DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
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REET REINART-OKUGBENI

Assay systems for characterisation of subtype-selective binding and functional activity of ligands on dopamine receptors

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Author's contribution:

Paper I: The author performed [^{3}H]SCH23390 binding experiments. AU was responsible for writing the manuscript.

Paper II: The author performed [^{3}H]SCH23390 binding experiments. AV was responsible for writing the manuscript.

Paper III: The author participated in planning of the experiments, performed ligand binding experiments and was responsible for writing the manuscript; ZG, SB and AS were responsible for synthesis of the ligands and writing the respective part of the manuscript.

Paper IV: The author created and characterised stable cell lines used in the study, and planned and performed ligand binding experiments; KA, KK and TK planned and performed synthesis of the ligands. The author and KA were equally responsible for writing the manuscript.

Paper V: The author created and characterised stable cell lines used in the study, planned and performed ligand binding experiments, and was responsible for writing the manuscript; AV performed functional assays; ZG and AS were responsible for synthesis of the ligands and writing the respective part of the manuscript.

ABBREVIATIONS

7-TMR	seven-transmembrane receptor; receptor with seven membrane-spanning domains
α-MEM	Alpha Minimum Essential Medium
AC	adenylate cyclase, adenylyl cyclase
ADA	adenosine deaminase, enzyme for the breakdown of adenosine
ATP	adenosine-5'-triphosphate
BacMam	a recombinant baculovirus for delivering genes of interest in to mammalian cells
BB	Brown's buffer used herein in [^{3}H]cAMP assays
B_{max}	maximal receptor density
BSA	bovine serum albumin
cAMP	3-',5'-cyclic adenosine monophosphate
CFP	cyan fluorescent protein (ECFP – enhanced CFP)
CHO-K1	a cell line derived from the original Chinese Hamster Ovary (CHO) cell line
DA	dopamine
DAR	dopamine receptor
DARPP-32	dopamine- and cAMP-regulated neuronal phosphoprotein, activated by PKA
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate Buffered Saline
DTT	dithiotreitol, a redox agent
EC_{50}	50% effective concentration of a ligand in functional assay
ED_{50}	50% effective viral dilution in cell-size based assay of baculovirus titration
EDTA	ethylenediaminetetraacetic acid, a chelating agent binding Ca^{2+} and Mg^{2+}
EGTA	ethyleneglycoltetraacetic acid, a chelating agent similar to EDTA but with higher affinity for Ca^{2+}
E_{max}	maximal response of a drug in functional assays; efficacy of a drug/ligand
Epac	exchange protein activated by cAMP
Epac2-camps	cAMP-biosensor protein based on cAMP-binding domain of Epac2 with ECFP and EYFP as a FRET-pair
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
FBS	foetal bovine serum
FCS	foetal calf serum
FP/FA	fluorescence polarisation/fluorescence anisotropy
FRET	Förster/fluorescence resonance energy transfer
FSK	forskolin
G418	geneticin

GDP	guanosine-5'-diphosphate
GPCR	G-protein-coupled receptor
G-protein	guanine nucleotide-binding protein
GTP	guanosine-5'-triphosphate
GTPγS	guanosine-5'-O-[gamma-thio]triphosphate
HEK293	Human Embryonic Kidney 293 cell line
IBMX	3-isobutyl-1-methylxanthine, a PDE inhibitor
IC_{50}	50% effective concentration of a ligand in a radioligand displacement assay
K_D	equilibrium dissociation constant of a labelled ligand determined by saturation assay
K_H	K_i of high-affinity binding site
K_i	equilibrium dissociation constant of unlabelled ligand determined by radioligand competition assay
K_L	K_i of low-affinity binding site
L-DOPA	L-3,4-dihydroxyphenylalanine
MOI	multiplicity of infection demonstrating the number of infectious viral particles per cell
NaBu	sodium butyrate
PDE	phosphodiesterase
PEP	phosphoenolpyruvate
PK	pyruvate kinase, enzyme catalysing the transfer of a phosphate group from PEP to ADP
PKA	protein kinase A, cAMP-dependent protein kinase
PMSF	phenylmethylsulfonyl fluoride, a serine protease inhibitor
RPMI	Roswell Park Memorial Institute medium
Sf9	clonal isolate of Sf21 cells derived from ovarian tissue of Fall Armyworm, *Spodoptera frugiperda*
TB	Tris-buffer with 50 mM Tris and HCl (pH 7.4), used herein for membrane preparation and in radioligand binding assays
Tris	tris(hydroxymethyl)aminomethane
WB	washing buffer with 20 mM K-phosphate and 100 mM NaCl (pH 7.4), used herein in radioligand binding assays
YFP	yellow fluorescent protein (EYFP – enhanced YFP)

INTRODUCTION

Dopamine plays an important role in central nervous system and in the periphery. It affects voluntary movement, feeding and reward, attention, working memory and learning, regulation of olfaction, hormone release, cardiovascular functions, immune system, and renal functions, among others.

Dopamine receptors belong to one of the major class of cell membrane receptors, the G-protein-coupled receptors, involved in chemical signal transmission between cells. They are targets for variety of drugs involved in diseases like schizophrenia, Parkinson's disease, depression and many others.

Developing methods that would help in understanding receptor function and signalling and facilitate the development of better drugs is a challenge for people from various disciplines – neuroscience, pharmacology, chemistry, molecular biology, physics *etc*.

Herein we report the progress of developing assay systems for subtype-selective characterisation of ligand binding and activity on three major dopamine receptor subtypes: D_1, D_2 and D_3 receptors.

1. LITERATURE REVIEW

1.1. The emergence of modern pharmacology and recognition of dopamine as a neurotransmitter

Pharmacology is a study of drugs (φάρμακον, pharmakon, "poison" in classic Greek; "drug" in modern Greek), more specifically the study of the interactions that occur between a living organism and chemicals that affect normal or abnormal biochemical function. It is difficult to state when humankind first started to use chemical substances as drugs (toxins) for human benefit, but among the first physiologists to study toxic substances, according to Gesztelyi *et al.*, was a French physiologist Claude Bernard (1813–1878) (Gesztelyi *et al.*, 2012). He carried out investigations with curare (common name for various arrow poisons originating from South America) and carbon monoxide. An important contribution to pharmacology was made by Mathieu Orfila (1787–1853) who studied forensic toxicology and is considered the father of toxicology. In one of his works he described the action of a large number of different poisons on dogs. He was not trying to explain the physiological action of the toxins or the pharmacology behind it; rather his observations reflected a toxicologist's point of view. The first to establish an institute of pharmacology was Rudolf Buchheim (1820–1879) and he did that in 1847 during his first year at the University of Tartu in Estonia (then University of Dorpat) (Kuchinsky, 1968). He turned a purely empirical pharmacology into an experimental science. A student of Buchheim, Oswald Schmiedeberg (1838–1921), who earned his medical doctorate from the University of Dorpat in 1866 with a thesis on the measurement of chloroform in blood, later became an assistant to Buchheim. In 1869 Schmiedeberg showed that muscarine, first isolated the same year from fly agaric (*Amanita muscaria*) (Schmiedeberg and Koppe, 1869), evoked the same effect on the heart as electrical stimulation of the vagus nerve (Scheindlin, 2001). Buchheim and Schmiedeberg are generally recognised as the founders of modern pharmacology (Gesztelyi *et al.*, 2012).

A corner stone in pharmacology was when receptors were recognised as specific binding sites on the cell membrane. Earlier in the 19th century receptors were still considered as something abstract, representing some sort of a switch that can produce tissue response when activated by certain drugs. The name "receptor" to describe the specific chemical structure on the cell receiving the agent evoking a cellular response was first used by Paul Ehrlich (1854–1915) in 1900 (Gesztelyi *et al.*, 2012). At about the same time John Newport Langley (1852–1925) proposed in 1905 that a "receptive substance" was the site of action of chemical mediators liberated by nerve stimulation (Rubin, 2007). It is believed that the receptor concept delivered by Langley was at least in part been attributed to ideas expressed by much forgotten Thomas Renton Elliott (1877–1961), whose mentor Langley was at the time. Elliott examined the role of epinephrine (adrenaline) on sympathetic nerve stimulation using variety of smooth

muscle preparations. Already in 1904 he proposed that "adrenaline might be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery" (Elliott 1904), referring to the existence of chemical rather than electrical neurotransmission. He also proposed that the "effector" stimulated by epinephrine was the "myoneural junction" and not the nerve endings or muscle fibers, suggesting the existence of chemical synapses. However, the findings of Elliott were set aside for years before the concept of chemical neurotramission was finally recognised as a mechanism for transmission of nerve impulses. Sir Henry Dale (1875–1968) in 1914 conducted experiments with derivatives of rye fungus ergot, containing acetylcholine (ACh), and found that it resembled the actions of muscarine, as well as the effects of parasympathetic nerve stimulation. It was Otto Loewi (1873–1961) who established the physiological relevance of ACh. In 1896 he took his doctor's degree at Strasbourg University studying the effects of various drugs on the isolated heart of the frog, a topic suggested by Oswald Schmiedeberg (Geison, 2008). In 1920 he conducted experiments with two frog hearts in a single bath. He showed that electrical stimulation of the vagus nerve of one heart was slowing down its rate, while causing the rate of the second heart also to diminish. He concluded that a substance liberated from the first heart was responsible for causing inhibition of the second heart; the substance was termed *vagusstoff* (later identified as ACh). He also demonstrated that atropine (an alkaloid of *Atropa belladonna*) blocked postsynaptic action of ACh on cardiac muscle without modifying its release by vagal nerve stimulation (Rubin, 2007). Atropine is now known as a competitive antagonist on muscarinic acetylcholine receptors. Otto Loewi, referred to as the "Father of Neuroscience", and Sir Henry Dale shared Nobel Prize in Physiology and Medicine in 1936 "for their discoveries relating to chemical transmission of nerve impulses". Dale and Loewi also conducted experiments with biogenic amines, epinephrine (adrenaline) and norepinephrine (noradrenaline), and showed similarities in their pharmacological properties with ACh. The glory for the discovery of norepinephrine as a neurotransmitter however belongs to Ulf von Euler (1905–1983). In the mid-1940s he differentiated epinephrine and norepinephrine using two fluorometric bioassays with different sensitivities to the two amines. Julius Axelrod (1912–2004) did experiments with tritiated norepinephrine to study its metabolic turnover and his studies resulted with proposing a mechanism for termination of catecholamine action and catecholamine uptake, which could be blocked by certain psychoactive drugs, such as cocaine. Cocaine is now known to block dopamine transporter (DAT) and inhibit uptake of monoamines such as serotonin, norepinephrine and dopamine (Fleckenstein *et al.*, 2000; Riddle *et al.*, 2008). In 1950s Sir Bernard Katz (1911–2003) studied neuromuscular transmission and demonstrated the role of extracellular calcium in regulation of the ACh released from presynaptic nerve terminals. This resulted in proposal of a vesicle hypothesis to account for the liberation of neurotransmitter. Katz, Axelrod and von Euler shared a Nobel Prize in 1970

"for discoveries relating to chemical transmission of nerve impulses" (Rubin, 2007).

Another biogenic amine, dopamine, first synthesised from levodopa (L-DOPA) in 1910, was long considered to be of limited physiological significance because of its modest activity on smooth muscle preparation, and it was mainly considered as a precursor of epinephrine and norepinephrine (Marsden, 2006). The discoveries described above, especially of von Euler and Axelrod however broke the ground to identify dopamine as a neurotransmitter. A Swedish scientist Arvid Carlsson (b. 1923) in 1957 was the first to propose that dopamine served as a central neurotransmitter (Carlsson *et al.*, 1957). It was stated before that epinephrine levels were much higher than that of dopamine in peripheral tissues, but Carlsson showed that the brain dopamine levels exceeded those of norepinephrine, suggesting more important role for dopamine as was generally believed. He also demonstrated that dopamine levels were depleted by reserpine (an indole alkaloid isolated from Indian Snakeroot; blocks vesicular monoamine transporters), which was reversed by administration of DOPA (3,4-dihydroxyphenylalanine, a precursor of dopamine). Carlsson observed that reserpine produced Parkinson-like symptoms in test animals, while administration of L-DOPA reduced the observed symptoms. He concluded that Parkinson's disease (PD) involves selective depletion of dopamine levels in the substantia nigra of the brain, which leads to failure in dopamine release. Paul Greengard's (b. 1925) work showed that dopamine acting on D_1 receptors activates the formation of cyclic adenosine monophosphate (cAMP), which in turn activates a cAMP-sensitive protein kinase that increases phosphorylation of a substrate known as DARPP-32. To appreciate their importance in the field Carlsson and Greengard together with Eric Kandel (b. 1929) shared a Nobel Prize in Physiology or Medicine in 2000 "for their discoveries concerning signal transduction in the nervous system" (Marsden, 2006; Iversen and Iversen, 2007; Rubin, 2007).

1.2. Dopamine receptors

In early 1970s, when the concept of receptor was still vague, an American pharmacologist Raymond Ahlquist (1914–1983) wrote: "To me they (receptors) are an abstract concept conceived to explain observed responses of tissues produced by chemicals of various structure" (Ahlquist, 1973). Earl Sutherland (1915–1974), a well-known American pharmacologist and biochemist, was less sceptical about a receptor being a molecular entity, but still, in 1967 he thought of it more of as a binding site on the enzyme adenylate cyclase, rather than an independent unit. A quotation from his 1967 publication: "... It seems likely that in most and perhaps all tissues the beta receptor and adenyl cyclase are the same" (Robison *et al.*, 1967). The first evidence for the existence of dopamine receptors (DARs) came in 1972 from studies showing that dopamine can acti-

vate adenylate cyclase (AC) (Kebabian *et al.*, 1972). Soon Philip Seeman identified a site that could be labelled by both dopamine and haloperidol (an antipsychotic and dopaminergic antagonist) (Seeman *et al.*, 1976). This site was later named the dopamine D_2 receptor in a paper that classified the dopamine receptors into D_1 and D_2 based on their pharmacological coupling to adenylate cyclase (Kebabian and Calne, 1979). Currently there are five different subtypes of DARs: D_1 to D_5, of which the subtype D_2 has two splice variants – D_{2S} and D_{2L}. The D_1 family constitutes D_1 and D_5 receptors, which both couple to $G_{s/olf}$ family of G-proteins activating AC and resulting in increase of cAMP. Whereas the D_2 family includes D_2, D_3 and D_4 receptors, coupled primarily to $G_{i/o}$ family of G-proteins and thus induce inhibition of AC (Rubin, 2007; Beaulieu and Gainetdinov, 2011). They all belong to the *Rhodopsin* family of G-protein-coupled receptors (discussed below).

The dopamine D_1 receptor is the most widespread of DA receptors, followed by the D_2 receptors. The D_3 dopamine receptor has a more limited pattern of distribution, restricted mainly to limbic areas of the brain. The D_4 dopamine receptor has the lowest level of expression in the brain having relatively higher expression in the limbic regions than in the striatum. The D_5 dopamine receptors are poorly expressed in multiple brain regions. In the periphery, all subtypes of dopamine receptors have been observed in varying proportions in the kidney, adrenal glands, sympathetic ganglia, gastrointestinal tract, blood vessels, and heart. D_1, D_2 and D_4 receptor have also been found in the retina (Missale *et al.*, 1998; Marsden, 2006; Beaulieu and Gainetdinov, 2011). In addition, receptor cloning has given more insight into the architecture of dopamine receptor subtypes (Bunzow *et al.*, 1988; Dal Toso *et al.*, 1989; Giros *et al.*, 1989, 1990; Grandy *et al.*, 1989; Chio *et al.*, 1990; Sunahara *et al.*, 1991; Van Tol *et al.*, 1991; Fu *et al.*, 1995). The D_1 and D_5 dopamine receptors are 80% homologous in their transmembrane domains, whereas the D_3 and D_4 dopamine receptors are 75% and 53% homologous, respectively, with the D_2 receptor. The NH_2-terminal domain has a similar number of amino acids in all of the dopamine receptors, whereas the COOH-terminal for the D_1-family receptors is seven times longer than that of the D_2-family receptors. Also the third intracellular loop, involved in G-protein coupling, is bigger in the D_2-like receptors (Missale *et al.*, 1998).

1.2.1. Dopamine receptor function

Dopamine has several functions in central nervous system including voluntary movement, feeding, affect, reward, sleep, attention, working memory, and learning. In the periphery, dopamine plays important physiological roles in the regulation of olfaction, retinal processes, hormonal regulation, cardiovascular functions, sympathetic regulation, immune system, and renal functions, among others.

Function of dopamine is strongly associated with the distribution and localisation of dopaminergic neurons and different subtypes of dopamine receptors. The dopaminergic system is divided into four following pathways:

1. The nigrostriatal dopaminergic pathway beginning from substantia nigra and projecting to striatum is the pathway connected to locomotor activity. This pathway is implicated in Parkinson's disease and tardive dyskinesia, which is one of the side-effects of antipsychotic drugs. Locomotor activity is primarily controlled by the D_1, D_2 and D_3 receptors. The activation of D_1 receptor has moderate stimulatory role on locomotion while the roles of D_2 and D_3 receptors are more complex because of both presynaptic and postsynaptic expression of these receptors. The effect is biphasic – activation of presynaptic D_2 receptors (at lower doses) causes decrease in locomotor activity and activation of postsynaptic D_2 receptors (at higher doses) increases locomotor activity. The activation of D_3 receptors is moderately inhibiting locomotion. The D_4 and D_5 receptors seem not to be involved in control of movement (Beaulieu and Gainetdinov, 2011).
2. The mesolimbic dopaminergic pathway is involved in reward and reinforcement mechanisms. The reward pathway begins from dopaminergic neurons in ventral tegmental area (VTA) of the midbrain projecting to nucleus accumbens (a part of ventral striatum), amygdala, hippocampus and prefrontal cortex. This pathway is heavily implicated in addiction, schizophrenia, bipolar disorder and depression. The D_1, D_2, and D_3 dopamine receptors are all involved in this pathway. Most of the clinically effective antipsychotic drugs mediate their action via blockage of D_2 receptors. Accumulating evidence, however, suggests that some roles attributed mainly to D_2 receptors might instead be modulated by D_3 receptors (Sokoloff *et al.*, 1992; Shafer and Levant, 1998; Joyce, 2001; Kiss *et al.*, 2008). D_3, D_4 and potentially D_5 receptors have some influence on cognitive function mediated by hippocampal regions, but still, specific roles of these receptors remain largely unknown. D_1 and D_2 receptors are involved in learning and memory, mediated primarily by prefrontal cortex (Beaulieu and Gainetdinov, 2011).
3. The mesocortical pathway connecting ventral tegmentum and cerebral cortex is essential for normal cognitive function and is involved in motivation and emotional response. This pathway and its function are closely associated with previously described mesolimbic pathway.
4. The tuberoinfundibular pathway, which connects hypothalamus to the pituitary gland, is connected to the regulation of hormone secretion. Pituitary D_2 DARs modulate prolactin secretion, kidney D_1 DARs mediate renin secretion, adrenal gland D_2 DARs regulate aldosterone secretion *etc.* (Beaulieu and Gainetdinov, 2011). Increased prolactin release, originating from this pathway, is a side effect of conventional antipsychotic drugs (Marsden, 2006).

1.2.2. Pharmacological properties of dopamine receptors

Dopaminergic ligands have remained an active area in the development of CNS drugs, agonists mainly in the treatment of Parkinson's disease and antagonist as antipsychotics in the treatment of schizophrenia. For quite a long time it has been believed that the therapeutic anti-parkinsonian effects of the dopamine precursor, L-DOPA, are primarily attributed to its stimulation of DA D_2 receptor whereas dyskinesia might be mediated by the over-stimulation of the D_1 receptor (Zhang *et al.*, 2008).

In schizophrenia the dopaminergic system is upregulated, as opposed to the down-regulation seen in PD. The observed dopamine supersensitivity in schizophrenia, leading to psychotic reactions, may be the result of enhanced sensitivity of D_2/D_3 receptors (Beaulieu and Gainetdinov, 2011). Most antipsychotic drugs are based on dopamine D_2 receptor antagonism. The hyperactivity of DA in subcortical structure is thought to associate with the positive symptoms, whereas D_1 receptor hypoactivity in the frontal cortical area has recently been suggested to attribute to the negative symptoms and impaired cognitive function. Low doses of selective D_1 receptor agonists (e.g. DHX, A77636, and SKF81297) have been shown to enhance cognitive function. First generation antipsychotic drugs (so called typical antipsychotics), such as chlorpromazine, haloperidol, flupentixol and others, are typically accompanied by pronounced side effects. Second generation antipsychotics (also called atypical antipsychotics), such as clozapine, sulpiride, aripiprazole and others, have fewer side effects and they have activity also on other GPCRs, especially on serotonin 5-HT_{2A} receptors (Zhang *et al.*, 2008).

Since the discovery of SCH23390 **(Figure 1)**, a benzazepine D_1 antagonist, a number of other 1-phenylbenzazepine agonists (i.e. SKF38393, SKF77434) and antagonists (i.e. SCH39166) have been developed. The structure of these compounds mimics the structure of the catecholamine dopamine and is widely recognised for predictable association with D_1 receptor activity. ^{3}H- and ^{11}C-labelled SCH23390 are widely used radioligands for D_1/D_5 receptors, but for their affinity also for 5-HT_2 receptors they are often replaced with SCH39166 for *in vivo* applications. SCH39166 has 25-fold lower affinity for 5-HT_2 receptors as well as slower rate of metabolism, possessing a more favourable signal-to-noise ratio (Banerjee and Prante, 2012). Various replacements in the phenyl ring of benzazepine can modulate affinity and selectivity of interactions with D_1 versus D_2 receptors. C-7 substituent (corresponding to the meta-OH of DA) can control pharmacological agonism versus antagonism at D_1 receptors. Halogen replacement of the 7-OH group results in D_1 antagonist activity (i.e. SCH23390, SCH83566, SCH39166) (Zhang, Neumeyer, *et al.*, 2007). A77636 is a full D_1 agonist which belongs to isochroman series (along with A68930). It increases locomotor activity and reduces Parkinson-like symptoms. ABT-431 has anti-Parkinson activity and also induces dyskinesia, similar to L-DOPA. ABT-431 has been studied also in other applications. For example it reduces cocaine-craving in humans and reverses haloperidol-induced

cognitive deficits in monkeys (Martin, 2011). Because of their adverse effects the overall advantage of D_1 agonists in PD treatment remains uncertain (Zhang *et al.*, 2008).

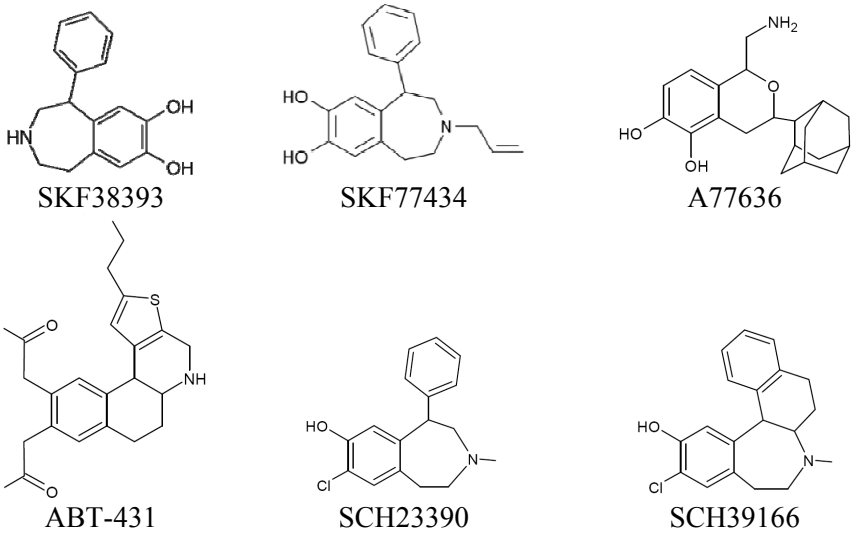


Figure 1. Dopamine D_1 receptor ligands (Zhang, Neumeyer, *et al.*, 2007; Martin, 2011)

Due to extensive homology between the binding sites it has been a challenge to develop selective ligands within the D_2 family of dopamine receptors. Aporphines are active both on D_1 and D_2 receptors and they constitute one of the largest groups of isoquinoline alkaloids. R-(–)-apomorphine **(Figure 2)**, known since the 19th century, is the prototype of aporphine DA agonists. Subcutaneously injected apomorphine is used as an anti-parkinsonian drug and its sublingual preparation has been used as a treatment for male erectile dysfunction. Apomorphine is often used in combination with L-DOPA, but its use is limited with poor bioavailability and short duration of action (Zhang, Neumeyer, *et al.*, 2007; Zhang, Zhang, *et al.*, 2007). A number of currently widely used antipsychotics (haloperidol, risperidon *etc.*) with D_2 antagonist activity belong to the family of aryl/heteroaryl-substituted piperazines/ piperidines. These compounds contain a linker between two functional groups, which generally provide additional activities at other neurotransmitter receptors, including serotonergic, muscarinic, α-adrenergic and histamine receptors. Recent efforts in developing D_2 antagonists with limited adverse neurological effects have focused on agents with only moderate D_2 antagonist activity or

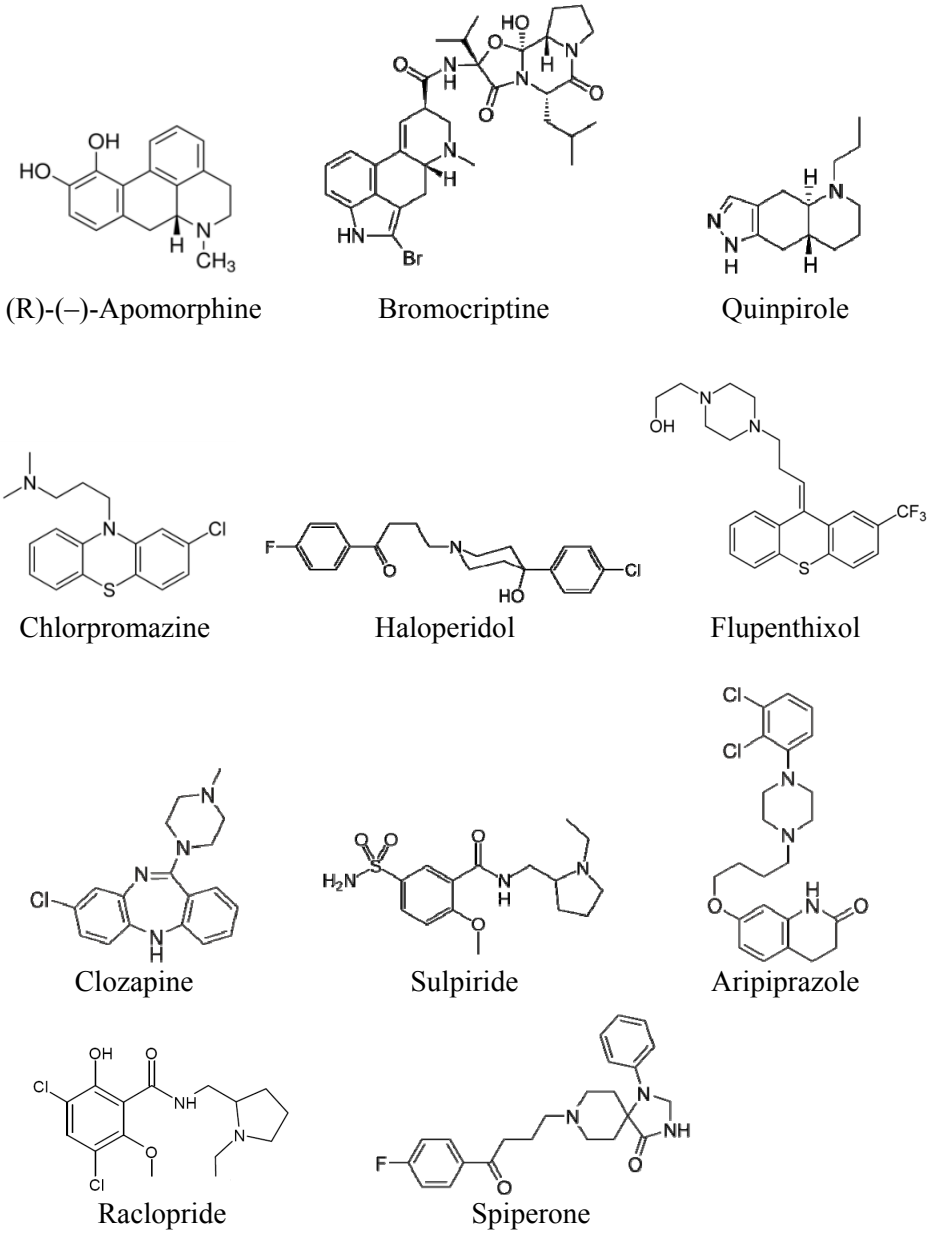
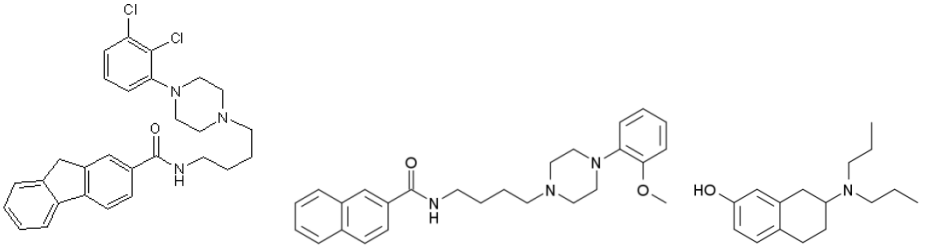


Figure 2. Dopamine D_2 receptor ligands (Prante *et al.*, 2010)

with D_2 partial-agonist effects, combined with interactions at other DA receptors (D_1, D_2 and D_4), serotonin (5-HT_{1A}, 5-HT_{2A} and 5-HT_3) receptors or α_1-adrenergic receptors. Dibenzazepines (clozapine, quetiapine *etc.*) as well have affinities on other neurotransmitter receptors, similar to the aryl/hetero-aryl-substituted piperazines/piperidines. Family of methoxybenzamides, used as antipsychotics, include some of the most widely used pharmacological tools such as sulpiride and raclopride. Trititated spiperone is well established in ligand binding (Prante *et al.*, 2010).

