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Biochemistry of dopamine D_2 receptors and its association with motivated behaviour

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Contribution of the author:

Paper 1: The author adapted the measurement method for D_2 receptor activation in striatal and accumbal tissue, performed biochemical experiments, analyzed the data and contributed to the writing of manuscript.

Paper 2: The author planned and conducted the D_2 receptor function measurement and was responsible for data analysis and participated in writing all the parts of the manuscript.

Paper 3: The author planned and conducted the D_2 receptor function measurement and was responsible for data analysis as well as writing the respective parts of the manuscript.

Paper 4: The author adapted both the ligand affinity and efficacy measurement methods for cell culture, conducted the experiments, analyzed the data and wrote the manuscript.

Paper 5: The author planned and conducted the D_2 receptor function measurement and was responsible for data analysis and participated in writing all the parts of the manuscript.

ABBREVIATIONS

[^{3}H]cAMP	[5',8'-^{3}H] adenosine-3',5'-cyclic monophosphate
[^{3}H]SCH23390	[N-methyl-^{3}H]R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5- tetrahydro-1H-3-benzazepine hydrochloride
[^{35}S]GTPγS	[^{35}S]-guanosine-5′-(γ-thio)-triphosphate
5-HT	5-hydroxytryptophan, serotonin
AC	adenylyl cyclase
ATP	adenosine-5'-triphosphate
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
cAMP	cyclic adenosine-3',5'-monophosphate
CHO	chinese hamster ovary
CNS	central nervous system
CVS	chronic variable stress
D_2^{High}	dopamine D_2 receptor high-affinity binding site
DA	dopamine, 3-hydroxytyramine
DARPP-32	dopamine and cAMP regulated phosphoprotein with molecular weight of 32 kilodaltons
DAT	dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
DTT	dithiothreitol
EB	exploration box
EDTA	ethylenediaminetetraacetic acid
EGTA	etylene glycol-bis-(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid
EPM	elevated plus maze test
FST	forced swimming test
GABA	γ-aminobutyric acid
GDP	guanosine-5'-diphosphate
GPCR	G protein-coupled receptor
GTP	guanosine-5'-triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HC	high chirpers
HE	high exploratory activity
HR	high responder
HRA	high rearing activity
HVA	homovanillic acid
IBMX	3-isobutyl-1-methylxanthine
LC	low chirpers
L-DOPA	L-3,4-dihydroxyphenylalanine
LE	low exploratory activity
LR	low responder
LRA	low rearing acitvity

MAO	monoamine oxidase
mRNA	messenger ribonucleic acid
NA	noradrenaline
NMR	nuclear magnetic resonance
PEP	phosphoenolpyruvate
PK	pyruvate kinase
RGS4	regulator of G protein signaling 4
Ro 20-1724	4-(3-butoxy-4-methoxybenzyl)-imidazolidin-2-one
Tris	tris(hydroxymethyl)aminomethane
USV	ultrasonic vocalization
VMAT-2	vesicular monoamine transporter 2

INTRODUCTION

The very basis of our behaviour lies in the chemical communication between the neurons in our nervous system. All the differences we see in the behaviour of humans as well as in rodents, monkeys, even in amoebae, are based on the differences in this communicational chemistry between the cells. Be it dopamine which can make us feel "high" or serotonin which can both provoke and relieve anxiety or cholecystokinin which signalizes that we have eaten enough – there are a wide variety of chemical languages and, of course, chemical ears in the form of receptors as well, to build up very different individuals. With the methods developed so far, from positron emission tomography to *in vitro* receptor biochemistry, the quantitative and qualitative chemical variability underlying behavioural differences, can be measured and analyzed.

The ultimate goal to study individual differences is to get to know something about *homo sapiens*, about us – especially about what makes us differentially susceptible to various disorders. That knowledge, in turn, may be a crucial key to the individualised pharmacotherapy, which is the new goal for pharmacology and, more generally, in the health systems nowadays.

2. LITERATURE OVERVIEW

2.1. Dopamine in central nervous system and its role in motivated behaviour

Dopamine (DA), 3-hydroxytyramine, derivative of amino acid tyrosine and precursor for the neurotransmitters noradrenaline and adrenaline, was in earlier times considered just a precursor for important neurotransmitters. The investigation of its own role in the brain began in 1950-s with demonstration of the presence of dopamine in brain (Carlsson *et al*. 1958), with especially high levels in striatum (Bertler and Rosengren 1959). With the development of immunohistochemical techniques, rat neurones containing dopamine and neuronal pathways projecting from substantia nigra to striatum and from ventral tegmental area to nucleus accumbens were visualized (Dahlström and Fuxe 1965).

Hereafter, at least seven distinct anatomical pathways for DA have been characterized in the central nervous system (CNS), three most important of them for psychotropic drug effects being nigrostriatal (from substantia nigra to striatum), meso(cortico)limbic (from ventral tegmental area to nucleus accumbens) and tuberoinfundibular (from arcuate nucleus of hypothalamus to median eminence) (Moore and Bloom 1978, Skagerberg and Lindvall 1985). In Parkinson's disease, death of dopamine-producing projection neurons in the substantia nigra results in loss of dopaminergic activity in nigrostriatal pathway (Bernheimer *et al.* 1973) The mesocorticolimbic pathway has been implicated in schizophrenia, particularly in psychosis (Bogerts 1999), as well as in drug addiction disorders (Nestler 2005).

DA controls a variety of functions both in CNS, including locomotor activity, cognition, motivation, emotion, endocrine regulation, as well as in peripheral nervous system (PNS), for example cardiovascular function, hormone release, renal function and gastrointestinal motility (Missale *et al.* 1998).

One of the most known functions of dopamine in CNS is of course its association with reward-related stimuli processing (Ungless 2004, Marsden 2006). DA release in nucleus accumbens is the most common correlate of actions of drugs of abuse, natural rewards or related conditioned cues (Di Chiara and Imperato 1988, Heffner *et al.* 1980, Becker *et al.* 2001) whereas DA in dorsal striatum is more important in mediating motor response selection and habit formation (Grillner *et al.* 2005, Faure *et al.* 2005).

2.1.1. Dopamine in mediation of reward-related behaviour and motivation

In 1954 experiments by Olds and Milner revealed that there are specific "centres" in the brain which are rewarding in the sense that rats were operantly responding to electrical stimulation of these areas, even to exclusion to all the other activity (Olds and Milner 1954).

That dopamine has something to do with mediating reward and drug reinforcement was proposed in late 1970s (Fibiger 1978, Wise 1978).

Very strong associations between mesocorticolimbic DA activity and natural rewards as food, sex, play *etc* have been demonstrated (Pfaus *et al.* 1995, Richardson and Gratton 1996) leading these investigators to propose that this system is the one responsible for generating evolutionally important, appropriate responses to natural rewards – and which is manipulated by drugs of abuse in the way which leads to addiction (Kelley and Berridge 2002). This "appropriate response" was once thought to be simple pleasure from rewarding stimuli, shaping a positive reinforcement theory (Wise 1985). Historically, the negative reinforcement theory actually firstly proposed seeking alleviation from the aversive state (withdrawal) as the main reason for drug dependence, but this theory failed to explain many aspects of reward-related behaviour, for example why animals readily self-administer different drugs in the absence of withdrawal symptoms and why there is a high tendency to relapse to drug-seeking behaviour long after withdrawal symptoms have subsided. On the other hand, the positive reinforcement theory was not able to explain why people still feel the desire for drugs in the conditions when their usual life is ruined because of these drugs that by themselves even do not produce the pleasant affective states anymore (Robinson and Berridge 1993). Both negative and positive reinforcement theories were taken into consideration in the incentive salience hypothesis, which deals with motivational *wanting* rather than *liking*, the last associated with pleasure from consumption of rewards, the former describing a motivational magnet quality of a stimulus that makes it a desirable and attractive goal and transforms it from being a mere sensory experience into something that commands attention, induces approach, and causes it to be sought out (Berridge and Robinson 1998). Now, one might propose that maybe potential rewards themselves have little to do with the DA-ergic activity in the mesolimbic system, but rather stimuli which *predict* them, therefore starting the "wanting" motivational machinery. Indeed, the reward prediction error hypothesis was presented by Schultz (Schultz *et al.* 1992, Schultz *et al.* 1997, Schultz 1998), relying on the fact that DA neurons fire to cues that predict rewards but not to already predicted hedonic rewards themselves. Whilst failing to discriminate between different rewards, dopamine neurons appear to emit an alerting message about the surprising presence or absence of rewards.

On the other hand, learning-based reward theories assume a malfunctioning stimulus-response associative learning with its sensitized cellular mechanism that leads to compulsive drug use and addiction (Di Chiara 1998, Berke and

Hyman 2000). Regarding reward prediction error hypothesis, an alternative explanation presumes that DA signal, either positive or negative, occuring after presentation of unexpected reward or omission of expected reward, respectively, facilitates the mobilization of behavioural and cognitive processing capacity toward any unexpected event of behavioural significance. This so-called behavioural-switching hypothesis (Redgrave *et al.* 1999) declares that DA has a more general role in associative learning. In nucleus accumbens, DA mediates the acquisition of appetitive response to motivationally important stimulus (Montague *et al.* 2004), while in dorsal striatum it participates in behavioural habit formation (Yin *et al.* 2008). Thus, the repetitive exposure of given stimulus, e. g., drug or drug cue, the transition is proposed to exist from reinforcement to habit formation, this means, from elevated DA activity in ventral striatum to dorsal striatum, respectively (Porrino *et al.* 2004).

Taken together, years of research have shown that mesocorticolimbic DA system should not be thought of as direct "natural reward system" hijacked by different drugs, but instead as a modulator of several functions related to motivated behaviour including behavioural activation, effort-related decision making, responsiveness to conditioned stimuli, learning, cognition – functions which are altered in DA-related diseases like Parkinson's disease, schizophrenia and depression (Salamone *et al.* 2005).

2.1.2. Dopamine and novelty related behaviour

In the fields of psychology, ethology and behavioural neuroscience, there are numerous models and hypotheses describing behavioural system which underlies appetitive motivation: approach system (Schneirla 1959), behavioural activation system (Gray 1987), search system (MacLean 1986), seeking system (Panksepp 1998), behavioural facilitation system (Depue and Collins 1999) *etc*. Activity in this motivational circuit promotes the feeling of excitation, positive engagement and desire for exploration, therefore determining the individuals' response to novelty, whereas neophobia serves as a controlling/inhibiting factor limiting the exploratory behaviour (Harro 2010, Wahlstrom *et al.* 2010). Thus, individual differences in responses to novelty indicate the underlying motivational state guiding one individual towards reward-seeking and the other towards risk-avoiding behaviour.

DA neurotransmission in limbic regions, including nucleus accumbens is largely implicated in mediating novelty-directed behaviour, as lesions in these brain areas halt exploration and approach behaviours (Koob *et al.* 1978), administration of DA-ergic agonists into these regions initiates novelty-seeking and goal-directed locomotor behaviour and DA-ergic antagonists do the opposite(Le Moal and Simon 1991, Wahlstrom *et al.* 2010).

From what is known so far about DA in striatum, it has been hypothesized that striatal DA neurons respond to primary rewards outside of the learning context, code reward prediction in response to cues that signal reward delivery

and provide an alerting message when reward is expected but not presented. As soon as the stimuli are no more new and the cues reliably predict the presence of rewards, striatal DA neurons fail to respond to these stimuli any longer, signaling therefore that reinforcement learning is complete. On a broader behavioural level, it means that DA potentiates exploratory processes in response to novel stimuli. (Samejima *et al*. 2005, Palmiter 2008)

Whereas novelty itself may act as a motivator, it has been proposed that it could also boost reward-directed behaviour and the possible DA-ergic activation to different stimuli, especially in hippocampus and striatum (Kakade and Dayan 2002, Wittmann *et al.* 2007, Wittmann *et al.* 2008, Guitart-Masip *et al.* 2010). As there is a strong association between exploratory behaviour, evoked by novel situation and objects, and reward-related behaviour, animal models based on individual differences in exploratory activity have been extensively used in the research of drug addiction (Piazza *et al.* 1989).

On the other hand, as the novelty always contains possible dangers and evokes neophobia, individual differences in novelty-related behaviours are a good starting point to study neurochemical and psychological mechanisms behind anxiety (Thiel *et al.* 1999, Landgraf *et al.* 2007, Mällo *et al.* 2007, Pawlak *et al.* 2008). Although it is the (altered) serotonin (5-hydroxytryptophan, 5-HT) neurotransmission which is thought to play a major role in anxiety, anxiety-related affective disorders and depression both in humans (Morilak and Frazer 2004, Lowry *et al.* 2008) as well as in corresponding animal models of these disorders (Griebel 1995) – the most famous player in the field being 5-HT transporter with its promotor region polymorphism, discovered by Klaus Peter Lesch (Lesch *et al.* 1996) –, DA has its own role in mediating passive reactions in anxiety-provoking situations. Whether it is due to the unique properties of midbrain DA neurons to be activated by both positive and negative reward-predicting stimuli (Matsumoto and Hikosaka 2009) or just dysfunction of midbrain DA system, leading to anhedonia and amotivation – amotivation to explore as well – (Martin-Soelch 2009), this remains an open question. Taken together, both 5-HT and DA appear as important mediators regarding anxiety disorders, hypothesis which is also supported by the latest *in vivo* imaging studies, showing e. g., the reduction of mesencephalic 5-HT transporter, mesencephalic and cingulate 5-HT_{1A} receptor and striatal DA D_2 receptor levels in patients with a variety of anxiety disorders (Nikolaus *et al.* 2010).

