Investigating the role of Kinases and Phosphatases in the constitutive trafficking of Metabotropic Glutamate Receptor 5 (mGluR5) in HEK293 Cells

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Certificate of Examination

This is to certify that the dissertation titled "Investigating the role of Kinases and Phosphatases in constitutive trafficking of Metabotropic Glutamate Receptor 5 (mGluR5) in HEK293 Cells" submitted by Mr. Kshitij Mohan (Reg. No.MS10039) for the partial fulfilment 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.

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(Supervisor)

Dated: April 24th, 2015

Declaration

The work presented in the dissertation has been performed 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 has been 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 bibliography.

Kshitij Mohan

Dated: April 24th, 2015

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)

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List of Figures

Abbreviations

AB-AM mix	Antibiotic Antimycotic mix
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CNS	Central Nervous System
cAMP	Cyclic Adenosine Monophosphate
DAG	Diacylglycerol
DHPG	Di-hydroxyphenylglycine
DMEM	Dulbecco's Modified Eagle Medium
FBS	Foetal Bovine Serum
GDP	Guanosine-5'-diphosphate
GPCR	G-Protein Coupled Receptor
GRK	G-Protein Coupled Receptor Kinase
GTP	Guanosine-5'-triphosphate
HEK293	Human Embryonic Kidney 293
IP3	Inositol 1,4,5-triphosphate
LTD	Long Term Depression
LTP	Long Term Potentiation
MEM	Minimum Essential Media
mGluR	Metabotropic Glutamate Receptor
NGS	Normal Goat Serum
NMDA	N-methyl-D-aspartate
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PP	Protein Phosphatase
RGS	Regulators of G Protein Signaling

Abstract

Glutamate is a major excitatory neurotransmitter in the central Nervous System, which binds to its post-synaptic receptors after Ca^{2+} mediated release into the synaptic cleft. Depending on the mode of action, glutamate receptors are either ionotropic – opens ion channels; or metabotropic – G Protein coupled receptors which elicit responses through second messenger pathways. mGluR5 belongs to group 1 metabotropic glutamate receptor family. They are implicated to play a crucial role in the phenomena of learning and memory, and mediate LTD in the hippocampus. They are present predominantly on the post synaptic neurons and are regulated by endocytosis, both ligand-mediated and constitutive (independent of ligand). It has been established that the mGluR5 is endocytosed to recycling compartment from where it recycles back to the surface in 3.5 hour. In this study, I have investigated the role of phosphatases in the trafficking of mGluR5. Additionally, I also studied whether basal phosphorylation by kinases is required for the endocytosis of the receptors. The study indicates that the phosphatases are involved in the trafficking of mGluR5 since inhibition of phosphatises prevented the recycling of mGluR5.

Contents

Chapter 1: Introduction

1.1 Glutamate Receptors

Glutamate is a major excitatory neurotransmitter present in the Central Nervous System. It is synthesized in the pre-synaptic neuron from glutamine with the help of the enzyme Glutaminase and stored in synaptic vesicles. When an action potential depolarizes the pre-synaptic neuron, it leads to the opening of Calcium ion channels. Increase in the concentration of Ca^{2+} ions leads to the fusion of synaptic vesicles containing glutamate with the plasma membrane of the pre-synaptic neuron and glutamate is released into the synaptic cleft. Upon Ca^{2+} mediated release of glutamate into the synaptic cleft, it binds to glutamate receptors [1]. Glutamate Receptors are transmembrane proteins and play major role in neurotransmission, synaptic plasticity and the development of the nervous system. Glutamate and other agonists bind to the receptors on their extracellular side. The binding of glutamate to the receptors present on the post-synaptic neurons trigger downstream signalling pathways and induce various intracellular responses. The type of response depends on the type of glutamate receptor [1,2,3]. Based on the function, structure and downstream signals they activate, Glutamate Receptors have been classified into:

a) Ionotropic Glutamate Receptors – These receptors are channels that get activated by the binding of glutamate. There are 3 ionotropic glutamate receptors, namely AMPA Receptors, NMDA receptors and Kainate Receptors, named after the agonists that activate them.

b) Metabotropic Glutamate Receptors – These are seven transmembrane G-Protein coupled Receptors which are activated by glutamate binding to them.

1.2 Metabotropic Glutamate Receptors

Metabotropic Glutamate Receptors (mGluRs) are seven transmembrane G-Protein Coupled Receptors which are activated when glutamate binds to them. The structure of metabotropic Glutamate receptor consists of seven transmembrane domains and the N-terminus region binds to glutamate and other agonists. Activation of mGluR elicits various slow but long term synaptic responses, mediated via a number of second

messengers. Depending on the structural, pharmacological and the second messenger pathways activated, metabotropic glutamate receptors have been categorized into three groups – Group I mGluRs (mGluR1 and mGluR5), Group II mGluRs (mGluR2 and mGluR3) and Group III mGluRs (mGluR4, mGluR6, mGluR7, mGluR8). Group1 mGluRs are G-Protein Coupled Receptors which are coupled to $G\alpha_{q/11}$. It activates Phospholipase C, which leads to the production of IP3 and DAG. Generation of IP3 leads to the opening of Ca^{2+} ion channels from ER, whereas DAG activates PKC and other signalling cascades. Thus, IP3 and DAG activate various downstream signalling and elicit intracellular responses in the post synaptic neuron. Group II and Group III mGluRs are coupled to $G\alpha_{i/o}$ and inhibit the synthesis of cyclic AMP [3, 4, 5, 6].

1.3 G-Protein Coupled Receptor Signalling

GPCRs are the largest category of transmembrane receptors in vertebrates. A huge proportion of cell signalling activities in vertebrates are mediated by GPCR signalling. It is estimated that more than half of the therapeutic drugs used to treat various diseases or conditions target one of the many GPCRs. There are approximately 1000 categories of GPCRs which have a conserved structure. GPCRs have a seven transmembrane spanning domain, an intracellular domain and a receptor binding domain. Based on their amino acid sequences, GPCRs are divided into three categories – A, B and C. Number of molecules such as neurotransmitters, hormones and external stimuli act as ligands for GPCRs and activate them. Binding of ligands to extracellular domain of GPCR leads to change in conformation of the transmembrane and intracellular domain. This change in the conformation of GPCR leads to the interaction of GPCR with heterotrimeric G-Protein. GTP replaces the GDP bound to the α-subunit of G-Protein. After this, GTP bound α-subunit of G-Protein and βγ-subunits dissociate and activate various effectors, which subsequently activate second messengers, which in turn activate the kinases, leading to the activation of downstream signalling pathways [8,9].

1.4 G Protein Coupled Receptor Regulation

1.4.1 Desensitization of GPCRs

In order to prevent the continuous signalling transduction and overstimulation of GPCRs, the GPCR signalling is regulated at two stages. The GPCR signalling can be regulated at the level of G-Protein, which involves replacing the GTP of the α-subunit of the G-Protein with the GDP. GTPase proteins known as RGS (Regulator of G-Protein Signalling) hydrolyze the α-subunit of G protein and thereby regulate the signalling. Another level at which the signalling via GPCR can be regulated is at the level of the receptor, which is referred to as the desensitization of the GPCRs. Desensitization of GPCR is a very common process which helps in the termination of the downstream signalling mediated by GPCRs. Desensitization of GPCRs lead to the inhibition of production of second messengers and hence terminates the GPCR signalling. The desensitization of receptors generally happens via two ways. First, Desensitization of GPCRs happens when G-Proteins are functionally uncoupled from the GPCRs. The second way in which desensitization occurs involves the internalization of the GPCRs into various intracellular compartments of the cell so that the ligands don't bind to the receptors and thereby, preventing the downstream signalling mediated through the receptors [8,9,10].

