DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
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Ligand binding to 5-HT_{1A} receptors and its regulation by Mg^{2+} and Mn^{2+}

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ABBREVIATIONS

[^{3}H]8-OH-DPAT	–	[^{3}H]-8-hydroxy-*N*,*N*-dipropyl-2-aminotetralin
[^{3}H]ipsapirone	–	7,7-dioxo-8-[4-(4-pyrimidin-2-ylpiperazin-1-yl)butyl]-7λ6-thia-8-azabicyclo[4.3.0]nona-1,3,5-trien-9-one
[^{3}H]QNB	–	[^{3}H]quinuclidinyl benzilate
[^{3}H]WAY100635	–	N-[2-[4-(2-[O-methyl-^{3}H]methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclohexane carboxamide
[^{125}I]-BH-MeO-N-PAT	–	^{125}I-[8-methoxy-2-[N-propyl-N-(3'-iodo-4'-hydroxyphenyl)-propionamido -N'- propylamino] tetralin]
[^{125}I]-p-MPPI	–	[^{125}I]-4-iodo-N-[2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl]-N-pyridin-2-yl-benzamide
[^{35}S]GTPγS	–	guanosine 5'-[gamma-^{35}S]-thiotriphosphate
5-HT	–	5-hydroxytryptamine, serotonin
5-HT$_{1A}$	–	serotonin$_{1A}$
7-TM	–	seven transmembrain
8-OH-DPAT	–	8-hydroxy-*N*,*N*-dipropyl-2-aminotetralin
ACh	–	acetylcholine
AGS	–	receptor-independent activators of G-protein signaling
α_H	–	proportion of high-affinity binding sites
B_{max}	–	maximal specific radioligand binding
BMY7378	–	8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride
BSA	–	bovine serum albumin
cAMP	–	cyclic adenosine monophosphate
CNS	–	central nervous system
DTT	–	dithiotreitol
EDTA	–	ethylene diamine tetraacetic acid
GABA	–	gamma-aminobutyric acid
GDP	–	guanosine diphosphate
GEF	–	guanine nucleotide exchange factor
GPCR	–	G-protein coupled receptor
Gpp(NH)p	–	5'-guanylylimidodiphosphate
GTP	–	guanosine-5'-triphosphate
GTPγS	–	guanosine 5'-O-[gamma-thio]triphosphate
IB	–	incubation buffer
iNOS	–	inductible nitric oxide synthase
IUPHAR	–	International Union of Basic and Clinical Pharmacology

K_D	–	equilibrium dissociation constant
k_{obs}	–	observational rate constant
k_{off}	–	dissociation rate constant
mRNA	–	messenger ribonucleic acid
NAD-299	–	(R)-3-N,N-dicyclobutylamino-8-fluoro-3,4-dihydro-2H-1-benzopyran-5-carboxamide hydrogen (2R,3R)-tartrate monohydrate
NAN-190	–	1-(2-methoxyphenyl)-4-(4-(2-phthalimido)butyl)piperazine
NE	–	norepinephrine
PIP2	–	phosphatidylinositol 4,5-bisphosphate
RGS	–	regulator of G-protein signalling
SEM	–	standard error of the mean
Sf9	–	Spodoptera frugiperda insect cell line
Tris	–	tris(hydroxymethyl)aminomethane
WAY100135	–	3-[4-(2-methoxyphenyl)piperazin-1-yl]-2-phenyl-N-tert-butyl-propanamide
WAY100635	–	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)cyclohexanecarboxamide
$\tau_{½inact}$	–	halftime of inactivation reaction
$\tau_{½off}$	–	halftime of dissociation reaction

1. SIGNAL TRANSDUCTION IN NEURONS

1.1. Receptors

Processing external environmental signals and signals from other cells is crucial for the living cycle and survival of a cell and understanding this communication has been a challenge for scientist for a long time. Until late 19th century it was believed that direct physical connections united the neurons helping them to communicate. Chemical transmission in synapse was demonstrated in historic experiments by Otto Loewi [Loewi, 1921] and now it is known that chemical transmission is the main communication method between nerves in the nervous system. Signaling between two neurons usually takes place in synapses, which are functional connections between neurons. A neuron typically has thousands of synapses allowing passing information from one cell to another. To start the transmission, synthesis of the neurotransmitter is necessary in the presynaptic nerve terminal.

There are several types of substances fulfilling the neurotransmitter criteria – for example a number of low molecular weight compounds (ACh, NE, 5-HT), amino acids (glutamate, glycine) and peptides (substance P, vasopressin, oxytocin), hormones and also light. Neurotransmitters are usually stored in vesicles in presynaptic neuron, and released in an exocytotic process specialized depending on the neuron. In neurons, receptor molecules for the released transmitters are localized on the postsynaptic part of the neuron, and are required for recognition of the chemical signal from the presynaptic nerve. Receptors can also be expressed on presynaptic side where they act as autoreceptors mediating further transmitter release. Exocytosis is followed by endocytosis, which recycles the transmitter from the synaptic cleft back to presynaptic neuron or the neurotransmitter is cleaved [Basic Neurochemistry, 2006].

Specific receptor molecules are used by all kinds of cells, to mediate external information to interior of the cell. Receptors are responsible for reacting to environmental stimuli such as odorants, hormones, neurotransmitters and other molecules. IUPHAR defines a receptor as "a cellular macromolecule, or an assembly of macromolecules, that is concerned directly and specifically in chemical signaling between and within cells" [Neubig *et al*. 2003]. In pharmacological means, „receptor" is a signal transducting molecule, which is activated by a native agonist. The main structural classes of receptors by IUPHAR are 1.0 – Ion-channel receptors; 2.0 – Seven transmembrane domain (G-protein coupled) receptors; 3.0 – Enzyme-associated receptors (with subunits having one membrane-inserted domain) and 4.0 – Transcriptional regulator receptors. The 2.0 structural class of seven transmembrane receptors has been further divided into three subclasses: 2.1 is the rhodopsin subclass including the vast majority of G-protein coupled receptors (GPCR); 2.2 is secretin receptor subclass and 2.3 is metabotropic glutamate/$GABA_B$ receptor subclass [Humphrey&Barnard 1998].

Although there are claims, that the term "G-protein coupled receptor (GPCR)" is outdated since the receptors of the rhodopsin subclass can often mediate intracellular biochemical pathways without the inclusion of G-proteins [Nygaard *et al*. 2009], in this thesis definition "GPCR" is used. GPCR's have 7 hydrofobic membrane spanning parts, which are connected with hydrophilic loops with the protein N-terminus outside and C-terminus inside the cell. Obtaining the crystal structure of β_2-adrenergic receptor [Cherezov *et al*. 2007] followed shortly by β1-adrenergic receptor [Warne *et al*. 2008] and A_{2A} adenosine receptor [Jaakola *et al*. 2008] crystal structures, has been a magnificent breakthrough. Before that the secondary structure and the crystal structure of bovine rhodopsin [Palczewski *et al*, 2000] were used to calculate and model GPCR properties. Crystal structures of receptors have helped further understand the roles of certain receptor regions. α-helix is the primary building block of GPCR consisting of 20–30 hydrophobic residues that form the membrane spanning part, which are connected with polar amino acid loops with variable size. GPCRs do not share many homological regions, but there are some highly conserved sequences, for example helix VIII and palmitoylation sites, but more importantly they share D(E)RY motif (in transmembrane helix III (TM-III)), CWxP motif (TM-VI) and NpxxY (TM-VII) motifs near intracellular part, which are considered important for interactions with G-proteins [Oldham &Hamm, 2006; Nygaard *et al*. 2009].

To date scientists have not reached a consensus about the question how many seven transmembrane G-protein coupled receptor genes are in the human genome. The sequencers of the first human genome counted 616 GPCR genes from rhodopsin, secretin and metabotropic glutamate class [Venter *et al*. 2001]. A year later Takeda and co-workers identified 948 GPCR genes [Takeda *et al*. 2002 (a)]. In 2003 Fredriksson counted 802 different GPCR genes [Fredriksson *et al*. 2003], couple of years later Zhang and colleagues claimed that there were 907 receptor genes [Zhang *et al*. 2006], however in the text they clarify that they included only the proteins with less than 500 amino acids. Therefore the estimation of the existence of roughly 1000 members seems plausible [Howard *et al*. 2001].

Rapidly growing information about the biochemistry of signal transduction allows us to conclude the following – receptors are main players in chemical communication between neurons and other cells. Exploring the properties of different receptors is highly beneficial for controlling progression of several diseases as well as understanding emotional and behavioral states of man. In 2003, 47 of top 200 drugs sold worldwide were targeting GPCR's, comprising almost 47% of sales in that list [Lundstrom&Chiu, 2006]. Constant interest of neuroscientists and pharmaceutical companies guarantees that studies of receptor mediated signaling between cells will be continued.

1.2. Neurotransmitter serotonin

Serotonin (5-HT) is a neurotransmitter mediating its effects through action on its transmembrane receptors in the central and peripheral nervous system as well as in non-neuronal tissues. 5-HT is thought to be one of the oldest neurotransmitters in evolution, its receptors are found from planaria and *c. Elegans* to drosophila and man. The major classes of 5-HT receptors probably evolved about 750 million years ago [Hannon&Hoyer, 2008]. Serotonin was first isolated from the enterochromaffin cells in the gastrointestinal tract by Ersparmer and colleagues in 1937, but was eventually crystallized and characterized from blood by Maurice Rapport [Rapport *et al*. 1948].

