DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
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Slow conformational changes in dopamine transporter interaction with its ligands

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“It is good to have an end to journey towards;
but it is the journey that matters in the end.”

Ursula K. Le Guin

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3. **Stepanov, V.**, Järv J. (2006) Slow isomerization step in the interaction between mouse dopamine transporter and dopamine re-uptake inhibitor N-(3-iodoprop-2E-enyl)-2β-carbo-[3H]methoxy-3β-(4'-methylphenyl)nortropane. Neurosci Lett., 410, 218–221

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ABBREVIATIONS

ADHD	–	attention-deficient hyperactivity disorder
[A*]	–	reporter ligand
CNS	–	central nervous system
DA	–	dopamine
DAT	–	dopamine transporter
GABA	–	γ-aminobutyric acid
GBR12909	–	1-(2-(bis-(4-fluorophenyl)methoxy)ethyl)-4-(3-phenyl-propyl)piperazine
GPCR	–	G protein-coupled receptor
K_A	–	dissociation constant
K_D	–	apparent dissociation constant
K_I	–	isomerisation "equilibrium" constant
k_{obs}	–	apparent association reaction constant
k_1	–	isomerisation constant
k_{-1}	–	reverse-isomerisation constant
MPP+	–	1-methyl-4-phenylpyridinium
NET	–	norepinephrine transporter
PE2I	–	N-(3-iodoprop-2E-enyl)-2β-carbomethoxy-3β-(4-methyl-phenyl)nortropane
PET	–	positron emission tomography
SERT	–	serotonin transporter
TM	–	transmembrane segment
[B]	–	unlabeled ligand
7-TM	–	7-transmembrane segment protein
^3H	–	tritium

I. INTRODUCTION

In the last fifty or so years the neurodegenerative diseases and drug abuse made a tremendous impact on the society as a whole, placing significant economic burden on the entire counties, if not the whole world. The challenge of fighting both neurodegenerative diseases and addiction conditions is the complexity of the underlying mechanisms that are involved in both of them (Pepper 2009; Garbutt 2009; Kogan 2007). Any attempt to interfere with brain function from outside quickly degenerates into "push-pull" situation, where gains are always off-set by some negative effect of the medication, like in the case of L-DOPA treatment for Parkinson's disease (Stowe 2008).

Despite the tremendous progress in life sciences that has significantly advanced our knowledge of the role of neurotransmitter systems in CNS function, including the phenomena involving dopaminergic nerve terminals, there are still many gaps that need to be filled and obstacles that have to be overcome before a complete picture emerges. Dopamine and the signal transduction pathways centered around it have been the focus of life scientists' attention for quite a while, as disturbances in functioning of dopaminergic system are linked to numerous pathologies, including schizophrenia, ADHD, Parkinson's disease and others neurodegenerative disorders (Leriche 2004; Seeman 2009; Jenner 2003). The main molecular components of this neurotransmitter system are dopamine receptors and dopamine transporters (DAT). If particular advances have been made in description of dopamine receptor families (Menelas & Davies 2001), still much is still known about the exact molecular structure, mechanism and specificity of action of the DAT protein, although its implication in a wide range of medical conditions and disorders have been recently well-documented (aan het Rot 2009; Zhou 2009; Seeman 1990; Ginovart 1997).

The X-ray crystallography confirmation of the Leu transporter structure was a significant breakthrough in the area, but even so, detailed elucidation DAT-substrate/inhibitor interaction mechanisms is not easy (Yamashita 2005; Singh 2007; Beuming 2008). The DAT protein is a major player in cocaine and amphetamine addictions and the negative impact of cocaine addiction on society is hard to overestimate (Goldstein 2009). Recent advances in diagnostic and treatment application of dopaminergic ligands have added to the need of more thorough characterisation of their interaction with the components of dopaminergic system and while the molecular biology is a potent tool for protein function investigation, the underlying molecular mechanisms protein interactions with small ligands can only be studied using methods of physical biochemistry. In this study we have undertaken the first attempt to use systematic kinetic analysis to characterize the ligand-protein interactions for DAT from rat/mouse brain striatum.

2. LITERATURE OVERVIEW

2.1. Dopamine transporter protein: structure and function

Dopamine transporter protein is a multi-domain trans-membrane protein belonging to the Na^+/Cl^- dependent transporter family (SCDNT), sharing several core characteristics with other members of the group like the 5-HT transporter (SERT), GABA transporter [GAT(1–3)], norepinephrine transporter (NET) and some others (Nelson 1998; Blakely 1992; Torres 2007; Yamashita 2005). Prime function of the DAT protein is to remove dopamine (structure of this ligand as well as some other compounds mentioned in this work are shown in Scheme 1, page 12) from the synaptic cleft after it has been released from presynaptic terminal during neuron firing. Rapid removal of signalling molecule (dopamine) after the active phase of signal transmission significantly reduces synapse "recycle time" and speeds up firing rate of the neuronal circuit. Comparison techniques and hydropathy analysis done in analogue with G protein-coupled receptors and other membrane proteins indicated early on that the transporter has 12 hydrophobic transmembrane (TM) segments, connected by 11 alternating intracellular and extracellular loops, with both C- and N-terminals located in the cytosol inside the cell, with total of approximately 620 amino acids residues (the exact number depends on the species in question (Chen 2000).

The recently published X-ray crystallographic data for the bacterial leucine transporter $LeuT_{Aa}$ from *Aquifex aeolicus* confirms those predictions and provides reasonable high-resolution structure analogue for DAT, supplemented by structural data for glutamate transporter analogue from *Pyrococcus horikoshii* (Yamashita 2005; Yernool 2004). Limited structural data is also available derived from comparison between DA_T and *Escherichia Coli* Na^+/H^+ antiporter (Ravna 2003; Dahl 2004). DA_T has been cloned from several mammal species, including human, monkey, rat, mouse and cow (Usdin 1991; Giros 1991; Giros 1992, for review on cloning of neurotransmitter transporters see Blakely 1992). Functional DAT proteins from *C. elegans* and *Eloria noyesi* also have been characterized (Kilty 1991; Chen 2006). Chen and co-workers result is quite interesting due the fact that *E. noyesi* actually feeds on the leaves of coca plants containing significant amounts of DA_T inhibitor cocaine, while cloned dopamine transporter has similar affinity for cocaine compared to other species (Chen 2006).

Dopamine transporters from different mammalian species display significant homology between species (up to 90%), the homology between DA_T of species belonging to different classes is smaller, for example DAT from *C. elegans* shares only 43% of its sequence with hDAT. Dopamine transporters taken as a general group also possess significant homology (reaching 50%) with other membrane monoamine transporters (SERT, GAT and NET showing numerous

conserved segments, possibly indicating that the said segments are important for transport function (Chen 2000; Usdin 1991; Miller 2001). Even with the lack of crystallographic structural data numerous site-directed mutagenesis studies were conducted and over the years allowed certain insight into functions of DAT. One of the most important facts site-directed mutagenesis established is that numerous binding sites exist within DAT for various types of ligands, both that are transported by DAT and also DAT inhibitors (Lin 1999; Uhl & Lin 2003; Ukairo 2005;).

Due to the high homology between DAT of different mammalian species, the findings of side-directed mutagenesis studies, in particularly those dealing cross-species conserved fragments can be broadly applied to any of the mammalian DAT proteins subtypes (Chen 2000, for extensive review see Voltz 2005). The NH_2 terminal plays significant role in the Na^+/Cl^- ion exchange processes in the catecholamine transporters, partially being responsible for the ionic dependence of dopamine uptake with the carboxyl-terminated tail part also playing some role in the transport process as its truncation from Pro^{597} downward results in bi-phasic dopamine uptake curves and decreased affinity for dopamine, while also significantly reducing high-affinity components of β-CFT binding in rDAT and hDAT (Syringas 2001; Lee 1996). In TM domains following substitutions of corresponding amino acids to alanine generally result in binding affinity decrease for cocaine-analogues while leaving dopamine affinity for hDAT relatively intact: TM2: Phe^{98}, Pro^{101}, Phe^{105}; TM3: Phe^{154}; TM4: Tyr^{251}; TM5: Tyr^{273}; TM6: Thr^{315}, Gln^{316}, Phe^{331}; TM7: Phe^{361}; TM8: Trp^{406}, Phe^{410}; TM9: Thr^{455}, Ser^{459}, Thr^{464}; TM1: Trp^{519}, Trp^{523}, Phe^{530}, while similar effects are less pronounced or absent in extracellular loops (Uhl & Lin 2003). Mutation at aforementioned residues also influence dopamine transport in mDAT, the Phe^{105} to Ala/Leu/Ile/Ser replacement creates transporters with low transport activity, while Phe^{105} to Tyr/Trp retained 75% of dopamine transport activity and high affinity for cocaine (Wu & Gu 2003). It is generally recognized that phenylalanine residues play significant role in selective influence of dopamine transport and cocaine analogs recognition: point mutations of Phe to Ala in positions 76, 98, 390 and 361 result in normal (wild-type) dopamine affinity for rDAT but reduce affinity of β-CFT (cocaine analogue) 3-to-8-fold; while Phe^{155} to Ala substitution reduced affinity of dopamine in rDAT more than 10-fold, the affinity decrease for β-CFT was significantly less in comparison (Lin 1999). Tryptophan residues in positions 162, 255 and 310 also play some role in dopamine transport process, as their substitution for alanine reduced dopamine affinity in uptake process, while replacement of Trp to Ala in positions 406, 496 and 523 reduced affinity for β-CFT without affecting dopamine uptake (Lin 2000). Affinity and uptake velocity are not affected in the same way by mutations introduced in DA_T: while affinity of DAT for transportable substance MPP^+ can be reduced via Tyr^{533} substitution for Phe, the transport velocity of the corresponding substance by the mutant transporter is increased; the same residue plays important role in the differential sensitivity

mechanism of rDAT and hDAT for cocaine and MPP^+, highlighting again the issue of multiple binding sites for different agents (Mitsuhata 1998).