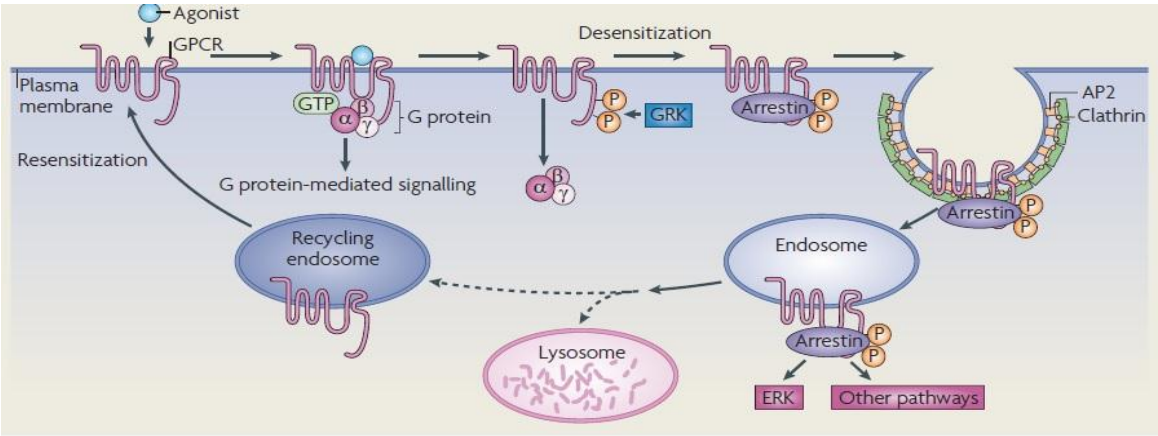


Fig1.1: Schematic depicting the desensitization, internalization and trafficking of G-Protein Coupled Receptors

(Source : *Stefanie L. Ritter and Randy A. Hall (2009). Fine tuning of GPCR activity by receptor interacting proteins. Nature Reviews Mol. Cell Bio* doi:10.1038/nrm2803)

One of the major ways in which GPCRs undergo desensitization is the activation – dependent desensitization, also known as homologous desensitization. When a GPCR is active, it not only activates the G-Proteins but also activates the G Protein Coupled Receptor Kinase (GRK). When GRK is activated, it specifically phosphorylates the activated GPCR. β-Arrestins recognise activated GPCRs which are in phosphorylated state. Subsequently, β-Arrestins associate with the activated GPCRs which are phosphorylated. β-Arrestins bind to the protein AP2 which acts as an adaptor between clathrin and β-Arrestin. β-Arrestins bind to clathrin as well. This binding of β-Arrestins with the phosphorylated GPCR, AP2 and Clathrin direct the GPCRs into the clathrin coated pits, leading to the subsequent internalization of the desensitized receptors [9].

Another way in which the GPCRs are desensitized is the heterologous desensitization. In heterologous desensitization, the desensitization is independent of the activation of GPCRs. The desensitization might be mediated by the phosphorylation of the GPCRs by kinases which are activated by second messengers or through various other kinsases, which act as negative feedbacks. Various kinases such as PKA, PKC, MAPK, etc also phosphorylate the GPCRs and lead to their desensitization. Other mechanisms involved in the desensitization of the GPCRs include reducing the responsiveness and reduction in the signalling efficiency of GPCR by negatively regulating the effectors and second messengers involved in the GPCR signalling by phosphorylating or chemically modifying them. Many GPCRs also undergo desensitization which doesn't involve the phosphorylation of the receptors [9,10].

1.4.2 Internalization of GPCRs

One of the crucial mechanisms of the desensitization and the regulation of the activity of GPCRs involve the subsequent endocytosis of the GPCRs. Once a GPCR is phosphorylated by GRK, the phosphorylated receptor is recognised by β-Arrestins. β-Arrestins associate with the phosphorylated receptors; and bind to AP2 (an adaptor protein which acts as an adaptor between β-Arrestin and Clathrin) and Clathrin. The association of β-Arrestins with the receptor target the desensitized receptors to clathrin coated pits and the receptors are subsequently internalized [9]. However, for many

GPCRs the internalization mechanisms have been found to be different than the one described above.

Following endocytosis of the receptors, they can have various fates depending on the type of the cell, type of receptor and the type of the ligand. A receptor, subsequent to its endocytosis, may be trafficked to the endosomes and recycling compartments. In this case, the receptors get recycled back to the cell surface. In endosome, the pH of the endosomal compartment changes and the endocytosed GPCRs are dephosphorylated. The dephosphorylated receptors then recycle back to the surface. GPCR receptors may also follow an alternative fate in which they are trafficked to the lysosome from early endosome. In lysosome, where the pH is acidic, the endocytosed receptors are degraded by various hydrolytic enzymes [8,9,10].

The GPCRs are not only internalized after agonist induced activation. The GPCRs also get endocytosed constitutively, which means that these receptors get internalized even without binding to the agonist. Constitutive endocytosis plays a major role in the maintenance of cell homeostasis. It is the mechanism by which the damaged receptors or the receptors with changed conformation are endocytosed, thereby maintaining a homeostasis. Constitutive endocytosis of the receptors keeps on happening at all the times in a cell, but the rate of constitutive endocytosis is very low as compared to agonist-dependent endocytosis [9].

1.5 Group 1 Metabotropic Glutamate Receptors

Metabotropic Glutamate Receptor, depending on their structural, pharmacological and the second messenger pathways activated, have been categorised into three groups – Group I mGluRs (mGluR1 and mGluR5), Group II mGluRs (mGluR2 and mGluR3) and Group III mGluRs (mGluR4, mGluR6, mGluR7, mGluR8).

Group 1 mGluRs consist of mGluR1 and mGluR5. They are mostly located on the post-synaptic neurons. The group1 mGluRs are linked to $G\alpha_{q/11}$ heterotrimeric G Protein. Upon the activation of Group I mGluR, it activates Phospholipase C, which leads to the production of IP3 and DAG. Generation of IP3 leads to the opening of Ca^{2+} ion channels

from ER, whereas DAG activates PKC and other signalling cascades. Thus, IP3 and DAG activate various downstream signalling and elicit intracellular responses in the post synaptic neuron [2, 3, 5, 6, 7].

Both the types of Group I metabotropic glutamate receptors – mGluR1 and mGluR5 – are differentially distributed in the brain. mGluR1 is strongly expressed in the Purkinje cells of cerebellar cortex, neurons of olfactory bulb and thalamus. The expression of mGluR5 is abundant in the hippocampus, cerebral cortex, nucleus accumbens and striatum. Group1 mGluRs have various roles in the central nervous system such as excitatory neurotransmission, synaptic plasticity and development. They have also been implicated in various diseases of the Central nervous system [3, 4, 5, 6].

1.6 Role of GroupI mGluR in synaptic plasticity

Group I mGluR mediated signalling plays a major role in synaptic plasticity in the central nervous system. mGluR signalling can regulate the synaptic strength of a synapse by regulating the number of NMDA and AMPA receptors present on the surface of the post-synaptic neuron. Group I mGluRs affect the synaptic plasticity by mediating both Long Term Potentiation (LTP) and Long Term Depression (LTD). The detailed molecular mechanisms of Group1 mGluR mediated LTD, both in cerebellum and in hippocampus, have been established. The activation of Group1 mGluR activates PLCβ and leads to the generation of IP3. IP3 opens the Calcium ion channels from the intracellular stores and increases the concentration of Ca^{2+}. The increase in the concentration of Ca^{2+} activate various signalling pathways, ultimately leading to the endocytosis and long term reduction in the number of AMPA Receptors [12]. The LTD mediated by GroupI mGluR in the hippocampus is independent of the activation of PLCβ and the subsequent increase in the concentration of Ca^{2+}. The activation of Group I mGluR leads to the rapid synthesis of cytoskeletal associated Arc protein, which play a crucial role in the endocytosis of the AMPARs and regulate the number of AMPA Receptors on the post synaptic neuron membrane. Arc is a cytoskeletal associated protein which also binds with other key proteins involved in the endocytosis of AMPARs, such as Dynamin2 and Endophilin [13]. The other proteins which are rapidly synthesised following the activation of GroupI

mGluR are STEP and MAP1b. These proteins downregulate the number of AMPA Receptors in the post synaptic neuron surface and therefore, mediate Long Term Depression [12, 13, 14].

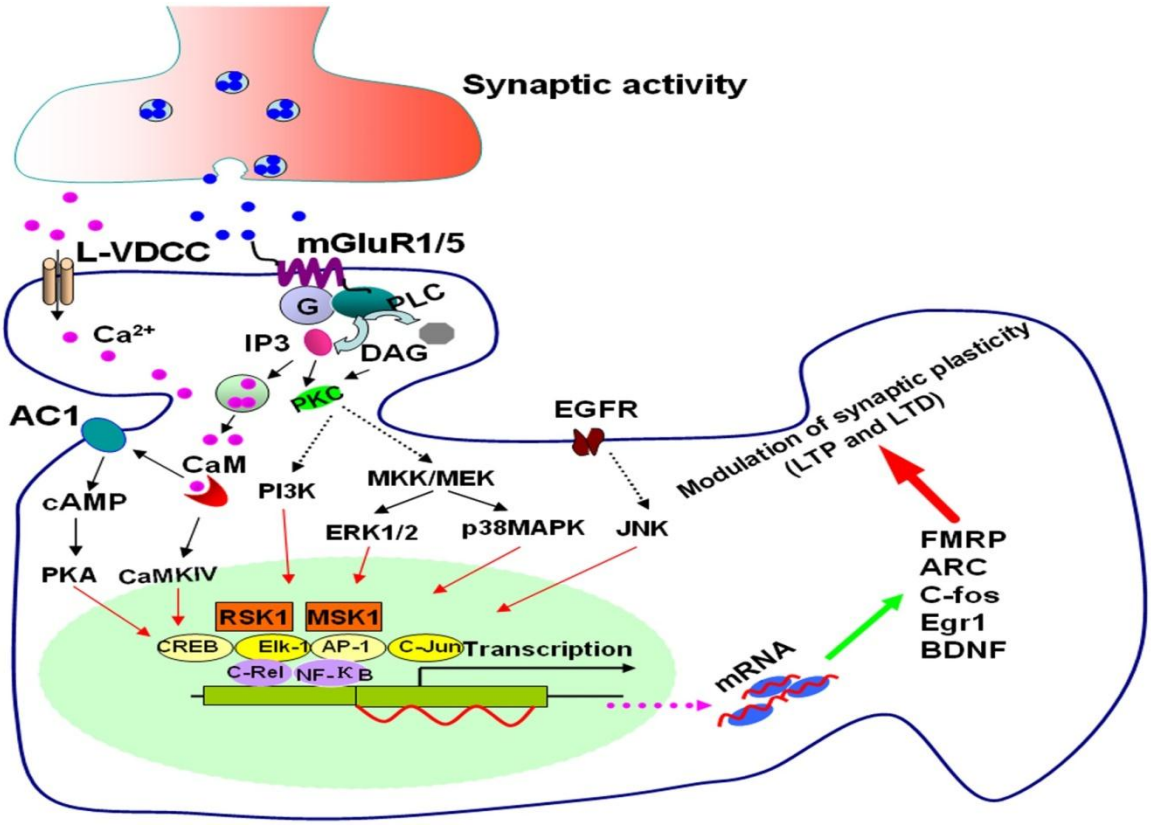


Figure 1.2 – The schematic showing the various pathways involved in the regulation of multiple synaptic activities activated by Group I mGluR