Most D_3 and D_4 receptor antagonists are piperazine analogues with variable selectivity for D_3 or D_4 over D_2 receptors. Developing D_3 and D_4 selective agonists has been less successful. Agonists 7-OH-DPAT (**Figure 3**) and its analogue 7-OH-PIPAT and partial agonist BP-897 are some of the few D_3-selective ligands, whereas BP-897 has been extensively used for the treatment of cocaine abuse (Prante *et al.*, 2010). Current D_3-selective antagonists (such as NGB2904) are far from ideal drug candidates due to their physicochemical characteristics, with many of them being poorly soluble and lipophilic and unfortunately there has been no great success yet to reduce the molecular weight and increase water solubility of D_3 antagonists (Micheli, 2011).



NGB2904 BP-897 7-OH-DPAT

Figure 3: Dopamine D_3 receptor ligands (Prante *et al.*, 2010)

The interest in selective D_4 receptor ligands was driven by the finding that the atypical neuroleptic drug clozapine preferentially binds to the D_4 receptor (Van Tol *et al.*, 1991). PD-168077 (**Figure 4**) was one of the first selective D_4 agonists (Glase *et al.*, 1997). It induces an improved memory performance, but is accompanied with dyskinesia; it also has proerectile effect as well as other D_4 receptor agonists ABT-724 and A-412997. Examples of D_4-selective antagonists include FAUC-213, L-745870 and U-101387. Not as many D_3-selective radioligands are available (i.e. [^{3}H]7-OH-DPAT and [^{11}C]GR218231) as there are D_4-selective radioligands (i.e. [^{3}H]A-369508 and [^{11}C]clozapine) for *in vitro* and *in vivo* use (Prante *et al.*, 2010). Very limited success has been met with development of selective D_5 receptor ligands that would not bind to its highly homologous and abundant D_1 receptors.

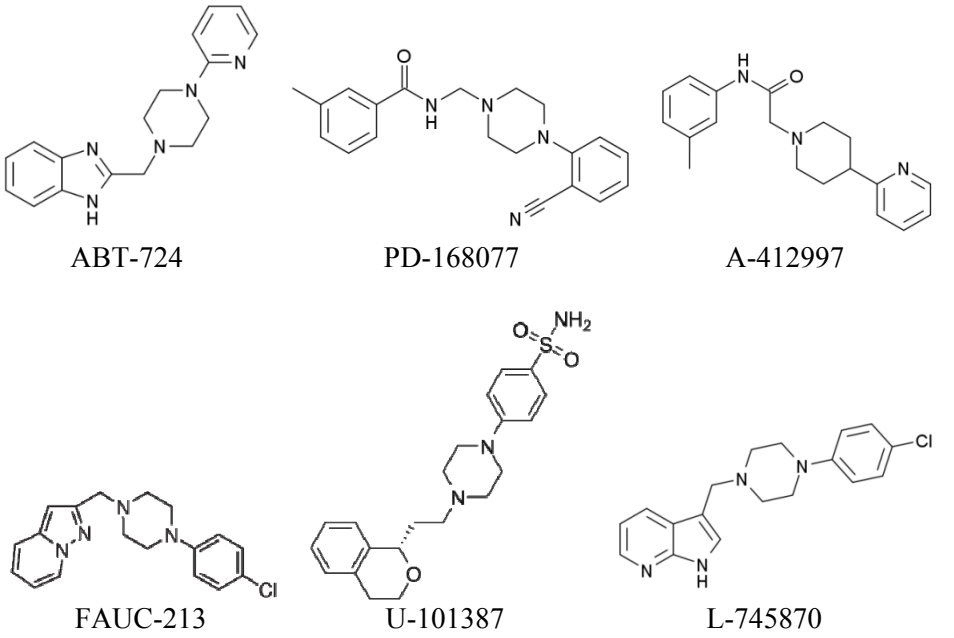


Figure 4. Dopamine D_4 receptor ligands (Prante *et al.*, 2010)

Many currently used dopaminergic drugs have several activities some of which can be seen as unwanted side-effects while others may possess therapeutic advantage. Dopaminergic agonists, generally used for treatment of PD and dyskinesia, are also used for pituitary tumours, hyperprolactinemia and related conditions (bromocriptine, cabergoline), erectile dysfunction (apomorphine), restless legs syndrome (ronipirole, pramipexole), type 2 diabetes (bromocriptine), bipolar disorder and depression (pramipexole, rotigotine) and hypertensive crisis (fenoldopam). In addition to treatment of schizophrenia certain D_2 receptor antagonists are used also in the treatment of nausea and vomiting, and in gastroparesis as a prokinetic drug to increase muscle contraction in the upper digestive tract, as well as in depression and bipolar disorder (Prante *et al.*, 2010; Beaulieu and Gainetdinov, 2011).

There is no consensus yet whether targeting dopamine receptors with more selective ligands would significantly improve their therapeutic response or not. Nevertheless, developing more subtype-selective ligands is still in the main focus of dopamine receptor pharmacology. The use of highly selective ligands is of immense importance for techniques such as positron emission tomography (PET) and single-photon emission computed tomography (SPECT), employed for imaging receptor distribution in patients *in vivo*, (Banerjee and Prante, 2012). In such applications selective ligands may help to clarify specific roles of receptor subtypes in diseases. As important as putting effort into development

of (selective) dopaminergic drugs is developing of new screening assays for the identification of compounds that are effective for dopamine-related disorders, and finding ways for detecting multiple signalling events downstream of receptor and G-proteins. This would help identify signalling pathways which are relevant for therapeutic effect and/or identify which pathways might lead to side effects.

Developing dopamine receptor subtype-selective ligands and furthermore, developing assay systems that could be used for characterisation of these new dopaminergic ligands and estimating their therapeutic potential on the level of cyclic AMP modulating activity, are both within the scope of this study.

1.3. Dopamine receptors as G-protein-coupled receptors

Dopamine receptors all belong to the family of G-protein-coupled receptors (GPCRs). GPCRs are cell membrane receptors, along with voltage- and ligand-gated ion channel receptors and nuclear hormone receptors (according to the International Union of Basic and Clinical Pharmacology, IUPHAR). G-protein-coupled receptors, also known as seven transmembrane receptors (7-TMRs), are the largest protein receptor superfamily in the body mediating cellular response to hormones and neurotransmitters, as well as being responsible for light, taste and olfactory responses. The structure of GPCRs became clearer by protein sequencing of rhodopsin, a protein responsible for sensation of light. At that time rhodopsin was still not considered as a protein from the group that we now know as G-protein-coupled receptors. Rather, the revealed seven transmembrane structure of rhodopsin as well as of bacteriorhodopsin was then thought of as a general feature of light-sensitive proteins (Ovchinnikov Yu, 1982). In 1986, when β_2-adrenergic receptor (Dixon *et al.*, 1986) and muscarinic acetylcholine receptor (Kubo *et al.*, 1986) were cloned it became clear that this structural feature is in fact common to all G-protein-coupled receptors, which was further supported by cloning the α_{2A}-adrenergic receptor the following year (Kobilka *et al.*, 1987). Many more GPCRs were cloned over the next years and some additional common features were found in their primary structure, i.e. N-linked glycosylation near the amino terminus, sites for regulatory phosphorylation on the inner loops and carboxy-terminal tail *etc.* (Lefkowitz, 2007). By switching portions of β_2- and α_2-adrenergic receptors, which were known to modulate adenylate cyclase activity via different types of G-proteins, G_s and G_i proteins, respectively, it was possible to identify receptor regions that were responsible for ligand binding specificity (the residues in the membrane spans) and regions responsible for G-protein coupling specificity (the carboxy-terminal portions of the third intracellular loop) (Kobilka *et al.*, 1988).

For decades many people made efforts to obtain high resolution receptor structures other than the ones based on modelling the primary amino acid sequences. The first crystal structure of a 7-TMR, resolved in 1990, was that of bacteriorhodopsin, a light-sensitive protein pump found in bacteria (Henderson *et al.*, 1990). Bacteriorhodopsin however is not a GPCR. Combining receptor crystallisation and X-ray technology it was finally possible to resolve the crystal structure of the first GPCR, rhodopsin (Palczewski *et al.*, 2000). It took a while before others succeeded to crystallise more demanding GPCRs, such as β-adrenergic receptors (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007), adenosine A_{2A} receptor (Jaakola *et al.*, 2008), dopamine D_3 receptor (Carlsson *et al.*, 2011), muscarinic M_2 (Haga *et al.*, 2012) and μ-opioid receptor (Manglik *et al.*, 2012). One of the most recent works with crystallised mutant adenosine A_{2A} receptor has shed light for the role of water molecules, sodium ions and lipids/cholesterol in GPCR stabilisation and function (Liu *et al.*, 2012).

On the basis of receptor sequence and structural similarities vertebrate GPCRs are divided into five families: *Rhodopsin* (family A), *Secretin* (family B), *Glutamate* (family C), *Adhesion* and *Frizzled/Taste2* (Lagerström and Schiöth, 2008; Rosenbaum *et al.*, 2009). According to IUPHAR the receptors are divided into families A, B, C, Frizzled and other 7-TMRs. For up-do-date details see http://www.iuphar-db.org. Major differences in structure among the families lie in the extracellular side, where the ligand binding takes place, and in the intracellular side, where functional coupling to intracellular effectors occur. The *Rhodopsin* family is the largest family of GPCRs and they represent the largest group of drug targets. Most of the receptors in this family have relatively small extracellular N-terminus whereas the ligand binding pocket is mainly confined to the transmembrane regions and extracellular loops. They bind amines (i.e. dopamine receptors as discussed earlier), peptides, lipid-like compounds, glycoproteins and nucleotides. Other four of the five GPCR families have long, structurally diverse N-termini, which also have certain role in ligand binding. More details can be found in (Lagerström and Schiöth, 2008; Rosenbaum *et al.*, 2009).

1.3.1. GPCR signalling

Most of the 7-TMRs signal the extracellular stimuli from hormones, neurotransmitters and other ligands via heterotrimeric guanine nucleotide binding proteins (G-proteins) – their direct signalling partners inside the cell. It also explains why the name G-protein-coupled receptors became widely accepted. During past years several G-protein-independent signalling pathways have been discovered (Charest *et al.*, 2007; Defea, 2008) and therefore the more flexible term "seven-transmembrane receptors" is being reintroduced (Lefkowitz, 2007; Kenakin and Miller, 2010).

However, the "primary" behaviour of GPCRs is still considered to be the one connected to G-protein activation and signalling; G-protein-independent sig-

nalling is connected to the "secondary" behaviour, such as dimerisation, oligomerisation, desensitisation, internalisation and interaction with other non-signalling membrane proteins. Secondary behaviour of GPCR signalling herein refers that these pathways are not yet as widely studied and/or as generally accepted; such signalling is seen as a new potential for drug discovery but for now it still remains largely an undiscovered area.

The extent of a drug response depends on the efficiency of the system, comprising factors such as the receptor density and the efficiency of receptor coupling to signalling partners. Ligand efficacy (or activity) describes the extent of a drug's response. Efficacy (E or E_{max}) is no longer considered as a universal property; rather the environment of the receptor dictates the apparent property of the drug (Kenakin, 2002). Therefore drugs could have several intrinsic activities. The term "intrinsic activity" was accommodated by E. J. Ariens in 1954 to describe the observation that not all agonists produce maximal response. The term "intrinsic activity" is highly related to the term "efficacy", which was first implemented by R.P. Stephenson in 1956, after his observation that a series of related alkytrimethylammonium compounds produced different maximal levels of guinea pig ileal contraction within a similar concentration range, reviewed in (Kenakin, 2010). In broader term efficacy is a combined property of receptor and ligand; a ligand causing the receptor to change its behaviour towards the cell (Kenakin, 2002). Ligands producing maximal cellular response have high efficacy (high intrinsic activity) and are termed "full agonists", ligands with lower efficacy are "partial agonists" and ligands with no efficacy are "(neutral) antagonists". In some cellular systems, namely, in constitutively active systems, it is possible to observe negative efficacy – this is when a ligand decreases basal response. Such ligands are termed "inverse agonists". A number of previously considered neutral antagonists behave as inverse agonists in a constitutively active system (Cai *et al.*, 1999; Rossier *et al.*, 1999; Ahmed *et al.*, 2006), which brought to the idea that ligands that would reverse a pathological constitutive activity might be a unique therapeutic application of inverse agonists (Kenakin, 2002; Tao, 2008). More information on other terms that are used to define ligand properties and functional response, but are out of the scope of the current thesis, include "protean agonism", "biased agonism", "functional selectivity", "agonist-directed trafficking", "ligand-directed signalling", "conformational selectivity" *etc.*, and are discussed in more detail in (Kenakin and Onaran, 2002; Gilchrist, 2007; Kenakin, 2007; Lane *et al.*, 2007; Neubig, 2007; Kenakin and Miller, 2010).

1.3.1.1. G-protein-dependent signalling of GPCRs

Heterotrimeric G-proteins

As stated above, GPCRs signal primarily via G-proteins. These heterotrimeric G-proteins consist of α-, β- and γ-subunits. The α-subunit is readily dissociable

upon activation by the receptor; β- and γ-subunits form a more stable dimeric complex. In humans, there are 21 Gα subunits encoded by 16 genes, 6 Gβ subunits encoded by 5 genes, and 12 Gγ subunits. Members of Gα family range in size from 39–52 kDa and most of them are post-translationally modified, palmitoylated and/or myristoylated, which helps them to be anchored in membrane. All six Gβ subunits are approximately 36 kDa in size and they need to form a complex with Gγ for proper folding and function. Gγ subunits are smaller, between 7 and 8 kDa and via post-translational prenylation with fatty acid moieties they anchor the Gβγ dimer in the membrane. Several different Gβγ dimers can interact with the same Gα isoform, suggesting that differential expression or subcellular location of the subunits may be an important determinant of signalling specificity (Oldham and Hamm, 2008).

Based on their function and primary sequence similarity of the Gα subunit of G-proteins, they are divided into four main classes: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12}$. A comprehensive overview of effectors activated by various G-protein isoforms can be found in (Kristiansen, 2004; Landry *et al.*, 2006; Birnbaumer, 2007). Here only some examples are given:

1. $G\alpha_s$ family increases the level of cAMP by activating all the 9 isoforms of AC. The main target of cAMP is cAMP-dependent protein kinase (PKA), but also exchange protein activated by cAMP (Epac) acting as a guanine exchange factor (GEF) for Rap GTPase, and cyclic nucleotide-gated (CNG) ion channels. Rap belongs to Ras-family of small monomeric GTP-binding proteins that regulate gene expression (Takai *et al.*, 2001).
2. $G\alpha_{i/o}$ family decreases cAMP levels by inhibiting AC isoforms 5 and 6, whereas $G\alpha_o$ might also inhibit AC1; $G\alpha_i$ and $G\alpha_o$ are almost 10-fold more abundant in cells than $G\alpha_s$ (Landry *et al.*, 2006), which is why $G\alpha_i$-signalling may often hinder $G\alpha_s$-signalling in heterologous systems.
3. $G\alpha_q$ family is known for increasing intracellular Ca^{2+} by activating phospholipase β (PLCβ) leading to production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG); IP_3 on the other hand migrates to endoplasmic reticulum (ER) and binds to its receptor leading to Ca^{2+} release from ER into cytosol. Depletion of Ca^{2+} stores from ER in turn triggers inflow of extracellular Ca^{2+} (Parekh and Penner, 1997). Both DAG and Ca^{2+} can switch on the kinase activity of protein kinase C (PKC), resulting in phosphorylation of various cellular proteins.
4. $G\alpha_{12}$ family activates Ras-GTPase activating protein (Gap1) and Rho family guanine nucleotide exchange factors (RhoGEFs). They can regulate mitogen-activated protein (MAP) kinase cascades.

The main effectors regulated by Gβγ-dimer are activation of PLCβ; inhibition of AC1, AC5 and AC6; activation of AC2, AC4 and AC7; activation of inwardly rectifying K^+ channels (GIRK/Kir) 3.1 and 3.4; inhibition of voltage-gated Ca^{2+} channels (Ca_V2); activation of phosphoinositide-3-kinase (PI3-kinase γ). PI3-kinase phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2), gene-

rating phosphatidylinositol (3,4,5)-triphosphate (PIP_3) (Kristiansen, 2004; Landry *et al.*, 2006).

G-protein activation

G-proteins are inactive in the heterotrimeric conformation (Gαβγ) where Gα binds GDP and the constitutive Gβγ dimer. Extracellular stimuli activate receptors by inducing a conformational change that permits G-protein binding and catalyses GDP release from Gα, thereby resulting in the formation of a stable high-affinity complex between the activated receptor and G-protein. Binding of GTP to Gα destabilises this complex, leading to a structural re-arrangement of Gα(GTP), Gβγ and the receptor. Both units, the Gα(GTP) and the Gβγ dimer, go on to interact with downstream effector proteins (discussed above). The cellular response is terminated when Gα hydrolyses GTP to GDP and re-associates with Gβγ, thus completing the cycle (Oldham and Hamm, 2006).

The rate limiting step in G-protein activation is the GDP-release, which may be regulated by non-receptor guanine nucleotide-exchange factors/proteins (GEFs, also known as GEPs) and guanine nucleotide dissociation inhibitors (GDIs) by accelerating or inhibiting GDP release, respectively (Siderovski and Willard, 2005; Oldham and Hamm, 2006). The formed receptor-G-protein complex is transient due to rapid binding of GTP, which concentration exceeds that of GDP by several-fold in cells. Binding of GTP causes a structural arrangement of Gα(GTP), Gβγ and the receptor that permits effector interactions while the Gβγ dissociates (Oldham and Hamm, 2006). G-protein subunits may as well remain closely associated following receptor activation (Bünemann *et al.*, 2003).

G-protein inactivation is regulated by GTPase activity which hydrolyses GTP to GDP. GTPγS, a commonly used analogue of GTP in pharmacological assays, has a sulphur atom instead of oxygen in the γ-phosphate position, rendering the molecule more resistant to hydrolysis and stabilising Gα in an active conformation (Zhang and Xie, 2012). GTPase activating proteins (GAPs) can accelerate the activity of GTPase and thereby inactivate G-proteins. Some examples of GAPs are PLCβ1, PDEγ, AC5 – all of which are known as G-protein activated effectors. Other than effector-mediated feedback inhibition, another class of GAPs has been identified, the regulators of G-protein signalling (RGS) with several members (Oldham and Hamm, 2008).

Heterotrimeric G-protein can easily accommodate a receptor dimer and it has been suggested that a GPCR dimer might provide the most appropriate footprint to bind a G-protein (Fotiadis *et al.*, 2006). Studies, however, show that dimerisation is not a requisite for receptor-G-protein interaction. A monomeric receptor also binds and activates G-proteins (Oldham and Hamm, 2008; Milligan, 2009). In the light of the above discussion, recently a crystal structure of the active state ternary complex composed of agonist-occupied monomeric β_2-adrenergic receptor and nucleotide-free G_s heterotrimer was published

(Rasmussen *et al.*, 2011), supporting the active receptor monomer theory. Receptor (homo-/hetero-) dimerisation may therefore merely provide signalling complexes that have unique ligand binding and G-protein coupling properties compared with the component monomeric receptors; dimerisation may add more possibilities for allosteric regulation (Oldham and Hamm, 2008; Canals *et al.*, 2011).

1.3.1.2. G-protein-independent signalling of GPCRs

The discovery of rhodopsin kinase (Shichi and Somers, 1978) and studies on β_2-adrenergic receptors lead to the discovery of two small families of proteins, the G-protein-coupled receptor kinases (GRKs) (Stadel *et al.*, 1983) and the β-arrestins (Lohse *et al.*, 1990), both of which regulate GPCRs similar to G-proteins in a stimulus-dependent fashion. These discoveries solved the problem of receptor desensitisation and revealed it as a universal regulatory mechanism common to all seven transmembrane receptors (Pitcher *et al.*, 1998; Lefkowitz and Shenoy, 2005).

After receptor is being activated and interacting with its G-protein, one of the GRKs begins to phosphorylate the receptor largely on its carboxyl terminal cytoplasmic tail. The phosphorylation promotes the β-arrestins to interact with the receptor leading to desensitisation of G-protein signalling by steric exclusion by the β-arrestins (Lohse *et al.*, 1990, 1992). Now there are at least 4 different β-arrestin genes, two of which are expressed only in retinal rods and cones, and seven members of the GRK family identified. The role of β-arrestins is much more complicated as it was initially suggested. They are also able to increase degradation of cAMP by recruiting phosphodiesterases (PDEs) to the receptor and mediate receptor internalisation after ubiquitination of the β-arrestin. In addition, the role of β-arrestin is not restricted to different desensitisation mechanisms. They are also involved in regulating activity of certain signalling pathways, like MAP kinase pathways (ERK1/2, P38 and JNK3), and AKT, PI3 kinase and others. For more details see (Lefkowitz and Shenoy, 2005; Lefkowitz, 2007). Drugs, such as lithium salts, for example, appear to act through inhibition of Akt (Beaulieu *et al.*, 2007), suggesting that some existing antidepressants may already selectively target the β-arrestin-dependent pathway over the G-protein-dependent pathway (Defea, 2008).

1.4. Methods for GPCR characterisation and ligand screening

Given the importance of GPCRs in health and disease they represent the largest family of drug targets. Development of assays for ligand screening remains in focus of drug discovery worldwide. Historically, drug discovery was physiology (or organ) based; now it is mainly a molecular-target based. Depending on the

purpose ligand binding assays or signalling-dependent functional assays are used. Many GPCRs have no known endogenous ligand (for instance the orphan GPCRs) and/or a high-affinity labelled ligand available, which is why some cases functional assays are the only alternative.

Classical techniques for measuring receptor/ligand interaction and G-protein activation are the radioligand binding and GTPase or [^{35}S]GTPγS assays, respectively. With better knowledge of GPCR signalling pathways and availability of new techniques cell-based functional assays have made a breakthrough during the past decades. Many GPCRs may have more than one signalling pathway and therefore measuring drug's effects on various pathways is often necessary to provide more accurate pharmacological profile of a drug.

When selecting a method it is important to consider whether to measure proximal or distal signalling step after GPCR stimulation. Events proximal to receptor activation (i.e. G-protein activation) reduce the incidence of false positives; events distal on the signalling cascade, however, enable to have better signal-to-noise ratio because of signal amplification by various intracellular effectors (AC, PKA, PLC *etc.*).

As important as choosing the most suitable method for testing a ligand, is also selecting the receptor source. For radioligand binding mainly cell membranes are used, although with the advent of fluorescent ligands intact cells may be preferred. For functional cellular assays live cells are used. Also it is important to consider the expression of different cellular proteins necessary or affecting receptor signalling.

1.4.1. Ligand binding assays

Ligand binding or receptor binding assay is the primary method for characterising direct interaction between a receptor and its ligand. It gives information about the affinity of ligands to the receptor, ligand association and dissociation rates and about the density of receptor in tissues or cells. The method requires high affinity and preferably selective ligands that can be chemically radiolabelled and used as tracers. Since the first radioligand binding in 1970 by Lefkowitz and colleagues, using an iodine-125 labelled adrenocorticotropic hormone to determine binding affinity for its receptors (Lefkowitz *et al.*, 1970), ^{3}H- and ^{125}I-labelled ligands have been widely used. Non-labelled ligands can be characterised by their ability to displace the binding of a radioligand to the target or to modulate the affinity of a radioligand for the target. Radioligand binding has been the main approach for screening of new drugs during the past decades.