Residues that are conserved throughout all Na^+/Cl^- dependent monoamine transporters are considered to be of special interest in relevance to molecular mechanism of substance recognition and transport, mutations at Asp^{79} in DA_T (conserved throughout aforementioned transporter family) differentiate mechanism of action of benztropine and other common DAT inhibitors of cocaine/mazindol type (Ukairo 2005). Other published data support topologically different or partially overlapping sites for different inhibitors of mDAT (or DAT in general for that matter), particularly for cocaine/MPP^+ and amphetamine-like ligands (Chen 2005). Other groups have pointed out that some of the amino acid residues, such as conserved cysteines (positions 180 and 189 in particular), while not affecting binding and transport in a significant way, have considerable influence on functional transporter expression, indicating that they might be actively involved in process of DA_T protein transportation and incorporation into the cellular membrane (Wang 1995; Chen 2005). Tyr^{335} has been shown to be involved in the regulation of the conformational states during transport phase (Loland 2002).

It was also shown that the entire protein (DAT) is not needed to archive at least some (or in some cases "wild-type") affinity for ligands, as separate part of the protein incorporated into cell membrane are capable of binding radioactively labeled compounds such as [^{125}I]DEEP and [^{125}I]RTI-82 (Vaughan & Kuhar, 1996). Zn^{2+} ions play significant role in the DAT function, as Zn^{2+} ion presence is necessary in the zinc-finger domain for proper transport functionality (Bjorklund 2007; Nørgaard-Nielsen 2006). The regulation of the DAT protein within the cell is both important and obscure topic of the on-going research. 4 DAT units appear to form tetramer within the cellular membrane and the up- and down-regulation of DAT activity and density within the cell is regulated in part by phosphorylation of the protein (Hastrup 2003; Gorentla 2009; Zhang 2009; Mortensen 2003). The cross-talk between DAT protein and GPCRs has also been noted (Bolan 2007). However, this area of research is hindered by the fact that there are many "unknowns" in the equation, such as possible existence of chaperone proteins or small proteins capable of attenuating DAT function (Mortensen 2003).

2.2. DAT in pathological conditions and its use in diagnostics

With dopamine transporter being the integral part of all neuronal circuits dependent dopamine for signal transduction and unclear regulatory mechanisms of the transporter function, the dopamine transporter can easily be involved in variety of pathological conditions in humans. Dopamine transporter levels correlate well with intensity of symptoms of attention deficit hyperactivity disorder (ADHD), drug addictions, schizophrenia, Huntington's and Parkinson's dis-

eases (Krause 2008; Meisenzahl 2007; Ginovart 2007; Nutt 2004; Rothman 2003). DAT has also been shown to play important role in processes of memory and memory impairment with age (Erixon-Lindroth 2005). In view of large amount of data accumulated implicating DAT in a variety of pathological neurological conditions and emerging data shedding light on its detailed structure, DAT presents a noteworthy target for drug development and further research (Rothman 2008; Runyon 2006; Dutta 2003).

As dopamine transporter levels correlate with symptom intensity, it is an obvious biomarker candidate for the diagnosis/monitoring of the conditions mentioned above. The positron emission tomography (PET) and single-photon emission tomography (SPECT) are an obvious choice for non-invasive imaging of the DAT. PET can be used to detect and quantitize ligands and corresponding receptors and transporters from nanomolar to picomolar range (Kessler 2003; Garcia-Alloza 2004; van Waarde 2000). A considerable number of ligands have been synthesised for PET imaging of DAT, most of them are either tropane derivates based around naturally occurring DAT ligand cocaine, or rimcazole and GBR12909 analogues (Newman 2002; Halldin 2003; Riss 2009; Elsinga 2006).

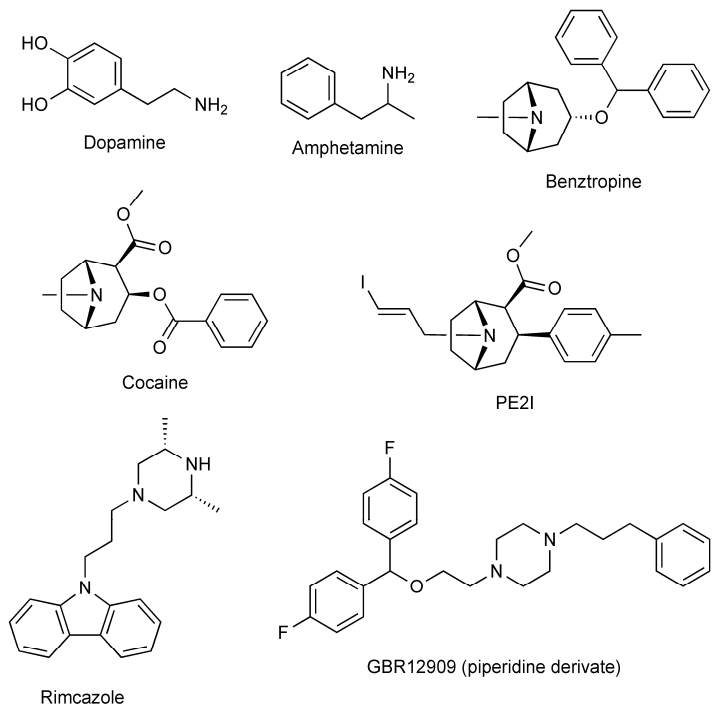
2.3. Dopamine re-uptake inhibitors and substrates

There is a wide range of structurally different compound classes that display affinity for DAT, many (if not all) also display significant affinity for other monoamine transporters (Glatt 2003; Rothman 2003; Runyon 2006; Riss 2009). It should be mentioned that there are two primary ways in which ligands modulate DAT: dopamine uptake inhibitors – that is cocaine and cocaine-analogues (both in structural and physiological term) disrupt dopamine re-uptake from synaptic cleft after its release from presynaptic neuron by docking with transporter and not being transported and therefore effectively stopping dopamine uptake, while amphetamine-like compounds are in effect "releasers" – they disrupt VMAT and DAT not only preventing dopamine re-uptake but contributing to increase of dopamine levels in the synaptic cleft (Rothman 2003; Riddle 2005).

The oldest dopamine uptake inhibitor known is cocaine (scheme 1 – cocaine), a natural compound found in the leaves of *Erythroxylum coca* - scrub bush native to South America, was discovered by the South American Indians in pre-Columbian era. The compound itself was isolated in 1860. Systematic work with cocaine analogs that affect CNS has begun in the 70's, starting with the work of Clarke and co-workers, where a phenyl group has been attached directly to the tropane cycle at C-3 position along with the elimination of cocaine ester fragment at C-3 has resulted in significant increase of biological activity (Clarke 1973). Since then tropane ring with (multi)-substituted aromatic group at C-3 has become one of the "corner-stones" for dopamine re-uptake inhibitor design (Ametamey 1995; Goodman 1994; Kim 2003; Newman 2002; Riss

2009). Two other classes of compounds that exhibit high selectivity and affinity for DAT are benztropines and piperidine derivatives (scheme 1 – benztropine and GBR12909) (Newman 2002; Foulon 1992; van der Zee 1980; Horn 1971; Simoni 2005). While all three groups can loosely be considered structural analogues of each other (along with rimcazole-type (scheme 1 - rimcazole) compounds (Izenwasser 1993) and tend to follow similar trends in term of affinity change with introduction of halogens and other substituents at aromatic fragments, their structure-activity relationship regarding affinity towards DAT can differ greatly. For 3-aryl tropanes the main requirement for high-affinity for DAT is that stereochemistry at C-2 and C-3 is β and the overall stereochemistry must be R(-)- (Carroll 1992; Wang 1993). DAT also appears to tolerate significant variation in the substituent at C-2 position; however no substituent at C-2 makes 3-aryl tropanes inactive at DAT (Carroll 1992; Davies 1993; Kozikowsky 1998; Kozikowsky 1995).

