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LIPID RAFTS AND G PROTEIN-COUPLED MONOAMINE RECEPTORS

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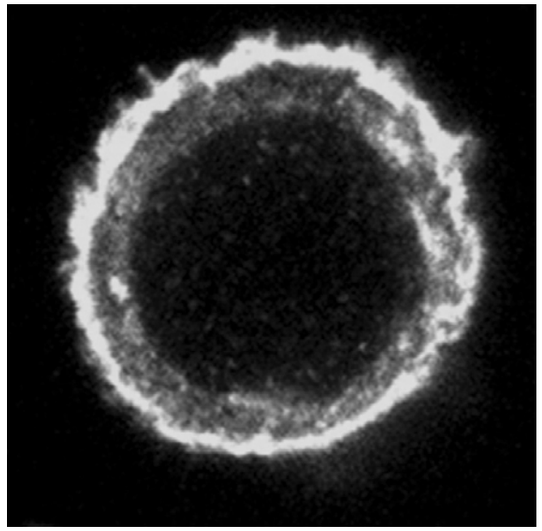
– A PHARMACOLOGICAL APPROACH



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Picture on cover: Immunostaining of a HeLa cell stably expressing 5-HT_7 receptors.

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For my grandmother, who
was always proud of me.

"To develop a complete mind: study the science of art; study the art of science. Learn how to see. Realize that everything connects to everything else."

Leonardo da Vinci

"Professionals built the Titanic - an amateur built the Ark."

Anon

ABSTRACT

The present work focused on lipid raft-mediated modulation of signaling and trafficking of serotonin (5-HT) and dopamine receptors. The 5-HT system is one of the most complex neurotransmitter systems, with a wide distribution both in the CNS and in the periphery. It is involved in the regulation of many biological functions as diverse as mood, metabolism and cardiovascular tone. 5-HT acts via at least 14 receptors, divided into seven subgroups according to sequence homology and mode of signal transduction. All of them are G protein-coupled receptors (GPCRs), except for the 5-HT_3 receptor, which is a ligand-gated ion channel.

The dopamine system is involved in the motor, cognitive and reward systems of the brain. Dopamine acts via five receptors, all GPCRs, divided into D_1-like and D_2-like receptors according to their mode of signal transduction. Dysregulation of the dopamine system is the underlying cause of addiction, schizophrenia and Parkinson's disease (PD).

The Singer-Nicholson fluidic mosaic model has for many years been the general model for the structure of biological membranes. In recent years this view has been revised with the discovery of liquid ordered microdomains, lipid rafts, enriched in cholesterol and sphingolipids. These microdomains play important roles in regulating signal transduction and trafficking events. A subgroup of lipid rafts, caveolae, is flask-shaped invaginations of the plasma membrane. They contain caveolin (Cav) proteins that can interact with other proteins, including GPCRs. Recently it was discovered that lipid rafts and caveolae participate in endocytosis and receptor trafficking. Although the exact mechanism for these processes is not yet fully understood, it seems clear that there can be alternative routes of receptor internalization than the classical endosomatic pathway via clathrin coated vesicles.

In the present work we found that decreases of cholesterol, gangliosides, sphingomyelin or Cav-1 (by RNA interference) levels, attenuated maximum agonist and antagonist binding to 5-HT_7 receptors. This could not be explained by a mere reduction in total receptor protein levels. Furthermore, signaling via 5-HT_7 receptors was attenuated by cholesterol depletion with HMG-CoA reductase inhibitors, statins, which are widely used in the treatment of atherosclerosis.

Reduction of cholesterol levels also attenuated signaling via 5-HT_{1A} receptors in primary neuronal cultures. Furthermore, we could detect dopamine D_1 receptors in low density regions in a sucrose gradient, suggesting lipid raft localization. This was further confirmed by the finding that reduction of cholesterol levels inhibited signaling via dopamine D_1 receptors in mouse striatal slices. These results give further indications that lipid raft play important roles in regulating monoamine GPCRs *in vivo*.

We have also found that Cav-1, a key protein in caveolae, regulates cell surface expression of 5-HT_7 receptors. Biochemical and cellular assays showed that 5-HT_7 receptors are located in low density regions in a sucrose gradient together with Cav-1, indicating lipid raft localization. Furthermore, it was found that 5-HT_7 receptors undergo agonist induced internalization and that this could be inhibited by reducing Cav-1 expression with RNA interference. The internalization could also be inhibited with genistein, known to block lipid raft-mediated internalization. In contrast, hypertonic sucrose solution could not inhibit internalization. Altogether this suggests that 5-HT_7 receptors are internalized through a clathrin-independent mechanism that is dependent on Cav-1.

SVENSK SAMMANFATTNING

I detta arbete studerades hur receptorer för de monoaminerga signalsubstanserna serotonin (5-HT) och dopamin regleras via s.k. lipid rafts. Serotoninsystemet är ett av de mest komplexa neurotransmittor-system som finns beskrivet och 5-HT finns distribuerat i många områden av det centrala och perifera nervsystemet. 5-HT är involverat i regleringen av många biologiska funktioner, såsom sinnesstämningar, metabolism och kardiovaskulärt tonus. Det finns minst 14 receptorer för 5-HT, som är indelade i sju grupper, med avseende på sekvenshomologi och signaleringsegenskaper. Dessa är alla G-proteinkopplade receptorer (GPCR), med undantag för 5-HT_3 receptorn som är en jonkanal.

Dopaminsystemet är involverat i motoriska och kognitiva funktioner i hjärnan, samt en essentiell del av hjärnans belöningssystem. Det finns fem receptorer för dopamin, alla GPCRer, uppdelade i två grupper, D_1- och D_2-lika. Indelningen grundar sig liksom för 5-HT-receptorer på likhet i sekvens och signaleringsegenskaper. Felaktig dopaminreglering är den underliggande orsaken till bl.a. substans-beroende, schizofreni och Parkinsons sjukdom.

Den generella bilden av biologiska membraner har länge varit den s.k. Singer-Nicholson fluidic mosaic model (ung. halvflytande mosaik). Detta synsätt har på senare år blivit reviderat med upptäckten av mikrodomäner i cellmembraner, s.k. lipid rafts, rika på mättade fettsyror och kolesterol. Dessa domäner spelar en viktig roll i cellsignalering och kontroll av transport av proteiner till och från cellytan. En undergrupp av lipid rafts, caveoler, innehåller caveolinproteiner, som genom sin speciella struktur skapar karakteristiska flaskformade inbuktningar i cellmembranet. Caveolinproteiner kan interagera med andra proteiner, inklusive GPCRer. Nyligen upptäcktes det att lipid rafts och caveoler kan mediera internalisering och endocytos. De exakta mekanismerna för detta är fortfarande inte helt klarlagda, men det verkar alltmer troligt att det kan finnas andra mekanismer för internalisering av receptorer än den klassiska endosomala vägen, som medieras av clatrin.

I detta arbete fann vi att minskning av nivåerna av olika lipid raft komponenter, som kolesterol, sphingomyelin, gangliosider eller Cav-1, ledde till minskad maximal agonist- och antagonistbindning till 5-HT_7 receptorer. Vidare fann vi också att signalering via 5-HT_7 receptorer påverkades av behandling med HMG-CoA reduktashämmare, statiner, som används kliniskt som behandling av åderförkalkning.

Minskning av kolesterolnivåer ledde också till reducering av signalering via 5-HT_{1A} receptorer i primära humana nervceller. Vidare kunde vi detektera dopamin D_1 receptorer i fraktioner med låg densitet i en sukrosgradient, vilket antyder att dessa receptorer är lokaliserade i lipid rafts. Denna uppfattning styrktes av att sänkning av kolesterolnivåer reducerade signalering via dopamin D_1 receptorer i striatala hjärnskivor från mus. Dessa resultat styrker uppfattningen att lipid rafts spelar en viktig roll för monoaminreceptorer *in vivo*.

Vi har även funnit att Cav-1, ett nyckelprotein i formationen av caveoler, reglerar uttryck av 5-HT_7 receptorer på cellytan. 5-HT_7 receptorer är lokaliserade i fraktioner med låg densitet i en sukrosgradient tillsammans med Cav-1, vilket är en indikation på att receptorerna är lokaliserade i lipid rafts. Vidare fann vi att 5-HT_7 receptorer internaliseras vid aktivering och att denna internalisering blockeras av minskade Cav-1 nivåer. Internaliseringen kunde också blockeras med genistein, som blockerar all internalisering via lipid rafts. Däremot hade behandling med hypertonisk sukros, som blockerar clatrinmedierad internalisering, ingen effekt på internalisering av 5-HT_7 receptorer. Tillsammans talar dessa resultat för att 5-HT_7 receptorer internaliseras via en mekanism som är beroende av Cav-1, men oberoende av clatrin.

LIST OF PUBLICATIONS

I. **Sjögren, B.**, Hamblin, M.W., Svenningsson, P. (2006) Cholesterol depletion reduces serotonin binding and signaling via human 5-$HT_{7(a)}$ receptors. *Eur. J. Pharm.* 552:1-10.

II. **Sjögren, B.**, Svenningsson P. (2007) Depletion of the lipid raft constituents, sphingomyelin and ganglioside, decreases serotonin binding at human 5-$HT_{7(a)}$ receptors in HeLa cells. *Acta Phys.* 190:47-53.

III. **Sjögren, B.**, Svenningsson P. (2007) Caveolin-1 affects binding and cell surface levels of human 5-$HT_{7(a)}$ receptors. *FEBS lett.* 581:5115-1521.

IV. **Sjögren, B.**, Csöregh, L., Svenningsson, P. Cholesterol reduction attenuates 8-OH-DPAT-mediated signaling in human primary neuronal cultures. *Submitted manuscript.*

V. **Sjögren, B.**, Zhang, X., Svenningsson, P. Dopamine D1 receptor-mediated signaling in brain slices from an animal model of Parkinsonism is modulated by sequestration of cholesterol. *Submitted manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

5-HT	5-Hydroxytryptamine, serotonin
5-CT	5-Carboxamidotryptamine
5-MeOT	5-Methoxytryptamine
8-OH-DPAT	8-hydroxy-2-di-n-propylamino-tetralin
AADC	Aromatic L-amino acid decarboxylase
AC	Adenylate cyclase
BBB	Blood brain barrier
cAMP	Cyclic adenosine monophosphate
Cav-1	Caveolin-1
Cav-2	Caveolin-2
Cav-3	Caveolin-3
CLICs	Clathrin independent carriers
CNS	Central nervous system
CP	Caudate Putamen
CREB	cAMP response-element binding protein
DAG	Diacyl glycerol
DIG	detergent-insoluble glycolipid-enriched membrane
DRM	Detergent-resistant membrane
ER	Endoplasmic reticulum
GPI	Glycosyl phosphatidyl inositol
GRK	G protein-coupled receptor kinase
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
IP_3	Inositol triphosphate
K_D	Dissociation constant
L-DOPA	L-3,4-dihydroxyphenylalanine
LSD	Lysergic acid diethylamide
MβCD	Methyl-β-cyclodextrin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
PDMP	(±)-*threo*-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride
PIP_2	Phospho inositol bisphosphate

PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PNS	Peripheral nervous system
NAc	Nucleus Accumbens
RISC	RNA induced silencing complex
SB269970	(*R*)-3-(2-(2-(4-Methylpiperidin-1-yl)-ethyl)pyrrolidine-1-sulfonyl)phenol
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS poly acryl amide gel electrophoresis
SKF-81297	R-[+]-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine
SN	Substantia nigra
SSRI	Selective serotonin re-uptake inhibitor
TIFF	Triton-insoluble floating fractions
VTA	Ventral tegmental area

1 INTRODUCTION

1.1 G PROTEIN COUPLED RECEPTORS (GPCRs) – AN OVERVIEW

GPCRs comprise the largest family of proteins, with over 1,000 members. The endogenous ligands for GPCRs range from neurotransmitters and peptides to hormones and even light. This family of receptors is the target for about 50% of the pharmaceutical drugs on the market today. The general structure of GPCRs is a seven transmembrane structure, with an extracellular N-terminal and an intracellular C-terminal (Fig. 1) (Dohlman et al., 1991).

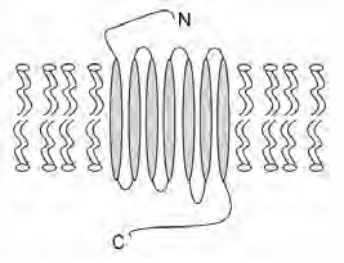


Figure 1. General structure of G protein-coupled receptors (GPCRs).

One of the difficulties when studying GPCRs is that they are hard to crystallize, due to their instability in detergent solutions. The first, and for a long time only, structure of a GPCR to be resolved was rhodopsin in the retina. However, recently the crystal structure of the β_2 adrenergic receptor was also resolved (Cherezov et al., 2007; Rasmussen et al., 2007). These receptors are both members of the rhodopsin-like, family A group of GPCRs. This family is by far the largest family of receptors and comprise of almost 200 proteins in addition to all the olfactory receptors. Two other groups, family B and C have also been identified, but these groups are much smaller. The family B group of receptors comprises of about 25 receptors and includes receptors for several hormones, such as secretin, glucagon and vasopressin. The family C receptors include the metabotropic glutamate receptors and $GABA_B$ receptors. These proteins have a very large extracellular N-teminal compared to the other families (Pierce et al., 2002).

1.1.1 Signal transduction via GPCRs

The common signal transduction pathway for GPCRs is by activating heterotrimeric G proteins. These proteins consist of one α-, one β- and one γ-subunit. The diversity in the signaling pathways via GPCRs is generated by the fact that there is, to date, 16 α-, five β- and 12 γ-subunits known, creating numerous possibilities of signaling complexes (Pierce et al., 2002). Generally the mode of signaling via a certain receptor is defined by the properties of the α-subunit in the G protein, and is described as G_s, for G proteins containing α_s, G_i for α_i and G_q for α_q-containing G proteins. The G proteins exist as

heterotrimeric entities in the non-stimulated system. Upon activation of a receptor, GDP is exchanged for GTP on the α-subunit causing it to detach from the βγ-subunits. These two proteins subsequently activate downstream signal transduction pathways.

A simplified overview of GPCR signaling pathways is presented in figure 2. Activation of $G_{\alpha s}$ leads to stimulation of adenylate cyclases (AC) and subsequent increases in intracellular cAMP levels. In contrast $G_{\alpha i}$ activation leads to inhibition of AC and reduction in the levels of cAMP. Activation of $G_{\alpha q}$ stimulates activation of phospholipase C (PLC), which eventually leads to release of Ca^{2+} into the cytosol, activating subsequent signaling cascades. In addition to the signaling effects via the α-subunits, the βγ-subunits also cause signaling events, such as activation of certain ion channels.

