DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
50

MODULATION OF SIGNAL TRANSDUCTION OF HEPTAHELICAL RECEPTORS BY OTHER RECEPTORS AND G PROTEINS

AIN UUSTARE

TARTU UNIVERSITY
PRESS

Department of Chemistry, University of Tartu, Estonia
Institute of Organic and Bioorganic Chemistry

Dissertation is accepted for the commencement of the degree of Doctor of Philosophy in Chemistry on April 13, 2006, by the Doctoral Committee of the Department of Chemistry, University of Tartu.

Supervisor: professor Ago Rinken

Opponent: Dr. pharm. Maija Dambrova, Riga

Commencement: June 1, 2006

ISSN 1406–0299
ISBN 9949–11–324–5 (trükis)
ISBN 9949–11–325–3 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr. 248

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I **Uustare A.**, Nasman J., Akerman KE., Rinken A. (2004) Characterization of M2 muscarinic receptor activation of different G protein subtypes. *Neurochem Int.* **44**, 119–24.

II **Uustare A.**, Vonk A., Terasmaa A., Fuxe K., Rinken A. (2005) Kinetic and functional properties of [3H]ZM241385, a high affinity antagonist for adenosine A2A receptors. *Life Sci.* 76, 1513–26.

III Alttoa A., Koiv K., Eller M., **Uustare A.**, Rinken A., Harro J. (2005) Effects of low dose N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine administration on exploratory and amphetamine-induced behavior and dopamine D2 receptor function in rats with high or low exploratory activity. *Neuroscience*. 132, 979–90.

IV **Uustare A**., Reinart R., Rinken A. Modulation of dopamine D1 receptor signalling by adenosine A1 receptors in Sf9 cells require expression of Gi proteins. Manuscript

Author's contribution

Paper I: Main person responsible for writing the paper, preformed experimental work except bacculovirus construction.

Paper II: Main person responsible for writing the paper, experimental work was preformed equally by three first authors.

Paper III: Preformed experiments of the biochemical part and was involved in writing of respective part of the manuscript.

Paper IV: Main person responsible for writing the paper, experimental work was preformed equally by the first two authors.

ABBREVIATIONS

γ[^{32}P]GTP	– guanosine 5'- [γ-^{32}P]- triphosphate
[^{35}S]GTPγS	– guanosine 5'-[γ-^{35}S]-thiotriphosphate
[^{3}H]CGS21680	– [^{3}H]2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethyl-carboxamidoadenosine
[^{3}H]DPCPX	– [propyl-^{3}H]8-cyclopentyl-1,3-dipropylxanthine
[^{3}H]NMS	– 1-[*N*-Methyl-^{3}H]scopolamine methyl chloride
[^{3}H]SCH23390	– [N-*methyl*-^{3}H]R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride
[^{3}H]ZM241385	– [2-^{3}H]-4-(2-(7-amino-2(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]-triazin-5-ylamino]-ethyl)phenol
7-TM	– Seven-transmembrane (receptors)
ADA	– adenosine deaminase
ADP	– adenosine diphosphate
BSA	– bovine serum albumine
cAMP	– cyclic adenosine monophosphate
cDNA	– complementary desoxyribonucleic acid
CGS21680	– 2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethyl-carboxamidoadenosine
CNS	– central nervous system
CPA	– N-(6)-cyclopenthyladenosine
DMPX	– dimethylpropargylxantine
DPCPX	– 8-Cyclopentyl-1,3-dipropylxanthine
DSP-4	– N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
EDTA	– ethylenediaminetetraacetic acid
EGTA	– ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
GABA	– γ-aminobutyric acid
GDP	– guanosine diphosphate
GEF	– guanine nucleotide exchange factor
GPCR	– G-protein coupled receptor
GRK	– G-protein coupled receptor kinase
GTP	– guanosine triphosphate
GTPγS	– guanosine 5'-[γ-thio]- triphosphate
HEPES	– 4-(2-hydroxyethyl)- 1-piperazineethane sulfonic acid
IBMX	– 3-isobutyl-1-methylxanthine
IUPHAR	– The International Union of Basic and Clinical Pharmacology
LC	– *locus coeruleus*
mAChR	– muscarinic acetylcholine receptor
MEM	– minimum essential medium
mRNA	– messenger ribonucleic acid

MRS1220	– 9-chloro-2-(2-furanyl)-5-[(phenylacetyl) amino][1,2,4]-triazolo(1,5-c)quinazoline
MSX-3	– phosphoric acid mono-(3-{8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydro-purin-3-yl}propyl)ester
PBS	– phosphate buffered saline
RGS	– regulator of G protein signaling
Ro 20-1724	– 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone
SKF38393	– 1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol
Tris	– tris(hydroxymethyl)aminomethane
ZM241385	– 4-(2-(7-amino-2(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]-triazin-5-ylamino]-ethyl)phenol

INTRODUCTION

Receptors

According to IUPHAR, receptor has been defined as "A cellular macromolecule, or an assembly of macromolecules, that is concerned directly and specifically in chemical signalling between and within cells" (Neubig *et al.* 2003). On the basis of different signal transduction principles, receptors can be divided into four major classes: 1 – ion channels and ligand-gated ion channels, 2 – seven-transmembrane domain, G protein coupled receptors, 3 – enzyme-associated receptors with subunits having one membrane-inserted domain and 4 – transcription factor receptors (Humphrey and Barnard 1998). Seven-transmembrane domain (7-TM) receptors are a class of receptor molecules transferring extracellular chemical signal to intracellular response system via activation of G proteins and/or other secondary messenger generating systems. These receptors are transmembrane proteins which have been proposed to have seven membrane-passing domains in their structure, corresponding to the hydrophobic areas in their polypeptide chain. There is a large amount of genes encoding that type of proteins (about 400 of them for neurotransmitter receptors and about thousand for olfactory and chemosensory receptors (Teller *et al.* 2001; Mombaerts 1999)). 7-TM receptors have been further classified on the basis of structure into three subclasses: 2.1 – rhodopsin receptor like, 2.2 – secretin receptor like and 2.3 – metabotropic glutamate and $GABA_B$ receptor like receptors (Humphrey and Barnard 1998). Subclass 2.1 is the largest subclass, including most of receptors for non-peptide neurotransmitters (with the exception of glutamate and GABA), particularly also all receptors dealt with in this work. Up to now, the only X-ray chrystal structure of a 7-TM receptor has been recorded for the light-sensitive 7-TM receptor rhodopsin (Palczewski *et al.* 2000; Teller *et al.* 2001), and the structures of other 7-TM receptors are presented assuming that they have similar structural elements. The existence of the seven transmembrane domains proposed from the sequence of primary structure was proven by X-ray structure analysis for the rhodopsin and therefore it has been assumed to be true also for the rest of the 2.1 subclass of 7-TM receptors. The existence of an additional eighth, cytoplasmatic helix has been shown at the carboxyterminal end of the transmembrane helix VII of rhodopsin and it has been proposed to exist in other 7-TM receptors as well (Palczewski *et al.* 2000). Also several indirect evidences like mutational studies have given results supporting the structures of receptors suggested on the basis of X-ray diffraction and hydropathy studies for receptors of this subclass.

The polypeptide chains of 7-TM receptors may be covalently modified, in extracellular loops glycosylated (Kristiansen 2004), while in intracellular loops are sometimes palmitylated (Papac *et al.* 1992). Phosphorylation of intracellular serine and threorine residues of the receptors have essential role in their signal

transduction regulation, leading for example to binding of arrestins and consequent desensitization and/or degradation (Sibley *et al.* 1984; Lohse *et al.* 1992; Lefkowitz and Shenoy 2005). From the intracellular side, 7-TM receptors are coupling to heterotrimeric G proteins (G proteins) that can be activated by the receptor. Since the direct activation of G proteins is the main signal transduction pathway for 7-TM receptors, these receptors have also been called G protein coupled receptors (GPCR-s). However, some of the latest studies have revealed several signal transduction pathways, where 7-TM receptors are connected to effectors without the help of heterotrimeric G proteins (Brzostowski and Kimmel 2001; Lefkowitz and Shenoy 2005). The exact mechanism how the receptors activate coupled G proteins and how this is regulated has remained to be found (Kristiansen 2004).

In addition to heterotrimeric G proteins, 7-TM receptors are found to interact with several other proteins (Nanoff *et al.* 1997; Klinger *et al.* 2002). Most studied proteins interacting with 7-TM receptors and/or their complexes with G proteins are arrestins (Lefkowitz and Shenoy 2005), GRK-s (G-protein coupled receptor kinases) (Penela *et al.* 2003) and RGS proteins (regulator of G protein signalling) (Bernstein *et al.* 2004; Georgoussi *et al.* 2005).

During the last decade more and more information has been accumulated that 7-TM receptors do not function as monomers, but form dimers or oligomers (Gomes *et al.* 2001). It has been shown for various receptors trough experiments of radioligand binding (Gomes *et al.* 2001), fluorescence and bioluminescence energy transfer (Canals *et al.* 2004), coimmunoprecipitation (Uberti *et al.* 2003). In addition to homooligomerization there has been proposed possible heterodimerization between several 7-TM receptors. For example, formation of heterodimers has been suggested between several subtypes of adenosine and dopamine receptor (Gines *et al.* 2000; Hillion *et al.* 2002; Ferre *et al.* 1991), while heterodimerization $GABA_{B1}$ and $GABA_{B2}$ receptor subtypes (belonging to 2.3 subclass of 7-TM receptors) has been shown to be required for the functioning of these receptors (Pin *et al.* 2004).

Within this dissertation, we have studied adenosine (A_1, A_{2A}), dopamine (D_1, D_2) and muscarinic (M_2) receptors, which all belong to the subclass 2.1 of 7-TM receptors, couple to G proteins and tend to form oligomers.

Ligand binding to 7-TM receptors

Ligands are molecules that specifically bind to extracellular side of the receptor. Ligands can be classified on the basis of their effect on receptor system, where (full) agonists fully activate the receptor pathway, partial agonists activate receptor system to a level that is lower than full agonists (neutral) antagonists block the receptor system and inverse agonists lower the constitutive activity of the receptor system if it is present. Activation is a change in conformation of

receptor molecule that affects other messenger molecules coupled to receptor, most notably the G proteins, and generates signal transduction. It has been proposed that receptors in the membrane can have several conformational states, at least one of which corresponds to activated state and at least one to inactive state. There are several approaches describing the role of ligands in activation of receptors. The extended ternary complex model expects equilibrium between active and inactive conformations of the receptor, where agonists preferentially bind and stabilize the former and inverse agonists the later one, while antagonist have no effect on this equilibrium, but block the binding other ligands. As an alternative approach, it has been proposed that active conformation(s) of the receptors are formed due to the agonist binding. There can be more than one active conformation, which have different efficacy to activate following steps of signal transduction, but also different selectivity in the activation of following pathways. For example, according to the extended ternary complex model, the receptor-ligand complexes containing different ligands have different affinity for G proteins (Kenakin 2004). Partial agonists, like full agonists, shift the balance towards activated conformation, but on lesser extent or to a less effective conformation, so maximal effect of receptor activation on the measured response is lower than that caused by full agonists. The level of agonism (full, partial or inverse) of a compound depends also on the level of constitutive activity of the system and so, there can be some ligands that are partial agonists in some system and inverse agonists in others (Jansson *et al.* 1998). Unfortunately, there is very little direct data about conformational changes connected with the ligand binding to receptors available, so the real structural background of this process is not yet clear (Kristiansen 2004). Therefore, several indirect methods have been used to measure the activation of receptor, starting from G protein activation and accumulation of secondary messengers to regulation of gene expression or other more physiological responses on the level of cell or organism (measurement of frog or guinea pig muscle contractions were classical approaches in pioneer studies of receptor mechanisms). The efficacy and potency of agonists can vary depending on the measurement method, as one or several steps in the signal transduction pathway can be 'limiting steps' (Kenakin 2004b).

Ligand binding measurements

For the characterization of ligand binding to receptors, labelled ligands forming stable complexes with receptors have been used; both equilibrium and kinetics of the complex formation with these compounds can be measured. Usually, radioactive isotopes (^{3}H, ^{125}I) are used to label the ligands, but step-by-step, fluorescent labels are being taken into use. The binding of a ligand to receptor is usually reversible and described as an equilibrium complex formation between

ligand molecule L and receptor molecule R (equation 1). In that case, the affinity of the ligand is described by equilibrium dissociation constant (K_D = [R][L]/[RL]) and kinetics are characterized by the parameters k_{obs} (pseudo-first order rate constant in conditions [L]>>[R]) and dissociation rate constant k_{-1}. The dependence of k_{obs} on ligand concentration is expected to follow equation $k_{obs} = k_{-1} + k_1[L]$ and to be connected with dissociation constant as $K_D = k_{-1}/k_1$).

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL$$

(Equation 1)

On the other hand, in several cases the dependence of k_{obs} on [L] has been found to be nonlinear (hyperbolic) (Järv *et al*. 1979; Lepiku *et al*. 1996; Oras *et al*. 2002). This has been explained by the isomerization of antagonist-receptor complex to a slow-dissociating state (Strickland *et al.* 1975) (equation 2), where RL is the fast formed complex and only the isomerized complex RL* can be measured by filtration methods.

$$R + L \overset{K_A}{\rightleftharpoons} RL \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL^*$$

(Equation 2)

The structural background of this isomerization and its significance *in vivo* is still unclear, but it has high impact on design of *in vitro* receptor determination experiments for many 7-TM receptors. These tight isomerized complexes allow direct characterization of interaction between receptors and labelled ligand. As there are only a limited number of this kind of labelled ligands available, the binding properties of other ligands can be characterized by their ability to compete with the 'tracer' ligands for the binding to the receptors (Cheng and Prusoff 1973). The binding of agonists to their receptors can also be modulated by the trimeric G proteins. In the absence of guanine nucleotides, the formation of ternary complex of agonist, receptor and G protein is proposed, which is stable over longer time and causes higher apparent affinity of agonist binding. This kind of high affinity agonist binding can usually not be detected, if the reaction medium contains GTP (or it's analogue GTPγS which cannot be hydrolyzed by G protein α subunit). The shift in agonist affinity caused by the GTPγS has widely been used as an indicator of receptor-G protein coupling.

