Constitutive Trafficking of metabotropic Glutamate Receptor 5 in HEK293 cells

Rishi Raj Trivedi

A dissertation submitted for the partial fulfilment of
BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali
April 2012

Certificate of Examination

This is to certify that the dissertation titled "Constitutive Trafficking of metabotropic Glutamate Receptor 5 in HEK293 cells " submitted by Mr. Rishi Raj Trivedi (Reg. No. MS07019) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Kavita Babu Dr. Mahak Sharma Dr. Samarjit Bhattacharyya
(Supervisor)

Dated: May 3rd, 2012

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Samarjit Bhattacharyya at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Rishi Raj Trivedi

Dated: May 3rd, 2012

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Samarjit Bhattacharyya

(Supervisor)

Acknowledgements

I am deeply grateful to Dr. Samarjit Bhattacharyya for introducing me to the field of *Neuroscience* and giving me the freedom to think and execute experiments. His patient guidance, enthusiasm, support, critical analysis and constructive suggestions all put together is an asset that I will always cherish in my memory. His attitude and assistance motivated me towards research. Samarjit, I sincerely thank you for the crucial suggestions and feedback, the time and support you have persistently dedicated to the project, and the continuous push for all of the members of Jit's lab to strive to achieve the best of our abilities. To Prof. N. Sathyamurthy for his constant support and encouragement. To Kavita and Mahak for their valuable suggestions about the mauscript and support.

I would like to convey my deep sense of gratitude and very special thanks to Mr. Prabhat Mahato (Rocky Sir) who instructed and guided me throughout my project, and showed his patience during initial phases of learning. He was always with me at the time of ups and downs during my research work. I am thankful to Saurabh Pandey (Guru Ji) for being such a nice lab mate who was always ready to provide help.

Also, a special token of thanks to my friends at IISER- Divya, Kiran, Vinii, Dhillu being great friends and making my experience a memorable one.

Finally, I also thank my parents for taking me to this level in my life; it was their blessings which always gave me courage to face all challenges and made my path easier.

List of Figures

Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid;
BSA	Bovine serum albumin
CNS	Central nervous system
cAMP	cyclic AMP
DAG	Diacylglycerol
DHPG	Di-hydroxy phenyl glysine
DMEM	Dulbecco's modified eagle's medium
EDTA	Ethylene diamine tetraacedic acid
EGFP	Enhanced green fluorescent protein
FBS	Fetal bovine serum
FXS	Fragile X-Syndrome
GDP	Guanosine-5'-diphosphate
GPCR	G-Protein Coupled Receptor
GRK	G-protein coupled receptor kinase
GTP	Guanosine-5'-triphosphate
HEK293 cells	Human embryonic kidney 293 cells
IP3	Inositol 1,4,5-triphosphate
LTD	Long term depression
LTP	Long term potentiation
NGS	Normal goat serum
NMDA	N-methyl-D- aspartate
PBS	Phosphate buffer saline
PEG	Polyethyleneglycol
PKC	Protein kinase C
PFA	Paraformaldehyde
PKA	Protein kinase C
PLC	Phospholipase C
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TE	Trypsin EDTA
PNK	Polynucleotide Kinase
RGS	Regulators of G protein signaling

Abstract

mGluR5b is a group 1 metabotropic glutamate receptor which belongs to the family of G-protein coupled receptors, responsible for mediating slower synaptic responses via second messenger pathway. mGluR5b present on the cell surface gets regulated by endocytosis which is a major component of either GPCR desensitization or resensitization or downregulation of the receptors. mGluR5b can get endocytosed in ligand-dependent as well as ligand independent (constitutive) manner. In this study, we have explored temporal regulation of the ligand-dependent and constitutive endocytosis pathways. Additionally we investigated the sub-cellular fate of the receptors subsequent to internalization. Our study suggests that mGluR5b enters into recycling compartment via constitutive endocytosis which is a slower process compared to ligand mediated endocytosis.

Contents

Chapter 1: Introduction

1.1 Glutamate receptors

Glutamate receptors are transmembrane proteins that bind specifically to glutamate on the extracellular side of the membrane [1]. Glutamate is the major excitatory neurotransmitter in the mammalian Central Nervous System (CNS) stored in synaptic vesicles in the presynaptic terminal, where it can be released in a calcium-dependent manner into the synapse. Upon binding of glutamate, glutamate receptors are activated which further transduce this signal into intracellular responses.

Based on their functional and pharmacological properties, glutamate receptors can be grouped into two main categories:

a) Ionotropic glutamate receptors (glutamate-gated cation channels)

b) Metabotropic glutamate receptors (glutamate activated G protein-coupled receptors)

The ionotropic receptors can be further categorized into NMDA receptors, AMPA receptors and Kainate receptors [2,3].

1.2 Metabotropic glutamate receptors (mGluRs)

Metabotropic glutamate receptors (mGluRs) are G protein coupled receptors, responsible for mediating slower synaptic responses via second messengers. The mGluR family contains seven members (mGluR1–7), which can be further classified into three subgroups based on primary sequence similarity, mode of intracellular signal transduction or physiological effects and pharmacology. Each mGluR has a large extracellular domain that binds glutamate and seven membrane-spanning domains [3, 4, 5, 6].

mGluR1 & mGluR5 subtypes make up the group I mGluRs. Group 1 mGluRs are mainly postsynaptic while other subgroups of mGluRs are also found on the presynaptic terminal. Group I mGluRs are coupled to phospholipase C (PLC) via a Gq coupling mechanism. Activation of PLC results in the production of inositol phosphates (IP3), which in turn leads to the release of Ca^{2+} from intracellular stores. PLC activation also results in diacylglycerol (DAG) production. DAG activates Protein Kinase C (PKC), a critically important kinase in many cellular systems [5, 7, 8, 11, 12, 13].

Although activation of group I mGluRs involves a G-protein coupled Ca^{2+}-dependent PLC-IP3 signaling pathway but other Ca^{2+}-independent cascades may also get activated via scaffolding proteins such as Homer and caveolin-1. Homer brings multiprotein signaling complexes in close proximity to mGluR5 and couples it to Ca^{2+}-independent ERK signaling pathway [9, 10, 11, 12, 13, 14].

The mGluRs are a diverse class of glutamate receptors, activating a wide variety of second messenger pathways at both pre- and postsynaptic sites. It is becoming clear that mGluRs share common regulatory mechanisms with one another and also with the larger family of GPCRs [11]. As with traditional GPCRs, phosphorylation of mGluRs mediates protein–protein interactions, G-protein coupling, receptor trafficking and desensitization. Several classes of protein kinases are activated by mGluRs. Similarly, mGluRs are directly phosphorylated by a variety of kinases.

1.3 Role of group I mGluRs in long-term potentiation and long-term depression

Mammalian brain can undergo activity or experience-dependent changes in synaptic structure and function known as synaptic plasticity. In the hippocampus, synaptic plasticity is divided into Long-term Potentiation (LTP) or Long-term Depression (LTD) depending on the increase or the decrease in the number of AMPA receptors present on the cell surface via exocytosis or endocytosis respectively. LTD in the hippocampus involves multiple mechanisms. In general, LTD is dependent on the activation of NMDA receptors and mGluRs. mGluR-LTD is NMDA receptor-independent which suggests that the two forms of LTD use completely different mechanisms as neither they obstruct each other nor they are mutually exclusive [15, 16].

Among the mGluR superfamily, group 1 mGluRs (mainly mGluR5) play the most important role in mediating LTD. Studies with DHPG (group I mGluR agonist) have shown that DHPG-LTD is not prevented by intracellular calcium chelator and does not dependent on extracellular Ca^{2+} [17, 18]. Thus, group I mGluR-LTD induction in the hippocampus is Ca^{2+}-independent and does not involve the typical intracellular signaling cascade that is normally associated with group I mGluR activation. There are some acute differences in mGluR-LTD

mediated by both the isoforms of group I mGluRs i.e. mGluR5-LTD can convert short-term depressions into LTD which is not the case with the mGluR1 LTD [22, 23].

Various mechanisms or combination of various mechanisms leads to mGluR-LTD response. These mechanisms include: 1) activation of all three Mitogen-Activated Protein Kinase (MAPK) subclasses: p38 MAPK [24], Jun N- terminal kinase (JNK) [25] and extra-cellular signal-regulated kinase (ERK) [26], 2) activation of postsynaptic Protein Tyrosine Phosphatases (PTPs) [27], and 3) activation of a member of the group S8A serine proteases namely subtilisin [28].

1.4 Role of mGluR5

mGluR5 primarily regulates the synaptic plasticity in forebrain region. It is involved in mediating hippocampal mGluR-LTD response. Investigating the molecular mechanisms underlying hippocampal mGluR-LTD is essential for understanding and treating diseases such as fragile X syndrome (FXS) and addiction in which synaptic plasticity abnormalities are displayed [29].

FXS is the leading known genetic cause of both mental retardation and autism spectrum disorders. FXS is caused by impaired synaptic glutamate signaling or by disrupted interactions between mGluRs and interacting proteins such as Homer 1a. In FXS, *fragile X mental retardation 1* (*fmr1)* gene gets silenced, which encodes the RNA-binding, translational represor fragile X mental retardation protein (FMRP). FMRP is very crucial for preventing mGluR5 stimulation leading to excessive AMPAR endocytosis via overactivation of signaling cascades. In the absence of FMRP, there is over expression of specific mRNAs in response to mGluR5 activation. It has been suggested that blocking or antagonizing the mGlu5 receptor pharmacologically, mGluR signaling could be modulated back to normal levels, which can be used as a cure to FXS [30, 31, 32].

