requires dynamic cytoskeletal actin turnover. *J Biol Chem.* 281(14):9773-80.
- Vuoriluoto M, Laine LJ, Saviranta P, Pouwels J, Kallio MJ. (2010). Spatio-temporal composition of the mitotic Chromosomal Passenger Complex detected using in situ proximity ligation assay. *Mol Oncol.* 5(1):105-11.
- Waldhoer M, Fong J, Jones RM, Lunzer MM, Sharma SK, Kostenis E, Portoghese PS, Whistler JL. (2005). A heterodimer-selective agonist shows in vivo relevance of G protein-coupled receptor dimers. *Proc Natl Acad Sci U S A.* 102(25):9050-5.
- Wang E, Ding YC, Flodman P, Kidd JR, Kidd KK, Grady DL, Ryder OA, Spence MA, Swanson JM, Moyzis RK. (2004). The genetic architecture of selection at the human dopamine receptor D4 (DRD4) gene locus. *Am J Hum Genet.* 74(5):931-44.
- Warne T, Moukhametzianov R, Baker JG, Nehmé R, Edwards PC, Leslie AG, Schertler GF, Tate CG. (2011). The structural basis for agonist and partial agonist action on a $\beta(1)$ -adrenergic receptor. *Nature.* 469(7329):241-4.
- Watts VJ, Vu MN, Wiens BL, Jovanovic V, Van Tol HH, Neve KA. (1999). Short- and long-term heterologous sensitization of adenylyate cyclase by D4 dopamine receptors. *Psychopharmacology (Berl).* 141(1):83-92.
- Weibrecht I, Leuchowius KJ, Clausson CM, Conze T, Jarvis M, Howell WM, Kamali-Moghaddam M, Söderberg O. (2010). Proximity ligation assays: a recent addition to the proteomics toolbox. *Expert Rev Proteomics.* 7(3):401-9.
- Weiner N, Langer SZ, Trendelenburg U. (1967). Demonstration by the histochemical fluorescence method of the prolonged disappearance of catecholamines from the denervated nictitating membrane of the cat. *J Pharmacol Exp Ther.* 157(2):284-9.
- Weis WI and Kobilka BK (2008) Structural insights into G-protein-coupled receptor activation. *Curr Opin Struct Biol* 18:734-740.
- Werner P, Hussy N, Buell G, Jones KA, North RA. (1996). D2, D3, and D4 dopamine receptors couple to G protein-regulated potassium channels in *Xenopus* oocytes. *Mol Pharmacol.* 49(4):656-61.

White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM and Marshall FH. (1998). Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature* 396:679-682.

Wise RA. (1996). Neurobiology of addiction. *Curr Opin Neurobiol.* 6:243-251.

Wreggett KA, Wells JW. (1995). Cooperativity manifest in the binding properties of purified cardiac muscarinic receptors. *J Biol Chem.* 270(38):22488-99.

Wu DF, Yang LQ, Goschke A, Stumm R, Brandenburg LO, Liang YJ, Holtt V and Koch T. (2008). Role of receptor internalization in the agonist-induced desensitization of cannabinoid type 1 receptors. *J Neurochem* 104:1132-1143.

Wu WL, Burnett DA, Spring R, Greenlee WJ, Smith M, Favreau L, Fawzi A, Zhang H, Lachowicz JE. (2005). Dopamine D1/D5 receptor antagonists with improved pharmacokinetics: design, synthesis, and biological evaluation of phenol bioisosteric analogues of benzazepine D1/D5 antagonists. *J Med Chem.* 48(3):680-93.

Wurtman RJ. (2002). Stress and the adrenocortical control of epinephrine synthesis. *Metabolism.* 51(6 Suppl 1):11-4.

Yamaguchi I, Harmon SK, Todd RD, O'Malley KL. (1997). The rat D4 dopamine receptor couples to cone transducin (Galphat2) to inhibit forskolin-stimulated cAMP accumulation. *J Biol Chem.* 272(26):16599-602.

Yan Z., Feng J., Fienberg A. A. and Greengard P. (1999). D(2) dopamine receptors induce mitogen-activated protein kinase and cAMP response element-binding protein phosphorylation in neurons. *Proc. Natl. Acad. Sci. USA* 96:11607-11612.

Yang JW, Jang WS, Hong SD, Ji YI, Kim DH, Park J, Kim SW, Joung YS. (2008). A case-control association study of the polymorphism at the promoter region of the DRD4 gene in Korean boys with attention deficit-hyperactivity disorder: evidence of association with the -521 C/T SNP. *Prog Neuropsychopharmacol Biol Psychiatry.* 32(1):243-8.

Yao WD, Spealman RD, Zhang J. (2008). Dopaminergic signaling in dendritic spines. *Biochem Pharmacol.* 75: 2055–2069.

Yilmaz Z, Kaplan AS, Levitan RD, Zai CC, Kennedy JL. (2012). Possible association of the DRD4 gene with a history of attention-deficit/hyperactivity disorder in women with bulimia nervosa. *Int J Eat Disord.* doi: 10.1002/eat.20986.

Yin HH, Lovinger DM. (2006). Frequency-specific and D2 receptor- mediated inhibition of glutamate release by retrograde endocanna- binoid signaling. *Proc Natl Acad Sci USA*. 103: 8251–8256.

Yoon S, Choi MH, Chang MS, Baik JH. (2011). Wnt5a-dopamine D2 receptor interactions regulate dopamine neuron development via extracellular signal-regulated kinase (ERK) activation. *J Biol Chem*. 286(18):15641-51.

Youdim MB, Buccafusco JJ. (2005). CNS Targets for multi-functional drugs in the treatment of Alzheimer's and Parkinson's diseases. *J Neural Transm*. 112(4):519-37.

Zawilska JB, Berezińska M, Rosiak J, Skene DJ, Vivien-Roels B, Nowak JZ. (2004). Suppression of melatonin biosynthesis in the chicken pineal gland by retinally perceived light - involvement of D1-dopamine receptors. *J Pineal Res*. 36(2):80-6.

Zhang L. J., Lachowicz J. E. and Sibley D. R. (1994). The D2S and D2L dopamine receptor isoforms are differentially regulated in Chinese hamster ovary cells. *Mol. Pharmacol*. 45:878-889.

Zheng W, Cole PA. (2002). Serotonin N-acetyltransferase: mechanism and inhibition. *Curr Med Chem*. 9(12):1187-99.

Zhu X and Wess J. (1998). Truncated V2 vasopressin receptors as negative regulators of wild-type V2 receptor function. *Biochemistry* 37:15773-15784.

Zimmermann T., Riedtdorf J., Girod A., Georget V. and Pepperkok R. (2002). Spectral imaging and linear un-mixing enables improved FRET efficiency with a novel GFP2-YFP FRET pair. *FEBS Lett*. 531:245-249.