Among paradigms that measure individual differences in novelty-related behaviour, the low and high responding rats (LR/HR, respectively) is probably the most known model, originally proposed to be used in studies on vulnerability to drug addiction (Piazza *et al.* 1989, Blanchard *et al.* 2009). Depending of what kind of novel environment (round alley, open field *etc*) is used to assess the reactivity, as well as other methodological aspects, behavioural and neurochemical measures may differ quite dramatically. Still, the LR animals are consistently more anxious in the elevated plus maze (EPM) test and exhibit more passive coping style in the forced swimming (FS) test (Kabbaj *et al.* 2000). Regarding their DA system, HR animals have higher *ex vivo* DA content

in nucleus accumbens and dorsal striatum (Antoniou *et al.* 2008), higher *in vivo* basal and evoked DA release in the nucleus accumbens (Hooks *et al.* 1992) and increased firing rate of DA ergic neurons in ventral tegmental area and substantia nigra (Marinelli and White 2000). This means that HE animals tend to have more active DA-ergic system which might be behind their higher locomotor response to novelty. However, HR rats have lower DA D_2 receptor expression in nucleus accumbens and striatum (Hooks *et al.* 1994, Dietz *et al.* 2008). Recently, HR and LR animals have been also selectively bred (Stead *et al.* 2006).

There are also other models considering inter-individual differences in novelty-related behaviour like low and high rearing activity (LRA/HRA) rats with higher extracellular DA levels in the nucleus accumbens of HRA animals (Thiel *et al*. 1999, Pawlak *et al.* 2008).

In the current study, exploration box, originally developed for assessing changes in exploratory behaviour after lesioning noradrenergic projections from the locus coeruleus (Harro *et al.* 1995, Otter *et al.* 1997), is used to evaluate individual differences in exploratory behaviour. In this paradigm, the animals have the opportunity of both to hide (in small dark home cage-like chamber) and to explore (in open field box-like arena with one known and three unknown objects). For separating animals with high vs low innate exploratory activity, a rat is observed for 15 minutes in two consecutive days, whereas the results from second day predict sufficiently well the activity during further tests (Mällo *et al.* 2007). The test gives almost bimodal distribution regarding the exploratory measures, with a) animals exhibiting low motivation to explore and high neophobia – both the core symptoms of depression – in one group and b) animals with high motivation to explore and low neophobia in another group (low/high exploring, LE/HE, respectively) (Alttoa *et al*., 2005, Mällo *et al.* 2007).

In behavioural tests, LE rats are more anxious in EPM, have more passive coping style in FS and acquire a more persistent association between neutral and stressful stimulus, while in the social interaction test they are equally active (Mällo *et al.* 2007). Neurochemically, LE rats have higher 5-HT transporter levels in the prefrontal cortex and higher citalopram-evoked 5-HT release in prefrontal cortex, as well as higher BDNF mRNA levels in the prefrontal cortex (Mällo *et al.* 2008). On the other hand, HE rats have higher basal and amphetamine-evoked DA release in striatum (Mällo *et al.* 2007) as well as higher proportion of DA D_2 receptor high-affinity binding sites D_2^{High} (Alttoa *et al.* 2009). Furthermore, the groups also have differences in their cerebral oxidative metabolism measured by cytochrome c oxidase histochemistry (Matrov *et al.* 2007).

Regarding the two major monoamine neurotransmitter systems, 5-HT and DA, it seems to be so that LE rats have more active/reactive 5-HT system, whereas HE rats might have more sensitive DA ergic neurotransmission. Recently, it has been shown that dopaminergic (super)sensitivity is accompanied by the modifications in the affinity states of the striatal DA D_2 receptors (Seeman *et al.* 2005, Seeman *et al.* 2006). This makes the D_2 receptors in stria-

tal area a very appetitive target for investigation in the LE/HE model but also in other models relevant to DA-ergic sensitivity, e.g., motivation, reward sensitivity *etc*.

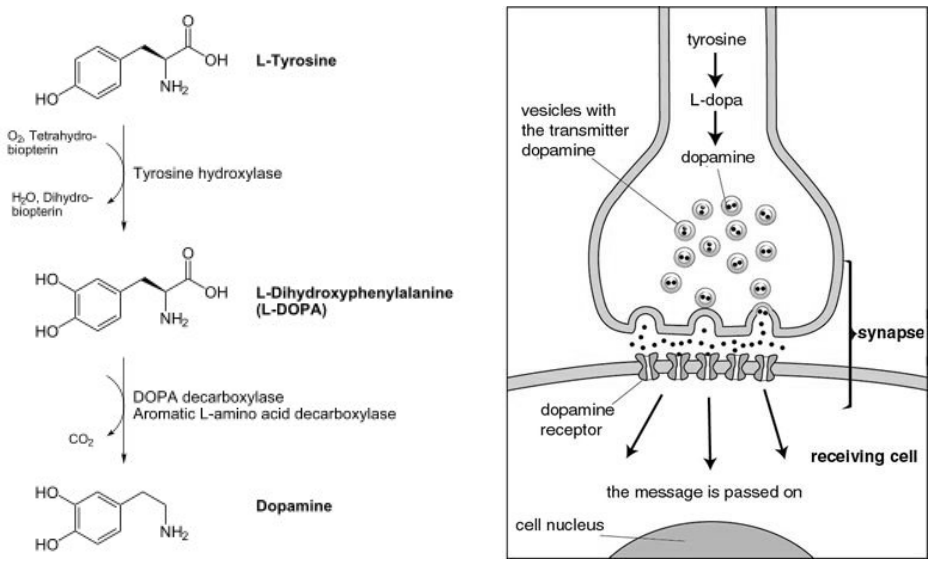
2.2. Dopaminergic signal transmission

Dopamine is one of the catecholamine neurotransmitters in the brain. It has features similar to many other small molecule neurotransmitters, especially other biogenic amines like noradrenaline (NA) and serotonin (5-HT): it is synthesized in nerve terminals, stored in small synaptic vesicles, released calcium-dependently into synaptic cleft and taken up and/or degraded by a specific transporter (dopamine transporter, DAT) or enzymes, respectively (Missale *et al*. 1998, Emilien *et al*. 1999, Le Foll 2010).

The first step of dopamine (as well as noradrenaline and adrenaline) biosynthesis (see Scheme 2.1) is the hydroxylation of the common precursor of the three abovementioned catecholamines, L-tyrosine, to L-3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (Nagatsu *et al*. 1964). L-DOPA has a very widespread use as an anti-Parkinsonian drug, because of its abilities to cross the blood-brain barrier, the property that DA does not possess (Abbott 2010). Tyrosine hydroxylase immunoreactivity is often used to characterize DA-ergic neurones/terminals in brain (Masserano and Weiner 1983).

The formation of DA from L-DOPA occurs by decarboxylation of the latter by the enzyme L-amino acid decarboxylase (Blaschko 1939). The transport of synthesized DA to synaptic vesicles occurs through monoamine vesicular transporters, on which act the DA-releasing drugs, e.g., reserpine and amphetamine (Hoffman *et al*. 1998, Zheng *et al*. 2006).

Upon the arrival of action potential to the nerve terminal and depolarization-dependent influx of Ca^{2+} ions into presynaptic area, the synaptic vesicles release DA into synaptic cleft via exocytosis where it diffuses towards the postsynaptic membranes and exerts its effects by acting on dopamine receptors. It should be mentioned that some of the DA receptors, especially D_2 receptors, also serve as presynaptic autoreceptors rather with negative feedback properties (Le Foll 2010). After completion of signal transmission, DA is taken up by specific transporter, DAT, which can be blocked by the well-known psychostimulants cocaine and amphetamine (Riddle *et al*. 2005). All the DA receptors are 7-transmembrane spanning G protein-coupled receptors (GPCR) and together with other components in DA signal transmission, including DAT, monoamine oxidase (MAO) *etc*, they represent a massive drug target system for substances against schizophrenia, depression, Parkinson's disease, attention deficit hyperactivity syndrome, Tourette's syndrome, migraine, drug dependence *etc* (Emilien *et al*. 1999, Volkow *et al.* 2009, Le Foll 2010).

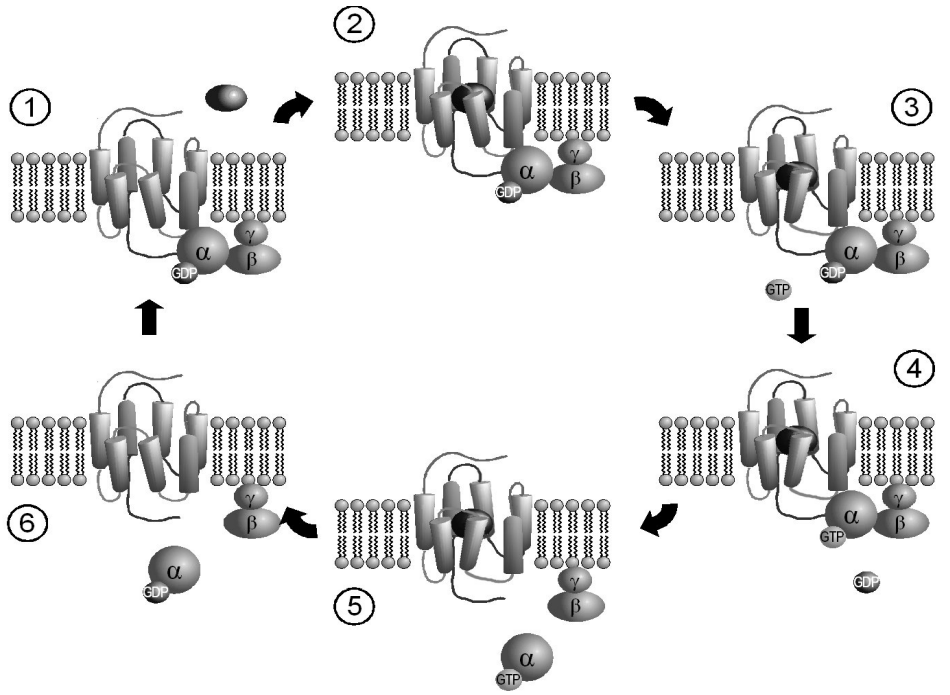


Scheme 2.1 Dopamine biosynthesis (left panel) and principles of signal transmission (right panel) Modified from http://www.unifr.ch/biochem/index.php?id=136.

2.2.1. Dopamine receptors

As all DA receptors are GPCRs, they share a common signal transmission mechanism (see Scheme 2.2) that begins with the agonist binding to the active site of the receptor inducing a conformational change in the receptor. This in turn alters the conformation within the heterotrimeric G protein, through which most of the functions of GPCRs are transmitted into the cellular environment. Conformational changes in G proteins cause a decrease in the affinity of guanosine-5'-diphosphate (GDP), bound to the α-subunit of heterotrimeric G protein in the resting state. GDP dissociates from the activated G protein and is replaced by the guanosine-5'-triphosphate (GTP). The α- and βγ- subunits then dissociate from each other and activate intracellular effectors, reassociating after the hydrolysis of GTP to GDP by GTPase part of G protein α-subunit, the reaction which, because of the high group transfer potential of GTP, gives the energy supply for activating intracellular effectors by G_α. (Bourne 1997, Cabrera-Vera *et al*. 2003, Brink *et al*. 2004, Kristiansen 2004, Milligan 2007)

G proteins are divided into four families based on the properties of G_α subunits: G_s proteins primarily activate adenylyl cyclase (AC) and therefore cause the elevation in intracellular second messenger cyclic adenosine-3',5'-monophosphate (cAMP) concentration, $G_{i/o}$ primarily inhibit AC, decreasing cAMP concentration, G_q proteins primarily activate phopholipase C pathways and $G_{12/13}$ regulate the small GTP binding proteins (Weng *et al*. 1998, Cabrera-Vera *et al*. 2003).



Scheme 2.2 Principle of the signal transmission cycle by GPCRs. Modified from http://xray.bmc.uu.se/lars/Practicals/Signal/trans_app.html.

In the last years, there have been numerous papers published about GPCR signal transmission mechanisms with new concepts and signaling counterparts described, including the growing research on GPCR homo- and heterooligomerization, multiple signaling states for GPCRs, allosteric regulation of GPCRs, GPCR interactions with ion (especially Ca^{2+}) channels, G-protein independent functions for 7-transmembrane receptors *etc* (for review, see Brzostowski and Kimmel 2001, Agnati *et al*. 2005, Perez and Karnik 2005, Waard *et al*. 2005, Eglen *et al*. 2007, Gilchrist 2007, Conn *et al*. 2009, Milligan 2009), but as these themes are more or less out of the scope of this thesis, they will not be discussed further here.

