

7TMR-RAFT: A Database for the Localization of Seven-Transmembrane Receptors to Lipid Rafts

By: Ramona Kaviani

Supervisor: Dr. Keyvan Sedaghat

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Department of Biology
Faculty of Science
Bahá'í Institute for Higher Education (BIHE)**

ABSTRACT

Seven-transmembrane receptors (7TMRs), also known as G-protein-coupled receptors (GPCRs), which are the target of a large number of drugs, are involved in many physiological and pathophysiological processes. It has been demonstrated recently that these receptors do not diffuse freely in the plasma membrane, but instead localize within distinct microdomains known as lipid rafts. These lipid rafts function as a platform for a range of cellular processes, such as receptor trafficking, signal transduction, internalization and desensitization. As a growing body of data indicates that GPCRs localize in lipid rafts, I have in this research project identified the GPCRs that have been reported to be associated with lipid rafts, and categorized their behavior in response to agonists, and the role of lipid raft localization in signal transduction, internalization and trafficking of the receptors. I have also developed a database, referred to as 7TMR-RAFT, (www.7tmr-raft.org) that supports searching and browsing of data pertaining to receptors associated with lipid rafts.

Key words: G-protein-coupled receptors, seven-transmembrane receptors, lipid raft, caveola, compartmentalization, signal transduction

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LIST OF ABBREVIATIONS

2-AG: 2-Arachidonoylglycerol
7TMRs: Seven-transmembrane receptors
A1R: Adenosine A1 receptor
Ach: Acetylcholine
ACs: Adenylyl cyclases
AD: Alzheimer's disease
ADA: Adenosine deaminase
AEA: Anandamide
ALS: Amyotrophic lateral sclerosis
ALXR: Lipoxin A4 receptor
AM: Alveolar macrophage
APC: Activated protein C
ASM: Airway smooth muscle
AT1: Angiotensine type 1
AT1Rs: AT1 receptors
AT2R: Angiotensine type 2
AVP: Vasopressin receptors
A β : Amyloid beta
B1R: Bradykinin B1 receptor
B2R: Bradykinin B2 receptor
BACE: β -Secretase
BAEC: Bovine aortic endothelial cell
BDNF: Brain-derived neurotrophic factor

BHK: Baby hamster kidney

BLT-1: B leukotriene receptor-1

BPH: Benign prostatic hyperplasia

C5a: Complement cleavage product

CASR: Calcium-sensing receptor

Cav-1: Caveolin-1

CCK: Cholecystokinin

CCPA: 2-chloro-N⁶-cyclopentyladenosine

CCR5: CC chemokine receptor type 5

CG: Chorionic gonadotropin

cGNRHR : Chicken gonadotropin releasing hormone receptor

CHO: Chinese Hamster Ovary

CLR: Caveolae-related lipid rafts

CNS: Central nervous system

CPu: Caudate putamen

CXCR4: -X-C chemokine receptor type 4

D1R: D1 dopamine receptor

DAG: Diacylglycerol

DIGs: Detergent-insoluble glycosphingolipid-cholesterol-enriched complexes

DLD-1 cell: Human colon cancer epithelial cell

DPCPX: 8-cyclopentyl-1, 3-dipropylxanthine

DRIP78: Dopamine receptor interacting protein of 78 kDa

EDG1: Endothelial differentiation gene 1

EGFR: Epidermal growth factor receptor

eNOS: Nitric oxide synthase

Env: Envelope

EPR: Electron paramagnetic resonance

fMLP: Formyl-methionyl-leucyl-phenylalanine

FPR: Formyl peptide receptor

FPRL-1: Formyl peptide receptor like-1

FRAP: Fluorescence recovery after photobleaching

FRET: Fluorescence resonance energy transfer

GABA: γ -aminobutyric acid

GalCer: Galactosyl-ceramide

GEM: Glycosphingolipid-enriched microdomains

GLP-1R: Glucagon-like peptide 1 receptor

GLP-2R: Glucagon-like peptide-2 receptor

GnRH: Gonadotropin releasing hormone

GnRHR: Gonadotropin releasing hormone receptor

GPAECs: Guinea pig aortic endothelial cells

GPCR: G protein-coupled receptor

GPI: Glycosylphosphatidylinositol

HAoSMC: Human aortic smooth muscle cells

hCG: Human chorionic gonadotropin

hD1Rs: Human D1 dopamine receptors

HEK293: Human embryonic kidney

HepG2: Liver hepatocellular carcinoma

HIV-1: Human immunodeficiency virus type-1

IGF-1: Insulin-like growth factor-1

IKK: Inhibitor- κ B

IP: Inositol phosphate

IP3: Inositol triphosphate

IPC: Ischemic preconditioning

IRS-1: Insulin receptor substrate-1

KOR: Kappa-opioid receptor

KW-6002: Istradefylline

LH: Luteinizing hormone

LHR: Luteinizing hormone receptor

LPA: Lysophosphatidic acid

LTB4: Leukotriene B4

LXA4: Lipoxin A4

MAchR: Muscarinic acetyl choline receptor

MALT: Mucosal associated lymphoid tissue

MAPK: Mitogen-activated protein kinase

MCH1R: Melanin-concentrating hormone receptor 1

MDCK: Madin-Darby Canine Kidney

mGluRs: Metabotropic glutamate receptors

mGnRHR 1: Mammalian type 1 gonadotropin releasing hormone receptor

Min 6: Mouse insulinoma cell line

MIP-1 β : Macrophage inflammatory protein 1 β

MLC: Myosin light chain

MOR: μ -Opioid receptor

n-3 PUFA: Omega-3 polyunsaturated fatty acid

NIH 3T3 cell: Mouse embryonic fibroblast cell line

NO: Nitric oxide

OT: Oxytocin

OTR: Oxytocin receptor

PAC1R: Pituitary adenylate cyclase-activating peptide receptor

PACAP: Pituitary adenylate cyclase-activating peptide

PAF: Platelet-activating factor

PAFR: Platelet-activating factor receptor

PARs: Protease-activated receptors

PBMC: Peripheral blood mononuclear cell

PC12 cells: Derived from pheochromocytoma of rat adrenal medulla

pCMVECs: Porcine cerebral microvascular endothelial cells

pCMVECs: Porcine microvascular endothelial cells

PDGFR β : Platelet-derived growth factor receptor β

PDMP: ($\pm$)-*threo*-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol

PG: Prostaglandin

PGI₂: Prostacycline

PLC: Phospholipase C

PMBC: Peripheral blood mononuclear cells

PrP^c: Prion protein

RAMP: Receptor activity modifying protein

RANTES: Regulated on activation normal T-cell express and secreted

R-PIA: *N*6-(*R*)-Phenylisopropyl adenosine

RPTCs: Renal proximal tubule cells

S1P1R: Sphingosine 1-phosphate receptor

Saos-2: Osteosarcoma cell line

SDF-1 α : Stromal cell-derived factor 1 α

SFKs: Src-family kinases

SHP-2: SH-2 domain-containing tyrosine phosphatase-2

SST: Somatostatin

SSTR: Somatostatin receptor

TAP: Tandem affinity purification

TF: Tissue factor

TGF: Tubuloglomerular feedback

TRH: Thyrotropin releasing hormone

TRHR: Thyrotropin-releasing hormone receptor

TSHR: Thyrotropin receptor

VCMCs: Vascular smooth muscle cells

YFP: Yellow fluorescent protein

α_1 AR: α_1 -Adrenoreceptors

β AR: β -Adrenoreceptor

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Chapter 1: Introduction

G-protein coupled receptors (GPCRs), also known as seven-transmembrane receptors (7TMRs), are the largest and most versatile family of membrane proteins. These receptors respond to a wide range of stimulants, such as neurotransmitters, hormones, ions, chemokines, photons of light, odors, etc ¹. In addition to their role in physiological regulation, they constitute 50-60 % of current drug targets ².

Upon GPCR activation, GPCRs couple to distinct classes of heterotrimeric G proteins that consist of the three subunits of α , β and γ . Receptor activation promotes the exchange of GDP for GTP from α -subunit of G protein. Indeed, free α -GTP and $\beta\gamma$ -subunits will form that can modulate the activity of diverse effector enzymes or ion channels ^{3,4} (Fig.1).

Based on sequence homology, the G_α subunit can be subdivided into four classes each of which regulates a particular effector system: G_s stimulation activates adenylyl cyclase and regulates certain Ca^{2+} channels; G_i inhibits adenylyl cyclase activity; $G_{12/13}$ regulates the GTP binding protein, and $G_{q/11}$ stimulates phospholipase C activity ^{3,4}. In addition to the role of G_α in regulating many signaling molecules, $G_{\beta\gamma}$ also interacts with many effector molecules such as GTPases, GPCRs, adenylyl cyclases, PI3 kinases, voltage-sensitive calcium channels and molecules involved in the mitogen-activated protein kinase (MAPK) pathway ³.

Until 1997, and based on the Singer-Nicolson fluid mosaic model ⁵, it was assumed that proteins and receptors diffuse freely in a sea of lipids in the plane of the cell membrane. Thus, according to this model GPCRs could diffuse randomly and freely in the membrane and activate G-proteins and effectors. However, in 1997, Simons and Ikonen presented a new model ⁶ for the cell membrane, being composed of distinct domains named “lipid rafts” that are rich in sphingolipids and cholesterol compared to the surrounding membrane ⁷ (Fig. 2). This new feature of the membrane contradicts the Singer-Nicolson fluid mosaic model because the lipid rafts prevent free diffusion of proteins and receptors in the cell membrane ⁶. It is now considered that proteins and receptors localize to lipid rafts, and that these lipid rafts function as platforms for a range of cellular signaling processes ^{4,8}. (Fig. 3).

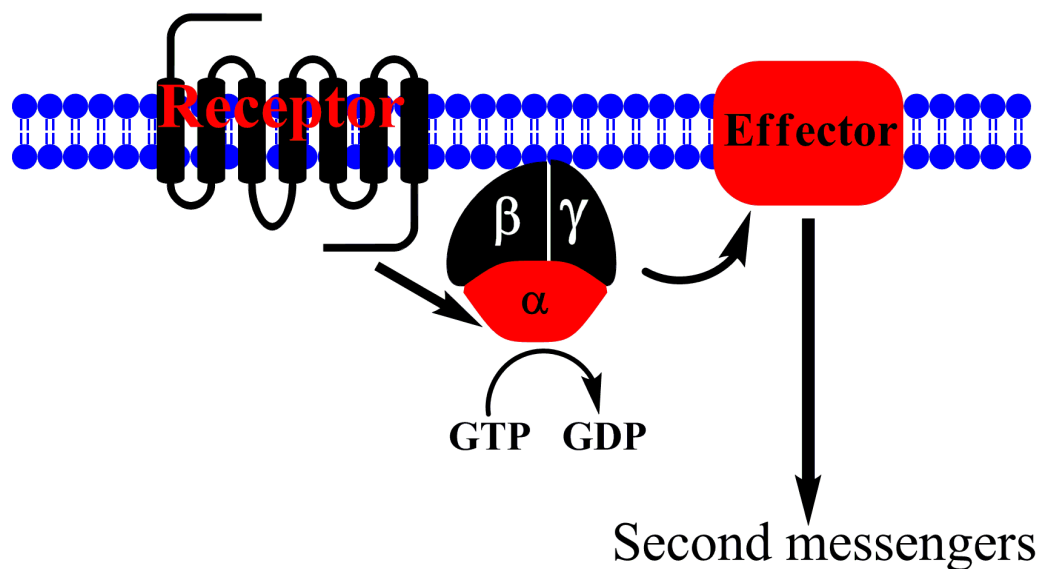


Figure 1. Signaling cascade of G protein-coupled receptors: GPCRs, heterotrimeric G proteins and membrane-bound effectors are three main components of this system.

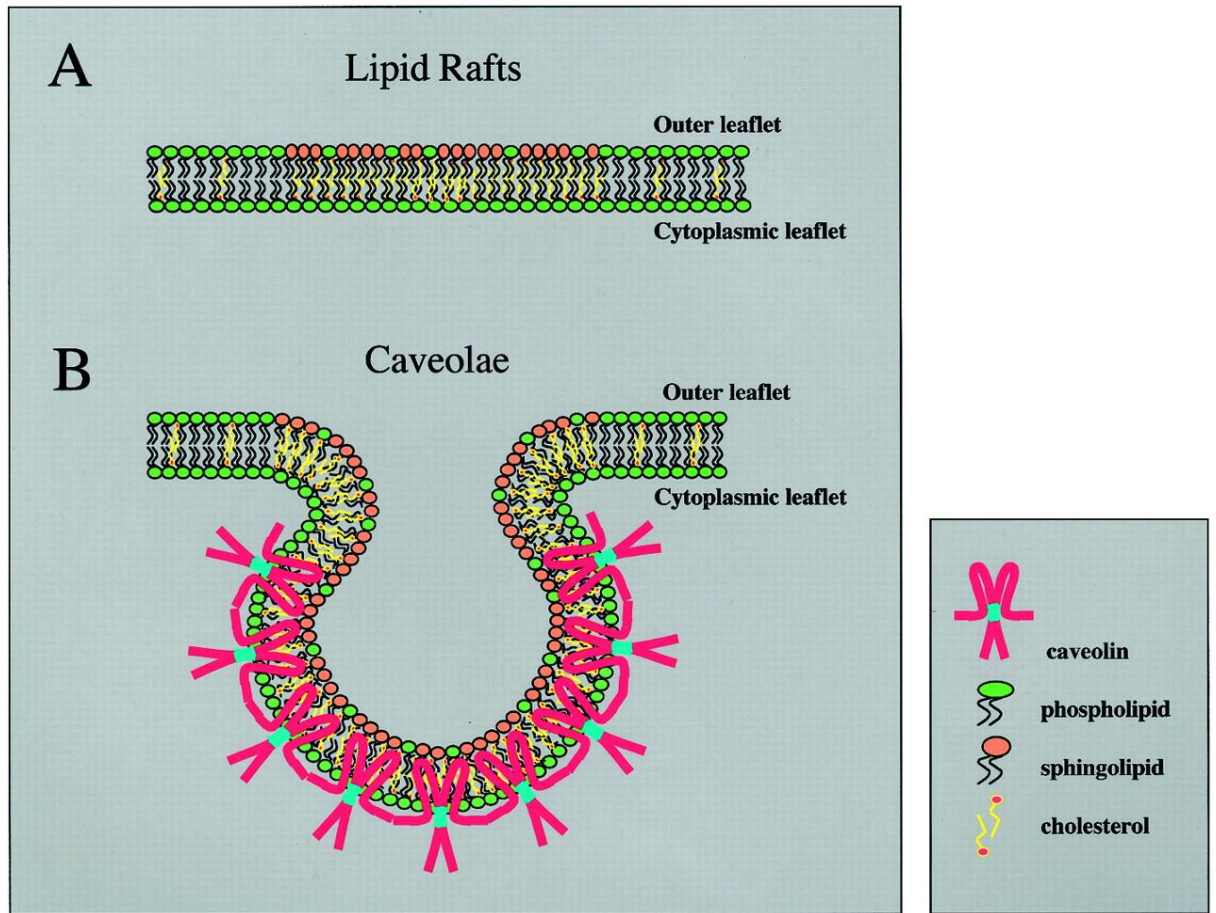


Figure 2. Cholesterol and sphingolipid-rich membrane microdomains (lipid rafts): A. Caveolin-free rafts. B. Caveolae.

Adapted from: Razani, B., Woodman, S.E. & Lisanti, M.P. Caveolae: from cell biology to animal physiology. *Pharmacol*

Rev 54, 431-67 (2002).⁷

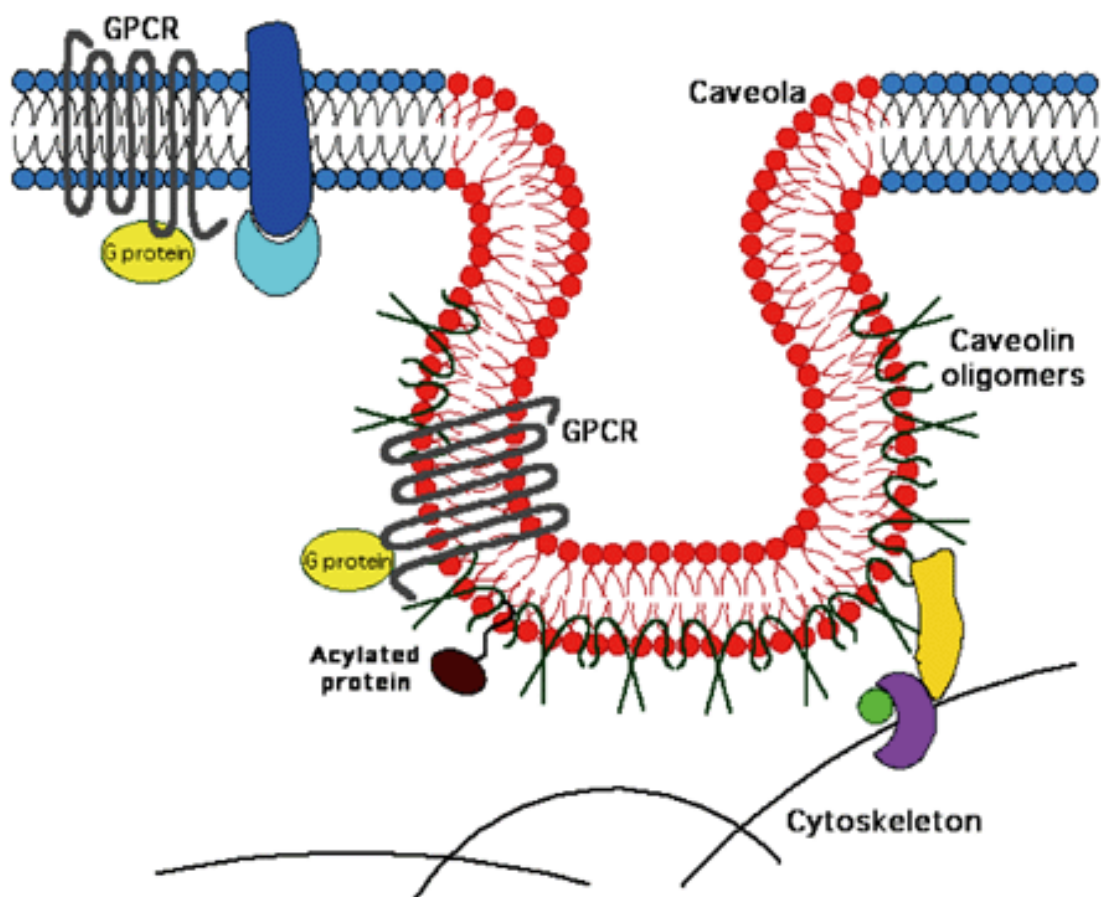


Figure 3. Schematic representation of the lipid and protein organization of a caveola: Sphingolipid- and cholesterol-rich domain is shown in red and nonraft membrane domains are in blue. Adapted from: Ostrom, R.S. & Insel, P.A. The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. *Br J Pharmacol* 143, 235-45 (2004) ⁴.

Lipid rafts

Lipid rafts are membrane microdomains that are rich in cholesterol and sphingolipids⁷. As sphingolipids are more saturated and possess longer lipid tails in, these membrane domains are thicker and less fluid compared to the rest of the surrounding membranes⁹. These physical properties lead to a concentration of specific membrane proteins within the lipid rafts while excluding others⁸.

Two types of lipid rafts exist: planar or non-caveolar lipid rafts and caveolae¹⁰ (Fig. 2). Both planar lipid rafts and caveolae differ from other membranes in their composition and in the way that the lipid-acyl chains packed together¹¹. The size of lipid rafts, published in different reports, ranges from 4 to 150 nm depending on the method and the cell type used for analysis¹². Each lipid raft contains approximately 3000 lipid molecules and 10-30 proteins^{11, 13}.

