Characterization of the 5-$HT_{7(a)}$ receptor: Specific receptor – G - protein interactions

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List of abbreviations:

AC – adenylate cyclase

AR – adrenergic receptor

cAMP – cyclic adenosine monophosphate

Cdc – cell division cycle

Cys – cysteine

DMEM – Dulbecco's modified eagle medium

e-NOS – endothelial nitric oxide synthase

ERK – extra cellular signal regulated kinase

FBS - fetal bovine serum

FCS – fetal calf serum

FITC – fluorescent iso- thiocyanate

GAP – growth and plasticity associated protein

GDP – guanosine- di-phosphate

GEF –guanine nucleotide-exchange factor

GFP – green fluorescent protein

GPCR – G- protein coupled receptor

GTP- guanosine-tri-phosphate

5-HT – 5-hydroxytrypthamine

IP - inositol –triphosphate

LF – Lipofectamine

LH/hCG – luteinizing hormone/ human chorion-gonadotropin

LPA – lisophosphatidic acid

MAP – mitogen activated protein

Met –methionine

MOR – μ opioid receptor

8-OH-DPAT – 8-hydroxy –(di-N-propylamino)-tetralin

PAGE – polyacrilamid gel electrophoresis

Pal – palmitate

PAT – protein acyl transferase

PBS- phosphate buffered saline

PCR – polimerase chain reaction

pfu – plaque forming units

PIP – phosphotidilinositol - bisphosphate

PK - protein kinase

PLC – phospholipase C

PMSF – phenylmethylsulphonyl fluoride

pPolh – Polyhidrine promoter

PPT – protein palmitoyl thioesterase

P/S – penicillin / streptomicine

RT – room temperature

SAPK – stress activated protein kinase

SDS – sodium dodecylsulphate

Ser – serine

SRE – serum response element

Sf – *Spodoptera frugeperda*

TMD – transmembrane domain

SUMMARY

Serotonin (5-hydroxytryptamine, or 5-HT) is very important neurotransmitter which regulates a variety of different physiological processes within the central Nervous System and at the periphery. Such effects are mediated through the large family of the specific 5-HT receptors. With the exception of the 5-HT_3 receptor, all other 5-HT receptors belong to the family of G – protein coupled receptors (GPCRs). Functionally, GPCRs act as biological switcher of branched network of signalling pathways by their interactions with heterotrimeric G-proteins. GPCRs are often subjected to the different post-translational modifications, which can modulate their activity and functions. One of such modifications is an attachment of 16-carbon fatty acid palmitate. Palmitoylation is unique between the post-translational modifications, because it is reversible and can be regulated. Functionally, palmitoylation of GPCRs can be involved in regulation of different processes including membrane targeting, interaction with G-proteins, basal and agonist-dependent activity as well as phosphorylation and desensitisation.

In the present study we demonstrated that the 5-$HT_{7(a)}$ receptor undergoes post-translational palmitoylation. By combining both palmitate incorporation and pulse-chase techniques we found that [^{3}H]-palmitate incorporation into the 5-$HT_{7(a)}$ receptor is dynamically changed after stimulation with agonist in dose-dependent and time-dependent manner. These effects were receptor-specific and did not represent metabolic effects.

We also determined cysteine residues Cys^{404}, Cys^{438} and Cys^{441} located in the carboxyl terminus of the receptor as potential palmitoylation sites. Surprisingly, by analysis of cysteine-deficient mutants we found that substitution of all three C-terminal cysteine residues did not completely abolish [^{3}H]-palmitate incorporation, suggesting that the 5-$HT_{7(a)}$ receptor palmitoylation is not restricted to the carboxyl-terminal domain.

Functional analysis revealed the critical role of the C-terminal palmitoylation for the modulation of receptor's constitutive activity. We also proposed a novel mechanism by which dynamic palmitoylation of proximal cysteine residue Cys^{404} may regulate both agonist-promoted and constitutive activity of the 5-$HT_{7(a)}$ receptor.

In addition, we demonstrated for the first time that the serotonin 5-$HT_{7(a)}$ receptor is coupled both biochemically and functionally with Gα12 subunit of heterotrimeric G-protein. We further determined that the 5-$HT_{7(a)}$ receptor-mediated stimulation of Gα12 protein resulted both in RhoA-dependent neurite retraction and cell rounding as well as in Cdc42-mediated filopodia formation. These findings suggest the important role of

serotonin in regulation of neuronal development in addition to its well-accepted function as neuromodulator.

1. INTRODUCTION

The human body is composed of trillions of individual cells. In order to coordinate the variety of different functions, these individual cells are required to interact with each other. Such cell-cell communications play a very important role in organisation of organs and systems in the functional organism. One of the principal means of communication between different cells is chemical signalling by signal substances like hormones. By this process cells within specific tissues or organs release a chemical signal, called a "ligand," which ultimately binds to and activates a protein molecule called a "receptor," which is located on the surface of the target. Every eukariotic cell contains receptors for different chemical and/or physical signals and receptor activation results in modulation different downstream effectors leading to cell-specific responses.

After binding of the ligand to the receptor, the receptor undergoes a conformational change which triggers a cascade of events transmitting the signal inside the cell. The specificity of response is determined by the specific ligand and receptor involved as well as availability of appropriate effectors. Therefore, the receptor activation may result in a lot of different responses, like changes in shape of the cell, the movement of the cell in a specific direction, the opening of channels on the cell surface allowing different ions to move in and out of the cell, the transmission of an electrical impulse that travels through the cell, or the secretion of some chemical that may in turn modulate the activity of another cell.

1.1. G-protein coupled receptors (GPCRs)

The receptors located on a cell surface can be classified into different superfamilies based upon their biochemical characteristics and structure. G-protein - coupled receptors (GPCRs) represent a major class of signal transduction proteins that modulate various biological functions such as vision, smell, taste and pain (Filipek et al., 2003; Hoon et al., 1999; Lindemann, 1996; Nelson et al., 2001). They are also involved in cell recognition and communication processes (Wilson and Bergsma, 2000). G-protein coupled receptors (GPCRs) are a superfamily of integral membrane proteins that are characterised by seven hydrophobic domains which are of sufficient length (typically 20-28 amino acid residues) to span the plasma membrane (see Fig.1.1). To date, members of the superfamily number in excess of 250 (Kolakowski, 1994) and include the opsins, the β-adrenergic receptors, odorant receptors and receptors for peptide hormones (Kolakowski, 1994; Watson and

Arkinstall, 1994). Sequence analysis has demonstrated that the GPCRs show poor sequence homology except within the hydrophobic domains.

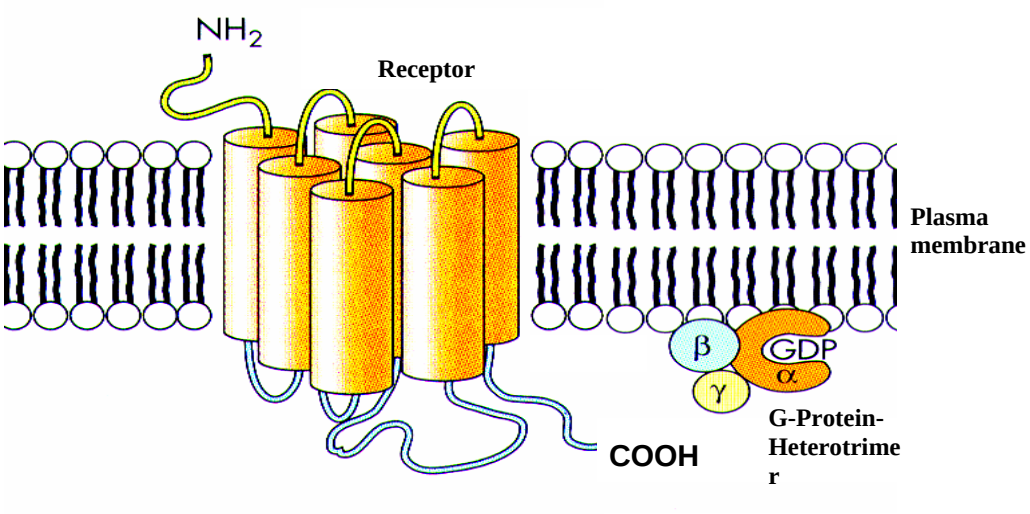


Figure 1. 1. Schematic structure of G-protein coupled receptor (GPCR).

Hydrophobic domains form seven transmembrane spanning (7-TMS) alpha-helices which are linked by alternate intra- and extracellular hydrophilic regions. In addition, the N-terminus of GPCRs, which is often glycosylated, is extracellular while the C-terminus is intracellular (reviewed by (Donnelly et al., 1994; Savarese and Fraser, 1992; Watson and Arkinstall, 1994). TMS domains are involved in cognate ligand binding and that they may be orientated in such a way that they form a ligand-binding pocket (Pardo et al., 1996). A number of GPCRs possess disulphide bridges within their extracellular domains which are believed to be involved in ligand binding and maintenance of the secondary structure in certain GPCRs (Cook and Eidne, 1997). In addition, conserved residues within each TMS domain are also thought to stabilize the GPCR structure (Savarese and Fraser, 1992). As it was demonstrated the intracellular loop between TMS V and TMS VI (loop 3), the C-terminus of GPCRs (Dohlman et al., 1991) and to a greater or lesser extent, other cytoplasmic domains (Watson and Arkinstall, 1994) are involved in interaction with G-proteins.

1.2. Heterotrimeric G-proteins

The first interaction partners in GPCR-mediated signalling are heterotrimeric G-proteins which have been first discovered by pioneer work of Alfred G. Gilman and Martin Rodbell (Gilman, 1987; Rodbell, 1995), Gilman and Rodbell found that G-proteins act as

signal transducers, transmitting the signals in the cell, and modulating different cellular effector systems (Rodbell, 1992).

Structurally, the heterotrimeric G-protein consists of alpha (molecular mass 39-46 kDa), beta (37 kDa) and gamma (8 kDa) subunits (Fig.1.1). On the basis of amino acidic similarity of alpha subunits G-proteins divided into four main classes: Gαi/0, Gαs, Gαq and Gα12/13 (Helmreich and Hofmann, 1996).

With the exception of G-proteins that were founded in sensory organs (α_t, α_{gust}, or α_{olf}) and a few types of a subunits that are predominantly expressed in haematopoietic cells (α_{16}) or in neuronal cells (α_0), most α subunits are expressed ubiquiterly. Therefore the individual cell usually expresses different types of α subunits (Neer, 1994).

In dependence of their α subunits, G-proteins activate different second messengers.

Gs-proteins. The members of this class (αs and α_{olf}) stimulate adenilate cyclase (AC) activity. Adenilate cyclase activation through the αs subunit of G-protein resulting in increase of intracellular cAMP level which is implicated in various cellular downstream cascades. The main downstream effecter in AC-mediated signalling is PKA, which modulate activity of different ion channels, for example Ca^{2+} channels (Mattera et al., 1989), Na^{+} channels (Schubert et al., 1989). $G\alpha_{olf}$ expresses exclusively in olfactory neuroepithelium (Pace and Lancet, 1986) and activates an olfactory-specific form of adenylate-cyclase (Jones and Reed, 1989).

Gi/0-proteins. This group of G-proteins is composed from different α-subunits (αi-1, αi-2, αi-3, α0, αt-1, αt-2, α_{gust}, αz) showing high sequence and functional homology. All these proteins inhibit adenilate cyclase (AC), leading to decrease of intracellular cAMP level. These proteins may regulate activity of K^{+} and Ca^{2+} channels, cGMP phosphodiesterase and also lead to MAP kinase activation (Cano and Mahadevan, 1995).

Gq-proteins. The family of Gq-proteins include αq, α11, α14, α15 and α16 subunits (Simon et al., 1991). They activate phospholipase C (PLC), which provokes the breakdown of phosphotidilinositol 4,5-bisphosphate (PIP) and generation of the intracellular second messengers, inositol 1,4,5-triphosphate (IP) and diacylglycerol (Berridge, 1993a; Berridge, 1993b). Diacylglycerol activates protein kinase C (PKC) which is one of the important effectors in intracellular signalling (el-Fakahany et al., 1988; Vicentini et al., 1985). In addition, Gαq directly stimulates the activity of Bruton's tyrosine kinase in lymphoma cells (Bence et al., 1997).

G12/13-proteins. This class of G-proteins is composed from α12 and α13 subunits which possess very low sequence homology to other G-proteins. Both activate Na^+/H^+ exchange (Voyno-Yasenetskaya et al., 1994), ERK (Voyno-Yasenetskaya et al., 1996) and are also implicated in the regulation of small GTP binding proteins (Buhl et al., 1995; Kozasa et al., 1998; Suzuki et al., 2003). Some experiments demonstrated G12 proteins activate Jun kinase/stress-activated protein kinase (JNK/SAPK) pathway (Vara Prasad, M., et al. 1995).

The β and γ subunits bind very tightly to each other and can be viewed as a functional monomer. Presently, five different β subunits (Watson et al., 1994) and six different γ subunits (Cali et al., 1992) are cloned, which can exist in different combinations. The βγ- complex acts as an anchor for α subunits by forming of heterotrimer. Moreover, βγ-subunits itself can modulate a great variety of effecter molecules such as K^+ channels, adenylyl cyclase, phospholipase Cβ (PLCβ), phospholipase A_2 (PLA_2), phosphoinositide 3-kinase (PI-3-kinase) and regulate their activity (Clapham and Neer, 1997; Morris and Malbon, 1999; Schwindinger and Robishaw, 2001). The βγ subunit may also act through Ras to activate mitogen-activated protein kinase (MAPK) pathways (Crespo et al., 1994; Faure et al., 1994).

1.3. G-protein-mediated signalling

Functionally, G-proteins become active when they bind GTP and are inactivated after hydrolysis of GTP to GDP (Neves et al., 2002; Simon et al., 1991).

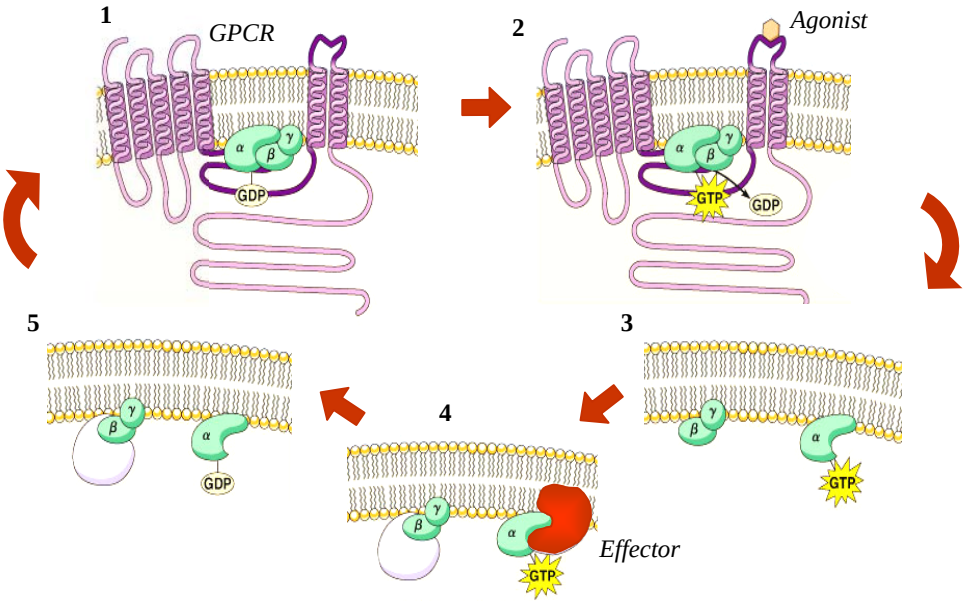


Figure 1. 2. G-protein activation cycle.

In the inactive state the α subunit of heterotrimeric G-protein bound GDP (Fig. 1.2), forming tight complex with β and γ subunits. Activation of receptor results in conformational change within the G-protein. This leads to release of GDP and binding of GTP molecule to the Gα subunit (GDP-GTP exchange, Fig. 1.2). Substitution of GTP for GDP causes additional conformational changes in G_α subunit leading to dissociation of GTP bounded G_α subunit from βγ (Fig. 1.2). Activated GTP bounded Gα subunit can modulate different downstream effectors (Fig. 1.2) like PLC or Adenylyl Cyclase (see below).

Since all Gα subunits possess the intrinsic GTPase activity, GTP becomes hydrolysed to GDP (Fig. 1.2). The presence of GDP on G_α subunit leads to re-establishing of heterotrimeric complex with βγ. In addition proteins named RGS can also accelerate the rate of GTP hydrolysis (Burns and Wensel, 2003; Martemyanov et al., 2003; Rahman et al., 2003).

Small GTPases represent another important class of GTP binding proteins. Similar to heterotrimeric G- proteins, small G- proteins are active when bound to GTP and inactive when bound to GDP. Guanine nucleotide-exchange factor (GEF) accelerates activation of small GTPases by binding to protein-GDP complex and causing dissociation of GDP.

In contrast to heterotrimeric G-proteins, small GTPases are monomers of 20-25 kDa mass. They exist in all eukariotes and are divided into five groups: the Ras, Rho/Rac/Cdc42, Rab, Sar1/Arf and Ran families (Balch et al., 1995).

The family of Rho GTPases (e.g. Rho, Rac, Cdc) are involved in regulation of different cellular processes, in particular cell morphology (Caron, 2003). Extensive studies of cytoskeletal dynamics have firmly established that Rho, Rac and Cdc42 control distinct morphogenic signalling pathways (Fig. 1.3), all of which are crucial for cell adhesion and/or motility (Etiene-Manneville and Hall, 2002). These proteins also take part in establishing of intracellular asymmetry (Butty et al., 2002), shape (Li et al., 2000), in single cell migration (Ridley, 2001; Small et al., 2002) and in coordinated cell migration (Nobes and Hall, 1999; Palazzo et al., 2001). It is now generally accepted that in most cell types, Cdc42 and Rac 1 signalling promote the formation of membrane protrusions, driven by the microtubule and actin cytoskeletal networks, whereas Rho activity is associated with acto-myosin-based cell contraction (Etiene-Manneville and Hall, 2002).

For example, in neuronal cells, activation of Rho results in neurite retraction and cell rounding, whereas activation of Rac and Cdc42 promotes cell spreading and neurite outgrowth (Leeuwen, 1998).

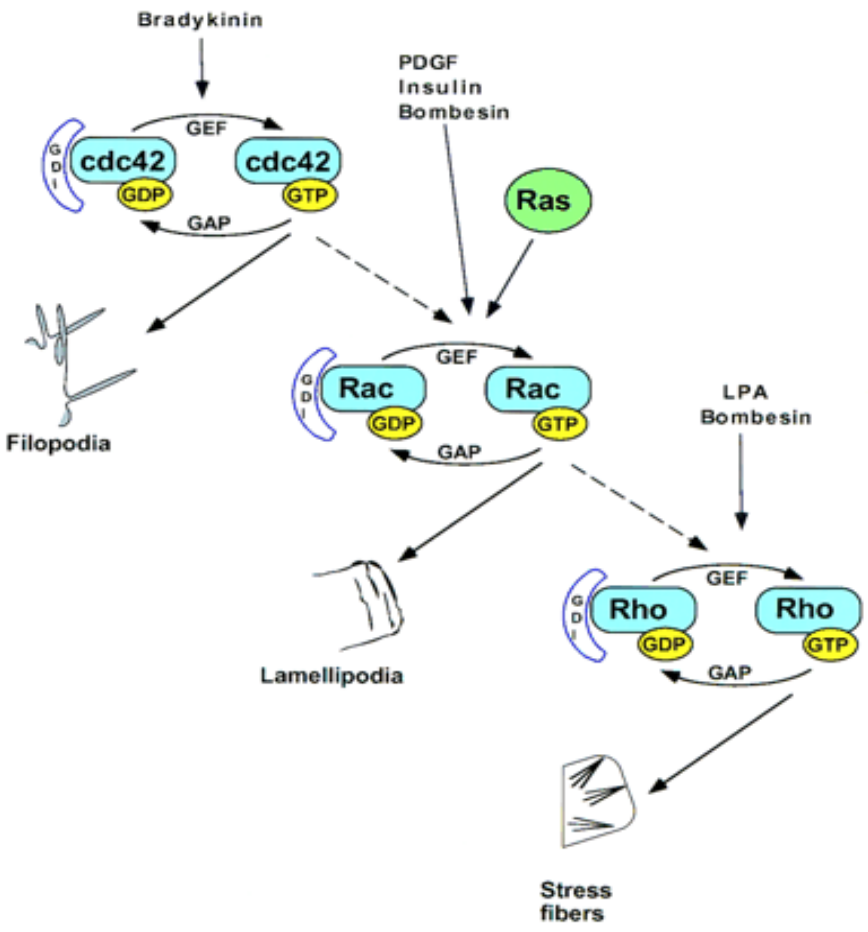


Figure 1. 3. Basic cycling of the Rho GTPases and cytoskeletal rearrangements.
Accepted from Li et al. (2000).

