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

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Author's contribution:

I Performed some of the experiments and contributed to drafting the manuscript

II Principal investigator, performed most of the experiments and wrote the manuscript

III Principal investigator, performed most of the experiments and wrote the manuscript

IV Principal investigator, performed most of the experiments and wrote the manuscript

ABBREVIATIONS

5-HT	5-hydroxytryptamine
5-HT_{1A}	5-hydroxytryptamine (serotonin) receptor 1A
8-OH-DPAT	8-hydroxy-N,N-dipropyl-2-aminotetralin
C12E10	Polyoxyethylene (10) lauryl ether
F_2FlAsH	4′,5′-bis(1,2,3-dithioarsolan-2-yl)-2′,7′-difluorofluorescein
FlAsH	4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein
FRET	Förster resonance energy transfer
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GMP	Guanosine monophosphate
GPCR	G-protein coupled receptor
GppNHp	Guanosine 5′-[β,γ-imido]triphosphate
G-protein	guanine nucleotide binding protein
GTPγS	Guanosine 5′–O-[gamma-thio]triphosphate
NAD 299	(3R)-3-(dicyclobutylamino)-8-fluoro-3,4-dihydro-2H-1-benzopyran-5-carboxamide hydrochloride
NAN-190	1-(2-methoxyphenyl)-4-(4-phthalimidobutyl)piperazine
RET	resonance energy transfer
Sf9	Spodoptera frugiperda 9
TCEP	tris(2-carboxyethyl)phosphine
WAY 100135	(S)-N-tert-butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide
WAY 100635	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)cyclohexanecarboxamide
βγM	dimer of β1 and StrepII and CCKACC labeled γ2-subunits

1. SIGNAL TRANSDUCTION BY GPCRS

1.1. GPCRs and transmembrane signaling

Chemical signal transmission between cells is mainly mediated by transmembrane proteins known as G-protein coupled receptors. These receptor proteins have extracellular ligand binding domains that recognize various signaling molecules such as peptide hormones, neurotransmitters like serotonin, acetylcholine – but they can also react to mechanical stimuli or even electromagnetic radiation (eg rhodopsin). Ligand binding to the receptor can initiate a conformational change in the receptor that alters its interactions with various intracellular effectors such as heterotrimeric G-proteins and arrestins. These intracellular proteins themselves also play an important role in regulating the activity of the receptor, in addition to participating in the transduction of receptor-mediated extracellular signals into intracellular responses.

In addition to their interactions with G-proteins all GPCRs share a common structural motif: they have seven transmembrane domains and an extracellular N-terminus and an intracellular C-terminus. GPCRs also undergo several post-translational modifications such as palmitoylation, phosphorylation, ubiquitination and glycosylation – which are important for receptor trafficking and effector interactions (Escriba et al., 2007). For example, GPCRs can be phosphorylated at multiple sites following agonist stimulation. This in turn can lead to arresting binding and receptor internalization and desensitization of the signaling pathway.

Between 300 and 800 GPCRs have been estimated to be present in the human genome (Bjarnadottir et al., 2006, Sharman et al., 2013), making it one of the largest protein families involved in signal transduction. There are currently six designated families of GPCRs, which are classified based mainly on their extracellular ligand binding domains (Sharman et al., 2013)

Class A (Rhodopsin-like) – tend to bind small ligands deep in their binding pockets.

Class B (Secretin receptor family) – tend to bind large peptides.

Class C (Metabotropic glutamate/pheromone) – have large “venus flytrap” ligand binding domains.

Adhesion – tend to have exceptionally long extracellular regions that facilitate cell and matrix interactions.

Frizzled – tend to have a cysteine-rich extracellular domain that has been implicated in Wnt binding.

Other

Although endogenous GPCR ligands tend to be activating (agonists), other types of ligands have been discovered and developed. For example: inverse agonists suppress constitutive receptor activity, thus inhibiting the ability of some receptors to spontaneously adopt their active conformations in the absence of agonists. Additional intermediate binding modes are also possible: (neutral) antagonists do not influence the active/inactive receptor equilibria, while partial agonists activate the receptor to a lesser extent than full agonists. Recent findings have also revealed the existence of biased agonism, where different ligands can elicit different downstream responses (Kenakin 2011). In addition to the aforementioned orthosteric ligands, receptors may also bind allosteric ligands that do not compete with the endogenous ligands and provide yet another approach to modulating receptor function. All of these types of ligands have found extensive use both in the clinic and the laboratory thanks to the comparatively easily accessible (and therefore druggable) extracellular ligand binding domains of GPCRs and their importance in the regulation of a vast array of physiological processes. Indeed numerous drugs have been developed that target GPCRs and a large proportion of the drugs in current clinical use (Overington et al., 2006) target these receptors. Despite this many GPCRs still have unidentified physiological functions and endogenous ligands (so called orphan receptors), so research into the role of GPCRs in various physiological processes is still very much ongoing.

1.2. 5-HT receptors

The monoamine 5-hydroxytryptamine (serotonin, 5-HT) in one of the first neurotransmitters discovered and it plays multiple roles in the mammalian CNS, including regulation of pain, mood, sleep and appetite (Nichols and Nichols 2008). In addition it has several major roles outside the CNS, eg. regulation of the gastrointestinal tract, hemostasis and blood clotting. Serotonin can be found in all bilateral animals, but it also often present in plants and fungi, making it one of the evolutionarily oldest signaling molecules.

Seven families of serotonin receptors have been classified in humans and many of these families contain multiple receptor subtypes. All of these serotonin receptors (except 5-HT_3 receptors, which are ligand-gated ion channels) are GPCRs. The 5-HT_1 family contains five subtypes denoted 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F} and all of them couple to $G_{i/o}$ proteins, while members of the other 5-HT receptor families may couple to $G_{q/11}$ or G_s. (Nichols and Nichols 2008)

In addition to serotonin receptors in the CNS, serotonin transporters also play an important role in modulating neurotransmission by terminating the signal via reuptake of the neurotransmitter. Many major classes of antidepressants target serotonin transporters, as inhibition of serotonin reuptake (which results in elevation of synaptic serotonin levels) is commonly thought to enhance mood.

The focus of this study was the 5-HT_{1A} receptor (a 46 kDa protein), which is the most widespread of all 5-HT receptors. It was also one of the first cloned receptors and one of the most thoroughly studied prototypical GPCRs. Several drugs have been designed to target this receptor (Lacivita et al., 2008) and activation by selective agonist (buspirone, tandospirone) has been found to relieve anxiety and depression. Some atypical antipsychotics also interact with this receptor and may play a role in their efficacy.

In addition to exploring the role of the 5-HT_{1A} receptor as a drug target, it has been implicated in some of the symptoms that result after overexposure to manganese, such as anxiety or irritability. It has also been shown that high affinity agonist binding to 5-HT_{1A} receptors is increased by Mn^{2+} in a tissue specific manner, where enhancement of agonist binding is especially pronounced in the hippocampus (Parkel et al., 2009). Mn^{2+} toxicity is also characterized by accumulation of the metal in the hippocampus, in addition to the basal ganglia and the cortex (Aschner 1999, Fitsnakis et al., 2006). It has been hypothesized that the tissue specific effects of Mn^{2+} could be explained by differences in G-protein coupling (postsynaptic 5-HT_{1A} receptors in the hippocampus have little receptor reserve (Meller et al., 2000), while Gi2 subunits are abundant (Aoki et al., 1992)) and investigating this possibility was one of the main aims of this thesis.

1.3. G-proteins

GPCRs like the 5-HT_{1A} receptor couple to heterotrimeric guanine nucleotide binding proteins (G-proteins), which are peripheral membrane proteins composed of α- (40–45 kDa), β-(~36 kDa), and γ-subunits (7–8 kDa). α- and γ-subunits are posttranslationally modified with lipid anchors that tether them to the plasma membrane. The β-subunit is also anchored as β- and γ-subunits form a tight dimer that does not disassociate under physiological conditions. There can be multiple combinations of β- and γ-subunits in these dimers as there are at least 6 β-subunit subtypes and at least 12 γ-subunit subtypes (Oldham and Hamm 2008). There are also at least 21 α-subunit subtypes (Oldham and Hamm 2008), which together with various βγ-subunit combinations can give rise to over a thousand different G-protein heterotrimers. Splice variants of α-subunits add even more possibilities to the list.

All α-subunits share similar structural motifs and are composed of a GTPase domain and an α-helical domain, with the guanine nucleotide-binding site situated between these two. The α-helical domain is believed to be responsible for sequestering the guanine nucleotide at the binding site, so is has to move in order to enable nucleotide exchange (Cherfils and Chabre 2003). The GTPase domain contains two flexible α-helices known as switch I and switch II, which change conformations when the α-subunit is activated.

1.4. G-protein activation and intracellular signaling

All heterotrimeric G-proteins bind GDP in their inactive state. When activated by a receptor their affinity for GDP is decreased and the nucleotide dissociates, followed by the association of GTP to the now active G-protein. Depending on the G-protein subtype, activation and GTP binding can be followed by dissociation of the heterotrimer into α- and βγ-subunits, which can both go on to interact with intracellular effector proteins. Subunit dissociation may, however, not be required for effector activation (Robishaw and Berlot 2004). Deactivation is accomplished by the intrinsic GTPase activity of the α-subunit, which hydrolyzes the nucleotide into GDP and returns the complex to the inactive state.

The activation and deactivation of G-proteins is also regulated by specialized proteins, which can, for example, either accelerate (GEFs) or inhibit (GDIs) GDP release or accelerate GTP hydrolysis (GAPs) (Siderovski and Millard 2005). Some GDIs may also inhibit heterotrimer reassociation by binding to the α-subunit and thus prolonging βγ-dependent signaling, while some G-protein effectors may have GAP activity. GPCRs themselves can also be viewed as GEFs.

Heterotrimeric G-proteins are classified based on the identity of the α-subunit and its effectors into several families (Milligan and Kostenis 2006). G_i family proteins (involved in inhibition of adenylate cyclase or activation of phosphodiesterases) are subdivided into $G_{i/o}$, G_t, G_{gust} and G_z. The G_s family is comprised of G_s and G_{olf}, which both activate adenylate cyclase. The G_q family is responsible for activation of phospholipase C and finally the $G_{12/13}$ family of α-subunits is involved in the activation of Rho family of small GTP-ases.

1.5. Role of Mg^{2+} and Mn^{2+} in G-protein mediated signal transmission

Guanosine nucleotides are normally complexed with divalent cations (usually Mg^{2+}) under physiological conditions and these divalent cations also play a crucial function in the activation/deactivation cycle of G-proteins: they are involved in GTP hydrolysis and nucleotide exchange. Divalent cations are also indirectly involved in receptor-G-protein interactions as addition of Mg^{2+} can promote GDP release, which in turn results in a transiently nucleotide-free G-protein. The latter is required for high affinity agonist binding (a state where the receptor is bound to a nucleotide-free G-protein) (Rinken 1996).

G_i protein α-subunits have two binding sites for divalent cations, one with a millimolar and the other with a nanomolar affinity (Malarkey 2008). The millimolar binding site is thought to be responsible for GDP/GTP exchange and high affinity agonist binding, while the nanomolar binding site is thought to be required for GTP hydrolysis. Mn^{2+} could substitute for Mg^{2+} in either of these binding sites (Freisinger and Sigel 2007) but its effects on high affinity agonist binding seem to point to competition at the millimolar binding site, as Mn^{2+} concentrations are required to be in that range for enhancement of high affinity agonist binding.

2. METHODS FOR THE CHARACTERIZATION OF RECEPTORS AND G-PROTEINS

In order to understand how GPCRs and G-proteins work on a molecular level it is generally necessary to be able to observe processes related to their functioning, in addition to characterizing them in more static terms such as based on their structure. This can be difficult as many of these proteins are present at relatively low concentrations (typically in the range of low pmol/mg total protein) in native tissues and therefore methods to study them under physiologically relevant conditions need to be very sensitive. Specificity can also be crucial as often several closely related receptors are expressed in the tissue or cell line being studied. Historically these issues were first addressed by using radiolabeled ligands (Paton and Rang 1965) that bind to distinct receptor populations, while in recent years fluorescent labeling has become even more popular. In addition to methods based on the binding of labeled ligands, assays may also use functional readouts such as generation of secondary messengers (Ca^{2+}, cAMP), activation genetically engineered biosensor constructs or even more basic responses such as cell survival or proliferation etc.

