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ANNI ALLIKALT

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Development of assay systems for studying ligand binding to dopamine receptors

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

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LIST OF ORIGINAL PUBLICATIONS

- **I** Dzirkale, Z., Rumaks, J., Svirskis, S., Mazina, O., **Allikalt, A.,** Rinken, A., Jekabsons, K. Muceniece, R., Klusa, V. (2013) Lunasin-induced behavioural effects in mice: Focus on the dopaminergic system. *Behavioural Brain Research* 256, 5–9.
- **II** Mazina, O., **Allikalt, A.,** Heinloo, A., Reinart-Okugbeni, R., Kopanchuk, S., Rinken, A. (2015) cAMP assay for GPCR ligand characterization: Application of BacMam Expression System. In *G Protein-Coupled Receptor Screening Assays* (pp. 65–77). Springer New York.
- **III** Tekko, T., Lakspere, T., **Allikalt, A.,** End, J., Kõlvart, K.R., Jagomäe, T., Terasmaa, A., Philips, M.-A., Visnapuu, T., Väärtnõu, F., Gilbert, S.F., Rinken, A., Vasar, E., Lilleväli, K. (2017) Wfs1 is expressed in dopaminoceptive regions of the amniote brain and modulates levels of D1-like receptors. *PLOS ONE* 12(3): e0172825.
- **IV** **Allikalt, A.,** Rinken, A., (2017) Budded baculovirus particles as a source of membrane proteins for radioligand binding assay: The case of dopamine D_1 receptor. *Journal of Pharmacological and Toxicological Methods* 86, 81–86.
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Author's contribution:

Paper I: The author participated in planning and performing the [^{3}H]SCH23390 binding experiments.

Paper II: The author planned and performed the experiments involving determination of virus titer with cell size-based assay.

Paper III: The author was involved in planning and performing the [^{3}H]SCH23390 binding experiments and wrote the corresponding part of the manuscript.

Paper IV: The author was the principal investigator responsible for the planning and performing the experiments as well as data analysis and writing of the manuscript.

Paper V: The author planned and performed all the ligand binding experiments, performed data analysis and was responsible for writing the manuscript.

ABBREVIATIONS

7TM	seven transmembrane
A68930	(1*R*,3*S*)-1-(aminomethyl)-3-phenyl-3,4-dihydro-1*H*-isochromene-5,6-diol
AB	assay buffer
ATP	adenosine triphosphate
BacMam	a recombinant baculovirus for delivering genes of interest into mammalian cells
Bacmid	baculovirus shuttle vector
B_{max}	maximal number of receptor binding sites
BSA	bovine serum albumin
BV	baculovirus
cAMP	3',5'-cyclic adenosine monophosphate
D_1-D_5	dopamine receptor subtypes
DA	dopamine
DPBS	Dulbecco's Phosphate-Buffered Saline
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EC_{50}	concentration of the sample that produces 50% of the maximal possible effect
EDTA	ethylendiaminetetraacetic acid
FA	fluorescence anisotropy
FRET	Förster/fluorescence resonance energy transfer
GDP	guanine diphosphate
GTP	guanine triphosphate
GPCR	G protein-coupled receptor
HB	homogenization buffer
HEK293	human embryonic kidney cells 293
IB	incubation buffer
IC_{50}	molar concentration of an unlabeled ligand that inhibits binding of a labeled ligand by 50%
ivp	infectious viral particles
K_D	equilibrium dissociation constant of a ligand determined directly in a binding assay using a labeled ligand
K_i	inhibition constant, refers to an equilibrium dissociation constant of an unlabeled ligand measured in competition with a labeled ligand
k_{off}	dissociation rate constant
k_{on}	association rate constant
L-DOPA	L-3,4-dihydroxyphenylalanine
LE300	7-methyl-6,7,8,9,14,15-hexahydro-5*H*-benz-[*d*]indolo[2,3-*g*]azecine

MNPA	2-methoxy-10,11-dihydroxy-*N*-propylnorapomorphine
MOI	multiplicity of infection
NAPS	*N*-(p-aminophenethyl)-spiperone
NPA	*R*(-)-*N*-propylnorapomorphine
SCH23390	(5*R*)-8-chloro-3-methyl-5-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine-7-ol
SCH39166	(6a*S*,13b*R*)-11-chloro-7-methyl-6,6a,7,8,9,13b-hexahydro-5*H*-benzo[*d*]naphtho[2,1-*b*]azepin-12-ol
Sf9	*Spodoptera frugiperda* cells
SKF38393	1-phenyl-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepine-7,8-diol
SKF81297	(1*R*)-6-chloro-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine-7,8-diol
SKF83566	8-bromo-3-methyl-5-phenyl-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepin-7-ol
TFI	total fluorescence intensity
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol
WB	washing buffer
Wfs1	wolframin
wt	wild-type

INTRODUCTION

Biopolymers like polysaccharides, nucleic acids and proteins are essential for all living organisms. Besides water, proteins are the most abundant type of molecules in the human body. Proteins are also the most versatile of all biomolecules, performing many functions required for life. Some proteins have catalytic activity and function as enzymes, others serve as structural elements, signal receptors, or transporters that carry specific substances into or out of the cells. This thesis focuses on the receptor proteins, specifically to G protein-coupled receptors that are located in the cell's plasma membrane. These proteins are responsible for detecting various chemical of physical signals outside the cell by binding specific chemical compounds, called ligands. Ligand binding to a receptor can initiate a conformational change in the structure of the protein, which in turn leads to a chain of biochemical events inside the cell.

Dopamine receptors, belonging to the family of G protein-coupled receptors, mediate several functions in the central nervous system, including control of locomotion, cognition, emotion, positive reinforcement, food intake and endocrine regulation. These receptors also have an important role in the periphery, where they modulate cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function and gastrointestinal motility. Abnormal dopaminergic signaling can lead to several neurological and psychiatric disorders and therefore these receptors are relevant targets in the pharmaceutical industry. Drugs that bind to dopamine receptors have been clinically used in the management of several diseases, such as schizophrenia, Parkinson's disease, bipolar disorder, Huntington's disease, attention deficit hyperactivity disorder and Tourette's syndrome.

Dopamine receptors have been in the center of G protein-coupled receptor research and numerous advancements have been made in understanding the structural, biochemical and functional properties of these proteins. However, much remains unclear and therefore developing methods that could help to gain more information about dopaminergic signaling and facilitate the development of better drugs is necessary. Thus, the aim of this study was to implement novel assay systems to characterize ligand binding to different subtypes of dopamine receptors. Experiments were carried out with several different receptors, including native receptors (tissue homogenates) and various recombinant protein expression systems (mammalian and insect cells, budded baculovirus particles) by using several methods to characterize receptor-ligand interactions with the emphasis on fluorescence-based methods.

1. LITERATURE REVIEW

1.1. G protein-coupled receptors

The ability of cells to receive and act on signals is fundamental to life for higher organisms. Usually, the signal represents information that is received by specific proteins and then converted into a cellular response. This process, where chemical or physical signal is transmitted through a cell as a series of molecular events, is called signal transduction. Proteins that are responsible for detecting these external signals are known as receptors, which have been broadly classified into six basic classes: transmembrane receptors that include G protein-coupled receptors (GPCRs), receptor tyrosine kinases, receptor guanylyl cyclases, gated ion channels, adhesion receptors and intracellular nuclear receptors (Nelson and Cox 2008). About 800 GPCRs have been identified in humans (Wacker *et al.* 2017). Approximately half of these mediate sensory functions, like olfaction (~400), taste (33), light perception (10) and pheromone signaling (5). The remaining ~350 GPCRs are binding natural ligands that range in size from small molecules to large proteins (Alexander *et al.* 2017).

Several classification schemes have been proposed to categorize GPCRs, but according to IUPHAR/BPS Guide to PHARMACOLOGY most of the human GPCRs can be divided into five classes: Glutamate (class C), Rhodopsin (class A), Adhesion, Frizzled/Taste2 (class F) and Secretin (class B) (Alexander *et al.* 2017). This is called the GRAFS classification system developed by the sequence similarities of GPCRs (Schiöth and Fredriksson 2005), in which rhodopsin constitutes the largest family that is further divided into four main groups (α, β, γ, δ) with 13 sub-branches. In general, the α-group includes GPCRs that bind amines and some peptides, the β-group includes only peptide binding GPCRs, the γ-group contains peptide, neuropeptide and opioid binding GPCRs and the δ-group includes a large group of olfactory receptors, purin receptors and glycoprotein receptors (Schiöth and Fredriksson 2005).

All GPCRs share common structural features, like an extracellular N-terminus, an intracellular C-terminus and seven transmembrane (7TM) α-helices connected by three intracellular and three extracellular loops (Palczewski *et al.* 2000). The term "7TM receptor" is often used as a synonym of GPCR to emphasize the existence of seven hydrophobic transmembrane domains. These transmembrane helices share the greatest homology between different GPCRs, while the most variability can be seen in N-terminus, followed by C-terminus and intracellular loop between transmembrane helices five and six. For example, the N-terminus is relatively short in monoamine and peptide receptors (10–50 amino acids) and much longer (350 – 600 amino acids) in glycoprotein hormone receptors and the glutamate family receptors (Kobilka 2007). While the extracellular domain is the least conserved, GPCRs have great homology at the cytoplasmic ends of the transmembrane helices, giving evidence of a conserved mechanism on activation and signal transduction (Mirzadegan *et al.* 2003).

For a particular cell the first step of signal transduction is a ligand interacting with a receptor. Precise molecular complementarity between the ligand and the receptor secures remarkable specificity of the signal transduction. The structure and function of GPCRs is similar in contrast to the structural diversity of the natural ligands, therefore it is only befitting that there are differences in sites and modes of ligand binding. Ligand binding domains have been determined for numerous GPCRs – many small ligands bind in the transmembrane region, but peptide hormones and proteins often bind to the N-terminus and extracellular loops (Ji *et al.* 1998). However, the location of the binding site does not only depend on the size of the ligand. For example, glycoprotein hormones, glutamate and Ca^{2+} are recognized by the large extracellular domain of the corresponding receptor (Pin *et al.* 2003).

Irrespective of the exact location of binding site the overall purpose of ligand binding remains the same. Upon ligand binding the GPCR undergoes a conformational change leading to subsequent activation of heterotrimeric G proteins (Rosenbaum *et al.* 2009). These guanine nucleotide-binding proteins (shortly G proteins) compose of three subunits: α, β and γ. In the inactive state guanine diphosphate (GDP) is bound to the α-subunit of a G protein. Conformational changes in the receptor catalyze the dissociation of GDP that is followed by the association of guanine triphosphate (GTP) with the α-subunit. Binding of GTP causes a dissociation of the G protein subunits from each other and from the receptor yielding a monomeric α-subunit and a βγ dimer. Both of these can now regulate the activity of the appropriate effectors, such as second-messenger-generating enzymes or specific ion channels (Gainetdinov *et al.* 2004). Hydrolysis of GTP to GDP and inorganic phosphate initiates the deactivation of G proteins, thus allowing reassociation of the subunits. GPCRs vary in their specificity to activate distinct G protein types. Based on the sequence homology of their α-subunit (Gilman 1987) GPCRs can be divided into four main families: $G_{i/o}$, $G_{s/olf}$, $G_{q/11}$, $G_{12/13}$ (Simon *et al.* 1991). $G_{s/olf}$ and $G_{i/o}$ transduce the signal via membrane-associated enzyme adenylate cyclase, either by activating ($G_{s/olf}$) or inhibiting ($G_{i/o}$) the enzyme. Hence, these G proteins influence the intracellular 3',5'-cyclic adenosine monophosphate (cAMP) levels. $G_{q/11}$ subunit activates phospholipase C, which in turn hydrolyzes phosphatidylinositol-4,5-bisphosphate into two second messengers – diacyl glycerol and inositol 1,4,5-trisphosphate. This causes the activation of protein kinase C and the intracellular Ca^{2+} mobilization. $G_{12/13}$ regulate cell processes through the use of guanine nucleotide exchange factors and therefore control the cell cytoskeleton remodeling and cell migration (Siehler 2007). Despite extensive studies, the GPCR and G protein coupling profile remains incomplete. Some GPCRs can only signal via single type of G protein, whereas many receptors can couple to a broader range of G protein families (Siehler 2007, Miyano *et al.* 2014).

This signaling cascade cannot be induced by all the ligands that bind to GPCRs. The ligands binding to the GPCR's active site (also called the orthosteric site) can be categorized based on their effect on the receptor function. A ligand that alters the receptor state resulting in a biological response upon

binding is called an agonist. Classical agonist increases receptor activity, while inverse agonist reduces it. Agonists are further divided as partial and full agonists, depending on the degree of effect produced. The effect of an agonist can be reduced by an antagonist, which upon binding does not result in cellular response. In addition to orthosteric ligands there are also allosteric ligands that increase or decrease the action of orthosteric ligands by binding to distinct allosteric sites on the receptor molecules (Neubig *et al.* 2003).

While GPCR signaling is essential, overstimulation can be deleterious, resulting in cellular toxicity or uncontrolled cellular growth. Therefore, a number of mechanisms exist for limiting GPCR signaling (Rajagopal and Shenoy 2018). GPCRs respond to agonists in a dose-dependent manner so that the concentration of the agonist is the primary control point for GPCR signaling (Gainetdinov *et al.* 2004). Hence, the signal attenuation includes removal of agonists from the extracellular fluid by dilution, uptake by transporters or enzymatic degradation (Böhm *et al.* 1997). However, one important feature of G protein signaling system is that it exhibits a memory of prior activation or signaling tone (Hausdorff *et al.* 1990). Thus, high activation of GPCR leads to a reduced ability to be stimulated in the future (desensitization), while low activation leads to an increased stimulation (sensitization). A good example is the "light receptor" rhodopsin, which adjusts to both dark and light within moments. This regulation can be achieved at the level of receptor itself in two main ways: by adjusting the signaling efficacy of receptors and by controlling the number of GPCRs present in the cell plasma membrane (Gainetdinov *et al.* 2004). Short-term desensitization occurs over minutes and is primary associated with phosphorylation of the agonist-activated receptors by G-protein receptor kinases followed by binding of β-arrestins that block further G protein-mediated signaling (Rajagopal and Shenoy 2018). Longer-term desensitization, referred to as downregulation, occurs over hours to days and involves agonist-induced receptor endocytosis, which can lead to either (i) dephosphorylation, resensitization and recycling to the membrane, (ii) targeting to lysosomes and degradation, (iii) activation of additional intracellular signaling pathways (Reiter and Lefkowitz 2006, Rajagopal and Shenoy 2018). Majority of GPCRs use clathrin-mediated internalization pathway that requires prior G protein receptor kinase mediated phosphorylation and subsequent β-arrestin recruitment (Shenoy and Lefkowitz 2003). Downregulation may even lead to decreased receptor mRNA levels that alters the rate of GPCR synthesis (Rajagopal and Shenoy 2018).

Individual GPCRs have unique combinations of signal-transduction activities involving G proteins as well as G protein-independent signaling pathways (Rosenbaum *et al.* 2009). It has been even proposed that the term GPCR should be abandoned in favor of 7TM receptors, because these proteins also signal without coupling to G proteins (Kobilka 2007). Nowadays it has become increasingly evident that the functions of abovementioned G protein receptor kinases and β-arrestins are not restricted to only desensitization and internalization. These two protein families also mediate the G protein-independent signaling of GPCRs (Reiter and Lefkowitz 2006). The list of described β-

arrestin-dependent signaling pathways has been growing rapidly and for example mitogen-activated protein kinases (JNK3, ERK1/2, p38 MAPK) can be activated via these proteins (Shenoy and Lefkowitz 2003). Interestingly, it has been demonstrated that some agonists can produce activation of some but not all available pathways. These are referred to as "biased" agonists, because they select which signaling pathways become activated upon binding to the receptor. Biased agonists have been shown to variously activate different G proteins and β-arrestins and have varying susceptibility to phosphorylation, desensitization and internalization (Kenakin 2009).