1.5 G-Protein Coupled Receptor Regulation

Regulation of GPCRs plays a fundamental role in maintaining physiologic homeostasis in the cells and a number of pathologic states are associated with alteration in the number or functional activity of certain GPCRs. GPCR downstream signaling events can be regulated at

the level of G-protein as well as at the level of receptor itself. At the level of G proteins, GPCR activity is attenuated by a family of proteins termed regulators of G protein signaling (RGS), which recognize specific members of G protein α subunits and accelerate the GTPase activity intrinsic to the α subunits of heterotrimeric G-proteins, thus playing crucial roles in the physiological control of G-protein signaling. At the level of receptors, a process termed desensitization regulates GPCR signaling [33, 34].

1.5.a Receptor Desensitization

Desensitization is a common feature of GPCRs, which serves to prevent receptor overstimulation. Receptor desensitization can result either from the functional uncoupling of receptors to the G-protein or by internalization of the receptors to intracellular compartments [33, 34].

This represents a change in the functional activity of receptors, which inhibits signal transduction to the effector molecules without changing the number of receptors in the cell. In the process of desensitization, generation of second messengers like IP3 and cAMP is rapidly attenuated [34].

It is now well established that phosphorylation of the receptor through a variety of kinases, viz., protein kinase A (PKA), protein kinase C (PKC) and G-protein coupled receptor kinases (GRKs), plays a critical role in the desensitization of most receptors [33, 35, 36].

Many G-protein coupled receptors can also desensitize through a phosphorylation-independent mechanism. Such desensitization can happen through various mechanisms that include agonist-induced receptor trafficking, phosphorylation-independent desensitization by GRKs and phosphorylation-independent arrestin binding [37].

Two types of desensitization have been investigated. Homologous desensitization is mediated by agonist-dependent activation of the same receptor, whereas heterologous desensitization is mediated by activation of different receptors. Homologous desensitization is initiated by a unique class of serine/threonine protein kinases, namely GRK as well as protein kinases A or C, whereas heterologous desensitization is initiated by second messenger-dependent kinases, such as PKA and PKC [38].

1.5.b Receptor Internalization: Major mechanism of desensitization or resensitization or downregulation

The regulation of the number of the receptors on the cell surface by endocytosis is a major component of GPCR desensitization. According to the conventional GPCR endocytosis model, agonist-dependent phosphorylation of receptors by GRKs is followed by binding of β-arrestins, which is required for the receptors to be targeted to clathrin-coated pits [33, 39].

On the other hand, receptors can get endocytosed constitutively, which is an agonist-independent process. This constitutive endocytosis occurs at low level all the time, and plays an important role in maintaining homeostasis in the cell [33].

Endocytosed receptors can have multiple fates. They can be trafficked towards endosomes or recycling compartment depending on the type of GPCR, type of ligand and cellular background. Some GPCRs are not recycled back towards the membrane but degraded following their passage from the early endosomes to the lysosomes. Acidification of endosomes and dephosphorylation of GPCRs allows their recycling to the membrane. This suggests that for many receptors internalization is not the mechanism used to desensitize the receptor. In fact, internalization seems to be necessary for the resensitization of many receptors [40, 41, 42].

1.5.c Down-regulation of GPCRs

After very prolonged (typically several hours to days) agonist-induced stimulation of receptors, the number of receptors present in cells is greatly reduced, so signal transduction via G proteins to effectors is strongly attenuated. This process is called receptor down-regulation because it involves reduction in the number of GPCRs rather than functional activity. Several mechanisms are involved in this process, including lysosomal degradation, ubiquitin-mediated proteolysis and destabilization of mRNA coding for GPCRs [42, 43].

1.5.d Regulation of group I mGluRs

Similar to many other GPCRs, the signal transduction pathways of mGluRs are regulated by multiple mechanisms acting at different levels. For example, after prolonged or repeated stimulation, Group I mGluRs undergo both homologous (agonist-dependent) and

heterologous (agonist-independent) desensitization.

mGluRs have been shown to desensitize in response to second messenger-dependent protein kinases, calmodulin kinase II, GRK, and the huntingtin-binding protein optineurin. These kinases contribute to the heterologous desensitization of the receptors [42, 44].

The homologous desensitization of agonist-activated mGluR is induced by GRK-mediated phosphorylation to facilitate the binding of β-arrestins to physically uncouple mGluRs from heterotrimeric G proteins. The agonist-stimulated internalization of mGluRs, in particular mGluR1, is partly regulated by PKC. PKC-mediated phosphorylation of mGluR5 has also been shown to regulate the interactions of the receptor with calmodulin. Another post-synaptic density protein named optineurin can interact with Group I mGluRs and function to inhibit mGluR1/5 G protein-coupling to PLC and IP3 signaling [45, 46, 47, 48].

1.6 Objective of the study

As described before, both mGluR1 and mGluR5 play crucial role in synaptic plasticity and in various neuropsychiatric disorders. Proper localization of the receptor within the cell and proper spatio-temporal regulation of the receptor is crucial for accurate signaling of the receptor as well as for generating mGluR-LTD. The process of receptor trafficking plays a critical role in controlling the localization and signaling of the receptor. Despite this obvious significance, very little is known about the trafficking of mGluR5 receptor. Previous studies have reported that mGluR5 receptor can get endocytosed on application of ligand. However the ligand-mediated as well as constitutive endocytosis of the receptor is not well understood. Constitutive endocytosis of mGluR5 is very important for basal activity within a cell and also for maintenance of homeostasis inside the cell. The main objective of this thesis was to study the constitutive and ligand-mediated endocytosis of mGluR5 receptor and also to investigate the sub-cellular fate of the receptors subsequent to internalization.

Chapter 2: Materials and Methods

2.1 MATERIALS

2.1.a Plasmids and constructs

Two constructs of mGluR5b were used for the study. myc-mGluR5b construct was obtained from Kathrine Roche (National Institute of Health, USA). In this construct, the full length mGluR5b cDNA was tagged with the myc at the N-terminus in pRK5 plasmid. In the other construct viz. mGluR5b-GFP (gift from Johana Montagomery, Auckland University, New Zealand), mGluR5b was tagged with GFP at the C-terminus and cloned in pcDNA3.1.

2.1.b Chemicals and reagents

Dulbecco's Modified Eagle medium (DMEM), Penicillin - streptomycin solution, Antibiotic–Antimycotic mix, Lipofectamine, OptiMEM, DPBS, Distilled Water, Trypsin-EDTA, Fetal bovine serum (FBS) were purchased from Invitrogen. Cycloheximide, ampicillin, PFA, poly-lysine, Fluoromount^{TM} Aqueous mounting medium were purchased from Sigma, USA. Fine chemicals were obtained from Life technologies (USA), Merck limited (USA). (RS)-3,5-DHPG was purchased from Tocris. Anti-myc mouse monoclonal antibody, anti-myc rabbit polyclonal antibody, anti-Rab11A mouse mnoclonal antibody, anti-LampI rabbit polyclonal antibodies were obtained from Abcam. Secondary antibodies viz., goat anti-mouse Alexa-568, goat anti-mouse Alexa-647, goat anti-mouse Alexa-488, goat anti-rabbit Alexa-568 and goat anti-rabbit Alexa-488 were purchased from Invitrogen (USA).

2.1.c Tissue culture wares

35 mm dishes, 60 mm dishes, 100 mm dishes, 24 well plates, 15 ml conical tubes, 50 ml conical tubes, 5 and 10 ml serological pipettes and cryotubes were from BD Falcon (USA) and Tarsons (India). Coverslips and glass slides were from fisher scientific (USA).

2.2 METHODS

2.2.1 Cell culture and transfection

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, antibiotic-antimycotic mix at 37°C, 5% CO2. Cells were transfected with the DNA when the confluency of the cells reached 65-70% on a 35 mm coverslip coated with 50 μg/ml poly-D-lysine. For the transfection, 2 μg of the DNA was mixed with 10 μg of lipofectamine in 1 ml OptiMEM and added to the cells for 5 hours. Expression was observed 24 - 36 hours after transfection.

2.2.2 Internalization studies

For internalization studies, two coverslips were used at each time point. While imaging typically 70-100 cells were randomly chosen and imaged in any one experiment and all experiments were repeated at least three times.

2.2.2.a Constitutive endocytosis assay

HEK293 cells were transfected with the myc- mGluR5b using lipofactamine as described before and experiments were performed 24 hours later. Transfected cells were blocked with 2% normal goat serum (NGS) and incubated with anti-myc mouse monoclonal antibodies (1:500) for 1 hour on ice,. Control cells were immediately fixed with 4% PFA in PBS for 15 min while other time point-cells were incubated at 37°C for 5 min, 15 min, 30 min and 60 min to allow constitutive internalization of the receptors. Subsequently, cells were fixed (without permeabilization) in 4% PFA at their respective time points for 15 min and then washed in PBS. To label the surface receptors, saturating concentration of first secondary antibody, goat anti-mouse Alexa-568 (1:100) was applied for 1 hour at 37°C and washed with PBS. Next, cells were permeabilized with 0.1% Triton X-100 for 30 min at room temperature. After washing cells were blocked with 2% NGS in PBS for 1 hour at 37°C followed by second secondary antibody,goat anti-mouse Alexa-647 (1:800) staining for 1 hour at 37°C. This antibody labeled the internalized receptors. The coverslips were mounted on glass slides and imaged under confocal microscope.