Quite in the opposite, the active stereoisomers of benztropine must have α-configuration at C-3 and S(+)- stereochemistry, otherwise they are inactive (see Newman 2002; Horn 1971). Introduction of substituents (like methyl/ethyl groups and halogens) into aromatic fragments of both 3-aryl tropanes and benztropines generally improves affinity for monoamine transporters and affects selectivity for specific transporters and receptors (Newman & Kulkarni 2002; van der Zee 1980; Kozikowsky 1998; Carroll 1991).



Scheme 1. Some of the examples of the ligands acting on the dopamine transporter.

The general rules for tropane-based cocaine analogues are as follows: the addition of (un)substituted aromatic cycle at C-3 with β-stereochemistry greatly improves ligand affinity towards monoamine transporters in general, while alkyl chain with substituents attached to basic nitrogen has significant impact on ligand specificity for a particular transporter (Kozikowsky 1998; Carroll 1991; Milius 1991).

However, while many of the 3-aryl tropanes are very potent dopamine reuptake inhibitors, in their majority they are relatively unselective and possess significant affinity towards other monoamine transporters. In view of the considerations above, numerous systematic studies have been undertaken in order to optimize structure for both affinity and selectivity with hope of finding better ligands for DAT that could be used in both *in vitro* and *in vivo* applications (Kozikowsky 1998; Milius 1991; Emond 1997; Bülow 2005).

Among numerous ligands that were evaluated for this purpose by several groups (see above) one in particular found expensive application for quantification of DAT density in *in vivo* applications – *N*-(3-iodoprop-2*E*-enyl)-2β-carbomethoxy-3β-(4'-methylphenyl)nortropane (scheme 1 – PE2I), originally synthesized by Emond and co-workers (Emond 1997). The ligand acquired the name PE2I and its behavior was studied extensively in both animal models and humans, labeled with Iodine-123 and 125, and also with carbon-11 and tritium (Emond 1997; Hall 1999; Emond 2008; Stepanov 2007). The compound *N*-(3-iodoprop-2E-enyl)-2β-carbomethoxy-3β-(4'-methylphenyl) nortropane (PE2I) synthesized by Emond and co-workers possesses both high affinity and selectivity for dopamine transporter (K_d=17 nM, 5-HTT/DAT=32 vs 0.1 for β-CIT), and as seen from number of articles received considerable attention due to its favourable profile as a dual use PET/SPECT ligand.

While carbon-11 and iodine-123 are useful isotopes for PET and SPECT imaging, they are hardly usable for *in vitro* studies, and the use of iodine-125 can also be problematic due to high biological risks associated with exposure to iodine-125, high penetration factor of the radiation given off and relatively short half-life, however high specific activity of iodine-125 is a definite plus. The fact that for typical *in vitro* applications specific activity in order of 70 Ci/mmol is often quite sufficient and tritium label is considered much less hazardous in terms of radiation (very low-penetrating β^- emissions compared to other isotopes often used), and long half-life of tritium ($t_{0.5}$=13.1 years) made labeling of PE2I with tritium at carboxyl-terminal a viable proposition for obtaining selective DAT ligand for *in vitro* use.

2.4. Principles of ligand interaction with G Protein-coupled receptors

The G protein-coupled receptors are one of the most numerous receptor classes, the GPCR superfamily consists of 6 classes (A to F) with over 350 GPCR encoded by human genome (Kolakowski 1994). The structure of the GPCR is well-established, the receptors themselves consist of 7 transmembrane domains spanning the cell membrane, interconnected by outlying fragments (Grigorieff 1996; Rasmussen 2007). The GPCR function though binding of response-inducing ligands (agonists) and subsequent activation of the receptor-bound G-protein though conformational changes in the receptor that in turns initiates a further signalling cascade (Gilchrist 2007).

The "mechanics" of the GPCR's were extensively studied, with muscarinic receptors receiving a "pharmacological definition" as early as 1914 (Dale 1914). Since original work of H. Dale several models of varying complexity were proposed, ranging from single-step association model, to highly complex models involving cooperative and allosteric interactions of multiple sites found on a functional receptor unit (De Amici 2009; Strickland 1974).

The mathematical complexity of the more sophisticated models, however, often leads to the appearance of a significant number of degrees of freedom, making those models difficult, if not impossible, to analyse (Christopoulos 2002). When attempting to use GPCR as a model for more complex ligand-binding protein, such as the DAT protein, we need to focus our attention at the core models that lie in the heart of more complex systems built around those basic mechanisms.

The simplest model of interaction of GPCR and its ligand that can in turn be applied to DAT and its ligands is simple one-step association:

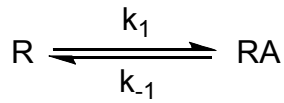


Figure 1. One-step association reaction scheme for protein-ligand interaction.

In this simplest scheme of all cases the R denotes protein molecule and A is the ligand. The k_1 is the rate constant of the association reaction - the on-rate constant, and k_{-1} is rate constant of dissociation reaction – the off-rate constant. Defining dissociation constant K_D as inverse equilibrium constant:

$$K_D = \frac{k_{-1}}{k_1} = \frac{[R] \cdot [A]}{[RA]} \tag{1}$$

and applying appropriate mathematical apparatus, the solution for equilibrium situation and kinetics can be obtained. Amount of receptor-ligand complex in relation to free ligand and total amount of receptor sites is defined by equation:

$$[RA] = \frac{[RA]_{MAX} \cdot [A]}{K_D + [A]} \tag{2}$$

This expression can be viewed as modified Michaelis-Menten equation, where $[RA]_{MAX}$ is the total amount of binding sites detectable and [A] is concentration of free ligand in solution. Kinetics of the association and dissociation processes are described by equations (3) and (4) respectively:

$$[RA] = [RA]_{MAX} \cdot \left(1 - e^{-k_{obs} \cdot t}\right) \tag{3}$$

$$[RA] = [RA]_{MAX} \cdot e^{-k_{-1} \cdot t} \tag{4}$$

In equations (3) and (4) $[RA]_{MAX}$ is maximum number of detectable binding sites, k_{obs} is the observed rate constant for association reaction and k_{-1} is rate constant of dissociation reaction. Dependence of k_{obs} on free ligand concentration is linear and is given by equation (5):

$$k_{obs} = k_1 \cdot [A] + k_{-1} \tag{5}$$

It is important to stress that in the case of one-step association the dependence of k_{obs} vs [A] is linear.

After a period of several decades when single-step model enjoyed universal acceptance in the academic circles, a significant body of evidence has been accumulated concerning the fact that receptor-ligand interactions do not follow scheme (1), shown beyond doubt by the fact that in some systems k_{obs} relation to free ligand deviate from linear and usually assumed hyperbolic shape (Järv 1979; Järv 1980; Schreiber 1984). This problem was analyzed earlier by Strickland and co-workers for enzyme reactions and resulted in introduction of two-step mechanism (Strickland 1974). According to this model receptor-ligand interaction has two steps: first – fast association of ligand with receptor, and second – a slow transformation of the previous complex into another complex. This model can be presented by the following scheme:

$$R \underset{}{\overset{K_A}{\rightleftharpoons}} RA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (RA)$$

Figure 2. Two-step model for protein-ligand interaction containing a slow isomerisation step.

where K_A is the "true" dissociation constant, and k_2 and k_{-2} are isomerization and "reverse"-isomerization rate constants for the (RA) complex. The RA complex is often refereed to as fast-dissociating complex and the (RA) as slow-dissociating one, reflecting the situation where the RA complex can not be determined by "slow" analysis methods such as filtration on glass-fibre filters or centrifugation, as these methods are only capable of detecting the meta-stable (RA) complex. In principle, RA can be visualized using "real-time" methods, such as florescent techniques (Swaminath 2003; Nakanishi 2006; Granier 2007). Similarly to the previously discussed model amount of (RA) is given by equation (6) that is similar to equation (2):

$$[(RA)] = \frac{\frac{[(RA)]_{MAX}}{K_I + 1} \cdot [A]}{K_D + [A]} \tag{6}$$

In this equation K_i is constant defined by relationship k_{-1}/k_1. The K_D in equation (6) also has different meaning if compared to that in equations (1) and (2):

$$K_D = \frac{K_A \cdot K_I}{K_I + 1} \tag{7}$$

where $K_A = [RA]/([A] \cdot [R])$. In case when k_{-1} is small and can be neglected K_D can be approximated as $K_A \cdot K_I$. The kinetic parameters of system described by Fig. 2 are represented by equation (8) and (9), corresponding to association and dissociation processes:

$$[RA] = [RA]_{MAX} \cdot \left(1 - e^{-k_{obs} \cdot t}\right) \tag{8}$$

$$[RA] = [RA]_{MAX} \cdot e^{-k_{-1} \cdot t} \tag{9}$$

It is important to stress here that the presence of the isomerisation step means that only isomerised protein-ligand complex can be detected under slow separation conditions, in contrast to the case described by Figure 1, where all sites occupied by the ligand are (theoretically) detected under assay condition.