GPCRs have fundamental roles in virtually all physiological functions (Reiter and Lefkowitz 2006) and are expressed throughout the human body (Wacker *et al.* 2017). Therefore, problems with GPCR mediated signal transduction can also cause various disorders. For example, these receptors have been implicated in many common diseases including allergies, depression, blindness, diabetes and various cardiovascular defects (Nelson and Cox 2008). As of November 2017, 134 GPCRs are targets for drugs approved in the United States or European Union (Sriram and Insel 2017). Particularly prominent therapeutics involving GPCRs include opioid analgesics, antihistamines, anticholinergics, antipsychotics, antimigraine drugs, antihypertensives and asthma drugs (Wacker *et al.* 2017). Around 700 approved drugs target GPCRs, implying that approximately 35% of approved drugs mediate their effects by modulating GPCR signaling pathways (Sriram and Insel 2018). This illustrates the importance of GPCR research and even though many aspects of signal transduction are already known, much remains unclear. Therefore, development of novel, sensitive analytical methods or implementation of existing techniques in an innovative way is of utmost importance.

1.1.1. Dopamine receptors

Catecholamines are natural GPCR ligands that contain a benzene ring with two adjacent hydroxyl groups (also called a catechol group) as well as an ethyleneamine side chain that may have additional *N*-substituents (Brady *et al.* 2011). Predominant catecholamine neurotransmitters in the brain are dopamine (DA), norepinephrine and epinephrine. Norepinephrine was the first of them to be recognized as a central neurotransmitter in 1954 by M. Vogt (Vogt 1954). At that time, DA was only thought to be a precursor of norepinephrine. In fact, dopamine is an intermediate in the biosynthesis of both norepinephrine and epinephrine. DA itself is synthesized from an amino acid tyrosine by the removal of a hydroxyl group to produce levodopa (L-DOPA). Decarboxylation of L-DOPA produces dopamine, which can then be converted to norepinephrine or further into epinephrine (Brady *et al.* 2011).

It was soon discovered by A. Carlsson and colleagues that DA can also function as an independent neurotransmitter (Carlsson *et al.* 1957). Interest in dopamine increased as it was realized that DA had an important role in certain

neurological and psychiatric disorders, like Parkinson's disease, schizophrenia, Tourette's syndrome, bipolar disorder, depression, hyperprolactinemia and attention deficit hyperactivity disorder (Missale *et al.* 1998, Strange and Neve 2013, Beaulieu *et al.* 2015). Dopamine has also been found to be essential in the brain reward system, hence it is also associated with drug dependence and addiction (Marsden 2006).

The first evidence that dopamine elicits its effect by binding to a cell-surface receptor came from the experiments showing the stimulation of adenylate cyclase by DA (Kebabian *et al.* 1972). The receptors described by Kebabian and colleagues are nowadays known and classified as dopamine D_1-like receptors. Soon a receptor that could bind dopamine and haloperidol was identified and called "neuroleptic/dopamine receptor" (Seeman *et al.* 1976). The paper also demonstrated that clinically effective antipsychotic drugs block binding of haloperidol. This receptor was later named as dopamine D_2 receptor (Kebabian and Calne 1979), which is a key target for several antipsychotic drugs. Kebabian and Calne also proposed that the two dopamine receptors that had been discovered belong to a separate class and hypothesized the existence of additional dopamine receptor subtypes (Kebabian and Calne 1979). Indeed, more dopamine receptor subtypes were discovered after the advancement in molecular biology enabled gene cloning of the receptors (Marsden 2006). Nowadays it is known that the effects of dopamine are mediated through five G protein-coupled receptors: D_1, D_2, D_3, D_4, D_5 (Gainetdinov *et al.* 2017). These subtypes are further divided into two families, D_1-like and D_2-like receptors, based on their sequence homology, pharmacological profile and by the ability to couple either with $G_{s/olf}$ or $G_{i/o}$ proteins. D_1-like receptors include D_1 and D_5 subtypes and the genes of these receptors do not contain introns in their amino acid coding regions (Missale *et al.* 1998). These two receptors activate the enzyme adenylate cyclase that catalyzes the formation of cAMP from ATP. Hence, activation of these receptors stimulates cAMP formation (Brady *et al.* 2011). Increase in the second messenger cAMP concentration may lead to the activation of protein kinase A (Walsh *et al.* 1968), modulation of cyclic nucleotide-gated ion channels (Fesenko *et al.* 1985, Kaupp and Seifert 2002) and exchange proteins activated by cAMP (De Rooij *et al.* 2000), to name a few. Contrary, D_2-like receptors (D_2, D_3, D_4) inhibit the enzyme adenylate cyclase and the formation of cAMP by coupling to $G_{i/o}$ protein. These subtypes also contain introns and alternative splicing may result in different isoforms of receptors, such as the short (D_{2S}) and long (D_{2L}) variants of D_2 receptor (Gainetdinov *et al.* 2017). D_2-like receptors are expressed both postsynaptically and presynaptically while D_1-like receptors are exclusively found on postsynaptic cells (Beaulieu and Gainetdinov 2011).

In addition to previously mentioned differences, D_1-like and D_2-like receptors share some structural dissimilarities, whereas members of the same family have considerable homology. The highest degree of amino acid sequence similarity is in the transmembrane domains: DA receptors share 31% sequence identity in that region and the similarity increases between members of the same

family (75% for D_1-like and 52% for D_2-like receptors) (Civelli 1993). The D_1-like receptors have a short intracellular loop 3 that is common for receptors coupling with $G_{s/olf}$, while the D_2-like receptors possess a long third intracellular loop as in many adenylate cyclase inhibiting GPCRs. Additionally, D_1-like receptors possess about seven times longer carboxyl terminus that is rich in serine and threonine residues and contains a cysteine residue near the beginning of the C-terminus. In D_2-like receptors, the cysteine residue is located in the end of carboxyl terminus (Gingrich and Caron 1993). This cysteine is conserved in all GPCRs and has been shown to be palmitoylated for anchoring the cytoplasmic tail to the membrane (Ovchinnikov *et al.* 1988, Ebersole *et al.* 2015). Additional conserved cysteine residues are in the extracellular loop 2 and 3, which are suggested to form a stabilizing intramolecular disulfide bridge in GPCRs (Dohlman *et al.* 1990). Compared to the carboxyl terminus, N-terminus has a similar number of amino acids in all dopamine receptor subtypes. *N*-glycosylation sites are also located in the N-terminus, but the number of potential glycosylation sites varies among different subtypes (Missale *et al.* 1998).

Dopamine receptor subtypes belonging to the same family have considerable homology in their structure, but still differ enough to have distinctive pharmacological profiles. For example, the main differences between D_1 and D_5 receptors are in the amino acid sequences of third intracellular loop and carboxyl terminus, which are therefore the key structural features causing the functional differences of these two receptor subtypes. Also, there is some considerable variation in the extracellular loop 2 region, which is much shorter in D_1 receptor (27 amino acids) than in D_5 (41 amino acids) (Missale *et al.* 1998). Differences between D_2-like receptors' structures can now be precisely characterized due to the existence of crystal structures for all the subtypes. The crystal structure of D_3 receptor (Chien *et al.* 2011) was reported seven years ago, while the structures for D_4 (Wang *et al.* 2017) and D_2 (Wang *et al.* 2018) have been obtained only recently and the structures of D_1-like receptors have not been published. These three crystal structures all represent inactive receptor states, binding either inverse agonists or antagonists. Compared to the other D_2-like receptors D_2 displays substantial structural differences in extracellular loops 1 and 2 and in the extracellular regions of transmembrane helices V, VI and VII. These and some additional key characteristics further described in Wang *et al.* 2018 are responsible for the unique ligand binding mode of D_2 receptor, where the ligand engages a deeper binding mode which has not been described for neither D_3 nor D_4 receptor.

Dopamine receptors are widely expressed in the central nervous system, where they are involved in the control of locomotion, cognition, emotion, positive reinforcement, food intake and endocrine regulation. Dopamine also has an important role in the periphery, where it modulates cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function and gastrointestinal motility (Missale *et al.* 1998, Beaulieu and Gainetdinov 2011). The previously described model of dopamine receptor signaling via activation or inhibition of adenylate cyclase is too simplistic to explain the functional fle-

xibility of these receptors. It is now known that dopamine receptors regulate multiple signaling pathways by interacting with various G proteins and by G protein-independent mechanisms, such as ion channels, receptor tyrosine kinases and β-arrestins (Beaulieu *et al.* 2015). For example, there are indications that both D_1 and D_2 receptors can transactivate the brain-derived neurotrophic factor receptor in neurons (Swift *et al.* 2011), regulate calcium channels through a direct protein-protein interaction (Kisilevsky and Zamponi 2008, Kisilevsky *et al.* 2008) and interact with Na^+-K^+-ATPase (Hazelwood *et al.* 2008). It has also been demonstrated that dopamine D_1-like receptors can regulate inositol trisphosphate-mediated signaling (Sahu *et al.* 2009, Medvedev *et al.* 2013) via coupling to $G_{q/11}$ proteins. D_2-like receptors' signaling is also largely mediated via βγ dimer of G protein leading to activation of phospholipase C and increase in cytoplasmic calcium concentration (Hernandez-Lopez *et al.* 2000), regulation of the activity of L- and N-type calcium channels (Yan *et al.* 1997, Hernandez-Lopez *et al.* 2000) as well as G protein-coupled inwardly rectifying potassium channels (Kuzhikandathil *et al.* 1998). Additionally, there is increasing evidence that G protein mediated signaling of dopamine receptors can even influence the phosphorylation and therefore functioning of ionotropic glutamate receptors (Beaulieu *et al.* 2015). D_2 receptors are also able to signal via G protein-independent pathways by interacting with β-arrestins, leading to inactivation of serine/threonine kinase Akt and activation on glycogen synthase kinase 3 (Beaulieu *et al.* 2004).

Further complexity in signal transduction comes from the fact that GPCRs can exist in oligomeric forms. This is also the case for dopamine receptors, which can form homodimers between two identical receptors and heterodimers by interacting with other members of the same family or with structurally divergent receptor families. These heterodimers can have different pharmacological, signaling and trafficking properties compared to the monomeric receptors (Angers *et al.* 2002). Members of dopamine receptor family are known to form following heterodimers: D_1-D_2, D_1-D_3, D_2-D_3, D_2-D_4 (Pou *et al.* 2012, Beaulieu *et al.* 2015). In some cases, existence of the dimers has been proved, but the biological function remains unclear and needs further investigation, whereas for some dimers the research has been more conclusive. For example, it is known that activation of D_1-D_2 complex has unique pharmacology that is distinct from of its single monomer, leading to the signaling via $G_{q/11}$ protein and successive release of Ca^{2+} from the internal store (Rashid *et al.* 2007). Also, there is increasing evidence that glutamate *N*-methyl-D-aspartate receptor can interact with both dopamine D_1 receptors (D_1-NMDA dimer) and D_2 receptors (D_2-GluN2B) (Beaulieu *et al.* 2015). These interactions seem to be physiologically relevant, because disruption of the D_1-NMDA complex led to working memory impairment (Nai *et al.* 2010) and disruption of D_2-GluN2B reduces cocaine-stimulated locomotor activity (Liu *et al.* 2006). Furthermore, many studies have shown that adenosine receptors can form heterodimers with dopamine receptors, resulting in D_1-A_1 and D_2-A_{2A} complexes (Ginés *et al.* 2000, Hillion *et al.* 2002). In both dimers activation of adenosine receptor can antagonize the

cAMP responses because adenosine and dopamine exert opposing effects in the brain. The list of receptors, which could form dimers with dopamine receptors is not conclusive and several additional dopamine receptor heterodimers have been described in Beaulieu *et al.* 2015. Taken together, the complex biology of dopamine receptor-mediated signal transduction mechanisms might represent a breakthrough for the development of innovative drugs for the treatment of DA related disorders.

1.1.1.1. Dopaminergic ligands

Dopaminergic drugs may modulate different parts of the dopaminergic signal transduction. For example, they may affect the enzymes catalyzing the synthesis of dopamine or degradation of DA, influence the DA release or reuptake, or bind to the dopamine receptors. Herewith, we concentrate on the ligands of dopamine receptors that are often in the focus of drug development. Usually agonists of dopamine receptors are known to treat the symptoms of Parkinson's disease, while the antagonists are used as antipsychotics in the treatment of schizophrenia. Depletion of DA in Parkinson's disease is commonly relieved by administration of L-DOPA. Unfortunately, its long-term use causes motoric complications such as dyskinesia (Zhang *et al.* 2008). Dopaminergic system is also involved in drug reinforcement and addiction (Volkow *et al*. 2009). A wide variety of addictive substances directly or indirectly affect dopaminergic signal transduction. Therefore, therapeutic interventions aimed at restoring normal dopaminergic signaling in drug users are of utmost importance. Moreover, some drugs that are designed to target other GPCRs may also affect dopaminergic system and therefore cause addiction among other side effects.

The most highly expressed dopamine receptor subtype is D_1 receptor which also plays a crucial role in a variety of cognitive functions and is implicated in substance abuse disorders. Although the first D_1-like selective antagonist SCH23390 was introduced more than three decades ago (Hyttel 1983), clinically useful D_1 ligands are rare. It is difficult to develop ligands that are specific for the D_1 and not to the D_5 receptor and all currently available ligands have similar affinity and potency to both D_1-like receptors (Nichols 2010). D_1-like receptors have high affinity for the benzazepine antagonists (SCH23390, SCH39166, SKF83566) that are also selective for D_1 and D_5 receptors. Another high affinity D_1 antagonist is LE300, which is structurally distinct from the benzazepines (Strange and Neve 2013).

Previous research indicates that D_1 agonists may be therapeutically useful in the treatment of Parkinson's disease (Li and Zhou 2013) and may also improve cognition and working memory in schizophrenia and age-related cognitive decline (Nichols 2010). The first known D_1-like agonist drug was apomorphine that can be easily synthesized from morphine. Interestingly, *N*-alkylation of apomorphine yields ligands with reduced affinity for D_1-like receptors and improved affinity for D_2-like receptors with *N*-propyl analog (NPA) having the

greatest selectivity. This observation seems to be a general property of dopaminergic ligands and has been referred to as the "propyl effect" (Nichols 2010). The discovery of a partial agonist SKF38393 was a breakthrough in DA research, as the molecule was highly selective for D_1-like receptors. Other benzazepine derivates (SKF81297, SKF38393) and dihydrexidine derivates (A86929, dihydrexidine, doxanthrine) are also D_1-like receptors' selective agonists (Strange and Neve 2013).

D_2-like receptor antagonists are divided into classical and atypical antipsychotics. The classical family includes phenothiazines (such as chlorpromazine), thioxanthenes (chlorprothixene), butyrophenones (haloperidol) and diphenylbutyl piperidines (pimozide) (Prante *et al.* 2010). These drugs are able to reduce the positive symptoms of schizophrenia but also have extrapyramidal side effects. In contrast, atypical drugs are able to influence positive and negative symptoms of schizophrenia and are less likely to produce side effects. Examples of these drugs include clozapine and olanzapine (Prante *et al.* 2010). Well-known D_2-like receptors' selective antagonists also include substituted benzamides (sulpiride, raclopride). Most of these antagonists have a similar affinity for all D_2-like receptors. However, selective antagonists for different D_2-like receptor subtypes have been developed. For example, L741626 is selective for D_2, NGB2904 for D_3 and L745870 for D_4 receptors (Strange and Neve 2013). Older D_2-like receptors agonistic drugs, such as bromocriptine and cabergoline are not selective and affect several subtypes. Now, several subtype selective agonists have been developed for D_2-like receptors, like sumanirole for D_2 and A412997 for D_4 receptors (Strange and Neve 2013). The maximal selectivity between different dopamine receptor subtypes has been seen for D_4 receptors' ligands with more than a 1000-fold higher affinity compared to the affinity for other subtypes (Beaulieu and Gainetdinov 2011).

A new promising direction for drug development is based on the functional selectivity. These biased ligands may be more efficacious drugs or have fewer side effects (Strange and Neve 2013). An interesting example of functional selectivity has been discovered with the D_1-D_2 heterodimer. An agonist SKF83959 activates the $G_{q/11}$ pathway leading to phospholipase C response without affecting $G_{s/olf}$-coupled D_1 receptors (Rashid *et al.* 2007). Another agonist SKF81297 can activate both $G_{q/11}$ and $G_{s/olf}$ pathway and a ligand SKF83822 activates only $G_{s/olf}$ pathway leading to activation of adenylate cyclase. These results indicate that the pharmacology of dopaminergic ligands is complicated and needs further investigation. For that, novel methods that could provide new insight about receptor-ligand interactions are required.