Ligand binding assays may be utilized for several purposes: for example to conduct basic research into receptor function, distribution and to discriminate between receptor subtypes. Ligand displacement assays also find widespread use in the development of novel receptor ligands and they may also even be employed to estimate endogenous ligand concentrations (or the presence of synthetic ligands) in native tissues.

2.1. Radioligand binding assays

Radioisotopes such as ^{3}H, ^{125}I or ^{32}P are ideal tools for labeling ligands in that isotopic replacement does not usually affect ligand binding to their target, whereas bulkier labels often need careful optimization to prevent loss of affinity. However, radioligand binding assays also have several drawbacks such as long read times, the requirement for a filtering step to separate bound and free ligand and also health hazards and disposal issues. These assays are also difficult to fully automate for HTS (even though homogeneous detection schemes such as SPA have been developed (Crane and Shih 2004)) and may also be quite expensive. The requirement for a filtration step also complicates kinetic analyses, whereas with optical methods ligand binding can often be monitored in real time. Fluorescent lables also make possible the observation of single-molecule binding events at very short timescales, while radioligands can be used as tracers to visualize receptor-releated processes deep inside living tissue (PET scans).

Radioligand binding assays are also somewhat limited in that they do not always discriminate between receptor ligands that cause different functional responses, ie agonists and antagonists. As ligands are often developed with the goal of generating functional responses, several approaches have been developed to overcome this limitation. Binding of the nonhydrolyzable nucleotide [^{35}S]GTPγS is one such method (Harrison and Traynor 2003). It is also perhaps the most direct and immediate downstream functional response that is common to all GPCRs, although nucleotide exhange kinetics depend on the G-protein that couples to the GPCR and these slow kinetics may render this assay unsuitable for G-proteins that do not belong to the Gi family. Still – G-protein based readouts are perhaps the most unambiguous methods for determining functional responses, as the response may be affected by other factors when measured further downstream in the signaling cascade. For example, even though a full agonist may activate a G-protein, the α- and βγ subunits that are released may have opposing effects on downstream effectors and lead to a response that more resembles that of a partial agonist or even an antagonist. An alternative G-protein based functional assay is based on the GTPase activity of G-proteins: hydrolysis of [^{32}P]GTP releases the radioactive γ-phosphate, which is separated from the labeled nucleotide by filtration. As mentioned previously, homogeneous detection schemes have also been developed for radioligands that enable their use in HTS, but hazardous waste and safety issues become even more pronounced in such large-scale screening projects. Therefore much attention has recently been paid to the development of fluorescent ligands.

2.2. Fluorescent ligand binding assays

The possibilities offered by fluorescent ligands have been recognized for decades and their uses have varied from being simple radioligand replacements to environment-sensitive probes that offer single-molecule level insight into the mechanisms, geometries and kinetics of receptor function and interaction with their binding partners. Fluorescent detection methods are also one of the few methods that can rival and sometimes even surpass radioassays in their sensitivity. As is the case with radioligands, it must also be possible to distinguish a bound fluorescent ligand from a free one and also specific binding from nonspecific binding. Absolute quantitation may also be a significant issue as the fluorescence of a fluorophore can be strongly dependent on its immediate molecular environment, its orientation, the specific geometry and optical properties of the detection instrument (Sridharan et al., 2014). Even when calibrated against a known fluorophore it is difficult to ensure that the fluorophores are placed under identical orientations in identical environments (biological samples can be very heterogenous). Whereas for radioligands, halflives are fundamental constants that are not affected by the environment,

although instrumental and experimental parameters may significantly influence the detected signal as well (eg by quenching of counting efficiency).

One of the reasons behind the increasing popularity of fluorescence-based methods is the increased availability of improved fluorophores that are bright, photostable and less sensitive to environmental parameters such as pH or the presence of a hydrophobic background that can result in significant nonspecific binding. Beside the choice of the fluorophore it is also critical to consider how they are conjugated to the ligand (often the fluorophore can be even bulkier than the ligand being labeled) as improper placement or linker lenght can lead to significant loss of affinity or even off-target effects, where the fluorophore itself can increase the affinity of the probe for undesired interactions and lead to false positive results.

There are still numerous limitations, such as the sensitivity to quenching agents, high background autofluorescence or scattering, to fluorescent detection methods. Some of these can be overcome by the use of a fluorophore with a long fluorescence lifetime (such as the lanthanides Eu or Tb) since autofluorescence, for example, tends to have a relatively short lifetime. Another approach to fluorescent detection is based on the nonradiative resonant energy transfer principle (RET), where – instead of relying on a single fluorophore – two optically active labels (with overlapping emission and excitation spectra) are used in tandem (Helms and Volkhard 2008). For example a FRET pair can be constructed between a fluorescent ligand and a fluorescently labeled receptor. In this case a RET signal becomes apparent only when the two fluorophores (or a fluorophore and a quencher) are in close proximity (generally less than 10 nm). If they are in close proximity the intensity of the donor signal is reduced and the intensity of the acceptor signal is increased and this change can be used as a sensitive method for measuring their distance from each other. Many different labeling strategies can be used for FRET, both inter- and intramolecular, in order to characterize the interaction of a protein with its various binding partners. An added advantage of this approach is that while labeling with a single environment-sensitive fluorophore often requires the use of purified receptor preparations in order to minimize background influence, RET methods can use less sensitive labels and still yield information about the environment thanks to altered donor-acceptor distances or geometries. This means that RET-based methods are generally better suited for studying receptors in their natural environment such as live cells (Sekar and Periasamy 2003).

RET-based methods are often based on the use of genetically engineered fluorescent proteins, which are bulkier than synthetic fluorophores by orders of magnitude. This can result in severe steric hindrance if placement of the fluorescent proteins are not carefully optimized for the protein interactions being studied, in addition to possibly affecting the localization, stability or even expression levels of the protein being labeled. Fluorescent proteins are also often less bright and photostable than small molecule fluorophores and they can

be subject to proteolytic cleavage. Therefore efforts have been made to engineer small-molecule fluorophores that can be used to specifically label proteins via a small genetically engineered binding sites. One such method is based on As-thiol interactions, where target proteins are modified to express multiple cysteine residues (often four or more) that bind biarsenical fluorescent ligands such as FlAsH (Griffin et al., 2001). Multiple FlAsH-analogues with different spectral properties and binding sites have been developed but their utility in cell-based assays has been met with limited success as nonspecific binding (which can be accompanied by significant changes in their fluorescent proteins) of these fairly hydrophobic proteins to cell membranes and endogenous cysteine rich proteins complicates the measurements (Stroffekova et al., 2001). It can also be difficult to ensure 1:1 stoichiometry of labeling. The environment-sensitive nature of some FlAsH-analogues, however, can be advantageous as the fluorescent label can be sensitive to changes in the conformation of the protein (Nakanishi et al., 2004) or the environment to which the protein is localized (eg cytoplasmic or membrane-bound).

2.3. Fluorescence polarization

Another approach to detecting binding of fluorescent ligands to their targets is based on the principle of fluorescence polarization: small fluorophores rotate rapidly in solution and therefore, if excited with linearly polarized light, emit it in random directions. Upon binding to a large protein target the rotation time is increased and the emitted light is no longer randomly polarized, which can be used as an indicator of binding. This method is homogeneous but depends on large changes in the populations of bound and free ligands, so fluorescence polarization assays need to be conducted in a mode where the concentrations of the target and labeled ligand are approximately equal and also close to their dissociation constant (Veikšina et al., 2014) This can be difficult for some targets that are expressed at low concentrations in native tissues, so methods to increase their abundance and/or decrease background have to be employed. Therefore much interest has been paid to expressing target proteins in cell lines, which can result in much higher expression levels and – depending on the expression system – lower background and crosstalk.

2.4. Heterologous expression systems

Multiple types of GPCRs and G-proteins are often expressed in mammalian cells, leading to possibilities for crosstalk due do, for example, competition for G-protein subunits or receptor heterodimerization (Rives et al., 2009, McGraw et al., 2006). These effects can make the investigations into the roles specific receptors or G-proteins difficult, even though overexpression strategies or treatment with bacterial toxins, use of fusion proteins etc can help to attain

selectivity. An alternative to GPCR signaling complex expression in mammalian cells is to introduce them into cell lines that have limited possibilities for crosstalk. Numerous such expression strategies have been employed with great success.

Bacterial and fungal expression systems were among the first (Bertin et al., 1992, King et al., 1990). Very high expression levels (important for structural studies, for example) can be attained at relatively low costs, but these single-celled organisms are often unable to correctly fold and posttranslationally modify mammalian proteins. This can significantly alter the functional properties of the proteins. Therefore multiple strains of bacteria and fungi have been created that express mammalian protein processing components, which can result in fully functional protein expression. Still, careful control experiments are needed to determine if the proteins are expressed and modified correctly and this can be very challenging if the protein being expressed is difficult to purify from (or characterize) in its native environment. Therefore, when very high expression levels are not critical, it is often more practical to use animal cell lines for protein expression.

One of the simplest protein expression systems using animal cells is based on cell lines derived from Spodoptera frugiperda (American fall army worm) pupal ovarian tissue that are infected with genetically engineered baculoviruses that drive heterologous protein expression using viral promoters (Luckow and Summers 1988) Relatively high protein yields are possible (milligrams/liter of culture medium), while the posttranslational modification machinery of insect cells usually results in the expression of fully active mammalian proteins (although glycosylation might be absent or incorrect in some cases) (Sutkowski et al., 1994, Taussig et al., 1993, Hepler et al., 1993). Insect cells can also be grown in serum free media and in suspension culture without need for CO_2 supplementation (in contrast to most mammalian cell lines), which have made them popular for large scale protein expression.

Another advantage of insect cells in comparison to mammalian cells is their lack of G-proteins that couple efficiently to mammalian GPCRs (Butkerait et al., 1995). Neither do they express GPCRs that are homologous to mammalian receptors or tend form oligomers with mammalian GPCRs, which could alter functional responses and ligand binding. Therefore mammalian signaling complexes can often be reconstituted in insect cells and studied in relative isolation – without the crosstalk that can be common in their native environment. Although limited coupling to insect G-proteins is possible in many cases: such as with the 5-HT_{1A} receptor, which can couple to endogenous inscet G_o-like proteins (Mulheron et al., 1994). Still – coupling is usually significantly less efficient and extensive than in mammalian cells and reconstitution of high-affinity agonist binding in insect cells requires coexpression of mammalian G-protein heterotrimers (Seifert et al., 1998, Lachance et al., 1999).

In addition to protein expression in insect cells, baculoviruses themselves can be used as a source of proteins for binding and even functional assays: budded baculoviruses envelope themselves with their host cell plasma membrane (Loisel et al., 1997, Saitoh et al., 2006). Using budded viruses also ensures that only fully formed and membrane-bound proteins are present in the assay, while simultaneously enriching these components in comparison to Sf9 cells. This can result in lower nonspecific binding and autofluorescence, which makes many assays based on fluorescent ligands (such as polarization based assays) more feasible (Veikšina et al., 2014). The cell-free nature of baculovirus preparations can also make the system more stable, while their ability to infect only insect cells means minimal safety precautions are needed in comparison to expression systems based on viruses that can infect mammalian cells.

3. AIMS

The overall aim of the thesis was to find new possibilities for studying G-protein mediated biochemical regulatory mechanisms in signal transduction. Special attention was paid on guanine nucleotide binding and its modulation by 5-HT_{1A} receptors and the bivalent cations Mg^{2+} and Mn^{2+}.

To achieve these general aims, several particular objectives were proposed:

- compare modulation of nucleotide binding by 5-HT_{1A} receptors and Mg^{2+} and Mn^{2+} in different brain regions and membranes of Sf9 cells with different G-proteins.

- monitor nucleotide binding to G-proteins by measuring changes in the binding parameters of fluorescent receptor ligands as they interact with 5-HT_{1A}-G-protein complexes in baculovirus particles of various compositions.

- expand possibilities for monitoring nucleotide binding to G-proteins using fluorescence-based approaches.