Muscarinic acetylcholine receptors

Two classes of receptors are activated by the neurotransmitter acetylcholine: nicotinic acetylcholine receptors that are multisubunit ion channels and muscarinic acetylcholine receptors (mAChR-s) that are 7-TM receptors. Muscarinic acetylcholine receptors are named after the substance muscarine, which was first separated from fly agaric mushroom (*Amanita muscaria*) by Schmiedeberg and Koppe at the University of Tartu (Schmiedeberg and Koppe 1869) and specifically activates all mAChR subtypes. There are five subtypes of mAChR-s: M_1, M_2, M_3, M_4 and M_5 (Caulfield and Birdsall 1998). All mAChR subtypes are expressed in the brain, but also several peripheral tissues contain one or a few subtypes. Most important functions of muscarinic signal transmission in CNS are motor control (among others through muscarinic neurons in striatum), memory, temperature and cardiovascular regulation, while in periphery mAChR-s control among others smooth muscle contraction, glandular secretion and cardiac rate and force (Caulfield and Birdsall 1998). The Alzheimer's disease is connected with the loss of cholinergic functions in the brain and therefore muscarinic ligands are in focus for the treatment of this disorder (Clader and Wang 2005; Greenlee *et al.* 2001). However, there have been great difficulties to achieve high subtype selectivity for muscarinic ligands and there are no selective ligands available having greater than tenfold selectivity for one subtype over all the others. Some ligands used in mAChR research are presented in Table 1. In addition to 'common' orthosteric binding site, the existence of an allosteric, modulatory binding site of mAChR-s has been proposed, for which several highly specific ligands like gallamine and alcuronium have been found (Birdsall *et al.* 2001). Also, a group of toxins from the poison of green mamba *Dendroaspis angusticeps* (peptides of 40-65 amino acid residues) have been shown to bind to mAChR-s with high affinity and some of them have very high subtype selectivity (Potter 2001; Karlsson *et al.* 2000).

The highest levels of M_1 mRNA and also the respective protein have been found in cerebral cortex, striatum and hippocampus, where they constitute about 50% of total mAChRs (Wei *et al.* 1994; Matsui *et al.* 2004; Myiakawa *et al.* 2001). M_1 knockout mice have a hyperactive phenotype and increased DA release in striatum (Wess 2003). M_2 mAChR are expressed high level in the thalamus, hypothalamus, pons and medulla. (Wei *et al.* 1994). In periphery, M_2 is the most important subtype in heart, where its activation causes bradycardia (Stengel *et al.* 2000). M_2 is also most widely spread subtype in the smooth muscle, where it modulates the contraction (Ehlert *et al.* 2003). There are no high level regions of expression of M_3 mAChR in brain, it is spread almost equally in all parts (Wei *et al.* 1994). In periphery the mediation of smooth muscle contractions is the most well known function of M_3 receptors, but control of salivary and other glands by these receptors and involvement in

functions of heart have also been suggested (Wang *et al.* 2004). M_3 knockout mice have lowered appetite and body weight, which is proposed to be connected with the role of M_3 mAChR in the release of appetite controlling peptide from hypothalamic neurons (Wess, 2003).

Table 1. Some ligands of muscarinic receptors (According to IUPHAR receptor database, http://www.iuphar-db.org/GPCR/index.html)

	M1	M2	M3	M4	M5
Antagonists:					
	pK_B				
atropine	9.0–9.7	9.0–9.3	9.0–9.7	9.1–9.6	8.9–9.7
4-DAMP	8.6–9.2	7.8–8.4	8.9–9.3	8.4–9.4	8.9–9.0
MT7	**9.8**	<6	<6	<6	<6
Pirenzepine	**7.8–8.5**	6.3–6.7	6.7–7.1	7.1–8.1	6.2–7.1
Tripitramine	8.4–8.8	**9.4–9.6**	7.1–7.4	7.8–8.2	7.3–7.5
Darifenacin	7.5–7.8	7.0–7.4	**8.4–8.9**	7.7–8.0	8.0–8.1
MT3	7.1	<6	<6	**8.7**	<6
Agonists:					
(+)-cis-dioxolane					
arecaidine propargyl ester					
carbamylcholine					
methylfurmethide					
oxotremorine-M					
Radioligands	K_d, nM				
[^{3}H]NMS	0.08–0.15	0.2–0.4	0.15–0.25	0.05–0.1	0.5–0.7
[^{3}H]QNB	0.015–0.06	0.02–0.05	0.03–0.09	0.02–0.08	0.02–0.06
[^{3}H]Pirenzepine	3–15				
[^{3}H]ACh		1–3		1–3	
[^{3}H]darifenacin			0.3		

Values of selective ligands are marked in **bold**.

The highest level of M_4 mAChR mRNA and corresponding protein has been found in striatum, where it is coexpressed with various subtypes of dopaminergic receptors (Wei *et al.* 1994; Matsui *et al.* 2004). M_4 knockout mice are lacking the muscarinic receptor agonists induced release of dopamine in striatum (Zhang *et al.* 2002). M_5 mAChR gene was the last to be found and expression of this receptor is lowest of the mAChR subtypes. As there are no tissues, where it predominates and no selective high-affinity ligands or toxins for this subtype, very little is known about pharmacology and physiology of M_5 mAChR. Some level of M_5 receptor mRNA has been be found in several parts of the brain (Wei *et al.* 1994) and in several arterial preparations (Wess 2003).

In the mice lacking M_5 mAChR, acetylcholine has no dilatory effect on cerebral arteries and also release of dopamine to striatum by dopaminergic neurons of ventral tegmental area has been decreased and the rewarding effects of opioids are lower (Matsui *et al.* 2004).

M_1 and M_3 and M_5 subtypes are preferentially coupled to $G_{q/11}$ family of G proteins, but coupling to G_i ad G_s families has also been shown in recombinant systems (Eglen and Nahorski 2000). M_2 and M_4 mAChRs are preferentially coupled to $G_{i/o}$ family of G proteins and their activation inhibits adenylate cyclase activity (Caulfield and Birdsall 1998), but coupling to G_s family and activation of adenylate cyclase at high agonist concentration has also been reported for M_2 mAChR at high receptor density (Tucek *et al.* 2001). In addition to inhibition of adenylate cyclase, these mAChR subtypes have been shown to open potassium and nonspecific cation channels (Wang *et al.* 2004).

Adenosine receptors

There are two main classes of receptors that bind extracellular purines: adenosine or P1 receptors recognizing adenosine and P2 receptors recognising phosphates of adenosine and uridine (Ralevic and Burnstock 1998). Four subtypes of adenosine receptor have been found to date: A_1, A_{2A}, A_{2B} and A_3, and all of these have a neuromodulatory function (Dunwiddie and Masino 2001). Native agonist for these receptors is adenosine that has a constant basal level present in blood. Increase in its level has been found in tissues by lack of oxygen, inflammation, during high synaptic activity in nervous system and in other stressful conditions (Ralevic and Burnstock 1998). Adenosine receptors are widely expressed and some level of adenosine dependent regulation can be found in almost all organs and tissues (Collis and Hourani 1993). Xanthines and their derivatives (for example caffeine and theophylline), have found to be antagonists for adenosine receptors, but general structural rules for the adenosine receptor ligands have not been defined (see Table 2). Some examples of ligands used in research of adenosine receptors are present in Table 2.

A_1 receptors are very widely spread in central nervous system, but also in other tissues (Ralevic and Burnstock 1998; Rivkees *et al.* 1995; Meyer *et al.* 2005). They preferentially couple to $G_{i/o}$ proteins and therefore cause inhibition of adenylate cyclase. In addition to G proteins, several other proteins including adenosine deaminase (ADA) (Torvinen *et al.* 2002; Ciruela *et al.* 1996), D_1 dopaminergic receptors (Gines *et al.* 2000) and a protein called *coupling co-factor*, that can keep the receptor in high affinity agonist binding state independently of G protein (Nanoff *et al.* 1997), have proposed to interact with the A_1 receptors and play crucial role in their signal transduction.

Table 2. Some ligands of adenosine receptors (According to IUPHAR receptor database, http://www.iuphar-db.org/GPCR/index.html)

	A_1	A_{2A}	A_{2B}	A_3
Antagonists:				
	Affinity, nM			
XAC	24	1*	6	92*
caffeine	12000**	2400**	13000**	80000**
theophylline	9000	1700*	7900*	86000*
DPCPX	**2.5**	1300*	17	4000*
enprofylline			**7000**	
MRS1220	305*	52*		**2**
Agonists in order of increase of potency for respective subtype:				
	NECA	NECA	**NECA**	**Cl-IB-MECA**
	CCPA	**CGS21680**	IB-MECA	NECA
	CPA	**HE-NECA**	R-PIA	R-PIA
	R-PIA	R-PIA	*CPA*	inosine
Radioligands	K_d, nM			
[^{3}H]CCPA	0.6			
[^{3}H]DPCPX	3.9			
[^{3}H]ZM241385		0.5-1	35	
[^{3}H]SCH58261		2		
[^{3}H]CGS21680		16		
[^{3}H]MRS1754			1.2	
[^{3}H]MRE 3008-F20				0.8

Values and names of selective ligands for respective subtype are marked in **bold**

* – data from Klotz, 2000

** – data from Fredholm *et al.* 1999

A_{2A} receptors are mainly bound to G_s subtype of G proteins, while in striatum coupling of these receptors to G_{olf} subtype has been proposed (Kull *et al.* 2000; Herve *et al.* 2001). A_{2A} receptors have the highest expression levels in brain in dopamine-rich regions: striatum, nucleus accumbens and olfactory tubercle (Ongini and Fredholm 1996), but lower levels of A_{2A} receptor can be found in almost all brain regions (Ralevic and Burnstock 1998). In periphery, A_{2A} receptors are located on immune tissues, platelets, vasculary smooth muscle and endothelium (Ralevic and Burnstock 1998). A_{2A} receptors have been shown to form homodimers (Canals *et al.* 2004) as well as heterodimers with D_2 dopaminergic receptor (Hillion *et al.* 2002; Ferre *et al.* 1991). Formation of heterodimers with sybtype 5 metabotropic glutamate receptors has also been proposed (Ferre *et al.* 2002). It has been found that A_{2A} receptor can keep guanine nucleotide indepenent constitutive activity, which could be associated with its very long C-terminal domain (Klinger *et al.* 2002).

A_{2B} receptors preferentially couple to G_s subtype as well, but coupling to $G_{q/11}$ family has been also found (Ralevic and Burnstock 1998). According to the distribution of A_{2B} receptor mRNA, these receptors are expressed in almost all cells of the body, but on rather low level (Ralevic and Burnstock 1998). A_{2B} receptor has rather low affinity for adenosine and desensitizes slowly, which may be important in pathological conditions where adenosine level has risen for a longer time period (Ralevic and Burnstock 1998). No specific agonist for this subtype has been found to date and specific antagonists (derivatives of 8-phenylanthine Carboxylic Acid Congeners (Kim *et al.* 2000) have become available only recently.

A_3 receptors are preferentially coupled to G proteins from the G_i family, but activation of $G_{q/11}$ proteins has also been found (Palmer *et al.* 1995). A_3 receptors are widely distributed over the organism (Ralevic and Burnstock 1998), but with low level and its exact functions are unclear (Lopes *et al.* 2003). A_3 receptors of different species do not have high level of homology (74% between human and rat), which also reflects in differences in ligand binding properties and complicates generation of new subtype selective ligands (Ralevic and Burnstock 1998).

Dopaminergic receptors

Up to date, five subtypes of dopaminergic receptors have been found and in addition the D_2 receptors are present as two splice variants D_{2S} and D_{2L}. On the basis of their pharmacological properties these receptors have been divided into D1 like receptors (D_1 and D_5 subtypes), which stimulate adenylate cyclase and D2 like receptors (D_{2S}, D_{2L}, D_3, D_4), which inhibit adenylate cyclase. High density of dopamineric receptors has been found in striatum, nucleus accumbens and olfactory tubercle, but they are also present in prefrontal cortex and some other parts of the brain (Weiner *et al.* 1991). The D_1 and D_2 (both S and L) subtypes are more widely spread in the brain, while D_3, D_4 and D_5 are less common (Emilien *et al.* 1999). Additionally, some dopaminergic receptors are expressed in the periphery, where they take part in control of blood vessel tone, heart rhythm and renal function (Emilien *et al.* 1999). The dopaminergic neurotransmission in the brain is mainly connected to motor activity, cognition and motivation (reinforcement and rewarding behavior) and to regulation of neuroendocrine function (Emilien *et al.* 1999). Motor activity is mostly bound to dopaminergic synaptic signalling in the (neo)striatum while motivation and rewarding behavior are connected to dopaminergic signalling in nucleus accumbens (Emilien *et al.* 1999). In the case of Parkinson's disease the dopaminergic neurons in substantia nigra are damaged causing greatly lowered dopaminergic output in striatum. Therefore ligands of dopaminergic receptors, but also compounds affecting other components of dopaminergic signal transduction system

(dopamine transporter, adenosine receptors) have possible application as drugs for Parkinson's disease (Emilien *et al.* 1999; Bara-Jimenez *et al.* 2003; Storch *et al.* 2004). Some representative ligands for dopaminergic receptor subtypes are presented in Table 3.

Table 3. Some ligands of dopaminergic receptors (According to IUPHAR receptor database, http://www.iuphar-db.org/GPCR/index.html)

	D_1	D_2	D_3	D_4	D_5
Antagonists:					
	K_i, nM				
(+)-butaclamol	0.3	0.3		88	0.4
haloperidol	15	**0.7**	3	2	30
clozapine	25	78	479	22–83	35
chlorpromazine	23	28	6	10	46
domperidone		**0.05**			
sulpiride	36000	1.8	20	830	29000
L745870				**1**	
NGD941				**3**	
Agonists in order of increase of potency for respective subtype:					
	SKF75670	**lisuride**	7-OH-DPAT	**PD168077**	fenoldopam
	fenoldopam	**bromocriptine**	**BP897**	apomorphine	SKF38393
	SKF38393	apomorphine	quinpirole	quinpirole	dopamine
	dopamine	7-OH-DPAT	apomorphine	dopamine	SKF75670
	apomorphine	quinpirole	dopamine	7-OH-DPAT	apomorphine
	7-OH-DPAT	dopamine	bromocriptine	bromocriptine	
Radioligands	K_d, nM				
[^{3}H]raclopride		2	3	2400	
[^{3}H]SCH23390	0.1–0.3				0.3
[^{3}H]Spiperone	220–450	0.5	0.6	0.07	4500

Values and names of selective ligands for respective subtype are marked in **bold**

D_1 dopaminergic receptors are mainly coupled to activation of adenylate cyclase via G_s and G_{olf}, and in striatum the G_{olf} appears to be dominating (Herve *et al.* 2001). D_1 receptors are highly expressed in striatum, nucleus accumbens, olfactory tubercle, prefrontal cortex, but also in substantia nigra (Weiner *et al.* 1991). In striatum they are located on GABA-ergic (striatonigral) neurons expressing substance P and dynorphin (Missale *et al.* 1998). In addition to G proteins, D_1 receptors interact with adenosine A_1 receptors (Gines *et al.* 2000) and DRIP-78 (Bockaert *et al.* 2004), and have the tendency to form homodimers (Lee *et al.* 2003).