While the traditional radioligand binding assays require washing and filtration steps, scintillation proximity assay (SPA) can be more easily scaled down and automated without any need for washing and filtration. In SPA, only the radiolabelled molecules binding to the GPCR immobilised on the surface of

SPA beads can activate the scintillation beads, which produce photons detectable with a scintillation counter (Wu and Liu, 2005).

Radioligand binding assay has been extensively employed in PET applications for imaging receptor distribution and densities in live brain. Some examples of imaging dopamine receptors as well as radiolabelling dopamine D_1 and D_2/D_3 antagonists SCH23390 and raclopride, respectively, are described in (Wagner *et al.*, 1983; Ehrin *et al.*, 1985; Halldin *et al.*, 1986; Nader *et al.*, 2006, 2008).

Non-radioactive alternatives have been developed for receptor binding assays. These include time-resolved fluorescence resonance energy transfer (TR-FRET) technology such as DELFIA™ TRF from PerkinElmer, LanthaScreen™ from Invitrogen and Tag-lite™ from Cisbio. All these methods require labelling of both the target GPCR and the ligand with an appropriate TR-FRET pair (i.e. a terbium cryptate fluorophore + red or green acceptor fluorophore), which upon interaction will produce a luminescent signal. For dopamine receptor assays Cisbio offers kits for D_1, D_2 and D_3 receptors including respective fluorescently labelled ligands and cell lines expressing the SNAP-receptor labelled with terbium cryptate (www.htrf.com).

More flexible non-radioactive alternatives use fluorescently labelled ligands with only the reporter ligand being labelled. In such assays it's possible to skip the filtration step by optical discrimination of solid-liquid phase partitioning of the fluorescent ligands (discrimination of fluorescence intensity from the cell surface versus from the surrounding buffer). Fluorescence polarisation/ anisotropy (FP/FA) is another methodology to discriminate fluorescence from bound versus unbound ligand. FP/FA is based on the principle that small molecules (fluorescently labelled free ligands) rotate faster in solution than larger molecules (i.e. labelled GPCRs buried in cell membranes) (Kunapuli, 2010; Veiksina *et al.*, 2010). Limited availability of tagged GPCR-expressing cell lines and suitable fluorophore-labelled ligands with sufficient affinity are the main reasons why the above described fluorescent techniques have not managed to push aside classical radioligand binding assays (Zhang and Xie, 2012).

1.4.2. Functional assays

Functional assays provide information on how a drug affects any receptor-connected cellular function of interest. Functional studies are used to distinguish agonists from antagonists, assess intrinsic activities or efficacies of ligands, detect which signalling pathway a ligand can activate and to what extent, reveal allosteric modulators on functional level and so on. In this chapter a selection of functional assays are described that are used in GPCR studies and ligand screening.

1.4.2.1. G-protein-dependent functional assays

GTPase assay

When GTP binds to Gα subunit, the affinity of Gα for Gβγ and the receptor is reduced, resulting in a release of Gα(GTP). This is the active form of Gα, which is able to initiate further cellular responses by interacting with downstream effectors. The GTPase activity of Gα is determined by addition of [γ-^{32}P]GTP to membrane samples. Later the Gα subunit cleaves GTP to GDP and inorganic phosphate ($^{32}P_i$) by its intrinsic GTPase activity and the released radioactivity is counted. GTPase assay is best suited for measuring $G\alpha_i$-coupled receptor mediated response since GTPase activity of $G\alpha_q$ and $G\alpha_s$ proteins is often very weak for proper detection (Schneider and Seifert, 2010). In addition to traditional radiometric GTPase assay colorimetric assays are on the market as well (from Innova Biosciences and others).

GTPγS binding assay

Similar to GTP, [^{35}S]GTPγS binds with high affinity to Gα and breaks the ternary complex leading to an active Gα species. However, in contrast to GTP, the γ-phosphate group in [^{35}S]GTPγS cannot be cleaved by the intrinsic GTPase activity of Gα since the bulky sulphur impedes the catalysis. Thus, [^{35}S]GTPγS–Gα is enriched in the cell membrane and can be determined after washing the sample, similar to a conventional filter binding assay or by SPA. Like the steady-state GTPase assay above, also the [^{35}S]GTPγS binding assay determines receptor activation at a very proximal point. [^{35}S]GTPγS binding assay is most suitable for $G\alpha_{i/o}$-coupled receptors because of higher expression of $G\alpha_i$ proteins in mammalian cells (Kunapuli, 2010; Schneider and Seifert, 2010). Now also fluorescent analogues are available: BODIPY-FL-GTPγS and Eu-GTP and applied for various purposes (Koval *et al.*, 2010; Tõntson *et al.*, 2012; Zhang and Xie, 2012).

cAMP assays

Binding of an agonist could either increase (via $G\alpha_s$) or decrease (via $G\alpha_{i/o}$) the synthesis of cyclic AMP, a second messenger first discovered by Earl Sutherland (Sutherland *et al.*, 1968). Screening for $G\alpha_s$-coupled receptors is rather straightforward, whereas it's much more complicated for $G\alpha_{i/o}$-coupled receptors, especially for $G\alpha_{i/o}$-coupled receptor antagonists, because in that case one needs to pre-stimulate AC with forskolin to inhibit the response with agonist and measure reversal of the agonist effect with antagonist (Zhang and Xie, 2012).

Variety of assays is designed to measure levels of cAMP. Current methodologies involve competition between endogenously produced cAMP and exogenously added labelled cAMP for binding with cAMP-binding protein (PKA-containing protein preparations) or anti-cAMP antibodies; as well as non-

competitive assays where endogenous cAMP binds to its cellular binding partner (such as PKA, Epac, cyclic nucleotide gated (CNG) ion channels), which is modified to produce a spectroscopic signal upon binding of cAMP (Hill *et al.*, 2010; Zhang and Xie, 2012).

Radiometric assays. The first [^{3}H]cAMP assays developed were based on cAMP binding to a rabbit anti-cAMP antibody (Steiner *et al.*, 1969) and to a cAMP-binding protein (presumably PKA) from bovine muscle (Gilman, 1970). Brown *et al.* used bovine adrenals instead of muscle tissue as a source of protein with a very high specificity for cAMP (Brown *et al.*, 1971). Nordstedt and Fredholm modified the methodology developed by Brown such that charcoal precipitation step was replaced with filtration through glass-fibre filters using a semi-automatic harvester (Nordstedt and Fredholm, 1990), which was a step towards high throughput assays. [^{3}H]cAMP assay was also used in the current study and is described in more detail in Methods and attached publications (**II, V**).

Another type of radiometric assays involves measuring the activity of the enzyme adenylate cyclase. In [α-^{32}P]ATP assay ATP is converted to [^{32}P]cAMP in an enzymatic reaction and [^{32}P]cAMP is quantified by liquid scintillation counting (Schneider and Seifert, 2010). A common commercial radiometric cAMP assay is the FlashPlate^{TM} cAMP assay from PerkinElmer using ^{125}I-labelled cAMP. Similar assay is designed for cGMP measurement, reviewed in (Hill *et al.*, 2010). The trend however is to replace radiometric assays with fluorescence or luminescence based homogenous assays.

Fluorescence/luminescence assays. There are several commercial cAMP assay kits available developed for high-throughput screening (HTS) purpose, such as HitHunter^{TM} by DiscoveRX, AlphaScreen^{TM} by PerkinElmer, cAMP-Glo^{TM} by Promega and many others employing luminescence and fluorescence detection (Kunapuli, 2010; Binkowski, 2011; Zhang and Xie, 2012). In academy various single-cell biosensors have been developed to investigate spatial and temporal dynamics of cAMP in living cells. The fluorescence imaging of cAMP dynamics was first reported using FlCRhR, a Förster resonance energy transfer (FRET) sensor consisting of fluorescein-tagged PKA-catalytic subunits and rhodamine-tagged regulatory subunits, which could be microinjected into living cells (Adams 1999). Upon binding of cAMP, the catalytic and regulatory subunits dissociate and reduction in FRET is observed. Several genetically encoded FRET-sensors have been developed since, but using genetically encoded fluorescent proteins as a FRET-pair, such as cyan and yellow fluorescence proteins (CFP and YFP, respectively), instead of injectable dyes. With genetically encoded sensors there was no longer a need for microinjection of the probe and it was possible to monitor FRET change in discreet microdomains (Zaccolo and Pozzan 2002). Soon monomolecular cAMP-sensors based on Epac protein were developed (DiPilato *et al.*, 2004; Nikolaev *et al.*, 2004; Ponsioen *et al.*, 2004). More details on different techniques for cAMP measurement can be found in (Hill *et al.*, 2010).

The application of genetically encoded biosensors can be improved by the use of viral vectors to facilitate the delivery of recombinant genes. Vectors of adenovirus, vaccinia virus and Semliki Forest virus are extensively used for protein expression in mammalian cells (Lundstrom, 2005). Baculovirus (an insect virus) based transfection system for mammalian cells (BacMam) is another good alternative for the above mammalian viruses; for more details see (Ames *et al.*, 2004, 2007). We have used BacMam system for mammalian cell expression of Epac2-camp sensor protein (Nikolaev *et al.*, 2004) and an improved version of Epac-camp sensor, the mTurquise based cAMP-sensor termed $^{T}Epac^{VV}$ (Klarenbeek *et al.*, 2011). The BacMam Epac2-cAMP sensor was used for studying the influence of Ca^{2+} and Mg^{2+} on ligand binding to melanocortin MC_1 receptor endogenously expressed in B16F10 murine melanoma cells (Mazina *et al.*, 2012) as well as for screening of 1- and 3-substituted apomorphine derivatives for their activation of D_1, D_{2L} and D_3 receptors expressed in HEK293 cells (unpublished data summarised in Results).

IP_3/IP_1 and Ca^{2+} assays

Stimulation of $G\alpha_q$-coupled receptors results in the activation of phospholipase C (PLC), which hydrolyses phosphatidylinositol bisphosphate (PIP_2) to form second messengers IP_3 and DAG. IP_3 is rapidly hydrolysed to IP_2 and IP_1. Traditional IP_3 assay for detecting PLC activity monitors [^{3}H]inositol incorporation (Schneider and Seifert, 2010; Zhang and Xie, 2012). A homogenous SPA assay is developed for the same purpose (Brandish *et al.*, 2003). Examples of non-radioactive antibody based IP_3 or IP_1 assays are AlphaScreen™ (PerkinElmer), HitHunter™ Fluorescence Polarization (DiscoveRx) and IP-One HTRF™ (Cisbio).

Ca^{2+} is another ubiquitous second messenger next to cAMP. $G\alpha_q$-coupled receptors produce ligand-dependent increase in intracellular Ca^{2+}. With the use of promiscuous G-proteins ($G\alpha_{15}$ or $G\alpha_{16}$) or using $G\alpha_q$ chimeras (Conklin *et al.*, 1993) it is possible to switch $G\alpha_{i/o}$, $G\alpha_s$ and $G\alpha_{12}$-coupled GPCR to induce a release in intracellular Ca^{2+} (Jacoby *et al.*, 2006; Oldham and Hamm, 2008). Compared to the second messenger cAMP, even a larger degree of signal amplification is expected with Ca^{2+} detection and therefore Ca^{2+} assays are one of the most sensitive and widely used assays (Mank *et al.*, 2006; Palmer and Tsien, 2006; Russell, 2011; Zhang and Xie, 2012). The first calcium indicators used for monitoring the dynamics of cellular calcium signalling were bioluminescent calcium-binding photoproteins, such as aequorin. It was followed by synthetic compounds developed by Roger Tsien and colleagues, such as quin-2, fura-2, indo-1, and fluo-3, of which Fura-2 was very popular among neuroscientists. Fura-2 is particularly useful because it allows more quantitative calcium measurements involving the ratioing of the signals obtained with alternating the excitation wavelengths. Roger Tsien was the one who also came up with protein-based genetically encoded calcium indicators (GECIs) (Grienberger and Konnerth, 2012). There's a wide variety of commercial calcium assays available

provided by Molecular Devices, Abcam, Life Technologies, PerkinElmer *etc.* Although not among the main pathways activated by DARs, there is conflicting evidence that D_1 and D_2 receptors might form functional dimers that signal via G_q proteins therefore leading to activation of PLC rather than modulation of cAMP synthesis via AC (Pollack, 2004; Rashid *et al.*, 2007; Verma *et al.*, 2010; Yano, 2012). It suggests that Ca^{2+} assays may be applicable also for studying dopamine receptor signalling. A critical review written by Charlton and Vauquelin gives a thorough overview about calcium detection with large degree of signal amplification with suggestions how to appreciate common artefacts and analyse complicated data appropriately (Charlton and Vauquelin, 2010).

Reporter gene assay

Common reporter-gene assays include expression of enzymes with activities linked to colorimetric, fluorescent or luminescent readouts, such as luciferase, β-galactosidase, secreted alkaline phosphatase (SAP), green fluorescent protein (GFP), β-lactamase and chloramphenicol acetyltransferase (CAT). There are reporter gene assays constructed both for detecting cAMP and Ca^{2+} signalling. Wide linearity, sensitivity and high signal-to-noise ratio are some of the advantages of reporter gene assays, while the requirement of substrate and engineered cell lines, as well as long incubation periods, difficulty to detect antagonists, and higher false positive rate due to the distal signalling event are limiting their use (Kunapuli, 2010; Zhang and Xie, 2012).

1.4.2.2. G-protein-independent functional assays

Receptor trafficking

Receptor trafficking (or internalisation) is a response to receptor activation and a common mechanism for receptor desensitisation. G-protein-coupled receptor kinases phosphorylate the receptor at threonine and serine residues. Cytosolic β-arrestins are recruited to the phosphorylated receptor, uncouple the receptor from G-proteins and target the receptor to clathrin-coated pits for endocytosis. With the development of imaging and high content screening (HCS) techniques receptor trafficking has become a quantifiable process. For monitoring the trafficking receptors are labelled with fluorescent ligands or with antibodies directed against an extracellular domain of the receptor or against an N-terminal epitope-tag. The primary antibody is detected with a fluorophore-labelled secondary antibody. For large scale screening the antibody-based immunofluorescent detection is not very convenient, therefore more often GPCRs are tagged with fluorescent proteins (like GFP or RFP). It is a widely used approach for GPCR deorphanisation (Zhang and Xie, 2012).

β-arrestin recruitment assay

β-arrestin recruitment as a negative regulation of GPCR signalling has been shown for almost all GPCRs. They can activate several signalling pathways (Src, ERK1/2, Akt) independently from G-proteins opening a new direction in GPCR screening and drug discovery. These assays employ GFP-tagged β-arrestins, which travel from cytoplasm to the membranes upon receptor activation and phosphorylation by GRKs. β-arrestin assays require modified cell lines expressing appropriately tagged β-arrestins and tagged GPCRs (Zhang and Xie, 2012).

MAPK activity assay

Mitogen-activated protein kinases (MAPKs) include three families of kinases – extracellular signal regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs) and p38s. Seven transmembrane receptors are connected to MAPKs either via classical G-protein-stimulated signalling pathways or via G-protein-independent β-arrestin pathway (Lefkowitz and Shenoy, 2005). Activation of the ERK signalling cascade results from activation of a wide range of GPCRs, including G_s-, G_i-, G_q- and $G_{12/13}$-coupled receptors, therefore ERK assays are considered quite a universal tool for studying GPCR activation. Some examples of commercial ERK activation assays are one from Millipore and Phospho-ERK from Cisbio.

Label-free assay systems

Label-free technology has the advantage of measuring GPCR activities in more native conditions without the need for labelling. Label-free whole-cell assays employ a sensor that converts ligand-induced (morphological) changes in living cells to optical, electrical, calorimetric, acoustic, magnetic or other signals. These assays require special consumables (i.e. plates with gold microelectrodes or optical gratings for electric and optical biosensor, respectively) and are prone to give false-positives and false-negatives (Zhang and Xie, 2012).

1.4.3. Receptor sources for study

As important as the assay for measuring ligand-receptor interactions or receptor activation events is the source of receptor, or more precisely, the system in which the interactions are studied. The first "pharmacological" experiments were performed on humans. A German pharmacist Friedrich Serturner (1783–1841), who isolated the first alkaloid from opium in 1805 administered a dose to himself and his three friends, the compound was named morphine, for Morpheus, Greek god of sleep (Scheindlin, 2001). More commonly, however, various test animals (mice, rats, dogs, pigs, guinea pigs) as well as isolated organs and tissues were used instead of human subjects. Animal tissues are now

replaced by immortalised modified cell lines of tumour origin (Reisine and Eglen, 2010). The advantages of using immortalised cell lines are numerous: easy to culture, relatively cheap, naive background, large and relatively homogenous expression of target proteins, and GPCRs can be stably expressed in those cells along with other proteins important for reporter readout making immortalised cell lines very versatile hosts.

Concerns related to clinical relevance of immortalised cell lines have forced scientists to turn more attention to using primary cells to study drug action (Nolan, 2007). For studying CNS receptors primary neuronal cells are particularly desired because they resemble the cellular environment found in human brain. Primary cells from transgenic animals with disease phenotype are used to study drug action on specific diseased cells (Zhao *et al.*, 2006; Eglen *et al.*, 2008; Viero *et al.*, 2008; Gullbo *et al.*, 2011). Due to limited availability of primary cells and need for highly sensitive detection methods (due to low expression of proteins of interest), primary cells are not used for drug HTS yet, but they are quite widely used for screening of toxicity (Davila *et al.*, 2004; Eglen *et al.*, 2008). Primary cells can't yet replace immortalised tumour cells or other widely used cell lines in drug research; rather they provide a complementing and physiologically more relevant model system.

When it comes to studying the structure and function of proteins, immortalised cell lines remain irreplaceable. For structural studies GPCRs have been expressed in bacteria and yeast, which provide higher yields of recombinant protein. Receptors expressed in bacteria often remain inactive because of problems with proper folding and/or post-translational modifications. Certain yeast strains, such as *Pichia Pastoris,* have been successfully used for high level and proper expression of GPCRs (Lundstrom, 2005). For studying GPCR function and for ligand screening insect and mammalian cell lines are widely used because they provide the most native-like environment for human GPCRs with similar or identical post-translational modifications necessary for ligand recognition and receptor signalling (Nettleship *et al.*, 2010).

Insect cells derived from pupal ovarian tissue of *Spodoptera frugiperda* (American fall army worm) are suitable when a better defined expression system without interfering GPCRs and with a limited set of G-proteins is preferred. First, the Sf21 cell line was isolated and later Sf9 cell line was derived from it. Sf9 cells have endogenous insect G-proteins, but these are mostly unable to couple to mammalian GPCRs and therefore Sf9 cells provide a low background in functional assays, increasing the sensitivity. Other benefits of Sf9 cells include high expression levels of recombinant GPCRs; they perform most of the post-translational modifications known for mammalian cells; and they provide several endogenous signal transduction components that are similar to pathways known from mammalian cells. For expression of the protein of interest the Sf9/baculovirus expression system is used. Insect cells are infected with a recombinant baculovirus in which a gene for viral protein polyhedrin (PH) is replaced with a gene of interest (Schneider and Seifert, 2010). The baculovirus

PH-promoter is active only in insect cells, but when it is replaced with a mammalian-active promoter (for instance, a cytomegalovirus (CMV) immediate early promoter) then the protein of interest can as well be expressed in mammalian cells. This is how BacMam system was developed. More details on BacMam system and its use in GPCR drug discovery and protein expression can be found in (Kost *et al.*, 2005; Ames *et al.*, 2007; Fornwald *et al.*, 2007). Our own recent application of BacMam system for expression of cAMP-biosensor in cultured melanocytes is reported in (Mazina *et al.*, 2012).

A widely used example of a mammalian cell line is HEK293. It was derived by transformation of human embryonic kidney (HEK) cells following exposure to fragments of human adenovirus type 5 (Ad5) DNA (Graham *et al.*, 1977). Number 293 designates Frank Graham's 293rd experiment. This permanently transformed cell line has incorporated Ad5 into chromosome 19 of the host genome. Although isolated from kidney tissue it should not be considered as a kidney cell line, rather it resembles cells with neuronal origin. Experimental evidence has shown that HEK293 cells express many mRNAs exclusively found only in neurones. This has led to suggest that Ad5 preferentially transformed early differentiating neurons in subset of embryonic kidney cells (Shaw *et al.*, 2002). HEK293 cells endogenously express various GPCRs known to couple with G_q, G_s and G_i proteins (Thomas and Smart, 2005; Atwood *et al.*, 2011), which suggests that they would provide a suitable expression system for functional studies of many types of GPCRs. The expression of dopamine receptors in HEK293 cells is negligible with only a minor expression of D_2 and D_4 receptor mRNA, as detected in an extensive microarray analysis (Atwood *et al.*, 2011). Proper expression of GPCR signalling machinery (Hellevuo *et al.*, 1993; Rich *et al.*, 2001), lack of significant expression of dopamine receptor subtypes (Thomas and Smart, 2005; Atwood *et al.*, 2011) and putative neuronal lineage (Shaw *et al.*, 2002; Vetter and Lewis, 2010) make HEK293 cells suitable for studying recombinant dopamine receptors. These arguments, as well as high transfection efficiency (Thomas and Smart, 2005), were the reasons why we selected HEK293 cell line as a host for dopamine D_1, D_{2L} and D_3 receptors when developing assay systems to characterise binding, functional activity and subtype selectivity of dopamine receptor ligands.

2. AIMS OF THE STUDY

General aim: to develop cellular assay systems for studying pharmacological properties of dopaminergic ligands.

Particular aims:

- To study interaction of dopamine D_1 and adenosine A_1 receptors in an insect origin model cell line.
- To characterise binding and functional activity of ligands on dopamine receptors in rat striatal tissue.
- To create and characterise mammalian origin stable cell lines with dopamine receptor D_1, D_{2L} and D_3 subtypes for ligand binding and receptor activation studies, whereas cyclic AMP is employed as an intracellular reporter molecule for measuring receptor activation.
- To apply the D_1, D_{2L} and D_3 dopamine receptor stable cell lines in conjunction with baculovirus-induced expression of fluorescent biosensor for cyclic AMP to study receptor activation and functional activity of new dopaminergic ligands.

3. MATERIALS AND METHODS

3.1. Cellular systems for dopamine receptors and preparation of cell membranes for assay

3.1.1. The Fall armyworm cell line (Sf9) expressing human adenosine A_1 and human dopamine D_1 receptors

Sf9 cells were grown in the Sf900 medium with L-glutamine supplemented with penicillin (50 U/mL) and streptomycin (50 µg/mL) at 27 °C in 500 mL flasks on a rotation shaker. The cultures were maintained at density $(0.5–3)\times10^6$ cells/mL. Recombinant baculoviruses for the receptors and G-protein subunits were prepared by Dr. Johnny Näsman (Kuopio University, Finland) as described earlier (Nasman *et al.*, 2001; Näsman *et al.*, 2002). For expression, the virus stocks of receptors (A_1 and/or D_1) and/or Gα subunits and/or Gβγ subunits were added to the cell suspension at confluence of $(2–3)\times10^6$ cells/mL. The cells were harvested 48–50 h after infection by centrifugation (1500 rpm, 10 min) and homogenised in the homogenisation buffer, containing 20 mM Tris–HCl (pH=7.4), 5 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 1 µg/mL aprotinin, 0.25 mM benzamidine, using Bandelin Sonopuls ultrasonic homogeniser (70 W, 70%, 3 passes 10 s each). The obtained suspension was centrifuged at low speed (1000×g, 10 min at 4 °C). The supernatant was centrifuged at 30,000×g for 40 min at 4 °C. The membranes were washed twice by re-suspension of pellet in homogenisation buffer and centrifugation. The final pellet was homogenised in assay buffer: 50 mM Tris-HCl (pH=7.4) (hereinafter referred as TB), additionally containing 5 mM $MgCl_2$, 1 mM EDTA, and in the experiments concerning cells expressing A_1 receptor, incubated for 30 min at 25 °C in the presence of 5 U/mL ADA (ADA, at least 1 U/mL, was kept in the reaction medium of all experiments with A_1 receptors).

3.1.2. Rat brain striatal tissue

Striatal tissues of Wistar rats were separated and collected as described previously (Mällo *et al.*, 2007). For biochemical experiments, the tissues were homogenised in 50 volume (ww/v) of ice cold 2.5 mM Tris-HCl buffer (pH=7.4) containing 2 mM EGTA by sonication (70 W 70% 3 passes, 10 s each). For AC assays, the suspension was diluted twice with TB containing 2 mM EGTA, divided into aliquots and stored at −80 °C until use. For radioligand binding assays, the membranes of the suspension were washed twice by centrifugation at 16,000 rpm for 40 min at 4 °C and homogenised in 30 volumes (ww/v) of binding buffer, TB containing 2 mM EGTA. The final suspension was divided into aliquots and stored at −80 °C until use.

3.1.3. Chinese hamster ovary cells (CHO-K1) expressing rat dopamine D_{2S} receptor and Ltk^- fibroblast cells expressing human D_1 receptors

Both the CHO-K1 and Ltk^- fibroblast cell lines stably expressing D_{2S} and D_1 receptors, respectively, were obtained from Professor K. Fuxe's laboratory at the division of Cellular and Molecular Neurochemistry, Department of Neuroscience, Karolinska Institute (Sweden) and grown on petri dishes at 37 °C in a humidified incubator in the presence of 5% CO_2, and membrane preparations for binding experiments prepared essentially as described in (Herm *et al.*, 2009).

Chinese hamster ovary cells (CHO-K1) stably expressing rat dopamine D_{2S} receptor, prepared as described in (Rinken *et al.*, 2001), were cultured in α-MEM, containing 10% FBS, penicillin (50 U/mL), streptomycin (50 µg/mL) and G418 (500 µg/mL). For radioligand binding experiments the cells were collected, washed in DPBS, and homogenised by sonication in assay buffer (TB containing 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA) and centrifuged at 30,000×g for 20 min at 4 °C. The membrane pellets were washed by re-homogenisation in assay buffer and centrifugation. The final pellets were re-suspended in assay buffer and stored at −80 °C until use.

Ltk^- fibroblast cells expressing human D_1 dopamine receptor were prepared as described in (Liu *et al.*, 1992; Ferré *et al.*, 1998), and routinely cultured at 37 °C with 5% CO_2 in DMEM supplemented with 10% FCS, 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 U/mL), G418 (200 µg/mL), as described in (Ferré *et al.*, 1998; Rinken, Ferré, *et al.*, 1999). For binding experiments cells were homogenised by sonication in TB and centrifuged at 30,000×g for 40 min at 4 °C; the membrane pellets were washed twice by re-homogenisation in TB and centrifugation; the final pellets were re-suspended in TB and stored at −80 °C until use.