1.4. Serotonin (5-hydroxytriptamine or 5-HT) receptors

Serotonin (5-hydroxytryptamine, or 5-HT) is one of the important neurotransmitters in nervous system. It acts through multiple receptors to mediate a great variety of responses in vertebrates and invertebrates. Serotonin has been implicated in the aetiology of numerous human diseases like depression, anxiety, social phobia, schizophrenia, and obsessive-compulsive as well as panic disorders (Lopez-Figueroa et al., 2004; Routledge and Middlemiss, 1996). In addition, migraine, hypertension, pulmonary hypertension, eating disorders, vomiting, and, more recently, irritable bowel syndrome (IBS) have been also suggested to be partly regulated by 5-HT (Crowell, 2001; Hamel, 1999; Sanger, 1996).

Work on 5-HT receptors began over 40 years ago when Gaddum and Picarelli (Gaddum and Picarelli, 1957), discovered two physiological actions of serotonin. Later

studies offered functional evidence for existence of additional receptor types (Tierney, 2000). Now, at least 14 serotonin receptors are cloned (Meneses, 1999). The 5-HT receptors are divided into seven distinct classes (5-HT_1 to 5-HT_7) based on their structural and functional characteristics. With the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel, all other 5-HT receptors belong to the GPCRs superfamily (Baez et al., 1995; Meneses, 1999).

The 5-HT_1 receptor class is comprised of five receptor subtypes (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}), which share 40 to 63 % sequence homology and couple preferentially to $G_{i/0}$ proteins to reduce the cAMP concentration by inhibition of AC (Albert et al., 1996; Boess and Martin, 1994; Saudou and Hen, 1994).

The 5-HT_2 receptors are coded by single gene, which undergoes alternative splicing to produce three isoforms: 5-HT_{2a}, 5-HT_{2b} and 5-HT_{2c} isoforms, which exhibit 46-50% overall sequence identity. These receptors couple preferentially to $G_{q/11}$ protein family to increase the hydrolysis of inositol phosphates and to elevate cytosolic Ca^{2+} concentration (Boess and Martin, 1994; Canton et al., 1996).

The 5-HT_3 receptors are placed within the ligand-gated ion channel receptor superfamily (Bruss et al., 1998). These receptors are found in neurones (of both central and peripheral origin), where they trigger rapid depolarisation due to a transient inward current, subsequent to the opening of non-selective cation channels (e.g. Na^+,Ca^{2+} influx, K^+ efflux) (Blier and Bouchard, 1993).

The 5-HT_4 receptors. For this subtype at least eight splice-variants were described. These receptors couple positively to adenylate cyclase to increase intracellular cAMP level (Bockaert et al., 1990; Dumuis et al., 1988). In addition to adenylate cyclase stimulation, direct coupling to potassium channels and voltage-sensitive calcium channel have been proposed as post-receptor events. Furthermore, it has been shown that 5-HT_{4a} receptor also implicate to activation of Gα13 signalling pathway (Ponimaskin et al., 2002).

The 5-HT_5 receptors are poorly investigated. It has been shown that human recombinant 5-HT_5 receptor produced an inhibition of forskolin-stimulated cAMP production indicating negative coupling to AC via G_i and G_0 (Erlander et al., 1993; Plassat et al., 1992; Wisden et al., 1993). However, several data suggest that this receptor may also stimulate AC (Carson et al., 1996).

The 5-HT_6 receptor has been cloned based on its sequence homology to known GPCRs of 5-HT family (Ruat et al., 1993a). This receptor is expressed endogenously in

neuronal tissue and positively coupled to adenylyl cyclase via G_s protein (Sebben et al., 1994).

The 5-HT$_7$ receptor is one of the most recently identified 5-HT receptor which was firstly cloned from the rat and later from human cDNA (Bard et al., 1993; Ruat et al., 1993b; Shen et al., 1993). The predicted amino acid sequences of the 5-HT$_7$ receptor isoforms display the characteristic seven putative membrane spanning regions (Heidmann et al., 1997; Lovenberg et al., 1993; Meyerhof et al., 1993; Stam et al., 1997; Tsou et al., 1994) of the G-protein coupled receptor superfamily. The receptor contains consensus sequences for two N-linked glycosylation sites in the predicted extracellular N-terminus (Bard et al., 1993; Lovenberg et al., 1993) and a number of putative sites for phosphorylation located in the third intracellular loop and in the cytoplasmic C-terminus (Bard et al., 1993; Heidmann et al., 1997; Meyerhof et al., 1993; Shen et al., 1993; Tsou et al., 1994). The 5-HT$_7$ receptor gene is located on human chromosome 10 (Gelernter et al., 1995) and contains two introns (Erdmann et al., 1996; Heidmann et al., 1997; Ruat et al., 1993b). The second intron corresponds to the C-terminus and provide for the generation of at least four splice variants including 5-HT$_{7(a)}$, 5-HT$_{7(b)}$, 5-HT$_{7(c)}$ and 5-HT$_{7(d)}$ (Heidmann et al., 1998; Jasper et al., 1997; Lovenberg et al., 1993).

1.4.1. Distribution of the 5-HT$_7$ receptors

In rat and guinea pig brain both the mRNA and receptor binding studies display a similar distribution of the 5-HT$_7$ (Gustafson et al., 1996; Stowe and Barnes, 1998). 5-HT$_7$ receptor expression is relatively high within regions of thalamus, hypothalamus and hippocampus with generally lower expression level in cerebral cortex and amygdale (Gustafson et al., 1996; Stowe and Barnes, 1998; To et al., 1995). Using reverse PCR, Plassat et al. (Plassat et al., 1993) detected 5-HT$_7$ m RNA in mouse brainstem, forebrain, cerebellum, embryo colliculi neurons, as well as in intestine and heart. In contrast, no expression of 5-HT$_7$ receptors was found in mouse spleen, liver, kidney or lung (Plassat et al., 1993). In the rat the highest levels of 5-HT$_7$ mRNA was obtained in hypothalamus and thalamus, as well as in brainstem and hippocampus. Lower level of expression was found in cerebral cortex, striatum, olfactory tubercle (Lovenberg et al., 1993; Meyerhof et al., 1993; Ruat et al., 1993b; Shen et al., 1993). Little or no transcripts were detected in cerebellum, pituitary, retina and most peripheral tissues with the exception of faint signals in the spleen (Shen et al., 1993), stomach and ileum (Ruat et al., 1993b).

1.4.2. Signal transduction through the 5-HT_7 receptor

The 5-HT_7 receptor stimulates adenilate cyclase activity though coupling to the heterotrimeric Gs-protein (Adham et al., 1998; Heidmann et al., 1998). Structurally, amino-acidic residues of third intracellular loop have been proposed to play important role in coupling with Gs protein as it was shown for other GPCRs (Obosi et al., 1997). Activation of 5-HT_7 receptor by agonists leads to the increase in cAMP production which in turn provokes calcium influx through T-type calcium channels (Lenglet et al., 2002b). Co-expression of the human 5-$HT_{7(a)}$ receptor with various isoforms of adenylil cyclase (AC) in human embryonic kidney 293 cells has demonstrated that this receptor activates not only a typical $G\tilde{s}$ sensitive AC5, but also two Ca^{2+}/calmodulin-sensitive ACs -AC1 and AC8 (Baker et al., 1998; Barnes and Sharp, 1999).

Physiological role of the 5-HT_7 is only poorly understood. It has been suggested that this receptor is involved in the control and regulation of the circadian rhythms since receptor agonists 5-CT and 8-OH-DPAT induced phase oscillations (Ehlen et al., 2001; Lovenberg et al., 1993). The other indirect evidence for this function is localisation of the 5-HT_7 receptor in suprachiasmic nuclei of hypothalamus – primer drivers of rhythm generation in mammalian (Moyer and Kennaway, 1999). The other possible receptor function is modulation of neuronal activity by inhibits of posthyperpolarisation in CA3 hippocampal nuclei (Gill et al., 2002). It has been also reported that aldosterone secretion in the rat adrenal gland may be regulated by the 5-HT_7 receptor (Contesse et al., 1994; Lenglet et al., 2002a). Recently, involvement this receptor in sleep and hypothermia has been demonstrated in "knock out" mouse (Hedlund et al., 2003).

1.5. Posttranslational modifications of G-protein coupled receptors

Proteins synthesized in a living cell are often subjected to various post-translational modifications such as phosphorylation, glycosylation. Posttranslational modifications add an additional level of complexity to receptor organization and play an important role in modulation of functions. The N-glycosilation of GPCRs takes a place within a consensus sequence on one or more asparagines residues. This is a complex process which was elucidated very early in the characterisation of receptors and is very good understood now (Li et al., 2000). In addition to glycosilation, GPCRs are also extensively phosphorylated by several kinases. The sites of phosphorylation have been mapped mainly to the carboxyl tail and to the third intracellular loop. Functionally, phosphorylation has been linked to regulatory processes, such as receptor desensitisation and internalisation (Ferguson et al.,

1998; Tsao and von Zastrow, 2001). In addition to these well-characterized modifications, GPCRs are often subjected to covalent modification with the different fatty acids.

Generally, there are three different types of lipid modification: modification with glycosylphosphatidylinositol (GPI- anchoring), prenylation and acylation.

- Addition of glycosylphosphatidylinositol (GPI) to C-terminus of proteins leads to anchoring to plasma membrane (Bhatnagar et al., 1997; Sharma et al., 2000); Udenfied and Kobulka, 1995).

- Prenylation is attachment of fifteen-carbon (farnesyl) or twenty-carbon (geranylgeranyl) isoprenoids to one or more cysteines located near the C-terminus of proteins by a chemically stable thioether bond (Bhatnagar et al., 1997).

- Acylation can be divided into two groups: myristoylation and palmitoylation. Protein N-myristoylation is the covalent attachment of a 14-carbon saturated fatty acid myristate to the N-terminal glycine residue localized within a consensus sequences of the protein. This is co-translational and stable modification which is catalysed by special enzyme N-myristoyl-transferase (NMT) (Raju and Sharma, 1999; Resh, 1999).

S-Palmitoylation is the attachment of long chain fatty acids (in particular 16-carbon palmitate) to one or several cysteine residues within the protein via a labile thioester linkage. Palmitoylation was first described in 1977 (Suzuki, M., et al., 1977) and represents wide spread modification, which was shown for protein of different origin (from virus, plant, yeast and insect to human) and functions.

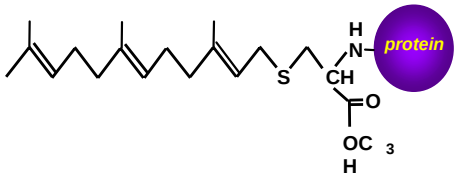
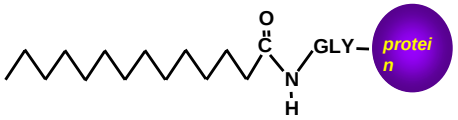
Modification	Type of the bond	Substrate
Prenylation (Farnesylation)	 Thioether; stable	Farnesyldiphosphat cytosolic protein (post-translational)
N-Myristoylation	 Amidic; stable	Myristoyl-CoA Nascent Polypeptide (co-translational)
Palmitoylation	 Thioester; labile	Palmitoyl-CoA Membrane-bound protein (post-translational)

Figure 1. 4. Types of fatty acid modifications.

1.5.1. Mechanism of acylation

There are two distinct views on the possible mechanism of protein palmitoylation. Several groups suggest non-enzymatically palmitoylation, while the others invoke a more traditional enzymatic mechanism by the enzyme a protein acyltransferase (PAT).

Non-enzymatic S-acylation

It has been shown that palmitoyl-CoA is capable to spontaneous S-acylation of thiol group on the cysteine residue in the absence of any cellular factors (Bano et al., 1998; Garbutt and Abraham, 1981). This can take place in the context of short peptides as well as of folded proteins. For example, short peptides derived from β- adrenergic receptor, p21 (K-ras), transferrin receptor, CD-4, SNAP-25 undergo non-enzymatic spontaneous S-acylation in the presence of the high concentrations of palmitoyl-CoA (Bharadwaj and Bizzozero, 1995; Bizzozero et al., 2001; Quesnel and Silvius, 1994). Under similar conditions rhodopsin (O'Brien et al., 1987) and G-protein αs subunit (Duncan and Gilman, 1996; Mollner et al., 1998) also can be modified by palmitate. However, since non-enzymatic palmitoylation need high concentration of Pal-CoA, it is difficult to assess the possible function of such modification *in vivo*.

Enzymatic palmitoylation

Majority of research data from the palmitoylation field suggest enzymatic nature of this modification. Two enzymes are involved in this process: protein palmitoyl (or acyl) transferases (PAT) which attach the palmitate to protein and protein palmitoyl (or acyl) thioesterases (PPT) which remove the lipids from polypeptide.

Protein Palmitoyltransferase Protein acyl transferase (PAT) activity has been detected in membrane fractions derived from different cell types (Berger and Schmidt, 1984; Mack et al., 1987) in addition to reports about PAT activity in membrane preparations from endoplasmic reticulum (ER), Golgi, or the plasma membrane (Berthiaume and Resh, 1995; Dunphy et al., 1996; Hiol et al., 2003; Liu et al., 1996; Ueno, 2000). More recent reports on protein palmitoyltransferases in *Saccharomices cerevisiae* and *Drosophila melanogaster* provided the first glimpse of enzymes that carry out protein palmitoylation (Linder and Deschenes, 2003).

Palmitoyl (acyl) protein thioesterases. A protein palmitoyl thioestarese (PPT)-1 was first purified in 1993 by Camp and Hofmann (Camp and Hofmann, 1993) and cloned one year late by the some groups (Camp et al., 1994). A related enzyme PPT-2 was cloned and characterized in 1997 by Soyombo and Hofmann (Soyombo and Hofmann, 1997). Both proteins are lysosomal enzymes which are responsible for the degradation of acylated peptides, but not for the turnover of palmitates on functional proteins. In contrast, another depalmitoylation enzyme, APT-1, was found to control the palmitate turnover on some palmitoylated proteins, including Gαs (Duncan and Gilman, 1998), eNOS (Yeh et al., 1999) and several viral proteins (Veit and Schmidt, 2001).

1.5.2. Sites of palmitoylation

Early studies indicated the presence of a PAT activity in the endoplasmic reticulum (ER) (Berger and Schmidt, 1985; Rizzolo and Kornfeld, 1988). However, most of the available evidence suggest that palmitoyation of nascent proteins occurs in a post-ER compartments, much possible in the early Golgi (Quinn et al., 1983). Palmitoylation activities in various Golgi subcompartments have also been confirmed for a number of substrates, including GAP-43 (McLaughlin and Denny, 1999), synthetic peptides (Schroeder et al., 1996), the luteinizing hormone/human choriogonadotropin (LH/hCG) receptor (Bradbury et al., 1997) and vaccinia virus proteins (Grosenbach et al., 2000).

Palmitoylation also occurs at the plasma membrane. This was confirmed in the case of erythrocytes which lack the machinery for the protein synthesis and transport (Seehafer

et al., 1988; Staufenbiel, 1988). Observations that palmitoylation of GPCRs and other signalling proteins can be regulated by agonist also confirm the fact that palmitoylation / depalmitoylation take a place at the cell surface (Mouillac et al., 1992; Ng et al., 1994b). Independent from the site of palmitoylation, palmitoyl-transferase activities are still to be membrane associated and no evidence for such activities in the cytosol has been reported (Cantrell and Borum, 1982; Hiol et al., 2003).

Comparison of the palmitoylated cysteines from diverse viral and cellular membrane glycoproteins reveals that whenever such proteins contain acylation sites, the sites are clustered around the borderline between transmembrane domain and cytoplasmic tail (Veit et al., 1991). Inspection of the aminoacids in the vicinity of the acylated cysteine residues of these proteins reveals no obvious consensus signal for acylation such as has been established, for instance, in the case of N-linked glycosylation (Ponimaskin and Schmidt, 1998; Veit et al., 1991; Yik and Weigel, 2002). The only structural theme common to the palmitoylated species is the occurrence of at least one cysteine residue usually within about four residues from the putative border between the inner leaflet of the lipid bilayer and the cytoplasm (Schmidt and Burns, 1989; ten Brinke et al., 2002). However, for several proteins was shown that the distance between potential acylation site and transmembrane domain (TMD) is not critical for palmitoylation. For instance, transforming growth factor α (Shum et al., 1996), cation-dependent mannose-6-phosphate receptor (Schweizer et al., 1996) and the major subunit of human asialoglycoprotein receptor H1 (Yik and Weigel, 2002) contain cysteine that are located at a position more than 20 residues distal to transmembrane border. It is assumed that the region between the cysteine and the transmembrane junction in these proteins could form a loop-like structure in order to bring the cysteine residue closer towards the plasma membrane (Schweizer et al., 1996).

Palmitoylation is seems to be not limited only to the C-termini of the receptors. Increasing number of experimental evidence suggest that this modification may also occur at the other receptor domains. For instance, mutation of all carboxyl-terminal cysteines by the rat μ-opioid receptor failed to affect palmitate incorporation, indicating that the palmitoylation sites resided outside this receptor domain (Chen et al., 1998). Moreover, in the case of the V_{1A} and V_2 vasopressin receptors (Hawtin et al., 2001), mutations of the cysteines on the carboxyl terminus reduced but not completely abolished palmitoylation, further supporting idea that additional sites can exist within other domains.

1.5.3. Functions of palmitoylation

Palmitoylation of many GPCRs has been shown to be regulated by agonist. Agonist-induced increase of palmitoylation was observed for β-adrenergic receptor (Mouillac et al., 1992), α_{2A}-adrenergic receptor (Kennedy and Limbird, 1994), muscarinic acetylcholine m2 receptor (Hayashi and Haga, 1997), as well as for the 5-hydroxytryptamine (5-$HT_{4(a)}$) receptor (Ponimaskin et al., 2001). In contrast, the palmitoylation of human A1 adenosine receptor did not change after receptor stimulation (Gao et al., 1999). Moreover, palmitoylation of the 5-HT_{1A} receptor has been recently shown to be a stable modification (Papoucheva et al., 2004).

By GPCRs palmitoylation has been shown to be involved in regulation of different functions (e.g. in the processing, targeting and in the expression of functional receptors on the cell surface). For vasopressin V_2 receptor it has been shown that mutations that removed cysteine 341 and 342 reduced expression of the receptor at the cell surface (Schulein et al., 1996). Similar data were obtained for δ-opioid and CCR5 chemokine receptor (Blanpain et al., 2001). Palmitoylation of the estrogen receptor ER α and β may play a critical role for the localisation of the receptor in caveolae and for the formation of the "steroid signalosome" (Acconcia et al., 2003).