Some structures of the abovementioned ligands are presented in **Figure 1**.

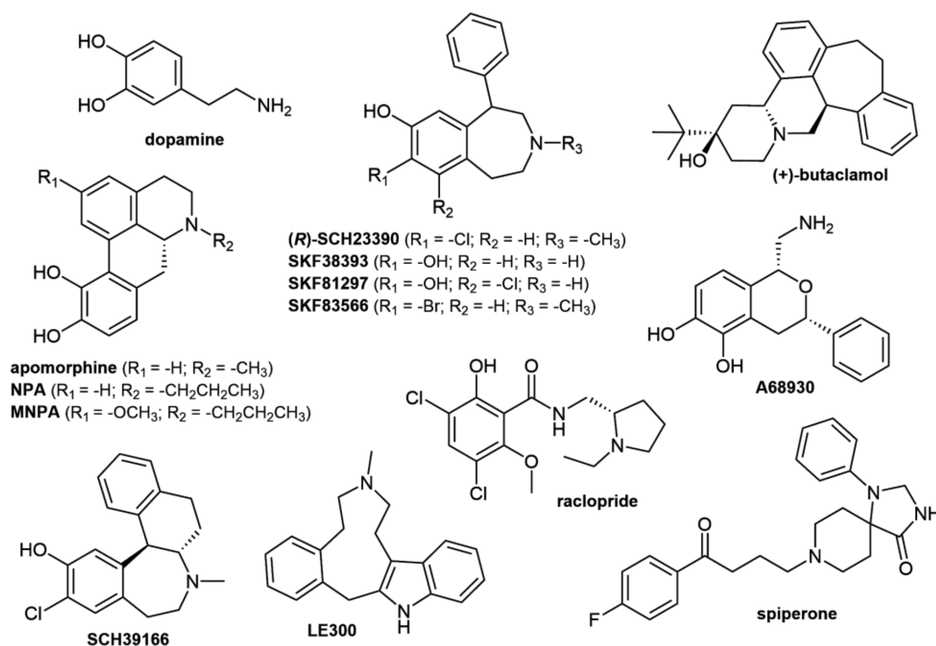

Figure 1. Examples of dopamine receptor ligands. These are the structures of the ligands that were used in the ligand binding experiments performed in this thesis. Stereochemistry is defined on structural formulas unless specified in name.

1.2. Ligand binding assays

The suggestion that chemical agents must be specifically bound to structures inherent to the living organisms before exerting an effect was first proposed by John Newport Langley in 1878 (Gesztelyi *et al.* 2012). At that time experiments were carried out on live animals or with animal tissues. For example, Langley studied the effect of pilocarpine (partial agonist of muscarinic acetylcholine receptors) on salivary secretion in the dog (Langley 1876). In these experiments he demonstrated that pilocarpine stimulated salivary production and atropine (antagonist) stopped it. Other similar tissue responses were used as a "signal" caused by certain chemical substances, like deceleration/acceleration of the heart rate, contraction of skeletal muscle etc. Experiments were also carried out with smaller organisms – Paul Ehrlich performed bacteriological investigations which lead him to believe that the cell protoplasm was supposed to have certain side-chains that were able to bind chemically the toxins produced by the bacteria (Maehle 2009). He later replaced the term "side-chain" with the term "receptor".

For a long time, the *in vivo* approach was the only method for characterizing and studying receptors. More knowledge about the interaction between a receptor and its ligands could be obtained from direct ligand binding assays,

which only became possible after introduction of radioligands in 1970s (Paton and Rang 1965, Lefkowitz *et al.* 1970). Receptor-ligand binding assays were among the earliest *in vitro* methods used to study receptor function and they continue to be an important tool in fundamental GPCR biology as well as in pharmacological industry for development and characterization of novel drug candidates (Flanagan 2016). In most cases, these assays rely on the use of labeled (radioisotope or fluorescent dye) ligands and therefore our focus will be on the corresponding methods. Ligand binding assay can be used to measure the concentration and localization of receptors in tissues or cells, to determine the affinity of a labeled ligand for the receptor of interest and to estimate kinetic parameters of ligand binding reaction, like association and dissociation rates (Zhang and Xie 2012). Additionally, binding parameters of unlabeled ligands could be characterized in competition binding experiments with a labeled ligand. However, ligand binding assay generally cannot be used to distinguish agonists from antagonists or inverse agonist, because direct interaction between a ligand and a receptor is measured instead of a physiological response (Flanagan 2016).

Fundamentally, receptor binding experiments can be divided into three basic types: kinetic experiments, where ligand binding is measured in time to determine association (k_{on}) and dissociation (k_{off}) rate constants; saturation binding experiments, where binding is measured at several concentrations of the labeled ligand at equilibrium to determine the affinity of the labeled ligand (K_d) and the number of receptor binding sites (B_{max}); competition binding experiments, where binding of labeled ligand is measured in the presence of incrementing series of concentrations of a unlabeled compound at equilibrium to determine the affinity of unlabeled compound (K_i) (Hein *et al.* 2005, Hulme and Trevethick 2010). Irrespective of the assay type, it is necessary to ensure that the labeled ligand is actually bound to the receptor. There are always two components of ligand binding (also called total binding): a specific component that describes ligand binding to the receptor of interest, and a nonspecific component that is affected by binding to other sites (Lazareno 2001). In addition to binding to the receptor, most ligands will bind to a greater or lesser extent to cell membranes, other proteins, plastic or glassware etc. (Flanagan 2016). Nonspecific binding is usually measured in the presence of an unlabeled ligand, which ideally only binds to the receptors of interest and therefore prevents binding of a labeled ligand to these specific sites. Specific binding cannot be directly measured and is rather calculated as a difference between total and nonspecific binding (Lazareno 2001).

Development of the binding assay includes several overlapping, interactive and recursive stages, like initial choices of receptor source, labeled ligand and assay conditions, optimization, validation, application to novel ligands and quantitative analysis of the results (Hulme and Trevethick 2010). However, the availability of labeled ligands greatly limits the application of ligand binding assays (Hein *et al.* 2005). Therefore, development and characterization of novel fluorescent- or radioligands is an important part of GPCR research.

1.2.1. Radioligand binding assay

Radioligand binding assay developed by Paton and Rang (Paton and Rang 1965) has been one of the most widely used methods in GPCR research. It is also simple to perform – a preparation of the receptor is incubated together with a radiolabeled ligand. After that, the receptor-bound fraction of the radioligand will be quantified since the free radioligand will be separated by filtration. There are various radioisotopes, like ^{3}H, ^{14}C, ^{32}P, ^{35}S or ^{125}I available that can be used for labeling ligands with minimal modifications of the chemical structure and therefore having no effect on the affinity of the ligand for the receptor. Many high affinity ligands are commercially available allowing to set-up an assay rather quickly. Among these, ^{3}H- and ^{125}I-labeled ligands are used most frequently (Hein *et al.* 2005). Iodinated ligands have high specific activity, short half-life (60 days) and high-energy γ-radiation making the assay very sensitive (Flanagan 2016). As a disadvantage, incorporating ^{125}I into the structure of the ligand may change its affinity, particularly if it is a low molecular weight compound (Hein *et al.* 2005). Therefore, iodine is more often used for labeling peptides. The key advantage of tritium is that it does not change the molecular structure of the ligand and therefore ^{3}H is most frequently used to label smaller compounds, such as dopaminergic ligands. These ligands can be used for long time due to the long half-life (12.3 years) of ^{3}H. Additionally, this isotope emits low-energy β-radiation that combined with low specific activity results in somewhat lower detection efficiency but is safer to use (Flanagan 2016). However, several ^{3}H atoms could be incorporated into the structure of the ligand and this enables to detect affinities in subnanomolar range.

While radioactivity-based assays are fast, sensitive, easy-to-use and reproducible they also have some drawbacks: hazardous to human health, produce radioactive waste, require special laboratory conditions and licenses and are rather expensive (de Jong *et al.* 2005). One of the major disadvantage, limiting the use of radioligand binding assay for high-throughput screening, is the need to separate free ligand from its receptor-bound form. To overcome this problem, scintillation proximity assay (Hart and Greenwald 1979) can be used. It is a homogeneous assay in which only the radioligand binding to GPCR immobilized on the surface of beads can activate the scintillation beads that produce photons detectable with a scintillation counter. Thus, this assay type enables to carry out binding experiments without washing or filtration steps (Zhang and Xie 2012).

Usually, the radioligand binding assay is performed with homogenized tissue or cell membrane preparations or with intact cells. In this case, free radioligand can be separated from the receptor-bound fraction by filtration or centrifugation. With detergent-solubilized receptors more specialized approaches, for example gel filtration (Rinken *et al.* 1994), must be used. Binding assays may also be performed with cells attached to culture dishes or with tissue slices attached on slides. After incubation, free radioligand can be simply removed with the medium. This can improve automation of the assay but contravenes with the theoretical requirement that both the receptor and ligand should be freely diffusible (Flanagan 2016).

1.2.2. Fluorescent ligand binding assay

Optical methods, like colorimetric, fluorescence or (chemo-/bio-)luminescence detection systems, have emerged as alternatives to radioactivity-based assays (de Jong *et al.* 2005). Among these, spectroscopic methods, for example assays based on color development, are not very sensitive and selective compared to fluorescence or luminescence measurements. An ideal assay for GPCR ligand screening should be easy-to-perform, sensitive, nonradioactive, robust, homogenous and easily adaptable to a microtiter plate for robotic automation (Zhang and Xie 2012). These criteria are met with fluorescence-based methods, which also enable monitoring of ligand binding in real time. During the last decades there has been a remarkable growth in the use of fluorescence in biochemistry due to fluorophores with enhanced brightness, greater photostability and improved physical properties like pH stability and water solubility (Hertzberg and Pope 2000). The choice of the fluorescent dye for labeling ligands is critical in assay development. For low molecular weight ligands, the size of the dye, use of a linker and its length as well as the position of the fluorophore are of major importance (de Jong *et al.* 2005). A bulky fluorophore may lead to significant changes in binding properties, e.g. result in lower affinity. This is the major limiting factor in development of fluorescence-based assays. Therefore, the labeled ligand should always be considered as a novel compound that should be thoroughly characterized.

Fluorescence measurements can provide information on a wide range of molecular processes, like the interaction of solvent molecules with fluorophores, rotational diffusion of biomolecules, distances between sites of biomolecules, conformational changes, and binding interactions (Lakowicz 2006). As with any method, fluorescence-based techniques also have some limitations: fluorescence emission can be quenched or scattered and high background autofluorescence interferes with detection of specific fluorescent signal. The latter can be reduced by using dyes with high excitation wavelength. Higher sensitivity and precision can be achieved with long lifetime lanthanides (Eu, Tb), because the background signals tend to have shorter life-times (de Jong *et al.* 2005). Promising results have been also obtained with Förster resonance energy transfer (FRET) based methods, but this usually requires labeling of both the receptor and the ligand (Rinken *et al.* 2018). Another possibility is to determine the binding of fluorescent ligand to the GPCR by monitoring decrease in its lateral mobility. This can be measured with fluorescence correlation spectroscopy, that detects fluctuations in fluorescence intensity upon diffusion of fluorescent ligand through a small detection volume (Briddon and Hill 2007). Additionally, receptor-bound fluorescent ligand also has less rotational freedom when compared with the free ligand and this can be detected by fluorescence anisotropy (FA) that is commonly used in biochemical measurements (Rinken *et al.* 2018). It is based on the phenomenon that upon excitation with polarized light, fluorophores whose dipole is parallel to the plane of polarized light will absorb and emit light. This will result in partially polarized

emission and the degree of polarization depends on the fluorophore's freedom of movement within its lifetime (Lakowicz 2006). Thus, binding of low molecular weight fluorescent ligand to a larger receptor causes an increase in FA that is calculated as follows:

$$FA = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities of the parallel and perpendicular components of the emitted light respectively. Denominator of the fraction ($I_{\parallel} + 2I_{\perp}$) corresponds to total fluorescence intensity (TFI) of the emitted light. One of the most critical steps in FA assay is labeling of the ligand – in addition to retaining high affinity, coupling of the fluorophore to the pharmacophore should be rigid enough so that the fluorophore cannot rotate freely upon fluorescent ligand binding to the GPCR. Moreover, properties of the fluorescent dye also have to meet the FA requirements. One important parameter to be considered is fluorophore's lifetime – for low molecular weight ligands it should remain between 2 – 5 ns. Other important properties of the fluorescent dyes include high extinction coefficient, quantum yield, emission wavelength and stability as well as low bleaching and nonspecific binding (Rinken *et al.* 2018). These criteria are largely met by the fluorescent dyes Cy3B and Bodipy FL that are employed in current thesis.

The homogeneous nature of FA assay makes it a so-called mix-and-measure type technique, which is often an advantage when it comes to assay automation and miniaturization for high-throughput applications. Additionally, high-quality data obtained from FA assay enables to determine the affinities of the fluorescent ligand and competitive ligands as well as kinetic parameters for labeled and unlabeled ligands. However, for good measurement window, the concentrations of the fluorescent ligand and the receptor need to be comparable to cause significant depletion of the labeled ligand as a result of the binding process (Nosjean *et al.* 2006). Such receptor concentrations are not present in native tissues and therefore overexpression systems need to be used.

1.3. Expression systems

One of the important aspects of ligand binding assays is the source of receptors as the system where the interactions are studied in. If we want to study the effects of drugs designed for humans, then the best choice would be testing in human subjects. Historically, experiments were often performed on humans. For example, in 1805 Friedrich Serturner administered a dose of morphine, an alkaloid isolated from opium, to himself and his friends (Scheindlin 2001). All of them experienced symptoms of severe opium poisoning for several days. Unfortunately, research involving humans is littered with a history of scandals and unethical experimentation. Nowadays, experimenting on humans in strictly

regulated and is usually carried out only with promising drug candidates during clinical studies. More commonly various test animals, especially mammals are used in pharmacological studies. Mice are often preferred because of their small size, ease of breeding, and short generation time (Scheindlin 2001). Also, rats, guinea pigs, rabbits and dogs are used, depending on the tests performed. Experiments could be performed on live animals, for example to test the toxicity of new drugs or to conduct behavioral experiments to investigate the effects of various compounds on central nervous system. More often pharmacology employs the use of isolated organs or tissues, which could also be used to perform ligand binding experiments on native receptors. However, most of the initial pharmacological screening is currently performed with various types of cells. Cells are a good model system providing a consistency and reproducibility of results that cannot always be achieved with the abovementioned options. Ligand binding experiments could be performed on live cells (intact cells) or on cell membrane homogenates.

The low natural abundance of GPCRs limits the use of native tissues for ligand binding assays. To overcome this problem, various overexpression systems that enable the production of recombinant protein have been developed and successfully applied. This can be done by introducing foreign genetic material into the chosen cell line. In the case of eukaryotic cells, the process is called transfection which can be further divided into two different types: transient and stable. Transient transfection is one of the most employed method for delivering genes inside the cell. With this method, foreign gene is expressed for a limited time and the gene is not incorporated into the cell's genome (Kim and Eberwine 2010). The mayor advantage of transient transfection is its quickness – it takes only a few days to go from a purified genetic material to the expressed protein (Andréll and Tate 2013). The drawback of this method is that it is difficult to achieve high transfection levels and invariable transfection efficiency. However, this is not a problem in some cases, for example in microscopy experiments where only transfected cells could be chosen for imaging. Contrarily, in stably transfected cells the foreign gene becomes part of the genome and therefore transgene expression is maintained even after replication (Kim and Eberwine 2010). Although creation of high producing stable line takes months to develop, after successful establishment the expression of protein is fast, robust and usually with high yield (Nettleship *et al.* 2010).

There are several different techniques available for introducing the protein encoding gene into a cell. These methods can be broadly classified into three groups: chemical, physical and biological. Some of the widely used chemical methods involve transfection reagents like cationic polymers, calcium phosphate and cationic lipids. Physical methods include electroporation and microinjection and biological approaches include the use of various viruses (Kim and Eberwine 2010). Ideally, DNA should be delivered with high efficiency, low cell toxicity and minimal effects on normal physiology. Other important criteria include ease of use and reproducibility. However, each of the

abovementioned methods possess its own advantages and disadvantages and thus must be chosen according to certain purpose and cell type.