Biosynthesis of 5-HT starts from L-tryptophan and transport of L-tryptophan to brain from the blood is necessary, the primary resource being dietary protein. Serotonergic neurons contain L-tryptophan-5-monooxygenase (also named tryptophane hydroxylase) which converts tryptophan to 5-hydroxytryptophan, which is converted to 5-HT by L-amino acid decarboxylase. The primary catabolic pathway of 5-HT is oxidative deamination by the enzyme monoamine oxidase.

Serotonin-containing neuronal cell bodies are restricted to discrete clusters of cells along the midline of the brainstem with axons reaching nearly every area of central nervous system. To date nine groups of 5-HT-containing cell bodies have been found in brain, named B_1-B_9 [Tork., 1990].

1.3. Serotonin receptors

Heterogeneity of 5-HT receptors was first noted in 1950s, when Gaddum and colleagues discovered that serotonin action on guinea pig ileum was partially blocked by morphine and partially by dibenzyline, therefore two receptor classes were proposed – 5-HT M and 5-HT D [Gaddum&Picarelli 1957]. In 1979 Peroutka and Snyder labeled two classes of brain 5-HT receptors – one binding [^{3}H]5-HT was named 5-HT_1 receptor and the other binding [^{3}H]spiperone was named 5-HT_2 receptor, while [^{3}H]LSD could label both types [Peroutka&Snyder, 1983]. Yet neither type was similar to Gaddum's M type in function and distribution, whereas 5-HT_2 resembled pharmacologically the D type. The receptors were named 5-HT_1-like, 5-HT_2 and 5-HT_3 (formerly M). After increased use of radioligand binding assays, autoradiography and second messenger studies, 5-HT_1 type was further divided to 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D} and 5ht_{1E}, with the 5-HT_{1C} resembling more the 5-HT_2 subtype, suggesting also further subdivision of the 5-HT_2 type. The situation was clarified with cloning techniques allowing further identification of 5-HT receptor subtypes. 5-HT_{1C} receptor was named 5-HT_{2C} due to higher similarity to 5-HT_2 subclass receptors. Molecular biology techniques led to the discovery of additional serotonin receptor subtypes and to date there are three major

families of serotonin receptors plus additional members (5-HT_4R, 5-HT_6R and 5-HT_7R).

5-HT_1 receptor family consists of 5 receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E} and 5-HT_{1F}) which are preferentially coupled to pertussis toxin sensitive $G_{i/o}$ protein, inhibiting adenylyl cyclase and leading to cease of cell firing. These receptors share 40–63% overall sequence identity in humans [Basic Neurochemistry 2006].

1.3.1. Serotonin$_{1A}$ (5-HT_{1A}) receptor

In the human genome the 5-HT_{1A} receptor gene is located on 5q11.2-q13 and is largely expressed throughout the central nervous system, and also in peripheral nervous system. 5-HT_{1A} receptor was the first serotonin receptor that was cloned and sequenced [Kobilka *et al*. 1987; Fargin *et al*. 1988].The receptor gene is intronless and produces a single peptide chain of 422 amino acids in human and rat and of 421 amino acids in mouse. For mapping the location of 5-HT_{1A} receptors, a range of 5-HT_{1A} ligands has been used in autoradiography, revealing higher receptor concentration in cortical and limbic brain areas (hippocampus, septum, cortical areas) and very low levels in basal ganglia and cerebellum, correlating almost identically with the receptor mRNA expression pattern. The pattern of 5-HT_{1A} receptor distribution is generally very similar across species, but it is somewhat different in cortical and hippocampal regions between human and rodents [Burnet *et al*. 1995; Barnes&Sharp, 1999].

In neurons, 5-HT_{1A} receptors are located postsynaptically in limbic areas, but in some brain regions (raphé nuclei) they act as autoreceptors on presynaptic part. Postsynaptic 5-HT_{1A} receptors inhibit adenylyl cyclase as well as open potassium channels. Presynaptic receptors function as somatodendritic auto-receptors modulating negative feedback of serotonergic neuronal activity. Presynaptic 5-HT_{1A} receptors in dorsal raphe nucleus are acting upon opening G-protein coupled K^+-channels without inhibition of adenylyl cyclase, leading to inhibition of cell firing [Basic Neurochemistry 2006].

5-HT_{1A} receptor has been shown to be able to couple to more than just one subtype of G-proteins. In hippocampus 5-HT_{1A} receptor seems to couple mainly to $G\alpha_o$ and weakly also to $G\alpha_{i3}$, whereas in cortex coupling to $G\alpha_o$ and $G\alpha_{i3}$ seems equally efficient. In anterior raphe the coupling seems to be mainly to $G\alpha_{i3}$ and in hypothalamus 5-HT_{1A} receptor seems to couple to $G\alpha_o$ and $G\alpha_{i3}$, but also to $G\alpha_{i1}$ and $G\alpha_z$ [la Cour *et al*. 2006]. In Sf9 cells 5-HT_{1A} receptor preferably couples to $G\alpha_{i1}$ and $G\alpha_z$ as indicated by [^{3}H]8-OH-DPAT binding [Butkerait *et al*. 1995].

The most common behavioral trait assigned to be regulated by 5-HT_{1A} receptor is anxiety, since knockout mice show anxious response [Olivier *et al*. 2001]. Therefore the main clinical potential of 5-HT_{1A} receptor agonists is considered to be regulation of anxiety and depression. 5-HT_{1A} receptors are

expressed very early in brain during embryonic life and they are considered to participate in brain maturation [Whitaker-Azmitia, 1991]. Prenatal stress has been shown to decrease density of 5-HT_{1A} receptors in hippocampus [van den Hove *et al*. 2006]. Hypothermic response and influence on long term memory have been shown to be related to 5-HT_{1A} receptors, also agonist 8-OH-DPAT has been shown to facilitate sexual behavior of male rats [Popova & Amstislavskaya, 2002; Morales-Otal *et al*. 2002]. Higher [Wong *et al*. 1993] but also lower [McBride *et al*. 1997] receptor density has been shown for alcohol-preferring rats in [^{3}H]8-OH-DPAT binding.

1.3.2. Other members of 5-HT_1 receptor family

5-HT_{1B} and 5-HT_{1D} receptors are also coupled to inhibition of cAMP formation and for some time they were considered to be just species variants of the same receptor. Presynaptic 5-HT_{1B} receptors modulate serotonin release, whereas postsynaptic receptors modulate release of other neurotransmitters (such as acetylcholine and dopamine). 5-HT_{1D} receptor mRNA is found only at low levels in basal ganglia, dorsal raphe nucleus and locus coeruleus. 5-ht_{1E} receptor was identified from homogenate of human frontal cortex, and its mRNA is found in cortex and caudate putamen. Due to lack of selective radioligands, this subtype has not been extensively studied and the true physiological role is to be revealed. In brain 5-ht_{1F} receptor mRNA is mainly found in cortex, hippo-campus and dentate gyrus, but the receptor is also expressed in peripheral nervous system.

1.3.3. 5-HT_2 receptor family

The 5-HT_2 receptor family comprises the subtypes 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. The family members are coupled to $G_{q/11}$ family of G-proteins modulating phospholipase C connected pathway. 5-HT_{2A} is found postsynaptically on serotonergic neurons, mainly in frontal cortex where it might be involved in higher cognitive function, but is also found in limbic system and basal ganglia. Activation of 5-HT_{2A} receptors results in increase of body temperature and secretion of adenocorticotropic hormone. 5-HT_{2A} may regulate also anti-psychotic drug action. 5-HT_{2B} is coupled to PIP_2 hydrolysis in clonal cells, but its role in CNS is still under investigation. In the brain, receptor mRNA is found in many regions, but it's protein product has been found in very low quantities.. The 5-HT_{2C} receptor is also found throughout the brain, mainly in limbic system and in the regions associated with motor function. mRNA of 5-HT_{2C} receptor gene undergoes splicing yielding an expression pattern for different splice variants in the brain [Burns *et al*. 1997].

1.3.4. 5-HT_3 receptor family

The 5-HT_3 receptor is the only ligand-gated ion channel in serotonin receptor family and it is located postsynaptically in the central and peripheral nervous system. There are 5 subunits' genes of 5-HT_3 receptors – 5-$HT_{3A, 3B, 3C, 3D, 3E}$ and additionally two pseudogenes – *HTR3C2* and *HTR3C4* in the human genome [Barnes *et al*. 2009]. The highest density of 5-HT_3 receptor in the brain is in the area postrema. 5-HT_3 receptor antagonists may have potential anxiolytic, antidepressant and cognitive effects but are also used for the treatment of nausea and vomiting in cancer patients receiving chemotherapy.