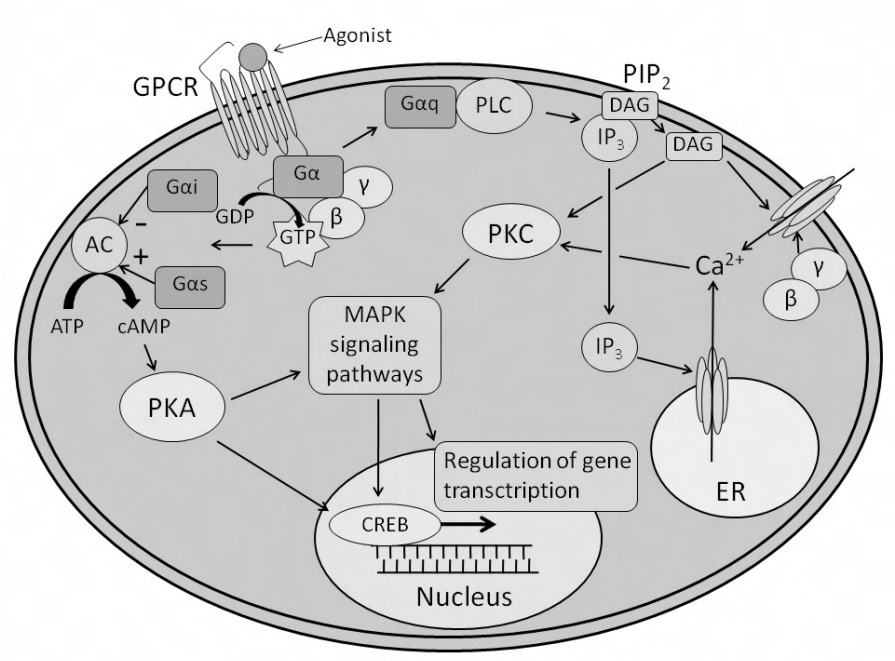


Figure 2. A simplified overview of GPCR signaling. $G_{\alpha s}$ and $G_{\alpha i}$ have opposing effects on Adenylate cyclase (AC), which produces cAMP from ATP. cAMP acts as a second messenger and activates downstream effectors, such as protein kinase (PKA), which can regulate transcription factors directly or indirectly via signaling cascades, such as MAPK pathways. $G_{\alpha q}$ activates phospholipase C (PLC), which cleaves phospho inositol bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 binds to the IP_3 receptor on the endoplasmatic reticulum (ER) and releases Ca^{2+} into the cytosol. DAG and the βγ-subunit can both activate Ca^{2+} channels in the plasma membrane, which releases Ca^{2+} from outside of the cell. Ca^{2+} (and DAG) can activate downstream effectors, such as protein kinase C (PKC), which can regulate transcription factors via signaling cascades, such as MAPK pathways.

1.1.2 Trafficking of GPCRs

Many mechanisms regulating receptor signaling have evolved in order to control the effects mediated via GPCRs. These include receptor desensitization and internalization. Certain protein kinases, such as PKA and PKC, play a role in the desensitization processes, as well as specific G protein-coupled receptor kinases (GRKs). By phosphorylating the C-terminal of a GPCR, these kinases are responsible for desensitizing the receptor and target it for internalization (Kohout and Lefkowitz, 2003). The first advances in the studies of GRK mechanisms were related to β_2-adrenergic receptors (Sibley et al., 1986). It was shown that the phosphorylation of the receptor by GRK-2 (also known as the β-adrenergic kinase 1 (βARK1)) was essential for internalization and further studies have since identified several other GRKs (Claing et al., 2002).

The next step in the internalization process is the association of β-arrestins to the phosphorylated receptor (Ferguson et al., 1996). This protein interacts with clathrin, thus targeting the receptor to internalization via clathrin-coated vesicles to endosomes, a process that is dependent on dynamin and the actin cytoskeleton. The internalized receptors are then destined for lysosomal degradation or recycling back to the membrane surface (Fig. 3) (Ferguson, 2001).

The process of desensitization and internalization of GPCRs can occur both as a negative feedback mechanism of agonist-induced homologous desensitization, or as heterologous inhibition of another receptor. The endosomatic pathway is by far the best characterized internalization mechanism. However, other routes for receptor internalization have recently emerged, for instance via uncoated vesicles or via caveolae, which will be described later (see section 1.4.3).

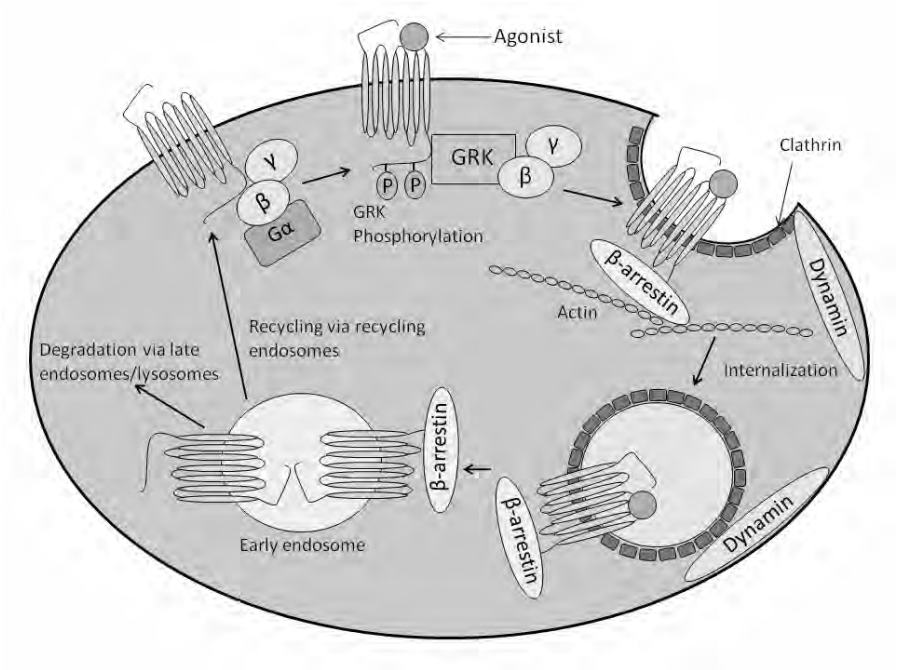


Figure 3. The model of GPCR desensitization and internalization via clathrin-coated vesicles. Agonist activation of a receptor induces signaling cascades that recruits G protein coupled receptor kinases (GRK), which phosphorylate the C-terminal tail of the receptor. This enables β-arrestin to bind the receptor and target it for internalization via clathrin coated vesicles. The receptor is transported to early endosomes where arrestin is released and the receptor can then be recycled to the plasma membrane or targeted for lysosomal degradation. Adapted from Pierce et al., 2002.

1.2 THE SEROTONIN SYSTEM

In evolutionary terms, serotonin (5-hydroxytryptamine; 5-HT) is one of the oldest neurotransmitters. It is synthesized from L-tryptophan both in the central and peripheral nervous system (CNS/PNS) and has been implicated in regulating several disease states, such as depression, anxiety and schizophrenia. It is also proposed to be involved in the pathology of migraine, eating disorders and hypertension. The serotonin system is one of the most diverse neurotransmitter systems and has been extensively characterized, although there is still much unknown about the role in the brain and in peripheral tissues.

1.2.1 Serotonin receptors

Although initially 5-HT had been long known to be an important mediator of neurotransmission, the molecular mechanisms of action remained unclear. The first reports were published in 1957, but it wasn't until 1976 that radioligand binding experiments could demonstrate the presence of binding sites specific for 5-HT. However, the nature of these binding sites was undefined until the first receptor for 5-HT, the 5-HT_{1A} receptor, was cloned in 1987. In the following years, several receptors for 5-HT, were cloned (Hoyer et al., 2002). To date 14 receptors for 5-HT have been characterized, making it one of the most complex neurotransmitter systems known. These receptors are divided into seven subclasses, according to sequence homology and modes of signaling (Barnes and Sharp, 1999; Hoyer et al., 2002). All belong to the rhodopsin-like family of GPCRs, with the exception of 5-HT_3 receptors, which are ligand-gated ion-channels. An overview of the 5-HT receptors is presented in figure 4.

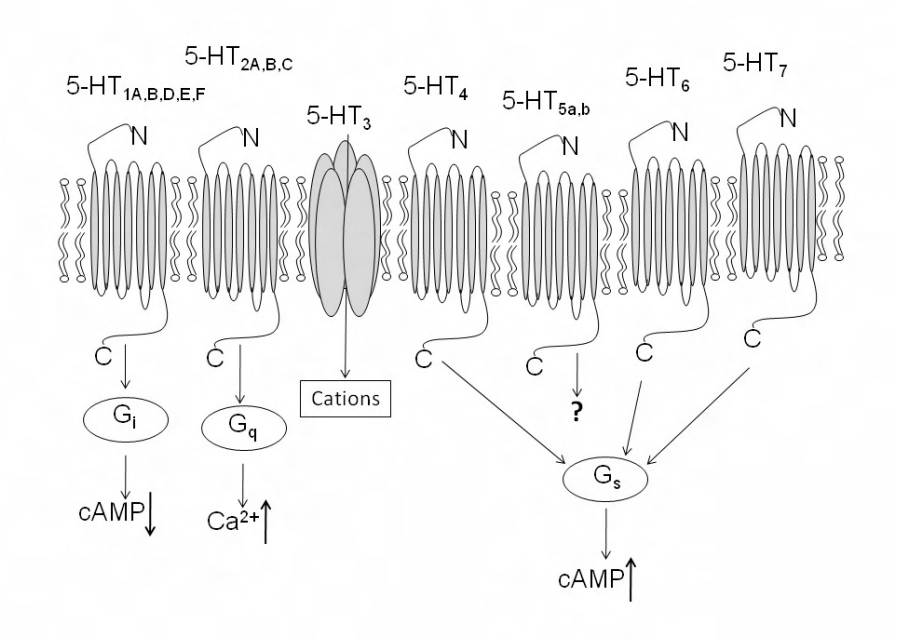


Figure 4. An overwiew of 5-HT receptors and their downstream signaling. All 5-HT receptors are GPCRs, with the exception of 5-HT_3 receptors, which are ligand-gated ion channels, permeable to cations. The five 5-HT_1 receptors are G_i coupled and inhibit the synthesis of cAMP. The 5-HT_2 receptors couple via G_q, which leads to intracellular increases in Ca^{2+}. The 5-HT_4, 5-HT_6 and 5-HT_7 are all coupled to G_s, leading to increases in cAMP. The coupling effectors for 5-HT_5 receptors still remain elusive.

1.2.1.1 5-HT$_1$ receptors

The 5-HT$_1$ receptors were the first ones to be identified and cloned, although this group has undergone some revision since its first discovery. This group now consists of five members, 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-ht$_{1E}$ and 5-ht$_{1F}$. The lower case letters for 5-ht$_{1E}$ and 5-ht$_{1F}$ indicate that the function of these receptors has yet to be demonstrated *in vivo*. All 5-HT$_1$ receptors are negatively coupled to adenylate cyclase, resulting in decreased production of cAMP (Fig. 4). The 5-HT$_{1C}$ receptor was a former member of this group, but has now been renamed 5-HT$_{2C}$, due to its higher sequence similarity and mode of signaling with the 5-HT$_2$ receptor family (Barnes and Sharp, 1999).

5-HT$_{1A}$ receptors

The first cloned receptor for 5-HT was the 5-HT$_{1A}$ receptor, and it is the most well characterized receptor for 5-HT (Albert et al., 1990). 5-HT$_{1A}$ receptors are located in many regions of the brain, both in neurons and glia, and play important roles in regulating emotional and cognitive states (Ogren et al., 2008; Pucadyil et al., 2005). In the CNS they are highly expressed at the somatodendritic level of serotonin neurons in the hippocampus and the raphe nuclei, where they act as autoreceptors to inhibit cell firing (Barnes and Sharp, 1999; Ogren et al., 2008). Postsynaptic 5-HT$_{1A}$ receptors are also abundant in many non-serotonin neurons, including hippocampus and cerebral cortex.

Many functions have been attributed to the 5-HT$_{1A}$ receptor, including regulation of feeding behavior and body temperature. Furthermore, 5-HT$_{1A}$ receptors play a role in anxiety-related disorders and it has been shown that stimulation of 5-HT$_{1A}$ receptors produces anxiolytic effects and that 5-HT$_{1A}$ receptor knockout mice display a higher degree of anxiogenic behaviors (Wood et al., 2002). It is also implicated that antagonists at 5-HT$_{1A}$ receptors could lead to faster onset of SSRI's. Since the effect of these drugs cause activation of 5-HT$_{1A}$ receptors due to increased 5-HT levels, the inhibitory effect on 5-HT release can be blocked by 5-HT$_{1A}$ receptor antagonists (Wood et al., 2002).

5-HT$_{1B}$ receptors

5-HT$_{1B}$ receptors (Hamblin et al., 1992) are distributed in regions of the basal ganglia, striatum and the frontal cortex and, like the 5-HT$_{1A}$ receptors, they are believed to function as terminal autoreceptors and heteroreceptors, regulating the release of other

neurotransmitters. In contrast to 5-HT_{1A} receptor knockouts, 5-HT_{1B} receptor knockout mice display a lower degree of anxiety and furthermore, 5-HT_{1B} receptor agonists have been shown to have an antidepressant effect (Hoyer et al., 2002).

5-HT_{1B} receptor agonists are used for the treatment of migraine. It is now believed that agonists at 5-HT_{1B} receptors, such as sumatriptan, act on receptors expressed in the smooth muscle of cerebral blood vessles, thus inducing vasoconstriction (Hamel, 1999).

5-HT_{1D} receptors

It was originally believed that the 5-HT_{1B} and 5-HT_{1D} receptor was one group and that the sequence variations observed were mere species differences. This notion was, however, later revised and these two receptors are now considered to be two subtypes of 5-HT_1 receptors with very high homology (Hoyer and Martin, 1997).

5-HT_{1D} receptors display a much lower level of expression compared to 5-HT_{1B} receptors. Similar to 5-HT_{1A} receptors, 5-HT_{1D} receptors have also been attributed the role of autoreceptors in the raphe nuclei, although this notion is yet to be confirmed experimentally (Hoyer et al., 2002; Roberts et al., 2001).

5-ht_{1E} and 5-ht_{1F} receptors

The least characterized 5-HT_1 receptors are the 5-ht_{1E} and 5-ht_{1F} receptors (Adham et al., 1993; Zgombick et al., 1992). So far no physiological roles have been attributed to either of these receptors and therefore they are assigned the lower case appellation (Hoyer and Martin, 1997).

The lack of selective radioligands has made attempts to determine the distribution of these receptors difficult. 5-ht_{1E} receptor mRNA has been detected in cortical regions and the amygdala. However, this distribution pattern might be revised if more selective radioligands become available (Barnes and Sharp, 1999). The 5-ht_{1F} receptor mRNA has been shown to be distributed in hippocampus, cortex and the dorsal raphe nucleus in the mouse and guinea pig brain (Adham et al., 1993). This receptor has been proposed to play a role in the aetiology of migraine although the exact mechanism is still unknown (Hamel, 1999).

1.2.1.2 5-HT_2 receptors

The 5-HT_2 receptors include three subtypes, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors. These are all G_q-coupled and activation of 5-HT_2 receptors results in the activation of

phospholipase C (PLC), leading to subsequent Ca^{2+} release from the ER. (Barnes and Sharp, 1999).