One of the most critical steps of recombinant protein expression is the choice of cell line, which should be selected according to the final goal bearing in mind that for a certain application one system may be more suitable than another. The closest alternative to GPCR native environment can be achieved in mammalian cells and therefore this system is widely used for functional studies in cases where correct post-translational modifications and natural signal transduction components are necessary for ligand recognition and receptor signaling (Nettleship *et al.* 2010). Correct *N*-glycosylation, post-translational machinery and molecular chaperones as well as a suitable lipid environment can be guaranteed while using mammalian cells (Andréll and Tate 2013). A lot of decisions need to be made while working with mammalian cell lines. First, a choice between immortalized cultured cell lines and primary cells must be made. For decades, cultured cell lines have played a critical role in scientific achievement, yet researchers have become increasingly concerned about the clinical relevance of these cells. Therefore, more attention is turned to primary cells that have been isolated directly from human or animal tissue. These cells are physiologically more relevant and generally maintain the characteristics (function, morphology, protein expression) of the parent tissue (Pappas 2010). Unfortunately, this great advantage is accompanied by some drawbacks: protocols for isolating primary cells are more complex, they have a finite life span, the cells are very sensitive and need optimized culture conditions and careful handling, growing might be more time-consuming and expensive (Bury *et al.* 2014, Pappas 2010). For these reasons, primary cells have not yet replaced immortalized cell lines that have undergone mutations allowing continuous division, such as tumor cells. The cultured cells are easy to grow and obtain, relatively cheap and GPCRs can be stably expressed with rather high expression levels. A widely used example of mammalian cells is human embryonic kidney (HEK293) cell line. It was derived from primary kidney cells four decades ago by transformation with fragments of adenovirus type five DNA (Graham *et al.* 1977). Although HEK293 cells are isolated from kidney, it has been shown to possess characteristic of neuronal cells and therefore this cell line is especially suitable for studying central nervous system receptors (Shaw *et al.* 2002). This cell line has many advantages, like quick reproduction, relatively easy maintenance, high efficiency of transfection and protein production, and reliable translation and processing of proteins (Thomas and Smart 2005). Furthermore, natural presence of mRNA for 28 GPCRs has been demonstrated in HEK293 cells, implying that corresponding signaling pathways are operational (Shaw *et al.* 2002).

In addition to mammalian cells, wide variety of cell lines derived from lower organisms are also available. Among these, insect, yeast and bacteria are used more frequently, especially if an expression system without interfering GPCRs and G proteins is preferred. For structural studies, GPCRs have been expressed in bacteria and yeast, which provide high levels of protein, are easy to scale-up and inexpensive to grow. Prokaryotic nature of the bacteria is associated with

drawbacks of the system, such as inability to perform necessary post-translational modifications, lack of G proteins, problems with protein folding, and in some cases low expression levels or truncated forms of the receptor. Yeast cells are capable to perform post-translational modifications similar to more complex eukaryotic cells, but the *N*-glycosylation of mammalian membrane proteins is inefficient, and existence of cell wall may hinder recovery of non-secreted proteins (Massotte 2003). Insect cells are often used to produce high amounts of GPCRs for crystallization studies, due to the easy and effective large-scale expression and eukaryotic protein processing capabilities. Researchers have found that it is easier to overexpress functional mammalian membrane protein in insect cells rather than in bacteria (Andréll and Tate 2013). Still, not all the post-translational modifications are similar to those of higher eukaryotes and special caution should be taken while working with glycoproteins (Kost *et al.* 2005). Efforts have been made to overcome this problem by developing an insect cell line that has a more mammal-like glycosylation pattern (Aumiller *et al.* 2012).

Insect cell expression system usually includes the use of invertebrate-specific viruses, called baculoviruses (BVs) and referred to as baculovirus expression vector system that is one of the most versatile eukaryotic expression systems available for protein production. The principal BV used for GPCR production is *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) with *Spodoptera frugiperda* (Sf9) cells derived from the ovarian tissue of fall armyworm as the host (Nettleship *et al.* 2010). First, protein encoding cDNA is inserted into a plasmid transfer vector under a strong polyhedrin promoter. Polyhedrin is naturally produced at very high levels to protect the BV particles from the environment and is nonessential for viral propagation in cell culture and can therefore be replaced by gene of interest (Massotte 2003). Next, site-specific transposition of the plasmid into a BV shuttle vector (bacmid) takes place (Luckow *et al.* 1993, Ciccarone *et al.* 1998). The bacmid is then propagated in *Escherichia coli* bacteria, purified and transfected into Sf9 cells to generate recombinant BVs. After that, the BV stock will be amplified to obtain a high-titer virus that can be used to infect cells for large-scale expression of the recombinant protein. One of the important steps in BV preparation is to determine the amount of infectious viral particles in high-titer virus prior protein production. This is necessary because one important infection parameter for protein expression is multiplicity of infection (MOI) that corresponds to the number of virus particles per cell. Optimal MOI should be established for each virus, medium, reactor and cell line to achieve the highest expression of active protein (Invitrogen Life Technologies 2013).

BVs infect many different insect species but do not propagate in any non-invertebrate hosts, including humans. The ease of use and low risk (Biosafety level 1) have been major factors in the widespread application of baculovirus-insect cell expression system (Kost *et al.* 2006). BVs enter the cells via facilitated endocytosis or fusion, followed by uncoating and replication of viral DNA and production of recombinant GPCRs on the surface of Sf9 cells. During

the BV infection cycle virus particles start to bud from the Sf9 cell, taking a part of the host's membrane, including membrane proteins with them. Thus, baculovirus-insect cell expression system represents a versatile tool for displaying recombinant GPCRs on the surface of Sf9 cells as well as on budded BV particles (Mäkelä and Oker-Blom 2008). These budded BVs are rod-shaped particles (approximately 200–400 nm in length and 40–50 nm in diameter) that could be separated from Sf9 cells by centrifugation and employed as a source of receptors for ligand binding assays (Veiksina *et al.* 2014). Furthermore, the GPCRs displayed on the surface of budded BV particles are in their native conformation, orientation and environment. Using these particles ensures that only fully matured and membrane-bound proteins are present in the assay. Still, it should be taken into consideration that the lipid composition of insect cells and mammalian cells differs, especially by the sterol content: mammalian cells contain predominantly cholesterol, while insect cells have ergosterol (Mäkelä and Oker-Blom 2008).

Even though BVs cannot replicate in mammalian cells, recombinant viruses containing mammalian cell-derived expression cassettes can function as gene delivering agents (Kost *et al.* 2006). This is called the BacMam system and it has many advantages such as high transduction rates, low cytotoxicity to host cells, expression level adjustment by amount of virus used, compatibility with various cell lines and ease of handling due to low biosafety level (**PAPER II**, Kost and Condreay 2002, Kost *et al.* 2006).

Finally, ligand binding experiments can also be performed with solubilized and purified receptors. Usually, a recombinant receptor is expressed in one of the previously described cell lines and then purified for further experiments. A serious obstacle is the requirement to extract the receptors from their native environment in the plasma membrane, coupled with the inherent instability of GPCRs in the detergents required for their solubilization (Jamshad *et al.* 2015). After purification it is often necessary to reconstitute them into a lipid structure such as a liposome. Upon success, receptors could be studied in a controlled environment and detailed information about molecular mechanisms underlying ligand binding, receptor activation and downstream signaling could be obtained. Recently, considerable progress has been made to visualize GPCRs and their signaling complexes at the structural level. According to GPCR database, 50 unique GPCR crystal structures are currently available and the number is increasing exponentially.

Dopamine receptors were the focus of this study, because these receptors are implicated in many neurological processes and abnormal signaling can lead to several neurological and psychiatric disorders. Our approach included development of novel assay systems to characterize ligand binding to different subtypes of dopamine receptors in various sources of receptors. Experiments were carried out with native GPCRs (tissue homogenates) and with various recombinant protein expression systems (mammalian and insect cells, budded baculovirus particles). Several methods were implemented to study receptor-ligand interactions with the emphasis on fluorescence-based assays.

2. AIMS OF THE STUDY

General aim of this study was to find novel possibilities to study the properties of dopamine receptors. The thesis mostly focused on dopamine D_1 receptors and implementing fluorescence-based methods to gain further insight about receptor-ligand interactions. The study design included several subtasks:

- To investigate possible link between expression of endoplasmic reticulum transmembrane protein wolframin and dopamine D_1-like receptor.
- To determine involvement of dopaminergic receptors in behavioural effects caused by the peptide lunasin.
- To validate the use of budded baculovirus particles as a source of receptors for radioligand binding assay.
- To implement fluorescence anisotropy-based assay for studying ligand binding to dopamine receptors.
- To characterize and quantify fluorescent ligand binding to dopamine receptors in live cells.

3. MATERIALS AND METHODS

3.1. Reagents and cell lines

Spodoptera frugiperda (Sf9) cells were obtained from Invitrogen Life Technologies and maintained as a suspension culture in a serum-free insect cell medium EX-CELL 420 (Sigma-Aldrich) without antibiotics at 27 °C in a non-humidified incubator. Human embryonic kidney cells (HEK293) stably expressing human wild-type dopamine D_1, D_{2L} or D_3 receptors were generated by Dr. Reet Reinart-Okugbeni as described in Reinart-Okugbeni *et al.* 2012. The cells were grown as an adherent monolayer on Petri dishes (Thermo Scientific, BioLite) in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose supplemented with 10% fetal bovine serum (both from Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin (NAXO, Smart Media) and 400 µg/ml geneticin (PAA Laboratories) at 37 °C in a humidified incubator with 5% CO_2. Density and viability of HEK293 and Sf9 cells were determined with the addition of 0.2% trypan blue (BioTop) using an Automated Cell Counter TC20™ (Bio-Rad Laboratories).

NaCl, KCl, KOH, KH_2PO_4, ethylendiaminetetraacetic acid (EDTA), $MgCl_2$, $CaCl_2$, NaOH, 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), HCl, dimethyl sulfoxide (DMSO), dithiothreitol (DTT) were from Applichem. Na-HEPES was obtained from Amresco, Pluronic F-127 was from Sigma-Aldrich and bovine serum albumin (BSA) was purchased from PAA Laboratories. Complete EDTA-Free Protease Inhibitor Cocktail was used according to the manufacturer's description (Roche Applied Science). Dopaminergic ligands apomorphine, dopamine and (+)-butaclamol were purchased from Sigma-Aldrich. 7-methyl-6,7,8,9,14,15-hexahydro-5*H*-benz-[*d*]indolo[2,3-*g*]azecine (LE300), (1*R*)-6-chloro-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine-7,8-diol (SKF81297), 1-phenyl-2,3,4,5-tetrahydro-1*H*-benzo[*d*]azepine-7,8-diol (SKF38393), (5*R*)-8-chloro-3-methyl-5-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine-7-ol (SCH23390), (6a*S*,13b*R*)-11-chloro-7-methyl-6,6a,7,8,9,13b-hexahydro-5*H*-benzo[*d*]naphtho[2,1-*b*]azepin-12-ol (SCH39166), (1*R*,3*S*)-1-(aminomethyl)-3-phenyl-3,4-dihydro-1*H*-isochromene-5,6-diol (A68930) and spiperone were from Tocris. *R*(-)-propylnorapomorphine (NPA) was obtained from Research Biochemicals International and 2-methoxy-10,11-dihydroxy-*N*-propylnorapomorphine (MNPA) was from PharmaSynth. Radioligands [^{3}H]SCH23390 (specific activity 84.3 Ci/mmol or 81.9 Ci/mmol), [^{3}H]Raclopride (74.0 Ci/mmol or 70.5 Ci/mmol), [^{3}H]WAY10063 (74.0 Ci/mmol) and [^{3}H]NMS (84.1 Ci/mmol) were from PerkinElmer. Total protein concentrations of the samples were determined with Bradford protein assay kit (Bio-Rad Laboratories) according to the manufacturer's protocol. The fluorescent ligand BodipyFL-SKF83566 (CellAura # 200773) specific for dopamine D_1 receptors was developed in the University of Nottingham, UK and was kindly provided by Professor Stephen Hill.

3.2. Receptor preparations

3.2.1. Mice brain hippocampal tissue

Wild-type (wt) C57BL/6 (Scanbur) and wolframin (Wfs1) knockout mice were housed under standard laboratory conditions as described in **PAPER III**. Wfs1-deficient mice do not suffer from gene inactivation and studies with these mice have been approved by the Estonian National Board of Animal Experiments. Obtaining mice tissue was performed after rapid execution and no manipulations with the animals occurred before. For radioligand binding experiments, the hippocampi were dissected on ice immediately after decapitation, frozen in liquid nitrogen and stored at -90 °C. All these procedures were carried out by Dr. Triin Tekko and her colleagues at the Institute of Biomedicine and Translational Medicine, University of Tartu.

Hippocampal membranes were prepared as described in Tõnissaar *et al.* 2008 with some modifications. Briefly, hippocampal tissue of wt or Wfs1 knockout mouse was homogenized in 1 ml of ice cold homogenization buffer (HB: 50 mM Tris-HCl, pH = 7.4) with a Bandelin Sonopuls sonicator for 3 × 10 s cycles. Membrane suspension was then collected by centrifugation at 30 000 × g for 20 min at 4 °C (Sigma 3K30, SIGMA Laborzentrifugen). After that, supernatant was discarded and the obtained pellet was rehomogenized in 1 ml of HB. This washing procedure was repeated three times and the final homogenization of membrane suspension was done in 50 ww/v of the incubation buffer (IB: 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, pH = 7.4). The final suspensions (20 mg tissue/ml) were stored at -90 °C until further use.

3.2.2. Sf9 cells and budded baculovirus particles

Recombinant viruses of dopamine D_1 and D_3 receptors were used in this work and constructed based on a Bac-to-Bac® Baculovirus Expression System (Invitrogen Life Technologies). The pcDNA3.1(+) expression vectors (Invitrogen Life Technologies) encoding human wild-type dopamine receptors D_1 or D_3 were purchased from the cDNA Resource Center. Both genes were cloned into pFastBac1 vectors under the strong polyhedrin promoter to achieve high-level protein expression. The obtained constructs were transformed into competent DH10Bac cells (Invitrogen Life Technologies) to generate recombinant bacmids. Purified and PCR-verified bacmids were then transfected into Sf9 cells with a transfection reagent ExGen 500 (Fermentas) according to the manufacturer's protocol. These low-titer viral stocks were collected and used to infect Sf9 cells (1.5×10^7 cells) to produce passage P1 BVs, which were further amplified to gain high-titer BVs that could be used for protein production.

3.2.2.1. Determination of baculovirus titers

BV titers were estimated with a viable cell size-based titration method (**PAPER II**). First, Sf9 were seeded to a 24-well cell culture plates (Thermo Scientific, BioLite) at a density of 2×10^5 cells/well in 250 µl of EX-CELL 420. After 30–60 minutes of incubation at 27 °C cells were infected with 250 µl of 3-fold serial dilutions of the harvested virus. Each virus dilution was pipetted to the plate in duplicates and the Sf9 cells were incubated with the virus for 24 hours. After that, cells along with the supernatant (500 µl) were transferred to a vial containing 9.5 ml of ISOTON II Diluent (Beckman Coulter) and average cell diameter was measured with Cell and Particle Counter (Z2 Series Coulter Counter, Beckman Coulter). To determine the concentration of infectious viral particles (ivp) a sigmoidal dose-response curve was fitted to a data obtained by plotting average cell diameter versus virus dilution factor. The virus titer was calculated by assuming that a cell can be infected by only one BV particle (Janakiraman *et al.* 2006, Laasfeld *et al.* 2017) with the following equation:

$$\text{Virus concentration (ivp/ml)} = \frac{N}{2 \times EC_{50} \times V} \quad (2)$$

where N – number of cells in single well at the time of infection (here 200 000); EC_{50} – virus dilution at which the average cell diameter has changed 50%; V – the solution volume of a single well (here 0.5 ml).

