DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
84

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
84

ARGO VONK

Determination of adenosine A_{2A}- and dopamine D_1 receptor-specific modulation of adenylate cyclase activity in rat striatum

TARTU UNIVERSITY
PRESS

Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Estonia

Dissertation is accepted for the commencement of the Degree of Doctor of Philosophy in Bioorganic chemistry on April 28, 2009 by the Doctoral Committee of the Institute of Chemistry, University of Tartu.

Supervisor: Professor Ago Rinken, dr. (med) (Chair of Bioorganic Chemistry of Institute of Chemistry, University of Tartu)

Opponent: Maija Dambrova, dr. (pharm) (Department of Medicinal Chemistry, Latvian Institute of Organic Synthesis)

Commencement: June 17, 2009 at Jakobi st. 2, room nr. 430

ISSN 1406–0299
ISBN 978–9949–19–127–7 (trükis)
ISBN 978–9949–19–128–4 (PDF)

Autoriõigus Argo Vonk, 2009

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr. 186

CONTENTS

LIST OF ORIGINAL PUBLICATIONS 6
ABBREVIATIONS 7
INTRODUCTION 9
G protein-coupled receptors 11
G proteins 12
Adenylate cyclase 13
The role of cAMP as a second messenger 14
Dopamine and adenosine signal transduction in striatum 16
Measurement of ligand affinity and receptors expression level by radioligand binding 18
Determination of adenylate cyclase activity 19
AIMS OF THE STUDY 21
MATERIALS AND METHODS 22
Chemicals 22
Measurement of cAMP accumulation in CHO cells 22
Striatal tissue preparations 23
Radioligand binding experiments 24
Adenylate cyclase activity assay 25
Data analysis 25
RESULTS & DISCUSSION 26
Tissue sample preparation and selection of homogenization buffer 26
Optimization of reaction buffer 26
Adenosine A_{2A} receptors 27
Dopamine receptors 28
Effect of variable chronic stress on rats with high and low exploratory activity 30
Gender-specific effects of tickling on dopaminergic and serotonergic signal transmission 31
CONCLUSIONS 33
REFERENCES 34
SUMMARY IN ESTONIAN 40
ACKNOWLEDGEMENTS 42

LIST OF ORIGINAL PUBLICATIONS

I. **Vonk A**., Uustare A., Rinken A. (2004) Modulation of activity of adenylate cyclase in rat striatal membranes by adenosine A_{2A} receptors. *Proc. Est. Acad. Sci. (Chem.)* **53**, 153–164.

II. Uustare A., **Vonk A**., Terasmaa A, Fuxe K., Rinken A. (2005) Kinetic and functional properties of [^{3}H]ZM241385, a high affinity antagonist for adenosine A_{2A} receptors. *Life Sci.* **76**, 1513–1526.

III. **Vonk A**., Reinart R., Rinken A. (2008) Modulation of adenylyl cyclase activity in rat striatal homogenate by dopaminergic receptors. *J. Pharmacol. Sci.* **108**, 63–70.

IV. Matrov D., **Vonk** A., Herm L., Rinken A., Harro J. (2009) Effect of chronic variable stress on rats with persistently low or high exploratory activity. *Stress* Submitted manuscript.

Author's contribution

Paper I: Performed experimental work, except for the radioligand binding experiments, and writing of the paper as main author.

Paper II: Performed cAMP accumulation measurement experiments and writing of the respective part of the paper.

Paper III: Performed experimental work, except for the [^{3}H]SCH 23390 binding experiments, and writing of the paper as main author.

Paper IV: Performed cAMP accumulation measurement experiments and writing of the respective part of the manuscript.

ABBREVIATIONS

$[^{35}S]GTP\gamma S$	$[^{35}S]$-guanosine-5′-(γ-thio)-triphosphate
$[^{3}H]$SCH 23390	[N-methyl-^{3}H]R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride
$[^{3}H]$ZM 241385	[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
A 77636	(1R-cis)-1-(aminomethyl)-3,4-dihydro-3-tricyclo[3.3.1.13,7]dec-1-yl-[14]-2-benzopyran-5,6-diol hydrochloride
AC	adenylate cyclase
ADA	adenosine deaminase
ADP	adenosine-5’-diphosphate
AKAP	the A kinase anchor protein
AMP	adenosine-5’-monophosphate
ATP	adenosine-5’-triphosphate
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
CaMK	calcium/calmodulin-dependent protein kinase
cAMP	cyclic adenosine-3’,5’-monophosphate
cGMP	cyclic guanosine-3’,5’-monophosphate
CGS 21680	4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride
CHO	Chinese hamster ovary
CNG	cyclic nucleotide gated ion channels
CNS	central nervous system
COMT	cathechol-O-methyltransferase
CVS	chronic variable stress
DA	dopamine
DAT	dopamine transporter
DHX	dihydrexidine
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis-(β-aminoethyl ether)-N, N, N’, N’-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPAC	exchange protein directly activated by cAMP
FRET	fluorescence resonance energy transfer
GABA	γ-aminobutyric acid
GAP	GTP-ase activating protein
GDP	guanosine-5’-diphosphate
GEF	guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine-5’-triphosphate
HC	high chirpers

HCN	hyperpolarization-activated, cyclic nucleotide gated ion channels
HE	high exploratory activity
IBMX	3-isobutyl-1-methylxanthine
LC	low chirpers
L-DOPA	3,4-dihydroxy-L-phenylalanine
LE	low exploratory activity
MAO	monoamine oxidase
MEM	minimum essential medium
MRS 1220	N-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-benzeneacetamide
MSX-3	3-(3-hydroxypropyl)-8-(m-methoxystyryl)-7-methyl-1-propargylxanthine phosphate disodium salt
PBS	phosphate buffered saline
PDE	phosphodiesterase
PEP	phosphoenolpyruvate
PK	pyruvate kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
RGS	regulator of G protein signaling
Ro 20-1724	4-(3-butoxy-4-methoxybenzyl)-imidazolidin-2-one
SERT	serotonin transporter
SKF 83566	8-bromo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol hydrochloride
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride

INTRODUCTION

The behavioral state of humans and animals is determined by the signal transmission between neurons of central nervous system. G protein coupled receptors (GPCRs) have significant role in interneuronal signal transmission as a point where reception of chemical signals from presynaptic neurons takes place. Disruptions in these interneuronal communication systems have been found to be the cause of many pathological conditions, like depression, a common affective disorder, where dysfunction of monoaminergic signal transmission has been proposed to be the main reason (Schildkraut 1965). However, the expanding research of depression has suggested a role for additional signal transmission systems like stress axis (hypothalamus-pituitary-adrenal axis) with its hormone transmitters (corticotrophin, adrenocorticotrophin and glycocorticoids), inflammatory agents and different neuropeptides (neuropeptide Y, substance P) as well.

Only in rare occasions neurochemical studies has been performed on human tissues, and therefore laboratory animals (usually rats and mice) are used for this purpose. However, to diagnose the depression in animals is impossible and therefore only certain dimensions of the latter are usually studied. There are many animal models describing different aspects of depression, e.g., sucrose consumption (anhedonia) (Willner *et al*. 1987), Porsolt's forced swimming test (which is rather correlative model to describe the effectiveness of antidepressants) (Porsolt *et al*. 1978), etc. In the same time, individual differences in animals' behavior can be studied to measure the individual's sensitivity to stress, which also characterizes its susceptibility to depression. For example, differences in exploratory behavior could be linked to the interplay between anxiety and motivation to explore/to experience the positive aspects of novelty, the balance which is impaired in the condition of depression. Responsiveness to tickling reflects individual's sensitivity to the pleasant stimuli, which is also severely decreased in depression, being one of the core symptoms of depression according to the DSM-IV (*Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*).

Dysfunctions in dopaminergic neurotransmission are proposed to be responsible for the development of depression (Harro & Oreland 2001). There are three different dopaminergic pathways in mammalian brain, which regulate different functions: the nigrostriatal pathway, the mesolimbocortical pathway and the tuberoinfundibular pathway. Nigrostriatal pathway begins from dopamine producing neurons in substantia nigra and these neurons project their axons to dopamine receptors containing neurons of striatum. This pathway is associated with regulation and execution of motor activity, e.g. regulation of the motivation to explore novel objects. The signaling intensity of nigrostriatal pathway has been found to be in correlation with the exploratory activity of rats (Alttoa *et al*. 2009). Mesolimbocortical pathway begins from ventral tegmental area and its neurons' axons end up in nucleus accumbens and also in brain cortical areas. The dopaminergic activity in mesolimbocortical pathway

mediates reinforcing effects of pleasant stimuli, including different drugs of abuse, novelty, etc (Di Chiara & Imperato 1988; Rebec *et al*. 1997). Tubero-infundibular pathway is associated with regulation of prolactin secretion from anterior pituitary.

The targets of nigrostriatal and mesolimbic pathways, the striatum and the nucleus accumbens, have high expression of dopamine D_1 and D_2 receptors (Missale *et al*. 1998). They are mainly expressed on GABAergic neurons and colocalization of these receptors is rare, as it has been estimated that only some 15–20% of striatal neurons may contain both receptors (Deng *et al*. 2006). Activation of D_1 and D_2 receptors separately in striatum has little effect on locomotor activity, but when both receptors are activated simultaneously the locomotor activity is increased significantly (Missale *et al*. 1998). Furthermore, it has been shown that D_1 and D_2 receptors are associated with different effectors in striatum (Lee *et al*. 2002). In nucleus accumbens D_2 receptors are mediating stimulant drug reinforcement and D_1 receptors are associated with permissive role (Missale *et al*. 1998). Considering the fact that dopaminergic neurotransmission in these regions is mediating motor and reinforcing aspects of motivationally important stimuli, it is of great importance to characterize these receptors in conditions of reduced motivation and positive affect (depression, stress) as well as look at the individual differences of these receptors signal transmission sensitivity in animals differently acting/reacting to salient stimuli (novelty, tickling) to exemplify the neurochemical basis of predisposition to depression.

In striatum and nucleus accumbens also adenosine receptors are highly expressed and evidence from behavioral up to radioligand binding level experiments indicate that antagonistic interactions exist between certain dopamine and adenosine receptor subtypes, specifically between D_1/A_1 and D_2/A_{2A} receptor pairs (Ferre *et al*, 1997; Fuxe *et al*, 1998). These receptors are associated mainly with modulation of adenylate cyclase (AC) activity, but D_1 and A_{2A} receptors stimulate and D_2 and A_1 receptors inhibit AC activity. These colocalized receptor pairs seem to form heterodimers (Hillion *et al*. 2002; Gines *et al*. 2000) and modulate their partners' properties by interactions on the receptor level (Ferre *et al*. 1994; Ferre *et al*., 1991).

To study the specific role of each dopamine and/or adenosine receptor subtype in behavior or in disease associated with dysfunction of dopaminergic or adenosinergic systems, it would be useful to study the signal transduction sensitivity of a given receptor system. The aim of current work was to develop a method for measuring D_1 and A_{2A} receptor-specific signaling in rat brain striatal tissue through measurement of AC activity modulation induced by these receptors and to use this method for characterization of these receptor signal transduction pathways in different animal models.

G protein-coupled receptors

In pharmacology the term "receptor" is defined as a protein which is able to recognize and translate a certain chemical signal into cell and alter the state of the cell accordingly (Kenakin *et al*. 1992). International Union of Pharmacology committee for receptor nomenclature and drug classification (NC-IUPHAR) has classified receptors into four families based on their structure: ion-channel receptors, seven-transmembrane domain (G protein coupled) receptors, enzyme-associated receptors and transcriptional regulator receptors (Humphrey & Barnard 1998). The seven-transmembrane domain receptors are the largest and most diverse family of receptors in mammalians, as nearly thousand of different G protein coupled receptors (GPCRs) have been proposed to exist in humans according to their genome (Takeda *et al*. 2002).

According to the recommendations of NC-IUPHAR receptor nomenclature, the GPCRs are named after their endogenous agonist or appropriate collective term when a family of related substances may interact with the receptor. Abbreviation of the endogenous agonist is used for naming a given GPCR, followed by numerical subscript, which describes the subtype of GPCR. For example in case of dopamine receptors letter D is used and appropriate number in subscript after D indicates specific subtype of dopamine receptor. Splice variants are indicated by the subscript letter in parentheses, e. g. $EP_{3(a)}$, $EP_{3(b)}$, etc. receptors. (Vanhoutte *et al*. 1996).

X-ray crystallographic analysis of rhodopsin (Palczewski *et al*. 2000) and β_2 adrenergic-receptor (Cherezov *et al*. 2007) have confirmed the assumption that the GPCR family receptors contain seven transmembrane domains, which are relatively hydrophobic and pass through plasma membrane (hence the name seven transmembrane domain receptors). These transmembrane domains are relatively conserved throughout the family of GPCRs (20–60%) and are connected through extracellular and intracellular loops, which are relatively hydrophilic and are more diverse between different GPCRs. Usually GPCRs contain also conserved cysteine residues located in different extracellular loops, which have found to form disulfide bonds stabilizing the structure of GPCRs. The N-terminal part of a GPCR, which usually contains several glycosylation sites, is located outside and C-terminal part of the receptor is inside of the cell. The intracellular part of the GPCR is responsible for transduction of signal to intracellular pathway, starting from heterotrimeric G proteins and leading to the second messenger systems. There are many sets of second messenger systems, which are responsible for the activation of different proteins inside the cell, having also feedback mechanisms to regulate the activity of receptors.

Although the main target of activated GPCRs are heterotrimeric G proteins, there are also many other proteins with which GPCRs interact, like arrestins, G proteins-coupled receptor kinases (GRKs), regulators of G protein signaling (RSG), other GPCRs, etc. Arrestins interact mainly with phosphorylated GPCRs and these interactions are connected with down regulation of signal and can lead to the internalization of the receptor. There is accumulating evidence

that GPCRs may dimerize or even oligomerize in plasma membrane (Park *et al*. 2004) and these complexes may be formed also between different GPCRs, where they modulate each other's function (Hillion *et al*. 2002).