- engineer fluorescence-labeled G proteins for sensing their activation and nucleotide binding

4. MATERIALS AND METHODS

4.1. Reagents and cell lines

Spodoptera frugiperda 9 (Sf9) cells were from Invitrogen Life Technologies or from Quattromed AS. Tris, HEPES, NaCl, EDTA, $MnCl_2$, $MgCl_2$ were from Applichem GmbH. Guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine 5'-O-[gamma-thio]triphosphate (GTPγS), Guanosine 5′-[β,γ-imido]triphosphate (GppNHp), ethanedithiol, tris(2-carboxyethyl)phosphine (TCEP), serotonin (5-HT), detergents and desthiobiotin were from Sigma-Aldrich GbmH. β-mercaptoethanol and digitonin were from Merck KGaA. Saponin was from Fisher Scientific. Bodipy-FL-NAN-190 (Catalogue # 201592) was from CellAura. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine were from Avanti Polar Lipids. Mastoparan, 1-(2-methoxyphenyl)-4-(4-phthalimidobutyl)piperazine (NAN-190), (3R)-3-(dicyclobutylamino)-8-fluoro-3,4-dihydro-2H-1-benzopyran-5-carboxamide hydrochloride, (NAD-299), (S)-N-tert-butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide (WAY-100135), N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridyl)cyclohexanecarboxamide (WAY-100635) and (±)-8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT) were from Tocris Bioscience. $AsCl_3$ was from Reachim. 4′,5′-bis(1,2,3-dithioarsolan-2-yl)-2′,7′-difluorofluorescein (F_2FlAsH) was synthesized according to published procedures (Spagnuolo et al., 2006). 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FlAsH) was from Toronto Research Chemicals. G-protein α-subunits (αq, αs_{long}, αs_{short}, αolf and α13) were from Kerafast Inc. Tetracysteine-labeled peptide (FLNCCPGCCMEP) was from Bachem AG. Fluorescein was from Lambert Instruments. [^{3}H]WAY-100635 (0.6 – 0.8 nM, 81 Ci/mmol) was from GE Healthcare Life Sciences. [^{3}H]8-OH-DPAT (0.8 – 1.2 nM, 106 Ci/mmol) and [^{35}S]-GTPγS) (1250 Ci/mmol) were from PerkinElmer Life and Analytical Sciences. OptiPhase HiSafe® 3 scintillation cocktail was from PerkinElmer Inc.

4.2. Preparation of membrane homogenates for radioligand binding studies

Brain tissue membranes: hippocampal and cortical tissue of Wistar rats was dissected and collected as described previously (Tonissaar et al., 2008) by prof. J. Harro's group. Bandelin Sonoplus sonicator (Bandelin electronic GmbH) was used to homogenize tissues in 50 mM Tris–HCl buffer (pH 7.4, IB), followed by incubation for 30 min at room temperature and centrifugation at 43,000 × g (Sorvall RC-5C, DuPont Instruments) for 20 min at 4°C. Pellets were re-suspended in IB and centrifuged for two more times. The final pellet was re-sus-

pended in the buffer and used for experiments immediately or stored at –80°C until use.

Sf9 cell membranes: Sf9 cells (Quattromed) were grown on the Sf900 medium at 27°C in suspension. The recombinant baculoviruses for G-protein subunits and 5-HT_{1A}R were prepared by prof. Johnny Näsman (Åbo Akademi, Finland) as described earlier (Nasman et al., 2001). For expression experiments Sf9 cells were infected with viruses coding for 5-HT_{1A} receptors, Gα- and Gβγ-subunits. Cells were harvested after 48 hours by centrifugation and homogenized by sonication, centrifuged and washed as described above for hippocampal and cortical membranes.

4.3. Expression of G-proteins (with or without 5-HT_{1A} receptors) in Sf9 cells for experiments with budded viruses or purified proteins

G-protein expressing baculoviruses were prepared as described in (Tõntson et al., 2012), 5-HT_{1A} expressing baculoviruses were a gift from Johnny Näsman, prepared as described in (Nasman et al., 2001). Sf9 cells (Invitrogen), grown in serum-free media, were simultaneously coinfected with 5-HT_{1A} (only for experiments with budded baculoviruses) and βγM-subunit expressing baculoviruses for control experiments with no heterologously expressed G protein α-subunits, or also with baculoviruses that were designed to express human G-protein α-subunits (either i1, i2 or i3). Sf9 cells were collected after cell viability had dropped down to approximately 50% by sedimenting the cells by centrifugation at 2000 × g for 5 min, after which the supernatants were centrifuged for 30 min at 40 000 × g (Sigma 3K30, SIGMA Laborzentrifugen GmbH) to sediment virus particles. The virus pellets were then resuspended in resuspension buffer (20 mM HEPES, pH=8.0, 10 mM NaCl, 1 mM EDTA, 2 mM β-mercaptoethanol and 2% DMSO) and frozen at –80 °C. Cell membranes for protein purification experiments were frozen as cell pellets at –80 °C.

4.4. Purification of G-proteins

For protein purification experiments the thawed cell pellets were homogenized in ice cold homogenization buffer (HB: 20 mM HEPES, pH = 8, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 5 μM GDP, 5 mM β-mercaptoethanol) and fresh protease inhibitors (diluted according to manufacturer's recommendations: Roche Complete EDTA-free, Roche) by sonication (Bandelin SonoPuls). Homogenates were then centrifuged for 30 min at 40 000 × g (Sigma 3K30) and the resulting membrane pellets resuspended in solubilization buffer (HB with 1% Na-cholate, 0.1% C12E10 and 0.5% dodecylsucrose) and shaken for 1 h at

4 °C at 250 rpm (ELMI DOS-20S, ELMI Ltd). The solubilized proteins were separated by centrifugation for 30 min at 40 000 × g (Sigma 3K30) and purified with affinity chromatography using Strep-Tactin Superflow high capacity resin (IBA GmbH) in Poly-Prep columns (Bio-Rad). The columns were washed with washing buffer (WB: 20 mM HEPES, pH = 8, 10 mM NaCl, 1 mM EDTA, 0.5% C12E10, 5 mM β-mercaptoethanol and optionally 2 mM MgCl2 and 5 μM GDP) and the G-proteins were eluted with elution buffer (WB + 2 mM desthiobiotin). Eluates were aliquoted, frozen and kept until use at 80 °C.

4.5. Analysis of purified G-proteins and quantitation of bound GDP

Protein fractions were analyzed using SDS–PAGE with 12% acrylamide Tricine gels (Schagger 2006). Tetracysteine-tagged proteins were visualized using F_2FlAsH-staining (Spagnuolo et al., 2006) on a Typhoon Trio fluorescence imager, followed by Ag-staining (Fermentas PageSilver). For fluorescent labeling 2 μM F_2FlAsH was added to SDS–PAGE samples during denaturation at 98 °C for 15 min, followed by an overnight incubation at 4 °C. Total purified protein was quantified by UV-absorbance at 280 nm (NanoDrop 1000, NanoDrop products). The amount of GDP that remained bound to our purified protein preparations was determined after heating the preparations for 30 min at 65 °C to denature the protein and release the nucleotide. The GDP concentration was determined by measuring the ability of these preheated solutions to inhibit Bodipy-FL-GTPγS binding to purified active G proteins, in comparison to dilutions of a standard solution of GDP for calibration.

4.6. Radioligand binding experiments

Suspensions of brain membranes (100 μg protein/sample) or Sf9 cell membranes (15 μg protein/sample) in 50 mM Tris–HCl buffer (pH 7.4) with 1 mM $MgCl_2$, $MnCl_2$ or EDTA were incubated with fixed concentrations of one of the radioligands – [^{3}H]WAY-100635 or [^{3}H]8-OH-DPAT – with a range of concentrations of displacing ligands or guanosine nucleotides. The reaction was terminated by rapid filtration through GF/B glass fiber filter (Whatman) and three washings with 3 ml ice-cold 20 mM K-phosphate-buffer (pH 7.4) containing 100 mM NaCl. Radioactivity content of the filters was measured using Beckman LS 1800 scintillation counter after overnight incubation of the filters in scintillation cocktail.

Binding of [^{35}S]GTPγS was measured in 50 mM Tris–HCl buffer (pH 7.4) with 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 ($5HT_{1A}R$ + G_i) membranes were incubated with 0.2 nM [^{35}S]GTPγS and

different concentrations of displacing guanosine nucleotides as described in (Pulges and Rinken 2008). The reaction was terminated, filtered on GF/B and bound radioactivity was measured as described above.

4.7. Fluorescence anisotropy measurements

All fluorescence anisotropy measurements were carried out at 28 °C in 96-well half area microtiter plates (Corning Product No.3993, Corning Life Sciences) in a Pherastar platereader (BMG LABTECH GmbH) as described in (Veiksina et al., 2010). Fluorescence anisotropies were measured using (polarized) excitation at 485 nm (20 nm bandwidth) and simultaneous dual (polarized) emission at 520 nm (20 nm bandwidth), which enabled recording of fluorescence emission intensities that are parallel and perpendicular to the plane of excitation light. Erythrosin B was used for fluorescence polarization calibration (Thompson et al., 2002).

Experiments with Bodipy-FL-NAN-190: The concentration of Bodipy-FL-NAN-190 was estimated by measuring absorbance at 508 nm using an Ultrospec 7000 (GE Healthcare Bio-Sciences AB) spectrophotometer. Before fluorescence anisotropy measurements, frozen baculovirus concentrates were rapidly thawed and resuspended by passing them through a 28G needle (10 passes) before adding them to the assay buffer (20 mM HEPES, pH=7.8, 100 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$ or $MnCl_2$, 2 mM β-mercaptoethanol and (for permeabilization of baculovirus particles) either 10 μg/ml of freshly prepared digitonin or saponin. Unless otherwise specified, 5-HT_{1A} receptor concentration was 2.33 nM and Bodipy-FL-NAN-190 concentration was 1.35 nM in the assays and all reagents, proteins and microtiter plates were kept on ice prior to the initiation of the measurements.

Experiments with Bodipy-FL-GTPγS: When Bodipy-FL-GTPγS was used as reporter ligand for the determination of nucleotide binding (McEwen et al., 2001), association and dissociation kinetics were measured using GDP-depleted G-proteins (1.5 μM stock solution) that were preincubated with or without 0.5 μM GDP for 2 h on ice (no added divalent cations during the preincubation) prior to the measurement. The baseline fluorescence anisotropy of the reporter Bodipy-FL-GTPγS (10 nM) was measured in 20 mM HEPES, pH = 7.8, 10 mM NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.1% C12E10 and either 2 mM $MgCl_2$ or $MnCl_2$ (FA buffer) for 30 min. The reactions were initiated by addition of the protein samples that had been preincubated on ice (final G-protein concentration 60 nM). After monitoring the association reaction for 60 min, the dissociation of Bodipy-FL-GTPγS was initiated by addition of 100 μM GTPγS or 100 μM GDP. The apparent affinities of GDP and GTPγS were determined by their abilities to displace Bodipy-FL-GTPγS binding. The wells were loaded with 100 μl of G-protein (30 nM) in FA buffer along with various concentrations of unlabeled nucleotides and mastoparan (10 μM) and incubated

for 1 h on ice. Then 5 nM (final concentration) Bodipy-FL-GTPγS was added and the reaction was monitored for 120 min. In experiments using mastoparan, an activator of G-proteins (Higashijima et al., 1988), each well also contained 1.4 µg of a mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine liposomes to enhance activation by mastoparan (Higashijima et al., 1990). The liposomes were prepared by sonication (Bandelin Sonopuls) for five cycles of 10 s and overnight shaking at 250 rpm (ELMI DOS-20S) at 4 °C and were used within a week of preparation.