Caveolae (originally termed as plasma membrane vesicles) are Ω-shaped invaginations of the plasma membrane that were first described by Palade in 1953¹⁴. They are known to contain a 21~22-kDa protein (VIP21) named caveolin^{15,16}. Caveolae have been detected in many cell types, but they are most abundant in endothelial cells, epithelial cells, adipocytes, fibroblasts, type I pneumocytes and muscle cell types^{17, 18}. Unlike caveolae, planar lipid rafts lack distinctive morphological features and do not contain caveolin. The presence of caveolae in neurons is controversial and it seems that neurons only contain non-caveolar rafts and flotillin, a protein that is analogous to caveolin¹⁰.

Caveolin, a major component and marker of caveolae, is a 178 amino acid protein consisting of three domains: an N-terminal domain of 101 amino acids, a membrane spanning segment of 33 amino acid residues and a C-terminal region of 44 amino acids^{19,20} (Fig. 4). The N-terminal domain contains a scaffolding domain (CSD) of ~20 amino acid residues that binds to caveolin-binding motifs [Φ X Φ XXXX Φ and Φ XXXX Φ XX Φ , where Φ is an aromatic amino acid (tryptophan, phenylalanine or tyrosine) and X is any amino acid] that exist within most caveolae associated proteins^{20, 21}.

The caveolin gene family has three distinct members: CAV-1, CAV-2 and CAV-3, which are responsible for encoding the proteins caveolin-1, caveolin-2 and caveolin-3, respectively²⁰. The level of caveolin expression has direct relation with the morphology of caveolae¹⁹. Caveolin-1 and caveolin-2 are expressed in epithelial and endothelial cells, fibroblasts, adipocytes and smooth muscles^{22, 23}. Although caveolin-2 is coexpressed with caveolin-1, there is evidence that their subcellular distribution is different: caveolin-1 is mostly localized to the cell surface while caveolin-2 is mostly found in the Golgi complex²⁴. Caveolin-3, also known as M-caveolin, is expressed in cardiac, skeletal and smooth muscle²³. However, Ikezu and coworkers showed that caveolin-3 expression is not limited to muscle cells but is also expressed in brain astroglial cells²⁵. Expression of caveolin-1 in cells lacking caveolae induces formation of caveolae²⁶. In agreement, disruption of caveolin-1 or caveolin-3 causes a loss of caveolae in cells where they normally exist^{27, 28}. These results demonstrate the importance of caveolin-1 and caveolin-3 in caveolar formation²⁹.

The mechanisms of raft association are not yet fully understood. It is proposed that three mechanisms may contribute to lipid raft targeting: 1) Post-translational modifications such as N-linked palmitoylation or myristoylation, or glycosylphosphatidylinositol (GPI) anchors, lead to protein partitioning into lipid rafts while proteins with prenyl groups tend to be excluded from this domain ^{4, 8, 30}. 2) Some proteins may be targeted to lipid rafts via a direct interaction with caveolin scaffolding domains by their caveolin binding motifs ^{4, 8}. 3) The structural components of hydrophobic residues of proteins increase the protein tendency to pack into an ordered lipid environment ⁴.

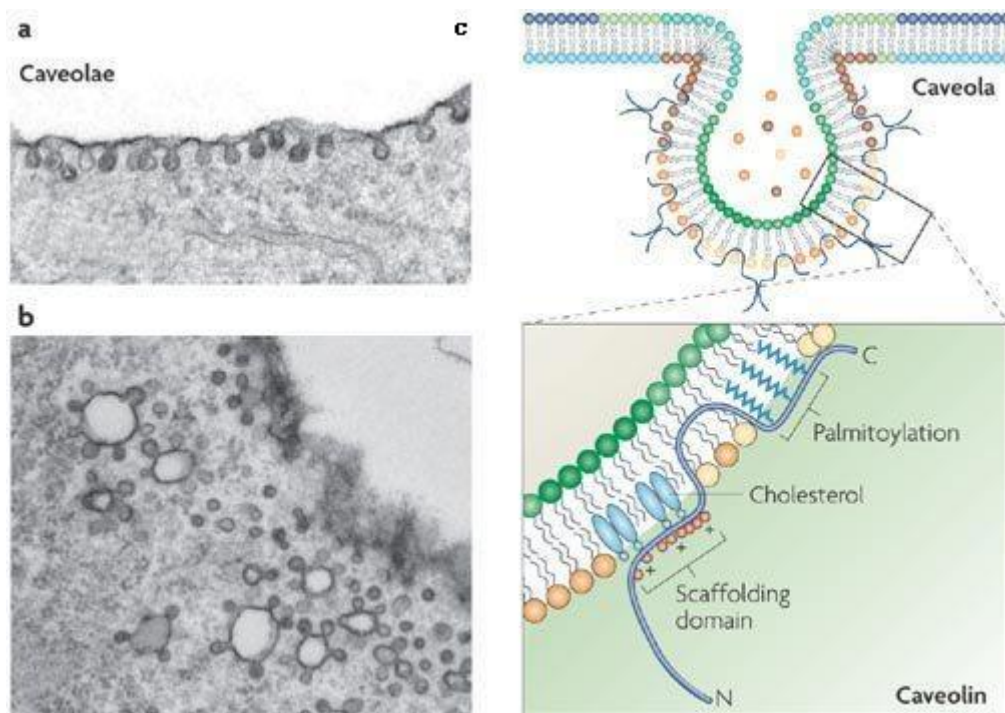


Figure 4. Caveolae and caveolin structures. Electron micrographs in a) and b) show caveolae in adipocytes labeled with an electron-dense marker; c) illustration of caveolin association with the plasma membrane, the cytoplasm-facing N and C terminals, and the hairpin intramembrane domain inserted within the lipid bilayer membrane. Positively charged (+) and aromatic amino acids (red circles) in the scaffolding domain might play a role in the interaction of caveolin with cholesterol. Adapted from: Parton, R.G. & Simons, K. The multiple faces of caveolae. *Nat Rev Mol Cell Biol* 8, 185-94 (2007)³¹.

Studying lipid rafts

Microscopic approaches can be useful tools for studying lipid rafts and caveolae. As an example, confocal microscopy has been used to detect colocalization of two proteins.

Indeed, colocalization with lipid rafts or caveolae markers, like flotilin or caveolin, allows deduction of the lipid raft or caveolae localization. The existence of lipid rafts and caveolae can also be defined by novel microscopic approaches, such as atomic force microscopy, fluorescence resonance energy transfer (FRET), and fluorescence recovery after photobleaching (FRAP) ³²⁻³⁵.

In addition to microscopic analyses, lipid rafts and caveolae can be isolated from cultured cells and tissues by biochemical approaches based on their insolubility in nonionic detergents (at 4° C) and their low buoyant density. Generally, biochemical methods for purifying lipid rafts and caveolae can be classified as detergent- and non-detergent-dependent. Herein, I briefly describe these two methods ^{33, 36, 37}.

Isolation of lipid rafts with nonionic detergents was first described by Brown and Rose in 1992 ³⁸. In general, cells are extracted in 0.5 to 1% Triton X-100 and then subjected to sucrose density gradient centrifugation. Although the most widely used detergent is Triton X-100, other detergents such as Brij96, Brij98, CHAPS, Lubrol WX, and Triton X-114 have been employed for this purpose. The purification of lipid rafts with detergents like Triton X-100 should be accomplished at 4° C because rafts are solubilised in Triton X-100 at 37° C. In all cases the rafts remain in the low density fractions of gradients ^{36, 37, 39}.

Although the detergent-dependent method is one of the most widely used, is easy and applicable for numerous types of cells, there are a number of problems regarding its use. The use of detergents is thought to induce artifacts and in addition, some proteins that

have been detected by other criteria to be associated with lipid rafts are solubilized in Triton X-100 ^{40, 41}.

There are two reported methods for isolating lipid rafts without using detergents which are based on gradient separation. One, which first developed by Song *et al.* in 1996, uses sodium carbonate buffer and homogenization to solubilize cell membranes ¹⁹. The resulting membrane fractions are then subjected to sucrose density gradient centrifugation. The other method described by Smart *et al.* in 1995, uses a two-step purification process ⁴². First, the plasma membrane is isolated from the remainder of the intracellular proteins by Percoll gradient centrifugation and the membrane fraction is then subjected to two rounds of OptiPrep density gradient centrifugation to obtain the lipid raft fraction ^{36, 37, 42-44}.

The function of lipid rafts can be assessed by tools that disrupt lipid rafts structure, such as through the use of methyl- β -cyclodextrin, which is a cholesterol depleting drug, or filipin, a cholesterol sequestering antibiotic. Treatment of cells with these raft-disrupting agents dissociates proteins from the lipid rafts and impairs the signaling or other cellular process in the rafts and in non-raft domains. The effect of β -cyclodextrin is nonspecific and may affect functions of both raft- and nonraft-proteins. Moreover, it can not only prevent caveolae-dependent internalization but may also interfere with clathrin-coated pit internalization ⁴⁵. Therefore, this method usually involves cholesterol replenishment after its extraction ^{8, 36}.

Lipid rafts and disease

There are a number of human diseases that are related to lipid rafts/caveolae. Here, I briefly explain some of them.

Cancer

There is a growing body of evidence that shows caveolin-1 inhibits cellular proliferation and acts as a tumor suppressor protein. Caveolin-1 is down-regulated in many human cancers, such as sarcomas, and lung, colon and ovarian carcinomas. However, in prostate carcinomas caveolin-1 is up-regulated in association with metastasis. It is known that the caveolin-1 and caveolin-2 genes are located in a region of the human chromosome that is frequently deleted in several human cancers, such as colon, renal, breast, prostate and ovarian carcinomas^{11, 46-49}.

Cardiovascular disease

Caveolae and caveolin are involved with the various functions of the cardiovascular system. Lipid rafts and caveolae are abundant in cardiomyocytes and regulate many ion channels and signal transduction pathways in the cardiovascular system. They also contain receptors that bind to LDL and HDL. Disruption of potassium channels that associate with caveolae and lipid rafts can cause hypertension and heart failure.

Likewise, nitric oxide synthase (eNOS) is important in cardiovascular homeostasis. Due to the role of caveolin-1 in regulating eNOS activity, it can be concluded that the caveolae and caveolin are involved in vascular abnormalities. Several Cav-1 and Cav-3 knockout studies have also demonstrated the role of caveolae in cardiovascular disease. Loss of Cav-1 and Cav-3 leads to hyperactivation of p42/44 MAPK. In cardiac fibroblasts hyperactivation of P42/44 MAPK in cardiac fibroblasts leads to growth factor secretion and consequently the hypertrophic response in neighboring cardiac myocytes.^{11, 50-53}

Prion disease

Prion disease is known to be promoted by post-translational conversion of the normal cellular prion protein, PrP^c, to a misfolded and infectious form (PrP^{Sc}) and to result in the formation of amyloid in brain. PrP is a GPI-anchored protein localized in lipid rafts/caveolae. This localization appears to involve conformational conversion of PrP^c to the abnormal PrP^{Sc} because the conversion is prevented by targeting the protein to clathrin-coated pits^{46, 54, 55}.

Alzheimer's disease

Alzheimer's disease (AD) is characterized by deposition of amyloid- β -peptide, which is produced by sequential cleavage of the amyloid precursor protein APP by enzymes β -secretase (BACE) and γ -secretase. It is considered that APP may associate with lipid rafts through its sphingolipid-binding domain. Several lines of evidence indicate that increasing the level of cholesterol enhances the risk of developing AD and

amyloid beta (A β) formation. In addition, cholesterol depletion inhibits A β formation in hippocampal neurons and decreases the risk of AD. A β fibrillogenesis is the major pathological event in AD that is thought to be related to the lipid rafts. Raft binding may cause conformational changes in soluble A β , leading to fibrillogenesis and amyloid plaque formation^{53, 54, 56}.

Insulin resistance and diabetes

Type 2 diabetes, which is characterized by insulin resistance and insulin deficiency, is thought to be associated with lipid rafts and caveolae. Initiated insulin signaling, including, autophosphorylation of the insulin receptor and phosphorylation of downstream signaling partners like the insulin receptor substrate-1 (IRS-1), is located in lipid rafts and caveolae. The insulin receptor directly interacts with caveolin-1 and caveolin-3 by its caveolin-binding motif. Caveolin-3 expression increases insulin-induced IRS-1 tyrosine phosphorylation whereas in Cav-3 knockout mice the insulin-induced activation of receptors and IRS-1 is attenuated. In addition, caveolae disruption by β -cyclodextrin inhibits insulin-induced glucose uptake, tyrosine phosphorylation of IRS-1 and insulin-dependent binding of IRS-1 to insulin receptors. Taken together, these results indicate the importance of lipid rafts and caveolae in the pathophysiology of insulin resistance^{47, 57, 58}.

Pathogens

Lipid rafts and caveolae are known as entry sites for many pathogens and toxins. For example, virus SV40 and influenza internalize into mammalian cells via lipid rafts and

caveolae. Moreover, caveolae may allow internalized *Escherichia coli* to survive by inhibiting fusion of phagosomes with lysosomes. Some toxins such as cholera toxin, aerolysin toxin and Shiga toxin are also proposed to enter cells through lipid rafts^{46, 54, 59}.

Several lines of evidences also show the importance of lipid rafts and caveolae in various stages of human immunodeficiency virus type-1 (HIV-1) infection, including: virus entry, regulation of signal transduction in HIV-1-infected cells and assembly of HIV-1 proteins^{54, 60}.

The HIV glycoprotein gp120 binds to its surface receptor CD4 and then to the co-receptors, CCR5 and CXCR4, all of which are located in lipid rafts. It seems that this raft clustering is essential for fusion of virus envelope to the cell membrane. In the case of sexually transmitted HIV-1, the virus should pass through epithelial cells that do not express CD4, CCR5 and CXCR4. It is proposed that HIV-1 is transported from the apical side to the basolateral membrane of epithelial cells by binding of gp120 and gp40 to the glycosphingolipid galactosyl-ceramide (GalCer)-enriched part of lipid rafts^{54, 60}.

Similar to many viruses, bacteria and parasites, HIV-1 infection may affect host cell signal transduction. Nef, an early HIV-1 gene product, whose expression correlates with pathogenicity of HIV-1, is a raft-associated protein. The roles of Nef proteins in contributing to the HIV-1 pathogenesis include: 1) downregulation of cell surface molecules, such as CD4, MHC class I and IL-2; 2) affecting signal transduction in cells; and 3) increasing viral replication. In addition, Nef association with lipid raft may affect

T cell activation by influencing the clustering of signaling molecules, such as Lck, Hck and Fyn^{54, 60}.

During infection, the viral envelop components become associated with lipid rafts. The HIV-1 envelope, Env, becomes palmitoylated in Golgi; a mechanism that is important for association with the lipid raft and assembly of the HIV-1 envelop. The HIV-1 the Gag protein which is necessary for assembly of virus-like particles is myristoylated like other raft-associated proteins. Therefore, both myristoylation of Gag protein and palmitoylation of the HIV-1 envelope are crucial for raft targeting of HIV-1 proteins. It is proposed that the HIV-1 Env associates with the lipid raft before moving to the plasma membrane. This targeting to lipid raft may be necessary for virion assembly and release from the plasma membrane^{54, 60}.

Objectives and significance of the present study

Lipid rafts have recently gained the attention of many scientists as these structures are found to contribute to diseases, such as Alzheimer's, Parkinson's, insulin resistance in type 2 diabetes, and viral infections such as HIV-1, measles and influenza^{54, 61}. Additionally, drugs used for treatment of psychiatric disorders, including schizophrenia and unipolar depression, accumulate in lipid rafts¹⁰.

Moreover, many cellular functions have been attributed to lipid rafts, including signal transduction, tumor suppression, membrane protein endocytosis and trafficking, lipid and cholesterol homeostasis and albumin transcytosis^{13,62,7}.

In order to better understand the physiological and clinical importance of GPCRs and the role of lipid rafts in receptor trafficking and signaling, it is necessary to explore the localization of GPCRs to these membrane microdomains. This can be helpful in gaining further insight into the pathophysiology of different diseases. For example, recent studies have shown that insulin resistance in type 2 diabetes is due to elimination of insulin receptors from lipid rafts as a result of glycosphingolipid gangliosid GM3 accumulation in these microdomains⁶¹.

Despite the important role of lipid rafts in regulating GPCR signaling and the growing data on the mechanisms by which these receptors move into and out of rafts under physiological and pathological conditions, little has been done to collate these findings into a systematic resource bank with easily-accessible data. Because of my interest in this field, I performed an extensive review of the current literature on this newly-discovered cellular structure, so as to identify GPCRs that have been demonstrated to be associated with lipid rafts. I hope that these data serve as an appropriate background and foundation for future studies on this hot topic of cellular biology.

1) The aim of the present study is to perform an extensive literature review to identify all GPCRs that are reportedly associated with lipid rafts, and to collect and summarize the data regarding the role of lipid rafts in receptor signaling and trafficking.

Another aspect that will be investigated is the behavior of GPCRs in response to activation, i.e. to identify those GPCRs that are localized to lipid rafts by default and those that may translocate to these microdomains upon activation. In addition, the role of GPCR localization in the pathophysiology of diseases will be investigated. Finally, as numerous questions regarding to how each GPCR associates with lipid rafts remain unaddressed, the sequences of these receptors will be examined to identify those that contain caveolin-binding motifs.

2) Another goal of this research is to create a website where the obtained information is provided in a user-friendly and freely-accessible manner to all the researchers interested in this field of biology. Similar websites summarizing other aspects of GPCR signaling have been created by well-known research groups to enable valuable information extracted from the literature to be presented to other scientists. We strongly believe that our work will be very useful for investigators studying GPCR signal transduction or lipid raft biology.

Chapter 2: Methodology

The data available in 7TMR-Lipid raft database was extracted from 109 PubMed published articles using “the name of receptor” AND “lipid raft”, “caveolae”, “caveolin” and “caveolin-binding motif” as keywords. The information system supporting the association of GPCRs with lipid rafts includes: 1) receptor species 2) functional consequence of GPCR localization to lipid rafts 3) methods of detecting raft association 4) mechanism of raft association 5) existence of caveolin-binding motif in receptor 6) cell type 7) cell species and 8) type of receptor expression in cells (endogenous vs transfection).

To facilitate access to the original articles, references shown in the 7TMR-Lipid raft database are linked to PubMed. Furthermore, this database supports both searching and browsing of G-protein coupled receptors. The search tool at top right side of each page makes it possible to search the content of the whole site, while the other search tool at the top left side of the browser page can be used to search for receptors by name. The receptors are arranged alphabetically and include receptors have experimentally been shown to be associated with lipid rafts. The website, which was developed using Drupal CMS, works with all browsers and does not need any specific requirements.

Chapter 3: Results

GPCR association with lipid rafts

There is a growing body of evidence which demonstrates that G protein-coupled receptors are associated with lipid rafts. Here, I briefly discuss the association of each receptor with the rafts and the effects of lipid rafts on the signaling, internalization and trafficking of the receptor. The information is summarized in Table 1 as well as in the 7TMR-RAFT database (www.7tmr-raft.org) that supports both searching and browsing of the receptors.