The parameter k_{obs} in the equation (8) is equal to:

$$k_{obs} = \frac{k_1 \cdot [A]}{K_A + [A]} + k_{-1} \tag{10}$$

It can be seen that while equation (5) gives linear dependence of k_{obs} from the free ligand concentration [A], the equation (10) represents hyperbolical rela-

tionship between the same values, and in perfect correlation with experimental data (Järv 1979; Järv 1980; Schreiber 1984; Järv 1990). This particular model including slow confromational change (isomerization step) was further developed by Järv and Eller to include second slow isomerization stage (Järv 1989). Cooperative effects observed in ligand-receptor interactions at muscarinic receptors, when binding of several ligands to single receptor increases speed of conformational transition from fast-dissociating complex to slow-dissociating one (Sillard 1987; Krejcí 2004).

The allosteric interactions in case of the GPCR are also assumed to play an important role in the protein-ligand interactions, contributing significantly to ligand specificity, pharmacological activity and affinity both *in* vivo and *in vitro* (Chistopoulos 2002; May 2007; De Amici 2009). The idea that several ligands of different types, that is agonist and antagonist, can bind simultaneously to single receptor was brought forward by Järv and co-workers (Järv 1980), when it was shown that presence of agonist does not affect equilibrium phase of antagonist-receptor association process characterized by K_A. This model was developed further in more recent publications with purpose of generalizing and expanding it (Järv 1995; Oras 1999). The model assumes that agonistic and antagonistic effects of drugs are related to their interaction with two distinct and separate binding sites that exist simultaneously on a single receptor molecule and possess different ligand specificity patterns. As these sites are simultaneously present on a single functional receptor assembly, the formation of a ternary complex between agonist, antagonist and receptor is possible, while all other classical effects such as displacement of one ligand by another are retained (Järv 1995).

3. OBJECTIVES OF DISSERTATION

The main objectives of this project were:

1. Adoption of the methods of kinetic analysis of GPCR-ligand interaction to the investigation of DAT-ligand interactions.
2. Development of a new selective tritium-labeled ligand for the dopamine transporter protein for in vitro use.
3. Investigation into mechanisms of interaction between different DAT inhibitors and substrates with the dopamine transporter protein.
4. Development of models for the abovementioned interactions and examination of their possible relevance for goal-oriented ligand design.

4. MATERIALS AND METHODS

4.1. Chemicals

The cocaine hydrochloride was obtained from Tamro AS, Estonia. The [^{3}H]PE2I (specific activity 74 Ci/mmol) was synthesized from [^{3}H]methyl iodide (Amersham UK) and des-metyl PE2I precursor (PharmaSynth AS, Estonia). The exact labeling and synthetic procedure for the synthesis of PE2I (N-(3-iodoprop-(2E)-enyl-2β-carbomethoxy-3β-4-(methylphenyl)nortropane) is described elsewhere (Stepanov 2007). Amphetamine hydrochloride was a gift from professor A. Zarkovski, University of Tartu. Other reagents, such as dopamine, HEPES and buffer salts were of highest commercial grade and purchased from Sigma-Aldrich, USA. The composition of assay buffer used throughout the study was as follows: 30 mM HEPES, 120 mM NaCl, 5 mM KCl, pH 7.40 at 25 C.

4.2. Dopamine transporter protein

The DAT protein used in the binding, displacement and kinetic assays was obtained as a membrane preparation of rat striatal membranes. For the preparation of the striatal tissue suspension female Winstar rats (3–5 months old) were decapitated, the striatum regions of the brain were rapidly dissected on a cold block, the tissue homogenized in the ice-cold incubation buffer and centrifuged at 30.000 g for 20 min 4 times, with supernatant discarded, and the resulting pellet resuspended in the incubation buffer after each centrifugation. Procedures involving live animals were carried out in accordance with Estonian Law and the European Council Directive of 24.11.1986 (86/609/EEC).

4.3. Equilibrium and kinetic assays

The membrane-bound [3H]PE2I was assayed by the conventional filtration method, using the Whatman GF/B filters, pretreated with 0.3% polyethyleneimine. In binding studies 0.2–0.4 mg striatal membrane fragments were incubated with various radioligand concentrations in the assay buffer at 25 °C during 30 min and rapidly filtered and washed with 10 ml of ice-cold phosphate buffer (100 mM NaCl, 20 mM phosphate, pH 7.4) using Brandel M-24S cell harvester. The filters were transferred into scintillation vials and 5 ml scintillation cocktail (ScintiSafe 3, Fisher Scientific, UK) was added. The filters were equilibrated in the cocktail for 12 h before counting. The filter-bound radioactivity was measured on a LKB Wallac 1219 Rackbeta liquid scintillation counter at 43% tritium counting efficiency with the scintillation cocktail used. Kinetics of [^{3}H]PE2I association with DAT sites on membrane fragments was

measured as described for [^{3}H]PE2I kinetic assay in our papers. The time course of the ligand association process was monitored by taking 100 µl aliquots from this reaction mixture. These aliquots were immediately diluted into 15 ml of ice-cold phosphate buffer to stop the process (time moments t) and rapidly applied onto GF/B filters. The filters were washed with 10 ml of ice-cold phosphate buffer and the bound radioactivity was measured as described above. The first samples were analyzed during 5–10 s after launching the process, and the time course of radioligand association was monitored during 3–15 min at 25 °C. Under the same assay conditions it was found that the non-specific radioligand binding was constant during the assay time, while its level depended linearly on radioligand concentration.

Kinetic analysis of association of non-radioactive GBR12909 was performed by using [^{3}H]PE2I as reporter ligand. Set-up of these experiments was as presented above, except that the reaction mixture contained various amounts of non-radioactive GBR12909 (from 0 to 43 nM) and constant concentration (3.5 nM) of radioactive PE2I was used. Shortly: 400 µl of mixture of GBR 12909 (various concentrations) and [^{3}H]PE2I (constant concentration in all runs) was added into 1800 µl of membrane homogenate to initiate the reaction ($t = 0$). Thereafter 100 µl aliquots were taken from this reaction mixture and diluted into 15 ml of ice-cold buffer to stop the process at the appropriate time moments (time t) and rapidly applied onto GF/B filters. The first data-points were collected after 5–10 s of reaction initiation and the process was followed during up to 15 min. The filter-bound radioactivity was counted as described above.

Kinetic analysis of the [^{3}H]PE2I association with striatal membranes in the absence and in the presence of cocaine, amphetamine and dopamine was performed by adding tracer and non-radioactive ligands simultaneously to the suspension of the rat striatal tissue and the process of the association of the [^{3}H]PE2I being monitored by taking 100 µL aliquots from the reaction mixture, filtering them on Whatman GF/B filters and washing with 15 ml of ice-cold phosphate buffer. The first samples were taken 5–15 seconds after initiating the process, and association was followed for 3–15 minutes (depending on the radioligand concentration). The influence of cocaine and amphetamine on [^{3}H]PE2I association with rDAT was measured varying both radioligand and non-radioactive ligand concentrations.

4.4. Kinetic formalism for [3H]PE2I binding with *r*DAT

The binding of unlabeled PE2I, the [^{125}I]PE2I and [^{11}C]PE2I has been shown to be specific and reversible in membrane preparations, brain slices and whole brain in vivo (Hall 1999; Emond 1997; Halldin 2003). Our work has also shown applicability of two-step binding models involving a slow isomerisation step developed for the GPCR's for the description of the [^{3}H]PE2I binding with rat

dopamine transporter (Stepanov, 2006). The binding of the [^{3}H]PE2I follows the following scheme (Fig. 3):

$$DAT \underset{}{\overset{K_A}{\rightleftharpoons}} DAT\text{-}A^* \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (DAT\text{-}A^*)$$

Figure 3. Two-step model containing a slow isomerisation step for DAT interaction with its ligand.

where DAT is the dopamine transporter and A* is the [^{3}H]PE2I. The fast equilibrium step is described by the dissociation constant K_A and the isomerization step by the kinetic constants k_1 and k_{-1}. The equilibrium binding process for such a model is described by the equation:

$$[(DAT - A^*)] = \frac{[(DAT - A^*)]_{MAX} \cdot [A^*]}{K_D + [A]} \tag{11}$$

and the kinetic behavior is governed by following expressions for the on- and off-rate reactions respectively:

$$[(DAT - A^*)] = [(DAT - A^*)]_{MAX} \cdot \left(1 - e^{-k_{obs} \cdot t}\right) \tag{12}$$

$$[(DAT - A^*)] = [(DAT - A^*)]_{MAX} \cdot e^{-k_{-1} \cdot t} \tag{13}$$

with relationship k_{obs} vs [A*] following hyperbolic curve:

$$k_{obs} = \frac{k_1 \cdot [A^*]}{K_A + [A^*]} + k_{-1} \tag{14}$$

It is important to note that this particular mechanism underpins the use of radioactive tracers in general as quick first-stage equilibrium, characterized by affinity constant K_A, does not allow the resulting complex DAT-A* to be determined by filtration, leaving only isomerised complex (DAT-A*) as measurable quantity.