There are no big differences between ligand binding properties of D_{2S} and D_{2L} receptor and only some differences in G protein subtype selectivity have

been found (Senogles *et al.* 1994). The D_2 receptors are highly expressed in striatum, nucleus accumbens, olfactory tubercle, hypothalamus, substantia nigra and cerebral cortex (Weiner *et al.* 1991). The D_2 receptors in striatum (mainly D_{2L} (Khan *et al.* 1998)) are usually expressed in enkephalin-expressing (striatopallidal) GABA-ergic neurons (Missale *et al.* 1998), coexpression in same neurons with D_1 subtype in striatum is low (Hersch *et al.* 1995). D_2 dopaminergic receptor has been shown to form homodimers (Lee *et al.* 2003) and to interact with several other proteins (Bockaert *et al.* 2004), including adenosine A_{2A} receptors (Hillion *et al.* 2002; Ferre *et al.* 1991) and proteins that link the receptor to cytoskeleton (Smith *et al.* 1999).

D_3 and D_4 receptors belong to the D2-like receptor family and have a high structural homology with the D_2 subtype, especially in the transmembrane regions. There are introns in the genes of D_3 and D_4 subtypes, just like in case of D_2 receptors, but no functioning alternatively spliced isoforms of these subtypes have been found. Pharmacologically, D_3 and D_4 subtypes are quite similar to D_2, but ligands with some degree of selectivity have been found during the recent years (Table 3). Higher levels of D_3 receptors have been found in nucleus accumbens, olfactory tubercle and Islands of Calleja, but lower levels are also present in other parts of the brain. D_4 receptors, in contrast, are not expressed in basal ganglia, but they can be found in cerebral cortex, hippocampus, hypothalamus and mesencephalon. (Missale *et al.* 1998).

D_5 subtype of dopaminergic receptors is structurally and pharmacologically very similar to D_1 subtype, initially these receptors were called D_{1b}. Like D_1 subtype, it is preferentially coupled to G_s subtype of G proteins. Up to now there are no selective ligands available, which would pharmacologically discriminate between D_1 and D_5 subtypes (Table 3). D_5 receptors are usually expressed in lower level than D_1 subtype and in many brain regions these receptors may be coexpressed. (Missale *et al.* 1998).

Interaction of adenosine and dopamine receptors in striatum

Neuromodulator adenosine has been found to have opposing effect to dopamine neurotransmission in central nervous system. For example adenosine receptor agonists inhibit and adenosine receptor antagonists potentiate motor activating effects of dopamine signalling (Ferre *et al.* 1997). Motor control and several other processes are controlled by dopaminergic signalling in the striatum and nucleus accumbens and the modulatory effects of adenosine are occurring in the same area. There are several subtypes of dopaminergic receptors (D_1, D_2, D_3) and adenosine receptors (mainly A_1 and A_{2A}) expressed in these areas, which can be involved in the regulation.

A_{2A} receptors are highly concentrated in striatum (Jarvis and Williams 1989) and are co-localized with dopamine D_2 receptors on striatopallidal GABA-ergic

neurons (Ferre *et al.* 1997). The effects of A_{2A} receptor agonist and antagonists have been shown to occur specifically on D_2 dopaminergic receptors (Ferre *et al.* 1997). Antagonistic effect of adenosine A_{2A} receptor activation on D_2 receptor signal transduction has also been shown on neuronal level, where A_{2A} agonists counteract the inhibitory effects of D_2 activation on GABA release from the neurons. On the cellular level, agonists of A_{2A} receptor have been shown to decrease the affinity of D_2 receptor agonists (Ferre *et al.* 1991; Dasgupta *et al.* 1996). A close co-localization of these two receptors has been shown (Hillion *et al.* 2002), which has led to the proposal that these two receptors may form heterodimers. A possible formation of heterodimers between D_3 and A_{2A} receptors has been also suggested to occur in transiently transfected HeLa cells (Torvinen *et al.* 2005).

Adenosine A_1 receptors are colocalized in striatum with both D_1 and D_2 receptors, but no A_1-specific modulation of D_2 receptor regulated motor activity has been found. On the other hand, A_1 receptors have been shown to specifically modulate D_1 receptor specific regulation of motor activity and A_1 agonists inhibit increase in GABA release caused by the D_1 receptor activation (Ferre *et al.* 1997). Activation of A_1 receptors have also been shown to lower the affinity of agonist binding to D_1 receptors in membrane preparations of fibroblast cells expressing both these receptors (Ferre *et al.* 1998). The formation of heterodimeric complexes between A_1 and D_1 receptors has been proposed on the data of receptor colocalization and coprecipitation in these cells (Gines *et al.* 2000).

G proteins – subtypes and activation

G proteins (guanine nucleotide binding regulatory proteins) form two big classes: monomeric 'small' G proteins that participate in several intracellular signalling pathways and heterotrimeric G proteins, which mediate signals from 7-TM receptors into cells. Herewith we use the term 'G proteins' for heterotrimeric G proteins, which consist of the α (G_α), β (G_β) and γ (G_γ) subunits, where β and γ form a dimer $\beta\gamma$, that does not dissociate under physiologic conditions. All three subunits have several subtypes: genes for 17 different Gα, 5 Gβ and 12 Gγ subunits have been described (Hur and Kim 2001). Guanine nucleotide binding and hydrolyzing site is located in the Gα subunit, where a helicase domain covers the bound nucleotide and holds back its dissociation while rather freely formed C- and N-terminal domains of this subunit are thought to be important for interactions with receptors (Cabrera-Vera *et al.* 2003). Some parts of the helicase domain are also found to take part in the selective receptor coupling (Cabrera-Vera *et al.* 2003; Heydorn *et al.* 2004).

Combining of all different α, β and γ subunits yields theoretically a very wide variety of trimeric complexes, but in practice there are several restrictions,

which limit the list. The βγ dimer is essential for G protein activation by the receptor (Fung 1983), but also activates by itself several effectors, like K^+ and Ca^{2+} specific ion channels, phospholipase C and adenylate cyclase and several protein kinases (Cabrera-Vera *et al.* 2003). Here clear subunit selectivity has not been found, but for example $G_{\beta 4}$ subunit is much more efficient and potent than $G_{\beta 1}$ subunit at coupling A_{2A} receptor to G_s protein (Murphree *et al.* 2002; McIntire *et al.* 2001), dimers containing β_5 subunit take preferentially part in signal transduction of $G_{q\alpha}$ (Lindorfer *et al.* 1998) and the $G_{\beta 1\gamma 1}$ dimer, that is present mainly in retina, has substantially lower affinity for some effectors like adenylate cyclase and phospholipase C (Ueda *et al.* 1994).

The selectivity for receptors and effectors is higher between the G_α subunit and therefore G proteins are classified into families on the basis of G_α subunit structure and properties. The four main families of G proteins (G_α subunits) are G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ (Offermanns 2003).

G_s (stimulating) family has been named after its ability to activate all membrane-bound isoforms of adenylate cyclase. (Offermanns 2003). This family contains $G_{olf\alpha}$ and two splice variants of $G_{s\alpha}$–$G_{s\alpha S}$ and $G_{s\alpha L}$ (Jones *et al.* 1990). All $G_{s\alpha}$ subunits can be ADP-ribosylated by cholera toxin, which leads to constitutive activation of corresponding G proteins (Jones *et al.* 1990).

The $G_{i/o}$ family has 8 subtypes: $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{o\alpha}$, $G_{z\alpha}$, $G_{gust\alpha}$, $G_{t\alpha r}$ and $G_{t\alpha c}$. Most of these proteins are sensitive to ADP-ribosylation by *pertussis* toxin that uncouples the Gα subunits from the receptor and disables their activation (Itoh *et al.* 1988). $G_{\alpha i1}$, $G_{\alpha i2}$ and $G_{\alpha i3}$ can cause inhibition of adenylate cyclase (i – inhibitory) and have quite similar structure and properties (Offermanns 2003). $G_{z\alpha}$ subunit like the $G_{i\alpha}$ subtypes, inhibits adenylate cyclase, but is not sensitive to *pertussis* toxin (Fong *et al.* 1988). $G_{\alpha o}$ is the most abundant subunit in neurons, but reports about its direct effectors are quite controversial and finally it has been suggested that most effects of G_o proteins are mediated by the βγ subunits (Offermans 2003). The $G_{t\alpha}$ subunits $G_{t\alpha r}$ and $G_{t\alpha c}$ belong to specific visual signal transduction protein transducin (Lerea *et al.* 1986), which mainly effects cyclic guanosyl monophosphate specific phosphodiesterase (Yarfitz and Hurley 1994). $G_{gust\alpha}$ (gustducin) takes part in signal transduction of taste receptors, its effector pathway is similar to transducin (Ruiz-Avila *et al.* 2003).

The $G_{q/11}$ family contains $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$ and $G_{\alpha 15/16}$ subtypes (Wilkie *et al.* 1991) that activate β isoforms of phospholipase C (Offermanns 2003).

The $G_{12/13}$ family contains $G_{12\alpha}$ and $G_{13\alpha}$ subunits (Strathmann and Simon 1991), that have no common effector pathway, but their role in activation of several effectors like guanine nucleotide exchange factors (GEFs) for Rho, a monomeric G protein (Riobo and Manning 2005) has been reported.

Selectivity of receptors for G protein subtypes

The classification of 7-TM receptors indicates preferred G proteins for each subtype of receptors, but most of the receptors can also activate other G proteins, albeit with lower efficacy. No 7-TM receptors preferring $G_{12/13}$ family to other G proteins have been found and these proteins are mainly activated by receptors, which are directed to $G_{q/11}$ family (Riobo and Manning 2005). There are also several other examples where receptors activate G proteins from different families. For example, α_{2A} adrenergic receptors mediate signal transduction via G proteins from both G_i and G_s families (Brink *et al.* 2000), D_1 dopamine receptor has been shown to activate G proteins from G_s and G_q families (Pacheco and Jope 1997).

On the other hand, there are several examples of receptors, which are very selective in activation of particular subtypes, even within one G protein family. For example, A_1 adenosine receptor has been shown to activate $G_{i\alpha3}$ and $G_{o\alpha}$ subtypes from the G_i family with different efficacy (Lorenzen *et al.* 1998), M4 mAChR distinguishes between the $G_{i\alpha2}$ and $G_{o\alpha}$ subtypes (Migeon *et al.* 1995), splice variants of D_2 dopamine receptor, D_{2S} and D_{2L} prefer $G_{i\alpha2}$ and $G_{i\alpha3}$ subtypes, respectively (Senogles 1994). In the case of α_{2A} adrenergic receptor the G protein subtype preference depends on the structure of activating ligand (Brink *et al.* 2000), which is caused by stabilization of different activated conformations of receptor by different agonists (Kenakin 2003).

Activation of G proteins, measuring of the activation level

According to current paradigm, the heterotrimeric G protein is in resting state as $\alpha\beta\gamma$ complex and the nucleotide-binding site of the α subunit contains GDP. The conformation change in 7-TM receptor that is triggered by agonist binding activates the G protein by lowering the GDP binding affinity of G_α subunit (Cabrera-Vera *et al.* 2003). After dissociation of GDP from the $\alpha\beta\gamma$ complex, GTP binds to G_α, causing a conformation change in this subunit and dissociation of the trimeric complex to G_α-GTP and $G_{\beta\gamma}$ subunits that both are able to activate effector proteins. Activation is terminated by hydrolyzing of GTP to GDP by nucleotidase activity of Gα subunit and following reformation of the $\alpha\beta\gamma$ complex. Dissociation of GDP from Gα is the rate-limiting step in this activation cycle (Cabrera-Vera *et al.* 2003).

The activation level of G protein or receptor-G protein complexes is usually measured as changes in second messenger concentrations caused by the modulation of activity of effector enzymes by G protein subunits, but more direct information can be obtained by measuring the changes in the rate of hydrolysis of GTP ($\gamma[^{32}P]$GTP) by Gα subunit (Gierschik *et al.* 1994), or in the affinity of

nucleotide binding affinity, (using [^{35}S]GTPγS) to Gα subunit (Wieland and Jakobs 1994) or in level of covalent binding of labelled GTP-derived affinity labels to Gα subunit using (Zor *et al.* 1995). In systems containing several subtypes of G proteins, most reliable results with these methods can be obtained for G proteins from the $G_{i/o}$ family, as the activation of other G proteins are shadowed by the high basal activity of the $G_{i/o}$ proteins (Milligan 2003). Therefore, it is often easier to describe the activation of G_s and $G_{q/11}$ family G proteins trough accumulation of corresponding second messengers, cyclic adenosine monophosphate (cAMP) and inositol phosphates, but in these cases the number of steps between activated receptor and measured response may complicate direct interpretation of obtained results. To increase the sensitivity and selectivity of direct G_α activation measurements, specific separation of subtypes of G_α-nucleotide complexes with immunoprecipitation methods has been proposed (Milligan 2003). Another possibility to measure activation of specific G protein subtypes is to use expression systems, where the level of other, interfering G proteins is very low and has no significant effect on the results, for example cells from the insect *Spodoptera frugiperda* (Sf9 cells) (Gille and Seifert 2003).

Using data from receptor research to interpret behavioral experiments

The behavior of organisms is mainly based on the signalling between neurons in central nervous system, certain aspects of behavior can be correlated with specific nerve signal transduction pathways. The properties of receptor-G protein complexes have a key role in signal transduction system and have impact up to the organism level and dysfunctions of these systems are connected with several pathologies. For example dopaminergic signalling from ventral tegmental area to striatum and nucleus accumbens is respectively connected with motor activity and positive reinforcement to novelty (Rebec *et al.* 1997) or drug taking (Di Chiara and Imperato 1988), malfunctions in this pathway are associated with motor disorders in Parkinson's disease and addiction disorders (Missale *et al.* 1998). Ventral tegmental area is innervated by noradrenergic neurons of locus coeruleus (LC), activity of these neurons has been shown to be important for adaption to changes in environment (Aston-Jones *et al.* 1991). Damaging of the adrenergic LC neurons results in lower activity of dopaminergic neurons and lower dopamine release in striatum (Lategan *et al.* 1992), which leads to increase in expression level of D_2 receptors in striatum (Harro *et al.* 2000). The absence of noradrenergic signalling from LC is also shown to cause D_2 receptor hypersensitivity to agonists (Weinshenker *et al.* 2002). LC is shown to have an important part in Parkinson's disease (Gesi *et al.* 2000). Noradrenergic nerve terminals of LC neurons can be specifically damaged or destroyed by using neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) (Lategan

et al. 1992), this has been used as a model for studying LC damages (or different levels of activity) in the nervous system. Hypersensitivity of dopaminergic receptors has also been observed when damaging nigrostriatal dopaminergic pathway with 6-hydroxydopamine (Missale *et al.* 1998), this hypersensitivity is not as much connected to increased receptor density, as to changes in interaction between D_2 dopaminergic receptors and G proteins (LaHoste and Marshall 1992; Terasmaa *et al.* 2000). We have studied the effect of partial LC damage caused by DSP-4 treatment on the signal transduction of D_2 receptors in striatum and nucleus accumbens.