(Source : Min Zhuo et.al, Frontiers in Pharmacology (http://journal.frontiersin.org/ Journal/10.3389/fphar.2012.00189/full)

1.7 Role of mGluR5

mGluR5 is a groupI metabotropic glutamate receptor, which is strongly expressed in the hippocampus, cerebral cortex, nucleus accumbens and striatum. Hippocampus is one of the most important areas in the memory formation. mGluR5 plays a major role in mediating synaptic plasticity in the hippocampus. The mechanism of mGluR5-mediated

LTD in hippocampus depends on the translational regulation of various proteins such as Arc, STEP and MAP1b which are involved in the endocytosis and regulation of the number of AMPA receptors on the post synaptic neuronal surface [12, 13, 14]. Apart from learning and memory, mGluR5 has also been implicated in various pathological conditions such as Fragile X Syndrome Mental Retardation, Goal-directed learning disorders and addiction to narcotic drugs [12].

Fragile X Syndrome Mental Retardation is caused due to excessive increase in mGluR5 mediated glutamate signalling at the synapses, which leads to enhanced Long Term Depression. In this condition, the *fmr1* gene, which codes for Fragile X Mental Retardation Protein (FMRP), gets silenced. When mGluR5 is activated, it activates the transcription of various mRNAs which encode crucial proteins involved in the endocytosis of AMPARs. FMRP regulates the transcription of these mRNAs. When FMRP protein is not produced, it leads to the excessive endocytosis of AMPARs. Understanding the detailed molecular mechanism of mGluR5-mediated LTD will help in providing a potential cure for the disease by targeting the receptor [12].

1.8 GroupI mGluR Receptor Regulation

1.8.1 Desensitization

The Group I mGluR mediated signaling is regulated at various levels, such as the functional uncoupling of G Proteins with the mGluRs and the desensitization followed by endocytosis of the mGluR itself, similar to typical GPCRs. The activity of Group I mGluR signalling is attenuated by the hydrolysis of the GTP bound to the G-Protein to GDP. RGS2 plays a role in the attenuation of mGluR1 signalling while RGS4 plays a role in the attenuation of mGluR5 signalling.

Similar to the desensitization of canonical GPCRs, mGluRs also undergo both homologous desensitization (agonist-dependent) and heterologous desensitization (agonist-independent). The homologous desensitization of GroupI mGluRs involve the phosphorylation of mGluRs by GRKs, followed by association of β-Arrestin and endocytosis via clathrin coated pits. The heterologous desensitization of GroupI mGluRs

involves various mechanisms, dependent and independent of receptor phosphorylation by PKC. Other kinases involved in the heterologous desensitization of mGluR1 and mGluR5 involve kinases activated by second messenger pathways such as PKA, Calmodulin Kinase and optineurin. GroupI mGluRs also undergo a phosphorylation-independent desensitization [15, 16, 17].

1.8.2 Endocytosis and Trafficking

Group I mGluRs are internalized after exposure to agonist. There are multiple mechanisms of Group I mGluR internalization, depending on various factors such as the type of GroupI mGluR (mGluR1 or mGluR5), size of the Group I mGluR splice variant and the trigger for the receptor internalization. In case of internalization triggered by homologous desensitization, the activation of mGluR1 activates GRK mediated phosphorylation of the receptor and increases the association of β-Arrestin with the receptors, leading to rapid endocytosis of the receptors. When internalization is triggered by heterologous desensitization, the internalization is mediated by the phosphorylation of the Group I mGluRs by various other kinases such as PKC, CAM Kinase and other kinases activated by the second messenger pathways. GroupI mGluRs also internalize via the carbachol induced endocytosis and lipid raft mediated endocytosis. Following ligand-dependent endocytosis, mGluR1 is trafficked to the recycling endosome where the receptor is dephosphorylated and recycled back to the surface. [20]

1.8.3 Constitutive Endocytosis

The Group I mGluRs (mGluR1 and mGluR5) also undergo internalization without being activated by their agonists, known as constitutive endocytosis. The receptors undergo constitutive endocytosis in order to maintain the homeostasis of the cell. Although the detailed mechanism of constitutive endocytosis of GroupI mGluRs is not completely known, but initial studies have established that the constitutive internalization of mGluR1 and mGluR5 receptors have certain differences [15, 17].

One of the mechanisms by which Group I mGluRs undergo constitutive endocytosis involves a Ral/Phospholipase D/RalGDS (Ral Guanine Nucleotide Dissociation Stimulator), in which the Ral/PLD2 act as adaptors. But the detailed mechanism of the internalization is not yet known. Bhattacharya et.al have also established that the constitutive endocytosis of mGluR5 is β-arrestin/Clathrin independent. Instead, mGluR5 internalizes via the Ral/PhsophilipaseD/RalGDS pathways, the details of which is not known. The factors that lead to the recruitment of the Ral/PhsophilipaseD/RalGDS complex and the downstream mechanisms after the assembly of these proteins which lead to endocytosis are also completely unknown. The figure below describes the current proposed model for the constitutive endocytosis of the Group I mGluRs [17, 18].

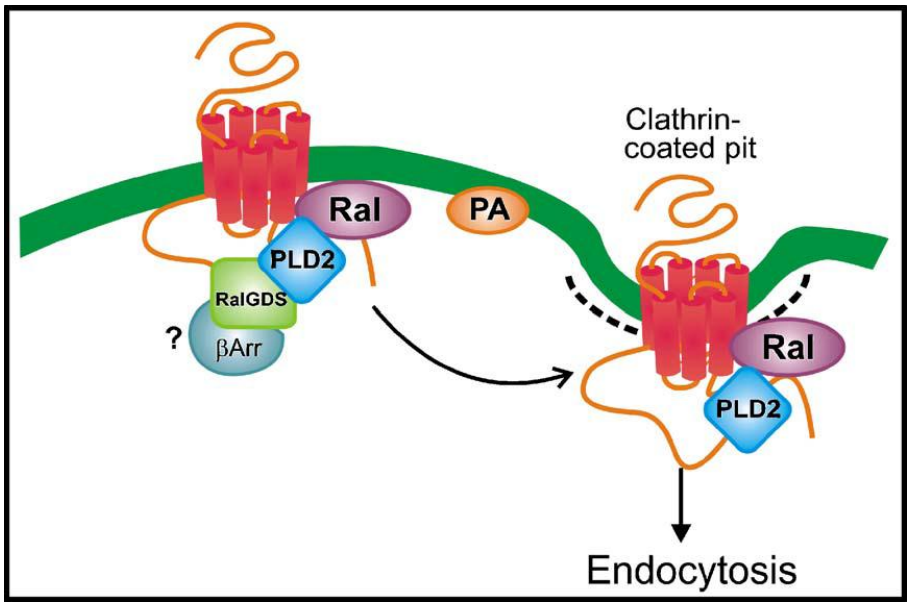


Fig 1.3 : Ral/PLD2 mediated constitutive endocytosis of Group I mGluRs. The groupI mGluRs undergo endocytosis mediated by Ral/PLD2, in which Ral/PLD2 act as adaptors. For the activity of Ral/PLD2 complex, PA (Phosphatidic Acid) formation is required.