2.2.2.b DHPG-mediated endocytosis assay for myc tagged receptors

HEK293 cells grown on coverslips, were transfected with the myc-mGluR5b using the lipofactamine as described before and experiments were performed 24 hours later. Transfected cells were blocked with 2% normal goat serum (NGS) in Plain DMEM and subsequently were incubated with anti-myc mouse monoclonal antibody for 15 min at 37°C. Cells were washed with DMEM and were treated with 100 uM DHPG for 5 minutes. After that, DHPG was washed and cells were chased in the absence of DHPG for various times (10 min and 25 min). For time course experiments, DHPG was applied for 1 min, 2 min and 5 min for initial time points and for subsequent time points DHPG was applied for 5 min followed by chase in absence of DHPG for varies times (10 min and 25 min). Cells were then fixed (without permeabilization) in ice cold 4% paraformaldehyde (PFA) in PBS for 15 min on ice and then washed in PBS. To visualize the surface receptors, saturating concentration of first secondary antibody i.e., goat anti-mouse Alexa-568 (1:100) in PBS was applied for 1 hour at 37°C. After that, cells were permeablized with 0.1% Triton X-100 for 30 min at room temperature. After washing the cells with PBS, cells were blocked with 2% NGS in PBS for 1 hour at 37°C followed by the application of second secondary antibody i.e., goat anti-mouse Alexa-647 (1:800) for 1 hour at 37°C to visualize the internalized receptors. The coverslips were then mounted on glass slides with Fluoromount after washing and imaged under confocal microscope.

2.2.2.c DHPG-mediated endocytosis assay for GFP-tagged receptors

HEK293 cells grown on coverslips, were transfected with the mGluR5b-GFP using the lipofactamine as described before and experiments were performed 24 hours later. Cells were treated with cycloheximide for 5 hours. Cells were washed with PBS and were treated with 100 uM DHPG for 5 minutes. After that, DHPG was washed and cells were chased in the absence of DHPG for various times (10 min and 25 min). For time course experiments, DHPG was applied for 1 min, 2 min and 5 min for initial time points and for subsequent time points DHPG was applied for 5 min followed by chase in absence of DHPG for varies times (10 min and 25 min). Cells were then fixed (without permeabilization) in ice cold 4% paraformaldehyde (PFA) in PBS for 15 min on ice and then washed in PBS. The coverslips

were then mounted on glass slides with Fluoromount after washing and imaged under confocal microscope.

2.2.3 Co-localization assay

HEK293 cells were transfected with the myc-mGluR5b DNA as described earlier using the lipofactamine. After 24 hours, transfected cells were blocked with 2% normal goat serum (NGS) and incubated with either anti-myc rabbit polyclonal antibody (1:500) or anti-myc mouse monoclonal antibody (1:500) for 1 hour on ice, washed with plain DMEM. To strip off all the receptors present at the cell surface, control cells were immediately treated with acidic solution i.e. acetic acid in NaCl solution for 3 min then fixed with 4% PFA in PBS for 15 min. For 30 min time point cells, they were incubated in plain DMEM for 30 min to allow the constitutive internalization of the receptors. These-cells were also acid stripped and fixed in 4% paraformaldehyde in PBS for 15 min and then washed in PBS. Next, cells were permeabilized with 0.1% Triton X-100 for 30 min at room temperature. After washing, cells were blocked with 2% NGS in PBS for 1 hour at 37˚C followed by staining with either mouse monoclonal primary antibody against Rab11(recycling compartment marker) for 4 hours at 37˚C or rabbit polyclonal primary antibody against LAMP1 (lysosomal marker) for 1 hour at 37˚C. Subsequently. secondary antibodies, goat anti-rabbit / goat anti mouse Alexa-568 (1:800) and goat anti-mouse / goat anti rabbit Alexa-488 (1:800) were applied for 1 hour at 37˚C to stain receptors and recycling compartment / lysosome. After washing with PBS, coverslips were mounted on glass slides and imaged under confocal microscope.

2.2.4 Confocal microscopy

Images were collected using a 63X oil immersion objective in Zeiss LSM 780 laser sacning confocal microscope. Series of optical sections were collected at step size of 0.43 μm. Digital offset and gain was set for individual experiment and was kept constant for the whole experiment. Alexa 488, Alexa 568, Alexa 647 and GFP excitation/emission were achieved using appropriate filter sets.

2.2.5 Image Analysis

All the images were analyzed using Image J software. Images were first z projected for maximum intensity projection. The images were then thresholded using identical values for a particular fluorophore and thresholded area were measured. The internalization index was measured by normalizing the internalized receptor with the total (surface + internal) receptors. In the case of GFP tagged mGluR5b receptors, only those cells that showed significant internal fluorescence were counted and each time point was compared with the control. For representation every image was adjusted using identical brightness and contrast.

Chapter 3: Results

3.1 Surface localization of tagged mGluR5b constructs in HEK293 cells

mGluR5b is a GPCR which belongs to group I family of mGluRs. mGluR5b is expressed at the membrane of post-synaptic cells and mediates second messenger response upon glutamate binding. To study the molecular and cellular mechanisms underlying the trafficking of mGluR5b we used two constructs of mGluR5b, one tagged with myc and another tagged with GFP. In myc-mGluR5b, myc was tagged at the N-terminus so that we can perform live cell antibody staining which will enable us for quantitation of the receptors (Figure 1A). We first checked whether both of these tagged receptors localize properly in HEK293 cells. For this purpose HEK293 cells were transfected with both the constructs and the expression was observed after 24 hours of transfection.

In case of myc-mGluR5b, after probing with anti-myc primary antibody, secondary antibody tagged with fluorophore Alexa-568 was used for visualization of the receptors. In the confocal image, localized expression of the receptors was observed at the cell surface (Figure 1B). Since live cells were stained with the primary antibody, the intracellular fluorescence was not visible.

mGluR5b-GFP also showed the surface localization but some amount of green fluorescence was also observed inside the cell due to continuous synthesis of the receptors tagged with GFP in endoplasmic reticulum and golgi apparatus.

Together these results suggest that myc as well as GFP tagging to the mGluR5b have not altered the localization of the receptors.

3.2 Constitutive endocytosis of myc-mGluR5b in HEK293 cells

GPCRs can get endocytosed constitutively (ligand-independent) as well as in ligand-dependent manner. Molecular players involved in these two mechanisms could be different depending on the type of GPCR. In case of mGluRs, ligand-dependent endocytosis has been explored in little detail whereas constitutive endocytosis is not well studied. Due to this reason we decided to look at the constitutive internalization of mGluR5b receptor. For this purpose, HEK293 cells were transfected with myc-mGluR5b and live cell antibody feeding assay was performed. In order to

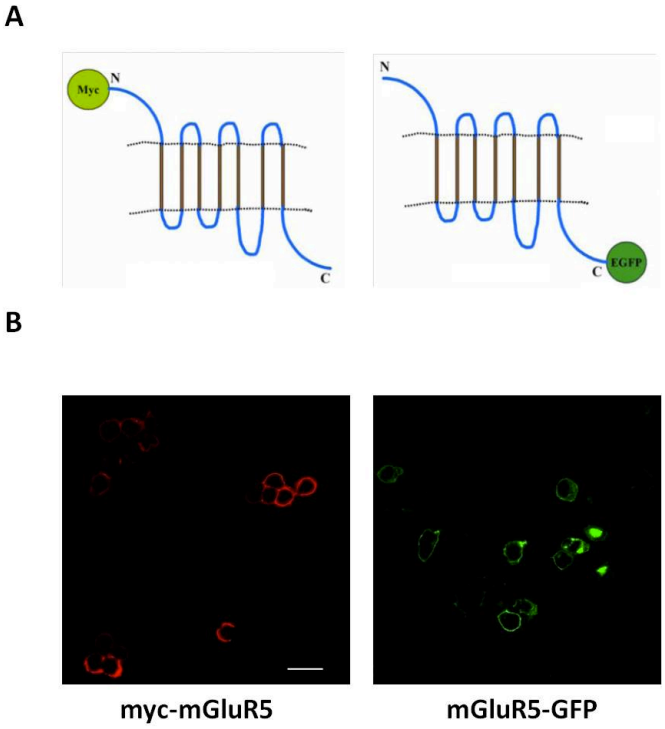


Figure 1: Expression of tagged mGluR5b receptors in HEK293 cells.
(A)Two different mGluR5b constructs are shown. In myc construct, myc is tagged at N-terminus while inGFP construct, mGluR5b was tagged with GFP at the C-terminus.Localized expression of the receptors was observed at the cell surface (B). In myc-mGluR5b, receptors were probed with anti-myc primary antibody and visualized with Alexa-568 tagged secondary antibody. mGluR5b-GFP also shown surface localization of the receptors with some internal fluorescence due to continuous synthesis of the receptors inside the cell. (scale bar = 10µm)

label all the primary antibody labeled surface receptors, saturating concentration of secondary antibody tagged with fluorophore Alexa 568 was used. Internal receptors were labeled by second secondary antibody tagged with Alexa 647 after permeablizing the cells. In order to study constitutive endocytosis, cells were incubated for 30 min at 37˚C subsequent to primary antibody staining. In control cells, receptors were present at the surface and there was negligible amount of fluorescence observed from the internalized receptors. After 30 min incubation at 37˚C, cells showed increased internal puncta due to constitutively endocytosed receptors (Figure 2A). Quantitation also showed that cells at 30 min (2.297±0.309) showed more internalization than control (1± 0.133)(Figure 2B). This result suggests that the receptor gets endocytosed constitutively in our experiments and indicate that our assay is robust enough to detect constitutive endocytosis of the receptor.