3.2.2.2. Production of receptors

For the generation of membrane preparations and budded baculoviruses, Sf9 cells were infected with a high-titer recombinant BV at a density of 2.0×10^6 cells/ml at multiplicity of infection MOI = 5. In case of Sf9 membrane preparations, cells were grown for 48 h until the viability of the cells had dropped to approximately 90%. After that, cells were harvested and centrifuged at 1000 × g for 5 min at room temperature. The pellet was stored at -90 °C until further preparation of membranes (described in 3.2.3).

To produce BV particles, cells were collected after the viability had decreased below 50% (approximately 72 hours) by centrifugation at 1000 × g for 10 minutes. After that, the supernatant was collected and centrifuged again for 45 minutes at 48 000 × g at 4 ºC. The pellets were first washed and then resuspended in assay buffer (AB: 11 mM Na-HEPES, 0.1% Pluronic F-127, 0.1 mg/ml BSA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM KCl, 135 mM NaCl and Protease Inhibitor Cocktail, pH = 7.4). Final volume of the AB was chosen so, that the BV preparation was concentrated 27 times in comparison of the initial cell suspension volume. The samples were then aliquoted and stored at -90 ºC until used for experiments.

3.2.3. HEK293 cells stably expressing dopamine receptors

Membrane preparations were produced from the HEK293 cells stably expressing either dopamine D_1, D_{2L} or D_3 receptors. HEK293 cells at approximately 95% confluency were harvested in Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca^{2+} and Mg^{2+} (NAXO, Smart Media) and centrifuged at 1000 × g for 5 minutes at room temperature. The pelleted cells were stored at -90 °C until preparation of membrane suspensions.

The cell membranes were prepared as described previously (Reinart-Okugbeni *et al.* 2012). Briefly, Sf9 or HEK293 cells (about 1.5×10^8 cells/tube) were melted on ice, washed with 20 ml of ice-cold HB and collected by centrifugation at 800 × g for 5 min at 4 °C. In case of Sf9 cells, protease inhibitor cocktail (Roche Applied Science) was added to the HB according to manufacturer's instructions. Next, the cells were resuspended in 20 ml of HB and homogenized with a homogenizer (Coleparmer Labgen 125) for at least 30 s. After that, membranes were collected by centrifugation at 30 000 × g for 20 min at 4 °C. The latter washing/centrifugation step was repeated once more, and the obtained membrane pellets were finally homogenized in IB so that a concentration of $c \approx 10^7$ cells/ml would be achieved. Obtained Sf9 and HEK293 membrane preparations were stored in 1 ml aliquots at -90 °C until further use.

3.3. Ligand binding assays

3.3.1. Radioligand binding assay

All the radioligand binding experiments were performed on round bottom 96-well plates (Greiner) and the reactions were carried out in a final volume of 250 µl per well. Assay buffer was IB supplemented with 1 mM of DTT just before the experiment. In the following subsections, preparation of samples and concentrations of reagents is described in detail for each particular membrane sample. However, all the procedures performed after the samples had already been carried to the assay plate remained the same. The plates with samples were incubated for 60 min (in case of saturation binding experiments) or for 90 min (competition binding experiments) at 25 °C. The reactions were stopped by separating free radioligand with rapid filtration through thick GF/B glass fiber filtermats (Perkin Elmer) using FilterMate Harvester (model D961962, PerkinElmer). Filters were then washed 5 times with an ice-cold washing buffer (WB: 20 mM K-phosphate, 100 mM NaCl, pH = 7.4), after which the filters were dried in a microwave oven at 800 W for 2 minutes. Solid scintillant MeltiLex™ B/HS was then impregnated into the filter using a MeltiLex™ Heat-sealer and filter-bound radioactivity was counted with a Wallac MicroBeta TriLux 1450 LSC Luminescence Counter (all from PerkinElmer). Total concentrations of the radioligand dilutions were determined in vials with 3 ml of liquid scintillation cocktail OptiPhase HiSafe (PerkinElmer).

3.3.1.1. Mice hippocampal membranes

In saturation binding experiments the hippocampal membranes of six mice from either wt or Wfs1 knockout group were pooled and used at a concentration of 20 mg tissue/ml. The membranes (3 mg tissue/well) were incubated with increasing concentrations of radioligand [^{3}H]SCH23390 in the absence (total binding) or in the presence (nonspecific binding) of 10 μM (+)-butaclamol. Data are obtained from three independent experiments performed in duplicates.

To estimate the number of binding sites of D_1-like receptors in individual wt and Wfs1 knockout mice, specific binding of 4 nM [^{3}H]SCH23390 was determined. For that, tissue concentration of 6.7 mg/ml (1 mg tissue/well) was used. All these experiments were carried out at least in duplicates for 22 wild-type and 24 Wfs1-deficient mice.

3.3.1.2. Sf9 and HEK293 cell membrane preparations and budded baculovirus particles

In saturation binding experiments, 150 μl of receptor preparations were added to a solution containing 50 μl of different concentrations of [^{3}H]SCH23390 (final 0.01–9 nM) and 50 μl of IB (for total binding) or 1 μM (+)-butaclamol (final concentration, for nonspecific binding). All 4 to 6 repetitive experiments were performed in duplicates.

For competition binding experiments, the receptor preparations were incubated with different serial dilutions of unlabeled ligands and with a fixed concentration of radioligand (near it's K_D value). The competition binding experiments (n = 2–3) were performed in triplicates. Inhibition constant (K_i) values were calculated from competition binding curves according to the Cheng-Prusoff equation (Cheng and Prusoff 1973) that enables to calculate the affinity for one-site non-cooperative binding model.

3.3.2. Fluorescence anisotropy assay

Fluorescence anisotropy experiments were performed with dopamine D_1 receptor selective fluorescent ligand BodipyFL-SKF83566. Concentration of the ligand was estimated by measuring absorbance at 507 nm using an UV-1800 spectrophotometer (Shimadzu). An assumption that extinction coefficient did not change when fluorophore was attached to the ligand was made in order to calculate the concentration of the ligand. After that, aliquoted stocks of the fluorescent ligand were stored at -20 °C in DMSO and further diluted in assay buffer before an experiment.

All the experiments were performed with black 96-well half area, flat bottom polystyrene NBS microtiter plates (Corning, Product No. 3993) as these have been found to give low background fluorescence as well as low adsorption of ligands onto the plastic surface (Veiksina *et al.* 2010). FA measurements were

carried out in a final volume of 100 µl/well at 27 °C using a PHERAstar plate reader (BMG LABTECH) with polarized excitation and dual emission, which allows to simultaneously record intensities that are parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the plane of excitation light. For BodipyFL-SKF83566 excitation at 485 nm (filter bandwidth 10 nm) and emission at 520 nm (filter bandwidth 10 nm) was used. Sensitivities of these two emission channels (G-factor) were corrected with erythrosine B as a standard (Thompson *et al.* 2002). Additionally, all the experiments included blank sample wells, where no fluorescence ligand was added, but same amount of BV was used as in other assay points.

In saturation binding assays two different concentrations of a fluorescent ligand were used in the presence of 8 µM (+)-butaclamol for nonspecific binding or in absence of (+)-butaclamol in case of total binding. Finally, serial dilutions of BV particles expressing dopamine D_1 receptors were added to all the wells to start the binding reaction. Altogether, three independent saturation binding experiments were carried out in duplicates.

To determine the kinetic properties ligand binding was initiated by addition of BV preparation containing D_1 receptors to a given concentration of fluorescent ligand (with or without 1 µM (+)-butaclamol) and the reaction was monitored in time. Dissociation was initiated after completion of the association reaction by addition of (+)-butaclamol (final c = 1 µM) or IB. Because the association and dissociation kinetics were fast no replicates were used and experiments were rather repeated several times (n = 5).

In competition binding experiments, fixed concentrations of the fluorescent ligand and BV particles were incubated with serial dilutions of various unlabeled ligands. Measurements were performed in duplicates and at least three independent experiments were carried out with each ligand.

Blank corrected fluorescence anisotropy was calculated from fluorescence emission intensities measured parallel and perpendicular to the plane of excitation light as described in detail in Veiksina *et al.* 2014. Software Aparecium 2.0 developed in our laboratory by Tõnis Laasfeld and available at http://www.gpcr.ut.ee/software.html was used for kinetic data acquisition and transformation. The affinity parameters (K_D of BodipyFL-SKF83566 and K_i values of unlabeled compounds) were calculated as described in Veiksina *et al.* 2014.

3.3.3. Fluorescent ligand binding to intact cells

These experiments were performed with a novel fluorescent ligand NAPS-Cy3B (**Figure 2**) synthesized by Mihkel Ilisson at the Chair of Organic Chemistry, University of Tartu. The ligand is a conjugate of the fluorophore Cy3B and D_2-type receptors' selective antagonist *N*-(p-aminophenethyl)-spiperone (NAPS). An amide formation reaction with Cy3B NHS-ester (GE Healthcare) and NAPS (Carbosynth Ltd) was carried out to couple the ligand with the

fluorescent dye. Desired compound was purified by HPLC, and its identity and purity were further confirmed by HPLC-MS (reaction yield 5.1 %).

HEK293 cells stably expressing dopamine receptors were grown as described previously in chapter 3.1. Trypsin-EDTA (0.005/0.002%, NAXO, Smart Media) was used for cell passaging to gain homogenous distribution of the cells on the Petri dish and to minimize the formation of cell aggregates. At the day of the experiment cells were seeded to a black 96-well Ibidi µ-plate at a density of 20 000 cells/well in 150 µl of DMEM. Cells were incubated at 37 °C for 5 h and after that the cell culture medium was replaced with the same volume of DPBS (with Ca^{2+} and Mg^{2+}, Sigma-Aldrich) followed by addition of ligands.

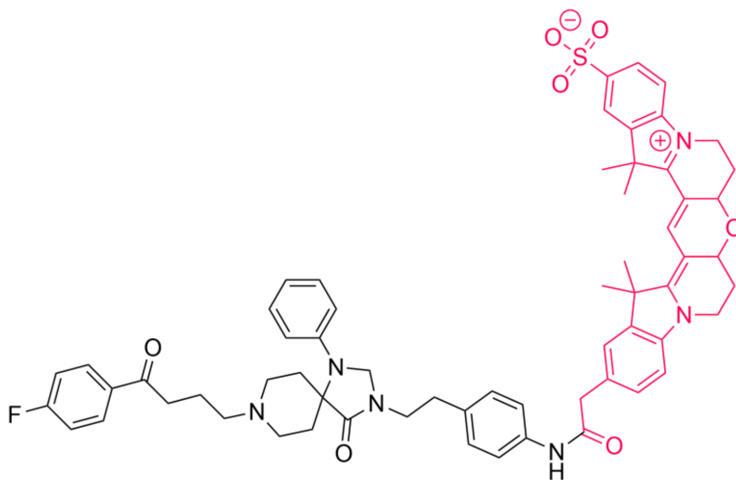

Figure 2. Chemical structure of the fluorescent ligand NAPS-Cy3B that is selective for D_2-like receptors. The ligand is a conjugate of *N*-(p-aminophenethyl)-spiperone (black) and the fluorophore Cy3B (red).

The concentration of the NAPS-Cy3B solution was determined spectrophotometrically by measuring absorbance at 574 nm with the assumption that extinction coefficient of the fluorophore remains unchanged when it is coupled to the ligand. Aliquoted stocks of the fluorescent ligand were stored at -20 °C in DMSO and further diluted in DPBS before an experiment. Final concentration of NAPS-Cy3B in the experiments was 1 nM and nonspecific binding was measured in the presence of 1 µM (+)-butaclamol. For the competition experiments, serial dilutions of unlabeled ligands (dopamine or (+)-butaclamol) were added to wells containing HEK293 cells and 1 nM NAPS-Cy3b. Bright-field and fluorescence microscopy images were obtained with Cytation 5 Imaging Multi-Mode Reader (BioTek), which has high-content imaging capabilities. Images were obtained using a LUCPLFLN 20× objective lens (WD 6.6, NA 0.45) (Olympus). For fluorescent images the excitation was performed with a 523 nm

LED cube together with the RFP filter having excitation at 531 nm (bandwidth 40 nm) and emission at 593 nm (bandwidth 40 nm). The average fluorescence intensity of membrane pixels was estimated using the membrane detection algorithm incorporated into software Aparecium 2.0 (http://www.gpcr.ut.ee/software.html).

All the data were analyzed using GraphPad Prism 5.0 (GraphPad Software) and are presented as mean ± S.E.M. if not stated otherwise. Statistically significant differences were determined by Student's t-test, where $P < 0.05$ was taken as the criterion of significance.

4. RESULTS AND DISCUSSION

4.1. Influence of wolframin expression to dopamine D_1-like receptor levels in mouse hippocampal membranes

Wolframin is a glycoprotein located in the membrane of endoplasmic reticulum and loss of its function causes a rare disease called Wolfram syndrome (Hofmann *et al.* 2003). The symptoms of this autosomal recessive neurodegenerative disorder include diabetes insipidus, diabetes mellitus, optic atrophy and deafness (Rigoli *et al.* 2011). Additionally, neurological complications and psychiatric disorders are frequently occurring among patients with Wolfram syndrome (Swift *et al.* 1990). The mechanisms causing these latter symptoms are largely unknown and therefore it is necessary to further investigate the reasons of abnormal brain functioning.

The radioligand binding assay is a sensitive assay, which provides quantitative about expression levels of GPCRs and affinities of different ligands for these receptors (Flanagan 2016). Here we have used this method to evaluate the receptor expression levels in hippocampal tissue. First, the expression patterns of wolframin in the brain could be investigated. It is known that Wfs1 is expressed in several rodent brain regions associated with control of behaviour and emotions (Takeda *et al.* 2001, Luuk *et al.* 2008). The behavioural responses to environmental stimuli are mediated via dopaminergic system and therefore we hypothesized that wolframin could affect the functioning of this pathway. Immunohistochemical study indicated that the expression pattern of wolframin is largely overlapping with that of D_1-like receptors, especially with D_1 subtype (**PAPER III**). To gain further knowledge about the influence of wolframin to the levels of dopamine D_1-like receptors, we used Wfs1 gene knockout mice (Luuk *et al.* 2009) together with wild-type mice as a control group. The number of binding sites of dopamine receptors in the mouse hippocampus were assayed with [^{3}H]SCH23390, which is a selective ligand for D_1-like receptors. As this radioligand has high affinity for D_1 (Zhou *et al.* 1990) as well as for D_5 receptors (Sunahara *et al.* 1991, Ricci and Amenta 1994) that are both expressed in hippocampus and have quite similar roles (Sarinana *et al*. 2014), the following conclusions are valid for both D_1-like receptors.

Mouse brain and consequently the hippocampus are rather small (~25 mg) and thus the hippocampal tissue of a single mouse is not sufficient for obtaining the radioligand binding curve. Therefore, several mice hippocampi were pooled for these experiments. The radioligand [^{3}H]SCH23390 bound to hippocampal membranes with high affinity, having K_D values of 0.31 ± 0.06 nM and 0.48 ± 0.08 nM for wt and Wfs1 gene knockout mice, respectively (**Figure 3A**). The number of binding sites was higher for Wfs1-deficient mice (B_{max} = 4.0 ± 1.3 fmol/mg tissue) than in the case of wild-type mice (B_{max} = 1.5 ± 0.1 fmol/mg tissue) (**Figure 3A**). To check the number of D_1-like receptor specific binding sites in individual mice, 4 nM concentration of [^{3}H]SCH23390

was used. At this concentration approximately 90% of available receptors are labeled and therefore adequate information about the number of total binding sites could be obtained. The number of detected binding sites for Wfs1 knock-out mice, 2.7 ± 0.6 fmol/mg tissue, was significantly higher ($P < 0.05$) than corresponding value of 1.1 ± 0.4 fmol/mg tissue for wt mice (**Figure 3B**). The number of binding sites determined in these latter experiments was lower, because all of the receptors could not be detected. However, both of these experiments demonstrate increased levels of D_1-like receptors in hippocampi of Wfs1-deficient mice.

