# Role of ubiquitination and deubiquitination in the group I metabotropic glutamate receptor trafficking

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***A thesis submitted for the partial fulfillment of the degree of***

***Doctor of Philosophy***


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# Declaration

I declare that the thesis entitled "Role of ubiquitination and deubiquitination in the group I metabotropic glutamate receptor trafficking" presents the work done by me under the supervision of Dr. Samarjit Bhattacharyya at Indian Institute of Science Education and Research (IISER) Mohali. This work is original and has not been done earlier by anyone else. The results presented in this thesis have not been submitted previously to this or any other University for a Ph.D or any other degree.

**Signature of the supervisor** **Signature of the candidate**

**(Dr. Samarjit Bhattacharyya)** **(Ravinder Gulia)**

**Place:** **Place:**

**Date:** **Date:**

# Certificate

I certify that this thesis entitled “Role of ubiquitination and deubiquitination in the group I metabotropic glutamate receptor trafficking” comprises research work carried out by Ravinder Gulia under my supervision at Indian Institute of Science Education and Research (IISER) Mohali for the degree of Doctor of Philosophy. The results presented in this thesis have not been submitted previously to this or any other University for a Ph.D or any other degree.

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## Abbreviations

- **AMPAR:** α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid receptor
- **AP5:** D-(-)-2-Amino-5-phosphonopentanoic acid
- **BSA:** Bovine serum albumin
- **CNS:** Central nervous system
- **cAMP:** cyclic AMP
- **DHPG:** 2,4- bis dihydroxy phenyl glycine
- **DAG:** Diacylglycerol
- **DNQX:** 6,7-Dinitroquinoxaline-2,3-dione disodium salt
- **DMEM:** Dulbecco's modified eagle's medium
- **EDTA:** Ethylene diamine tetraacedic acid
- **FBS:** Fetal bovine serum
- **GRK:** G-protein coupled receptor kinase
- **$IP_3$**: Inositol 1,4,5-triphosphate
- **$PIP_2$:** Phosphatidylinositol 4,5-bisphosphate
- **mGluR:** Metabotropic glutamate receptor
- **NMDAR:** N-methyl-D-aspartate receptor
- **PBS:** Phosphate buffer saline
- **PKA:** Protein kinase A
- **PKC:** Protein kinase C
- **PLC:** Phospholipase C
- **PFA:** Paraformaldehyde
- **PCR:** Polymerase chain reaction
- **DIV:** Days *in vitro*
- **DUBs:** Deubiquitinating enzymes
- **USPs:** Ubiquitin-specific proteases

- **FUDR:** 5-Fluoro-2'-deoxyuridine (FUDR)
- **NEM:** N-Ethylmaleimide
- **PEI:** Polyethylenimine
- **PYR-41:** 4-[4-[(5-Nitro-2-furanyl)methylene]-3,5-dioxo-1-pyrazolidinyl]benzoic acid ethyl ester
- **DMSO:** Dimethyl sulfoxide
- **AB-AM mix:** antibiotic-antimycotic mix
- **LTP:** Long-term potentiation
- **LTD:** Long-term depression
- **NGS:** Normal goat serum
- **TTX:** Tetrodotoxin

*Chapter 1*

# Introduction

For multicellular organisms, to ensure that tissues, organs, and organ systems function appropriately, it is essential that the cells have the ability to sense the changes and respond to the external environment. Cells communicate with each other *via* chemical messengers released into the extracellular space. To perceive this change in the microenvironment and to respond correctly, cells have receptors either on the cell surface or inside the cell. Many important physiological processes are regulated by the coordinated actions of receptor-mediated signaling pathways. In addition to the intracellular receptors that frequently act as transcription modulators, a large variety of cell surface receptors also play an important role in this process by sensing the extracellular cues and initiating the intracellular signaling pathways. Cell surface receptors are membrane-bound proteins that interact with a variety of ligands and initiate various intracellular signaling pathways. These signaling pathways, in turn, mediate or modulate a wide variety of functions ranging from the maintenance of the cardiovascular and immune systems to the neuronal system in the brain.

## 1.1. G-protein coupled receptors (GPCRs)

G-protein coupled receptors (GPCRs) are the most extensive and indispensable surface molecules present on the cell membrane and are responsible for maintaining the homeostasis of living cells. These receptors cross the membrane seven times and are therefore also known as seven-transmembrane receptors. These proteins have an extracellular N-terminus exposed to the extracellular milieu, which is responsible for sensing various signaling molecules such as nucleotides, amino acids, lipids, peptide, photons, etc. (Joost and Methner, 2002; Foord et al., 2005). They also have three extracellular loops, three intracellular loops and a carboxy-terminal tail **(Figure 1.1).** Completion of the human genome project suggests that about 4% of the human and mouse genomes code for GPCRs (Bjarnadóttir et al., 2006). About 800 GPCRs have been identified in humans, of which around 50% (~400) are responsible for olfactory function, around 30 for taste, 10 for light perception and 5 for pheromone signaling (Mombaerts, 2004; Tang et al., 2012). GPCRs are classified into six classes on the basis of their sequence homology, type of the ligand that they interact with and the G-protein coupling (Kolakowski, 1994; Bockaert and Pin, 1999; Fredriksson et al., 2003; Foord et al., 2005). Rhodopsin family or class A, the largest

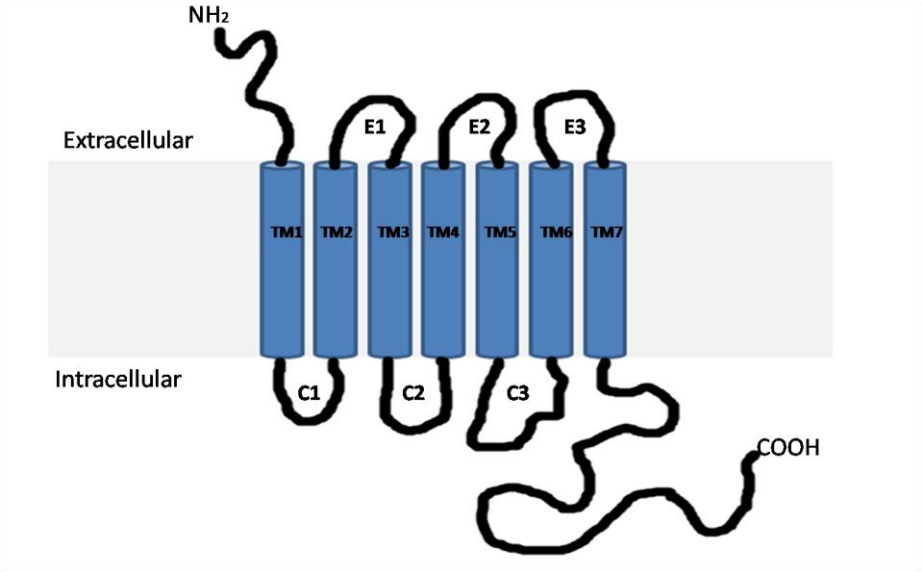


**Figure 1.1. Diagrammatic representation of the G-protein coupled receptors**

G-protein coupled receptors (GPCRs) are seven transmembrane receptors with an extracellular amino terminal domain and an intracellular carboxy terminal domain. These receptors have three extracellular loops (E1, E2 and E3), three intracellular loops (C1, C2 and C3) and a carboxy terminus tail.