3.1.4. Human embryonic kidney cells (HEK293) stably expressing human dopamine D_1, D_{2L} and D_3 receptors

HEK293 cells, obtained from American Type Culture Collection, were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were grown at 37 °C in a humidified incubator with 5% CO_2. The pcDNA3.1+ expression vectors (Invitrogen), containing the desired gene of human wild type dopamine receptor (*DRD1*, *DRD2L* and *DRD3*), purchased from the Missouri S&T cDNA Resource Center (www.cdna.org). For transfection, cells were seeded on 6-well plates, cultured 24 hours to reach ~90% confluence and transfected with 4 µg of DNA per well using Lipofectamine™ 2000 according to manufacturer's instructions. To obtain stable lines cells were maintained and passed for 2 weeks in the presence of 800 µg/mL G418. After about 12 days four to six G418-resistant colonies per each receptor subtype were selected and transferred to individual wells on a

24-well plate. After five further passages, passing cells gradually from 24-well plate to 6-well plate and finally to a petri dish (d=90 mm), the clonal cultures were tested for receptor expression by radioligand binding. Henceforth the cells were maintained in the presence of 400 µg/mL of G418.

For radioligand binding experiments cells were grown on petri dishes until almost confluent, collected in DPBS, centrifuged at 800×g at room temperature and the pellet stored at −80 °C. The frozen pellets were melted on ice and washed by homogenisation with a tissue homogeniser (Coleparmer Labgen 125) for 30 s in ice-cold DPBS and centrifugation at 800×g for 5 min at 4 °C. The pellet was re-homogenised in TB and centrifuged at 30,000×g for 20 min followed by a second re-suspension and homogenisation step. The latter homogenisation and centrifugation steps were repeated once and the final pellet was homogenised in assay buffer (TB supplemented with 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT). The membrane preparations were stored in 1 mL aliquots (a' 10×10^6 cells/mL) at −80 °C until use.

3.2. Baculoviruses for expression of cAMP-biosensor in mammalian cells (BacMam Epac2-camps)

3.2.1. Preparation and production of the BacMam Epac2-camps baculoviruses

The Epac2 protein-based cAMP-biosensor BacMam system (BacMam Epac2-camps) was constructed as described in (Mazina *et al.*, 2012). Shortly, the expression vector for pcDNA3.1(+)-EYFP- epac2B(murine)-ECFP was received from Professor M. J. Lohse (The Institute of Pharmacology and Toxicology, University of Würzburg, Germany). The Epac2-camps construct, under the control of the cytomegalovirus promoter, was cloned into the pFastBac1 vector using the restriction enzymes *Bst*1107I (*Bst*Z17I) and *Bsp*68I (*Nru*I) for pcDNA3.1(+) and *Eco*105I (*Sna*BI) and *Ksp*AI (*Hpa*I) for pFastBac1, respectively. The polyhedrin promoter was removed from the pFastBac1 vector to ensure low promoter interference during virus amplification. The obtained pFastBac-Epac2-camps construct was transformed into DH10Bac competent cells for the production of recombinant bacmid DNA. PCR-verified bacmid DNA was then transfected into Sf9 cells using four equivalents of transfection reagent ExGen 500 (Fermentas) to prepare BacMam Epac2-camps virus stocks according to the Invitrogen Life Technologies Bac-to-Bac expression system manual. P1 viral stocks were amplified and viral titer determined in plaque assay or in cell size-based assay (described below). To obtain large amount of high titer baculovirus the P1 virus was further amplified in 120–200 mL of Sf9 cell suspension at a density of $(1–1.5)\times10^6$ cells/mL at MOI approximately 0.1. The obtained P2 or P3 viral stock was centrifuged at 40,000×g for 30 minutes at 4 °C and the viral pellet concentrated 10-fold in DPBS by re-suspending the

pellet in 1:10 of starting volume. The obtained 10× BacMam Epac2-camps virus was stored at 4 °C in 500 µL aliquots and used for expression of Epac2-camps protein in HEK293 cells (described below).

3.2.2. Baculovirus titration

3.2.2.1. Plaque assay

The assay was performed according to Invitrogen Life Technologies' Instruction Manual ("Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques," 2002), with some modifications. Shortly, Sf9 cells were seeded on 6-well plates at 1×10^6 cells/well in 1 mL of ExCell 420 cell culture medium and allowed to adhere for 30–60 minutes. 500 µL of culture medium was removed from wells before addition of 500 µL of 2× viral dilutions (final dilutions between 1×10^{-4} and 1×10^{-9}). The cells were incubated for further 1 h with the virus and then the entire medium was removed from wells and immediately replaced by 2 mL of ~1% agarose solution (prepared in SF900 (1.3x) medium by autoclaving and kept at 37–42 °C until use). After the gel was hardened the plates were sealed with parafilm and incubated for 6–10 days at 28 °C. Viral plaques were then counted and used for calculating viral stock concentration as plaque forming units per mL (pfu/mL).

3.2.2.2. Cell size-based assay

The cell size-based assay for measuring viral titers described in (Janakiraman *et al.*, 2006) was simplified for routine in-lab use. Shortly, Sf9 cells were seeded on 24-well plates at 0.2×10^6 cells/well in 250 µL of ExCell 420 cell culture medium and allowed to adhere for 30–60 minutes. Then 250 µL of viral dilutions (3-fold serial dilutions of harvested viral supernatant or of concentrated virus with final dilutions between $1\times10^{-0.3}$ and 1×10^{-5}) were added to wells. The cells were incubated in the presence of virus for 24 h and thereafter the average cell diameter was counted using a Beckman Coulter cell counter (2^{TM} Series COULTER COUNTER® Cell and Particle Counter). The average cell diameter versus log(viral dilution) was plotted on graph and viral concentration as infectious units per mL (IU/mL) was calculated from a sigmoidal dose-response curve, using **Equation 1**:

$$Viral\ concentration\ (IU/mL) = \frac{\frac{1}{ED_{50}}\times 50\%\ of\ infected\ cells}{V} \qquad (1),$$

where V – sample volume in wells (here 0.5 mL); ED_{50} – 50% effective viral dilution corresponding to viral dilution at which the average cell diameter has changed 50%; *50% of infected cells* – 50% of the cells in wells at the time of infection (here 0.1×10^6 cells) given that the number of cells ~ the number of

infective viral particles and the proportion of secondary infection is minimal (O'Reilly *et al.*, 1994).

To present viral titers in commonly used plaque forming units (pfu/mL) the titers in IU/mL, calculated according to **Equation 1**, were transformed using a linear in-lab correlation equation (**Equation 2**) obtained from plotting titers of different viral stocks as IU/mL versus pfu/mL, derived experimentally in cell size-based and plaque assays, respectively.

$$log\, Y = 0.92 \times log\, X + 0.67 \quad (2),$$

where Y is the desired viral titer in pfu/mL, and X is the experimentally derived viral titer in IU/mL calculated according to **Equation 1**. Viral titers determined this way were used for calculating MOI values for amplification of virus (described above) and for expression of biosensor protein in mammalian cells (described below).

3.3. Radioligand binding assays

3.3.1. Saturation and competition binding

All the reactions were carried out in a final volume of 250 µL in test tubes or in multiwell plates in assay buffer (TB supplemented with salts specified above in "Cellular systems for dopamine receptors and preparation of cell membranes for assay").

In saturation binding experiments 150 µL of membrane homogenates were added to a solution containing 50 µL of radioligand at varying concentrations (ranging from 0.1 to 10 nM) and assay buffer (to determine total binding of radioligand) or competing ligand at saturating concentration (to determine radioligand binding to non-specific binding sites). In competition binding experiments appropriate volume of membrane homogenates were added to a solution containing 50 µL of radioligand at fixed concentration (near its K_D value) and 50 µL of competing ligand at varying concentrations (typically in the range of 6 and 8 logarithmic units, depending on a ligand) and/or GTPγS at fixed concentration (10 µM or 100 µM) where necessary. Reaction mixtures were incubated at 25 °C or 30 °C for 60 to 90 minutes (see details in corresponding papers). All the reactions were stopped by filtration through thick GF/B glass fibre filtermats using Brandel M-24 Cell Harvester (Brandel) or FilterMate Harvester (Perkin Elmer) and filters washed 3 to 5 times with ice cold washing buffer (WB: 20 mM K-phosphate, 100 mM NaCl, pH=7.4). In cases where Brandel Cell Harvester was used filters were impregnated overnight in scintillation cocktail OptiPhase HiSafe (Wallac PerkinElmer Life Sciences) and the radioactivity content of filters was measured by RackBeta 1219 liquid scintillation counter. In cases where FilterMate Harvester was used the filters were dried in a microwave oven at 800W for 2 min and impregnated using sheets of

solid scintillant MeltiLex™ B/HS and a MeltiLex™ Heatsealer (both of Wallac). Filter-bound radioactivity was counted using a Wallac MicroBeta TriLux 1450 LSC Luminescence Counter (Perkin Elmer).

3.4. Functional assays

3.4.1. [^{35}S]GTPγS assay

Reactions were carried out in test tubes in a final volume of 250 µL in TB supplemented with 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA. Activation of dopamine D_{2S} receptors stably expressed in CHO-K1 cells was measured by incubating [^{35}S]GTPγS (final concentration 0.2 nM) with GDP (final concentration 10 µM), appropriate concentrations of compound and membrane suspension of CHO-K1 cells for 90 min at 25 °C. The reaction was terminated by filtration through GF/B filters using Brandel cell harvester and the filters washed with 3 mL of ice-cold WB. Filters were impregnated overnight in OptiPhase HiSafe scintillation cocktail and radioactivity counted as described above using RackBeta 1219 liquid scintillation counter.

3.4.2. [^{3}H]cAMP competition assay

3.4.2.1. cAMP accumulation in rat brain homogenates

The assay was carried out in 1.5 mL Eppendorf tubes in a final volume of 150 µL in 30 mM Tris-HCl buffer (pH=7.4) containing 5 mM $MgCl_2$, 1 mM ATP, 10 µM GTP, 0.75 mM EGTA, 7.5 mM KCl, 100 mM NaCl, 0.1 mM IBMX, 0.1 mM Ro20-1724, 100 µg/mL bacitracin, 0.03% BSA, and ATP regenerating system (10 mM PEP and 30 µg/mL PK). Shortly, 50 µL of test ligand at suitable concentrations was added to a 100 µL solution containing homogenised tissue (approx. 16 µg protein/mL) and mixed on ice. The reaction was started by transferring the tubes to a 30 °C water bath, followed by 15-min incubation. The reaction was terminated by adding a solution containing EDTA (final concentration 25 mM) and subsequent boiling of samples for 5 min.

The content of accumulated cAMP in the samples was measured by competition binding with [^{3}H]cAMP to cAMP binding protein, essentially as described in (Nordstedt and Fredholm, 1990), in a final volume of 300 µL in Brown's Buffer (BB: 50 mM Tris-HCl (pH=7.4 at 4 °C), 8 mM theophyllin, 10 mM EDTA, 100 mM NaCl and 6 mM DTT. Shortly, 100 µL of cAMP-binding protein (16-fold dilution of stock in BB; stock prepared as described in (Brown *et al.*, 1971)) was added to 100 µL of [^{3}H]cAMP (at ~2 nM final concentration) and 100 µL of sample at suitable dilution. For cAMP standard curve known concentrations of cAMP were added in the assay instead of the test samples. The reaction was terminated by filtration through GF/B filters using Brandel cell harvester and the filters washed three times with 3 mL of ice-cold WB.

Filters were impregnated overnight in OptiPhase HiSafe scintillation cocktail and radioactivity counted as described above using RackBeta 1219 liquid scintillation counter.

3.4.2.2. cAMP accumulation in intact HEK293 cells

HEK293 cells expressing the desired dopamine receptor subtypes were seeded on 96-well cell culture plates two to three days before the assay at a density of $(0.02–0.05)\times10^6$ cells/well in 100 µL of DMEM without G418. cAMP accumulation assay was carried out in a final volume of 100 µL in DPBS. Shortly, on the day of assay the cell culture medium was replaced by 50 µL DPBS w/o Ca^{2+} and Mg^{2+}, pre-warmed to 37 °C. Thereafter 50 µL of increasing concentrations of 2× ligand solutions in DPBS with 2 mM $MgCl_2$ and 200 µM IBMX, pre-warmed to 37 °C, were added to cells and incubated for 15 min at 37 °C. In case of the D_{2L} and D_3 receptors, the added mixture also contained forskolin (FSK) at a final concentration of 32 µM. Reaction was stopped by addition of 50 µL of ice cold 1.2 M perchloric acid and incubation on ice for 1 h. The mixture in wells was neutralised by addition of 50 µL of 1.2 M KOH and the plates were then stored at −20 °C until the [^{3}H]cAMP assay.

[^{3}H]cAMP assay was carried out in multiwell plates in a final volume of 150 µL in BB. The above described frozen samples were thawed and diluted in BB. 50 µL of [^{3}H]cAMP (at 10 nM final concentration) and 50 µL of cAMP-binding protein (10-fold dilution of stock in BB; stock prepared as described by Brown *et al.* (Brown *et al.*, 1971)), were added to a 50 µL of diluted sample and incubated at 2–8 °C for 1 h. For cAMP standard curve known concentrations of cAMP were added in the assay instead of the test samples. After incubation the assay mixtures were filtered through GF/B filters using FilterMate harvester and filters washed three times with WB, dried and impregnated with solid scintillation material MeltiLex using MeltiLex® HeatSealer. Radioactivity was counted with a PerkinElmer Wallac MicroBeta TriLux 1450 LSC Luminescence Counter.

3.4.3. BacMam Epac2-camps cAMP-biosensor assay

The amount of 10× BacMam virus (prepared as described above in "Preparation and production of the BacMam Epac2-camps baculovirus") suitable for transfecting HEK293 cells was determined empirically. Typically 250 µL of 10× virus in 4 mL of serum-free RPMI medium per petri dish was used (at 50–75% of cell confluency, corresponding to $(4–6)\times10^6$ cells/dish). The cells were seeded 2 to 3 days before transfection at about $(0.8–1.5)\times10^6$ cells/dish in routine cell culture medium (DMEM + 10% FBS + 100 U/mL penicillin + 100 µg/mL streptomycin) without G418. On the day of transfection the cell culture medium was aspirated from cells and replaced by 4 mL of serum-free

RPMI medium and 250 µL of 10× BacMam Epac2-camps virus. After 2–3 hours of incubation in serum-free RPMI medium 8 mL of routine culture medium was added, supplemented with sodium butyrate (NaBu) at 5 mM final concentration to boost the production of sensor protein (Palermo *et al.*, 1991). To allow the expression to take place the cells were further incubated for 20–24 hours before the assay.

On the day of cAMP assay, cells were removed from the dish by trypsination and seeded on black clear-bottom 96-well cell culture plates in 80 or 90 µL/well of DPBS with Ca^{2+} (1.2 mM) and Mg^{2+} (0.5 mM) at a cell density ranging from 30,000 to 60,000 cells/well (typically cells from one petri were seeded on one or two 96-well plates). Cells were allowed to adhere for 2 h and then used in the assay. All reactions were carried out in a final volume of 100 uL.

3.4.3.1. cAMP assay for G_s-coupled dopamine receptors (D_1 DARs)

Background fluorescence intensities were detected in non-stimulated cells by excitation at 427(20) nm (ECFP excitation) and measuring dual emission of the excited fluorophores at 480(20) nm (ECFP emission) and 530(20) nm (EYFP emission) using PHERAstar plate reader (BMG LABTECH GmbH), temperature set to 37 °C. For agonist dose-response 10× ligand solutions (8-fold serial dilutions in milliQ water) were added to wells (a' 10 µL/well) in a final volume of 100 uL and fluorescence emission was measured at every five minutes. For antagonist response cells (80 µL/well) were incubated with antagonist (a' 10 µL/well) for 10 minutes before stimulation with agonist (a' 10 µL/well) and fluorescence emission was measured as before with an exception that background fluorescence was measured after 10 minute incubation with antagonists, immediately before stimulating with agonist.

3.4.3.2. cAMP assay for $G_{i/o}$-coupled dopamine receptors (D_{2L} and D_3 DARs)

Background fluorescence intensities were detected as stated above. For agonist dose-response agonist serial dilutions containing FSK (at final concentration 10 µM) were added to wells and fluorescence emission measured as stated above. For antagonist response cells were incubated with antagonist solution containing 10 µM FSK for 10 minutes before stimulation with agonist and fluorescence emission measured as before with an exception that background fluorescence was measured after 10 minute incubation with antagonists, immediately before stimulating with dopamine.

3.4.3.3. Calculating the change in FRET upon ligand stimulation

Sigmoidal dose-response curves were presented as a change in Förster resonance energy transfer (ΔFRET) between two fluorophores upon ligand stimulation at different concentrations. ΔFRET was calculated as described in (Mazina *et al.*, 2012) according to **Equation 3**:

$$\Delta FRET = \frac{\frac{YFP_0}{CFP_0} - \frac{YFP_t}{CFP_t}}{\frac{YFP_0}{CFP_0}} \quad (3),$$

where YFP_0 and CFP_0 represent background fluorescence intensities measured at 530 nm and 480 nm, respectively, upon excitation at 427 nm, and YFP_t and CFP_t represent the respective fluorescence intensities measured at time point t of ligand stimulation. The pEC_{50} values presented in **Table 4** and **6** are calculated based on ΔFRET at 10 minutes of incubation with the test ligands.

3.5. Data analysis

All pharmacological data were analyzed by means of non-linear least squares regression analysis using the GraphPad Prism™ 4.03 or 5.00 (GraphPad Software Inc., San Diego, CA, USA). Data from radioligand saturation binding were fit to a one-site binding hyperbola to calculate B_{max} and K_D values. Data from competition binding were fit to a sigmoidal one-site or two-site binding curve and Cheng-Prusoff equation (Cheng *et al.*, 1973) was used to convert IC_{50} values into K_i, K_H and K_L values. Data from functional assays were normalised to 100% response and fit to sigmoidal dose-response curves. ΔFRET values were calculated using MATLAB 7.8.0 (R2009a) (MathWorks, Natick, Massachusetts, USA).

4. RESULTS AND DISCUSSION

4.1. D_1 and A_1 receptor-receptor interaction in Sf9 cells

Antagonistic interaction and co-localisation of adenosine A_1 and dopamine D_1 receptors has been shown in cultured cells as well as in natural neuronal cells (Ferré *et al.*, 1994, 1998; Ginés *et al.*, 2000). These findings have suggested formation of heterodimers between these receptors as one possible mechanism of their antagonistic modulation. The aim of our study was to investigate this antagonistic modulation in a simplified model system, in an insect cell line (Sf9) with baculovirus-expressed human adenosine A_1 and dopamine D_1 receptors along with their appropriate G-protein subtypes ($G\alpha_s$, $G\alpha_{i1}$ and $\beta_1\gamma_2$). Sf9 cells enable high-level expression of recombinant proteins and the absence of constitutively active receptors provides an excellent signal-to-noise ratio in functional assays. While mammalian cells often express several different GPCRs and G-proteins, which makes it difficult to selectively study only certain interaction partners, Sf9 cells are a relatively "clean" system (Schneider and Seifert, 2010).

To mimic the situation in striatum, various combinations of receptors and G-proteins were co-expressed. A strong coupling between A_1 and G_i proteins was detected, as estimated from radioligand displacement curves and the effect of 100 μM GTPγS on agonist affinity. GTPγS has the ability to disrupt the receptor-G-protein complex and thereby reduce the fraction of receptors that bind agonist with high affinity (Gazi *et al.*, 2003; Strange, 2008). However, GTPγS had no effect on agonist binding to D_1 receptors, indicating weaker or no coupling between these receptors and G_s proteins in Sf9 cell membranes. To further assess coupling between the receptor and its respective signalling system, agonist response to cAMP accumulation was tested. Activation of dopamine D_1 receptors with dopamine and SKF38393 increased cAMP accumulation, while activation of A_1 receptors with N^6-cyclopentyladenosine (NCPA) inhibited forskolin-activated accumulation of cAMP, indicating proper coupling of both D_1 and A_1 receptors with their respective second messenger systems. Co-stimulation of A_1 and D_1 receptors with NCPA and dopamine, respectively, had no effect on dopamine affinity nor binding kinetics of D_1 antagonist [^{3}H]SCH23390 in $A_1D_1G_iG_s$ expressing Sf9 cell membranes. In cAMP accumulation assay the D_1 receptor activation was reversed by NCPA only when the cells were co-expressed with G_i proteins, indicating the role of G_s and G_i proteins in the antagonistic interaction. It shows that in our model system the antagonistic A_1-D_1 receptor interaction could be related to other than previously proposed direct A_1-D_1 receptor heterodimer formation in which case A_1 receptor agonists have been shown to affect D_1 receptor agonist binding affinities (Franco *et al.*, 2007). On HEK293 cell line Cao *et al.* (2007) has demonstrated that the A_1 agonist effect on D_1 agonist-induced synthesis of cyclic AMP is

more likely a receptor-receptor interaction related phenomenon and not connected to G_i proteins, which is contrary to our results (Cao *et al.*, 2007). Whether the G_i protein-related antagonistic effects we observed were specific to Sf9 cells is currently unresolved. However, based on our results the antagonistic interaction between A_1 and D_1 receptors might not be direct as it requires their respective G-proteins to be present for the effect to be observed.

4.2. D_1 and D_2 receptor ligand binding and cAMP accumulation in rat brain tissue

Dopamine D_1 and D_2 receptors are co-expressed in brain and several studies show that in striatum there is a substantial physical and functional interaction between the two dopamine receptor subtypes (Surmeier *et al.*, 1998; Aizman *et al.*, 2000; Rashid *et al.*, 2007). Our aim was to elaborate a [^{3}H]cAMP-based methodology for measuring signal transduction in homogenate of rat striatum. The expression and density of D_1 and D_2 receptors was confirmed by saturation binding of [^{3}H]SCH23390 (K_D = 0.75 ± 0.08 nM, B_{max} = 62 ± 2 fmol/mg tissue) and [^{3}H]raclopride (K_D = 2.3 ± 0.2 nM, B_{max} = 21 ± 1 fmol/mg tissue), respectively, as well as by displacement of the above radioligands by D_1 and D_2 receptor specific ligands. Inhibition of [^{3}H]SCH23390 binding by antagonists were best described by a single-binding-site model with Hill coefficients close to unity, while the effect of agonists fit better to a two-binding-site model. Activation of G-proteins with GTPγS decreased the fraction of D_1 receptor high affinity binding sites from 44–64% to the level of 27–43% for different agonists (DA, DHX and A77636), without affecting affinities of the ligands. In addition to that, a substantial fraction (51%) of high affinity binding sites of quinpirole binding in competition with [^{3}H]raclopride was detected, which taken together suggests proper coupling of both the D_1 and D_2 receptors with their respective G-proteins. Despite that only the D_1 receptor-specific activation of AC was detected in [^{3}H]cAMP assay while D_2-specific inhibition remained fully hidden. The reason behind our observation is not clear because D_2 receptor activation in rat striatum has successfully been detected before using [^{35}S]GTPγS assay (Rinken, Finnman, *et al.*, 1999; Odagaki and Toyoshima, 2006). In conclusion we successfully implemented the [^{3}H]cAMP radioassay for detecting D_1 receptor-specific signal transduction while D_2 receptor activation remained fully hidden with no apparent interference on the D_1 receptor mediated cAMP accumulation.

4.3. Development of D_1, D_{2L} and D_3 receptor assay systems for ligand screening

For ligand screening it's important to use assay systems that would express appropriate receptors together with their signalling partners and with little or no interference from other receptors that would mask the signal of interest. We started out with model systems available at the time, obtained from our co-operation partners. In case of dopamine receptors these included brain tissues of rats and mice, Sf9 cells with transient expression of receptors and G-proteins of interest, and Ltk^- fibroblast and CHO-K1 cells with stable expression of human D_1 and rat dopamine D_{2S} receptors, respectively. However, when screening for ligands and their subtype-selectivity, more comparable cellular systems are preferred. Therefore we aimed to develop cell lines with stable expression of human dopamine D_1, D_{2L} and D_3 receptors that would be based on the same mammalian host cell line. After several unsuccessful attempts to obtain stable lines with chinese hamster ovary cells (CHO-K1) we switched to human embryonic kidney (HEK293) cells that are known for their high transfection efficiency (Thomas and Smart, 2005). See for discussion on other benefits of HEK293 cell line in the Literature Review above. From only 4 to 10 isolated clones per receptor subtype we managed to obtain 4 to 6 receptor-positive clonal cell lines. The level of receptor expression for D_{2L} and D_3 receptors was similar in each of the tested clones, whereas for D_1 receptors the receptor expression varied significantly between the clones. The ligand binding properties were highly similar in both high- and low-expression clonal cell lines of D_1 receptors, but modulation of cyclic AMP in [^{3}H]cAMP assay was significantly improved in the high-expressing clone with a better signal-to-noise ratio, so we decided to use this clone in our further experiments. The obtained HEK293 cells stably expressing individual human dopamine D_1, D_{2L} and D_3 receptor subtypes were then thoroughly characterised for their ligand binding and functional properties before application in ligand screening.

4.3.1. Ligand binding to dopamine D_1, D_{2L} and D_3 receptors

The dopamine D_1 receptor expression was determined by the specific binding of a D_1 receptor tritiated antagonist [^{3}H]SCH23390 with $K_D = 0.48 \pm 0.06$ nM and $B_{max} = 570 \pm 20$ fmol/10^6 cells. For comparison, in rat striatal homogenate the affinity of [^{3}H]SCH23390 binding was with $K_D = 0.75 \pm 0.08$ nM (**II**) and in Sf9 cell membranes with $K_D = 1.1 \pm 0.1$ nM (**I**).

The dopamine D_{2L} and D_3 receptor expression was determined by the specific binding of a D_{2L} and D_3 receptor non-selective antagonist [^{3}H]raclopride with a $K_D = 0.66 \pm 0.07$ nM, $B_{max} = 164 \pm 4$ fmol/10^6 cells, and $K_D = 0.83 \pm 0.13$ nM, $B_{max} = 111 \pm 4$ fmol/10^6 cells, for D_{2L} and D_3 receptors, respectively. These affinities are in agreement with data published earlier for these

radioligands and receptor subtypes (Hidaka *et al.*, 1996; Lepiku *et al.*, 1997; Cao *et al.*, 2006). For comparison, in rat striatal homogenate the affinity of [^{3}H]raclopride binding was with K_D = 2.3 ± 0.2 nM (**II**). Subtype identity was further confirmed by displacement experiments with different selective and non-selective dopaminergic ligands (dopamine, apomorphine, SCH39166, sulpiride, NGB2904 and others, data not shown).