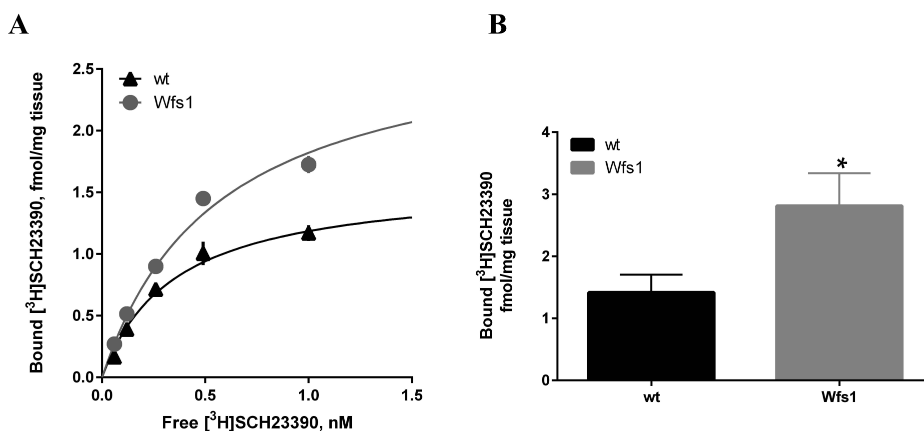

Figure 3. Comparison of specific binding of radioligand [^{3}H]SCH23390 to hippocampal membranes of wt and Wfs1 knockout mice. A – Saturation binding curve of [^{3}H]SCH23390 binding to pooled samples of six wt or Wfs1 knockout mice. The membrane suspensions (3 mg/well) were incubated with different concentrations of [^{3}H]SCH23390 for 60 min and bound radioactivity was measured as described in Materials and Methods. Data presented as mean ± S.E.M. from a representative experiment (n = 3) performed in duplicates. B – The level of [^{3}H]SCH23390 binding sites of individual wt (n = 22) and Wfs1 (n = 24) knockout mice determined in hippocampal membrane suspensions (1 mg/ml) incubated with 4 nM radioligand. Data presented as mean ± S.E.M. of all the mice tested. *$P < 0.05$ vs wt mice.

Previous experiments with Wfs1-deficient mice have shown that various points in dopaminergic signalling cascade are altered compared to wt mice. For example, functional studies indicated that wolframin is necessary for normal dopamine secretion in the striatum (Matto *et al.* 2011) and for dopamine transporter expression in midbrain (Visnapuu *et al.* 2013). Additionally, Wfs1-deficient mice demonstrate abnormal responses to dopamine agonists (Visnapuu *et al.* 2013, Luuk *et al.* 2009). All these results indicate impaired functioning of dopaminergic system in the animal model of Wolfram syndrome. Our study suggests that alterations in dopaminergic signalling are caused, at least in part, by the upregulation of D_1-like dopamine receptor expression in Wfs1-deficient

mice (**PAPER III**). This could in turn influence the activity of ligands and should be tested in functional assays. On one hand, higher dopamine receptor expression levels might enhance signalling in Wfs1 gene knockout mice. On the other hand, higher number of binding sites might be a compensatory mechanism for maintaining normal dopaminergic signal transduction due to lower levels of dopamine in the synaptic cleft. Similar effect has been seen in Parkinson's disease model, when 6-hydroxydopamine is injected to animals to induce degeneration of dopamine neurons (Ungerstedt 1968), resulting in increased expression levels and activity of dopamine D_2-type receptors (Terasmaa *et al.* 2000).

Clearly, the role of wolframin in dopaminergic signalling needs further investigation, especially concerning the postsynaptic signalling pathways. One of the possibilities is to generate primary cultures from hippocampal tissue of Wfs1-deficient and wt mice, which could later be used as a source of receptors in functional assays. A widely used method is GTPγS binding assay. However, this is typically suitable for GPCRs coupled to $G_{i/o}$ proteins, like dopamine D_2-type receptors, and would not be the method of choice for D_1-type receptors. Another option would be to monitor a change in the concentration of second messenger molecule cAMP. This could be carried out with a Förster resonance energy transfer (FRET)-based biosensor system that allows real-time detection of changes in cellular cAMP concentrations (Mazina *et al.* 2012, **PAPER II**). Unfortunately, functional assays were outside the focus and time-frame of this study but could be carried out in the future.

4.2. Determining the effect of lunasin to dopamine receptors

Lunasin is a 43-amino acid peptide isolated from soybeans (Odani *et al.* 1987, Galvez and de Lumen 1999). The peptide has an unusually high proportion of aspartic acid residues and nine out of ten of these residues are located in the carboxyl terminus forming a polyaspartyl sequence. The structure also contains a cell adhesion motif RGD (Odani *et al.* 1987). The biological activity of lunasin was first discovered in 1999 when A. Galvez together with his colleagues discovered that the peptide binds to chromatin and arrests cell division. Since then, many health-promoting properties of lunasin have been described. For example, its cancer-preventing activity (de Lumen 2005, Hernandez-Ledesma *et al.* 2009a), anti-inflammatory and antioxidant effects (Hernandez-Ledesma *et al.* 2009b, de Mejia and Dia 2009) and cholesterol-lowering properties (Galvez 2012, Gu *et al.* 2017). It is also known that upon oral administration lunasin can enter the target tissues in an intact and bioactive conformation and is able to cross the blood-brain barrier (Hsieh *et al.* 2010). This is presumably due to naturally occurring protease inhibitors in soy and other lunasin containing seeds, that protect lunasin from digestion making it bioavailable. This in turn generates a possibility that lunasin may also affect nervous system. Thus, we carried out experiments to study the effect of lunasin on the behavioural

responses in mice (**PAPER I**). It was seen that administration of lunasin induced motionlessness and catalepsy. Catalepsy is also a symptom of Parkinson's disease and mostly caused by dopamine receptors antagonism. Therefore, the influence of lunasin to behaviour caused by known dopaminergic drugs was determined. It was seen that lunasin reduces hyperlocomotion caused by administration of amphetamine as well as apomorphine-induced climbing. The obtained results indicate that the effects of lunasin to behaviour occur, at least in part, via dopamine receptors and therefore pharmacological properties of lunasin on dopamine receptors were evaluated.

In these experiments membrane preparations of HEK293 cells stably expressing either dopamine D_1 or D_2 receptor were used. Radioligand binding studies demonstrated modest affinity of lunasin for dopamine D_1 receptor with K_i = 60 ± 15 µM (**Figure 4**). Dopamine was used as a control (**Figure 4**) and the determined affinity (K_i = 36 ± 7 µM) was in good agreement with the previously published data from our workgroup (Reinart-Okugbeni *et al.* 2012). However, no dose-dependent decrease in binding of radioligand [^{3}H]Raclopride upon increasing concentration of lunasin was observed for dopamine D_2 receptors (data not shown).

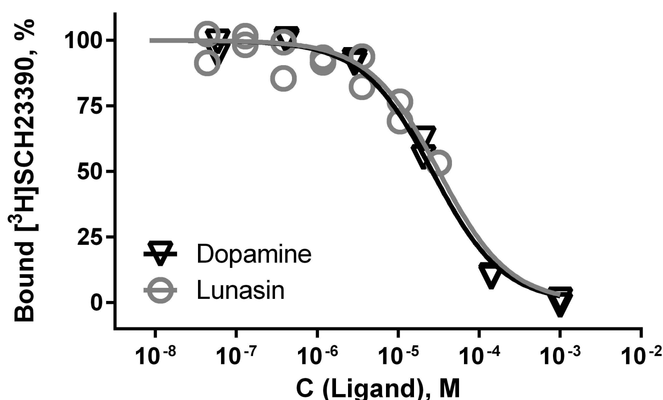

Figure 4. Displacement of [^{3}H]SCH23390 by dopamine or lunasin at dopamine D_1 receptors in HEK293 cell membranes. Serial dilutions of either dopamine or lunasin were incubated with radioligand (c = 1 nM) and membrane preparations for 90 min at 25 °C. Results were obtained from two independent experiments performed in duplicates. Data on the graph are from a representative experiment.

To further investigate lunasin's effect on dopamine receptors we wanted to test the biological activity of the peptide in FRET-based biosensor assay that allows real-time detection of changes in cellular cAMP levels (**PAPER II**). It was observed that lunasin inhibited the cAMP formation initiated by agonist apomorphine (10 nM) in HEK293 cells expressing dopamine D_1 receptor. The apparent pIC_{50} value was 6.1 ± 0.3 (n = 5) for lunasin and 7.61 ± 0.03 (n = 5)

for D_1-specific antagonist SCH39166 used as a control. Neither agonistic nor antagonistic properties of lunasin were seen in cells expressing D_2 receptors. The data obtained from radioligand binding studies and cAMP assay indicate that D_1 receptor, rather than D_2, may play an essential role in mediating lunasin's effects. In addition to dopaminergic system, other non-identified mechanisms may be involved in the behavioural effects of lunasin.

4.3. Implementing budded baculoviruses as a source of dopamine receptors for radioligand binding assay

It has been proved that baculovirus particles that have budded from Sf9 cells have active receptors on their surface. For example, it has been shown that BVs are a good source of melanocortin 4 receptors (Veiksina *et al.* 2014) and serotonin 5-HT_{1A} receptors (Tõntson *et al.* 2014) in fluorescence anisotropy-based assay. Generally, this expression systems results in correct folding of proteins, high expression levels and post-translational modifications similar to mammalian proteins. The ease of use and low risk of biohazard (Biosafety level 1) are also major advantages of this expression system (Kost *et al.* 2006). In **PAPER II** we have optimized and described all the steps required to generate, harvest and titrate BVs in order to obtain high-quality receptor preparation. This enables different laboratories to achieve comparable results, which is of utmost importance in terms of reproducibility.

Since dopamine receptors are in the focus of drug discovery and several fluorescent ligands have been synthesized for D_1- and D_2-like receptors (Monsma *et al.* 1989, Bakthavachalam *et al.* 1991, Tabor *et al.* 2017) then we decided to implement fluorescence anisotropy-based assay to characterize ligand binding to these receptors. For that, we focused on dopamine D_1 receptor and produced a high-titer BV that could be used to infect a large number of insect cells. It was seen that MOI = 5 should be optimal to achieve sufficient dopamine D_1 receptor expression. Once the budded baculoviruses were obtained we decided to characterize these in a radioligand binding assay. The assay is usually carried out with tissue or cell membrane preparations, which allow rapid separation of the free radioligand from the receptor-bound fraction. However, these sources of receptor cannot be considered a homogenous system, rather a mixture of lipoparticles with very different shapes and sizes with the additional uncertainty about the orientation of receptors in these particles (Bailey *et al.* 2009). Alternatively, receptors could be solubilized and purified followed by reconstitution into artificial lipid vesicles (Haga *et al.* 1985). This method enables to achieve a homogeneous receptor preparation but requires high amounts of protein and is labor-intensive. As a compromise, BVs could be used as a source of receptor, since the size of BV particles is uniform, and the orientation of receptor remains the same as it was in Sf9 cell membrane. The suitability of BVs for the radioligand binding assay was tested in **PAPER IV**, where the obtained results were also compared with the receptors in "classical"

membrane preparations (HEK293 and Sf9 cells). Total protein concentrations of all these preparations were determined with the Bradford assay kit and remained in the range on 0.4–0.7 mg/ml. The samples were further diluted for the radioligand binding experiments to achieve comparable level of binding and to avoid problems with ligand depletion.

First, saturation binding experiments were carried out to evaluate the number of binding sites and the affinity of the radioligand. The binding of [^{3}H]SCH23390 was with high affinity and saturable with the nonspecific binding remaining below 25% of the level of total binding for all the receptor preparations studied. The K_D was similar for Sf9 cells and BV particles containing D_1 receptors, being 1.7 ± 0.2 nM and 1.8 ± 0.2 nM respectively. These values are in good agreement with the results obtained previously for D_1 receptors expressed in Sf9 cells (He *et al.* 2003, Uustare *et al.* 2006), but somewhat higher than the K_D of 0.5 ± 0.1 nM determined in HEK293 cell membranes. The number of binding sites again coincided for Sf9 cells and BV particles, having B_{max} values of 9.2 ± 0.5 pmol/mg and 9.8 ± 1.7 pmol/mg protein respectively, giving additional evidence that the receptors in these two preparations have similar properties. The specific binding determined in HEK293 membranes was slightly lower with the B_{max} = 6.3 ± 0.4 pmol/mg. Even though it should be taken into account that receptors expressed in insect cells have a different environment than in mammalian cells, the obtained results demonstrate that BVs could be used as a membrane preparation in radioligand binding assay. High expression level of dopamine D_1 receptors could be achieved with BVs and this makes these an attractive choice for the pharmacological screening of ligands.

Competition binding experiments were carried out to compare the general pharmacological profile of these three different membrane preparations expressing dopamine D_1 receptors. Altogether, five known dopaminergic agonists and four antagonists were tested and all of the ligands resulted a concentration-dependent inhibition of [^{3}H]SCH23390 binding to D_1 receptors in all of the studied preparations. The pIC_{50} values (**Table 1**) obtained from the competition binding curves and corresponding K_i values were in good agreement with each other and with the previously reported results of these drugs (Gainetdinov *et al.* 2017).

Table 1. pIC_{50} values of dopamine D_1 receptor agonists and antagonists determined in competition binding experiments performed with Sf9 or HEK293 membrane preparations or with BVs. Serial dilutions of unlabeled compounds together with radioligand [^{3}H]SCH23390 were incubated with different receptor preparations for 90 min at 25 °C. Results were obtained from at least two independent experiments performed in triplicates. The data is presented here as mean ± S.E.M.

	pIC_{50}		
	HEK293	**Sf9**	**BV**
Agonists			
Apomorphine	5.89 ± 0.22	6.16 ± 0.18	5.99 ± 0.20
Dopamine	4.29 ± 0.24	4.95 ± 0.10	5.04 ± 0.10
MNPA	5.20 ± 0.10	5.61 ± 0.10	5.31 ± 0.12
SKF81297	7.04 ± 0.14	6.83 ± 0.10	6.86 ± 0.10
A68930	7.25 ± 0.10	8.00 ± 0.23	7.61 ± 0.22
Antagonists			
LE300	8.00 ± 0.24	7.98 ± 0.13	8.21 ± 0.16
(+)-butaclamol	8.84 ± 0.10	8.74 ± 0.10	8.90 ± 0.35
SCH23390	8.74 ± 0.19	8.36 ± 0.18	8.82 ± 0.10
Spiperone	5.69 ± 0.10	6.06 ± 0.11	6.26 ± 0.10

These experiments indicated that budded BVs are a suitable source of dopamine D_1 receptors. However, baculoviruses have been widely implemented to study other GPCRs and therefore we decided to test a selection of receptors in radioligand binding studies. In addition to dopamine D_1 receptors we have obtained similar results with D_3 receptors (with radioligand [^{3}H]Raclopride) (data not shown), serotonin 5-HT_{1A} receptors (with [^{3}H]WAY100635), M_1 and M_2 muscarinic acetylcholine receptors (with [^{3}H]NMS) (**Figure 5**). The pharmacological properties of these receptors remained unchanged and we are confident that the same approach can be applied for other GPCRs as well. Another important aspect is that BVs provide a homogenous system, which allows to use considerably longer incubation times, if necessary, to reach an equilibrium, without risk of aggregation or precipitation (**PAPER IV**). All the data demonstrate that budded baculoviruses are a suitable source of membrane receptors for the radioligand binding studies.

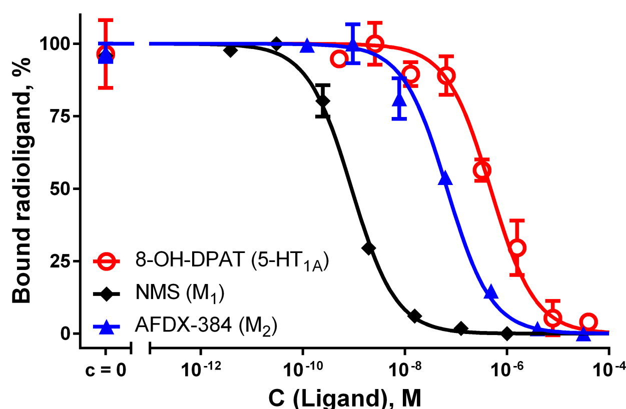

Figure 5. Competition binding experiments performed on budded baculovirus particles containing either serotonin 5-HT$_{1A}$ (○), M$_1$ (♦) or M$_2$ (▲) muscarinic acetylcholine receptors. Serial dilutions of unlabeled ligands 8-OH-DPAT (in case of 5-HT$_{1A}$), NMS (M$_1$) and AFDX-384 (M$_2$) were incubated with radioligand (1.6 nM [^{3}H]WAY10063 for 5-HT$_{1A}$ and 0.6–0.7 nM [^{3}H]NMS for muscarinic acetylcholine receptors) along with BV preparation for 90 minutes at 25 °C. The data is presented as mean ± S.E.M. from a representative experiment performed in triplicates.