Adenosine receptors

There are four Adenosine receptors: A1, A2A, A2B and A3. Adenosine A1 receptor (A1R) mediates a broad range of signaling cascades by binding to G_o / G_i proteins⁶³. Activation of the A1 receptor regulates many signaling proteins such as adenylate cyclase, phospholipase C (PLC), as well as Ca^{2+} and K^+ channels^{63,64}. Several mouse and rat knock-out studies have shown that A1R is involved in many physiological processes, such as antinociception, analgesia, ischemic precondition of heart and other tissues, neuronal hyperpolarisation, neuroprotection, anticonvulsant activity, presynaptic inhibition, reduction of sympathetic and parasympathetic activity, bradycardia and mediation of tubuloglomerular feedback (TGF)^{65,66}.

The localization of A1R to caveolae was first reported in ventricular cardiomyocytes by Lasley *et al* ⁶⁷. By using a detergent-free method to isolate caveolae from unstimulated ventricular cardiomyocytes, A1R was primarily localized to caveolae. However, after agonist CCPA (2-chloro-N⁶-cyclopentyladenosine) treatment, the receptor translocated from caveolae to the non-raft domain of the plasma membrane. This effect was blocked by the receptor antagonist DPCPX (8-cyclopentyl-1, 3-dipropylxanthine), showing its agonist-dependent manner ⁶⁷. In addition immunoelectron microscopy revealed the association of A1R with caveolin-3 in caveolae ⁶⁷.

However, studies performed on kidney epithelial cells and smooth muscle cell cultures gave contradictory results ^{68, 69}. Isolation of caveolae using a detergent-free method, indicated small amounts of A1R in unstimulated cells ⁶⁸, while activation of A1R by the agonist R-PIA (N⁶-(R)-phenylisopropyl Adenosine) led to caveolae-dependent internalization of the receptor ^{68,69}. A1R forms a complex with adenosine deaminase (ADA), a protein that acts as a receptor activity modifying protein (RAMP), and the complex aggregates in caveolae and then translocate to the intracellular compartment ^{68, 69}. Confocal microscopy analysis has demonstrated the returning back pathways for A1R and ADA. A1R recycles to the non-caveolar plasma membrane region where as ADA returns back to the caveolae-like structures. In addition, pull-down experiments have indicated the interaction between C-terminal domain of adenosine A1R and caveolin-1 ⁶⁹.

There is evidence that K_{ATP} localization in the caveolin-rich membrane domain is essential for A1R-mediated regulation of K_{ATP} channels in cardiomyocytes. This could

be explained by the fact that A1R translocates out of caveolae after 15min stimulation while the effect of A1R on K_{ATP} channels is acute and occurs before its translocation⁷⁰. There is also evidence showing the importance of A1R association with caveolae in cardiomyocytes; A1R modifies MAPK activity in caveolin-rich membranes.⁷¹ In addition, it has been demonstrated that A1R activation by CPA promotes PKC ϵ and PKC δ targeting to caveolae. This effect could be important for cardioprotection⁷².

The other type of adenosine receptor, A2A, also mediates multiple physiological actions. Evidence suggests that the A2A receptor plays a role in regulating blood pressure, in mediating pain through peripheral tissues, and in neuroprotection following ischemic damage⁶⁵. According to such roles, increasing evidence implicates the A2A receptor as a potential therapeutic target, with A2A agonists and antagonists representing drugs for a wide range of diseases, such as immunological, neurodegenerative, psychiatric and inflammatory disorders^{73,74}. For example, istradefylline (KW-6002), an adenosine A2A antagonist, is now in phase III trials for Parkinson's disease⁷⁵.

Using double immune florescence and confocal laser microscopy in a Chinese Hamster Ovary (CHO) cell line, it was shown that caveolin-1 and the A2A and D2 receptors or most probably the A2A-D2 heterodimer cointernalized upon agonist treatment. The same result was also observed for the caveolin-1 interaction and internalization with the D1 and A2A receptors⁷⁶.

In another study, using the detergent-dependent isolation method it was shown that adenosine the A2A receptor, Trk receptor and active src-family kinase (SFK) exist in

both lipid raft and non lipid raft fractions. Coimmunoprecipitation analysis also revealed the association between A2A, Trk receptor and SFK in both lipid raft and non lipid raft fractions. This finding could be very important in the treatment of amyotrophic lateral sclerosis (ALS), as the susceptibility of motor neurons to excitotoxic insults will be limited by signal inhibition of Trk activation by the brain-derived neurotrophic factor (BDNF). This could be possible by disruption of lipid rafts with cholesterol depleting agents⁷⁷.

Adrenergic receptors

Adrenoreceptors, which exist in almost all peripheral tissues and many neuronal populations, mediate the central and peripheral actions of epinephrine and norepinephrine. Both epinephrine and norepinephrine are involved in controlling blood pressure, airway reactivity and myocardial contractile rate among other things. Moreover, adrenoreceptors are targets for many synthetic drugs. Agonists and antagonists interacting with these receptors serve as a means of treatment for many diseases such as asthma, cardiac arrhythmia, congestive heart failure, hypertension, depression and glaucoma⁷⁸.

Adrenoreceptors are divided into two main groups: α and β -adrenoreceptors. The α -adrenoreceptors are subdivided to the α_1 and α_2 -adrenoreceptor while the β -adrenoreceptors (β AR) are subdivided to β_1 , β_2 and β_3 ⁶⁶.

Using detergent-free isolation methods it was demonstrated that α_1 -adrenoreceptors (α_1 ARs) and their signaling molecules, G_q and PLC- β subtypes, are localized in caveolin-enriched membrane domains in vascular smooth muscle cells and rat cardiac myoblast tissue (H9C2 cells). Immunoprecipitation also showed that α_1 AR and G_q were bound to caveolin-3⁷⁹. However, these results do are in contrast to the study of Dreja *et al.* which, using subcellular sucrose density fractionation, was unable to detect, cavolin-3 was and found that α_1 AR did not colocalize with caveolin-1 in smooth muscle cells in rat tail arteries suggesting that this discrepancy may have been due to the different tissues or cell types used⁸⁰.

Using detergent-free purification, Schwencke *et al.* showed that β_1 and β_2 adrenoreceptors were located in caveolin-enriched membrane domain of COS-7 cells. The β -adrenoreceptors colocalize with caveolin-1 in unstimulated COS-7 cells and have high potential affinity to bind caveolin-1⁸¹. Previously, it was shown that β -adrenoreceptor internalized via non-clathrin coated vesicles in human epidermoid carcinoma A431 cells⁸².

Moreover, Rybin *et al.* used two detergent-free isolation methods (one based on a sucrose gradient and the other on OptiPrep gradient) to demonstrate β AR localization to lipid rafts in both cardiomyocytes and quiescent cardiac fibroblasts. β_1 ARs were distributed along all membrane fractions, including internal membranes, caveolae and non-caveolae membrane fractions whereas β_2 ARs localization was limited to caveolae in both type of cells. Upon agonist treatment β_2 ARs moved out of caveolae while β_1 ARs localization was not altered⁴³. Cholesterol depletion by cyclodextrin increased agonist-induced cAMP accumulation, showing that caveolae negatively regulates cAMP

accumulation. Hence, one of the potential mechanisms that could be postulated is that caveolin-3, which coimmunoprecipitated with adenylyl cyclase suppressed the activity of cAMP⁴³. However, in another study of Xian *et al.* it was shown that disruption of caveolae by filipin leads to translocation of β 2ARs out of the caveolin 3-enriched domains and an increase in contraction rate but with no effect on cAMP accumulation. The discrepancy between these two studies, about the effect of filipin on cAMP accumulation, may result from technical differences. Nevertheless, the increase in contraction rate in response to filipin may result from distribution of β 2AR to the plasma membrane, thereby providing greater accessibility of signaling molecules, such as PKA, G_s or specific channels, to the receptor. In contrast, filipin does not have any effect on β 1ARs signaling⁸³.

In addition, caveolae disruption by cyclodextrin indicates that caveolae negatively control cAMP accumulation in agonist-stimulated cells⁴³. This could be important in developing therapeutic agents regarding that effect the activity of β AR in mediating cAMP formation by adenylyl cyclases (ACs) in diseases, such as cystic fibrosis, asthma and congestive heart failure⁸⁴.

The localization of β 3_aARs to caveolae was also assessed recently, using filipin III treatment of CHO cells and brown adipocytes. Caveolae disruption either by filipin III or by β 3_aARs caveolin-binding motif mutation causes accumulation of cAMP in CHO-K1 cells while filipin III treatment of brown adipocytes diminishes agonist-induced cAMP production indicating that this localization is important for β 3_aARs signaling in brown adipocytes.⁸⁵ This is consistent with the study of El-Yazbi *et al.* who demonstrated the reduced function of all three subtypes of β -AR in small intestine of

cav-1 knockout mice. This could be explained by the reduction of PKA activity due to the role of caveolae in target protein recruitment ⁸⁶.

Angiotensin II type 1 receptors

The actions of Angiotensin II are mediated by angiotensin type 1 (AT1) and angiotensin type 2 (AT2) receptors. AT1 receptors (AT1Rs) are primarily coupled to Gq/11 and mediate signal transduction through stimulation of phospholipase C, phospholipase D, phospholipase A2, calcium channel and inhibition of adenylyl cyclase ⁸⁷. Many studies that were done on human showed that AT1 receptor contributes to physiological functions such as regulation of arterial blood pressure, aldosterone, ADH and endothelin secretion, smooth muscle contraction, proliferation and migration, renal function among others ^{66,87}.

Using detergent-free isolation, Ishizaka *et al.* showed that the AT1 receptor was not present in caveolin-enriched membrane fraction of unstimulated vascular smooth muscle cells (VSMCs) but moved to caveolae upon agonist stimulation ⁸⁸. In addition, coimmunoprecipitation showed that the AT1 receptor interacts with caveolin-1 in agonist treatment ⁸⁸. It was later demonstrated that microtubules are essential in translocating the AT1 receptor to caveolae ⁸⁹. Using Cav-1 SiRNA indicated the essential role of caveolin-1 in targeting AT1R to caveolin-enriched membrane fraction which is necessary for ROS production, Rac1 activation, Sos-1 tyrosine phosphorylation, Ras-dependent Akt phosphorylation and VSMC hypertrophy ⁹⁰. It has

also been demonstrated a scaffolding role of caveolin-1 in transactivating epidermal growth factor receptor (EGFR) by angiotensin II. EGFR initially binds to caveolin and is present in caveolin-enriched microdomain. After angiotensin II stimulation, the AT1R translocates to this microdomain while EGFR exits. It was suggested that cholesterol-enriched microdomain, caveolin-1 and focal adhesion play an important role in spatial and temporal organization of AT1R signaling in VSMCs ^{90, 91}.

In another study using both detergent-free and detergent-dependent isolation methods, the proportion of AT1R in caveolae was minor in both basal condition and Ang II stimulated COS-7 cells. This was contrary to Ishizaka's work as the AT1R concentration did not change in stimulated cells. This incompatibility could be explained by different cell types under studies ⁹². It was also revealed that caveolin-binding motif on cytoplasmic tail of AT1R plays an important role in routing the receptor to plasma membrane, its internalization and its interaction to accessory protein dopamine receptor interacting protein of 78 kDa (DRIP78) which regulates the expression and functions of receptor. In addition, the caveolin binding motif of AT1 receptor is necessary for efficient activation of phospholipase C ⁹².

However, using electron microscopy Wyse *et al.* showed that the Ang II-stimulated AT1 receptor moved to cyclodextrin-sensitive non-caveolar lipid raft in baby hamster kidney (BHK) cells. They also suggested that AT1 receptor forms a complex with cav-3 in an initial stage of AT1R maturation in endoplasmic reticulum. Caveolin act as molecule chaperone and play a central role in transporting the AT1 receptor to plasma membrane and the receptor surface expression ⁹³.

Bradykinin receptors

Bradykinin (BK) is a peptide hormone which acts through two GPCRs, bradykinin B1 and B2 receptors. B2 receptor (B2Rs) which is ubiquitously expressed and desensitized rapidly participated in acute phase of inflammation and pain response whereas B1 receptor (B1R) which generally does not express in normal tissues and desensitized slowly is involved in chronic phase of inflammatory and pain process. In addition, B1R may provide a protective effect in brain during inflammatory diseases such as multiple sclerosis^{94, 95}.

Using the conjugated B1 receptor fused with yellow variant green fluorescent protein (YFP), it was shown that B1R-YFP distributed over the plasma membrane in the unstimulated stably transfected human embryonic kidney (HEK293) cells. However, after agonist treatment the B1R-YFP aggregates in multiple groups associated with plasma membrane and colocalizes with caveolin-1. Using confocal microscopy it was also indicated that β -Cyclodextrin which cause cholesterol depletion and caveolar disruption, inhibited the agonist-induced distribution of receptor to membrane-associated aggregates⁹⁶.

Using detergent-free and detergent-dependent isolation methods, De weerd *et al.* indicated that B2 bradykinin receptor together with $G\alpha_q$ and $G\alpha_i$ were present in caveolae in DDT1-MF-2 smooth muscle cells and addition of agonist recruit these three signaling components into caveolae in an agonist-dependent manner. Their data was

also supported by immunoelectron microscopy that was showed the BK receptor complex migrated from cytosol or non-caveolar region to caveolae after phosphatidylinositol hydrolysis suggesting that this translocation may not be essential for initial stage of receptor signaling but may play a role in receptor internalization and desensitization by holding G_{α} subunits in GDP-bound state⁹⁷.

Haasemann *et al.* also demonstrated the agonist-dependent translocation of Bradykinin B2 receptor in human epithelial carcinoma cell line by using immunocytochemical labeling with antibody. In this study it was shown that bradykinin B2 receptors moved to caveolae upon agonist treatment. Moreover, the confocal microscopy analysis revealed the colocalization of bradykinin B2 receptor with caveolin-1 in agonist-treated cells but not in unstimulated cells. After prolonged bradykinin treatment, B2 receptor detached from plasma membrane and fused to other cell organelles showing that B2R internalization via caveolae also connects with other endocytic pathway⁹⁸.

Contrary to Haasemann *et al.* result in another study using detergent-free method showed that under basal condition B2R and STAT3 transcription factor localized partially in lipid raft whereas Tyrk2 tyrosine kinase is localized entirely. After BK treatment of the bovine aortic endothelial cells (BAECs) the B2R and STAT3 translocated out of caveolae in an agonist-dependent manner⁹⁹.

In a study using immunoblotting, Lamb *et al.* assessed the lipid raft association of bradykinin B1 and B2 receptor in HEK293 cells. Both receptors in addition to their agonist exist in caveolae-related lipid rafts (CLR). However, B1 receptor is not enriched

in CLR since B2 receptor is enriched and targets to it specifically. Both receptors contain caveolin binding motif in addition to a site for palmitoylation in their c-terminal domains. This receptor tail was only responsible to CLR targeting of B2R but not B1R¹⁰⁰.

The effect of Bradykinin receptor in lipid raft was not only limited to internalization or desensitization but also the regulation of signaling. As an example colocalization of B2R and metalloendopeptidase EP24.15/16 in terminal endings of neurons could work as microenvironment for enzymatic regulation of BK-induced receptors activation that may contribute to control pain sensation¹⁰¹.

Calcium-sensing receptors

Calcium ions are essential for several physiological functions, including blood coagulation, bone mineralization, regulation of enzymatic activity and cell proliferation. Accordingly, extracellular calcium should be controlled strictly^{102, 103}.

Calcium-sensing receptor (CASR), sensing the extracellular level of calcium, regulates parathyroid hormone secretion, Ca^{2+} excretion in kidney, bone remodeling, hormonal secretion, cell proliferation and differentiation. The CASR is also potentially a therapeutic target for treatment of osteoporosis and hyperparathyroidism¹⁰²⁻¹⁰⁴.

Association of CASR with caveolae was shown in bovine parathyroid cells by detergent-free method ¹⁰². The receptors colocalized with signaling molecules including eNOS, PKC and $G_{\alpha q/11}$. Activation of CASR by high level of Ca^{2+} increases tyrosine phosphorylation of caveolin-1 in caveolin-enriched membrane domain suggesting that CASR signaling, mediating the inhibitory effect of Ca^{2+} on parathyroid hormone secretion localizes in caveolae ¹⁰².

Although CASR is determined to colocalize with both Caveolin-1 and Caveolin-2 in osteosarcoma cell line (Saos-2), it only forms the complex with Caveolin-1 ¹⁰³. Caveolin-1 binding with CASR up-regulates receptor function which could be important in CASR-related disorders or pathophysiological process of bone remodeling ¹⁰³.

Recently, it has been demonstrated that CASR predominantly localizes in caveolae in cardiomyocytes using detergent-free purification method ¹⁰⁵. Activation of CASR by Ca^{2+} stimulates several downstream signaling pathways, including ERK1/2 and PI3K/AKT which play a cardioprotective role in ischemic preconditioning (IPC). These signaling molecules are enriched in caveolae in cardiomyocytes where IPC significantly increases the phosphorylation level. CASR antagonist NPS2143 treatment was shown to abolish IPC-induced cardioprotection and downstream protective signaling pathways confirming that CASR activation mediates IPC-induced cardioprotection by a process involved caveolae. Additionally, it has been suggested that IPC induces CASR internalization via caveolae ¹⁰⁵.

Cannabinoid receptors

The cannabinoid receptors are divided to two subtypes CB1 and CB2 receptors. CB1 receptor is neuromodulatory receptor expressed in cerebellum, hippocampus, cortex and basal ganglia whereas CB2 mainly expressed peripherally ¹⁰⁶.

The endocannabinoid system consisting of CB1 and CB2 receptors, two endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) and regulatory proteins involves in several biological processes such as vascular relaxation, cell proliferation and apoptosis in cancer cells ^{107, 108}.

The CB1 receptor was found to localize in lipid raft by Optiprep density gradient fractionation and immunocytochemistry analysis in human breast cancer MDA-MB-231 cells ¹⁰⁸. This localization is dependent on agonist binding as the amount of receptor localized in lipid raft decreased after agonist anandamide (AEA) and also M β CD treatment ^{108, 109}.

Association of cannabinoid receptor with lipid raft affects many cellular processes such as internalization, signaling and cellular trafficking ^{110, 111}. Agonist-induced internalization is inhibited in HEK293 cells treated with filipin suggesting that internalization partially occur via caveolae in parallel to clathrin-coated pathway ^{112, 113}. However, internalization in neuroblastoma cells that naturally express CB1 is not caveolae-dependent ¹¹².

Cholesterol depletion by M β CD abolishes the antiproliferative activity of the cannabinoid receptor antagonist SR141716 suggesting that SR141716 inhibits the cell growth in human breast cancer through a lipid raft/caveolae-dependent mechanism. Additionally, AEA needs lipid rafts to inhibit cell proliferation. Overall, these findings indicate that endocannabinoid could serve as therapeutic target for the treatment of human cancers specially breast cancer ¹¹⁰.

Due to the fact that lipid raft can control the CB1R but not CB2R, developing a therapeutic drug which is targeted to lipid raft can be used for disorders such as Alzheimer's disease, Parkinson's disease and schizophrenia that result from upregulation or downregulation of CB1R but not CB2R ^{109, 110, 114}.

CC chemokine receptors

CC chemokine receptor type 5 (CCR5) which is mainly expressed in macrophage, dendritic cells and T cells has gained attention recently by many scientist as coreceptor for HIV entry ^{115, 116}. HIV envelope gp120 binds to CD4 receptor on the host cells leading to conformational changes of gp120. This alternation facilitates binding of gp120 to chemokine receptors. Therefore the chemokine receptor blockage could prevent HIV entry ¹¹⁷. For example Maraviroc is the first CCR5 inhibitor approved by FDA in 2007. Furthermore, Vicriviroc another CCR5 inhibitor is now in phase III trials ¹¹⁷. CCR5 function is not limited to virus entry but also involves inflammation and autoimmunity ¹¹⁸.