Dopamine receptor-G-protein coupling in the cell lines was estimated by measuring the effect of GTPγS on agonist binding. For D_1 and D_{2L} receptors there was a significant rightward shift in the dopamine binding curve in the presence of GTPγS, while the effect was negligible in case of D_3 receptors (see **Figure 5**).

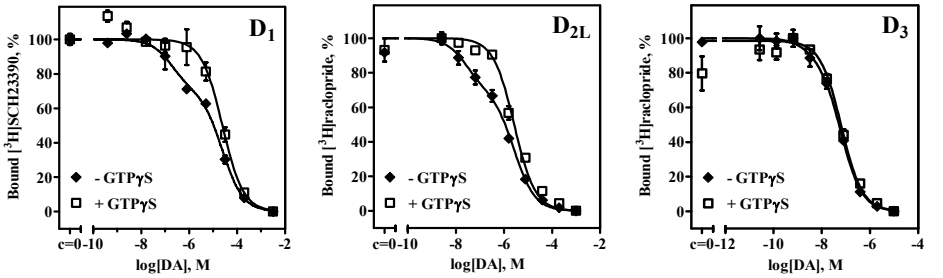


Figure 5. GTPγS effect on agonist binding to dopamine D_1, D_{2L} and D_3 receptors in HEK293 cell membranes. Data on graph are from a single representative experiment with affinity constant values and fractions of high affinity binding (α_H) for D_1: K_H = 76 nM, K_L = 9.9 μM, α_H (– GTPγS) = 29 ± 3%, α_H (+ GTPγS) = 1.8 ± 4.3% ; D_{2L}: K_H = 11 nM, K_L = 1.1 μM, α_H (– GTPγS) = 30 ± 3%, α_H (+ GTPγS) = 2.5 ± 2.7% ; D_3: K_H = 1.6 nM, K_L = 33 nM, α_H (– GTPγS) = 11 ± 5% , α_H (+ GTPγS) = 1.5 ± 2.3% .

The fraction of agonist high affinity binding varied between the receptor subtypes with values of 29%, 30% and 11% for D_1, D_{2L} and D_3 receptors, respectively. In all cases GTPγS completely disrupted the high affinity binding of dopamine. For comparison, in rat striatal homogenate the fraction of high affinity binding was higher, 63% and 51% for binding of dopamine to D_1 and quinpirole to D_2/D_3 receptors, respectively (**II**). In striatum GTPγS decreased the high affinity binding approximately two fold, but did not completely disrupt it. It is interesting to note that for D_1 and D_{2L} receptors the values of high and low affinity binding in HEK293 cells differ in the range of 2 logarithmic units, whereas for D_3 receptors it is much less, in the range of 20 fold difference. In striatum the differences of agonist's high and low affinity binding lie somewhere in between, in the range of 50-fold for DA binding to D_1 receptors and 25-fold for quinpirole binding to D_2/D_3 receptors (**II**). The observed relative insensitivity to GTPγS suggests that the D_3 receptors maintain relatively high affinity agonist binding even in the absence of G-proteins (Freedman *et al.*,

1994; Vanhauwe *et al.*, 1999). In many cases D_3 receptors have failed to give proper functional response in recombinant systems suggesting improper coupling of receptors and their putative $G_{i/o}$ proteins, interaction with different G-protein subtypes or lack of suitable isoforms of adenylate cyclase (Robinson and Caron, 1997; Newman-Tancredi *et al.*, 1999; Zaworski *et al.*, 1999). Functional coupling of D_3 receptors and G-proteins in our HEK239 cells still needed to be confirmed in functional assays.

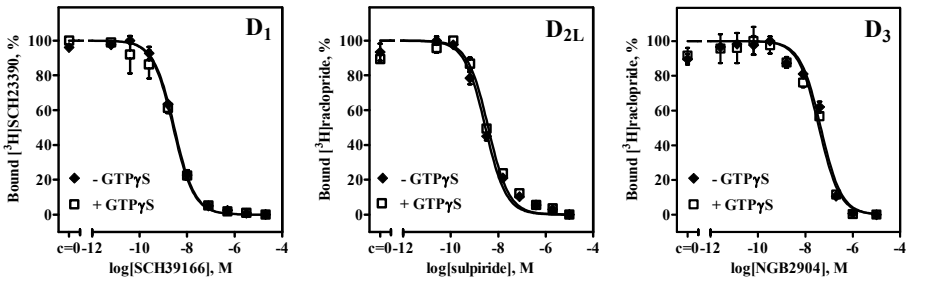


Figure 6. GTPγS effect on antagonist binding to dopamine D_1, D_{2L} and D_3 receptors in HEK293 cell membranes. Data on graph are from a single representative experiment with affinity constant values for D_1 (SCH39166): K_i (– GTPγS) = 1.2 nM and K_i (+ GTPγS) = 1.0 nM; D_{2L} (sulpiride): K_i (– GTPγS) = 1.2 nM and K_i (+ GTPγS) = 1.6 nM; D_3 (NGB2904): K_i (– GTPγS) = 22.9 nM and K_i (+ GTPγS) = 19.1 nM.

The affinities of D_1-selective and D_2-non-selective antagonists, SCH39166 and sulpiride, on binding to D_1 and D_{2L} receptors, respectively, were in agreement with data published earlier (Freedman *et al.*, 1994; Wu *et al.*, 2005) (Freedman *et al.*, 1994), whereas the affinity of D_3-selective antagonist NGB2904 was approximately 10 times weaker than reported (Xi and Gardner, 2007) (see **Figure 6)**. The observed low affinity may be attributed to high lipophilicity of NGB2904 and poor water solubility in aqueous environment (Heidbreder and Newman, 2010), which distorted our results. GTPγS had no significant effect on antagonist binding.

4.3.2. Modulation of cAMP levels by dopamine D_1, D_{2L} and D_3 receptors

In addition to characterising receptor binding affinity of ligands, measured in radioligand binding assays, it's also important to estimate ligand's ability (potency and efficacy) to trigger or block cellular or physiological response of interest. In previous cellular systems (Sf9, CHO-K1 and Ltk^- fibroblast cells and rat brain tissue) we measured ligand activity on the level of G-protein activation (by [^{35}S]GTPγS assay) or cAMP accumulation (by [^{3}H]cAMP assay). To confirm that dopamine receptors in the newly developed cell lines couple

properly to their respective signalling machinery, we decided to measure receptor-mediated cAMP-accumulation using two different methods. [^{3}H]cAMP radioassay gave good results with D_1 and D_{2L} receptors, whereas measuring D_3 receptor-mediated cAMP accumulation was problematic (Argo Vonk, unpublished data). The apparent weak coupling between D_3 receptors and G-proteins, revealed by insensitivity to GTPγS in ligand binding assay, observed also elsewhere (Robinson and Caron, 1997), and small D_3-specific effect on cAMP-response may be related to lack or shortage of proper partners for D_3 receptor signalling or to low sensitivity of the [^{3}H]cAMP radioassay. Despite the setbacks with D_3 receptor signalling in [^{3}H]cAMP assay we went on with applying an alternative method and measured cAMP regulation in dynamic mode using BacMam Epac2-camps cAMP-biosensor, which can detect local changes in cAMP concentration and therefore may provide a more sensitive assay. This system had worked well in B16F10 cells in activation of endogenous G_s-coupled melanocortin MC_1 receptors (Mazina *et al.*, 2012). Detection of G_i-coupled receptor-mediated cAMP-response, however, is more complicated compared to G_s-mediated response while it requires adenylate cyclase to be activated with forskolin to be able to detect agonist-mediated inhibition of cAMP synthesis. HEK293 cells with recombinant D_{2L} or D_3 receptors were therefore a good opportunity to test the applicability of our cAMP-biosensor assay with G_i-coupled receptors.

During the optimisation of experimental conditions we found that for better biosensor expression it was necessary to include a histone deacetylase inhibitor, sodium butyrate (NaBu), in the biosensor expression step. NaBu is generally accepted for increasing the expression of recombinant proteins (Palermo *et al.*, 1991). The optimal concentration of NaBu in our system was 5 mM, which provided sufficient level of biosensor expression without causing potential side effects (Prasad and Sinha, 1976; Kruh, 1981; Aranda *et al.*, 1990).

Another critical factor for BacMam-baculovirus induced receptor expression is the amount of infective virus particles used per number of cells, also called multiplicity of infection (MOI). In our case MOI suitable for sufficient expression of cAMP-biosensor in HEK293 cells ranged between 100 and 200. Such high MOIs necessitated the viral suspensions to be concentrated prior to transfection of HEK293 cells so that the volume of virus stock to be added to cells could be reduced. In case of B16F10 cells up to 10 times lower MOIs produced sufficient biosensor expression and therefore the virus concentration step could be omitted (Mazina *et al.*, 2012). In routine practice we used 250 µL of P3-amplified and 10×-concentrated BacMam Epac2-camps baculovirus (can be stored at 4 °C in DPBS for several months) per dish of HEK293 cells at 60–80% confluence (~4–6 mln cells). However, appropriate MOI for different cell lines should be tested empirically.

By varying different media (blank RPMI, blank DMEM, DMEM with FBS) and time of transduction, it was concluded that the best biosensor expression and cAMP-response was obtained by initiating the baculovirus transduction in

1/3 volume of blank RPMI for 2–3 hours before addition of 2/3 volume of routine culture medium (DMEM + FBS + strep/pen, supplemented with 5 mM NaBu) and further incubation for at least 20 hours.

We also tested the effect of Ca^{2+} and Mg^{2+} supplement in assay medium (commercial DPBS with Ca & Mg, PAA Laboratories GmbH), as well as the effect of phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX). As the presence of Ca^{2+} (at 1.2 mM) and Mg^{2+} (at 0.5 mM) significantly increased signal-to-noise ratio it was included in all the further assays. IBMX, a drug that decreases cAMP degradation by inhibiting PDE activity, on the other hand, reduced the signal-to-noise ratio dramatically and distorted the results, and was therefore excluded from the assay.

To avoid problems related to poor adherence of HEK293 cells on assay plate, it was necessary to perform the biosensor expression on petri dish a day prior to assay and to transfer the cells to 96-well plates 2–3 hours before the assay. For comparison, in case of strongly adhering cells such as B16F10 the biosensor expression could be performed directly on 96-well cell culture plate and on the next day the culture medium could simply be replaced with assay medium.

In optimised assay conditions forskolin caused a clear dose-response curve in all of the new dopamine D_1, D_{2L} and D_3 receptor-expressing stable cell lines. An example with D_1 receptor expressing cells (**Figure 7**) shows that forskolin induced the highest increase in cAMP, while elevation of cAMP was lower in case of non-selective agonists, dopamine and apomorphine, and D_1-selective full agonist A77636. D_1-selective antagonist SCH39166 alone had no effect on modulating cAMP level. Maximal response (ΔFRET) of D_1 receptor agonists varied between days of assay, remaining mostly between 10–15%.

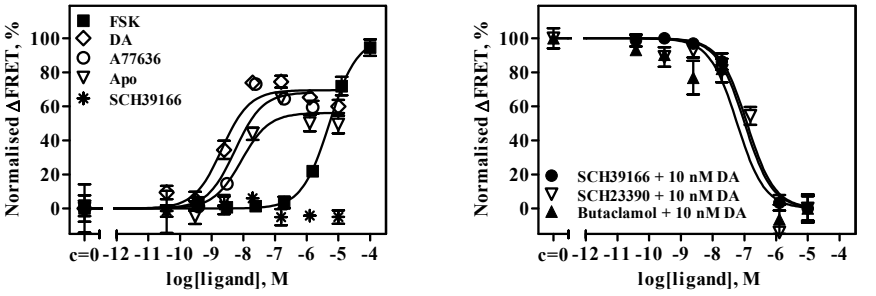


Figure 7. Effects of dopaminergic agonists and antagonists on modulation of cAMP level in HEK293 cells with recombinant expression of D_1 receptors. Graphs showing data from representative experiments performed in triplicates at 10 min of ligand treatment. The pEC_{50} values of tested compounds: FSK, 5.29 ± 0.07 ($\%E_{max}$ = 100 ± 4); DA, 8.68 ± 0.16 ($\%E_{max}$ = 70 ± 4); A77636, 8.27 ± 0.16 ($\%E_{max}$ = 68 ± 4); Apo = 8.07 ± 0.20 ($\%E_{max}$ = 56 ± 4); SCH39166, 6.99 ± 0.11; SCH23390, 6.92 ± 0.11; butaclamol, 7.23 ± 0.17.

The antagonists' potency to block D_1 receptor activation was measured by pre-incubating the cells first with serial dilutions of antagonists for 10 min and then measuring the response of 10 nM dopamine after 10 min stimulation. All the antagonists, the D_1-selective SCH39166 and SCH23390 and non-selective butaclamol, blocked dopamine-induced cAMP-response in a similar manner (**Figure 7** on the right).

In B16F10 cells with endogenous expression of MC_1 receptors we could clearly discriminate between full and partial agonistic responses (Mazina *et al.*, 2012), however, in HEK293 cells with over-expressed dopamine receptors, the response of partial agonists was not significantly different from the response of dopamine. Similar effects have been observed before in systems with high signal amplification (i.e. in cAMP and Ca^{2+} assays) and/or in systems with high receptor expression levels (Niedernberg *et al.*, 2003). To be able to detect partial agonistic responses in cAMP-assay a solution might lie in using cell lines with lower level of receptor expression. This in turn would decrease the signal window of the assay, as was seen by activation of endogenous β-adrenergic receptors present in our D_1 receptor-overexpressed HEK293 cells (**Figure 8**). The maximum level of cAMP-response mediated by the endogenous β_2-receptors and by the overexpressed D_1 receptors differ significantly, which could be explained by differences in receptor expression levels. To confirm that the measured signals indeed reflect specific D_1 and/or β receptor activation events we measured the effects of D_1 and β-adrenergic antagonists. D_1 antagonist SCH39166 efficiently blocked the D_1 receptor activation by dopamine, while a β-adrenergic antagonist propranolol was not able to affect dopamine-induced cAMP accumulation. The activation of endogenous β_2-adrenergic receptors by a β-adrenergic agonist isoproterenol was blocked by β-adrenergic antagonist

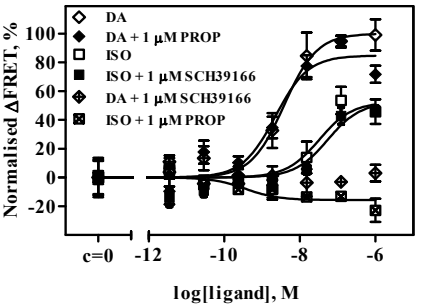


Figure 8. Effect of D_1 and β-adrenergic antagonists on modulation of cAMP level by dopaminergic and β-adrenergic agonists. HEK293 cells with recombinant expression of D_1 receptors are pre-incubated with D_1 antagonist SCH39166 or β-antagonist propranolol (PROP) at fixed concentration before addition of agonists, dopamine (DA) or isoproterenol (ISO), or treated with the agonists alone. Graph showing data from a representative experiment performed in triplicates at 10 min of agonist treatment.

propranolol and not by SCH39166. These results confirm that by using selective ligands we can efficiently discriminate between the cAMP accumulations triggered by activation of endogenous β-adrenergic and recombinant dopamine D_1 receptors.

It is more complicated to measure cAMP-response mediated by G_i-coupled receptors, as discussed earlier. To be able to detect inhibition of cAMP-synthesis upon receptor activation it is first necessary to increase intracellular cAMP level by directly activating AC with forskolin. In our system the optimal concentration of forskolin in G_i-coupled dopamine receptor assays was 10 μM at which the signal-to-noise ratio of agonist response was the highest. At these conditions 1 μM dopamine decreased the forskolin-activated cAMP-level by approximately 50%, although the overall cAMP-response "window" (ΔFRET) was in average three times smaller than could be seen in G_s-coupled dopamine D_1 receptor assay. In such system we were able to demonstrate that activation of D_3 receptors by dopamine and apomorphine reproducibly inhibited cAMP signal (**Figure 9** left), similar to that observed with D_{2L} receptors (data not shown). Also we demonstrated that the D_3-selective agonist 7-OH-PIPAT efficiently decreased cAMP level and the D_3-selective antagonist NGB2904 had no effect at least up to a concentration of 1 μM (**Figure 9** right).

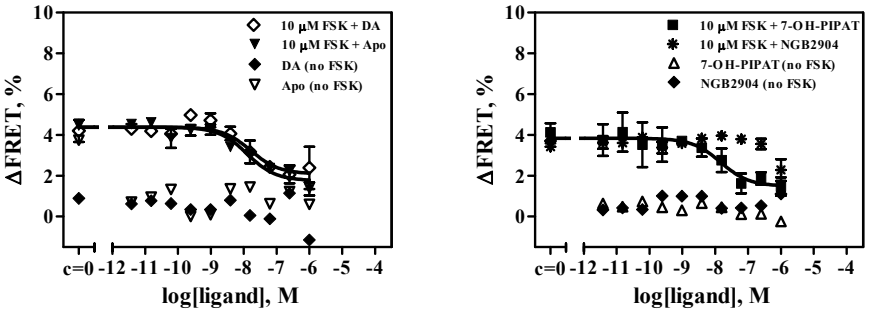


Figure 9. Effects of non-selective dopaminergic agonists (DA, Apo) and D_3-selective agonist (7-OH-PIPAT) and antagonist (NGB2904) on modulation of cAMP level in HEK293 cells expressing D_3 receptors. Graph showing data from a representative experiment performed in triplicates at 10 min of ligand treatment in the presence or absence of FSK.

As a result, despite the observed GTPγS insensitivity and weak coupling between D_3 receptors and G-proteins in our model system and inconsistent results obtained with [^{3}H]cAMP assay, we successfully managed to monitor receptor-mediated cAMP response of all the three dopamine receptor subtypes using the cAMP-biosensor assay.

Herewith we conclude that we developed and characterised a set of stable cell lines expressing individual D_1, D_{2L} and D_3 receptors, ready to be used for ligand screening purposes in ligand binding and cAMP assays.

4.4. Subtype-selective screening of dopamine receptor ligands

Our main focus is developing and applying modern techniques for studying specific interactions/events on the GPCR signalling pathway, but we have also created strong contacts with research groups whose main interest is the synthesis of biologically active compounds. In cooperation with Professor Attila Sipos from the University of Debrecen, Hungary, we have a history of screening for dopaminergic activity of apomorphine derivatives. More recently we started screening of azabicyclo-derivatives with dopaminergic potential in cooperation with Professor Tõnis Kanger from the Tallinn University of Techonolgy, Estonia.

4.4.1. Binding of 3-azabicyclo heptane derivatives

Professor Tõnis Kanger and his co-workers have developed a specific scheme for the synthesis of 3-azabicyclo[3.2.0]heptanes with a general structure shown in **Figure 10**.

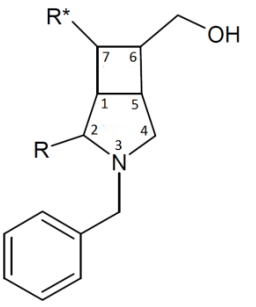


Figure 10. Structure of 3-azabicyclo[3.2.0]heptane derivatives with substituents at position 2 and 7.

Previously it's been reported that 3-azabicyclo[3.1.0]hexane **1** derivatives with sulphonamide (Micheli, Hamprecht, *et al.*, 2010) or triazol (Micheli, Arista, *et al.*, 2010) are selective dopamine D_3 antagonists. 3-azabicyclo[3.2.1]octane **2** benzamide derivative SSR181507 (Depoortere *et al.*, 2003) and indolyl-substituted bicyclic compounds (Paul *et al.*, 2008) are D_2 antagonists. Benzamides

of 3-azabicyclo[3.3.1]nonane **3** were found to be non-selective and showed nearly identical binding to dopamine D_2 and D_3 receptors (Mach *et al.*, 1999).

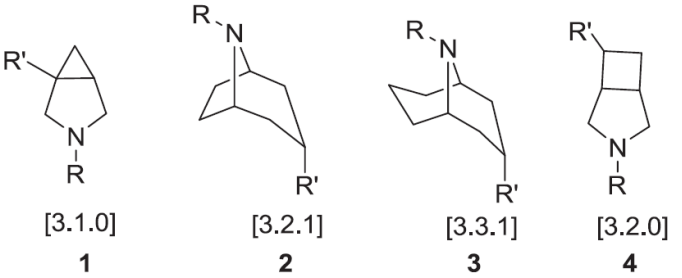


Figure 11. Azabicyclo scaffolds with different ring sizes found among dopamine receptor modulators (from **IV).**

Compounds with a 3-azabicyclo[3.2.0]heptane structure **4** have been shown to bind dopamine D_2-like receptors (Drescher *et al.*, 2006) and could be beneficial as antipsychotic drugs used i.e. for treating schizophrenia or related disorders (Beaulieu and Gainetdinov, 2011). Therefore a set of 3-azabicyclo[3.2.0]heptane derivatives with various substitutions at positions 2 and 7 were synthesised (**Figure 10** and **12**) and their binding affinity tested on dopamine receptor subtypes D_1, D_{2L} and D_3. All the compounds were obtained in two enantiomeric forms, A and B, resolved by lipase-catalyzed kinetic resolution using an immobilised lipase B of *Candida antarctica* (Novozym 435) as an acylating agent. In **Figure 12** the enantiomeric form A is presented.

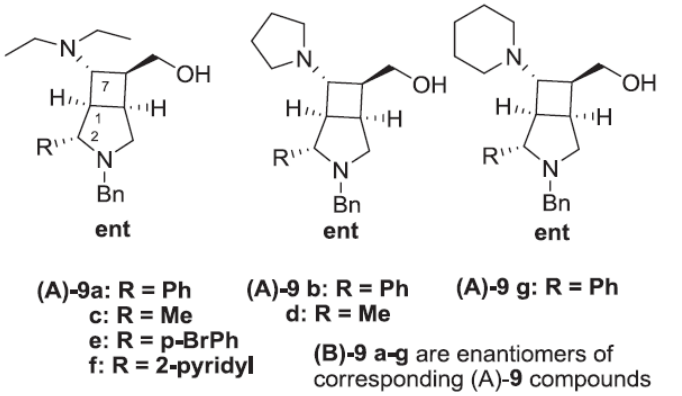


Figure 12. Structures of the synthesised 3-azabicyclo[3.2.0]heptane derivatives with substituents at position 2 and 7 (from **IV**).

The (B)-enantiomers had substantially higher affinities compared to (A)-enantiomers and all the compounds had better affinity for dopamine D_{2L} and D_3 receptors, in accordance with previously published results with similar scaffolds. The studied substitutions mainly affected binding affinities for D_2-like receptors, while the affinities for D_1 receptors remained in the range of 20 and 40 μM, with an exception of compounds **9c**, **9d** and **9f,** which affinity for D_1 receptors was significantly reduced. This also affected the observed selectivity profile of these compounds with compound **(B)-9c** reaching to more than 40-fold selectivity for D_{2L} and more than 80-fold selectivity for D_3 receptors compared to D_1 receptors. The compounds' affinities were generally in a micromolar range; however, compound **(B)-9b** with pyrrolidinyl at position 7 and phenyl at position 2 had substantially higher affinity, reaching to below 300 nM affinities for D_{2L} and D_3 receptors and resulting in the most D_{2L}- and D_3-selective compound of the series.

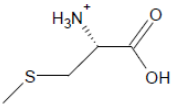
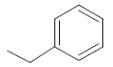
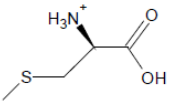
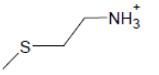
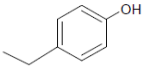
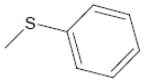
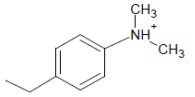
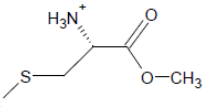
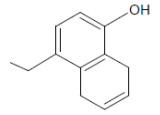
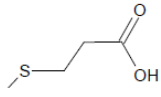
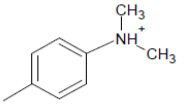
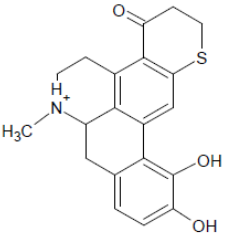
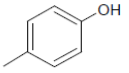
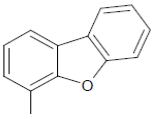
4.4.2. Binding and cAMP-modulating activity of 1-, 2- and 3-substituted apomorphines

Apomorphine is one of the earliest pharmacological tools used to characterise DA receptors. Acting as an agonist on D_1 and D_2 dopamine receptors, it is used in the treatment of Parkinson's disease, as well as in erectile dysfunction. Its use however is greatly limited by poor bioavailability and short duration of action. Recent studies have focused on improving the pharmacokinetic and pharmacodynamics properties of apomorphine and apomorphine's binding affinity and functional activity, as well as its selectivity for dopamine receptor subtypes (Zhang, Neumeyer, *et al.*, 2007; Zhang, Zhang, *et al.*, 2007).

Important elements in the apomorphine core structure (**Figure 13**) include: a) protonated quaternary amine function that plays critical role in the insertion of the molecule to the binding site of the receptor protein, forming a stable ion-ion interaction with Asp-114 of TM helix 3 of D_2 receptor; b) absolute configuration of C-6a, as R-(–)-enantiomers are known to be dopamine agonist and S-(+)-counterparts have antagonist features; c) phenolic hydroxyl groups at positions 10 and 11 of which the latter determines dopamine activation; d) substitutions at positions 2 and 3, which affect dopamine receptor binding ability, for instance D_2 receptor binding affinity is greatly increased by phenyl and 4-hydroxyphenyl substituents at position 2 (Zhang, Zhang, *et al.*, 2007).

In current study we tested the binding and functional activity of 2-substituted (**III**), 1-substitued (**V**) and 3-substituted apomorphine analogues with a general formula shown in **Figure 13** and with substitutions as depicted in **Table 1**.

Table 1. Structures of substituents R^1, R^2 and R^3 of the studied apomorphines

No.	1-substituted Apomorphines	No.	2-substituted Apomorphines	No.	3-substituted Apomorphines
	Hydrogen at R^2 and R^3; R^1:		Hydrogen at R^1 and R^3; R^2:		Hydrogen at R^1 and R^2; R^3:
1–1 (18)		**2–1 (4a)**		**3–1**	
1–2 (19)		**2–2 (4b)**		**3–2**	
1–3 (20)		**2–3 (4c)**		**3–3**	
1–4 (21)		**2–4 (4d)**		**3–4**	
1–5 (22)		**2–5 (4e)**		**3–5**	
1–6 (23)		**2–6 (4f)**		**3–6**	
		2–7* (4g)		**3–7**	
				3–8	

*For ease of interpretation compound No. **2–7** is depicted in its full formula. Numbers in brackets designate the numeration of respective compounds as depicted in the corresponding paper.