(Source : *Gurpreet K. Dhami and Stephen S.G.Fergusson (2006) Regulation of metabotropic Glutamate receptor signalling, desensitization and endocytosis Pharmacology & Therapeutics 111 (2006) 260–271*)

1.9 Objective of the Study

GroupI mGluRs play a major role in synaptic signalling and modulation of synaptic plasticity. mGluR5 is a key player involved in the Long Term Depression (LTD) in the hippocampus. As stated before, apart from mediating signalling and plasticity, mGluR5 is also implicated in various neurological disorders such as Fragile X Mental Retardation Syndrome, addiction to narcotic drugs and goal-directed learning disorders. Understanding the mechanisms of desensitization, internalization and trafficking of mGluR5 is crucial to understand the behaviour of this receptor in the central nervous system which in turn will help us to unravel the role of this receptor in synaptic plasticity and various other neuropsychiatric disorders. Despite GroupI mGluRs being a crucial player in synaptic plasticity and various neuropsychiatric disorders, the mechanisms of their endocytosis, localisation and trafficking – both ligand mediated as well as constitutive – is not known in detail. Previous studies done by Trivedi R. et al have shown that the mGluR5 upon constitutive endocytosis, goes to the recycling compartment, from where the receptors recycle back to the surface in 3.5 hours in HEK293 cells. Another set of studies have shown that the Kinases are involved in the ligand mediated endocytosis of mGluR5b and the subsequent recycling of the mGluR5b back to the surface is dependent on Phosphatases (Mahato et.al, unpublished results). The aim of the study is to understand the mechanisms of constitutive endocytosis and trafficking, and to investigate whether the protein kinases and phosphatases play a role in the process.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell Lines

The experiments were performed on HEK293 (Human Embryonic Kidney) Cells and Neuro-2A cell lines, which were purchased from National Centre for Cell Sciences Pune (India) and stored in liquid nitrogen at -196°C.

2.1.2 Plasmids and Constructs

The myc-mGluR5b construct was used for the studies. myc was tagged at the N-terminus of full length cDNA of mGluR5b in pRK Plasmid. The mGluR5b construct used for the studies was gifted by Dr. Katherine Roche, NIH, USA.

2.1.3 Reagents and Chemicals

Antibiotic – Antimycotic Mix, DPBS, Dulbecco's Modified Eagle Medium, MEM, Lipofectamine, Opti-MEM, 0.05% Trypsin-EDTA, Distilled Water, FBS (Fetal Bovine Serum) were purchased from Invitrogen, USA. Ampicillin (Na^+ Salt) was purchased from HiMedia®, India. CalphostinC, Endothall, Fluoromount™, H89, PFA, Polylysine were brought from Sigma, USA. R,S-3,5-DHPG was purchased from Tocris Bioscience, UK. Normal Goat Serum was purchased from GeNei™, India. Anti-myc mouse monoclonal antibody ab18185 was purchased from abcam®, UK. Secondary antibodies goat-anti mouse Alexa-568 and goat-anti mouse Alexa-647 were purchased from Life Technologies™, USA. Plasmid isolation Mini Kit and Plasmid Isolation Midi Kit were purchased from Qiagen® Gmbh, Germany.

2.1.4. Tissue Culture Wares

100 mm tissue culture dishes were purchased from Thermo Scientific®. 24 well tissue culture plates, 15 mL conical tubes, 50 mL conical tubes, serological pipettes of 5 mL and 10 mL were purchased from Falcon®, USA and Tarsons®, India. Coverslips were from Fisher Scientific, USA and Glass slides from Globe Scientific, US.

2.2 Methods

2.2.1 Cell Culture and Transfection

Both HEK293 and Neuro2A cells were maintained in 100 mm cell culture dish in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Foetal Bovine Serum and Antibiotic – Antimycotic mix at 37°C and 5% CO_2. The cells were plated in 24 well plates, on coverslips coated with 50 µg/ml poly-D-lysine. The cells were transfected at the confluency of 60-80%. 2 µg of DNA was dissolved in 200 µL of Plain DMEM. 10 µL of Lipofectamine was dissolved in 200 µL of Plain DMEM. Both the solutions were mixed and incubated at 37°Cfor 30 minutes, following which 600 µL of plain DMEM was added to make the final volume of the transfection mix to 1 mL. The cells were incubated in the transfection mix at 37°C, 5% CO_2. The incubation time was 3.5 hours in case of Neuro2A cells and 4.5-5 hours in case of HEK293 cells. The expression was observed after 24-30 hours in HEK293 cells. In Neuro2A cells, the DMEM (with 10% FBS) was removed after 24 hours and DMEM with 1 % FBS was added to induce differentiation. The cells were incubated for further 24 hours to differentiate. Expression was observed subsequently.

2.2.2 DHPG mediated Endocytosis assay in Neuro2A cells

Neuro2A cells were grown on coverslips and transfected using the method described above. The experiments were performed after differentiation of the cells. The cells were incubated in blocking solution 2% NGS (in Plain DMEM) for 15 minutes and then incubated with anti-myc mouse monoclonal antibody (1:500) at 37°C for 15 minutes. The cells were treated with a solution containing 100µM DHPG in plain DMEM for 5 minutes at 37°C. The cells were washed twice to remove DHPG and chased for 25 more minutes. This was followed by fixation of cells (without permeabilization) with chilled 4% PFA for 15 minutes on ice. Three washes of PBS were given. In order to observe the surface receptors, the cells were incubated in saturating concentration of goat anti-mouse Alexa-568 (1:100) secondary antibody in PBS for 1 hour at 37 °C. The cells were then permeabilized with 0.1% Triton-X for 40 minutes at room temperature. Three washes of PBS were given and the cells were incubated in the second secondary antibody goat anti-

mouse Alexa-647 (1:800) at 37°C for 1 hour in order to observe the receptors which have endocytosed. The cells were given three washes of PBS and the coverslips were mounted on glass slides using Fluoromount™. The imaging was done in the Zeiss inverted fluorescence microscope (Axio Observer Z1).

2.2.3 Constitutive Endocytosis Assay in HEK293 Cells

HEK293 cells were grown on coverslips and transfected with mGluR5b using the method as discussed in section 2.2.1 and the experiments were performed after 24 hours. The old media was removed and the cells were incubated in Plain DMEM for 30 minutes. The 24 well plate containing the cells were transferred on ice and incubated in 2% Normal Goat Serum (in Plain DMEM) on ice for 5 minutes. The cells were then incubated with anti-myc mouse monoclonal antibody (1:500) for 1 hour on ice. The cells were incubated at 37°C for the 30 minute in plain DMEM. While the cells in control condition were fixed (without permeabilization) with chilled 4% PFA for 15 minutes on ice right after incubation, the cells with 30 minute time-point were fixed after 30 minutes. After this, the cells were given three washes of PBS and incubated in saturating concentration of goat anti-mouse Alexa-568 (1:100) secondary antibody for 1 hour at 37°C. To visualise the internalized receptors, the cells were permeabilized by 0.1% Triton-X for 40 minutes at room temperature and subsequently incubated in second secondary antibody, viz., goat anti-mouse Alexa-647 for 1 hour at 37°C. Three washes of PBS were given to the cells. The coverslips were then mounted on glass slides using Fluoromount™. The imaging was done in the Zeiss inverted fluorescence microscope (Axio Observer Z1). In case of the experiments where drugs were used to study the role of Kinases in constitutive endocytosis, the drugs were applied 30 minutes before the incubation in primary antibody and the drugs were present throughout the course of experiments. The drugs used were 500nM CalphostinC (inhibits Protein Kinase C) and 10µM H89 (inhibits Protein KinaseA).

2.2.4 Trafficking Assay in HEK293 cells

HEK293 cells were transfected with mGluR5b as described in the section 2.2.1. The constitutive endocytosis in HEK293 cells was induced as described in the endocytosis assay in the above section (section 2.2.3). Since it has already been established that the mGluR5b recycles back to the surface in 3.5 hours,(Constitutive internalization and recycling of metabotropic glutamate receptor5, Rishi Raj Trivedi and Samarjit Bhattacharyya, *Biochem. Biophys. Res. Commun. 427, 185–190.),* the cells were chased for an additional time-point of 3.5 hours. The cells were incubated in primary antibody, fixed, incubated in Alexa-568 secondary antibody to label the surface receptors, permeabilised by Triton-X 100, incubated in Alexa-647 conjugated secondary antibody to label the internalized receptors and the coverslips were mounted using Fluoromount™ as described in the previous section (Section 2.2.3). In the experiments which included studying the effects of drugs which inhibit Protein Phosphatases during recycling, the drugs were applied 30 minutes before the incubation in primary antibody. The drugs were present throughout the course of the experiments. The drugs used were 50µM endothall (inhibitor of both PP2A and PP1), 100nM Okadaic Acid (inhibitor of both PP2A and PP1), 5nM Okadaic Acid (inhibitor of only PP2A, not PP1), 1µM CyclosporinA and 1µM FK506 (inhibitors of PP2B).

2.2.5 Fluorescence Microscopy

Imaging was performed in the inverted fluorescent microscope (Zeiss Axio Observer Z1) using a 63X oil immersion objective. Digital parameters were set at the beginning of an experiment and the same values were used for the entire experiment. The excitation and emission for Alexa-568 and Alexa-647 were done using the appropriate filter sets in the microscope.