The first evidence for the existence of DA receptors in the CNS came in 1972 from biochemical studies showing that DA was able to stimulate adenylyl cyclase (for review, see Kebabian and Calne 1979). Since then, five fully functional and genotypically unique metabotropic dopamine receptors have been cloned from mammalian species including humans (Le Crom *et al*. 2003). They are divided into two families: D1- (including D_1 and D_5 receptors) and D2-family (including D_2, D_3 and D_4 receptors), based upon similarities in sequence, pharmacology and ability to stimulate or inhibit AC activity mediated via coupling to $G_{\alpha s}$ or $G_{\alpha i/o}$ proteins (Missale *et al.* 1998, Le Foll 2010, Strange 2010). The D1-family receptor genes do not contain introns in their coding regions whereas D2-family receptor genes are interrupted by introns, arising the

possibility of alternative splicing which result, in the case of D_2 receptor, in two isoforms, D_{2L} and D_{2S}, with somewhat different subcellular localization and pharmacology (Guiramand *et al*. 1995, Emilien *et al*. 1999, Usiello *et al*. 2000, Takeuchi and Fukunaga 2003).

D1-family of DA receptors. This family of DA receptors, consisting of D_1 and D_5 receptors, primarily couples to G_s type of G proteins, activating therefore the cAMP producing effector AC (Missale *et al*. 1998, Le Foll 2010, Strange 2010). The D_1 and D_5 receptors share 80% identity in their transmembrane domains, being also pharmacologically very similar so that currently there is no ligand that can discriminate between them (Missale *et al*. 1999, Strange 2010). Still, the D_1 receptors are far more abundant in the (rat) brain than D_5 receptors; the presence of D_1 receptor mRNA and protein have been demonstrated in dorsal and ventral striatum, globus pallidus, olfactory bulb, amygdala, hypothalamus, thalamus and frontal cortex of rat brain (Fremeau *et al*. 1991, Levey *et al*. 1993). In addition to coupling to AC and therefore cAMP accumulation, it has been shown that in rat Ltk^- cells D_1 receptor is able to activate the phospholipase C pathway (Yu *et al*. 1996). Numerous pharmacological and knock-out studies have been shown the involvement of D_1 receptor signaling in locomotor activity, learning, drug reinforcement and addiction; it is possible that D_1 ligands could have some utility in the treatment of Parkinson's disease (Emilien *et al*. 1999, Dalley and Everitt 2009, Le Foll 2010).

D2-family of DA receptors. D_2, D_3 and D_4 receptors, belonging to this family, preferentially couple to G_i type of G proteins, inhibiting the activity of AC and therefore the accumulation of cAMP. Among all the DA receptors, the first one cloned was the D_2 receptor (Bunzow *et al*. 1988). This receptor is also the most targeted protein in pharmacotherapy concerning DA-ergic system, the examples being antipsychotic and anti-Parkinsonian drugs (Le Foll 2010, Strange 2010). As the D_2 receptor is also in the center of the current study, receptor localization, signal transmission and its association with different conditions are discussed in more detail in the next chapter.

The other members of D2-family, D_3 and D_4 receptors are located predominantly in the limbic regions of the brain with the D_3 receptor being expressed in some brain areas associated with motor functions, e.g., dorsal striatum (putamen) as well (Landwehrmeyer *et al*. 1993, Rivera *et al*. 2003). In addition to the classical cAMP pathway, D_3 and D_4 receptors have been shown to couple to G protein-regulated potassium channels (Werner *et al*. 1996) and D_4 receptors to inhibit an L-type calcium current (Mei *et al*. 1995). These receptors have mainly been associated with reward and emotional learning processes (Le Foll *et al*. 2005, Laviolette *et al*. 2005).

The members of D2 family are pharmacologically very similar, too, and although partially selective agonists are available for some of the subtypes and receptor knock-out animals serve as a behavioural and physiological tool to study the importance of different DA receptors (Waddington *et al*. 2005), subtype-selective agonists and antagonists are highly desired in the field of drug

discovery and receptor biochemistry, to get a more precise knowledge about how each of the DA receptor subtypes (dys)functions in organism.

2.2.2. Dopamine D_2 receptors

The D_2 receptor is the predominant D2-family receptor subtype in the brain, located, similarly to the D_1 receptor, at high levels in typical DA rich brain areas including ventral and dorsal striatum, amygdala, hippocampus and hypothalamus. However, in contrast to D_1 receptor mRNA, D_2 receptor mRNA is less abundant in cortical areas, but highly expressed in the DA cell bodies in substantia nigra and ventral tegmental area (Meador-Woodruff *et al*. 1989, Landwehrmeyer *et al*. 1993, Levey *et al*. 1993). In addition, colocalization of these two major DA-ergic receptors is not common, at least in the striatal region, as it has been estimated that only some 15–20% of striatal neurons may contain both receptors (Deng *et al*. 2006).

The D_2 receptor exists as two alternatively spliced isoforms differing in the insertion of a stretch of 29 amino acids in the third intracellular loop, resulting in the short and long isoforms of D_2 receptor, D_{2S} and D_{2L}, respectively (Monsma *et al*. 1989). The splice variants of the D_2 receptor are differently distributed with D_{2S} predominating in the cell bodies and and projection axons of the DA ergic cell groups in midbrain and hypothalamus and D_{2L} being more strongly expressed by neurons in the striatum and nucleus accumbens. Among these splice variants, the D_{2S} receptor is the likely DA autoreceptor controlling DA release, whereas the D_{2L} isoform is primarily a postsynaptic receptor expressed in striatal areas in medium-sized GABA (γ-aminobutyric acid) ergic and large cholinergic neurons (Khan *et al*. 1998). D_2 receptor mRNAs prominently segregate in the enkephalin-containing neurons (Le Moine *et al*. 1990, Curran and Watson 1995).

D_2 receptors couple mainly to different members of G_i family of G proteins, whereas the coupling efficiency depends on D_2 receptor isoform, G protein expression level, brain area, cell system and agonist nature (Sidhu and Niznik 2000, Cordeaux *et al*. 2001, Rinken *et al*. 2001, Gazi *et al*. 2003, Nickolls and Strange 2004). In addition, differences between signal transmission mechanisms of pre- and postsynaptic D_2 receptors have to be taken into account (De Mei *et al*. 2009).

The best characterized intracellular effect of activation of D_2 receptors is the inhibition of AC and, therefore, cAMP synthesis. But the influence of D_2 receptors on voltage-dependent potassium currents, protein kinase C and Ca^{2+} release and arachidonic acid release has also been shown (Liu *et al*. 1992, Castellano *et al*. 1993, Schinelli *et al*. 1994).

On the behavioural level, there is a myriad of evidence that D_2 receptor is involved in the processes like locomotor activity, motivation, cognition, reward, addiction, depression, (emotional) memory *etc*. Some of the results from knockout and pharmacological studies are shortly summarized below.

Studies with knock-out mice have revealed that D_2 receptor knock-out animals display Parkinsonian-like locomotor impairment (Baik *et al*. 1995), they are insensitive to the hypolocomotor and hypothermic effects of D_2/D_3 agonists (Boulay *et al*. 1999) and to the cataleptic effects of haloperidol (a D_2 antagonist) (Boulay *et al*. 2000), they exhibit reduced ethanol-conditioned place preference (Cunningham *et al*. 2000) and reduced locomotor activity and slower acquisition of a place-learning task (Tran *et al*. 2002). They have abnormal synaptic plasticity in the striatum (Calabresi *et al*. 1997) and they do not exhibit autoinhibition of dopamine release (Benoit-Marand *et al*. 2001, Rouge-Pont *et al*. 2002), the DAT activity in these animals is decreased (Dickinson *et al*. 1999) and they have altered GABAergic neurotransmission (An *et al*. 2004).

Studies from behavioural/pharmacological studies show that the level of $D_{2/3}$ receptors is reduced in the striatum of human cocaine, heroin, alcohol and methamphetamine addicts (Volkow *et al*. 1997) and monkeys chronically exposed to cocaine self-administration (Nader *et al*. 2002, Nader *et al*. 2006) but on the other hand, cocaine self-administration produces a persistent increase of D_2 high affinity binding sites D_2^{High} in rat striatum (Briand *et al*. 2008) or, interpreted alternatively, affects the cooperation between D_2 receptor dimers (Franco *et al*. 2010). The impulsivity in 5-choice serial reaction time task inversely correlates with the $D_{2/3}$ receptor availability in nucleus accumbens and predicts the high rate of cocaine self-administration in rats (Dalley *et al*. 2007) whereas overexpression of D_2 receptors in nucleus accumbens of rats reduces alcohol self-administration (Thanos *et al*. 2001). In accordance with these results, the high responding rats to novelty (HR, see Chapter 2.1.2) that are supposed to be more impulsive, have lower D_2 receptor expression in striatum and nucleus accumbens (Hooks *et al*. 1994, Dietz *et al*. 2008).

Intra-accumbal administration of high dose of the D_2 receptor antagonist sulpiride elevates the accumbal DA level and intake of natural reward sucrose, similarly to the cocaine, probably acting through D_2 presynaptic autoreceptors (Hajnal and Norgren 2001).

Striatal DA denervation with unilateral 6-hydroxydopamine, one of the animal models of Parkinson's disease, increases the D_2 receptor binding sites and decreases the GDP binding affinity in rat striatum, leading to the D_2 receptor supersensitivity (Terasmaa *et al*. 2000a).

In the inescapable stress-induced learned helplessness, a widely used depression model in animal, D_2 receptor antagonist sulpiride enhanced the failure behaviour, suggesting an adaptive and/or protective role for D_2 receptor in the inescapable stress (Wang *et al*. 2007). Socially isolated Flinders Sensitive Line rats, a genetic animal depression model, demonstrate significantly lower D_2 receptor mRNA levels in all striatal areas, including nucleus accumbens, compared to the control Sprague-Dawley rats (Bjørnebekk *et al*. 2007).

The association of D_2 receptor function with psychosis and the action of antipsychotics (which are all, at least partially, blocking D_2 receptor signal transmission), have been in the center of research for already more than 30 years now beginning with Philip Seeman's work who called the receptor

"neuroleptic/dopamine receptor", as it could be labelled by both dopamine and the antipsychotic (neuroleptic) haloperidol (Seeman *et al*. 1976). Since then, it has been shown that most individuals with schizophrenia are supersensitive to dopamine. Animal models of psychosis show that a variety of risk factors, genetic and nongenetic, are associated with behavioral supersensitivity to dopamine, reflected in elevated levels of dopamine D_2^{High} receptors (Seeman 2010) or, based on the analysis by Franco *et al*. (2010), in affected cooperation between D_2 receptor dimers.

2.2.3. Characterization of signal transduction through dopamine D_2 receptors

The methods for characterizing GPCRs could roughly be divided into structural and dynamical. The structural methods like X-ray christallography and nuclear magnetic resonance (NMR) describe the construction of proteins; methods employing fluorescence and radioactivity rather measure the behaviour of receptors in the system: their ligand binding properties, interactions with each other and with intracellular signaling counterparts *etc*. However, this kind of measurement methods-based distinction does not hold anymore, as both christallography and NMR methods are used for characterization of ligand binding properties and bio-NMR even for measuring intracellular processes whereas fluorescent methods serve as an excellent tool in structural biology.

The dynamical methods could also be divided into two with the first describing processes accompanying the ligand binding to receptors: ligand binding kinetics, affinity, competition with other ligands, receptor cooperativity and oligomerization *etc*. The second part describes what is happening inside the cell once the receptor has been activated/deactivated: nucleotide exchange on G proteins, further signal transmission steps like cAMP accumulation, activation of different ion channels, expression of immediate early genes (c-fos, c-jun). Considering the first part, measuring only the ligand binding to the receptor does not give any information about activation or deactivation of intracellular pathways that lead to the physiological response. However, this kind of analysis is important to get the first information about the ligand affinity and kinetics. On the other hand, studying very faraway steps in signal transmission cascade, e. g., expression of c-fos, one should consider the indirect interaction between many receptors and signaling pathways which may complicate the interpretation of the results.

The receptor-dependent activation of G proteins and activation-induced exchange of GDP to GTP on the G protein α-subunit is one of the earliest activated receptor-mediated events. This means that it can be used to provide traditional pharmacological parameters: potency, efficacy, affinity, without the influence of amplification or other modulation that may occur when analyzing parameters further downstream of the receptor (Harrison and Traynor 2003).

The classical *in vitro* [^{35}S]GTPγS binding assay measures the level of activated G proteins following agonist binding to GPCR. As in this assay format the hydrolysis-resistant [^{35}S]GTPγS is used, the G protein is prevented from reforming as a heterotrimer and the amount of activated G proteins can be measured as the amount of [^{35}S]-label incorporated (Harrison and Traynor 2003, Milligan 2003). Physiologically more relevant is the [^{35}S]GTPγS binding auto-radiography, where [^{35}S]GTPγS binding is measured in tissue sections where receptor-G protein complexes are supposedly functional. Moreover, this method gives additional information about the anatomical resolution of G protein activation and thus, the functional receptor distribution (Sim *et al*. 1995, Sóvágó *et al*. 2001).