For several receptors, the mutation of palmitoylated cysteines may result in accelerated degradation**,** as it has been shown for the human adenosine A_1 receptor. In this case the non-palmitoylated receptor mutant was degraded much more faster as that obtained for the wild type protein (Gao and Nuss, 1998).

In addition to processing and targeting, palmitoylation may be implicated into modulation of the receptor activity.

Mutations in palmitoylation sites of β_2-adrenergic receptor lead to uncoupling with Gαs and consequently to reduced adenylate cyclase activation (Moffett et al., 1993; O'Dowd et al., 1989). Similarly, palmitoylation-deficient mutant of muscarinic acetylcholine receptor m2 possesses reduced ability to activate $G\alpha_0$ and $G\alpha_{12}$ (Hayashi and Haga, 1997). Mutations of palmitoylation sites of human somatostatin receptor type 5 lead to a reduced coupling to adenylate cyclase (Hukovic et al., 1998). In contrast, the palmitoylation of rhodopsin receptor has been shown to produce only a little effect on its ligand-induced signalling (Karnik et al., 1993). Moreover, signalling through α_{2A} adrenergic receptor (Kennedy and Limbird, 1993), the LH/hCG receptor (Kawate and Menon, 1994; Kawate et al., 1997), the dopamine D_1 receptor (Jin et al., 1999), the human adenosine A_1 receptor (Gao et al., 1999), and the human thyrotropin receptor (Tanaka et

al., 1998) was not significantly changed after mutation of palmitoylation sites. Palmitoyl-deficient mutant of 5-$HT_{4(a)}$ receptor was shown to have higher basal activity in compare with the native protein (Ponimaskin et al., 2001). In contrast, mutations of acylation sites in case of 5-HT_{1A} disrupts coupling with Gαi protein and consequently abolish adenylate cyclase inhibition (Papoucheva et al., 2004).

Other important function of palmitoylation is regulation of receptor desensitisation. For example, palmitoylation mutant of β_2-adrenergic receptor was shown to undergo very efficient basal phosphorylated; this mutant incorporated four times more phosphate than the wild-type (Moffett et al., 1993). For this receptor palmitoylation has been proposed to play a role of molecular switcher regulating the accessibility of phosphorylation sites involved in the desensitisation of the receptor (Qanbar and Bouvier, 2003).

Taken together, an analysis of the functions of palmitoylation is necessary for each individual receptor to understand its signalling mechanism.

1.6. Questions for dissolving

Despite the accumulation of knowledge about physiological importance of the 5-HT receptors, it is still unclear by which mechanisms the serotonin receptor functional activity is regulated and which signalling pathways may be activated by these receptors. Since palmitoylation represents common feature playing regulatory role for diverse proteins we were interested to investigate:

- whether the 5-$HT_{7(a)}$ receptor modified with palmitic acid
- what a functional role such a modification can play

In addition we also analysed which G-proteins may be activated by the 5-$HT_{7(a)}$ receptor and examined corresponded signalling pathways.

2. EXPERIMENTAL PROCEDURES

2.1. Materials

Chemicals

Applied Biosystems, (Branchburg, New Jersey USA) *AmpliTaq Gold PCR Kit*

Amersham Biosciences, *ECL™ Western Blotting Detection Reagents, 5-hydroxy[3H] tryptamine trifluoroacetate (107 Ci/mmol)*

Calbiochem, (Darmstadt, Germany), *Cycloheximide*

Genomed, (Bad Oeynhausen, Germany), *"Jetsorb" Gel Extraktion Kit*

Gibco/BRL, (Berlin, Germany), *FCS, Penicillin/Streptomycin solution*

Hartmann Analytic GmbH, (Braunshweig, Germany), *[9,10-^{3}H] Palmitic acid (30-60 Ci/mmol).*

ICN, (Eschwege, Germany), *Tran [^{35}S]-label methionine (1000Ci/mmol).*

Invitrogene Ltd., (Paisley, Scotland), *Oligonucleotides primers, Cellfectin reagent, Lipofectamin 2000 reagent, TC-100 insect cells medium, and fetal calf serum (FCS), S.O.C. Medium.*

Kodak, (Rochester, New York), *Kodak X-Omat AR film*

New England Biolabs, (Schwalbach, Germany), *Enzymes used in molecular cloning*.

Nunc, (Wiesbaden, Germany), *Cell culture dishes*

PerkinElmer Life Sceince, (Koeln, Germany), *[^{35}S] GTPγS (1300 Ci/mmol.)*

Qiagen GmbH, (Hilden, Germany), *QIAGEN Plasmid Maxi Kit (10)*

Roth, (Karlsruhe, Germany), *Ampicillin Natriumsalt*, *Temed P.A., Acrylamide, Bis-Acrylamide, 2-Merkapthoethanol, Glycin, X-gal, IPTG.*

Serva, (Heidelberg, Germany), *Ammoniumpersulfate (for protein gels), Bromphenolblue*

Sigma, (Deisenhofen, Germany), *5- Hydroxytryptamine, 8-OH-DPAT*, *Protein A-Sepharose CL-4B beads, Gentamicin Sulfate, Kanamicine Sulfate, SDS, Ethidiumbromide, PMSF, GDP, GTP, Bacto-Tryptone, Bacto-Yeast extract, Bacto-Agar*.

Sigma diagnostics, (St.Louis, USA), *Protein Standard Kit*

All chemicals were purchased from Roth (Karlsruhe), if it no other marked.

Whatman, (Clifton, USA), *96- well microplates with Whatman Hydrophilic GF/C Filter*

Buffers compositions

1) Extra-cellular buffer: 150 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$,1 mM $MgCl_2$, pH 7.4, 330 mOsm.

2) PBS: 140 mM NaCl, 3mM KCl, 2mM KH_2PO_4, pH 7.4

2) RIPA-buffer: 1% Triton-X-100, 1% Natriumdesoxycholat, 0.1% Natriumdodecylsulfate (SDS), 0.15 M Natriumchloride, 20 mM Tris/HCl (pH 7.4), 10 mM EDTA, 1mM Phenylmethylsulfonylfluoride (PMSF), 10 mM Jodacetamid.

3) NTEP-buffer: 0.5% NP-40, 150 mM Natriumchloride, 50 mM Tris/HCl (pH 7.9), 5 mM EDTA, 10 mM Jodacetamide, 1mM PMSF.

4) SDS-Gel Elektrophores buffer: 25 mM Tris/HCl (pH 8.3-8.5), 192 mM Glycin, 0.1% SDS.

5) Protein-load-gel buffer (3x): 93.7 mM Tris/HCl (pH 6.8), 30% Glycerin, 9% SDS, 1.5 % bromphenolblue.

6) Tris-HCl/SDS buffer for proteingelelectrophoresis (4x): 0.5 M Tris-HCl, pH 6.8, 14 mM SDS.

7) Tris-HCl/SDS buffer for proteingelelectrophoresis (4x): 1.5 M Tris-HCl, pH 8.8, 140 mM SDS

8) 1x TE – buffer: 0.01 M Tris-HCl, pH 8.0, 7.6 or 7.4, 1 mM Na_2EDTA (pH 8.0)

9) 50x TAE-buffer: 2 M Tris-HCl, 0.05 M Na_2EDTA (pH 8.0), 1M glacial acetic acid

10) Blot buffer: 25 mM Tris/HCl, pH 8.3, 192 mM glycine, 20% (v/v) methanol.

11) Blocking solution: 5% (w/v) low fat milk powder in PBS / 0.05% Tween

12) Antibody incubation buffer: PBS / 0.05% Tween

13) Fixation solution: 3.0% w/v formaldehyde in PBS

14) Permeabilization solution: 0.5% v/v Tritone-X-100, 0.5 M NaCl, 50 mM PBS, pH 7.4

Media for bacteria

YT: 0.8 % (w/v) Bacto-Tryptone, 0.5 % (w/v) Bacto-Yeast extract and 0.25 % (w/v) NaCl, pH 7.0

SOC: 2 % (w/v) Bacto-Tryptone, 0.5 % (w/v) Bacto-Yeast extract, 0.05 % (w/v) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, pH 7.0

YT-agar: 1.5 % agar (w/v) in liquid YT medium

All media were sterilized by autoclaving.

Antibodies

Anti- *c-Myc* - a mouse monoclonal IgG_1 antibodies were from **Santa Cruz Biotechnology**,

Anti *Gαi* – an affinity purified rabbit polyclonal antibodies raised against a peptide mapping at the carboxyl terminus of Gαi-3 of rat origin.

Anti *Gαs* – an affinity purified rabbit polyclonal antibodies raised against a peptide mapping within the amino terminal domain of Gαs of human origin.

Anti-*Rabbit Ig Peroxidase Conjugate* were from **Sigma.**

Anti-*mouse Ig, horseradish peroxidase linked whole antibody* were from **Amersham Pharmacia Biotech.**

Cells strains and plasmid DNAs

pTracer^{TM}-CMV2 DNA, pcDNA3.1/Myc-His(-), Subcloning Efficiency^{TM} DH5α^{TM} Chemically Competent *E.coli* were from **Invitrogen**

pFastBac vector was from **Life Technologies**

pECFP, pEYFP, pEGFP plasmid DNA were from **Clontech**

MAX Efficiency DH10Bac^{TM} Competent cells were from **Life Technologies**

Cos.7 cells from African green monkey kidney

N1E-115 neuronal cells from mouse neuroblastoma

NIH3T3 fibroblastoid cells in monolayer from Swiss mouse embryo

Primery Mouse hippocampal Neurones were isolated from embryonic mice and cultured as mixed glial cells.

Sf.9 insect cells line from *Spodoptera frugiperda*

Oligonucleotide's sequences

Cys 404-Ser:

5' CTA CTC CAG TCC CAG TAC CGG 3'

5' CCG GTA CTG GGA CTG GAG TAG 5'

Cys 438-Ser:

5' CTA CAA AAC TCT GAC CAC TG 3'

5' CAG TGG TCA GAG TTT TGT AG 3'

Cys 441-Ser:

5' GAC CAC TCT GGG AAA AAA GG 3'

5' CCT TTT TTC CCA GAG TGG TC 3'

Cys 438-441-Ser :

5' CTA CAA AAC TCT GAC CAC TCT GGG AAA AAA GG 3'

5' CCT TTT TTC CCA GAG TGG TCA GAG TTT TGT AG 5'

Oligonucleotide for Myc-tagged epitope containing **HindIII site***:*

5’ CC***A AGC TTC*** GCC ACC ATG GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG ATG GAC GTT AAC AGC AGC 3’

FastBac sense primer:

5’ TAT TCC GGA TTA TTC ATA CCG 3’

FastBac anti-sense primer:

5’ GTG GTA TGG CTC ATT ATG ATC C 3’

2.2. Methods

2.2.1. Cell culturing

Insect cells

Sf.9 cells were grown in 75-mm^2 flasks with 10 ml of TC-100 medium supplemented with 10% of FCS and 1% of S/P at 28°C. For sub-culturing, medium was removed, 5 ml of fresh TC-100 medium was added and cells were scraped from the flask, re-suspended and transferred into new flask.

Mammalian cells

N1E-115 and Cos.7 cells were cultured in DMEM supplemented with 10% of FCS and 1% of penicillin/streptomycin on 10 cm plates. For sub-culturing of N1E, cells were scraped from plates in the 5 ml of growing medium with a rubber policeman, re-suspend in a fresh medium and put into complete DMEM medium. For splitting of Cos. 7 cells, medium was removed, cells were treated with 0.05% trypsin (w/v)/0.02% EDTA (w/v) until they no longer adhered to the plate. Trypsin/EDTA was removed, cells were re-suspend in 5 ml of fresh DMEM medium and divided on the new plates.

NIH3T3 cells were cultured in DMEM supplemented with glutaMAX II, 10%FCS, 1% penicillin/streptomycin on 10 cm plates. For passages, cells were trypsinized, then re-suspended in fresh medium and put onto the new plate.

All mammalian cells were grown at 37°C, 5% CO_2, 95% humidity and split every 3-4 days.

2.2.2. Cell transfection procedure

2.2.2-1. Transfection of Cos.7 cells by electroporation

Cos.7 cells grown on 150 mm dishes were washed with PBS and then treated with Tripsin-EDTA (5 ml/dish). After that, cells were resuspended in 10 ml of DMEM medium

and transferred into 50 ml Falcon tubes. After centrifugation (1000 rpm, 5 min), medium was removed and cells were resuspended in Electroporation buffer EP (50 mM KH_2PO_4, 20 mM CH_3CO_2K, 20 mM KOH, 26.7 mM $MgSO_4$, pH 7.4) to obtain 10^7 cells/ 100 μl of buffer. 500 ng of the 5-$HT_{7(a)}$ cDNA was diluted in 200 μl of 5 x EP buffer containing 15 μg of pRK5 plasmid DNA. After combining of cells suspension with cDNA solution, final mixture was staining for 15-30 min at RT. Electroporation procedure was performed in gene pulsar cuvettes upon next conditions: 280 V and 1000 F at t_{const}=30 msec. After electroporation cells were placed into 15 ml Falcon tubes with 12 ml of DMEM medium with supplements and dispersed then into 12-well clusters with 1 ml of cells suspension into each one to grow up. Six hours after transfection medium was changed on DMEM without FBS, with [^{3}H]-adenosine (2 μCi/ml) and cells were grown overnight.

2.2.2-2. Transfection of mammalian cells with Lipofectamine 2000 reagent

All basic procedures were performed according to the manufactures recommendations. DNA was diluted in appropriate volume of Opti-MEM 1 Reduced Serum medium without serum and antibiotics. Lipofectamine 2000 was diluted in appropriate volume of Opti-MEM 1 medium. After mixing and 5 min incubation, diluted DNA was combined with the diluted Lipofectamine 2000, mixed gently and incubated at RT for 20 minutes to allow the formation of DNA – Lipofectamine complexes. The complexes were added to cells and incubated at 37°C in a CO_2 incubator for 3-4 hour. After that medium was re-placed by the appropriate growth medium. Depended on the amount of the cells, different ration of LF 2000 and DNA were used (Table 1).

Culture vessel	*Volume of plating medium*	*DNA (μg) and Dilution Volume (μl)*	*Lipofectamine 2000 (μl) and Dilution Volume (μl)*
96- well	100 μl	0.2 μg in 25 μl	0.5 μl in 25 μl
24-well	500 ml	0.8 μg in 50 μl	4.0 μl in 100 μl
35-mm	2 ml	4.0 μg in 250 μl	10 μl in 250 μl

Table 2. 1. Ration of DNA and Lipofectamine used for transfection of mammalian cells.

2.2.3. Working with DNA

2.2.3-1. DNA gel electrophoresis

Size of DNA (or DNA fragments) was analysed by the agarose gel electrophoresis. The DNA bands were visualised under ultraviolet light of 302 nm after incubation of the gel in ethidium bromide (0.5 μg/ml).

Gels were prepared according to the size of the DNA fragments of interest as follows:

Agarose concentration (%)	*Resolving size range (kb)*
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-4
2	0.1-3

Table 2. 2. Relationship between agarose concentration and resolving DNA size.

2.2.3-2. Restriction, purification and ligation of DNA

Five to 10 μg of plasmid DNA or 1 to3 μg of PCR products were used for restriction digestion with appropriate endonucleases. Total volume of reaction mixture was 40 μl. Assay conditions were defined according to the restriction enzyme.

For the DNA purification DNA band was cut from the agarose gel after electrophoresis and put into Eppendorf tube. Purification procedure was performed with Gel-Extraction-Kit "Jetsorb" from Genomed**.** DNA containing agarose was incubated with Natriumjodid at 50°C for 15 minutes to dissolve agarose completely. In parallel special DNA-binding suspension (Silicamatrix) was added to the mixture. After incubation, suspension was pelleted by centrifugation and washed one time with low-salt buffer and two times with high-salt buffer. DNA was eluted from Silicamatrix with TE-buffer at 50°C.

For ligation vector DNA and cDNA fragments were mixed in an Eppendorf tube with Ligation buffer, water and ligase in volume of 10 μl. Reaction was performed at room temperature for 2 hours. Ligation mixture was used then for the transformation of *E.coli* competent cells.

2.2.3-3. Transformation of *E.coli* competent cells

Competent cells were refrozen on the ice and placed into 12-ml polypropylene tubes. 10 μl of ligation reaction was added to the 100 μl of competent cells and mix by gently tapping. The mix was incubated on ice for 30 minutes, cells were heat shocked for 45 seconds at the 37°C and then placed on ice for 2 minutes. Nine hundreds μl of pre-warmed SOC medium was added to each vial and tubes were incubated at 37°C for 1 hour at 225 rpm in a shaking incubator. After that cells were collected by centrifuge at 3000 rpm for 3 minutes. Cell's pellet was re-suspended in 100 μl of YT medium and spread on LB agar plate with appropriate antibiotics and bacteria were growth overnight at 37°C.

2.2.3-4. Plasmid mini- and maxi preparations

Small-, medium- and large-scale plasmid extractions were performed using Plasmid mini/midi- and maxi-prep kits from QIAGEN according to the manufactures recommendations. The basic principle involve alkaline lyses of the cell wall, degradation of RNA by RNAse, binding of plasmid DNA to a silica-gel matrix and removing of proteins and chromosomal DNA with high salt.

2.2.3-5. Construction of the recombinant baculovirus

The gene encoding 5-$HT_{7(a)}$ receptor was kindly provided by Dr. Isabel Bermudez, School of Biological and Molecular Science, Oxford University. The 5-$HT_{7(a)}$ cDNA was cleaved by EcoRI endonuclease and 1.35 kb fragment containing the gene was cloned into pFastBac donor plasmid (Life technologies). The resulting plasmid was transfected into DH10Bac (Invitrogene) *Escherichia coli* cells containing bacmid and helper DNA (Fig.2.1.). Recombinant bacmid DNA was then purified with a "*Quiagen plasmid maxi kit*", according to manufactural protocol, checked for the presence and orientation of gene by PCR with receptor-specific primers and transferred into Sf.9 cells with Cellfectin reagent (Fig.2.1). Finally, recombinant virus was purified and amplified to $1x10^8$ pfu per ml.