3.3 Kinetics of the mGluR5b internalization in HEK293 cells

Previous experiment suggested that mGluR5b gets constitutively endocytosed, which can be detected at 30 min time point using live cell antibody feeding assay. We next wanted to investigate how fast the mGluR5b receptor gets constitutively endocytosed. For this we performed time course experiment of mGluR5b internalization in order to study the kinetics of the constitutive internalization. HEK293 cells were transfected with myc-mGluR5b. After incubating with primary antibody on ice for 1 hour, control cells were immediately fixed with 4% PFA, while other cells were incubated at 37°C for respective durations before fixation in order to allow constitutive endocytosis.

We found that control cells showed small amount of internal fluorescence, which did not increase significantly at 5 min. At 15 min, internal puncta started appearing due to the presence of the constitutively internalized receptors, which further increased at 30 min (Figure 3A). Quantitation, also suggested that the value of internalization in cells at control (1± 0.133) is almost similar to cells at 5 min (1.035±0.130) that got increased at 15 min (1.771±0.299) and 30 min (2.297±0.309)(Figure 3B). We also measured the internalization of the receptor at 1 hour and no significant difference between the internalization at 30 min at 1 hr was observed (data not shown). These results suggest that the constitutive endocytosis of the myc-mGluR5b receptor begins after 5 min and saturates at 30 min in HEK293 cells.

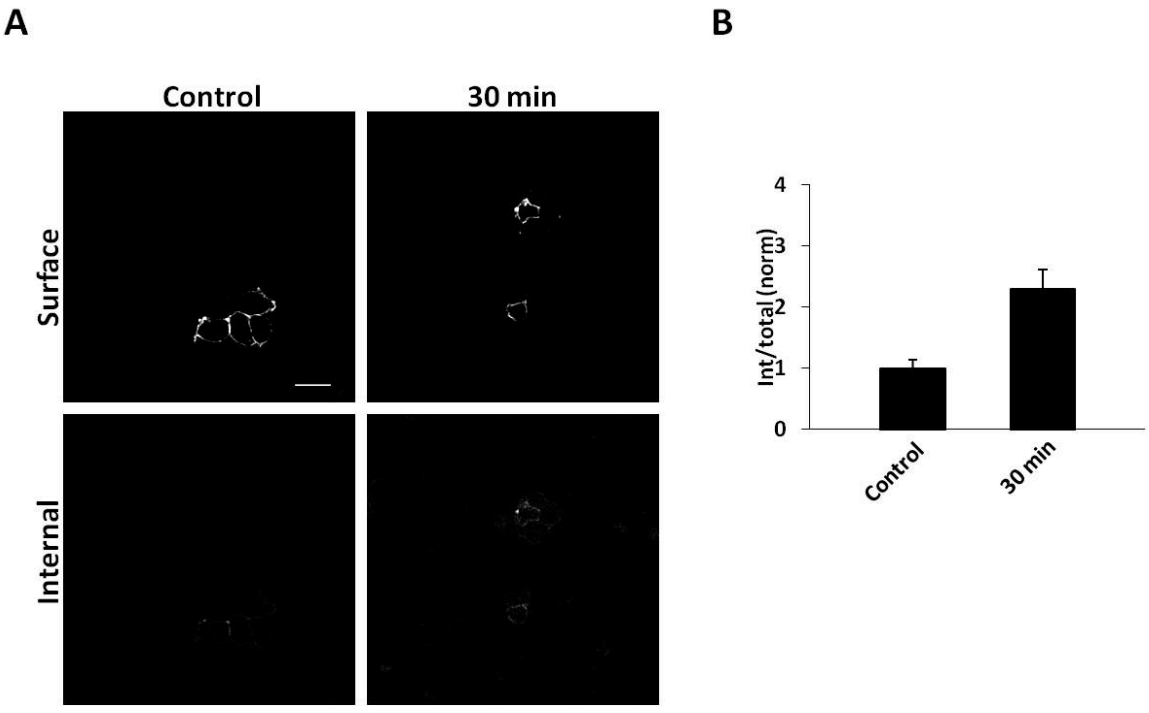


Figure 2: Constitutive endocytosis of myc-mGluR5b.
(A) In control cells, receptors were present at the surface and there was negligible amount of fluorescence observed from the internalized receptors. After 30 min incubation at 37˚C internal puncta were observed due to constitutively endocytosed receptors inside the cells. (B) Quantitation of the constitutively endocytosis receptors also showed significant increase in the internalization index. (scale bar=10μm)

3.4 Ligand-dependent endocytosis of mGluR5b in HEK293 cells

As stated before, most of the GPCR gets endocytosed primarily in ligand dependent fashion, which is also true for mGluR5b. We wanted to investigate whether ligand dependent endocytosis of myc-mGluR5b and mGluR5b-GFP occurs in HEK293 cells or not. For this experiment we chose DHPG as agonist. In case of myc-mGluR5b construct, live cell antibody feeding assay was done. Cells were then treated with DHPG for 5 min and were chased for 25min in the absence of DHPG as described in Method section. Control cells didn't receive any DHPG treatment. Surface and internal fluorescence is shown at both the time points in Figure 4A. As expected control cells showed very less internal fluorescence suggesting that significant amount of endocytosis has not happened and most of the receptors were localized at the membrane. At 30 min, cells showed very high internal fluorescence suggesting that receptors got endocytosed on DHPG application (Figure 4A). Quantitative analysis also revealed that there was approximately 3-fold increase in the receptor internalization at 30 min (3.014±0.313) than in control (1±0.100) (Figure 4B). The same endocytosis experiment was performed at 1 hours time point, which showed that there is no significant difference in 1 hour and 30 min time point cells in term of the degree of internalization of the receptors (data not shown).

In case of mGluR5b-GFP construct, following the transfection, HEK293 cells were treated with cycloheximide for 5 hours to clear internal fluorescence. After 5 hours, in control, most of the cells showed surface expression of GFP and very little/no internal fluorescence whereas a minority of the cells still showed some internal fluorescence because these cells are probably refractory of the cycloheximide. The number of cells showing internal fluorescence got increased when they were observed at 15 min time point. Cells were counted for only surface and surface along with internal expression of cells. It was found that in control, 73% cells showed clearance in internal fluorescence but 27% cells still showed internal fluorescence. This value of 27% cells

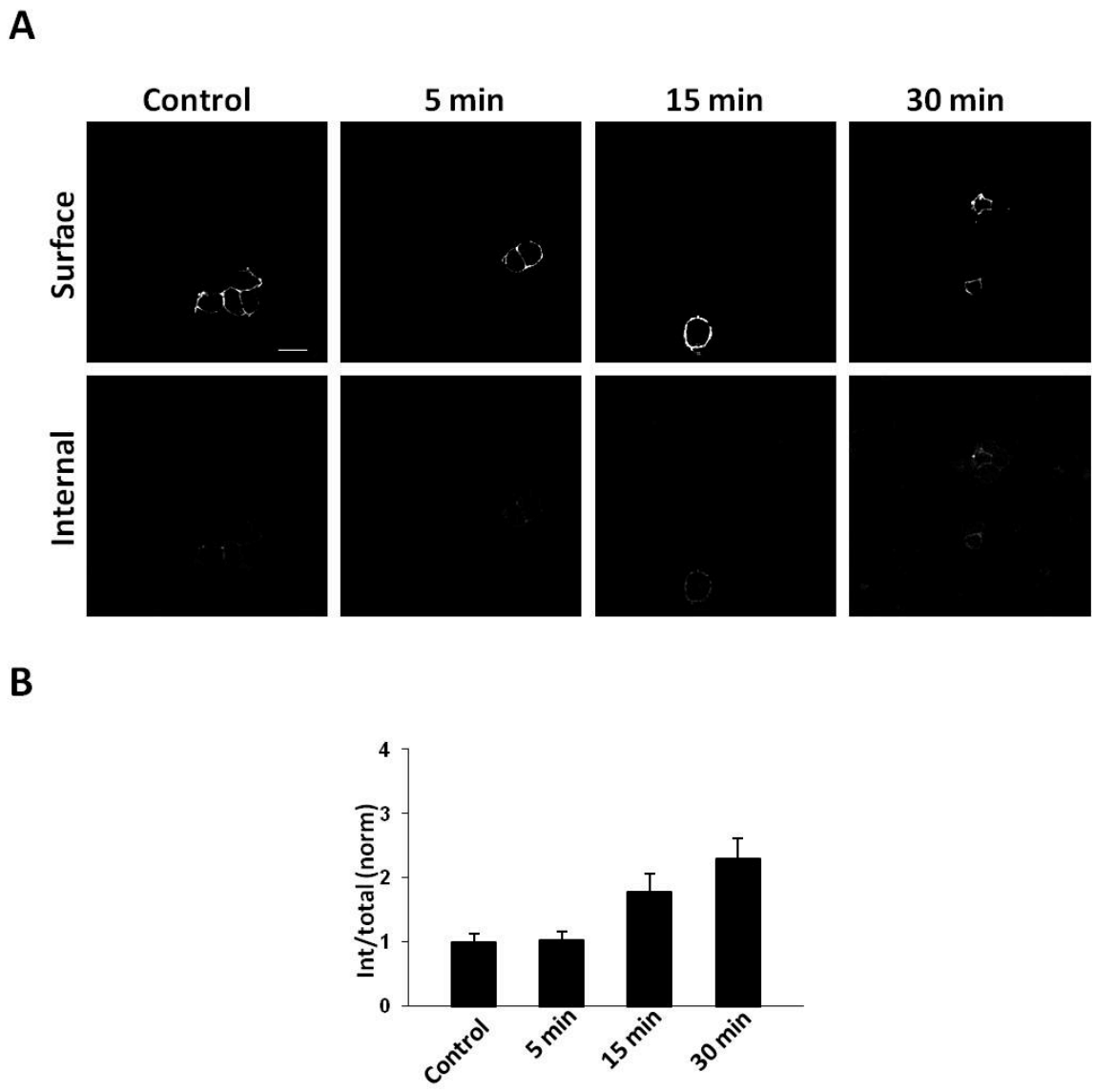


Figure 3: Time course of constitutive endocytosis of myc-mGluR5b.
(A) Control cells show very less internal fluorescence. Cells were incubated at 37˚C for 5 min, 15 min, and 30 min without the application of ligand (DHPG) to observe internal florescence due to constitutively endocytosed receptors. Control cells showed very less internal fluorescence, which did not increase significantly at 5 min. At 15 min, internal puncta started appearing which further increased at 30 min. (B) Quantitation, also suggested that the value of internalization in cells at control is almost similar to cells at 5 min that got increase at 15 min and 30 min. (scale bar = 10μm)