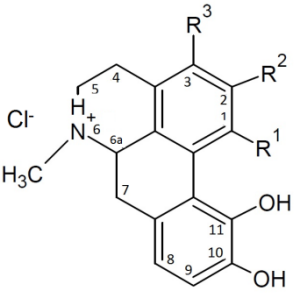


Figure 13. General structure of apomorphine with substituents at position 1, 2 and 3. In case of apomorphine R^1, R^2 and R^3 represent hydrogen atoms.

Pharmacological properties of the 2-subsituted apomorphines were studied using cell membranes of Ltk^- fibroblast and CHO-K1 cells, available at the time, with recombinant expression of human D_1 and rat dopamine D_{2S} receptors, respectively. Later, the 1-substituted and 3-substituted apomorphines were studied on human dopamine D_1, D_{2L} and D_3 receptors expressed in HEK293 cells. In all cases the compounds' affinities were measured in competition assay with [^{3}H]SCH23390 (for D_1R) and [^{3}H]raclopride (for $D_{2L}R$ and D_3R) and compound's functional response was measured either with [^{35}S]GTPγS, [^{3}H]cAMP or cAMP-biosensor assay.

4.4.2.1. 2-substituted apomorphines

Very different substituents at position 2 of apomorphine core structure (**Figure 13**) have been studied. Here we are exploring the effect of 2-thioether substitutions on the compound binding to D_1 and D_2 receptors and potency to activate D_2 receptors. The results for 2-substituted apomorphines are summarised in **Table 2**.

Table 2. Binding and functional data of 2-substituted apomorphine derivatives

	Radioligand competition assay			[^{35}S]GTPγS assay	
	K_i (nM) ± SEM		*Selectivity*	*EC_{50} (nM)*	*Efficacy (%)*
Comp.	D_1	D_{2S}	D_{2S}/D_1	D_{2S}	
2–1	4200 ± 1500	200 ± 50	21	211 ± 90	60 ± 5
2–2	141 ± 32	55 ± 14*	2.5	198 ± 5.2	62 ± 6
2–3	1300 ± 550	9.6 ± 2.2*	135	16 ± 8	112 ± 14
2–4	2200 ± 8	580 ± 150	47	820 ± 140	65 ± 7
2–5	380 ± 120	10 ± 2	38	24 ± 6	94 ± 7
2–6	150 ± 80	400 ± 95	0.4	148 ± 30	29 ± 6
2–7	140 ± 45	415 ± 110	0.3	383 ± 36	68 ± 6
Apo	72 ± 5.6	11.5 ± 0.7	6.3	30 ± 8	100

Data from **III** (Reinart *et al.*, 2011). K_i characterises the ability of a compound to inhibit [^{3}H]SCH23390 (D_1) or [^{3}H]raclopride (D_{2S}) binding to the corresponding dopamine receptor subtypes in membrane preparations of Ltk^- fibroblast or CHO-K1 cells, respectively. Efficacy represents the level of [^{35}S]GTPγS binding in comparison with the effect of apomorphine. Selectivity is the ratio of the compound's inhibition constant values (K_i) for designated receptor subtypes.

In this set of compounds the amino and/or carboxylic functions were inserted at position 2 of apomorphine core structure via a 2-thioether linkage. In general the 2-thioether function itself is unfavourable for the proper positioning of the ligand to the binding pocket, but the presence of the amino-group on the side chain at position 2 (compound **2–3** and **2–5** in **Table 2**.) increased affinity and selectivity for D_2 receptors. Compounds **2–3** and **2–5** were the only compounds which behaved as equipotent D_{2S} receptor full agonists with apomorphine in [^{35}S]GTPγS assay. In general, all the compounds had higher affinity and selectivity for D_{2S} receptors, but compounds **2–6** and **2–7** had an increased affinity and slight preference for D_1 receptors. It has been shown that the binding site at the C-2 position can tolerate a substituent with different properties (Zhang, Neumeyer, *et al.*, 2007). The 2-alkylthio-N-n-propylnoraporphine series (2-methyl-, 2-ethyl-, 3-propylthio-N-n-propylnorapomorphines) shows high D_2 affinity, but poor oral bioavailability in behavioral assays. The highest D_2 receptor affinity (3.7 nM) has been shown with the smallest, 2-methylthio, substituent (Tóth *et al.*, 2006).

4.4.2.2. 1-substituted apomorphines

The importance of substitutions at position 2 of apomorphine core structure and the existence of a lipophilic cleft in the binding site of D_2 receptor near position 2 (Neumeyer, 1985) led to further investigate the electronic and steric inter-

actions in the proximity of that cleft. Therefore 1-substituted apomorphines were synthesised. For testing dopaminergic activities of the 1-substituted apomorphines the above described human D_1, D_{2L} and D_3 receptor expressing HEK293 cells were used. Ligand potencies to activate D_1 and D_{2L} receptors were assessed in [^{3}H]cAMP assay, while measuring D_3 receptor activation with this assay was complicated and the results controversial as described above. However, the cAMP-biosensor assay was sensitive enough to reproducibly detect activation of all three receptor subtypes. Therefore, for comparison, the potency values of 1-substituted apomorphines determined by both [^{3}H]cAMP and cAMP-biosensor assays are disclosed herein.

To summarise the ligand binding results, substitutions at position 1 mainly affected compounds' affinities for D_1 receptors, while only a minor effect was seen on the affinities for D_{2L} and D_3 receptors. 1-hydroxymethyl function in compound **1–3** dramatically decreased affinity for D_1 receptor, thereby increasing the selectivity for the D_{2L} and D_3 receptors to a level of more than 20- and 50-fold, respectively. The 4-hydroxybenzyl moiety in compound **1–4** on the other hand increased affinity for D_1 receptors, completely abolishing the selectivity for D_{2L} and D_3 receptors (**Table 3**).

In our model systems all the compounds behaved as full agonists relative to apomorphine both in radiometric [^{3}H]cAMP and in fluorescence cAMP-biosensor assays (data not shown). Potency values measured in [^{3}H]cAMP assay correlate well with affinity values measured in radioligand competition assay and these results are discussed in more detail in (**V**). Herein more focus is paid on the comparison of two cAMP detection methods used for measuring compound potencies.

The potency values reflected by cAMP-biosensor assay, which measures not total cAMP, like the [^{3}H]cAMP assay, but dynamic changes in cAMP concentration, are mostly higher for all compounds and the D_1 to D_{2L} selectivity profile differs from the one determined with [^{3}H]cAMP assay (**Table 4** and **V**). The most D_{2L}-potent and D_{2L}-selective compound **1–3** in [^{3}H]cAMP and radioligand binding assays has lost its selectivity in cAMP-biosensor assay. The same loss of selectivity applies also for dopamine, which has an average binding affinity for D_3 receptor much higher than for D_{2L} and D_1 receptors, but its potency values do not differ that much between the receptor subtypes. It may be explained by receptor states (G-protein coupled versus uncoupled receptors), which bind agonist with different affinities and trigger intracellular signalling depending on the availability of receptors in G-protein-coupled state. Therefore, even if a small fraction of receptors (receptors in high affinity state) are activated, the average potency of a ligand may be significantly higher compared to its average binding affinity. In general, the 1-substituted apomorphines' affinity and potency values between receptor subtypes correlate quite well, with some exceptions. For instance, compounds **1–1 (18)** and **1–6 (23)** more potently activated D_1 receptors compared to activation of D_{2L} receptors in cAMP-biosensor

assay, whereas in [^{3}H]cAMP assay and radioligand binding assay the situation was the opposite.

Large difference in potency values of the same ligand measured by different assays is nothing exceptional. Why sometimes we can't see the same order of potencies for different agonist in different assays arises from differences in equilibrium versus non-equilibrium assays and small system amplification versus large system amplification. Fast on-rate ligands with low affinity might have better potency in non-equilibrium conditions when measured in amplified systems (Ca^{2+}, cAMP assays) compared to slower on-rate ligands but higher affinity ligands; likewise, in non-amplified systems at equilibrium conditions (GTPγS assay) the higher affinity and slower on-rate ligand can have better potency (Charlton and Vauquelin, 2010). In ideal case all ligands should also be tested for their on- and off-rates, in addition to receptor binding affinity in equilibrium conditions and functional response in various assays (in non-amplified and highly amplified systems). In summary, the cAMP-biosensor assay less efficiently reflects differences in ligand potencies compared to [^{3}H]cAMP assay and the potency values obtained with [^{3}H]cAMP assay correlate better with the ligand binding data in **Table 3**. However, [^{3}H]cAMP assay detects total cAMP

Table 3. Binding data of 1-substituted apomorphine derivatives

		Radioligand competition assay					
	-R[1]	*K_i (nM) ± SEM*			*Selectivity*		
Comp.	substituent	D_1R	$D_{2L}R$	D_3R	D_1/D_{2L}	D_1/D_3	D_{2L}/D_3
1–1	Me	187 ± 46	54 ± 10	23 ± 5	3.4	8.2	2.4
1–2	Bn	168 ± 34	77 ± 20	55 ± 9	2.2	3	1.4
1–3	OH-Me	980 ± 160	43 ± 13	17 ± 4	22	57	2.6
1–4	4-OH-benzyl	40 ± 11	47 ± 9	28 ± 6	0.8	1.4	1.7
1–5	4-*N,N*-dimethyl-amino-benzyl	135 ± 39	79 ± 31	53 ± 12	1.7	2.6	1.5
1–6	(4-OH-1-naphtyl)-methyl	437 ± 83	170 ± 64	130 ± 30	2.6	3.4	1.3
Apo	H	492 ± 49	53 ± 15	17 ± 3	9.3	30	3.2
DA	n/a	12000 ± 3000	2100 ± 200	15 ± 4	1.7	0.9	26.8

K_i characterises the ability of a compound to inhibit [^{3}H]SCH23390 (D_1) or [^{3}H]raclopride (D_{2L} and D_3) binding to the corresponding human dopamine receptor subtypes in membrane preparations of HEK293 cells. Selectivity is the ratio of the compound's inhibition constant values (K_i) for designated receptor subtypes.Values are represented as mean ± SEM of the apparent inhibition constant (K_i) from at least three independent experiments carried out in duplicate or triplicate.

in the system, which may not reflect dynamic and localised cAMP changes in cell taking place upon ligand stimulation, the changes which may be sufficient for activating downstream signalling. The cAMP-biosensor used in this study is distributed evenly in the cytosol (Nikolaev *et al.*, 2004) and the observed change in FRET therefore reflects any change in cAMP concentration in any cellular compartment. Even if the total change in cAMP remains rather small and hard to detect with methods detecting total cAMP in system, a localised increase in cAMP concentration would be more easily traceable with cAMP-biosensor.

Table 4. Functional data of 1-substituted apomorphine derivatives

		cAMP-biosensor assay					
	-R^1	*pEC_{50} ± SEM*			*Selectivity*		
Comp.	substituent	D_1R	$D_{2L}R$	D_3R	D_1/D_{2L}	D_1/D_3	D_{2L}/D_3
1–1	Me	8.49 ± 0.23	7.96 ± 0.29	8.16 ± 0.21	0.3	0.5	1.6
1–2	Bn	7.26 ± 0.44	8.31 ± 0.33	7.87 ± 0.25	11.3	4.0	0.4
1–3	OH-Me	7.82 ± 0.24	8.43 ± 0.37	8.59 ± 0.31	4.1	5.9	1.4
1–4	4-OH-benzyl	8.33 ± 0.27	8.33 ± 0.25	8.19 ± 0.22	1.0	0.7	0.7
1–5	4-*N,N*-dimethyl-aminobenzyl	6.95 ± 0.21	7.10 ± 0.08	7.67 ± 0.40	1.4	5.2	3.7
1–6	(4-OH-1-naphtyl)-methyl	7.63 ± 0.24	7.49 ± 0.07	7.68 ± 0.24	0.7	1.1	1.5
Apo	H	8.52 ± 0.12	8.70 ± 0.12	8.56 ± 0.09	1.0	1.5	1.5
DA	n/a	8.78 ± 0.12	8.53 ± 0.13	8.71 ± 0.04	0.8	0.6	0.7

pEC_{50} characterises the concentration of a compound to generate 50% of the receptor-dependent change in cAMP concentration. Selectivity is the ratio of the compound's potency values (EC_{50}) for designated receptor subtypes. Values are represented as mean ± SEM from at least three independent experiments carried out in duplicate or triplicate

4.4.2.3. 3-substituted apomorphines

Currently very little information is available about the effect of 3-substituted apomorphines on the binding and activation of dopamine receptors. To further study the steric and electronic interactions of apomorphine derivatives with dopamine receptor binding and function a new set of 3-substituted apomorphines has been synthesised, some of which are published in (Sipos *et al.*, 2007).

Biological assays with 3-substituted derivatives (presented in **Table 5**) revealed many interesting properties of the compounds. The 4-OH-Phenyl substituent, when located at position 2 is known to trigger high affinity binding for

D_2 and D_3 receptors (K_i values 1.0 nM and 3.2 nM, respectively) compared to apomorphine, having very little effect on the affinity for D_1 receptors (K_i value 167 nM) (Sondergaard *et al.*, 2005), which results in an increased selectivity for D_{2L} and D_3 receptors. At position 1 the 4-OH-Phenyl substituent (compound **1–4** in **Table 3**) has no significant effect on D_{2L} (K_i 47 nM) and D_3 (K_i 28 nM) receptor binding, while the affinity for D_1 (K_i 40 nM) receptor is increased 10-fold compared to apomorphine. The affinity for D_1 receptor (K_i 4.6 nM) is even more increased when the 4-OH-Phenyl substituent is located at position 3, as in compound **3–7** (**Table 5**), resulting in complete loss of selectivity for D_{2L} (K_i 9.2 nM) and D_3 (K_i 4.9 nM) receptors. It was discussed here earlier that the 4-OH-Phenyl function at position 2 most likely allows formation of a tight interaction between the OH-group and a complementary amino acid residue in the binding pocket favouring binding to D_2 receptors. Here it seems that the phenyl-group is also important at position 1 and particularly at position 3 for providing a suitable distance for the OH-group to interact with the D_1 receptor, whereas the binding to D_{2L} receptor is less affected, compare compounds **3–3** and **3–7**. Interestingly, reducing the space between the hydrogen donating OH-group and the compound's core structure at position 3 (in compound **3–3**) has tremendously increased affinity for D_3 receptors (K_i 0.27 nM), while the affinity for D_{2L} receptors (K_i 2.5 nM) is much less affected. However, at position 1 the hydroxymethyl group had no effect on the affinity for D_{2L} and D_3 receptors (see compound **1–3** in **Table 3**). From **Table 5** it is seen that the compound **3–3** has a very unique selectivity profile with its submicromolar affinity for D_1 (K_i 130 nM) receptor and subnanomolar affinity for D_3 receptors (K_i 0.27 nM), resulting in more than 500-fold selectivity for D_{2L} receptors and up to 5000-fold selectivity for D_3 receptors, compared to its affinity for D_1 receptors. Bromine in compound **3–2** had very little effect on D_1 and D_{2L} receptor binding, while binding to D_3 receptors was highly increased, resulting in the most intriguing D_3-selective compound of the series with more than 80-fold selectivity over D_{2L} receptors. The more electronegative chlorine in compound **3–1** slightly increased the binding to D_1 receptors, but the affinity for D_{2L} and D_3 receptors was decreased, which resulted in complete loss of selectivity. Phenyl and 4-*N,N*-dimethylaminophenyl groups at position 3 (compounds **3–5** and **3–6**, respectively) both increased affinity for D_1 receptors approximately 6-fold, whereas at position 1 analogous substitutions, benzyl and 4-*N,N*-dimethylaminobenzyl (compounds **1–2** and **1–5**), resulted in 3-fold increase of affinity for D_1 receptors. At position 1 the benzyl (compound **1–2)** and 4-*N,N*-dimethylaminobenzyl (compound **1–5**) functions slightly decreased the affinity for D_3 receptors, while at position 3 the phenyl-group (compound **3–5**) had no effect and the 4-*N,N*-dimethylaminophenyl (compound **3–6**) slightly decreased affinity for D_3 receptors.

The distinct selectivity profiles of 3-substituted apomorphines **3–2** and **3–3** observed in radioligand competition assays (**Table 5**) were lost in cAMP-bio-sensor assay (**Table 6**). However, all the compounds behaved as highly potent

full agonists with higher potencies towards D_{2L} and D_3 receptors. An exception was 4-OH-phenyl substituted compound **3–7**, which had both higher affinity and higher potency for D_1 receptors. Compound **3–3** with a very low affinity for D_1 receptor behaved as a very potent ligand in cAMP-biosensor assay. Similar effects are seen with dopamine. It suggests that some ligands can more potently activate the receptor and trigger signalling even if their average observed binding affinity (composed of low and high affinity binding components) is low. Another factor that has a great impact on the high potency values observed in cAMP-biosensor assay is the high signal amplification.

Taken together, the results with 1-, 2- and 3-substituted apomorphines show that substitutions at position 2 mainly affect binding to D_2 (and/or D_3) receptors, while substitutions at positions 1 affect mainly binding to D_1 receptors. The D_3 receptor binding is mainly affected by substitutions at position 3 with hydroxyl and bromine being the most potent and increasing the affinity for D_3 receptors to subnanomolar level. In functional assays measuring cAMP response the 1- and 3- substituted compounds behave as fully potent dopaminergic ligands on all three dopamine receptor subtypes.

Table 5. Binding data of 3-substituted apomorphine derivatives

		Radioligand competition assay					
	-R^3	*K_i (nM) ± SEM*			*Selectivity*		
Comp.	substituent	D_1R	$D_{2L}R$	D_3R	D_1/D_{2L}	D_1/D_3	D_{2L}/D_3
3–1	Cl	109 ± 16	54 ± 20	40.6 ± 6.1	2.0	2.7	1.3
3–2	Br	443 ± 49	19 ± 10	0.22 ± 0.04	24	2010	86
3–3	OH	1330 ± 100	2.5 ± 0.6	0.27 ± 0.02	530	4930	9.3
3–4	Me	130 ± 17	27.2 ± 4.9	18.1 ± 0.3	4.8	7.2	1.5
3–5	Ph	55.4 ± 4.9	13.7 ± 3.3	11.7 ± 4.2	4.0	4.7	1.2
3–6	4-*N,N*-dimethyl-amino-Ph	59.1 ± 6.3	45 ± 14	30.2 ± 1.3	1.3	2.0	1.5
3–7	4-OH-Ph	4.6 ± 1.8	9.2 ± 2.5	4.9 ± 0.5	0.5	0.9	1.9
3–8	4-dibenzo-furanyl	46 ± 10	21 ± 6	27.4 ± 4.1	2.2	1.7	0.8
Apo	H	373 ± 45	20.8 ± 0.4	14 ± 3	18	27	1.5
DA	n/a	10400 ± 1200	1990 ± 690	38.6 ± 5.6	5.2	270	52

K_i characterises the ability of a compound to inhibit [^{3}H]SCH23390 (D_1) or [^{3}H]raclopride (D_{2L} and D_3) binding to the corresponding human dopamine receptor subtypes in membrane preparations of HEK293 cells. Selectivity is the ratio of the compound's inhibition constant values (K_i) for designated receptor subtypes. Values are represented as mean ± SEM of the apparent inhibition constant (K_i) from at least three independent experiments carried out in duplicate or triplicate.

Table 6. Functional data of 3-substituted apomorphine derivatives

Comp.	-R^3 substituent	cAMP-biosensor assay					
		pEC_{50} ± SEM			*Selectivity*		
		D_1R	$D_{2L}R$	D_3R	D_1/D_{2L}	D_1/D_3	D_{2L}/D_3
3–1	Cl	7.93 ± 0.08	8.13 ± 0.21	8.24 ± 0.11	1.6	2.0	1.3
3–2	Br	7.84 ± 0.19	8.48 ± 0.15	8.65 ± 0.08	4.4	6.4	1.5
3–3	OH	8.34 ± 0.04	8.55 ± 0.07	8.75 ± 0.05	1.6	2.6	1.6
3–4	Me	8.24 ± 0.06	8.56 ± 0.2	8.50 ± 0.17	2.1	1.8	0.9
3–5	Ph	7.74 ± 0.04	8.52 ± 0.27	8.41 ± 0.15	6.1	4.7	0.8
3–6	4-*N,N*-dimethyl-amino-Ph	8.00 ± 0.06	8.37 ± 0.13	8.41 ± 0.15	2.3	2.6	1.1
3–7	4-OH-Ph	8.42 ± 0.09	8.33 ± 0.2	8.31 ± 0.22	0.8	0.8	1.0
3–8	4-dibenzo-furanyl	7.39 ± 0.07	7.68 ± 0.21	7.65 ± 0.27	1.9	1.8	0.9
Apo	H	8.52 ± 0.12	8.70 ± 0.20	8.56 ± 0.14	1.5	1.1	0.7
DA	n/a	8.78 ± 0.12	8.53 ± 0.21	8.71 ± 0.07	0.6	0.8	1.5

pEC_{50} characterises the concentration of a compound to generate 50% of the receptor-dependent change in cAMP concentration. Selectivity is the ratio of the compound's potency values (EC_{50}) for designated receptor subtypes. Values are represented as mean ± SEM from at least four independent experiments carried out in triplicate.

5. CONCLUSIONS

The thesis describes the process of developing assay systems with recombinant dopamine receptors for characterisation of subtype-selective binding and functional activity of ligands. The binding and function of dopamine receptors was studied in various model systems such as Sf9 insect cell line, striatal tissue of rat brain, CHO and fibroblast cell lines, and HEK293 cells.

Sf9 cells were used for studying the interaction between dopamine D_1 and adenosine A_1 receptors. In this cell line, A_1-D_1 receptor antagonistic interaction was revealed only by the modulation of cyclic AMP levels and only when the receptors were expressed together with both G_i and G_s proteins.

High expression of dopamine D_1 and D_2 receptor expression was shown in rat striatum with proper ligand binding and G-protein coupling profiles. Nevertheless, the D_2 receptor-mediated signalling could not be measured in [^{3}H]cAMP assay, whereas the activation of D_1 receptor was clearly detected. Therefore [^{3}H]cAMP assay was shown to be applicable for selective detection of D_1 receptor signalling events in rat striatal tissue, whereas [^{35}S]GTPγS assay could discriminate the D_2 receptor activation, as shown earlier.

Binding properties and functional activity of newly synthesised ligands was initially studied on D_1 and D_2 receptors expressed in Ltk$^-$ fibroblast and CHO-K1 cell lines, respectively. HEK293 cell-based dopamine D_1, D_{2L} and D_3 receptor stable cell lines were developed to obtain more comparable assay systems. Functional properties of the receptors were studied on the level of G-protein activation (the effect of GTPγS on agonist affinity) and activation of AC (modulation of cAMP levels). While GTPγS effectively modulated agonist binding to D_1 and D_{2L} receptor, the effect on D_3 receptor binding was negligible, suggesting a weak interaction of D_3 receptors and G-proteins. This was further supported by [^{3}H]cAMP assay in which proper agonist response to D_1 and D_{2L} receptor activation was reflected, whereas the D_3 receptor activation could not be detected. Using our BacMam cAMP-biosensor assay we could reliably detect modulation of cAMP levels by all the three receptor subtypes, suggesting that the D_3 receptors indeed were effectively coupled to their G-proteins in spite of deficient sensitivity to GTPγS and lack of cAMP-response in [^{3}H]cAMP assay.

The dopamine receptor stable cell lines were used in screening for new dopaminergic ligands in cooperation with research groups involved in organic synthesis. Major focus was in screening of various apomorphine derivatives. Several compounds with nanomolar affinities for D_2 and D_3 receptors were identified, along with two compounds with subnanomolar affinity for D_3 receptors - the 3-bromine substituted and the 3-hydroxy substituted apomorphine, which also possessed the highest D_3/D_2 selectivity (~90-fold) and the highest D_2/D_1 selectivity (~500-fold), respectively. The highest D_3/D_1 selectivity was observed with the same compounds, 2000-fold and 5000-fold, respectively. To the best of our knowledge these two compounds are the most D_3-selective of apomorphines published so far and with the best D_3 receptor

binding affinity. In functional assays most of the tested apomorphine derivatives behave as full agonists. In addition, the new cell lines were used to screen for binding of 3-azabicyclo[3.1.0]hexane derivatives obtained by diastereoselective chemoenzymatic synthesis. Most of these compounds bind with significantly better affinity to D_2 and D_3 receptors compared to D_1 receptors, but the binding affinities remained in micromolar or submicromolar range. In summary, we identified some promising scaffolds, which is an additional step towards new selective and potent dopaminergic drugs.

6. SUMMARY IN ESTONIAN

Katsesüsteemid ligandide sidumisomaduste ja funktsionaalse aktiivsuse alatüüp-selektiivseks iseloomustamiseks dopamiini retseptoritel

Dopamiin (DA) ehk 3-hüdroksütüramiin on närvivirgatsaine, mida organism sünteesib aminohappest türosiin. DA ja talle vastavaid retseptoreid (DAR) leidub nii perifeerses kui kesknärvisüsteemis (KNS) ning koos mõjutavad need mitmeid organismile olulisi funktsioone, nagu liigutuste koordineeritust, söömist, und, tähelepanu, mälu, õppimist, motivatsiooni jne. Perifeerse süsteemi DAR-d osalevad muuhulgas haistmis-, nägemis- ja hormonaalse regulatsiooni protsessides ning mõjutavad südameveresoonkonna ja neerude tööd. Mitmed KNS haigused, sh Parkinsoni tõbi (PD) ja skisofreenia, on seotud DA või DAR-te taseme ja funktsiooni häiretega. Kui PD korral on tegemist DA signaaliradade alatalitlusega, siis skisofreenikute ajus on olukord vastupidine ehk DA signaalirajad on ülereguleeritud ja ülitundlikud.

Ravimid, mis suudavad dopamiinergilisi häireid kontrolli all hoida või leevendada on sageli kas DA imiteerivad ühendid (DAR agonistid) või DA toimet blokeerivad ühendid (DAR antagonistid). DA retseptoreid on viis erinevat alatüüpi, mis kõik erinevad teineteisest nii füsioloogilise funktsiooni ja farmakoloogiliste omaduste kui paiknemise poolest. D_1-perekonna (D_1 ja D_5) DAR-te farmakoloogilised omadused erinevad oluliselt D_2-perekonna (D_2, D_3, D_4) omadest. D_1 retseptorite aktiveerimisel suureneb rakus tsüklilise adenosiin-monofosfaadi (cAMP) sünteesi reguleeriva ensüümi adenülaadi tsüklaasi (AC) aktiivsus ning D_2 retseptorite aktiveerimisel see väheneb, avaldudes vastavalt kas cAMP taseme tõusus või languses. Ka dopamiinergiliste ligandide ehk DAR-eid äratundvate ühendite sidumisomadused perekondade vahel erinevad enam kui perekonna sees.