5-HT_{2A} receptors

The 5-HT_{2A} receptor is widely distributed, both in central and peripheral tissues, such as various smooth muscle tissues. It has been proposed that the 5-HT_{2A} receptor could be a target for the treatment of hypertension, because of its affinity for the antihypertensive drug ketanserin. However, this is debated since ketanserin also has high antagonistic affinity for the α_1 adrenergic receptor (Barnes and Sharp, 1999). In the brain the 5-HT_{2A} receptor is located in the cortex and regions of the basal ganglia. Because of its affinity for atypical antipsychotics, such as clozapine, olanzapine and risperidone, it has been implicated in the treatment of schizophrenia (Hoyer et al., 2002).

5-HT_{2B} receptors

Although initially denoted the 5-HT_{2F} receptor, the 5-HT_{2B} receptor was cloned in 1992 and later renamed to become the third and most recently discovered member of the 5-HT_2 receptor family (Kursar et al., 1992). The distribution of 5-HT_{2B} receptor mRNA and the corresponding protein is much more restricted than that of for instance 5-HT_{2A} and 5-HT_{2C} receptors and the levels of expression is much lower. The distribution in the rat brain is restricted to a few areas, namely the cerebellum, lateral septum, dorsal hypothalamus and medial amygdala (Duxon et al., 1997).

There is still much to be discovered regarding the physiological functions mediated via 5-HT_{2B} receptors. It is however known that activation of 5-HT_{2B} receptors mediates vasorelaxation in isolated blood vessels from pig and contraction of longitudinal muscle isolated from human, although this has not yet been demonstrated *in vivo* (Kennett et al., 1996; Kennett et al., 1994).

Agonism at the 5-HT_{2B} receptor can cause valvular heart disease as demonstrated by the recent withdrawal of the anti-obesity drug fenfluramine and the dopamine receptor agonist pergolide, used in the treatment of Parkinson's disease, as they were both shown to be potent agonists at 5-HT_{2B} receptors (Roth, 2007).

5-HT_{2C} receptors

The 5-HT_{2C} receptor was one of the first 5-HT receptors identified (Hoyer, 1988). Initially believed to be a member of the 5-HT_1 receptor family (then identified as the 5-

HT_{1C} receptor), the 5-HT_{2C} receptor was later re-classified and became a member of the 5-HT_2 receptor family.

5-HT_{2C} receptors are widely distributed throughout the brain, for instance in areas of cortex, basal ganglia and limbic structures, such as Nucleus accumbens (NAc), hippocampus and amygdala. Unlike the other 5-HT_2 receptors, the 5-HT_{2A} receptor has not been detected outside the CNS (Barnes and Sharp, 1999). There are several physiological responses believed to be mediated via activation of 5-HT_{2C} receptors, such as hypolocomotion, anxiety and hyperthermia. 5-HT_{2C} receptors have also been implicated in the regulation of food intake and weight control due to the fact that 5-HT_{2C} receptor antagonists can increase food intake in rats (Bonhaus et al., 1997). To further support this notion it has also been shown that chronic treatment with 5-HT_{2C} receptor agonists reduces weight gain and food intake in rats (Hayashi et al., 2005).

1.2.1.3 5-HT_3 receptors

In the late 1980's it was discovered that 5-HT exerted effects via a ligand gated ion channel, that shared common features of other ion channels of this type, such as GABA receptors (Derkach et al., 1989). The 5-HT_3 receptor was later cloned (Maricq et al., 1991) and two subunits have now been identified, 5-HT_{3A} and 5-HT_{3B}. Both subunits are essential to form the pentameric complex that makes up the ion channel.

The 5-HT_3 receptor is the only known ion-channel in the 5-HT receptor family. It is permeable to cations and has allosteric sites for several compounds. So far, no 5-HT_3 receptor subtypes have been identified, but significant species differences have been reported (Hoyer et al., 2002).

The highest expression of 5-HT_3 receptors is found in the dorsal vagal complex in the brainstem. This could explain the proposed involvement in chemotherapy- and radiotherapy-induced vomiting and nausea. 5-HT_3 receptors are also expressed in lower levels in forebrain regions, such as hippocampus, amygdala and superficial layers of the cortex, but there are substantial differences in expression pattern between human and rodent, leading to discrepancies in pharmacological properties (Barnes and Sharp, 1999). It has been shown that activation of 5-HT_3 receptors causes increased dopamine release. It is also proposed that antagonists for the 5-HT_3 receptor cause similar effects as known antipsychotics and anxiolytics, and that this receptor is involved in cognitive enhancement. However both of these notions still remain to be confirmed clinically (Hoyer et al., 2002).

1.2.1.4 5-HT$_4$ receptors

The 5-HT$_4$ receptor was first discovered in the late 1980's (Bockaert et al., 1990). However this receptor was not cloned until 1995 (Gerald et al., 1995) and it was then discovered that there are two isoforms of the receptor, 5-HT$_{4S}$ and 5-HT$_{4L}$, later renamed 5-HT$_{4(a)}$ and 5-HT$_{4(b)}$. The 5-HT$_{4(a)}$ isoform is widely distributed in the rat brain, except for the cerebellum, whereas the 5-HT$_{4(b)}$ isoform is restricted to the striatum. Later studies have shown the presence of several other splice variants of the 5-HT$_4$ receptor that are abundantly expressed in rat, mouse and human and with some species-specific variations (Vilaro et al., 2002). These are distributed in several brain areas as well as in peripheral tissues, such as the gut and in the atrium of the heart (Barnes and Sharp, 1999).

5-HT$_4$ receptors have been proposed to be involved in the regulation of contraction of the colon and oesophagus by triggering acetylcholine release. Agonists at the 5-HT$_4$ receptor are also believed to mediate contractile effects in the heart. In the CNS, stimulation of 5-HT$_4$ receptors seems to enhance synaptic transmission by modulating other neurotransmitters, such as dopamine, acetylcholine and GABA. By this mechanism, 5-HT$_4$ receptors are implicated to have a role in memory enhancement (Hoyer et al., 2002). Recently, it was proposed that agonists at the 5-HT$_4$ receptor could be putative antidepressants with a faster onset than traditional treatments, such as SSRI's. It was found that traditional antidepressant effects, such as desensitization of 5-HT$_{1A}$ autoreceptors and hippocampal neurogenesis appeared in rats after only three days of treatment with a 5-HT$_4$ receptor agonist, compared to 2-3 weeks for the SSRI citalopram (Lucas et al., 2007).

1.2.1.5 5-HT$_5$ receptors

The least characterized receptor for 5-HT is the 5-HT$_5$ receptor. Two subtypes of this receptor, 5-HT$_{5A}$ and 5-HT$_{5B}$, have been identified (Erlander et al., 1993; Matthes et al., 1993). The physiological effector system for 5-HT$_{5A,B}$ receptors remains elusive, although a negative coupling to cAMP has been implicated in transfected cells (Francken et al., 2000). The sequence homology to other 5-HT receptors is low, except for the well conserved putative transmembrane domains III and IV. The distribution of the two 5-HT$_5$ receptor subtypes is still largely unknown, although mRNA analysis has suggested widespread distribution in both rat and mouse brain (Barnes and Sharp, 1999). However, there have been no reports on functional 5-HT$_5$ receptors *in vivo*, and the function of these receptors is still unknown.

1.2.1.6 5-HT$_6$ receptors

The 5-HT$_6$ receptor was first identified in rat and later in human and is a G$_s$-coupled receptor that stimulates cAMP production (Kohen et al., 1996; Ruat et al., 1993a). It has high affinity for the hallucinogenic drug LSD as well as several antidepressants and antipsychotics, including clozapine. However, 5-HT$_6$ receptors display lower affinity for 5-HT compared to other 5-HT receptors (Branchek and Blackburn, 2000).

The rat and human 5-HT$_6$ receptor is mainly distributed in the CNS with the highest mRNA levels in the striatum and olfactory tubule. Moderate to low expression was also found in the cortex, amygdala and the hippocampus. In the periphery 5-HT$_6$ receptor mRNA was barely detectable, with a faint signal in the adrenals (Barnes and Sharp, 1999; Branchek and Blackburn, 2000; Ruat et al., 1993a). The 5-HT$_6$ receptor protein distribution is very similar to the distribution of 5-HT$_6$ receptor mRNA (Mitchell and Neumaier, 2005).

5-HT$_6$ receptors are proposed to have a modulating role, interacting with several other neurotransmitters, such as GABA, acetylcholine and dopamine. They are therefore suggested to be indirectly linked with mechanisms mediated via these neurotransmitters (Mitchell and Neumaier, 2005). Through these interactions, 5-HT$_6$ receptors are suggested to play a role in regulating memory consolidation, depression, anxiety and regulation of body weight, although the exact importance for these receptors remains unclear (Branchek and Blackburn, 2000). Recent data suggest that 5-HT$_6$ receptor agonists may have antidepressant properties and that 5-HT$_6$ receptor antagonists can block the antidepressant effects of fluoxetine (Svenningsson et al., 2007).

1.2.1.7 5-HT$_7$ receptors

The 5-HT$_7$ receptor is the most recent addition to the 5-HT receptor family and the human, rat and mouse receptor were cloned in parallel by several groups (Bard et al., 1993; Lovenberg et al., 1993; Ruat et al., 1993b; Shen et al., 1993; Tsou et al., 1994). Like 5-HT$_4$ and 5-HT$_6$ receptors, 5-HT$_7$ receptors are positively coupled to AC, via G$_s$ proteins. Shortly after the receptor was cloned it was also discovered that there are four isoforms of the 5-HT$_7$ receptor. The 5-HT$_{7(a)}$, 5-HT$_{7(b)}$ and 5-HT$_{7(d)}$ isoforms are expressed in human, whereas the 5-HT$_{7(a)}$, 5-HT$_{7(b)}$ and 5-HT$_{7(c)}$ are expressed in rodents. These isoforms differ in the length and amino acid composition of the predicted intracellular C-terminal (Heidmann et al., 1997; Heidmann et al., 1998; Krobert and Levy, 2002). There is no apparent difference in pharmacological properties

between the isoforms, although studies have shown that the 5-$HT_{7(d)}$ displays a different internalization pattern (Guthrie et al., 2005).

5-HT_7 receptors are widely expressed in different cell types in the central nervous system and in peripheral tissues. The highest levels of 5-HT_7 receptor mRNA in the brain is found in the thalamus, hypothalamus and hippocampus and moderate to low levels in cortical regions (Hedlund and Sutcliffe, 2004; Varnas et al., 2004). This distribution correlates well with the proposed functions of 5-HT_7 receptors. Genetic and pharmacological studies have provided evidence that 5-HT_7 receptors may be involved in regulation of emotions, thermoregulation, circadian rhythmicity and memory processes (Hedlund and Sutcliffe, 2004; Vanhoenacker et al., 2000). Furthermore, 5-HT_7 receptor knock-out mice display a shifted circadian rhythm, a phenomenon that can be mimicked by using 5-HT_7 receptor antagonists (Guscott et al., 2005).

Because of its distribution to cerebral vascular smooth muscle cells, the 5-HT_7 receptor has also been proposed to be involved in vasodilation and therefore is a possible target for the treatment of migraine (Hamel, 1999).

1.3 THE DOPAMINE SYSTEM

The dopamine system is involved in a number of processes in the CNS and is one of the most abundant neurotransmitters in the brain. Since its discovery over 40 years ago, several disease states have been linked to a dysfunctional regulation of dopamine. Among these are schizophrenia, Parkinson's disease (PD), addiction and ADHD.

Dopamine is synthesized from the aromatic amino acid tyrosine. Tyrosine hydroxylase (TH) converts it to L-3,4-dihydroxyphenylalanine (L-DOPA) and a subsequent decarboxylation by the enzyme aromatic L-amino acid decarboxylase (AADC) results in dopamine (Vallone et al., 2000).

Four dopaminergic pathways projects from areas of the brain where dopamine is synthesized: i) The nigrostriatal pathway, which projects from the substantia nigra (SN) pars compacta to the dorsal striatum, ii) The mesolimbic pathway, which projects from the VTA and innervates the ventral striatum, NAc and the olfactory tubercle as well as the limbic system, iii) The mesocortical projections that originate from the VTA and innervates the frontal cortex and iv) the tuberoinfundibular pathway, which projects from nuclei in the hypothalamus, innervating the hypothalamic-hypophyseal system (Dahlstrom and Fuxe, 1964).

Dopamine released in the dorsal striatum regulates motor behaviors (Missale et al., 1998). In the ventral striatum dopamine is involved in the reward system and thus

stimulation of dopamine leads to feelings of satisfaction. Many addictive drugs, target dopamine in this area, both by activating specific receptors or inhibiting dopamine re-uptake, such as cocaine, causing higher dopamine levels, and enhancing the feeling of reward.

Parkinson's disease (PD) is a severe neurodegenerative disease, where there is a substantial loss of dopaminergic neurons in the SN pars compacta (Hornykiewicz and Kish, 1987). This leads to a reduction in dopamine output to the dorsal striatum resulting in an impairment of motor function, which causes symptoms like rigidity and tremor. To date the most effective treatment of PD is by L-DOPA treatment, which replaces the lost dopamine (Carlsson et al., 1957). However, as the neurodegeneration progresses the dose of L-DOPA must be increased and this gives rise to a number of side effects, including dyskinesia and hallucinations (Lang and Lozano, 1998a; Lang and Lozano, 1998b).

1.3.1 Dopamine receptors

In similarity to the serotonin system there are also several receptors for dopamine, which all belong to the GPCR family. They are divided into D_1-like and D_2-like receptors according to sequence homology and signal transduction mechanisms. An overview of dopamine receptor subtypes is presented in figure 5.

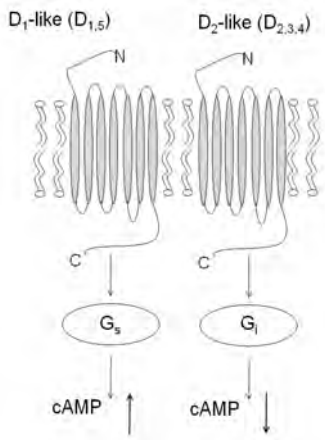


Figure 5. An overview of dopamine receptor subtypes. The D_1-like receptors (D_1 and D_5) are positively coupled to cAMP via G_s proteins, whereas the D_2-like receptors (D_2, D_3 and D_4) are negatively coupled to cAMP via G_i.

1.3.1.1 D_1-like receptors

The dopamine D_1-like receptors consist of the D_1 and D_5 receptors. These receptors are positively coupled to AC, and the activation of D_1-like receptors leads to intracellular increases in cAMP. The dopamine D_1 receptor was first cloned in 1990 (Dearry et al., 1990; Monsma et al., 1990; Sunahara et al., 1990; Zhou et al., 1990) and

the D_5 receptor (alternatively termed D_{1A} or $D_{1\alpha}$) was cloned the year after (Sunahara et al., 1991). In comparison the D_1 receptor displays up to 20-fold higher affinity for dopamine than the D_5 receptor.