If we want to adapt this model for description of the simultaneous interaction of two ligands with DAT protein while assuming that both ligands compete for the same binding site on the protein, two approaches are possible. The first is to assume that both ligands are capable of inducing isomerisation of the DAT-ligand complex (Fig. 4):

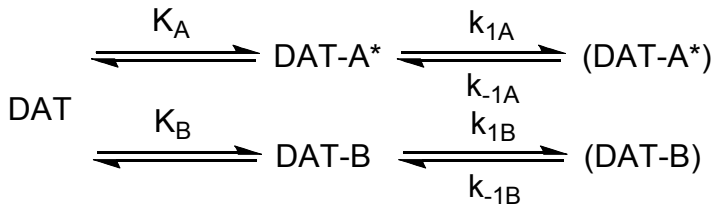


Figure 4. Model for the competitive interaction of two ligands with DAT, where both ligands are capable of including a slow isomerisation of the DAT-ligand complex.

and the second is that only one of the ligands is capable of such behavior (Fig. 5):

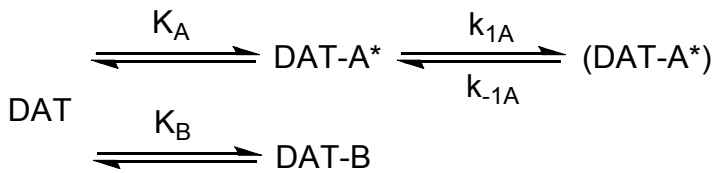


Figure 5

The case presented in Fig. 4, where both ligands induce isomerisation upon binding with DAT can be analyzed in relation to the radioactive tracer ligand A* (in our case the [^{3}H]PE2I) yielding following parameters:

$$[(DAT-A^*)] = \frac{[(DAT-A^*)]_{MAX}}{\frac{K_A \cdot K_{IA}}{[A]} \cdot \left(\frac{[B]}{K_B} + \frac{[B]}{K_B \cdot K_{IB}} + 1 \right) + (K_{IA} + 1)} \quad (15)$$

$$k_{obs} = \frac{k_{1A} \cdot [A^*]}{K_A + [A^*]} + \frac{k_{1B} \cdot [B]}{K_B + [B]} + const \quad (16)$$

As it can be seen, displacement of the labeled tracer by the unlabeled ligand can be observed with increase of the unlabeled ligand concentration (15) and k_{obs} vs [A*] relationship is described by the sum of two hyperbolic curves (16), indicating the apparent increase in association rate for the labeled tracer in the presence of increasing concentrations the unlabelled ligand, the later can be attributed to the fact that the increase of the amount of the (DAT-B) complex leads to the depletion of the available free DAT pool.

The second case is presented in Fig 5. In this scheme only one ligand (A*) is capable of inducing isomerisation of the transporter-ligand complex and the

second ligand is incapable of producing such effect upon binding. The rate equations can be solved for the labeled ligand, yielding the following solutions.

$$[(DAT - A^*)] = \frac{[(DAT - A^*)]_{MAX}}{\frac{K_A \cdot K_{IA}}{[A]} + \frac{K_A \cdot K_{IA} \cdot [B]}{K_B \cdot [A]} + K_{IA} + 1} \quad (17)$$

$$k_{obs} = \frac{k_{1A} \cdot [A^*]}{K_A \cdot \left(1 + \frac{[B]}{K_B}\right) + [A^*]} + k_{-1A} \quad (18)$$

If the unlabeled ligand is unable to cause isomerisation of the ligand-transporter complex, the k_{obs} for the labeled tracer decreases dose-dependently with the increase in the concentration of the unlabeled ligand, following the equation (18). At the same time we would obviously observe standard dose-dependent decrease of amount of the radioactive tracer bound to DAT if concentration of the unlabeled ligand increases, as two ligands are in a direct competition for the binding site on the transporter protein (17).

Therefore if we assume that DAT protein possesses a single site at which various ligands compete with each other, the models describing such competition can be easily proved by study of the influence of unlabelled ligand concentration on the apparent rate of association of the labeled ligand. If both ligands cause isomerisation, the k_{obs} for the labeled tracer ([^{3}H]PE2I on our case) should dose-dependently increases with the increase in the unlabeled ligand concentration. If the unlabeled ligand is unable to cause isomerisation of the ligand-transporter complex, the k_{obs} for the labeled tracer decreases dose-dependently with the increase of the concentration of the unlabeled ligand.

5. RESULTS AND DISCUSSION

5.1. Equilibrium binding and displacement of [3H]PE2I at rDAT

The [^{3}H]PE2I was found to bind to membrane preparation of rat striatum tissue specifically and reversibly, the non-specific binding was of [^{3}H]PE2I was linearly dependent on the [^{3}H]PE2I concentration, and at equilibrium conditions the binding of [^{3}H]PE2I followed the common binding isotherm:

$$\left[\left(DAT - \left[^3H\right]PE2I\right)\right]_{EQ} = \frac{\left[\left(DAT - \left[^3H\right]PE2I\right)\right]_{MAX} \cdot \left[\left[^3H\right]PE2I\right]}{K_D + \left[\left[^3H\right]PE2I\right]} \quad (19)$$

where $[(DAT\text{-}[^3H]PE2I)]_{EQ}$ is the specific radioligand binding at equilibrium, $[(DAT\text{-}[^3H]PE2I)]_{MAX}$ is the maximum value of the specific binding and K_D is the apparent dissociation constant. The resulting picture (Fig. 6)

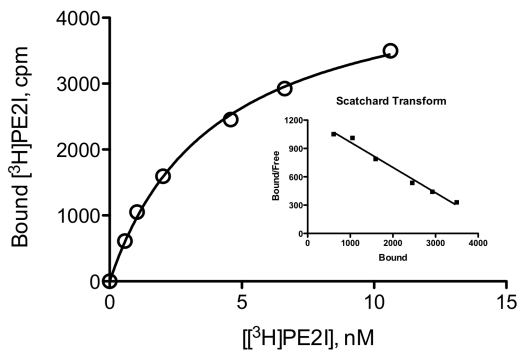


Figure 6. Specific binding of [^{3}H]PE2I to the rat dopamine transporter. Isert: Scatchard transform of the binding data, showing a homogeneous population of binding sites.

shows that [^{3}H]PE2I binds to a homogenous population of binding sites with affinity of 2.0 to 4.0 nM, which is in agreement with previously published data for unlabeled PE2I (Emond 1997; Stepanov 2006). The ligand is also displaced by from the complex with the DAT by various other ligands knows to have affinity for the DAT protein (Fig. 7).

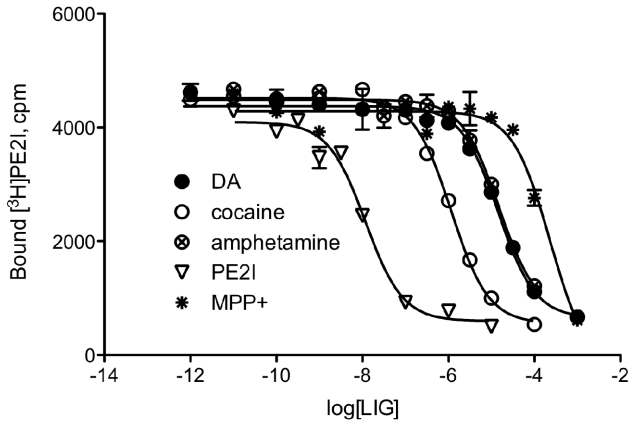


Figure 7. Displacement of the [^{3}H]PE2I from the rDAT-[^{3}H]PE2I complex by various DAT active compounds. See graph legend for explanation.

The K_i values for the [^{3}H]PE2I binding inhibition for those ligands are summarised in Table 1 below. The K_i values obtained from the displacement studies are in good agreement with data published by others.