The big disadvantage of the [^{35}S]GTPγS assay in natural tissues is that, in the presence of many receptors and all the families of G-proteins, only these receptors which couple with G_i-family of G proteins are measurable because of the high background of [^{35}S]GTPγS binding to G_i proteins (Milligan 2003). To overcome that problem and eliminate the G_i proteins, it is possible to use pertussis toxin which selectively disrupts the G_i-GPCR interaction by ADP-ribosylation of G_i proteins, or use the immunological methods like immuno-precipitation or scintillation proximity assay which are developed for [^{35}S]GTPγS binding assay as well (Gurdal *et al*. 1997, Chakrabarti *et al*. 2005, DeLapp *et al*. 2004, la Cour *et al*. 2007). However, usually these methods bring additional steps into the analysis (pertussis toxin treatment, many washing and centrifugation steps in immunoprecipitation) or they are too capricious to use in the natural tissue membranes.

However, when G_i-bound GPCRs are present at high expression levels in the tissue under investigation, the [^{35}S]GTPγS binding assay is robust and re-producible and gives much more information about signal transduction *process* than a simple determination of receptor and/or G protein levels (either the levels of mRNA or protein). D_2-receptor dependent [^{35}S]GTPγS binding assay has succesfully been used by our group as well as by others, in both striatal as well as CHO cell membranes (Rinken *et al*. 1999, Terasmaa *et al*. 2000b, Roberts *et al*. 2004, Odagaki and Toyoshima 2006, Lin *et al*. 2006, Rudissaar *et al*. 2008).

2.3. Modulators of dopaminergic signal transmission

Changes in DA-ergic signal transmission in the dorsal and ventral striatum have consistently been shown in association with reward and motivational processes. However, DA release, its effect on DA receptors and the intracellular signaling cascade following DA-ergic activation, are modulating and modulated by many other neurotransmitters, peptides *etc*, present in striatum and related brain areas.

Striatal GABAergic output neurons (95% of striatal neurons) can be divided into two different subtypes: the striato-pallidal GABAergic neurons which contain the peptide enkephalin and express the dopamine D2-family receptors

and the striato-nigral neurons containing the peptides dynorphin and substance P and expressing D1-family receptors. The remaining 5% of striatal neurons are striatal interneurons, either choline- or GABAergic (Alexander and Crutcher 1990, Kawaguchi 1997).

Reciprocal modulations of dopamine and glutamate (an amino acid that is a universal excitatory neurotransmitter) play a major integrative role in the striatum in the control of motor activity, emotional and motivational processes as well as in learning mechanisms. For example, several glutamatergic agonists have been shown to potentiate DA release, preferentially through ionotropic glutamate receptors (Krebs *et al*. 1991, Carrozza *et al*. 1992). Additionally, DA signaling through D_2 receptor act as modulator of glutamate-dependent long term depression (a form of synaptic plasticity, cellular learning), increasing the likelihood that it will occur (Pawlak and Kerr 2008). For more comprehensive review of DA-glutamate interactions, see, for example (David *et al*. 2005). The interaction between D_2 and one of the metabotropic glutamate receptors, mGlu5, has also been shown on the receptor biochemistry level (Cabello *et al*. 2009).

In pharmacotherapy, especially from the perspective of treatment possibilities in Parkinson's disease, the antagonistic interactions between dopamine and adenosine systems, demonstrated both in behavioural and receptor level, are very important (Hillion *et al*. 2002, Tronci *et al*. 2006, for review, see Ferre *et al*. 2008).

Other systems, including 5-HT (Zhou *et al*. 2005, Di Matteo *et al*. 2008), cholecyctokinin (Rotzinger *et al*. 2002, Alttoa and Harro 2004), opioidergic (Herz 1998, Häggkvist *et al*. 2010), cannabinoidergic (Kearn *et al*. 2005, López-Moreno *et al*. 2008) *etc* have been shown to interact with the DA-ergic signal transmission

2.3.1. The vigilance-promoting noradrenergic system and dopaminergic neurotransmission

One of the most studied neurotransmitter systems, modulating DA-ergic signal transmission, is the noradrenergic system originating from locus coeruleus.

The locus coeruleus is a nucleus comprised mostly of noradrenaline (NA) containing neurons that project to very different areas in brain, including the DA-ergic regions (Berridge and Waterhouse 2003). It has been proposed that the enhanced firing of locus coeruleus NA neurons promotes attention and orienting to important environmental stimuli by having a profound influence on the cognitive processes of attention, perception and memory (Aston-Jones and Bloom 1981, Sara 2009). Exposure to novelty has been shown to increase the extracellular levels of NA in prefrontal cortex (Feenstra *et al*. 2000). Dysregulation of locus coeruleus NA-ergic projections has been suggested to be the major initial trigger in the pathogenesis of depression (Harro and Oreland 2001).

The locus coeruleus NA-ergic system regulates the activity of DA neurotransmission in ventral tegmental area by modulating the firing rate of DA-ergic neurons (Grenhoff and Svensson 1993, Arencibia-Albite *et al*. 2007, Guiard *et al*. 2008). Lesioning the locus coeruleus-originating NA-ergic projections with the selective neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) decreases basal and/or stimulated DA release in nucleus accumbens (Lategan *et al*. 1992, Häidkind *et al*. 2002) and striatum (whilst upregulating the D_2 receptors in striatum and probably sensitizing their signal transmission there (Harro *et al*. 2003). The latter could explain why these animals are behaviourally hypersensitive to amphetamine (Harro *et al*. 2000).

The interaction between locus coeruleus NA-ergic and striatal DA-ergic system also operates indirectly via prefrontal cortex. Treatment with DSP-4 causes the decrease in NA content but increase in its efflux measured by in vivo microdialysis in frontal cortex (Hughes and Stanford 1998). Altered NA release in prefrontal cortex, in turn, can modulate DA neurotransmission in nucleus accumbens (Ventura *et al*. 2003 and 2005).

2.4. The role of dopaminergic signal transmission in the effects of amphetamine and other psychostimulants

Amongst all the drugs of abuse that have the common feature to elevate the DA-ergic signaling in nucleus accumbens and related areas, triggering the cascade of molecular adaptations and synaptic plasticity underlying drug addiction, psychostimulants act directly on the DA system. They interact with the protein responsible for DA clearance, dopamine transporter (DAT), blocking the uptake of DA back to nerve terminals and therefore causing the elevation of DA in synaptic cleft. Behaviourally, this kind of action causes the increase in locomotor activity, sexual activity *etc*., and sometimes in aggression and eventually in behavioural stereotypy. Repeated administration of psychostimulants leads to behavioural sensitization, which is expressed as an increased locomotor response to the drug after a period of chronic use. Behavioural sensitization is often used as an animal model of different aspects of addiction like drug craving and compulsive drug-seeking behaviour (White and Kalivas 1998, Everitt and Wolf 2002, Schmitt and Reith 2010).

Regarding the action on DAT, psychostimulants can be divided into two groups: blockers (inhibitors) and substrates.

DAT blockers, e. g., cocaine, inhibit the DA uptake process, binding to either the DA binding site or to an allosterical site on DAT. The blocking of DAT has repeatedly been shown to lead to insertion of DAT molecules into presynaptic membrane. In studies with DAT-expressing cells, the incubation with cocaine increases the plasmalemmal DAT expression (Daws *et al*. 2002, Little *et al*. 2002). Chronic administration of cocaine upregulates striatal DAT expression in

rhesus monkeys (Beveridge *et al*. 2009), increased DAT expression has also been shown in postmortem analyses of brain tissue from human cocaine addicts (Little *et al*. 1999) and synaptosomes prepared from such a tissue exhibit greater [^{3}H]DA uptake than the synaptosomes from controls (Mash *et al*. 2002).

DAT substrates, e. g., amphetamine and its more potent congener methamphetamine, are actively translocated by DAT. They can either up- or down-regulate the DAT expression, depending on the duration of substrate exposure. Incubation of DAT-expressing cells with amphetamine leads to a significant reduction in cellular [^{3}H]DA uptake and DAT expression (Saunders *et al*. 2000, Kahlig *et al*. 2004). In brain tissues, a difference between striatal and accumbal DAT regulation have been demonstrated, as 15 min preincubation with amphetamine decreased the [^{3}H]DA uptake by striatal but not accumbal synaptosomes of rat brain tissue (Richards and Zahniser 2009). The upregulation of DAT levels in response to amphetamine is rather a very fast process, occuring within the seconds after amphetamine exposure.

In addition of the substrate-like action of amphetamine, it regulates also the vesicular monoamine transporter 2 (VMAT-2), releasing DA into presynaptic area. This increase in DA concentration promotes the DAT to work in the reversed mode – to transport DA from nerve terminals to synaptic cleft. However, it is possible that the regulation on VMAT-2 is indirect and mediated by D_2 autoreceptor activation (Brown *et al*. 2002, Riddle *et al*. 2005). D_2 receptors are overall the most studied presynaptic receptors among DAT-regulating GPCRs. Activation of D_2 autoreceptors attenuates DA-ergic neurotransmission via different pathways, e.g., inhibition of tyrosine hydroxylase, but the evidence suggest that D_2 receptor activation also reduces extracellular DA concentration by acute upregulation of DAT. Upregulation of surface DAT by D_2 activation requires G_i-family G proteins because pretreatment with pertussis toxin abolishes the increase in DAT radiotracer [^{3}H]CFT (2 beta-carbomethoxy-3- beta-(4-fluorophenyl)-N-[3H]methyltropane)) binding after incubation with a D_2 agonist (Meiergerd *et al*. 1993, Mayfield and Zahniser 2001, Wu *et al*. 2002).

Although both amphetamine and cocaine share the ability to bind to the transporters of other monoamines, NA and 5-HT, too, it is their action on DAT which is most central for both motor and reinforcing properties of these psychostimulants (White and Kalivas 1998).

All the drugs of abuse, as well as all the novel and possibly important stimuli, cause DA release in nucleus accumbens and general alterations in DA-ergic signaling in striatal areas. Therefore, psychostimulants, as direct DA releasers serve as a very good tool to study the neurobiology underlying these reward-related processes. The neurobiological changes accompanying the behavioural sensitization, for example, could reflect the neurochemistry of addicted brain, leading to the discovery of effective anti-addictive drugs. Differences in DA-ergic signal transmission between individuals have been shown to correlate with the drug addiction vulnerability and impulsivity. However, often these

differences are revealed only after stressing the DA-ergic system; the last could again be easily done with chronic administration of psychostimulants.

Treament with amphetamine has been shown to increase D_2 receptor mRNA levels in the dorsal striatum of mice (Giordano *et al*. 2006), on the other hand, no changes in D_1 nor D_2 mRNA levels were detected in rat striatum after acute or chronic amphetamine administration (Richtand *et al*. 1997). On the protein level, D_2^{High} levels are elevated in amphetamine-sensitized rats (Seeman 2009), while administration of $D_{2/3}$ receptor antagonist sulpiride potentiates the effect of amphetamine on striatal DA levels (Jaworski *et al*. 2001).

Concerning the intracellular signaling following activation of DA receptors, the levels of $G_{i/o}$ proteins in striatum have been found to decrease after chronic psychostimulant treatment (Striplin and Kalivas 1993), an effect that is in correlation with the decrease in regulator of G protein signaling 4 (RGS4) mRNA (Schwendt *et al*. 2006). Reduction of both binding and inhibition of forskolin-induced cAMP accumulation by D_2 receptor in rat nucleus accumbens has been shown during amphetamine sensitization (Chen *et al*. 1999). Recently, a very important role has been proposed for dopamine and cAMP regulated phosphoprotein with molecular weight of 32 kilodaltons (DARPP-32) in psychostimulant (as well as many other drugs of abuse) mediated actions (Svenningsson *et al*. 2005). Amphetamine administration causes the increase in immediate early gene c-fos expression only in D_1 expressing striatal neurons but when amphetamine is administered to rats in novel environment, c-fos increases both in D_1 and D_2 expressing neurons (Badiani *et al*. 1999), the result which shows the importance of novelty in reward-related stimuli processing.