The known distribution of dopamine D_1 receptors is based on data from *in situ* hybridization, Northern blot, PCR analysis and receptor binding autoradiography. These studies have shown high expression of D_1 receptors in different areas of the brain, including the caudate putamen (CP) and NAc in the striatum, as well as in the olfactory tubercle (OT), cerebral cortex and amygdala (Monsma et al., 1990). D_1 receptors have also been detected in the SN pars reticulata, although no mRNA has been detected in this area. This indicates that D_1 receptors are synthesized in striatal neurons and transported to the SN (Jackson and Westlind-Danielsson, 1994).

The distribution of dopamine D_5 receptors is more restricted than that of dopamine D_1 receptors. mRNA for the D_5 receptor has been detected in the hippocampus, lateral mammillary nucleus, the parafascicular nucleus of the striatum (Jackson and Westlind-Danielsson, 1994) and in the subthalamic nucleus (Svenningsson and Le Moine, 2002).

1.3.1.2 D_2-like receptors

The D_2-like receptors include the D_2, D_3 and D_4 receptors. They are all negatively coupled to AC and therefore activation of these receptors results in decreased cAMP production. The first receptor from this group to be cloned was the D_2 receptor (Bunzow et al., 1988). It distributes mainly to brain areas such as OT, CP and NAc, as well as the SN pars compacta and the VTA. In contrast to dopamine D_1 receptors, which are mainly postsynaptic, the D_2 receptor is also located presynaptically (Vallone et al., 2000). In the periphery, D_2 receptors are also distributed to the retina, kidney, cardiovascular system and pituitary gland (Jackson and Westlind-Danielsson, 1994).

The dopamine D_3 receptor (Sokoloff et al., 1990) is specifically distributed to limbic areas, such as NAc, but in contrast to D_1 and D_2 receptors it is hardly expressed in the dorsal striatum. It has also been found at low levels in the hippocampus and various levels of the cortex (Missale et al., 1998).

Dopamine D_4 receptors (Van Tol et al., 1991) display a different expression pattern compared to other dopamine receptors. High levels of expression have been found in frontal cortex, amygdala, hippocampus and hypothalamus, whereas only low levels of expression has been found in the basal ganglia (Missale et al., 1998).

1.4 LIPID RAFTS

The Singer-Nicolson fluidic mosaic model of cell membranes was for many years considered applicable to all cell types. Proteins were embedded in a phospholipid bilayer more or less evenly distributed in the cell membrane (Singer and Nicolson, 1972). This view has lately been altered with the discovery of microdomains, structurally ordered and rich in saturated lipids, such as cholesterol and sphingolipids. These microdomains, now referred to as lipid rafts, seem to play important roles in regulating several functions in biological membranes, such as signaling events and membrane traffic. Although there has been some debate over the existence of these rafts and their importance (Munro, 2003), there is now compelling evidence that lipid rafts are microdomains in the plasma membrane, about 50 nM in diameter, that are important regulators of cell signaling events (Simons and Ikonen, 1997).

1.4.1 Structure and function of lipid rafts

The components of lipid rafts display high degree of saturation in the acyl side chains and because of this they create clusters rich in cholesterol and sphingomyelin, which are separated from the surrounding membrane (Brown and London, 1998b). The common feature of these microdomains is their insolubility in non-ionic detergents (Brown and London, 1998a) and they are therefore referred to as detergent-resistant membranes (DRMs) or detergent-insoluble glycolipid-enriched membranes (DIGs). Furthermore, these detergent-insoluble complexes have the ability to float on low density sucrose gradients, which has become a powerful tool to study the composition of lipid rafts, and they are therefore also referred to as Triton-insoluble floating fractions (TIFFs) (Simons and Ikonen, 1997; Simons and Toomre, 2000).

There has been some debate over the composition of lipid rafts in native cell membranes. One argument is the fact that the use of different detergents results in the extraction of different components of rafts (Chamberlain, 2004). Nevertheless, lipid rafts are proposed to play a role in clustering receptors and proteins involved in signal transduction, thus creating a signal transduction "platform", making signaling events more effective. Furthermore, they form attachments to cytoskeletal proteins, and are involved in trafficking (see section 1.4.3). Certain proteins localize to lipid rafts because of specific binding motifs in their structure, such as glycosyl phosphatidyl inositol (GPI)-anchors (Brown and Rose, 1992), or by modifications of their amino acid sequence, for instance by palmitoylation or myristoylation (Wang et al., 2001). These motifs have been found in several proteins detected within lipid rafts, but do not

seem to be absolutely essential for raft association. Theoretically, by compartmentalization of two proteins involved in receptor signaling, such as a GPCR and a corresponding G protein, the signaling becomes more effective (Ostrom, 2002). Moreover, the opposite scenario can also be imagined, in that some proteins are excluded from lipid rafts and thereby are not able to efficiently participate in signaling events.

1.4.2 Caveolae – a subgroup of lipid rafts

A subgroup of lipid rafts is caveolae, which are flask-shaped invaginations of the plasma membrane. In contrast to planar lipid rafts, these microdomains can be visualized with electron microscopy and these invaginations were identified more than 50 years ago (Yamada, 1955). In addition to cholesterol and sphingomyelin, caveoale contain cholesterol-binding caveolin proteins. Caveolin-1 (Cav-1) was the first protein of this family to be discovered. Originally designated VIP-21, Cav-1 is the most important of the three known caveolin proteins to maintain the structure of caveolae. It is often co-expressed together with Cav-2 in several cell types. In contrast, Cav-3 is specific to muscle cells (Lisanti et al., 1994; Quest et al., 2004).

Cav-1 has a very distinct structure with a cytoplasmic N-terminal and a scaffolding domain (residues 82-101) which is responsible for interactions with other proteins. Overlapping with the scaffolding domain is the oligomerization domain (residues 61-101) which forms attachments to other caveolin proteins. Furthermore there is a transmembrane domain (residues 102-134) responsible for forming attachments to the plasma membrane (Cohen et al., 2004). Ultimately, this creates a characteristic hairpin structure of the protein.

Cav-1 interacts with many proteins through the scaffolding domain and specific caveolin binding motifs have been identified in proteins interacting with Cav-1. The general sequence for this interaction is ΦXΦXXXXΦ, ΦXXXXΦXXΦ or ΦXΦXXXXΦXXΦ, where Φ is an aromatic amino acid (Trp, Phe or Tyr) and X is any amino acid. This motif has been found in several proteins interacting with Cav-1, such as certain G protein α-subunits (Couet et al., 1997).

Caveolin proteins cluster together in large protein complexes to form the characteristic invaginations in the plasma membrane. A general structure of caveolae is presented in figure 6. Since caveolin binds directly to cholesterol, caveolae are sensitive to cholesterol depletion, causing them to flatten.

Caveolae are present in many cell types and are especially abundant in adipocytes, endothelial cells and fibroblasts. Interestingly, neither neurons nor lymphocytes express functional caveolae (Liu et al., 2002). However, recent reports suggest that, despite the lack of caveolae, some populations of neurons do express caveolin proteins (Gaudreault et al., 2005; Masserini et al., 1999). The function of these non-caveolar caveolin proteins remain to be determined, but it seems that these proteins can play an important part in the regulation of cellular functions also outside of caveolae (Head and Insel, 2007). For instance, Cav-1 has been suggested to modulate neuronal plasticity. Overexpression of Cav-1 led to a decrease in neurite outgrowth and an increase of neurite density in primary hippocampal neurons (Gaudreault et al., 2005). Furthermore, a recent study found that Cav-1 knockout mice have reduced brain weight in adult animals compared to wild type without affecting the number of neurons in different brain regions. These animals also displayed impaired motor functions and were less active than wild type mice, suggesting that Cav-1 might play a role in regulating motor control (Trushina et al., 2006).

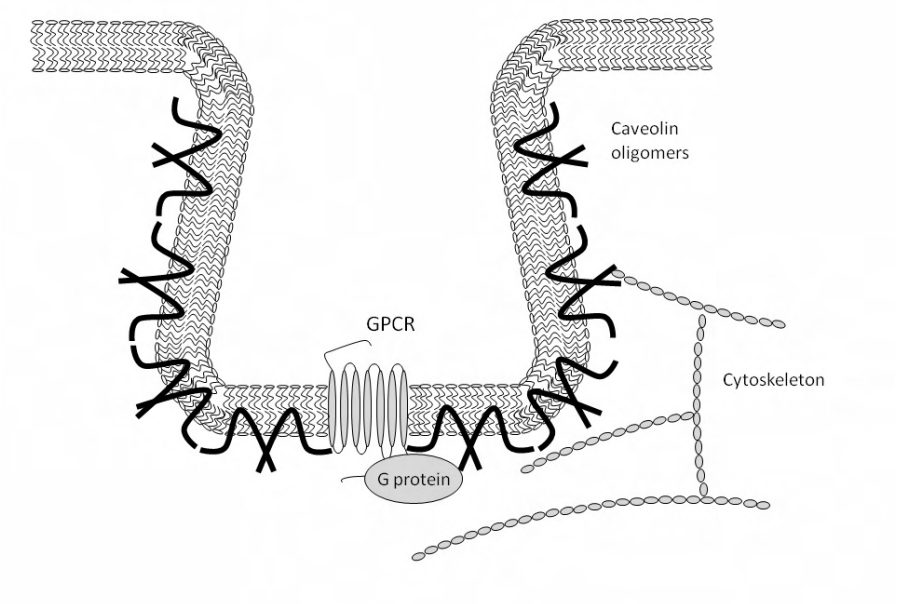


Figure 6. The general structure of caveolae, which are flask-shaped invaginations of the plasma membrane. Shown in black are caveolin proteins, forming oligomers. Also shown is a GPCR with an associated G protein. GPCRs can be targeted to caveolae via direct interaction with caveolin or by modifications in the C-terminal tail, such as palmitoylation or myristoylation. Adapted from Ostrom and Insel, 2004.

1.4.3 Lipid raft-mediated receptor trafficking

As described in section 1.1.2, GPCRs undergo both constitutive and agonist-induced internalization. This is a clathrin-dependent mechanism, and is an important regulator of signal transduction. Recently, alternative hypotheses of internalization mechanisms have evolved, involving lipid rafts and/or caveolae. These processes are clathrin-independent, but can be dynamin-dependent (Le Roy and Wrana, 2005). The mechanism for this process is not yet fully understood, but it's becoming clear that receptors and proteins can undergo internalization via different paths than the classical endosomal route.

One common view on clathrin-independent endocytosis is that it is a highly regulated process and the rate of constitutive internalization is very low. Furthermore, this internalization process is insensitive to hypertonic sucrose (which inhibits clathrin-dependent endocytosis) and sensitive to cholesterol depletion. The first studies of clathrin-independent endocytosis revealed a pathway that was also independent on dynamin. Several GPI-anchored proteins have been shown to be internalized via this pathway and it was proposed that this anchor is required for this to occur. A new type of uncoated vesicles, termed clathrin independent carriers (CLICs), seemed to mediate this internalization. As GPI-anchors are also a common motif for lipid raft associated proteins, this new pathway is suggested to be mediated via lipid rafts (Fig. 7) (Kirkham and Parton, 2005).

Of the clathrin-independent pathways the caveolae-mediated pathway has been the most extensively studied (Fig. 7). The structure of caveolae led to early theories that they could be involved in internalization through budding from the plasma membrane to form a homologue to clathrin-coated vesicles, caveosomes (Nichols et al., 2001). Caveolae-mediated internalization shares some common features with the endosomatic pathway. They are both dependent on dynamin, which can be constitutively located at the neck of caveolae or recruited in response to specific signals. Hence, cavolae-mediated endocytosis can be blocked by overexpression of a dominant negative dynamin II mutant (K44A) (Le and Nabi, 2003; Pelkmans et al., 2002). However, since clathrin-dependent endocytosis also depends on dynamin, the dynamin mutant is ineffective in determining the internalization pathway.

Some studies have also indicated that actin plays a role in the vesicular transport (Pelkmans et al., 2002). It has been proposed that local actin polymerization is essential for the internalization via caveolae. Both actin and dynamin are here proposed to participate in the budding of caveolae from the cell surface (Pelkmans et al., 2002).

Another common feature is that like clathrin-mediated endocytosis, caveolae-mediated endocytosis is dependent on protein phosphorylation, possibly on Cav-1 itself. The exact mechanism for this is not yet fully understood (Kirkham and Parton, 2005). Nevertheless it is possible to block caveolae budding with tyrosine kinase inhibitors, such as genistein, and stimulate it with phosphatase inhibitors (Nabi and Le, 2003; Parton and Richards, 2003).

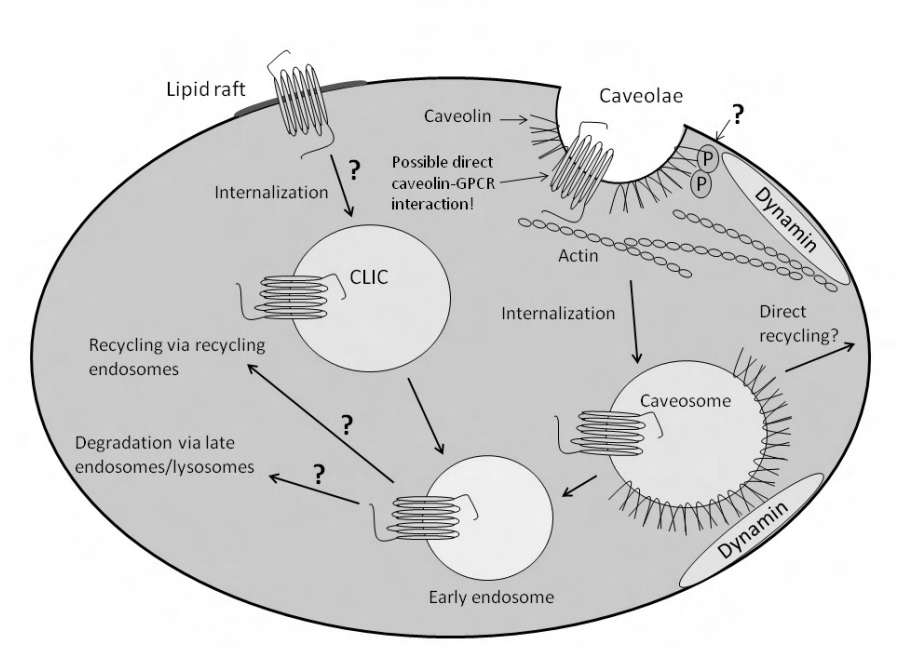


Figure 7. Internalization of receptors mediated via lipid rafts or caveolae. Proteins can be associated with these pathways through specific motifs in their sequence (e.g. GPI anchors) or by a direct interaction with caveolin. Lipid raft-mediated endocytosis is independent on clathrin and dynamin and mediated via a clathrin independent carrier (CLIC). The exact mechanism for this pathway is not known. Caveolae mediated internalization is dependent on actin and dynamin, and possibly facilitated by a direct phosphorylation of caveolin proteins. Receptors are internalized via a homologue to clathrin-coated vesicles, caveosomes, and both pathways converge in early endosomes.