subfamily of GPCRs, includes mainly olfactory receptors and receptors for a variety of ligands such as a peptides, neurotransmitters, hormones, pheromones etc. This family has been further subdivided into 19 subgroups based on the phylogenetic analysis (Joost and Methner, 2002). Class B or secretin family of receptors preferentially couple to the $G_{\alpha s}$ protein to activate the adenylyl cyclase pathway (Hollenstein et al., 2014). Ligands for this family of receptors include polypeptide hormones of 27-141 amino acid residues, viz.,. glucagon, glucagon-like peptides (GLP-1, GLP-2), glucose-dependent insulinotropic polypeptide (GIP), secretin, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP) and growth-hormone-releasing hormone (GHRH) etc. These receptors show a number of conserved proline residues within the seven transmembrane segments that are thought to be essential for the conformational shape of these receptors (Conner et al., 2005). The glutamate family or class C includes metabotropic glutamate receptors, a calcium sensing receptor, $GABA_B$ receptors, pheromone receptors and various taste receptors. Metabotropic glutamate receptors are characterized by a large venus fly-trap like N-terminus ligand binding pocket (Mombaerts, 2004; Trzaskowski et al., 2012). Class D members include $G_{\alpha i}$-associated pheromone receptors. Class E receptors are specific cyclic AMP (cAMP) receptors, comprising of a distinct family of G-protein coupled receptors of slime molds. cAMP receptors orchestrate the genes which are responsible for the development of *Dictyostelium discoideum* and also coordinate the aggregation of single cells to form a multicellular entity (Louis et al., 1994). Frizzled family or class F consists of 10 Frizzled proteins and Smoothened. It is the most conserved class of receptors among all GPCRs. Frizzled is activated by the secreted lipoglycoproteins of the Wnt family, whereas Smoothened is indirectly activated by the Hedgehog family of proteins acting on the transmembrane protein, Patched (PTCH) (Huang and Klein, 2004).

GPCRs bind an astoundingly diverse set of ligands, viz., proteins, small molecules, hormones, drugs, photons etc. These receptors are complex signaling machines. Much of this complexity arises due to their conformational and positional dynamism and their diversity in binding to different types of ligands. The affinity for a ligand also varies from receptor to receptor. As a result, these receptors can adopt a series of fleeting conformations that are influenced by their association with the ligands, other receptors, signaling and regulatory proteins, by post-translational modifications, and by environmental cues. This conformational flexibility

determines the capacity of these receptors to engage with the signaling machinery (Geppetti et al., 2015). There are various kinds of ligands which can bind to GPCRs and which may or may not activate the downstream signaling pathways. The ligands which activate the receptor are called "agonists", whereas ligands which do not activate the receptors upon binding are called "antagonists". Ligands that activate the receptor to a lesser extent than agonists are known as "partial agonists". Many ligands decrease the GPCRs basal activity upon binding, and they are called "inverse agonists". Around 35 - 40% of the total available drugs in the market target GPCRs, because of their involvement in various physiological processes and diseases. Majority of these drugs act as antagonists towards the receptors (Foord et al., 2005; Schlyer and Horuk, 2006).

GPCRs transduce the extracellular signal *via* interaction with the heterotrimeric G-protein complex. G-proteins are composed of three different subunits viz., α, β and γ. In an inactive stage, $G_{\alpha}$ of the G-protein is associated with GDP and exists as a complex with $G_{\beta\gamma}$. Binding of a GPCR either with the agonists or partial agonists is thought to cause a conformational change of the receptor. This in turn, leads to the action of the receptor as a GEF (guanine nucleotide exchange factor) and due to which, the GDP of the $G_{\alpha}$ gets exchanged with GTP. Subsequently, activated heterotrimeric G-protein dissociates into GTP bound $G_{\alpha}$ monomer and $G_{\beta\gamma}$ stable dimer subunits. Both the activated $G_{\alpha}$ and $G_{\beta\gamma}$ subunits further activate or inhibit the activity of a series of downstream effecters, such as, kinases, phosphatases, ion channels, nucleotide cyclases etc., which in turn modulates various cellular processes (Neer, 1994). Due to its intrinsic GTPase activity, the $G_{\alpha}$ subunit subsequently hydrolyzes the bound GTP to GDP, resulting in the inactivation of the $G_{\alpha}$ and its reassociation with the $G_{\beta\gamma}$ dimer to form an inactive heterotrimeric G-protein complex (McCudden et al., 2005). This reassociation of the $G_{\beta\gamma}$ subunits with the GDP bound $G_{\alpha}$ terminates all effector interactions with the receptor. Thus, as per the standard model of GPCR signaling, the lifetime of the $G_{\alpha}$ subunit in the GTP-bound state determines the duration of the signaling by both $G_{\alpha}$-GTP and free $G_{\beta\gamma}$ subunits (McCudden et al., 2005; Black et al., 2016) **(Figure 1.2).** The GTP hydrolysis is also regulated by another family of proteins called "regulator of G-protein signaling (RGS)"(Xie and Palmer, 2007). There are ~25 RGS proteins encoded in the human genome and these proteins play a critical role in the regulation of the G-protein mediated activity.