Manes *et al.* was firstly reported the association of CCR5 with lipid raft by using detergent-dependent isolation method. It was revealed that after the insulin-like growth factor-1 (IGF-1) treatment of MCF-7 cells, the number of CCR5 in detergent-insoluble glycosphingolipid cholesterol-enriched complexes (DIG) were increased ¹¹⁹. Furthermore immunofluorescence microscopic analysis showed that CCR5 and the lipid raft marker ganglioside GM1 colocalized partly in Hela cells ¹²⁰. In another study using the same method, Nguyen *et al.* indicated that CCR5 did not colocalize with GM1 in normal condition. However, after macrophage inflammatory protein 1 β (MIP-1 β) binding, the functional receptors localize in lipid rafts on the T-cell surface ¹²¹.

Using disrupters of caveolae, filipin and nystatin it was shown that CCR5 internalized in a caveolae-dependent manner in CHO cells. Filipin inhibited MIP- α induced internalization while nystatin inhibited MIP- α and CCL5 (regulated on activation normal T-cell express and secreted [RANTES]) induced internalization ¹²². This is contrary to Signoret *et al.* result which believes that caveola is not responsible for CCR5 internalization ¹²³. Using immunofluorescence microscopy analysis did not show any association between CCR5 and caveolin-enriched structure in CHO cells either before or after CCL5 treatment ¹²³.

Similar to most GPCR, CCR5 contains Cys residues (in the case of CCR5, Cys-321, Cys-323, Cys-324) in the carboxyl terminal tail that are modified by palmitoylation ¹²⁰. Venkatesan *et al.*, using several methods including detergent extraction, copatching experiment with lipid raft marker GM1 and cholesterol extraction, revealed that palmitoylation of carboxyl terminal tail of CCR5 is a determinant for membrane raft localization ¹¹⁸. After prolonged treatment of agonist RANTES, CCR5 undergoes

internalization via vesicles mostly colocalized with caveolin¹¹⁸. CCR5 palmitoylation is also involved in CCR5 cell surface expression, intracellular trafficking and signaling cascade^{120, 124}.

Association of CD4, CCR5 and HIV-1 envelope gp120 with lipid raft raises the possibility that raft-association of CCR5 is involved in HIV entry in to cells^{125, 126}. Using confocal microscopy was shown that in the absence of HIV-1, CCR5 and GM1 distributed randomly through plasma membrane. However HIV-1 incubation of PM1 T cells leads to aggregation of CCR5 in lipid rafts. Unbinding the HIV-1 envelope gp120 from lipid raft in the presence of M β CD supports the idea that HIV receptors localization in lipid raft is responsible for virus entry¹²⁶.

In consistent, using four pharmacological agents including M β CD, filipin and nystatin that disrupt lipid raft by sequestering or removing cholesterol and Lavastatin which inhibits cholesterol, the inhibition of HIV entry and reverse transcription in macrophages was indicated¹²⁷.

In contrast, Percherancier *et al.* presented opposite results indicating that even in the absence of CCR5 and CD4 from lipid raft, HIV-1 still enter primary T-cells. Indeed, they believe the receptor localization in lipid rafts is not required for HIV-1 entry in to T-cells. However, the results revealed that cholesterol extraction by M β CD, inhibits HIV-1 entry into cells; explaining the important role of plasma membrane cholesterol content but not receptor localization to lipid raft¹²⁸. Using a mechanism disrupting lipid raft without altering cholesterol content will be useful to avoid these conflicting data.

Another type of CC chemokine receptor is CCR6 which is mostly expressed on memory T-cell, B-cell, dendritic cells and lymphoid tissues. It is known that the receptor is involved in regulating leukocyte recruitment in inflammatory disease and maintenance of mucosal associated lymphoid tissue (MALT) homeostasis ¹²⁹.

It is recently found that lipid raft plays an important role in CCR6 signaling. M β CD treatment of Jurkat cells prior the stimulation, impaired CCR6-induced chemotaxis suggesting that lipid raft is essential for receptor activation and signaling. Proteomic analysis showed that many proteins mostly involved in cytoskeleton regulation recruit to lipid raft upon CC6 activation ¹²⁹.

CXC chemokine receptors

CXC chemokine receptor type 4 (CXCR4) is an alpha-chemokine receptor expressed in different cell types including lymphocytes, peripheral blood lymphocytes, monocytes and overexpressed in variety of cancers such as human breast cancer, ovarian cancer, cervical cancer, pancreatic cancer, small-cell lung cancer and prostate cancer ^{130, 131}.

CXCR4 is another HIV coreceptor that indicates CD4 alone could not be sufficient for virus infection. Association of CXCR4 with lipid raft and its role in HIV infection is assessed by many experiments. Using immunoelecteron microscopy and confocal microscopy it was shown that small amount of CXCR4 localizes in glycosphingolipid-enriched microdomains (GEM) of HEK293 cells and peripheral blood mononuclear

cells (PMBC) and Sup T1 lymphoblastoid cells in the absence of HIV envelope gp120 while gp120 treatment recruit the receptor to GM3-enriched microdomains where can form CD4-gp120-CXCR4 complex^{125, 132}. Therefore, these microdomains could serve as a target to prevent or block HIV-1 infection¹³².

Another study that used detergent-dependent method and confocal microscopy suggested that CD4 localized almost exclusively in lipid raft but CXCR4 was excluded from raft in both H9 leukemic T cells and PBMC. However, the result was somehow different in response to SDF-1 α and HIV-1 gp120. They illustrated that the localization of CD4 and CXCR4 were not changed either in presence of stromal cell-derived factor 1 α (SDF-1 α) or gp120 in H9 cells. This discrepancy may be due to the difference in their methods; percents of detergent used and temperature¹³³.

Association of CXCR4, CD4 and CCR5 to membrane raft was also detected in PM1 T cells by detergent-dependent isolation method and confocal microscopy¹²⁶. Chemokine (SDF-1 α or CXCL12) binding to CXCR4 occurred in lipid raft¹³⁴. Inhibition of HIV infection by M β CD treatment illustrates the importance of lipid raft integrity and HIV receptors localization in HIV infection¹²⁶. This can be helpful for development of a new strategy for treatment of HIV infection by interfering with the HIV receptor localization in lipid rafts¹²⁶.

Using immunocytochemistry it was also shown that CXCR4 localized to lipid raft in human progenitor cell line KG1a. Under basal condition the receptor distributed uniformly over plasma membrane. However, in response to SDF-1, the receptor migrates to the lipid raft at leading edge of endothelium cells. Disruption of lipid raft

prevents receptor internalization and SDF-1-induced migration confirming the role of lipid raft in the receptor surface expression and signaling¹³⁵.

In addition to the role in HIV infection, CXCR4 association in lipid raft is essential for dimerization of CXCR4 receptors¹³⁰. Due to the fact that CXCR4 dimerization is required for the receptor signaling, disruption of lipid rafts by M β CD stops CXCR4-mediated cell migration¹³⁰. Treatment of cells with omega-3 polyunsaturated fatty acid (n-3 PUFA) which disrupts lipid rafts similar to M β CD, displaces CXCR4 and down-regulates receptor activity¹³⁶.

Up-regulation of chemokine receptors like CXCR4 causes the migration of cancerous cells to other tissues which is an important factor in metastasis. Therefore, n-3 PUFA could be a blocking agent in cancer metastasis especially in breast cancer suggesting that diet rich in omega-3 fatty acids may be beneficial for breast cancer patients¹³⁶.

Receptor up-regulation also involves in other disease such as Alzheimer's disease, atherosclerosis and other cancers. Therefore, disruption of lipid raft could be helpful for treatment of many diseases¹³⁶.

CXCR1 is another chemokine receptor expressed mainly in neutrophils and dendritic cells^{137, 138}. It conducts leukocytes to the site of inflammation. Chemokine binding like interleukin-8 to the receptor activates G-protein signaling pathways such as Ca²⁺ influx and PI3K activation^{139, 140}.

Using FRET it was demonstrated that interleukin-8 induces CXCR1 movement to lipid raft in CX1-HEK cells. FRAP measurement also confirmed that activated receptor displayed restricted mobility in plasma membrane while inactive receptor diffuse rapidly. This also strengthens the result that activated receptor associates with lipid raft¹⁴⁰.

Lipid raft disruption by M β CD impaired G-protein-dependent signaling such as Ca^{2+} response and PI3K activation but it does not remarkably affect ligand-induced receptor phosphorylation¹⁴⁰.

Cholecystokinin (CCK) receptors

Cholecystokinin receptors, binding the hormones cholecystokinin or gastrin, distribute mainly in central nervous system (CNS) and gastrointestinal tract^{141, 142}. CCK receptors contribute to the regulation of several physiological functions in brain including depression, anxiety, cognition, analgesia and satiety^{141, 142}. The peripheral functions also include stimulation of gallbladder contraction and secretion of pancreatic amylase in the pancreas¹⁴¹.

Using detergent-dependent method Roettger *et al.* showed that CCK1 receptor associates with caveolae in CHO cells¹⁴³. The receptor internalizes via caveolae in addition to clathrin endocytic pathway¹⁴³. However, the association of CCK1 receptor

to caveolae could not be detected in pancreatic acinar cells using electron microscopy¹⁴⁴.

Association of CCK receptor with caveolae may have a role in pathogenesis of cholesterol-induced gallbladder hypomotility. Muscle cells of gallbladder with cholesterol stones contain excess cholesterol mainly localized in caveolae, resulting enhancement of caveolin-3 expression. Increasing caveolin-3 expression promotes targeting CCK1 receptor to caveolae that may cause reduction of ligand binding and inhibiting $G\alpha_{i3}$ function¹⁴⁵.

Chemotactic peptide receptors

Chemotactic peptide receptors include several receptors such as formyl peptide receptors (FPRs), complement cleavage product (C5a), leukotriene and lipoxin receptors¹⁴⁶.

To assess whether formyl peptide receptor and B leukotriene receptor-1 (BLT-1) localized to lipid raft, Sitrin *et al.* used detergent Brij58-dependent method for membrane fractionation experiments¹⁴⁷. Using this method it was shown that BLT-1 receptor resides in low density fractions and coisolates with lipid raft marker flotillin-1 in human neutrophils. However, FPR was distributed in high density non-raft fraction coisolating with a non raft marker Cdc42. These results were also confirmed by using

FRET indicating that BLT-1 but not FPR localized to lipid raft. The localization of neither BLT-1 nor FPR was changed upon agonist treatment ¹⁴⁷.

Treatment of cells with M β CD abolishes the intracellular Ca²⁺ mobilization and ERK1/2 phosphorylation in response to leukotriene B4 (LTB4) but not formyl-methionyl-leucyl-phenylalanine (fMLP), suggesting that lipid raft integrity is required for LTB4-induced signaling but not for fMLP-mediated activation ¹⁴⁷. This is inconsistent with Xue *et al.* study indicating that FPR clustering is dependent on receptor activation ¹⁴⁸. This study showed by confocal microscopy that FPR colocalized with lipid raft marker GM1 in U937 promonocytic leukemia cell line. However, in the absence of ligand, the receptors clustering was decreased suggesting that lack of ligand led to loss of active form which promotes receptor exclusion out of lipid raft ¹⁴⁸.

In a recent study using detergent-free method, Serezani *et al* showed that after IgG-RBC but not LTB4 treatment, BLT-1 moved from non-lipid raft fractions to lipid raft microdomain in rat pulmonary alveolar macrophage (AM) ¹⁴⁹. Using confocal microscopy also confirmed that immunoreceptor Fc γ RI as well as BLT-1 and another class of leukotriene receptor CysLT1 translocate to lipid raft upon Fc γ R engagement ¹⁴⁹. During Fc γ R engagement, phosphorylation of BLT-1 by Src kinase mediates the association of BLT-1 with Fc γ RI and G α i3 in lipid raft microdomain. This was the first evidence for direct interaction of a GPCR and immunoreceptor ¹⁴⁹.

Disruption of lipid raft by M β CD and 2 β HPCD diminished LTB4-induced enhancement of phagocytosis, antimicrobial activity and signaling indicating the role of

lipid raft integrity in AM signaling and microbicidal activity of BLT-1 but not CysLT1

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Formyl peptide receptor like-1 (FRL-1) is another chemotactic peptide receptor subfamily which has 70% homology with FPR. FRL-1 can be activated by two different ligands, Trp-Lys-Tyr-Met-Val-Met-CONH₂ (WKYMVM) and lipoxin A₄ (LXA₄). FRL-1 is known to involve in modulation of inflammatory response, regulation of host defense against pathogenic infection, removal of damaged cells and in various disease like Alzheimer's disease, prion disease, amyloidosis and AIDS ^{150, 151}.

It is identified recently that lipid raft is required for FRL-1 mediated signaling. M β CD treatment of both CHO and human glioblastoma U87 cells suppresses the WKYMVM-induced phosphorylation of inhibitor- κ B (IKK), ERK and Akt suggesting the critical role of raft microdomain in FRL-1-mediated IKK activation. As IKK is an onset kinase for NF κ B signaling cascade, this finding can offer potential therapeutic drug for FRL-1-related diseases like Alzheimer's disease ¹⁵². Although the localization of FRL-1 was not assessed in this study, it was shown that FRL-1 signaling molecules including c-Src, G α_{i2} and ERK localized in lipid raft and WKYMVM induced recruitment of ERK to this microdomain ¹⁵².

Lipoxin A₄ receptor (ALXR), which was first identified as formyl peptide receptor like-1 based on sequence homology was shown to localize in lipid raft in HEK293 cells using detergent-dependent isolation method ¹⁵³. Immunoblotting also revealed that ALXR resides in the low density fraction where caveolin-1, flotillin-1, SH-2 domain-containing tyrosine phosphatase-2 (SHP-2) and platelet-derived growth factor receptor β

(PDGFR β) also existed. Moreover, immunoprecipitation showed that SHP-2 associated with ALXR in lipid raft and this association increased by LXA4-induced receptor activation¹⁵³.

Treatment of cells by M β CD displayed that lipid raft integrity is necessary for LXA4 transinhibition of PDGFR β . In this new proposed model, LXA4 binding to ALXR residing in lipid raft promotes SHP-2 recruitment to and reactivation in lipid raft. In a time-dependent manner, SHP-2 released from ALXR and can bind to PDGFR β localized in lipid raft to reduce its mitogenic activity¹⁵³.

Dopamine receptors

The D1 dopamine receptor (D1R), the most abundant subtype of dopamine receptor in the brain, is expressed in hippocampus, striatum, nucleus accumbens, glial cells and astrocytes. D1 receptors involve in many neural processes like memory, learning, voluntary movement and reward¹⁵⁴. Dopamine receptors are target for the main drugs used in pharmacotherapy of neurological and psychiatric disorders such as Parkinson's disease, Huntington's disease, schizophrenia, bipolar disorder and Tourette's syndrome^{155, 156}.

Association of D1 dopamine receptor with lipid raft was assessed for the first time by Yu *et al.* using several experimental techniques¹⁵⁷. Detergent-free isolation methods showed two pools of human D1Rs (hD1Rs), the high molecular mass hD1Rs located in

lipid raft where caveolin-2 and flotillin-1 also exist and the low molecular mass hD1Rs localized in non-raft plasma membrane in HEK293 cells ¹⁵⁷. Using Coimmunoprecipitation and confocal microscopy it was found that association of hD1Rs with caveolin-2 β increased upon agonist fenoldopam treatment in HEK293 and rat renal proximal tubule cells (RPTCs). Caveolin-2 cofractionated with signaling molecules like Gas, G $\beta\gamma$, PKAc and PKA_{RII β} . Fenoldopam treatment also stimulated the adenylyl cyclase activity in lipid raft ¹⁵⁷. The stimulatory effect of fenoldopam on cAMP accumulation is inhibited by caveolin-2 antisense suggesting that caveolin-2 regulated D1R-induced signal transduction ¹⁵⁷.

In another study, using detergent-free isolation method it was shown that under basal condition, the majority of dopamine D1 receptor existed in noncaveolar plasma membrane of rat proximal tubules ¹⁵⁸. However, after dopamine treatment, the receptor was recruited to caveolar plasma membrane where also rich in Na-K-ATPase. Coimmunoprecipitation also illustrated that dopamine increased the interaction between caveolin-2 and D1 receptors ¹⁵⁸.

Kong *et al.* also used detergent-free method to determine localization of D1R in caveolae ¹⁵⁹. Under basal condition, D1 receptors were found in both caveolin-enriched and noncaveolin-enriched fractions in COS-7 cells. Agonist SKF 81297 treatment led to translocation of receptor to caveolin-enriched fractions ¹⁵⁹. Caveolin-1 binding motif mutation and M β CD treatment abolished D1 receptor internalization suggesting that integrity of caveolae and caveolin-1 binding motif is critical for receptor internalization. In addition, caveolae disruption enhanced agonist-induced cAMP production suggesting the regulatory role of caveolae in D1R signaling and recycling ¹⁵⁹.

Endothelin receptors

Endothelin receptors which are widely expressed in all tissues have two subtypes ET_A and ET_B. ET_A receptor predominantly exists in smooth muscle of blood vessels is mainly responsible for vasoconstriction. However, ET_B receptor is present in endothelial cells that line blood vessels and play a role in the release of endothelium-derived relaxing factor such as nitric oxide (NO). In addition, ET_B receptor is known as "clearing receptors" to clean endothelin from circulation ^{160, 161}.

Coimmunoprecipitation of ET_A and its bound ligand with caveolin showed that ET_A receptor colocalized with caveolin in caveolae in COS cells and the localization is important in signal transduction ¹⁶². Endothelin-1 activation of ERK1/2 via ET_A receptor requires EGF-R transactivation and intact caveolae in mesangial cells ¹⁶³. Moreover, agonist induced ET_A receptor internalization via either caveolae or clathrin-coated pits depends on the oxidative states of cholesterol in cell membrane ¹⁶⁴.

ET_B receptor and the signaling molecules such as Shc, Grb2 and small G protein Rho were also demonstrated to localize in caveolae in primary astrocytes using detergent-free methods ¹⁶⁵. Caveolae disruption by filipin III abolishes ET-1-induced tyrosine phosphorylation of proteins like FAK and ERK-2 phosphorylation, suggesting the importance of caveolar structure integrity in FAK and ERK activation. In addition, prevention of stress fiber and focal adhesion formation in the presence of filipin III, revealed that caveolae play crucial role in these structural formation ¹⁶⁵. Nonetheless, it

is recently approved that filipin III is not specific for lipid raft disruption as it causes disturbance of sterol-containing membranes leading cellular components leakage. Thus, filipin III could not be a good indicator to assess the effect of ET_B receptor localization in ERK1/2 activation due to its diverse effects ¹⁶⁶. Accordingly, analyzing ERK 1/2 activation pattern comparing both COS-7 cells where caveolae naturally exist and caveolae-free HEK293 cells revealed no significant difference between ET_B constructs suggesting that caveolae localization of ET_B receptor might increase ERK1/2 signaling but not the activation patterns ¹⁶⁶.

Using detergent-dependent method Yamaguchi *et al.* has also shown that free-ligand ET_B receptor interacts directly with caveolin-1 in HEK293 cells both in vitro and in vivo. Caveolin-1 targets the receptor to caveolae while ET-1 removes them from this microdomain ¹⁶⁷. Filipin treatment decreased agonist-induced ERK1/2 phosphorylation suggesting the role of caveolin-1 in binding to receptor and keeping the ligand-free ET_BR in caveolae, thereby controls ET signal transduction ¹⁶⁷. Despite that, Grossman *et al.* believe that detergent-dependent method is not a good candidate for localization of receptor in lipid raft due to generation of lipid raft cluster artificially ¹⁶⁶. They demonstrated that, in COS-7 cells, N-terminal truncated ET_B receptor localized to caveolae using detergent-free method whereas full-length or glycosylation-deficient receptor distributed among plasma membrane. In HEK293 cells that are naturally caveolae-free due to lack of caveolin-1 expression, ET_B receptors localized to caveolae artificially only when they fused to caveolin-2. Therefore, ET_B receptor localization in caveolae depends on the cell type and post-translational modifications ¹⁶⁶.