Table 1. Unlabeled ligand affinities obtained from displacement data (Fig. 7)

Ligand	logK_i	K_i
PE2I	–8.41±0.07	4.0 nM
Cocaine	–6.41±0.04	380 nM
Amphetamine	–5.30±0.10	5 μM
Dopamine	–5.32±0.05	5 μM
MPP+	–4.12±0.10	76 μM

It can be seen that transportable DAT substrates possess quite low affinity for the DAT protein as determined by displacement studies – such low affinity can be explained by the fact that binding of a transportable substrate must be rather weak to allow for rapid transition though the "pore" during the active transport phase and dissociation from the protein after transport has taken place and/or by the fact that residence time of a transportable substrate at DAT is very small due the act of transportation constantly occurring.

5.2. Kinetics of [^{3}H]PE2I binding to *r*DAT in the absence of competing ligands

The binding of [^{3}H]PE2I to the rDAT apparently contains a slow isomerisation step that most likely constitutes a conformational change in the transporter protein that takes place after initial ligand binding occurs. The exact reason for such conformational change is unclear at the moment, but apparently ligands with low-nanomolar affinity seem to be able to induce this change and such behaviour is similar in case of many different GPCRs (Järv 2005). The presence of the slow isomerisation step is made apparent by the fact that dependence of the apparent rate constant for the [^{3}H]PE2I association versus free [^{3}H]PE2I concentration is hyperbolic:

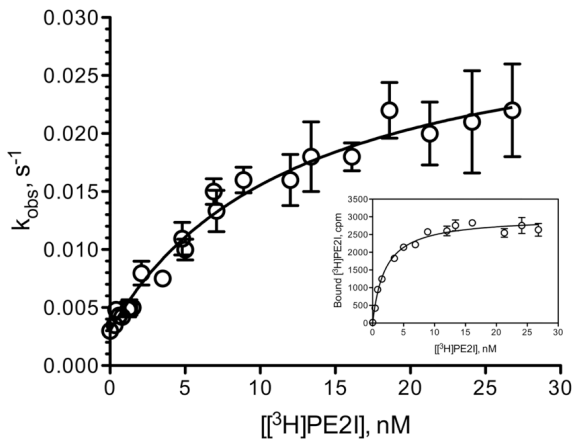


Figure 8. Hyperbolic dependence of the k_{obs} vs [^{3}H]PE2I concentration for [^{3}H]PE2I association reaction with rDAT. Insert: Binding curve obtained from kinetic data (B_{max} asymptote values).

yielding the following kinetic parameters: K_A= 12.5±2.1 nM, k_{on}= 0.028±0.002 s^{-1}, k_{off}= 0.003±0.0005 s^{-1} for a two-step binding mechanism (Fig. 3). The off-rate constant for the [^{3}H]PE2I is not affected by the [^{3}H]PE2I concentration (Stepanov 2006).

5.3. Kinetics of [^{3}H]PE2I binding to *r*DAT in the presence of GBR12909, cocaine and amphetamine

Our previous detailed kinetic analysis of the [^{3}H]PE2I interaction with mouse and rat dopamine transporters revealed the presence of a slow isomerization step in this process, allowing us to use [^{3}H]PE2I as a reporter ligand for investigation into non-labeled ligand binding mechanism. The kinetic analysis of cocaine, (+)-amphetamine and GBR12909 interaction with rDAT binding sites was made by analyzing effects of various concentrations of these unlabeled ligands on the kinetic behaviour of the radioactive tracer, specifically the impact of the non-radioactive ligand concentration on the k_{obs}, and consequently on K_A and k_{on} values.

The impact of unlabeled GBR12909 was to increase the observed k_{obs} value of the [^{3}H]PE2I association process with DAT in a dose-dependent manner, indicating that both GBR12909 and [^{3}H]PE2I are capable of inducing isomerisation (Stepanov 2008). The K_D for the GBR12909 from the displacement data was found to be 7.5±0.4 nM. The analysis of GBR12909 impact on [^{3}H]PE2I kinetics yielded the following parameters for the GBR12909 interaction with DAT: K_A=34±11 nM, k_1=0.033±0.005 s^{-1}. This allows us to calculate the K_D value for GBR12909 from the kinetic data, yielding approximately 4 nM. This is in good agreement with the displacement data. These data are in perfect agreement with observation made earlier by Do-Rego and co-workers regarding GBR12783, where its impact on the dopamine uptake indicated that a slow isomerisation step is present (Do-Rego 1999).

Influence of cocaine and amphetamine was investigated by two complementary approaches. Firstly binding kinetics of the [^{3}H]PE2I to rDAT was studied by keeping [^{3}H]PE2I concentration constant (4.0 nM) and varying concentration of the unlabeled ligand, respectively cocaine or amphetamine. It can be seen (Fig. 9 and 10) that kinetic curves obtained were well described by the exponential rate equation:

$$\left[(DAT - A^*)\right] = \left[(DAT - A^*)\right]_{MAX} \cdot \left(1 - e^{-k_{obs} \cdot t}\right) + NS \qquad (20)$$

where [L] is the concentration of the radioligand, permitting calculation of R_{max} and k_{obs} at different concentrations of the unlabeled ligands.

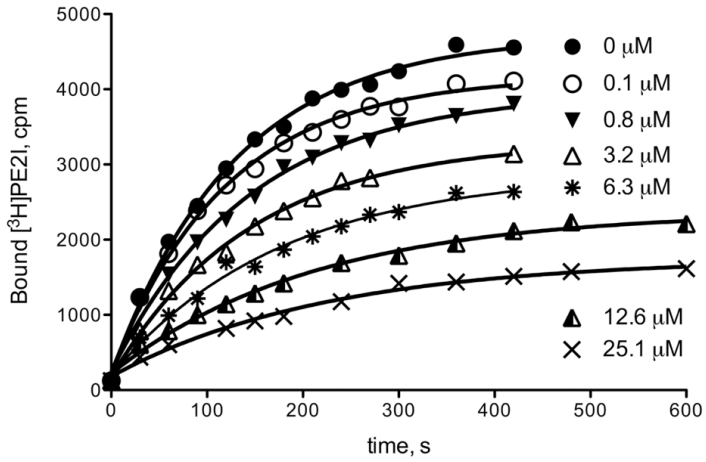


Figure 9. Time-course of the [^{3}H]PE2I binding to rDAT in the presence of (+)-amphetamine.

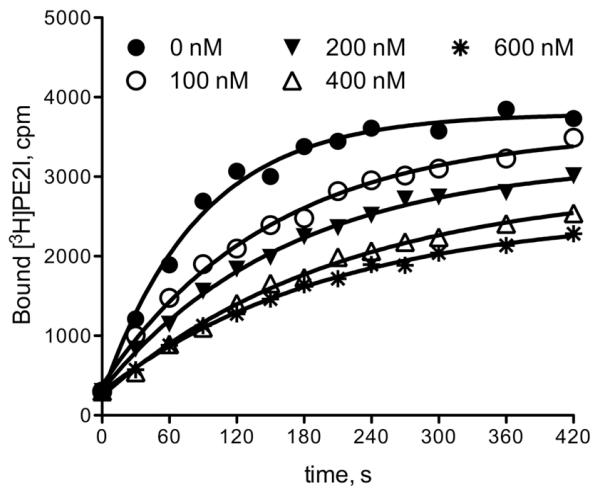


Figure 10. Time-course of the [^{3}H]PE2I binding to rDAT in the presence of cocaine.

It was found that increase in concentration of both cocaine and amphetamine at decreased the k_{obs} and B_{max} for the [^{3}H]PE2I association process in a dose-dependent manner (Fig. 11 and 12):

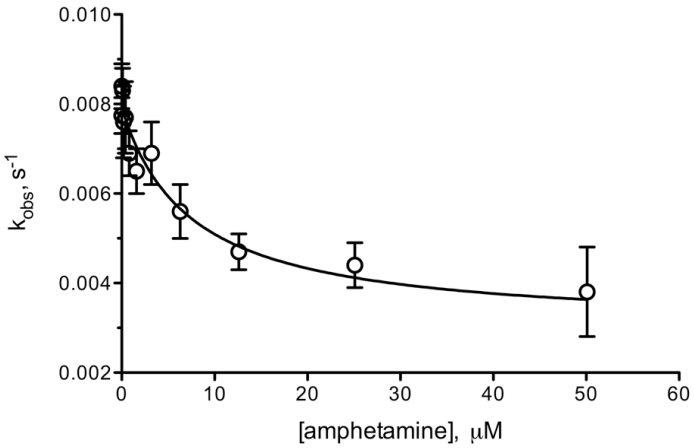


Figure 11. Influence of various amphetamine on the observed on-rate (k_{obs}) constant of the [^{3}H]PE2I association with rDAT at the constant concentration of [^{3}H]PE2I.