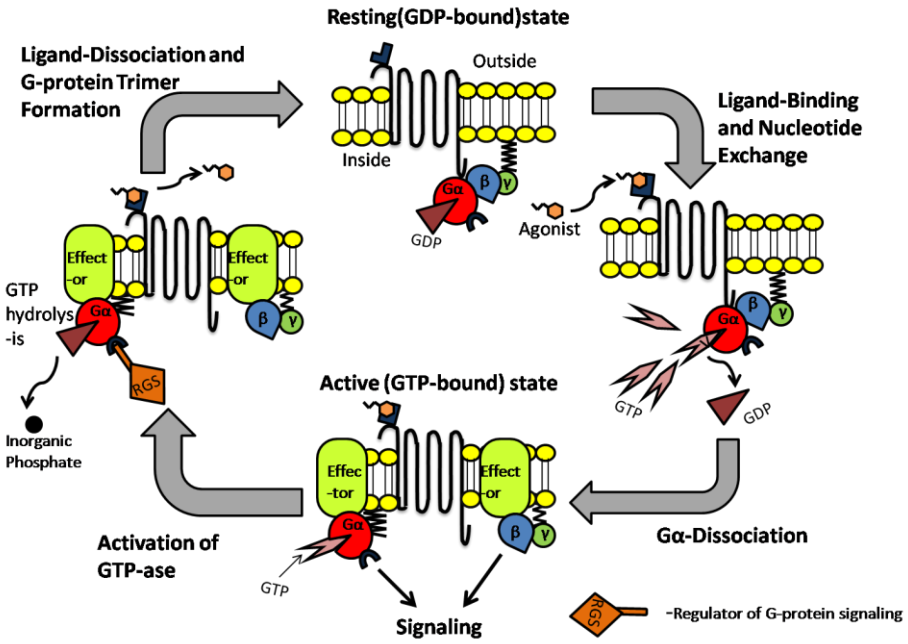


**Figure 1.2. Mechanisms for the activation of the G-protein coupled receptors**: The GPCR gets activated upon agonist/ligand binding and subsequently, the inactive form of the G-protein interacts with the receptor. The activated receptor acts as a guanosine nucleotide exchange factor (GEF) and as a result the GDP bound to the $G_\alpha$ is replaced by GTP. Subsequently, the α-subunit of the G-protein dissociates from the βγ subunit. Activated $G_\alpha$ can then interact with an effector like phospholipase C (PLC) or adenylate cyclase, which results in the initiation of the second-messenger cascade. The dissociated βγ subunit also transduces various intracellular signaling. Hydrolysis of GTP takes place by the activity of the $G_\alpha$ subunit and allows the reformation of the inactive G-protein complex.

The specificity and complexity in the GPCR signaling comes from the presence of a diverse array of G protein subunits. 17 genes have been discovered till today in the human genome encoding at least 23 types of $G_{\alpha}$ proteins. These subunits have been further subdivided into 4 subclasses ($G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$), based on the sequence similarity and on the type of the signaling cascade initiated by them upon activation (Wettschureck and Offermanns, 2005; Xie and Palmer, 2007). $G_{\alpha s}$ and $G_{\alpha i/o}$ regulate the adenylate cyclase pathway; $G_{\alpha s}$ acts as a stimulatory signal, whereas $G_{\alpha i/o}$ acts as an inhibitory signal. Adenylate cyclase catalyzes the conversion of the adenosine triphosphate (ATP) into cyclic-adenosine monophosphate (cAMP). The cAMP level in the cell regulates the activity of various ion channels and also the activity of protein kinase A (PKA) (Serezani et al., 2008; Godinho et al., 2015). Upon activation, $G_{\alpha q/11}$ activates the phospholipase C (PLC), which subsequently cleaves phosphatidylinositol 4, 5-bisphosphate ($PIP_2$) into inositol 1,4,5-triphosphate ($IP_3$) and membrane-bound diacylglycerol (DAG). $IP_3$ binds to its receptors present on the endoplasmic reticulum (ER) membrane, which results in the release of $Ca^{2+}$ from the ER and thus causes a rise in the intracellular $Ca^{2+}$. DAG and $Ca^{2+}$ are responsible for the activation of protein kinase C (PKC) which phosphorylates many target proteins leading to various cellular responses (Dhami and Ferguson, 2006a). $G_{\alpha 12/13}$ activates Rho GEF, which in turn activates Rho factors which are responsible for the cytoskeleton regulation. At least 6 different $G_{\beta}$ and 12 different $G_{\gamma}$ subunits have been discovered and have been shown to play very important roles in the regulation of various ion channels along with the activation of some isoforms of PLC and $IP_3$ (Vanderbeld and Kelly, 2000). In comparison to the variety of GPCRs and a diverse array of G-proteins that exist in nature; the number of downstream effectors are rather limited. It is likely that a particular receptor may interact independently with many G-proteins within the same class or occasionally with the other classes of G-proteins, depending on their availability in the vicinity of the receptor (Offermanns et al., 1994).

## 1.2. G-protein coupled receptor desensitization and trafficking

The balance between the "on and off" states of the receptor signaling plays a crucial role in the coordinated response. A critical feature of the GPCR signaling is the memory of any prior exposure of the receptor to the ligand and influence of that prior experience on their ability to get

stimulated in future. Continuous or repeated exposure to the ligand leads to a decreased sensitivity of the receptor towards the ligand. This phenomenon is called "desensitization" of the receptor. Desensitization is an important feedback mechanism that protects the cells from acute or chronic overstimulation of the receptor (Beaumont et al., 1998; Dale et al., 2000a; Kelly et al., 2008; Bhattacharyya, 2016). GPCRs undergo desensitization by various mechanisms or combination of multiple mechanisms. These mechanisms include: 1) uncoupling of the receptor from the G-protein involved due to the modifications of the receptor such as phosphorylation and arrestin binding, ubiquitination etc. 2) sequestration of the receptors in the endocytic compartments, 3) down-regulation of the receptors by lysosomal degradation (Lefkowitz, 1998; Ferguson, 2001; Kohout and Lefkowitz, 2003; Woolf and Linderman, 2003; Gainetdinov et al., 2004; Kelly et al., 2008; Luttrell and Gesty-Palmer, 2010). Short term desensitization occurs over minutes and is mainly due to the β-arrestin preventing the G-protein interaction with the receptor. On the other hand, degradation of the receptor in the lysosome subsequent to the ligand-mediated internalization of the receptor and decrease in the receptor mRNA level through unclear mechanisms are known to be responsible for the long-term desensitization or downregulation (Rajagopal and Shenoy, 2017). Any impairment in the desensitization process of the receptors results in the overstimulation of the cell, which might have pathological consequences (Freedman and Lefkowitz, 1996; Rajagopal and Shenoy, 2017). Many receptors also regain the ability to couple with the G-proteins after some time subsequent to the removal of the ligand (Pippig et al., 1995; Zhang et al., 1997; Ferguson et al., 1998; Oakley et al., 1999; Anborgh et al., 2000; Trapaidze et al., 2000; Roosterman et al., 2004). This process is called "resensitization" of the receptor. The time course for resensitization of GPCRs also varies depending on the type of the receptor and the type of the system.