MATERIALS AND METHODS

Cell cultures and transfection

Chinese hamster ovary cell line (CHO-K1) stably expressing double hemagglutinin-tagged dog adenosine A_{2A} receptors, prepared by Maria Torvinen (Karolinska Institute, Stockholm, Sweden) and characterized as described earlier (Torvinen *et al.* 2002; Torvinen *et al.* 2004) were grown to adherence and maintained in a-MEM without nucleosides, containing 10% foetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and geneticin (G-418, 500 μg/ml) at 37°C in a 5% CO_2 – 95% air atmosphere with saturated humidity on plastic Petri dishes.

Sf9 cells for expression of A_1 and D_1 receptors were grown in the Sf900 medium with L-glutamine (Gibco), supplemented with penicillin (50 U/ml, Gibco) and streptomycin (50 μg/ml, Gibco). Sf9 cells for expression of M_2 receptors were grown in Grace's insect medium supplemented with lactalbumin hydrolysate and yeastolate (Life Technologies). Additional supplements were 8% (v/v) foetal bovine serum (Life Technologies), 100 U/ml penicillin and 80 U/ml streptomycin (Sigma–Aldrich Fine Chemicals). In both cases, the cells were grown at 27°C in 500 ml flasks on a rotation shaker. The cultures were maintained at density $(0.5–3)\times10^6$ cells/ml. Expression of particular receptors and G proteins was initiated by addition of high-titer virus stocks to the culture of cells as described in (I, IV). All infections were allowed to proceed for 48–50 h and then the cells were harvested by centrifugation (1500 rpm, 10 min).

The recombinant baculoviruses for the receptors and G protein subunits were prepared by Dr. Johnny Näsman (Kuopio University, Finland) as described earlier (Näsman *et al.* 2001, Näsman *et al.* 2002). The cDNA-s for the human adenosine A_1 receptor and for the human dopamine D_1 receptor in pcDNA3 were obtained from the laboratory of Prof. K. Fuxe (Karolinska Institutet, Sweden), transfer vectors harbouring the genes for M_2 mAChR, $G\alpha_s$, $G\alpha_{i1}$, $G_{\alpha o}$ and $\beta_1\gamma_2$, all of bovine origin, were gifts from Prof. T. Haga (Gakushin University, Tokyo, Japan).

Preparation of membranes

CHO cells

For radioligand binding experiments the cells were collected, washed with PBS, and homogenized by sonication in homogenization buffer (HB, 20 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.5). The crude homogenate was centrifuged at 40,000 g for 20 min at 4°C and the pellet homogenized in HB. The resulting membrane suspension, unless otherwise indicated, was incubated with

adenosine deaminase (ADA, EC 3.5.4.4, Roche Diagnostics GmbH, 5 U/ml) for 30 min at 37°C to remove endogenous adenosine. The membranes were centrifuged and homogenized two more times as above but without ADA treatment. The final pellet was resuspended in HB (0.2 mg protein/ml) and was used directly for binding experiments.

Sf9 cells

The collected cells were homogenized by sonication in the Sf9 homogenization buffer (Sf9HB), containing 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM EGTA, 0.1 mM phenyl methyl sulphonyl fluoride, 1 μg/ml aprotinin, 0.25 mM benzamidine. The cell nuclei and big cell particles were separated by centrifugation at 1000×g for 10 min at 4°C and membranes were collected by centrifugation of the supernatant fraction at 30,000×g for 40 min at 4°C. The membranes were washed twice by resuspension of pellets in Sf9HB and centrifugation and the final homogenizate was stored at –80°C. In experiments with A_1 receptors, the membranes were also incubated with 5 U/ml ADA for 30 min at 25°C in the binding assay buffer (BAB), containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA before the final homogenization.

Striatum and nucleus accumbens

Rats were decapitated and respective brain regions were dissected by prof. J. Harro as described in the (III). Tissues were homogenized by homogenization in 30 vol (ww/v) of Tris-HCl (pH=7.4). The membranes were collected by centrifugation at 40,000 g for 20 min at 4°C and were washed by homogenization and centrifugation for two more times. For the experiments with D_2 stimulated [^{35}S]GTPγS binding, the final pellets of striatal membranes were homogenized in 90 vol (ww/v) and nucleus accumbens membranes in 450 vol (ww/v) of the incubation buffer (20 mM K-HEPES, 7 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.4). For the experiments with [^{3}H]ZM241385, all homogenizations were done in HB.

Radioligand binding experiments

We have used [2-^{3}H]-4-(2-(7-Amino-2(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]-triazin-5-ylamino]-ethyl)phenol ([^{3}H]ZM241385, 21 Ci/mmol, Tocris Cookson Ltd.), [propyl-^{3}H]8-Cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX, 124 Ci/mmol), [N-*methyl*-^{3}H]R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride ([^{3}H]SCH23390, 71.0 Ci/mmol),

and 1-[*N*-Methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS, 79.0 Ci/mmol, all from Amersham Biosciences) as radioligands for A_{2A} adenosine, A_1 adenosine, D_1 dopamine and M_2 muscarinic receptors, respectively.

A_{2A} adenosine receptors were characterized by incubating membranes (100 μg protein/500 μl) in incubation buffer (IB, 20 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA pH 7.4) with appropriate concentrations of [^{3}H]ZM241385 (0.04 to 4 nM) for 45 min at 25°C. In displacement experiments the non-labelled compounds (from 1 pM to 100 μM, depending on ligand studied) were incubated with 1.4 nM [^{3}H]ZM241385 for 60 min at 25°C. A_1 adenosine and D_1 dopamine receptors were characterized by incubation of membranes in BAB (20 μg protein/point) with increasing concentrations of radioligand (0.1–10 nM for [^{3}H]DPCPX, 0.2–7 nM for [^{3}H]SCH23390) or in displacement experiments the different concentrations of the ligand of interest (from 1 pM to 100 μM) with a fixed concentration of radioligand (2.5 nM [^{3}H]SCH23390 or 5.0 nM [^{3}H]DPCPX) for 60 minutes at 25°C. Muscarinic M_2 receptors were characterized by incubation of membranes (30 μg protein/ 250 μl) in the HEG buffer, containing 20 mM Na-HEPES (pH 7.4), 30 mM NaCl, 5mM $MgCl_2$, 1 mM EDTA, 0.5 mg/ml BSA, with different concentrations of [^{3}H]NMS (10pM to 5 nM) or, in the case of displacement experiments, with 1.35nM [^{3}H]NMS and other ligands (from 100 pM to 100 μM) for 90 min at 25 °C. The reactions were terminated by rapid filtration through GF/B glass-fibre filters (Whatman Int. Ltd., Madistone, UK) using a Brandel cell harvester and three washes of 5 ml of ice-cold washing buffer containing 20 mM Tris-HCl and 100 mM NaCl (pH 7.5). The filters were kept overnight with 5 ml of scintillation cocktail OptiPhase HiSafe®3 (Wallac Perkin Elmer Life Sciences) and radioactivity content was measured using a Beckman LS 1800 scintillation counter. Non-specific binding was determined in the presence of 0.5 mM dimethylpropargylxantine (DMPX), 1 μM DPCPX (8-Cyclopentyl-1,3-dipropylxanthine, Tocris Bioscience), 1 μM butaclamol (Sigma-Aldrich Fine Chemicals) and 1 μM atropine, for A_{2A} adenosine, A_1 adenosine, D_1 dopamine and M_2 muscarinic receptors, respectively.

Association kinetics experiments were started by addition of the radioligand at a time moment 0 to a membrane suspension, at timed intervals aliquots were taken and filtered on GF/B as described above. Dissociation kinetics were measured after preincubation of membranes with radioligand for 45 min at 25°C and dissociation was initiated by addition of excess of unlabelled competitive ligand or by 20 times dilution of reaction medium. At timed intervals aliquots from the incubation medium were filtered on GF/B and the bound radioactivity was determined as described above.

The binding of guanosine 5'-[γ-^{35}S]-thiotriphosphate ([^{35}S]GTPγS 1250 Ci/ mmol, Amersham Biosciences) to G_i and G_o proteins expressed in Sf9 cells was measured by incubating membrane suspension (15 μg protein/250 μl) with 0.2 nM radioligand and different concentrations of competitive nucleotides in

HEG buffer for 90 min at 25°C. Isotope dilution method was used to determine the total amount of GTPγS binding sites: 0.2 nM [^{35}S]GTPγS was mixed with increasing concentrations of unlabelled GTPγS (up to 1 μM) to have a saturating concentration of this nucleotide. The modulation of [^{35}S]GTPγS binding (0.2 nM) by muscarinic receptors was measured in the presence of 10 μM GDP and different concentrations of muscarinic ligands. To measure inverse-agonist effects of antagonists, the incubation buffer containing 20 mM Na-HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 0.5 mg/ml BSA (final [Na^+] ≈10 mM) was used.

D_2 receptor specific modulation of [^{35}S]GTPγS binding was carried out as described earlier (Rinken *et al.* 1999). In brief, the membranes (500 μg of striatal or 150 μg of nucleus accumbens membranes per tube) were incubated with 0.2 nM [^{35}S]GTPγS and different concentrations of GDP (3 mM — 1 μM) and 1 mM dopamine or 10 μM butaclamol for 90 min at 30°C. The reaction was stopped and the radioactivity content of the filters was counted as described above.

cAMP accumulation

The receptor-specific activation of adenylate cyclase was determined by measurement of accumulation of cyclic adenosine monophosphate (cAMP) in cells. CHO cells were sown onto 24-well Petri dishes (NUNC) 24 h before experiments and the cell medium was supplemented with ADA (3 U/ml). The cells were washed with serum-free α-MEM medium and incubated in 400 μl serum-free α-MEM medium containing 1.5 U/ml ADA for 30 min at 37°C. The ligand dilutions and 100μM 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone, (Ro 20-1724, Torcis Cookson Ltd.) in 100 μl PBS per well were added and the incubation carried out for 15 minutes at 37°C. The reaction was terminated with addition of ice-cold $HClO_4$ (final 0.4 M). After 1h incubation on ice, the cell lysates were neutralized with KOH, centrifuged at 16,000 g for 2 min and the cAMP content of the supernatant fractions were determined by the modified protein-binding method (Nordstedt and Fredholm 1990).

Sf9 cells (10 ml, (2–3) x 10^6 cells/ml) were infected with baculovirus stocks of receptor(s) and/or G protein subunits, dispensed to a 24-well petri dishes and infections were allowed to proceed for 48 hours. Then the cells were washed with PBS and incubated for 30 min with 400 μl PBS containing 1.5 U/ml ADA. The adenosine and/or dopamine receptor ligands together with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) in 100 μl PBS were then added to the wells and the cells were incubated for additional 15 min at 27°C. The reactions were stopped and amount of cAMP produced was measured as described above. The inhibitory effects of A_1 receptor agonists were measured after activation of

adenylate cyclase with the receptor-independent activator forskolin (15 μM, Tocris Bioscience).

All data were analyzed using the commercial program GraphPad PRISM™ (GraphPad Software Inc.). Data are presented as mean ± S.E.M. of at least two independent determinations carried out in duplicates. Statistical significance of differences was determined by Student-Newman-Keuls test, where $P<0.05$ was taken as a criterion of significance.

RESULTS

Binding properties of A_{2A} specific antagonist [^{3}H]ZM241385 to rat striatal membranes and membranes of CHO cells transfected with dog A_{2A} receptor

[^{3}H]ZM241385 binding affinity to striatal membranes was K_d=0.14±0.01 nM and to CHO cell membranes 0.23±0.03 nM. The densities of the binding sites were 1620±40 and 360±15 fmol/mg protein, respectively. The presence of at least 30 mM NaCl was required for the high affinity binding of [^{3}H]ZM241385 to appear. A_{2A} receptor agonist [^{3}H]CGS21680 had comparable affinity to the binding sites in striatal and CHO membranes as well (K_d was 68±3 nM in striatum and 78±3 nM in CHO membranes, respectively) and the amount of binding sites labelled was same as that for the antagonist (1650±60 fmol/mg protein in striatum, 400±30 fmol/mg protein in CHO).

To break down possible internal adenosine, the membrane preparations were treated with adenosine deaminase (ADA). This treatment had no effect on the radioligand binding to striatal membranes (K_d was 0.17±0.03 in untreated and 0.14±0.01 in treated membranes), but substantially increased its affinity to CHO membranes (K_d in untreated membranes was 0.49±0.07 and in treated membranes 0.23±0.03), which indicates presence of endogenous adenosine and/or lack of sufficient ADA in the CHO cell membrane preparations. In all the following experiments, CHO membranes were pretreated with ADA.

The [^{3}H]ZM241385 binding kinetics were rather fast: dissociation half-life was ~3 min (rate constants were k_{-1} = 0.20±0.01 min^{-1} and k_{-1} = 0.25±0.04 min^{-1} in striatal and CHO membranes, respectively), association of 0.8 nM ligand had half-life 1.2 min, which means that a steady state was reached in about 6 minutes. More detailed research of [^{3}H]ZM241385 binding kinetics was carried out on the striatal membranes. Increase in radioligand concentration caused an increase in association rate and at the highest measurable concentration 5.9 nM, k_{obs} was 3.8±0.8 min^{-1} and respective half-life was 11±2 s. The dependence of observed rate constant k_{obs} on concentration was linear, which refers to simple one-step binding equilibrium (equation 1). The calculated value of the second order rate constant was k_1=0.42±0.01 min^{-1}M^{-1} and the dissociation constant from kinetic parameters $K_d=k_{-1}/k_1$=0.48±0.04 nM, which is higher than the value from the equilibrium binding experiment. This discrepancy and high deviation of k_{obs} values at high radioligand concentration allowed us to use a more complex reaction scheme that includes an isomerization step (equation 2) and has been used to describe the binding of antagonists to other 7-TM receptors. The fitting of data to the two-step reaction scheme yielded an isomerization rate constant value k_i=6.9±1.2 min^{-1} and a K_A value 8.5±2.2 nM, K_d calculated from these values was $K_d=K_A(k_{-i}/k_i)/(1+k_{-i}/k_i)$=0,24 nM, which

matcher better with the dissociation constant found from the equilibrium binding experiments.