2.2.6 Image Analysis

ImageJ, a public-domain software developed by the National Institutes of Health, was used to analyze the images procured from the fluorescence microscope. All the images

were first converted from 16-bit type to 8-bit type. The images for each channel (for Alexa-568 and Alexa-647) were stacked together. For a particular fluorophore, a particular threshold was set, which was constant for the entire experiment. After thresholding, the thresholded area for a given fluorophore – Alexa-568 or Alexa-647 – was measured. **Internalization index** (also known as **endocytosis index**), defined as the ratio of internalized receptors and the total number (internalized + surface) of receptors, was calculated. The data was normalised with their respective controls. The representative images were produced by adjusting the image parameters identically for all images in a particular experiment in the adobe photoshop software.

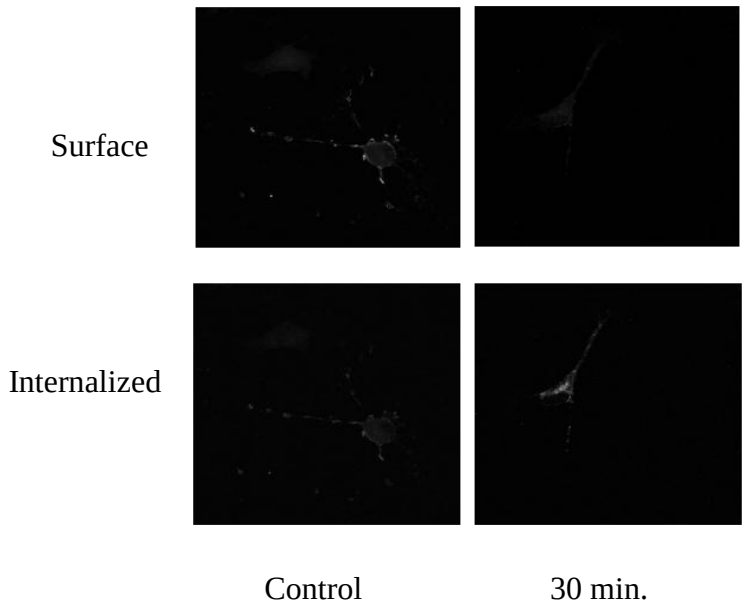
$$Internalization\ Index = \frac{Internalized\ receptors}{Internalized\ receptors + Surface\ receptors}$$

Chapter 3 : Results

3.1 Ligand mediated endocytosis of mGluR5b in Neuro2A cells

Neuro2A cell line, a neuroblastoma cell line from mouse, was used to study the ligand mediated endocytosis of mGluR5b. The Neuro2A cells were transfected with myc-mGluR5b and then the ligand mediated endocytosis assay was performed as described previously. Mouse monoclonal antibody against myc was used as the primary antibody. The secondary antibody tagged with fluorophore Alexa-568 was used which stained all the receptors on the surface. After permeabilization with Triton-X 100, the cells were incubated with second secondary antibody tagged with the fluorophore Alexa-647, which stained all the internalized receptors. After 30 minutes of incubation at 37°C, the cells showed more number of internal punctae due to the internalization of the receptors. The quantitation of the mGluR5b in this endocytosis assay performed in Neuro2A cells showed that the cells at 30 minutes showed significant endocytosis than control. The Endocytosis index or the internalization index, as defined previously was **2.45 ± 0.35** for the cells at 30 minutes, while for the control, the internalization index was **1 ± 0.15 (Fig. 3.1).** This result suggests that upon exposure to the ligand DHPG, the metabotropic Glutamate Receptor 5 gets endocytosed in 30 minutes and the assay can be used to study the ligand mediated endocytosis of mGluR5b in this cell line.

A



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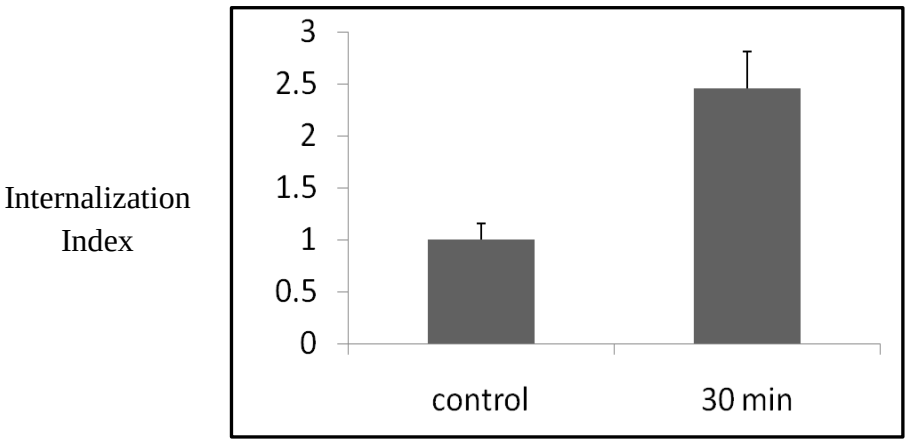


Figure 3.1 – Ligand mediated endocytosis of mGluR5b in Neuro2A cells

A)In control condition, the surface fluorescence was much higher than the internal fluorescence as most of the receptors were on the surface. After the incubation of cells with the ligand, the internal fluorescence was very high at 30 min while the surface fluorescence was low due to the endocytosis of the mGluR5b receptors.

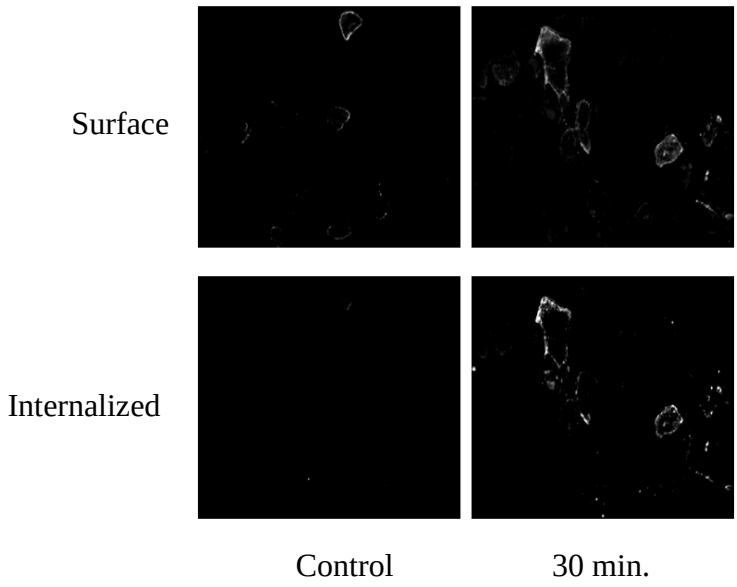
B)Quantitation of the endocytosis of mGluR5b in the ligand mediated endocytosis assay showed significant endocytosis (**2.45±0.35)** as compared to the control (1±0.15).

3.2 Constitutive endocytosis of mGluR5b in HEK293 cells

As discussed in section 1.8.3, the Group I mGluRs (mGluR1 and mGluR5) also undergo endocytosis without being activated by their agonists, the process known as constitutive endocytosis. The mechanism of constitutive endocytosis and subsequent trafficking of mGluR5 is not known in details. Previous studies have established that the constitutive endocytosis of mGluR5b in HEK293 cells as well as in primary neurons is independent of Arrestin and Clathrin (Bhattacharya, M. et.al.), but the detailed mechanism is not known.

To study the constitutive endocytosis of mGluR5b in HEK293 cells, the myc-mGluR5b construct was used. HEK293 cells were transfected with myc-mGluR5b and the constitutive endocytosis assay was performed as described previously. Mouse monoclonal antibody against myc was used as the primary antibody. The secondary antibody tagged with fluorophore Alexa-568 was used which stained all the receptors on the surface. After permeabilization with Triton-X 100, the cells were incubated with second secondary antibody tagged with the fluorophore Alexa-647, which stained the internalized receptors. After 30 minutes of incubation at 37°C, the cells showed more number of internal punctae due to the internalization of the receptors. Quantitation of mGluR5b receptors subsequent to the constitutive internalization showed that the internalization index for the cells at 30 minutes was **1.47±0.16** as compared to the control cells which had an internalization index of **1±0.11 (Fig. 3.2).** This result suggests that the mGluR5b gets constitutively endocytosed in 30 minutes in HEK293 cells and this assay can be performed to study the trafficking of mGluR5b upon constitutive endocytosis.