Subsequent to the activation of these receptors, various protein kinases like protein kinase A (PKA), protein kinase C (PKC) and G-protein coupled receptor kinases (GRKs) phosphorylate the receptor (Bouvier et al., 1988; Alaluf et al., 1995; Gereau and Heinemann, 1998a; Francesconi and Duvoisin, 2000a; Sallese et al., 2000; Choi et al., 2011; Ko et al., 2012; Bhattacharyya, 2016; Mayor Jr et al., 2017). Phosphorylation of the receptor promotes the binding of β-arrestin, which uncouples the receptor from the G-protein involved, leading to the desensitization of the receptor. In addition to the above mentioned kinases, GPCRs act as

substrates for other kinases as well. For example, casein kinases have been reported to phosphorylate some GPCRs that result in the desensitization of the receptor (Budd et al., 2000). G-protein coupled receptor kinases (GRKs) phosphorylate only the agonist-activated receptors. In mammals, the GRK family consists of seven GRKs (GRK1-7) and they have been sub-divided into 3 subgroups (GRK1/7, GRK2/3, GRK 4/5/6) based on their sequence and functional similarities (Premont et al., 1996; Lodowski et al., 2006). GRK1 and GRK7 are found to be expressed predominantly in the retinal tissues, whereas GRK4 is primarily localized in the testes (Ferguson, 2001). GRK2/3/5/6, on the other hand, expresses ubiquitously and accounts for the regulation of most GPCRs in the system. GRKs have similar structural organization; a central catalytic domain, an N-terminus thought to be required for the substrate recognition and a C-terminal domain for the targeting of the kinase at the plasma membrane. The specificity in the GPCR recognition by GRKs is largely based on the activation state of the receptors and the relative abundance of the specific GRKs in a given cell type. Although GRKs show some preference over the phosphorylation sites on the receptors, several studies have also suggested that they may choose other sites to phosphorylate in case the preferred site is mutated. The second messenger-dependent protein kinases (PKA, PKC etc.) phosphorylate the activated receptors as a feedback mechanism, but they can also phosphorylate the receptor that has not been exposed to the agonist (Hausdorff et al., 1989; Lohse et al., 1990). In addition to the receptors, these kinases also phosphorylate the downstream effectors and thereby regulate GPCR signaling (Hausdorff et al., 1989; Lohse et al., 1990; Kohout and Lefkowitz, 2003).

Subsequent to the phosphorylation of GPCRs, another class of regulatory proteins called, arrestins get recruited. Binding of arrestin to the receptor results in the uncoupling of the receptor from the G-protein involved and that leads to the desensitization of the receptor (Ferguson et al., 1996b; Krupnick and Benovic, 1998; Kohout and Lefkowitz, 2003; Black et al., 2016). The binding of arrestin to the receptor also plays a key role in the recruitment of other adaptor proteins which results in the internalization of the receptor (Ferguson et al., 1996b; Mundell et al., 2001; Paing et al., 2002). The mechanism of β-arrestin mediated desensitization and trafficking of $\beta_2$-adrenergic receptor ($\beta_2$-AR) has been studied in detail (Moore et al., 2007). The activation of $\beta_2$-AR by the ligand causes the localization of arrestin to the plasma membrane. The activated $\beta_2$-AR and arrestin complex redistributes to the clathrin-coated pits and arrestin

dissociates subsequent to the internalization of the receptor (Wolfe and Trejo, 2007). In contrast, many reports have suggested that the internalization of some GPCRs is arrestin-independent. For example, M1, M2, M3 and M4 muscarinic cholinergic receptors have been shown to internalize upon over-expression of dominant negative arrestin and dynamin mutants (Pals-Rylaarsdam et al., 1997; Lee et al., 1998). The member of the serotonin receptor family, 5-$HT_{2A}$ has also been reported to internalize in arrestin and dynamin-independent manner (Bhatnagar et al., 2001). In case of PAR1, both constitutive and ligand-mediated internalization processes have been shown to take place *via* arrestin-independent pathways in mouse embryonic fibroblast (MEF) derived from arrestin2 and arrestin3 knockout mice (Kohout et al., 2001; Paing et al., 2002) **(Figure 1.3).**

It has been observed that the mechanism of internalization and the subcellular fate of the receptor subsequent to the internalization depend on the type of the receptor, type of the ligand and the cellular background (Bhattacharyya, 2016). Some GPCRs go to the recycling compartment and recycle back to the cell surface, whereas others land in the lysosome for degradation, like the PAR1 receptor (Trejo and Coughlin, 1999). Due to the intense research in the past few decades, our understanding of the biological importance of GPCR endocytosis expanded rapidly. Initially, the endocytosis of GPCRs was believed to be the primary mechanism for the desensitization of the receptors, since internalization physically separates the receptors from the G-proteins (Sibley and Lefkowitz, 1985; Ferguson, 2001). However, this hypothesis was challenged by the following observations: 1) for many receptors, the receptor desensitization proceeds more rapidly than the endocytosis of the receptor, 2) desensitization profile of the $\beta_2$-adrenergic receptor remained unaltered even after blocking the endocytosis by various pharmacological and chemical inhibitors (Yu et al., 1993; Pippig et al., 1995; Ferguson et al., 1998). These results together suggested that for many receptors endocytosis is not the mechanism to desensitize the phosphorylated receptor subsequently binds β-arrestin which leads to the desensitization of the receptor. The desensitized receptor internalizes *via* dynamin and clathrin-dependent pathway. Subsequently, the receptor resensitizes due to the dephosphorylation and recycles back to the cell surface. Alternatively, the internalized receptor is targeted for degradation in the lysosomes receptor. In fact, internalization is necessary for the resensitization and downregulation of many GPCRs. The idea that internalization is necessary for the receptor resensitization came from several observations: 1) $\beta_2$-adrenergic receptors isolated from the light endosomal fractions were