3. AIMS OF THIS STUDY

- To examine the dopaminergic signal transmission through D_2 receptors in rats having different behavioural phenotypes regarding their behaviour in motivationally important situations
- To study the effects of repeated administration of dopamine releaser amphetamine on the behavioural phenotype and D_2 receptor biochemistry in rats with different novelty-related behaviour
- To study the influence of noradrenergic denervation with toxin DSP-4 on the behavioural phenotype and D_2 receptor biochemistry in rats with different novelty-related behaviour
- For the abovementioned purposes, to develop a method for characterization of sensitivity of DA neurotransmission through DA D_2 receptors in the membranes of (dorsal) striatum and nucleus accumbens, employing D_2-dependent [^{35}S]GTPγS binding activation assay
- To characterize newly synthesized potential D_2 receptor specific ligands using [^{35}S]GTPγS binding activation assay in the cell culture expressing DA D_2 receptors

4. MATERIALS AND METHODS

4.1. Chemicals

N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) was purchased from Astra-Zeneca, Södertälje, Sweden; D-amphetamine sulphate from Sigma, St. Louis, MO, USA; [^{35}S]GTPγS ([^{35}S]-guanosine-5′-(γ-thio)-triphosphate)with specific activity of 1250 Ci/mmol from PerkinElmer, Mechelen, Belgium; [Methoxy-^{3}H]Raclopride (74 mCi/mmol) from PerkinElmer, Boston, MA, USA; [N-methyl-^{3}H]R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride ([^{3}H]SCH23390) with specific activity of 71 Ci/mmol from GE Life Sciences; [5',8'-^{3}H] adenosine-3',5'-cyclic monophosphate ([^{3}H]cAMP, 48 Ci/mmol) from Amersham Life Sciences; isopenthane, cyclic adenosine-3',5'-monophosphate (cAMP), 4-(3-butoxy-4-methoxybenzyl)-imidazolidin-2-one (Ro 20-1724), dithiothreitol (DTT), guanosine 5'-diphosphate (GDP) lithium salt, dopamine, butaclamol from SigmaAldrich, USA; Tris-(hydroxymethyl)-aminomethane (Tris), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), bovine serum albumine (BSA), $MgCl_2$, NaCl, KH_2PO_4, KCl from AppliChem, Darmstadt, Germany; scintillation cocktail OptiPhase HiSafe from Wallac PerkinElmer Life Sciences, Cambridge, UK; ethylenediaminetetraacetic acid (EDTA) from Merck; isobutylmethylxanthine (IMBX) from Tocris Bioscience, Bristol, UK; phosphoenolpyruvate (PEP), pyruvate kinase (PK) from Roche Diagnostics; RPMI-1640 from GIBCO.

4.2. Animals

Male and female Wistar and male Sprague-Dawley rats were from Scanbur BK AB, Sweden. For animal housing and handling, see Procedures, Animal handling.

4.3. Procedures

4.3.1. Animal handling

Animals were housed individually or four per cage in plastic cages with food (Lactamin R35, Sweden) and water ad libitum. Room temperature was maintained at 21±2°C and 12:12 h light darkness cycle was applied. All procedures were carried out in compliance with the European Communities Council Directive (86/609/EEC) and approved by the Ethics Committee of the University of Tartu.

4.3.2. Drug administration

DSP-4 and D-amphetamine sulphate were dissolved in distilled water and administered intraperitoneally, in the doses of 10 mg/kg and 0.5 mg/kg, respectively. Solutions were prepared freshly before experiments. Control animals received an injection of distilled water.

4.3.3. Chronic variable stress procedure

The CVS procedure used was developed on the basis of the previous experiments (Harro *et al*. 1999 and 2001). Various stressors of different duration were applied daily. Each stressor was applied once during the weekly cycle; then stressors were repeated in the same order during the consecutive cycles. The stressors applied were: cold (4°C) water and wet bedding, imitation of the intraperitoneal injection, stroboscopic light, tail pinch with a clothes-pin placed 1 cm distal from the base of tail, cage tilt at 45°, movement restriction in a small cage, and strong illumination (900 lx) during the dark phase. Control rats remained undisturbed in their cages except for daily weighing and weekly sucrose preference testing until the commencement of behavioural experiments.

4.3.4. Collection of tissues for biochemical experiments

The animals were sacrificed by decapitation, brains were dissected on ice-cold plate according to the atlas of Paxinos and Watson and tissue samples were immediately frozen in isopenthane/dry ice, and then subsequently stored in freezer at −80 °C until biochemical experiments the measurement D_2 receptor-stimulated [^{35}S]GTPγS binding. Details about the time between completion of behavioural experiments and decapitation are presented in corresponding Papers.

4.3.5. Growing and maintenance of cell cultures

Chinese hamster ovary cells (CHO-K1 cells; CCL61, American Type Culture Collection, Rockville, MD, USA) stably expressing rat dopamine D_{2S} receptor and Ltk$^-$-fibroblast cells expressing D_1 dopamine receptors were obtained from Professor K. Fuxe's laboratory at the division of Cellular and Molecular Neurochemistry, Department of Neuroscience, Karolinska Institute (Sweden). Cells were grown and maintained in RPMI-1640 medium at 37°C and 5% of CO_2 in the atmosphere.

4.4. Behavioural methods

4.4.1. Exploration box test

The test was conducted, as described in (Harro *et al.* 1995 and Otter *et al.* 1997). The exploration box consisted of an open area part (0.5×1m, side walls 40 cm) and a little (20×20×20 cm) covered compartment with wood shavings on its floor. The little compartment was directly attached to the open area part through an 20×20 cm opening. The open area was divided into 8 squares and, situated throughout the area, there were 3 novel (glass jar, cardboard box and wooden handle) and 1 familiar (food pellet) objects.

The test begins with placing the rat into little compartment, facing away from the opening to open field. A test lasted for 15 min and the following behavioural events were counted: 1) latency before entering open part, 2) number of entries into open part, 3) time spent exploring in the open part, 4) number of lines crossed, 5) object investigations made, 6) rearings, and, summing the last three measures, also 7) sum of the exploratory events.

4.4.2. Open field test

For open field test, the same exploration box was used, but in this time, the passage between small compartment and open area was closed.

15 min after amphetamine injection, the rat was placed into one corner (always the same corner for all animals) of the open area. The test lasted for 15 min and 1) number of lines crossed, 2) object investigations made, 3) rearings and 4) sum of previous three events were counted.

4.4.3. Sucrose consumption

Sucrose consumption tests were carried out both in light and dark phase and lasted 1 h. Food and water were available freely all the time, except for the hour before the start of sucrose consumption measurement. In the test box, there were two bottles, one filled with 1% sucrose solution and the other with water. Sucrose and water consumption was measured by weighing the bottles before and at the conclusion of the test. Sucrose preference was measured by calculating the proportion of sucrose consumption out of total liquid consumption.

4.4.4. Induction of 50 kHz ultrasonic vocalizations

The single-housed rats were given daily tickling sessions (15 sessions of stimulation over 2 min every day, for details see Paper III) for 3 weeks, and 50-kHz and 22-kHz calls and body weight were daily measured. The calls in the

50-kHz and 22-KHz range that were elicited during the stimulation, and made audible to the experimenter as specific “chirps” via the ultrasonic detector, were manually counted. The rats were divided into groups with high and low levels of 50-kHz ultrasonic vocalizations (USVs) by the median split of the average response on Days 12–14 of tickling, providing the HC and LC groups mentioned above. The control animals remained single-housed through that time and received no handling except for weekly weighing.

4.5. Biochemical methods

4.5.1. Preparation of membranes from rat striatum and accumbens for [^{35}S]GTPγS analysis

The striatal and accumbal tissues were homogenized in 5ml of homogenization buffer (50mM Tris–HCl, pH 7.4) by Bandelin Sonopuls sonicator (three passes, 10 s each). The membrane fragments were collected by centrifugation at 30 000×g for 20 min at 4 °C and washed by homogenization and centrifugation for two more times. The final pellet was resuspended in incubation buffer A (20mM K-HEPES, 7mM $MgCl_2$, 100mM NaCl, 1mM EDTA, 1mM DTT, pH 7.4) for D_2 receptor specific [^{35}S]GTPγS binding activation measurement.

4.5.2. D_2 receptor-stimulated [^{35}S]GTPγS binding in rat striatal and accumbal membranes

Binding of [^{35}S]GTPγS was carried out as described in Rinken *et al.* 1999, with slight modifications. The membranes (200 μg of accumbal and 500 μg of striatal tissue per tube) were incubated with 0.2 nM [^{35}S]GTPγS and different concentrations of GDP (3 mM to 1 μM) and 1mM DA or 10μM butaclamol in buffer A in the volume of 250 μl for 90 min at 30 °C. The reaction was stopped by rapid filtration through GF/B glass fiber filters using a Brandel cell harvester and the filters were washed three times with 3ml of ice-cold 20mM K-phosphate buffer (pH 7.4) containing 100mM NaCl. The radioactivity content of the filters was counted in 4ml of scintillation cocktail with a RackBeta 1219 liquid scintillation counter.

4.5.3. Preparation of membranes from rat striatum and accumbens for cAMP accumulation analysis

The striatal and accumbal tissue were homogenized in 50ml/g (striatum) or 100 ml/g (accumbens) ice cold 50 mM Tris-HCl (pH 7.4) buffer, containing 2 mM EGTA by Bandelin Sonopuls sonicator (three passes, 10 s each). For AC

assays, the suspension was diluted twice with 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EGTA, divided into aliquots and stored at −80°C until use.

4.5.4. Adenylyl cyclase assay

The assay was carried out in a reaction medium containing 30 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM ATP, 10 μM GTP, 0.75 mM EGTA, 7.5 mM KCl, 100 mM NaCl, 0.1 mM IBMX, 0.1 mM Ro20-1724, 100 μg/ml bacitracin, 0.03% BSA, and ATP regenerating system (10 mM PEP and 30 μg/ml PK). The reaction was started by transferring tubes containing membrane homogenate (approx. 17 μg tissue per point) with the ligand of interest from an ice bath to a 30°C water bath, followed by a 15-min incubation. The reaction was terminated by adding a solution containing EDTA (final concentration of 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 (Vonk *et al*. 2008). Bound radioactivity was determined by rapid filtration through GF/B filters using a Brandel cell harvester and three washes of 3 ml of ice-cold washing buffer containing 100 mM NaCl and 20 mM K-phosphate buffer (pH 7.4) as described previously. Nonspecific binding of [^{3}H]cAMP was determined in the absence of the binding protein.

4.5.5. Preparation of membranes from D_2 receptor expressing CHO cells

Cells were collected by scraping them off from dishes, washed and homogenized by Bandelin Sonopuls sonificator in raclopride binding buffer B (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4) and centrifuged at 30 000 g for 20 min at 4 °C. The membrane pellets obtained were re-homogenized in B and centrifuged once more. The final pellets were re-suspended in B (2.5 Petri dishes per ml) and stored at –80 °C until use.

4.5.6. Competition experiments with [^{3}H]raclopride in D_2 receptor expressing CHO cell membranes

Binding affinities of compounds to D_2 dopamine receptors were measured by incubation of 1.1 nM [^{3}H]raclopride and appropriate concentrations of compounds (0.1 nM....0.1 mM) with membrane suspension of CHO cells in B for 90 min at 25 ºC. The reaction was stopped by filtration through GF/B filters using Brandel cell harvester and the filters were washed with 3 mL of ice-cold washing buffer (20 mM K-phosphate buffer, 100 mM NaCl, pH 7.4). Filters were incubated in scintillation cocktail OptiPhase HiSafe overnight and the

radioactivity content of filters was measured by RackBeta 1219 liquid scintillation counter.

4.5.7. D_2 receptor-stimulated [^{35}S]GTPγS binding in D_2 receptor expressing CHO cells

The membranes from CHO cell line were prepared and stored as described above.

D_2 receptor activation properties of compounds were measured by incubating 0.2 nM [^{35}S]GTPγS with 10 μM GDP, appropriate concentrations of compounds, and membrane suspension of CHO cells in buffer B in the volume of 250 μl for 90 min at 25 ºC. The reactions were stopped and bound radioactivity was determined as described above.

4.5.8. Preparation of membranes from D_1 receptor expressing Ltk$^-$-fibroblast cells

For experiments with D_1 receptors, fibroblast membranes were prepared as described in 4.5.5, with slight modifications: cells were washed and homogenized in 50 mM Tris-HCl (pH=7.4) instead of B and 40 minutes of centrifugation time was used. The final pellets were suspended in 50 mM Tris-HCl (2 Petri dishes per ml) and stored at –80 °C until use.

4.5.9. Competition experiments with [^{3}H]SCH23390 in D_1-receptor expressing fibroblast cell membranes

Binding affinities of compounds to D_1 dopamine receptors were measured by incubation of 2 nM [^{3}H]SCH23390 with appropriate concentrations of compounds in membrane suspension of Ltk$^-$-fibroblast cells in B without sodium- and potassium chloride for 60 min at 25 ºC. The reactions were stopped and bound radioactivity was determined as described above.

4.6. Data analysis

All biochemical data were analysed by means of non-linear least squares regression method using a commercial program GraphPad PRISM^{TM} 4.0 (GraphPad, San Diego, CA, USA). Behavioural data was analysed in program StatView (Adept Scientific Plc, Letchworth, UK) using ANOVA, with repeated measures added, as appropriate. Post hoc comparisons were done by Fishers PLSD test. In correlation analysis, Pearson correlation coefficients were used. For all the details, see respective Papers.

5. RESULTS AND DISCUSSION

5.1. Dopamine D_2 receptor-mediated [^{35}S]GTPγS binding activation in rat striatal and accumbal membranes

To characterize dopamine-dependent activation of G_i-proteins, striatal and accumbal membrane preparations were either activated (dopamine) or deactivated (butaclamol) with dopaminergic ligands and the potency of GDP to compete with [^{35}S]GTPγS was measured as done in by Rinken *et al*. 1999. DA decreased the affinity of GDP by ca 0.2 log units, thereby increasing the [^{35}S]GTPγS binding, measured at the same GDP concentration, compared to the butaclamol-deactivated state (Fig 6.1). In later discussion sections, these effects are called DA effect on GDP affinity and DA-dependent (or D_2-receptor mediated) [^{35}S]GTPγS binding, respectively.