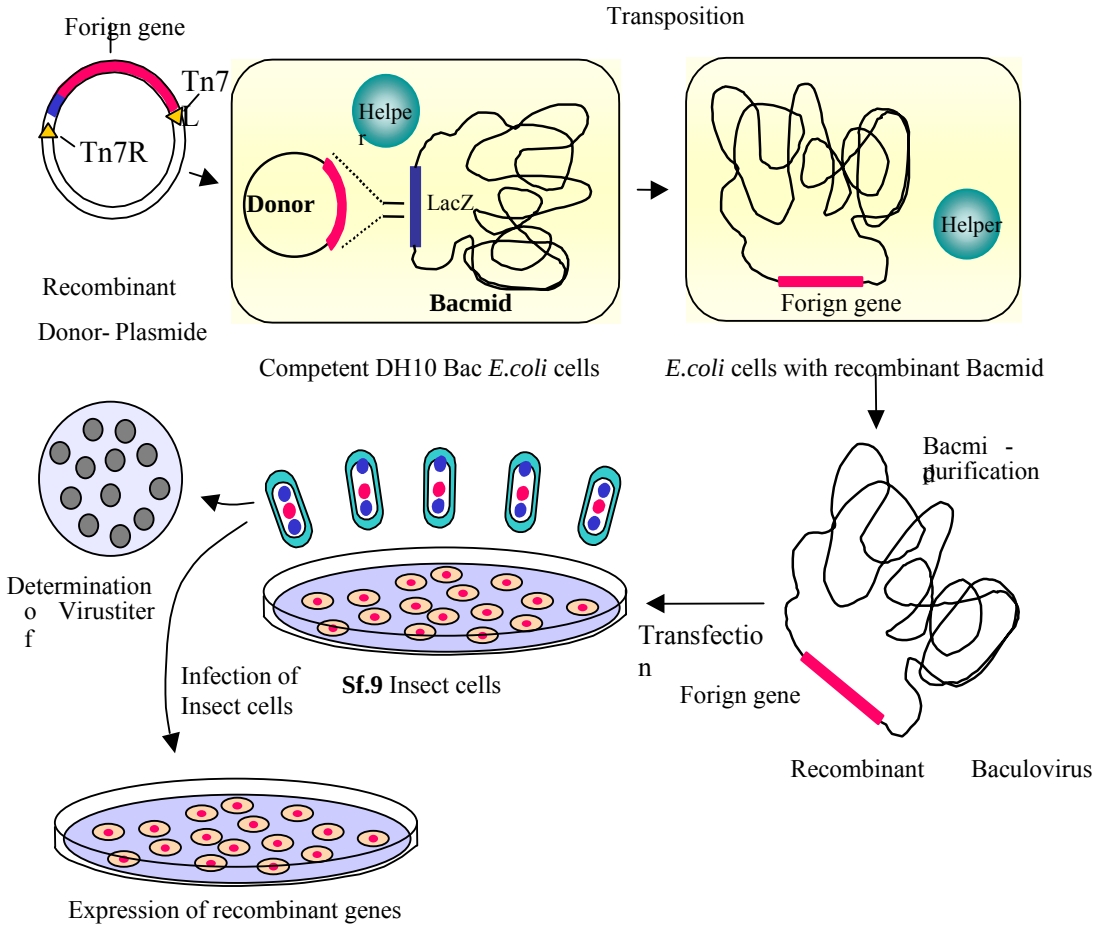


Figure 2. 1. Principle of Baculovirus expression system.

2.2.3-6. Construction of the 5-HT$_{7(a)}$ receptor cysteines mutants

To generate the receptor mutants we designed specific primers in which nucleotides sequence encoding for the cysteines residues were replaced by serines. The mutants were created by overlap extension PCR method (Fig. 2.2). For this, two partial cDNA (strains A and B) for every mutant were separately amplified by PCR from the wild type cDNA using corresponded overlapping sense (a1) or antisense (b1) primers and pFastBac sense (a2) or antisense (b2) primers. The two PCR products were purified from agarose gel, mixed and then used as a template for the second PCR. The second PCR reaction was performed using pFastBac sense and antisense primers. Resulting PCR products were purified, cut by HindIII endonuclease and cloned into pFastBac or pcDNA 3.1 vector. Substitutions of cysteines by serines were verified by double-stranded DNA sequencing.

The standard reaction mixture (50 µl final volume):

50–100 pmol forward primer in dH_2O

50-100 pmol reverse primer in dH_20

1 x nucleotide mix (200 µM dATP, dCTP, dGTP, dTTP)

1 x PCR buffer without $MgCl_2$ (20 mM Tris/HCl, pH 8.4, 50 mM KCl)

2.5 mM $MgCl_2$

20 ng template DNA in 10 mM Tris/HCl, pH 8.5

1 U DNA polymerase (AmpliTaqGold DNA polimerase from "Applied Biosystems" was used in all experiments)

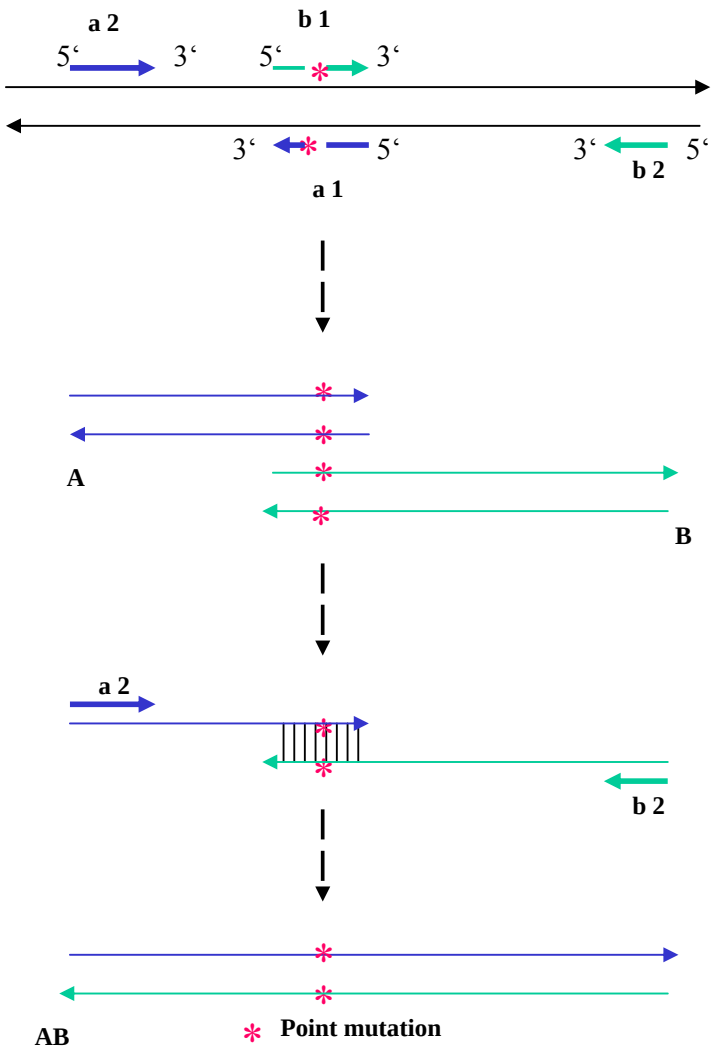


Figure 2. 2. Principle of Overlap-Extension PCR method.

2.2.4. Handling with proteins

2.2.4-1. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The principle of polyacrylamide gel electrophoresis is separation of a large range of proteins of varying molecular weights under the influence of an electrical field within the continuous, cross-linked polymer matrix. Here, the polymer is polyacrylamide and the cross-linking agent is bis-acrylamide. Cross-linking is catalysed through a free radicals produced by the addition of ammonium peroxide and TEMED (1,2-Bis-(dimethylamino)-etane).

Two phases gels was used for a separation: stacking gel with a low level of cross-linkage and low pH, allowing protein bands to enter the gel and collect without smearing, and a separating gel with a higher pH, in which the proteins are separated on the basis of size. The proteins were loaded on the top of the gel and migrated under the influence of an applied electrical field from the cathode to the anode. To define the molecular weight, molecular weight marker was loaded and separated in parallel.

2.2.4-2. Western blot

For the western blot procedure a semi-dry set-up was employed. After completion of electrophoresis, the polyacrylamide gel was removed, pre-equilibrated in blot buffer and placed on top of three sheets of Whatman filter paper and a nitrocellulose membrane (0.2 μm pore diameter). After carefully removing any air bubbles present, additional three pre-equilibrated sheets of Whatman filter paper were applied on the top. The blotting was carried out in the Blotting chamber from "Biometra" by applying of 3 mA per 1cm^2 of the gel for 1 h.

After transfer, membrane was treated with blocking solution for 2 h at RT or overnight at 4°C to saturate protein binding sites and incubated with primary antibody (1:1000 dilution) in 10 ml PBS/Tween buffer for 1 h at RT. After three washes, the membrane was incubated with the second antibody, covalently coupled with horseradish peroxidase (goat anti-mouse diluted 1: 5000).

For the detection ECL Western Blotting Detection reagents (Amersham Biosciences) was used. Equal volumes of detection solution 1 and detection solution 2 were mixed to a final volume allowing to cover the membrane (ca. 2 ml). Mixed detection reagent was added on to the membrane, incubated for 1 minute at RT and after that

covered with a sheet of SaranWrap. The proteins were visualised by the fluorography using Kodak-X-OMAT AR films.

2.2.4-3. Metabolic labelling and immunoprecipitation

Sf.9 cells were grown in TC-100 medium supplemented with 10% (v/v) fetal calf serum and 1% (w/v) penicillin/streptomycin) (complete TC-100). For expression Sf.9 cells ($1,5x10^6$) grown on 35 mm dishes were infected with recombinant baculovirus encoding 5-$HT_{7(a)}$ receptor at a multiplicity of infection of 10 plaque forming units per cell. After 48 h, Sf.9 cells were labelled with Tran [^{35}S]-label (50 μCi/ml in TC-100 medium) or [^{3}H]-palmitic acid (300 μCi/ml in TC-100 medium) for periods indicated in figure legends. In some cases 5-HT or SB 269970 were added to the final concentrations as indicated in figure legends. To block protein synthesis, cycloheximide (50 μg/ml) was added 10 min before incubation with [^{3}H]-palmitate or [^{35}S]-methionine. After labelling cells were washed once with ice-cold PBS and lysed in 600 μl of NTEP buffer. Insoluble material was pelleted (5 min, 20 000 g) and antibodies AS9458 raised against C-terminal peptide of the 5-$HT_{7(a)}$ receptor were added to the resulting supernatant in dilution 1:60. After overnight agitation at 4°C, 30 μl Protein A-Sepharose CL-4B was added and samples were incubated with gentle rocking for 2 h. After a brief centrifugation pellet was washed twice with cold NTEP buffer and the immuno-complexes were released from beads by incubation for 30 min at 38 °C in non-reducing Laemly buffer. Radiolabelled polypeptides were analysed by SDS/PAGE (12% w/v gel) and detected by fluorography with Kodak X-Omat AR films.

2.2.5. Treatment with hydroxylamine

After SDS/PAGE gels, containing 5-$HT_{7(a)}$ receptor labelled with [^{35}S]-methionine or [^{3}H]-palmitate were fixed in 10% v/v acetic acid, 10% v/v methanol and then washed in water twice for 15 min. Then the gels were treated overnight with 1M hydroxylamine (pH 7.5) or 1 M Tris-HCl (pH 7.5) as control. After that, the gels were washed in water and rocked in DMSO for 30 min to wash out cleaved fatty acids. The gels were again washed two times with water for removing DMSO and then processed for fluorography.

2.2.6. Fatty acid analysis

[^{3}H]-palmitate-labelled 5-$HT_{7(a)}$ protein was purified by immunoprecipitation and subjected to SDS/PAGE and fluorography. The acyl protein band was excised from the

gel, soaked in water twice for 30 min and then dried under vacuum in desiccators. Fatty acids were cleaved by treatment of the dried gel slices with 6 M HCl for 16 h at 110°C in tightly sealed ampoules. Fatty acids were then extracted three times with hexane; the upper phases were pooled and concentrated. Separation into individual fatty acid species was performed on RP-18-TLC plates (Merck) with acetonitrile/acetic acid (1:1, v/v) as the mobile phase. Radiolabelled fatty acids were detected by fluorography after the plates had been sprayed with En^{3}Hance (DuPont). For identification of fatty acid species, radiolabelled marker fatty acids ([^{3}H]myristate, [^{3}H]-palmitate and [^{3}H]-stearate) were run on the same plate in parallel.

2.2.7. cAMP accumulation assay

Adenylyl cyclase activity was measured by determining conversion of [^{3}H]-ATP to [^{3}H]-cAMP from total cells lysates. For this DMEM with [^{3}H]-adenine was removed one day after transfection and 900 μl (or 1 ml for the control) of HBS, containing 10% pargylyne, 10% ascorbate and 1.6 mM phosphodiesterase inhibitor RO-20-1724 was added to the each plate. 100 μl of 5-HT solution (concentration range from 1 nM to 1 mM) was added to the cells. After 10 min incubation at 37°C reaction was stopped by aspiration of the medium and adding of 1 ml of ice-cold 5% three chloric acid (TCA). Cells were scraped, transferred into tubes and 100 μl of 200 mM mix of ATP and cAMP was added into each tube following by centrifugation at 1700 rpm for 10 minutes. Supernatant was placed into columns containing Resin, total ATP was eluted with a water and analysed by liquid scintillation counter.

2.2.8. GTPγS binding assay

Sf.9 cells were grown in TC 100 medium supplemented with 10% FCS and 1% P/S on 60 mm dishes. For the assay cells were infected with 1-5 m.o.i per cell by recombinant baculoviruses, encoding for the 5-HT$_{7(a)}$ receptor, Gαs and Gβγ subunits. 48 h after infection cells were scraped from dishes, washed with 0.9% NaCl supplemented with 2 μg/ml aprotenin and 100 μM PMSF and re-suspend in 2 ml of 20 mM Hepes- 1 mM EDTA (pH 8.0), containing 100 μM PMSF, 2 μg/ml aprotenin and 10 μg/ml leupeptin. Cells were homogenised with Teflon pestle and non-broken cells were removed by centrifugation at 100 g for 5 min. Supernatant was transferred into new tubes and centrifuged at 16 000 g for 30 min at 4°C. Membrane pellet was washed one time with Hepes-EDTA and centrifuged at 16 000 g for 30 minutes. After that membrane fraction

was resuspended in Hepes-EDTA to adjust the protein concentration to 3 mg/ml and samples were stored at –80°C.

Membranes from Sf.9 cells expressing the 5-$HT_{7(a)}$ receptor wild type or acylation-deficient mutants and G- protein α subunits (Gi, Gs, G12, G13) together with $\beta_1\gamma_2$ subunits were resuspended in 55 µl of 50 mM Tris/HCl (pH 7.4) containing 2 mM EDTA, 100 mM NaCl, 3 mM $MgCl_2$, and 1 µM GDP. After adding [^{35}S]-GTPγS (1300 Ci/mmol) to a final concentration of 30 nM, samples were incubated for 5 min at 30°C in the absence or presence of agonist (1 µM 5-HT). The reaction was terminated by adding of 50 mM Tris/HCl (pH 7.5), containing 20 mM $MgCl_2$, 150 mM NaCl, 0.5 % Nonidet P-40, 200 µg/ml aprotinin, 100 µM GDP and 100 µM GTP for 30 min on ice. The samples were agitated for 1 h at 4°C with 5 to 10 µl of appropriate Gα subunits-directed antiserum preincubated with 100 µl of 10 % suspension of protein A-sepharose. Immunoprecipitates were washed three times, boiled in 0.5 ml of 0.5 % SDS, then 4 ml of Ecolite+ was added and radioactivity was measured by by scintillation spectrometry.

2.2.9. Ligand binding assay

Membranes were prepared from transiently transfected Cos.7 cells plated on 15-cm dishes and grown in DMEM supplemented with 10% FCS for 6 h and then for 20 h in DMEM without FCS. The cells were washed twice with PBS, scraped with a rubber policeman, harvested in PBS, and centrifuged at 4°C (900g for 4 min). The pellet was resuspended in buffer containing 10 mM HEPES (pH 7.4), 5 mM EGTA, 1 mM EDTA, and 0.32 M sucrose and homogenized 10 times with a glass-Teflon homogeniser at 4°C. The homogenate was centrifuged at 20000 g for 20 min and the membrane pellet was resuspended in 50 mM HEPES (pH 7.4) to obtain 5 mg/ml protein solution which was stored at –80°C until using.

Binding assays were performed in 96-well, round-bottom microtiter plates with total reaction volumes 200 µl, containing 20 µg of membrane preparation and different concentrations of [^{3}H]-5-HT with or without 10 µM 5-HT in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM ascorbate, 0,1% BSA and 100 µM GTP. The reaction mix was incubated at 23 °C for 60 min and transferred to the Whatman Hydrophilic GF/C Filter, pre-soaked in 0.3% polyethylenimine, washed with approximately 4 ml/well with an ice-cold buffer containing 50 mM Tris-HCl (pH 7.0 at RT) and 2 mM $MgCl_2$. The filters were dried and counted in a Top-Count liquid scintillation cocktail counter (Packard).

2.2.10. Indirect Immunofluorescence

Sf.9 cells $2.5x10^5$ were placed on the coverslip and infected by recombinant baculovirus or wild-type baculovirus. 48 h after infection cells were washed three times with PBS and then fixed with paraformaldehyde (3% w/v in PBS) for 15 min. Un-reacted paraformaldehyde was quenched with 100 mM glycine for 15 min, and cells were again washed with PBS. In several experiments cells were permeabilised with Triton X-100 (0.1 % v/v in PBS), blocked with 5% w/v BSA in PBS and incubated with primer antibodies raised against 5-$HT_{7(a)}$ receptor or against c-*myc*-epitop in dilution 1:500 for 60 min at 37°C. Samples were washed with PBS and incubated with secondary antibodies (Fluor 488, Alexa, Netherlands) diluted 1:1000 in PBS for 60 min at 37°C. Coverslips were placed in 90 % v/v glycerol and analysed under fluorescence microscope with appropriate filter at 63x magnification. The results were recorded with a digital camera RTE/CCD-782 (Princeton Instruments).

2.2.11. Transfection and morphological analysis of N1E-115 cells

N1E-115 cells were grown in Dulbecco´s modified Eagle´s medium (DMEM) containing 10% fetal calf serum and 1% penicillin/streptomycin at 37°C under 5% CO_2. For transient transfection, cells were seeded at low-density ($5x10^5$) into 35-mm dishes and transfected with 1 µg of pTracer, pTracer/5-$HT_{7(a)}$-WT using Lipofectamine 2000 Reagent (Invitrogen). At 14-16 h post-transfection the cells were washed and incubated for 24-36 h in serum-free DMEM to induce morphological differentiation. Agonist-induced changes in shape were monitored using the LSM510 (Zeiss) confocal microscope with appropriate "GFP" filter setting. Experiments were performed at 37°C in bicarbonate/CO_2 buffered DMEM. Cells were either scored rounded, flattened, or flattened with neurite the length of at least twice the cell body diameter ("neurite-bearing"). For each transfection, the percentage of rounded, flattened and neurite-bearing cells was calculated from at least 400 green cells counted. The experiments were performed in duplicate per transfection and morphologies were scored without prior knowledge of the dish identities. An average percentage was calculated from at least three independent experiments.

2.2.12. Live cell imaging

NIH3T3 cells $8x10^4$ were plated on the cover-slip and transfected with appropriate plasmid DNA. Cells were starved for 14-16 h in DMEM w/o FCS and analysed by laser scanning confocal microscopy (LSM 510-META, Zeiss). Experiments were performed at

37°C in circulated bicarbonate/CO_2 buffered extra-cellular buffer. Images were collected with an objective 63x with water immersion under lambda mode setting after recording of corresponding reference spectra. Images were scanned every 5 min during 1 h. After two scanning cycles extra-cellular buffer was supplemented with specific agonist (10 μM 5-HT or 8-OH-DPAT).

3. RESULTS

3.1. Cloning and expression of the 5-HT$_{7(a)}$ receptor

The cDNA encoding the mouse 5-HT$_{7(a)}$ gene was cut by the EcoRI endonuclease and sucbcloned into pFastBac plasmid. To detect receptor expression, Myc-tagged 5-HT$_{7(a)}$ receptor was created by PCR with primers containing specific nucleotide sequences encoding Myc-tag epitope and Hind III restriction site. Resulting PCR-product was sub-cloned into pFastBac vector into Hind III site (Fig. 3.1) and nucleotide sequence of final product was determined by sequencing analysis.

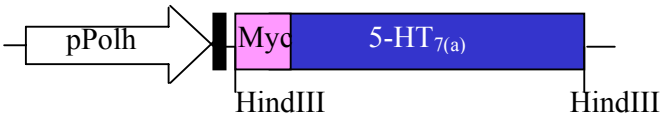


Figure 3. 1. Construction of the 5-HT$_{7(a)}$ gene subcloned into pFastBac-vector.