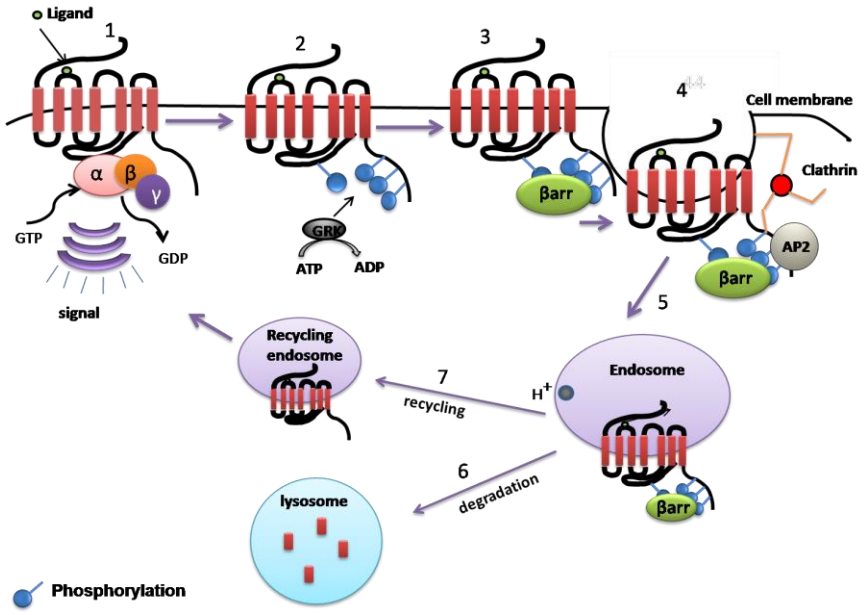


**Figure 1.3. Schematic representation of the GPCR trafficking.** Activation of the GPCR by the agonist results in the phosphorylation of the receptor by the activated kinases. The phosphorylated receptor subsequently binds β-arrestin which leads to the desensitization of the receptor. The desensitized receptor internalizes *via* dynamin and clathrin-dependent pathway. Subsequently, the receptor resensitizes due to the dephosphorylation and recycles back to the cell surface. Alternatively, the internalized receptor is targeted for degradation in the lysosomes.

less phosphorylated than the receptors isolated from the plasma membrane fraction (Sibley and Lefkowitz, 1985), 2) the endosomal fractions were found to be enriched in GPCR specific phosphatases activity (Sibley and Lefkowitz, 1985; Pitcher et al., 1995), 3) internalization defective mutants were not able to resensitize although their signaling and desensitization properties were intact (Barak et al., 1994) and 4) resensitization of the GPCRs was blocked upon inhibition of the endocytosis using pharmacological and chemical inhibitors (Pippig et al., 1995; Garland et al., 1996; Hasbi et al., 2000). It has been reported that for many GPCRs such as, m3AChR, delta opioid receptor, $\beta_2$-adrenergic receptors, μ-opioid receptor, PTHR, endothelin A receptor, cholecystokinin receptor, endocytosis is necessary for their resensitization process (Lutz et al., 1993; Giannini and Boulay, 1995; Pippig et al., 1995; Garland et al., 1996; Edwardson and Szekeres, 1999; Wolf et al., 1999; Bremnes et al., 2000; Hasbi et al., 2000). Internalization is also necessary for downregulation of many GPCRs. For example, protease activated receptors and endothelin B receptors, upon internalization, predominantly targeted to lysosomes for degradation (Trejo and Coughlin, 1999; Bremnes et al., 2000). Thus, GPCR trafficking plays a key role in regulating the GPCR turn over and also maintenance of signaling and homeostasis in the cell.

### 1.3. Role of ubiquitination in the trafficking of GPCRs

Ubiquitination is a reversible post-translational modification known to regulate the signaling and trafficking of many GPCRs (Hicke and Riezman, 1996; Galan and Haguenauer-Tsapis, 1997; Terrell et al., 1998; Marchese and Benovic, 2001; Shenoy et al., 2001b; Tanowitz and von Zastrow, 2002; Haglund et al., 2003; Moriyoshi et al., 2004; Komander, 2009; Alonso et al., 2011; Hislop and von Zastrow, 2011; Lahaie et al., 2016; Skieterska et al., 2017). Ubiquitin (Ub) is a 76 amino acid residue, an 8.5 kDa peptide, highly conserved across all phyla. Ubiquitin binds covalently to the lysine residues in the target proteins through a sequential three-step process involving E1 activating enzyme that activates ubiquitin in an ATP-requiring reaction, to generate a high-energy thiol ester intermediate, E1-S~ubiquitin. Subsequently, ubiquitin conjugating enzyme E2 transfers the activated ubiquitin from E1 *via* an additional high energy thiol ester intermediate, E2-S~ubiquitin. Finally, the ubiquitin ligase enzyme, E3, transfers

ubiquitin to a lysine side chain of the substrate. E3s specify the timing and substrate selection of the ubiquitination reactions (Hershko and Ciechanover, 1998; Glickman and Ciechanover, 2002; Ciechanover, 2003; Hicke et al., 2005; Yang et al., 2007; Komander, 2009). There are a large number of E3 Ub ligases and based on the differences in their functional domains, they are further divided into three families-RING, HECT and U box (Komander, 2009; Caballero and Marchese, 2011) **(Figure 1.4).** Ubiquitin is attached *via* the C-terminal glycine residue to the ε-$NH_2$ lysine side chain of the target protein through isopeptide bond or through α-$NH_2$ group forming peptide bond. In 2004, Aaron Ciechanover, Avram Hershko and Irwin Rose were awarded the Nobel Prize in Chemistry for their discovery and characterization of ubiquitin. Modified target proteins can undergo various kinds of ubiquitinations such as monoubiquitination, multi-monoubiquitination and polyubiquitination. Attachment of a single ubiquitin moiety to the target protein results in the monoubiquitination. Similarly, attachment of a single ubiquitin moiety to the target protein at multiple sites results in the multi-monoubiquitination. On the other hand, ubiquitin itself can undergo ubiquitination to form a distinct isopeptide linked ubiquitin chain resulting in the polyubiquitination. The ubiquitin protein contains 7 lysine residues (K6, K11, K27, K29, K33, K48 and K63) and an amino-terminus methionine (Met or M1) that can serve as an acceptor site for additional ubiquitin molecules generating polyubiquitinated proteins. Thus, ubiquitin chains can be connected to either of the lysines present at positions K6, K11, K27, K29, K33, K48, K63 or M1, resulting in the formation of various types of ubiquitin chains (Haglund et al., 2003; Ciechanover and Ben-Saadon, 2004; Hicke et al., 2005; Komander, 2009; Mabb and Ehlers, 2010; Dores and Trejo, 2012; Komander and Rape, 2012) **(Figure 1.5)**. Historically, ubiquitin has been reported to be involved in protein degradation because it serves as a tag for recognition by the proteasomal machinery (Hershko and Ciechanover, 1998; Glickman and Ciechanover, 2002). A cytosolic protein that is old or damaged or a protein that has undergone regulated degradation such a cyclin and some transcription factors are tagged with polyubiquitin chains, which are then recognized by the regulatory 26S proteasome. Recently it has been reported that these modifications regulate various functions inside the cell, such as, endocytosis of membrane receptors, DNA repair, histone activity, protein degradation etc (Mabb and Ehlers, 2010).