G proteins

Guanine nucleotide binding regulatory proteins (G proteins) are divided into two major groups; the small monomeric G proteins and the big heterotrimeric G proteins. Small G proteins are not directly coupled with GPCRs, although they are involved in regulation of intracellular signaling of cells. Heterotrimeric G proteins function mainly through coupling with GPCRs and mediate the signals received from receptors to specific intracellular targets, like adenylate cyclase (AC), phospholipase C (PLC), etc. Heterotrimeric G proteins are comprised from three subunits; α, β and γ. 27 different α-subunits (including splice variants), 5 β-subunits and 14 γ-subunits has been discovered up to now (Downes & Gautam 1999; Landry *et al*. 2006). The molecular weights of α-, β- and γ-subunits are in range of 39–52 kDa, 35–39 kDa and ~8 kDa, respectively. α-subunit contains nucleotide binding site and possesses GTPase activity. The selectivity of coupling with GPCRs and direction of generation of the response are usually determined by this subunit. The α-subunits of G proteins are divided into four families based on their structure and coupling with effectors: these are G_s, $G_{i/o}$, G_q and $G_{11/12}$ families. G_s-family has three members: α_{olf} and two α_s splice variants (α_{sLong} and α_{sShort}). $G_{i/o}$-family includes α_{i1-3}, α_{o1-2}, α_{t1-2}, α_z and α_{gust}. To family of G_q belongs α_q, α_{11}, α_{14-16}, and $G_{12/13}$-family has α_{12} and α_{13}. G_s-family proteins can be ADP-ribosylated by *cholera* toxin, which activate them constitutively. *Pertussis* toxin ADP-ribosylates α_i, α_o and α_t proteins that inactivates and also uncouples them from GPCRs. α_t and α_{gust} are ADP-ribosylated by both toxins, other α-subtypes are insensitive towards these toxins. β and γ subunits of G protein form a tight complex that does not dissociate under physiological conditions and its presence is essential for effective G protein activation by receptors (Fung 1983).

In the resting state the heterotrimeric G protein is in αβγ complex with guanosine-5'-diphosphate (GDP) bound to the α subunit. Activation of coupled receptor induces conformational changes in G protein that invokes the dissociation of GDP from α subunit and association of guanosine-5'-triphosphate (GTP). The dissociation of GDP from α subunit is the rate limiting stage in G protein activation cycle (Cabrera-Vera *et al*. 2003). The nucleotide exchange on α-subunit may be additionally regulated by guanine nucleotide exchange factors (GEFs), which increase the rate of nucleotide exchange. The binding of GTP will lead to the dissociation of α-subunit from βγ complex, whereas both of them are signal carriers, interacting with their effectors and modulating their activity. In many cases α-subunit and βγ complex have lipid anchors attached to them to keep them close to plasma membrane. During the modulation of effector's activity the GTPase part of α-subunit hydrolyzes the bound GTP to

GDP, which is followed by re-association of α and βγ complex so that the G protein becomes inactive again. Certain proteins called GTP-ase activating proteins (GAPs) can speed up the hydrolysis of GTP bound to α-subunit of activated G protein, thus shortening the effect on the effectors. There are some reports that activated G proteins do not dissociate into α and βγ subunits *in vivo* (Bünemann *et al*. 2003).

There are several effector pathways that can be activated through G proteins. Members of the G_s-family α subunits stimulate the activity of plasma membrane bound AC isoforms, while α_{i1-3} and α_z subtypes inhibit AC activity. AC catalyses the synthesis of a second messenger cAMP, which activates, among others, protein kinase A (PKA) – a kinase that phosphorylates different proteins, regulating their activity in this way. α_{o1-2} subtypes are very abundant in neurons but the reports about their effectors are quite controversial. α_{t1-2} subtypes are involved in visual signal transduction, where they activate cyclic guanyl-3', 5'-monophosphate (cGMP) specific phosphodiesterases (PDEs), thus decreasing the cGMP concentration. The $G_{q/11}$-family α-subunits activate β isoform of enzyme phospholipase C (PLC_β), which hydrolyzes inositolphospholipids, thus generating two second messengers: inositolphosphates that are responsible for release of Ca^{2+} from intracellular stores and diacylglycerol that activates protein kinase C (PKC). α_{11} and α_{12} are associated with activation of GEFs and monomeric G protein Rho, respectively.

In addition to α-subunits, βγ-subunits have been found to be regulators of certain effectors activities inside the cell as well. One of those effectors is PLC_β, which activity is stimulated by βγ-subunits. Also AC activity is modulated by βγ-subunits and depending on the isoform of AC βγ-subunits either stimulate or inhibit its activity. Some K^+ and Ca^{2+} channels can also be activated by βγ complex.

Adenylate cyclase

Already in 1957 T. W. Rall and E. W. Sutherland published the finding that in tissues cyclic adenosine-3', 5'-monophosphate (cAMP) is synthesized from adenosine-3'-triphosphate (ATP) (Rall & Sutherland 1958), but the protein responsible for the catalysis of this reaction was cloned and characterized in 1989 (Krupinski *et al*. 1989). According to the enzyme nomenclature AC is numbered EC 4.6.1.1. To date 10 different genes have been found that are responsible for encoding ACs, and from some of these genes several splice variant are produced. The molecular weight of ACs varies between 118–140 kDa. 9 AC isoforms are membrane integrated proteins, which are marked as AC1 to AC9 (roman numbers are also often used), and one is a cytosolic soluble protein termed as sAC (soluble AC). Here only the properties of membrane integrated ACs, which are responsible for receiving and transduction of signals from GPCRs, will be discussed. Membrane bound ACs are relatively homologous to each other and they consist from two relatively hydrophobic regions termed as M1 and M2, each of which has six membrane spanning

domains connected by short intracellular and extracellular loops, and three relatively hydrophilic regions termed C1, C2 and N-terminal domains. Both the C1 and C2 domains are divided into a and b regions. The C1a and C2a regions, which are relatively conserved within the AC family, are essential for the enzymatic activity. The C1b and C2b regions have found to have binding sites for many regulators of AC activity. Structural details of catalytic unit of AC have been uncovered by X-ray crystallography and the binding sites of enzymatic activity regulators and substrate have been located (Tesmer *et al.* 1997). AC is enzymatically active when C1a and C2a domains form a complex with each other and between them a site for ATP binding and cAMP synthesis is generated. α-subunits of G_s- and G_i-family regulate the activity of AC by modulation of the complex formation between C1a and C2a domains. Activated α_i inhibit AC activity by decreasing the affinity of C1a domain towards C2a domain, while α_s increases the complex formation. βγ-subunits of G proteins are also shown to modulate the activity of AC by binding to C1b domain. βγ complexes stimulate the activity of AC isoforms 2, 4, 5, 6 and 7 and inhibit isoforms 1, 3 and 8. Forskolin, a diterpene purified from Indian Coleus plant, activates AC directly by mechanism that is similar to α_s — by increasing the complex formation between C1a and C2a domains. The N-terminal of ACs is highly variable and it has been proposed that they have some role in regulation of enzymatic activity (Simpson *et al*. 2006; Lai *et al*. 1999).

It is known that for effective catalysis of cAMP synthesis the presence of Mg^{2+} is crucial. Furthermore, X-ray crystallographic studies have shown that two Mg^{2+} are recruited into catalytic site of the enzyme. Mg^{2+} can be replaced by Mn^{2+}. Also Ca^{2+} and Zn^{2+} bind to catalytic site, but most of the AC isoforms activity is inhibited by binding of these ions.

Many kinases have found to phosphorylate ACs, like protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin kinase (CaMK), etc. Depending on the amino acid position that is phosphorylated, AC activity is either stimulated or inhibited. Phosphorylation of AC isoforms 5 and 6 by PKA leads to the inhibition of these ACs. There are also many glycosylation and nitrosylation sites found in AC, but they have no or little direct influence on the enzyme activity.

Usually multiple isoforms of AC are expressed in one cell. In central nervous system (CNS) all AC isoforms are expressed, but some of them are detected predominantly in specific areas, like AC3 in olfactory cilla and AC5 in basal ganglia (Chern 2000).

The role of cAMP as a second messenger

Cyclic adenosine-3', 5'-monophosphate (cAMP) was the first intracellular second messenger discovered. In 1950s E.W. Sutherland studied how epinephrine activates phosphorylase in liver and found that cAMP is one of components responsible for this signal transduction. cAMP has been found to

mediate its effects through activation of PKA, cyclic nucleotide-regulated ion channels and exchange protein directly activated by cAMP (EPAC). In addition, some isoforms of phosphodiesterases (PDEs), enzymes that degrade cAMP itself, have been found to be activated by cAMP.

PKA is an enzyme that phosphorylates certain serine and threonine amino acids in different proteins inside the cell, regulating their activity in this way. In inactive state the holoenzyme of PKA consist from four subunits, two of which are catalytic and other two regulatory subunits. Each regulatory subunit has two cAMP binding sites and after cAMP binding these subunits dissociate from the complex and catalytic subunits become enzymatically active. There are a lot of proteins which activity is dependent on phoshporylation state and many of them are phosphorylated by PKA. Some of the PKA phosphorylation targets are for example Ca^{2+} and K^{2+} channels, certain isoforms of AC, many PDE isoforms, glycogen synthase, pyruvate kinase, etc.

The activation of PDE by PKA phosphorylation is one of the negative feedback mechanisms of cAMP/PKA pathway, where high concentration of cAMP induces activation PDEs, which in turn degrades cAMP and thus reduces its concentration in cells. PKA also phosphorylates and inhibits the activity of AC isoforms 5 and 6, which is another negative feedback mechanism of cAMP/PKA pathway.

Cyclic nucleotide-regulated channels are comprised from two different types of channels, cyclic nucleotide-gated (CNG) channels and hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels. Activities of those channels are regulated by binding of cAMP and/or cyclic guanosine-3',5'-mono-phosphate (cGMP). CNG channels are directly activated by cyclic nucleotides, while HCN channels are activated by hyperpolarization and cyclic nucleotides enhance the channel activity.

EPAC proteins are guanine nucleotide exchange factors (GEFs) of Ras-superfamily GTPases that are activated by cAMP binding. The small G proteins, which in inactive state have GDP bound with them, are activated by GDP replacement with GTP that is induced by other proteins called GEFs. To date two isoforms of EPAC has been discovered, named EPAC1 and EPAC2. The latter has two cAMP binding sites and EPAC1 has one cAMP binding site. EPAC proteins are implicated in several diverse cellular responses like secretion, cell adhesion, apoptosis, etc.

The role of PDEs inside the cell is to regulate the concentration of cyclic nucleotides. 21 genes are known to encode PDEs in humans and with splice variants 96 different isoforms of PDEs have been discovered. PDE isoforms are divided into 11 families, based on their specificity towards cAMP and cGMP, regulation of activity, localization in cell, etc. PDE families 4, 7 and 8 are cAMP-specific PDEs, PDE families 5, 6 and 9 have cGMP-specificity and PDEs of families 1, 2, 3, 10 and 11 are non-specific towards cAMP/cGMP. (Conti & Bevao 2007).

cAMP signaling inside the cell is usually compartmentalized. This idea arose already three decades ago, when it was shown that prostaglandin E stimulated

cAMP accumulation in cardiac myocytes but didn't mimic the effects of β-adrenergic receptor-dependent phosphorylation of substrates essential for contractility (Hayes *et al*. 1979). It was proposed that distinctive cAMP micro-domains must exist in cells that are responsible for PKA activation and also that cAMP is not completely freely diffusible in cytosol of cells (Steinber & Brunton 2001). The microdomains of cAMP are generated with the help of PDEs, which are specifically located within the microdomains to regulate the cAMP concentration (Conti & Bevao 2007). Additionally PKAs are also located specifically in these domains fixed with A-kinase anchor proteins (AKAPs), which allow phosphorylation of only specific target proteins that are within the current cAMP microdomain (Dodge-Kafka *et al*. 2006). This means that although many GPCRs are coupled with the same subtype of G proteins that modulate the AC activity, the physiological responses of these GPCRs may be quite different.

Dopamine and adenosine signal transduction in striatum

Striatum is a part of basal ganglia, and it is anatomically divided into caudate nucleus and putamen. There are mainly GABAergic neurons in striatum, which project to globus pallidus and substantia nigra. These neurons contain post-synaptically high levels of adenosine and dopamine receptors, which may form common signal transduction modulation complexes. Striatum has important role in regulation of motor functions and cognitive processes. Dysfunction of dopaminergic signaling in striatum is associated with many pathological conditions like depression, schizophrenia, etc. Loss of dopaminergic signaling in striatum is the cause of Parkinson disease. To get better understanding of the role of each receptor in different pathological conditions of brain, each receptors effect on the function of striatal neurons needs to be studied separately.

Dopamine (DA) is predominant catecholamine neurotransmitter in the mammalian brain, which regulates many different functions in central nervous system, but also in periphery. DA is synthesized in axon terminals by two-step process from tyrosine. In the first step tyrosine hydroxylase produces dihydroxyphenylalanine (L-DOPA) and in the second step DOPA decarboxylase removes carboxyl group of L-DOPA. Generated DA is transported into storage vesicles by specific vesicular transporters, from where it is released into synaptic cleft via action potential triggered calcium-dependent exocytosis to activate postsynaptic DA receptors. DA is removed from synaptic cleft by back-transportation into presynaptic terminal via specific DA transporters (DAT) and, additionally, is degraded by monoamine oxidase (MAO) or cathecol-O-metyltransferase (COMT). The groups of neurons producing DA in brain are marked as A8 – A15 areas, which are positioned in midbrain (A8 – A10), inter-brain (A11 – A14) and cerebrum (A15), and these cell project to many areas of

brain. The signal transmission from A9 (substantia nigra) and A10 (ventral tegmential area) cells groups, making up the nigrostriatal and the mesolimbo-cortical pathway, have been studied most extensively.