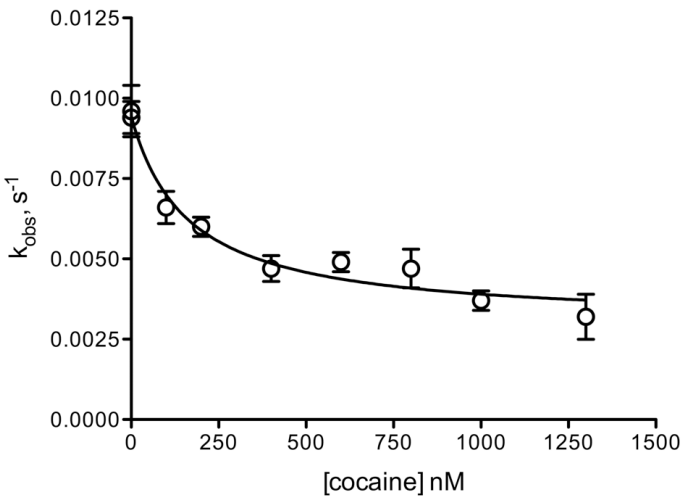


Figure 12. Influence of various cocaine on the observed on-rate (k_{obs}) constant of the [^{3}H]PE2I association with rDAT at the constant concentration of [^{3}H]PE2I.

Curve-fitting of the data above yields affinity constants of 6.1±1.1 μM and 125±20 nM for amphetamine and cocaine, respectively. It also confirms that neither cocaine nor amphetamine are able to induce isomerisation step after binding to the dopamine transporter protein (see Section 4.4, analysis of competitive models of DAT interaction with two ligands).

The influence of cocaine and amphetamine on [^{3}H]PE2I association kinetics was also studied assaying rDAT under a number of [^{3}H]PE2I concentrations in the presence of the certain fixed concentrations of the unlabeled ligand (Fig. 13 and 14).

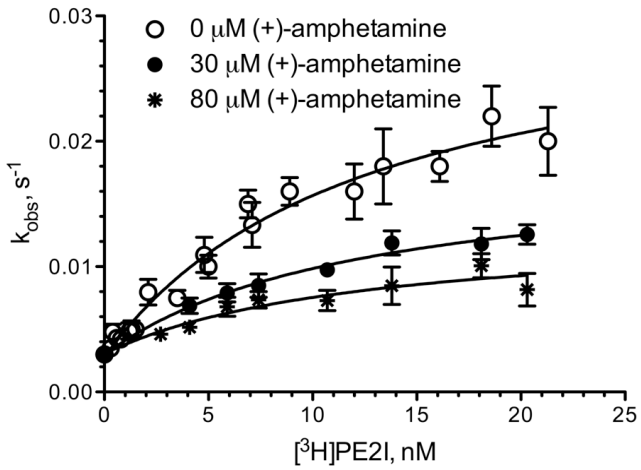


Figure 13. Influence of constant concentration of (+)-amphetamine on the observed on-rate (k_{obs}) constant of the [^{3}H]PE2I association with rDAT at various concentration of [^{3}H]PE2I.

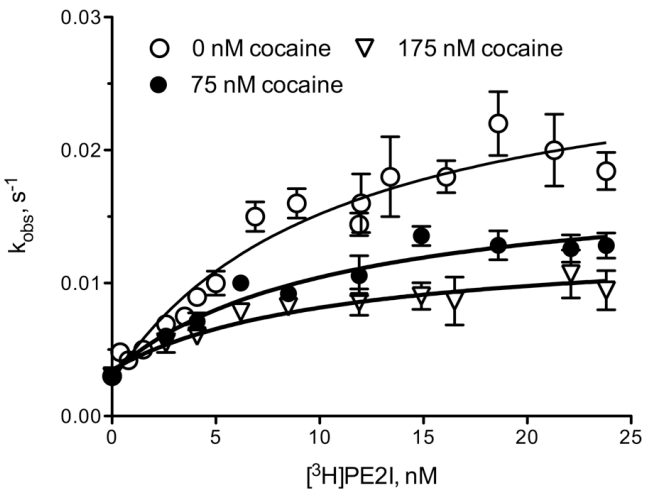


Figure 14. Influence of constant concentration of cocaine on the observed on-rate (k_{obs}) constant of the [^{3}H]PE2I association with rDAT at various concentration of [3H]PE2I.

The general hyperbolic dependence of the k_{obs} vs free [^{3}H]PE2I concentration remained unchanged in the presence of cocaine and amphetamine respectively, however, the presence of unlabeled ligand decreased k_{on} values in a dose-dependent manner, while corresponding K_A value of the tracer ligand used remained unaffected (Table 2).

Table 2. Kinetic parameters of the [^{3}H]PE2I binding to rDAT in the presence of amphetamine and cocaine.

amphetamine, μM	[^{3}H]PE2I k_{on}, s^{-1}	[^{3}H]PE2I k_{off}, s^{-1}	[^{3}H]PE2I K_A, nM
0	0.029±0.003	0.0028±0.0004	12.5±2.4[#]
30	0.016±0.002	0.0029±0.0007	
80	0.010±0.002	0.0030±0.0006	
cocaine, nM	[^{3}H]PE2I k_{on}, s^{-1}	[^{3}H]PE2I k_{off}, s^{-1}	[^{3}H]PE2I K_A, nM
0	0.026±0.003	0.0026±0.0006	10.7±2.3[##]
75	0.015±0.003	0.0033±0.0008	
175	0.010±0.003	0.0035±0.0008	

[#] – value is the same across all sets, P=0.93, F(DFn, DFd) 0.071 (2,28)
[##] – value is the same across all sets, P=0.62, F(DFn, DFd) 0.49 (2,29)

The results show that for [^{3}H]PE2I K_A is in fact independent of the concentration of the unlabeled ligand used in these experiments. This is in direct violation with both models for competitive interaction of ligands with dopamine transporter presented earlier in Section 4.4. In turn, this indicates that the interaction mechanism between DAT, [^{3}H]PE2I and cocaine/amphetamine is more complex than that presented in Figure 5. Analyzing appropriate kinetic models it can be shown that K_A for labeled tracer can remain unaffected in the presence of the unlabeled ligand only in the case where unlabeled ligand does not compete with the tracer for the binding site on the dopamine transporter (Fig. 15).

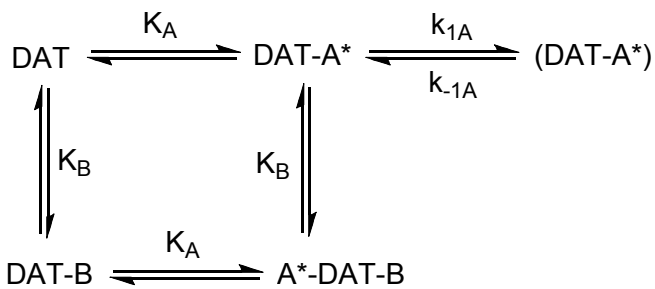


Figure 15. Non-exclusive interaction of two ligands with rDAT through ternary complex formation.

The constant value of K_A for tracer ligand [^{3}H]PE2I in the presence of high concentration cocaine and amphetamine indicates that [^{3}H]PE2I binding to the DAT is in no way attenuated by the binding of cocaine or amphetamine to the same transporter protein molecule. In other words the binding is completely non-competitive. This conclusion raises a very important question – if both ligands are capable of simultaneous binding to the protein, then how can radioligand displacement be observed? The answer to this question is very straightforward. By definition, in this assay system we cannot determine complexes that result from fast equilibrium interactions, such as DAT-A*, DAT-B and A*-DAT-B. Only (DAT-A*) can be determined by slow separation methods such as filtration or centrifugation. If the formation of the ternary complex A*-DAT-B disrupts slow conformational change, and this assumption does not violate the proposed kinetic models, we would observe decrease in [^{3}H]PE2I binding to DAT protein in a dose-dependent manner upon addition of the second ligand:

$$[(DAT-A^*)] = \frac{\frac{[(DAT-A^*)]_{MAX}\cdot[A^*]}{\left(\left(\frac{[B]}{K_B}+1\right)\cdot K_I+1\right)}}{\frac{\left(K_A\cdot K_I\cdot\left(\frac{[B]}{K_B}+1\right)\right)}{\left(\left(\frac{[B]}{K_B}+1\right)\cdot K_I+1\right)}+[A^*]} \quad (5)$$

The kinetic approach described above allows us to reliably determine a number of important binding parameters for a given ligand and elaborate the details of the particular mechanism of interaction between ligand and its target. In our case there is compelling evidence that PE2I possesses quite different binding mechanism compared to that of cocaine and amphetamine. It also suggests that inhibition of DAT can proceed by at least two different mechanisms – competitive and non-competitive inhibition.