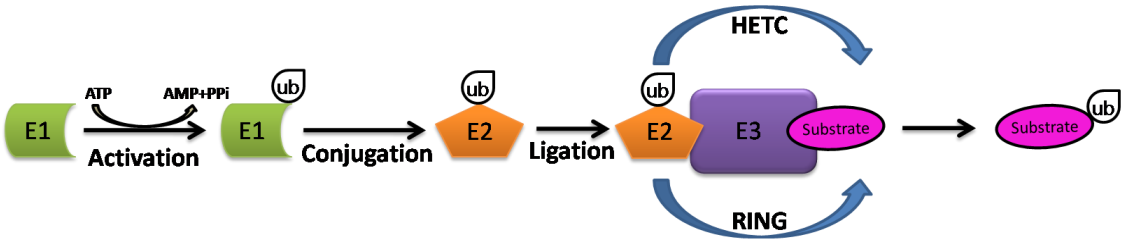


**Figure 1.4. Mechanisms of the ubiquitination pathway.** Ubiquitination of a substrate is the result of the sequential action of three enzymes, viz., E1, E2, and E3. Initially, ubiquitin is activated by the ubiquitin-activating enzyme E1 and is transferred to the E1 enzyme active site in an ATP dependent manner. After that, ubiquitin is transferred to the second enzyme, E2 (ubiquitin-conjugating enzyme). Finally, the enzyme E3 (ubiquitin ligase) recognizes, binds the target substrate, and labels it with the ubiquitin.

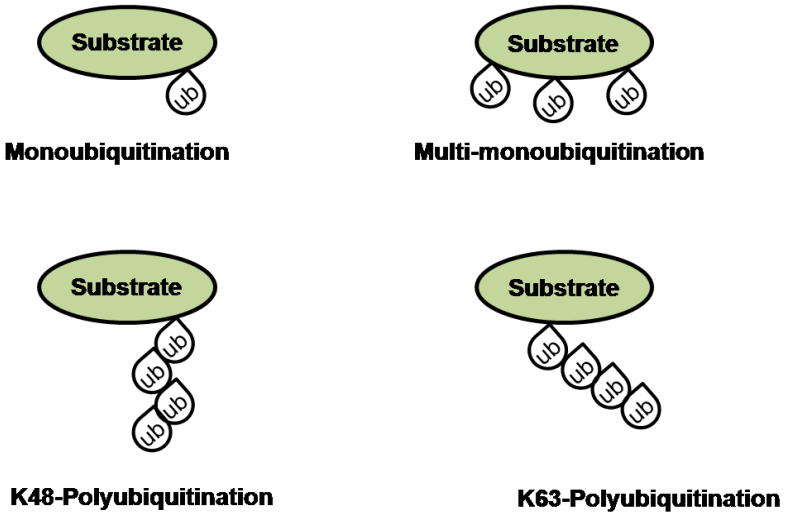


**Figure 1.5. Diagrammatic representation of the various types of ubiquitination.**

In last few years, attention has been given on how ubiquitination regulates the signaling and trafficking of receptors at the membrane. Many membrane proteins are known to get ubiquitinated subsequent to the ligand application, including receptor tyrosine kinases (RTKs) and GPCRs, where ubiquitination has been implicated in the regulation of their signaling and internalization (Hicke and Riezman, 1996; Terrell et al., 1998; Haglund et al., 2003; Acconcia et al., 2009; Caballero and Marchese, 2011; Kennedy and Marchese, 2015). Ubiquitination acts as a sorting signal to facilitate the trafficking of many mammalian GPCRs from endosomes to lysosomes for degradation (Marchese et al., 2008). But for a number of GPCRs, ubiquitination plays many other roles (Tanowitz and von Zastrow, 2002). In case of the ste2 receptor (yeast GPCR), ubiquitination of the lysine residue at the C-terminus of the receptor serves as a signal for the ligand-mediated endocytosis (Hicke and Riezman, 1996). In case of $\beta_2$-adrenergic receptor, μ-Opioid (MOR) receptor and chemokine receptor (CXCR4), ubiquitination upon ligand-mediated activation of the receptor sorts the receptor for degradation (Marchese and Benovic, 2001; Hislop and von Zastrow, 2011; Xiao and Shenoy, 2011). Ubiquitination-dependent internalization and sorting of membrane receptors requires recognition of the ubiquitinated protein by downstream adaptors involved in the trafficking machinery of cells, such as AP-2, Epsin, Eps-15, Hrs, STAM (Piper et al., 2014). All these endocytic adaptor proteins contain ubiquitin-interacting motifs (UIM) and sort the ubiquitinated cargo from early to the late endosome (Polo, 2012). During sorting of these GPCRs, another class of ubiquitination recognizing proteins, called deubiquitinases, play critical role determining the fate of the receptors (Jean-Charles et al., 2016).

In the human genome, around 100 genes code for the deubiquitinases (DUBs), which mediate the reversal of the ubiquitination process, but the functional importance of these DUBs is least understood. DUBs are required in the cell for three major purposes: 1) Ubiquitin can be transcribed from several genes as a linear fusion of multiple ubiquitin molecules; therefore generation of free ubiquitin requires DUB activity, 2) DUBs can remove ubiquitin chains from post-translationally modified proteins, leading to the stabilization of cytosolic proteins or regulating the signaling and trafficking route of internalized receptors and 3) DUBs can edit the form of ubiquitin modification by trimming ubiquitin chains (D'Andrea and Pellman, 1998; Clague and Urbé, 2006; Komander, 2010; Clague et al., 2012; Clague and Urbé, 2017). DUBs also maintain the free ubiquitin homeostasis in the cell. They are broadly categorized into 5