Five different DA receptors have been found to exist that are termed as D_1, D_2, D_3, D_4 and D_5, and all of them are GPCRs. Additionally D_2 receptor has two splice variants, long and short D_2 receptor (termed as D_{2L} and D_{2S}, respectively), which differ by the length of 3rd intracellular loop. Pharmacologically DA receptors are divided into two subfamilies based on the G protein coupling. The D1-family includes D_1 and D_5 receptor subtypes, which function primarily by stimulating the activity of AC. The D2-family consists from D_2, D_3 and D_4 receptors, and these receptors inhibit AC activity. In addition, DA receptors have found to couple also other signal transduction pathways, like G_q-coupled activation of PLC, which regulates the intracellular Ca^{2+} concentration (Jin *et al.* 2001).

Adenosine system is somewhat different from the dopamine system, as adenosine is synthesized both intracellularly and extracellularly and its concentration between these two environments is regulated by specific transporters present in plasma membrane, which pass adenosine through in both directions, so that the concentration of adenosine would be kept relatively similar inside and outside of the cell. Adenosine is synthesized by ecto-5'-nucleodidase, which dephosphorylates adenosine-5'-monophosphate (AMP), and also by hydrolysis of S-adenosyl-homocysteine. The tonic level of adenosine in cat and rodent brain have been estimated to be around 30 – 300 nM (Svenningsson *et al.* 1999). Adenosine deaminase (ADA) is an enzyme that degrades adenosine into inosine, thus keeping the concentration of adenosine under control. It is dominantly expressed in the cell but is present also on the extracellular surface of plasma membrane.

Adenosine modulates cellular processes mainly through four adenosine receptors, which are termed as A_1, A_{2A}, A_{2B} and A_3 receptor, all of them being GPCRs. A_1 and A_3 receptors are coupled with G_i-family proteins and inhibit AC activity. A_{2A} and A_{2B} receptors on the contrary lead to the stimulation of AC activity upon activation. Adenosine receptors have also been found to modulate activity of other effectors than AC as well, e.g. G protein-gated inwardly rectifying K^+-channels (Ito *et al.* 1995) and N-type Ca^{2+}-channels (Mynlieff *et al.* 1994).

The highest expression of DA receptors in brain have been found in striatum, where dominantly D_1 and D_2 subtypes of DA receptors are expressed, but there are also other subtypes of DA receptors in lower levels (Missale *et al.* 1998). D_1 receptor is present mainly in striato-nigral neurons that contain also neuropeptide substance P and dynorphin, while D_2 receptor is preferentially localized in striato-pallidal neurons containing enkephalin (Le Moine & Bloch 1995). Colocalization of D_1 and D_2 receptors in striatal neurons is low (Deng *et al.* 2006). High levels of adenosine A_1 and A_{2A} receptors are present in brain. Furthermore, it has been shown that A_{2A} receptor is colocalised with D_2 receptor in striato-pallidal neurons (Svenningsson *et al.* 1997) and A_1 receptor with D_1

receptor in striato-nigral neurons (Ferre *et al*. 1996). Additionally, there are findings that adenosine and DA receptors in striatum have antagonistic interaction in receptor level. It has been proposed that A_{2A}/D_2 receptors (Hillion *et al*. 2002) and A_1/D_1 receptors (Gines *et al*. 2000) form heterodimers. Moreover, in rat striatal membranes A_1 receptor ligands affect the ligand binding characteristics of D_1 receptor (Ferre *et al*. 1994) and A_{2A} receptor ligands affect the ligand binding characteristics of D_2 receptor (Ferre *et al*. 1991).

One of the targets of dopaminergic mesolimocortical pathway, nucleus accumbens, has also high expression of DA D_1 and D_2 subtype receptors and also adenosine A_1 and A_{2A} subtype receptors and antagonistic interaction between these receptors have been also found (Ferre *et al*. 1996a; Ferre *et al*. 1994a).

Measurement of ligand affinity and receptors expression level by radioligand binding

Many GPCRs have ligand binding site within the extracellular side of the receptor, which is formed between its transmembrane domains. Binding of an agonist to the receptor induces conformational changes in the receptor, which in turn leads to the activation of coupled G protein inside the cell. Agonist can be full or partial agonist, depending on whether maximal response of downstream signaling is achieved or only partial. Antagonist binding to receptor on contrary does not lead to the activation of G proteins and also blocks binding of some other ligand to the receptor. Inverse agonists inhibit constitutive activity of receptors.

The binding of ligand to receptor is usually described by reversible one step bimolecular reaction, where ligand/receptor complex (RL) is formed between ligand (L) and receptor (R). The affinity of ligand towards receptor is often characterized by equilibrium dissociation coefficient (K_D=[L]*[R]/[RL]). The appliance of one step ligand binding mechanism can be controlled by studying the dependence of observed association rate coefficient (k_{obs}) from ligand concentration. According to the one-step binding scheme in the pseudo-first order conditions ([L]>>[R]) the k_{obs} is linearly dependent from ligand concentration, but in some occasions the dependence have been found to be hyperbolical (Järv *et al*. 1979; Lepiku *et al*. 1996; Oras *et al*. 2002). This discrepancy has been explained with the presence of additional isomerization step of ligand/receptor complex, following the binding of the ligand (Strikland *et al*. 1975). In this two-step scheme the fast equilibrium binding of ligand is followed by slower isomerization of ligand/receptor complex and with filtration methods only the amount of isomerized complex can be determined.

As the expression level of receptors in tissues and cells is usually low, the determination of ligand's affinity and the number of binding sites (B_{max}) in sample requires very sensitive methods, and therefore labeling of ligands with

radioactive isotopes (^3H, ^{125}I) is used. Nowadays also fluorescent labels are used for ligand labeling, but as these labels usually have significant influence on the affinity of ligand towards receptors, fluorescent methods have not yet replaced the radioligand binding method.

The binding properties of non-labeled ligands are usually measured by their ability to compete with labeled ligands in binding to receptor. According to the simplest binding competition scheme, where labeled and non-labeled ligands compete in binding for the same biding site, the affinity of non-labeled ligand can be calculated using Chen-Prusoff equation (Cheng & Prusoff 1973), which is as follows: $K_i=IC_{50}/(1+([*L]/K_D])$, where K_i is the affinity coefficient of non-labeled ligand, IC_{50} is the concentration of non-labeled ligand that caused 50% inhibition of labeled ligand binding, *L is the concentration of labeled ligand and K_D is the affinity of labeled ligand towards receptor.

The agonist binding to receptor can be modulated by G proteins, but the mechanism of this modulation is not yet fully clear. It is proposed that if G proteins are in guanosine nucleotide free state then ternary complex of agonist, receptor and G protein is formed, which is stable over longer time and higher agonist apparent affinity is observed. If some nucleotide is bound to the G protein of the complex, the high affinity binding for agonist is lost.

Determination of adenylate cyclase activity

Many GPCRs regulate different intracellular processes through modulation of intracellular concentration of cAMP via regulation of AC activity. Studying the GPCR mediated modulation of intracellular cAMP concentration can give essential information about the status of the receptor/G protein/AC system. Over the decades several methods for measurement of cAMP concentration in tissue or cell lysates and also inside the intact cells have been elaborated.

One of the earliest method developed for measurement of cAMP concentration is binding competition method, where cAMP from sample and [^3H]cAMP or [^{125}I]cAMP compete in binding to the protein that specifically binds cAMP (Brown *et al*. 1971; Gilman 1970) or to the cAMP-specific antibody (Steiner et al. 1969). The amount of labeled cAMP/protein complex formed in the assay is dependent from the cAMP concentration present in sample. The labeled cAMP/protein complex can be separated from unbound labeled cAMP by filtration, centrifugation or precipitation methods and then its quantity can be determined through measurement of radioactivity. Here it is important that the cells are lysed and PDEase activity inhibited before conducting the cAMP assay. The cAMP-binding protein for this kind of experiments has been purified from different tissues, like adrenal (Brown *et al*. 1971) and muscle tissue (Gilman 1970). Most of the commercially available cAMP assays kits are based on the antibody system, but instead of radioactive labeling of cAMP different kinds of ELISA-based methods are utilized.

As an alternative approach, the substrate of AC is labeled and its turnover is followed. Here usually [α-^{32}P]ATP is used, but also fluorescent labeling of ATP has been applied (Cunliffe *et al.*, 2006). After the termination of reaction, the non-reacted ATP and produced cAMP are separated chromatographically or using capillary electrophoresis, and their concentrations are determined.

In case of intact cells the labeled substrate cannot be directly added to the cells, instead the cells are preincubated with [^{3}H]adenine and inside of the cell [^{3}H]ATP is synthesized from [^{3}H]adenine. After the cAMP formation reaction the cells are lysed and accumulated [^{3}H]cAMP is separated chromatographically from other tritium labeled molecules and its concentration can be determined then.

All previous methods require relatively large population of cells for the effective AC activity measurement, but recently several sensitive cAMP sensors have been elaborated that permit measurements in single cell. These cAMP sensors are based on downstream targets of cAMP, like PKA, EPAC and CNG (Nikolaev & Lohse 2006). The PKA and EPAC based sensors are designed to work on fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) principles, where binding of cAMP changes energy transfer between donor and acceptor fluorophore of the protein. With CNG based sensor Ca^{2+} dyes can be used, as Ca^{2+} flow into cells through CNG ion channels is cAMP-dependent, or patch-clamp technique can be applied to measure changes in cAMP concentration.

The measurement of cAMP concentration can be used to characterize the potencies of ligands that bind to the receptors associated with AC activity modulation. Potencies of agonists are described with EC_{50} value, which is the concentration of agonist at which 50% of the maximal effect is achieved. In the case of antagonist, presence of agonist is needed for estimation their affinity from functional experiments. The inhibition effect produced by antagonist is directly dependent on its binding affinity towards receptor, and from concentration-dependent inhibition of agonist-induced effect, the affinity of antagonist can be determined. The antagonist's affinity coefficient measured from functional experiment is termed as K_b, which according to the modified Cheng-Prusoff equation can be calculated as $K_b=IC_{50}/(1+([A]/EC_{50}))$, where IC_{50} is the concentration of antagonist that produces 50% inhibition of agonist's effect, [A] is agonist concentration and EC_{50} is agonist's potency (Cheng 2001).

Another frequently used method to determine antagonist's K_b value from functional experiments is construction of Schild plot, for which agonist concentration-response curves are measured in presence of different concentrations of antagonist (Arunlakshana & Schild 1959). If antagonist competes with agonist in binding for the same site in receptor, then linear dependence with slope equal to unity is observed between log(DR-1) vs. log(antagonist conc.) plot, where DR is the ratio between agonist's EC_{50} value in presence of certain concentration of antagonist and agonist's EC_{50} value in absence of antagonist.

AIMS OF THE STUDY

The aims of the study were to develop and validate a method for measuring signal transduction of dopamine- and adenosine receptors subtype-specifically in rat brain striatum by determination of AC activity, and to applay this method for studying relationships between behavior and signal transduction sensitivity of aforementioned rececptors in different animal (rats) models. The particular objectives were:

- The development of a method for determination dopamine D_1 receptor-specific modulation of AC activity in rat brain striatal tissue.
- The development of a method for determination adenosine A_{2A} receptor-specific modulation of AC activity in rat brain striatal tissue.
- Determination of sensitivity of dopaminergic signal transduction on rats with high and low exploratory activity and effect of stress regimen on this.
- Determination of gender-specific effects of tickling on dopamine signal transduction.

MATERIALS AND METHODS

Chemicals

[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]ZM 241385, 21 Ci/mmol) was purchased from Tocris Cookson Ltd., [N-methyl-^{3}H]R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride ([^{3}H]SCH 23390) and [5',8'-^{3}H] adenosine-3',5'-cyclic monophosphate ([^{3}H]cAMP, 48 Ci/mmol) were obtained from Amersham Biosciences. [^{3}H]raclopride (74 Ci/mmol) from Perkin Elmer Life Sciences. 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronami-dosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS 21680), 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylami-no]ethyl)phenol (ZM 241385), N-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-benzeneacetamide (MRS 1220), (1R-cis)-1-(aminomethyl)-3,4-dihydro-3-tricyclo[3.3.1.13,7]dec-1-yl-[14]-2-benzopyran-5,6-diol hydrochloride (A 77636), 8-bromo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol hydrochloride (SKF 83566), (R)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH 23390), dihydrexidine (DHX), isobutylmethylxanthine (IBMX) and forskolin were obtained from Tocris Cookson Ltd. Adenosine deaminase (ADA, EC 3.5.4.4, 5 mg/ml), phosphoenolpyruvate (PEP), pyruvate kinase (PK) and guanosine-5'-(3-thio)-triphosphate (GTPγS) were purchased from Roche Diagnostics. Guanosine-5'-diphosphate (GDP), cyclic adenosine-3',5'-monophosphate (cAMP) and 4-(3-butoxy-4-methoxybenzyl)-imidazolidin-2-one (Ro 20-1724), butaclamol, bovine serum albumin (BSA) and dopamine (DA) were obtained from Sigma-Aldrich Chemie Gmbh· 3-(3-hydroxypropyl)-8-(m-methoxystyryl)-7-methyl-1-propargylxanthine phosphate disodium salt (MSX-3) was from Pharmaceutical Institute of the University of Bonn. Cell culture media and reagents were from GIBCO™. All other reagents were of analytical grade from regular suppliers.

Measurement of cAMP accumulation in CHO cells

The Chinese hamster ovary cells stably expressing dog adenosine A_{2A} receptors were seeded onto 24-well plates 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 of ADA for 30 min at 37ºC. The ligand dilutions and the PDE inhibitor Ro 20-1724 (final concentration 100 μM) in 100 μl PBS per well were added and the incubation carried out for 15 min at 37ºC. The reaction was terminated with addition of ice-cold $HClO_4$ (final concentration 0.4 M). After 1 h 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 & Fredholm 1990). Briefly, the cAMP from sample was incubated with [^{3}H]cAMP and cAMP-binding protein, after which the bound radioactivity was separated by rapid filtration through GF/B glass-fiber filters (Whatman Int. Ltd.) using Brandell cell harvester followed by three washes of 3 ml of ice-cold phosphate buffer (20 mM, pH 7.5) containing 100 mM NaCl. The filters were kept overnight with scintillation cocktail OptiPhase HiSafe 3 (Wallac Perkin Elmer Life Sciences) and the radioactivity content was measured using scintillation counter (Beckman LS 1800 or LKB Wallac 1219 Rackbeta).