5.4. Ternary complex formation and its implications for ligand behaviour at rDAT

The ternary complex formation between [^{3}H]PE2I and cocaine/amphetamine has significant implications for the entire field of monoamine transporter research and in particular for the dopamine transporter-related investigations. The formation of the said complex shows that DAT inhibition can be achieved by two different ways, and furthermore, the behaviour of the ligand in question is not in an obvious relation with its structure – PE2I and cocaine both share tro-

pane backbone, yet inhibit DAT by different mechanisms, and GBR12909, while lacking the tropane cycle in its structure, behaves similar to PE2I. In view of the latest data regarding DAT protein and its interaction with ligands, results described above are also relevant in light of the discussion about multiple binding sites and drug addiction treatments. The previous paradigm of binding of DAT inhibitors and substrates to the different parts of the dopamine transporter has been recently questioned following the conclusion that dopamine and cocaine bind to the same site on DAT (Beuming 2008).

Kinetic measurements do not allow "definitive" answer to the question as to whenever two "isomerising" ligands share the same binding site or not – we can only determine whenever two ligands form a ternary complex or not and thus show that they do not share same binding site (if the complex is formed), however the proof of either of the binding modes does allow us certain insight into the problem and some elbowroom for speculation. In this discussion we proceed from the following. Firstly, we have revealed that some ligands are capable to induce isomerisation (in this case PE2I and GBR12909) and some are not (cocaine, amphetamine and probably dopamine, following our recent results). Secondly, other results have shown that cocaine and dopamine share the same binding site (Beuming 2008). Thirdly, we have also revealed that PE2I and cocaine/amphetamine form a ternary complex with DAT. Further it can be assumed that binding sites for dopamine and amphetamine overlap to a very significant extent in any case, as both molecules are transportable by the DAT. In such a case the fact that dopamine and cocaine sites overlap should mean that cocaine and amphetamine sites overlap as well, and as both ligands bind to the same sites and yet both form ternary complex with DAT and [^{3}H]PE2I, we can surmise that there is a definite possibility that isomerising ligands exhibit binding to a distinctly different site.

This conclusion may have significant implication for design of dopaminergic drugs: while it is not possible to design analogue of cocaine that would act as cocaine antagonist for the treatment of cocaine abuse and bind to the same site as cocaine, the presence of the second site, occupancy of which results in strong DAT inhibition, must be taken into account when designing high-affinity DAT inhibitors. This also means that the isomerisation step must be considered in the target-oriented ligand design, as its presence significantly increases affinity of the ligand for the target, as the occupancy of the binding site is no longer dependent only on the affinity of the ligand for the binding site, but also on the kinetic properties of the isomerised complex.

CONCLUSIONS

In this dissertation slow conformational changes, which accompany binding of specific ligands with the dopamine transporter protein (DAT), were discovered and the kinetic mechanism of this binding process was analysed in conjunction with kinetic models describing similar phenomena for the G protein-coupled receptors. This analysis allowed characterisation of both fast equilibrium step and slow isomerisation stages of the ligand binding process. A new radioligand [^{3}H]PE2I was developed for the purpose of selective assay of DAT in samples containing SERT and NET transporters. This ligand was also proven to be a superior tool for DAT assay in vitro analysis if compared with the currently used [^{3}H]GBR12909.

The results obtained extend our understanding about the mechanism of interaction between DAT and its inhibitors, demonstrating that although the binding of such ligands occurs reversibly, at least two kinetically distinguishable steps can be identified in this process for several potent inhibitors. As the "isomerisation" phenomenon seems to be quite general for the G-protein coupled receptors with 7-transmembrane segments, it can be assumed that similar situation may occur in the case of the neurotransmitter transporters having 12 transmembrane segments, and therefore may have general meaning for functioning of these membrane-integrated proteins.

The presence of the slow isomerisation step in drug-protein interaction pathway may have significant meaning for biochemical and pharmacological properties of this ligand, as its apparent binding effectiveness can be increased through the isomerization step. This also means that drug selectivity, which is determined through effectiveness of its binding with different target sites, also depends on the presence or absence of the isomerisation step. Therefore it is important to reveal the elements of ligand structure that are responsible for engender of the isomerisation step and quantitative characteristics, as this information may guide the effect-directed drug design more effectively.

The method of kinetic analysis was extended to the competition kinetics experiments and this approach was used for cocaine and amphetamine. These studies revealed that dopamine transporter could be inhibited not only by competitive mechanism, as it has been previously/generally assumed, but also by hitherto unknown mechanism of non-exclusive ligand binding. The data obtained allowed us to stipulate that at least two distinct sites exist on the functional DAT unit, each site is being capable of binding corresponding molecule and the binding of said ligand (inhibitor) at one site does not exclude binding of another ligand (inhibitor or substrate) at the second site. However, binding of the second ligand to the same transporter unit interferes with slow isomerisation step as well as with neurotransmitter transport. The possibility of non-exclusive mechanism of DAT inhibition also opens new interesting perspectives of DAT ligand design and may have significant implications for general understanding of monoamine transports inhibition mechanisms.

SUMMARY IN ESTONIAN

Ligandide seostumisega kaasnevad dopamiini transporteri aeglased konformatsioonimuutused

Käesolevas doktoritöös uuriti aeglasi konfirmatsiooni muutusi, mis ilmnevad spetsiifiliste ligandide seostumisel dopamiini transporterile ning analüüsiti nende nähtuste kineetilist mehhanismi ja võimaliku tähendust, lähtudes G-valkudega konjugeeritud retseptorite jaoks analoogiliste nähtuste kirjeldamiseks loodud kineetilistest mudelitest. Teostatud kineetika uuringud lubasid kirjeldada ligandide seostumise kiiret tasakaalulist staadiumit ja järgnevat aeglast etappi. Transportervalgu määramiseks aju membraanifragmentides sünteesiti uudne radioligand [^{3}H]PE2I, mis tagab mõõtmiste selektiivsuse uuritavas preparaadis leiduvate SERT ja NET transporterite juuresolekul. See radioligand osutus oluliselt paremaks töövahendiks kui seda on üldiselt kasutatav radioligand [^{3}H]GBR12909.

Saadud tulemused võimaldavad täielikumalt mõista DAT ja dopamiini transporti pidurdavate ligandide vahelist toimet, võimaldades esmakordselt eristada mitme tugeva inhibiitori korral kiiret ja aeglast sidumise staadiumit. Kuivõrd sarnane "isomerisatsiooninähtus" on osutunud universaalseks mehhanismiks G-valkudega konjugeeritud retseptorite korral, mida iseloomustab 7 transmembraanse segmendi olemasolu, siis võib oletada, et sarnase nähtuse ilmnemine transportvalkude korral, milles on 12 trans-membraanset fragmenti, võib samuti osutuda üldiseks seaduspäraks.

On oluline, et isomerisatsiooni avaldumine ligandi sidumisel võib oluliselt mõjutada selle aine biokeemilisi ja farmakoloogilisi omadusi, sest selle staadiumi kaudu on võimalik oluliselt suurendada ligandi sidumise näivat efektiivsust. Sidumise kaudu on aga omakorda võimalik mõjutada ligandi toime selektiivsust. Seetõttu on selektiivsete ligandide loomisel oluline mõista, millised ligandi struktuuri elemendid määravad isomerisatsiooni ilmnemise ja seda staadiumit kirjeldavad kvantitatiivsed parameetrid.

Kineetilise analüüsi meetodid leidsid rakendust ka kokaiini ja amfetamiini sidumise mehhanismi kineetilistel uuringutel, kus kasutati radioaktiivse ligandina samuti [^{3}H]PE2I-d. Need katsed näitasid, et dopamiini transporteri inhibeerimisel esineb lisaks tuntud ja üldtunnustatud konkurentsele mehhanismile veel selles töös avastatud „mitte-välistav" ligandide sidumise mehhanism. Selle mehhanismi kohaselt peab funktsionaalselt aktiivse DAT pinnal olema vähemalt kaks sidumiskohta, kusjuures ligandi sidumine neist ühele ei välista teise ligandi sidumist teisele kohale. Samal ajal aga on mõjutatud aeglane isomerisatsiooni staadium ja samuti transportfunktsiooni täitmine. Selline mitte-välistava seostumise ja DAT funktsioonide vahelise sõltuvuse olemasolu avab põhimõtteliselt uue viisi ligandide loomiseks ning avardab oluliselt meie arusaamu monoamiinide transporterite inhibeerimise viisidest.

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5. Stepanov, V., Jarv, J. Non-overlapping binding sites for substrates and inhibitors at the dopamine transporter protein. *Manuscript. Submitted to Neurochemistry International.*

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