A)



B)

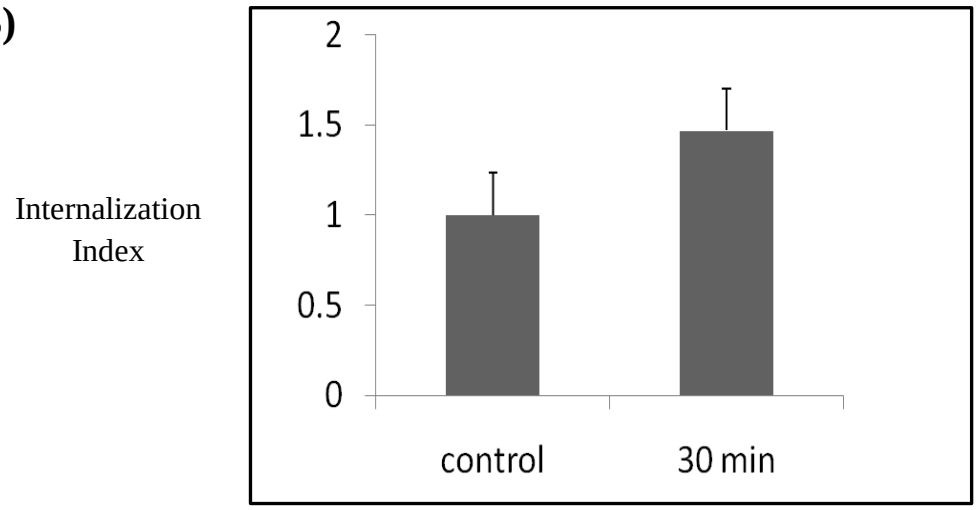
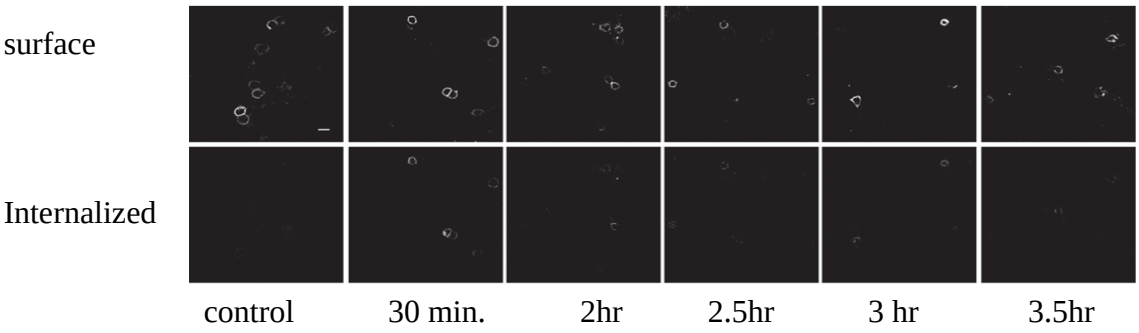


Figure 3.2 – Constitutive endocytosis of mGluR5b in HEK293 cells

A) In control condition, the surface fluorescence was much higher than the internal fluorescence, which is negligible, as most of the receptors were on the surface. After the incubation of cells for 30 minutes, the internal fluorescence went up while the surface fluorescence went down due to the constitutive endocytosis of the mGluR5b receptors.

B) Quantitation of mGluR5b constitutive endocytosis showed significant endocytosis of the receptor (**1.47±0.16)** at 30 minutes as compared to the control **(1±0.11).**

A



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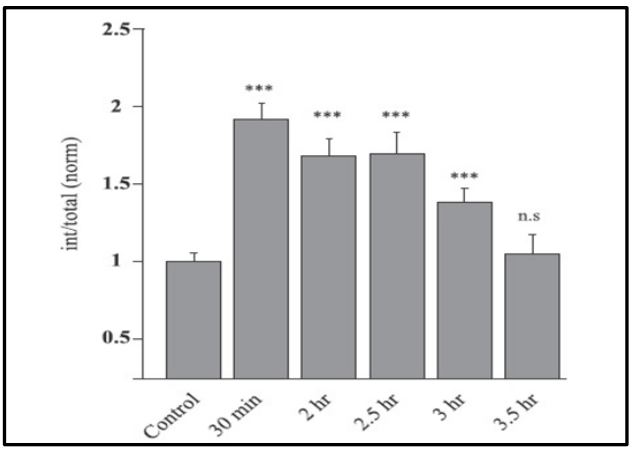


Figure 3.3 – Recycling of mGluR5b after constitutive endocytosis in HEK293 cells

A) The mGluR5b receptors, subsequent to the constitutive endocytosis, recycle back to cell surface. The internal fluorescence is highest at 30 minutes, which decreases as time progresses and at 3.5 hour condition, the surface fluorescence is higher and the internal fluorescence is negligible, similar to the control condition. This suggests that mGluR5b recycles back to cell surface after constitutive endocytosis in 3.5 hour.

B) Quantitation of mGluR5b also suggests the recycling of mGluR5 back to cell surface.

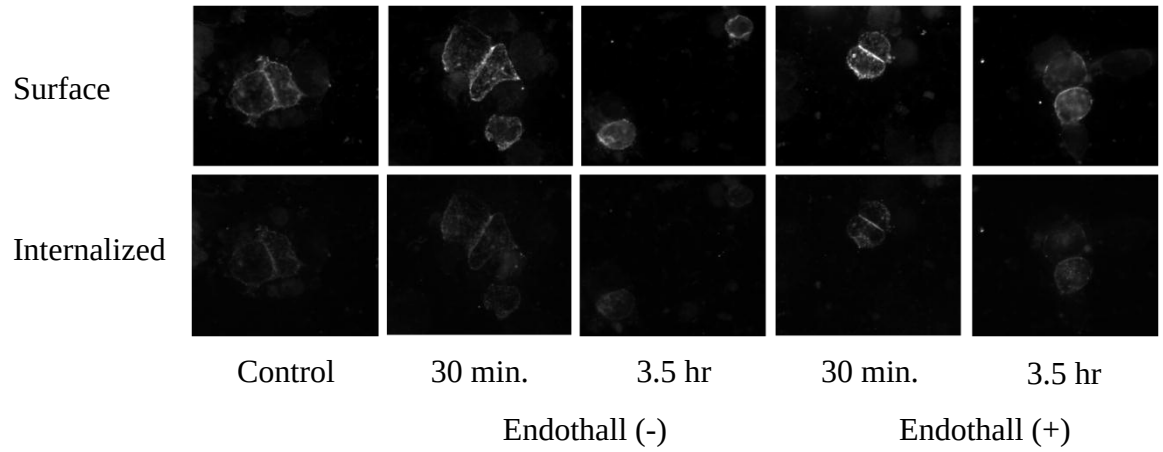
Source: Trivedi R. R. and Bhattacharyya S. (2012) Constitutive internalization and recycling of metabotropic glutamate receptor 5 (mGluR5). Biochem. Biophys. Res. Commun. 427, 185–190

3.3 Effect of Endothall (50 µM) on mGluR5b Trafficking (Constitutive) in HEK293 Cells

It has been established that the mGluR5b receptors, after constitutive endocytosis, recycle back to the surface (Trivedi et.al.). The receptors recycle back to the surface in 3.5 hours after constitutive endocytosis (Fig. 3.3) while it recycles back to surface in 2.5 hours in case of ligand-mediated endocytosis (Mahato et.al, unpublished results). The recycling of many GPCRs is dependent on Phosphatases which dephosphorylate the receptors before recycling. The role of phosphatases, if any, in the trafficking of mGluR5 after constitutive endocytosis is still not known. Mahato et.al (unpublished results) have shown that the recycling of mGluR5b subsequent to DHPG-mediated endocytosis is dependent on Protein phosphatases PP2A and PP2B. Whether these phosphatases are also involved in the recycling of mGluR5b subsequent to constitutive endocytosis is not known. The experiment aims at understanding the role of phosphatases in recycling of mGluR5 after constitutive endocytosis by using pharmacological inhibitors of PP1 and PP2A. The drug Endothall, at concentration of 50 µM, specifically inhibits both the protein phosphatases PP1 and PP2A. HEK293 cells were transfected with myc-mGluR5b. Recycling assay and immunocytochemistry were performed subsequently in presence of the drug as per the methods discussed in Chapter 2. Since it has been shown that the mGluR5b constitutively internalizes in 30 minutes and recycles back to cell surface in 3.5 hours, the time points chosen were control (0 min), 30 minutes and 3.5 hours.

Imaging and quantitation studies suggested that although Endothall (50 µM) didn't affect the endocytosis of the receptor since the endocytosis index at 30 minutes in case of cells treated with endothall (**1.44±0.08**) was similar to the endocytosis index of cells at 30 minute which were not treated with endothall (**1.30±0.11**). In case of 3.5 hour time point, the cells which were not treated with endothall showed normal recycling (**1.07±0.12**), while recycling was blocked at 3.5 hour in the cells treated with endothall (**1.54±0.23**) **(Fig. 3.4).** This result suggests that the protein phosphatases PP1 and PP2A are involved in the recycling of mGluR5b after constitutive endocytosis in HEK293 cells.