Striatal tissue preparations

For [^{3}H]ZM 241385 binding experiments – Rat striatum was homogenized by Bandelin Sonopuls (three passes, 10 s each; Bandelin Electronic GmbH, Germany) in ice-cold homogenization buffer (HB, 20 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.5) and centrifuged at 40,000×g for 20 min at 4ºC. The membrane pellet was washed by resuspension in HB and centrifugation two more times. The final suspension was divided into aliquots and stored at -80ºC until use.

For [^{3}H]SCH 23390 binding experiments – Rat striatum was homogenized in 50 volume (v/w) of ice-cold Tris-HCl buffer (2.5 mM pH 7.4) containing also 2 mM EGTA. The suspension was washed twice by centrifugation at 16,000 rpm for 40 min at 4ºC and homogenized in 30 volumes (v/w) of Tris-HCl buffer (50 mM, pH 7.4) containing also 2 mM EGTA. The final suspension was divided into aliquots and stored at –80ºC until use.

For [^{3}H]raclopride binding experiments – The membranes were prepared as described in Harro *et al.* 2003.

For AC activity experiments – Washed membranes were prepared from rat striatum by homogenization of striatal tissue in 60 volumes (v/w) of Tris-HCl buffer (50 mM, pH 7.4), followed by centrifugation at 20,000×g for 40 min at 4ºC. Resulting membrane pellet was resuspended in the same amount of Tris-HCl buffer (50 mM, pH 7.4) and centrifuged for the second time at the same conditions. The homogenization and centrifugation step was repeated one more time and final pellet was resuspended in assay buffer (AB, 30 mM Tris-HCl (pH 7.4), 8.3 mM $MgCl_2$, 0.1 mM Ro 20-1724, 0.75 mM EGTA, 7.5 mM KCl and 100 mM NaCl), divided into aliquots and stored at -80ºC until use.

The crude homogenate was prepared by homogenization of striatal tissue by sonication in 65 volumes (v/w) of AB, divided into aliquots and stored at –80ºC until use.

The crude homogenate without sodium-, potassium-, magnesium salts and Ro 20-1724 was prepared by homogenization of striatal tissue by sonication in 50 volumes (v/w) of Tris-HCl buffer (2.5 mM, pH 7.4) containing also 2 mM EGTA. The homogenate was diluted with the same volume of Tris-HCl buffer

(50 mM, pH 7.4) containing also 2 mM EGTA, divided into aliquots and stored at -80ºC until use.

Radioligand binding experiments

[^{3}H]ZM 241385 binding experiments – Equilibrium binding assays were performed by incubating membranes (100 µg protein/500 µl) in incubation buffer (IB, 20 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, pH 7.4) with appropriate concentrations of [^{3}H]ZM 241385 (0.4 to 4 nM) for 45 min at 25ºC. The reaction was stopped by rapid filtration through GF/B glass fiber filter (Whatman) and four washings with 3 ml ice-cold washing buffer (20 mM potassium phosphate buffer pH 7.5, 100 mM NaCl) and bound radioactivity was measured as described above. Non-specific binding was determined in the presence of 0.5 mM dimethylpropargylxanthine (DMPX). Displacement experiments were performed by incubating membranes (70 µg protein/ml) in Tris-HCl buffer (30 mM Tris-HCl, pH 7.4) containing also 100 mM NaCl, 10 mM $MgCl_2$ and 0.75 mM EGTA with 2.3 nM [^{3}H]ZM 241385 and non-labeled ligands (CGS 21680 in concentration range 10 pM to 10 µM, ZM 241385 in concentration range 10 pM to 10 µM, MSX-3 in concentration range 100 pM to 30 µM) with and without the presence of 30 µM GTPγS for 60 min at 30ºC. The reaction was terminated by rapid filtration, the filters were kept overnight with scintillation cocktail OptiPhase HiSafe 3 (Wallac Perkin Elmer Life Sciences) and the radioactivity content was measured using scintillation counter (Beckman LS 1800 or LKB Wallac 1219 Rackbeta).

[^{3}H]SCH 23390 binding experiments – In saturation binding experiments homogenate of rat striatal membranes (1.9 mg tissue/ml) in 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EGTA was incubated with increasing concentrations of [^{3}H]SCH 23390 (0.06 – 7 nM) for 45 min at 30°C. The reaction was terminated by rapid filtration and the radioactivity content was measured as describe above. Non-specific binding of [^{3}H]SCH 23390 was determined with 1 µM SKF 83566. In displacement binding experiments, different concentrations of the ligand were incubated with fixed concentration of [^{3}H]SCH 23390 (1.7 – 2.1 nM in different experiments) for 45 min at 30°C.

[^{3}H]raclopride binding experiments – The suspension of membranes (5.4 mg tissue/ml) was incubated with different concentrations of [^{3}H]raclopride (0.1 – 6 nM) for 60 min at 25°C, and the incubation was terminated by rapid filtration as described above. Non-specific binding of [^{3}H]raclopride was determined with 10 µM (+)-butaclamol. In displacement binding experiments, different concentrations of the ligand were incubated with 2.8 nM [^{3}H]raclopride for 60 min at 25°C. The reaction was terminated by rapid filtration and the radioactivity content was measured as describe above.

Adenylate cyclase activity assay

The assay was carried out in a reaction medium containing 30 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM ATP, 10 µM GTP, 0.75 mM EGTA, 7.5 mM KCl, 100 mM NaCl, 0.1 mM IBMX, 0.1 mM Ro 20-1724, 100 µg/mL bacitracin, 0.03% BSA and ATP regenerating system (10 mM PEP and ~30 µg/mL PK), if not stated otherwise. The reaction was started by placing tubes containing membrane homogenate (2 to 12 µg protein/ml) with the ligand of interest from ice bath to 30°C water bath and incubated for 15 min. Reaction was terminated by addition of a solution containing EDTA (final concentration 25 mM) and subsequent boiling of samples for 5 min. The content of accumulated cAMP in samples was measured as described in section "measurement of cAMP accumulation in CHO cells".

Data analysis

All data, except for data from behavioral experiments, were analyzed by means of nonlinear least squares method using program GraphPad PRISM™ (GraphPad Software, Inc.). Radioligand binding and cAMP accumulation determination experiments data are presented as mean ± SEM of at least two independent determinations carried out at least in duplicates. The statistical significance of difference was determined by the Student-Newman-Keuls test, where $P<0.05$ was taken as the criterion of significance, except for behavioral studies, were data was analyzed with two-factor ANOVA (Exploration × Stress) or with two-factor ANOVA for repeated measures (Exploration × Stress × Time). Subsequent pair-wise comparisons were made with Fisher's LSD test. Statistical significance was set at $p < .05$. All statistics of behavioral experiments were made using SPSS 16 software (SPSS Inc. Chicago, USA).

RESULTS & DISCUSSION

Tissue sample preparation and selection of homogenization buffer

The modulation of second messenger generation is usually preferred to study in intact cells, but this is rarely an option when GPCR induced modulation of second messenger generation is studied in animal tissues. In current study the determination of AC activity was done using a method described in Valkna et al. 1995, but the conditions were optimized for the measurement of modulation of AC activity induced by adenosine and dopamine receptors in rat striatum.

In the homogenate of rat striatum, where homogenization buffer consisted from 30 mM Tris-HCl (pH 7.4), 0.1 mM Ro 20-1724, 8.3 mM $MgCl_2$, 7.5 mM KCl and 100 mM NaCl, the basal cAMP accumulation level was 44 ± 2 fmol/min per µg tissue. The washout of the cytosolic part of cells by centrifugation resulted with threefold reduction of basal cAMP accumulation, but also accumulation of cAMP in presence of forskolin and A_{2A} receptor agonist CGS21680 decreased threefold. Omitting the magnesium, sodium and potassium salts and Ro 20-1724 from homogenization buffer, leaving 26 mM Tris-HCl and 2 mM EGTA, led to the increase of basal accumulation of cAMP, but also CGS21680-specific accumulation of cAMP increased 1.6 fold, reaching the level of 25 ± 2 fmol/min per µg tissue. In the following experiments, homogenate of rat striatum in 26 mM Tris-HCl buffer (pH 7.4) with 2 mM EGTA was used as a tissue sample.

Optimization of reaction buffer

In GPCR signal transduction system Mg^{2+} plays crucial role in activation of G proteins (Hildebrandt *et al.* 1991) and also in catalysis of cAMP synthesis (Birnbaumer & Rodbell 1969). However, depending on the step of signal transduction of interest, the optimal concentration of Mg^{2+} required is quite different.

In the absence of $MgCl_2$ in reaction buffer no cAMP production was observed. At 1 mM $MgCl_2$ already significant cAMP accumulation in basal conditions occurred and increased hyperbolically with the increase of Mg^{2+} concentration. The half-maximal level of basal accumulation of cAMP was achieved at 6.5 mM concentration of Mg^{2+}. In parallel, the dependence of A_{2A}- and D_1 receptor-specific cAMP accumulation from $MgCl_2$ concentration was studied. A_{2A} receptor-specific cAMP accumulation, induced by CGS 21680, reached the plateau at 3 mM $MgCl_2$ and started to decrease at concentrations above 10 mM. The highest relative effect (CGS 21680-induced effect *vs.* basal) was achieved at 3 mM $MgCl_2$. Similar optimum for effect induced by D_1

receptor was found to be at 5 mM $MgCl_2$ and these experimental conditions were used in following experiments.

GTP/GDP exchange is an important step in GPCR signal transduction and therefore also the concentrations of these nucleotides are important factors (Rinken *et al*. 2001). Replacement of 10 µM GTP in reaction buffer with 1 µM non-hydrolysable GTP analogue GTPγS increased the basal accumulation of cAMP but no increase in CGS 21680 induced cAMP accumulation was observed. Furthermore, addition of 1 µM GDP to reaction buffer or changes in GTP concentration didn't cause increase of receptor-specific effect in both studied receptor-systems.

Adenosine A_{2A} receptors

In striatal homogenate the A_{2A} receptor agonist CGS 21680 caused concentration-dependent increase in accumulation of cAMP. The maximal level of cAMP accumulation achieved in presence of 100 µM CGS 21680 was 22 ± 2 fmol/min per µg tissue, and the stimulation of cAMP accumulation was characterized with the pEC_{50} = 6.3 ± 0.2 and Hill slope was 1.1 ± 0.3. To avoid the influence of endogenous adenosine, striatal homogenate was pretreated with adenosine deaminase (ADA, 10 U/ml), but no significant effect on CGS 21680 dependent cAMP accumulation was observed. As ADA by itself is highly expressed in rat striatum (Yamamoto *et al*. 1988) and is tightly associated with the adenosine receptors (Preston *et al*. 2000; Herrera *et al*. 2001), it can be proposed that endogenous adenosine is cleaved in homogenate of striatum by endogenous ADA. Furthermore, the fact that A_{2A} receptor-specific antagonist ZM 241385 didn't affect the basal accumulation of cAMP in striatal homogenate confirms the lack of influence of endogenous adenosine. But ZM 241385 caused concentration-dependent inhibition of cAMP accumulation induced by 10 µM CGS21680. Assuming that ZM 241385 and CGS 21680 compete in binding for common binding site in the receptor, the K_b value for antagonist was calculated to be 1.2 nM (0.5 – 2.6 nM, CI 95%). Hill slope of ZM 241385's inhibition curve was 1.1 ± 0.4. Similarly behaved also another A_{2A} receptor antagonist MSX-3, for which K_b value of 2.4 nM (0.7 – 7.5 nM, 95% CI) was obtained.

Binding of $[^3H]$ZM 241385 to rat striatal membranes was saturable and described with a K_d = 0.14 ± 0.01 nM and B_{max} = 1620 ± 40 fmol/mg protein. All studied A_{2A} receptor ligands inhibited concentration-dependently the binding of $[^3H]$ZM 241385 to striatal membranes. In case of CGS 21680 the K_i value was 45 nM (30 – 69 nM, 95 % CI) and Hill slope of the $[^3H]$ZM 241385 binding inhibition curve was 0.79 ± 0.12. Activation of G proteins with 100 µM GTPγS had no significant effect on the affinity of CGS 21680, but increased the Hill slope value of the inhibition curve. For antagonists of A_{2A} receptors following K_i values were obtained: 0.8 nM (0.6 – 1.1 nM, 95 % CI) for ZM 241385 and 50 nM (37 – 72 nM, 95 % CI) for MSX-3. The Hill slopes of

antagonists' concentration-dependent curves of [^{3}H]ZM 241385 binding inhibition were not significantly different from unity. Addition of GTPγS had no effect on antagonists' abilities to inhibit [^{3}H]ZM 241385 binding.

In intact CHO cells, expressing dog A_{2A} receptors, CGS 21680 also induced stimulation of cAMP accumulation, the pEC_{50} of stimulation curve was 7.82 ± 0.08 and Hill slope was 0.76 ± 0.06. All studied antagonists of A_{2A} receptors (ZM 241385, MSX-3 and MRS 1220) were able to fully inhibit this effect in a concentration-dependent manner. From the Schild plots, created using data from CGS 21680 concentration-dependent curves in presence of different concentrations of antagonist, following K_b values for antagonists were obtained; 6.6 nM (3.2 – 14.3 nM, 95% CI) for ZM 241385, 39.7 nM (19.5 – 63.1 nM, 95% CI) for MSX-3 and 16.8 nM (6.5 – 28.8 nM, 95% CI) for MRS 1220.