Experiments with F_2FlAsH: The apparent affinities of GDP, GMP, GTPγS and GppNHp were determined by their abilities to modulate F_2FlAsH fluorescence anisotropy in the presence of αβγM heterotrimers. For experiments with nucleotide-depleted αi-heterotrimers, 5 nM F_2FlAsH was used to label approximately 40 nM G-proteins and nucleotide affinities were determined at 2h from the start of the experiment. For experiments with αq, αs_{long}, αs_{short}, αolf and α13, 12.5 nM α-subunits were preincubated on ice with 15 nM βγM for 60 min, to allow the subunits to associate, before addition of 15 nM F_2FlAsH and nucleotides. Nucleotide affinities for αs_{long}+βγM, αs_{short}+βγM, αq+βγM and αolf+βγM were determined at 6h from the start of the experiment and for α13+βγM at 14h. All measurements (unless specified otherwise) were made in 20 mM HEPES buffer, pH=7.8, with 1 mM EDTA, 2 mM $MgCl_2$, 10 mM NaCl, 2 mM β-mercaptoethanol, 1 mM TCEP and 0.1% C12E10. All reagents, proteins and microtiter plates were kept on ice prior to the initiation of the measurements. In experiments where F_2FlAsH and G-proteins were subjected to heat denaturation, microtiter plates that had been measured in the platereader for 6 h were sealed with AbsorbMax adhesive film (Excel Scientific Inc) and heated at 70 °C for 1h in an Eppendorf Thermomixer Comfort (Eppendorf AG) with shaking at 400 rpm. The plates were then cooled to room temperature, unsealed and remeasured in the platereader for an additional 30 min. Data was then collected at 30 min from the start of the measurement.

4.8. Spectrophotometric measurements of F_2FlAsH-complexes

Fluorescence emission spectra of free F_2FlAsH (5 nM) and its complexes with G-proteins (12.5 nM αolf with or without 10 µM GTPγS, or just 15 nM βγM subunits) were determined using a Perkin-Elmer LS 55 luminescence spectrometer (PerkinElmer Inc) with excitation at 480 nm and a 10 nm emission slit width at 100 nm/min scan speed.

4.9. Fluorescence lifetime measurements of F_2FlAsH-complexes

Ffluorescence lifetimes were determined in the frequency domain by using an imaging attachment (LIFA-X, Lambert Instruments) consisting of a signal generator, Multi-LED excitation source with a 3 W light emitting diode (477 nm LED), and an intensified CCD Li^2CAM-X with GEN-III GaAs photocathode. The CCD was mounted on the side port of an iMIC inverted digital fluorescence microscope (Till Photonics GmbH). Multi-LED was fiber coupled to the epicondenser of iMIC. The filter cube comprised of a BrightLine HC 475/35 nm (Semrock) exciter, a zt 491 rdcxt dichroic (Chroma) and a BrightLine HC 525/45 nm (Semrock) emitter. For all samples and references a series of images with an exposure time of 150 ms was taken at 11 modulating frequencies (from 1–120 MHz, with increasing LED AC from 0.1 until 2.5 V) and 12 phase-shifts between LED and image intensifier per every modulating frequency. Photons were collected with 4 × UPLSAPO objective (Olympus). Measurements were made in two independent experiments using 50 nM F_2FlAsH in the presence or absence of either 60 nM βγM or αolf and α13 (with or without 10 μM GTPγS) or 600 nM of tetracysteine-labeled peptide (FLNCCPGCCMEP). F_2FlAsH and its complexes were preincubated at 28 °C for 6 h before lifetime measurements at room temperature.

4.10. Data analysis

All data were analyzed using nonlinear regression with GraphPad Prism (GraphPad Software Inc.).

Data analysis for radioligand binding experiments: Statistical comparison of non-linear regression models were performed by F-test as suggested by Draper and Hunter (Draper and Hunter 1967). Statistical significance of differences was determined by Student-Newman-Keuls test.

Data analysis for experiments with Bodipy-FL-NAN-190: Fluorescence anisotropy data was blank corrected for each experiment before data was pooled and fitted for determination of K_d and receptor concentration as described in (Veiksina et al., 2014). Fluorescence anisotropy data for Bodipy-FL-NAN displacement by serotonergic agonists and antagonists was pooled and normalized before fitting. Data in Tables 2, 3 and 4 were fitted without preliminary blank correction and/or normalization. Apparent potency values (pEC_{50}) were calculated using three or four parameter competitive binding equations and are presented as mean ± SD.

Data analysis for experiments with Bodipy-FL-GTPγS: Background corrected fluorescence anisotropies were calculated using data from at least two representative parallel experiments by subtracting background (no added fluorophores) parallel and perpendicular fluorescence intensities from the corresponding parallel and perpendicular sample intensities. For estimation of

association and dissociation kinetics the baseline anisotropy of free Bodipy-FL-GTPγS was additionally subtracted from sample anisotropies (to correct for volume effects caused by addition of proteins and GTPγS). Apparent affinity values (pIC_{50}) were calculated using three parameter competitive binding equations and data measured at approximately 60 min from the addition of Bodipy-FL-GTPγS. The statistical significance of the effects of Mn^{2+} and mastoparan was evaluated using the F-test. Protein purity was estimated using ImageJ 1.43 (National Institute of Health) by analyzing Ag-stained gels.

Data analysis for experiments with F_2FlAsH: Fluorescence anisotropy data was baseline (F_2FlAsH-G-protein complex with no added nucleotides) corrected for each experiment before data was pooled and fitted for determination of nucleotide affinities. Kinetic curves were also baseline corrected to show the appearance of nucleotide-sensitivity. Apparent affinity values ($logEC_{50}$) were calculated using three parameter competitive binding equations (Y=Bottom + [(Top-Bottom)/(1+10^(X-logEC$_{50}$))]), where X corresponds to logarithm of molar concentration of nucleotide.

5. RESULTS AND DISCUSSION

5.1. Radioligand based assays for characterizing receptor-G-protein coupling in native tissue homogenates and Sf9 cell membranes

The ability of Mn^{2+} to stabilize high affinity agonist binding has been known for a long time for several receptors. In case of 5-HT_{1A} receptors, the effects of Mn^{2+} depend on the region of the brain, showing enhanced high-affinity agonist binding in hippocampal membranes, whereas in cortical and Sf9-cell membranes no differences between Mg^{2+} and Mn^{2+} have been reported. These differences between tissues could be explained by selective receptor-G-protein coupling, but the exact mechanisms remain unknown. Therefore our initial experiments into 5-HT_{1A} and G-protein coupling started with radioligand binding experiments using membrane homogenates from specific rat brain regions or Sf9 cells (that expressed 5-HT_{1A} receptors and G-proteins), in order to make a detailed comparison of the effects of nucleotides and bivalent cations on receptor-G-protein complexes expressed in these tissues and to provide a control for developing further model systems with more defined compositions.

Our results showed that that the agonist 5-HT had superior affinity in displacing [^{3}H]WAY-100635 (a neutral antagonist) bound to rat hippocampal membranes in the presence of Mn^{2+}, in comparison to Mg^{2+}. A two-site binding model was statistically preferred, with the apparent affinities of 5-HT being 1.6 nM and 230 nM for high- and low- affinity binding sites, respectively. In the presence of Mn^{2+} the proportion of high affinity sites (87%) was significantly higher than in the presence of Mg^{2+} (66%). Addition of 30 μM GTPγS diminished almost all of the high affinity binding in the presence of Mg^{2+} (7%), while Mn^{2+} could retain a substantial amount (31%) of high-affinity binding in hippocampal membranes. In cortical membranes the enhancement of agonist binding by Mn^{2+} did not appear. Also, in this tissue preparation 30 μM GTPγS diminished all high affinity agonist binding sites even in the presence of Mn^{2+}. Thus, in hippocampal membranes, but not in cortical membranes, Mn^{2+} generated more high-affinity agonist binding sites than Mg^{2+} did.

In preparations of Sf9 cell membranes expressing 5-HT_{1A} receptors and Gi2 proteins (but not in membranes expressing Gs proteins), the agonist 8-OH-DPAT displaced [^{3}H]WAY-100635 binding also according to the two-site model, having apparent affinities of 1.5 nM and 97 nM for high- and low-affinity binding sites, respectively. The proportions of high-affinity binding sites were similar in the presence of Mn^{2+} and Mg^{2+} and in both cases these binding sites were lost in the presence of 30 μM GTPγS. However, if 1 μM GTPγS was used to activate G-proteins, 36% of high-affinity binding was retained in Sf9 cell membranes in the presence of Mn^{2+}, in comparison to 49% in cortical and 61% in hippocampal preparations. No high affinity binding was detected in any of these preparations the presence of Mg^{2+} when 30 μM GTPγS

was used. Thus enhanced agonist binding to 5-HT_{1A} receptors in the presence of Mn^{2+} appeared also in other tissue preparations, when different concentrations of GTPγS were used for activation of the system.

In summary, these results revealed Mn^{2+}-dependent differences in the GTPγS-sensitivity of high-affinity agonist binding between brain regions. In the presence of 30 μM GTPγS high-affinity agonist binding was not detected in cortical and Sf9 membranes, but not all receptors were turned into low-affinity state in hippocampal membranes. After lowering the concentration of GTPγS to 1 μM, high-affinity agonist binding appeared in the presence of Mn^{2+} also in cortical and Sf9 membranes, but not in the presence of Mg^{2+}. It shows that in different preparations 5-HT_{1A} receptors have no principal differences in sensitivity to Mn^{2+}, but the effects of nucleotides and the presence of either Mg^{2+} or Mn^{2+} seem to have crucial role in this regulation

Further experiments investigated the influence of nucleotides on high-affinity agonist binding to 5-HT_{1A} receptors by measuring as the ability of 8-OH-DPAT to displace binding of [^{3}H]WAY-100635 to hippocampal membranes in presence of varying concentrations of guanosine nucleotides and bivalent ions. Here, in the presence of Mn^{2+}, GTPγS inhibited high-affinity agonist binding with a pIC_{50} of 6.3, while in the presence of Mg^{2+} the pIC_{50} was 7.0. Similar differences appeared also in the case of GDP, which inhibited high-affinity agonist binding with pIC_{50} of 4.4 and 3.3 in the presence of Mg^{2+} and Mn^{2+}, respectively. In a separate experiment we measured how these ions, in combination with nucleotides, influence direct binding of [^{3}H]8-OH-DPAT. Here both nucleotides studied were less potent by almost an order of a magnitude in inhibiting [^{3}H]8-OH-DPAT binding when Mn^{2+} was present in comparison to experiments with Mg^{2+} (Table 1). A similar tendency was found also in cortical membranes, although the difference was smaller; remaining in the range of 0.5 logarithmic units (Table 1). Thus, Mn^{2+} decreased potencies of nucleotides for inhibiting agonist high-affinity binding to 5-HT_{1A} receptors and this effect was more clearly revealed in hippocampal preparations. These results also suggests that the role of Mn^{2+} in regulating nucleotide binding (and therefore high affinity agonist binding) is mainly realized through the G-proteins that couple with 5-HT_{1A} receptors in various tissues. Therefore direct measurements of how Mg^{2+} and Mn^{2+} influence nucleotide binding properties of G-proteins were undertaken by measuring nucleotide potencies in displacing [^{35}S]GTPγS binding in hippocampal, cortical and Sf9 membranes. In this assay the nucleotides were more potent than they were at inhibiting agonist high-affinity binding and the potency decreasing effect of Mn^{2+} appeared only for GDP, but not for GTPγS, in all preparations studied (Table 1). In comparison to modulation of high affinity agonist binding, Mn^{2+} had only a moderate influence on the nucleotide's properties in inhibiting [^{35}S]GTPγS binding. This indicates that the main fraction of G-proteins labeled with [^{35}S]GTPγS in brain membranes is not directly connected with 5-HT_{1A} receptors and other approaches suitable for characterization of the molecular mechanisms behind

the regulation of this receptor system by bivalent cations had to be found, since it seemed that efficient coupling of the 5-HT_{1A} receptor and G-proteins was necessary to detect specific regulation by Mg^{2+} and Mn^{2+}.

Table 1. Influence of magnesium and manganese on the potencies of guanosine nucleotides (pIC_{50}) to inhibit [^{3}H]8-OH-DPAT (0.5 nM) and [^{35}S] GTPγS (0.2nM) binding to preparations of different brain areas

		Hippocampus		Cortex	
		GDP	GTPγS	GDP	GTPγS
vs. [^{3}H]8-OH-DPAT	Mg^{2+}	4.0±0.3[a]	6.5±0.1	3.8±0.4	6.1±0.1
	Mn^{2+}	3.0±0.1	5.5±0.3	3.3±0.2	5.6±0.3
vs. [^{35}S]GTPγS	Mg^{2+}	7.6±0.1	8.9±0.1	8.0±0.1	9.1±0.1
	Mn^{2+}	7.2±0.1	8.8±0.1	7.4±0.1	9.1±0.1

[a] pIC_{50} values presented are the negative logarithm of concentrations of corresponding nucleotides causing 50% of inhibition of 0.5nM [^{3}H]8-OH-DPAT or 0.2nM [^{35}S]GTPγS binding to corresponding membrane preparation.