Modulation of cAMP accumulation in CHO cells expressing A_{2A} receptors

Selective A_{2A} receptor agonist CGS21680 caused a concentration-dependent increase in cAMP accumulation in CHO cells expressing A_{2A} receptors. Maximal increase was 600% over the basal level and EC_{50} of CGS21680 was 15±3 nM. Other agonists adenosine and N-cyclopentyladenosine caused a similar activation with EC_{50} values of 51±10 nM and 250±70 nM, respectively. Adenosine A_2 receptor antagonists ZM241385, MRS1220 and MSX-3 were tested for their ability to inhibit the effect of CGS21680. None of them had an effect on basal level of cAMP accumulation, but inhibited the effect of CGS21680 in a concentration-dependent manner (IC_{50} values in the presence of 100 nM agonist were 54±28, 81±26 and 175±89 for ZM241385, MRS1220 and MSX-3, respectively). All antagonist inhibited agonist effects in a competitive manner, as the respective Schild plots had slopes close to unity and calculations revealed K_i values 6.6 nM, 16.8 nM and and 39.7 nM for ZM241385, MRS1220 and MSX-3, respectively. The K_i for ZM241385 was considerably greater than its dissociation constant in the equlibrium binding experiments, but close to the K_A value (8.5±2.2 nM), which describes the first binding step in the two-step binding reaction scheme discussed above. The physiological potency of antagonist is determined under fast equilibrium conditions, which do not favour the formation of isomerised RL^* complex and the ability of antagonist to compete with agonist is determined by the K_A value. On the other hand, the non-isomerized antagonist-receptor complexes dissociate too fast to be measured in the radioligand binding assay, where only isomerised complexes are detected. Using the K_i value from the adenylate cyclase inhibiton assay (K_i=6.6 nM) as K_A in the equation $K_d=K_A(k_{-i}/k_i)/(1+k_{-i}/k_i)$ gives a K_D value of 0.20 nM, which is in very good agreement with the data from the [^{3}H]ZM241385 binding assay.

It would have been interesting to see if A_{2A} receptor antagonists have same effect on cAMP accumulation in striatal membranes, but unfortunately the adenylate cyclase assay in membrane preparations is more complicated (especially with adenosine receptors, since rather high concentration of nucleotides is required in the assay buffer) and less sensitive. We have worked out a reliable methodology to measure cAMP accumulation in brain membranes (Vonk *et al*. 2004), but testing A_{2A} antagonists there still needs to be done.

Expression of A_1 adenosine and D_1 dopaminergic receptors in Sf9 cells with different G proteins

Sf9 cells were used as expression system with low level of native G proteins and no $G_{i/o}$ family proteins that would couple to respective mammalian receptors. Infection of the Sf9 cells with baculoviruses encoding D_1 and A_1 receptors caused appearance of specific binding sites for D_1 antagonist [^{3}H]SCH23390 and A_1 antagonist [^{3}H]DPCPX respectively. No specific binding of either ligand could be detected on uninfected cells. Affinities of [^{3}H]SCH23390 and [^{3}H]DPCPX were K_d=1.1±0.1 nM and K_d=2.2±0.8 nM respectively. Densities of D_1 and A_1 antagonist binding sites were 2.2±0.1 pmol/mg and 2.9±0.4 pmol/mg respectively, which indicates an expression level similar to that usually achieved in Sf9 cells. Coexpression of $G_{s\alpha}$, $G_{i\alpha1}$ or $\beta_1\gamma_2$ subunits had no significant influence on binding properties of radiolabelled antagonists.

D_1 receptor agonists SKF38393 and dopamine displaced [^{3}H]SCH23390 binding with affinities K_i=0.35 μM and 1.3 μM respectively and GTPγS (100 μM) had only small effect on the affinities, decreasing them to the level of K_i=0.45 μM for SKF38393 and K_i=1.7 for dopamine. This indicates a weak influence of coexpressed G_s protein on the agonist binding properties of D_1 receptor. Low level of high affinity agonist binding to G_s coupled receptors in Sf9 expression system has also been described before (Gille and Seifert, 2003). The effect of coexpressed $G_{i\alpha1}$ on the agonist affinity of A_1 receptor was considerably greater – A_1 agonist CPA displaced the binding with very high affinity (K_{iH}=0.30 nM) with no detectable amount of low affinity sites. Presence of 100 μM GTPγS caused appearance of a low affinity site (K_{iL}= 190 nM) and fraction of high affinity sites was decreased to 0.31±0.06, that confirms a strong coupling between A_1 receptors to G_i proteins in our system. Presence of ADA in the incubation medium was required for high affinity agonist binding as it has been aslo in the case of CHO cells.

The association and dissociation kinetics of [^{3}H]SCH23390 for complexes with D_1 receptors in Sf9 membranes were measured. Dissociation half-life was 5.0±0.7 min (k_{-1} = 0.13±0.01 min^{-1}). The apparent first-order association rate constant k_{off} was linearly dependent on ligand concentration, which refers to simple bimolecular binding reaction mechanism. Calculated second order rate constant for the association reaction was k_{on}=0.20±0.02 min^{-1}nM^{-1} and binding constant calculated from kinetic data K_d=k_{off}/k_{on} was 0.71±0.17 nM, which was in good agreement with the constant found from equilibrium binding data.

D_1 receptor agonists dopamine and SKF38393 increased accumulation of cAMP in Sf9 cells expressing the D_1 receptor in a concentration-dependent manner with EC_{50} values 190 nM and 53 nM, respectively. This indicates that despite the weak effect of G_s on agonist binding, there is a coupling of D_1

receptor to G_s protein. Activation of A_1 receptor with CPA had no effect on the basal cAMP accumulation, but inhibited the forskolin-activated accumulation.

Both radioligand binding and functional assays confirm the expression of D_1 and A_1 receptors in Sf9 cells treated with the respective baculoviruses and also correct coupling of these receptors to G proteins and adenylate cyclase.

Interactions between A_1 and D_1 receptor systems in Sf9 cells

We used the Sf9 cells coexpressing A_1 and D_1 receptors with G_i and G_s proteins that are described above. In this system the A_1 receptor agonists had no significant influence on the ability of dopamine to displace [^{3}H]SCH23390 binding, where the pIC_{50} of dopamine was 5.35±0.06 in the absence and 5.32± 0.07 in the presence of 10 nM CPA. A small decrease of high affinity fraction of dopamine binding has been described earlier in a different expression system (Fibroblast Ltk^- cells expressing A_1 and D_1 receptors) (Ferre *et al.* 1998), but it did not occur in our system. One reason may be, that necessary level of high-affinity agonist binding sites could not be achieved in our system. There is also a possibility that the modulated association and dissociation kinetics compensate each other, as it has been found for [^{3}H]quinpirole binding (Lepiku *et al*. 1997) and therefore we have studied the influence of adenosine receptor activation on the kinetic properties of [^{3}H]SCH23390 binding to D_1 receptors in Sf9 cell membranes. 10 μM CPA had no significant influence on the dissociation rate of [^{3}H]SCH23390 from the membranes of Sf9 cells expressing $A_1D_1G_iG_s$, and this process did not depend on the initiating ligand used, whether it was a dopaminergic antagonist (10 μM butaclamol, k_{off}= 0.13±0.01 min^{-1}) or agonist (1 mM dopamine, k_{off}=0.13±0.02 min^{-1}). The rate constant of [^{3}H]SCH23390 dissociation in the absence of CPA was 0.12±0.01 min^{-1}.

As an alternative approach we have studied the modulation between receptors on the level of generation of secondary messengers (cAMP) and checked the influence of different components of signal transduction pathway. We used Sf9 cells coexpressing D_1 and A_1 receptors and G_s protein with and without G_i protein. In the cells expressing both receptors and both G proteins, SKF38393 increased the accumulation of cAMP and CPA inhibited this increase. In the cells expressing both receptors but only G_s and no G_i, CPA had no effect on the increase in cAMP accumulation by SKF38393. As can be seen here, presence of the inhibitory G protein (G_i) was required to achieve the modulation of the dopaminergic AC activation by adenosine receptor agonist. This indicates the lack of direct regulation between these receptors in Sf9 cells and occurring of antagonistic modulation of signal transduction between these receptors on the second messenger level. However, this does not disprove the possible direct interactions between A_1 and D_1 receptors, but rather indicate that these are not detectable in Sf9 cell system.

Coupling of the M_2 mAChR with G_{i1} and G_o type of G proteins in Sf9 cells

Infection of the Sf9 cells with baculovirus encoding M_2 mAChR caused the appearance of specific [^{3}H]NMS binding sites in the membranes prepared from the infected cells. The binding of [^{3}H]NMS was saturable with a K_d of 0.34±0.14 nM and density of the binding sites was 1.4±0.5 pmol/mg membrane protein. Coexpression of $G_{i\alpha1}$ or $G_{o\alpha}$ together with βγ subunits of G proteins had no significant effect on the binding properties of [^{3}H]NMS binding sites. The number of [^{35}S]GTPγS binding sites was 106±6 and 63±4 pmol/mg membrane protein in membranes coexpressing M_2 mAChR and G_i (M_2/G_i) or G_o (M_2/G_o), respectively. In membranes of cells without G protein encoding viruses, there was 28±3 pmol/mg protein of [^{35}S]GTPγS binding sites.

The displacement curves of [^{3}H]NMS by muscarinic antagonists atropine and scopolamine were with a Hill coefficient close to unity and not affected by the presence of 30 μM GTPγS. Displacement curves of muscarinic agonists and partial agonists were more shallow (Hill coefficients 0.34–0.69) and the model of two binding sites was preferred over the one binding site model by the F-test. Presence of 30 μM GTPγS shifted the curves towards the lower affinity state of the competitive ligands. There were no significant differences in agonist affinities and in the fraction of their high affinity sites between the M_2/G_i and M_2/G_o preparations.

The binding affinities of GTPγS and GDP were measured from their abilities to displace [^{35}S]GTPγS binding in membranes of cells expressing corresponding G proteins. The apparent affinity of GTPγS in M_2/G_i membranes was 12±0.6 nM and in M_2/G_o membranes 6±0.4 nM and muscarinic ligands had no significant effect on the affinities of GTPγS in these membranes. However, the affinity of GDP was 91±37 nM in M_2/G_i membranes and 154±13 nM in M_2/G_o membranes and all muscarinic agonists studied decreased the affinity of GDP in these preparations. Thus, in the presence of 300 μM carbamoylcholine, the affinity of GDP was dropped to very similar level for both preparations (805 nM in M_2/G_i and 810 nM in M_2/G_o).

Since the activation of muscarinic receptors modulated the binding of [^{35}S]GTPγS only in the presence of GDP, where the decrease in the affinity of GDP caused increase of [^{35}S]GTPγS binding, we measured muscarinic activation of G proteins in the presence 1 μM GDP. All muscarinic agonists and partial agonists studied increased the binding of [^{35}S]GTPγS in a concentration-dependent manner having similar potencies for the M_2/G_i and M_2/G_o complexes. The total amount of basal and activated [^{35}S]GTPγS binding was different between the membrane preparations due to different expression level of $G_{i1\alpha}$ and $G_{o\alpha}$ proteins, but there were no significant differences in relative efficacies of studied ligands (compared to carbachol activation) between the preparations

of different G proteins. Muscarinic antagonists atropine, scopolamine and NMS behaved as inverse agonists, causing concentration-dependent decrease in [^{35}S]GTPγS binding with potencies that were in good agreement with their affinities from the displacement curves. These inverse agonist effects could be observed only in the presence of low concentrations of NaCl, as higher ionic strength of solution destabilizes the receptor-G protein complexes and leads to disappearance of its intrinsic activity. In all these experiments no significant differences between G_{i1} and G_o proteins in coupling mutual regulation with M_2 receptors in Sf9 cell membranes appeared.

The influence of exploratory activity and partial LC denervation on the dopaminergic modulation of GDP binding affinity

The Wistar rats used in this work were separated into groups of high and low exploratory activity (HE and LE, respectively) using exploration box as described in (Harro, 1993). This experimental setup relies on both neophobia and rewarding for novel environments.

When levels of monoamine neurotransmitters were measured in different regions of brain, the only detected difference between HE and LE groups was in the level of serotonin and it's metabolite 5-hydroxyindolacetic acid in frontal cortex and striatum, but no differences in dopamine levels could be detected in any region studied.

After partial LC lesion was generated by administration of DSP-4 (10 mg/kg), the noradrenaline level decreased in frontal cortex (by 29% and 25% respectively) and hippocampus (by 18% and 16% respectively) in both HE and LE rats. These parts of brain get noradrenaline input only from LC and so this change is a good measure of LC denervation level. Neurotoxin treatment also decreased levels of dopamine and its metabolites in nucleus accumbens, but the change was significant only for LE rats.

DSP-4 treatment had no effect on the exploratory activity of HE rats, but in LE rats it prevented the increase in exploratory activity, that would be caused by being accustomed to the exploration box. Administration of amphetamine (0,5 mg/kg) increased locomotor activity in all animals. The DSP-4 treatment had no effect on the amphetamine-dependent increase of locomotor activity in LE rats, but lowered it in HE rats.

In context of the present dissertation we have tested the role of exploratory activity of rats and the influence of DSP-4 treatment on the dopaminergic regulation of guonosine nucleotide binding in membrane preparations of striatums and nucleus accumbenses. We have found that DSP-4 treatment caused increase in [^{35}S]GTPγS binding to rat striatal membranes in the presence of 0.1 mM GDP for LE animals and decrease in HE animals. DSP-4 treatment

decreased also dopamine dependent change in GDP affinity, but only in HE rats. This decrease may be related to decreased effect of amphetamine in DSP-4 treated HE rats. In preparations of nucleus accumbenses, no significant differences in properties of [^{35}S]GTPγS and GDP binding between animal groups studied could be found.

CONCLUSIONS

- The new labelled selective adenosine A_{2A} receptor antagonist [^{3}H]ZM241385 binds with receptors reversibly having K_D values 0.14±0.01 nM and 0.23± 0.03 nM in striatal and CHO membranes, respectively. The potency of ZM241385 to inhibit cAMP accumulation caused by A_{2A} agonists in CHO cells was characterized with K_i value 6.6 nM.
- It was proposed that K_D characterizes isomerisation of the receptor-ligand complexes describing radioligand binding, while K_i corresponds to formation of the the first fast equilibrium complex determining physiological potency in competition between agonists and antagonists.
- Adenosine A_1 and Dopamine D_1 receptors expressed in Sf9 cells had ligand binding properties close to respective values in brain membranes. Activation of the A_1 receptors with CPA did not affect the ligand binding to co-expressed D_1 receptors, but inhibited dopaminergic accumulation of cAMP, if the G_i proteins were coexpressed with the receptors. No direct influence of A_1 receptor activation on D_1 receptor properties could be found in our system.
- Muscarinic agonists and partial agonists had equal potency and relative efficacy in activation of G_i and G_o subtypes of G proteins coexpressed with M_2 mAChR coexpressed in Sf9 cells. All studied muscarinic antagonists behaved as inverse agonists in our system, inhibiting the basal [^{35}S]GTPγS binding with equal efficacy for both G_i and G_o proteins.
- In the studies about the role of exploratory activity of rats and influence of DSP-4 treatment on the dopaminergic regulation of guanosine nucleotide binding in rat brain we have found that DSP-4 treatment caused an increase in [^{35}S]GTPγS binding to rat striatal membranes for LE animals and decrease in HE animals. DSP-4 treatment decreased also dopamine dependent change in GDP affinity, but only in HE rats, which may be related to decreased sensitivity on amphetamine of DSP-4 treated HE rats.