Uustare *et al*. 2005 have shown that there is a discrepancy between dissociation constant values of [^{3}H]ZM 241385 that were obtained from equilibrium binding experiment (K_d = 0.14 ± 0.01 nM) and calculated from its dissociation- and association rate constants (K_d = 0.48 ± 0.04 nM). It was proposed that the binding of [^{3}H]ZM 241385 occurs instead of the simple one step equilibrium reaction by the scheme including at least two steps, where fast binding to the receptor is followed by isomerization step (Järv *et al*. 1979). According to this model ligand's dissociation constant is determined by equation:

$$K_d = K_A * K_i / (1 + K_i)$$

where K_A is the dissociation constant of binding and K_i describes the isomerization step. Analyzing kinetic data of [^{3}H]ZM 241385 binding according to the two step binding model revealed rough estimation of binding and isomerization parameters, which could not be directly determined: $k_i = 6.9 \pm 1.2\ min^{-1}$ and K_A = 8.5 ± 2.2 nM. The latter parameter is in good agreement with value obtained for ZM 241385 in the inhibition of cAMP accumulation in CHO cells. From this could be assumed that the antagonist-mediated inhibition of agonist-induced effect in the cells is determined by the first step of antagonist binding. Taking into account the dissociation constant value of ZM 241385 obtained from AC activity modulation assay in calculation of K_d value of ZM 241385 according to the two step binding model reveals a K_d value 0.20 nM, which is in very good agreement with value obtained from radioligand binding experiment (K_d = 0.14 ± 0.01 nM).

Dopamine receptors

Dopamine (DA) D_1 as well as D_2 receptors are highly expressed in rat striatum (Levey *et al*. 1993; Deng *et al*. 2006) and both of them modulate AC activity, but in opposite directions. The aim was to characterize and filter out the

dopaminergic receptors subtype selective signal transduction pathways in striatum.

D2-family selective agonist quinpirole (at concentration up to 10 μM) had no effect on basal, nor on forskolin (100 nM) and nor on CGS 21680 (1 μM) stimulated accumulation of cAMP. Presence of D_2 receptor in striatal preparation was confirmed by specific binding of [^{3}H]raclopride, which is a D_2 receptor-specific radioligand. [^{3}H]raclopride binding to striatal membranes was characterized with K_d = 2.3 ± 0.2 nM and B_{max} = 21 ± 1 fmol/mg tissue, which are in good agreement with data published previously (Dewar *et al*. 1989). Quinpirole was ineffective at modulating cAMP accumulation, but caused concentration-dependent inhibition of [^{3}H]raclopride binding, indicating its binding to D_2 receptors.

DA, an endogenous agonist of all dopamine receptor subtypes, caused concentration-dependent stimulation of cAMP accumulation in striatal homogenate. This stimulation effect was effectively inhibited by 1 μM D_1 receptor-specific antagonist SCH 23390, but not by D2-family selective antagonist sulpiride at concentrations up to 10 μM. Both antagonists (SCH 23390 and sulpiride) had no effect on basal accumulation of cAMP. DA also stimulated cAMP accumulation in the presence of 100 nM forskolin and this effect was inhibited by SCH 23390.

Also other agonists of D_1 receptor studied (DHX and A 77636) stimulated cAMP accumulation and their effects were inhibited by SCH 23390. Maximal DA induced cAMP accumulation was 11 ± 1 fmol/min per μg tissue, while DHX's and A 77636's maximal effects were 122 ± 12 % and 140 ± 9 % of that of DA, showing that DA is a partial agonist to D_1 receptor in current system. The pEC_{50} values of D_1 receptor agonists, characterizing their stimulation of cAMP accumulation, were 4.7 ± 0.1 for DA, 6.4 ± 0.1 for DHX and 8.7 ± 0.1 for A 77636, and these values are in good agreement with their potency values reported earlier (DeNinno *et al*. 1991; Mottola *et al*. 1992).

In addition to SCH 23390 also other antagonists of D_1 receptor (SKF 83566 and butaclamol) inhibited DA induced cAMP accumulation concentration-dependently in striatal homogenate. Assuming that a simple competitive model describes the antagonists' induced inhibition of cAMP accumulation, for D_1 receptor antagonists following K_b values were obtained: 0.9 nM (0.6 – 1.3 nM, 95 % CI) for SCH 23390, 1.0 nM (0.6 – 1.7 nM, 95 % CI) for SKF 83566 and 4.5 nM (2.3 – 7.5 nM, 95 % CI) for butaclamol.

All ligands of D_1 receptor studied here inhibited D_1 receptor-specific radioligand [^{3}H]SCH 23390 binding to striatal membranes. The radioligand binding to striatal membranes was characterized by K_d value 0.75 ± 0.08 nM and B_{max} was 62 ± 2 fmol/mg tissue. Fitting data of agonists' displacement curves to a model predicting two independent binding sites revealed two sets of dissociation constants for all agonists, the high-affinity binding sites proportion varying between 44 to 64 %. Activation of G proteins by 100 μM GTPγS decreased the proportion of high affinity binding to a 22–43% level, but had no significant influence on agonists' affinities. Agonists' binding affinities

corresponding to low affinity binding (K_{low} 16 µM for DA, 383 nM for DHX and 6.6 nM for A 77636) were in good agreement with their potencies to stimulate accumulation of cAMP (EC_{50} 26 µM for DA, 435 nM for DHX and 1.8 nM for A 77636).

All studied antagonists of D_1 receptor displaced [^{3}H]SCH 23390 binding according to the model of one binding site, giving K_i values 0.46 nM (0.40–0.54 nM, 95 % CI) for SCH 23390 and 1.23 nM (0.89 - 1.66 nM, 95 % CI) for SKF 83566. Addition of GTPγS to the reaction medium had no effect on antagonists' affinities to inhibit [^{3}H]SCH 23390 binding.

Although both DA receptors, D_1 and D_2 receptors, are highly expressed in rat striatum (Levey *et al*. 1993; Deng *et al*. 2006), only effect of D_1 receptor on AC activity was detected using homogenate of rat striatum. It has been shown in mice striatum that D_2 receptor modulates the level of cAMP via AC isoform 5 (Lee *et al*. 2002). From the other side, D_2 receptor preferentially couple to α_o subtype of G proteins (Gazi *et al*. 2003) and they have practically no influence on AC5 activity in vitro (Taussig *et al*., 1994). Another possibility is that D_2 receptors in current sample are coupled to effectors other than AC, like PLC (Rashid *et al*. 2007), but this pathway is not connected with the activation of $G_{i/o}$-family proteins. In the same time effective $G_{i/o}$-family proteins activation by D_2 receptor in rat striatal membranes have been shown through [^{35}S]GTPγS binding assay (Rinken *et al*. 1999). Thus the reasons why D_2 receptor-specific modulation of AC activity did not occur in striatal homogenate remains to be studied.

Nevertheless, clear D_1 receptor-specific effect on AC activity was detected as its ligands modulated the cAMP accumulation and D_1-specific radioligand [^{3}H]SCH 23390 binding with similar affinities. Thus, we can conclude that the proposed assay system is suitable for characterization of D_1 receptor-specific signal transduction in rat striatal preparations, and the effects of D_2 receptor remain fully hidden. Current methodology has been used for characterization of D_1 receptor signal transduction system in striatum and nucleus accumbens of rats, whose sensitivity to stress, exploration activity and response to tickling were studied (Manuscript of paper IV; Herm *et al*., 2007).

Effect of variable chronic stress on rats with high and low exploratory activity

The aim of this part was to investigate the influence of chronic variable stress (CVS) (Katz *et al*. 981; Katz 1982) on different behavioral (exploratory, hedonic and anxiety-related) and neurochemical (DA D_1 and D_2 receptors signal transmission and serotonin transporter expression) characteristics of rats pre-selected on the basis of their exploratory activity. Animals with high (HE) and low (LE) exploration activity in the exploration box test (Otter *et al*. 1997) were submitted to 5 week long CVS, during which they were weekly tested for

preference and intake of 1% sucrose solution, and after the end of CVS additional behavioral tests were conducted. Decelerated weight gain and lower intake and preference of sucrose solution were observed in animals submitted to CVS regimen compared to control group rats. LE/stressed and LE/control group rats both showed near zero activity in exploratory box test. CVS abolished the decrease in exploratory activity of HE animals during the experiment course and increased significantly social interaction time with previously unknown partner of both HE and LE animals. After the behavioral experiments rats were anesthetized, decapitated and their brains dissected to conduct various biochemical experiments. D_1 receptor-specific cAMP accumulation in nucleus accumbens was significantly higher in stressed animals compared to control group, while in striatum no differences between stressed and control animals were found. This finding is in line with studies that show decrease of DA levels (Gambarana *et al.* 1999; Scheggi *et al.* 2002) and increase in D_1-specific radioligand binding in nucleus accumbens, but not in striatum, of animals submitted to a chronic stress regimen (Scheggi *et al.* 2002). In striatum of HE group rats significantly higher level of D_1 receptor-specific cAMP accumulation was observed than in LE group rats. It has been demonstrated that in HE group animals extracellular DA level is higher in striatum compared to LE (Mällo *et al.* 2007). These results indicate that the higher dopaminegic signal output in striatum is one of the factors that increase exploratory activity in rats. No statistical differences of D_2 receptor induced [^{35}S]GTPγS binding to striatal- or accumbal membranes nor change in GDP affinity was found between different groups. Binding of serotonin transporter (SERT)-specific radioligand ([^{3}H]citalopram) to rat brain cortex membranes was not statistically different between different rat groups. These results are comparable with the previous study (Tõnissaar *et al.* 2008), where CVS had no influence on D_2 receptor-mediated signal transduction and serotonin transporter density in rats pre-selected on the basis of their sociability profile.

Gender-specific effects of tickling on dopaminergic and serotonergic signal transmission

Tickling of rats can be viewed as a reward because rats elicit ultrasonic sounds (~50 kHz) in response to tickling, which are associated with positive affective state, since they are emitted also during play, sex and administration of drugs of abuse (Panksepp *et al.* 2001). In the following study it was investigated whether tickling affects dopaminergic and serotonergic signal transmission in rat brain. Male and female Wistar rats were divided into high (HC) and low chirpers (LC) (sounds made at 50 kHz) groups according to their responses to tickling and into control group (non-tickled rats). Tickling of rats resulted with enhanced D_1 receptor-specific cAMP accumulation in nucleus accumbens of both sex rats, but in case of male rats the difference of D_1 receptor signaling between control and tickled groups was larger. No statistically significant differences in D_1

receptor-specific cAMP accumulation between male and female control groups and between HC and LC from both sexes were found. D_2 receptor induced $[^{35}S]GTP\gamma S$ binding was significantly lower in female control group than in male control rats. No differences in D_2 receptor induced $[^{35}S]GTP\gamma S$ binding between male control and tickled groups were observed. While tickling significantly enhanced D_2 receptor induced $[^{35}S]GTP\gamma S$ binding (and thus the sensitivity of signal transduction) in female rats, in males it had no influence in this regard. Binding of SERT-specific radioligand ($[^3H]$citalopram) to brain cortex membranes was also significantly lower in female control rats than in male controls. Female LC, but not HC, rats had significantly higher $[^3H]$citalopram binding compared to control group. In case of male rats there was no statistical difference in $[^3H]$citalopram binding level between control and tickled groups.

The reason for the lower D_2 receptor induced $[^{35}S]GTP\gamma S$ binding in striatum and $[^3H]$citalopram binding in brain cortex of female control rats compared to male controls may be that the expression of D_2 receptors in striatum and of SERT in brain cortex is suppressed by the hormonal status of female rats. Overall these results indicate that tickling stimulates the D_1 receptor signaling output in nucleus accumbens but doesn’t have specific effects on D_2 receptor signaling in striatum and on serotonergic system in brain cortex.

CONCLUSIONS

The aims of current work were to develop a method for measuring dopamine- and adenosine receptors' subtype-specific signaling in rat brain striatal homogenate through determination of AC activity, and to applay this method for studying relationships between behavior and signal transduction sensitivity of aforementioned receptors in different animals (rats) models.

- Highest receptor-specific cAMP accumulation was achieved in crude striatal homogenate without additional washings using homogenization buffer that contained only Tris-HCl and EGTA (pH 7.4), and no additional monovalent ions were used during the preparation.
- Mg^{2+} ions were essential in assay medium for receptor-specific modulation of AC activity. The optimal concentrations of Mg^{2+} for adenosine A_{2A}- and dopamine D_1 receptor-specific assays were between 3 to 5 mM, where best ratio of receptor-specific cAMP accumulation to total cAMP accumulation was achieved.
- Endogenous agonists in tissue had no direct effect on AC activity in studied system, as antagonists of adenosine A_{2A}- and dopamine D_1 receptor had no effect on basal cAMP accumulation. Additional pretreatment of striatal homogenate with adenosine deaminase also had no effect, neither on basal nor on A_{2A} receptor-mediated cAMP accumulation.
- All studied adenosine A_{2A}-specific ligands modulated AC activity in striatal homogenate with potencies that were in good agreement with their potencies to inhibit [^{3}H]ZM 241385 binding.
- Kinetic analysis of [^{3}H]ZM 241385 binding revealed that it includes in addition to fast equilibrium also isomerization of antagonist-receptor complex to a slow-dissociating state. The potency of ZM 241385 to inhibit the activation of AC is determined by the fast equilibrium stage of complex formation.
- All studied dopamine D_1-specific ligands were able to modulate AC activity in striatal homogenate, but none of the D_2-specific ligands studied. The presence of D_2 receptors in striatum was confirmed by specific binding of [^{3}H]raclopride to striatal membranes. All studied dopamine D_1-specific ligands modulated AC activity in striatal homogenate with potencies that were in good agreement with their potencies to inhibit [^{3}H]SCH 23390 binding. It indicates that under given experimental conditions, only dopamine D_1 receptor mediated stimulation of AC activity can be measured in homogenate of rat striatum, while dopamine D_2 receptor effects remain fully hidden.
- Comparison of animals with high (HE) and low (LE) exploration activity revealed higher level of D_1 receptor-specific cAMP accumulation in striatum of HE group rats, while in nucleus accumbens no differences was found. Chronic variable stress (CVS) affected D_1 receptor-specific cAMP accumulation in nucleus accumbens, but not in striatum.
- Tickling of rats resulted with enhanced D_1 receptor-specific cAMP accumulation in nucleus accumbens of all rats, but in the case of male rats the effect was larger.

REFERENCES

Alttoa A., Seeman P., Kõiv K., Eller M., Harro J. (2009) Rats with persistently high exploratory activity have both higher extracellular dopamine levels and higher proportion of D(2) (High) receptors in the striatum. *Synapse* **63**, 443–6.

Arunlakshana O., Schild H. O. (1959) Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother*. **14**, 48–58.