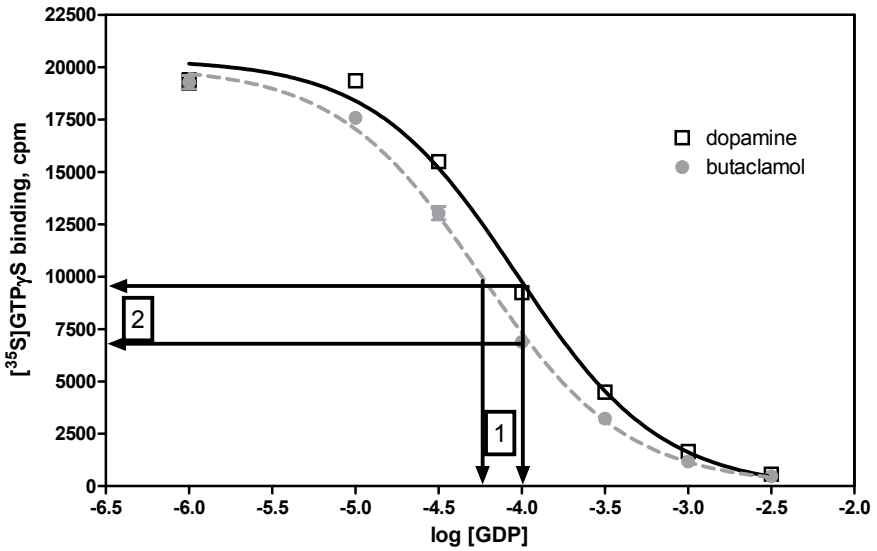


Figure 6.1. Influence of DA on the binding of [^{35}S]GTPγS in the the presence of different concentrations of GDP in rat striatal membranes. Arrows indicate the effects of 1) 1 mM dopamine on GDP affinity and 2) 1 mM dopamine on [^{35}S]GTPγS binding. The pEC_{50} value for GDP in the presenece of 10 μM butaclamol was 4.24 ±0.3 and in the presence of 1 mM dopamine 4.03 ± 0.4.

It has been shown before that the presence of agonist does not significantly change the affinity of GTPγS, which means that all the apparent effects are caused by altered GDP binding affinity. The amount of bound [^{35}S]GTPγS (at fixed GDP concentration) reflects the signal transmission sensitivity of the activated complex, therefore, the higher the DA-dependent [^{35}S]GTPγS binding

is, the higher or more sensitive is also signal transmission. The difference between [^{35}S]GTPγS binding in the presence of DA and butaclamol is variable at different GDP concentrations, approaching zero at very high (all [^{35}S]GTPγS displaced by GDP regardless of the presence of agonist/antagonist) and very low (all GDP displaced by [^{35}S]GTPγS regardless of the presence of agonist/ antagonist) GDP concentrations. The maximal agonist effect was determined in striatal membranes at 100 mM and in accumbal membranes at 32 mM GDP.

It should be mentioned that competitive binding process between GDP and [^{35}S]GTPγS will not reach the equilibrium in the conditions used, probably due to the slow dissociation kinetics of nucleotides (Rinken *et al*. 1999). So all the experimental data points are actually kinetic one-point measurements and therefore the experimental conditions should be kept as constant as possible because GDP potency to compete with [^{35}S]GTPγS depends on time.

As dopamine and butaclamol have a considerable affinity for all the dopamine receptors coupled to G_i-proteins, the contribution of signals from D_3 and D_4 receptors should also be considered. The expression of D_4 receptors in striatum and nucleus accumbens is negligible, but the expression of D_3 dopamine receptor in nucleus accumbens has been reported (Landwehrmeier *et al*. 1993), so in nucleus accumbens there could be an additional component of D_3-mediated [^{35}S]GTPγS binding whereas in striatum we measure a relatively pure D_2-receptor effect.

5.2. D_2 receptor signal transduction in Wistar rats with low or high exploratory activity

In the work by Alttoa *et al.* 2005 it has been found that there is a tendency towards more sensitive dopamine D_2 signal transmission in high exploring (HE) animals in striatum, compared to low exploring (LE) animals, manifesting in higher dopamine-dependent [^{35}S]GTPγS binding and lower GDP affinity in HE rats.

This kind of tendency have appeared in most of our studies concerning experiments with LE/HE rats so far, (for example see Fig 6.2), but the effect never reached statistical significance.

However, it should be mentioned that the tendency for more sensitive D_2 receptor signal transduction in HE rats was not the only parameter which was different between LE and HE animals, regarding DA neurotransmission in the Paper 2.

Also, dopamine levels in striatum were significantly lower in HE animals compared to LE animals (25.3 ± 2.85 pmol/mg vs 34.4 ± 2.05 pmol/mg), and also the ratios between dopamine and its main metabolites DOPAC and HVA (DA/DOPAC and DA/HVA), reflecting the possibly higher DA turnover in HE rats (see Paper 2).

Considering also the fact that that HE rats have higher basal and stimulated DA release and higher proportion of D_2 high affinity agonist binding sites in striatum (Alttoa *et al*. 2009), these results together point to the higher DA-ergic activity of high explorers in their striatal area, which may be the cause of the higher locomotor acitvity of the rats with this phenotype. Higher DA activity, in turn, could make the HE animals more susceptible to rewards, including drugs of abuse, especially those ones acting directly through DA system like psycho-stimulants amphetamine and cocaine.

5.3. D_2 receptor signal transduction in amphetamine treated animals

One of the main characteristics of psychostimulant actions is development of behavioural sensitization after repeated administration of a drug. The HE animals with their higher DA-ergic signal transmission were thought to be more susceptible to the effects of psychostimulant amphetamine, both in behavioural and receptor biochemistry level.

But on the contrary, our results indicated that repeated administration of amphetamine *desensitized* both the locomotor activity of HE rats in open field as well as D_2-mediated [^{35}S]GTPγS binding activation to striatal membranes of HE rats (Fig 6.3). It should be noted that the decrease of locomotor activity in HE rats after chronic amphetamine treatment was not caused by increase in stereotypic behaviour.

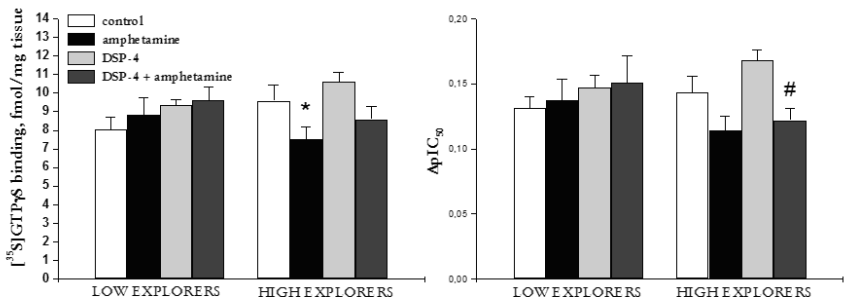


Figure 6.2 DA-dependent activation of [^{35}S]GTPγS binding in the presence of 0.1 mM GDP (left panel) and the DA-dependent change in GDP affinity (right panel) in rat striatal membranes. Amphetamine desensitized the DA-dependent [^{35}S]GTPγS binding in HE animals, regardless of DSP-4 treatment.$^{*,\#}$ $p<0.05$ vs respective control.

Dopamine D_2 receptors are mostly inhibiting presynaptic autoreceptors or, more precisely, presynaptic inhibitory functions are prevalent in the case of psycho-stimulant caused DA release which inhibits firing of DA-ergic cells, effect which is blocked by D_2 antagonist raclopride (Shi *et al*. 2000). HE animals have higher

D_2^{High} receptor proportion, so one might assume that the DA negative feedback signaling in the striatum of HE animals is enhanced, compared to LE animals. The reason of this sensitive negative feedback system might rely in the adaptation to the higher basal and evoked DA release in the striatum of HE rats. The D_2 (in striatopallidal neurons) receptor ablation has been shown to inhibit both locomotor and drug reward processes (Durieux *et al*. 2009), thus the sensitive D_2 receptor signal transmission might be the basis of higher locomotor activity of HE animals. The chronic administration of amphetamine desensitizes this negative feedback system through D_2 receptors which, in turn, might explain the desensitization of the locomotor activity in amphetamine-treated HE rats.

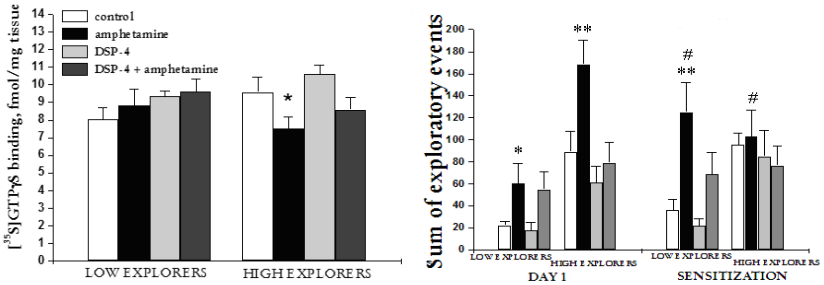


Figure 6.3. Correlation between locomotor desensitization (left panel) and down-regulation of DA-dependent [^{35}S]GTPγS binding in rat striatal membranes (right panel) of high exploring animals. Behavioural sensitization was evident in LE rats. Both behavioural sensitization and desensitization to amphetamine were absent in DSP-4 treated animals (for discussion, see chapter 6.4).

On the other hand, our results have shown that chronic cocaine administration has no effect on HE nor LE animals' D_2 signal transduction sensitivity (data not shown). As cocaine has a different mechanism of action: it is only a blocker of DAT whereas amphetamine is a substrate of DAT going inside presynaptic terminal and acting on vesicular monoamine transporter, therefore releasing even more DA from synaptic vesicles to synaptic cleft (Riddle *et al.* 2005), this result seems logical, because cocaine has a weaker impact on DA-ergic signaling than amphetamine. Additionally, cocaine has a considerable affinity to serotonin transporter and the release of serotonin can attenuate the effects of DA (Rothman and Baumann 2006).

5.4. D_2 signal transduction after lesioning of noradrenergic system with neurotoxin DSP-4

To assess the influence of NA-ergic input on DA-ergic neurotransmission and locomotor activity of LE and HE rats, animals were treated with the neurotoxin DSP-4 which selectively disrupts the NA-ergic projections originating from locus coeruleus.

No effect of partial lesioning of locus coeruleus originating NA-ergic projections on D_2 receptor signal transmission was found (Paper 2), although the up-regulation of D_2 receptor number in non-preselected rats had been demonstrated 1 month after the treatment with 10 or 50 mg/kg DSP-4 (Harro *et al.* 2003). However, the desensitizing effect of amphetamine on HE animals remained also after DSP-treatment (Figure 6.2). So, in the D_2 receptor-G protein signal transduction level, there was no effect of partial lesioning of NA-input from locus coeruleus. Still, in behavioural level, only these LE/HE animals who had an intact input from locus coeruleus, developed behavioural sensitization/ desensitization to amphetamine (Fig 6.3). It could be assumed that DSP-4 influences amphetamine-caused behaviours in non-D_2-receptor signal transmission-dependent way, at least in the striatum. However, repeated amphetamine treatment caused significant elevation of striatal dopamine content in DSP-4 treated HE rats (see Table 1 in Paper 2) compared to control and only amphetamine treated animals. This effect, which, in fact, makes HE rats neurochemically more similar to LE control rats in this context, could account for the abolishment of behavioural desensitization.

5.5. D_2 receptor signal transduction sensitivity and sucrose consumption

Novelty can be considered as a natural reward source, due to expectation of something good from novel situations/objects. However, all the unknown and new contexts also contain possible dangers and therefore the anxiety component should always be taken into account.

Measuring sucrose consumption and preference in animals' home cage minimizes the influential anxiety parameter and may give more direct associations between reward susceptibility and its biochemical mechanism, e. g., dopaminergic signal transduction sensitivity.

We have studied the susceptibility of Sprague-Dawley rats to the natural reward sucrose and found that they preferred sucrose over water and the consumption and preference was more pronounced in the dark phase of light-dark cycle as it could be expected for rats, who are nocturnal animals. Supporting this idea, a recent paper shows that both natural and drug-related reward vary in a diurnal fashion, as does tyrosine hydroxylase (L-DOPA-synthesizing

enzyme) protein level in nucleus accumbens and ventral tegmental area (Webb *et al*. 2009).