One problem when studying clathrin-independent internalization is that there are several points of convergence in the process. For instance, both CLICs and caveosomes are linked to early endosomes, why the ability to study very early carriers is needed to visualize these pathways. Furthermore, some lipid raft-associated proteins can utilize different internalization pathways, making the use of these proteins as markers for lipid raft-mediated endocytosis difficult (Hanzal-Bayer and Hancock, 2007). For instance cholera toxin B can internalize via both clathrin coated vesicles and caveolae as well as

via a pathway that is independent of clathrin and dynamin and sensitive to cholesterol (Kirkham et al., 2005). Furthermore, the endothelin type A (ET_A) receptor has been shown to switch from a caveolae-mediated to a clathrin-dependent internalization pathway following cholesterol oxidation in Chinese hamster ovary cells (Okamoto et al., 2000). This indicates that certain proteins can internalize via different routes depending on available components of the internalization machinery.

1.4.4 The role of lipid rafts in GPCR function

The function of several GPCRs has been shown to be regulated via lipid rafts and caveolae. It is apparent that lipid rafts and caveolae are important modulators of GPCR function (Chini and Parenti, 2004). Furthermore, several proteins important for GPCR signaling events, such as various G proteins and adenylyl cyclase subtypes have been shown to localize to lipid rafts. A summary of GPCRs that have been shown to be associated with lipid rafts is shown in table 1. Notable is that this list may not be complete as new associations emerge rapidly.

The present work will focus on the importance for lipid rafts in the regulation of monoamine receptor function. Early work on monoamine receptors was done on β_1- and β_2 adrenergic receptors in cardiac myocytes. Rybin and co-workers observed that, although these two receptors are co-localized in these cells and both couple to G_s and subsequent activation of adenylate cyclase, there were discrepancies in signaling efficiency, not conclusive with a random membranous distribution of receptors and G proteins. It was shown that β_2 receptors are exclusively located in caveolae, whereas β_1 receptors are more evenly distributed both in caveolae and non-caveolae fractions. Furthermore it was observed that certain adenylate cyclase subtypes where also shown to be allocated in caveolae, possibly explaining the discrepancies in signaling efficiency between β_1 and β_2 adrenergic receptors (Rybin et al., 2000). Later it was also shown that $G_{\alpha s}$ proteins are internalized upon agonist stimulation of β_2 adrenergic receptors in a clathrin-independent manner, a process suggested to be mediated by lipid raft-dependent mechanisms (Allen et al., 2005).

Early studies showed that the affinity of 5-HT to 5-HT_1 receptors expressed in NCB-20 cells was increased by gangliosides (Dawson and Berry-Kravis, 1984). This increase could not be explained by a direct binding of 5-HT to gangliosides at the concentrations at which it labels the receptor. Another observation came a few years later when it was shown that stimulation of 5-HT_{1A} receptors increases the rate of synthesis of gangliosides in a number of different cell types (Singh et al., 1996).

Table 1. A summary of GPCRs suggested of being associated with lipid rafts/caveolae.

RECEPTOR	REFERENCE(S)
5-HT_{1A}	(Pucadyil and Chattopadhyay, 2004; Pucadyil and Chattopadhyay, 2005; Renner et al., 2007; Singh et al., 1996)
5-HT_{2A}	(Bhatnagar et al., 2004; Dreja et al., 2002)
Adenosine A_1	(Gines et al., 2001; Lasley et al., 2000)
α_1 adrenergic	(Dreja et al., 2002; Fujita et al., 2001)
β_1 adrenergic	(Liu et al., 2007; Rybin et al., 2000)
β_2 adrenergic	(Allen et al., 2005; Liu et al., 2007; Ostrom et al., 2002; Rybin et al., 2000)
Angiotensin AT1	(Wyse et al., 2003)
Bradykinin B_1	(Lamb et al., 2002)
Bradykinin B_2	(de Weerd and Leeb-Lundberg, 1997; Haasemann et al., 1998; Lamb et al., 2002)
Dopamine D_1	(Kong et al., 2007; Yu et al., 2004)
Endothelin ET_A	(Chun et al., 1994; Okamoto et al., 2000)
Endothelin ET_B	(Teixeira et al., 1999; Yamaguchi et al., 2003)
$GABA_B$	(Becher et al., 2004; Becher et al., 2001)
Gonadotropin releasing hormone (GnRH)	(Navratil et al., 2003)
m2 muscarinic acetylcholine	(Feron et al., 1997)
Neurokinin 1	(Monastyrskaya et al., 2005)
μ-opioid	(Zhao et al., 2006)
Somatostatin SST2	(Krisch et al., 1998; Mentlein et al., 2001)
Sphingosine EDG-1	(Igarashi and Michel, 2000)

Further evidence for the involvement of lipid rafts for the function of 5-HT_{1A} receptors emerged with the recent observation that cholesterol sequestration with methyl-β-cyclodextrin (MβCD) modulates both agonist and antagonist binding to 5-HT_{1A} receptors in bovine hippocampal membranes, an effect that can be reversed by adding exogenous cholesterol (Pucadyil and Chattopadhyay, 2004; Pucadyil and Chattopadhyay, 2005). Moreover, 5-HT_{1A} receptors have been shown to distribute to detergent-resistant membrane fractions together with Cav-1 in stably transfected NIH-3T3 cells and this localization is dependent on receptor palmitoylation (Renner et al., 2007).

Cav-1 has also been shown to interact with both stably transfected and endogenously expressed 5-HT_{2A} receptors (Bhatnagar et al., 2004). This interaction facilitated the

interaction between 5- HT_{2A} receptors and G_q proteins and silencing of Cav-1 by RNA interference nearly abolished signaling via 5-HT_{2A} receptors.

Dopamine D_1 receptors have also been suggested to be associated with lipid rafts. An early study suggested that membranous cholesterol may be involved in radioligand binding and signaling via dopamine D_1 receptors (Maguire and Druse, 1989). A more recent study (Yu et al., 2004) was performed in HEK-293 cells and rat renal proximal tubule cells and showed that Cav-2 interacts with dopamine D_1 receptors. Cav-2 was also involved in D_1 receptor signaling and adenylyl cyclase was activated to a greater extent in lipid rafts than in non-raft membranes in response to a D_1 receptor agonist (Yu et al., 2004). Another study also found an interaction between D_1 receptors and Cav-1 in COS-7 cells and in rat brain. But in contrast to the previous study, they found that caveolae localization attenuates cAMP signaling via D_1 receptors (Kong et al., 2007). Hence, additional studies are needed to determine the effects of lipid raft localization for dopamine D_1 receptors.

2 GENERAL AIMS

Based on the outlined background information on lipid raft-involvement in GPCR function, the general aims of this work were:

1. to investigate whether lipid raft components, such as cholesterol, sphingolipids and gangliosides, are important for agonist and antagonist binding to serotonin receptors, using a cell line stably transfected with the human 5-$HT_{7(a)}$ receptor as a model system.

2. to investigate whether cholesterol levels affect signaling via serotonin receptors, using a cell line stably transfected with the human 5-$HT_{7(a)}$ receptor as a model system.

3. to examine the role of Cav-1 in regulating agonist binding and membranous localization of serotonin receptors using a cell line stably transfected with the human 5-$HT_{7(a)}$ receptor as a model system.

4. to examine the role of cholesterol in regulating signaling via endogenously expressed serotonin receptors, using human primary neuronal cell cultures as a model system.

5. to examine the role of cholesterol in regulating signaling via endogenously expressed dopamine receptors, using striatal brain slices from mouse as a model system.

3 COMMENTS ON METHODOLOGY

The methods used in this work are described in detail in the accompanying papers and are listed below (Table 2). One major part of this work involves the utilization of radioligand binding assays and this will therefore be discussed in more detail below. Some details on the usage of siRNA are also discussed, as well as considerations about density gradient centrifugation for purification of DRM's.

Several compounds acting on 5-HT or dopamine receptors were used in this work and are listed in table 3. Some compounds were only used in the characterization of the utilized cell line (paper I) and are not listed here. Several approaches were used to alter levels of lipid raft components in the different model systems. Reagents are described in table 4.

Table 2. Methods used in the present work.

METHOD	**PAPER**
Culture of HeLa cells stably transfected with the human 5-$HT_{7(a)}$R	I, II, III
Culture of human neuronal cell cultures	IV
SDS-PAGE and Western blot	I, II, III, IV, V
Radioligand binding in HeLa cells	I, II, III
MTT viability assay	I, II, III, IV
Cholesterol assay	I, II, IV, V
Studies of intracellular protein phosphorylation	I, III, IV, V
siRNA transfection	III
Sucrose density gradient purification of DRM's	III, V
Sub-cellular fractionation	III
6-hydroxydopamine lesioning	V
Incubation and stimulation of striatal slices	V

Table 3. Agonists and antagonists used in the present work.

NAME	MODE OF ACTION
5-HT	Endogenous agonist of all 5-HT receptors
5-CT	5- $HT_{1A,B,D}$, 5-HT_5 and 5-HT_7 receptor agonist
5-MeOT	5-$HT_{1A,B,D}$, 5-HT_{2A-C}, 5-HT_4, 5-HT_6 and 5-HT_7 receptor agonist
8-OH-DPAT	5-$HT_{1A/7}$ receptor agonist
SB269970	Selective 5-HT_7 receptor antagonist
SKF-81297	Selective D_1 receptor agonist

Table 4. Reagents used to affect lipid raft constituent levels.

NAME	MODE OF ACTION
Methyl-β-cyclodextrin	Cholesterol sequestering agent
Mevastatin	HMG-CoA reductase inhibitor
Mevalonate	HMG-CoA reductase inhibitor
Fumonisin B_1	sphingosine N-acyltransferase inhibitor
PDMP	Glucosylceramide synthase inhibitor
siRNA Cav-1	Post-transcriptional silencing of Cav-1 protein

3.1 RADIOLIGAND BINDING ASSAYS

Basic methodology

The use of radioligand binding is a powerful tool in classical receptor pharmacology and in the current project this method was utilized in a major part of the work (Papers I-III). In practice, a radiolabelled agonist or antagonist was incubated with cell suspension until equilibrium was reached. The samples were subjected to rapid filtration using a semiautomatic cell harvester (Hall and Thor, 1979), separating the large protein-ligand complexes from unbound ligand. The filters were incubated in scintillation fluid overnight at room temperature and finally counted in a Rackbeta 1209 scintillation counter. The data from all binding experiments were analyzed using non-linear regression in the GraphPad Prism 4.0 software.

In theory a receptor can exist in several different activation states, depending on conformational states or whether a ligand or G protein is bound or not. Ligand receptor interactions can be described by the ternary complex model. Very simply this can be described by:

$$A + R = AR$$

where A is an agonist and R is a receptor. The ternary model has later been modified to the extended ternary model, taking into consideration that receptors can undergo activation independent of an agonist, and can also be affected by allosteric modulators, giving a wide range of activation states (Kenakin, 2004).

Saturation binding assays

In saturation binding experiments the direct interaction of a tracer ligand and a receptor can be observed. The number of available binding sites and the affinity of the ligand for the receptor can be directly determined. The prerequisite is that there is an available radioligand for the receptor. The radioligand is equilibrated with the receptor in a range of concentrations, yielding the total binding to the receptor. Nonspecific binding is determined in the presence of a high concentration of an unlabelled ligand for the receptor, which blocks the binding of the radioligand to the receptor. By subtracting the nonspecific binding from the total binding, the specific binding can be determined:

$$Total\ binding - Nonspecific\ binding = Specific\ binding$$

The total and nonspecific binding can be defined as:

$$Total\ binding = \frac{[A]B_{max}}{[A] + K_D} + k[A] \qquad Nonspecific\ binding = k[A]$$

Where k is a constant defining concentration relationship for nonspecific binding, [A] is the concentration of the radioligand, B_{max} is the total number of binding sites and K_D is the dissociation constant. The total and nonspecific binding are the experimentally determined variables. Thus the specific binding must be calculated from these two entities. From the previous definitions the specific binding [AR] can thus be expressed as:

$$[AR] = \frac{[A]B_{max}}{[A] + K_D}$$

The specific binding will increase as the concentration of radioligand increases. However, as the number of available binding sites is limited the curve plotted will be saturated. For that reason the data are often transformed to a straight line, referred to as

a Scatchard plot, where K_D = -1/slope and B_{max} is the intercept of the x-axis. However the values obtained from a linear transform may not correspond to the true values. Hence, the use of nonlinear regression is more reliable when determining maximum binding and affinity for a receptor-ligand complex.

3.2 SUCROSE DENSITY GRADIENT CENRIFUGATION

The emerging data on lipid rafts is accompanied by methods of purification of these microdomains. Recently, several methods for the purification of liquid ordered microdomains have been proposed, many of which include sucrose density gradient centrifugation with, or without detergents. In this work (paper III, V) we utilized the method of Triton X-100 extraction according to previously published studies (Li et al., 1995; Sargiacomo et al., 1993). Briefly, HeLa cells were harvested, homogenised and re-suspended in 25 mM HEPES pH 7.1, 150 mM NaCl, 1% Triton X-100. Mouse brain tissue was sonicated in MBS (25 mM MES pH 6.5, 150 mM NaCl) with 1% Triton X-100. The lysates were incubated for 30 min on ice before adjusting the sucrose concentration to 45% (90% sucrose in MBS). A discontinuous gradient (35% and 5% sucrose in MBS) was formed over the lysate and the gradients were centrifuged for 24 h at 140,000×*g* at 4°C in an SW-41 rotor. Fractions were then taken from the top of the gradient, resulting in 12 fractions. An opaque band corresponding to DRM's could be seen in the 5%/35% boundary. The fractions were subjected to SDS-PAGE and western blot with appropriate antibodies. An overview of the procedure is briefly described in figure 8.

There are some considerations when choosing which type of method for lipid raft purification to use. In this work several modifications were tested before deciding on the final protocol. The usage of Triton X-100 extraction is the most commonly used, but there are a number of protocols using other detergents, such as CHAPS, Brij98 or Lubrol WX (Chamberlain, 2004). Some reports suggest that the detergent of choice influences the number and type of proteins being isolated from the detergent-insoluble fractions. There are also detergent-free methods for lipid raft purification. This protocol utilizes sodium carbonate buffer at high pH (pH 11) and additional homogenization and sonication steps (Song et al., 1996). Whether this method or a detergent-based method is to be used is still debatable. However, it is important to consider the alternatives before deciding which protocol to use.

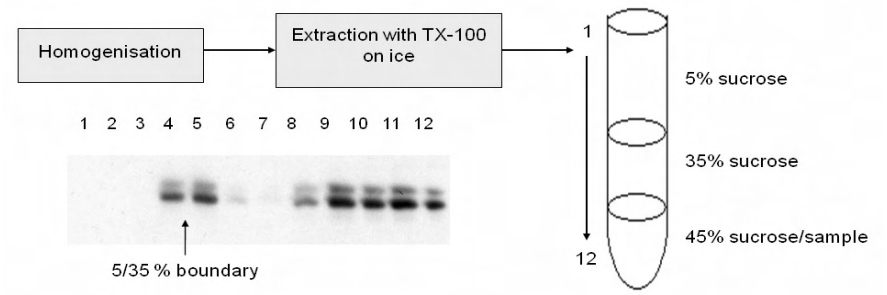


Figure 8. Flow scheme of sucrose density gradient fractionation for purification of DRM's. Cells or tissue is homogenized, followed by solubilisation of membranes in Triton X-100. The discontinuous sucrose gradient is then formed above the sample and the gradient is subjected to high-speed centrifugation. Fractions are taken from the top of the gradient and subjected to SDS-PAGE. The western blot above is an example from paper III.