Glucagon receptors family

The glucagon-like peptide 1 receptor (GLP-1R) belongs to class B GPCRs and is known to affect β -cell function and glucose homeostasis. GLP-1R stimulates cAMP, PI3K and ERK1/2 signaling. Activation of GLP-1R signaling could be used therapeutically in treatment of diabetic peripheral neuropathy and other neurodegenerative diseases such as Alzheimer's disease. GLP-1 receptor agonists are also being assessed for clinical use as antidiabetic agents¹⁶⁸⁻¹⁷⁰.

The real time confocal microscopy illustrated that glucagon-like peptide 1 receptor existed in both plasma membrane and mobile cytosolic compartment in HEK293 cells, transiently expressing receptor and caveolin, and mouse insulinoma (MIN6) cell line. Cell fractionation using detergent-dependent method showed the localization of GLP-1R in Triton-insoluble fraction where caveolin-1 also was present. Immunoprecipitation experiments and confocal fluorescence microscopy analysis also confirmed that GLP-1R interaction with caveolin-1 is necessary for cellular trafficking because blocking this interaction by caveolin binding motif mutation of GLP-1R and dominant-negative caveolin-1 abolish receptor membrane expression¹⁷¹.

Upon agonist treatment of HEK293 cells which stably expressed GLP-1R, the receptor internalized in association with caveolin-1 via a dynamin-dependent but β -arrestin2-independent mechanism¹⁷¹. It was demonstrated that lipid raft integrity is necessitated for GLP1R-induced ERK1/2 activation which involves epidermal growth factor receptor transactivation. Thus, involvement of lipid raft in receptors expression,

signaling and regulation could help us in developing therapeutic agents for treatment of type 2 and perhaps type 1 diabetes ¹⁷¹.

Another subtype of glucagon receptor family named Glucagon-like peptide-2 receptor (GLP-2R) expressed in human endocrine cells, human and rodent myofibroblasts, murine enteric neurons and particular regions of CNS ^{172, 173}. The receptors inhibit apoptosis in transfected cells and have cytoprotective effect on intestinal injuries. The trophic and anti-apoptotic actions of GLP-2R signaling have proposed the potential usage of GLP-2 agonists for intestinal disorder treatment ^{170, 173}.

Using detergent-dependent isolation method followed by western blot analysis it was shown that glucagon-like peptide-2 receptor localized in both Triton X-100-soluble and -insoluble fractions of transfected DLD-1 and BHK cells. GLP-2 treatment increased transiently human Glucagon-like peptide-2 receptor (hGLP-2R) colocalization with caveolin-1 ¹⁷².

Cholesterol depletion agent, M β CD and cholesterol sequestration agent, filipin prevented the agonist-induced internalization of hGLP-2R suggesting that receptor required lipid raft for endocytosis. Using clathrin-coated pit inhibitor and mutant dynamin constructs it was demonstrated that hGLP-2R internalization and desensitization occur independently of clathrin- and dynamin-1-dependent pathways ¹⁷².

Treatment of transfected DLD-1 cells with cholesterol disruptor M β CD and clathrin-coated pit inhibitor did not alter receptor-induced cAMP accumulation suggesting that

GLP-2-stimulated adenylyl cyclase activity is independent of receptor internalization¹⁷².

Glutamate receptors

Glutamate receptors are divided to two categories ionotropic and metabotropic. Ionotropic glutamate receptors consist of AMPA, NMDA and Kainate receptors whereas metabotropic glutamate receptors belong to seven transmembrane receptors classified into three groups I, II and III¹⁷⁴.

Metabotropic glutamate receptors (mGluRs) involve in modulatory actions on neurotransmitter release, synaptic plasticity and neuronal excitability. Therefore, they can be used therapeutically in anti inflammation, excitotoxicity reduction and apoptotic and necrotic cell death inhibition¹⁷⁴⁻¹⁷⁶.

Using immunocytochemistry (detergent-free) and coimmunoprecipitation it was shown that metabotropic glutamate receptors colocalize with both cavolin-1 and caveolin-2 in BHK-1 α cells suggesting that mGluR1 α associated with caveolin-enriched membrane domain¹⁷⁷. Agonist-induced activation of mGluR1 increased ERK MAPK phosphorylation in caveolin-enriched membrane but not in other fractions¹⁷⁷. This is inconsistent with another study that showed enhanced ERK1-2 phosphorylation in neurons of caveolin-1 knockout mice suggesting that caveolin-1 modulates mGluR1-

dependent activation of ERK-MAPK signaling¹⁷⁸. This could be very important in synapse formation and plasticity¹⁷⁸.

mGluR1 has two caveolin binding motifs located in first and third intracellular loops and play an important role in direct interaction of mGluR with caveolin-1^{178, 179}. Using Tandem affinity purification (TAP) cassettes, coimmunoprecipitation and pull down assays, it was shown that caveolin-1 and mGluR1 interact with each other in Madin-Darby Canine Kidney (MDCK) and HEK293 cells^{178, 179}. The association of mGluR1 in HEK293 was also confirmed by Triton X-100 detergent-dependent isolation method¹⁷⁸. In addition, in vivo association of mGluR5 with lipid raft was found using detergent-dependent isolation method and immunoblotting¹⁷⁸.

Caveolin-1 interaction with mGluR1 regulates receptor cell surface expression and rate of internalization. Caveolin-binding motif disruption increases the rate of receptor internalization. Moreover, knockdown expression of caveolin-1 by SiRNA, strengthen receptor internalization. Therefore, it was assumed that caveolin-1 transiently stabilizes mGluR on the cell surface¹⁷⁸. Due to the importance of mGluR1 in spine morphogenesis and synaptic function, regulation of mGluR signaling, surface expression and endocytosis by caveolin-1 could be an influential mechanism to regulate synaptic efficiency¹⁷⁸.

In addition to the role of clathrin-coated pits in mGluR1 internalization, it is found that mGluR1 internalization occurs at least in part, via caveolae/raft pathway. Lipid raft disruption by M β CD and nystatin decreases receptor endocytosis suggesting that mGluR1 can internalize via caveolae/lipid raft in HEK293 cells and neurons¹⁷⁸.

Gonadotropin releasing hormone receptors

The Gonadotropin releasing hormone receptor (GnRHR) is very critical for reproductive function as its binding to gonadotropin releasing hormone (GnRH) stimulates the synthesis and secretion of luteinizing hormone (LH), follicle stimulating hormone (FSH) and gonadotropic hormones^{180, 181}. It mediates its actions through several signaling pathways including MAPK and PKC activation^{180, 182}. Today the GnRHR antagonist is widely used in prostate cancer treatment. In addition, GnRHR agonist and antagonist have potential to be used therapeutically in breast and ovarian cancer¹⁸³.

Navratil *et al.* assessed the membrane localization of mammalian type 1 gonadotrophin releasing hormone receptor (mGnRHR1) which has a unique structure among GPCRs and other types of GnRHR in lacking the cytoplasmic carboxy terminal tail^{184, 185}. By the aid of both detergent-free and detergent-dependent isolation methods, they showed that GnRH receptor localizes to low density membrane fraction where the flotillin but not caveolin was detectable¹⁸⁶. It means that GnRH receptor and its downstream signaling intermediates including RAF1, ERK and G_{αq} localize to non-caveolar lipid raft in gonadotroph derived α-T3-1 cells^{186,187}. This localization is agonist-independent and leads to regulation of MAPK pathways like ERK that are essential for fertility controlling¹⁸⁸. The same experiment using both detergent-free and detergent-dependent isolation methods has similar result in CHO cells except that caveolin-1 is expressed in low density membrane fractions¹⁸⁶.

In contrast to mammalian GnRH receptor, the nonmammalian GnRHR has long carboxyl terminal tail thus rapid internalization. In a study that was done on chicken gonadotropin releasing hormone receptor (cGnRHR), the receptor internalization was inhibited using disrupters of caveolae (filipin, mβCD and dominant negative caveolin-1 mutant) suggesting that cGnRH receptor internalized in dynamin and caveolar-dependent manner in both COS-7 and HEK293 cells. Mutation analysis also indicated the important role of Cys³²⁸ and threonine doublet (Thr³⁶⁹ Thr³⁷⁰) in receptor internalization. Although it was not shown in this study, it was suggested that Cys³²⁸ palmitoylation may target the cGnRH-R to caveolae¹⁸⁹.

Histamine receptors

Histamine H1 receptor a subtype of histamine receptors is a prominent mediator of allergy and inflammation. H1 receptor is distributed in various tissues such as mammalian brain, adrenal medulla, cardiovascular system, genitourinary system, endothelial cells, and lymphocytes¹⁹⁰. This receptor mainly binds to G_{q/11} leading to production of inositol triphosphate (IP3) and diacylglycerol (DAG). H1 receptor mediates several physiological actions including smooth muscle contraction, prostacyclin synthesis, catecholamine secretion from adrenal medulla and neurotransmission^{190, 191}.

Self *et al* were the first to investigate the internalization pathways of human histamine H1 receptor in CHO-K1 cells¹⁹². Preincubation of cells with hypertonic

sucrose which inhibits clathrin-mediated endocytosis had no effect on histamine-induced H1 receptor internalization. In contrast, lipid raft/caveolae disrupter agents like filipin or nystatin prevented histamine-induced internalization of H1 receptors¹⁹². Under basal condition, H1 receptor colocalized with caveolin-1. However, after stimulation of cells with histamine, no caveolin-1 internalization was detected in parallel with H1 receptor proposing that the receptor may internalize via lipid rafts rather than caveolae¹⁹².

Luteinizing hormone receptors

The luteinizing hormone receptor (LHR) or lutropin/choriogonadotropin receptor is a member of glycoprotein receptors which is found in testis, ovary and also extragonadal organs like uterus. It is known that LHR plays a key role in proliferation and differentiation of testis and ovary. Binding of LH or chorionic gonadotropin (CG), LH receptor independently activates two G-protein-dependent signaling pathways, adenylyl cyclase and PLC^{193, 194}.

Western blot analysis showed that in the absence of ligands, LH receptors localized in high density caveolin-free membrane fractions of CHO. Binding of human chorionic gonadotropin (hCG) hormone leads to movement of receptors in to lipid raft¹⁹⁵.

Consistent with these results, Smith *et al.* used detergent-dependent cell fractionation method to demonstrate that after hCG binding, functional but not non-functional LH

receptor translocated to lipid raft ¹⁹⁶. Disruption of lipid raft by M β CD, reduced signaling but not blocked it completely suggesting that raft integrity was not necessary for cAMP production, but it can increase signaling efficiency ¹⁹⁶.

Palmitoylation site of LH receptor has been shown to be essential for raft localization. Mutation of C-terminus palmitoylation sites of the receptor blocked the movement of LH receptor to lipid rafts upon hCG treatment ¹⁹⁷. However, it has no significant effect on cAMP signaling corroborating that raft localization is not critical for cAMP production ¹⁹⁷.

Lysophosphatidic acid receptors

Lysophosphatidic acid (LPA) is a lipid growth factor which acts through activating G protein-coupled receptors LPA1R-LPA5R. LPA signaling regulates many cellular responses such as cell proliferation and growth, cell survival and death, cell mobility and gene expression ¹⁹⁸⁻²⁰⁰.

Immunoprecipitation and western blot experiments revealed that LPA1R colocalized with caveolin-1 in porcine cerebral microvascular endothelial cells (pCMVECs) nuclear fraction whereas the receptors were dually compartmentalized into caveolae and clathrin-coated pits in cell membrane ²⁰⁰.

There are conflicting data regarding the role of caveolae in LPA signaling. In pCMVEC cells, caveolae disruption by M β CD and filipin did not modify LPA1R-induced cyclooxygenase-2 and nitric oxide synthase RNA and protein expression indicating that caveola is not important for LPA activity²⁰⁰. In brown pre-adipocytes, LPA-stimulated ERK1/2 phosphorylation did not change in Cav-1(-/-) compared to wild type cells suggesting that Cav-1 is not required for LPA-induced ERK1/2 phosphorylation²⁰¹.

In contrast, treatment of COS-7 cells with M β CD, reduced the LPA-induced ERK1/2 phosphorylation. Therefore in COS-7 cells, caveola was found to be essential for LPA signaling²⁰².

Melanin-concentrating hormone receptors

The melanin-concentrating hormone receptor 1 (MCH1R) is one of the subtypes of melanin-concentrating hormone receptors expressed in many regions of brain including cortex, midbrain, pons, hypothalamus and thalamus. MCH1R is known to involve in regulating stress, emotion, motivation, and appetite. Indeed, MCHR antagonism might be a potential approach for treatment of anxiety, depression and obesity^{203, 204}.

MCH1R has been shown by detergent-free methods to reside in lipid rafts containing cavelin-1 in stably transfected CHO-K1 cells under basal conditions²⁰⁵. There was no significant change in receptor localization after MCH treatment.

Coimmunoprecipitating studies also confirmed that MCH1R directly interacts with caveolin-1 under basal conditions²⁰⁵.

Disruption of caveolae by M β CD weakened MCH-mediated ERK signaling suggesting that caveolae can serve as mediator for MCH-mediated ERK1/2 signaling²⁰⁵.

Melatonin receptors

Melatonin receptor is a G protein-coupled receptor which is activated by pineal hormone melatonin. There are two distinct classes of melatonin receptors: One, MT1 that is expressed in both central nervous system and peripheral tissues and second is MT2 which is expressed in retina and CNS²⁰⁶.

Using indirect immunofluorescence and confocal microscopy, it was shown that MT1 receptor colocalized with Cav-1 suggesting that MT1 receptor exists in caveolae in MCF-7 human breast cancer cells²⁰⁶.

Considering the involvement of MT1 signaling in MCF-7 human breast cancer cells, caveolae can serve as potential platform for regulation of MT1 signaling and therefore breast cancer therapy²⁰⁷.

Muscarinic receptors

Muscarinic acetyl choline receptor (mAChR), one of the two major types of cholinergic receptors consists of five subtypes M1-M5. mAChRs mediate peripheral response including cardiac rate modulation, smooth muscle contraction and glandular secretion. Moreover, they involve in temperature regulation, cardiovascular regulation and memory²⁰⁸.

Studies have shown the translocation of M2 muscarinic receptor to low density gradient fraction after agonist treatment^{209, 210}. Using immunofluorescence and immunoperoxidase staining it was also indicated that muscarinic acetylcholine receptor randomly distributed in human embryonal fibroblast cells. Prolonged agonist treatment leads to internalization of the receptor by noncoated-multivesicular structures²¹¹. However, Feron *et al.* revealed for the first time the nature of these structures in adult rat ventricular myocytes²¹². By the aid of detergent-free method and immunoprecipitation it was demonstrated that following agonist carbachol treatment, M2 muscarinic receptors translocate to caveolae containing caveolin-3, endothelial isoform of eNOS, p21ras, G proteins and receptor associated tyrosine kinase^{212, 213}. This translocation leads to 25-30% dynamin-dependent sequestration and internalization of mAChR that may negatively control NO signaling²¹⁴. Moreover, norepinephrine stimulation increase the amount of p21ras and G proteins in caveolae, indicating the importance of caveolae in signal transduction thus the potential role in physiological and pathological conditions²¹³.

Recently, M3 muscarinic receptor and $G_{q/11}$ was also demonstrated to localize in caveolae in human and canine airway smooth muscle (ASM). Caveolae disruption by cyclodextrin and caveolin-1 inhibition by SiRNA and cell-permeable caveolin-1 CSD peptide illustrate that caveolae and caveolin-1 play an important role in acetylcholine (ACh)-induced Ca^{2+} mobilization leading to ASM contraction^{191, 215}. Loss of cav-1 gene expression impairs M3 muscarinic cholinergic activity due to both interruption of ACh release and M3 receptor-mediated signaling in mouse urinary bladder smooth muscle²¹⁶. However, cholesterol desorption from human urinary bladder inhibits cholinergic contraction but no significant inhibition was seen in mouse or rat urinary bladder suggesting the different role of caveolae in M3 muscarinic receptor signaling among different species²¹⁷.

Neurokinin 1 receptors

Neurokinin 1 (NK1) receptor is a subtype of tachykinin receptors which binds to substance P. It is widely expressed in both central and peripheral nervous system. In CNS, NK1 receptor involves in several behavioral reactions, nociception, and autonomic reflex. In PNS, it mediates smooth muscle contraction, neurogenic inflammation and immune responses. Indeed, NK1 receptor has been implicated in several diseases such as anxiety, depression, arthritis, cystitis and emesis^{218, 219}.

Using detergent-dependent isolation methods it was found that both endogenous NK1 receptor in HAoSMC and transiently expressed receptor in HEK293 and HepG2

cells localized in lipid raft ²²⁰. The presence of NK1 receptor in lipid rafts was also confirmed by FRET in HEK293 cells ²²¹. The receptor colocalized with raft-associated proteins like annexin-6, Lyn and Gαq but the caveolin interaction was not crucial suggesting that receptors localized in non-caveolar raft ²²⁰.

Following receptor stimulation by Substance P, the activated PKC moved to lipid raft where NK1 receptor was present. Indeed, phosphorylation of C-tail of receptor by PKC was facilitated. Lipid raft disruption by MβCD or filipin reduced NK1 receptor mediated signaling. MβCD treatment abolishes NK1-induced ERK activation and phosphorylation after exposing to SP ²²⁰.

In another study, the existence of NK1 receptor in lipid raft was detected in HEK293 cells by computer simulation of the electron paramagnetic resonance (EPR) spectra and the GHOST consolidation procedure. In these methods, information is obtained from structure and properties of membrane domain ²²².

Using dominant-negative mutants of dynamin (dynk44A) and β-arrestin (beta-ar319-418), the receptor internalization was reduced whereas this effect was not significant using dominant-negative caveolin-1 (Cav-1 S80E). These observations suggest that receptor internalization occurs predominantly via clathrin-dependent pathway ²²².

Using BRET technology, it was shown that in the presence of caveolin S80E as well as MβCD, interaction of NK1 with β-arrestin was reduced suggesting that NK1R/β-

arrestin complexes form in lipid raft/caveolae, hence these microdomains play an important role in receptor desensitization ²²².

Therefore, we can conclude that regulation of NK1 receptor signaling by affecting lipid rafts may be a useful approach for treatment of relevant diseases such as depression, anxiety and inflammatory diseases.

Opioid receptors

Opioid receptors mediate physiological and pharmacological effects of endogenous opioid peptides and of opioid drugs ^{37, 223}. They are mainly coupled to G_i/G_0 and affect several signaling effectors such as adenylyl cyclase inhibition, p42/p44 MAP kinase activation, Ca^{2+} conductance reduction and K^+ conductance enhancement ³⁷. There are at least 3 types of opioid receptors including μ -, δ - and κ -opioid receptors which mainly distributed in central nervous system and several peripheral tissues such as kidney, spleen, lung, adrenal, small and large intestine, ovary, uterus and testis. δ -opioid receptor also predominantly expressed in heart ²²⁴.

μ -opioid receptor (MOR) was found to colocalize with caveolin-1 and adenylyl cyclase 5/6 in lipid rafts/caveolae of HEK293 cells using detergent-free method. The majority of MOR remains in lipid raft after agonist etorphine or morphine treatment. M β CD treatment inhibited etorphine-induced MOR internalization and also adenylyl cyclase superactivation ²²⁵. However, adenylyl cyclase superactivation after agonist treatment is dependent on receptor localization in lipid raft/caveolae but not on the agonist-induced receptor internalization ²²⁵.