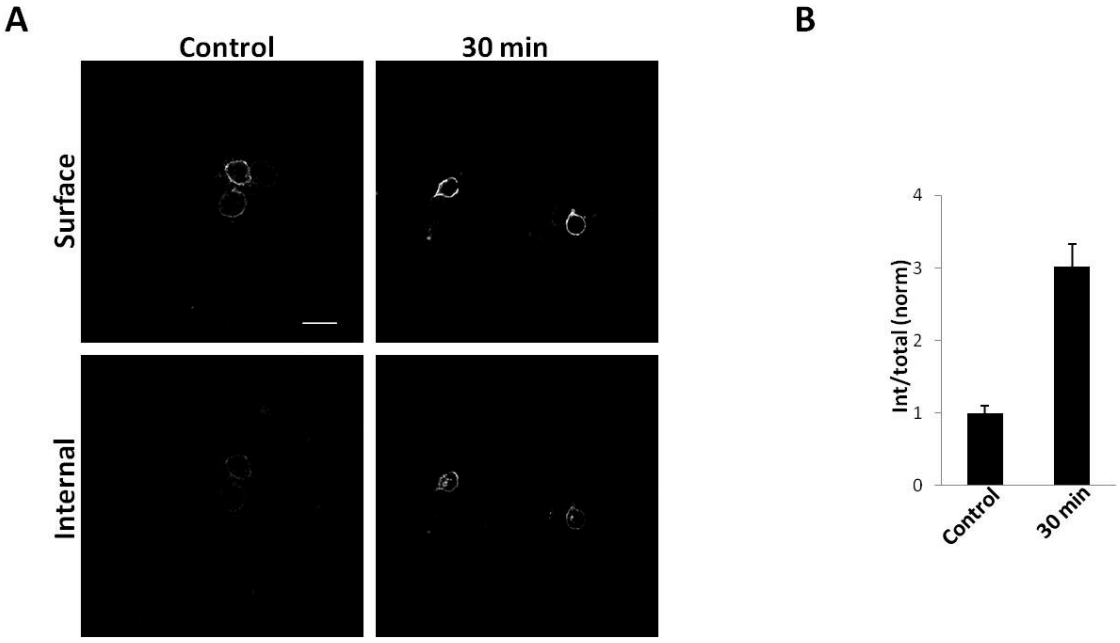


Figure 4: Ligand mediated endocytosis of myc-mGluR5b.
(A) In control cells, receptors were present at the surface and fluorescence was observed from the receptors that were present only at the surface. In 30 min time-point, cells were treated with for 5 min and were chase for 25 min in the absence of DHPG. At 30 min, cells showed very high internal fluorescence. (B) Quantitative analysis also revealed that there was approximately 3-fold increase in the receptor internalization at 30 min than in control cells. (scale bar=10μm)

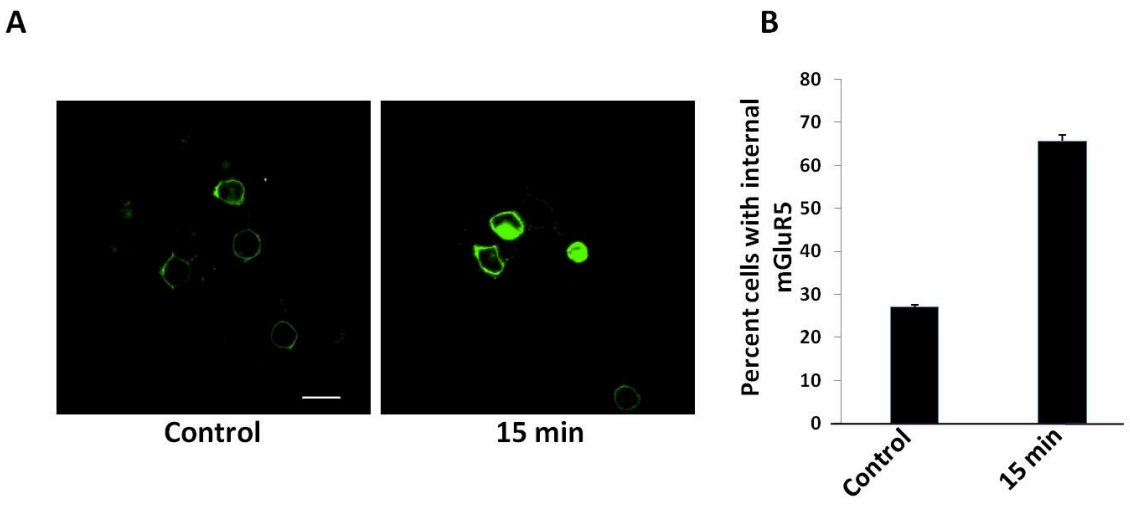


Figure 5: Ligand mediated endocytosis of mGluR5b-GFP.
(A) Upon transfection, cells were treated with cycloheximide for 5 hours. In control, most of the cells showed surface expression of GFP and very little/no internal fluorescence. The number of cells showing internal fluorescence got increased when they were observed at 15 min time point. Cells at 15 min were treated with DHPG for 5 min followed by 10 min chase. (B) Cells were counted for only surface and surface along with internal expression of cells. In control, 27% cells showed internal fluorescence which further increased to 68.5% when DHPG was applied in cell at 15 min time point. (scale bar=10μm)

showing internal mGluR5b further increased to 68.5% when DHPG was applied for 5 min in the cells at 15 min time point (Figure 5B). This result suggests that significant proportion of cells induced endocytosis of GFP tagged receptors on DHPG application.

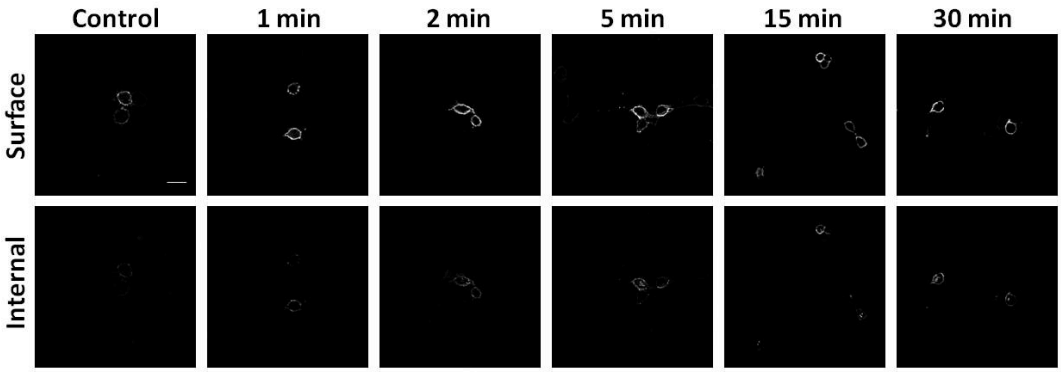
Together these results suggest that both myc and GFP tagged receptors got endocytosed by ligand and their internalization pattern was also found to be similar which indicates that tagging did not alter the trafficking properties of the receptors.

3.5 Kinetics of ligand-dependent endocytosis of mGluR5b in HEK293 cells

Earlier experiment suggested that both myc-mGluR5b and mGluR5b-GFP get endocytosed upon ligand application. Next, we wanted to observe the agonist induced internalization kinetics of both the mGluR5b constructs. For this purpose, time course experiment was performed at 6 time points- control, 1 min, 2 min, 5 min, 15 min, and 30 min. Control cells were not treated with DHPG. At 1 min, 2 min, and 5 min time points, DHPG was applied for 1, 2, and 5 min respectively. While for 15 min and 30 min time-points, DHPG was added for 5 min followed by 10 min and 25 min chase respectively.

Live cell antibody feeding assay was performed in HEK293 cells transfected with myc-mGluR5b. As expected, in control, expression of the receptors was observed only at the surface of cells. At 1 min, cells started showing internal fluorescence due to endocytosed receptors, which further increased at 2 min. At 2 min time point internal fluorescence was observed close to the surface due to newly internalized receptors. At 5 min, internal fluorescence got dispersed and receptor puncta was observed inside the cells, which got increased further at 15 min, suggesting that endocytosis is still going on. At 30 min, expression of the internalized receptors was observed close to the surface as well as inside the cells (Figure 6A). Quantitation also supported the qualitative observation as the value of internalization got increased as we allowed the endocytosis to happen from control cells (1±0.100) to 1 min (1.547±0.220), 2 min (1.901±0.385), 5 min (2.145±0.356), 15 min (2.446±0.290). Maximum internalization was quantified in cells at 30 min (3.014±0.313) (Figure 6B). This experiment suggests that ligand mediated endocytosis of myc-mGluR5b is a fast process and starts right after the application of ligand.