Therefore we decided to continue our studies using budded baculoviruses from Sf9 cells, where 5-HT_{1A} receptors could be reconstituted with specific G-protein subunits. Budded baculoviruses were chosen as the basis for this model system as they represent a concentrated and well-defined source of receptors and are therefore especially suited for fluorescence-based assays (Veikšina et al., 2014). By investigating the effects of Mn^{2+} and nucleotides on partial agonist binding in this model system, we aimed to selectively address receptor-G-protein coupling.

5.2. Characterizing receptor-G-protein coupling in budded baculovirus particles with fluorescent ligands

Our experiments using budded baculoviral particles as a model system for studying 5-HT_{1A} receptor coupling to G-proteins began with an initial characterization of Bodipy-FL-NAN-190, which is a novel 5-HT_{1A} specific fluorescent ligand. It is especially interesting for fluorescence anisotropy based applications as the fluorophore in conjugated to the ligand using a restrained linker, which should result in a large change in anisotropy upon binding to its target. Indeed the results showed large specific changes in both fluorescence intensity and anisotropy when Bodipy-FL-NAN-190 was added to 5-HT_{1A} receptors expressed in baculovirus particles, while there was no significant specific anisotropy change when virus particles without 5-HT_{1A} receptors were

used. The binding of Bodipy-FL-NAN-190 was rapid: association curves quickly reached a plateau and were fitted as a single phase exponential functions (although the reaction was not pseudo first order) and resulted in an apparent $t_{½}$ of 44.8 s. Dissociation kinetics were likewise rapid and single phase, with an apparent $t_{½}$ of 244 s. As both association and dissociation kinetics were rapid a steady-state analysis of Bodipy-FL-NAN-190 binding could be used for estimation of K_d of the ligand, which resulted in a value of 0.30 nM. This is in agreement with affinity proposed in IUPHAR-DB for NAN-190 (Newman-Tancredi et al., 1998).

5.2.1 Pharmacological characterization of the baculovirus-based model system

As Bodipy-FL-NAN-190 could be displaced from 5-HT_{1A} receptor expressing baculovirus particles using serotonergic ligands, it was used to characterize the binding of various such ligands in the presence or absence of GTPγS (and, in some cases, to compare the effects of Mg^{2+} and Mn^{2+}) to further characterize this model system.

Antagonist displacement experiments yielded single-site binding curves with the pIC_{50} values (Table 2) of the studied ligands being close to their previously reported vales. Addition of GTPγS had limited effects on ligand binding and did not substantially alter the pIC_{50} values, but it did appear to lower the fluorescence anisotropy of Bodipy-FL-NAN-190 in the absence of competing ligands. This suggests that Bodipy-FL-NAN-190 binding is agonist-like ([^{3}H]NAN-190 has also been reported to behave like a partial agonist in binding experiments (Rydelek-Fitzgerald et al., 1990), where nucleotides decreased the B_{max} of [^{3}H]NAN-190 by over 20%) and that GTPγS decreases high affinity Bodipy-FL-NAN-190 binding probably by limiting the amount of nucleotide-free G-proteins that are able to associate with the 5-HT_{1A} receptor.

Displacement experiments with 5-HT_{1A} agonists however yielded two-site binding curves, with the pIC_{50} values measured also being close to their previously reported values (Table 2) (Newman-Tancredi et al., 2001). The effect of GTPγS was again mainly apparent in the absence of significant amounts of competing ligands, but here we could additionally study nucleotide effects on the relative proportions of high and low affinity binding sites. The effects of GTPγS on the proportion of high affinity agonist binding sites were small in the absence of coexpressed αi-subunits, indicating limited coupling of insect G-proteins to 5-HT_{1A} receptors. When we coinfected our previously described 5-HT_{1A} receptor and $\beta_1\gamma_2$-subunit expressing Sf9 cells with baculoviruses encoding αi-subunits, we saw significantly larger nucleotide effects on high affinity Bodipy-FL-NAN-190 binding (Table 3). The proportion of high affinity binding sites for αi-subunit containing baculovirus particles (in the presence of Mg^{2+}, with shared pIC_{50} values for GTPγS treated and untreated datasets) was between 36% and 53% and addition of GTPγS lowered this down

to 19–32%, whereas when no mammalian α-subunits were coinfected the proportion of high affinity binding sites was 19% in the absence and almost unaltered in the presence of GTPγS. This result indicates that all three mammalian αi-subunits can couple to 5-HT_{1A} receptors in budded baculovirus particles and this can enhance high affinity agonist binding.

Table 2. Apparent potencies of ligands in competition with 1.35 nM Bodipy-FL-NAN-190 at 5-HT_{1A} receptors in budded baculoviruses

Ligand	Control		+ 10 μM GTPγS	
	pIC_{50}^{H} pIC_{50}^{L}	$\alpha_H^{\#}$	pIC_{50}^{H} pIC_{50}^{L}	$\alpha_H^{\#}$
WAY 100135	7.3±0.1*		7.3±0.1*	
WAY 100635	8.20±0.03*		8.00±0.04*	
NAD-299	8.50±0.03*		8.40±0.04*	
NAN-190	7.7±0.1*		7.7±0.1*	
5-HT	7.8±0.2 6.1±0.1	19±2	7.8±0.2 6.1±0.1	15±2
(±)8-OH-DPAT	8.6±0.2 6.9±0.1	33±5	8.6±0.2 6.9±0.1	17±5[§]

pIC_{50}^{H} and pIC_{50}^{L} values were calculated from displacement curves against 1.35 nM Bodipy-FL-NAN-190 according two- or one-site binding models. Data are mean ± SD of pooled data of 2 independent experiments carried out in duplicates.

* One-site model was preferred over two-site model by F-test ($P<0.05$)

[#] Fraction (%) of high-affinity binding sites obtained when IC_{50} values were shared between data sets obtained in presence and absence of 10 μM GTPγS

[§]: $P<0.001$ – different from the $\alpha_H^{\#}$ value in the absence of GTPγS

As Mn^{2+} has been shown to elicit tissue specific effects on 5-HT_{1A} signaling, which is thought to be connected with coupling of specific Gi protein subtypes with these receptors, we decided to additionally study the effects of Mn^{2+} on 5-HT binding to the four baculovirus preparations expressing various α-subunits in order to see whether the effect is connected with some particular α-subtype. The effects of Mn^{2+} on high affinity Bodipy-FL-NAN-190 binding were significant. There was an increase in the proportion of high affinity binding sites for all four receptor preparations containing different G-proteins that we investigated (Table 3), both in the absence and presence of GTPγS. For baculovirus particles not expressing mammalian α-subunits the effect of GTPγS on high affinity agonist binding seemed even less pronounced in the presence of Mn^{2+} than in the presence of Mg^{2+}. This result indicates that 5-HT_{1A} receptors are more sensitive to Mn^{2+} effects in the presence of αi-subunits, but these

effects could not be clearly separated in this system due to a background of insect G-proteins that also seemed to interact with the receptor.

Table 3. Influence of GTPγS on the proportion of 5-HT high affinity binding sites in the presence of 1 mM Mg^{2+} or Mn^{2+}

Gα	α_H[#]			
	Mg^{2+}	Mn^{2+}	Mg^{2+}+GTPγS	Mn^{2+}+GTPγS
i1	53±2	62±2***	32±3	40±2*
i2	36±3	50±2**	19±3	30±3***
i3	50±2	60±2***	28±2	40±2***
control	19±2	25±2*	15±2	24±2*

[#] Fraction (%) of high-affinity binding sites were calculated from displacement curves of 5-HT against 1.35 nM Bodipy-FL-NAN-190. Control experiments were carried out in the absence of coexpressed human α-subunits). Data were fitted to a two-site binding model, where high and low affinity pIC_{50} values were shared between all data sets and had values 7.78±0.03 and 6.11±0.02, respectively. Data are mean ± SD of pooled data of 2 independent experiments carried out in duplicates.
*: P<0.05, **: P<0.01, ***: P<0.001 – different from the α_H[#] value in the presence of Mg^{2+}.

5.2.2. The effects of nucleotides on high affinity Bodipy-FL-NAN-190 binding to 5-HT_{1A} receptors

As guanine nucleotides clearly altered the ability of agonists to displace Bodipy-FL-NAN-190 from 5-HT_{1A} receptors, we used this nucleotide-dependent effect on high affinity Bodipy-FL-NAN-190 binding for the characterization of functional complexes between 5-HT_{1A} receptors and different G-proteins. We determined the apparent pEC_{50} values for GDP and GTPγS (in the presence of Mg^{2+} or Mn^{2+}) to modulate Bodipy-FL-NAN-190 binding in four different baculovirus preparations, expressing either αi-proteins or no mammalian α-subunits. Nucleotide free G-proteins have been shown to be essential for high affinity agonist binding (Rinken et al., 2001, Yao et al., 2009), so the addition of GDP would be expected to switch the receptor into a low affinity binding state, while GTPγS would be expected to functionally sequester the G-protein α-subunit from the signaling complex as activation by the nonhydrolyzable nucleotide would limit receptor- α-subunit interactions. Thus we could study the effect of Mn^{2+} on both GDP-saturated ternary complexes and on signaling complexes where there should be no αi-subunits present (nucleotide binding to α-subunits is also affected by Mn^{2+}). For all four baculovirus preparations studied we found that the fluorescence anisotropy of Bodipy-FL-NAN-190 was higher in the presence of Mn^{2+} than Mg^{2+}, especially in the absence of added nucleotides, while the fluorescence anisotropy of the free ligand itself seemed unaffected by Mn^{2+}.

The pEC_{50} values with which GDP and GTPγS modulated Bodipy-FL-NAN-190 binding showed that insect G-proteins, when coupled to 5-HT_{1A} receptors, have a higher apparent affinity for GTPγS than αi-proteins and lower apparent affinity for GDP (Table 4). We found no significant differences between the pEC_{50} values for GTPγS in the presence of either Mg^{2+} or Mn^{2+} for all four baculovirus preparations (containing either human or just insect α-subunits) studied. This is consistent with the theory that GTPγS uncouples the receptor from the G-protein and therefore the effects that divalent cations may have on the G-protein α-subunits cannot be detected at the receptor level. However there were clear differences between Mg^{2+} and Mn^{2+} on effects of GDP, as Mn^{2+} significantly lowered apparent pEC_{50} values of GDP for all baculovirus preparations studied. Similar results were obtained also in our previous experiments using [^{35}S]GTPγS to investigate 5-HT_{1A} receptors in rat brain membrane homogenates. These findings suggests that Mn^{2+} may accelerate GDP release from the ternary complex, which would lead to increased high affinity Bodipy-FL-NAN-190 binding by increasing the proportion of nucleotide free G-proteins, which seems to be connected with the higher fluorescence anisotropy signals of Bodipy-FL-NAN-190 in the absence of added nucleotides. The overall results also indicate that Bodipy-FL-NAN-190 is a fluorescent ligand that is well suited for investigating 5-HT_{1A} receptors and also their complexes with G-proteins, since as a partial agonist it is sensitive to the presence of nucleotide-free G-proteins.

Table 4. Apparent pEC_{50} values of nucleotides in the presence of 1 mM Mg^{2+} or Mn^{2+} to inhibit high affinity agonist binding to 5-HT_{1A} receptors in baculoviruses with different human G-protein α-subunits

Gα	GDP		GTPγS	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
i1	6.2±0.1	5.7±0.1**	8.6±0.1	8.4±0.1
i2	8.1±0.3	6.3±0.2***	8.4±0.2	9.0±0.1
i3	6.7±0.1	5.5±0.2***	8.7±0.2	8.9±0.1
control	5.7±0.2	4.3±0.2*	10±0.5	9.6±0.3

pEC_{50} values were calculated from concentration-effect curves, where nucleotides modulated binding of 1.35 nM Bodipy-FL-NAN-190 to corresponding baculovirus preparations (2.33 nM 5-HT_{1A}). Control experiments were carried out in the absence of coexpressed human α-subunits). Data are mean ± SD of pooled data of 2 or 4 independent experiments carried out in duplicates.
*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$ – different from the pEC_{50} value in the presence of Mg^{2+}.