Comparison of signal transduction sensitivity of D_2 receptors in nucleus accumbens with sucrose intake and preference of rats in dark phase revealed significant positive correlations with Pearson coefficients $r^2 = 0.35$, $p < 0.01$ and $r^2 = 0.27$, $p < 0.05$, respectively (Fig 6.4). On the other hand, a negative correlation between first-test sucrose preference and dopamine-dependent G-protein activation was found in striatum (data not shown, see the Paper 1). As the first sucrose consumption test is influenced by some degree of novelty (a new bottle, a new taste), striatal D_2 receptor function could be related to animals' behaviour in novel situation (similar to that examined in the Paper 2), while accumbal D_2 receptor function is correlated with already developed reward sensitivity. With the current study design, it is impossible to say, whether the D_2 receptor signaling sensitivity in nucleus accumbens is *influencing* or *influenced by* sucrose consumption properties, but they are related nevertheless. Considering that the differences in D_2 receptor signal transduction sensitivity were already present before sucrose preference tests, the greater sensitivity of D_2 receptors could reflect the more sensitive DA neurotransmission in general, which, in turn, leads to greater DA release after reward cues (or reward itself) and the more sensitive behavioural response – sucrose consumption. On the other hand, it have been discussed before that D_2 receptors serve as presynaptic autoreceptors, controlling DA release and probably uptake, too, through interactions with DAT. Positive correlation between reward (amphetamine in the referred case) "wanting" and DA release potential (amount?) in ventral striatum has been demonstrated (Leyton *et al*. 2002). Thus, if there is an association between sucrose preference/consumption (resulting from higher level of "wanting") and DA release potential, the more sensitive D_2 receptor signal transduction could serve as an adaptive mechanism for the more effective clearance of DA.

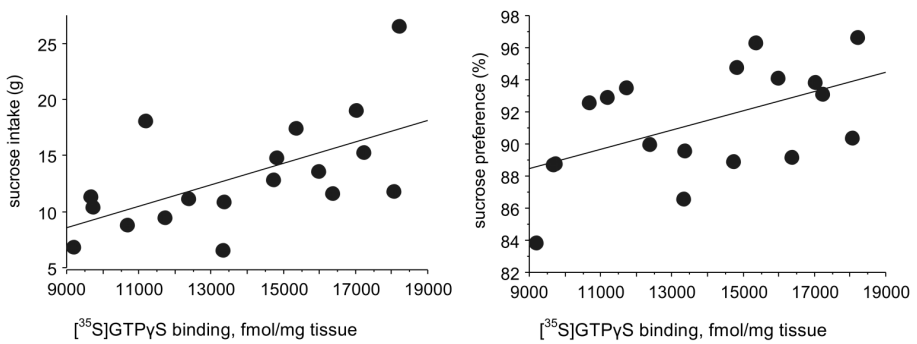


Figure 6.4. Regression analysis of DA-induced [^{35}S]GTPγS binding in nucleus accumbens and mean sucrose intake (left panel, $r^2 = 0.35$, $p < 0.01$) or preference (right panel, $r^2 = 0.27$, $p < 0.05$) in four sucrose preference tests during dark phase.

5.6. D_2 receptor signal transduction sensitivity and tickling

Motivation and reward-directed behaviour could be divided into two components: motor and subjective. To investigate further which role dopamine signal transmission through D_2 receptor has in motivation and reward processing, one would like to dissociate the motor component from rewarding situation. This could be done easily by manipulating the animals in a way that mimics rough-and-tumble play in juvenile rats, in a word, by tickling. It has been shown that tickling is perceived as rewarding by rats because it elicitis positive-emotion showing 50 KHz calls in these animals (Panksepp and Burgdorf 2003). 50 KHz calls are also emitted during psychostimulant administration, sex, play *etc* (Knutson *et al.* 1998 and 1999).

In Paper 3 we studied whether D_2 receptor signal transmission sensitivity is associated with the responsivness to tickling in young rats.

After continuous sessions of tickling of young Wistar male and female rats, two separate groups emerged in both sexes, differing from each other by the degree they responded to tickling: low and high chirpers (LC/HC), emitting less or more 50 KHz ultrasonic calls, respectively, while tickled. Both groups were similar in anxiety tests, but HC-rats were more passive in exploration box test. In male rat groups, there were no differences in D_2-dependent G-protein activation in control, LC and HC groups in striatum (Fig 6.5). This could point to the possibility that D_2 receptor signal transmission sensitivity in striatum is mediating and manipulated by the motor aspects of reward-related behaviour and in the absence of motor requirements, the susceptibility to rewards is mediated by other signaling systems.

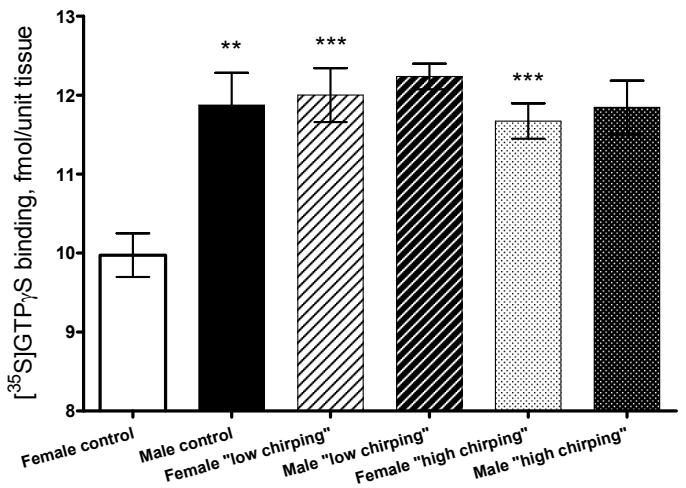


Figure 6.5. DA-dependent binding of [^{35}S]GTPγS in rat striatal membranes. **,*** $p<0.01$, 0.001 vs female control.

Among females, control female rats had dramatically lower D_2 receptor signal transduction sensitivity, which disappeared after tickling (Fig 6.6).

As D_2 receptor functions and availability might be under the control of hormonal cycles in females, a phenomenon which have been demonstrated on female Cynomolgus monkeys (Czoty *et al*., 2009), it remains an open question whether tickling manipulated directly with dopamine signaling sensitivity or with some hormone system which in turn upregulated the D_2 receptor-G-protein coupling sensitivity.

It is interesting to mention that measuring accumbal dopamine signal transmission through D_1 receptor, in secondary messenger cAMP accumulation assay, tickling had indeed influences on D_1 receptor function and this was more pronounced in male group (Fig 6.6). So, differentiation between motor and subjective reward processing could already be found in the level of dopamine signal transmission, one process mediated preferentially through D_2- and other through D_1-originating G-protein dependent pathways.

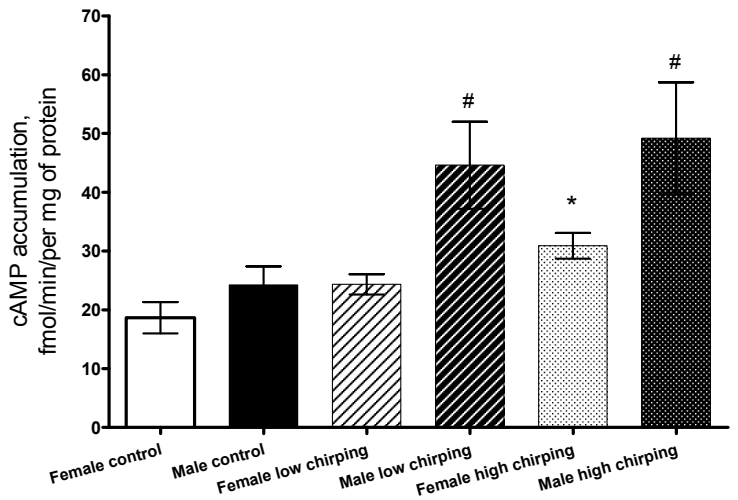


Figure 6.6. DA-dependent cAMP accumulation in rat accumbal membranes. $^{\#,*}$ $p<0.05$ vs respective control.

5.7. D_2 receptor signal transduction sensitivity and stress

So far, we have considered dopamine signal transduction through D_2 receptors in different contexts of *positive* reward presentation: with the anxiety component included or excluded, motor component bigger or smaller *etc*.

Very important question in psychology is, how the individual phenotypes influence the result what different life events have on a person. In depression, for example, one of the most characteristic feature is the loss of interest and

motivation plus developing anhedonia. The loss of motivation is greatly associated with dopamine signal transmission in brain limbic areas, including striatum and nucleus accumbens. In laboratory animals, the harsh human life events leading to depression, are mimicked by different stress regimes. In Paper 5 we examined the influence of CVS procedure to LE and HE rats and questioned, whether stimuli with opposite valence to reward has some (negative) effect on D_2 receptor signal transduction sensitivity.

The more anxious LE rats seemed to be more influenced by stress in the conventional measure of anhedonia, the sucrose consumption test. Still, in the FST, they developed more active coping style after stress which indicates that even when stress has a bigger influence on LE rats, some of those influences were adaptive. On the other hand, stress had no influence on D_2 receptor signal transduction sensitivity in striatum and nucleus accumbens, either in LE or HE rats. Considering that HE rats possessed a more reactive DA system but stress had a bigger influence on LE-rats, it is not surprising that we cannot detect any changes in this receptor signaling pathway. Still, D_1 receptor signaling through cAMP accumulation in nucleus accumbens was enhanced by stress procedure (Fig 6.7), mimicking the situation with tickling. This could reflect the possibility that both rewarding/reward-predicting as well as *stressful* (and stress-predicting) stimuli require a common subjective processing to acquire a proper incentive value. This process is probably mediated, at least partly, by D_1 receptor signaling in nucleus accumbens. D_2 receptors, in turn, seem to participate in processes where the motivated motor activation is required. As CVS consists from procedures which rats cannot control and (preventively) react to, it resembles the tickling, although here we consider punishment rather than reward. But again, we see the different contribution of D_2 and D_1 receptors to the overall DA signal transmission in mediating stimulus processing. This, in turn, might lead to the requirement of subtype-specific D_1 and D_2 agonist and antagonist to be used in the pharmacological manipulations in different reward-related behavioural experiments to elucidate the behavioural roles of D_1 and D_2 receptors. However, as mentioned above, the selectivity between DA receptor subtypes is difficult to achieve.

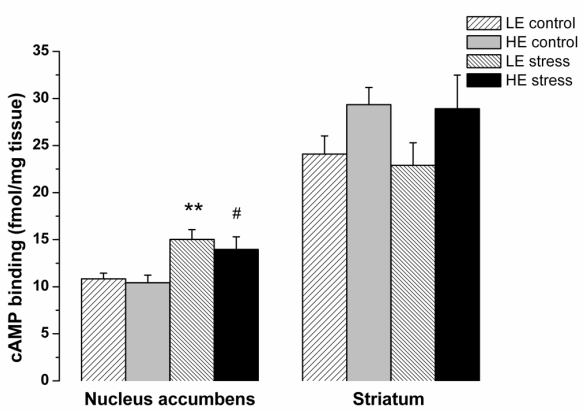
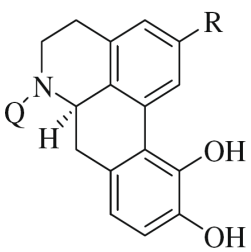


Figure 6.7. The effect of CVS on D_1-dependent cAMP accumulation in nucleus accumbens and striatum of LE and HE rats. ** – $p<0.01$ vs. LE control; # – $p<0.05$ vs. HE control.

5.8. Subtype-specific ligands for D_2 receptors

D_2 receptors are important in different conditions, including addictive disorders, depression, but also Parkinson's disease and schizophrenia. On one hand, subtype-specific high affinity ligands are desired to serve as tools for pharmacological research on the field of these disorders to characterize the receptor subtype-specific physiological effects; on the other hand, selective drugs are needed for treatment of abovementioned conditions.

In the field of drug design, subtype selectivity, affinity and efficacy (in the case of agonist) are the main milestones in the way to the synthesis of effective substance with possibly little side effects and possibly large specific effects. In the case of D_1 and D_2 receptors, regarding the similarities in their structure and anatomical distribution, it is complicated to synthesize ligands which have clear selectivity of one over the other subtype (Lan *et al.* 2006). For us the starting point to synthesize dopaminergic ligands was norapomorphine which was substituted in the positions N6 and 2 (Q and R), indicated in Figure 6.8.



	R	Q
1	H	Me
2	Me	Et
3	Ph	Et
4	4-OH-Ph	Et
5	Me	Pr
6	Ph	Pr
7	4-OH-Ph	Pr

Figure 6.8. Norapomorphine (1) and its derivates, which were synthesized and characterized in Paper 4. Me-methyl; Et-ethyl; Pr-propyl; Ph-phenyl; 4-OH-Ph-4-hydroxyphenyl.

For characterization of the affinity, potency and efficacy of synthesized compounds, the D_2 receptor expressing chinese hamster ovary (CHO) cell line was used as a more pure testing system compared with brain membranes. The [^{35}S]GTPγS binding activation assay was adapted to this system, measuring the DA dose response curve (for [^{35}S]GTPγS binding activation) in the presence of 10 μM GDP, where the activation of 150 % over baseline was achieved (Fig 6.9).