The pFastBac plasmid containing cDNA encoding for the Myc-tagged 5-HT$_{7(a)}$ receptor was used for generation of recombinant Baculovirus.

To analyse expression and post-translational modification of the 5-HT$_{7(a)}$ receptor we used recombinant baculovirus expression system. This system is widely used to express heterologous genes in cultured insect cells, mainly Sf.9 cells, since it allows abundant expression of recombinant protein. Moreover the recombinant proteins are processed, modified, targeted to their appropriate cellular locations, and posses functional similarity to their authentic counterparts. To monitor the expression and intracellular distribution of the recombinant receptor, infected Sf.9 cells were first subjected to immunofluorescence. The results shown in Fig. 3.2 demonstrate that the 5-HT$_{7(a)}$ receptor expressed in Sf.9 is mainly exposed on the cell surface.

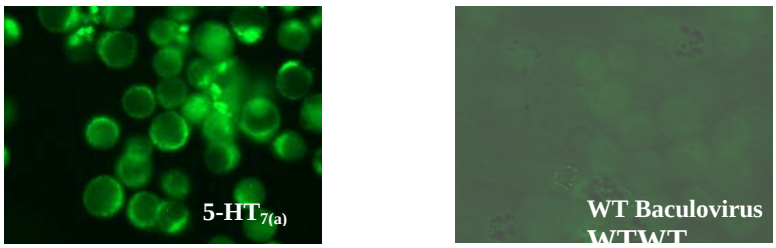


Figure 3. 2. Expression of the 5-HT$_{7(a)}$ receptor. Sf.9 cells infected with recombinant (left) or wild-type (right) baculovirus were fixed 48 h post infection and stained with antibody directed against the 5-HT$_{7(a)}$ receptor. After that, cells were analysed by confocal laser scan microscopy (Zeiss, LSM-510). Immunostaining shows cell surface distribution of the receptor.

As seen in Fig. 3.3, labelling with [^{35}S]-methionine followed by immunoprecipitation with anti-Myc antibody and SDS-PAGE analysis revealed a single protein band with molecular mass of the 5-HT$_{7(a)}$ receptor. No specific bands were found in immunoprecipitates of non-infected or of Sf.9 cells infected with the wild type baculovirus (Fig. 3.3, left panel). This emphasizes that the immunoprecipitated 46-kDa protein shown in Fig. 3.3 is indeed the 5-HT$_{7(a)}$ receptor.

3.2. Hydrophobic modification of the 5-HT$_{7(a)}$ receptor

To examine whether the 5-HT$_{7(a)}$ receptor is acyl-modified, Sf.9 cells infected with recombinant or wild type baculovirus were metabolically labelled with [^{3}H]-palmitic acid. The resulting fluorogram (Fig. 3.3, right panel) demonstrates that the 5-HT$_{7(a)}$ receptor effectively incorporated [^{3}H]-palmitate and that the labelled polypeptide co-migrated with the [^{35}S]-methionine labelled 5-HT$_{7(a)}$ protein.

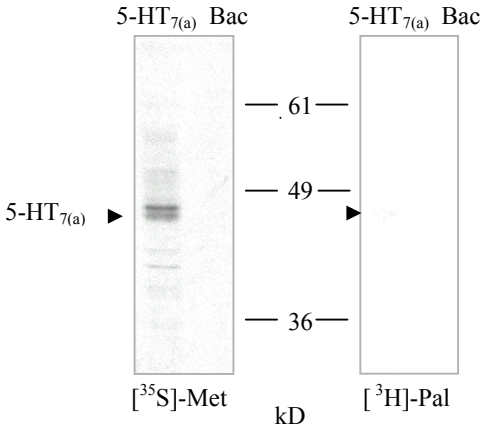


Figure 3. 3. Palmitoylation of the 5-HT$_{7(a)}$ receptor. Sf.9 cells expressing the 5-HT$_{7(a)}$ receptor were labelled with [^{3}H]-palmitate or [^{35}S]-methionine. Receptor was immunoprecipitated with anti-Myc antibodies and subjected to SDS/PAGE and to fluorography. Cells infected with a wild-type baculovirus (Bac) were used as a control. The fluorogram is representative of four independent experiments.

To distinguish between amide-type and ester type fatty acid linkages the chemical nature of the fatty acid bond in the 5-HT$_{7(a)}$ receptor was determined. In contrast to the amide bond, S-ester and hydroxyester linkages are sensitive to the presence of 2-mercaptoethanol (Schmidt et al., 1979). To investigate whether the fatty acid was attached to the 5-HT$_{7(a)}$ receptor by a S-ester bond, proteins labelled with [^{3}H]-palmitic acid were subjected to 2-mercaptoethanol treatment. The results shown in Figure 3.4 A demonstrate that [^{3}H]- palmitate-derived radioactivity bound to the protein was sensitive to heating with buffer supplemented with increasing concentrations of 2-mercaptoethanol. This suggests

that the 5-HT$_{7(a)}$ receptor contains exclusively S-ester –linked acyl groups and no fatty acids linked by an amide bond, which would have been resistant to such treatment.

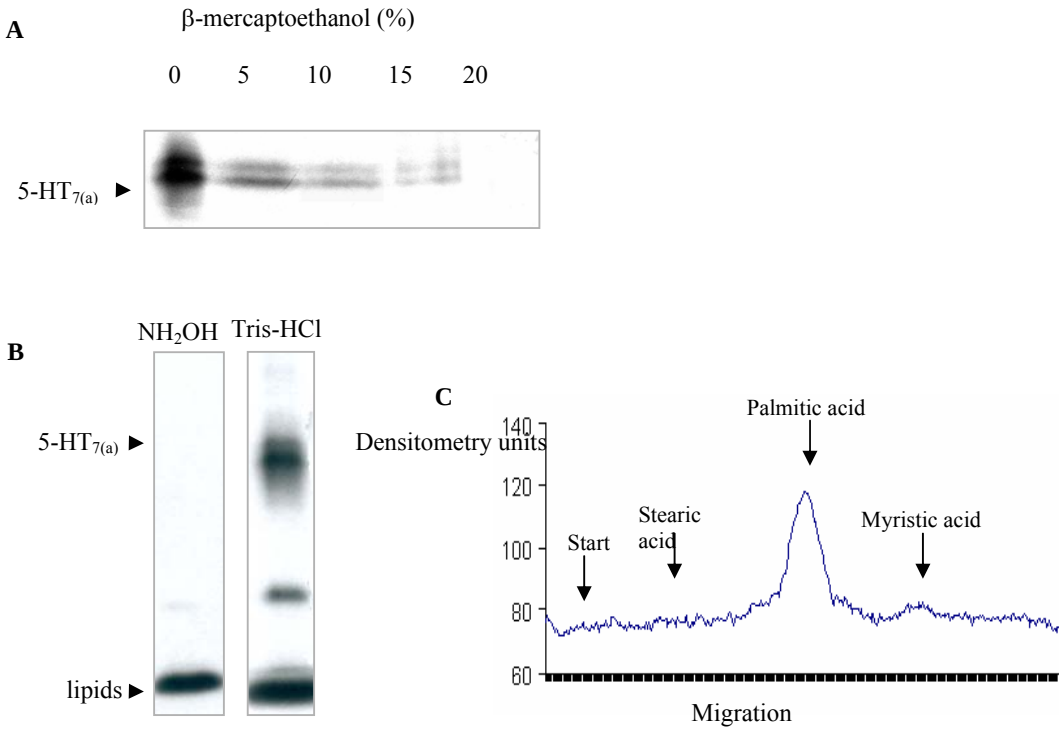


Figure 3. 4. Fatty acid analysis.
A. Sf.9 cells expressing the 5-HT$_{7(a)}$ receptor were labelled with [^{3}H]-palmitate. After immunoprecipitation, samples were treated with increasing concentration of β-mercaptoethanol for 30 min at 37°C followed by SDS/PAGE and fluorography. The data shown are representative of three experiments.
B. After labelling with [3H]-palmitate cells, expressing the 5-HT$_{7(a)}$ receptor were lysed, immunoprecipitated and subjected to SDS/PAGE. Gel was treated with 1 M Tris/HCl (pH 7.4) or 1 M hydroxylamine (pH 7.4). The resulting fluorogram is representative of three independent experiments.
C. Receptor-bound fatty acids were analysed by thin - layer chromatography (TLC). The fluogram obtained from the RP 18 TLC plate after two days exposure was analysed with Gel-Pro Analyser software, version 3.1.

The S-ester bond can be distinguish from the hydroxyester one by its sensitivity to treatment with hydroxylamine (Kaufman et al., 1984). After treatment of gels containing fatty-acid labelled 5-HT$_{7(a)}$ protein with neutral hydroxylamine, this reagent cleaved the palmitate-derived label from the receptor, whereas labelled lipids remained unaffected (Fig. 3.4 B). This sensitivity to neutral hydroxylamine and reducing agent indicates that fatty acid is bound to the 5-HT$_{7(a)}$ receptor via an S-ester-type linkage.

To prove the identity of the fatty acids bound to the 5-HT$_{7(a)}$ receptor after labelling with [^{3}H]-palmitate, proteins were subjected to fatty acid analysis. Fatty acids were hydrolysed from gel-purified the 5-HT$_{7(a)}$ receptor and separated into the individual fatty

acids species by TLC. Radio-chromatogram scanning of the TLC plates revealed that the 5-$HT_{7(a)}$ receptor contained only palmitic acid (Fig. 3.4 C).

3.2.1. Palmitoylation of the 5-$HT_{7(a)}$ receptor is agonist-dependent

Palmitate can be attached to the proteins in constitutive or in the reversible manner. To test whether palmitoylation of the 5-$HT_{7(a)}$ receptor is dynamic modification, receptor was labelled with [^{3}H]-palmitate during 60 minutes in the absence or in the presence of increasing concentrations of agonist 5-HT. The intensity of incorporated radioactivity was valuated from fluorograms exposed on gels containing immunoprecipitated the 5-$HT_{7(a)}$ receptor after labelling. The data obtained demonstrate that treatment with agonist results in dose-dependent increase in incorporation of [^{3}H]- palmitate into receptor (Fig. 3.5). It should be also noted that such increase in labelling can not be explained due to increase in the amount of newly synthesized receptor. Indeed, as shown in the Figure 3.5. B, the incorporation of [^{35}S]- methionine into the 5-$HT_{7(a)}$ receptor was even decreased after the application of agonist.

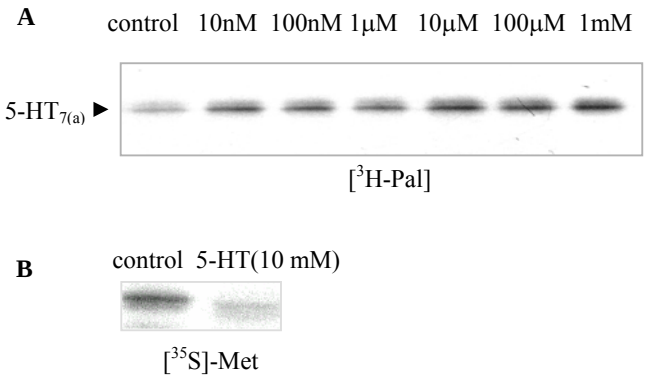


Figure 3. 5. Agonist-promoted incorporation of [^{3}H]-palmitate into the 5-$HT_{7(a)}$ receptor.
A. Sf.9 cells expressed the 5-$HT_{7(a)}$ receptor were labelled with [^{3}H]-palmitate for 60 min in the absence or in the presence of increasing concentrations of 5-HT. The receptor was immunoprecipitated, separated by SDS/PAGE and subjected to fluorography.
B. The effect of 5-HT treatment on receptor synthesis.

To exclude the influence of possible changes in the protein synthesis, cells expressed receptor were labelled with [^{3}H]-palmitate or [^{35}S]-methionine in the presence or in the absence of protein synthesis blocker cycloheximide. As it is seen in resulting fluorogram (Fig. 3.6), block of the protein synthesis by cycloheximide has no effect on the 5-$HT_{7(a)}$ receptor palmitoylation. More important, incorporation of [^{3}H]-palmitate still remains agonist-promoted. This result demonstrates that palmitoylation of the 5-$HT_{7(a)}$

receptor is post-translational process and indicate that previously synthesized receptor is available for repeated rounds of palmitoylation/ depalmitoylation.

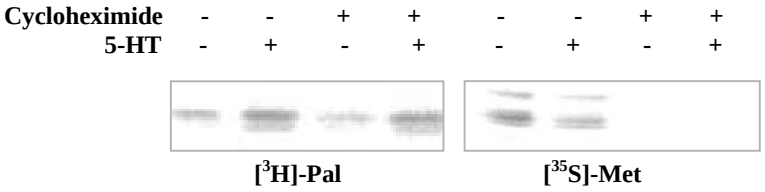


Figure 3. 6. Palmitoylation of the 5-HT$_{7(a)}$ receptor is independent from the protein synthesis.
Sf.9 cells expressing the 5-HT$_{7(a)}$ receptor were labelled for 60 min with [^{3}H]-palmitate or [^{35}S]-methionine in the absence (-) or in the presence (+) of cycloheximide (50 μg/ml). In parallel, 100 nM 5-HT or water was added. After SDS/PAGE, radiolabel incorporation was detected by fluorography. Experiment was repeated three times.

3.2.2. Dynamic of the 5-HT$_{7(a)}$ receptor palmitoylation

In order to obtain more information about the dynamic of receptor palmitoylation, Sf.9 cells expressed the 5-HT$_{7(a)}$ were incubated with [^{3}H]-palmitic acid for 5, 20, 40 and 60 min. As shown in Fig. 3.7, intensity of radioactivity of immunoprecipitated receptor increases steadily as a function of time reflects a basal palmitate turnover. To determine whether the activation of receptor influenced its palmitoylation state, the [^{3}H]- palmitate incorporation was analysed after agonist stimulation. Results of this experiment reveal that palmitoylation of the 5-HT$_{7(a)}$ receptor was significant increased in the presence of 5-HT as compared with non-stimulated receptor (Fig. 3.7).

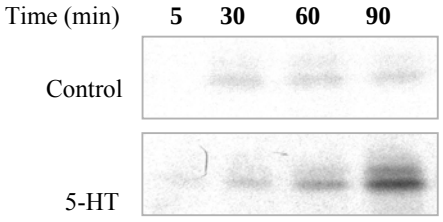


Figure 3. 7. Time course of the incorporation of [^{3}H]-palmitate into the 5-HT$_{7(a)}$ receptor after agonist treatment. Insect cells expressing the 5-HT$_{7(a)}$ receptor were labelled with [^{3}H]-palmitate in the presence of 100 nM 5-HT for the periods indicated. Data was obtained after fluorography followed by immunoprecipitation and SDS/PAGE and represents of three independent experiments

3.2.3. Identification of acylation site(s) on the 5-HT$_{7(a)}$ receptor palmitoylation

Taken together, these data suggest that palmitoylation of the 5-HT$_{7(a)}$ receptor is dynamic process and agonist stimulation increase the rate of receptor palmitate turnover. Given that the 5-HT$_{7(a)}$ receptor contains covalently-bound palmitate, the next step was to identify sites of acylation. The 5-HT$_{7(a)}$ receptor possesses three cysteine residues (Cys^{404}, Cys^{438} and Cys^{441}) within its intracellular C-terminal domain and these cysteines can represent palmitoylation sites. To identify which of these cysteines can be palmitoylated, a number of substitution mutants (Cys^{404}-Ser, Cys$^{438-441}$ and Cys$^{404-438-441}$), in which different cysteine residues were replaced by serines was constructed.

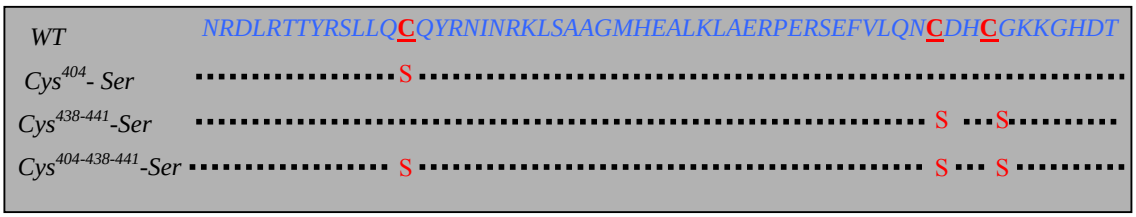


Figure 3. 8. C-terminal sequence of the 5-HT$_{7(a)}$ receptor with substituted cysteine residues. Schematic view of the 5-HT$_{7(a)}$ mutants. The cytoplasmic, carboxyl-terminal sequences of the 5-HT$_{7(a)}$ receptor and three substitution mutants are given in a single-letter code. The amino acid numbers for four cysteine residues are indicated.

All resulting mutants were expressed by Baculovirus expression system and proteins were detected by Western blot analysis (Fig. 3.9).

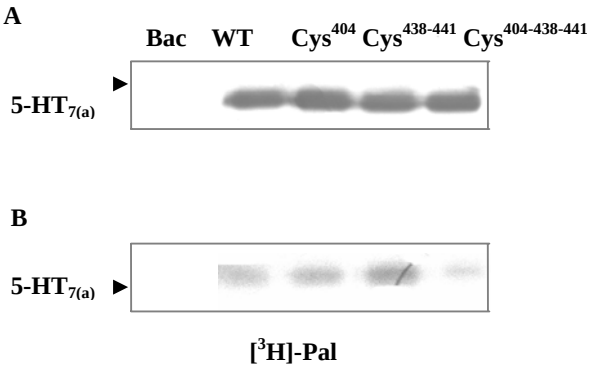


Figure 3. 9. Expression and acylation of wild type and mutants of the 5-HT$_{7(a)}$ receptor in Sf. 9 cells.
A. Insect cells expressed wild type (wt) and different C-terminal cysteines mutants of the 5-HT$_{7(a)}$ receptor were lysed, separated by SDS/PAGE and then subjected to Western-blot analysis.
B. Sf.9 cells expressed the 5-HT$_{7(a)}$ receptor wild type and cysteine mutants were labelled with [^{3}H]-palmitate. Proteins were immunoprecipitated with Myc-antibodies and subjected SDS/PAGE and subsequent fluorography. Data shown are representative of two independent experiments.

To determine the site(s) of palmitoylation, individual mutants were metabolically labelled either with [^{3}H]-palmitate or with [^{35}S]-methionine. Labelling with [^{35}S]-methionine shows that all mutants were expressed at the levels comparable with those of the 5-HT$_{7(a)}$ receptor wild type. Resulting fluorogram after labelling with [^{3}H]-palmitate demonstrated that replacement each of single cysteine and replacement of several cysteines in different combinations results in decreased palmitoylation. However, even after replacement of all three C-terminal cysteines by serine (Cys$^{404\text{-}438\text{-}441}$-Ser mutant) receptor was still palmitoylated (Fig. 3.9). Therefore, it can be concluded that in addition to the C-terminal cysteine residues, the 5-HT$_{7(a)}$ receptor is palmitoylated on additional cysteine located somewhere else. Similarly results has been previously reported for Gαs subunit (Kleuss and Krause, 2003) and for vasopressin V$_1$a receptor (Hawtin et al., 2001).