5.3. Methods for characterization of nucleotide binding to purified G-proteins

In previous experiments receptor-G-protein coupling was investigated in a relatively natural environment: measurements were conducted with the proteins localized to the plasma membrane (in either cell membrane homogenates or budded viruses). In those cases the samples contained numerous proteins that could have participated in receptor-G-protein interactions and thus unambiguous conclusions about the specific roles of receptor-coupled G-proteins were difficult to reach. Therefore a study of the properties of purified G-proteins was also undertaken, where opportunities for crosstalk are significantly reduced and the possibility could be discounted that the receptor could contribute selectively to bivalent cation sensitivity or nucleotide-binding properties of various αi-subunits. For the following experiments G-protein heterotrimers were expressed in Sf9 cell membranes, which were then solubilized and purified using affinity chromatography to yield protein preparations of over 90% homogeneity. The tandem affinity chromatography approach, based on StrepII-tagged γ2-subunits, also enabled partial depletion (approximately 0.6 mol of GDP per 1 mol of G-protein was retained) of the heterotrimers of endogenous GDP.

5.3.1 Characterization of G-proteins using Bodipy-FL-GTPγS

Bodipy-FL-GTPγS has been proposed as a promising reporter for characterization of nucleotide binding to heterotrimeric G-proteins (McEwen et al., 2001). Our results showed that addition of purified heterotrimeric G-proteins to Bodipy-FL-GTPγS indeed caused increases in both fluorescence intensity and fluorescence anisotropy while the baseline fluorescence anisotropy of free Bodipy-FL-GTPγS was stable. Upon addition of purified GDP-depleted G-proteins, a rapid ($\tau_{1/2}$ < 1 min) increase in anisotropy was detected, which was followed by a slower ($\tau_{1/2}$ ~ 10–20 min) decline. The latter process followed, at least within the first hour, the kinetics of a first order reaction and so is probably connected with thermal inactivation of nucleotide-free G-proteins (Ferguson et al., 1986). Indeed, incubation of the protein preparations for an hour at 28 °C before addition of Bodipy-FL-GTPγS also caused a significant loss in signal intensity. The large transient peaks disappeared and the maximum signals reached only ~20–30% of their previous values. The binding of Bodipy-FL-GTPγS was also slower, having properties characteristic of GDP-release limited association (Senogales et al., 1990, Carty et al., 1990). Similarly, when the nucleotide-depleted G-proteins were preincubated with GDP, the initial transient peak in binding was depressed and a more stable but lower signal was detected.

Rapid association rates of [^{35}S]GTPγS have previously been detected only for nucleotide-free G-protein preparations (Ferguson et al., 1986), while in membrane preparations the half-life of this reaction is approximately one hour (Rinken et al.,

1999). It could therefore be proposed that the initial large pool of easily available but unstable binding sites corresponds to a nucleotide-free fraction of the purified protein. As the G-proteins used were at least 40 % nucleotide-free, they could indeed become partially inactivated during the experiments.

5.3.2. Modulation of nucleotide binding by Mn^{2+}, mastoparan and 5-HT_{1A} receptors

When Mg^{2+} was replaced with Mn^{2+} in experiments using GDP-depleted α_i-subunits, this resulted in overall lower transient peaks and also lower stable binding levels. However, some G-protein subtype-specific effects were also revealed. When comparing the apparent thermal denaturation kinetics of G-proteins, we observed that Mn^{2+} decelerated the loss of Bodipy-FL-GTPγS binding to α_{i1}, but accelerated it for α_{i2} (Table 5). In displacement experiments, where proteins were preincubated with different concentrations GDP or GTPγS, the apparent potencies of these nucleotides to inhibit Bodipy-FL-GTPγS binding were decreased up to one order of magnitude by Mn^{2+} (Table 6) in comparison with Mg^{2+}. This effect was most pronounced for GDP binding to the α_{i1}- and α_{i2}-subunits (Table 6). In the presence of mastoparan (a receptor mimetic) the apparent nucleotide affinities decreased, but the differences in pIC_{50} values achieved a statistically significant level only for the α_{i2} subunit. Mastoparan also significantly accelerated the apparent denaturation rate of α_{i2} and the effects were additive with the effects of Mn^{2+} for this subunit. In contrast to other subtypes, Mn^{2+} had no significant influence on Bodipy-FL-GTPγS binding to α_{i3}.

Table 5. Rate constants for GDP-depleted G-protein – Bodipy-FL-GTPγS complex decompositions

	k_I (min^{-1}) ± SD[a]					
	α_{i1}		α_{i2}		α_{i3}	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
–lipo[b]	0.070±0.004	0.059±0.002**	0.043±0.002	0.055±0.002***	0.052±0.002	0.061±0.006
+ lipo[b]	0.071±0.007	0.057±0.003*	0.046±0.004	0.052±0.002	0.051±0.006	0.057±0.003
+lipo + mast[b]	0.064±0.007	0.058±0.003	0.043±0.006	0.071±0.003***###	0.059±0.005	0.065±0.004

[a] first order rate constants (mean ± SD) measured from 12 to 70 minutes after addition of the G-protein preparation to Bodipy-FL-GTPγS

[b] inactivation was measured in the presence (+lipo) or absence (-lipo) of liposomes (14 μg/ml) or in the presence of liposomes and mastoparan (10 μM) (+lipo + mast)

* P<0.05, ** P<0.01, *** P<0.001 vs. corresponding value in the presence Mg^{2+}

P<0.001 vs. corresponding value without mastoparan

The apparent opposing effects of Mn^{2+} on nucleotide-depleted and nucleotide-saturated purified G-protein preparations indicate that in fact Mn^{2+} enhances nucleotide release from the proteins in both cases. Mn^{2+} makes nucleotide-saturated G-proteins initially more available to Bodipy-FL-GTPγS by accelerating nucleotide exchange, so GDP can be replaced by the fluorescent nucleotide. Mn^{2+} also makes nucleotide-saturated G-protein preparations less stable, which could indicate that G-proteins spend more time in the apparently unstable nucleotide free state (which would promote high affinity agonist binding). Indeed – in experiments where the reaction medium did not contain enough nucleotides to saturate the G-proteins, Mn^{2+} also inhibited Bodipy-FL-GTPγS binding in comparison to Mg^{2+}. Taken together, the experiments done in the presence of either Mg^{2+} or Mn^{2+} seem to indicate that the latter shifts the G-protein-nucleotide binding equilibrium in favor of the nucleotide free state of the α-subunit, rather than either the GDP- or the GTPγS-bound states, which seem more stable in the presence of Mg^{2+}.

Table 6. Apparent affinities of GDP and GTPγS to purified protein preparations

	pIC_{50}[a]					
	α_{i1}		α_{i2}		α_{i3}	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
			GDP			
–lipo[b]	8.12±0.12	7.72±0.11*	8.18±0.07	7.63±0.13***	8.00±0.07	7.68±0.16*
+ lipo[b]	8.49±0.20	7.73±0.19*	8.42±0.18	7.84±0.17*	8.05±0.06	7.32±0.20**
+lipo + mast[b]	8.57±0.14	7.37±0.21***	8.26±0.36	7.24±0.35	7.98±0.09	7.71±0.14
			GTPγS			
+ lipo[b]	8.16±0.06	7.71±0.16**	8.43±0.09	7.85±0.16**	7.92±0.10	7.72±0.22
+lipo + mast[b]	7.96±0.10	7.80±0.21	8.13±0.12$^{\#}$	7.90±0.25	7.67±0.14	7.42±0.26

[a] negative logarithm of concentration of the nucleotide causing 50 % of inhibition of 5 nM Bodipy-FL-GTPγS binding (mean ± SD) measured after 60 minutes incubation

[b] the affinities were measured in the presence (+lipo) or absence (-lipo) of liposomes (14 μg/ml) or in the presence of liposomes and mastoparan (10 μM) (+lipo + mast)

* P<0.05, ** P<0.01, *** P<0.001 vs. corresponding value in the presence Mg^{2+}

$^{\#}$ P<0.05 vs. corresponding value without mastoparan

Comparison of the effects of nucleotides on purified G-proteins and G-proteins coexpressed with 5-HT_{1A} receptors in baculovirus particles showed that GDP had an effect at much higher concentrations in receptor-coupled G-proteins, which indicates functional coupling of G-proteins and 5-HT_{1A} receptors in the baculovirus preparations. Also – as Mn^{2+} had the largest effect on GDP binding in both baculovirus preparation expressing αi2 subunits and on the purified αi2

subunits themselves – it could be proposed that the effects of Mn^{2+} on high affinity agonist binding in the hippocampus could be the result of enhanced coupling of 5-HT_{1A} receptors to αi2 subunits that have been depleted of GDP by Mn^{2+}.

5.4. Fluorescent labeling of G-proteins for characterization of nucleotide binding

In addition to measuring fluorescent nucleotide binding to purified proteins, it would also be advantageous to be able to use fluorescent approaches for direct measurements of nucleotide binding to G-proteins in living cells or membrane homogenates. However, no good fluorescent nucleotides have been available for monitoring G-protein activation in such environments (Gille and Seifert 2003) and instead measurement of G-protein heterotrimer association and dissociation using FRET-based approaches have more commonly been employed (Adjobo-Hermans et al., 2011, Frank et al., 2005). Therefore an attempt was made to design new fluorescently-labeled G-proteins for measurements in more natural environments (mammalian cells) and – in addition to the abovementioned StrepII and CCKACC tagged γ2-subunits – three CCPGCC-tagged α-subunits (i2, s_{long}, q) were engineered to enable dual orthogonal labeling with biarsenical fluorophores like FlAsH and ReAsH (Chen et al., 2007). Initial experiments with these putative FRET constructs (using purified heterotrimers) however yielded unexpected results: even when the FRET pair was labeled with a single biarsenical fluorophore a clear nucleotide-dependent signal emerged. Further experiments were conducted to investigate this effect and to see if it could be developed as a novel assay for measuring nucleotide binding to G-proteins, beginning with the characterization of some of the photophysical changes that accompany F_2FlAsH binding to G-proteins.

Fluorometric measurements showed that the fluorescence intensity of F_2FlAsH was increased up to twofold by the addition of G-protein α-subunits, with no significant shift in emission or absorbance maxima in comparison to free F_2FlAsH. Measurement of the fluorescence lifetime of F_2FlAsH complexes (Table 7) showed that free F_2FlAsH exhibited a two component exponential decay rate with a shorter component of 1.0 ns and a longer component of 4.3 ns, with the shorter lifetime component comprising 34% of the signal. Addition of a tetracysteine labeled peptide decreased the proportion of the faster decaying component down to 11%, as did the addition of βγM, which decreased the 1.0 ns component down to 25% of the total. When αolf subunits were added to F_2FlAsH the proportion of the faster decaying component was decreased to 12% in the absence of GTPγS, but when the nucleotide was present we observed a fluorescence decay rate that was best described by a 3-component fit with a previously undetected very rapidly decaying component ($\tau<0.1$ ns, 17% of the signal), while the proportion of the 1.0 ns component was increased to 31%.

These results indicate that the nucleotide-dependent changes in fluorescence anisotropy, which will be described in the following paragraphs, could be the result of quenching of F_2FlAsH by guanine nucleotides that bind to G-protein α-subunits in close proximity to the fluorophore. This quenching would (by decreasing the time available for rotation) increase fluorescence anisotropy. However, if the reaction is accompanied by a change in the rotational correlation time (as a result of altered binding or conformational changes) it would have the opposite effect on steady state fluorescence anisotropy measurements. Thus, the final results would depend on the extent of changes in fluorescence lifetime in comparison to changes in the rotational correlation time.