1.3.5. Other 5-HT receptors

5-HT_4, 5-HT_6 and 5-HT_7 receptors stimulate adenylyl cyclase via the G_s family of G-proteins. 5-HT_4 subtype can be found in olfactory tubercle, striatum, substantia nigra and hippocampus and it is located postsynaptically mediating release of several neurotransmitters. There are at least nine splice variants of the 5-HT_4 receptor. 5-HT_6 receptor mRNA has been found in striatum, nucleus accumbens, hippocampus, cerebral cortex and olfactory tubercle, but its function in intact tissue has not been characterized due to lack of a selective agonist. There are at least four known splice variants of 5-HT_7 receptor (5-HT_{7a}-5-HT_{7d}) which have not been shown to differ in their pharmacology. 5-HT_7 receptor is expressed in hippocampus, cortex, septum, thalamus, hypothalamus and amygdala. Four splice isoforms of 5-HT_7 receptor are known. 5-HT_7 receptor is involved in regulation of mammalian circadian clock.

There are also two types of orphan receptors in serotonin receptor family – 5-HT_{1P} and 5-ht_5 (the latter has two subtypes – 5-ht_{5A} and 5-ht_{5B}). In rat and mouse, the 5-ht_{5a} receptor mRNA is located in several brain regions, for example cerebral cortex, hippocampus and amygdala, and 5-ht_{5b} mRNA has been found in the hippocampus and habenula and dorsal raphe nucleus of rat and human. In recombinant systems 5-ht_{5a} receptor is coupled to adenylyl cyclase inhibition whereas the functional role of 5-ht_{5b} is unknown. 5-HT_{1P} receptor has been described in periphery and has high affinity for 5-HT and it mediates slow depolarization of particular myenteric neurons.

2. RECEPTOR INTERACTIONS WITH LIGANDS

2.1. Receptor-ligand interactions

In addition to known endogenous ligands, receptors can interact with other molecules, which bind specificly and modulate their activity. Receptor ligands are divided largely into two groups – antagonists and agonists, of which agonists are additionally divided to full agonists and partial agonists. While an antagonist is defined as a molecule that upon binding to a receptor interferes with a biologically active molecule (such as a hormone or agonist), an agonist is a molecule that produces its own effect upon binding to a receptor [Kenakin, 2004]. Agonists possess a property called efficacy, which describes their ability to activate a biochemical pathway. Partial agonists are molecules that have smaller efficacy for a certain biochemical pathway then full agonist. There are also other theories of agonist-receptor interaction, for example the ternary complex model [De Lean *et al*. 1980]. This model assumes that receptor exists in two different conformational states – active (activates G-protein) and inactive – and agonist stabilizes the active receptor conformation while inverse agonist stabilizes the inactive one. Antagonist binding does not interfere with the equilibrium between active and inactive conformations and partial agonists shift the equilibrium toward active conformation but to a lesser degree than full agonists. Derivatives of ternary complex model allow formation of more receptor conformations. Biochemical experiments have shown that different agonists for the same receptor may also activate different cellular pathways, called stimulus trafficking or biased agonism, proving that there may be several active receptor conformations. The shortcoming of ternary complex model is that it does not include the equilibrium between guanosine nucleotides and G-proteins on ligand binding to receptor. Therefore the catalytic kinetic model has been introduced including interactions between agonist, receptor, G-protein and nucleotide. In this model the equilibrium between the active and inactive states is also modified by G-protein and nucleotides and not only by agonist.

Many receptors have been shown to possess another ligand binding site for allosteric modulators in addition to orthosteric site. A number of molecules are known to act on allosteric site of different receptors, and many of them are known as drugs with high selectivity. Allosteric modulators usually do not have an effect on biochemical pathway on their own, but either enhance or inhibit agonist's activity. Allosteric modulators are known for receptors such as $GABA_A$, glutamate NMDA, muscarinic M_2, opioid receptors, etc. [Gao & Jacobson, 2006]

2.2. Radioligands

Radioligands are radioactively labeled ligands that can bind to a receptor. First radioligands with high specific activity and high affinity were introduced in 1970s, allowing measuring of the number and affinity of binding sites in a given biological sample, also to perform kinetic experiments. Over the years different radioactive isotopes have been used for labeling, such as ^{3}H, ^{32}P, ^{35}S and ^{125}I. All ligands can theoretically be used as radioligands (agonists, antagonists) but a constant drive is to develop ones with higher affinity. High affinity antagonists are especially useful for several studies, since they only block the receptor and do not activate a cellular response mechanism during the experiment. Furthermore, antagonist binding is not usually influenced by receptor-G-protein coupling, allowing measurements of the whole receptor pool in sample, although in some cases it could be biologically more meaningful to measure agonist binding. Usually, radioligand binding to receptors has expressed as a simple reversible reaction:

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL$$

and the affinity of the ligand to receptor is calculated as the equilibrium dissociation constant $K_D=[L]x[R]/[LR]$ of this reaction. In these cases the observational kinetic constant ($k_{obs}=k_{-1}+k_{+1}[L]$) is measured under ligand excess over the receptor ([L]>>[R]), and k_{obs} is in linear relationship with ligand concentration. However, more complex binding equilibriums have been described [Järv *et al*. 1979; Lepiku *et al*. 1996; Oras *et al*. 2002], where relationship between k_{obs} and ligand concentration is hyperbolic and additional conformational changes of the RL complex are proposed.

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RL^*$$

In some cases the relationship between k_{obs} and ligand concentration can be even sigmoidal revealing more complex interactions [Sillard *et al*. 1985].

2.3. Serotonin$_{1A}$ receptor ligands

A number of 5-HT$_{1A}$ receptor ligands for studying the receptor has been synthesized over the years and many of them have been labeled with radioactive isotopes. The most common ligand that has been used for 5-HT$_{1A}$ receptor studies is agonist 8-OH-DPAT. [^{3}H]8-OH-DPAT has been one of the most widely used 5-HT$_{1A}$ receptor radioligands, but since it is an agonist, it only binds to receptors in high-affinity state at the concentrations used. Development of antagonists (BMY7378, NAN-190, etc.) has been problematic due to insufficient selectivity and some of them (NAN-190, WAY100135) have been shown to act as partial agonists under certain conditions. Two antagonists developed quite recently have shown promising binding characteristics – WAY100635 [Hume *et al*. 1994; Laporte *et al*. 1994; Khawaja *et al*. 1995; Gozlan *et al*. 1995] and NAD-299 [Johansson *et al*. 1997; Jerning *et al*. 1998], the former been extensively used since it became commercially available. The biggest benefit of WAY100635 is that its tritiated form was the first selective 5-HT$_{1A}$ receptor antagonist on the market and it has been used in many studies making it possible to receive information about the whole receptor pool in the experiment [Gozlan *et al*. 1995]. Before [^{3}H]WAY100635, several other radioligands were used – [^{3}H]5-HT, [^{3}H]ipsapirone, [^{125}I]-BH-MeO-N-PAT, [^{125}I]-p-MPPI, all of which were problematic considering the selectivity and/or affinity.

3. G-PROTEINS

3.1. G-protein function

In 1978 the group led by Alfred Gilman identified a GTP-binding component that was necessary for adenylyl cyclase regulation [Ross *et al*. 1978], which gave birth to the term "G-protein" meaning a "GTP binding regulatory protein". Now it is known, that G-proteins play the central role in signal transduction, regulating a variety of cellular processes acting as a receptor mediated on/off switch directing extracellular signal to cell's interior. Mammalian G-proteins are divided into two major categories – small G-proteins and heterotrimeric G-proteins. Heterotrimeric G-proteins consist of three subunits, namely α, β and γ, which after G-protein activation dissociate to α-subunit and βγ-dimer which can be separated only by denaturation.

The heterotrimeric G-proteins in mammalian nervous system have been categorized by their α-subunit functions to families of G_s, G_i, G_q and G_{12}. 27 Gα, 5 Gβ and 13 Gγ subunit genes have been described in the human genome [Venter *et al*. 2001]. G_i-subcategory receives its name from the ability to inhibit adenylyl cyclase, while G_s-proteins stimulate adenylyl cyclase. The main pathway for the G_q family of G-proteins is activation of phospholipase C pathway and the G_{12} family regulates activity of RH-Rho-GEF proteins. β and γ subunits have specific cellular distribution and are shared by different α-subunits. Although most β subunits can interact with most γ subunits, the formation of all possible complexes has not been found. In addition to Gα-subunits, Gβγ-subunits are also modulating the activity of several bio-chemical pathways.

Gα-subunits have GTPase domain that binds and hydrolyses GTP and helical domain that buries the nucleotide within the core of the protein. The helical domain is the most divergent between G-proteins, and could determine the specificity for interactions with a receptor. Gβ-subunit has β-propeller structure and couples to γ-subunit using the γ-subunit's N-terminus. [Pierce *et al*. 2002]

G-protein subunits are modified by covalent addition of long-chain fatty acids. These moieties help the G-proteins to attach themselves to lipid membrane to interact with other membrane proteins. All Gα-subunits but $G\alpha_t$ are palmitoylated, members of $G\alpha_i$ family are also myristoylated, γ-subunits are modified on their C-terminal cystein residues by isoprenylation [Cabrera-Vera *et al*. 2003]. These covalent modifications may also regulate nucleotide exchange of G-protein [Cao & Huang, 2005].