A



B

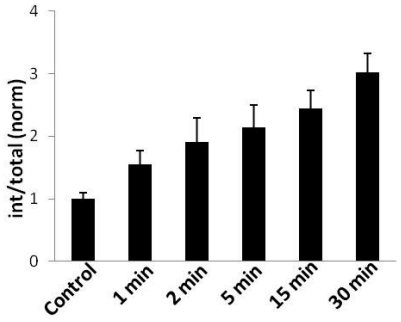


Figure 6: Time course of Ligand mediated endocytosis of myc-mGluR5b.
(A) Kinetics of ligand (DHPG) mediated endocytosis was studied at 1min, 2 min, 5 min, 15 min and 30 min. Control cells were not treated with DHPG. In control cells, expression of the receptors was observed only at the surface of cells. At 1 min, cells started showing internal fluorescence which further increased at 2 min, 5 min, 15 min and 30 min subsequently. At 30 min, expression of the internalized receptors was observed close to the surface as well as inside the cells. (B) Quantitation also supported the qualitative observation and the value of internalization got increased as we allowed the endocytosis to happen from control cells to 1 min, 2 min, 5 min, 15 min. Maximum internalization was quantified in cells At 30 min. (scale bar = 10μm)

Next, we wanted to observe whether GFP tagging has altered the kinetics of the receptor internalization, time course endocytosis experiment was performed on HEK293 cells transfected with mGluR5b-GFP. In control, most of the cells showed only surface expression of the receptors but not the internal expression which did not change at 1 min time point. At 2 min, we observed internal GFP fluorescence suggesting that internalization had started which got increased further at 5 min. At 5 min, expression of internalized receptors was observed in the shape of a blob. Receptors remained localized inside the cell at 15 min and 30 min time point.

Cells were counted for internalized receptor expression. It was found that in control, only 27% cells still showed internal fluorescence. Upon ligand treatment at 1 min 30% cells showed internal GFP expression, which got increased to 63.5% at 2 min and 68.5% in cells at 5 min time point. 64.5% cells showed internal fluorescence at 15 min and 62% of them showed internalized receptors at 30 min (Figure 7B). These results suggest that GFP tagged receptors also follow similar kinetics as observed in case of myc tagged receptors.

Together these results also suggest that ligand-mediated endocytosis is faster than the constitutive endocytosis.

3.6 Internalized receptor mGluR5b receptors enter recycling compartment subsequent to internalization

After getting endocytosed, GPCRs can have multiple sub-cellular fates. Upon internalization they can enter into endosomes and then go to the recycling compartment. For some GPCR, recycling is not possible and they enter into the lysosome get degraded in the lysosome . The fate of the receptor is dependent on various factors like type of GPCR, ligand and cell type. For the resensitization of the receptors many times acidification of recycling endosomes and dephosphorylation of the GPCRs is required. Our earlier results suggest that mGluR5b can get endocytosed constitutively, even without the application of ligand. But the fate of the receptor

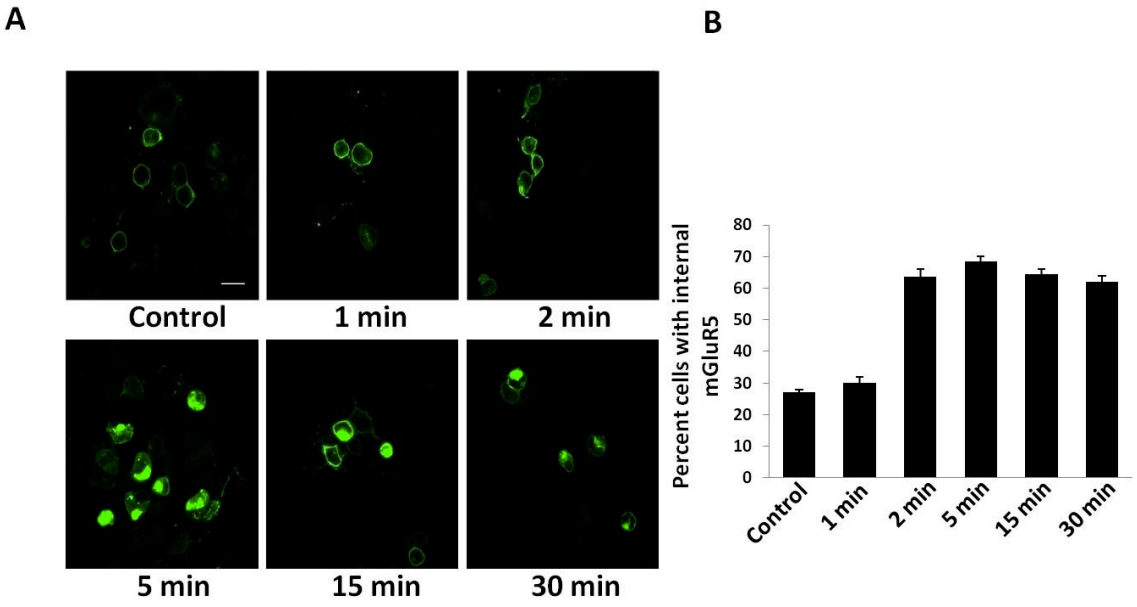


Figure 7: Kinetics of Ligand mediated endocytosis of mGluR5b-GFP.
(A) kinetics of the receptor internalization for GFP-tagged receptor was observed at 1 min, 2 min, 5 min, 15 min and 30 min time point. In control, most of the cells showed only surface expression of the receptors but not the internal expression which did not change at 1 min time point. At 2 min, we observed internal GFP fluorescence which got increased further at 5 min. At 15 min and 30 min, cells showed localized expression of the receptors inside the cell. (B) Cells were counted for internalized receptor expression. Percent of cells with internal GFP expression got increased from control to higher time points. (scale bar = 10μm)

after the endocytosis is not known. So we investigated the fate of the receptors by performing co-localization studies with recycling endosome marker-Rab11 and lysosomal marker-Lamp1.

In constitutive endocytosis experiment maximum internalization was observed at 30 min. To observe maximum co-localization with Rab11 and Lamp1, we chose 30 min time point for co-localization study. HEK293 cells were transfected with myc-mGluR5b. After 24 hours of transfection, myc-mGluR5b receptors were labeled with anti-myc mouse monoclonal antibody or anti-myc rabbit polyclonal antibody followed by respective secondary antibody tagged with Alexa 568. For staining of Rab11 and Lamp1, mouse monoclonal and rabbit polyclonal primary antibody was used respectively. Subsequently appropriate secondary antibody tagged with Alexa 488 was used to visualize these markers (as described in the method section).

As expected, control cells did not show observable internalized receptors in both the co-localization experiment. Interestingly, after 30 min of constitutive internalization myc-mGluR5b was seen to be co-localized with Rab11 (Figure 8), whereas not much co-localization was observed with Lamp1 (Figure 9).

Together these results suggest that constitutively endocytosed mGluR5b goes to recycling compartment subsequent to internalization while very little or no receptors enter into the lysosome.

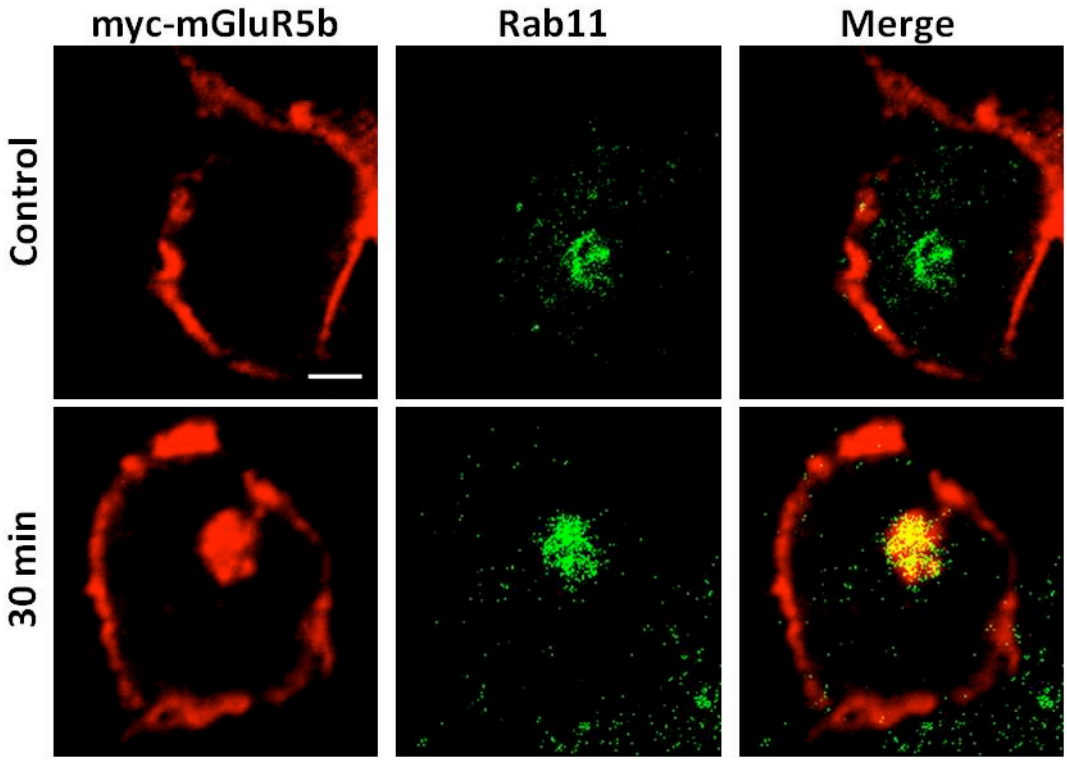


Figure 8: Myc-mGluR5b localizes at recycling compartment upon constitutive internalization. After 24 hours of transfection, myc-mGluR5b receptors were labeled with anti-myc primary antibody followed by staining with secondary antibody tagged with Alexa-568 (Red). To visualize recycling compartment, cells were incubated in anti-rab11 primary antibody followed by secondary antibody tagged with Alexa 488 (Green). Control cells did not show observable internalized receptors while after 30 min of constitutive internalization myc-mGluR5b was seen to be co-localized with Rab11. (scale bar = 2 μm)