Table 7. Fractions of F_2FlAsH and its complexes' fluorescence with a lifetime of 1 ns

	Fraction $\tau_{(1.0\ ns)}$[a] %	χ^2
F_2FlAsH	34±3	1.9
F_2FlAsH+GTPγS	29±1	3.7
CCPGCC-peptide	11±2	3.8
βγM	25±2	4.6
αolf	12±5	1.67
αolf+GTPγS	56±4[b]	12[b]
α13	37±4	6.7
α13+GTPγS	38±3	2.5

[a]Fluorescence lifetimes were fitted to equations (1–3) with two components (1.0 ± 0.2 ns and 4.3 ± 0.1 ns) and fractions corresponding to 1.0 ns are reported as mean ± SD

[b]A better fit was obtained by including a third very fast component corresponding to 17% of fluorescence, but with a lifetime that was below the measurement range of the instrument ($\tau < 0.1$ ns)

5.4.1. The effects of nucleotides on the fluorescence anisotropy of F_2FlAsH complexes with various G-protein sybtypes

Investigations into the kinetics and nucleotide-dependence of F_2FlAsH binding to various G-protein subunits showed that addition of purified α-subunits to F_2FlAsH (in the presence of βγM-subunits) caused an increase in fluorescence anisotropy. The magnitude of these increases depended on the α-subunit subtype and it could be attenuated by the addition of GTPγS for all 8 α-subunits (αi1, αi2, αi3, αs_{long}, αs_{short}, αolf, αq, α13) studied.

When F_2FlAsH was added to purified wild type α-subunits in the absence of tetracysteine-tagged βγM subunits, a large GTPγS-sensitive signal was also evident and this time-dependent increase in nucleotide-sensitive fluorescence anisotropy was practically unaltered by the presence of βγM-subunits. These results indicate that F_2FlAsH interacts directly with G-protein α-subunits and

nucleotide-dependent changes in anisotropy are not the results of α-subunit binding to F_2FlAsH-labeled βγM subunits in a way that alters the latter's fluorescent properties. In the case of βγM complexes with αs_{short}, αs_{long} and αolf, addition of GTPγS depressed the fluorescence anisotropy signal close to F_2FlAsH-labeled βγM-levels, which could be interpreted (based of fluorescence lifetime measurements) as possible quenching of the fluorophore by the guanine nucleotide in close-proximity to the binding site of F_2FlAsH, or possibly also by some conformational rearrangements of the G-protein heterotrimers (or just α-subunits) that made the F_2FlAsH-binding sites on α-subunits less favorable for F_2FlAsH interactions upon nucleotide binding. These conformational rearrangements may have even led to complete loss of binding for some α-subunits, although the appearance of a sub 0.1 ns fluorescence lifetime component (Table 7) in the presence of GTPγS would not be explained by this. Additionally: as G-protein α-subunits may aggregate under certain conditions (Agnati et al., 2010), it is possible that nucleotide-dependent changes may have been caused by dissociation of α-subunit oligomers (GTPγS is thought to disaggregate α-subunits). These oligomers could have presented multiple cysteine residues to F_2FlAsH in close proximity (in the absence of added nucleotides), so F_2FlAsH could have cross-linked these α-subunits (leading to large increases in fluorescence anisotropy).

In contrast to αs_{short}, αs_{long} and αolf the fluorescence anisotropy remained higher (in comparison to free F_2FlAsH or F_2FlAsH-βγM) for αq and α13 in the presence of GTPγS. This seems to indicate that F_2FlAsH was still able to associate with αq and α13 in their nucleotide-bound states – but as this interaction with F_2FlAsH had limited nucleotide sensitivity, the F_2FlAsH-binding site on these subunits is probably not close enough or oriented properly for quenching (or other mechanisms that lower fluorescence anisotropy such as disaggregation or conformational rearrangements) by guanine nucleotides. Additionally: low nucleotide sensitivity could also be caused by slower nucleotide binding to αq and α13.

5.4.2. As-cysteine interactions as a possible mechanism for F_2FlAsH binding to G-proteins

F_2FlAsH could interact with G-protein α-subunits through either nonspecific hydrophobic interactions or through more specific binding modes such as As-thiol interactions. Our results support the latter and indicate that G-protein α-subunits compete with high affinity for F_2FlAsH binding with other cysteine-rich proteins or dithiol motifs present in the reaction medium. For example, the presence of 2 mM β-mercaptoethanol in the assay buffer (in comparison to experiments done in the presence of 5 μM β-mercaptoethanol) had only a small effect on nucleotide-sensitive changes in fluorescence anisotropy. Dithiols (200μM ethanedithiol) could, however, block the interaction of F_2FlAsH with

G-protein α-subunits. These results seem to indicate that F_2FlAsH binds to multiple closely located cysteine residues on G-protein α-subunits. As no such residues are present in the highly conserved guanine nucleotide binding site of G-proteins (Cabrera-Vera et al., 2010), it appears that F_2FlAsH acts as an allosteric sensor of nucleotide binding. Unfortunately we were not able to directly determine the affinity of F_2FlAsH for G-protein α-subunits as a non-fluorescent biarsenical ligand was not available to us for measuring nonspecific F_2FlAsH binding.

We also tested whether nonfluorinated FlAsH (Lumio Green) would associate in a nucleotide-sensitive manner with G-protein α-subunits. These results were comparable to experiments done using F_2FlAsH, indicating that the formation of nucleotide-sensitive complexes with G-protein α-subunits is not a unique property of F_2FlAsH but a more general interaction with biarsenical ligands. We also tested whether fluorescein could associate with G-proteins in a nucleotide-dependent manner (presumably through hydrophobic interactions): no changes in fluorescence anisotropy caused by the addition of G-proteins could be detected; neither could we detect any nucleotide effects. This further supports the hypothesis that FlAsH analogues bind to cysteine residues on α-subunits via high affinity As-thiol interactions.

5.4.3. F_2FlAsH labeled G-proteins as an assay for measuring nucleotide binding

As guanine nucleotides had robust effects on the fluorescence anisotropy of F_2FlAsH-αβγM complexes we utilized this system as a novel assay for the characterization of nucleotide binding to G-proteins. We note that due to slow nucleotide-exchange kinetics and low nucleotide-affinities of some α-subunits, traditional orthosteric ligands (labeled nucleotides) would have been limited in their applicability, whereas F_2FlAsH – as an allosteric probe – could be used to monitor their nucleotide-bound states without being as limited by the nucleotide exchange requirement.

Eight different α-subunits in combination with F_2FlAsH and βγM were studied to reveal the effects of GDP, GMP, GTPγS and GppNHp on these complexes. Depending on the α-subunit, large differences in signal amplitudes, nucleotide affinities and even in the type of the effect (increase or decrease of fluorescence anisotropy) upon addition of guanine nucleotides, was seen. The four nucleotides tested had a similar order in their apparent binding affinities for all of the G-proteins studied, with GTPγS having the highest and GMP the lowest affinity (Table 8). GDP was equipotent with GTPγS and GppNHp for αi-complexes, but less potent for all other G-protein preparations tested. Similar trends were seen for the amplitude of the nucleotide-dependent change in fluorescence anisotropy, with GTPγS causing the greatest change, while for GDP and GMP the changes were smaller for most α-subunits (with the exception of αi2 and αi3). This could indicate that GTPγS binding to α-subunits

can cause a conformational rearrangement that brings the quenching guanine moiety closer to the F_2FlAsH binding site than either GDP or GMP – or alternatively: GTPγS leads to disaggregation of α-subunit oligomers while GDP and GMP cause smaller conformational rearrangements.

Proteins of the Gi family differed from the other five G-protein subtypes tested in several ways and there were also some specific differences between the αi-subunits themselves: these three subunits (purified in nucleotide-depleting conditions) exhibited striking differences between the direction of the effects of activating guanine nucleotides (nonhydrolyzable GTP-analogues) and GDP or GMP (thought to preferentially bind to and stabilize inactive G-protein conformations). In comparison to experiments done without added nucleotides, the addition of GDP and GMP caused an increase in fluorescence anisotropy, whereas the addition of GTPγS and GppNHp resulted in a decrease. This could indicate that the nucleotide-depleted αi-subunit preparations partially denatured at 28 °C and could no longer interact with F_2FlAsH (when no additional GDP or GMP was present), whereas the addition of GDP or GMP could stabilize the nucleotide-free pool of α-subunits and preserve their F_2FlAsH binding ability, thus increasing the signal. Alternatively the stabilizing effects of GDP and GMP could be connected with the stability of the G-protein heterotrimer itself: GDP-bound αi-subunits would be expected to be associated with βγM-subunits, which would reduce their rotational mobility, while GTPγS treatment would be expected to (fully or partially) dissociate the heterotrimer and lead to a decrease in fluorescence anisotropy. Disaggregation of αi-subunits themselves by GTPγS is also a possible mechanism behind the different effects of activating and nonactivating guanine nucleotides as αi-subunits are thought to be especially likely to aggregate (Agnati et al., 2010). Additionally, as the affinity of these three Gi protein preparations for GDP and GTPγS had been measured previously using a Bodipy-FL-GTPγS-based nucleotide displacement assay in a similar environment, we could directly compare the results from the two methods: the affinity of αiβγM complexes for GDP and GTPγS determined using Bodipy-FL-GTPγS was in close agreement with the values obtained by using the F_2FlAsH-based assay. Also, our estimation of the apparent affinity of GTPγS for αs-subunits is similarly close to previously published values determined using assays based on the displacement of fluorescent nucleotides (McEwen et al., 2001) and high concentrations (10-30 μM) of GTPγS have previously been found necessary to activate αq and α13 (Chan et al., 2011), which is reflected by our results: these two proteins had the lowest apparent affinity for GTPγS out of all the G-protein subtypes studied.

There may be several reasons why the effects GDP and GMP had on αi-subunits differed from other G-protein subtypes. One reason may be that αs_{short}, αs_{long}, αq, αolf and α13-proteins were not purified in nucleotide-depleting conditions, so they could have been saturated with GDP. These 5 α-subunits had all been purified using GST-Ric8 association (Chan et al., 2011) instead of StrepII-labeled γ2-subunits, as was the case for αi-heterotrimers. So these two

purification approaches may have yielded protein preparations that had significantly different compositions in terms of nucleotide and cofactor content and also in G-protein subunit stoichiometry.

Table 8. Apparent affinities of nucleotides in F_2FlAsH-αβγM complexes

	pEC_{50}[a]			
	GTPγS	GppNHp	GDP	GMP
αs_{long}	7.47 ± 0.09	6.56 ± 0.07	4.72 ± 0.18	ND
αs_{short}	7.06 ± 0.04	6.42 ± 0.04	4.85 ± 0.05	>–3
α13	5.65 ± 0.13	5.36 ± 0.14	4.98 ± 0.23	ND
αq	6.27 ± 0.05	5.96 ± 0.07	5.87 ± 0.06	>–3
αolf	6.43 ± 0.04	5.84 ± 0.05	5.00 ± 0.03	>–3
αi1	8.19 ± 0.05	8.08 ± 0.04	8.19 ± 0.16	5.89 ± 0.12
αi2	8.02 ± 0.09	7.84 ± 0.14	8.17 ± 0.13	4.45 ± 0.07
αi3	7.87 ± 0.06	7.93 ± 0.07	8.08 ± 0.10	5.29 ± 0.13

[a] negative logarithm of concentration of the nucleotide causing 50 % of fluorescence anisotropy changes of F_2FlAsH-αβγM complexes (mean ± SD), determined after 6 h incubation for αs_{long}, αs_{short}, αq and αolf, after 14 h for α13, after 2 h for αi1, αi2 and αi3.
Data is from two to three independent experiments carried out in duplicate. Data were pooled and then fitted to three parameter competitive binding equations.

When comparing the effects of nucleotides on F_2FlAsH-G-protein complexes, it was apparent that F_2FlAsH-αiβγM protein complexes had the highest fluorescence anisotropy in the presence of GDP or GMP, while the same was true for F_2FlAsH-αs_{short}, αs_{long}, αq, αolf and α13 complexes in the absence of any added nucleotides. It has been suggested that αi-subunits in their GDP-bound state could have multiple binding sites for βγ-subunits (Wang et al., 2009), which could also (in addition to inhibition of denaturation of nucleotide-free αi-subunits) explain the increase in fluorescence anisotropy upon GDP and GMP binding to αi-subunits: βγM subunits were probably present at a slight excess in the αiβγM protein preparations, so they could form complexes with at least some αiβγM-heterotrimers. The added mass of a second βγM binding to the G-protein heterotrimer would be expected to further decrease the rotational mobility of the F_2FlAsH-G-protein complex and result in a slightly higher anisotropy. Thus absence of any GDP or GMP-induced increase in the fluorescence anisotropy of αs_{short}, αs_{long}, αq, αolf and α13 complexes with F_2FlAsH and βγM could be explained by the lack of a second βγM-subunit binding site on these α-subunits.