Table 1. Classification of G-proteins and their respective signaling pathways [Pierce *et al*. 2002; Cabrera-Vera *et al*. 2003]

Family	Subtype	Effector
G_s	$G\alpha_{s(S)}$	↑adenylyl cyclase, calcium channels
	$G\alpha_{s(L)}$	↑GTPase of tubulin ↑src tyrosine kinase
	$G\alpha_{olf}$	↑adenylyl cyclase, calcium channels, src tyrosine kinase
G_i	$G\alpha_{i1}$ $G\alpha_{i2}$ $G\alpha_{i3}$	↓adenylyl cyclase, ↑src tyrosine kinase, Rap 1 GTPase activating protein, GRIN 1 and 2
	$G\alpha_{oA}$ $G\alpha_{oB}$	↑GTPase of tubulin ↑src tyrosine kinase
	$G\alpha_z$	Ca^{2+} and K^+ channels, Rap 1 GTPase activating protein
	$G\alpha_T$	↑cGMP-phosphodiesterase
	$G\alpha_g$	Phosphodiesterase
G_q	$G\alpha_q$ $G\alpha_{11}$ $G\alpha_{14,15,16}$	↑phospholipase C, ↑Bruton's tyrosine kinase, LARG Rho-GEFs
G_{12}	$G\alpha_{12}$ $G\alpha_{13}$	↑Na^+/H^+ exchanger, ↑phospholipase D, ↑p115Rho- GEF, ↑iNOS, PDZ-RhoGEF, LARG Rho-GEF, E-Cadherin

↑ – activation; ↓ – inhibition; GEF – guanine nucleotide exchange factor; iNOS – inducible nitric oxide synthase.

3.2. G-protein activation and nucleotide exchange

Although some reports have shown that 7TM receptors can activate cellular pathways without the inclusion of G-proteins, GPCR's mainly deliver the signal to G-protein, through conformational change in the orientation between receptor's helices [Nygaard *et al*. 2009]. GPCRs are also known to interact directly with other proteins such as G-protein coupled receptor kinases, RGS proteins and arrestins [Pierce *et al*. 2002].

The simplified understanding of the G-protein activation cycle which is a part of the main signal transduction pathway by 7TM receptors, starts at the resting state when G-protein is in its heterotrimeric form with GDP bound to α-subunit. After the receptor attached to G-protein binds a neurotransmitter (agonist), a conformational change in receptor follows. Change in receptor is transferred to G-protein via C-terminus of G-protein (since antibodies targeting that region block receptor-G-protein signaling), and G-protein adopts the change in receptor conformation allowing GDP to dissociate. GDP dissociation is considered to be the rate limiting step of G-protein activation. Nucleotide binding pocket is occupied by GTP, which lowers the affinity of α-subunit to βγ-dimer losing the hydrophobic binding pocket used by βγ-dimer and the

G-protein dissociates. Both, α-subunit and βγ-dimer are biologically active, influencing activity of adenylyl cyclase, ion channels and other enzymes. α-subunits possess GTPase activity hydrolyzing the bound GTP to GDP allowing the G-protein to re-associate and couple to receptor when ligand has dissociated from the receptor, and the system reaches the resting state. In addition to GPCR-s, RGS-proteins affect G-protein stimulating GTPase activity while AGS-proteins influence nucleotide exchange on Gα [Blumer *et al*. 2005]. After the signal transduction from outside the cell to inside, the activated receptor is often desensitized, meaning the weakening of the signal transmission under long lasting stimulation. Desensitization is initiated by phosphorylation of the receptor on cytoplasmic side by protein kinases and can happen in long and in short term. In case of short term desensitization the receptor signaling is blocked by binding arrestin to the phosphorylated site, whereas in case of long term desensitization, down regulation is connected with arrestin-mediated internalization.

Although the crystal structure of five 7TM receptors and several G-proteins are known, there are still some uncovered issues to the sequence of events and players in the nucleotide exchange in G-protein. Ligand binding to receptor takes place about 40 Å away from the peptide sequence that is coupled to G-protein and is thought to initiate changes in G-protein conformation. Additionally, the receptor coupled sequence is about 30 Å away from nucleotide binding site in G-protein. C-terminus of G-protein is considered to be the main player in this interaction, since mutations in C-terminus have been shown to mediate receptor-G-protein selectivity [Conklin *et al*. 1993], but additional regions are possibly also needed [Cerione *et al*. 1985]. Gβγ has been shown to participate in nucleotide exchange as well [Rondard *et al*. 2001], making things more complicated, since it is unclear whether receptor or Gβγ acts with Gα first [Oldham *et al*. 2006; Nanoff *et al*. 2006]. It is also possible, that simultaneous interaction of both receptor and Gβγ in required for full activation of G-protein [Johnston&Siderovski, 2007] and that βγ sequence that initiates guanosine exchange on one Gα, could be the exchange inhibitor for another [Johnston *et al*. 2005]. Mutations in G-protein have been used to determine the amino acids in binding pocket responsible for binding guanosine nucleotides. It has been widely accepted that residues in α5 helix are the main component interacting with ribose part of the nucleotides affecting basal nucleotide release rates and mediating receptor-catalyzed nucleotide exchange. The phosphate backbone is known to form a complex with Mg^{2+} in solution at cellular concentrations, therefore it is unknown if guanosine nucleotides enter G-protein in complex with Mg^{2+}, or Mg^{2+}-ion binds later to G-protein-guanosine nucleotide complex.

Radioactively labeled guanosine nucleotides have been used as G-protein ligands for studying nucleotide exchange and G-protein activation cycle. While GDP can be used in its natural form, use of GTP would be inefficient since it would be hydrolyzed to GDP by GTPase activity of Gα shortly after binding.

Therefore several of non-hydrolyzable GTP analogues have been synthesized like GTPγS and Gpp(NH)p, which are also available in radiolabeled form and widely used for characterization of nucleotide binding properties of G-proteins.

3.3. Role of Mg^{2+} and Mn^{2+} in nucleotide exchange

Mg^{2+} has been found to play a key role in signal transduction mechanism of GPCR. Two Mg^{2+} binding sites have been shown on $G\alpha_s$ and $G\alpha_{i1}$ in GDP and GTPγS bound conformation [Malarkey *et al*. 2008]. The binding constants are in nanomolar and in millimolar range for the different sites. It has been thought, that binding of Mg^{2+} with equilibrium constant in nanomolar range is stabilizing GTPase function of G-protein, while at the site with equilibrium constant in millimolar range, Mg^{2+} is responsible for nucleotide exchange. Two regions, namely switch I and switch II of Gα contribute the residues necessary for interactions with Mg^{2+} and beta/gamma phosphate of guanosine triphosphate. When GDP is bound to Gα, Mg^{2+} remains in the same place, however switch I does not make a contact with Mg^{2+} anymore. Mg^{2+} binds to Gα with low affinity when GDP is bound to protein, but Mg^{2+} is needed to release the βγ-subunit from heterotrimer and for the full activation of G-protein [Coleman & Sprang, 1998].

Higashijima and co-workers have shown that binding of Mg^{2+} inhibits GTPγS dissociation from $G\alpha_o$ and $G\alpha_i$ as well as from GDP-free oligomeric G_o and G_i protein. It was also shown that Mg^{2+} locks GTPγS into $G\alpha_o$, as the addition of Mg^{2+} blocks GTPγS dissociation but 10 mM $MgSO_4$ did not accelerate the rate of GTPγS binding to $G\alpha_o$ and $G\alpha_i$, and created very small increase in number of GTPγS binding sites [Higashijima *et al*. 1987]. They also claim that Mg^{2+} binding to $G\alpha_o$-GTPγS is very fast with kinetic constant 10^6 M^{-1} min^{-1}. In case of GDP, addition of Mg^{2+} accelerated GDP dissociation from heterotrimeric G_o protein but did not change the rate of dissociation from $G\alpha_o$. Altogether these data propose that Mg^{2+} is able to inhibit GDP affinity and binding kinetics to G-protein [Higashijima *et al*. 1987]. In case of GTPγS, Mg^{2+} inhibits nucleotide dissociation, however it should be noted that some authors have considered GTPγS a very slowly dissociating nucleotide from G-protein [Chidiac *et al*. 1999]. GTPγS release could also be agonist-dependent [Kupprion *et al*. 1993] and presence of agonist could affect also GDP affinity [Shiozaki & Haga, 1992].

There are hints in the literature, that Mn^{2+} changes nucleotide exchange dynamics [Mohini *et al*. 1985; Hall *et al*. 1985; Harikumar&Chattopadhyay, 1998; Arro *et al*. 2001]. More specifically, Mn^{2+} was shown to accelerate GDP release under some conditions [Quist *et al*. 1999]. Although discussed in many papers, no systematic study reports on manganese effects on guanosine nucleotide exchange were available to us.

3.4. Manganese in organism

Manganese is an important trace element in organisms and it is found in variety of tissues. Manganese is required for proper functioning of several organisms' physiological functions, such as metabolism, immune system function, regulation of cellular energy, bone growth and blood clotting. As an important cofactor of several enzymes in the brain it is involved in neurotransmitter synthesis and metabolism. Manganese is known in 11 oxidation states, of which Mn^{2+} and Mn^{3+} are found in living organisms.