Käesoleva töö eesmärgiks oli luua ning töösse rakendada rakukultuuridel põhinevad mudelsüsteemid erinevate sünteetiliste ligandide sidumisomaduste ja aktiivsuse testimiseks dopamiini D_1, D_2 ja D_3 retseptoritel, võimaldades sel viisil tuvastada ja iseloomustada uute DAR agonistide ja antagonistide farmakoloogilisi omadusi katseklaasi tasemel ja saadud tulemuste põhjal leida uusi alatüüp-selektiivseid ja efektiivseid ühendeid. Töö algfaasis uuriti dopamiini retseptorite sidumisomadusi erinevates rakulistes süsteemides, nende hulgas putuka- ja imetajarakkudes ekspresseeritud dopamiini retseptoritel ning roti aju preparaadis endogeenselt ekspreseeritud dopamiini retseptoritel.

Sf9 rakke kasutati dopamiini D_1 ja adenosiini A_1 retseptorite vaheliste interaktsioonide uurimiseks. A_1-D_1 retseptorite antagonistlik toime avaldus vaid cAMP taseme muutuses ning mõlema retseptori aktivatsiooniks vajalike G-valkude (α_s ja α_i) olemasolul.

Ligandi sidumise ja GTPγS toime katsetega näidati, et dopamiini D_1 ja D_2 retseptorid roti aju juttkehas on seotud neile vastavate G-valkudega. Retsepto-

rite funktsionaalsuse testimisel [^{3}H]cAMP meetodil andis efekti vaid D_1-retseptorite aktiveerimine. Seega leiti, et [^{3}H]cAMP metoodika sobib roti aju juttkeha D_1 retseptorite selektiivse aktivatsiooni uurimiseks, kuid D_2 retseptorite aktivatsiooni mõõtmiseks tuleks kasutada näiteks [^{35}S]GTPγS meetodit.

Uute ühendite dopamiinergilise aktiivsuse testimiseks olid algselt meie käsutuses vaid imetaja päritolu Ltk$^-$ ja CHO-K1 rakuliinid, mis ekspresseerisid vastavalt D_1 ja D_2 retseptoreid. Samas tekkis vajadus luua rakuliinid kus, a) kõik retseptorid oleksid võimalikult sarnases rakukeskkonnas, b) kõigi retseptori alatüüpide aktiivsust saaks mõõta signaaliraja samal tasandil, nt cAMP kaudu.

Sellest tulenevalt loodi HEK293 rakkudel põhinevad dopamiini D_1, D_{2L} ja D_3 retseptoreid stabiilselt ekspresseerivad rakuliinid ning iseloomustati saadud rakuliinide farmakoloogilisi omadusi erineval tasemel. Retseptorite tugevat seost G-valkudega näitasid ligandi sidumise katsed, kus GTPγS juuresolek mõjutas oluliselt agonisti sidumisafiinsust D_1 ja D_2 retseptorile, samas kui D_3 retseptori korral täheldati vaid nõrka GTPγS efekti. Nõrk interaktsioon D_3 retseptori ja G_i valkude vahel avaldus ka [^{3}H]cAMP katses, millega ei õnnestunudki D_3 retseptori aktivatsiooni reprodutseeruvalt määrata, samas kui D_1 ja D_2 retseptorite aktivatsioon oli [^{3}H]cAMP meetodiga edukalt detekteeritav. Kasutades cAMP taseme mõõtmiseks cAMP-biosensorit (BacMam Epac2-camps), õnnestus määrata kõigi kolme dopamiini retseptori alatüübi spetsiifilisi signaale ja seega ka ligandide funktsionaalsust.

Koostöös sünteesiga tegelevate uurimisrühmadega kasutati saadud katsesüsteeme uute ligandide sidumisafiinsuse ja funktsionaalsete omaduste skriinimiseks. Suure osa käesolevas töös testitud ainetest võtsid enda alla uued apomorfiini derivaadid. Töö käigus leiti mitmeid nanomolaarse afiinsusega D_2-selektiivseid ühendeid. Samuti tuvastati paar subnanomolaarse afiinsusega ning märkimisväärse D_3-selektiivsusega agonisti (3-bromo ja 3-hüdroksü apomorfiinid), mis meile teadaolevalt on seni avaldatud apomorfiini derivaatidest kõige D_3-selektiivsemad ja kõige parema D_3 retseptori afiinsusega ühendid. Funktsionaalsetes katsetes käitusid enamus testitud ühenditest täisagonistidena. Lisaks apomorfiini derivaatidele testiti ka uudse diastereoselektiivse kemoensümaatilise sünteesi teel saadud 3-asabitsüklo[3.2.0]heptaani derivaatide sidumisomadusi. Näidati, et nimetatud ühendid seostusid oluliselt paremini D_2 ja D_3 retseptoritele võrreldes D_1 alatüübiga, samas jäid nende sidumisafiinsused, paari erandiga, võrdlemisi tagasihoidlikuks – mikromolaarsesse suurusjärku. Kokkuvõttes tuvastati ligandide skriinimisel mitmeid paljulubavaid struktuurielemente, mis on täiendav samm uute selektiivsete ja efektiivsete dopamiinergiliste ravimkandidaatide arendamisel.

7. REFERENCES

Ahlquist, R.P. (1973). Adrenergic receptors: a personal and practical view. *Perspectives in Biology and Medicine* 17, 119–122.

Ahmed, M., Muntasir, H.A., Hossain, M., Ishiguro, M., Komiyama, T., Muramatsu, I., Kurose, H., Nagatomo, T. (2006) Beta-blockers show inverse agonism to a novel constitutively active mutant of beta1-adrenoceptor. *Journal of Pharmacological Sciences* 102, 167–172.

Aizman, O., Brismar, H., Uhlén, P., Zettergren, E., Levey, a I., Forssberg, H., Greengard, P., Aperia, A. (2000) Anatomical and physiological evidence for D1 and D2 dopamine receptor colocalization in neostriatal neurons. *Nature Neuroscience* 3, 226–230.

Ames, R., Fornwald, J., Nuthulaganti, P., Trill, J., Foley, J., Buckley, P., Kost, T., Wu, Z., Romanos, M. (2004) BacMam recombinant baculoviruses in G protein-coupled receptor drug discovery. *Receptors and Channels* 10, 99–107.

Ames, R.S., Kost, T.A., Condreay, J.P. (2007) BacMam technology and its application to drug discovery. *Reporter* 1669–1682.

Aranda, A., Toro, M.J., Usera, F., Pascual, A. (1990) Regulation by butyrate of the cAMP response to cholera toxin and forskolin in pituitary GH1 cells. *European Journal of Biochemistry* 187, 683–689.

Atwood, B., Lopez, J., Wager-Miller, J., Mackie, K., Straiker, A. (2011) Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* 12, 14.

Banerjee, a, Prante, O. (2012) Subtype-Selective Dopamine Receptor Radioligands for PET Imaging: Current Status and Recent Developments. *Current Medicinal Chemistry* 19, 3957–3966.

Beaulieu, J.-M., Gainetdinov, R.R. (2011) The Physiology, Signaling, and Pharmacology of Dopamine Receptors. *Pharmacological Reviews* 63, 182–217.

Beaulieu, J.-M., Gainetdinov, R.R., Caron, M.G. (2007) The Akt-GSK-3 signaling cascade in the actions of dopamine. *Trends in Pharmacological Sciences* 28, 166–172.

Binkowski, B. (2011) GloSensor ™ Technology: Overview GloSensor ™ Technology. Last received September 30, 2012 from www.promega.com

Birnbaumer, L. (2007) Expansion of signal transduction by G proteins. The second 15 years or so: from 3 to 16 alpha subunits plus betagamma dimers. *Biochimica et Biophysica Acta* 1768, 772–793.

Brandish, P.E., Hill, L.A., Zheng, W., Scolnick, E.M. (2003) Scintillation proximity assay of inositol phosphates in cell extracts: High-throughput measurement of G-protein-coupled receptor activation. *Analytical Biochemistry* 313, 311–318.

Brown, D.L., Albano, J.D., Ekins, R.P., Sgherzi, A.M. (1971) A simple and sensitive saturation assay method for the measurement of adenosine 3':5'-cyclic monophosphate. *Biochemical Journal* 121, 561–562.

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

Bünemann, M., Frank, M., Lohse, M.J. (2003) Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proceedings of the National Academy of Sciences of the United States of America* 100, 16077–16082.

Cai, G., Gurdal, H., Smith, C., Wang, H.Y., Friedman, E. (1999) Inverse agonist properties of dopaminergic antagonists at the D(1A) dopamine receptor: uncoupling of the D(1A) dopamine receptor from G(s) protein. *Molecular Pharmacology* 56, 989–996.

Canals, M., Sexton, P.M., Christopoulos, A. (2011) Allostery in GPCRs: "MWC" revisited. *Trends in Biochemical Sciences* 36, 663–672.

Cao, Y., Sun, W.-C., Jin, L., Xie, K.-Q., Zhu, X.-Z. (2006) Activation of adenosine A1 receptor modulates dopamine D1 receptor activity in stably cotransfected human embryonic kidney 293 cells. *European Journal of Pharmacology* 548, 29–35.

Cao, Y., Xie, K.-Q., Zhu, X.-Z. (2007). The enhancement of dopamine D1 receptor desensitization by adenosine A1 receptor activation. *European Journal of Pharmacology* 562, 34–38.

Carlsson, A., Lindquist, M., Magnusson, T. (1957) 3,4-Dihydroxyphenylalanine and 5-Hydroxytryptophan as Reserpine Antagonists. *Nature* 180, 1200.

Carlsson, J., Coleman, R.G., Setola, V., Irwin, J.J., Fan, H., Schlessinger, A., Sali, A., Roth, B.L., Shoichet, B.K. (2011) Ligand discovery from a dopamine D3 receptor homology model and crystal structure. *Nature Chemical Biology* 7, 769–778.

Charest, P.G., Oligny-Longpré, G., Bonin, H., Azzi, M., Bouvier, M. (2007) The V2 vasopressin receptor stimulates ERK1/2 activity independently of heterotrimeric G protein signalling. *Cellular Signalling* 19, 32–41.

Charlton, S.J., Vauquelin, G. (2010) Elusive equilibrium: the challenge of interpreting receptor pharmacology using calcium assays. *British Journal of Pharmacology* 161, 1250–1265.

Cheng, Y.-C., Prusoff, W.H., Yung-Chi, C. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochemical Pharmacology* 22, 3099–3108.

Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G.F., Thian, F.S., Kobilka, T.S., Choi, H.J., Kuhn, P., Weis, W.I., Kobilka, B.K., Stevens, R.C. (2007) High-Resolution Crystal Structure of an Engineered Human 2-Adrenergic G Protein Coupled Receptor. *Science* 318, 1258–1265.

Chio, C.L., Hess, G.F., Graham, R.S., Huff, R.M. (1990) A second molecular form of D2 dopamine receptor in rat and bovine caudate nucleus. *Nature* 343, 266–269.

Conklin, B.R., Farfel, Z., Lustig, K.D., Julius, D., Bourne, H.R. (1993) Substitution of three amino acids switches receptor specificity of Gq[alpha] to that of Gi[alpha]. *Nature* 363, 274–276.

Davila, J.C., Cezar, G.G., Thiede, M., Strom, S., Miki, T., Trosko, J. (2004) Use and application of stem cells in toxicology. *Toxicological sciences an official journal of the Society of Toxicology* 79, 214–223.

Defea, K. (2008) Beta-arrestins and heterotrimeric G-proteins: collaborators and competitors in signal transduction. *British Journal of Pharmacology* 153, S298–S309.

Depoortere, R., Boulay, D., Perrault, G., Bergis, O., Decobert, M., Françon, D., Jung, M., Simiand, J., Soubrié, P., Scatton, B. (2003) SSR181507, a dopamine D2 receptor antagonist and 5-HT1A receptor agonist. II: Behavioral profile predictive of an atypical antipsychotic activity. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology* 28, 1889–1902.

DiPilato, L.M., Cheng, X., Zhang, J. (2004) Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proceedings of the National Academy of Sciences of the United States of America* 101, 16513–16518.

Dixon, R.A., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., Frielle, T., Bolanowski, M.A., Bennett, C.D., Rands, E., Diehl, R.E., Mumford, R.A., Slater, E.E., Sigal, I.S., Caron, M.G., Lefkowitz, R.J., Strader, C.D. (1986) Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature* 321, 75–79.

Drescher, K., Haupt, A., Unger, L., Turner, S.C., Braje, W., Grandel, R. (2006) Azabicycloheptyl compounds suitable for treating disorders that respond to modulation of the dopamine D3 receptor. *Chemical Abstracts*.

Eglen, R.M., Gilchrist, A., Reisine, T. (2008) An overview of drug screening using primary and embryonic stem cells. *Combinatorial Chemistry High Throughput Screening* 11, 566–572.

Ehrin, E., Farde, L., de Paulis, T., Eriksson, L., Greitz, T., Johnström, P., Litton, J.-E., Nilsson, J.L.G., Sedvall, G., Stone-Elander, S., Ögren, S. (1985) Preparation of 11C-labelled raclopride, a new potent dopamine receptor antagonist: Preliminary PET studies of cerebral dopamine receptors in the monkey. *The International Journal of Applied Radiation and Isotopes* 36, 269–273.

Ferré, S., Popoli, P., Giménez-Llort, L., Finnman, U.B., Martínez, E., Scotti de Carolis, A., Fuxe, K. (1994) Postsynaptic antagonistic interaction between adenosine A1 and dopamine D1 receptors. *Neuroreport* 6, 73–76.

Ferré, S., Torvinen, M., Antoniou, K., Irenius, E., Civelli, O., Arenas, E., Fredholm, B.B., Fuxe, K. (1998) Adenosine A1 receptor-mediated modulation of dopamine D1 receptors in stably cotransfected fibroblast cells. *The Journal of Biological Chemistry* 273, 4718–4724.

Fleckenstein, A.E., Gibb, J.W., Hanson, G.R. (2000) Differential effects of stimulants on monoaminergic transporters: pharmacological consequences and implications for neurotoxicity. *European Journal of Pharmacology* 406, 1–13.

Fornwald, J. a, Lu, Q., Wang, D., Ames, R.S. (2007) Gene expression in mammalian cells using BacMam, a modified baculovirus system. *Methods in Molecular Biology* 388, 95–114.

Fotiadis, D., Jastrzebska, B., Philippsen, A., Müller, D.J., Palczewski, K., Engel, A. (2006) Structure of the rhodopsin dimer: a working model for G-protein-coupled receptors. *Current Opinion in Structural Biology* 16, 252–259.

Franco, R., Lluis, C., Canela, E.I., Mallol, J., Agnati, L., Casadó, V., Ciruela, F., Ferré, S., Fuxe, K. (2007) Receptor-receptor interactions involving adenosine A1 or dopamine D1 receptors and accessory proteins. *Journal of Neural Transmission* 114, 93–104.

Freedman, S.B., Patel, S., Marwood, R., Emms, F., Seabrook, G.R., Knowles, M.R., McAllister, G. (1994) Expression and pharmacological characterization of the human D3 dopamine receptor. *The Journal of Pharmacology and Experimental Therapeutics* 268, 417–426.

Fu, D., Skryabin, B.V., Brosius, J., Robakis, N.K. (1995) Molecular cloning and characterization of the mouse dopamine D3 receptor gene: an additional intron and an mRNA variant. *DNA and Cell Biology* 14, 485–492.

Gazi, L., Nickolls, S. a, Strange, P.G. (2003) Functional coupling of the human dopamine D2 receptor with G alpha i1, G alpha i2, G alpha i3 and G alpha o G proteins: evidence for agonist regulation of G protein selectivity. *British Journal of Pharmacology* 138, 775–786.

Geison, G. L. (2008) Loewi, Otto. *Complete Dictionary of Scientific Biography*. Last received September 30, 2012 from http://www.encyclopedia.com

Gesztelyi, R., Zsuga, J., Kemeny-Beke, A., Varga, B., Juhasz, B., Tosaki, A. (2012) The Hill equation and the origin of quantitative pharmacology. *Archive for History of Exact Sciences* 66, 427–438.

Gilchrist, A. (2007) Modulating G-protein-coupled receptors: from traditional pharmacology to allosterics. *Trends in Pharmacological Sciences* 28, 431–437.

Gilman, a G. (1970) A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proceedings of the National Academy of Sciences of the United States of America* 67, 305–312.

Ginés, S., Hillion, J., Torvinen, M., Le Crom, S., Casadó, V., Canela, E.I., Rondin, S., Lew, J.Y., Watson, S., Zoli, M., Agnati, L.F., Verniera, P., Lluis, C., Ferré, S., Fuxe, K., Franco, R. (2000) Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. *Proceedings of the National Academy of Sciences of the United States of America* 97, 8606–8611.

Giros, B., Martres, M.P., Sokoloff, P., Schwartz, J.C. (1990) Gene cloning of human dopaminergic D3 receptor and identification of its chromosome. *Comptes rendus de lAcademie des sciences Serie III Sciences de la vie* 311, 501–508.

Giros, B., Sokoloff, P., Martres, M.P., Riou, J.F., Emorine, L.J., Schwartz, J.C. (1989) Alternative splicing directs the expression of two D2 dopamine receptor isoforms. *Nature* 342, 923–926.

Glase, S.A., Akunne, H.C., Georgic, L.M., Heffner, T.G., MacKenzie, R.G., Manley, P.J., Pugsley, T.A., Wise, L.D. (1997) Substituted [(4-Phenylpiperazinyl)- methyl]-benzamides: Selective Dopamine D4 Agonists. *Journal of Medicinal Chemistry* 40, 1771–1772.

Graham, F.L., Smiley, J., Russell, W.C., Nairn, R. (1977) Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5. *Journal of General Virology* 36, 59–72.

Grandy, D.K., Marchionni, M.A., Makam, H., Stofko, R.E., Alfano, M., Frothingham, L., Fischer, J.B., Burke-Howie, K.J., Bunzow, J.R., Server, A.C. (1989) Cloning of the cDNA and gene for a human D2 dopamine receptor. *Proceedings of the National Academy of Sciences of the United States of America* 86, 9762–9766.

Grienberger, C., Konnerth, A. (2012) Imaging Calcium in Neurons. *Neuron* 73, 862–885.

Gullbo, J., Fryknäs, M., Rickardson, L., Darcy, P., Hägg, M., Wickström, M., Hassan, S., Westman, G., Brnjic, S., Nygren, P., Linder, S., Larsson, R. (2011) Phenotype-based drug screening in primary ovarian carcinoma cultures identifies intracellular iron depletion as a promising strategy for cancer treatment. *Biochemical Pharmacology* 82, 139–147.

Haga, K., Kruse, A.C., Asada, H., Yurugi-Kobayashi, T., Shiroishi, M., Zhang, C., Weis, W.I., Okada, T., Kobilka, B.K., Haga, T., Kobayashi, T. (2012) Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. *Nature* 482, 547–551.

Halldin, C., Stone-Elander, S., Farde, L., Ehrin, E., Fasth, K.J., Långström, B., Sedvall, G. (1986) Preparation of 11C-labelled SCH 23390 for the in vivo study of dopamine D-1 receptors using positron emission tomography. *International Journal Of Radiation Applications and Instrumentation. Part A: Applied Radiation and Isotopes* 37, 1039–1043.

Heidbreder, C. a, Newman, A.H. (2010) Current perspectives on selective dopamine D(3) receptor antagonists as pharmacotherapeutics for addictions and related disorders. *Annals of the New York Academy of Sciences* 1187, 4–34.

Hellevuo, K., Yoshimura, M., Kao, M., Hoffman, P.L., Cooper, D.M.F., Tabakoff, B. (1993) A Novel Adenylyl Cyclase Sequence Cloned from the Human Erythroleukemia Cell Line. *Biochemical and Biophysical Research Communications* 192, 311–318.

Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E., Downing, K.H. (1990) Rhodopsin and bacteriorhodopsin: structure-function relationships. *Journal of Molecular Biology* 213, 899–929.

Herm, L., Berényi, S., Vonk, A., Rinken, A., Sipos, A. (2009) N-Substituted-2-alkyl- and 2-arylnorapomorphines: novel, highly active D2 agonists. *Bioorganic and Medicinal Chemistry* 17, 4756–4762.

Hidaka, K., Tada, S., Matsumoto, M., Ohmori, J., Tasaki, Y., Nomura, T., Usuda, S., Yamaguchi, T. (1996) In vitro pharmacological profile of YM-43611, a novel D2-like receptor antagonist with high affinity and selectivity for dopamine D3 and D4 receptors. *British Journal of Pharmacology* 117, 1625–1632.

Hill, S.J., Williams, C., May, L.T. (2010) Insights into GPCR pharmacology from the measurement of changes in intracellular cyclic AMP; advantages and pitfalls of differing methodologies. *British Journal of Pharmacology* 161, 1266–1275.

Iversen, S.D., Iversen, L.L. (2007) Dopamine: 50 years in perspective. *Trends in Neurosciences* 30, 188–193.

Jaakola, V.-P., Griffith, M.T., Hanson, M.A., Cherezov, V., Chien, E.Y.T., Lane, J.R., IJzerman, A.P., Stevens, R.C. (2008) The 2.6 Angstrom Crystal Structure of a Human A2A Adenosine Receptor Bound to an Antagonist. *Science* 322, 1211–1217.

Jacoby, E., Bouhelal, R., Gerspacher, M., Seuwen, K. (2006) The 7-TM G-Protein-Coupled Receptor Target Family. *ChemMedChem* 1, 760–782.

Janakiraman, V., Forrest, W.F., Chow, B., Seshagiri, S. (2006) A rapid method for estimation of baculovirus titer based on viable cell size. *Journal of Virological Methods* 132, 48–58.

Joyce, J.N. (2001) Dopamine D3 receptor as a therapeutic target for antipsychotic and antiparkinsonian drugs. *Pharmacology and Therapeutics* 90, 231–259.

Kebabian, J.W., Calne, D.B. (1979) Multiple receptors for dopamine. *Nature* 277, 93–96.

Kebabian, J.W., Petzold, G.L., Greengard, P. (1972) Dopamine-sensitive adenylate cyclase in caudate nucleus of rat brain, and its similarity to the "dopamine receptor". *Proceedings of the National Academy of Sciences* 69, 2145–2149.

Kenakin, T. (2002) Efficacy at G-protein-coupled receptors. *Nature reviews. Drug discovery* 1, 103–110.

Kenakin, T. (2007) Functional selectivity through protean and biased agonism: who steers the ship? *Molecular Pharmacology* 72, 1393–1401.

Kenakin, T. (2010) The Evolution of Receptors: From On-Off Switches to Microprocessors, in: Gilchrist, A. (Ed.), GPCR Molecular Pharmacology and Drug Targeting. A John Wiley & Sons, Inc., Publication, New Jersey, pp. 1–26.

Kenakin, T., Miller, L.J. (2010) Seven Transmembrane Receptors as Shapeshifting Proteins: The Impact of Allosteric Modulation and Functional Selectivity on New Drug Discovery. *Pharmacological Reviews* 62, 265–304.

Kenakin, T., Onaran, O. (2002) The ligand paradox between affinity and efficacy: can you be there and not make a difference? *Trends in Pharmacological Sciences* 23, 275–280.

Kiss, B., Laszlovszky, I., Horváth, A., Némethy, Z., Schmidt, E., Bugovics, G., Fazekas, K., Gyertyán, I., Agai-Csongor, E., Domány, G., Szombathelyi, Z. (2008)

Subnanomolar dopamine D3 receptor antagonism coupled to moderate D2 affinity results in favourable antipsychotic-like activity in rodent models: I. neurochemical characterisation of RG-15. *Naunyn-Schmiedeberg's Archives of Pharmacology* 378, 515–528.

Klarenbeek, J.B., Goedhart, J., Hink, M.A., Gadella, T.W.J., Jalink, K. (2011) A mTurquoise-Based cAMP Sensor for Both FLIM and Ratiometric Read-Out Has Improved Dynamic Range. *PLoS ONE* 6, e19170.

Kobilka, B.K., Kobilka, T.S., Daniel, K., Regan, J.W., Caron, M.G., Lefkowitz, R.J. (1988) Chimeric alpha 2-,beta 2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science* 240, 1310–1316.

Kobilka, B.K., Matsui, H., Kobilka, T.S., Yang-Feng, T.L., Francke, U., Caron, M.G., Lefkowitz, R.J., Regan, J.W. (1987) Cloning, sequencing, and expression of the gene coding for the human platelet alpha 2-adrenergic receptor. *Science* 238, 650–656.

Kost, T. a, Condreay, J.P., Jarvis, D.L. (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nature Biotechnology* 23, 567–575.

Koval, A., Kopein, D., Purvanov, V., Katanaev, V.L. (2010) Europium-labeled GTP as a general nonradioactive substitute for [(35)S]GTPgammaS in high-throughput G protein studies. *Analytical Biochemistry* 397, 202–207.

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

Kruh, J. (1981) Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Molecular and Cellular Biochemistry* 42, 65–82.

Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., Mishina, M., Haga, T., Haga, K., Ichiyama, A., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T., Numa, S. (1986) Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature* 323, 411–416.

Kuchinsky, G. (1968) The Influence of Dorpat on the Emergence of Pharmacology as a Distinct Discipline. *Journal of the History of Medicine and Allied Sciences* XXIII, 258–271.

Kunapuli, P. (2010) Ultra-High-Throughput Screening Assays for GPCRs, in: Gilchrist, A. (Ed.), GPCR Molecular Pharmacology and Drug Targeting. John Wiley & Sons, Inc., New Jersey, pp. 300–323.

Lagerström, M.C., Schiöth, H.B. (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nature Reviews Drug Discovery* 7, 339–357.

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

Lane, J.R., Powney, B., Wise, A., Rees, S., Milligan, G. (2007) Protean Agonism at the Dopamine D2 Receptor: (S)-3-(3-Hydroxyphenyl)-N-propylpiperidine Is an Agonist for Activation of Go1 but an Antagonist/Inverse Agonist for Gi1, Gi2, and Gi3. *Molecular Pharmacology* 71, 1349–1359.

Lefkowitz, R.J. (2007) Seven transmembrane receptors: something old, something new. *Acta Physiologica* 190, 9–19.

Lefkowitz, R.J., Roth, J., Pastan, I. (1970) Radioreceptor assay of adrenocorticotropic hormone: new approach to assay of polypeptide hormones in plasma. *Science* 170, 633–635.

Lefkowitz, R.J., Shenoy, S.K. (2005) Transduction of receptor signals by beta-arrestins. *Science* 308, 512–517.

Lepiku, M., Järv, J., Rinken, A., Fuxe, K. (1997) Mechanism of modulation of [3H]raclopride binding to dopaminergic receptors in rat striatal membranes by sodium ions. *Neurochemistry International* 30, 575–581.