3.3. Functional importance of the C-terminal receptor palmitoylation

Since Sf.9 cells have no detectable expression of most mammalian receptors, co-expression of receptor and different G- proteins followed by measurement of agonist-promoted binding of [^{35}S]-GTPγS to the Gα subunit allows to assess the selectivity of receptor – G-protein coupling (Barr et al., 1997). Using this approach interaction of the 5-HT$_{7(a)}$ receptor with G- proteins belonging to different families was analysed. For that Sf.9 cells were co-infected with baculoviruses encoding the 5-HT$_{7(a)}$ receptor wild type in combinations with G-$\beta_1\gamma_2$ and different types of Gα subunits: αs, αi, α12 and α13. Membranes isolated from infected cells were incubated with radioactive-labelled [^{35}S]-GTPγS in the presence or in the absence of agonist (5-HT). G$_\alpha$ subunits were then immuno-precipitated with appropriate Gα specific antibodies and bound [^{35}S]-GTPγS was measured by scintillated counter. The amount of [^{35}S]-GTPγS bound to Gα subunits was used as direct indicator of G- protein activation.

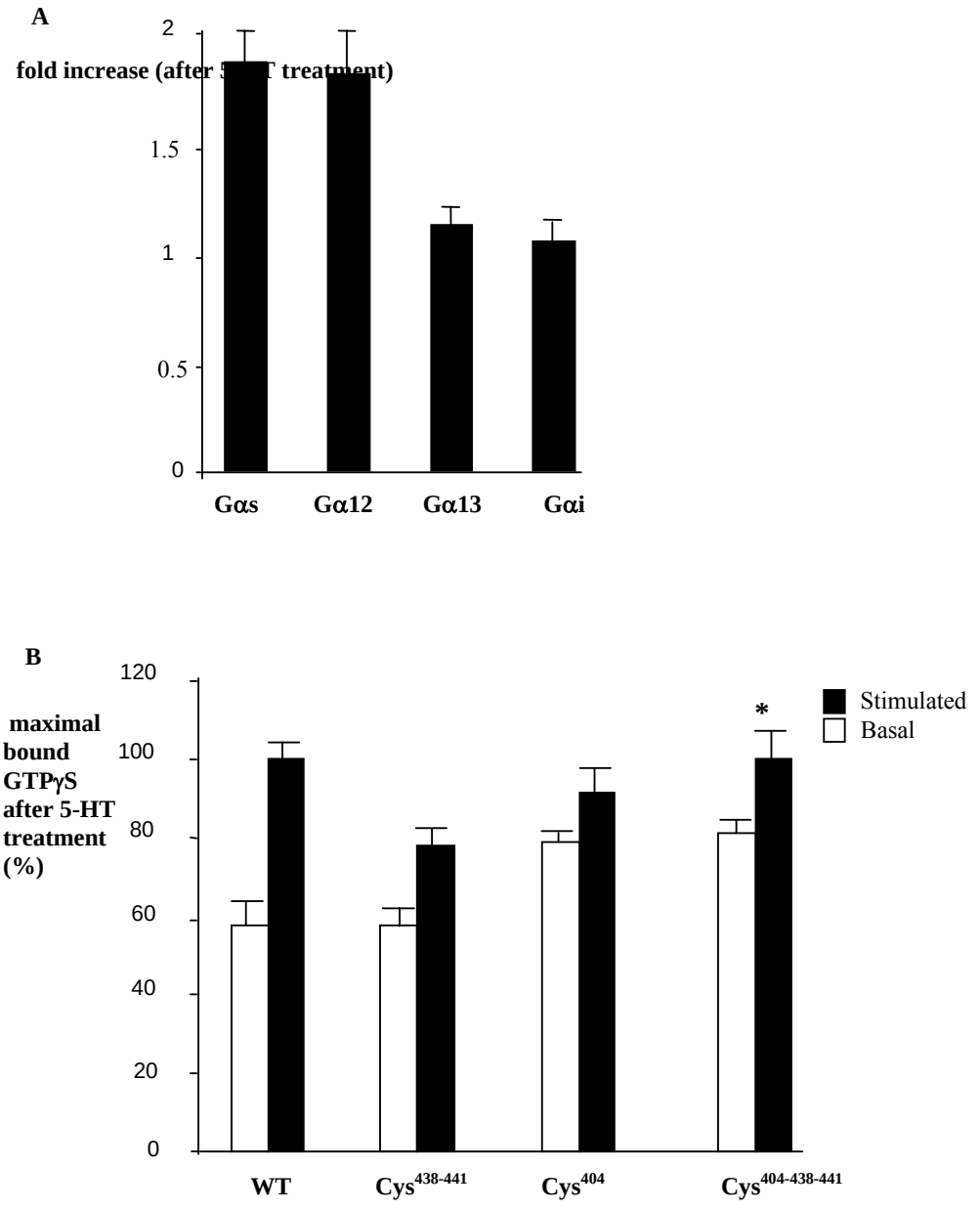


Figure 3. 10. A. Coupling of the 5-HT$_{7(a)}$ receptor with different G proteins. Membranes prepared from Sf.9 cells expressed the 5-HT$_{7(a)}$ receptor and different G proteins were incubated with [^{35}S]-GTPγS in the presence of 100 nM 5-HT or vehicle (H_2O) for 10 min at 37°C. Immunoprecipitation was performed with appropriate antibodies against different Gα-subunits. Data represent means ± S.E. as fold increase over basal level and represent three independent experiments.

B. $G_{\alpha s}$ coupling with the 5-HT$_{7(a)}$ receptor wt and mutants. Membranes prepared from insect cells expressed the 5-HT$_{7(a)}$ receptor wild type and different C-terminal cysteine mutants were incubated with [^{35}S]-GTPγS in the presence or absence 100 nM 5-HT, immunoprecipitated with $G_{\alpha s}$ antibodies and subjected to scintillation analysis. Data represent means ± S.E. of three independent experiments.

As shown in the Fig. 3.10 A, no interaction of the receptor with Gαi and Gα13 was detected. In contrast, [^{35}S]-GTPγS binding effectively increased when the 5-HT$_{7(a)}$ receptor was co-expressed with Gαs protein, even in the absence of agonist (Fig.3.10 A). Activation of the receptor by 5-HT resulted in almost 2-fold increase in the [^{35}S]-GTPγS binding in comparison with a control, demonstrating that the 5-HT$_{7(a)}$ receptor interacts with Gs protein.

Next we analysed potential of Gαs for coupling to different cysteine-deficient mutants of the 5-HT$_{7(a)}$ receptor. As shown in Fig. 3.10 B, activation of mutated receptors led to increase in [^{35}S]-GTPγS bounding to Gs protein at the same level as it was demonstrated for the wild type receptor. However, analysis of the constitutive agonist-independent activity for the single mutants revealed that Cys^{404} –Ser mutant couples with Gαs approximately 1.5 time more effectively as compared with the receptor wild type (Fig. 3.10 B). In contrast, agonist-independent binding of [^{35}S]-GTPγS to Gs obtained for Cys$^{438-441}$-Ser mutant was similar to the wild type. The substitution of all three C-terminal cysteine in mutant Cys$^{404-438-441}$-Ser results in increased constitutive activity as it was demonstrated for the single Cys^{404}–Ser mutant. These results indicate that acylation of proximal to plasma membrane cysteine in position 404 is critically involved in regulation of the receptor constitutive activity.

The experiments with insect cells demonstrated the possible involvement of palmitoylation in regulation of basal constitutive activity (Fig. 3.10). Therefore, we next tested agonist-independent activation of acyaltion-deficient 5-HT$_{7(a)}$ mutants in the mammalian cell system. The cDNAs encoding for the receptor wild type and mutants forms were cloned into pcDNA 3.1(-) palsmid (Invitrogen), transfected into Cos.7 cells and ability each of the proteins to stimulate intracellular cAMP formation was examined.

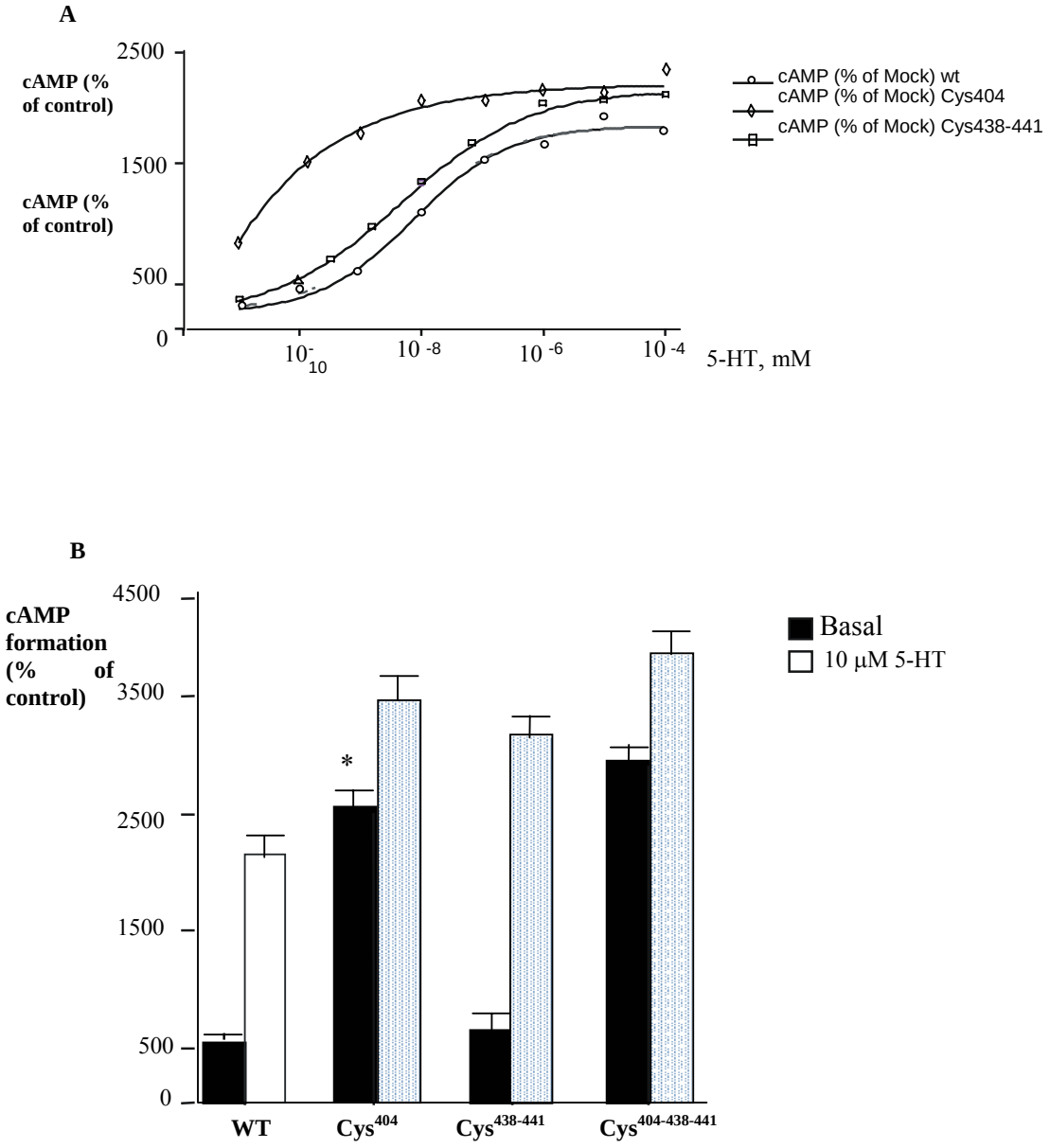


Figure 3. 11. A. Kinetic curves of cAMP accumulation for the 5-HT$_{7(a)}$ receptor wild type and cysteine mutants. Cos.7 cells transfected with the wild type or mutated 5-HT$_{7(a)}$ receptor were assayed for cAMP accumulation and data from three independent experiments were analysed for kinetic of the accumulation.

B. cAMP accumulation in response to stimulation of the 5-HT$_{7(a)}$ receptor. Cos.7 cells expressing wild type or mutated the 5-HT$_{7(a)}$ receptors were assayed for cAMP production. Level of cAMP accumulation was measured after 15 min of incubation in the presence or absence of agonist and defined as convergence of [^{3}H]-ATP to [^{3}H]-cAMP. Data represent percent increase over the control. Each value represents the mean $\pm$ S.E. from three independent experiments.

The wild type 5-$HT_{7(a)}$ receptor expressed in these cells demonstrated high level of constitutive activity in compare with mock-transfected cells. Analysis of basal cAMP accumulation for receptors in which Cys^{404} was replaced by serine (Cys^{404}-Ser or $Cys^{404-438-441}$-Ser) revealed almost five-fold increase in cAMP production as compared to the wild type (Fig. 3.11 B). In contrast, agonist-independent cAMP formation for $Cys^{438-441}$–Ser mutant as well as for Cys^{438}–Ser and Cys^{441}–Ser constructs was similar to the wild type. Application of the agonist results in 4 to 5-fold increase in the cAMP production after expression of the receptor wild type. Substitution of proximal cysteine (Cys^{404}) reduced this effect, leading to the decrease in relative efficacy of 5-HT to stimulate receptor activity over basal conditions. As shown in Fig. 3.11 A, the high basal activity obtained for Cys^{404} and $Cys^{404-438-441}$ mutants the same time leads to a decrease in concentration of agonist which is needed to achieve saturation level of cAMP production from 10^{-6} to 10^{-8} M. Moreover, analysis of the kinetic reflected that accumulation of cAMP in dose-response experiments differs for the receptor wild type and Cys^{404}- Ser mutant (Fig. 3.11 A).

Taken together, both experiments (GTPγS binding and cAMP accumulation assay) demonstrate that acylation of proximal cysteine residue (Cys^{404}) plays critical role in modulation of receptor constitutive activity.

3.4. Pharmacological characterisation of the wild type and mutants 5-$HT_{7(a)}$ receptor

Ligand binding affinity of the 5-$HT_{7(a)}$ receptor wild type and its palmitoyl-deficient mutants was analysed by saturation binding assay in membrane preparations isolated from transiently transfected Cos.7 cells.

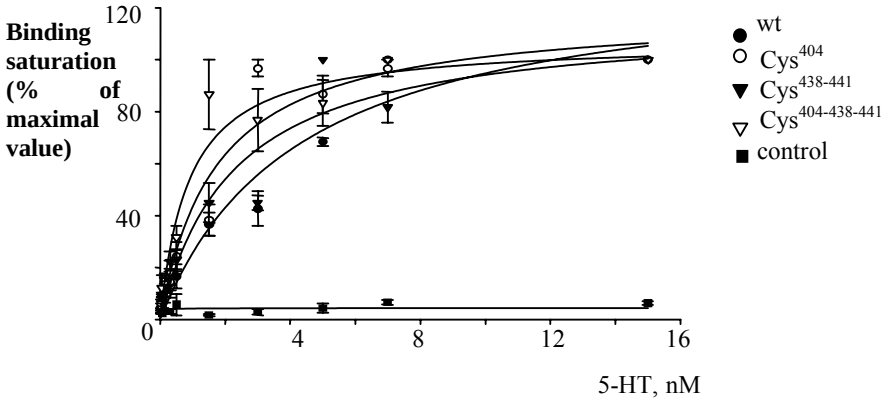


Figure 3. 12. Binding of [^{3}H]-5-HT to the receptor wt and cysteine mutants. Binding was performed in membranes prepared from Cos.7 cells transiently expressed of the 5-$HT_{7(a)}$ receptor wild type or different C-terminus cysteine mutants. Saturation curves for receptor wt and mutants represent fitted results from three independent experiments. Data were analysed by SigmaPlot programm.

As shown in Table 1 and Fig. 3.12., the affinity of [^{3}H]-5-HT for the wild type 5-$HT_{7(a)}$ receptor (K_D = 4.3) was quite different from that obtained for the Cys^{404}-Ser (K_D = 1.75), $Cys^{404-438-441}$–Ser (K_D = 0.9) and $Cys^{438-441}$–Ser mutants (K_D = 2.3), further confirming the importance of palmitoylation for the regulation of the receptor's basal activity.

	WT	**$Cys^{438-441}$**	**Cys^{404}**	**$Cys^{404-438-441}$**
K_D	4,30±0,91	2,30±0,60	1,75±0,37	0,90±0,21
B_{max}	135±11,8	115,6±9,3	119,8±7,5	107,3±6,2

Table 3. 1. Membrane fractions were prepared from Cos. 7 cells expressing the 5-$HT_{7(a)}$ receptor. Saturation analysis was performed using [^{3}H]-5-HT as radioligand. Saturation curves were analysed by non-linear regression to generate apparent dissociation constants, K_D (nM), and maximum number of receptors, B_{max} (fmol/mg of protein). Data shown represent mean ± S.E. from three independent experiments.

3.5. The 5-$HT_{7(a)}$ receptor signalling through the G12 pathway activation

Using the [^{35}S]-GTPγS binding assay we found that the 5-$HT_{7(a)}$ receptor couples effectively not only with Gαs but also with Gα12 proteins (Fig. 3.10 A). Activation of Gαs was predictable and consistent with data obtained before (Adham et al., 1998). Activation of the Gα12 by the 5-$HT_{7(a)}$ receptor was a novel observation and therefore this new pathway was analysed in more details.

3.5.1. The 5-$HT_{7(a)}$ receptor and morphological changes: N1E-115 cells

The prominent downstream effectors in G12-mediated signalling are small GTPases of the Rho family. The Rho GTPase family, which regulates the actin cytoskeleton, has been shown to be involved in processes of neurite outgrowth. Neuroblastoma N1E-115 cells express multiple G-protein coupled receptors and these cells show Gα12/Gα13 mediated activation of RhoA with subsequent growth cone collapse and neurite retraction in response to lysophosphatidic acid (LPA) and thrombin (Kranenburg et al., 1999; Tigyi et al., 1996). Given that the 5-$HT_{7(a)}$ receptor couples to Gα12 subunits, we used N1E-115 cells as a model to analyse the role of the 5-$HT_{7(a)}$ receptor in the regulation of neuronal morphology. Because N1E-115 cells also express endogenous 5-HT_3 receptor, which is a cation channel, we initially determined if a specific agonist of a 5-

$HT_{7(a)}$ receptor, 8-OH-DPAT, could induce morphological changes in the cells. The N1E-115 cells acquire a flattened morphology and begin to extend neurites after serum removal. To induce cell rounding, LPA treatment of the cells transfected with pTracer plasmid (Invitrogen) was used as a positive control. Data showed that in non-stimulated N1E-115 cells, approximately 70% of the cells were flattened, 20% were rounded and another 10% displayed neurite outgrowth. Stimulation of the cells with LPA induced pronounced neurite retraction – the amount of neurite-bearing cells decreased from 10 to 3%. Also LPA induced rounding of the ~ 80% of cells. Stimulation of the cells transfected with the control plasmid with 8-OH-DPAT did not cause any changes in the cell morphology (Fig. 3.13)