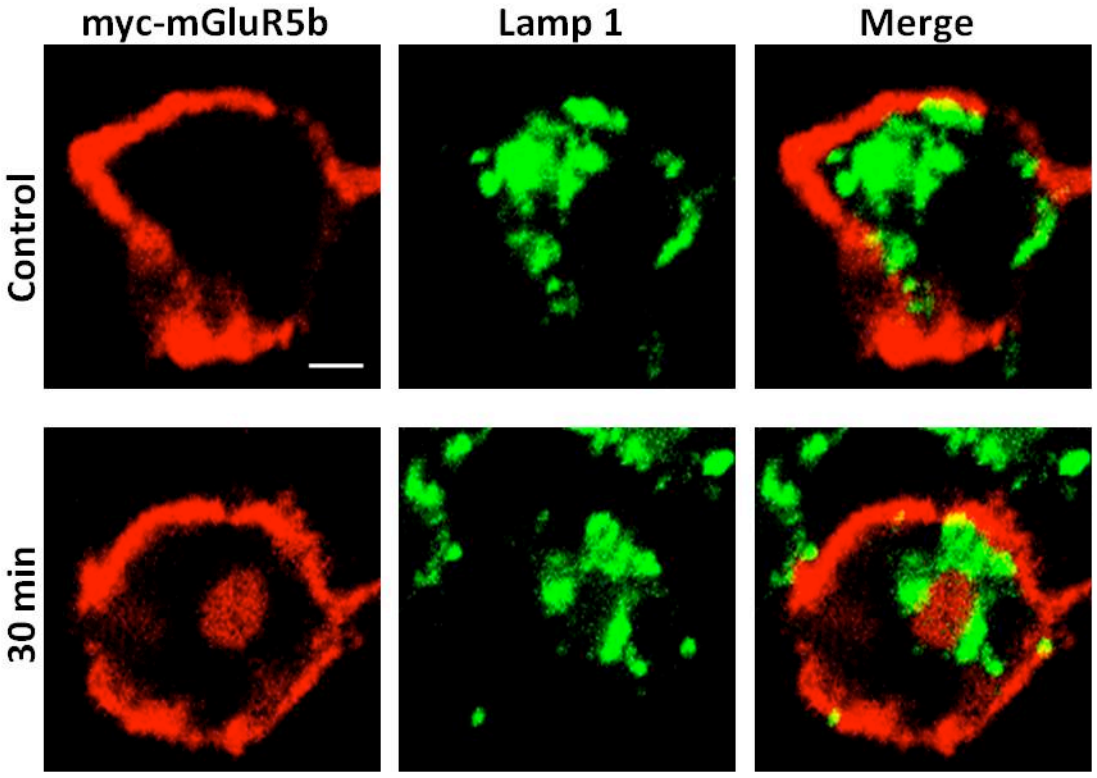


Figure 9: Myc-mGluR5b does not enter the lysosomal compartment upon constitutive endocytosis.
myc-mGluR5b receptors were labeled with anti-myc and anti-LAMPI primary antibody followed by staining with Alexa-568 (red) and Alexa 488 (green) conjugated secondary antibody to visualize receptors and lysosomal compartment. Neither control cells showed observable internalized receptors nor after 30 min of constitutive internalization myc-mGluR5b was seen to be co-localized with LAMP 1. (scale bar = 2 μm)

Discussion

Discussion

Endocytosis of G-protein coupled receptors is major mechanism of desensitization or resensitization or downregulation the receptors [33, 39, 40, 41]. Among group I mGluRs, mGluR1 has been shown that it undergoes both agonist-dependent and agonist-independent endocytosis for its regulation [8, 49]. For mGluR5b, people have studied only ligand (DHPG) mediated endocytosis, but not much is known about the constitutive endocytosis of mGluR5b. In this study, we have investigated DHPG-dependent endocytosis of mGluR5b as well as we have explored whether it gets constitutively endocytosed [50].

Previous work has shown that internal or surface receptors could be measured by various techniques like biotinylation assays [51], surface fluorescence measurement, and internal fluorescence measurement [52]. All these methods do not take into account the variability in the receptor expression among the cells, which is the main disadvantage, as we cannot compare surface/internal expression from one cell to the others without normalizing to the total receptor. To overcome this, we have used dual labeling assay in our endocytosis experiment that enables us to normalize the expression within the cell and allows us to compare the expression between the cells without any perturbation. Another advantage of this technique is, it allows the staining of the surface receptors only and thus fluorescence from the internalized receptors is minimal in the control cells. This technique also allows us to measure fraction of the receptors that are getting endocytosed by using two different secondary antibodies tagged with different fluorophores.

In this study, we observed that DHPG-induced endocytosis of mGluR5b is faster than the constitutive endocytosis of the receptor. It suggests that triggering mechanism of the ligand-mediated vs. constitutive endocytosis might be very different because both of them are happening at different time scale. It is known that for many GPCRs, ligand mediated endocytosis involves activation of second messenger pathway, while in constitutive endocytosis, it may require basal level or no response of second messengers.

Therefore, It can be hypothesized that in constitutive endocytosis, either build up of the activity of kinases takes longer time or the constitutive endocytosis happens in a kinase independent fashion as the kinetics of process of ligand-independent receptor internalization

is slower than the ligand dependent one. According to another hypothesis, there might be some conformational changes of the receptor involved, which is a very slow process responsible for the delayed constitutive endocytosis response.

We have also shown that in constitutive endocytosis, receptor enters into the recycling compartment predominantly and not much observable amount of receptors were visible in the lysosome as seen by the LAMPI staining. It suggests that the existing receptors are probably reused again after a cycle of endocytosis and majority of them are not replaced by newly synthesized receptors. It also raises the question of spatial regulation of the recycled and newly synthesized receptors. It would be interesting to study where do the recycled as well as newly synthesized receptors localize in a neuronal membrane upon delivery.

It should be mentioned here that in this study, we have shown only the spatio-temporal localization of the endocytosed receptors inside the cell, but we have not studied the kinetics of receptor recycling. Various GPCR show different time of exit from the recycling compartment and recycling is thought to be the mechanim of resensitization for many of them. For example, some GPCRs show delayed recycling and some recycled back as soon as they enter into the recyclosome. This fast versus slower recycling depends on the sequence of the GPCRs at the C-terminus end and third intracellular loop. It would be mandatory to investigate what fraction of mGluR5b receptors recycles back to the cell membrane and how fast/slow is this recycling process.

Interestingly, in the lab it has been observed that in case of ligand-mediated endocytosis of mGluR5b, receptors also enter into the recycling compartment (personal communication with Prabhat Mahato). This data suggests that probably constitutive as well as ligand mediated endocytosis of this receptor may follow the same route of trafficking. It would be very important to study the molecular players in both of these endocytosis processes.

References

1. Gether U (2000) Uncovering molecular mechanisms involved in activation of G protein- coupled receptors. Endocr Rev 21:90-113.
2. Pin, J. P., & Duvosin, R. (1995). The metabotropic glutamate receptors: structure and functions. Neuropharmacology 34, 1–26.
3. Nakanishi, S. (1994). Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity. Neuron 13, 1031–1037.
4. Dale, L. B., Babwah, A. V., & Ferguson, S. S. G. (2003). Mechanisms of metabotropic glutamate receptor desensitization: role in the patterning of effector enzyme activation. Neurochem Int 41, 319–326.
5. Conn, P. J., & Pin, J. P. (1997). Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37, 205–237.
6. Pin, J. P., & Bockaert, J. (1995). Get receptive to metabotropic glutamate receptors. Curr Opin Neurobiol 5, 342–349.
7. Dale, L. B., Babwah, A. V., Bhattacharya, M., Kelvin, D. J., & Ferguson, S. S. G. (2001a). Spatial-temporal dynamics of metabotropic glutamate receptor- mediated inositol 1,4,5-triphosphate, Ca2+, and protein kinase C oscillations. J Biol Chem 276, 35900–35908.
8. Dale, L. B., Bhattacharya, M., Seachrist, J. L., Anborgh, P. H., & Ferguson, S. S. G. (2001b). Agonist-stimulated and tonic internalization of metabo- tropic glutamate receptor 1a in human embryonic kidney 293 cells: agonist- stimulated endocytosis is a`-arrestin1 isoform-specific. Mol Pharmacol 60, 1243–1253.
9. Nakahara K, Okada M and Nakanishi S (1997) The metabotropic glutamate receptor mGluR5 induces calcium oscillations in cultured astrocytes via protein kinase C phosphorylation. Journal of Neuro- chemistry 69: 1467–1475.
10. Wess J (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recogni- tion. FASEB Journal 11: 346–354.
11. Nishizuka Y. 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665.