Accumulation of manganese in organism has been shown to cause neuronal damage in the midbrain and the development of symptoms resembling Parkinson disease. Manganese overexposure can be work-related (welding, mining, battery assembly, etc.) but also can result from liver disease or contaminated drinking water. The normal brain manganese concentration is in the range of 10 μM [Sumino *et al*. 1975], but in case of intoxication the concentration increase might be more than 10-fold, as suggested from primate and rat studies [Ingersoll *et al*. 1999; Lai *et al*. 1999; Roels *et al*. 1997]. The main understanding of how overexposure to manganese causes Parkinsonian symptoms is that the high concentrations of manganese damage and kill dopaminergic neurons [Prabhakaran *et al*. 2008], claiming that disturbances in dopaminergic signal transmission is the main cause of symptoms. However, dissimilarities between manganism and Parkinsonism include less frequent resting tremor, more frequent dystonia, particular propensity to fall backwards and most of all – manganism is insensitive to levodopa treatment [Parenti *et al*. 1986].

Several groups have shown that manganese causes free radical formation and produces reactive oxygen species and oxidative stress [Zhang *et al*. 2009; Milatovic *et al*. 2009; Gunter *et al*. 2006], inhibition of astrocyte function [Giordano *et al*. 2009; Hazell 2002] or even astrocyte death [Gonzalez *et al*. 2008] probably through selective changes in expression of a number of genes [Sengupta *et al*. 2007], mitochondrial dysfunction [Gunter *et al*. 2009; Gonzalez *et al*. 2008; Zhang *et al*. 2008], disturption of calcium dynamics in mitochondria and endoplasmatic reticulum [Tjalkens *et al*. 2006] and also it has been shown to accumulate in nucleus of the neurons [Kalia *et al*. 2008]. Opposing the studies above, manganese has been shown to exhibit neuroprotective action under some conditions [Sziràki *et al*. 1995; Worley *et al*. 2002]. Also the genomic background of the workers suffering from manganese overexposure has been studied, since it has been known that not all individuals, as well as animals, respond to manganese overexposure in the same way. Interestingly, proposed candidate genes were not specific to dopaminergic signal transduction [Curran *et al*. 2009].

In addition to vulnerable dopamine system, a few studies have shown that other neurotransmitter systems are influenced by manganese intoxication, such as glutamate [Fitsanakis *et al*. 2006; Xu *et al*. in press 2009; Takeda *et al*. 2002

(b)], GABA [Anderson *et al*. 2008; Fitsanakis *et al*. 2006; Takeda *et al*. 2002 (b)] and cholinergic [Finkelstein *et al*. 2007].

Relatively little is known about how manganese influences serotonergic system. Involvement of serotonin receptors and also hippocampus has been suggested by group led by Michael Aschner [Finkelstein *et al*. 2007]. Furthermore, it has been shown that Mn^{2+} is able to influence ligand binding to 5-HT_{1A} receptor in some, but not in all brain regions [Hall *et al*. 1985; Harikumar& Chattopadhyay, 1998; Arro *et al*. 2001; Milligan *et al.* 2001]. Hall and co-workers showed that a clear effect was detected to the high-affinity binding component of 8-OH-DPAT and only in hippocampal membranes, whereas no effect was seen on cortical or striatal preparations. Ability of Mn^{2+} to stabilize [^{3}H]8-OH-DPAT high affinity binding in expense of the low-affinity sites was noticed by Mongeau and others [Mongeau *et al*. 1992]. In case of α_2 noradrenergic receptors, larger number of high-affinity binding sites for agonist was detected in presence of Mn^{2+}, but the number of low affinity binding sites remained unaltered [Rouot *et al*. 1980; U'Prichard & Snyder, 1980]. Similar effect has been shown for other receptor agonists, for example adenosine A_{2a} receptor [Villalobos *et al*. 2001], substance P receptor [Mohini *et al*. 1985] and muscarinic M_1 receptor ligands [Ladner&Lee, 1999]. However, Mn^{2+} does not influence antagonist binding [Chang & Snyder, 1980; Ladner&Lee 1999] until 10 mM concentration [Harikumar&Chattopadhyay, 2001]. Although reported in many papers, the background of the effect of Mn^{2+} has remained unclear.

4. AIMS OF THE STUDY

The aims of this thesis were:

- Determination of kinetic properties of [^{3}H]WAY100635 binding for characterization of 5-HT_{1A} receptors in different samples.
- Comparison of Mg^{2+} and Mn^{2+} in regulation of ligand binding to 5-HT_{1A} receptors in different tissues.
- Determination of tissue-specific mechanisms of regulation of signal transduction of 5-HT_{1A} receptor by bivalent ions Mg^{2+} and Mn^{2+}.

5. MATERIALS AND METHODS

5.1. Membrane preparations

Wistar rats were decapitated and respective brain regions were dissected by prof. J. Harro as described before [Alttoa *et al*. 2005]. Hippocampal and cortical tissues were homogenized in 30 vol (ww/v) of 50 mM Tris–HCl buffer, pH 7.4 (IB) by Bandelin Sonoplus sonificator (2 passes, a´ 10 s), incubated for 30 min at room temperature and centrifuged at 43,000g for 20 min at 4°C. The membrane pellet was washed by re-suspending in 50 mM Tris–HCl buffer pH = 7.4 followed by centrifugation for two more times. The final pellet was re-suspended in 30 vol (ww/v) of the buffer and stored at –80°C until use.

Sf9 cells (Quattromed AS, Tartu, Estonia) were grown as described in [Uustare *et al*. 2006]. The recombinant baculoviruses for G protein subunits and 5-HT_{1A}R were prepared by prof. Johnny Näsman (Åbo Academi University, Finland) as described earlier [Näsman *et al*. 2002]. Cells were harvested by centrifugation (10 min at 1500 rpm), and homogenized by sonification, and centrifuged and washed as described above for hippocampal and cortical membranes. The final pellet was stored in –80°C until further use.

5.2. Radioligand binding

In saturation binding experiments the suspension of hippocampal membranes (100 μg protein) in the IB containing 0.1 mg/ml bovine serum albumin (BSA) was incubated with different concentrations (0.009–0.71 nM) of N-[2-[4-(2-[O-methyl-^{3}H]methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclohexane carboxamide ([^{3}H]WAY100635) (81 Ci/mmol; Amersham Pharmacia Biotech) in total 1 ml reaction volume at 25°C for appropriate time. For [^{3}H]-8-hydroxy-*N*,*N*-dipropyl-2-aminotetralin ([^{3}H]8-OH-DPAT) binding (0.01–1.8 nM, 106 Ci/mmol; Perkin Elmer Life Sciences, Inc) the incubation time was 60 minutes and the volume of reaction was 250 μl. $MgCl_2$, $MnCl_2$ and EDTA were used in 1 mM concentrations unless noted otherwise. The reaction was terminated and bound radioactivity was separated by filtering through GF/B (Whatman) glass microfibre filter and washing three times with ice-cold 3 ml of 20 mM potassium-phosphate-buffer (pH = 7.4) containing 100 mM NaCl. The radioactivity content of the filters was counted in 5 ml of scintillation cocktail OptiPhase HiSafe®3 (Wallac Perkin Elmer Life Sciences) by Beckman LS 1800 scintillation counter. The specific binding was determined as the difference between total and nonspecific binding (nonspecific binding measured in presence of 10 μM WAY100135 or 8-OH-DPAT). In displacement experiments, a range of concentrations of displacing ligands were incubated with fixed concentration (0.5–0.9 nM) of [^{3}H]WAY100635 for 90 min at 25°C or

(0.2–0.8 nM) [^{3}H]8-OH-DPAT for 60 min at 25°C and the reaction was terminated as described above.

5.3. Kinetic experiments

Association kinetic experiments were started by addition of [^{3}H]WAY100635 (final concentration from 0.08–4.5 nM) to a membrane suspension in the incubation buffer (final volume depending on the radioligand concentration between 2.8 and 12 ml). At time intervals aliquots (200–1000 µl depending on radioligand concentration) were taken and filtered on GF/B as described above. Parallel incubations with corresponding concentration of [^{3}H]WAY100635 and 10 µM 5-HT or WAY100135 were used to estimate non-specific binding.

Dissociation kinetics were measured after preincubation of membranes with 0.5 nM [^{3}H]WAY100635 for 90 min at 25°C. Dissociation was then initiated by addition of non-labelled 5-HT and at timed intervals aliquots (200 µl) were filtered on GF/B filters and the bound radioactivity was determined as described above.

5.4. [^{35}S]GTPγS binding and displacement

In [^{35}S]GTPγS binding experiments the suspension of hippocampal membranes (50 µg/ml) in the incubation buffer, containing 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 3 mM $MgCl_2$, 200 µM GDP, 1 mM dithiotreitol (DTT) was incubated with 0.2 nM [^{35}S]GTPγS at 30°C for 90 min in the presence of appropriate concentrations of ligands as described in [Rinken *et al*. 1999]. For determination of antagonistic properties 10 µM 5-HT was used to achieve maximum effect of 5-HT_{1A}-specific [^{35}S]GTPγS binding over basal and appropriate concentrations of competitive antagonists were used. The reactions were terminated by filtration through GF/B glass microfibre filter as described above.