4.4. Fluorescence anisotropy-based assay for studying ligand binding to dopamine receptors

Radioligand binding assay is a good choice for determining the binding affinity of the ligands as well as the number of binding sites. However, it is a so-called endpoint-based method and therefore gives limited information about the binding process. For example, it is more difficult to accurately measure reaction kinetics, due to the necessity of sampling each time point separately. Fluorescence-based assays, like fluorescence anisotropy, have emerged as promising methods that allow on-line monitoring of binding reactions without the need to take individual samples in time. One limiting factor of the FA assay is that it requires high receptor concentration due to its ratiometric nature, which means that FA signal depends on the concentration of both the free and the receptor-bound fluorescent ligands. Therefore, the concentrations of the receptor and the fluorescent ligand need to be comparable. As the previous results demonstrated, high expression levels of D$_1$ receptor can be achieved with BVs, we further tried to implement these BVs for FA assay.

Binding of fluorescent ligands can lead to change in FA as well as in total fluorescence intensity and both of these signals could be used to monitor ligand binding. Among the available fluorescent ligands, we have found that BodipyFL-SKF83566 is the most promising candidate for the characterization of ligand binding to D$_1$ receptors (**PAPER V**). Addition of D$_1$ receptor BVs to BodipyFL-SKF83566 caused a concentration-dependent increase in TFI. How-

ever, this increase did not depend on receptor activity as there were no significant differences in the TFI measured for total and nonspecific binding (determined in the presence of 1 μM (+)-butaclamol). It can be suggested that this increase in TFI could be caused by autofluorescence of BVs as similar increase was also detected in the absence of the BodipyFL-SKF83566.

However, it was observed that addition of BV preparation containing D_1 receptors to the fluorescent ligand caused a time-dependent increase in FA values (span ≈ 0.1) that reached a plateau within 3 minutes (**Figure 6**). Approximately half of this FA change could be attributed to the specific binding to the dopamine D_1 receptors as blocking the receptors with 1 μM (+)-butaclamol also resulted in an increase in FA compared to the signal obtained for the free fluorescent ligand without BVs. After the association of BodipyFL-SKF83566 to D_1 receptors was completed (10 min after starting the reaction) dissociation reaction was started by addition of 1 μM (+)-butaclamol. Decrease in FA, corresponding to the dissociation of fluorescent ligand from the D_1 receptors, was observed, and it reached the level of nonspecific binding within about 7 minutes (**Figure 6**). These results indicate that the change in FA could be attributed to binding of BodipyFL-SKF83566 to dopamine D_1 receptors and it can be monitored in real-time. The fluorescent ligand binds to dopamine D_1 receptors reversibly and the fast association and dissociation kinetics (half-lives approximately 40 and 70 seconds, respectively) allow to quickly reach an equilibrium.

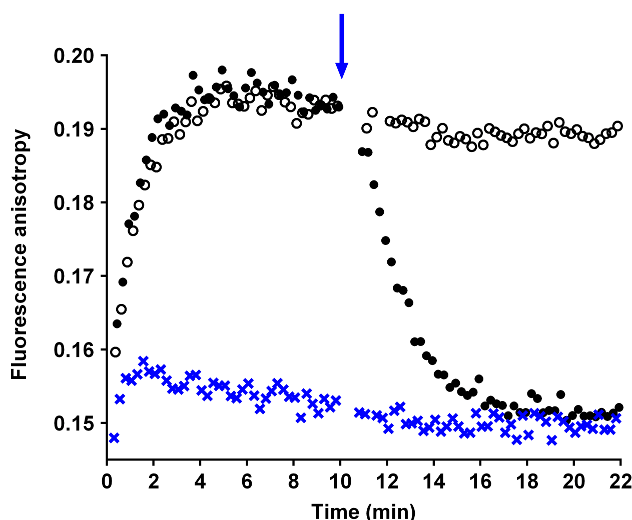

Figure 6. Time course of FA change caused by binding of BodipyFL-SKF83566 to dopamine D_1 receptors in budded baculovirus particles. The reaction was initiated by addition of D_1 receptors to 4.6 nM BodipyFL-SKF83566 in the absence (○,●) and presence (×) of 1 μM (+)-butaclamol. After 10 min (indicated with an arrow) the measurement was paused and dissociation was initiated by addition of 1 μM (+)-butaclamol (●) or an equivalent volume of assay buffer (○). Data are from a representative set of five independent experiments performed.

Another important aspect of the fluorescent ligand is the affinity, which was measured with two separate methods (**PAPER V**). First, the ability of BodipyFL-SKF83566 to compete with radioligand [^{3}H]SCH23390 in membranes of HEK293 cells stably expressing D_1 receptors was tested. BodipyFL-SKF83566 caused a concentration-dependent inhibition of [^{3}H]SCH23390 binding which could be characterized with a $pIC_{50} = 7.79 \pm 0.01$ and a corresponding $K_i = 6.0 \pm 1.8$ nM. To directly determine the affinity of BodipyFL-SKF83566 in the FA assay, total and nonspecific binding (with 8 μM (+)-butaclamol) were measured at two different concentrations of the fluorescent ligand (2.3 nM and 18.4 nM) by varying the amount of D_1 receptor BVs. The binding parameters were obtained by fitting the data to equations adapted for FA assay (Veiksina *et al.* 2014) and the K_D value of 5.19 ± 0.15 nM was obtained. This is in very good agreement with the affinity determined in radioligand binding assay and coincides with the affinity previously determined for similarly modified SKF83566 (Bakthavachalam *et al.* 1991). The nanomolar range affinity is sufficient for using BodipyFL-SKF83566 as a reporter ligand in the FA assay.

As binding of BodipyFL-SKF83566 to dopamine D_1 receptors can be directly monitored we used it to characterize the binding properties of various dopaminergic ligands. For that, ligands with up to four orders of magnitude difference in affinities were chosen. All of these competition binding experiments yielded one-site binding curves with the pIC_{50} and corresponding K_i values close to the previously reported affinities (**Table 2**). Similar dose-response curves were obtained when these unlabeled ligands were also tested in radioligand binding experiments with D_1 receptor BVs. The pIC_{50} and K_i values determined from these experiments coincided with the results from the FA assay (**Table 2**). A linear correlation between pK_i values was obtained from the affinities determined for the same ligands with the two aforementioned methods (**Figure 7**). The coefficient of determination (R^2) was 0.96 and the slope did not significantly differ from unity nor y-intercept from zero. These results suggest that BodipyFL-SKF83566 is a suitable reporter ligand for FA assay and the assay system described here is readily applicable for high-throughput screening of novel dopaminergic ligands.

Table 2. Binding affinities of dopaminergic ligands for dopamine D_1 receptors in baculovirus particles determined in radioligand binding assay in competition with [^{3}H]SCH23390 or in fluorescence anisotropy assay with BodipyFL-SKF83566. The values are expressed as mean ± S.E.M. of three independent experiments performed in duplicates (FA assay) or two measurements performed in triplicates (radioligand binding assay).

	Radioligand binding		Fluorescence anisotropy	
	pIC_{50}	K_i[a], nM	pIC_{50}	K_i[b], nM
Agonists:				
Dopamine	5.04 ± 0.10	6770 ± 110	5.09 ± 0.10	5300 ± 880
Apomorphine	5.99 ± 0.20	840 ± 360	5.44 ± 0.10	1710 ± 150
A68930	7.61 ± 0.22	20.0 ± 9.3	7.24 ± 0.10	38.5 ± 5.5
SKF81297	6.86 ± 0.10	99 ± 11	6.70 ± 0.10	119 ± 21
SKF38393	5.99 ± 0.10	665.9 ± 2.5	5.20 ± 0.10	1850 ± 370
NPA	5.59 ± 0.10	1663 ± 46	5.65 ± 0.10	1210 ± 110
Antagonists:				
SCH39166	8.52 ± 0.14	2.05 ± 0.63	8.17 ± 0.10	4.8 ± 1.6
(+)-butaclamol	8.90 ± 0.35	1.23 ± 0.84	8.62 ± 0.27	1.70 ± 0.76
LE300	8.21 ± 0.16	4.8 ± 1.6	8.24 ± 0.19	3.7 ± 1.7
Spiperone	6.26 ± 0.10	390 ± 31	5.85 ± 0.10	622 ± 86
SCH23390	8.82 ± 0.10	1.08 ± 0.21	8.06 ± 0.12	3.61 ± 0.60

[a] K_i were calculated from displacement curves according to the Cheng-Prusoff equation (Cheng and Prusoff 1973).
[b] K_i were calculated by fitting the data to a set of equations implemented previously for fluorescence anisotropy competition experiments (Veiksina *et al.* 2014).

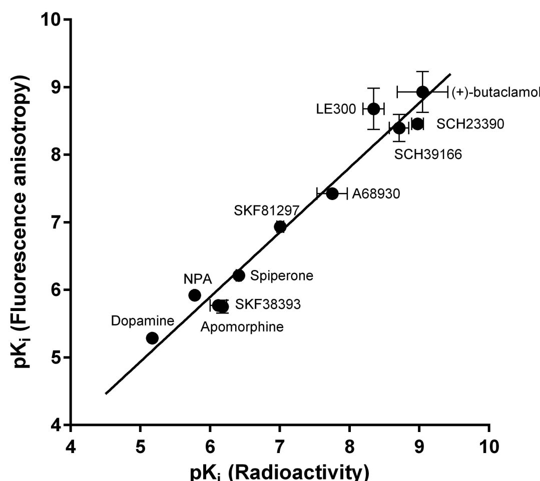

Figure 7. Comparison of apparent affinities of dopaminergic ligands in fluorescence anisotropy assay with apparent affinities measured in radioligand binding assay. BodipyFL-SKF83566 was used as a reporter ligand in FA assay and [^{3}H]SCH23390 was applied in the radioligand binding assay. Results (mean ±S.E.M.) were obtained from three independent experiments performed in duplicates (FA assay) or at least two measurements performed in triplicates (radioligand binding assay) after 90 min of incubation. $R^2 = 0.96$

As demonstrated above, fluorescent ligand BodipyFL-SKF83566 binding to D_1 receptors could be monitored on-line (**Figure 6**). It has been demonstrated earlier that kinetic parameters of the unlabeled ligand can be calculated from kinetic data of competition binding curves (Rinken *et al.* 2018). Unfortunately, kinetics of the studied dopaminergic ligands (**Table 2**) were too fast for this instrumental setup to reliably estimate kinetic constants for these ligands. However, monitoring the change of apparent pIC_{50} values in time during the competition experiments, temporary over- or underestimation of pIC50 could be observed (**PAPER V**). For example, there was a temporary overestimation of the affinity of dopamine, which indicates that this ligand is faster than BodipyFL-SKF83566, and the underestimation of SCH23390's affinity indicates that the competitor has slower binding kinetics than the fluorescent ligand (**PAPER V**). This information gives general estimation about the competitor's kinetic properties even though exact rate constants could not be determined. Of course, real kinetic constants can be obtained for all these ligands, but it requires special equipment with an automatic dispensing and shaking system as well as smaller number of samples. As this would give information about the binding mechanism of a particular ligand, but is not usable for general screening studies, it remained outside the scope of the current study. Our assay set-up with 96-well plates and conventional plate spectrofluorimeter equipped with a polarizer (Veiksina *et al.* 2015) does not allow to follow the fast ligand binding kinetics, where half-lives are shorter than a minute.

Usually the overall cost of the assay depends mostly on the amount of receptor and ligands needed. In the FA assay, 96-well half-area plates are used, which allow considerably smaller sample volumes per well than feasible in radioligand binding experiments were filtration and washing steps are required. This is an important advantage when high molecular weight ligands with low affinity are tested. For example, radioligand displacement experiments with lunasin resulted in 50% inhibition of radioligand [^{3}H]SCH23390 binding upon highest lunasin concentration (32 μM) used (**Figure 4**). Since we already showed that FA assay with BodipyFL-SKF83566 is a suitable method for the characterization of ligand binding to dopamine D_1 receptors, we also used the method to validate the results of lunasin. The smaller assay volume enabled to use up to 100 μM concentrations of lunasin in competition binding experiments and therefore it was possible to more accurately determine the lower plateau of the lunasin's binding curve. **Figure 8** demonstrates the concentration-dependence of FA for lunasin and dopamine (used as a control) and the obtained pIC_{50} values were 4.81 ± 0.05 for lunasin and 4.93 ± 0.02 for DA. Consequent K_i values were calculated as described in Veiksina *et al.* 2014 and resulted in K_i = 12.2 ± 2.5 μM and K_i = 5.66 ± 0.58 μM for lunasin and DA, respectively. These results are in good agreement with the estimated affinity of lunasin measured in **PAPER I**.

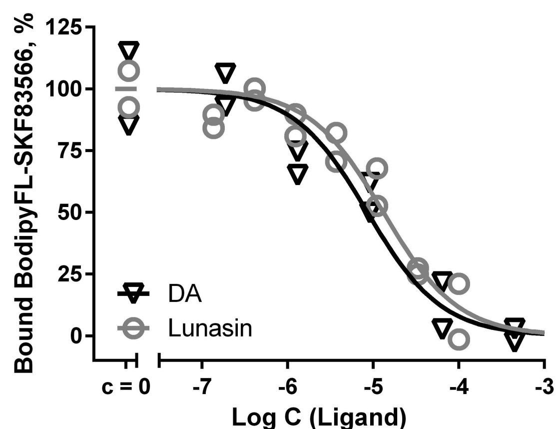

Figure 8. Displacing fluorescent ligand BodipyFL-SKF83566 by dopamine or lunasin at dopamine D_1 receptors in budded baculovirus particles. Serial dilutions of either dopamine or lunasin were incubated together with BodipyFL-SKF83566 (c = 2.3 nM) and BV particles at 27 °C. Fluorescence anisotropy was measured 90 min after start of the reaction. Results were obtained from two independent experiments performed in duplicates. Data on graph are from a single representative experiment.

4.5. Visualization of ligand binding to dopamine receptor in live cells

In addition to dopamine D_1 receptors we also tried to develop assay systems to study D_2-like receptors. As there were no commercially available ligands for these receptors, we decided to find a suitable candidate by ourselves. Fluorescent ligand NAPS-Cy3B appears to be a promising reporter ligand because NAPS has been shown to retain its affinity even after coupling to a fluorescent dye (Monsma *et al.* 1989) and fluorophore Cy3B is known to be a suitable choice for receptor studies (Veiksina *et al.* 2010).

First, NAPS-Cy3B was tested in a FA assay with BVs expressing D_3 receptors. Addition of D_3 receptor BVs to the fluorescent ligand did not cause a significant change in TFI, while clear receptor-specific change in FA could be detected. However, even after the optimization of measurement conditions (like concentrations of the fluorescent ligand and the receptor, incubation time) the signal window remained below 0.03, which is too low to reliably measure ligand binding to D_3 receptors. We then determined the affinity of NAPS-Cy3B in a competition assay with radioligand [^{3}H]Raclopride in HEK293 cells stably expressing D_3 receptors and the corresponding results were $pIC_{50} = 8.95 \pm 0.28$ and $K_i = 0.71 \pm 0.38$ nM. Hence, the affinity of this fluorescent ligand should be sufficient for FA assay and is not responsible for the small specific signal. This could be caused by the properties of the fluorescent ligand – if the fluorophore is able to rotate even after the fluorescent ligand has bound to the receptor then the increase in FA upon ligand binding could be negligible.