3.3 TRANSFECTION OF HELA CELLS WITH SIRNA AGAINST CAV-1

Small interfering RNAs (siRNAs) are double-stranded RNA molecules that are able to silence a single gene in a sequence-specific manner by specific degradation of the target mRNA. In recent years siRNA has become a useful tool in the studies of protein function (Cheng et al., 2003; Scherer and Rossi, 2003). Briefly, short, double stranded RNA fragments (usually 19-21 base pairs) are introduced to the cells. The siRNA binds to the target mRNA and the duplex is recognized by the RNA induced silencing complex (RISC) complex, which binds the RNA duplex and targets it for degradation (Fig. 9). This method can be used in cell cultures as well as *in vivo* (Fire et al., 1998) and has been proposed as a potential therapeutic method for the treatment of specific forms of cancer and infectious diseases, such as HIV (Cheng et al., 2003).

In this work (paper III) we used siRNA construct targeting two parts of Cav-1 mRNA. These constructs had been successfully used in a previous study (Sunaga et al., 2004) and were composed as follows:

CAV1-1: 5'-AGACGAGCUGAGCGAGAAGCA-3' (sense)
5'-UGCUUCUCGCUCAGCUCGUCU-3' (antisense)
CAV1-2: 5'-CAUCUACAAGCCCAACAAC-3' (sense)
5'-GUUGUUGGGCUUGUAGAUG-3' (antisense).

HeLa cells stably transfected with the human 5-$HT_{7(a)}$ receptor were utilized in the study, investigating the role of Cav-1 in regulating the agonist binding and cell surface expression of 5-HT_7 receptors. The amount of siRNA was initially titrated to determine the appropriate concentration and a cell viability assay was also performed. The transfections were performed with Oligofectamine® (Invitrogen) according to the manufacturer's description. Furthermore, several time points between 24 and 72 h after transfection were investigated to determine the appropriate incubation time. In the final protocol cells were transfected with 100 nM of each construct and were allowed to recover for 48 h before assay.

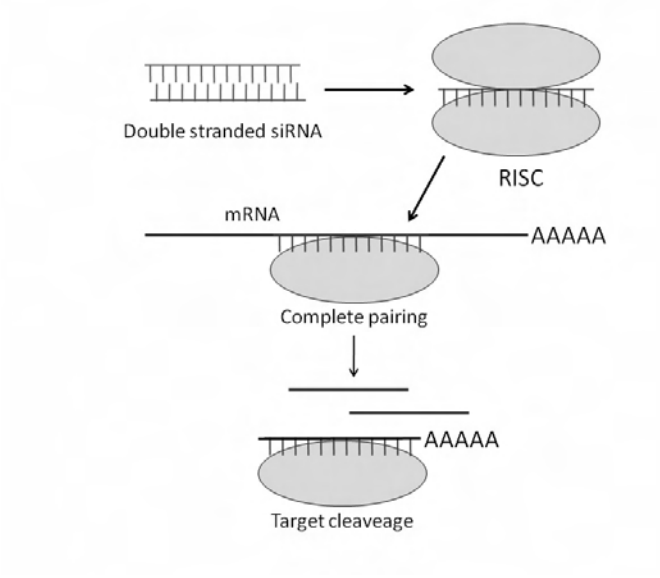


Figure 9. The mechanism of siRNA mediated silencing of genes. siRNA is introduced to the cells, where it binds the target mRNA. This complex is recognized by the RNA induced silencing complex (RISC) which binds and cleaves the target mRNA, thus targets it for degradation. Adapted from Cheng et al. 2003.

4 RESULTS

4.1 5-HT$_7$ RECEPTORS ARE DEPENDENT ON LIPID RAFT INTEGRITY

The initial work was to investigate whether different properties of 5-HT$_7$ receptor function was dependent on lipid raft integrity (Papers I-III). An initial characterization of a HeLa cell line stably transfected with the human 5-HT$_7$ receptor showed that these receptors displayed a similar pharmacology as has been reported in other studies for both cloned (Krobert et al., 2001; Shen et al., 1993) as well as natively expressed (To et al., 1995) 5-HT$_7$ receptors (Paper I).

4.1.1 Ligand binding to 5-HT$_7$ receptors is affected by cholesterol depletion

To investigate effects of cholesterol depletion on ligand binding to 5-HT$_7$ receptors, we used combined treatment with fumonisin B_1, mevastatin and mevalonate (MFM) to inhibit cholesterol synthesis (Paper I). This treatment resulted in a significant reduction in cholesterol levels in the studied cells without causing toxic actions on cell viability.

In saturation binding experiments, it was found that MFM treatment caused a strong reduction of maximum binding of both [^{3}H]5-HT and the selective 5-HT$_7$ receptor antagonist [^{3}H]SB269970 (Lovell et al., 2000) to 5-HT$_7$ receptors (Fig. 10). This could be reversed by adding back exogenous cholesterol, indicating that the effects seen were caused by reduced cholesterol levels and not unspecific effects of the MFM treatment. Neither MFM nor cholesterol had any effects on the affinity of [^{3}H]5-HT or

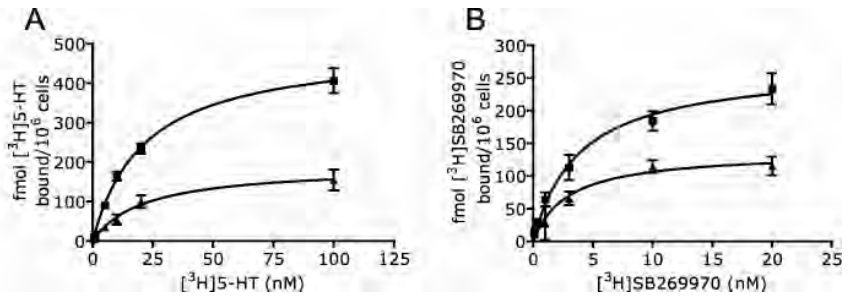


Figure 10. Effects of MFM treatment on maximum agonist and antagonist binding to 5-HT$_7$ receptors. **A.** MFM treatment reduces maximum [^{3}H]5-HT binding to 5-HT$_7$ receptors. **B.** MFM treatment reduces maximum [^{3}H]SB269970 binding to 5-HT$_7$ receptors. ■ control; ▲ MFM.

[^{3}H]SB269970 binding to 5-HT$_7$ receptors indicating that cholesterol is important for the membranous expression of 5-HT$_7$ receptors but is not essential for maintaining different affinity states of the receptors.

To further confirm the effects of cholesterol depletion on ligand binding to 5-HT$_7$ receptors a common cholesterol sequestering agent methyl-β-cyclodextrin (MβCD) (Ohtani et al., 1989) was also used. However, this treatment has been criticized because of its toxic effects on cell viability (Ulloth et al., 2007). Indeed, we found that MβCD treatment caused a dose-dependent reduction in cell viability. However, at the concentration used in the radioligand binding experiments, MβCD caused no significant toxic actions.

We found that treatment with MβCD significantly reduced cholesterol in the studied cells and this caused a significant reduction in the maximum [^{3}H]5-HT- and [^{3}H]SB269970-binding to 5-HT$_7$ receptors. Similar to the effects of MFM treatment, the addition of exogenous cholesterol caused a significant reversal of the effect of MβCD on [^{3}H]5-HT and [^{3}H]SB269970 binding to 5-HT$_7$ receptors (Paper I).

This work showed that cholesterol depletion, both by sequestration of cholesterol and by cholesterol synthesis inhibition, reduces maximum agonist and antagonist binding to recombinant 5-HT$_7$ receptors. Following MFM treatment, but not MβCD treatment, we found a reduction of 5-HT$_7$ receptor immunoreactivity. Hence the effects on reduced maximum ligand binding cannot be completely explained by a mere reduction in total 5-HT$_7$ receptor protein levels.

4.1.2 Ligand binding to 5-HT$_7$ receptors is affected by depletion of gangliosides and sphingolipids

Lipid rafts are rich, not only in cholesterol, but also in gangliosides and sphingolipids and these components play an important part in maintaining lipid raft integrity (Simons and Ikonen, 1997). Since it was evident that cholesterol depletion affected radioligand binding to 5-HT$_7$ receptors, the studies were continued by investigating these two other lipid raft components (Paper II).

The cells were depleted either of sphingomyelin by using Fumonisin B$_1$ (Naslavsky et al., 1999), or depleted of gangliosides, using the potent glucosylceramide synthesis inhibitor, PDMP (Nagafuku et al., 2003). Similar to the effects of cholesterol depletion, treatment with either Fumonisin B$_1$ or PDMP reduced the maximum [^{3}H]5-HT binding to 5-HT$_7$ receptors, but without affecting cholesterol levels. This suggests that sphingomyelin and gangliosides regulate [^{3}H]5-HT binding to 5-HT$_7$ receptors in a

cholesterol-independent manner. Neither of the treatments had any significant effects on the affinity of [^{3}H]5-HT for 5-HT$_7$ receptors and, in contrast to MFM treatment, no change in total 5-HT$_7$ receptor protein levels could be detected after treatment either with Fumonisin B$_1$ or PDMP.

4.1.3 Signaling via 5-HT$_7$ receptors is attenuated by cholesterol depletion

As described in the introduction, 5-HT$_7$ receptors are primarily G$_s$-coupled and activate adenylyl cyclase, which causes subsequent increases in cAMP production and activation of protein kinase A (PKA) signaling pathways (Bard et al., 1993; Lovenberg et al., 1993; Tsou et al., 1994; Adham et al., 1998). The phosphorylation state of the transcription factor CREB at Ser^{133} is known to be a phosphosubstrate for PKA, and a point of convergence for several signaling pathways that are likely to be activated via 5-HT$_7$ receptors in transfected cells (Adham et al., 1998; Kvachnina et al., 2005; Mayr and Montminy, 2001; Norum et al., 2005; Tsou et al., 1994; Zhu et al., 1994). We found that 5-HT caused very strong increases in the phosphorylation states of both Ser^{133}-CREB and Ser^{63}-ATF-1, which were significantly counteracted by MFM treatment (Fig. 11; Paper I).

The effect of cholesterol depletion on 5-HT$_7$ receptor signaling was less pronounced than the effect on [^{3}H]5-HT binding to 5-HT$_7$ receptors. This discrepancy could perhaps be explained by the presence of spare receptors, a common phenomenon in recombinant cell lines (Kenakin, 1997).

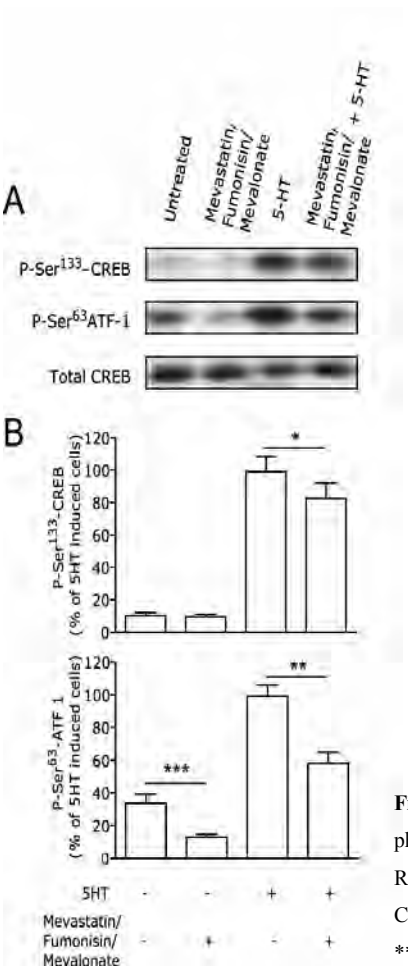


Figure 11. Cholesterol depletion reduces 5-HT induced phosphorylation of Ser^{133}-CREB and Ser^{63}-ATF-1. **A.** Representative immunoblots. **B.** Quantification of Ser^{133}-CREB. **C.** Quantification of Ser^{63}-ATF-1. *p<0.05; **p<0.01; ***p<0.001 with Student's t-test.

4.1.4 Cav-1 is involved in ligand binding and membrane expression of 5-HT$_7$ receptors

To further strengthen our hypothesis that 5-HT$_7$ receptors are dependent on lipid raft integrity we investigated the influence of Cav-1 on 5-HT binding and cell surface levels of 5-HT$_7$ receptors. These two proteins were found to be co-localized in detergent-insoluble fractions of a discontinuous sucrose gradient, suggesting that they are both localized in lipid rafts in the HeLa cell line stably transfected with the human 5-HT$_7$ receptor (Fig. 12; Paper III).

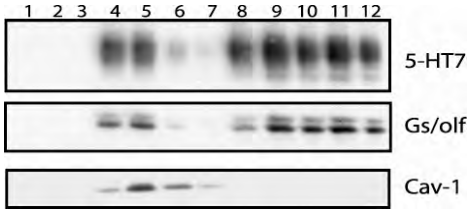


Figure 12. Sucrose density gradient fractionation on HeLa cells. The 5-HT$_7$ receptor, Cav-1 and $G_{s/olf}$ are all located in the low-density, detergent-insoluble fractions 4 and 5.

As discussed in the introduction, most GPCRs undergo desensitization followed by receptor internalization in response to agonist stimulation (von Zastrow, 2003). The desensitization leads to a shift towards increased affinity (Gether and Kobilka, 1998). We found that pre-treatment of the cells with 5-HT caused a significant reduction in the maximum [^{3}H]5-HT binding to 5-HT$_7$ receptors, suggesting that 5-HT$_7$ receptors are internalized upon agonist stimulation. Moreover, the affinity for [^{3}H]5-HT at 5-HT$_7$ receptors was significantly increased by 5-HT pre-treatment, indicating a shift to a low-affinity state of the receptors.

By using siRNA technique we could significantly reduce Cav-1 expression and this caused a significant decrease in maximum [^{3}H]5-HT binding to 5-HT$_7$ receptors in intact cells without affecting total 5-HT$_7$ receptor protein levels. Similar to the effects seen after depletion of cholesterol, gangliosides or sphingolipids, no significant change in affinity was found after Cav-1 siRNA knockdown.