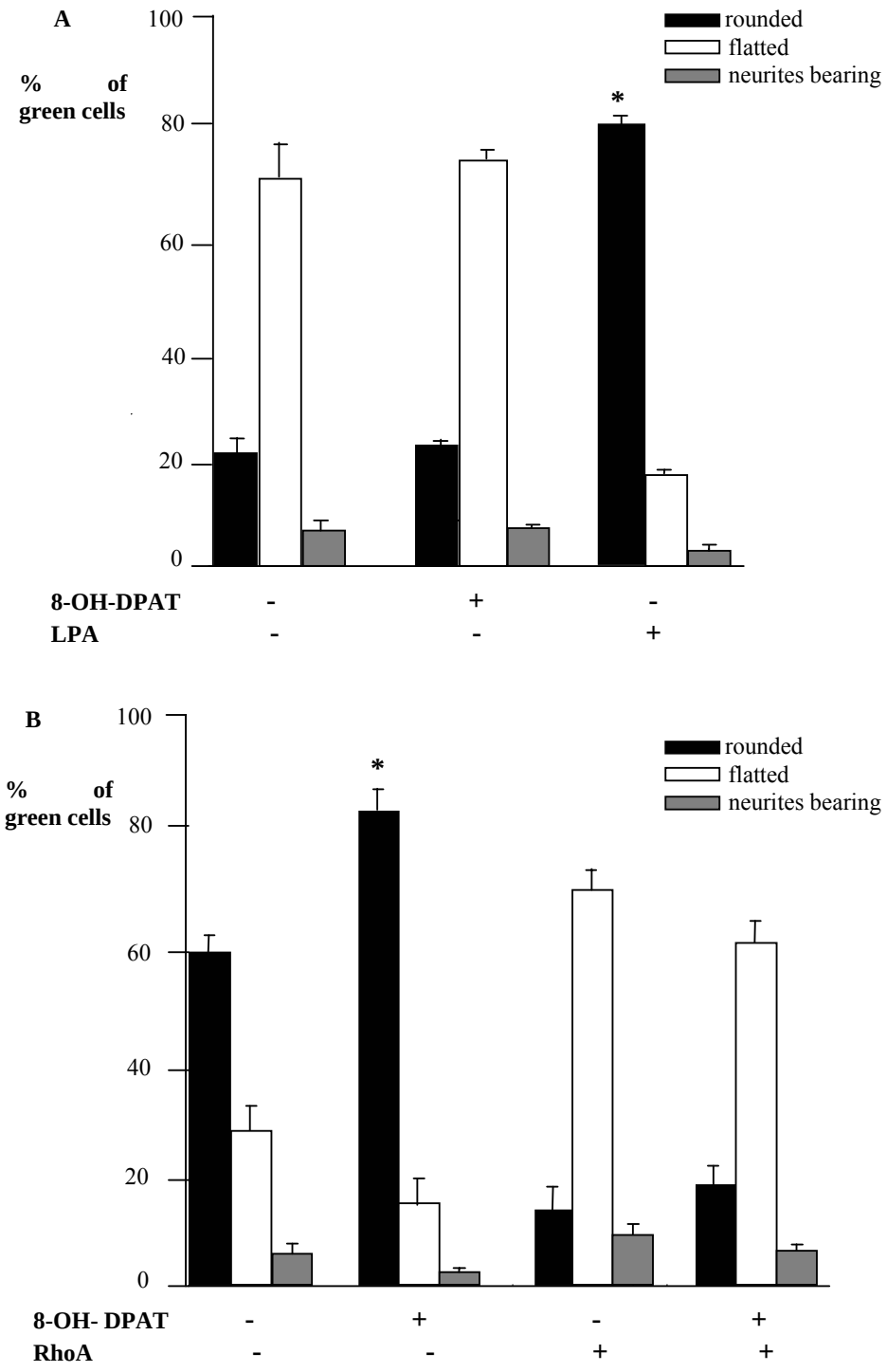


Figure 3. 13. Morphological analysis of the transfected neuroblastoma cells (N1E-115).
A. N1E-115 cells were transfected with pTracer vector and then treated either with 8-OH-DPAT or with LPA. Data show percentage of different cell forms. Each data represents the mean ± S.E. from three independent experiments. A statistically significant increase obtained for the number of rounded cells after stimulation with LPA in compare with round-formed cells amount for basal level is noted (*, $p <$ 0,0001).

B. N1E-115 cells were transfected with the 5-$HT_{7(a)}$-pTracer DNA alone or cotransfected with dominant-negative RhoA mutant (N 19 Rho).
Data shown represent percentage of different cell forms. Each data represents the mean ± S.E. from three independent experiments. A statistically significant increase obtained for round-formed N1E cells after stimulaion with 8-OH-DPAT in compare with round-formed cells in basal level is noted (*, $p <$ 0,0011).

To analyse the possible role of the 5-$HT_{7(a)}$ receptor in controlling neurite behaviour, N1E-115 neuroblastoma cells were transiently transfected with the 5-$HT_{7(a)}$ receptor wild-type subcloned into pTracer vector. This vector allows scoring the transfected cells only by the parallel expression of the green fluorescence protein (GFP). Sixteen hours after transfection, cells were serum-starved for an additional 24 hours and then stimulated with 8-OH-DPAT. Correct protein expression of the transfected cDNAs was verified by immunoprecipitation analysis (not shown). As shown in the Fig. 3.13 B, expression of the 5-$HT_{7(a)}$ receptor induced significant changes in the cell shape even without agonist stimulation: 20$\pm$2.7% of rounded cells after transfection with pTracer and 60$\pm$3.4% after transfection with the 5-$HT_{7(a)}$ receptor wt, suggesting that the native 5-$HT_{7(a)}$ receptor expressed in these cells possesses a high G12-mediated basal constitutive activity. Stimulation of the cells expressing wild type 5-$HT_{7(a)}$ receptor with 8-OH-DPAT increased the amount of rounded cells to 80.5$\pm$3% (Fig. 3.13 B).

To assess whether RhoA is required for the contractility in neuronal cells induced by the 5-$HT_{7(a)}$-dependent activation of Gα12 we co-transfected dominant-negative RhoA (N19) together with the wild-type 5-$HT_{7(a)}$ receptor. As shown in insert of Fig. 3.13 N19 RhoA significantly reduced both constitutive as well as 8-OH-DPAT-promoted cell rounding, suggesting that the 5-$HT_{7(a)}$ receptor operated via Rho GTPases to induce cytoskeletal contraction.

3.5.2. The 5-$HT_{7(a)}$ receptor and morphological changes: NIH3T3 cells

Biochemical measurements (e.g. pull down assay and gene reporter assay) performed in NIH3T3 cells demonstrated that the 5-$HT_{7(a)}$ receptor can activate another member of Rho GTPases family - Cdc42 in addition to RhoA (data not shown). NIH3T3 cells are known to express Cdc42 in high amount and are therefore often to analyse the Cdc42 activity, which is reflected through the filopodia and lamellopodia formation (Kozma et al., 1995). To analyse the cell shape changes induced by the 5-$HT_{7(a)}$ receptor in real-time, we have constructed GFP-tagged 5-$HT_{7(a)}$ receptor. The functional activity of the GFP-tagged receptors was confirmed using SRE assay. GFP-5-$HT_{7(a)}$ stimulated SRE to the extent similar to their wild type counterparts (data not shown), suggesting that GFP-tagged receptors were functionally active. NIH3T3 cells transfected with the 5-$HT_{7(a)}$ receptor were stimulated with 10 μM 5-HT and observed under the microscope (LSM 510-Meta, Zeiss) for 60 min. In the cells expressing the 5-$HT_{7(a)}$ receptor, serotonin generated the formation of the elongated filopodia in a time-dependent manner (Fig. 3.14).

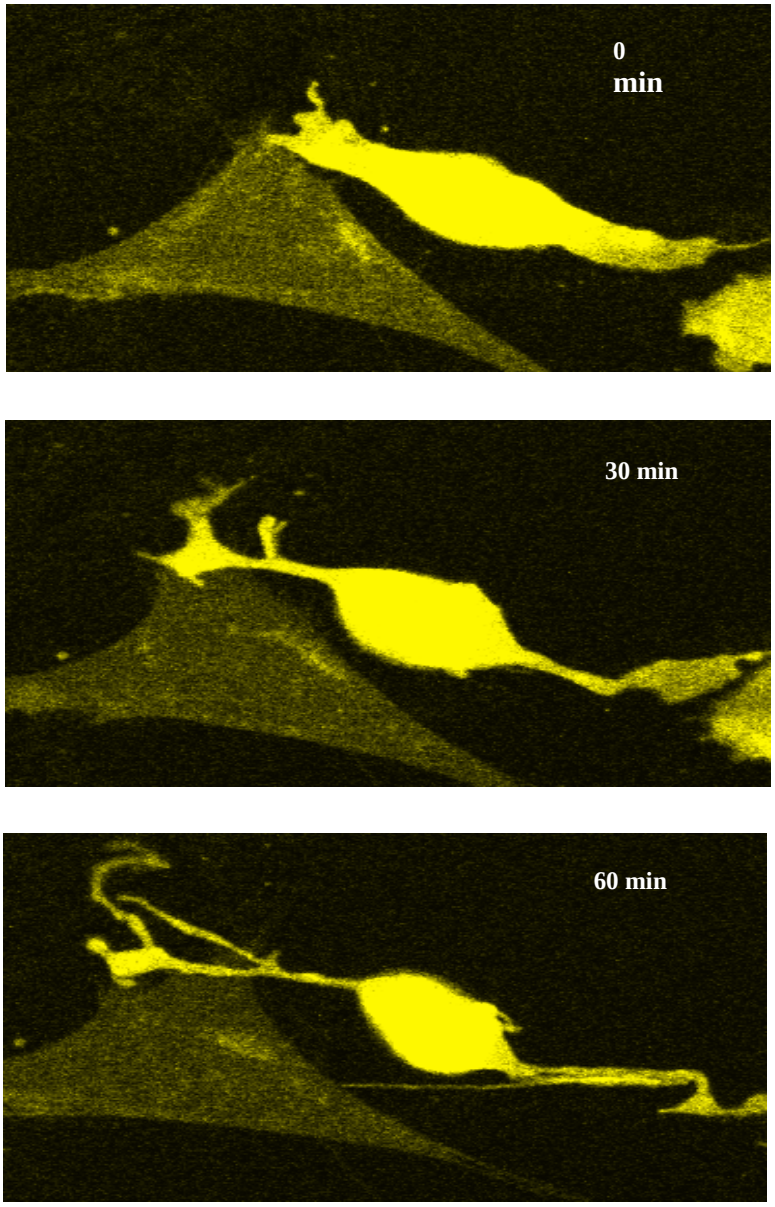


Figure 3. 14. 5-HT stimulated filopodia outgrowth. NIH3T3 cells transfected with pTracer plasmid encoding for the 5-$HT_{7(a)}$ receptor were treated with 10 μM 5-HT and monitored under confocal microscopy during 60 min.

To analyse the role of the 5-$HT_{7(a)}$ receptor in actin reorganisation, NIH3T3 cells transfected with YFP-tagged 5-$HT_{7(a)}$ receptor were stained with FITC-phalloidin to determine the actin organisation. As shown in Fig. 3.15 stimulation of the cells with 10 μM serotonin for 30-60 min resulted in reorganisation of the actin cytoskeleton and in the formation of filopodia-like protrusions in the receptor-expressing cells. In the cells that did

not express the 5-HT$_{7(a)}$ receptor serotonin did not induce changes in the actin cytoskeleton, suggesting that the observed changes were dependent on the 5-HT$_{7(a)}$ receptor.

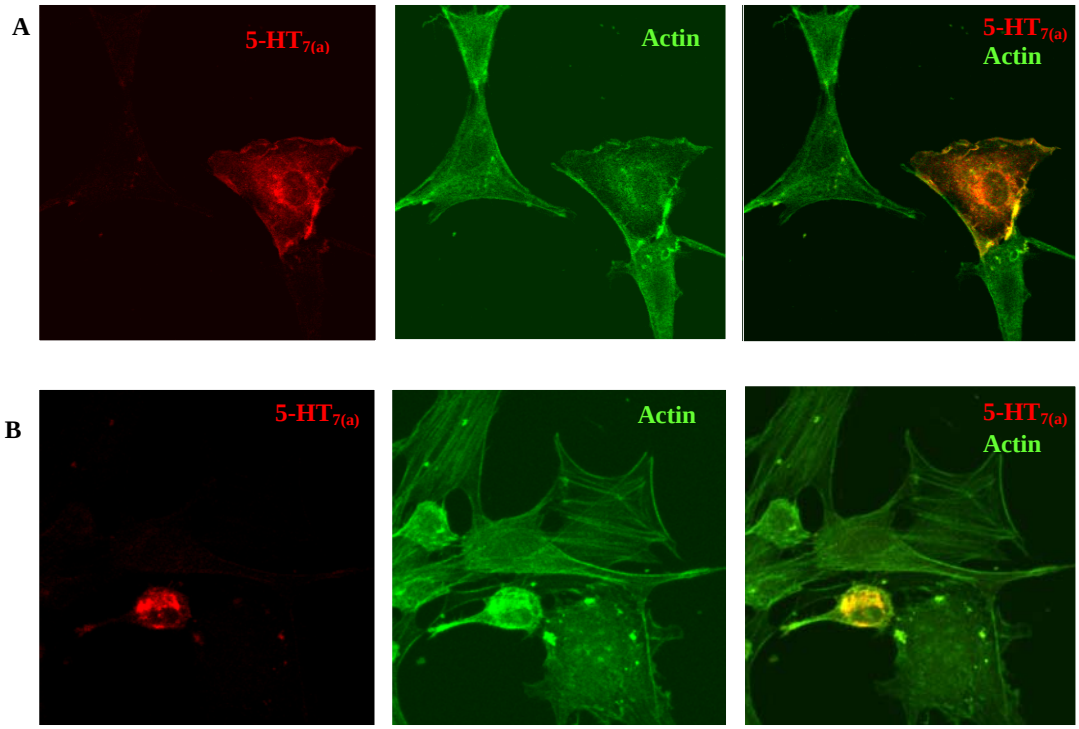


Figure 3. 15. The 5-HT$_{7(a)}$ receptor induced reorganisation in the cytoskeleton.
A. F-actin organisation in cells expressed the 5-HT$_{7(a)}$ receptor. NIH3T3 cells were transfected with pEYFP-5HT$_{7(a)}$ plasmid DNA and stained after fixation with anti-phalloidin immunoglobuline conjugated with FITC.
B. F-actin reorganiastion after treatment with 5-HT$_{7(a)}$ agonist. NIH3T3 cells expressing pEYFP-5-HT$_{7(a)}$ were treated for 60 min with 100 μM 8-OH-DPAT, fixed and stained with FITC conjugated phalloidin antibodies.

3.5.3. Distribution of the 5-HT$_{7(a)}$ receptor and Gα12 protein in hippocampal neurons

With finding that the 5-HT$_{7(a)}$ receptor binds to Gα12 subunit, we next analysed distribution both of the receptor and G-protein in neuronal cells. Cultures of hippocampal neurons were prepared from 1-3 days old C57BL/6J mice. Thereafter, neurons were transfected with GFP-5-HT$_{7(a)}$ receptor with transfection efficiency of 1-2%. Twenty-four hours after transfection, neurons were fixed, permeabilised, and endogenous Gα12 was detected by staining with appropriate antibodies. Confocal microscopy of the neuronal cultures showed that GFP-tagged receptor and endogenous Gα 12 subunits are located both in the cell body and neurite (Fig. 3.16). We have detected a high degree of co-localisation between Gα12 and the 5-HT$_{7(a)}$ receptor, which is corroborated our previous results demonstrating that the 5-HT$_{7(a)}$ receptor is coupled to Gα12.

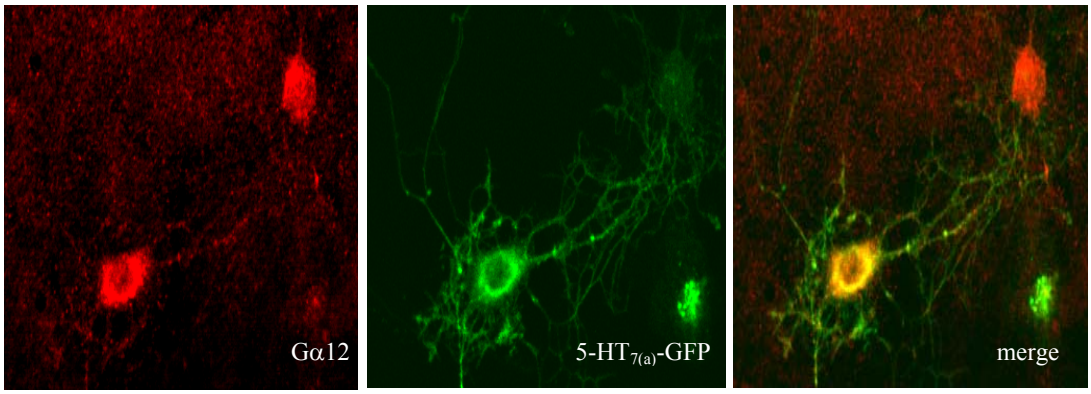


Figure 3. 16. Colocolisation of the 5-HT$_{7(a)}$ receptor and Gα_{12} subunit in neurons. Neuronal cells from the hippocampus of mice were transfected with pcDNA 3.1(-) plasmid encoding for the 5-HT$_7$-GFP protein. 36 h post-transfection cells were fixed with paraformaldehide and stained with Gα12 antibodies. Gα_{12} staining was visualised by exposure to Alexa fluor 546 antibodies. Localisation both proteins was analysed by confocal microscopy.

4. DISCUSSION

4.1. Expression and palmitoylation of the 5-$HT_{7(a)}$ receptor

The 5-$HT_{7(a)}$ receptor belongs to the family of the G-protein coupled receptors (GPCRs). Structurally, GPCR possess seven transmembrane domains linked by alternating intracellular (*i*1-*i*3) and extracellular (*e*1-*e*4) loops. The extracellular receptor surface, including the N-terminus, is known to be critically involved in ligand binding. The intracellular receptor surface, including C-terminal domain and intracellular loops (in particular *i*2 and *i*3), is known to be important for G-protein recognition and activation. The GPCRs often undergo to the different post-translational modifications, like glycosylation and phosphorylation. Functionally, such modifications have been linked to regulatory processes, such as ligand binding desensitisation and internalisation (Ferguson et al., 1998; Tsao and von Zastrow, 2001). In addition, many signalling proteins are subjected to the lipid modifications, such as prenylation, myristoilation or palmitoylation. Among various modifications with fatty acids, a covalent attachment of the 16-C fatty acid palmitate is more functional interesting because it is reversible and may be regulated.

In this study we present evidence that the 5-$HT_{7(a)}$ receptor expressed by the baculovirus system is covalently modified by palmitic acid. The fact that the label is sensitive to reducing agents, as shown by treatment with β-mercaptoethanol and hydroxylamine (Fig. 3.4), indicates that fatty acids are attached through a thioester linkage to the free sulfhydryl group on cysteine residue(s).

Palmitoylation of G- protein-coupled receptors (GPCRs) seems to be a general feature of these signaling molecules; approximately 80% of all known receptors contain the potentially palmitoylable cysteine residue(s) downstream of their seventh transmebrane domain (Probst et al., 1992). However, regulation of receptor palmitoylation in response to receptor stimulation has been sporadically addressed but not yet been critically reviewed. The results of experiments presented here demonstrate that palmitoylation of the 5-$HT_{7(a)}$ receptor is a dynamic process which can be modulated by agonists. Results of incorporation experiments demonstrate that the increase in palmitoylation most likely does not result solely from acylation of newly synthesized receptors since the rate of agonist-promoted palmitoylation was approximately 3 times faster than with untreated controls (Fig. 3.5 and 3.7). More important is the observation that this increase in palmitoylation is paralleled by a slight decrease in the rate receptor synthesis. These effects of agonist

treatment are receptor-specific and did not result from general metabolic effects, since 5-HT neither influenced total protein synthesis nor did palmitate incorporation at any of the labeling conditions applied. Although the detailed mechanism involved in the regulation of palmitoylation/depalmitoylation cycles of the 5-$HT_{7(a)}$ receptor is still unknown, our results indicate that the exchange of non-labeled and [^{3}H]-labeled palmitate is accelerated upon agonist treatment. These observations do not preclude a simultaneous change in stoichometry, but taken together with the results obtained from the cycloheximide experiments, the above data strongly suggest that the biological activation of the 5-$HT_{7(a)}$ receptor enhances the palmitate exchange on this polypeptide.

Palmitate turnover implies that at any given point palmitoylated and non-palmitoylated forms of the 5-$HT_{7(a)}$ receptor are presented in the cell and are available for repeated cycles of palmitoylation/depalmitoylation.