In another study done by Zheng *et al.* it was shown both in vitro and in vivo that following agonist etrophine but not morphine treatment MOR moves from lipid raft to non raft fractions. For agonist-induced translocation, the receptor should dissociate from G α i2 and then bind to β -arrestin. M β CD treatment attenuated the etrophine- and morphine-meidated adenylyl cyclase inhibition and morphine-induced but not etrophine-induced ERK phosphorylation suggesting that receptor localization in lipid raft is necessary for these G-protein-dependent signalings, while etrophine-induced ERK phosphorylation required receptor movement to non-raft domain ²²⁶.

The δ -opioid receptor involves in mood regulation, analgesia and morphine tolerance. δ -opioid receptor was shown to localize in caveolae and associate with caveolin-3 by detergent-free method and coimmunoprecipitation experiments in adult rat ventricular myocytes ²²⁷. Caveolae disruption by M β CD attenuates cytoprotection induced by IPC and δ -opioid receptor agonist, suggesting the importance of receptor compartmentation in caveolae for cardiac protection from ischemic damage ²²⁷. These results also confirmed by another study showing that δ -opioid-induced IPC is dependent on caveolin-3 expression ²²⁸.

Caveolin-1 and caveolin-3 expression in neuroblastoma $\times$ glioma NG108-11 hybrid cells attenuate the inhibition of N-type Ca²⁺ channels induced by δ -opioid receptor stimulation. Indeed, this effect could be important for regulation of electrical excitability in neurons ²²⁹.

Using detergent-free methods, Huang *et al.* showed that the majority of δ -opioid receptors reside in lipid rafts in caveolin-deficient neuronal cells (NG108-15) and

caveolin-enriched non-neuronal cells (CHO) and the majority of total opioid receptors present in rat caudate putamen (CPu) were also located in low density membrane domains. After full agonist treatment, a portion of δ -opioid receptors, which may undergo internalization, moved out of lipid rafts independent of receptor coupling to $G_{i/o}$ protein but dependent on receptor activation²²³.

Cholesterol reduction by M β CD treatment attenuates δ -opioid receptor-mediated G protein signaling in rat brain and NG108-15 cells but increased it in CHO cells. This discrepancy between cells might be due to high level of caveolin-1 that inhibits G protein, in non-neuronal cells (CHO) but not in neuronal cells (CPu and NG108-15). Cholesterol depletion reduced the association between $G\alpha_i$ and caveolin-1, which cause disinhibition of $G\alpha_i$, thereby increasing signaling in CHO cells²²³.

Contradictory with the above results, Levitt *et al.* showed by the aid of detergent-dependent isolation methods that δ -opioid receptors resided in caveolin-deficient fractions, the same fractions as a non raft marker, transferrin receptors exists in HEK293 cells while μ -opioid receptors resided in both caveolin-poor and enriched fractions²³⁰. This discrepancy with previous results may be due to different methods used for isolation of membrane microdomains²³⁰. Moreover, cholesterol depletion by M β CD did not alter adenylyl cyclase sensitization or inhibition by δ -opioid agonists²³⁰. Using same methods and identical cell system, μ -opioid receptor agonist-mediated adenylyl cyclase inhibition and sensitization were attenuated by M β CD²³⁰. Despite the fact that this signaling change is the result of cholesterol reduction, it cannot be concluded that the effect is due to lipid raft destruction²³⁰.

Kappa-opioid receptor (KOR) which produces physiological and pharmacological effects such as water diuresis, dysphoria, analgesia and antipruritic effects is found to reside in lipid rafts by detergent-free method ³⁷. The endogenous KOR in human placenta and stably expressed in CHO cells located in lipid rafts. Disruption of lipid raft by M β CD, increased phosphorylation of p42/44 MAPK indicating that KOR localization in lipid rafts greatly affects G proteins coupling to receptor ³⁷.

Oxytocin receptors

The mammalian hormone and neurotransmitter oxytocin (OT) acts through oxytocin receptor. The typical functions of oxytocin are stimulation of uterine smooth muscle contraction during parturition and milk ejection throughout lactation. OT receptor also involves in some behaviors such as learning and memory, stress and anxiety, sexual and maternal behaviors ²³¹.

Oxytocin receptor (OTR) is found in many tissues including uterus, heart, kidney, pancreas and thymus. It is also abundant in several brain regions such as thalamus, hypothalamus, limbic system, basal ganglia, brain stem and spinal cord ^{231, 232}.

Using both detergent-free and Triton X-100 extraction methods, it was shown that about 1-15% of oxytocin receptor localized to cholesterol-sphingolipid enriched

membrane domain (raft) in HEK293 cells ²³³. It was also demonstrated that small amount of the OTR exists in caveolin-1 enriched microdomains of MDCK cells ²³⁴. The receptors associated with lipid rafts exhibit 3-fold higher stability compared to receptors localized in cholesterol-poor high density fractions ²³³.

There is evidence that localization of OTR in caveolae plays an important role in regulating cell proliferation. OTR targeted to caveolae upon fusion to caveolin-2 leads to enhanced cell proliferation while excluding from this microdomain inhibits the mitogenic activity and cell growth ²³⁴. Therefore, the integrity of lipid raft is necessary for preserving this signaling effect ^{234, 235}. Interestingly, oxytocin receptor and caveolin-1 expression and colocalization increase in aging and benign prostatic hyperplasia (BPH) pointing to the proliferative effect of oxytocin on caveolae-associated oxytocin receptors ²³⁶. Nevertheless, the result of Whittington study on human prostate is totally different ²³⁷. Caveolar disruption by cyclodextrin impedes the inhibitory effect of OT in PrSC cells in the presence of testosterone while it does not affect OT-induced cell proliferation in PC-3 cells. It is noteworthy to consider here that under normal condition, OT inhibits cell proliferation in PrSC cells but stimulates cell proliferation in PC-3 cells suggesting differential effects of OT based on cell type ²³⁷.

P2Y receptors

P2Y receptors, a family of purinergic receptors regulate the effector systems including phospholipase C and adenylyl cyclase by binding to G proteins. They involve

in many biological functions such as immune regulation, lipogenesis regulation, platelet aggregation and smooth muscle proliferation^{238, 239}.

The presence of endothelial P2Y receptor in cholesterol-enriched domain such as caveolae was proposed for the first time in guinea pig aortic endothelial cells (GPAECs)²⁴⁰. Treatment of cells with M β CD and filipin disrupted the P2Y signaling suggesting that these microdomains are essential for agonist-specific response to intracellular calcium rising²⁴⁰. Attenuation of P2Y signaling by RNAi-mediated Cav-1 knockdown in C6 cells also supported the importance of Cav-1 and caveolae in P2Y-mediated signaling²⁴¹.

Quinton *et al.* for the first time determined the importance of lipid raft localization of P2Y₁₂ G α_i -coupled in ADP-mediated platelet aggregation and adenylyl cyclase down-regulation²⁴². M β CD treatment reduced P2Y₁₂-mediated inhibition of cAMP production and platelet aggregation indicating that lipid raft is required for ADP-mediated platelet activation²⁴².

Using detergent-dependent methods it was shown that in resting cells, P2Y₁₂ receptor homooligomers localized in lipid raft²⁴³. Both in vivo treatment of clopidogrel and its active metabolite, Act-Met leads to disruption of oligomers and partitioning of dimers and monomers out of lipid rafts resulting in the lost ability of the receptor to bind to ligand. Regarding the role of P2Y₁₂ in platelet aggregation, this mechanism describes in vivo antithrombotic and antiaggregating activities of clopidogrel²⁴³.

P2Y2 receptor was found to localize in lipid raft microdomains of NG 108-15 cells using detergent-dependent and immunoblotting methods. Treatment of cells with cyclodextrin and $G_{q/11}$ inhibitor inhibited P2Y2-induced PLC activation and Ca^{2+} mobilization and UTP-mediated cell migration suggesting that lipid rafts are essential for P2Y2-mediated $G_{q/11}$ -PLC- Ca^{2+} signaling in NG 108-15 cells ²³⁸.

Pituitary adenylate cyclase-activating peptide receptors

Pituitary adenylate cyclase-activating peptide (PACAP) which is known to involve in neuronal differentiation and proliferation, mediates its action by binding to PACAP type one receptor (PAC1R). PACAP receptor is widely distributed in both peripheral tissues and central nervous system including endocrine glands, pancreas, hypothalamus, pineal glands and granule layer of the cerebellum ^{244, 245}.

Zhang *et al.* used detergent-dependent isolation method to show that PACAP stimulation increased the existence of PAC1R in caveolin-enriched detergent-insoluble microdomains of PC12 cells ²⁴⁶. Using confocal microscopy also exhibited that PAC1R distributed uniformly in PC12 cells whereas receptors in PACAP-stimulated cells appeared in patched patterns. Additionally the level of PAC1R colocalization with GM1 elevated after PACAP stimulation. Taken together, these results demonstrated that PACAP stimulation induced translocation of PAC1R into lipid rafts/caveolae ²⁴⁶.

Perturbation of caveolae by different methods such as treatment of cells with filipin or nystatin and caveolin-1 siRNA, inhibited PACAP-induced neurite outgrowth indicating the importance of lipid rafts/caveolae in PACAP-induced neuritogenesis.

PAC1R translocation to lipid rafts/caveolae increased the efficiency of receptor coupling to AC. Indeed, cAMP production increased and consequently activated the PACAP-induced neurite outgrowth²⁴⁶. However, May *et al.* showed that filipin and nystatin treatment had little effect on PACAP-mediated Akt activation in sympathetic neurons²⁴⁷.

Platelet-activating factor receptors

Platelet-activating factor (PAF) is an active phospholipid mediator which mediates its action by binding to PAF receptor (PAFR). PAFR implicated in many pathologic processes such as inflammatory process, anaphylaxis, asthma, streptococcal infection, skin disease and CNS disorders^{248, 249}.

Using detergent-free isolation methods, Poisson *et al.* demonstrated that PAFR resided in lipid raft microdomains in CHO, HEK293, COS7 and A431 cell lines. PAFR physically interacts with caveolin-1 via its caveolin-binding motif which is important in PAFR-mediated signaling including inositol phosphate (IP) production and MAPK activation²⁴⁸.

Lipid raft/caveolae disruption by M β CD treatment reduced IP production, intracellular Ca²⁺ flux and ERK phosphorylation in both cells expressing caveolin-1 (A431, HEK293) and cells lacking it (Ramos B cells), but it has no effect in monocytes. Therefore, PAFR localization in lipid raft/caveolae may be important for PAFR signaling in many cells²⁴⁸.

Prostanoid receptors

There are eight subtypes of prostanoid receptors including DP, FP, IP, TP, EP1, EP2, EP3 and EP4. They are activated by prostaglandin (PG) D₂, PGE₂, PGF_{2α}, PHI₂, PGH₂, prostacycline (PGI₂) and thromboxane A₂. Prostanoid receptors play an important role in physiological and pathophysiological conditions such as inflammation, bone resorption, systemic blood pressure regulation, pain and allergy^{250, 251}.

Using detergent-free methods and immunofluorescence microscopy, Liu *et al.* found that a distinct portion of Prostanoid IP receptor but not EP2 receptor, localized in lipid rafts in COS7 cells²⁵². AC6 overexpression increased IPR-agonist-mediated enhancement in cAMP production indicating that AC6 colocalized with IPR in lipid raft²⁵².

In another study, using detergent-free isolation method and immunoblotting analysis it was shown that prostanoid EP2 and EP4 receptors localized in non-raft, non-caveolin rich fractions while IPR and β₂AR colocalized with AC6/AC5 in lipid rafts²⁵³.

Protease-activated receptors

Protease-activated receptors (PARs) mediate several physiological and pathological process including inflammation, homeostasis, thrombosis, cell proliferation, vascular development and cancer progression. There are 4 subtypes of PARs: PAR-1, PAR-2, PAR-3 and PAR-4. They are mainly expressed in endothelial cells, smooth muscle cells, immune cells, platelets and fibroblasts^{254, 255}.

The predominant mediator of thrombin, PAR-1 and its signaling components have been shown by detergent-free and immunoaffinity isolation to reside in lipid raft in endothelial cells²⁵⁶. Consistently, Lu *et al.* using detergent-free, immunoprecipitation and confocal microscopy found that PAR-1 partially localizes in caveolae and physically interact with cavolin-1 in COS7 cells and the agonist treatment does not alter the receptor localization²⁵⁷.

Disruption of lipid rafts in BAECs by filipin attenuates PAR-1 stimulated myosin light chain (MLC) phosphorylation which is necessary for actin-myosin interaction and stress fiber formation. In contrast, caveolin-1 knock down did not affect MLC phosphorylation and cell morphology suggesting that lipid raft, but not caveolae, is necessary for PAR-1-induced signaling pathways and endothelial cytoskeletal reorganization²⁵⁶.

PAR-2 which is resistant to thrombin was shown to exist mainly in lipid rafts under basal condition by western immunoblotting of epidermis²⁵⁸. Following receptor activation and also barrier abrogation, the receptor internalized from lipid raft to

cytoplasm²⁵⁸. The localization of PAR-2 also was shown in human breast carcinoma cell line MDA-MB-231 by detergent-dependent isolation method²⁵⁹. Using dual immunofluorescence confocal microscopy it was shown that PAR-2 as well as tissue factor (TF) colocalized with caveolin-1 in caveolae²⁵⁹.

The activation of PARs occurs through the proteolytic cleavage of extracellular N-terminal segment of inactive receptor²⁵⁴. Lipid raft/caveolae is required for cleavage of PARs by activated protein C (APC) or TF-VIIa (coagulation factor VIIa forms a complex with TF)^{259, 260}. As an example, membrane compartmentalization of PAR-1 in caveolae is critical for activation of receptor protective signaling by APC in endothelial cells^{260, 261}.

Serotonin 5-HT receptors

The serotonin 5-HT receptors are divided to 6 classes of G protein-coupled receptors including 5-HT1, 5-HT2, 5-HT4, 5HT5, 5-HT6 and 5-HT7 and one ligand-gated ion channel 5-HT3²⁶².

Using detergent-free method, Dreja *et al.* for the first time showed that serotonin receptor 5HT-2A localized in caveolae⁸⁰. Cholesterol depletion by cyclodextrin reduced agonist response while the response restores by exogenous cholesterol that renovates caveolae. As the level of 5-HT2A receptor and caveolin-1 were not affected by cholesterol extraction, it was suggested that localization of receptor in caveolae may be responsible for agonist response⁸⁰.

5-HT_{1A} receptor which plays a crucial role in psychiatric disorders was shown that partially associates with lipid raft. Using copatching assay and gradient centrifugation it was shown that notable fraction of 5-HT_{1A} receptor colocalized with lipid raft marker GM1 in NIH-3T3 and CHO cells. Receptor palmitoylation plays an important role in this localization and signaling²⁶³.

5-HT₇ receptor responsible for regulation of circadian rhythms, vasodilatation, depression, emotions, memory process and thermoregulation is known to depend on lipid raft integrity for its function. Treatment of Hela cells with fumonisin B1 which depletes sphingomyelin and is a potent glucosylceramide synthesis inhibitor, ($\pm$)-*threo*-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) reduced 5-HT binding to 5-HT₇ receptor. This reduction is not dependent on neither cholesterol nor receptor level as both treatment did not change cholesterol or receptor level suggesting that the lipid raft integrity is responsible for actions of 5-HT via 5-HT₇ receptor²⁶⁴.

Somatostatin receptors

Somatostatin is an abundant neuropeptide which has inhibitory effect on many cellular functions such as proliferation, secretion and motility. It mediates its action through five subtypes of somatostatin receptors (SSTR1-SSTR5). Somatostatin (SST) and somatostatin receptor (SSTR) also connect to several pathological conditions such as Alzheimer's disease, neuroendocrine dysfunctions and some types of cancers²⁶⁵.

Different SSTRs are expressed in various normal and tumor cells. As an example, SST2 receptor, the most common sst receptors is expressed in pancreas, spleen, vessels, pituitary glands, adrenal glands and prostatic stroma and neuroendocrine tumors. It is also expressed in breast carcinoma, lymphomas, medulloblastomas, paragangliomas and hepatocellular carcinomas ^{265, 266}. Mentlein *et al.* were the first to report that SST2 receptor internalizes via caveolin-positive endosomal vesicles in human glioma cells using electron microscopy and biochemical approaches ²⁶⁷. Although it was shown before that SST2 receptor internalizes via uncoated endosomal caveolin-free vesicles ²⁶⁸.

Sphingosine 1-phosphate receptors

Sphingosine 1-phosphate receptor (S1P1R) also known as endothelial differentiation gene 1 (EDG1) is strongly expressed in lymphoma cells ²⁶⁹. It plays an important role in regulation of cell migration, cell proliferation, cell differentiation and angiogenesis ^{270, 271}.

Sphingosine 1-phosphate receptor has been shown by detergent-free method and coimmunoprecipitation to reside in caveolae ²⁷². Although the majority of S1P1R exists in caveolae, agonist S1P treatment increase the concentration of receptor in caveolae meaning that agonist induces S1P1R translocation from noncaveolar fraction to caveolae in transiently transfected COS-7 cells ²⁷². However, this is inconsistent with the Kohno study that showed that S1P1R accumulated in caveolin-enriched microdomains in stably transfected CHO cells in agonist-independent manner. The reason for this dissimilarity is not yet known but may depend on type of transfection or

cell type ²⁷⁰. It is known that N-glycans of receptors (specific glycosylation site at asparagine 30) play an important role in targeting the S1P1R to caveolae ²⁷⁰.

S1P activates eNOS while caveolin-1 overexpression inhibits eNOS activation and S1P-induced phosphorylation of S1P1R. This inhibitory effect of caveolin can help us in regulating the sphingolipid-dependent signaling in vascular wall ²⁷².

Thyrotropin receptors

Thyrotropin receptor (TSHR) is a member of glycoprotein hormone receptor found on the basolateral membrane of thyroid epithelial cells ²⁷³. In response to TSH, the receptor coupled to G_s leading to enhanced cAMP levels ²⁷⁴. The TSHR plays a crucial role in development, growth and function of thyroid ²⁷⁵.

Thyrotropin receptor has been shown to reside in lipid raft of CHO cells using confocal microscopy. The receptors move out of lipid raft upon activation ²⁷⁶. Although, Costa *et al.* demonstrated that TSH receptors did not exist in sphingolipid-cholesterol domain/caveolae either in thyrocytes or CHO cells using detergent-dependent isolation methods, ²⁷⁷ localization of TSHR in CHO and thyrocyte lipid rafts were proved by another study using detergent-free and coimmunoprecipitation methods ²⁷⁸. It was shown that multimeric TSHR preferentially resides in lipid rafts whereas TSHR monomers found in both lipid raft and non-raft fractions ²⁷⁸. Treatment of cells with M β CD did not alter the cAMP accumulation induced by TSH. This can be explained by the fact that M β CD was not able to remove all monomeric thyrotropin receptors, suggesting that TSHR-associated with lipid raft were not changed enough by M β CD to

disrupt signaling^{277, 278}. However, further investigation showed that cAMP accumulation increased by caveolin-1 knockdown suggesting that lipid rafts attenuate $G\alpha_s$ -coupled TSHR signaling²⁷⁹.

Thyrotropin-releasing hormone receptors

Thyrotropin-releasing hormone receptor (TRHR) is a mammalian G-protein coupled receptor presents in brain and pituitary^{280, 281}. Thyrotropin releasing hormone (TRH) which binds to TRHR controls the synthesis and release of thyrotropin and prolactin from anterior pituitary²⁸². TRH binding to TRHR activates phospholipase C and phosphoinositidase²⁸².