A



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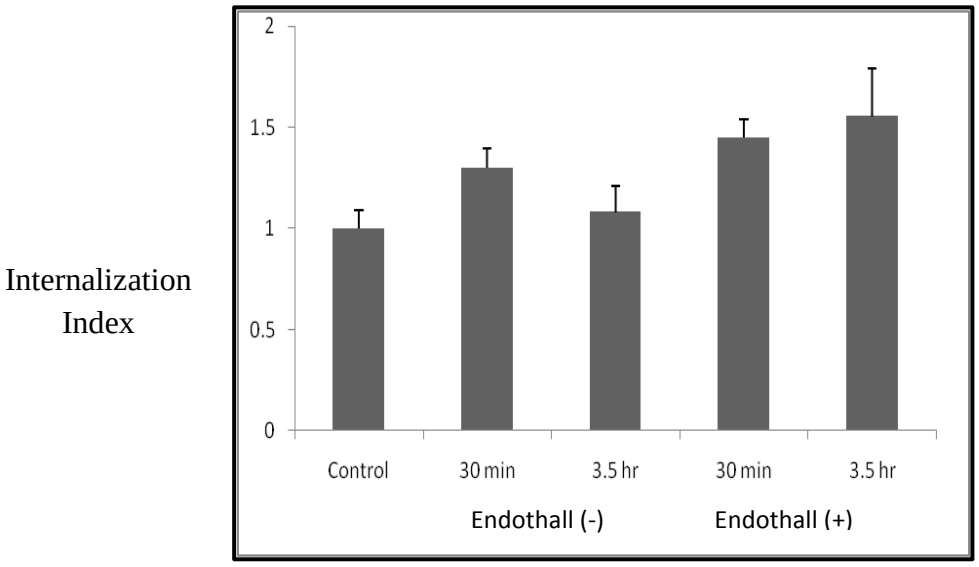


Figure 3.4 – Effect of Endothall (50µM) on mGluR5b trafficking (Constitutive) in HEK293 Cells

A) When the cells are treated with Endothall (50µM), an inhibitor of PP2A and PP1 for the entire period of the experiment, the internal punctae at 3.5 hr in the Endothall (+) cells is similar to the 30 min time point, suggesting that endothall blocks the recycling of mGluR5b subsequent to the constitutive endocytosis.

B) Quantitation of mGluR5b in the experiment at various time points also suggests that endothall prevents the recycling of mGluR5b back to the surface.

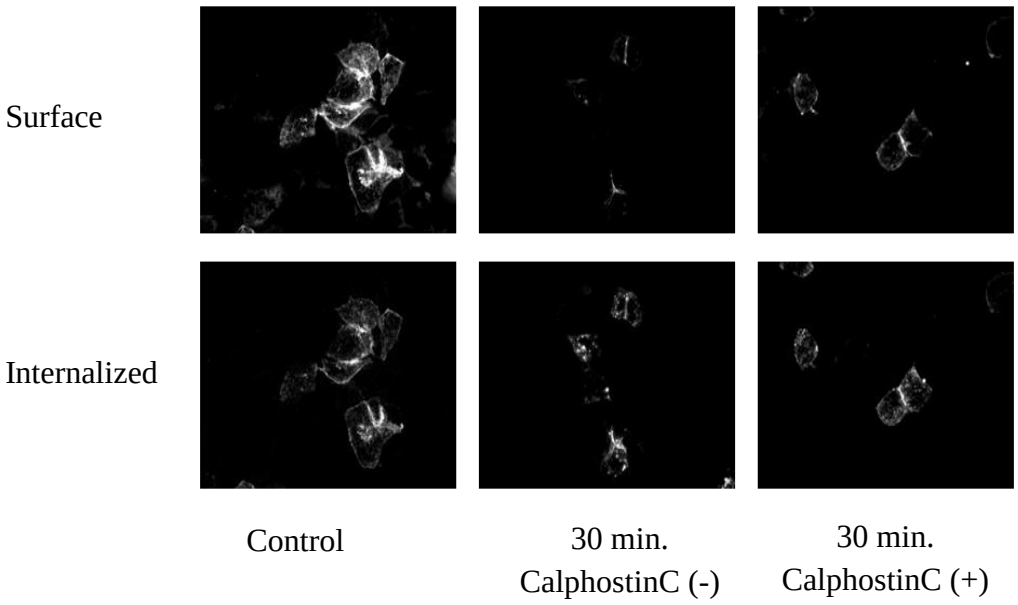
3.4 Effect of Calphostin C (500nM) on the constitutive endocytosis of mGluR5b in HEK293 Cells

Many GPCRs are desensitized by the phosphorylation of the receptors by GRK, PKC and other protein kinases activated by the various second messenger pathways. Subsequent to the phosphorylation, these G Protein Coupled Receptors recruit β-Arrestin and undergo internalization via clathrin mediated endocytosis. The endocytosis of mGluR5 after its activation by ligand follows the same mechanism, but the mechanism of constitutive endocytosis of mGluR5 is not known. Bhattacharya M. et al have found that the constitutive endocytosis of mGluR5 is independent of β-Arrestin and clathrin, but the role of kinases in constitutive endocytosis is not known. The previous experiment has suggested that phosphatases PP1 and PP2A are involved in the recycling of mGluR5 after constitutive endocytosis. So, it further needed to be investigated whether basal phosphorylation plays a role in the constitutive endocytosis of mGluR5 in HEK293 cells. To study the role of kinases in constitutive endocytosis of mGluR5, the major protein kinases PKA and PKC which are well established in the ligand mediated endocytosis of Group I mGluRs were pharmacologically inhibited and the effect on constitutive endocytosis was studied.

CalphostinC, at concentration of 500 nM, specifically inhibits Protein Kinase C (PKC). In order to study the role of PKC in the constitutive endocytosis of mGluR5b in HEK293 cells, the cells were transfected with myc-mGluR5b. Endocytosis assay and immunocytochemistry were performed in presence of the drug subsequently as per the methods discussed in Chapter 2. Since it has been shown that the mGluR5b constitutively internalizes in 30 minutes, the time points chosen were control (0 min) and 30 minutes.

Imaging and quantitation suggests that CalphostinC (500 nM) doesn't affect the constitutive endocytosis of the receptor. The endocytosis index at 30 minutes in case of cells treated with CalphostinC (**2.13±0.10**) was similar to the endocytosis index of cells at 30 minute which were not treated with CalphostinC (**2.28±0.14**) **(Fig. 3.5).** This result suggests that the protein kinase PKC is not involved in the constitutive endocytosis of mGluR5 in HEK293 cells.

A



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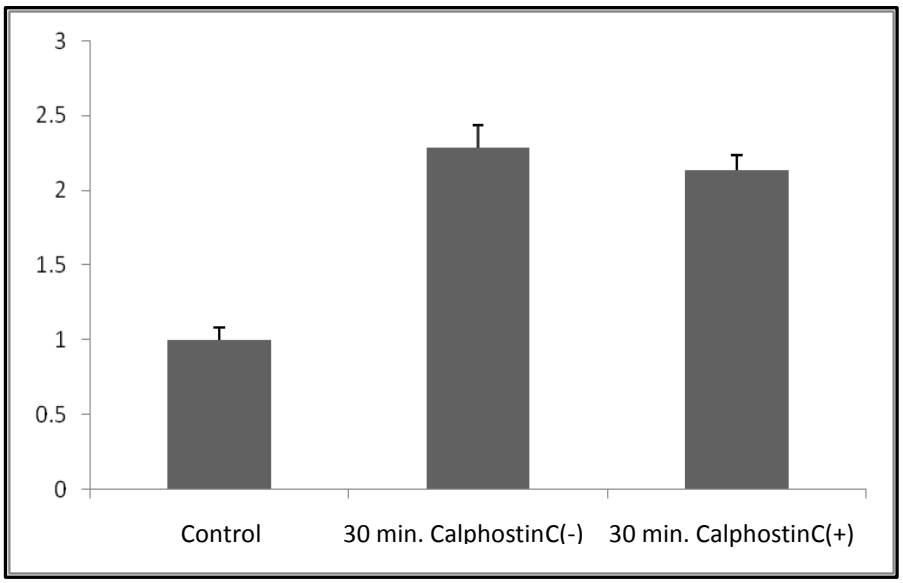

Figure 3.5 – Effect of Calphostin C (500nM) on the constitutive endocytosis of mGluR5b in HEK293 Cells

A) The cells at 30 minute which were not treated with CalphostinC show much greater internal fluorescence than surface. Also, the cells at 30 minute which were treated with CalphostinC, show high internal fluorescence suggesting that treating the cells with CalphostinC doesn't affect endocytosis of mGluR5b

B) Quantitation results also suggest that treating the cells with Calphostin C doesn't affect the endocytosis of mGluR5b. The endocytosis index at 30 minutes in the cells treated with CalphostinC is **2.13±0.10** while the endocytosis index at 30 minutes in cells which were not treated with CalphostinC is **2.28±0.14**

3.5 Effect of H89 (10 μM) on the constitutive endocytosis of mGluR5 in HEK293 Cells

Another key protein kinase which is involved in the ligand mediated endocytosis of some GPCRs and GroupI mGluR is PKA. The role of PKA in the constitutive endocytosis of GroupI mGluR and mGluR5 in particular is not known. In order to study whether PKA plays any role in the constitutive endocytosis of mGluR5b in HEK293 cells, the constitutive endocytosis was investigated by in presence of 10 μM H89 which specifically inhibits PKA.