Binding parameters of nucleotides were measured by their ability to inhibit [^{35}S]GTPγS binding. Experiments were carried out in 50 mM Tris–HCl buffer (pH 7.4) with added 1 mM DTT and 100 mM NaCl in presence of 1 mM $MgCl_2$ or $MnCl_2$. 30 µg protein/sample of brain membranes or 5 µg protein/sample of Sf9 cell (serotonin$_{1A}$ receptor + Gi protein) membranes were incubated with 0.2 nM [^{35}S]GTPγS and a range of concentrations of displacing guanosine nucleotides (GDP, GTPγS). The reaction was terminated by filtration and the bound radioactivity was determined as described above.

5.5. Data analysis

All data were analyzed by means of non-linear least squares regression method using a commercial program GraphPad PRISM^{TM} (GraphPad, San Diego, CA, USA). Data are presented as mean ± SEM of at least two independent determinations carried out in duplicates. Statistical significance of differences was determined by Student–Newman– Keuls test, where $P < 0.05$ was taken as a criterion of significance.

6. RESULTS

6.1. Characterization of [^{3}H]WAY100635 binding

Addition of [^{3}H]WAY100635 to the rat hippocampal preparation caused time-dependent increase in specific binding of the radioligand. Data was fitted to the exponential equation derived for pseudo first-order reaction to homogenous population of binding sites, revealing apparent association rate constant for 0.5 nM [^{3}H]WAY10063 to be k_{obs} =0.10 ± 0.01 min^{-1}. The specific binding of [^{3}H]WAY100635 could be reversed with the excess of non-labelled competitive ligand and off-rate constant k_{off} = (7.8 ± 1.1) × 10^{-3} min^{-1} was obtained for this process. This means that full dissociation of the radioligand and achievement of equilibrium at all radioligand concentration requires at least 7.5 hours ($\tau_{½off}$ = 89 ± 11 min). With independent experiments we found that loss of the specific binding sites of [^{3}H]WAY100635 in the hippocampal membranes in the absence of ligands was still slower than the radioligand dissociation ($\tau_{½inact}$ = 26.4 ± 5.6 h), but it had to be taken into account in interpretation of the results. As a compromise, we used 240-minutes long incubation for [^{3}H]WAY100635 binding experiments, since within that time the receptor inactivation(< 10%) can be neglected and equilibrium is achieved at most of concentarations of the studied radioligand. Binding parameters for [^{3}H]WAY100635 in hippocampal membranes under these conditions were K_D=87 ± 4 pM and B_{max}=15.1 ± 0.2 fmol/mg protein.

More detailed kinetic analysis of [^{3}H]WAY100635 binding showed that at concentrations up to 2.9 nM the obtained apparent association rate constants increased linearly with the increase of [^{3}H]WAY100635 concentration, indicating that the process follows regularities of simple bimolecular reaction between the ligand and the receptor and the second order on-rate constant of this process was k_1=0.14 ± 0.01 min^{-1} nM^{-1}. At higher ligand concentrations the reaction rate increased rapidly and it could not be reliably measured with conventional radioligand binding methods. These data show that [^{3}H]WAY100635 binding is not a simple bimolecular reaction. There are several possibilities for that kind of binding profile – for example conformational changes of receptor-ligand complex or ligand binding cooperativity. In case of cooperativity, binding of several ligand molecules to the receptor would accelerate conformational changes between the firstly bound ligand and the receptor and could speed up the isomerization process. This sort of behavior has been described for a muscarinic antagonist [^{3}H]QNB [Sillard et al., 1985]. Second possibility is that binding sites on the receptor are tandemly arranged and therefore binding of more than one antagonist molecule(s) would interfere with the dissociation of the first bound antagonist as it has been demonstrated for the melanocortin system [Kopanchuk et al., 2006].

In competition experiments all used 5-HT$_{1A}$ receptor ligands fully displaced [^{3}H]WAY100635 binding and their potencies were in general agreement with

the affinities of these ligands to the receptor reported earlier. Displacement curves for antagonists had Hill coefficients close to unity, while displacement curves of agonists were shallow with the Hill coefficients close to 0.5 and two-binding site model was preferred by t-test in fitting of these data. In the presence of 30 μM GTPγS, which was used to activate G-proteins, agonists' apparent potencies were decreased and Hill coefficients were increased on their displacement caused by reduction of the proportion of high-affinity binding sites (Table 2). Removal of Mg^{2+} from the reaction medium with EDTA had also decreasing influence on the agonist binding potencies, while antagonists' binding properties were not affected by these modulators (Table 2).

Table 2. Inhibition parameters of some 5-HT_{1A} receptor ligands from [^{3}H]WAY100635 displacement experiments measured in hippocampal membranes in presence of Mg^{2+} or EDTA and with or without GTPγS

Ligand	K_i (K_{iH} and K_{iL}, nM; (α_H))*			
	Control		+ GTPγS	
	Mg^{2+}	EDTA	Mg^{2+}	EDTA
5-HT	2.1 ± 0.4 (0.63 ± 0.04) 110 ± 42	10 ± 2 (0.40 ± 0.03) 780 ± 180	(N.D. †) 525 ± 136	(N.D.) 758±219
8-OH-DPAT	0.21 ± 0.03 (0.78 ± 0.03) 22 ± 12	2.1 ± 0.7 (0.67 ± 0.11) 35 ± 23	0.2 ± 0.1 (0.22 ± 0.03) 22 ± 3	(N.D.) 15 ± 1
S14506	1.0 ± 0.2	1.5 ± 0.4	2.6 ± 0.3	3.0 ± 0.5
WAY100635	0.11 ± 0.01	0.11 ± 0.01	0.19 ± 0.01	0.12 ± 0.01
WAY100135	2.5 ± 0.2	3.9 ± 0.4	4.6 ± 0.3	4.3 ± 0.3
NAN-190	2.5 ± 0.2	6.8 ± 1.0	6.7 ± 1.0	7.9 ± 1.4

* –K_i values were calculated from displacement curves against 0.2 nM [^{3}H]WAY100635 with corrections of Cheng-Prusoff [Cheng&Prusoff 1973]. In the case of preferred two-site model the K_{iH} and K_{iL} values and the fraction of high-affinity binding sites (in parentheses) are presented.; † – not detected.

In addition to ability to inhibit [^{3}H]WAY100635 binding, 5-HT caused also concentration-dependent activation of [^{35}S]GTPγS binding to hippocampal membranes, achieving activation of 66 ± 2% over the basal level in the presence of 200 μM GDP with potency EC_{50} = 1.6 ± 0.3 μM. Here the antagonists studied inhibited this activation and their apparent affinities were calculated to be in this experiments for WAY100635 K_i = 7.0 ± 3.1 nM, for NAN-190 K_i = 80 ± 34 nM and for WAY100135 K_i = 109 ± 31 nM. The results from radioligand binding and competitive binding experiments did not coincide with the results of functional experiment. Similar discrepancy has been found also earlier for adenosine A_{2A} receptors [Uustare *et al*. 2005], where it was shown that in displacement experiments only isomerized complex of the radioligand with the

receptor is measured due to fast dissociation of un-isomerized complexes on the filter. In functional experiment, which is in principle non-equilibrial system, the formation of first un-isomerized agonist-receptor and antagonist-receptor complexes regulate [^{35}S]GTPγS binding on G-proteins, therefore different potencies of antagonists are obtained in comparison with equilibrial experiments.

6.2. Regulation of ligand binding by $MnCl_2$ and $MgCl_2$

Magnesium is known as an essential component for generation of agonist high-affinity binding to 5-HT_{1A} receptors, but in some cases manganese was even more efficient in this system. There were no significant differences in [^{3}H]8-OH-DPAT binding affinity in hippocampal membranes in the presence of 1 mM $MgCl_2$ or $MnCl_2$ (K_D = 0.28 ± 0.03 in presence of either salt), whereas in the presence of manganese considerably higher number of binding sites was detected (B_{max} = 31.6 ± 1.1 fmol/mg tissue) in comparison with the radioligand binding in the presence of magnesium (B_{max} = 22.1 ± 0.9 fmol/mg tissue). However, in the similar experiment with cortical membranes, statistically significant difference in number of high-affinity binding sites for [^{3}H]8-OH-DPAT was not found – B_{max} = 7.6 ± 0.2 fmol/mg tissue with 1 mM $MgCl_2$ and B_{max} = 8.2 ± 0.4 fmol/mg tissue with 1 mM $MnCl_2$ (p=0.31). Antagonist binding, measured by specific binding of [^{3}H]WAY100635 to these membranes was not significantly affected in the case of either tissues by these ions. This allowed to use [^{3}H]WAY100635 as the tracer ligand for the characterization of agonist binding to 5-HT_{1A} receptors. Also in this assay format the bigger stabilizing effect of Mn^{2+} on agonist high-affinity binding to 5-HT_{1A} receptors in rat hippocampal membranes in comparison with Mg^{2+} was confirmed. Here we found that 5-HT had 93 ± 2% of binding sites in high-affinity state in the presence of 1 mM $MnCl_2$ in comparison with 70 ± 1% with 1 mM $MgCl_2$ and 28 ± 1% after removal of these bivalent ions with 1 mM EDTA. In cortical membranes Mg^{2+} and Mn^{2+} were equipotent for generation of agonist high-affinity binding, and the level of high-affinity binding sites for agonist remained on the level of 71 ± 10%. Since there are no reports of differences in rat 5-HT_{1A} receptor structure between expression sites, which could be a reason of different Mn^{2+}/Mg^{2+} effects in different tissues, the involvement of G-proteins or other molecules further down the biochemical pathway can be considered. As Mg^{2+} is reported to be essential for nucleotide binding to G protein and for generation of high-affinity agonist binding same sites could also be targets for Mn^{2+} and therefore we studied the influence of these ions on nucleotide exchange as the next step.