High affinity of NAPS-Cy3B to dopamine D_3 receptors determined in a radioligand competition assay indicates tight binding of the fluorescent ligand to these receptors and therefore it should be also possible to characterize NAPS-Cy3B binding to D_3 receptors in live cells. To verify this, we performed fluorescence microscopy experiments with live HEK293 cells stably expressing dopamine D_3 receptors. The cells were incubated with NAPS-Cy3B in the absence (total binding) and in the presence of 1 μM (+)-butaclamol (nonspecific binding). There was a clear difference in binding patterns of total (**Figure 9A**) and nonspecific binding (**Figure 9B**) – in the first case, accumulation of NAPS-Cy3B into the membrane could be detected, whereas no clear membrane labeling could be seen for nonspecific binding. We also performed experiments with HEK293 cells without any stable transfection and saw no significant binding of NAPS-Cy3B to cell membranes. The data indicate that NAPS-Cy3B binds specifically to dopamine D_3 receptors expressed in HEK293 cells.

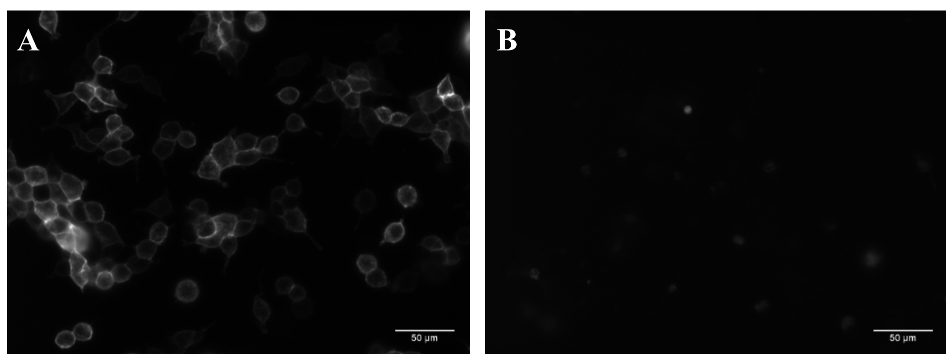

Figure 9. Fluorescence microscopy images of 1 nM fluorescent ligand NAPS-Cy3B binding to HEK293 cells stably expressing D_3 receptors. Total binding (A) was determined in the absence and nonspecific binding (B) was obtained in the presence of 1 μM (+)-butaclamol after 90 minutes of incubation. The number of cells per well was 25000. Scale bar corresponds to 50 μm.

Since the obtained results indicated that NAPS-Cy3B can be successfully used in fluorescence microscopy experiments, we further tried to implement this technique in competition binding experiments. A clear concentration-dependent decrease in fluorescence intensity could be seen in experiments with the unlabeled antagonist (+)-butaclamol (**Figure 10**). Higher concentrations of (+)-butaclamol resulted in lower fluorescence intensity while at lower concentrations the unlabeled ligand could not compete with NAPS-Cy3B for binding to dopamine D_3 receptors. To use this method for screening of novel ligands, quantitative information needs to be acquired from the fluorescent images. Simple analysis, such as calculating the average intensity of the fluorescence images, gives poor results. Higher receptor-ligand complex concentration does give rise to the average intensity. However, differences in confluence

also change the average intensity of the image to a large extent and the exact number of cells in each image frame is practically impossible to control. Therefore, an approach that takes into account only the fluorescence intensity of membrane pixels is preferred. Here, an algorithm developed by Tõnis Laasfeld as a "Membrane Tools" module of Aparecium 2.0 software was used for quantification. As a result, the relative amount of fluorescent ligand NAPS-Cy3B bound to the D_3 receptors in HEK293 cell membranes could be quantified. This enables us to obtain dose-response curves for unlabeled dopaminergic ligands (**Figure 11**) and to determine the corresponding pIC_{50} values, which were 6.63 ± 0.28 for dopamine and 7.67 ± 0.17 for (+)-butaclamol. Additionally, no specific binding and its changes could be detected when wt HEK293 cells were used in this kind of experiment (**Figure 11**).

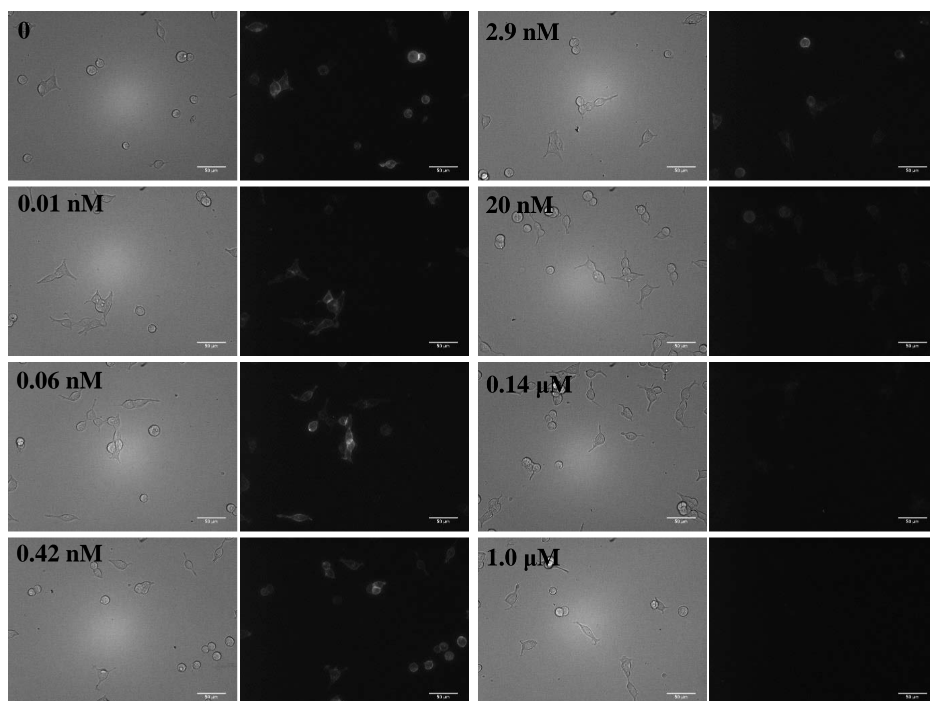

Figure 10. Inhibition of fluorescent ligand NAPS-Cy3B binding by (+)-butaclamol. HEK293 cells (20000 cells/well) stably expressing dopamine D_3 receptors were incubated with 1 nM NAPS-Cy3B and given concentrations of (+)-butaclamol for 90 minutes. Bright field (left) and fluorescence (right) images were taken after 90 minutes of incubation with ligands as described in Methods. Scale bar corresponds to 50 µm.

The fluorescence microscopy assays performed with live mammalian cells that are more similar to native environment of GPCRs than BV particles can provide additional data about dopaminergic ligand binding process. NAPS-Cy3B has a high affinity to D_2-like receptors and spectral properties suitable for following processes in live cells. Unfortunately, its antagonistic properties make it impossible to characterize agonist-dependent effects like desensitization and endocytosis. Thus, future aim would be to obtain a fluorescent agonist which could provide additional information about dopaminergic receptors. When employing high-end fluorescent microscopes, these processes could be even detected at a single molecule level.

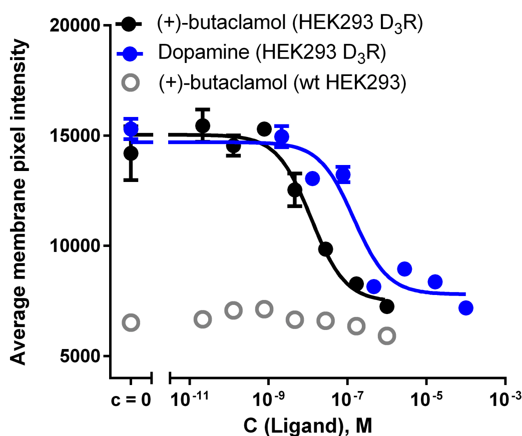

Figure 11. Competition binding curves of NAPS-Cy3B obtained from fluorescence microscopy experiments. Measurements were performed with either wt HEK293 cells (○) or HEK293 cells stably expressing D_3 receptors (●,●). HEK293 cells (20000 cells/ well) were incubated with 1 nM NAPS-Cy3B and serial dilutions of either dopamine (●) or (+)-butaclamol (●). The average fluorescence intensity of membrane pixels was estimated using the membrane detection algorithm incorporated into software Aparecium 2.0.

5. CONCLUSIONS

Dopamine is an important neurotransmitter that mediates its functions through five different types of G protein-coupled receptors on the cellular plasma membrane. Dysfunction of dopaminergic signalling is connected with several neurological and psychiatric disorders, like Parkinson's disease, schizophrenia, Tourette's syndrome, bipolar disorder, depression, hyperprolactinemia and attention deficit hyperactivity disorder. Therefore, acquiring further information about the signal transduction process mediated by dopamine receptors is essential.

This thesis focuses on the development of novel assay systems to characterize ligand binding to different subtypes of dopamine receptors. Experiments were carried out with native G protein-coupled receptors (tissue homogenates) and with various recombinant protein expression systems (mammalian and insect cells, budded baculovirus particles). Additionally, several methods were implemented to study receptor-ligand interactions with the emphasis on fluorescence-based methods.

Hippocampal membranes of wild-type and wolframin knock-out mice were tested in radioligand binding experiments to investigate possible link between the expression of wolframin and dopamine D_1-like receptors. We have determined by $[^3H]$SCH23390 binding that membranes of wolframin knock-out mice have higher number of dopamine D_1-like receptors than the membranes of wild-type mice. This upregulation of D_1-like receptors may be connected with physiological abnormalities of Wolfram syndrome.

We have studied the binding properties of lunasin, a bioactive peptide isolated from soybeans, to dopaminergic receptors. Radioligand binding assay as well as fluorescence anisotropy-based assay demonstrated micromolar range affinity of the peptide to D_1 receptors, while no effect to D_2 receptors even at submillimolar concentrations was observed.

We have shown that budded baculovirus particles can be used as a source of receptors for radioligand binding experiments. Obtained ligand binding parameters for D_1 receptors in baculovirus particles were in good agreement with the results of "classical" membrane preparations (HEK293 and Sf9 cells). Same approach can also be applied for other receptors, as we have obtained similar results with D_3 receptors, serotonin 5-HT_{1A} receptors, M_1 and M_2 muscarinic acetylcholine receptors.

The fluorescent ligand BodipyFL-SKF83566 is suitable for the characterization of ligand binding to D_1 receptors in baculovirus particles. This assay system enables to measure ligand binding in real time, so that more information about ligand binding kinetics is received. Obtained binding parameters were in good correlation with the parameters measured in radioligand binding assay. Homogeneous nature of the fluorescence anisotropy assay makes it readily applicable for high-throughput screening of novel dopaminergic drugs.

For D_2-like receptors, a new high-affinity fluorescent ligand NAPS-Cy3B was synthesized. Even though it was not a good reporter ligand for fluorescence anisotropy-based assay, due to its modest signal window, it can be used in fluorescence microscopy experiments with live cells stably expressing D_3 receptors. In these experiments a clear concentration-dependent decrease in NAPS-Cy3B binding was observed upon increasing concentrations of unlabeled antagonist (+)-butaclamol. To reliably quantify binding of NAPS-Cy3B to D_3 receptors we have developed an algorithm that takes into account only the fluorescence intensity of membrane pixels. As a result, affinity of the competitor could be estimated. An advantage of this assay system is that it enables to study ligand binding to dopaminergic receptors in live mammalian cells, which is very similar to the native environment of GPCRs.

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7. SUMMARY IN ESTONIAN

Dopamiini retseptoritele ligandi sidumise uurimist võimaldavate katsesüsteemide arendamine

Dopamiin on organismis oluline virgatsaine, mis vahendab närvisignaali ülekannet organismis seostudes raku membaanis paiknevatele G-valguga seotud retseptorite perekonda kuuluvatele dopamiini retseptoritele. Häired dopamiinergilises signaaliülekandes on seotud mitmete neuroloogiliste ja psühhiaatriliste haigustega, nagu näiteks Parkinsoni tõbi, skisofreenia, Tourette'i sündroom, bipolaarne häire, depressioon, hüperprolaktineemia ning tähelepanupuudulikkusega hüperaktiivsus. Seetõttu on dopamiini retseptorite signaaliülekande põhjalikum uurimine jätkuvalt päevakorral.

Käesolev doktoritöö keskendub uudsete katsesüsteemide arendamisele, mis võimaldavad uurida ligandide seostumist erinevatele dopamiini retseptorite alatüüpidele. Eksperimente viidi läbi nii natiivsete retseptoritega (homogeniseeritud koed), kui ka erinevate rekombinantse valgu ekspressioonisüsteemidega (imetaja- ja putukarakud, pungunud bakuloviiruste osakesed). Retseptorite ja ligandide vaheliste interaktsioonide iseloomustamiseks rakendati nii klassikalist radioligandi sidumist kui ka mitmeid fluorestsentsil põhinevaid meetodeid.

Metsik-tüüpi ning wolframiini geeni puudulikkusega hiirte hipokampustest valmistatud membraanpreparaate võrreldi radioligandi sidumiskatsetes, eesmärgiga uurida võimalikku seost wolframiini ekspressiooni ja dopamiini D_1-tüüpi retseptorite vahel. Radioligandi [^{3}H]SCH23390 seostumiskatsed näitasid, et wolframiini-puudulikel hiirtel on rohkem dopamiini D_1-tüüpi retseptoreid kui tavalistel hiirtel. Kõrgem D_1-tüüpi retseptorite ekspressioonitase võib olla otseselt seotud Wolframi sündroomiga.

Antud töö käigus uuriti ka lunasiini, sojaubadest eraldatud peptiidi, sidumisomadusi dopamiini retseptoritele. Nii radioligandi sidumiskatsete kui ka fluorestsentsanisotroopia eksperimentide tulemusena nähti, et lunasiin seostub D_1 retseptoritele mõõduka, mikromolaarses suurusjärgus afiinsusega. Seevastu lunasiini seostumist D_2 retseptorile ei täheldatud.

Töö käigus tõestati, et pungunud bakuloviiruse osakesi on võimalik kasutada D_1 retseptorite allikana radioligandi sidumiskatsete läbiviimiseks. Testitud ligandide seostumisparameetrid langesid hästi kokku tavapärastest membraanpreparaatidest (HEK293 ja Sf9 rakud) saadud tulemustega. Samasugust lähenemist saab kasutada ka teiste G-valguga seotud retseptorite jaoks, sest radioligandi katseid pungunud bakuloviirustega on sooritatud ka dopamiini D_3, serotoniini 5-HT_{1A} ja muskariinsete M_1 ning M_2 atsetüülkoliini retseptoritega.

Fluorestsentsligand BodipyFL-SKF83566 osutus hästi sobivaks fluorestsentsanisotroopia katsete läbiviimisel D_1 retseptoreid sisaldavate bakuloviirustega. Loodud katsesüsteem võimaldab jälgida ligandi sidumist reaalajas, iseloomustades seega ka paremini protsessi kineetikat. Ligandide jaoks määratud seostumisparameetrid olid heas kooskõlas radioligandi sidumiskatsetes saadud

tulemustega. Antud katsesüsteemi ülesehitus omab potentsiaali dopamiinergiliste ravimikandidaatide kiirsõeluuringute läbiviimisel.

Lisaks D_1 retseptoritele, sooviti välja töötada ka fluorestsanisotroopial põhinev katsesüsteem D_2-tüüpi retseptorite uurimiseks. Selleks sünteesiti uus, kõrge afiinsusega antagonist NAPS-Cy3B. Tagasihoidlik signaaliaken ei võimaldanud antud fluorestsentsligandi usaldusväärselt rakendada fluorestsentsanisotroopia katsetes. Küll aga sobis NAPS-Cy3B hästi fluorestsentsmikroskoopia katsete läbiviimiseks. See võimaldab uurida ligandi sidumist D_3 retseptoritele otse elusatel imetajarakkudel. Konkureeriva antagonisti (+)-butaklamooli kontsentratsioonist sõltuv NAPS-Cy3B sidumine rakkudele näitas fluorestsentsligandi spetsiifilist seostumist D_3 retseptoritele. Seostumise kvantifitseerimiseks loodud algoritm võimaldab arvesse võtta vaid membraanipikslite fluorestsentsintensiivsust. Antud katsesüsteemiga saab hinnata erinevate märgistamata ühendite afiinsust D_3 retseptorile elusates rakkudes.

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