REFERENCES

Alexander S. P., Mathie A., Peters J. A. (2005) 7 TM Receptors, *Br. J. Pharmacol.* **144 Suppl 1**, S4–S62.

Aston-Jones G., Shipley M. T., Chouvet G., Ennis M., van Bockstaele E., Pieribone V., Shiekhattar R., Akaoka H., Drolet G., Astier B. (1991) Afferent regulation of locus coeruleus neurons: anatomy, physiology and pharmacology. *Prog. Brain Res.* **88**, 47–75.

Bara-Jimenez W., Sherzai A., Dimitrova T., Favit A., Bibbiani F., Gillespie M., Morris M. J., Mouradian M. M., Chase T. N. (2003) Adenosine A(2A) receptor antagonist treatment of Parkinson's disease. *Neurology* **61**, 293–6.

Bernstein L.S., Ramineni S., Hague C., Cladman W, Chidiac P., Levey A. I., Hepler J. R. (2004) RGS2 binds directly and selectively to the M1 muscarinic acetylcholine receptor third intracellular loop to modulate Gq/11alpha signaling. *J. Biol. Chem.* **279**, 21248–56.

Birdsall N. J., Lazareno S., Popham A., Saldanha J. (2001) Multiple allosteric sites on muscarinic receptors. *Life Sci.* **68**, 2517–24.

Bockaert J., Fagni L., Dumuis A., Marin P. (2004) GPCR interacting proteins (GIP), *Pharmacol. Ther*. **103**, 203–21.

Brink C. B., Wade S. M., Neubig R. R. (2000) Agonist-directed trafficking of porcine alpha(2A)-adrenergic receptor signaling in Chinese hamster ovary cells: l-isoproterenol selectively activates G(s). *J. Pharmacol. Exp. Ther.* **294**, 539–47.

Brzostowski J. A., Kimmel A. R. (2001) Signaling at zero G: G-protein-independent functions for 7-TM receptors. *Trends Biochem. Sci.* **26**, 291–7.

Cabrera-Vera T. M., Vanhauwe J., Thomas T. O., Medkova M., Preininger A., Mazzoni M. R., Hamm H. E. (2003) Insights into G protein structure, function, and regulation. *Endocr. Rev.* **24**, 765–81.

Canals M., Burgueno J., Marcellino D., Cabello N., Canela E. I., Mallol J., Agnati L., Ferre S., Bouvier M., Fuxe K., Ciruela F., Lluis C., Franco R. (2004) Homodimerization of adenosine A2A receptors: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J. Neurochem*. **88**, 726–34.

Caulfield M. P. and Birdsall N. J. (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.* **50**, 279–290.

Cheng Y., Prusoff W. H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099–108.

Ciruela F., Saura C., Canela E. I., Mallol J., Lluis C., Franco R. (1996) Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. *FEBS Lett.* **380**, 219–23.

Clader JW, Wang Y. (2005) Muscarinic receptor agonists and antagonists in the treatment of Alzheimer's disease. *Curr. Pharm. Des*. **11**, 3353–61.

Collis M. G., Hourani S. M. (1993) Adenosine receptor subtypes. *Trends Pharmacol. Sci.* **14**, 360–6.

Dasgupta S., Ferre S., Kull B., Hedlund P. B., Finnman U. B., Ahlberg S., Arenas E., Fredholm B. B., Fuxe K. (1996) Adenosine A2A receptors modulate the binding characteristics of dopamine D2 receptors in stably cotransfected fibroblast cells. *Eur. J. Pharmacol.* **316**, 325–31.

Di Chiara G., Imperato A. (1998) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. U S A.* **85**, 5274–8.

Dunwiddie T. V., Masino S. A. (2001) The role and regulation of adenosine in the central nervous system. *Annu. Rev. Neurosci.* **24**, 31–55.

Eglen R. M., Nahorski S. R. (2000) The muscarinic M(5) receptor: a silent or emerging subtype? *Br. J. Pharmacol.* **130**, 13–21.

Ehlert F. J. (2003) Contractile role of M2 and M3 muscarinic receptors in gastrointestinal, airway and urinary bladder smooth muscle. *Life Sci.* **74**, 355–66.

Emilien G., Maloteaux J. M., Geurts M., Hoogenberg K., Cragg S. (1999) Dopamine receptors — physiological understanding to therapeutic intervention potential. *Pharmacol. Ther.* **84**, 133–56.

Ferre S., Fredholm B. B, Morelli M., Popoli P., Fuxe K. (1997) Adenosine-dopamine receptor-receptor interactions as an integrative mechanism in the basal ganglia. *Trends Neurosci.* **20**, 482–7.

Ferre S., Karcz-Kubicha M., Hope B. T., Popoli P., Burgueno J., Gutierrez M. A., Casado V., Fuxe K., Goldberg S. R., Lluis C., Franco R., Ciruela F. (2002) Synergistic interaction between adenosine A2A and glutamate mGlu5 receptors: implications for striatal neuronal function. *Proc. Natl. Acad. Sci. U S A.* **99**, 11940–5.

Ferre S., Torvinen M., Antoniou K., Irenius E., Civelli O., Arenas E., Fredholm B. B., Fuxe K. (1998) Adenosine A1 receptor-mediated modulation of dopamine D1 receptors in stably cotransfected fibroblast cells. *J. Biol. Chem.* **273**, 4718–24.

Ferre S., von Euler G., Johansson B., Fredholm B. B., Fuxe K. (1991) Stimulation of high-affinity adenosine A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. *Proc. Natl. Acad. Sci. U S A.* **88**, 7238–41.

Fong H. K., Yoshimoto K. K., Eversole-Cire P., Simon M. I. (1988) Identification of a GTP-binding protein alpha subunit that lacks an apparent ADP-ribosylation site for pertussis toxin. *Proc. Natl. Acad. Sci. U S A.* **85**, 3066–70.

Fredholm B. B., Battig K., Holmen J., Nehlig A., Zvartau E.E. (1999) Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.* **51**, 83–133.

Fung B. K. (1983) Characterization of transducin from bovine retinal rod outer segments. I. Separation and reconstitution of the subunits. *J. Biol. Chem.* **258**, 10495–502.

Garritsen A., Simonds W. F. (1994) Multiple domains of G protein beta confer subunit specificity in beta gamma interaction. *J. Biol. Chem.* **269**, 24418–23.

Georgoussi Z., Leontiadis L., Mazarakou G., Merkouris M., Hyde K., Hamm H. (2006) Selective interactions between G protein subunits and RGS4 with the C-terminal domains of the mu- and delta-opioid receptors regulate opioid receptor signaling. *Cell. Signal.* **18**, 771–82.

Gesi M., Soldani P., Giorgi F. S., Santinami A., Bonaccorsi I., Fornai F. (2000) The role of the locus coeruleus in the development of Parkinson's disease. *Neurosci. Biobehav. Rev.* **24**, 655–68.

Gierschik P., Bouillon T., Jakobs K.H. (1994) Receptor-stimulated hydrolysis of guanosine 5'-triphosphate in membrane preparations. *Methods. Enzymol.* **237**, 13–26.

Gille A., Seifert R. (2003) Co-expression of the beta2-adrenoceptor and dopamine D1-receptor with Gsalpha proteins in Sf9 insect cells: limitations in comparison with fusion proteins. *Biochim. Biophys. Acta.* **1613**, 101–14.

Gines S., Hillion J., Torvinen M., Le Crom S., Casado V., Canela E. I., Rondin S., Lew J. Y., Watson S., Zoli M. Agnati L. F., Verniera P., Lluis C., Ferre S., Fuxe K., Franco R. (2000) Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. *Proc. Natl. Acad. Sci. U S A*. **97**, 8606–11.

Gines S., Hillion J., Torvinen M., Le Crom S., Casado V., Canela E. I., Rondin S., Lew J. Y., Watson S., Zoli M., Agnati L. F., Verniera P., Lluis C., Ferre S., Fuxe K., Franco R. (2000) Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. *Proc. Natl. Acad. Sci. U S A.* **97**, 8606–11.

Gomes I., Jordan B. A., Gupta A., Rios C., Trapaidze N., Devi L. A. (2001) G protein coupled receptor dimerization: implications in modulating receptor function. *J. Mol. Med.* **79**, 226–42.

Greenlee W., Clader J., Asberom T., McCombie S., Ford J., Guzik H., Kozlowski J., Li S., Liu C., Lowe D., Vice S., Zhao H., Zhou G., Billard W., Binch H., Crosby R., Duffy R., Lachowicz J., Coffin V., Watkins R., Ruperto V., Strader C., Taylor L., Cox K. (2001) Muscarinic agonists and antagonists in the treatment of Alzheimer's disease. *Farmaco.* **56**, 247–50.

Harro J. (1993) Measurement of exploratory behavior in rodents. *Methods. Neurosci.* **14**, 359–377.

Harro J., Merikula A., Lepiku M., Modiri A. R., Rinken A., Oreland L. (2000) Lesioning of locus coeruleus projections by DSP-4 neurotoxin treatment: effect on amphetamine-induced hyperlocomotion and dopamine D2 receptor binding in rats, *Pharmacol. Toxicol.* **86**, 197–202.

Hersch S. M., Ciliax B. J., Gutekunst C. A., Rees H. D., Heilman C. J., Yung K. K., Bolam J. P., Ince E., Yi H., Levey A. I. (1995) Electron microscopic analysis of D1 and D2 dopamine receptor proteins in the dorsal striatum and their synaptic relationships with motor corticostriatal afferents. *J. Neurosci.* **15**, 5222–37.

Herve D., Le Moine C., Corvol J. C., Belluscio L., Ledent C., Fienberg A. A., Jaber M., Studler J. M., Girault J. A. (2001) Galpha(olf) levels are regulated by receptor usage and control dopamine and adenosine action in the striatum. *J. Neurosci.* **21**, 4390–9.

Heydorn A., Ward R. J., Jorgensen R., Rosenkilde M. M., Frimurer T. M., Milligan G., Kostenis E. (2004) Identification of a novel site within G protein alpha subunits important for specificity of receptor-G protein interaction. *Mol. Pharmacol.* **66**, 250–9.

Hillion J., Canals M., Torvinen M., Casado V., Scott R., Terasmaa A., Hansson A., Watson S., Olah M. E., Mallol J., Canela E. I., Zoli M., Agnati L. F., Ibanez C. F., Lluis C., Franco R., Ferre S., Fuxe K. (2002) Coaggregation, cointernalization, and codesensitization of adenosine A2A receptors and dopamine D2 receptors. *J. Biol. Chem.* **277**, 18091–7.

Humphrey P. P., Barnard E. A. (1998) International Union of Pharmacology. XIX. The IUPHAR receptor code: a proposal for an alphanumeric classification system. *Pharmacol. Rev.* **50**, 271–7.

Itoh H., Toyama R., Kozasa T., Tsukamoto T., Matsuoka M., Kaziro Y. (1988) Presence of three distinct molecular species of Gi protein alpha subunit. Structure of rat cDNAs and human genomic DNAs. *J. Biol. Chem.* **263**, 6656–64.

Jansson C. C., Kukkonen J. P., Nasman J., Huifang G., Wurster S., Virtanen R., Savola J. M., Cockcroft V., Akerman K. E. (1998) Protean agonism at alpha2A-adrenoceptors. *Mol. Pharmacol.* **53**, 963–8.

Järv J., Hedlund B., and Bartfai T. (1979) Isomerization of the muscarinic receptor * antagonist complex. *J. Biol. Chem.* **254**, 5595–5598.

Jarvis M. F., Williams M. (1989) Direct autoradiographic localization of adenosine A2 receptors in the rat brain using the A2-selective agonist, [3H]CGS 21680. *Eur. J. Pharmacol.* **168**, 243–6.

Jones D. T., Masters S. B., Bourne H. R., Reed R. R. (1990) Biochemical characterization of three stimulatory GTP-binding proteins. The large and small forms of Gs and the olfactory-specific G-protein, Golf. *J. Biol. Chem.* **265**, 2671–6.

Karlsson E., Jolkkonen M., Mulugeta E., Onali P., Adem A. (2000) Snake toxins with high selectivity for subtypes of muscarinic acetylcholine receptors. *Biochimie* **82**, 793–806.

Kenakin T. (2003) Ligand-selective receptor conformations revisited: the promise and the problem. *Trends. Pharmacol. Sci.* **24**, 346–54.

Kenakin T. (2004) Principles: Receptor theory in pharmacology. *Trends Pharmacol. Sci.* **25**, 186–92.

Kenakin, T. A Pharmacology Primer, Elsevier Academic Press, 2004. (b)

Khan Z. U., Mrzljak L., Gutierrez A., de la Calle A., Goldman-Rakic P. S. (1998) Prominence of the dopamine D2 short isoform in dopaminergic pathways. *Proc. Natl. Acad. Sci. U S A.* **95**, 7731–6.

Kim Y. C., Ji X., Melman N., Linden J., Jacobson K. A. (2000) Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A(2B) adenosine receptors. *J. Med. Chem.* **43**, 1165–72.

Klinger M., Freissmuth M., Nanoff C. (2002) Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. *Cell. Signal.* **14**, 99–108.

Klotz K. N. (2000) Adenosine receptors and their ligands, *Naunyn Schmiedebergs Arch. Pharmacol.* **362**, 382–91.

Kristiansen K. (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol. Ther.* **103**, 21–80.

Kull B., Svenningsson P., Fredholm B. B. (2000) Adenosine A(2A) receptors are colocalized with and activate g(olf) in rat striatum. *Mol. Pharmacol.* **58**, 771–7.

LaHoste G. J., Marshall J. F. (1992) Dopamine supersensitivity and D1/D2 synergism are unrelated to changes in striatal receptor density. *Synapse* **12**, 14–26.

Lategan A. J., Marien M. R., Colpaert F. C. (1992) Suppression of nigrostriatal and mesolimbic dopamine release in vivo following noradrenaline depletion by DSP-4: a microdialysis study. *Life Sci.* **50**, 995–9.

Lee C., Murakami T., Simonds W. F. (1995) Identification of a discrete region of the G protein gamma subunit conferring selectivity in beta gamma complex formation. *J. Biol. Chem.* **270**, 8779–84.