The involvement of G-proteins in Mn^{2+} and Mg^{2+} regulation is indicated by the fact that in displacement of [^{3}H]WAY100635, 30 μM GTPγS turned most of the 8-OH-DPAT high-affinity binding sites to low affinity state in presence of $MgCl_2$ (α_H= 7 ± 4%), but considerable amount of high-affinity binding sites was still detected in presence of 1 mM $MnCl_2$ (α_H=31 ± 7%). In rat cortical membranes different regulation after addition of 30 μM GTPγS was not found and receptors were turned into low-affinity state for agonist in presence of either Mg^{2+} or Mn^{2+}. However, if 30 μM GTPγS was substituted for 1 μM GTPγS, different regulation of $MnCl_2$ and $MgCl_2$ appeared also in cortical membranes and was detected in hippocampal preparations as well. There were no high-affinity agonist binding sites with of 1 mM $MgCl_2$, but in presence of 1 mM $MnCl_2$ their proportion was on the level of 61 ± 1% in hippocampal membranes and 49 ± 2% in cortical membranes. This kind of sensitivity to GTPγS concentration refers directly to involvement of G-proteins and/or regulators of nucleotide exchange in augmentation of agonist high-affinity binding.

Different sensitivity of G proteins in different tissues can be caused by different subtypes of the trimers. Therefore we created baculovirus system for expression of 5-HT_{1A} receptors in Sf9 cells with or without G-proteins' sub-types/subunits. The presence of the receptors were confirmed by specific and high-affinity binding of [^{3}H]WAY100635 (K_D = 0.27 ± 0.03 nM and $B_{max}(G\alpha_i)$ = 63 ± 4 fmol/μg protein, $B_{max}(G\alpha_s)$ = 7.0 ± 0.9 fmol/μg protein). Sf9 cells expressing serotonin$_{1A}$ receptor without additional G-proteins showed no high-affinity agonist binding in presence of 1 mM $MgCl_2$ or $MnCl_2$ (pIC_{50} values 6.33 ± 0.05 and 6.36 ± 0.04, respectively). Co-infection of Sf9-cells with baculovirus of 5-HT_{1A} receptor and baculovirus of Gs-protein caused a slight increase in agonist affinity but differences did not appear between Mg^{2+} and Mn^{2+} (pIC_{50}=6.96 ± 0.07 and pIC_{50}=7.04 ± 0.09 in presence of these cations, respectively). When Sf9-cells were co-infected with baculoviruses for 5-HT_{1A} receptor and Gi proteins ($G\alpha_{i1}$ and $G\beta_1\gamma_2$), 8-OH-DPAT exhibited high-affinity binding in presence of either cation (with Mg^{2+} (pIC_{50} = 8.36 ± 0.05) and with Mn^{2+} (pIC_{50} = 8.73 ± 0.06)). No significant difference appeared in number of high-affinity binding sites – α_H= 81 ± 4% with $MnCl_2$ and α_H=70 ± 4% with $MgCl_2$. Since Mg^{2+} and Mn^{2+} differed in their influence on agonist high-affinity binding in hippocampal and cortical membranes, the effect of GTPγS was measured only in preparations expressing Gi_1 with the receptor. In presence of 30 μM GTPγS neither Mg^{2+} nor Mn^{2+} could produce detectable amount of high-affinity binding sites. Yet in presence of 1 μM GTPγS and $MnCl_2$, 36 ± 3% high-affinity binding sites were detected.

6.3. Regulation of nucleotide binding by $MnCl_2$ and $MgCl_2$

It could be expected that properties of the signaling system through 5-HT_{1A} receptor would differ in brain tissue and in Sf9 cells, due to clear over-expression of receptor and G-proteins in transgenic cell line with markedly altered membrane composition and peculiarities of the post-translational machinery in the cell. But it also seems that different signaling complexes form in different parts of brain (hippocampus and cortex) for 5-HT_{1A} receptor, having unique sensitivity for guanosine nucleotides in presence of Mn^{2+}. Therefore we measured nucleotide potencies in these brain regions in presence of $MgCl_2$ or $MnCl_2$.

High-affinity agonist binding has been used as a parameter for characterization of nucleotide binding to G proteins. We measured the influence of nucleotides on the potency of 10 nM 8-OH-DPAT to displace binding of 0.5 nM [^{3}H]WAY100635 to hippocampal membranes in presence of $MgCl_2$ or $MnCl_2$. In the presence of 1 mM $MnCl_2$, GTPγS inhibited agonist binding with $pIC_{50} = 6.3 \pm 0.1$, while in the presence of 1 mM $MgCl_2$ with $pIC_{50} = 7.0 \pm 0.1$. Similar difference appeared for GDP, which inhibited high-affinity agonist binding with $pIC_{50} = 4.4 \pm 0.1$ and $pIC_{50} = 3.3 \pm 0.1$ in the presence of $MgCl_2$ and $MnCl_2$, respectively. In a separate experiment we measured how these ions in combination with nucleotides influence direct binding of [^{3}H]8-OH-DPAT, excluding possible alteration of results by the presence of antagonist. GDP was less potent to inhibit [^{3}H]8-OH-DPAT binding when Mn^{2+} was present ($pIC_{50}=4.0 \pm 0.3$ with Mg^{2+} and $pIC_{50}=3.0 \pm 0.1$ with Mn^{2+}), and similar tendency was found for GTPγS, which had $pIC_{50}=6.5 \pm 0.1$ with Mg^{2+} and $pIC_{50}=5.5 \pm 0.3$ with Mn^{2+}. In cortical membranes the same trend where nucleotides were less potent in the presence of Mn^{2+} in comparison with Mg^{2+}, was detected, although the difference was smaller, remaining in the range of 0.5 logarithmic units for GDP and GTPγS. These differences between brain regions point once again to the involvement of different G-protein-coupled complexes for the signal transduction of 5-HT_{1A} receptors in different brain regions.

We also measured how $MgCl_2$ and $MnCl_2$ influence the nucleotides' potencies to displace [^{35}S]GTPγS binding in hippocampal, cortical and Sf9 membranes. Obtained data indicated that in these assays the nucleotides are much more potent than in inhibition of agonists' high-affinity binding and potency decrease effect of manganese appeared only for GDP, but not for GTPγS in all preparations studied (Table 3).

Table 3. Inhibition of [^{35}S] GTPγS binding by GDP and GTPγS in hippocampal, cortical and Sf9-cell preparations

	-log IC_{50}*					
	Hippocampal membranes		Cortical membranes		Sf9 (5-HT_{1A} + Gi)	
	GDP	GTPγS	GDP	GTPγS	GDP	GTPγS
Mg^{2+}	7.6 ± 0.1	8.9 ± 0.1	8.0 ± 0.1	9.1 ± 0.1	7.4 ± 0.1	8.7 ± 0.1
Mn^{2+}	7.2 ± 0.1	8.8 ± 0.1	7.4 ± 0.1	9.1 ± 0.1	7.1 ± 0.1	8.6 ± 0.1

* – Negative logarithm of the concentration causing 50% inhibition of 0.2 nM [^{35}S]GTPγS binding.

These results show that Mn^{2+} inhibits potency of GDP on the regulation of signal transduction via 5-HT_{1A} receptors, while its role is not distinctively clear for GTPγS properties. Influence on GDP binding is the most pronounced in experiments where nucleotides inhibit [^{3}H]8-OH-DPAT binding being the most sensitive in hippocampal membranes and showing tenfold lower ability of GDP to inhibit agonist high affinity binding when Mg^{2+} is substituted with Mn^{2+}. Since GDP dissociation is considered to be the rate-limiting step of nucleotide exchange and G-protein activation, even slight disturbances in G-protein-GDP complex equilibrium influence the overall signal transduction via a receptor. Our results show that Mn^{2+} has great impact on 5-HT_{1A} receptor system, but we were not able to fully characterize this regulation.