Experiments assessing the subcellular distribution of TRHR reported variable results. Dromata *et al.* for the first time showed that TRHR stably expressed in HEK293 cells moved to low-density vesicular fractions from plasma membrane upon agonist treatment²⁸². Although the nature of these low density fractions was not determined, later it was shown that caveolae disruption by β -cyclodextrin prevented $G_{q/11}\alpha$ tyrosine phosphorylation indicating that intact caveolae are needed for TRH-induced signaling²⁸³. However, Rudajev *et al.* using three different methods of cell fractionation (detergent extraction, alkaline sonication and mechanical homogenization) later demonstrated that the majority of TRHR is excluded from detergent-resistant membrane domain/caveolae in the same cells²⁸⁴.

Vasopressin receptors

Vasopressin receptors (AVP) are activated by three receptor subtypes, V1a and V1b receptors that activate phospholipase C and V2 receptor which stimulates adenylyl cyclase. Vasopressin receptors play an important role in regulating water homeostasis. They involve in nephrogenic diabetes insipidus ²⁸⁵.

To our knowledge, the localization of vasopressin receptors in lipid rafts has not been reported yet. Moneer *et al.* showed that V1a is not concentrated in lipid raft using membrane fractionation and immunoblotting ²⁸⁶. However, Dreja *et al.* have shown that M β CD treatment reduces the AVP signaling in endothelium of tail artery ⁸⁰. It was revealed later that lipid raft is required for AVP-induced trafficking in thick ascending limb of loop of Henle ²⁸⁷.

γ -aminobutyric acid (GABA) receptors

γ -aminobutyric acid is the major inhibitory neurotransmitter which mediates its action through GABA_A, GABA_B and GABA_C receptors. Both GABA_A and GABA_C are ionotropic receptors whereas GABA_B is metabotropic. GABA_B receptors are G protein-coupled receptors distributing within central nervous system and also peripheral autonomic terminals ^{288, 289}.

Using detergent-dependent method following lipid raft isolation by sucrose gradient fractionation, Becher *et al.* for the first time found that GABA_B receptors associate with lipid raft in rat cerebellum²⁹⁰. Localization of G_{αo} and G_{αi} in lipid raft might indicate that lipid raft in rat cerebellum cluster GABA_B receptor and its downstream signaling molecules²⁹⁰.

Using the same method, it was demonstrated that GABA_BR associates with lipid raft in CHO cells as well, indicating that GABA_B receptor localization to lipid raft is not depend on the cell type²⁹¹. Comparing the receptor signaling between whole membrane fractions and lipid raft-enriched fractions, showed the reduction in agonist-induced GTPγS binding suggesting that GABA_B receptor localization to lipid raft may inhibit receptor signaling²⁹¹.

Table 1. G protein-coupled receptors that associate with lipid rafts.

Receptor	Receptor organism	Functional implication	Methods used to detect raft association	Mechanism of raft association	Caveolin-binding motif	Cell type	Species	Receptor expression
Adenosine A1 receptor	Rat ⁶⁷ Pig ⁶⁸ Syrian hamster ⁶⁹	Agonist stimulation leads to translocation out of ⁶⁷ or into caveolae and caveolae-dependent internalization ^{68, 69} .	Detergent-free ^{67, 68} Electron microscopy ⁶⁹ Confocal microscopy ⁶⁹	Bound caveolin-3 ⁶⁷ and caveolin-1 ⁶⁹	Yes ⁶⁸	Ventricular cardiomyocyte ⁶⁷ Kidney epithelial cell ⁶⁸ Smooth muscle cell line ⁶⁹	Rat ⁶⁷ Pig ⁶⁸ Syrian hamster ⁶⁹	Endogenous ¹⁻³
Adenosine A2 _A receptor	Dog ⁷⁶ Rat ⁷⁷	Internalization via raft-dependent mechanism ⁷⁶	CM ⁷⁶ , Detergent-dependent ⁷⁷	Bound caveolin-1 ⁷⁶		CHO ⁷⁶ Neonatal spinal cord ⁷⁷	Chinese hamster ⁷⁶ Rat ⁷⁷	Stable transfection ⁷⁶ Endogenous ⁷⁷
Angiotensin II type 1 receptor	Rat ^{88, 93} Human ⁹²	Agonist stimulation leads to translocation to caveolae ⁸⁸ or non-caveolin lipid raft ⁹³ or with no effect on localization ⁹² . Caveolin-binding mutation reduces plasma membrane expression, agonist-dependent internalization and phospholipase C production ⁹² .	Detergent-free ^{88, 93} , Detergent-dependent ⁹³ EM ⁹²	Bound caveolin-1 ⁸⁸	Yes ⁹³	VSMC ⁸⁸ BHK ⁹³ HEK cells ⁹³ COS-7 cells ⁹²	Rat ⁸⁸ Hamster ⁹³ Human ⁹³ Monkey ⁹²	Endogenous ⁸⁸ Transient transfection ^{92, 93}
B-Leukotriene receptor	Human ¹⁴⁷ Rat ¹⁴⁹	Agonist treatment does not alter receptor localization ¹⁴⁷ . MβCD treatment abolishes LTB ₄ -induced Ca ²⁺	Detergent-dependent; FRET ¹⁴⁷ Detergent-free ¹⁴⁹	n.a		Neutrophil ¹⁴⁷ Alveolar macrophage ¹⁴⁹	Human ¹⁴⁷ Rat ¹⁴⁹	Endogenous ^{147, 149}

		mobilization and ERK1/2 phosphorylation ¹⁴⁷ . After IgG-RBC treatment receptor moved to lipid raft ¹⁴⁹ .						
Bradykinin B1 receptor	Rabbit ⁹⁶ Human ¹⁰⁰	Agonist induced receptor translocation to caveolae ⁹⁶ . Receptor localization in CLR not changed in response to agonist ¹⁰⁰ .	CM ⁹⁶	Putative caveolin binding motif ¹⁰⁰	Yes ¹⁰⁰	HEK293 ^{96, 100}	Human ^{96, 100}	Stable transfection ^{96, 100}
Bradykinin B2 receptor	Human ¹⁰⁰ Hamster ⁹⁷	Agonist BK induces movement of receptor, Gαq and Gαi to caveolae ^{97, 98} . BK treatment leads to translocation of receptor out of caveolae ⁹⁹ or had no effect on localization ¹⁰⁰ .	Detergent-free ^{97, 99} Detergent-dependent ⁹⁷ CM ⁹⁸ Immunoblotting ¹⁰⁰	Putative caveolin binding motif ¹⁰⁰	Yes ¹⁰⁰	DDT1-MF-2 smooth muscle cell ⁹⁷ Epithelial carcinoma cell line ⁹⁸ Bovine aortic endothelial cells ⁹⁹ HEK293 ¹⁰⁰	Hamster ⁹⁷ Human ^{100, 98} , Bovine ⁹⁹	Transient transfection ¹⁰⁰ Stable transfection ⁹⁸ Endogenous ⁹⁷
Ca sensing receptor	Bovine ¹⁰² Human ¹⁰³	IPC increases ERK1/2 phosphorylation in caveolin-3-enriched fraction ¹⁰⁵	Detergent-free ^{102, 105} Detergent-dependent ¹⁰³	Bound caveolin-1 ¹⁰³ and caveolin-3 ¹⁰⁵	Yes ¹⁰³	Parathyroid cells ¹⁰² Saos-2 cells ¹⁰³ Cardiomyocytes ¹⁰⁵	Bovine ¹⁰² Human ¹⁰³ Mouse ¹⁰⁵	Endogenous ^{102, 103}
Cannabinoid CB1 receptor	Human ¹⁰⁸ Mouse ¹¹² Human ¹¹³	Agonist treatment decreases the receptor associated with lipid rafts ¹⁰⁸ . Receptor partially internalized via caveolae ¹¹² .	Detergent-free based on Opti prep density gradient ¹⁰⁸ CM ¹⁰⁸			Breast cancer MDA-MB-231 cells ¹⁰⁸ HEK-293 ^{112, 113}	Human ^{108, 112, 113}	Endogenous ¹⁰⁸ Stable transfection ^{112, 113}
CC chemokine receptor CCR5	Human ¹²⁰⁻¹²²	Functional receptor localizes into lipid raft upon agonist treatment ¹²¹ . CCR5 internalized via caveolae-dependent way ¹²² .	Detergent-dependent ¹¹⁹ Immunofluorescence microscopy ¹²⁰	Palmitoylation of C-terminal domain ¹¹⁸		Human breast carcinoma MCF-7 cells ¹¹⁹ Hela cells ¹²⁰ ; HEK-293 cells ¹²⁰ CEM NK T cells ¹²¹ CHO cells ¹²²	Human ¹¹⁹⁻¹²¹ Chinese hamster ¹²²	Transient transfection ¹²⁰ Stable transfection ^{121, 122}
CC chemokine CCR6	Human ⁸⁸	MβCD treatment impaired receptor-	Detergent-dependent ⁸⁸			Jurkat cells ⁸⁸	Human ⁸⁸	Stable transfection ⁸⁸

		induced chemotaxis ⁸⁸						
Cholecystokinin receptor type 1	Rat ¹⁴³	Receptor partially internalized via caveolae ¹⁴³	Detergent-dependent ¹⁴³ EM ¹⁴³	include palmitoylation site in c-terminal ¹⁴³		CHO cells ¹⁴³	Chinese hamster ¹⁴³	Stable transfection ¹⁴³
CXC chemokine receptor type one (CXCR1)		Ligand induced receptor movement to lipid raft ¹⁴⁰ .	FRET ¹⁴⁰ FRAP ¹⁴⁰	n.a		CX1-HEK cells ¹⁴⁰	Human ¹⁴⁰	Stable transfection ¹⁴⁰
CXC chemokine receptor type four (CXCR4)	Human ^{125, 132}	Gp120 recruits receptor to lipid raft ¹³² . MβCD treatment inhibits receptor internalization and HIV infection ^{126, 134, 135} .	CM ^{125, 126, 132} EM ¹³² , Detergent-dependent ¹²⁶ CM ¹³⁵			HEK293 ¹²⁵ PBMC ¹²⁵ Sup T1 lymphoblastoid ¹³² PM1 T cells ¹²⁶ Hematopoietic progenitor cell line KG1a ¹³⁵	Human ^{125, 126, 132, 135}	Endogenous ^{125, 132} Stable expression ¹³⁵
Dopamine D1 receptor	Human ^{157, 159} , rat ¹⁵⁸	Agonist treatment causes the receptor shift to caveolar plasma membrane ¹⁵⁷⁻¹⁵⁹ . Caveolin-2 antisense reduces cAMP accumulation ¹⁵⁷ . Caveolin-1 binding motif mutation and MβCD treatment abolish receptor internalization ¹⁵⁹ .	Detergent-free ¹⁵⁷⁻¹⁵⁹ CM ¹⁵⁷ BRET ¹⁵⁹	Interaction with Caveolin-2 ¹⁵⁷ ; Bound caveolin-1 ¹⁵⁹	Yes ¹⁵⁹	HEK293 ^{157, 159} Proximal tubular cells ^{157, 158} COS-7 ¹⁵⁹	Human ^{157, 159} Rat ¹⁵⁸	Stable transfectant ¹⁵⁷ COS-7: transient transfection ¹⁵⁹ , endogenous ¹⁵⁸
Endothelin A receptor	Rat ¹⁶² Human ¹⁶⁴	Receptor localization in caveolae is important in signal transduction ¹⁶² . Agonist induces receptor internalization ¹⁶⁴ .	Coimmunoprecipitation ¹⁶²	Bound caveolin ¹⁶²		COS cells ¹⁶² CHO cells ¹⁶⁴	Chinese hamster ¹⁶⁴	Transient transfection ¹⁶² Stable transfection ¹⁶⁴
Endothelin B receptor	Rat ¹⁶⁵ Plasmid ¹⁶⁷	Filipin treatment inhibits signaling ¹⁶⁵ . Receptor moves out of caveolae upon agonist treatment ¹⁶⁷	Detergent-free ¹⁶⁵ Detergent-dependent ¹⁶⁷ Detergent-free ¹⁶⁶	Bound caveolin-1 ¹⁶⁷	No ¹⁶⁷	Primary astrocytes ¹⁶⁵ HEK293 ¹⁶⁷	Rat ¹⁶⁵	Endogenous ¹⁶⁵ Stable transfection ¹⁶⁷
Formyl	Human	MβCD	CM ¹⁴⁸			U937	Human	Stable

peptide receptor	¹⁴⁸	treatment reduces receptor internalization and abolished Ca^{2+} mobilization in response to ligand ¹⁴⁸				promonocytic leukemia cell line ¹⁴⁸	¹⁴⁸	transfection ¹⁴⁸
Formyl peptide receptor like-1	Human ¹⁵²	MβCD treatment suppresses WKYMVM-induced IKK, ERK and Akt phosphorylation ¹⁵²	Detergent-dependent ¹⁵²			U87 astrocytoma cells ¹⁵² CHO cells ¹⁵²	Human ¹⁵² Chinese hamster ¹⁵²	Endogenous ¹⁵² Stable expression ¹⁵²
GABA _B receptor	Rat ²⁹⁰ Hamster ²⁹¹	Receptor localization in lipid raft may inhibit signaling ²⁹¹	Detergent-dependent ^{290, 291}	n.a		Cerebellum ²⁹⁰ CHO cells ²⁹¹	Rat ²⁹⁰ Chinese hamster ²⁹¹	Endogenous ²⁹⁰ stable expression ²⁹¹
Glucagon-like peptide 1 (GLP-1) receptor	Mouse ¹⁷¹	Receptor interaction with caveolin-1 is necessary for cellular trafficking. Receptor-induced ERK1/2 activity depends on lipid raft integrity. Agonist induced receptor internalization in association with caveolin-1 ¹⁷¹	Detergent-dependent ¹⁷¹	Interact with caveolin-1 ¹⁷¹	Yes ¹⁷¹	Insulinoma MIN6 cells ¹⁷¹ HEK293 cells ¹⁷¹	Human ¹⁷¹ Mouse ¹⁷¹	Endogenous ¹⁷¹ Stable expression ¹⁷¹ Transient expression ¹⁷¹
Glucagon-like peptide 2 (GLP-2) receptor	Human ¹⁷²	MβCD treatment inhibited receptor internalization and desensitization but not adenyly cyclase activity ¹⁷²	Detergent-dependent ¹⁷² Western blot ¹⁷²			DLD-1 cells ¹⁷² BHK cells ¹⁷²	Human ¹⁷² Hamster ¹⁷²	Transient transfection ¹⁷²
Histamine H1 receptor	Human ¹⁹²	Filipin or nystatin prevented histamine-induced internalization of receptor ¹⁹²	CM ¹⁹²			CHO-K1 ¹⁹²	Chinese hamster ¹⁹²	Stable expression ¹⁹²
Luteinizing hormone (LH) receptor	Rat ¹⁹⁶	After human chorionic gonadotropin binding, receptors move	Detergent-dependent ¹⁹⁶			CHO cells ¹⁹⁶	Chinese hamster ¹⁹⁶	Stable expression ¹⁹⁶

		to lipid raft. ^{195, 196} . MβCD treatment reduced signaling ¹⁹⁶ .						
Lipoxin A4 receptor	Human ¹⁵³	MβCD treatment reduces LXA4-dependent PDGFRβ dephosphorylation ¹⁵³ . LXA4 binding to receptor promotes SHP-2 recruitment to lipid raft.	Detergent-dependent ¹⁵³	n.a		HEK293 ¹⁵³	Human ¹⁵³	Stable Overexpression ¹⁵³
Lysophosphatidic acid receptor	Pig ²⁰⁰	MβCD treatment reduces LPA-induced ERK1/2 phosphorylation ²⁰² .	Immunoprecipitation ²⁰⁰ Western blot ²⁰⁰			PCMVECs ²⁰⁰ COS-7 cells ²⁰²	Pig ²⁰⁰	Endogenous ²⁰⁰
M2 muscarinic receptor	Rat ^{212, 214}	Receptor translocates to caveolae upon agonist treatment ²¹² ; Possibly internalization by raft – dependent mechanism ²¹⁴ .	Detergent-free ^{212, 214} Immunoprecipitation ²¹²	n.a		Ventricular myocyte ²¹² Cardiac myocyte ²¹⁴ COS-7 cells ²¹⁴	Rat ^{212, 214}	Endogenous ²¹² Cardiac myocyte: endogenous ²¹⁴ COS-7: transient transfection ²¹⁴
M3 muscarinic receptor	Human ^{191, 215} Canine ²¹⁵	Caveolae disruptions by cyclodextrin and caveolin-1 inhibition impair Ca ²⁺ regulation ^{191, 215} .	Caveolae disruption ^{191, 215}	n.a		Airway smooth muscle ^{191, 215}	Human ^{191, 215} Canine ²¹⁵	Endogenous ^{191, 215}
Mammalian GnRH receptor	Mouse ¹⁸⁶	MβCD treatment attenuates GnRHR activation of ERK and induction of c-fos gene expression ¹⁸⁶ .	Detergent-free, detergent-dependent ¹⁸⁶	n.a		Gonadotrope-derived α-T3-1 cell, CHO cells ¹⁸⁶	Mouse ¹⁸⁶ Chinese hamster ¹⁸⁶	α-T3-1 cell: Endogenous ¹⁸⁶ CHO: Stable transfection ¹⁸⁶
Melanin-concentrating hormone receptor (MCHR)		MβCD treatment impaired MCH-mediated ERK signaling ²⁰⁵ .	Detergent-free ²⁰⁵	Bound caveolin-1 ²⁰⁵		CHO-K1 cells ²⁰⁵	Chinese hamster ²⁰⁵	Stable transfection ²⁰⁵
Melatonin receptor	Human ²⁰⁶		Immunofluorescence and confocal microscopy ²⁰⁶			MCF-7 breast cancer cells ²⁰⁶	human	Endogenous ²⁰⁶
Metabotropic glutamate receptor type		Agonist increases signaling in	Detergent-free ¹⁷⁷ CM ^{177, 178}	Caveolin binding motif ¹⁷⁸ ,	Yes ^{178, 179}	BHK-1α cells ¹⁷⁷ HEK293 ¹⁷⁸	Hamster ¹⁷⁷ Human	Permanently expressed ¹⁷⁷