groups: 1) ubiquitin C-terminal hydrolase (UCH), 2) ubiquitin-specific protease (USP), 3) ovarian tumor domain (OTU), 4) Machado-Joseph disease (MJD), and 5) Jab1/Mpn/Mov34 (JAMM). UCH, USP, OTU and MJD are also known as cysteine proteases, containing a thiol group in the active site, whereas, JAMMs are metalloproteases (Nijman et al., 2005). Most DUBs catalyse a proteolytic reaction between a Lys ε-amino group and a carboxyl group corresponding to the C terminus of ubiquitin. DUBs are highly specific at multiple levels to distinguish between many ubiquitin like molecules, isopeptides ( through the ε-amino group), linear peptides ( through α- amino group) and between different types of ubiquitin linkages (Komander, 2009; Komander et al., 2009). The mechanism of the substrate specificity of deubiquitinases is not yet explored. DUBs also control the dynamics of ubiquitin-mediated signaling events (Clague et al., 2013). Deubiquitination plays a critical role in the learning and memory formation. In mice, the ubiquitin C-terminal hydrolase L3 (UCH-L3) is required for the formation of working memory but hippocampal LTP is not affected in mice lacking UCH-L3 (Wood et al., 2005). The parathyroid hormone receptor (PTHR), subsequent to the activation, gets internalized and recycles back in 2 hr. It has been reported that activation of PTHR up-regulates the level of USP2 (Ubiquitin-specific protease 2), favoring the balance towards the rapid deubiquitination and recycling of PTHR (Alonso et al., 2011). Agonist stimulation of the $\beta_2$-AR leads to the ubiquitination and lysosomal degradation of the receptor, but over-expression of USP33 and USP20 counteracts these effects and promotes receptor recycling and resensitization. Additionally, knockdown of both USPs 33 and 20 abolishes receptor recycling and resensitization but enhances ubiquitination as well as lysosomal degradation. Thus, USPs 20 and 33 act as novel regulators that dictate the post-endocytic fate of internalized $\beta_2$-ARs (Shenoy et al., 2001a; Shenoy, 2007; Berthouze et al., 2011). For metabotropic γ-aminobutyric acid receptor (GABAB), USP14 is responsible for the deubiquitination of the receptor and dictates the post-endocytic fate of the receptor (Lahaie et al., 2016). The mice lacking USP14 exhibit increased GABA receptor levels at Purkinje cell surface and increased post-synaptic current (Lappe-Siefke et al., 2009). Ubiquitination controls the most complex aspects of cell physiology and is reversed by the action of a large family of deubiquitinating enzymes (DUBs). Therefore, the deubiquitinating enzymes are emerging as an attractive possible therapeutic target for a number of disease conditions **(Figure 1.6)**.

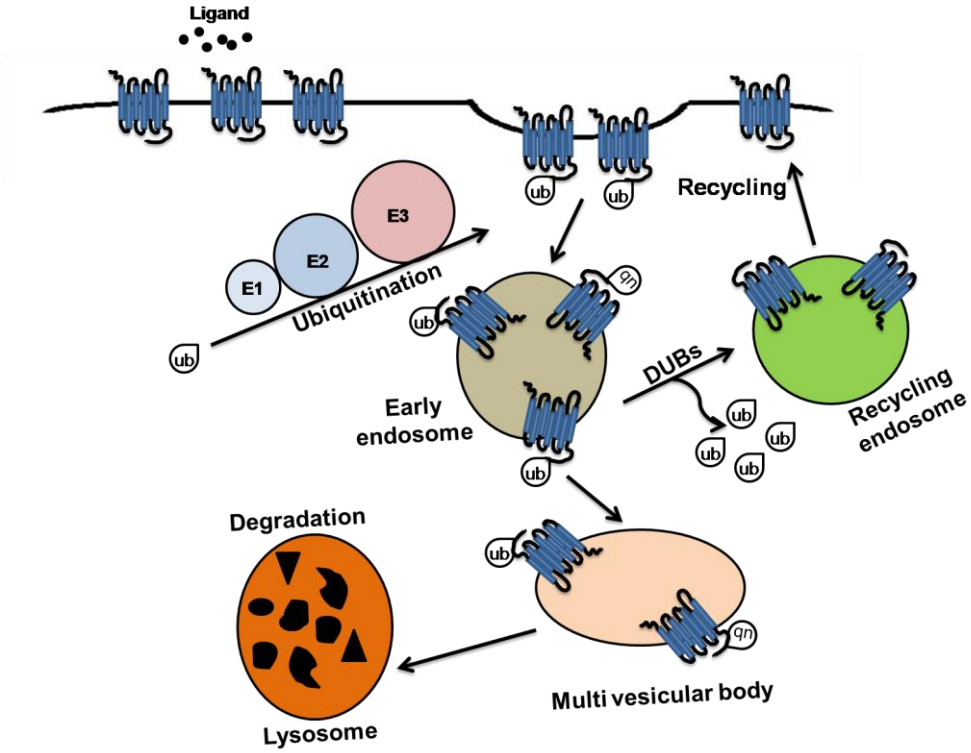


**Figure 1.6. Role of ubiquitination and deubiquitination in the ligand-dependent trafficking of GPCRs.** Upon stimulation by the ligand, some GPCRs get ubiquitinated. Ubiquitination acts as signal for the internalization and sorting of many GPCRs. Balance between the ubiquitination and deubiquitination dictates the fate of the receptor subsequent to the internalization.

## 1.4. Glutamate receptors

L-glutamate (Glu) acts as a major excitatory neurotransmitter in the mammalian brain, resulting in the excitation or stimulation of the post-synaptic neurons (Fonnum, 1984; Erecińska and Silver, 1990; Conn and Pin, 1997; de Bartolomeis and Szumlinski, 2012; Bhattacharyya, 2016). Although glutamate is a non-essential amino acid, primarily involved in the intermediate metabolism and other functions, it is also involved in more than 90% of the excitatory synapses in the human brain. Glutamate has been reported to be synthesized in the CNS through two major pathways: 1) the *de novo* synthesis of glutamate from glucose and amino acid derivatives *via* energy metabolism and 2) synthesis from glutamine as part of the glutamate-glutamine cycle by the activity of the principal enzyme, glutaminase (Erecińska and Silver, 1990; Rousseaux, 2008). The optimal concentration of glutamate inside the brain is maintained by the glutamate transporters, viz., vesicular glutamate transporters (VGLUTs) and excitatory amino acid transporters (EAATs), present in the neurons, astrocytes, and the blood-brain barrier (Danbolt, 2001; Shigeri et al., 2004; Koch and Larsson, 2005; Beart and O'shea, 2007). VGLUTs are multimeric $H^+$/Glu antiporters and play an important role in the transport of the cytoplasmic glutamate into the synaptic vesicles (Fremeau Jr et al., 2004; Shigeri et al., 2004; Takamori, 2006; Wallén-Mackenzie et al., 2010). Excitatory amino acid transporters (EAATs), on the other hand, are sodium-dependent glutamate antiporters, known to transport the extracellular glutamate present in the synaptic clefts and at the extrasynaptic sites actively into the neurons and glial cells, thus protecting the brain from excessive glutamate signaling which causes excitotoxicity (Shigeri et al., 2004; Holmseth et al., 2012; Underhill et al., 2014; Zhou et al., 2014). Glutamate transduces its signal by binding with the receptors present in the synaptic and extrasynaptic regions. It activates two types of receptors in the CNS, viz., ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) (Dale et al., 2000a; Dale et al., 2001; Mundell et al., 2001; Dhami and Ferguson, 2006a; Niciu et al., 2012; Bhattacharyya, 2016) **(Figure 1.7)**.