Birnbaumer L., Rodbell M. (1969) Adenyl cyclase in fat cells. II. Hormone receptors. *J. Biol. Chem*. **244**, 3477–82.

Brown B. L., Albano J. D., Ekins R. P., Sgherzi A. M. (1971) A simple and sensitive saturation assay method for the measurement of adenosine 3':5'-cyclic monophosphate. *Biochem. J.* **121**, 561–2.

Bünemann M., Frank M., Lohse M. J. (2003) Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proc. Natl. Acad. Sci. U S A*. **100**, 16077–82.

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.

Cheng H. C. (2001) The power issue: determination of KB or Ki from IC50. A closer look at the Cheng-Prusoff equation, the Schild plot and related power equations. *J. Pharmacol. Toxicol. Methods* **46**, 61–71.

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.

Cherezov V., Rosenbaum D. M., Hanson M. A., Rasmussen S. G., Thian F. S., Kobilka T. S., Choi H. J., Kuhn P., Weis W. I., Kobilka B. K., Stevens R. C. (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* **318**, 1258–65.

Chern Y. (2000) Regulation of adenylyl cyclase in the central nervous system. *Cell Signal*. **12**, 195–204.

Conti M., Beavo J. (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu. Rev. Biochem.* **76**, 481–511.

Cunliffe J. M., Sunahara R. K., Kennedy R. T. (2006) Detection of adenylyl cyclase activity using a fluorescent ATP substrate and capillary electrophoresis. *Anal. Chem.* **78**, 1731–8.

Deng Y. P., Lei W. L., Reiner A. (2006) Differential perikaryal localization in rats of D1 and D2 dopamine receptors on striatal projection neuron types identified by retrograde labeling. *J. Chem. Neuroanat.* **32**, 101–16.

DeNinno M. P., Schoenleber R., Perner R. J., Lijewski L., Asin K. E., Britton D. R., MacKenzie R., Kebabian J. W. (1991) Synthesis and dopaminergic activity of 3-substituted 1-(aminomethyl)-3,4-dihydro-5,6-dihydroxy-1H-2-benzopyrans: characterization of an auxiliary binding region in the D1 receptor. *J. Med. Chem*. **34**, 2561–9.

Dewar K. M., Montreuil B., Grondin L., Reader T. A. (1989) Dopamine D2 receptors labeled with [^{3}H]raclopride in rat and rabbit brains. Equilibrium binding, kinetics, distribution and selectivity. *J. Pharmacol. Exp. Ther*. **250**, 696–706.

Di Chiara G., Imperato A. (1988) 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.

Dodge-Kafka K. L., Langeberg L., Scott J. D. (2006) Compartmentation of cyclic nucleotide signaling in the heart: the role of A-kinase anchoring proteins. *Circ. Res.* **98**, 993–1001.

Downes G. B., Gautam N. (1999) The G protein subunit gene families. *Genomics* **62**, 544–52.

Ferré 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., O'Connor W. T., Svenningsson P., Bjorklund L., Lindberg J., Tinner B., Stromberg I., Goldstein M., Ogren S. O., Ungerstedt U., Fredholm B. B., Fuxe K. (1996) Dopamine D1 receptor-mediated facilitation of GABAergic neurotransmission in the rat strioentopenduncular pathway and its modulation by adenosine A1 receptor-mediated mechanisms. *Eur. J. Neurosci.* **8**, 1545–53.

Ferre S., Popoli P., Tinner-Staines B., Fuxe K. (1996a) Adenosine A1 receptor-dopamine D1 receptor interaction in the rat limbic system: modulation of dopamine D1 receptor antagonist binding sites. *Neurosci. Lett*. **208**, 109–12.

Ferré S., Popoli P., Giménez-Llort L., Finnman U. B., Martínez E., Scotti de Carolis A., Fuxe K. (1994) Postsynaptic antagonistic interaction between adenosine A1 and dopamine D1 receptors. *Neuroreport.* **6**, 73–6.

Ferré S., O'Connor W. T., Snaprud P., Ungerstedt U., Fuxe K. (1994a) Antagonistic interaction between adenosine A2A receptors and dopamine D2 receptors in the ventral striopallidal system. Implications for the treatment of schizophrenia. *Neuroscience* **63**, 765–73.

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.

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.

Fuxe K., Ferré S., Zoli M., Agnati L. F. (1998) Integrated events in central dopamine transmission as analyzed at multiple levels. Evidence for intramembrane adenosine A2A/dopamine D2 and adenosine A1/dopamine D1 receptor interactions in the basal ganglia. *Brain Res. Brain Res. Rev.* **26**, 258–73.

Gambarana C., Ghiglieri O., Masi F., Scheggi S., Tagliamonte A., De Montis M. G. (1999) The effects of long-term administration of rubidium or lithium on reactivity to stress and on dopamine output in the nucleus accumbens in rats. *Brain Res*. **826**, 200–9.

Gazi L., Nickolls S. A., Strange P. G. (2003) Functional coupling of the human dopamine D2 receptor with G alpha i1, G alpha i2, G alpha i3 and G alpha o G proteins: evidence for agonist regulation of G protein selectivity. *Br. J. Pharmacol.* **138**, 775–86.

Gilman A. G. (1970) A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. U S A*. **67**, 305–12.

Hayes J. S., Brunton L. L., Brown J. H., Reese J. B., Mayer S. E. (1979) Hormonally specific expression of cardiac protein kinase activity. *Proc. Natl. Acad. Sci. U S A.* **76**, 1570–4.

Ginés S., Hillion J., Torvinen M., Le Crom S., Casadó V., Canela E. I., Rondin S., Lew J. Y., Watson S., Zoli M., Agnati L. F., Verniera P., Lluis C., Ferré 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.

Harro J., Terasmaa A., Eller M., Rinken A. (2003) Effect of denervation of the locus coeruleus projections by DSP-4 treatment on [3H]-raclopride binding to dopamine D(2) receptors and D(2) receptor-G protein interaction in the rat striatum. *Brain Res.* **976**, 209–16.

Harro J., Oreland L. (2001) Depression as a spreading adjustment disorder of monoaminergic neurons: a case for primary implication of the locus coeruleus. *Brain Res. Brain Res. Rev.* **38**, 79–128.

Herm L., Vonk A., Mällo T., Matrov D., Harro J., Rinken A. (2007) Gender-specific effects of tickling on serotonergic and dopaminergic signal transmission in rats. *Eur. Neuropsychopharmacol.* **17** (Supplement 4), S256.

Herrera C., Casadó V., Ciruela F., Schofield P., Mallol J., Lluis C., Franco R. (2001) Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. *Mol. Pharmacol.* **59**, 127–34.

Hildebrandt J. D., Day R., Farnsworth C. L., Feig L. A. (1991) A mutation in the putative Mg(2+)-binding site of Gs alpha prevents its activation by receptors. *Mol. Cell Biol.* **11**, 4830–8.

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.

Ito H., Hosoya Y., Inanobe A., Tomoike H., Endoh M. (1995) Acetylcholine and adenosine activate the G protein-gated muscarinic K+ channel in ferret ventricular myocytes. *Naunyn Schmiedebergs Arch. Pharmacol.* **351**, 610–7.

Jin L. Q., Wang H. Y., Friedman E. (2001) Stimulated D(1) dopamine receptors couple to multiple Galpha proteins in different brain regions. *J. Neurochem.* **78**, 981–90.

Järv J., Hedlund B., Bartfai T. (1979) Isomerization of the muscarinic receptor * antagonist complex. *J. Biol. Chem.* **254**, 5595–8.

Katz R. J., Roth K. A., Carroll B. J. (1981) Acute and chronic stress effects on open field activity in the rat: implications for a model of depression. *Neurosci. Biobehav. Rev.* **5**, 247–51.

Katz R. J. (1982) Animal model of depression: pharmacological sensitivity of a hedonic deficit. *Pharmacol. Biochem. Behav.* **16**, 965–8.

Lee K. W., Hong J. H., Choi I. Y., Che Y., Lee J. K., Yang S. D., Song C. W., Kang H. S., Lee J. H., Noh J. S., Shin H. S., Han P. L. (2002) Impaired D2 dopamine receptor function in mice lacking type 5 adenylyl cyclase. *J. Neurosci.* **22**, 7931–40.

Kenakin T. P., Bond R. A., Bonner T. I. (1991) Definition of pharmacological receptors. *Pharmacol. Rev.* **44**, 351–62.

Krupinski J., Coussen F., Bakalyar H. A., Tang W. J., Feinstein P. G., Orth K., Slaughter C., Reed R. R., Gilman A. G. (1989) Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. *Science* **244**, 1558–64.

Lai H. L., Lin T. H., Kao Y. Y., Lin W. J., Hwang M. J., Chern Y. (1999) The N terminus domain of type VI adenylyl cyclase mediates its inhibition by protein kinase C. *Mol. Pharmacol.* **56**, 644–50.

Landry Y., Niederhoffer N., Sick E., Gies J. P. (2006) Heptahelical and other G-protein-coupled receptors (GPCRs) signaling. *Curr. Med. Chem.* **13**, 51–63.

Le Moine C., Bloch B. (1995) D1 and D2 dopamine receptor gene expression in the rat striatum: sensitive cRNA probes demonstrate prominent segregation of D1 and D2 mRNAs in distinct neuronal populations of the dorsal and ventral striatum. *J. Comp. Neurol.* **355**, 418–26.

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

Levey A. I., Hersch S. M., Rye D. B., Sunahara R. K., Niznik H. B., Kitt C. A., Price D. L., Maggio R., Brann M. R., Ciliax B. J. (1993) Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. *Proc. Natl. Acad. Sci. U S A*. **90**, 8861–5.

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.

Mottola D. M., Brewster W. K., Cook L. L., Nichols D. E., Mailman R. B. (1992) Dihydrexidine, a novel full efficacy D1 dopamine receptor agonist. *J. Pharmacol. Exp. Ther*. **262**, 383–93.

Mällo T., Alttoa A., Kõiv K., Tõnissaar M., Eller M., Harro J. (2007) Rats with persistently low or high exploratory activity: behaviour in tests of anxiety and depression, and extracellular levels of dopamine. *Behav. Brain. Res*. **177**, 269–81.

Mynlieff M., Beam K. G. (1994) Adenosine acting at an A1 receptor decreases N-type calcium current in mouse motoneurons. *J. Neurosci.* **14**, 3628–34.

Nikolaev V. O., Lohse M. J. (2006) Monitoring of cAMP synthesis and degradation in living cells. *Physiology (Bethesda)*. **21**, 86–92.

Norsted 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.

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

Otter M. H., Matto V., Sõukand R., Skrebuhhova T., Allikmets L., Harro J. (1997) Characterization of rat exploratory behavior using the exploration box test. *Methods Find. Exp. Clin. Pharmacol*. **19**, 683–91.

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.

Panksepp J., Knutson B., Burgdorf J. (2001) The role of brain emotional systems in addictions: a neuro-evolutionary perspective and new 'selfreport' animal model. *Addiction* **97**, 459–469.

Park P. S., Filipek S., Wells J. W., Palczewski K. (2004) Oligomerization of G protein-coupled receptors: past, present, and future. *Biochemistry* **43**, 15643–56.

Porsolt R. D., Anton G., Blavet N., Jalfre M. (1978) Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur. J. Pharmacol*. **47**, 379–91.

Preston Z., Lee K., Widdowson L., Freeman T. C., Dixon A. K., Richardson P. J. (2000) Adenosine receptor expression and function in rat striatal cholinergic interneurons. *Br. J. Pharmacol*. **130**, 886–90.

Rall T. W., Sutherland E. W. (1958) Formation of a cyclic adenine ribonucleotide by tissue particles. *J. Biol. Chem.* **232**, 1065–76.

Rashid A. J., So C. H., Kong M. M., Furtak T., El-Ghundi M., Cheng R., O'Dowd B. F., George S. R. (2007) D1-D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of Gq/11 in the striatum. *Proc. Natl. Acad. Sci. U S A*. **104**, 654–9.

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., Terasmaa A., Raidaru G., Fuxe K. (2001) D2 dopamine receptor-G protein coupling. Cross-regulation of agonist and guanosine nucleotide binding sites. *Neurosci. Lett*. **302**, 5–8.

Rinken A., Finnman U. B., Fuxe K. (1999) Pharmacological characterization of dopamine-stimulated [35S]-guanosine 5'(gamma-thiotriphosphate) ([35S]-GTPgammaS) binding in rat striatal membranes. *Biochem. Pharmacol.* **57**, 155–62.

Scheggi S., Leggio B., Masi F., Grappi S., Gambarana C., Nanni G., Rauggi R., De Montis M. G. (2002) Selective modifications in the nucleus accumbens of dopamine synaptic transmission in rats exposed to chronic stress. *J. Neurochem*. **83**, 895–903.

Schildkraut J. J. (1965) The catecholamine hypothesis of affective disorders: a review of supportive evidence. *Am. J. Psychiatry* **122**, 509–22.

Simpson R. E., Ciruela A., Cooper D. M. (2006) The role of calmodulin recruitment in Ca2+ stimulation of adenylyl cyclase type 8. *J. Biol. Chem.* **281**, 17379–89.

Steinberg S. F., Brunton L. L. (2001) Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. *Annu. Rev. Pharmacol. Toxicol.* **41**, 751–73.

Steiner A. L., Kipnis D. M., Utiger R., Parker C. (1969) Radioimmunoassay for the measurement of adenosine 3',5'-cyclic phosphate. *Proc. Natl. Acad. Sci. U S A.* **64**, 367–73.

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.

Svenningsson P., Le Moine C., Fisone G., Fredholm B. B. (1999) Distribution, biochemistry and function of striatal adenosine A2A receptors. *Prog. Neurobiol*. **59**, 355–96.

Svenningsson P., Le Moine C., Kull B., Sunahara R., Bloch B., Fredholm B. B. (1997) Cellular expression of adenosine A2A receptor messenger RNA in the rat central nervous system with special reference to dopamine innervated areas. *Neuroscience* **80**, 1171–85.

Takeda S., Kadowaki S., Haga T., Takaesu H., Mitaku S. (2002) Identification of G protein-coupled receptor genes from the human genome sequence. *FEBS Lett.* **520**, 97–101.