6. CONCLUSIONS

In summary our studies into the role of G-proteins in the regulation of 5-HT_{1A} mediated signal transduction led to the following conclusions:

- 5-HT_{1A} receptor signaling complexes in the rat brain are differentially regulated by Mn^{2+}, which caused an increase of high-affinity agonist binding in rat hippocampal membranes in comparison with experiments in the presence of Mg^{2+}, but not in rat cortical membranes or Sf9 cell membranes expressing 5-HT_{1A} receptors and Gi1 heterotrimers.
- Activation of G-proteins with 30 μM GTPγS turned all 5-HT_{1A} receptors in the above-mentioned tissue preparations into a low-affinity state for agonist binding in the presence of Mg^{2+}, but not in the presence of Mn^{2+} in rat hippocampal membranes. However, at 1 μM GTPγS, a substantial amount of high affinity agonist binding in the presence of Mn^{2+} was detected also in cortical membranes and Sf9 cells, but not with Mg^{2+}.
- Comparison of the abilities of GDP and GTPγS to modulate high affinity agonist binding to 5-HT_{1A} receptors indicated that both nucleotides were almost 10-fold less potent in the presence of Mn^{2+} in comparison to Mg^{2+}. This means that by inhibiting guanosine nucleotide binding to G-proteins in complex with 5-HT_{1A} receptors, Mn^{2+} acts as an enhancer for agonist binding.
- Bodipy-FL-NAN-190 was found to be well suited reporter ligand for characterization of ligand binding to 5-HT_{1A} receptors expressed in budded baculovirus particles, as binding is accompanied by large increases in fluorescence intensity and anisotropy.
- The fluorescence anisotropy assay based on Bodipy-FL-NAN-190 binding to baculovirus particles was suitable for the characterization of binding properties of non-labeled serotonergic ligands, but also for characterizing the abilities of different G proteins to promote high affinity agonist binding. There were no significant differences between αi1 and αi3 subtypes, while ligand binding in the presence of αi2 had higher sensitivity to GDP and Mn^{2+}.
- StrepII-tagged γ_2-subunits were an expedient construct for the purification of heterotrimeric G-proteins and also for depleting them of bound nucleotides. Nucleotide depletion greatly accelerated the denaturation rates of α_i-subunits.
- Fluorescence anisotropy measurements with Bodipy-FL-GTPγS enabled on-line monitoring of the binding of this ligand and revealed differences in the sensitivities of nucleotide-depleted and saturated Gαi-proteins to Mn^{2+}– and mastoparan: in comparison to Mg^{2+}, Mn^{2+} inhibited nucleotide binding to all αi-heterotrimers studied and accelerated nucleotide release. Mn^{2+} had stabilizing effect on the nucleotide free state of the αi1 subunit, whereas both Mn^{2+} as well as activation by mastoparan destabilized the αi2 subunit.

- The biarsenic fluorescent ligand F_2FlAsH binds to various wild-type G-protein α-subunits (αi1, αi2, αi3, αs_{long}, αs_{short}, αolf, αq, α13) via high affinity As-cysteine interactions. This allosteric probe enables on-line monitoring of the nucleotide bound states of α-subunits via changes in fluorescence anisotropy and intensity of their F_2FlAsH-complexes.
- Addition of nucleotides to F_2FlAsH-labeled α-subunits caused concentration-dependent effects on their fluorescence anisotropy. Nucleotide affinities for GTPγS, GppNHp, GDP and GMP were comparable to values obtained in assays based on the displacement of labeled nucleotides.
- GDP and GMP seemed to stabilize nucleotide-depleted F_2FlAsH-αi-subunit complexes, whereas for all other α-subunits tested the fluorescence anisotropy decreased in their presence. GTPγS and GppNHp decreased fluorescence anisotropy for F_2FlAsH-α-subunit complexes for all G-protein subtypes studied.

7. SUMMARY IN ESTONIAN

G-valkude alatüüpide regulatsioon retseptorite, guaniinnukleotiidide ja Mn^{2+} poolt

Käesoleva töö eesmärgiks oli uurida signaaliülekande mehhanisme, mida vahendavad G-valk seotud retseptorid: valgud, mille kaasabil aktiveerivad raku väljaspoolt tulevad (sageli keemilised) signaalid raku sisemuses olevaid heterotrimeerseid guaniinnukleotiide siduvaid valke (G-valke). G-valk seotud retseptorid kuuluvad inimese suurimasse (erinevatel hinnangutel kuni 800 liikmega) integraalsete membraanvalkude perekonda ning nad osalevad väga paljudes füsioloogilistes protsessides (olles seega olulisteks ravimimärklaudadeks): alustades embrüonaalsete rakkude diferentseerumisest kuni kesknärvisüsteemi täppisregulatsioonini välja. Nende poolt aktiveeritavaid G-valkude perekonda kuulub samas vaid paarkümmend liiget, mis samuti interakteeruvad võrdlemisi tagasihoidliku hulga rakusiseste efektoritega.

Serotoniin 1A retseptor (5-HT_{1A}R) kuulub G-valk seotud retseptorite perekonda ning omab mitmeid rolle kesknärvisüsteemis nagu meeleolu, une ja seksuaalkäitumise regulatsioon. Samuti on välja pakutud hüpotees, et see retseptor võib omada rolli mangaani mürgistuse korral tekkivate sümptomite väljakujunemisel: biokeemilised mõõtmised on näidanud, et mangaan omab teatud ajupiirkondades märgatavat mõju agonistide retseptoriga seostumisele. See piirkonna-spetsiifiline regulatsioon näib olevat seotud vastavates kudedes leiduvate G-valkude mangaani poolt mõjutatavate nukleotiidide seostumise mehhanismidega. Nende mehhanismide väljaselgitamine ning selleks vajalike uudsete mõõtmismeetodite välja töötamine oligi antud töö kitsamaks eesmärgiks.

Esmased mõõtmised rakendasid klassikalisi radioaktiivselt märgistatud ligandidel põhinevaid meetodeid, et uurida 5-HT_{1A}R-it tema võrdlemisi loomulikus keskonnas: imetaja kesknärvisüsteemi koepreparaatides. Lisaks võrdlesime ajukudedes saadud tulemusi mõõtmistega, kus rakendati heteroloogselt putukarakkudes ekspresseeritud retseptoreid. Tulemused näitasid, et sõltuvalt aktiveerivate nukleotiidide kontsentratsioonist omas mangaan mõju kõrge afiinsusega agonisti sidumisele kas ainult roti hipokampuses või lisaks ka teistes koepreparaatides – kui G-valkude aktivatsiooniks kasutati madalat nukleotiidi kontsentratsiooni. Samuti omas mangaan suurt mõju nukleotiidide võimele moduleerida kõrge afiinsusega agonisti seostumist, vähendades näivaid nukleotiidide afiinsust ligi 10 kordselt.

Edasised mõõtmised kasutasid samuti putukarakkudest pärit retseptoreid kuid mõõtmised teostati uudse fluorestsentsligandiga: Bodipy-FL-NAN-190-iga. Esmalt iseloomustasime selle ligandi omadusi, et veenduda tema sobivuses 5-HT_{1A}R-i uurimiseks. Samuti kasutasime 5-HT_{1A}R-allikana bakuloviirusosakesi, mis võimaldasid mõõtmisi reaalajas rakendades fluorestsentsanisotroopia meetodit. Ekspresseerides viirusosakestes koos retseptoriga erinevaid

Gi-valkude alaühikuid õnnestus mõõta mangaani selektiivset mõju erinevate retseptor-G-valk komplekside funktsioneerimisele. Tulemused näitasid, et mangaan näis omavat suurimat mõju i2-alaühikule ning et Bodipy-FL-NAN-190 seostumine bakuloviirustes ekspresseeritud 5-HT_{1A} retseptorile on sobilik mudelsüsteem selle retseptori G-valk komplekside iseloomustamiseks.

Olles eelnevalt uurinud mangaani mõju nukleotiidide G-valkudele seostumisele 5-HT_{1A} retseptori juuresolekul teostasime järgmised mõõtmised biokeemiliselt oluliselt paremini defineeritud mudelsüsteemiga: kasutades puhastatud G-valke. Nende valkude saamiseks töötasime välja puhastamismetoodika, mis ühtlasi võimaldas saada nukleotiidivabasid G-valke. Puhastatud valkudega katsed võimaldasid määrata nende alatüüpide vahelisi erinevusi olukorras, kus retseptori mõju oli välistatud. Selleks kasutasime fluorestsentsmärgistatud nukleotiidide, mille seostumist jälgisime fluorestsentsanisotroopia meetodil. Taaskord näitasid tulemused, et mangaani mõju on suurim i2-alaühikule, vähendades nii nukleotiidide afiinsust kui ka selle valgu enda stabiilsust. Samas i3-alaühikule mangaani märgatav mõju puudus. i1-alaühiku puhul oli väike efekt olemas (mangaan vähendas nukleotiidide afiinsust) kuid katiooni mõju valgu denaturatsioonile näis olevat vastupidine i2-alaühikuga. Seega võib järeldada, et mangaani mõju kõrge afiinsusega agonistide seostumisele 5-HT_{1A}R-iga on tihedalt seotud mangaani poolt tekitatud nukleotiidivaba G-valgu sisaldusega retseptor-G-valk kompleksis.

Viimase eesmärgina plaanist uurida G-valkude rolli signaaliülekandes oli proovida mõõta G-valkude selektiivset aktivatsiooni imetajarakkudes, mida fluorestsentsnukleotiididel põhinevate meetoditega oleks väga komplitseeritud (kui mitte võimatu) teha. Selleks disainisime mitmeid fluorestsentsmärgistamist-võimaldavaid G-valke, et mõõta elavates rakkudes nende aktivatsiooni kasutades Försteri resonantsenergia ülekande printsiipi. Kontrollkatsed saadud valkudega aga näitasid, et märgistamiseks kasutatav biarseenne fluoroforoor seostub samuti ka natiivsete G-valkude α-alaühikutega (kõrge afiinsusega arseen-tsüsteiin interaktsioonide kaudu), mis võimaldasid täiesti uudsel viisil jälgida nukleotiidide seostumist ning G-valkude aktivatsiooni *in vitro*. Seda isegi G-valkude puhul, millele jälgimine traditsiooniliste ortosteeriliste ligandidega on nukleotiidide aeglase dissotsiatsioonikineetika tõttu raskendatud.

Biarseensete ligandidel põhineva meetodi abil uurisime kaheksat erinevat G-valgu α-alaühikut. Mõõdetud nukleotiidide afiinsused langesid kokku kirjanduse andmetega, mis põhinesid märgistatud nukleotiidide seostumisel. Samuti näitasid tulemused, et G-valkude alatüüpide vahel on suuri erinevusi ning Gi valgud eristusid selgelt teiste G-valkude perekondade liikmetest kuna nende puhul sõltus signaali muutuse suund lisatud nukleotiidi tüübist, samas kui kõigi teiste α-alaühikute puhul viis kõikide nukleotiidide lisamine signaali vähenemiseni (tõenäoliselt fluorestsentsligandi emissiooni kustutamise kaudu).

Kokkuvõtvalt näitasid meie tulemused, et 5-HT_{1A} retseptori ning tema G-valk komplekside uurimiseks saab edukalt kasutada mitmeid fluorestsentsmeetodeid, mis võimaldavad mõõtmisi homogeenses keskkonnas ning reaalajas.

Kasutades bakuloviirustel põhinevat ekpressioonisüsteemi saab hõlpsalt ekspresseerides koos retseptoriga erinevaid G-valkude alatüüpe ja uurida alatüüpselektiivseid retseptor-G-valk interaktsioone ning nende tundlikkust guaniinnukleotiidele ning divalentsetele katioonidele.

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