Lee S. P., O'Dowd B. F., Rajaram R. D., Nguyen T., George S. R. (2003) D2 dopamine receptor homodimerization is mediated by multiple sites of interaction, including an intermolecular interaction involving transmembrane domain 4, *Biochemistry* **42**, 11023–31.

Lefkowitz R. J., Shenoy S. K. (2005) Transduction of receptor signals by beta-arrestins, *Science* **308**, 512–7.

Lepiku M., Rinken A., Jarv J., Fuxe K. (1996) Kinetic evidence for isomerization of the dopamine receptor-raclopride complex. *Neurochem. Int.* **28**, 591–5.

Lepiku M., Rinken A., Jarv J., Fuxe K. (1997) Modulation of [3H]quinpirole binding to dopaminergic receptors by adenosine A2A receptors. *Neurosci. Lett.* **239**, 61–4.

Lerea C. L., Somers D. E., Hurley J. B., Klock I. B., Bunt-Milam A. H. (1986) Identification of specific transducin alpha subunits in retinal rod and cone photoreceptors. *Science* **234**, 77–80.

Lindorfer M. A., Myung C. S., Savino Y., Yasuda H., Khazan R., Garrison J. C. (1998) Differential activity of the G protein beta5 gamma2 subunit at receptors and effectors. *J. Biol. Chem.* 1998 **273**, 34429–36.

Lohse M. J., Andexinger S., Pitcher J., Trukawinski S., Codina J., Faure J. P., Caron M. G., Lefkowitz R. J. (1992) Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems. *J. Biol. Chem.* **267**, 8558–64.

Lopes L. V., Rebola N., Costenla A. R., Halldner L., Jacobson M. A., Oliveira C. R., Richardson P. J., Fredholm B. B., Ribeiro J. A., Cunha R. A. (2003) Adenosine A3 receptors in the rat hippocampus: Lack of interaction with A1 receptors. *Drug. Dev. Res.* **58**, 428–438.

Lorenzen A., Lang H., Schwabe U. (1998) Activation of various subtypes of G-protein alpha subunits by partial agonists of the adenosine A1 receptor. *Biochem. Pharmacol.* **56**, 1287–93.

Matsui M., Yamada S., Oki T., Manabe T., Taketo M. M., Ehlert F. J. (2004) Functional analysis of muscarinic acetylcholine receptors using knockout mice. *Life Sci.* **75**, 2971–81.

McIntire W. E., MacCleery G., Garrison J. C. (2001) The G protein beta subunit is a determinant in the coupling of Gs to the beta 1-adrenergic and A2a adenosine receptors. *J. Biol. Chem.* **276**, 15801–9.

Meyer P. T., Elmenhorst D., Bier D., Holschbach M. H., Matusch A, Coenen H. H., Zilles K., Bauer A. (2005) Quantification of cerebral A1 adenosine receptors in humans using [18F]CPFPX and PET: an equilibrium approach. *Neuroimage* **24**, 1192–204.

Migeon J. C., Thomas S. L., Nathanson N. M. (1995) Differential coupling of m2 and m4 muscarinic receptors to inhibition of adenylyl cyclase by Gi alpha and G(o)alpha subunits. *J. Biol. Chem.* **270**, 16070–4.

Milligan G. (2003) Principles: extending the utility of [35S]GTP gamma S binding assays. *Trends Pharmacol. Sci.* **24**, 87–90.

Missale C., Nash S. R., Robinson S. W., Jaber M., Caron M. G. (1998) Dopamine receptors: from structure to function. *Physiol. Rev.* **78**, 189–225.

Miyakawa T., Yamada M., Duttaroy A., Wess J. (2001) Hyperactivity and intact hippocampus-dependent learning in mice lacking the M1 muscarinic acetylcholine receptor. *J. Neurosci.* **21**, 5239–50.

Mombaerts P. (1999) Seven-transmembrane proteins as odorant and chemosensory receptors. *Science* **286**, 707–11.

Murphree L. J., Marshall M. A., Rieger J. M., MacDonald T. L., Linden J. (2002) Human A(2A) adenosine receptors: high-affinity agonist binding to receptor-G protein complexes containing Gbeta(4). *Mol. Pharmacol.* **61**, 455–62.

Nanoff C., Waldhoer M., Roka F., Freissmuth M. (1997) G protein coupling of the rat A1-adenosine receptor — partial purification of a protein which stabilizes the receptor-G protein association. *Neuropharmacology.* **36**, 1211–9.

Näsman J., Kukkonen J. P., Akerman K. E. (2002) Dual signalling by different octopamine receptors converges on adenylate cyclase in Sf9 cells. *Insect Biochem. Mol. Biol.* **32**, 285–93.

Näsman J., Kukkonen J. P., Ammoun S., Akerman K. E. (2001) Role of G-protein availability in differential signaling by alpha 2-adrenoceptors. *Biochem. Pharmacol.* **62**, 913–22.

Neubig R. R., 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. *Pharmacol. Rev.* **55**, 597–606.

Nordstedt C., Fredholm B. B. (1990) A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Anal. Biochem.* **189**, 231–4.

Offermanns S. (2003) G-proteins as transducers in transmembrane signalling. *Prog. Biophys. Mol. Biol.* **83**, 101–30.

Ongini E., Fredholm B. B. (1996) Pharmacology of adenosine A2A receptors. *Trends Pharmacol. Sci.* **17**, 364–72.

Oras A., Kilk K., Kunapuli S., Barnard E. A., Jarv J. (2002) Kinetic analysis of [35S]dATP alpha S interaction with P2y(1) nucleotide receptor. *Neurochem. Int.* **40**, 381–6.

Pacheco M. A., Jope R. S. (1997) Comparison of [3H]phosphatidylinositol and [3H]phosphatidylinositol 4,5-bisphosphate hydrolysis in postmortem human brain membranes and characterization of stimulation by dopamine D1 receptors. *J. Neurochem.* **69**, 639–44.

Palczewski K., Kumasaka T., Hori T., Behnke C. A., Motoshima H., Fox B. A., Le Trong I., Teller D. C., Okada T., Stenkamp R. E., Yamamoto M., Miyano M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science.* **289**, 739–45.

Palmer T. M., Gettys T. W., Stiles G. L. (1995) Differential interaction with and regulation of multiple G-proteins by the rat A3 adenosine receptor. *J. Biol. Chem.* **270**, 16895–902.

Papac D. I., Thornburg K. R., Bullesbach E. E., Crouch R. K., Knapp D. R. (1992) Palmitylation of a G-protein coupled receptor. Direct analysis by tandem mass spectrometry. *J. Biol. Chem.* **267**, 16889–94.

Penela P., Ribas C., Mayor F. Jr. (2003) Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cell. Signal.* **15**, 973–81.

Pin J. P., Kniazeff J., Binet V., Liu J., Maurel D., Galvez T., Duthey B., Havlickova M., Blahos J., Prezeau L., Rondard P. (2004) Activation mechanism of the heterodimeric GABA(B) receptor. *Biochem Pharmacol.* **68**, 1565–72.

Potter L. T. (2001) Snake toxins that bind specifically to individual subtypes of muscarinic receptors. *Life Sci.* **68**, 2541–7.

Ralevic V., Burnstock G. (1998) Receptors for purines and pyrimidines. *Pharmacol. Rev.* **50**, 413–92.

Rebec G. V., Grabner C. P., Johnson M., Pierce R. C., Bardo M. T. (1997) Transient increases in catecholaminergic activity in medial prefrontal cortex and nucleus accumbens shell during novelty. *Neuroscience* **76**, 707–14.

Rinken A., Finnman U. B., Fuxe K. (1999) Pharmacological characterization of dopamine-stimulated [^{35}S]GTPgS binding in rat striatal membranes. *Biochem. Pharmacol.* **57**, 155–162.

Riobo N. A., Manning D. R. (2005) Receptors coupled to heterotrimeric G proteins of the G12 family. *Trends Pharmacol. Sci.* **26**, 146–54.

Rivkees S. A., Price S. L., Zhou F. C. (1995) Immunohistochemical detection of A1 adenosine receptors in rat brain with emphasis on localization in the hippocampal formation, cerebral cortex, cerebellum, and basal ganglia. *Brain Res.* **677**, 193–203.

Ruiz-Avila L., Wong G. T., Damak S., Margolskee R. F. (2001) Dominant loss of responsiveness to sweet and bitter compounds caused by a single mutation in alpha –gustducin. *Proc. Natl. Acad. Sci. U S A.* 98, 8868–73.

Schmiedeberg O., Koppe R. (1869) *Das Muscarin. Das Giftige Alkaloid des Fliegenpilzes (Agaricus Muscarius L.)* Verlag von F. E. W. Vogel, Leipzig, 1869.

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**, 23120–7.

Sibley D. R., Peters J. R., Nambi P., Caron M. G., Lefkowitz R. J. (1984) Desensitization of turkey erythrocyte adenylate cyclase. Beta-adrenergic receptor phosphorylation is correlated with attenuation of adenylate cyclase activity. *J. Biol. Chem.* **259**, 9742–9.

Smith F. D., Oxford G. S., 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**, 19894–900.

Stengel P. W., Gomeza J., Wess J., Cohen M. L. (2000) M(2) and M(4) receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle in vitro. *J. Pharmacol. Exp. Ther.* **292**, 877–85.

Storch A., Ludolph A. C., Schwarz J. (2004) Dopamine transporter: involvement in selective dopaminergic neurotoxicity and degeneration. *J. Neural. Transm.* **111**, 1267–86.

Strathmann M. P., Simon M. I. (1991) G alpha 12 and G alpha 13 subunits define a fourth class of G protein alpha subunits. *Proc. Natl. Acad. Sci. U S A.* **88**, 5582–6.

Strickland S., Palmer G., Massey V. (1975) Determination of dissociation constants and specific rate constants of enzyme-substrate (or protein-ligand) interactions from rapid reaction kinetic data. *J. Biol. Chem.* **250**, 4048–52.

Terasmaa A., Andbjer B., Fuxe K., Rinken A. (2000) Striatal dopamine denervation decreases the GDP binding affinity in rat striatal membranes. *Neuroreport* **11**, 2691–4.

Torvinen M., Gines S., Hillion J., Latini S., Canals M., Ciruela F., Bordoni F., Staines W., Pedata F., Agnati L. F., Lluis C., Franco R., Ferre S., Fuxe K. (2002) Interactions among adenosine deaminase, adenosine A(1) receptors and dopamine D(1) receptors in stably cotransfected fibroblast cells and neurons. *Neuroscience* **113**, 709–19.

Torvinen M., Kozell L. B., Neve K. A., Agnati L. F., Fuxe K. (2004) Biochemical identification of the dopamine D2 receptor domains interacting with the adenosine A2A receptor. *J. Mol. Neurosci*. **24**, 173–80.

Torvinen M., Marcellino D., Canals M., Agnati L. F., Lluis C., Franco R., Fuxe K. (2005) Adenosine A2A receptor and dopamine D3 receptor interactions: evidence of functional A2A/D3 heteromeric complexes. *Mol. Pharmacol.* **67**, 400–7.

Tucek S., Michal P., Vlachova V. (2001) Dual effects of muscarinic M2 receptors on the synthesis of cyclic AMP in CHO cells: background and model. *Life Sci.* **68**, 2501–10.

Uberti M. A., Hall R. A., Minneman K. P. (2003) Subtype-specific dimerization of alpha 1-adrenoceptors: effects on receptor expression and pharmacological properties. *Mol. Pharmacol.* **64**, 1379–90

Ueda N., Iniguez-Lluhi J. A., Lee E., Smrcka A. V., Robishaw J. D., Gilman A. G. (1994) G protein beta gamma subunits. Simplified purification and properties of novel isoforms. *J. Biol. Chem.* **269**, 4388–95.

Vonk A., Uustare A., Rinken A. (2004) Modulation of activity of adenylate cyclase in rat striatal membranes by adenosine A2A receptors. *Proc. Estonian Acad. Sci. Chem.* **53**, 153–164.

Wang Z., Shi H., Wang H. (2004) Functional M3 muscarinic acetylcholine receptors in mammalian hearts. *Br. J. Pharmacol.* **142**, 395–408.

Wei J., Walton E. A., Milici A., Buccafusco J. J. (1994) m1-m5 muscarinic receptor distribution in rat CNS by RT-PCR and HPLC. *J. Neurochem.* **63**, 815–21.

Weiner D. M., Levey A. I., Sunahara R. K., Niznik H. B., O'Dowd B. F., Seeman P., Brann M. R. (1991) D1 and D2 dopamine receptor mRNA in rat brain. *Proc. Natl. Acad. Sci. U S A*. **88**, 1859–63.

Weinshenker D., Miller N. S., Blizinsky K., Laughlin M. L., Palmiter R. D. (2002) Mice with chronic norepinephrine deficiency resemble amphetamine-sensitized animals. *Proc. Natl. Acad. Sci. U S A*. **99**, 13873–7.

Wess J. (2003) Novel insights into muscarinic acetylcholine receptor function using gene targeting technology. *Trends Pharmacol. Sci.* **24**, 414–20.

Wieland T., Jakobs K. H. (1994) Measurement of receptor-stimulated guanosine 5'-O-(gamma-thio)triphosphate binding by G proteins. *Methods Enzymol.* **237**, 3–13.

Wilkie T. M., Scherle P. A., Strathmann M. P., Slepak V. Z., Simon M. I. (1991) Characterization of G-protein alpha subunits in the Gq class: expression in murine tissues and in stromal and hematopoietic cell lines. *Proc. Natl. Acad. Sci. U S A*. **88**, 10049–53.

Yarfitz S., Hurley J. B. (1994) Transduction mechanisms of vertebrate and invertebrate photoreceptors. *J. Biol. Chem.* **269**, 14329–32.

Zhang W., Yamada M., Gomeza J., Basile A. S., Wess J. (2002) Multiple muscarinic acetylcholine receptor subtypes modulate striatal dopamine release, as studied with M1-M5 muscarinic receptor knock-out mice. *J. Neurosci.* **22**, 6347–52.

Zor T., Halifa I., Kleinhaus S., Chorev M., Selinger Z. (1995) m-Acetylanilido-GTP, a novel photoaffinity label for GTP-binding proteins: synthesis and application. *Biochem. J.* **306**, 253–8.

SUMMARY IN ESTONIAN

HEPTAHELIKAALSETE RETSEPTORITE SIGNAALI ÜLEKANDE MÕJUTAMINE TEISTE RETSEPTORITE JA G VALKUDE POOLT

Käesolevas töös uuriti mõningate heptahelikaalsete (7-TM) retseptorite ligandide sidumise ja signaali ülekande regulatsiooniga seotud aspekte.