Taussig R., Tang W. J., Hepler J. R., Gilman A. G. (1994) Distinct patterns of bidirectional regulation of mammalian adenylyl cyclases. *J. Biol. Chem*. **269**, 6093–100.

Tesmer J. J. G., Sunahara R. K., Gilman A. G., Sprang S. R. (1997) Crystal Structure of the Catalytic Domains of Adenylyl Cyclase in a Complex with Gsalpha′GTP**γ**S. *Science* **278**, 1907–16.

Tõnissaar M., Herm L., Eller M., Kõiv K., Rinken A., Harro J. (2008) Rats with high or low sociability are differently affected by chronic variable stress. *Neuroscience* **152**, 867–76.

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.
Vanhoutte P. M., Humphrey P. P., Spedding M. (1996) X. International Union of Pharmacology recommendations for nomenclature of new receptor subtypes. *Pharmacol. Rev.* **48**, 1–2.
Valkna A., Juréus A., Karelson E., Zilmer M., Bartfai T., Langel U. (1995) Differential regulation of adenylate cyclase activity in rat ventral and dorsal hippocampus by rat galanin. *Neurosci. Lett*. **187**, 75–8.
Willner P., Towell A., Sampson D., Sophokleous S., Muscat R. (1987) Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology* **93**, 358–64.
Yamamoto T., Staines W. A., Dewar K., Geiger J. D., Daddona P. E., Nagy J. I. (1988) Distinct adenosine deaminase-containing inputs to the substantia nigra from the striatum and tuberomammillary nucleus. *Brain. Res*. **474**, 112–24.

SUMMARY IN ESTONIAN

Adenosiini A_{2A}- ja dopamiini D_1 retseptori vahendusel toimuva adenülaadi tsüklaasi aktiivsuse regulatsiooni määramine roti aju juttkehas

Käesoleva dissertatsiooni raames töötati välja metoodika, mille abil mõõta adenosiini A_{2A}- ja dopamiini D_1 retseptori poolt vahendatavat adenülaadi tsüklaasi (AC) aktiivsuse modulatsiooni roti aju juttkeha homogenaadis, kasutades selleks tsüklilise adenosiin-3', 5'-monofosfaadi (cAMP) kontsentratsiooni muudu määramist. Juurutatud metoodika abil uuriti rottide erinevate käitumuslike iseärasuste seoseid D_1 retseptori signaaliülekanderadade tundlikkusega aju juttkehas ja naalduvas tuumas.

Koeproovi valmistamise optimeerimisel leiti, et parimaid tulemusi annab juttkeha homogenaat, mille puhul pole kasutatud endogeensete ligandide väljapesemist tsentrifuugimise ja rehomogeniseerimise teel, ning mille homogeniseerimispuhver sisaldas vaid Tris-HCl ning EGTA (pH 7.4). Homogenaadi eeltöötlemine adenosiin-deaminaasiga ei mõjutanud A_{2A} retseptori-spetsiifilist cAMP akumulatsiooni, mis näitab, et endogeenne adenosiin ei mõjuta AC aktiivsust antud preparaadis. Retseptor-spetsiifilise AC aktiivsuse regulatsiooni mõõtmiseks on vajalik Mg^{2+} juuresolek, kusjuures viimase optimaalseks kontsentratsiooniks leiti vahemik 3 kuni 5 mM.

Adenosiin A_{2A} retseptori agonist CGS 21680 põhjustas kontsentratsioonsõltuvalt AC aktiiveerimise juttkeha homogenaadis ja antagonist ZM 241385 inhibeeris seda efekti. Ligandide võime moduleerida AC aktiivsust oli heas kooskõlas nende võimega inhibeerida [^{3}H]ZM 241385 sidumist juttkeha membraanidele. [^{3}H]ZM 241385 seostumise kineetiline analüüs näitas, et sidumine toimub kaheastmelise reaktsiooniskeemi järgi, kus sidumise kiiresti püsituvale tasakaalu staadiumile järgneb isomeriseerumine aeglaselt dissotsieeruvaks kompleksiks. Seejuures on antagonisti AC aktiivsuse inhibeerise võime ära määratud juba tema sidumise kiire tasakaalu staadiumiga.

Ka dopamiini D_1 retseptori ligandid olid võimelised mõjutama AC aktiivsust juttkeha homogenaadis, kuid mitte dopamiini D_2 retseptori-spetsiifilised ligandid. D_2 retseptorite olemasolu selles koepreparaadis leidis kinnitust [^{3}H]raklopriidi sidumisega ning uuritud ligandide võimega efektiivselt [^{3}H]raklopriidi sidumist inhibeerida. Sellest võib järeldada, et käesoleval juhul D_2 retseptorid kas ei aktiveeri piisaval hulgal AC inhibeerivaid G valke või need G valgud ei ole antud tingimustel funktsionaalsed. Samas D_1 retseptori ligandid mõjutasid AC aktiivsust juttkeha homogenaadis ja inhibeerisid D_1 retseptori antagonisti [^{3}H]SCH 23390 sidumist juttkeha membraanidele afiinsustega, mis olid omavahel heas kooskõlas.

D_1 retseptori-spetsiifilist signaaliülekannet mõõdeti kõrge ja madala uudistamisaktiivsusega rottide aju juttkehas ja naalduvas tuumas ning samuti uuriti kas stress mõjutab antud retseptori signaaliülekannet nimetatud ajuosades. Rotid

selekteeriti madala ja kõrge uudistamisaktiivsusega gruppideks vastavalt nende käitumisele uudistamiskasti testis ja mõlemas grupis rakendati pooltele rottidele stressirežiimi. Eksperimentide tulemusena leiti, et kõrgema uudistamisaktiivsusega rottidel on ka D_1 retseptori signaaliülekande efektiivsus juttkehas statistiliselt olulisel määral suurem võrreldes väheuudistavate rottidega, samas aga naalduvas tuumas statistiliselt olulist erinevust ei leitud. Stress jällegi mõjutas oluliselt D_1 signaaliülekannet just naalduvas tuumas, kuid mitte juttkehas. Lisaks uuriti rottide kõditamisele reageerimise seost D_1 signaali ülekandega naalduvas tuumas. Lähtudes kõditamise peale ~50 kHz sagedusel esile toodud häälitsuste arvule jaotati eraldi isas-ja emasrotid palju- ja vähepiiksujate rühma ning lisaks moodustati ka mõlemast soost kontrollrühm, keda ei kõditatud. Tulemusena leiti, et D_1 retseptori signaaliülekanne naalduvas tuumas ei ole oluliselt erinev vähe- ja paljupiiksuvatel rottidel (nii isas- kui ka emasloomade puhul), küll aga leiti, et kõditamine suurendab D_1 retseptori signaaliülekande tundlikkust ning isasloomade puhul on see mõju suurem.

ACKNOWLEDGEMENTS

Current work has been done at the Chair of Bioorganic Chemistry of the Institute of Chemistry, University of Tartu. I am thankful to everybody who has contributed in some way to my research and also to current thesis.

Most of all, I would like to thank my supervisor, prof. Ago Rinken, for his guidance and for upholding positive attitude and hope in me even in situations where nothing seemed to go right with the experiments.

My special thanks go to Laura Herm, who willingly shared her vast knowledge of behavioral sciences, which was vital for writing respective parts in current thesis.

I'm grateful to Ain Uustare and Reet Reinart for conducting most of the radioligand binding experiments, results of which have been major help for writing of scientific papers and also current thesis, and also to Raili Remmel who had enough time to grow CHO cells for certain experiments.

Also I would like to thank my family for their support (especially for the financial side of it).

Current work has been supported by the Estonian Science Foundation (grant No. 4603 and 6492), by the Estonian Ministry of Education and Science (0182734s06 and 0182643), by a grant (QLG3-CT-2001-01056) from the European Commission and the EU Framework 6 Integrated Project NEWMOOD (LSHM-CT-2004-503474).

PUBLICATIONS

CURRICULUM VITAE

I. Personal details

1. First and family name: Argo Vonk
2. Date and place of birth: October 20th, 1979, Suure-Jaani, Estonia
3. Citizenship: Estonian
4. Marital status: Unmarried
5. Address: Ringpuiestee 2–2, Suure-Jaani

Telephone: +372 566 18452
E-mail: vonk@ut.ee

6. Current post: Extraordinary researcher in Institute of Chemistry, University of Tartu

7. Education:

1998–2002 Student in University of Tartu (chemistry), *B.Sc.* in bioorganic chemistry in 2002.
2002–2004 Graduate student in Institute of Organic and Bioorganic Chemistry, University of Tartu. *M.Sc.* in bioorganic chemistry in 2004.
2004–present PhD student in Institute of Chemistry, University of Tartu, supervisor prof. Ago Rinken

8. Languages: English (good), Russian (satisfactory)

9. Employment history:

2006–2007 extraordinary researcher in Institute of Organic and Bioorganic Chemistry, University of Tartu.
2008–present extraordinary researcher in Institute of Chemistry, University of Tartu.

II. Research

1. Major research fields:
   – Determination of adenosine A_{2A}- and dopamine D_1 receptor-specific modulation of adenylate cyclase activity in rat brain tissue and in specific cells lines.
   – In cooperation with the research group of J. Harro, the characterization of dopamine D_1 receptor-specific signal transduction in brain of different animal (rat) models.
   – Testing of new possible dopamine D_1 receptor's ligands through their ability to inhibit $[^3H]$SCH 23390 binding to D_1 receptor.

2. List of Publications:

   Scientific articles:

   Vonk A., Uustare A., Rinken A. (2004) Modulation of activity of adenylate cyclase in rat striatal membranes by adenosine A_{2A} receptors. *Proc. Est. Acad. Sci. (Chem.)* **53**, 153–164.

   Uustare A., **Vonk A.**, Terasmaa A, Fuxe K., Rinken A. (2005) Kinetic and functional properties of [^{3}H]ZM241385, a high affinity antagonist for adenosine A_{2A} receptors. *Life Sci.* **76**, 1513–1526.

   Vonk A., Reinart R., Rinken A. (2008) Modulation of adenylyl cyclase activity in rat striatal homogenate by dopaminergic receptors. *J. Pharmacol. Sci.* **108**, 63–70.

   Herm L., Berényi S., **Vonk A.**, Rinken A., Sipos A. (2009) N-Substituted-2-Alkyl- and 2-Arylnorapomorphines: Novel, Highly Active D_2 Agonists. *Bioorg. Med. Chem*. in press.

   Presentations at conferences:

   Argo Vonk, Ago Rinken. "Dopaminergc modulation of adenylyl cyclase in rat striatum membranes is determined by D_1 receptors". 29th Estonian Chemistry Days, 2005. Abstracts of Scientific Conference, page 136.

   Argo Vonk, Reet Reinart, Ago Rinken. "Dopaminergic modulation of adenylyl cyclase in rat striatum is determined by D_1 receptors". 31-st FEBS Congress "Molecules in Health and Disease", 2006. Late Abstract Book, page 45 (PP-1243).

   Argo Vonk, Reet Reinart, Ago Rinken. "Dopaminergic regulation of adenylyl cyclase activity in rat striatal homogenate". Biochemical Society Focused Meeting "Family resemblances? Ligand binding and activation of family A and B G-protein-coupled receptors", 2007. Program & Abstracts, page 11 (P026).

   Laura Herm, **Argo Vonk**, Tanel Mällo, Denis Matrov, Jaanus Harro, Ago Rinken. "Gender-specific effects of tickling on serotonergic and dopaminergic signal transmission in rats". The Journal of the European College of Neuropsychopharmacology, 2007, 17 (S4), page S256 (P.1.c.025)

   Argo Vonk, Reet Reinart, Ain Uustare, Ago Rinken. "Measurement of adenosine A_{2A}- and dopamine D_1 receptors mediated modulation of adenylyl cyclase activity in homogenate of rat brain striatum" 30th Estonian Chemistry Days, 2007. Abstracts of Scientific Conference, pages 193–4.

CURRICULUM VITAE

I. Üldandmed

1. Ees ja perekonnanimi: Argo Vonk
2. Sünniaeg ja koht: 20 oktoober 1979, Suure-Jaani, Eesti
3. Kodakondsus: Eesti
4. Perekonnaseis: Vallaline
5. Aadress: Ringpuiestee 2–2, Suure-Jaani

Telefon: +372 566 18452

e-mail: vonk@ut.ee

6. Praegune töökoht, amet: Tartu Ülikooli Keemia Instituudi erakorraline teadur
7. Haridus:

1998–2002 Tartu Ülikooli keemia eriala üliõpilane, bakalaureuse kraad (*B. Sc.*) bioorgaanilises keemias 2002 aastal.

2002–2004 Tartu Ülikooli bioorgaanilise keemia magistrant, magistrikraad (*M.Sc.*) bioorgaanilise keemias 2004 aastal.

2004–praegu Tartu Ülikooli bioorgaanilise keemia doktorant, juhendaja prof. Ago Rinken

8. Keelteoskus: Inglise keel (hea), vene keel (rahuldav)

9. Teenistuskäik:

2006–2007 Tartu Ülikooli Orgaanilise ja Bioorgaanilise Keemia Instituudi erakorraline teadur

2008–praegu Tartu Ülikooli Keemia Instituudi erakorraline teadur

II. Teaduslik ja arendustegevus

1. Peamised uurimisvaldkonnad:
   - Adenosiini A_{2A} ja dopamiini D_1 retseptorite kaudu toimiva adenülaadi tsüklaasi aktiivsuse moduleerimise määramine roti aju koepreparaatides ning spetsiifilistes rakuliinides.
   - Erinevates loommudelites (rotid) dopamiini D_1 retseptorite signaaliülekande kirjeldamine aju juttkehas ja naalduvas tuumas koostöös J. Harro töörühmaga.
   - Dopamiini D_1 retseptorite võimalike uute ligandide testimine nende võime kaudu inhibeerida [^{3}H]SCH 23390 sidumist antud retseptoritele.

2. Publikatsioonide loetelu:

Teaduslikud artiklid:

Vonk A., Uustare A., Rinken A. (2004) Modulation of activity of adenylate cyclase in rat striatal membranes by adenosine A_{2A} receptors. *Proc. Est. Acad. Sci. (Chem.)* **53**, 153–164.