12. Tateyama M, Kubo Y. 2006. Dual signaling is differentially activated by different active states of the metabotropic glutamate receptor 1a. Proc Natl Acad Sci U S A 103:1124–1128.
13. Thandi S, Blank J.L., Challiss R.A. 2002. Group-I metabotropic glutamate receptors, mGlu1a and mGlu5a, couple to extracellular signal-regulated kinase (ERK) activation via distinct, but overlapping, signalling path- ways. J Neurochem 83:1139–1153.
14. Tu JC, Xiao B, Yuan JP et al. (1998) Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. Neuron 21: 717–726.
15. Bashir Z.I. and Collingridge G.L. (1994) An investigation of depotentiation of long-term potentiation in the CA1 region of the hippocampus. *Exp Brain Res* 100:437–443.
16. Bashir Z.I., Jane D.E., Sunter D.C., Watkins J.C., and Collingridge G.L. (1993b) Metabo- tropic glutamate receptors contribute to the induction of long-term depression in the CA1 region of the hippocampus. *Eur J Pharmacol* 239:265–266.
17. Schoepp D.D. and Conn P.J. (1993) Metabotropic glutamate receptors in brain function and pathology. *Trends Pharmacol Sci* 14:13–20.
18. Schoepp D.D., Goldsworthy J, Johnson B.G., Salhoff C.R., and Baker S.R. (1994) 3,5- dihydroxyphenylglycine is a highly selective agonist for phosphoinositide-linked metabotropic glutamate receptors in the rat hippocampus. *J Neurochem* 63:769– 772.
19. Schnabel R, Kilpatrick I.C., and Collingridge G.L. (1999a) An investigation into signal transduction mechanisms involved in DHPG-induced LTD in the CA1 region of the hippocampus. *Neuropharmacology* 38:1585–1596.
20. Schnabel R, Kilpatrick I.C., and Collingridge G.L. (2001) Protein phosphatase inhibitors facilitate DHPG-induced LTD in the CA1 region of the hippocampus. *Br J Pharmacol* 132:1095–1101.
21. Bolshakov VY and Siegelbaum SA (1994) Postsynaptic induction and presynaptic expression of hippocampal long-term depression. *Science* 264:1148–1152.
22. Fitzjohn S.M., Kingston A.E., Lodge D, and Collingridge G.L. (1999) DHPG-induced LTD in area CA1 of juvenile rat hippocampus; characterisation and sensitivity to novel mGlu receptor antagonists. *Neuropharmacology* 38:1577–1583.

23. Fitzjohn S.M., Palmer M.J., May J.E., Neeson A, Morris S.A., and Collingridge G.L. (2001) A characterisation of long-term depression induced by metabotropic glutamate receptor activation in the rat hippocampus in vitro. *J Physiol* 537:421–430.
24. Huang C.C., You J.L., Wu M.Y., and Hsu K.S. (2004b) Rap1-induced p38 mitogen-activated protein kinase activation facilitates AMPA receptor trafficking via the GDI.Rab5 complex. Potential role in (S)-3,5-dihydroxyphenylglycene-induced long term depression. *J Biol Chem* 279:12286–12292.
25. Li X.M., Li C.C., Yu S.S., Chen J.T., Sabapathy K, and Ruan D.Y. (2007b) JNK1 contrib- utes to metabotropic glutamate receptor-dependent long-term depression and short term synaptic plasticity in the mice area hippocampal CA1. *Eur J Neurosci* 25:391–396.
26. Gallagher S.M., Daly C.A., Bear M.F., and Huber K.M. (2004) Extracellular signal-regulated protein kinase activation is required for metabotropic glutamate recep- tor-dependent long-term depression in hippocampal area CA1. *J Neurosci* 24: 4859 – 4864.
27. Huang C.C. and Hsu K.S. (2006) Sustained activation of metabotropic glutamate receptor 5 and protein tyrosine phosphatases mediate the expression of (S)-3,5-dihydroxyphenylglycine-induced long-term depression in the hippocampal CA1 region. *J Neurochem* 96:179–194.
28. MacGregor D.G., Mallon A.P., Harvey A.L., Young L., Nimmo H.G., and Stone T.W. (2007) Group S8A serine proteases, including a novel enzyme cadeprin, induce long- lasting, metabotropic glutamate receptor-dependent synaptic depression in rat hippocampal slices. *Eur J Neurosci* 26:1870–1880.
29. Bear M.F., Huber K.M., and Warren S.T. (2004) The mGluR theory of fragile X mental retardation. *Trends Neurosci* 27:370–377.
30. Huber K.M., Gallagher S.M., Warren S.T., and Bear M.F. (2002) Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proc Natl Acad Sci U S A* 99:7746 –7750.
31. Todd P.K., Mack K.J., and Malter J.S. (2003) The fragile X mental retardation protein is required for type-I metabotropic glutamate receptor-dependent translation of PSD-95. *Proc Natl Acad Sci U S A* 100:14374 –14378.

32. Zalfa F, Eleuteri B, Dickson KS, Mercaldo V, De Rubeis S, di Penta A, Tabolacci E, Chiurazzi P, Neri G, et al. (2007) A new function for the fragile X mental retardation protein in regulation of PSD-95 mRNA stability. *Nat Neurosci* 10:578 – 587.
33. Ferguson S.S.G. 2001. Evolving concepts in G-protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharma- col Rev 53:1–24.
34. Clark R.B. Receptor desensitization. *Adv Cyclic Nucl Prot Phos Res* 1986;20:151–209.
35. Gereau R.W., Heinemann S.F.. (1998). Role of protein kinase C phosphory- lation in rapid desensitization of metabotropic glutamate receptor 5. Neuron 20:143–151.
36. Krupnick, J. G., & Benovic, J. L. (1998). The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu Rev Pharmacol Toxicol 38, 289–319.
37. Dhami, G. K., Anborgh, P. H., Dale, L. B., Sterne-Marr, R., & Ferguson, S. S. G. (2002). Phosphorylation-independent regulation of metabotropic glutamate receptor signaling by G protein-coupled receptor kinase 2. J Biol Chem 277, 25266–25272.
38. Chuang T.T., Iacovelli L., Sallese M., et al. (1996) G protein-coupled receptors: heterologous regulation of homologous desensitization and its implications. *Trends Pharmacol Sci*.17:416 – 421.
39. Gainetdinov R.R., Premont R.T., Bohn L.M., Lefkowitz R.J., Caron M.G. (2004). Desensitization of G-protein-coupled receptors and neuronal functions. Annu Rev Neurosci 27:107–144.
40. Tsao P.I., von Zastrow M (2001) Diversity and specificity in the regulated endocytic membrane trafficking of G-protein-coupled receptors. Pharmacol Ther 89:139-147.
41. Tsao P., von Zastrow M (2000a) Downregulation of G protein-coupled receptors. Curr Opin Neurobiol 10:365-369.
42. Dhami, G. K. & Ferguson, S. S. G. (2006) Regulation of metabotropic glutamate receptor signaling, desensitization and endocytosis. Pharmacology & Therapeutics 111, 260–271.
43. Koenig JA, Edwardson JM. (1997) Endocytosis and recycling of G pro- tein-coupled receptors. *Trends Pharmacol Sci*;18:276– 287.

44. Bhattacharya, M., Babwah, A. V., Godin, C., Anborgh, P. H., Dale, L. B., Poulter, M., et al. (2004). Ral and phospholipase D2-dependent pathway for constitutive metabotropic glutamate receptor endocytosis. J Neurosci 24, 8752–8761.
45. Mundell, S. J., Matharu, A. L., Pula, G., Roberts, P. J., & Kelly, E. (2001). Agonist-induced internalization of the metabotropic glutamate receptor 1a is arrestin- and dynamin-dependent. J Neurochem 78, 546–551.
46. Mundell, S. J., Pula, G., Carswell, K., Roberts, P. J., & Kelly, E. (2003). Agonist-induced internalization of metabotropic glutamate receptor 1A: structural determinants for protein kinase C- and G protein-coupled receptor kinase-mediated internalization. J Neurochem 84, 294–304.
47. Mundell S. J., Pula G., McIlhinney R. A., Roberts P. J., & Kelly E. (2004). Desensitization and internalization of metabotropic glutamate receptor 1a following activation of heterologus G37q/11-coupled receptors. Biochemistry 43, 7541–7551.
48. Dale, L. B., Bhattacharya, M., Anborgh, P. H., Murdoch, B., Bhati, M., Nakanishi, S., et al. (2000). G protein-coupled receptor kinase-mediated desensitization of metabotropic glutamate receptor 1A protects against cell death. J Biol Chem 275, 38213–38220.
49. Mundell, S.J., Matharu, A., Pula, G., Roberts, P.J. & Kelly, E.(2001).Agonist-induced internalization of the metabotropic glutamate receptor 1a is arrestin- and dynamin-dependent. Journal of Neurochemistry, 78, 546±551
50. Nash MS, *et al*. (2002) Determinants of metabotropic glutamate receptor-5-mediated Ca2 and inositol 1,4,5-trisphosphate oscillation frequency. Receptor density versus agonist concentration. *J Biol Chem* 277:35947–35960.
51. Ribeiro F.M., Ferreira L.T., Paquet M., Cregan T., Ding Q., Gros R., Ferguson S.S.G. (2009). Phosphorylation-independent regulation of metabotropic glutamate receptor 5 desensitization and internalization by G protein-coupled receptor kinase 2 in striatal neurons. J Biol Chem 284:23444–23453
52. Barak L. S., Ferguson S. S. G., Zhang J., Martenson C., Meyer T., Caron M. G. (1997). Internal Trafficking and Surface Mobility of a Functionally Intact β_2 Adrenergic Receptor-Green Fluorescent Protein Conjugate. Mol. Pharmacol. 51:177–184.