1		caveolin-enriched membrane ¹⁷⁷ and also induces internalization via caveolae/raft ^{178, 179} .	Detergent-dependent ¹⁷⁸ TAP ¹⁷⁸ Pull down assay ¹⁷⁸	¹⁷⁹		MDCK ¹⁷⁸ Neurons ¹⁷⁸ Cerebellum ¹⁷⁹	¹⁷⁸ Canine ¹⁷⁸ Mouse ¹⁷⁸ Rat ^{178, 179}	HEK293: transient transfection ¹⁷⁸ MDCK: transient transfection ¹⁷⁸ Neurons: endogenous ¹⁷⁸
Neurokinin 1 (NK1) receptor	Human ^{220, 222}	MβCD and filipin treatments decrease receptor signaling such as ERK activation and phosphorylation ²²⁰ . Caveolin mutation and MβCD treatment reduced NK-1R/β-arr2 interaction ²²² .	Detergent-dependent ²²⁰ FRET ²²¹ EPR in combination with GHOST consolidation procedure ²²²			HAoSMC ²²⁰ HEK-293 ²²⁰⁻²²² HepG2 ²²⁰	Human ^{220, 222}	Endogenous ²²⁰ Transient transfection ^{220, 221} Stable expression ²²²
Non-mamalian GnRH receptor	Chicken ¹⁸⁹	Caveolae-dependent internalization ¹⁸⁹	Immunoprecipitation ¹⁸⁹	Presumably palmitoylation ¹⁸⁹		COS-7 ¹⁸⁹ HEK293 ¹⁸⁹	Monkey ¹⁸⁹ Human ¹⁸⁹	Transient transfection ¹⁸⁹
Oxytocin receptor	Human ^{233, 234}	Agonist-induced localization in lipid raft increases or inhibits cell proliferation ^{234, 237} .	Detergent-free ²³³ Detergent-dependent ^{233, 234}	Caveolin-2 binding ²³⁴		HEK293 cells ²³³ MDCK ²³⁴	Human ²³³ Canine ²³⁴	Stable expression ^{233, 234}
P2Y12 receptor	Human ^{242, 243}	MβCD treatment reduced P2Y12-mediated inhibition of cAMP production and platelet aggregation ²⁴² .	Detergent-dependent ²⁴³			HEK-293 ²⁴³ Platelets ²⁴²	Human ^{242, 243}	Endogenous ²⁴² Transient expression ²⁴³
P2Y2 receptor	Mouse ²³⁸	MβCD treatment inhibited P2Y2-induced PLC activation, Ca ²⁺ mobilization and UTP-mediated cell migration ²³⁸ .	Detergent-dependent ²³⁸			Neuroblastoma × glioma NG 108-15 cells ²³⁸	Mouse × Rat ²³⁸	Endogenous ²³⁸
Pituitary adenylate cyclase-activating peptide receptor type	Rat ²⁴⁶	PACAP stimulation induced receptor translocation into lipid	Detergent-dependent ²⁴⁶ CM ²⁴⁶			PC12 cells ²⁴⁶	Rat ²⁴⁶	Endogenous ²⁴⁶

one (PAC1R)		raft/caveolae leading to cAMP production and neurite outgrowth ²⁴⁶ .						
Platelet-activating factor receptor (PAFR)	Human ²⁴⁸	MβCD treatment reduced PAF-induced IP production, Ca ²⁺ mobilization and ERK phosphorylation ²⁴⁸ .	Detergent-free ²⁴⁸	Putative palmitoylation site ²⁴⁸	Yes ²⁴⁸	Stable CHO cells ²⁴⁸ Transient HEK293 cells ²⁴⁸	Chinese hamster ²⁴⁸ human ²⁴⁸	Stable expression ²⁴⁸ Transient expression ²⁴⁸
Protease-activated receptor type one (PAR-1)	Bovine ²⁵⁶ Human ^{257, 260}	Filipin treatment attenuates MLC phosphorylation and stress fiber formation ²⁵⁶ . Agonist treatment did not change receptor localization in raft ²⁵⁷ . PAR-1 signaling by APC is lost in caveolin-1 deficient cells ²⁶⁰ .	Detergent-free ^{256, 257} Immunoaffinity isolation ²⁵⁶ Immunoprecipitation ²⁵⁷ CM ²⁵⁷	bound caveolin-1 ²⁵⁷		Aortic endothelial cells ²⁵⁶ HEK-293 ²⁵⁷ COS-7 cells ²⁵⁷ Endothelial cells ²⁶⁰	Bovine ²⁵⁶ Human ^{257, 260}	Endogenous ^{256, 260} HEK-293: stable transfection ²⁵⁷ , COS-7 cells: transient transfection ²⁵⁷
Protease-activated receptor type two (PAR-2)	Mouse ²⁵⁸ Human ²⁵⁹	Receptor activation and barrier abrogation leads to receptor internalization ²⁵⁸ . MβCD and filipin treatment in addition to caveolin-1 silencing reduced TF-VIIa-mediated cleavage of PAR-2 and IL-8 gene expression ²⁵⁹ .	Western immunoblot ²⁵⁸ Detergent-dependent ²⁵⁹ Immunofluorescence confocal microscopy ²⁵⁹			Epidermis ²⁵⁸ Breast carcinoma cell line MDA-MB-231 ²⁵⁹	Mouse ²⁵⁸ Human ²⁵⁹	Endogenous ^{258, 259}
Prostanoid IP receptor	Human ²⁵²	IPR/AC signaling compartmentalized in lipid raft ²⁵² .	Detergent-free Immunofluorescence microscopy ²⁵²			COS-7 cells ²⁵²	Monkey ²⁵²	Transient transfection ²⁵²
Somatostatin receptor	Human ²⁶⁷	Receptor internalized via caveolin-positive endosomal vesicles after	EM, biochemical technique ²⁶⁷			Glioma cells ²⁶⁷	Human ²⁶⁷	Endogenous ²⁶⁷

		ligand binding ²⁶⁷ .						
Sphingosine 1-phosphate receptor	Human ²⁷² Murine ²⁷⁰	Agonist induced translocation to the caveolae, caveolin overexpression inhibited receptor function ²⁷² .	Detergent-free ²⁷²	Bound caveolin ²⁷² , N-glycan ²⁷⁰		COS-7 ²⁷² CHO cells ²⁷⁰	Chinese hamster ²⁷⁰	Transient transfection ²⁷² Stable transfection ²⁷⁰
Thyrotropin receptor	Human ^{276, 278}	Receptors move out of raft upon activation ²⁷⁶ . MβCD treatment did not alter cAMP accumulation ²⁷⁸ . Cav-1 knockdown increased cAMP accumulation ²⁷⁹ .	CM ²⁷⁶ Detergent-free ²⁷⁸	n.a		CHO cells ^{276, 278} Thyrocytes ²⁷⁸	Chinese hamster ^{276, 278} Rat ²⁷⁸	Stable transfection ^{276, 278}
Thyrotropin-releasing hormone receptor	Rat ^{282, 283}	Agonist induced receptor movement in to low density vesicular fraction ²⁸² . MβCD treatment inhibited G _q /G ₁₁ α tyrosine phosphorylation ²⁸³ .	Sucrose gradient centrifugation and immunoblotting ²⁸²	n.a		HHEK293 cells ²⁸²⁻²⁸⁴	Human ²⁸²⁻²⁸⁴	Stable transfection ²⁸²⁻²⁸⁴
Vasopressin receptor		MβCD treatment reduces AVP function ⁸⁰ . Lipid raft is required for AVP-induced trafficking ²⁸⁷ .				Endothelium of tail artery ⁸⁰ Thick ascending limb of the loop of Henle ²⁸⁷	Rat ^{80, 287}	
α1-adrenoceptor	Rat ⁷⁹	n.a	Detergent-free ⁷⁹	Bound caveolin-3 ⁷⁹		Smooth muscle cells, cardiac myoblast H9C2 cells ⁷⁹	Rat ⁷⁹	Endogenous ⁷⁹
β2-adrenoceptor	Rat ²⁹² Mouse ⁸³	Agonist treatment leads to receptor movement out of caveolae ⁴³ . Caveolae disruption by filipin or cyclodextrin increases cAMP accumulation	Detergent-free ^{43, 81}	Bound caveolin-1 ⁸¹	Yes ⁸⁴	COS-7 cells ⁸¹ Cardiomyocytes ²⁹² Cardiac fibroblasts ⁴³ Neonatal myocytes ⁸³	Rat ^{43, 292} Mouse ⁸³	Endogenous ^{83, 292} Transient transfection ⁸¹

		and contraction rate ^{43, 83} .						
β 1-adrenoceptor	Rat ²⁹² Mouse ⁸³	No functional implication observed upon agonist treatment or raft disruption ^{43, 83} .	Detergent-free ^{43, 81}	Bound caveolin-1 ⁸¹	Yes ⁸⁴	COS-7 cells ⁸¹ Cardiomyocytes ²⁹² Cardiac fibroblasts ⁴³ Neonatal myocytes ⁸³	Rat ^{43, 292} Mouse ⁸³	Endogenous ^{83, 292} Transient transfection ⁸¹
β 3a-adrenoceptor	Mouse ⁸⁵	Filipin treatment alters accumulation of c-AMP ⁸⁵ .	Caveolae disruption ⁸⁵	Putative caveolin binding domain ⁸⁵	Yes ⁸⁵	CHO-K1, brown adipocytes ⁸⁵	Chinese hamster ⁸⁵ Mouse ⁸⁵	Endogenous ⁸⁵
δ -opioid receptor	Rat ²²⁷ Mouse ^{223, 229}	M β CD treatment attenuates cardiac protection from ischemic damage ²²⁷ . Cav1 and cav3 expression attenuates N-type Ca ²⁺ channels inhibition induced by receptor agonist ²²⁹ . Agonist induced receptor movement out of lipid raft ²²³ . M β CD treatment attenuates receptor-mediated signaling in neuronal cells but increased it non-neuronal cells ²²³ .	Detergent-free ^{223, 227} Immunoprecipitation ²²⁷ Detergent-dependent fractionation ²²⁹	Associates with caveolin-3 ²²⁷	Yes ²²³	Ventricular myocytes ²²⁷ Neuroblastoma x glioma NG108-15 hybrid cell ^{223, 229} CHO cells ²²³	Rat ²²⁷ Chinese hamster ²²³ Mouse x rat ²²⁹	Endogenous ^{223, 227, 229} Stable transfection ²²³
κ -opioid receptor	Human ³⁷	M β CD treatment increased p42/44 MAPK phosphorylation ³⁷ .	Detergent-free ³⁷		Yes ³⁷	Placenta ³⁷ CHO cells ³⁷	Human ³⁷ Chinese hamster ³⁷	Endogenous ³⁷ Stable transfection ³⁷
μ -opioid receptor	Rat ^{225, 226} , Mouse ²³⁰	M β CD treatment inhibited receptor internalization and adenylyl cyclase superactivation ²²⁵ . Agonist etorphine induce receptor movement out of raft ²²⁶ . M β CD	Detergent-free ^{225, 226} CM ⁸¹ Detergent-dependent ²³⁰		Yes ²²³	HEK-293 ^{225, 226, 230}	Human ^{225, 226}	Stable expression ^{225, 226, 230}

		treatment attenuated etrophine- and morphine-mediated adenyly cyclase inhibition and morphine-induced but not etrophine-induced ERK phosphorylation ²²⁶ .						
5-HT _{1A} receptor	Mouse ²⁶³	Signaling reduces in cyclodextrin treated and palmitoylated-deficient cells ²⁶³	Gradient centrifugation Copatching assay ²⁶³	Palmitoylation ²⁶³		NIH-3T3 cells ²⁶³ CHO cells ²⁶³	Mouse ²⁶³ Chinese hamster ²⁶³	Stable transfection ²⁶³ Transient transfection ²⁶³
5-HT _{2A} receptor	Rat ⁸⁰	MβCD treatment reduced agonist response ⁸⁰ .	Detergent-free ⁸⁰	n.a		Tail artery ⁸⁰	Rat ⁸⁰	Endogenous ⁸⁰
5-HT ₇ receptor	Human ²⁶⁴	Fumonisin B1 and PDMP treatment decrease remarkably binding of 5-HT to 5-HT ₇ receptor ²⁶⁴ .		n.a		HeLa cells ²⁶⁴	Human ²⁶⁴	Stable transfection ²⁶⁴

Chapter 4: Discussion and conclusion

Although still there are many unanswered questions regarding the role of lipid rafts/caveolae, the association of G-protein-coupled receptors (GPCRs) with these microdomains, as well as the mechanisms of this association, a growing body of evidence proposes some new paradigms regarding the behavior of GPCRs in response to agonist and their function in relation to lipid rafts.

Some receptors are known to be localized in lipid rafts and their localization does not change upon agonist treatment^{92, 205, 257}. In the case of AT1, mammalian GnRH and MCHR1, the agonist-independent localization of receptors in lipid rafts is responsible for mediating the signaling pathways that mainly include MAPK, PKC, ERK1/2 and RAF1^{92, 186, 205}. Such agonist-independent compartmentalization of receptors in lipid rafts also involves cellular processes such as internalization, for example, the AT1 and μ -opioid receptors that have been shown to internalize via lipid rafts/caveolae^{92, 225}.

It is also found that many receptors move to lipid rafts upon agonist binding. Receptors, such as adenosine A1R, bradykinin B2R, chemokine CXCR4, muscarinic M2R, dopamine D1R and PAR-2, translocate to the lipid rafts upon agonist binding and internalize via caveolae-dependent pathways^{68, 69, 97, 98, 135, 159, 214, 258}.

Other receptors move to lipid rafts in response to agonists and activate specific signaling cascades, such as ROS, RAC1, Akt, Ca^{2+} responses, PI3K, cAMP and $\text{G}_q/\text{G}_{11}\alpha$ signaling. Furthermore, receptors that move to lipid rafts in an agonist-

dependent manner and whose localization is necessary for activation of signaling process include the AT1, CXCR4, LH, TRH, dopamine D1 and PAC1 receptors^{90, 91, 135, 157, 196, 246, 283}. However, in the case of muscarinic M2 receptors, receptor localization in lipid rafts is important in the negative control of NO signaling²¹⁴.

In addition to the importance of receptor localization in lipid rafts for proper signaling receptor compartmentalization may also be necessary for other aspects of receptor function. For example, association of CXCR4 with lipid raft is necessary for receptor dimerization¹³⁰. Regarding to the role of receptor dimerization in CXCR4-mediated signaling, receptor localization plays an important role in signal transduction¹³⁶.

Additionally, lipid rafts are found essential for mediating processes such as modification of the N- or C-terminal tails of receptors. For example, lipid rafts/caveolae are essential for N-terminal truncation of PAR1 that is needed for its activation²⁶⁰. Therefore, localization of PAR1 receptors to lipid rafts is necessary for activation of receptors and PAR1-mediated signaling by APC²⁶⁰. Another example of the importance of lipid rafts in receptor modification and signal transduction is the NK1 (Neurokinin 1) receptor²²⁰. After NK1 stimulation by SP, PKC enters the lipid rafts where NK1 also exists and facilitate phosphorylation of the C-tail of the receptor thereby uncoupling it from the G protein²²⁰.

There are also a number of receptors that move out of the lipid rafts upon agonist treatment. For example β 2-adrenergic receptors move out of lipid rafts following agonist treatment and are internalized by clathrin coated vesicles⁴³. However, in the case of the cannabinoid CB1 receptor, only a portion of the receptors moves out of lipid

rafts in response to the agonist^{108, 112}. As CB1 was shown to internalize in parallel via both the caveolae-dependent pathway and clathrin coated vesicles, it can be concluded that a portion of receptors that move out of lipid rafts undergo internalization via clathrin-coated pits¹¹².

It is thus clear that, localization of receptors in lipid rafts or caveolae usually increases the efficiency of signaling by restricting the movement of receptors and signaling molecules, and increasing their concentration in such microdomains. However, there are some exceptions where the signaling is down regulated in lipid rafts. For instance, β 2-AR translocation out of caveolae increases the cardiac contraction rate suggesting that the signaling molecules are more available when out of lipid rafts to interact with receptors^{43, 83}. Moreover, GABA_B receptor-mediated signaling is also down-regulated when the receptors are located in lipid rafts compared to whole membrane²⁹⁰.

In addition to the role of lipid rafts in receptor internalization and signal transduction, receptor compartmentalization also plays an important role in cellular trafficking and surface expression of receptors. For example, caveolin-1 stabilizes surface expression of the metabotropic Glutamate receptor¹⁷⁸.

Comparing the results from different receptor localization studies is difficult because of several reasons. First, receptor localization and response upon agonist binding may depend on the cell type or the endogenous or heterologous receptor system. For example, the A1 Adenosine receptor moves into lipid rafts in kidney epithelial cells upon agonist treatment but is excluded from lipid rafts in ventricular myocytes^{67, 68}. Furthermore, observed receptor translocation into or out of lipid rafts may be dependent

on the time chosen for the experiments, the biochemical procedures (detergent-dependent vs. detergent-independent) and the methods used for lipid raft analysis.

Association of G protein coupled receptors (GPCRs) with lipid rafts is found to regulate the functions of these receptors. The concentration of receptors, signaling molecules and effectors in lipid rafts make these membrane microdomains a platform for efficient and rapid signaling. Receptors preferentially bind and activate a certain family of G proteins and this may be due to colocalization of the receptor and specific G proteins in a particular microdomain, such as lipid rafts/caveolae. Thus, the specificity and fidelity of GPCR signaling may be the result of their compartmentalization in lipid rafts.

Understanding the mechanisms of therapeutic agents and their association with lipid rafts provides the possibility of designing new and more efficient drugs. Recently, it was found that Gs α localization in detergent-resistant microdomains was altered following chronic antidepressant treatment²⁹³. Thus, Gs α concentrated in Triton-insoluble membrane domains (lipid rafts/ caveolae) moved with adenylyl cyclase to Triton-X100-soluble membrane domains upon antidepressant treatment. This effect increased adenylyl cyclase coupling to Gs α which mediate the action of chronic antidepressant treatment²⁹⁴⁻²⁹⁶.

As lipid rafts affect a broad range of processes, such as receptor-mediated signal transduction, surface expression, dimerization, internalization and desensitization, it is possible to develop lipid raft-targeted drugs for treatment of GPCR-mediated diseases. Understanding differences in localization of various receptors in the same cell or the same receptor in different tissues can help in the development of drugs that can

specifically target the receptors in specific tissues. Interestingly, caveolar internalization pathways can progress without fusion to lysosomes where the acidic compartment may attenuate the efficacy of therapeutic agents like small molecules or proteins ²⁹⁷. Therefore, caveolae can be exploited for delivering drugs, and imaging reagents and therapy vectors ²⁹⁷. However, further research is required to precisely determine how receptors associate with lipid rafts and the functional implications of such associations in cells. Numerous studies are now focusing on caveolin knockout animals and their involvement in human diseases such as cancer, cardiomyopathy, diabetes, muscular dystrophy, and restrictive lung diseases ^{298, 299}. However, there is still limited, if any, evidence regarding the involvement of GPCRs dislocation from lipid rafts/caveolae in human diseases. Hence, we need to intensify our efforts to study lipid rafts and gain further insights into the precise function of these microdomains in the cell in general and in GPCR signal transduction in particular.

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خلاصه

گیرنده های seven transmembrane (7TMR) یا گیرنده های G-protein coupled (GPCR) که به عنوان هدف برای تعداد زیادی از داروها محسوب می شوند، در بسیاری از فرایندهای فیزیولوژیکی و پاتوفیزیولوژیکی نقش مهمی را ایفا می کنند. اخیراً به اثبات رسیده است که این گیرنده ها بطور آزادانه در غشای سلول حرکت نمیکنند بلکه آنها در نواحی میکروسکوپی (میکرودومین) از غشاء سلولی که غنی از کلسترول و اسفینگومیلین هستند و lipid raft نامیده می شوند قرار می گیرند و لذا حرکت محدودی خواهند داشت. Lipid rafts در بسیاری از فرایندهای سلولی شامل انتقال سیگنال، ورود گیرنده ها به داخل سلول و در نتیجه غیر فعال ساختن آنها شرکت می کنند. با توجه به گسترش سریع روزافزون اطلاعات و یافته های مرتبط با این موضوع، نیاز به یک پایگاه اطلاعاتی دقیق و معتبر در این زمینه، ما را بر آن داشت تا به تحقیق و جمع آوری اطلاعات لازم بپردازیم و آنها را بصورت یک بانک الکترونیکی در اختیار محققین و جویندگان این علم قرار دهیم. نتایج این تحقیق بطور خلاصه مشتمل بر معرفی گیرنده های GPCR که ارتباطشان با lipid raft گزارش شده است، مکانیسمی که باعث ورود گیرنده به این میکرو دومین ها میشود، روشهای مختلف آزمایشگاهی که برای اثبات وجود گیرنده ها در lipid rafts استفاده شده و نقش این میکرو دومین ها در رفتار گیرنده و نحوه انتقال سیگنال توسط آن میباشد در بانک الکترونیکی (7TMR-RAFT) (www.7tmr-raft.org) ذخیره گردیده است.