Uustare A., **Vonk A.**, Terasmaa A, Fuxe K., Rinken A. (2005) Kinetic and functional properties of [^{3}H]ZM241385, a high affinity antagonist for adenosine A_{2A} receptors. *Life Sci.* **76**, 1513–1526.

Vonk A., Reinart R., Rinken A. (2008) Modulation of adenylyl cyclase activity in rat's triatal homogenate by dopaminergic receptors. *J. Pharmacol. Sci.* **108**, 63–70.

Herm L., Berényi S., **Vonk A.**, Rinken A., Sipos A. (2009) N-Substituted-2-Alkyl- and 2-Arylnorapomorphines: Novel, Highly Active D_2 Agonists. *Bioorg. Med. Chem*. in press.

Konveretside ettekanded:

Argo Vonk, Ago Rinken. "Dopaminergc modulation of adenylyl cyclase in rat striatum membranes is determined by D_1 receptors". 29th Estonian Chemistry Days, 2005. Abstracts of Scientific Conference, page 136.

Argo Vonk, Reet Reinart, Ago Rinken. "Dopaminergic modulation of adenylyl cyclase in rat striatum is determined by D_1 receptors". 31-st FEBS Congress "Molecules in Health and Disease", 2006. Late Abstract Book, page 45 (PP-1243).

Argo Vonk, Reet Reinart, Ago Rinken. "Dopaminergic regulation of adenylyl cyclase activity in rat striatal homogenate". Biochemical Society Focused Meeting "Family resemblances? Ligand binding and activation of family A and B G-protein-coupled receptors", 2007. Program & Abstracts, page 11 (P026).

Laura Herm, **Argo Vonk**, Tanel Mällo, Denis Matrov, Jaanus Harro, Ago Rinken. "Gender-specific effects of tickling on serotonergic and dopaminergic signal transmission in rats". The Journal of the European College of Neuropsychopharmacology, 2007, 17 (S4), page S256 (P.1.c.025)

Argo Vonk, Reet Reinart, Ain Uustare, Ago Rinken. "Measurement of adenosine A_{2A}- and dopamine D_1 receptors mediated modulation of adenylyl cyclase activity in homogenate of rat brain striatum" 30th Estonian Chemistry Days, 2007. Abstracts of Scientific Conference, pages 193–4.

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS

1. **Toomas Tamm.** Quantum-chemical simulation of solvent effects. Tartu, 1993, 110 p.
2. **Peeter Burk.** Theoretical study of gas-phase acid-base equilibria. Tartu, 1994, 96 p.
3. **Victor Lobanov.** Quantitative structure-property relationships in large descriptor spaces. Tartu, 1995, 135 p.
4. **Vahur Mäemets.** The ^{17}O and ^{1}H nuclear magnetic resonance study of H_2O in individual solvents and its charged clusters in aqueous solutions of electrolytes. Tartu, 1997, 140 p.
5. **Andrus Metsala.** Microcanonical rate constant in nonequilibrium distribution of vibrational energy and in restricted intramolecular vibrational energy redistribution on the basis of slater's theory of unimolecular reactions. Tartu, 1997, 150 p.
6. **Uko Maran.** Quantum-mechanical study of potential energy surfaces in different environments. Tartu, 1997, 137 p.
7. **Alar Jänes.** Adsorption of organic compounds on antimony, bismuth and cadmium electrodes. Tartu, 1998, 219 p.
8. **Kaido Tammeveski.** Oxygen electroreduction on thin platinum films and the electrochemical detection of superoxide anion. Tartu, 1998, 139 p.
9. **Ivo Leito.** Studies of Brønsted acid-base equilibria in water and non-aqueous media. Tartu, 1998, 101 p.
10. **Jaan Leis.** Conformational dynamics and equilibria in amides. Tartu, 1998, 131 p.
11. **Toonika Rinken.** The modelling of amperometric biosensors based on oxidoreductases. Tartu, 2000, 108 p.
12. **Dmitri Panov.** Partially solvated Grignard reagents. Tartu, 2000, 64 p.
13. **Kaja Orupõld.** Treatment and analysis of phenolic wastewater with microorganisms. Tartu, 2000, 123 p.
14. **Jüri Ivask.** Ion Chromatographic determination of major anions and cations in polar ice core. Tartu, 2000, 85 p.
15. **Lauri Vares.** Stereoselective Synthesis of Tetrahydrofuran and Tetrahydropyran Derivatives by Use of Asymmetric Horner-Wadsworth-Emmons and Ring Closure Reactions. Tartu, 2000, 184 p.
16. **Martin Lepiku.** Kinetic aspects of dopamine D_2 receptor interactions with specific ligands. Tartu, 2000, 81 p.
17. **Katrin Sak.** Some aspects of ligand specificity of P2Y receptors. Tartu, 2000, 106 p.
18. **Vello Pällin.** The role of solvation in the formation of iotsitch complexes. Tartu, 2001, 95 p.

19. **Katrin Kollist.** Interactions between polycyclic aromatic compounds and humic substances. Tartu, 2001, 93 p.
20. **Ivar Koppel.** Quantum chemical study of acidity of strong and superstrong Brønsted acids. Tartu, 2001, 104 p.
21. **Viljar Pihl.** The study of the substituent and solvent effects on the acidity of OH and CH acids. Tartu, 2001, 132 p.
22. **Natalia Palm.** Specification of the minimum, sufficient and significant set of descriptors for general description of solvent effects. Tartu, 2001, 134 p.
23. **Sulev Sild.** QSPR/QSAR approaches for complex molecular systems. Tartu, 2001, 134 p.
24. **Ruslan Petrukhin.** Industrial applications of the quantitative structure-property relationships. Tartu, 2001, 162 p.
25. **Boris V. Rogovoy.** Synthesis of (benzotriazolyl)carboximidamides and their application in relations with *N*- and *S*-nucleophyles. Tartu, 2002, 84 p.
26. **Koit Herodes.** Solvent effects on UV-vis absorption spectra of some solvatochromic substances in binary solvent mixtures: the preferential solvation model. Tartu, 2002, 102 p.
27. **Anti Perkson.** Synthesis and characterisation of nanostructured carbon. Tartu, 2002, 152 p.
28. **Ivari Kaljurand.** Self-consistent acidity scales of neutral and cationic Brønsted acids in acetonitrile and tetrahydrofuran. Tartu, 2003, 108 p.
29. **Karmen Lust.** Adsorption of anions on bismuth single crystal electrodes. Tartu, 2003, 128 p.
30. **Mare Piirsalu.** Substituent, temperature and solvent effects on the alkaline hydrolysis of substituted phenyl and alkyl esters of benzoic acid. Tartu, 2003, 156 p.
31. **Meeri Sassian.** Reactions of partially solvated Grignard reagents. Tartu, 2003, 78 p.
32. **Tarmo Tamm.** Quantum chemical modelling of polypyrrole. Tartu, 2003. 100 p.
33. **Erik Teinemaa.** The environmental fate of the particulate matter and organic pollutants from an oil shale power plant. Tartu, 2003. 102 p.
34. **Jaana Tammiku-Taul.** Quantum chemical study of the properties of Grignard reagents. Tartu, 2003. 120 p.
35. **Andre Lomaka.** Biomedical applications of predictive computational chemistry. Tartu, 2003. 132 p.
36. **Kostyantyn Kirichenko.** Benzotriazole — Mediated Carbon–Carbon Bond Formation. Tartu, 2003. 132 p.
37. **Gunnar Nurk.** Adsorption kinetics of some organic compounds on bismuth single crystal electrodes. Tartu, 2003, 170 p.
38. **Mati Arulepp.** Electrochemical characteristics of porous carbon materials and electrical double layer capacitors. Tartu, 2003, 196 p.
39. **Dan Cornel Fara.** QSPR modeling of complexation and distribution of organic compounds. Tartu, 2004, 126 p.

40. **Riina Mahlapuu.** Signalling of galanin and amyloid precursor protein through adenylate cyclase. Tartu, 2004, 124 p.
41. **Mihkel Kerikmäe.** Some luminescent materials for dosimetric applications and physical research. Tartu, 2004, 143 p.
42. **Jaanus Kruusma.** Determination of some important trace metal ions in human blood. Tartu, 2004, 115 p.
43. **Urmas Johanson.** Investigations of the electrochemical properties of polypyrrole modified electrodes. Tartu, 2004, 91 p.
44. **Kaido Sillar.** Computational study of the acid sites in zeolite ZSM-5. Tartu, 2004, 80 p.
45. **Aldo Oras.** Kinetic aspects of dATPαS interaction with $P2Y_1$ receptor. Tartu, 2004, 75 p.
46. **Erik Mölder.** Measurement of the oxygen mass transfer through the air-water interface. Tartu, 2005, 73 p.
47. **Thomas Thomberg.** The kinetics of electroreduction of peroxodisulfate anion on cadmium (0001) single crystal electrode. Tartu, 2005, 95 p.
48. **Olavi Loog.** Aspects of condensations of carbonyl compounds and their imine analogues. Tartu, 2005, 83 p.
49. **Siim Salmar.** Effect of ultrasound on ester hydrolysis in aqueous ethanol. Tartu, 2006, 73 p.
50. **Ain Uustare.** Modulation of signal transduction of heptahelical receptors by other receptors and G proteins. Tartu, 2006, 121 p.
51. **Sergei Yurchenko.** Determination of some carcinogenic contaminants in food. Tartu, 2006, 143 p.
52. **Kaido Tämm.** QSPR modeling of some properties of organic compounds. Tartu, 2006, 67 p.
53. **Olga Tšubrik.** New methods in the synthesis of multisubstituted hydrazines. Tartu. 2006, 183 p.
54. **Lilli Sooväli.** Spectrophotometric measurements and their uncertainty in chemical analysis and dissociation constant measurements. Tartu, 2006, 125 p.
55. **Eve Koort.** Uncertainty estimation of potentiometrically measured ph and pK_a values. Tartu, 2006, 139 p.
56. **Sergei Kopanchuk.** Regulation of ligand binding to melanocortin receptor subtypes. Tartu, 2006, 119 p.
57. **Silvar Kallip.** Surface structure of some bismuth and antimony single crystal electrodes. Tartu, 2006, 107 p.
58. **Kristjan Saal.** Surface silanization and its application in biomolecule coupling. Tartu, 2006, 77 p.
59. **Tanel Tätte.** High viscosity $Sn(OBu)_4$ oligomeric concentrates and their applications in technology. Tartu, 2006, 91 p.
60. **Dimitar Atanasov Dobchev**. Robust QSAR methods for the prediction of properties from molecular structure. Tartu, 2006, 118 p.

61. **Hannes Hagu**. Impact of ultrasound on hydrophobic interactions in solutions. Tartu, 2007, 81 p.
62. **Rutha Jäger.** Electroreduction of peroxodisulfate anion on bismuth electrodes. Tartu, 2007, 142 p.
63. **Kaido Viht.** Immobilizable bisubstrate-analogue inhibitors of basophilic protein kinases: development and application in biosensors. Tartu, 2007, 88 p.
64. **Eva-Ingrid Rõõm.** Acid-base equilibria in nonpolar media. Tartu, 2007, 156 p.
65. **Sven Tamp.** DFT study of the cesium cation containing complexes relevant to the cesium cation binding by the humic acids. Tartu, 2007, 102 p.
66. **Jaak Nerut.** Electroreduction of hexacyanoferrate(III) anion on Cadmium (0001) single crystal electrode. Tartu, 2007, 180 p.
67. **Lauri Jalukse.** Measurement uncertainty estimation in amperometric dissolved oxygen concentration measurement. Tartu, 2007, 112 p.
68. **Aime Lust.** Charge state of dopants and ordered clusters formation in CaF_2:Mn and CaF_2:Eu luminophors. Tartu, 2007, 100 p.
69. **Iiris Kahn**. Quantitative Structure-Activity Relationships of environmentally relevant properties. Tartu, 2007, 98 p.
70. **Mari Reinik.** Nitrates, nitrites, N-nitrosamines and polycyclic aromatic hydrocarbons in food: analytical methods, occurrence and dietary intake. Tartu, 2007, 172 p.
71. **Heili Kasuk.** Thermodynamic parameters and adsorption kinetics of organic compounds forming the compact adsorption layer at Bi single crystal electrodes. Tartu, 2007, 212 p.
72. **Erki Enkvist.** Synthesis of adenosine-peptide conjugates for biological applications. Tartu, 2007, 114 p.
73. **Svetoslav Hristov Slavov**. Biomedical applications of the QSAR approach. Tartu, 2007, 146 p.
74. **Eneli Härk.** Electroreduction of complex cations on electrochemically polished Bi(*hkl*) single crystal electrodes. Tartu, 2008, 158 p.
75. **Priit Möller.** Electrochemical characteristics of some cathodes for medium temperature solid oxide fuel cells, synthesized by solid state reaction technique. Tartu, 2008, 90 p.
76. **Signe Viggor.** Impact of biochemical parameters of genetically different pseudomonads at the degradation of phenolic compounds. Tartu, 2008, 122 p.
77. **Ave Sarapuu.** Electrochemical reduction of oxygen on quinone-modified carbon electrodes and on thin films of platinum and gold. Tartu, 2008, 134 p.
78. **Agnes Kütt.** Studies of acid-base equilibria in non-aqueous media. Tartu, 2008, 198 p.
79. **Rouvim Kadis.** Evaluation of measurement uncertainty in analytical chemistry: related concepts and some points of misinterpretation. Tartu, 2008, 118 p.

80. **Valter Reedo.** Elaboration of IVB group metal oxide structures and their possible applications. Tartu, 2008, 98 p.
81. **Aleksei Kuznetsov.** Allosteric effects in reactions catalyzed by the cAMP-dependent protein kinase catalytic subunit. Tartu, 2009, 133 p.
82. **Aleksei Bredihhin.** Use of mono- and polyanions in the synthesis of multisubstituted hydrazine derivatives. Tartu, 2009, 105 p.
83. **Anu Ploom.** Quantitative structure-reactivity analysis in organosilicon chemistry. Tartu, 2009, 99 p.