Liu, W., Chun, E., Thompson, A.A., Chubukov, P., Xu, F., Katritch, V., Cherezov, V., Stevens, R.C. (2012) Structural Basis for Allosteric Regulation of GPCRs by Sodium Ions. *Science* 337, 232–236.

Liu, Y.F., Civelli, O., Zhou, Q.Y., Albert, P.R. (1992) Cholera toxin-sensitive 3',5'-cyclic adenosine monophosphate and calcium signals of the human dopamine-D1 receptor: selective potentiation by protein kinase A. *Molecular Endocrinology* 6 , 1815–1824.

Lohse, M.J., Andexinger, S., Pitcher, J., Trukawinski, S., Codina, J., Faure, J.P., Caron, M.G., Lefkowitz, R.J. (1992). Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems. *Journal of Biological Chemistry* 267, 8558–8564.

Lohse, M.J., Benovic, J.L., Codina, J., Caron, M.G., Lefkowitz, R.J. (1990) beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science* 248, 1547–1550.

Lundstrom, K. (2005) Structural genomics of GPCRs. *Trends in Biotechnology* 23, 103–108.

Mach, R.H., Hammond, P.S., Huang, Y.S., Yang, B.O., Xu, Y.P., Cheney, J.T., Freeman, R., Luedtke, R.R. (1999) Structure-activity relationship studies of N-(9-benzyl)-9-azabicyclo[3.3.1]nonan-3-beta-yl benzamide analogues for dopamine D-2 and D-3 receptors. *Medicinal Chemistry Research* 9, 355–373.

Manglik, A., Kruse, A.C., Kobilka, T.S., Thian, F.S., Mathiesen, J.M., Sunahara, R.K., Pardo, L., Weis, W.I., Kobilka, B.K., Granier, S. (2012) Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. *Nature* 485, 0–6.

Mank, M., Reiff, D.F., Heim, N., Friedrich, M.W., Borst, A., Griesbeck, O. (2006) A FRET-Based Calcium Biosensor with Fast Signal Kinetics and High Fluorescence Change. *Biophysical Journal* 90, 1790–1796.

Marsden, C.A. (2006) Dopamine: the rewarding years. *British Journal of Pharmacology* 147, S136–S144.

Martin, Y.C. (2011) The Discovery of Novel Selective D1 Dopaminergic Agonists: A-68930, A-77636, A-86929, and ABT-413. *International Journal of Medicinal Chemistry* 2011, 1–8.

Mazina, O., Reinart-Okugbeni, R., Kopanchuk, S., Rinken, A. (2012) BacMam System for FRET-Based cAMP Sensor Expression in Studies of Melanocortin MC1 Receptor Activation. *Journal of Biomolecular Screening* 17, 1096–1101.

Micheli, F. (2011) Recent advances in the development of dopamine D3 receptor antagonists: a medicinal chemistry perspective. *ChemMedChem* 6, 1152–1162.

Micheli, F., Arista, L., Bonanomi, G., Blaney, F.E., Braggio, S., Capelli, A.M., Checchia, A., Damiani, F., Di-Fabio, R., Fontana, S., Gentile, G., Griffante, C., Hamprecht, D., Marchioro, C., Mugnaini, M., Piner, J., Ratti, E., Tedesco, G., Tarsi, L., Terreni, S., Worby, A., Ashby, C.R., Heidbreder, C. (2010) 1,2,4-Triazolyl

azabicyclo[3.1.0]hexanes: a new series of potent and selective dopamine D(3) receptor antagonists. *Journal of Medicinal Chemistry* 53, 374–391.

Micheli, F., Hamprecht, D., Bonanomi, G., Di Fabio, R., Donati, D., Gentile, G., Heidbreder, C., Prandi, A., Tarsi, L., Terreni, S. (2010) [3-azabicyclo[3.1.0]hex-1-yl]phenyl-benzenesulfonamides as selective dopamine D3 antagonists. *Bioorganic and Medicinal Chemistry Letters* 20, 5491–5494.

Milligan, G., 2009. G protein-coupled receptor hetero-dimerization: contribution to pharmacology and function. *British Journal of Pharmacology* 158, 5–14.

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

Mällo, T., Matrov, D., Herm, L., Kõiv, K., Eller, M., Rinken, A., Harro, J. (2007) Tickling-induced 50-kHz ultrasonic vocalization is individually stable and predicts behaviour in tests of anxiety and depression in rats. *Behavioural Brain Research* 184, 57–71.

Nader, M.A., Czoty, P.W., Gould, R.W., Riddick, N.V. (2008) Review. Positron emission tomography imaging studies of dopamine receptors in primate models of addiction. *Philosophical Transactions of the Royal Society of London – Series B: Biological Sciences* 363, 3223–3232.

Nader, M.A., Morgan, D., Gage, H.D., Nader, S.H., Calhoun, T.L., Buchheimer, N., Ehrenkaufer, R., Mach, R.H. (2006). PET imaging of dopamine D2 receptors during chronic cocaine self-administration in monkeys. *Nature Neuroscience* 9, 1050–1056.

Nasman, J., Kukkonen, J.P., Ammoun, S., Akerman, K.E. (2001) Role of G-protein availability in differential signaling by alpha 2-adrenoceptors. *Biochemical Pharmacology* 62, 913–922.

Nettleship, J.E., Assenberg, R., Diprose, J.M., Rahman-Huq, N., Owens, R.J. (2010) Recent advances in the production of proteins in insect and mammalian cells for structural biology. *Journal of Structural Biology* 172, 55–65.

Neubig, R.R. (2007) Missing links: mechanisms of protean agonism. *Molecular Pharmacology* 71, 1200–1202.

Neumeyer, J.L. (1985) Synthesis and Structure-Activity Relationships of Aporphines as Dopamine Receptor Agonists and Antagonists, in: Phillipson, J. D., Roberts M. F., Zenk, M.H. (Ed.), The Chemistry and Biology of Isoquinoline Alkaloids. Springer-Verlag, Berlin, Germany, pp. 146–170.

Newman-Tancredi, a, Cussac, D., Audinot, V., Pasteau, V., Gavaudan, S., Millan, M.J. (1999) G protein activation by human dopamine D3 receptors in high-expressing Chinese hamster ovary cells: A guanosine-5'-O-(3-[35S]thio)- triphosphate binding and antibody study. *Molecular Pharmacology* 55, 564–574.

Niedernberg, A., Tunaru, S., Blaukat, A., Harris, B., Kostenis, E. (2003) Comparative analysis of functional assays for characterization of agonist ligands at G protein-coupled receptors. *Journal of Biomolecular Screening* 8, 500–510.

Nikolaev, V.O., Bünemann, M., Hein, L., Hannawacker, A., Lohse, M.J. (2004) Novel single chain cAMP sensors for receptor-induced signal propagation. *The Journal of Biological Chemistry* 279, 37215–37218.

Nolan, G.P. (2007) What's wrong with drug screening today. *Nature Chemical Biology* 3, 187–191.

Nordstedt, C., Fredholm, B.B. (1990) A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Analytical Biochemistry* 189, 231–234.

Näsman, J., Kukkonen, J.P., Akerman, K.E.O. (2002) Dual signalling by different octopamine receptors converges on adenylate cyclase in Sf9 cells. *Insect Biochemistry and Molecular Biology* 32, 285–293.

Odagaki, Y., Toyoshima, R. (2006) Dopamine D2 receptor-mediated G protein activation assessed by agonist-stimulated [35S]guanosine 5'-O-(gamma-thiotriphosphate) binding in rat striatal membranes. *Progress in Neuropsychopharmacoly and Biological Psychiatry* 30, 1304–1312.

Oldham, W.M., Hamm, H.E. (2006) Structural basis of function in heterotrimeric G proteins. *Quarterly Reviews of Biophysics* 39, 117–166.

Oldham, W.M., Hamm, H.E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nature Reviews Molecular Cell Biology* 9, 60–71.

Ovchinnikov Yu, A. (1982) Rhodopsin and bacteriorhodopsin: structure-function relationships. *FEBS Letters* 148, 179–191.

O'Reilly, D.R., Miller, L.., Lucknow, V.A. (1994) Baculovirus Expression Vectors: A Laboratory Manual. Oxford University Press, New York.

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., Fox, B.A., Trong, I.L., Teller, D.C., Okada, T., Stenkamp, R.E., Yamamoto, M., Miyano, M. (2000) Crystal Structure of Rhodopsin: A G Protein-Coupled Receptor. *Science* 289, 739–745.

Palermo, D.P., DeGraaf, M.E., Marotti, K.R., Rehberg, E., Post, L.E. (1991) Production of analytical quantities of recombinant proteins in Chinese hamster ovary cells using sodium butyrate to elevate gene expression. *Journal of Biotechnology* 19, 35–47.

Palmer, A.E., Tsien, R.Y. (2006) Measuring calcium signaling using genetically targetable fluorescent indicators. *Nature Protocols* 1, 1057–1065.

Parekh, A.B., Penner, R. (1997) Store depletion and calcium influx. *Physiological Reviews* 77, 901–930.

Paul, N.M., Taylor, M., Kumar, R., Deschamps, J.R., Luedtke, R.R., Newman, A.H. (2008) Structure-activity relationships for a novel series of dopamine D2-like receptor ligands based on N-substituted 3-aryl-8-azabicyclo[3.2.1]octan-3-ol. *Journal of Medicinal Chemistry* 51, 6095–60109.

Pitcher, J. a, Freedman, N.J., Lefkowitz, R.J. (1998) G protein-coupled receptor kinases. *Annual Review of Biochemistry* 67, 653–692.

Pollack, A. (2004). Coactivation of D1 and D2 dopamine receptors: in marriage, a case of his, hers, and theirs. *Science's STKE: signal transduction knowledge environment*, pe50.

Ponsioen, B., Zhao, J., Riedl, J., Zwartkruis, F., van der Krogt, G., Zaccolo, M., Moolenaar, W.H., Bos, J.L., Jalink, K. (2004) Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. *EMBO Reports* 5, 1176–1180.

Prante, O., Dörfler, M., Gmeiner, P. (2010) The Dopamine Receptors, in: Neve, K.A. (Ed.), Dopamine Receptor Subtype-Selective Drugs: D2-Like Receptors. Humana Press, Totowa, NJ, pp. 101–135.

Prasad, K.N., Sinha, P.K. (1976) Effect of sodium butyrate on mammalian cells in culture: a review. *In Vitro* 12, 125–132.

Rashid, A.J., So, C.H., Kong, M.M.C., Furtak, T., El-Ghundi, M., Cheng, R., O'Dowd, B.F., George, S.R. (2007) D1–D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of Gq/11 in the striatum. *Proceedings of the National Academy of Sciences* 104 , 654–659.

Rasmussen, S.G.F., Choi, H.-J., Rosenbaum, D.M., Kobilka, T.S., Thian, F.S., Edwards, P.C., Burghammer, M., Ratnala, V.R.P., Sanishvili, R., Fischetti, R.F., Schertler, G.F.X., Weis, W.I., Kobilka, B.K. (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* 450, 383–387.

Rasmussen, S.G.F., DeVree, B.T., Zou, Y., Kruse, A.C., Chung, K.Y., Kobilka, T.S., Thian, F.S., Chae, P.S., Pardon, E., Calinski, D., Mathiesen, J.M., Shah, S.T.A., Lyons, J.A., Caffrey, M., Gellman, S.H., Steyaert, J., Skiniotis, G., Weis, W.I., Sunahara, R.K., Kobilka, B.K. (2011) Crystal structure of the b 2 adrenergic receptor – Gs protein complex. *Nature* 477, 549–555.

Reinart, R., Gyulai, Z., Berényi, S., Antus, S., Vonk, A., Rinken, A., Sipos, A. (2011) New 2-thioether-substituted apomorphines as potent and selective dopamine D2 receptor agonists. *European Journal of Medicinal Chemistry* 46, 2992–2999.

Reinart-Okugbeni, R., Ausmees, K., Kriis, K., Rinken, A., Kanger, T. (2012) Chemo-enzymatic synthesis and evaluation of 3-azabicyclo[3.2.0]heptane derivatives as dopaminergic ligands. *European Journal of Medicinal Chemistry* 55, 255–261.

Reisine, T., Eglen, R.M. (2010) High-Throughput GPCR Screening Technologies and the Emerging Importance of the Cell Phenotype, in: Gilchrist, A. (Ed.), GPCR Molecular Pharmacology and Drug Targeting. John Wiley & Sons, Inc., New Jersey, pp. 191–208.

Rich, T.C., Fagan, K. a, Tse, T.E., Schaack, J., Cooper, D.M., Karpen, J.W. (2001) A uniform extracellular stimulus triggers distinct cAMP signals in different compartments of a simple cell. *Proceedings of the National Academy of Sciences of the United States of America* 98, 13049–13054.

Riddle, E.L., Fleckenstein, A.E., Hanson, G.R. (2008) Role of Monoamine Transporters in Mediating Psychostimulant Effects, in: Rapaka, R.S., Sadée, W. (Eds.), Drug Addiction. Springer New York, pp. 169–177.

Rinken, A., Ferré, S., Terasmaa, A., Owman, C., Fuxe, K. (1999) Serotonergic agonists behave as partial agonists at the dopamine D2 receptor. *NeuroReport* 10, 493–495.

Rinken, A., Finnman, U., Fuxe, K. (1999) Pharmacological characterization of dopamine-stimulated [35S]-guanosine 5'(gamma-thiotriphosphate) ([35S]GTPgammaS) binding in rat striatal membranes. *Biochemical Pharmacology* 57, 155–162.

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

Robinson, S.W., Caron, M.G. (1997) Selective Inhibition of Adenylyl Cyclase Type V by the Dopamine D3 Receptor. *Molecular Pharmacology* 52, 508–514.

Robison, G.A., Butcher, R.W., Sutherland, E.W. (1967) Adenyl cyclase as an adrenergic receptor. *Annals of the New York Academy of Sciences* 139, 703–723.

Rosenbaum, D.M., Rasmussen, S.G.F., Kobilka, B.K. (2009) The structure and function of G-protein-coupled receptors. *Nature* 459, 356–363.

Rossier, O., Abuin, L., Fanelli, F., Leonardi, A., Cotecchia, S. (1999) Inverse agonism and neutral antagonism at alpha(1a)- and alpha(1b)-adrenergic receptor subtypes. *Molecular Pharmacology* 56, 858–866.

Rubin, R.P. (2007) A Brief History of Great Discoveries in Pharmacology: In Celebration of the Centennial Anniversary of the Founding of the American Society of Pharmacology and Experimental Therapeutics. New York 59, 289 –359.

Russell, J.T. (2011) Imaging calcium signals in vivo: a powerful tool in physiology and pharmacology. *British Journal of Pharmacology* 163, 1605–1625.

Scheindlin, S. (2001) A brief history of pharmacology. Modern Drug Discovery. *From Concept to Development* 4, 87–88.

Schmiedeberg, O., Koppe, R. (1869) Das Muscarin. Das Giftige Alkaloid des Fliegenpilzes (Agaricus Muscarius L.), seine Darstellung, chemischen Eigenschaften, physiologischen Wirkungen, toxicologische Bedeutung und sein Verhältniss zur Pilzvergiftung im allgemeinen. Verlag von F. C. W. Vogel, Leipzig.

Schneider, E.H., Seifert, R. (2010) Sf9 cells: a versatile model system to investigate the pharmacological properties of G protein-coupled receptors. *Pharmacology and Therapeutics* 128, 387–418.

Seeman, P., T, L., Chau-Wong, M., Wong, K. (1976) Antipsychotic drug doses and neuroleptic/dopamine receptors. *Nature* 261, 717–719.

Shafer, R.A., Levant, B. (1998) The D3 dopamine receptor in cellular and organismal function. *Psychopharmacology* 135, 1–16.

Shaw, G., Morse, S., Ararat, M., Graham, F.L. (2002) Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. *The FASEB Journal* 16, 869–871.

Shichi, H., Somers, R.L. (1978) Light-dependent phosphorylation of rhodopsin. Purification and properties of rhodopsin kinase. *Journal of Biological Chemistry* 253, 7040–7046.

Siderovski, D.P., Willard, F.S. (2005) The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. *International Journal of Biological Sciences* 1, 51–66.

Sipos, A., Debreceni, S., Szabó, R., Gyulai, Z., Berényi, S. (2007) Synthesis of 3-Alkyl and Arylapomorphines. *Synthetic Communications* 37, 2549–2558.

Sokoloff, P., Martres, M.P., Giros, B., Bouthenet, M.L., Schwartz, J.C. (1992) The third dopamine receptor (D3) as a novel target for antipsychotics. *Biochemical Pharmacology* 43, 659–666.

Sondergaard, K., Kristensen, J.L., Palner, M., Gillings, N., Knudsen, G.M., Roth, B.L., Begtrup, M. (2005) Synthesis and binding studies of 2-arylapomorphines. *Organic and Biomolecular Chemistry* 3, 4077–4081.

Stadel, J.M., Nambi, P., Shorr, R.G., Sawyer, D.F., Caron, M.G., Lefkowitz, R.J. (1983) Catecholamine-induced desensitization of turkey erythrocyte adenylate cyclase is associated with phosphorylation of the beta-adrenergic receptor. *Proceedings of the National Academy of Sciences* 80, 3173–3177.

Steiner, A.L., Utiger, R., Parker, C. (1969) Radioimmunoassay for the measurement of adenosine 3',5'-cyclic phosphate. *Proceedings of the National Academy of Sciences of The United States of America* 64, 367–373.

Strange, P.G. (2008) Agonist binding, agonist affinity and agonist efficacy at G protein-coupled receptors. *British Journal of Pharmacology* 153, 1353–1363.

Sunahara, R.K., Guan, H.C., O'Dowd, B.F., Seeman, P., Laurier, L.G., Ng, G., George, S.R., Torchia, J., Van Tol, H.H., Niznik, H.B. (1991) Cloning of the gene for a human dopamine D5 receptor with higher affinity for dopamine than D1. *Nature* 350, 614–619.

Surmeier, D.J., Yan, Z., Song, W.J. (1998) Coordinated expression of dopamine receptors in neostriatal medium spiny neurons. *Advances in Pharmacology* 42, 1020–1023.

Sutherland, E., Robison, G., Butcher, R. (1968) Some aspects of the biological role of adenosine 3',5'-monophosphate (cyclic AMP). *Circulation* 37, 279–306.

Takai, Y., Sasaki, T., Matozaki, T. (2001) Small GTP-Binding Proteins. *Physiological Reviews* 81, 153–208.

Tao, Y.-X. (2008) Constitutive activation of G protein-coupled receptors and diseases: insights into mechanisms of activation and therapeutics. *Pharmacology Therapeutics* 120, 129–148.

Thomas, P., Smart, T.G. (2005) HEK293 cell line: A vehicle for the expression of recombinant proteins. *Journal of Pharmacological and Toxicological Methods* 51, 187–200.

Van Tol, H.H., Bunzow, J.R., Guan, H.C., Sunahara, R.K., Seeman, P., Niznik, H.B., Civelli, O. (1991) Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. *Nature* 350, 610–614.

Dal Toso, R., Sommer, B., Ewert, M., Herb, A., Pritchett, D.B., Bach, A., Shivers, B.D., Seeburg, P.H. (1989) The dopamine D2 receptor: two molecular forms generated by alternative splicing. *The European Molecular Biology Organization Journal* 8, 4025–4034.

Tóth, M., Berényi, S., Csutorás, C., Kula, N.S., Zhang, K., Baldessarini, R.J., Neumeyer, J.L. (2006) Synthesis and dopamine receptor binding of sulfur-containing aporphines. *Bioorganic and Medicinal Chemistry* 14, 1918–1923.

Tõntson, L., Babina, A., Võsumaa, T., Kopanchuk, S., Rinken, A. (2012) Characterization of heterotrimeric nucleotide-depleted Gαi-proteins by Bodipy-FL-GTPγS fluorescence anisotropy. *Biochemical and Biophysical Research Communications* 524, 93–98.

Vanhauwe, J.F., Fraeyman, N., Francken, B.J., Luyten, W.H., Leysen, J.E. (1999) Comparison of the ligand binding and signaling properties of human dopamine D(2) and D(3) receptors in Chinese hamster ovary cells. *The Journal of Pharmacology and Experimental Therapeutics* 290, 908–916.

Veiksina, S., Kopanchuk, S., Rinken, A. (2010) Fluorescence anisotropy assay for pharmacological characterization of ligand binding dynamics to melanocortin 4 receptors. *Analytical Biochemistry* 402, 32–39.

Verma, V., Hasbi, A., O'Dowd, B.F., George, S.R. (2010) Dopamine D1-D2 receptor Heteromer-mediated calcium release is desensitized by D1 receptor occupancy with or without signal activation: dual functional regulation by G protein-coupled receptor kinase 2. *The Journal of Biological Chemistry* 285, 35092–35103.

Vetter, I., Lewis, R.J. (2010) Characterization of endogenous calcium responses in neuronal cell lines. *Biochemical Pharmacology* 79, 908–920.

Viero, C., Kraushaar, U., Ruppenthal, S., Kaestner, L., Lipp, P. (2008) A primary culture system for sustained expression of a calcium sensor in preserved adult rat ventricular myocytes. *Cell Calcium* 43, 59–71.

Wagner, H.N., Burns, H.D., Dannals, R.F., Wong, D.F., Langstrom, B., Duelfer, T., Frost, J.J., Ravert, H.T., Links, J.M., Rosenbloom, S.B., Lukas, S.E., Kramer, A.V., Kuhar, M.J. (1983) Imaging dopamine receptors in the human brain by positron tomography. *Science* 221 , 1264–1266.

Wu, S., Liu, B. (2005) Application of Scintillation Proximity Assay in Drug Discovery. *BioDrugs* 19, 383–392.

Wu, W.-L., Burnett, D.A., Spring, R., Greenlee, W.J., Smith, M., Favreau, L., Fawzi, A., Zhang, H., Lachowicz, J.E. (2005) Dopamine D1/D5 Receptor Antagonists with Improved Pharmacokinetics: Design, Synthesis, and Biological Evaluation of Phenol Bioisosteric Analogues of Benzazepine D1/D5 Antagonists. *Journal of Medicinal Chemistry* 48, 680–693.

Xi, Z.-X., Gardner, E.L. (2007) Pharmacological Actions of NGB 2904, a Selective Dopamine D3 Receptor Antagonist, in Animal Models of Drug Addiction. *CNS Drug Reviews* 13, 240–259.

Yano, H. (2012) Deconstructing G Protein-Coupled Receptor Dimer Pharmacology: Case Studies in Dopamine D1 and D2 Receptors. PhD dissertations, Academic Commons of Columbia University.

Zaworski, P.G., Alberts, G.L., Pregenzer, J.F., Im, W.B., Slightom, J.L., Gill, G.S. (1999) Efficient functional coupling of the human D3 dopamine receptor to Go subtype of G proteins in SH-SY5Y cells. *British Journal of Pharmacology* 128, 1181–1188.

Zhang, A., Neumeyer, J.L., Baldessarini, R.J. (2007) Recent progress in development of dopamine receptor subtype-selective agents: potential therapeutics for neurological and psychiatric disorders. *Chemical Reviews* 107, 274–302.

Zhang, A., Zhang, Y., Branfman, A.R., Baldessarini, R.J., Neumeyer, J.L. (2007) Advances in development of dopaminergic aporphinoids. *Journal of Medicinal Chemistry* 50, 171–181.

Zhang, J., Xiong, B., Zhen, X., Zhang, A. (2008) Dopamine D1 receptor ligands: where are we now and where are we going. *Medicinal Chemistry* 29, 272–294.

Zhang, R., Xie, X. (2012) Tools for GPCR drug discovery. *Acta Pharmacologica Sinica* 33, 372–384.

Zhao, S., Maxwell, S., Jimenez-Beristain, A., Vives, J., Kuehner, E., Zhao, J., O'Brien, C., De Felipe, C., Semina, E., Li, M. (2006) Generation of embryonic stem cells and transgenic mice expressing green fluorescence protein in midbrain dopaminergic neurons. *European Journal of Neuroscience* 19, 1133–1140.

8. AKNOWLEDGEMENTS

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Reinart, R., Gyulai, Z., Berényi, S., Antus, S., Vonk, A., Rinken, A. and Sipos, A. (2011) New 2-thioether-substituted apomorphines as potent and selective

dopamine D2 receptor agonists. *European Journal of Medicinal Chemistry* 46, 2992–2999.

Mazina, O., **Reinart-Okugbeni, R**., Kopanchuk, S. and Rinken, A. (2012) BacMam System for FRET-Based cAMP Sensor Expression in Studies of Melanocortin MC1 Receptor Activation. *Journal of Biomolecular Screening,* 17, 1096–1101.

Reinart-Okugbeni, R., Ausmees, K., Kriis, K., Rinken, A. and Kanger, T. (2012) Chemoenzymatic synthesis and evaluation of 3-azabicyclo[3.2.0]-heptane derivatives as dopaminergic ligands. *European Journal of Medicinal Chemistry* 55, 255–261.

Reinart-Okugbeni, R., Vonk, A., Uustare, A., Gyulai, Z., Sipos, A. and Rinken, A. (2012) 1-Substituted Apomorphines as Potent Dopamine Agonists. *Submitted for publication in Bioorganic and Medicinal Chemistry Letters.*

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Uustare, A., **Reinart, R**. and Rinken, A. (2006) Modulation of dopamine D1 receptor signaling by adenosine A1 receptors in Sf9 cells requires expression of Gi proteins. *Neuroscience Letters* 406, 169–173.

Vonk, A., **Reinart, R**. and Rinken, A. (2008) Modulation of adenylyl cyclase activity in rat striatal homogenate by dopaminergic receptors. *Journal of Pharmacological Sciences* 108, 63–70.

Reinart, R., Gyulai, Z., Berényi, S., Antus, S., Vonk, A., Rinken, A. and Sipos, A. (2011) New 2-thioether-substituted apomorphines as potent and selective dopamine D2 receptor agonists. *European Journal of Medicinal Chemistry* 46, 2992–2999.

Mazina, O., **Reinart-Okugbeni, R**., Kopanchuk, S. and Rinken, A. (2012) BacMam System for FRET-Based cAMP Sensor Expression in Studies of Melanocortin MC1 Receptor Activation. *Journal of Biomolecular Screening,* 17, 1096–1101.

Reinart-Okugbeni, R., Ausmees, K., Kriis, K., Rinken, A. and Kanger, T. (2012) Chemoenzymatic synthesis and evaluation of 3-azabicyclo[3.2.0]-heptane derivatives as dopaminergic ligands. *European Journal of Medicinal Chemistry* 55, 255–261.

Reinart-Okugbeni, R., Vonk, A., Uustare, A., Gyulai, Z., Sipos, A. and Rinken, A. (2012) 1-Substituted Apomorphines as Potent Dopamine Agonists. *Submitted for publication in Bioorganic and Medicinal Chemistry Letters.*

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