7. CONCLUSIONS

The conclusions from this work can be summarized as follows:

- [^{3}H]WAY100635 binding kinetics is described as a simple bimolecular reaction between 5-HT$_{1A}$ receptor and [^{3}H]WAY100635 up to 2.9 nM ligand concentration. At higher [^{3}H]WAY100635 concentrations rapid increase of reaction rate takes place, possibly revealing cooperative behavior of the receptor-ligand complex.
- The affinities of antagonists determined from competition with [^{3}H]WAY100635 did not coincide with their potencies to inhibit 5-HT-dependent activation of [^{35}S]GTPγS binding indicating that different steps of ligand binding determine the potencies in these experiments.
- [^{3}H]WAY100635 binding to 5-HT$_{1A}$ receptors is with very high affinity and slow. Therefore this radioligand is excellent for determining 5-HT$_{1A}$ receptor binding sites, but it's applicability in equilibrium studies is strongly limited
- Mn^{2+} in millimolar concentration caused increase of high-affinity agonist binding to rat hippocampal membranes in comparison with experiments in the presence of Mg^{2+}, but not in rat cortical membranes.
- G_{i1} proteins are required to be co-expressed with 5-HT$_{1A}$ receptors to achieve high affinity agonist binding and its moderate modulation by Mn^{2+} ions.
- The potencies of guanosine nucleotides in inhibition of high-affinity agonist binding to 5-HT$_{1A}$ receptors in all studied preparations was clearly higher in presence of Mg^{2+} than with Mn^{2+}, meaning that by inhibiting guanosine nucleotide binding, Mn^{2+} acts as an enhancer for agonist binding.
- Different sensitivity to ions and regulation of nucleotide binding in hippocampal and cortical membranes proposes that 5-HT$_{1A}$ receptors in these tissues are coupled to different signal transduction system (subtypes of G proteins or regulatory proteins) but further studies are necessary to find out the most sensitive target of Mn^{2+} modulation.

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SUMMARY IN ESTONIAN

Ligandi sidumine 5-HT_{1A} retseptorile ja selle modulatsioon Mg^{2+} ja Mn^{2+} poolt.

Paljud seitsme transmembraanse heeliksiga G-valguga seotud retseptorid on farmaatsiatööstusele huvipakkuvaks märklauaks, sest nad on võtmeks terve rea organismis toimuvates biokeemiliste protsesside regulatsioonis. Serotoniini$_{1A}$ (5-HT_{1A}) retseptori vastu tuntakse huvi enamasti tema seotuse tõttu depressiooni ja ärevustunde kujunemisel. Samuti on rottidel tehtud uuringud on näidanud retseptori seotust seksuaalkäitumise ja alkoholilembusega.

Antud töö esimeses osas iseloomustasime 5-HT_{1A} retseptori spetsiifilise antagonisti [^{3}H]WAY100635 sidumist roti aju preparaatide membraanidele. Katsetulemused näitasid, et ligandi sidumise kineetika on aeglane ning radioligandi sidumiskatses on tasakaalu saavutamiseks vaja reaktsioonisegu inkubeerida vähemalt 7,5 tundi 25°C juures. Selle aja jooksul toimub aga retseptorpreparaadi inaktivatsioon, mistõttu pakkusime optimaalse kompromissina välja 4-tunnise inkubatsiooniaja, mille jooksul on saavutatud vähemalt 90% reaktsiooni sügavus kõikidel ligandi kontsentratsioonidel, samas kaotamata oluliselt proovi bioloogilist aktiivsust. Radioligandi seostumise kineetika mõõtmised näitasid, et madalamatel ligandi kontsentratsioonidel on reaktsiooni kiirus lineaarses sõltuvuses radioligandi kontsentratsioonist, mis on iseloomulik bimolekulaarsele üheetapilisele tasakaalulisele reaktsioonile. Kõrgematel ligandi konsentratsioonidel toimub aga reaktsiooni oluline kiirenemine ja ilmneb sidumise positiivne koperatiivsus, kus ligandi sidumine mõjutab järgmise ligandi ja retseptori vahelist interaktsiooni. Sidumise mitmeetapilisust ja komplekset mehhanismi kinnitas funktsionaalsete katsete tulemuste võrdlemine sidumiskatsete tulemustega. Need tulemused näitavad, et [^{3}H]WAY100635 on hea ligand 5-HT_{1A} retseptorite kontsentratsiooni määramiseks, kuid selle rakendatavus ligandide afiinsuse määramiseks tasakaalulises katses on vägagi piiratud.

Töö teises osas uuriti Mg^{2+} ja Mn^{2+}-ioonide mõju 5-HT_{1A} retseptoriga seotud signaaliülekandele. 1 mM Mn^{2+} suurendas spetsiifilise agonisti [^{3}H]8-OH-DPAT sidumist roti hippokampuse membraanidele 43% võrreldes sidumisega 1 mM Mg^{2+} juuresolekul, kuid ei mõjutanud oluliselt ligandi sidumise afiinsust. Samas aga roti ajukoore membraanides sellist erinevust ei esinenud. Neid tulemusi kinnitasid ka [^{3}H]WAY100635 väljatõrjumiskatsed agonistidega, kus Mn^{2+} juuresolekul oli kõrge afiinsusega sidumiskohtade fraktsioon hipokampuse membraanides oluliselt suurem kui Mg^{2+} sisaldusega reaktsioonis, kuid jällegi ei saadud sellist erinevust Mn^{2+} ja Mg^{2+} vahel ajukoore membraanides. Seejuures ei täheldatud nende ioonide olulist mõju [^{3}H]WAY100635 sidumisele kummagi aju piirkonna preparaadi korral. Kui aga väljatõrjumiskatsetes kasutati G-valkude aktiveerimiseks 30 μM GTPγS-i, kadus agonisti kõrge afiinsusega sidumine ajukoore preparaadis, kuid Mn^{2+} juuresolekul jäi hippokampuses osa

retseptoreid agonistile kõrge afiinsusega olekusse. Kui aga kasutati 1 µM GTPγS-i, ilmnes Mn^{2+}-ga agonisti kõrge afiinsusega sidumine ka ajukoore membraanpreparaadis, kuid Mg^{2+} juuresolekul ei esinenud seda kummaski preparaadis. Tundlikkus GTPγS-i kontsentratsioonile viitab G-valkude otsesele osalusele agonisti kõrge afiinsusega sidumiskohtade erinevas mõjutamises Mg^{2+}-i ja Mn^{2+}-i poolt. Selle mehhanismi täpsemaks uurimiseks kasutati Sf9 rakke, milles ekspresseeriti bakuloviiruste abil 5-HT_{1A} retseptorid kas koos imetajate G-valkudega ($G\alpha_i$ + $G\beta_1\gamma_2$ või $G\alpha_s$ + $G\beta_1\gamma_2$) või ilma G-valkudeta. Kõrge afiinsusega [^{3}H]WAY100635 sidumine toimus kõigile nendele preparaatidele, kuid kõrge afiinsusega agonisti sidumine saavutati ainult preparaadis, kus 5-HT_{1A} retseptorid olid ekspresseeritud koos G_i-valguga. Samas ei täheldatud ka selles preparaadis Mn^{2+} erisust Mg^{2+}-st ei [^{3}H]8-OH-DPAT sidumisel ega [^{3}H]WAY100635 väljatõrjumisel 8-OH-DPAT-iga nii nukleotiidide puudumisel kui ka 30 µM GTPγS juuresolekul. Küll aga säilis 8-OH-DPAT 36 ± 3% kõrge afiinsusega sidumiskohtadest ainult Mn^{2+} juuresolekul, kui G-valkude aktiveerimiseks kasutati 1 µM GTPγS. Need tulemused näitavad, et Sf9 rakkudes moodustunud 5-HT_{1A}R-G_i kompleks on oma tundlikkuses Mn^{2+}/Mg^{2+} ioonidele sarnane roti ajukoores toimiva 5-HT_{1A}R kompleksidega, kuid erinev hipokampuses esinevatest kompleksidest. Need erinevused võivad olla põhjustatud erinevate G-valgu alatüüpide osalemisest 5-HT_{1A} retseptori kaudu toimuvas signaaliülekandes erinevates aju osades, aga ka täiendavate regulaatorite esinemisest roti hippokampuses. G-valkude spetsiifilisuse uurimiseks mõõdeti nukleotiidide mõju 5-HT_{1A} retseptorkompleksile erinevates katsesüsteemides. Mn^{2+}-ioonid vähendasid oluliselt nukleotiidide võimet inhibeerida agonisti kõrge afiinsusega sidumist, kusjuures see mõju oli suurem GDP kui GTPγS puhul ja suurem hippokampuse, kui ajukoore preparaatide korral. Samas olid need nukleotiidid oluliselt efektiivsemad [^{35}S]GTPγS sidumise inhibeerimisel, kus Mn^{2+} eristuv mõju avaldus vaid GDP sidumisele. Antud tulemused näitavad, et erinevad G valgud on seotud 5-HT_{1A} retseptorite signaaliülekandega roti aju erinevates osades, mistõttu ka nende süsteemide tundlikkus Mn^{2+} ioonidele on erinev.

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PUBLICATIONS

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2005–2009	Estonian Biocentre, extraordinary researcher
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Main reserch areas

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Genomic background of mental retardation. Copy-number variation and epigenetic markers in genomic DNA in patients with mental retardation.

Development of detection platform to discriminate bacterial strains based on tmRNA as a biological marker.

List of publications

Sven Parkel and Ago Rinken (2004) Kinetics of [^{3}H]WAY100635 binding to 5-HT_{1A} receptors in rat hippocampal membranes; Proc. Estonian Acad. Sci. Chem, 53(3), pp. 116–124

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Peamine teadustegevus

Signaaliülekanne reteptorite ja G-valkude vahel. Signaaliülekande modulatsioon metalliioonide Mg^{2+} ja Mn^{2+} poolt.

Genoomse DNA koopiaarvu variatsioonide ja vaimse arengu mahajäämuse vaheliste seoste uurimine.

tmRNA kasutamine bakteridiagnostikas.

Publikatsioonide loetelu

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Erialane enesetäiendus

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