H89, at concentration of 10 μM, specifically inhibits Protein Kinase A (PKA). In order to study the role of PKA in the constitutive endocytosis of mGluR5b in HEK293 cells, the cells were transfected with myc-mGluR5b. Endocytosis assay in presence of the drug and immunocytochemistry were performed subsequently as per the methods discussed in Chapter 2. Since it has been shown that the mGluR5b constitutively internalizes in 30 minutes, the time points chosen were control (0 min) and 30 minutes.

Imaging and quantitation suggests that inhibiting PKA doesn't affect the constitutive endocytosis. The endocytosis index at 30 minutes in case of cells treated with H89 (**2.18±0.13**) was similar to the endocytosis index of cells at 30 minute which were not treated with CalphostinC (**2.08±0.13**) (**Fig. 3.6**). This result suggests that the protein kinase PKA is not involved in the constitutive endocytosis of mGluR5 in HEK293 cells.

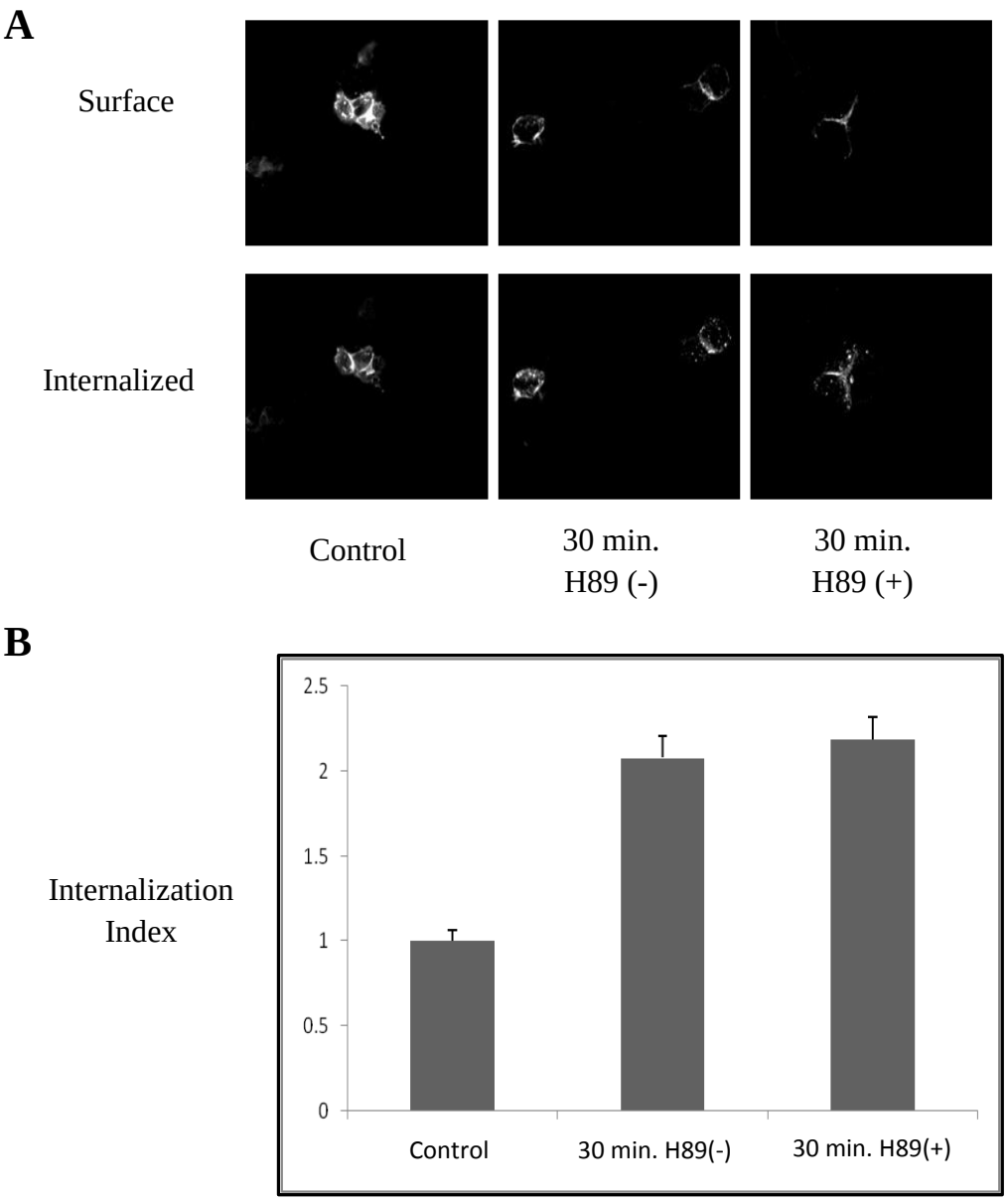

Figure 3.6 – Effect of H89 (10 µM) on the constitutive endocytosis of mGluR5 in HEK293 Cells

A) The cells at 30 minute which were not treated with H89 show greater internal fluorescence than surface. Also, the cells at 30 minute which were not treated with H89, show high internal fluorescence suggesting that treating the cells with H89 doesn't affect endocytosis of mGluR5

B) Quantitation results also suggest that treating the cells with H89 doesn't affect the endocytosis of mGluR5. The endocytosis index at 30 minutes in the cells treated with H89 is **2.18±0.13** while the same at 30 minutes in cells which were not treated by H89 is **2.08±0.13**

Chapter 4 : Discussions

Discussions

Group1 mGluRs undergo both ligand mediated as well as constitutive endocytosis. While the mechanisms of ligand mediated endocytosis and the subsequent recycling has been somewhat studied, very less is known about the mechanisms of constitutive endocytosis and subsequent recycling of mGluR5. In this project, I have investigated the role of Kinases and Phsophatases in constitutive endocytosis and in the subsequent recycling of the mGluR5b.. A previous study by Bhattacharya M. et al has established that the endocytosis of mGluR5 is independent of β-Arrestin and Clathrin; and instead, depends on a Ral/PhsophilipaseD/RalGDS machinery. In case of canonical GPCRs also, it is established that while ligand mediated endocytosis requires Kinases – either GRK or kinases activated by second messenger pathways – the constitutive endocytosis may or may not be dependent on the phosphorylation of the receptor by the kinases. In this project, it was found that inhibiting Protein Kinase C (PKC) by CalphostinC didn't affect the constitutive endocytosis of mGluR5b in HEK293 cells. Similarly, inhibition of Protein Kinase A (PKA) by H89 also didn't affect the endocytosis of mGluR5b in HEK293 cells.

It can be hypothesized that the mechanisms of ligand mediated endocytosis of mGluR5b and constitutive endocytosis of mGluR5b are different. There is a possibility that the constitutive endocytosis of mGluR5b is either independent of Kinases and depends on slow conformational and structural changes in the receptors or some other kinase(s) that we have not studied is involved in the constitutive endocytosis of the receptor. Another probable hypothesis that can be attributed to the slower timescale of the constitutive trafficking of mGluR5b might be that some kinases might be involved in the constitutive endocytosis, but the activity of Kinases in this case is extremely slow compared to the activity in ligand mediated endocytosis.

The studies on ligand mediated endocytosis and trafficking of mGluR5b in HEK293 as well as in neuronal cell lines have shown that subsequent to endocytosis, the receptors are recycled back to the surface. In this case also, the time taken for recycling back to the surface is faster in case of recycling after ligand mediated endocytosis as compared to the recycling after the constitutive endocytosis of mGluR5b. Also, the recycling of mGluR5b

back to the surface in case of ligand mediated endocytosis is dependent on protein phosphatases (Mahato et al, *unpublished results*). In this project, it was found that inhibiting protein phosphatases PP1 and PP2A by endothall prevented the recycling of mGluR5b after constitutive endocytosis in HEK293 cells.

Hence, based on the above results, it seems that the recycling of mGluR5b is dependent on Phosphatases both in the case of ligand mediated endocytosis as well as constitutive endocytosis. Since it has been established that the mGluR5 receptors go to recycling compartment after constitutive endocytosis, it can be hypothesised that the dephosphorylation of the mGluR5b in the endosome is essential for the recycling of the mGluR5 back to the surface. Therefore, it can be proposed that that the mechanisms of constitutive and ligand mediated endocytosis are different as the former is independent of PKC while the ligand mediated endocytosis is affected by the inhibition PKC. But once the receptor has been internalized, the mechanisms of recycling back to the surface might be same as Protein Phosphatases play a role in the recycling of mGluR5 in both the cases.

It would be interesting to study the role of various other kinases in the process of constitutive endocytosis of mGluR5. Studying whether phosphorylation of the receptor is mandatory for the constitutive endocytosis or does it happen independent of receptor phosphorylation is crucial in understanding the mechanism of constitutive endocytosis. As we know that the constitutive endocytosis of mGluR5 is independent of beta-arrestin and clathrin, understanding the key molecular players and the role of Ral/PLD2/RalGDS in the constitutive endocytosis will be crucial in deciphering the mechanisms of the constitutive endocytosis of mGluR5, about which not much is known yet.

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