The regulated turnover of protein palmitoylation was first reported for the peripheral membrane protein p21-ras (Magee et al., 1987). Similar results have been reported for other proteins involved in signal transduction, including α-subunits of heterotrimeric G- protein [reviewed in (Mumby, 1997)] and eNOS (Liu et al., 1995). For example, stimulation of palmitate incorporation has been observed for Gαq, Gαs, Gαi and Gα0 upon activation through serotonin receptors (Chen and Manning, 2000; Gurdal et al., 1997). Stimulation of the β_2-adrenergic receptor also results in an increased turnover of palmitate on Gαs (Mumby et al., 1994; Wedegaertner and Bourne, 1994). Although quite common, regulated palmitate turnover does not seem to be a general feature, since palmitoylation of Gαz and Gα12 is not affected by stimulation D2 dopamine and PAR1 thrombin receptors, respectively (Morales et al., 1998; Ponimaskin et al., 1998). The dynamic nature of palmitoylation obtained here for the 5-$HT_{7(a)}$ receptor parallels previous observations on increased turnover of palmitate in β_2-adrenergic (β_2AR) and dopamine D1 receptors upon receptor stimulation (Loisel et al., 1996; Moffett et al., 1993; Ng et al., 1994a). In addition, it has been reported for the endothelin A receptor and for the A1 adenosine receptor that palmitoylation may also be involved in coupling with G- protein and receptor proteolysis, respectively (Gao et al., 1999; Okamoto et al., 1997).

In order to gain better insights into the molecular mechanics of the 5-$HT_{7(a)}$ signaling and to search for the specific biological functions of dynamic palmitoylation of the 5-$HT_{7(a)}$ receptor, the generation and functional analysis of its acylation-deficient mutants was chosen as a specific tool.

4.2. Potential sites of the 5-$HT_{7(a)}$ receptor palmitoylation

From analysis of the primary structure of acylated GPCRs, it is known that palmitoylation occurs exclusively on cysteine residues located at the C-terminal juxta-membrane portion of the receptors (Ponimaskin and Schmidt, 1998; Morello and Bouvier, 1996). The 5-$HT_{7(a)}$ receptor possesses three cysteine residues within its C-terminal cytoplasmic domain, Cys^{404}, Cys^{438} and Cys^{441}. Of the three cysteine residues at the C-terminal cytoplasmic domain of the receptor, Cys^{404} is highly conserved among GPCRs and also correspond to the site that has been shown to be palmitoylated. However, a substitution of this cysteine residue with serine does not completely abolished palmitoylation. Replacement of additional Cys^{438} alone or in combination with $Cys^{438-441}$ also did not significantly affected the relative palmitoylation efficiency of the resulting mutants as compared with the wild-type. To our surprise, simultaneous substitution of all cysteine residues ($Cys^{404-438-441}$) within the C-terminal domain still results in detectable [^{3}H]-palmitic acid incorporation, demonstrating that palmitoylation of the 5-$HT_{7(a)}$ receptor is not restricted to this locus. This finding is at odds with the observations that palmitoylation sites of rhodopsin and β_2-adrenergic, α_{2A}-adrenergic, luteinizing hormone/human choriogonadotropin, endothelin A, thyrotropin and 5-$HT_{4(a)}$ receptors (Mouillac et al., 1992; Kennedy and Limbird, 1993; Tanaka et al., 1998, Ponimaskin et al., 2002) are cysteine residues in the proximal portion of the C-terminal domain. However, our results are in accordance with recent findings on $serotonin_{1B}$ and $serotonin_{1D}$ receptors by O'Dowd et al. The $serotonin_{1D}$ receptor was shown to be palmitoylated despite of its lack of cysteine residues in the C-terminal domain. The $serotonin_{1B}$ receptor was palmitoylated; however, a mutant in which the only cysteine residue in the C-terminal domain was substituted with alanine was still palmitoylated. In addition, the μ-opioid receptor (MOR) has been also shown to be palmitoylated not only via the cysteines in the C-terminal tail domain and where this palmitate is located in the MOR has not been identified as yet (Chen, Schahabi et al., 1998).

4.3. Functional role of the 5-$HT_{7(a)}$ receptor palmitoylation

Functional analysis of mutant GPCRs lacking the acylation site have failed to reveal a common functional role for receptor palmitoylation. Moreover, mutagenesis of the palmitoylated cysteine residues is often associated with different, sometimes opposite effects on the functional receptor activities. Therefore, by using acylation-deficient mutants

of the 5-$HT_{7(a)}$ receptor we analysed the possible role of dynamic palmitoylation in different receptor properties, including coupling with heterotrimeric G-proteins, ligand-binding efficiency, modulation of downstream signal cascades as well as modulation of basal constitutive receptor activity. Evaluation of agonist-promoted binding of [^{35}S]GTPγS with G-proteins belonging to different families as well as agonist-promoted cAMP production revealed that the recombinant 5-$HT_{7(a)}$ receptor communicates with $G\alpha_s$ but not with $G\alpha_i$ (Fig. 3.10. A). This observation is in line with the current notion that native as well as heterologously expressed 5-$HT_{7(a)}$ receptors couple positively to adenylate cyclase catalysing cAMP production (Adham et al., 1998; Heidmann et al., 1998). Analysis of acylation-deficient 5-$HT_{7(a)}$ mutants revealed that C-terminal mutants are indistinguishable from the wild-type in their ability to interact with Gs (Fig. 3.10 B) and to stimulate adenylyl cyclase activity (Fig. 3.11) after agonist stimulation. The lack of any effect of palmitoylation on the coupling of the 5-$HT_{7(a)}$ receptor with G-proteins and on the downstream effectors, parallels recent reports on palmitoylation of the 5-$HT_{4(a)}$ receptor. The 5–$HT_{4(a)}$ receptor couples to both Gs as well as to G13 (Ponimaskin et al., 2002) and mutations of cysteine residues 328, 329 and 386, resulting in non-palmitoylated receptor, have no effect on coupling with either classes of G-proteins after agonist stimulation (Ponimaskin et al., 2002). This differs from the features reported for rhodopsin, β2-adrenergic (β2AR) as well as for endothelin types A and B (ET_A and ET_B) receptors. Recent works on rhodopsin indicate that chemical depalmitoylation enhances light-dependent GTPase activity of G_t and strongly decreases the light-independent activity of opsin-*atr* (Pepperberg et al., 1995; Sachs et al., 2000). Similary, functional characterisation of non-palmitoylated β2AR and the ET_B receptor revealed that palmitoylation is essential for agonist-stimulated coupling to Gs and to both Gq- and Gi-proteins, respectively (Morello and Bouvier, 1996; O'Dowd et al., 1989). These opposing findings suggest that palmitoylated cysteine residues may play differing roles at different receptor-G-protein interfaces. This may be due to the different subunit composition of Gs, Gq, Gt and Gi/o or, alternatively, to the differing receptor structures presented to the appropriate G-proteins.

The most distinct finding of the present study was the observation that C-terminal palmitoylation modulates the agonist-independent constitutive 5-$HT_{7(a)}$ receptor activity. Spontaneous or constitutive GPCR activity has been convincingly described for 10 years in the pioneering work of Costa and Lefkowitz (Costa and Herz, 1989; Cotecchia et al., 1990). Now it is well established that GPCRs can reach their active state even in the absence of agonist, as a result of a natural shift in the equilibrium between their inactive

and active conformations (Lee et al., 2000). The capacity of a native receptor to spontaneously isomerize from an inactive form (R) to an active form (R*) is certainly a very important pharmacological characteristic because this may explain part of its physiological and possible pathological behavior, as well as the effect of drugs classified as inverse agonists (able to reverse the isomerization because of their higher affinity for R than for R*) on the receptor. Although the agonist-independent signaling has been observed for a wide variety of GPCRs (in particular after their overexpression in heterologous cells), molecular constrains involved in the regulation of receptor constitutive activity remain poorly understood. There are some data to indicate that specific sequences within the third intracellular loop *i*3, as well as composition of the C-terminal tail of GPCRs, may be essential for the isomerization of receptors from the R to the R* form (Leurs et al., 1998).

What could be a possible scenario in which the C-terminal palmitoylation of the 5-$HT_{7(a)}$ receptor modulate its constitutive activity? It has been proposed that palmitoylation of GPCRs may provide a lipophilic membrane anchor to create an additional fourth intracellular loop in the carboxyl-terminal region of the receptor (Ross, 1995; Bouvier et al., 1995). More recently, direct evidence for this has been obtained for rhodopsin (Moench et al., 1994; Palczewski et al., 2000). Therefore, C-terminal palmitoylation of the 5-$HT_{7(a)}$ receptor may result in the formation of the additional intracellular loop (Fig. 4.1).
Functionally, this conformation could be critical for determining the basal level of agonist-independent receptor activation. When cysteine residue 404 becomes depalmitoylated (by basal or agonist-promoted palmitate-turnover), this loop could be destroyed and, from a functional point of view, such a change in the conformation could lead to the significant increase of the receptor's constitutive activity by shifting R-->R* equilibrium towards the R* state.

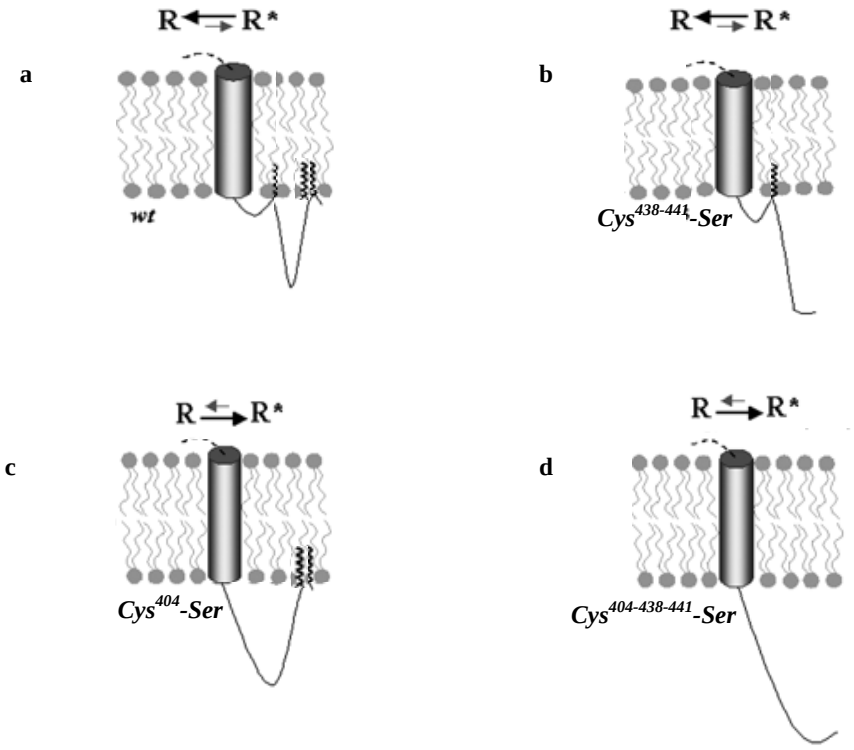


Figure 4. 1. Proposed mechanism for the modulation of the 5-HT$_{7(a)}$ receptor constitutive activity by palmitoylation. The seventh transmembrane domain as well as carboxyl-terminal cytoplasmic tail of the 5-HT$_{7(a)}$ receptor are schematically shown. Depending on the number of cysteine residues modified, palmitoylation could result in the formation of two (*a*), one small (*b*), one large (*c*), or no (*d*) intracellular loops. In the present model, every conformation could be changed to one of the remaining three forms by basal or agonist-promoted palmitate turnover.

4.4. The 5-HT$_{7(a)}$ receptor-mediated activation of Gα12 subunit of heterotrimeric G-protein

It is widely accepted that native as well as heterologously expressed 5-HT$_7$ receptors couple positively to adenylyl cyclase catalysing cAMP production. By reconstitution of the 5-HT$_{7(a)}$ receptor and different G-proteins in Sf.9 cells we have also directly demonstrated that the recombinant 5-HT$_{7(a)}$ receptor communicates with Gα$_s$ but not with Gαi or Gα13 subunits (Fig. 3.10 A). In addition, in the present study we have shown for the first time that the serotonin 5-HT$_{7(a)}$ receptor is also coupled both biochemically and functionally to Gα12 subunit of heterotrimeric G- protein followed by parallel activation of two small GTPases, Cdc42 and RhoA.

A precise pattern of neuronal connections is essential for the function of the adult nervous system. During embryonic development, neuronal growth cones navigate along specific pathways, delineated by multiple molecular guidance cues, to reach their appropriate distant targets. Neurite outgrowth and growth cone motility are among the

many aspects of neuronal development that can be affected by specific neurotransmitters. Serotonin (5-HT) is one of neurotransmitters, which may affect the neurite outgrowth besides its well-established role in neuronal communication. However, the effect of 5-HT varied substantially among the cell types and systems that were analysed. For instance, addition of 5-HT to the growing neurite from the snail (*Helisoma*) neurons caused an abrupt cessation of their elongation (Goldberg et al., 1991; Haydon et al., 1984). Depletion of 5-HT in the snail *Achatina fulica* resulted in axonal sprouting of buccal ganglion neurons (Baker et al., 1993). Other experiments have demonstrated that application of 5-HT induces growth cone collapse in chick dorsal root ganglion as well as in cerebral giant cells of *Lumnaea stagnalis* (Igarashi et al., 1995; Koert et al., 2001). In addition, 5-HT inhibited neurite outgrowth from retinal neurons of goldfish (Lima and Schmeer, 1994). While all of the above data are consistent with the assumption that 5-HT acts to decrease or arrest neuritic outgrowth, several other experiments have provided opposite results. For example, in the sphinx moth exposure to 5-HT enhances neurite growth from antennal lobe neurons (Mercer et al., 1996). Likewise, in the rat brain application of 5-HT increased the dendritic differentiation of calretenin positive neurons in the cerebral cortex (Liu and Lauder, 1991) and promoted the neurite outgrowth from thalamic neurons (Lieske et al., 1999).

The molecular mechanisms underlying the inhibitory as well as the growth-promoting effects of 5-HT on neurite outgrowth are poorly understood. It has been reported that activation of the Rho family of small GTPases (RhoA, Rac1 and Cdc42) through a G12/13-initiated pathway may modulate various pathways that affect cell locomotion through dynamic regulation of the actin cytoskeleton. Thus, RhoA stimulates the formation of stress fibres (Ridley and Hall, 1992), Rac reorganizes actin into lamellipodia (Ridley et al., 1992) and Cdc42 induces the formation of filopodia (Nobes and Hall, 1995). Rho GTPases are broadly expressed in multiple neural tissues (Olenik et al., 1999; Yamamoto et al., 1988) and initial studies showed that Rac1 is essential for lamellipodia formation (Luo et al., 1994), axon outgrowth, and dendritic spine formation (Luo et al., 1996). Expression of dominant negative forms of Rac1 and Cdc42 during *Drosophila* neural development inhibits neurite formation, whereas expression of their constitutively active forms promotes neurite outgrowth (Threadgill et al., 1997). More recent findings also support the involvement of Rac and Cdc42 in growth cone protrusion (Lee et al., 2000). Conversely, Rho-induced stress fibre formation leads to growth cone collapse and neurite retraction, presumably by sequestering actin for incorporation into

stress fibres that would otherwise be available for lamellipodia and filopodia formation (Lee et al., 2000; Li et al., 2000; Sebok et al., 1999).

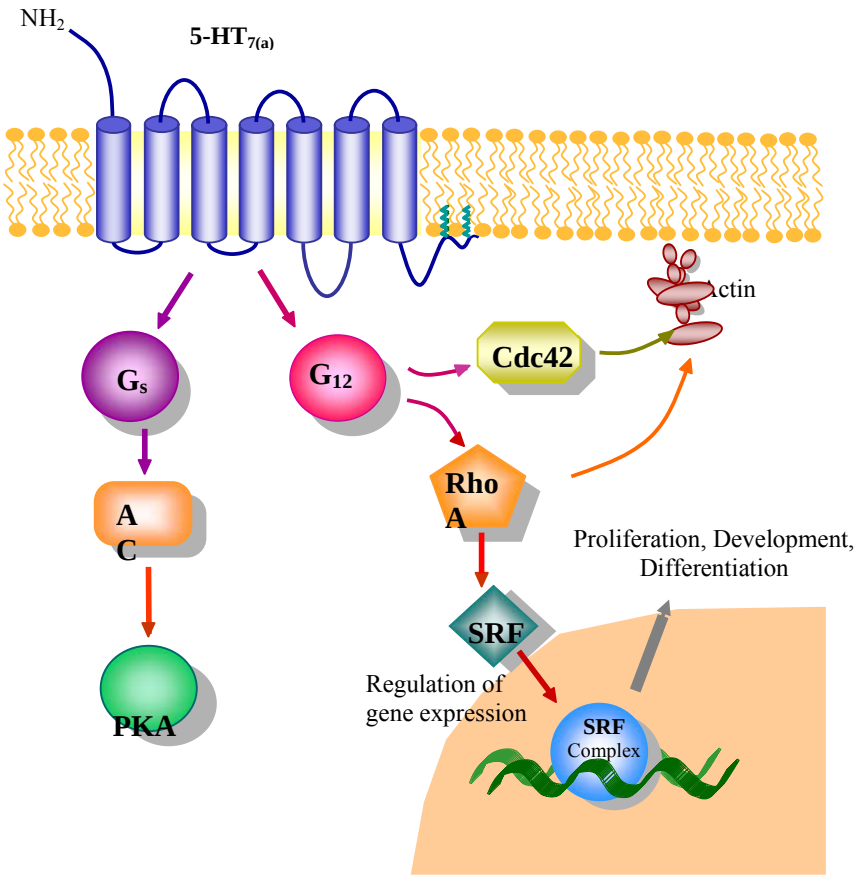


Figure 4. 2 Activation different signalling pathways through the 5-HT$_{7(a)}$ receptor.

The present data showing that the 5-HT$_{7(a)}$ receptor directly activates Gα12 leading to the activation RhoA as well as Cdc42 proteins and could therefore provide the molecular basis for serotonin-induced RhoA-dependent cell rounding as well as for Cdc42-mediated stimulation of neurite outgrowth. In this way, Gα12-mediated signalling by 5-HT may play an important role in the development and plasticity functions of the nervous system.

5. CONCLUSIONS

The mouse 5-hydroxytriptamine 7(a) (5-$HT_{7(a)}$) receptor is one of the G-protein coupled serotonin receptors (GPCRs) which participates in various physiological functions including circadian rhythm, sensory processing and limbic processes regulation. Moreover, a possible involvement of the 5-$HT_{7(a)}$ receptor in migraine and in the modulation of sympathetic afferent pathways under the clinical relevance of this receptor.

The present study demonstrates that the 5-$HT_{7(a)}$ receptor is post-translationaly modified with palmitate in reversible and agonist-dependent manner. By site-directed mutagenesis we found that palmitic acids are attached not only to C-terminal cysteine residues, representing most common sites of palmitoylation, but also on the cysteine located in intracellular loop. Functional analysis revealed that palmitoylation is critically involved in regulation of the receptor constitutive activity by affecting both ligand binding and interaction with G- proteins.

In addition, by analysis of signalling pathways we have found that the 5-$HT_{7(a)}$ receptor interacts not only with heterotrimeric Gs- but also with G12- protein. By activation of G12-mediated signalling, the 5-$HT_{7(a)}$ receptor activates two members of the Rho family of small GTPases RhoA and Cdc42. We also demonstrate that agonist-induced activation of the 5-$HT_{7(a)}$ receptor resulted both in RhoA-dependent neurite retraction and cell rounding as well as in Cdc42-mediated filopodia outgrowth. In this way, Gα12-mediated signalling by the 5-$HT_{7(a)}$ receptor may play important role in the control of neuronal architecture and neuronal plasticity.

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