5-HT did not reduce maximum [^{3}H]5-HT binding to 5-HT$_7$ receptors in cells transfected with Cav-1 siRNA, suggesting that the agonist-induced reduction in cell surface expression of 5-HT$_7$ receptors is mediated by a Cav-1-dependent mechanism. Cav-1 siRNA did not counteract the agonist-induced lowering of affinity of [^{3}H]5-HT towards 5-HT$_7$ receptors, indicating a lack of participation in Cav-1 in the desensitization of 5-HT$_7$ receptors. The idea that internalization of 5-HT$_7$ receptors is

dependent on lipid raft integrity was further confirmed by the observation that genistein, a tyrosine kinase inhibitor and blocker of lipid raft-mediated internalization (Pang et al., 2004), also inhibited the 5-HT-induced reduction in maximum [^{3}H]5-HT binding to 5-HT$_7$ receptors. In contrast, treatment with hypertonic sucrose, known to inhibit clathrin-mediated endocytosis (Heuser and Anderson, 1989) failed to inhibit the 5-HT-induced reduction in maximum [^{3}H]5-HT binding to 5-HT$_7$ receptors.

Further evidence that Cav-1 is involved in both constitutive and agonist stimulated cell surface expression of 5-HT$_7$ receptors came from subcellular fractionation experiments. Although Cav-1 siRNA knockdown had no effect on the total levels of 5-HT$_7$ receptor protein, the amounts of both monomeric and dimeric 5-HT$_7$ receptors in the membrane fraction were significantly reduced. Similarly, treatment with 5-HT significantly reduced the amounts of both monomeric and dimeric membranous 5-HT$_7$ receptors. In agreement with the data from the radioligand binding studies, no additive effects of Cav-1 siRNA and 5-HT treatment were found on 5-HT$_7$ receptors in the membrane fraction (Fig. 13).

Altogether our data indicate that 5-HT-mediated actions on cell surface expression of 5-HT$_7$ receptors in HeLa cells involves a caveolae/raft-dependent endocytosis pathway that is independent of clathrin (Le Roy and Wrana, 2005; Nabi and Le, 2003).

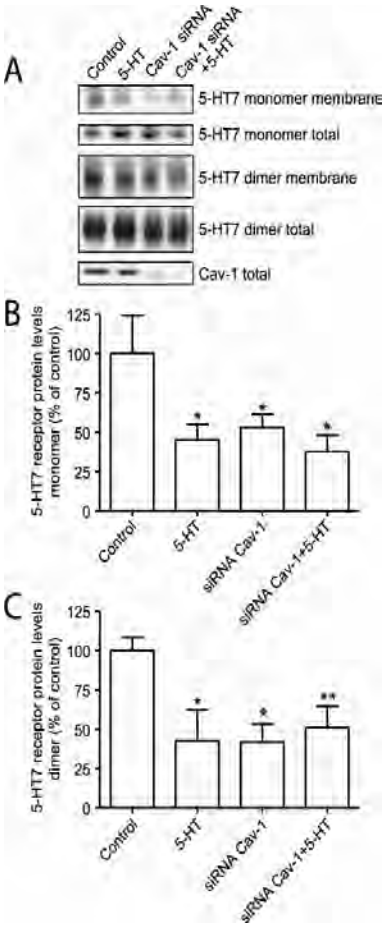
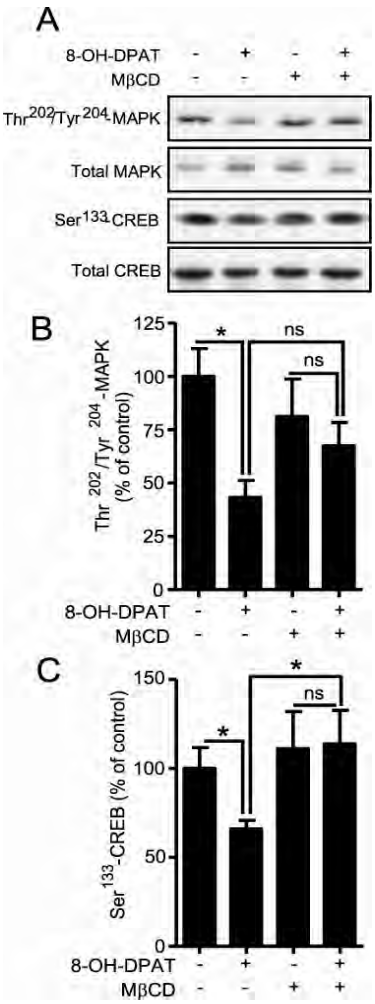


Figure 13. 5-HT, Cav-1 siRNA and both treatments combined results in lowered membranous 5-HT$_7$ receptor protein levels. **A.** Representative immunoblots. **B.** Quantification of monomeric 5-HT$_7$ receptors in the membrane fraction. **C.** Quantification of monomeric 5-HT$_7$ receptors in the membrane fraction. *p<0.05; **p<0.01; ***p<0.001 with One-way ANOVA followed by Newman-Keul's post hoc test.

4.2 5-HT$_{1A}$ RECEPTOR SIGNALING IS ATTENUATED BY CHOLESTEROL REDUCTION

The studies were continued by investigating the involvement of cholesterol levels for endogenous serotonin receptors (Paper IV). 5-HT$_{1A}$ receptors were detected by immunoblotting in human primary neuron cultures whereas 5-HT$_7$ receptors were not. The 5-HT$_{1A/7}$ receptor agonist, 8-OH-DPAT, caused a reduction in Thr^{202}/Tyr^{204}-MAPK and Ser^{133}-CREB phosphorylation (Fig. 14). The effects were concluded to be mediated via 5-HT$_{1A}$ receptor activation, because of its known inhibitory effects on MAPK and CREB phosphorylation (Barnes and Sharp, 1999), and the fact that no 5-HT$_7$ receptors could be detected in the neuronal cell cultures.



Reduction of cholesterol levels by treatment with MβCD prior to 8-OH-DPAT stimulation significantly counteracted the reduction in Ser^{133}-CREB phosphorylation (Fig. 14A, C), giving an indication that cholesterol levels are important for 5-HT$_{1A}$ receptor signal transduction. We could also detect a trend for MβCD treatment to counteract the 8-OH-DPAT-mediated reduction of Thr^{202}/Tyr^{204}-MAPK (Fig. 14A, B). From these data we draw the conclusion that cholesterol levels are important for 5-HT$_{1A}$ receptor signal transduction.

Figure 14. Stimulation of human neuronal cultures with 8-OH-DPAT results in decreased phosphorylation of CREB and MAPK. **A.** Representative immunoblots. **B.** Effects of 8-OH-DPAT and MβCD on Thr^{202}/Tyr^{204}-MAPK. **C.** Effects of 8-OH-DPAT and MβCD on Ser^{133}-CREB. *p<0.05 with One-way ANOVA followed by Newman-Keul's post hoc test for pairwise comparison.

4.3 DOPAMINE D_1 RECEPTOR SIGNALING IS AFFECTED BY CHOLESTEROL SEQUESTRATION

Earlier studies have suggested that the dopamine D_1 receptor is dependent on lipid raft integrity, although the results are conflicting (Kong et al., 2007; Yu et al., 2004). In this study (Paper V) we confirmed the presence of dopamine D_1 receptors in low-density, detergent-insoluble fractions from mouse brain (Fig. 15), along with Cav-1 and $G_{s/olf}$, two proteins known to associate with lipid rafts, as well as the AMPA receptor subunit GluR1. However, two cytosolic proteins, MEK and MAPK, were only found in the high-density, detergent-soluble fractions.

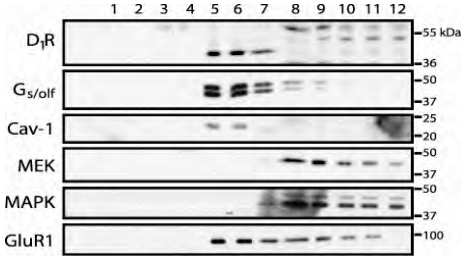


Figure 15. Sucrose density gradient fractionation on mouse brain tissue. The dopamine D_1 receptor, Cav-1, $G_{s/olf}$ and the AMPA receptor subunit GluR1 are all predominantly located in the low-density, detergent-insoluble fractions 5 and 6. The two signaling proteins MAPK and MEK are located in the high-density, detergent-soluble fractions.

Studies on D_1 receptor-mediated intracellular protein phosphorylation were conducted in mouse striatal slices. The dopamine D_1 selective agonist SKF-81297 caused a significant induction of Thr^{202}/Tyr^{204}-MAPK and Ser^{845}-GluR1 phosphorylation (Fig. 16A-C). Pretreatment with MβCD caused a small but significant decrease of total cholesterol levels. This reduction had no effect on basal phosphorylation levels of MAPK or GluR1, but significantly counteracted D_1 receptor agonist-induced phosphorylation of Ser^{845}-GluR1 (Fig. 16B). It also tended to counteract Thr^{202}/Tyr^{204}-MAPK phosphorylation (Fig. 16C).

Previous studies have indicated that endogenous cholesterol may play a role in the aetiology of Parkinson's disease (PD) (de Lau et al., 2006). We therefore also studied effects of cholesterol reduction on dopamine D_1 receptor signal transduction in slices from unilaterally 6-OHDA lesioned animals, a model of PD. 6-OHDA lesion virtually abolished (95.3% reduction) the expression of tyrosine hydroxylase (TH; Fig 16D). No major differences were detected in the 6-OHDA lesioned hemisphere compared to the

intact hemisphere. MβCD treatment significantly counteracted D_1 receptor agonist-induced phosphorylation of Thr^{202}/Tyr^{204}-MAPK (Fig. 16F) and there was a tendency to decrease Ser^{845}-GluR1 phosphorylation (Fig. 16E).

In summary, this study shows that levels of cholesterol play a role in regulating dopamine D_1 receptor signaling, both under normal conditions and in an animal model of PD.

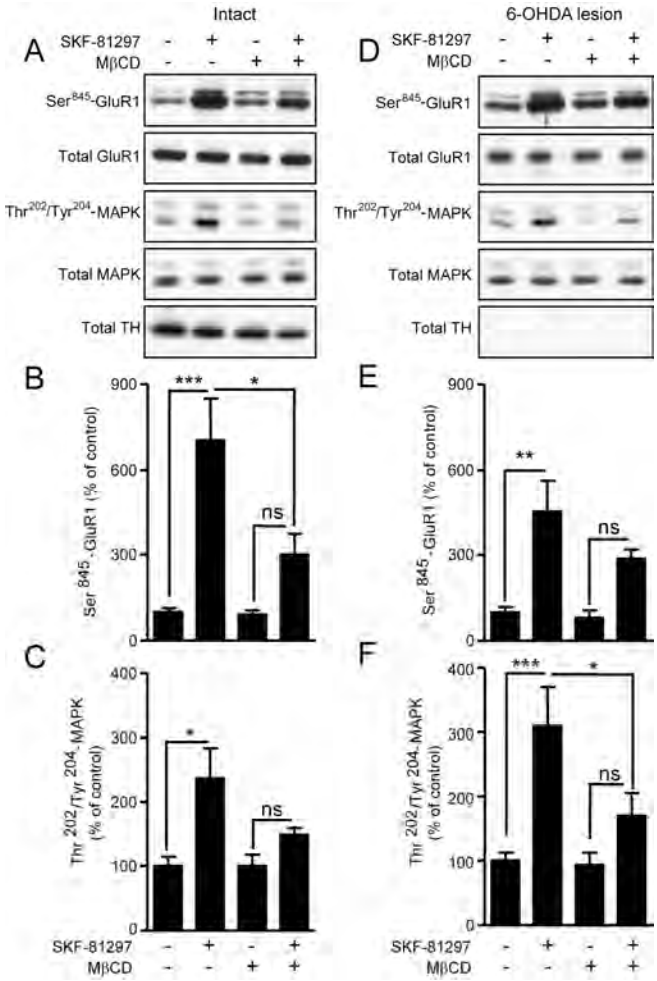


Figure 16. Effects of cholesterol reduction on dopamine D_1 receptor signaling. **A-C.** Intact hemisphere of striatum. **D-F.** 6-OHDA lesioned hemisphere of striatum. *p<0.05; **p<0.01; ***p<0.001 with One-way ANOVA followed by Newman-Keul's post hoc test for pairwise comparison.

5 GENERAL DISCUSSION

As the list of GPCRs that are affected by lipid rafts in the plasma membrane continues to grow it is becoming increasingly clear that these microdomains are indeed important modulators of physiological responses. The initial theories that lipid rafts function as mere signaling platforms have been further confirmed with the discovery that not only receptors but also G proteins and downstream signaling proteins such as adenylate cyclase subtypes, PKC and MAPK also localize in these microdomains (Oh and Schnitzer, 2001; Ostrom and Insel, 2004). This opens up the possibility for diversity in signal transduction pathways. The predominant signaling pathway can be determined by co-localizing certain receptors and downstream effectors in the same microdomain, while excluding others. Furthermore, there are examples of agonist-induced translocation of receptors in or out of lipid rafts. One example is the β_2 adrenergic receptor, which resides predominantly within caveolae in cardiac myocytes. Upon agonist stimulation, the receptor migrates out of these microdomains, and activates downstream effects (Rybin et al., 2000). In the same study it was found that adenylate cyclase subtypes V/VI also resides within caveolae and that cyclodextrin treatment increases β_2 adrenergic receptor-induced cAMP production. This suggests a role of caveolae in regulating the efficacy of signal transduction.

5.1 CHOLESTEROL REGULATES 5-HT AND DOPAMINE RECEPTORS

In the present work the focus has been on the importance of lipid raft components in the regulation of receptors for 5-HT and dopamine. One finding is that recombinantly expressed 5-HT_7 receptors are dependent on lipid raft integrity. The reduced maximum agonist and antagonist binding seen after depletion of cholesterol indicated at an early stage that lipid rafts may be important modulators for 5-HT_7 receptor function (Paper I). This was further confirmed by the fact that depletion on sphingomyelin or gangliosides reduced maximum radioligand binding in a similar manner (Paper II).

The most common way of lowering cholesterol levels in biological assays is by the cholesterol sequestering agent methyl-β-cyclodextrin (MβCD). However, this reagent has been found to be toxic at high concentrations and is a fairly crude way of reducing cholesterol. Indeed we found a dose-dependent reduction in HeLa cell viability after treatment with MβCD. We therefore also investigated the use of cholesterol synthesis inhibitors. With this treatment we utilized compounds that have medical relevance. In

fact, mevastatin (also named, compactin and ML-236B) was the first potent, specific inhibitor of HMG CoA reductase and its derivatives, lovastatin and simvastatin, are widely used in the clinic as cholesterol lowering agents. Indeed we could detect the same reduction in maximum agonist and antagonist binding to 5-HT_7 receptors with HMG-CoA reductase inhibitors as with MβCD, further strengthening the notion that cholesterol levels are important for 5-HT_7 receptor ligand binding.

Not only ligand binding but also signaling via 5-HT_7 receptors was attenuated by cholesterol depletion as demonstrated with studies of phosphorylation of the two signaling proteins CREB and ATF-1 after agonist stimulation (Paper I). However the magnitude of reduction was not as pronounced as in the ligand binding studies. This could be explained by the fact that in this system we utilized a recombinant cell line with high expression of receptors, resulting in the presence of spare receptors, which could explain the discrepancies between the results (Kenakin, 1997).

We have also found that cholesterol levels are important for signaling via natively expressed human 5-HT_{1A} receptors (paper IV). In contrast to the HeLa cells, no toxic effects of the MβCD treatment were found in the human neuronal cultures, suggesting that different cell types display different sensitivity to this treatment.