Adenosiini A_{2A} retseptori spetsiifilise antagonisti [^{3}H]ZM241385 sidumist roti aju juttkeha ja A_{2A} retseptorit ekspresseerivatest CHO rakkudest valmistatud membraanpreparaatidele ning nimetatud ligandi võimet blokeerida adenülaadi tsüklaasi (AC) aktivatsiooni kirjeldati **Artiklis II**. Selle ligandi sidumise kineetika kirjeldamiseks aju juttkeha membraanidele pakuti välja kaheastmeline reaktsiooniskeem, mille kohaselt moodustab [^{3}H]ZM241385 retseptoriga algselt kiire tasakaaluga kompleksi, mis järgnevalt muundub aeglaselt dissotsieeruvaks isomeriseerunud kompleksiks. Seejuures määrab esimene tasakaal antagonisti võime blokeerida füsioloogilist vastust, aga isomeriseerunud komplekse on võimalik määrata radioligandi sidumise katsetes.

Muskariinsete M_2 atsetüülkoliini retseptorite võimet moduleerida G_i ja G_o valkude aktiivsust Sf9 rakkudes kirjeldati **Artiklis I**. Võrreldes muskariinsete retseptorite täis- ja osaliste agonistide ning antagonistide võimet konkureerida märgistatud antagonisti [^{3}H]NMS sidumisega M_2 retseptorile G_i või G_o sisaldavates membraanpreparaatides ja [^{35}S]GTPγS sidumise mõjutamist nende ligandide poolt mõlemates preparaatides, olulisi erinevusi nende G valkude vahel ei täheldatud. Samas näidati, et uuritud muskariinsed antagonistid skopolamiin ja atropiin käitusid uuritavates süsteemides pöördagonistidena, inhibeerides [^{35}S]GTPγS sidumist G_i ja G_o valkudele võrdsel määral.

Dopamiini D_1 retseptorite ligandi sidumise omaduste ja signaali ülekande moduleerimist adenosiini A_1 retseptorite poolt Sf9 rakkudes kirjeldatakse **Käsikirjas IV.** A_1 ja D_1 retseptorite korrektset ekspressiooni Sf9 rakkudes kinnitasid nii ligandide sidumisomadused kui funktsioneerimine, mis olid võrreldavad ajukudedes mõõdetutega. A_1 retseptori agonist tsüklopentüüladenosiin (CPA) ei mõjutanud ei agonistide afiinsust D_1 retseptorile ega märgistatud D_1 antagonisti [^{3}H]SCH23390 dissotsiatsioonikineetikat, kuid inhibeeris D_1 agonistide poolt stimuleeritud AC aktiivsust. Samas oli selle efekti ilmnemiseks vajalik G_i valgu ekspressioon ning otsest A_1 retseptori toimet D_1 retseptorile selles süsteemis ei täheldatud.

GDP afiinsuse muutust kui retseptori aktiveerimise mõõtu kasutati, et uurida D_2 dopaminergilise retseptori signaali ülekande erinevusi käitumise järgi selekteeritud ja erinevalt töödeldud rottide ajus ja seda kirjeldatakse **Artiklis III**. Rotid jagati kõrge ja madala uudistamisaktiivsusega gruppideks (vastavalt HE ja LE), kus mõlemas grupis tekitati osale rottidest osaline noradrenergiliste neuronite kahjustus 10 mg/kg DSP-4 manustamisega. DSP-4 töötlus suurendas [^{35}S]GTPγS sidumist LE grupi rottide ajude juttkehadest valmistatud membraa-

nidele, samas kui HE rottidel see sidumine sama töötluse tulemusena vähenes. Samuti vähendas DSP-4 töötlus HE rottidel dopamiinist sõltuvat GDP afiinsuse muutust juttkeha membraanides, mis võib olla ka seotud amfetamiini vähenenud psühhostimulatoorse mõjuga nendel rottidel. Rottide akumbens tuumadest valmistatud membraanpreparaatides ei olnud statistiliselt olulisi erinevusi GDP ja [^{35}S]GTPγS sidumisparameetrites.

ACKNOWLEDGEMENTS

The present study has mainly been preformed at the Institute of Organic and Bioorganic Chemistry of the University of Tartu, some experimental work has also been done at Department of Physiology at Uppsala University. I am very grateful to everybody who helped me in my research. In particular, I would like to thank:

My supervisor, professor Ago Rinken, not only for his great professional guidance, but also for his patience with me.

Professor Karl Åkerman for giving me a chance to work in his laboratory, where I learned a lot and I would also like to thank all the nice people in his group.

Dr. Johnny Näsman who has helped me with problems concerning insect cells and baculoviruses.

Dr. Maria Torvinen for professional help with some cell lines.

All the people of the laboratory 152, especially Raili for her help with cell culture work and Laura for helping me to understand behavioural science.

The work was also supported by Estonian Science Foundation grant 6492 (ETF 6492).

PUBLICATIONS

CURRICULUM VITAE

1. General

1. Name:	Ain Uustare
2. Date of Birth:	5. january, 1978
Place of Birth:	Tallinn, Estonia
3. Citizenship:	Estonian
4. Marital Status:	Married Wife: Marika Uustare, s. 21.09.1981
5. Address:	Aida 11–30, 51004 Tartu, tel. 5620 8086, e-mail deffi@ut.ee
6. Current occupation:	Researcher in University of Tartu, Institute of Organic and Bioorganic Chemistry Jakobi 2, 51014, Tartu Phone: +372 737 5296
7. Education:	
1996–2000	Student in University of Tartu, *B.Sc.* (chemistry) in 2000
2000–2001	Graduate student in University of Tartu, *M.Sc.* (bio-organic chemistry) in 2001
2001–2006	PhD Student in University of Tartu, Institute of organic and bioorganic chemistry
8. Languages spoken:	English, german
9. Posts Held:	
1997–1998	ab Assistant in University of Tartu, Institute of Chemical Physics
2001–30.09.2002	Extraordinary researcher in University of Tartu, Institute of organic and bioorganic chemistry
1.10.2002–present	Researcher in University of Tartu, Institute of organic and bioorganic chemistry

II. Professional Activities

1. Main research areas

Interactions between adenosine (A_1 and A_{2A}) and dopamine (D_1 and D_2) receptors, using different cell lines (Sf9, CHO, fibroblasts) as expression system.

Details of signal transduction between receptors and G proteins. We are mainly using Sf9 cell line expressing M_2 receptor with various G proteins and we are studying receptors selectivity between G protein subtypes and balances in agonist and GDP binding.

Measurement of dopaminergic signal transduction efficacy trough modulation of [^{35}S]GTPγS binding (in cooperation with the group of prof. Jaanus Harro)

2. List of Publications

Scientific articles in international journals

Lund, P., Shariatmadari, R., **Uustare, A**., Detheux, M., Parmentier, M., Kukkonen, J. P., Åkerman, K. E. O. The Orexin OX1 Receptor Activates a Novel Ca2+ Influx Pathway for Coupling to Phospholipase C. J. Biol. Chem., 2000, vol. 275, No. 40, 30806–30812.

Uustare, A., Näsman, J., Åkerman, K. E. O., Rinken, A. Characterization of M2 muscarinic receptor activation ofdifferent G protein subtypes. Neurochem. Int., 2004, vol. 44, Issue 2, 119–124.

Uustare, A., Vonk, A., Terasmaa, A., Fuxe, K., Rinken A. Kinetic and functional properties of [3H]ZM241385, a high affinity antagonist for adenosine A2A receptors. Life Sci., 2005, vol. 76, issue 13, 1513–1526.

Alttoa A., Koiv K., Eller M., **Uustare A.**, Rinken A., Harro J. Effects of low dose N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine administration on exploratory and amphetamine-induced behavior and dopamine D(2) receptor function in rats with high or low exploratory activity. Neuroscience. 2005, vol. 132, issue 4, 979–90.

Other scientific articles

Arro, A., **Uustare, A.**, Harro, J., Rinken, A. Modulation of [3H]-8-OH-DPAT Binding to Rat Brain Membranes by Metal Ions. Proc Estonian Acad. Sci. Chem., 2001, 50, 1, 27–37.

Vonk, A., **Uustare, A.**, Rinken A. Modulation of activity of adenylate cyclase in rat striatal membranes by adenosine A2A receptors. Proc Estonian Acad. Sci. Chem., 2004, 53, 4, 153–164.

3. Honors, Grants and Fellowships

"Boston A2A '06 Travel Fellowship Award" to attend the meeting "Targeting Adenosine A_{2A} Receptors in Parkinson's Disease and other CNS Disorders"in Boston May 17–19, 2006

"FEBS Fellowship" for The 2006 FEBS Forum for Young Scientists and 31st FEBS Congress in Istanbul, 22–29 June 2006

4. Other Scientific and Organisatory activities:

I am a member of Estonian Biochemical Society and selected to be a member of board of Estonian Biochemical Society since 22. of February, 2005

I have held an oral presentation on the Estonian Chemistry Days in 2001 and a poster presentation on the Estonian Chemistry Days in 2005

III. Professional self-improvement

14.06.2005–22.06.2005 I took part in IBRO (*International Brain Research Organization)* course “IBRO Course in Neuroscience” in Tallinn

CURRICULUM VITAE

I. Üldandmed

1. Ees, ja perekonnanimi:	Ain Uustare
2. Sünniaeg:	5. jaanuar, 1978
Sünnikoht:	Tallinn, Eesti
3. Kodakondsus:	Eesti
4. Perekonnaseis:	Abielus abikaasa: Marika Uustare, s. 21.09.1981, TÜ keskkonnatehnoloogia magistrant
5. Aadress:	Aida 11–30, 51004 Tartu, tel. 5620 8086, e-mail deffi@ut.ee
6. Praegune töökoht:	Tartu Ülikooli orgaanilise ja bioorgaanilise keemia instituudi teadur, Jakobi 2, 51014, Tartu Telefon: 737 5296
7. Haridus:	1996–2000 Tartu Ülikooli keemia üliõpilane, bakalaureusekraad bioorgaanilises keemias 2000. aastal. 2000–2001 Tartu Ülikooli bioorgaanilise keemia magistrant, *M.Sc.* (bioorgaanine keemia) 2001. aastal 2001–2006 Tartu Ülikooli bioorgaanilise keemia doktorant, juhendaja prof. Ago Rinken
8. Keelteoskus:	inglise, saksa
9. Teenistuskäik:	1997–1998 Tartu Ülikooli Keemilise Füüsika instituudi laborant. 2001–30.09.2002 TÜ Orgaanilise ja bioorgaanilise keemia instituudi erak. teadur. 1.10.2002–k.a. TÜ Orgaanilise ja bioorgaanilise keemia instituudi teadur

II. Teaduslik ja arendustegevus

1. Peamised uurimisvaldkonnad.

Adenosiini (A_1 ja A_{2A}) ja dopamiini (D_1 ja D_2) retseptorite vahelise interaktsiooni uurimine, kasutades erinevates rakuliinides (Sf9, CHO, fibroblastid) ekspresseeritud retseptoreid.

Retseptori ja G valgu vahelise signaali ülekande peenmehhanismi uurimine. Uurimissüsteemina on kasutatud peamiselt Sf9 valke, milles on ekspresseeritud M_2 muskariinset retseptorit koos erinevate G valgu alatüüpidega, uurime retseptori võimet eristada G valgu alatüüpe ning agonistide ja GDP sidumise vahelise tasakaalu probleeme.

Dopaminergilise signaaliülekande efektiivsuse mõõtmine [^{35}S]GTPγS sidumise modulatsiooni teel koostööna prof. Jaanus Harro grupiga.

2. Publikatsioonide loetelu

Teaduslikud artiklid rahvusvahelise levikuga väljaannetes

Lund, P., Shariatmadari, R., **Uustare, A**., Detheux, M., Parmentier, M., Kukkonen, J. P., Åkerman, K. E. O. The Orexin OX1 Receptor Activates a Novel Ca2+ Influx Pathway for Coupling to Phospholipase C. J. Biol. Chem., 2000, vol. 275, No. 40, 30806–30812.

Uustare, A., Näsman, J., Åkerman, K. E. O., Rinken, A. Characterization of M2 muscarinic receptor activation ofdifferent G protein subtypes. Neurochem. Int., 2004, vol. 44, Issue 2, 119–124.

Uustare, A., Vonk, A., Terasmaa, A., Fuxe, K., Rinken A. Kinetic and functional properties of [3H]ZM241385, a high affinity antagonist for adenosine A2A receptors. Life Sci., 2005, vol. 76, issue 13, 1513–1526.

Alttoa A., Koiv K., Eller M., **Uustare A.**, Rinken A., Harro J. Effects of low dose N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine administration on exploratory and amphetamine-induced behavior and dopamine D(2) receptor function in rats with high or low exploratory activity. Neuroscience. 2005, vol. 132, issue 4, 979–90.

Muud teaduslikud artiklid

Arro, A., **Uustare, A.**, Harro, J., Rinken, A. Modulation of [3H]-8-OH-DPAT Binding to Rat Brain Membranes by Metal Ions. Proc Estonian Acad. Sci. Chem., 2001, 50, 1, 27–37.

Vonk, A., **Uustare, A.**, Rinken A. Modulation of activity of adenylate cyclase in rat striatal membranes by adenosine A2A receptors. Proc Estonian Acad. Sci. Chem., 2004, 53, 4, 153–164.

3. Saadud uurimistoetused ja stipendiumid

"Boston A2A '06 Travel Fellowship Award" to attend the meeting "Targeting Adenosine A_{2A} Receptors in Parkinson's Disease and other CNS Disorders"in Boston May 17–19, 2006

"FEBS Fellowship" for The 2006 FEBS Forum for Young Scientists and 31st FEBS Congress in Istanbul, 22–29 June 2006

4. Muu teaduslik ja organisatsiooniline tegevus:

Olen Eesti Biokeemia Seltsi liige ja Eesti Biokeemia Seltsi juhatuse liige alates 22. veebruarist 2005. Osalesin Eesti Biokeemia Seltsi kevadkooli läbiviimisel 29–30 aprillil 2005.

Olen teinud suulise ettekande Eesti Keemiapäevadel aastal 2001 teemal "M_2 muskariinsete retseptorite interaktsioonid G_o ja G_i tüüpi G-valkudega" ja

poster-ettekande Eesti keemiapäevadel aastal 2005 teemal “Retseptor-retseptor interaktsiooni uurimine A_1 adenosiini ja D_1 dopamiini retseptorite vahel”

Osalesin aastal 2003. ja 2005. aastal Tartu Ülikooli keemia eriala bakalaureusetööde kaitsmise komisjonis.

III. Erialane enesetäiendus

14.06.2005–22.06.2005 osalesin IBRO (*International Brain Research Organization)* kursusel “IBRO Course in Neuroscience” Tallinnas