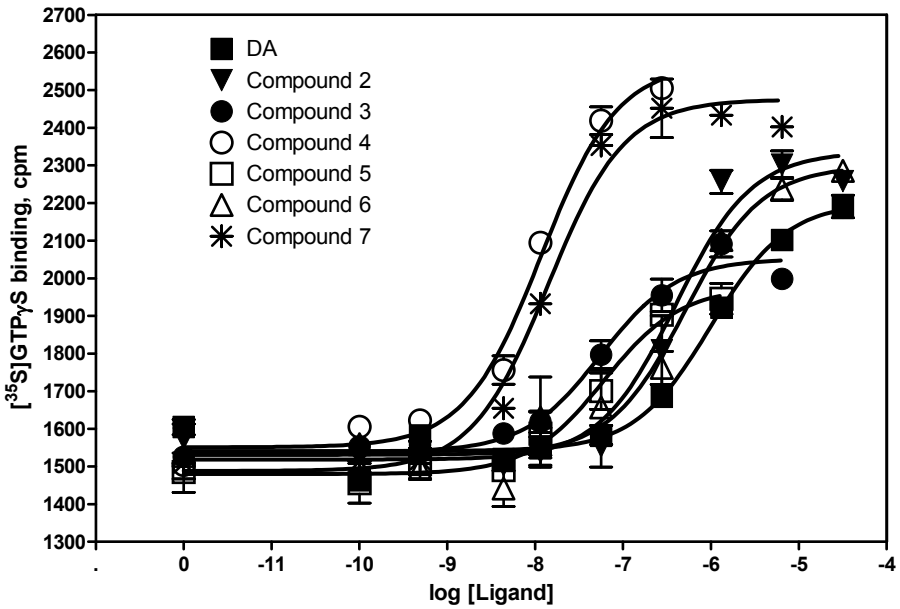


Figure 6.9. Activation of [^{35}S]GTPγS binding by different concentrations of DA and synthesized coumpounds in the presence of 10 μM GDP in the membranes from D_2 receptor expressing CHO cell line. DA pEC_{50} was 6.00 ± 0.08 and the maximal effect of DA on [^{35}S]GTPγS binding was taken as 100% for normalization. Data from two representative experiments.

Additionally, the [^{3}H]raclopride and [^{3}H]SCH23390 binding and competition assays were adapted for D_2 expressing CHO cell line and for D_1 expressing Ltk^- -fibroblast cell line (data not shown).

Most of the compounds studied, had high affinity for D_2 receptors expressed in CHO cell line, full or partial agonist behaviour in [^{35}S]GTPγS binding activation analysis and clear D_2 over D_1 selectivitiy. 2-(4-hydroxyphenyl)-substituted compounds had highest affinity, subtype selectivity and full agonist properties (Table 6.1).

As a result of this work, new D_2-ergic agonists were synthesized, some of them with nanomolar affinity, high efficacy and selectivity over D_1 receptors, altogether potentially suitable for further and more specific use in measuring D_2 receptor signal transduction in receptor biochemistry level. However, more thorough characterization of these ligands, concerning their activity on other DA receptor subtypes and also 5-HT receptor subtypes is needed before introducing them in studies on brain tissues. Additionally, assessment of pharmacokinetic profiles of these ligands has to be done for estimating their suitability in behavioural analyses.

Table 6.1. Binding affinities of dopamine, norapomorphine and synthesized compounds to DA D_1 and D_2 receptors expressed in CHO cell line and their potencies and efficacies for D_2 receptors.

Compound	D_2			D_1	D_2/D_1
	Vs [^{3}H]Raclopride *Ki* (nM)	Activation of [^{35}S]GTPγS binding EC_{50} (nM)	Efficacy (%)	*Vs* [^{3}H]SCH23390 *Ki* (nM)	Specificity (fold)
Dopamine	197 ± 67	1425 ± 813	100	124 ± 23	0.6
1	11.5 ± 0.7	53 ± 2.9	58 ± 1	72 ± 5.6	6.3
2	115 ± 47	480 ± 28	112 ± 3	1340 ± 516	12
3	14.0 ± 2.8	51 ± 5.2	72 ± 15	31 ± 1.4	2.2
4	1.5 ± 0.1	8.7 ± 0.6	112 ± 6	124 ± 2.8	83
5	9.2 ± 0.9	40 ± 8.5	65 ± 4	278 ± 5.6	30
6	192 ± 46	577 ± 93	100 ± 5	669 ± 172	3.5
7	2.0 ± 0.1	12.0 ± 2.9	99 ± 16	94 ± 15	46

SUMMARY

To study the sensitivity of DA ergic signal transmission through D_2 receptors in rat dorsal and ventral striatum, the DA-dependent [^{35}S]GTPγS binding assay was adapted for using in rat striatal and accumbal membrane preparation.

Signal transmission sensitivity was assessed in different rewarding/motivating contexts. Correlation between D_2 signal transmission sensitivity in rat accumbal membranes and preference for natural reward sucrose was found. In this situation, both subjective and motor (to approach the bottle, to choose sucrose over water) component was present in reward-related behaviour. On the contrary, when the motor component was absent and reward (as tickling) was administered passively, also the correlation between D_2 signal transmission sensitivity and response to reward was absent.

When the motivator/reward was a novelty, differences between signal transmission sensitivity in low and high exploring rats emerged after chronic manipulation with their DA-ergic system with the direct DA releaser amphetamine. Chronic administration of amphetamine desensitized the D_2 receptor signal transmission in the striatum of HE rats which was in the good correlation with the behavioural desensitization, seen in these rats.

To summarize this part, the D_2 receptor signal transmission sensitivity seems to mediate motivated/reward-related behaviour in the cases, when motivated motor events are required. On the contrary, in the other works from our group it has been shown that D_1 receptor signal transmission in striatal areas seems to be correlated rather with subjective processing of both positive and negative reward.

This, in turn, rises the need for DA receptor subtype-specific agonists and antagonists for pharmacological manipulations in behavioural studies in different motivational contexts to assess more specifically the roles of D_1 and D_2 receptors' contribution into the motivated/reward-related behaviour. In the current studies, a set of new norapomorphine-based potential D_2-ergic ligands were characterized in terms of affinity, efficacy and selectivity between D_2 and D_1 in specific cell lines. As a result of this work, potential ligands with 4-hydroxyphenyl substitution in the position 2 in apomorphine backbone were introduced.

SUMMARY IN ESTONIAN

Dopamiin D_2 retseptorite biokeemia ja selle seos motiveeritud käitumisega

Dopamiin ehk 3-hüdroksütüramiin on olulise rolliga virgatsaine nii perifeerses kui kesknärvisüsteemis. Ilmselt tuntuim funktsioon, mille vahendamisel on dopamiinil keskne osa, on sõltuvus- ning hüvekäitumine, mida viimastel aegadel on hakatud vaatlema kui motiveeritud ning õpitava käitumise üht komponenti. Dopamiini vabanemine aju naalduvas tuumas ning sellega külgnevatel aladel, näiteks selgmises juttkehas, on üks enim uuritud korrelaate sõltuvuskäitumise katsetes. Korduvalt on näidatud dopamiini vabanemist vastuseks erinevate narkootikumide (amfetamiin, heroiin, etanool) manustamisele või nendega seotud teiseste stiimulite esitamisele – aga dopamiin vabaneb ka näiteks vastuseks potentsiaalselt olulistele keskkondlikele stiimulitele, seda eriti siis, kui stiimulid on looma jaoks uudsed. Rottide uudsuskäitumine ning individuaalsed erinevused selles on olnud aluseks mitmete inimkäitumise oluliste aspektide nagu sõltuvuskäitumine, impulsiivsus, depressioon *etc*, neurobioloogiliste korrelaatide uuringutes.

Dopamiini signaaliülekanne rakku toimib läbi 7-transmembraansete G-valkudega seotud retseptorite. Retseptorite aktiveerimisel toimub nendega seonduvatel G-valkudel neile puhkeolekus seostunud guanosiin 5'-difosfaadi (GDP) dissotsiatsioon ning guanosiin 5'-trifosfaadi (GTP) assotsiatsioon. Assotsiatsiooni määra – ning sellega seotult signaaliülekande tundlikkust – on võimalik mõõta kui asendada GTP radioaktiivse analoogiga, kus viimasel fosfaatrühmal on üks hapnik asendatud radioaktiivse väävliga. Sellist ühendit pole G-valgud suutelised hüdrolüüsima ning kui eraldada akumuleerunud [^{35}S]GTPγS-G valk kompleksid reaktsioonikeskkonnast, ongi võimalik signaaliülekande määra kvantitatiivselt hinnata.

Viiest dopamiini retseptorist, mis jagunevad kahte perekonda (D1-perekond D_1 ja D_5 retseptoritega ning D2-perekond D_2, D_3 ja D_4 retseptoritega, mis vastavalt aktiveerivad ja inhibeerivad sekundaarset virgatsainet adenosiin 3',5'-tsüklilist monofosfaati tootvat ensüümi adenülüül tsüklaasi), on seoses sõltuvusning motiveeritud käitumisega ilmselt enim uuritud D_2 retseptorit, mille võime funktsioneerida nii pre- kui postsünaptilise retseptorina annab talle olulise rolli dopamiinergilise signaaliülekande moduleerimises. Mitmes rottide uudistamisaktiivsuse mudelis on näidatud tundlikumat dopamiini signaaliülekannet ning suuremat D_2 retseptori kõrge afiinsusega sidumiskohtade arvu suurema uudistamisaktiivsusega isenditel, samuti on positronemissioontomograafiaga demonstreeritud muutunud D_2 retseptorite sidumispotentsiaali ravimisõltlastel.

Käeoleva töö eesmärgiks oli uurida roti aju dopamiinergilist signaaliülekannet läbi D_2 retseptori erineva käitumusliku seadumusega rottidel. Uuriti nii uudsusega seotud käitumuslike seadumuste (LE/HE mudel) kui hüvituskäitumisega seotud seadumuste (suhkrutarbimine; LC/HC mudel) seost D_2 retseptori signaaliülekandega aju naalduvas tuumas ning selgmises juttkehas,

samuti uuriti stressi mõju D_2 retseptorite signaaliülekandele. Lisaks analüüsiti D_2 retseptori signaaliülekande tundlikkust LE/HE mudelis tingimustel, kus neile oli manustatud (korduvalt) amfetamiini (vabastab dopamiini sünapsisse) või (ühekordselt) noradrenergilist neurotoksiini DSP-4. Selleks kohandati edukalt [^{35}S]GTPγS sidumisanalüüs mõõtmaks D_2-sõltuvat G valkude aktivatsiooni aju-membraanides ning rakendati antud meetodit ka uute arvatavate D_2-selektiivsete ravimite iseloomustamiseks hiina hamstri munasarja rakkudes.

Leiti, et D_2 retseptorite signaaliülekande tundlikkus korreleerub sarrustile reageerimise tundlikkusega juhul, kui rotil on vaba valik hüvitit (suhkrulahus) tarbida ning selleks tuleb teha ka motoorseid pingutusi. Kui eraldada hüvekäitumise katses motoorne komponent (rotte kõdistades), kaob ka korrelatsioon. Uudsusega seotud käitumiskatses ei olnud küll algselt vahet madala ning kõrge uudistamisaktiivsusega (LE/HE) rottide D_2 retseptori signaaliülekande tundlikkuses, kui vahe ilmnes pärast korduvat dopamiinergilist manipulatsiooni amfetamiiniga, mis näitas, et nö stressisituatsioonis adapteerub dopamiinergiline signaaliülekanne läbi D_2 retseptori neil rottidel erinevalt, nimelt nii, et HE loomadel vähendab amfetamiini korduvmanustamine D_2 retseptori signaaliülekande tundlikkust ning see on hästi korrelatsioonis ka käitumusliku desensitisatsiooni tekkega.

Nii kõdistamisele kui stressile reageerimise puhul avaldus selge vahe D_2 ning D_1 retseptori signaaliülekande tundlikkuse muutustes, mis viitab nende retseptorite erinevale osalusele sarrustatud käitumise eri aspektide vahendamisel, mis omakorda tõstatab vajaduse alatüüp-spetsiifiliste ligandide järgi, uurimaks vastavate retseptorite rolli *in vivo*, käitumiskatsetes.

D_2 retseptorit ekspresseerivates hiina hamstri munasarja rakkudes oli [^{35}S]GTPγS sidumise analüüsi näol tegemist hästi reprodutseeritava analüüsimeetodiga, mis sobis hästi kirjeldamaks uusi potentsiaalselt D_2-ergilisi ligande, mis olid sünteesitud lähtudes norapomorfiinist. Asendis 2 4-hüdroksüfenüülrühma omavad ligandid osutusid küllaltki afiinseteks ning efektiivseteks, samuti suhteliselt selektiivseteks (võrreldes D_1-ga). Kui need ligandid peaksid osutuma D_2-selektiivseteks ka võrdluses teiste retseptoritega ning nende farmakokineetilised parameetrid oleksid sobivad, saaks sünteesitud ühendeid vaadelda potentsiaalsete kandidaatidena nii ravimitööstusele kui ka käitumiskatsetesse D_2-spetsiifiliste efektide uurimiseks.

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PUBLICATIONS

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