The present results on the effects of cholesterol depletion on signaling via 5-HT_{1A} receptors (Paper IV) are in agreement with previous studies on the 5-HT_{1A} receptor. It has been demonstrated that both agonist and antagonist binding to 5-HT_{1A} receptors is attenuated by cholesterol depletion in bovine hippocampal membranes (Pucadyil and Chattopadhyay, 2004; Pucadyil and Chattopadhyay, 2005). Furthermore, a recent study showed that 5-HT_{1A} receptors partition into rafts in stably transfected NIH-3T3 cells and that this localization is disrupted by cholesterol depletion (Renner et al., 2007). The localization of 5-HT_{1A} in lipid rafts depended on palmitoylation of the C-terminal tail of the receptor.

MβCD treatment also affected signaling via dopamine D_1 receptors, expressed in the mouse striatum (Paper V). In accordance with previous data (Snyder et al., 2000; Valjent et al., 2000) we found that MAPK and the AMPA receptor subunit GluR1 are phosphorylated upon D_1 receptor activation in mouse striatum. We also detected dopamine D_1 receptors in the low density fractions of a sucrose density gradient, in agreement with other studies (Kong et al., 2007; Yu et al., 2004). In contrast to previously reported data (Ostrom and Insel, 2004), we did not detect MAPK in the detergent-insoluble fractions. This could be due to methodological differences between

our study and previous reports. For instance the detergent of choice could affect the protein composition of the detercted DRM's (Chamberlain, 2004).

In this study we found that even small decreases of cellular cholesterol can induce dramatic effects on intracellular signaling. The cholesterol decreases in these experiments were only 15 %, compared to both HeLa cells and primary neuronal cultures, were the reduction in cholesterol levels was much more pronounced. Nevertheless, we found that MβCD treatment reduced dopamine D_1 agonist-induced phosphorylation of both MAPK and GluR1, suggesting that these receptors are localized in lipid rafts. Moreover, in the 6-OHDA lesioned hemisphere of striatum, a model of PD, there was no significant differences in the effects caused by MβCD treatment. This would be expected, since the dopamine receptors in striatum should not be affected in PD. Rather the dopamine released from neurons projecting from substantia nigra pars compacta is diminished. However, theoretically the receptor properties could be changed due to reduced endogenous stimulation. These changes could involve an up-regulation of receptor number as well as changes in affinity of existing receptors.

Previous studies on the relation between dopamine D_1 receptors and lipid rafts have focused on the interaction between the D_1 receptor and caveolins (Kong et al., 2007; Yu et al., 2004). Recently, it was found, in COS-7 cells and rat brain, that D_1 receptors are found in lipid rafts and interact with Cav-1 via a binding motif in transmembrane domain 7 (Kong et al., 2007). Through this interaction, Cav-1 regulates both trafficking and signaling of the D_1 receptor in COS-7 cells. In contrast to our present data, it was found that pretreatment with MβCD potentiates D_1 receptor-mediated signal transduction.

Conversely, another study found that D_1 receptors located in low-density membrane fractions are able to activate adenylate cyclase to a greater extent than receptors in high density fractions. Furthermore, co-localization between D_1 receptors and Cav-2 was increased after treatment with the D_1 receptor agonist fenoldopam, in both HEK-293 and RTPC cells. In better agreement with our present data this study also showed that signaling via D_1 receptors is attenuated by siRNA-mediated knockdown of Cav-2 protein levels (Yu et al., 2004). Certainly, there is a difference in cell background between these two studies, which could explain the discrepancies in the results obtained. The two studies have also studied the importance of different caveolin proteins. Furthermore, there could be species differences involved, further adding to the different conditions. Nevertheless, published studies and our present data all indicate

that dopamine D_1 receptor function is dependent on lipid raft integrity and can be added to the growing list of G protein-coupled receptors (Insel et al., 2005) that are regulated by these cholesterol-containing microdomains. Further studies are needed to determine the mechanisms of lipid raft involvement with certainty.

In neither the human primary neuronal cultures nor the striatal mouse slices was it possible to use HMG-CoA reductase inhibitors, due to the prolonged treatment that is necessary for these reagents. We were therefore limited to the use of MβCD, which gives effects on cholesterol levels after short-term treatment. It would be relevant to investigate clinically used statins in primary cultures, tissue or even whole animals to ascertain central effects of statin treatment on signaling via 5-HT and dopamine receptors.

Statins are widely used as cholesterol lowering agents to treat atherosclerosis and cardiovascular disease. However, it has been suggested that these agents can give side effects unrelated to their effect on plasma cholesterol. Lipophilic statins, such as simvastatin and lovastatin can pass through the blood brain barrier (BBB), which could explain possible central side effects. In contrast, the hydrophilic compound pravastatin does not pass the BBB (Saheki et al., 1994). It has been suggested that statins can control neuroinflammation and could therefore be useful in early treatment of multiple sclerosis (MS) and Alzheimer's disease (Adamson and Greenwood, 2003). Furthermore, recent reports suggest a role of lipid and cholesterol metabolism in the pathogenesis of PD. Higher serum levels of total cholesterol were associated with a significantly decreased risk of PD (de Lau et al., 2006). Consistent with this is our finding that signaling via dopamine D_1 receptors is impaired by reduced cholesterol levels (Paper V), although there could be a number of other mechanisms underlying the effect of cholesterol levels in the pathogenesis of PD.

Several studies have also suggested a connection between cholesterol levels and depression and anxiety (reviewed in (Papakostas et al., 2004)). It has been found that reduced serum cholesterol was correlated with major depressive disorder and that one possible mechanism was a direct effect on the 5-HT system in the brain. Both reduced levels of 5-HT and a downregulation of 5-HT receptors or decreased activity of the 5-HT transporter as a consequence of reduced membranous cholesterol has been indicated. The connection between these studies and our current data on the 5-HT_7 and 5-HT_{1A} receptors is still elusive, but it's notable that our results correlate well with the proposed hypotheses for the involvement of cholesterol in 5-HT related pathological states.

Many studies concerning lipid rafts and their importance for GPCR signaling have been performed in recombinant cell lines. The possibilities to treat these cells and use different genetic manipulations are greater, as well as the fact that the endogenous pool of receptors and proteins are usually better defined. However, a limitation of these studies is that they give only an indication of the importance of lipid rafts in receptor signaling *in vivo*. More studies need to be performed in native system in order to determine the real importance for liquid ordered microdomains. A recent study showed that lipid rafts play an important role also in intact neurons (Hering et al., 2003). Specifically, lipid rafts were shown to be important for maintenance of normal synapse density and morphology, as well as AMPA receptor stability in rat hippocampal neurons. The studies on ligand binding to 5-HT_{1A} receptors in bovine hippocampal membranes, as previously mentioned, also increase the knowledge on how lipid rafts regulate endogenous GPCR function (Pucadyil and Chattopadhyay, 2004; Pucadyil and Chattopadhyay, 2005).

5.2 CAVEOLAE AND 5-HT_7 RECEPTOR TRAFFICKING

Trafficking of receptors is an important process that regulates cell surface expression and signaling capacity of certain receptor-effector pathways. It is now clear that the classical endosomatic pathway, although by far the best characterized, is not the only route of internalization that can occur. Clathrin-independent internalization can occur via caveolae or lipid rafts (see section 1.4.3). However, lipid raft localization of receptors does not seem to be a static situation. Therefore it is premature to assume that mere localization within lipid rafts or caveolae indicates lipid raft-mediated internalization.

As exemplified earlier by β_2 adrenergic receptors, there are several examples of migration in or out of lipid rafts upon agonist stimulation and the internalization route can therefore be divided into four possible scenarios: i) The receptor resides in lipid rafts and is internalized via lipid raft-dependent pathways. One example of this is the endothelin receptor, ET_A (Chun et al., 1994). ii) The unstimulated receptor resides in non-raft areas of the membrane, but migrates to lipid rafts upon agonist stimulation and is then internalized via this pathway. This is the case for somatostatin receptor, sst2 (Krisch et al., 1998; Mentlein et al., 2001). iii) The opposite scenario is also possible, where the activation of a receptor induces migration out of lipid rafts and the receptor is internalized via the classical endosomatic pathway, as is the case for the β_2 adrenergic receptor (Rybin et al., 2000). iv) Finally, there is a possibility that a receptor is located

outside of lipid rafts in the resting state. Upon activation it migrates to lipid rafts to activate certain signaling pathways. Finally it moves out again to be internalized via conventional clathrin coated vesicles, as has been reported for the Angiotensin II receptor (Wyse et al., 2003).

From the present work it seems evident that constitutive cell surface expression of 5-HT_7 receptors is dependent on lipid raft integrity and that agonist stimulated internalization of the receptors is mediated via a mechanism that is dependent on Cav-1 and independent on clathrin. We have found no evidence for agonist-induced migration in or out of lipid rafts, although it is possible that this could occur. Sucrose density gradient fractionation (Paper III), demonstrated that 5-HT_7 receptors are present in both high and low density fractions, indicating that in order to be internalized via a lipid raft-mediated pathway the receptors need to migrate from the high density to the low density fraction at some point. However, it is not resolved when and via which mechanism this would occur. It can also not be excluded that the presence of 5-HT_7 receptors in the high density fraction could be a consequence of overexpression of the receptors and that lower expression levels would give a more clear distribution pattern.

As discussed in the introduction, caveolae-mediated internalization of receptors is dependent on dynamin and the actin cytoskeleton. Whether these two components play a role in 5-HT_7 receptor internalization is unclear, but probable if 5-HT_7 receptors are internalized via caveolae. The dynamin dependence of 5-HT_7 receptor internalization has not been studied in this work. However, by using the dynamin II dominant negative K44A mutant we would not be able to distinguish between caveolae-mediated and clathrin-mediated endocytosis since these are both dependent on dynamin. We did, however, confirm that hypertonic sucrose could not inhibit the reduction in agonist-induced reductions in maximum 5-HT binding to 5-HT_7 receptors, indicating that the agonist-induced receptor internalization is not dependent on clathrin. Furthermore the fact that genistein could inhibit the same phenomena suggests a lipid raft-mediated internalization process. Taken together, when combined with the cell fractionation and radioligand binding data after Cav-1 siRNA transfection, it is probable that internalization of 5-HT_7 receptors occurs via a caveolae-dependent mechanism. We can therefore propose a model of 5-HT_7 receptor internalization as demonstrated in figure 17.

Previous studies have demonstrated that siRNA-mediated knockdown of Cav-1 reduces signaling via 5-$HT_{(2A)}$ receptors and that this is a result from a direct interaction between Cav-1 and the 5-$HT_{(2A)}$ receptor (Bhatnagar et al., 2004). In the present work we demonstrated similar effects of siRNA-mediated knockdown of Cav-1 on 5-HT_7 receptors. We could not confirm a direct interaction for 5-HT_7 receptor, but did find both proteins in low density fractions on a sucrose gradient (Paper III). This might not be a direct evidence for caveolar co-localization, since this method does not distinguish between caveolae and lipid rafts. Nevertheless, in combination with the results on radioligand binding and subcellular receptor localization after siRNA-mediated knockdown of Cav-1 we propose that, at least in these HeLa cells, 5-HT_7 receptors are part of a larger complex bound to Cav-1.

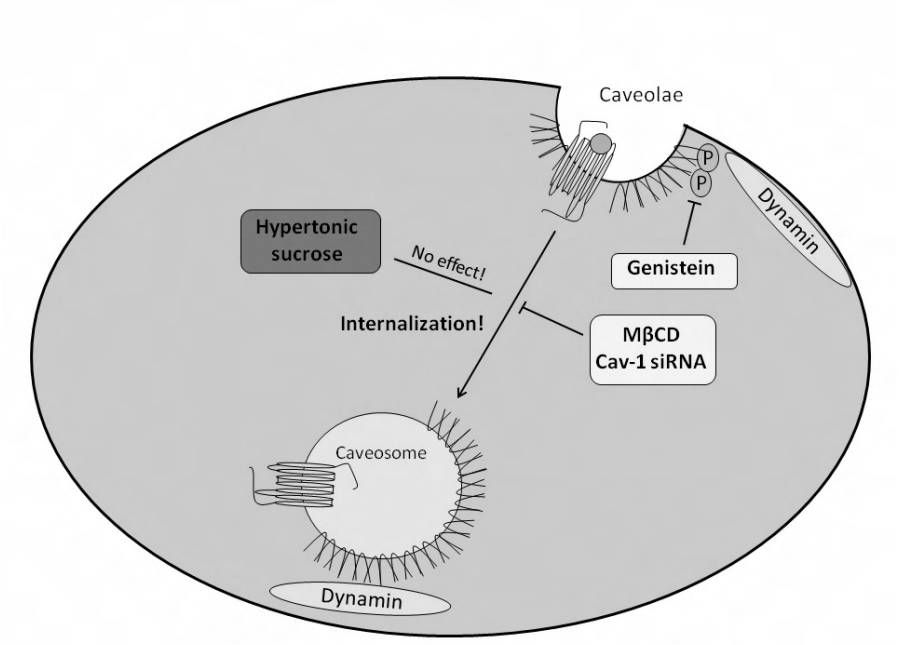


Figure 17. Our proposed model of 5-HT_7 receptor internalization in HeLa cells. The localization in caveolae seems evident and that 5-HT_7 receptors are internalized via a caveolae-dependent mechanism. The role of dynamin is elusive, although it seems probable that the internalization is also dynamin-dependent. In addition to their inhibiting effect on 5-HT_7 receptor internalization, MβCD and Cav-1 siRNA are also affects constitutive membrane expression of 5-HT_7 receptors. Treatment with PDMP to reduce sphingomyelin, Fumonisin B_1 to reduce gangliosides or HMG-CoA reductase inhibitors to reduce cholesterol levels also affect constitutive membrane levels of 5-HT_7 receptors, although the precise role in 5-HT_7 receptor internalization remains to be elucidated.

6 CONCLUDING REMARKS

From the data presented in this thesis we can draw some general conclusions:

- Cholesterol in the plasma membrane is an important regulator of agonist and antagonist binding to 5-HT_7 receptors.

- Other lipid raft components, namely gangliosides and sphingolipids, are also involved in the regulation of agonist and antagonist binding to 5-HT_7 receptors.

- Cholesterol depletion attenuates signaling via 5-HT_7 receptors.

- Some 5-HT_7 receptors co-localize with Cav-1 in low-density fractions of a sucrose density gradient.

- Together these data suggests that 5-HT_7 receptors localize in lipid rafts/caveolae.

- Cav-1 is important for constitutive membrane expression of 5-HT_7 receptors.

- 5-HT_7 receptors undergo agonist-induced receptor internalization via a pathway that is dependent on Cav-1 and independent on clathrin.

- Signaling via natively expressed human 5-HT_{1A} receptors is attenuated by cholesterol depletion.

- Dopamine D_1 receptors co-localize with Cav-1 in low-density fractions of a sucrose density gradient, suggesting lipid raft localization.

- Signaling via natively expressed mouse dopamine D_1 receptors is attenuated by sequestering of cholesterol.

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