**Ionotropic glutamate receptors (iGluRs):** They are the longest known and best studied glutamate receptors. These are ion channels, which are permeable to cations (Hollmann and Heinemann, 1994). In general, all iGluRs consist of four subunits and each subunit contains four

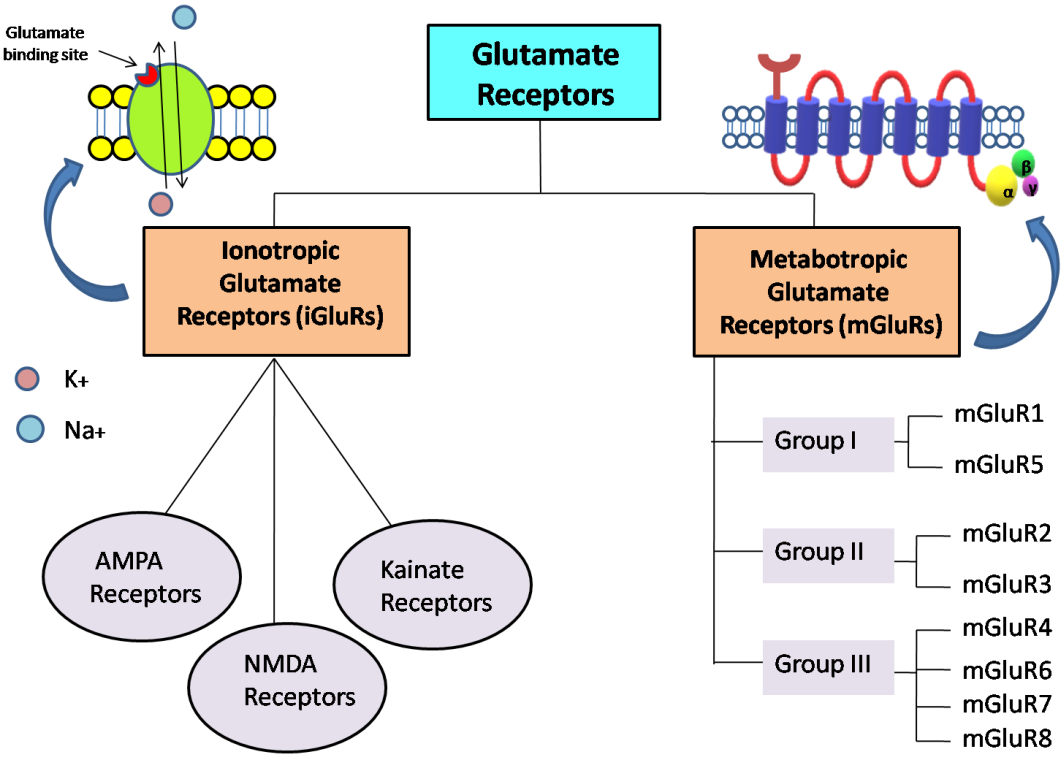


**Figure 1.7. Diagrammatic depiction of glutamate receptors**

well-conserved domains, including an extracellular N-terminal domain followed by a ligand binding domain, a transmembrane domain spanning the cell membrane four times and an intracellular carboxy-terminal domain. One of the most intriguing features of iGluRs is their diverse ion channel properties which come from several permutation combinations of the subunits that make the ion channels. On the basis of their pharmacological and electrophysiological properties and their agonist preference, these are subdivided into three types: α -amino-3-hydroxy-5-methyl- 4- isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and Kainate (KA) receptors (Huganir and Nicoll, 2013; Bhattacharyya, 2016).

**1. AMPA receptors (AMPARs):** AMPA receptors are the most widely distributed receptors and mainly localized at the post-synaptic membrane. They exist as heteromers on the synapse. These receptors, upon binding with glutamate, induce fast excitatory neurotransmission. They were initially identified as "Quisqualate receptors" because they can bind quisqualate with a higher affinity than glutamate, their natural ligand (Honoré et al., 1982). After that, they have been renamed as AMPA receptors on the basis of their selective agonist binding, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid. Each AMPAR subunit contains agonist binding sites; agonist occupancy at two sites is sufficient for channel opening. These receptors consist of four subunits; GluA1, GluA2, GluA3, and GluA4. All of the isoforms can undergo RNA editing and exist as flip/flop splice isoforms (Sommer et al., 1990; Greger et al., 2002; Derkach et al., 2007). These receptors exist as heterotetrameric ion channels, consisting of "dimer of dimers" (Shi et al., 1999; Song and Huganir, 2002; Greger et al., 2007). These subunits have similar topology but their intracellular C-terminal tail varies from each other which are responsible for their differential binding to the post-synaptic density proteins, localization, and trafficking. The GluA2 subunit has been reported to bind PICK1, a scaffolding protein through its PDZ domain, whereas GluA1 subunit binds preferentially with SAP91, another scaffolding protein (Leonard et al., 1998; Citri et al., 2010; Fiuza et al., 2017). The trafficking of these AMPA receptors plays a key role in regulating the synaptic efficacy by either enhancing or decreasing the neurotransmission across the synapse (Carroll et al., 2001; Malinow and Malenka, 2002; Malenka and Bear, 2004; Kauer and Malenka, 2007; Citri and Malenka, 2008a; Bhattacharyya et al., 2009; Bhattacharyya, 2016). Insertion of AMPARs at the synapse or removal of AMPARs

from the synapse is a direct cellular correlate of long-term potentiation (LTP) or long-term depression (LTD) respectively.

**2. NMDA receptors (NMDARs):** NMDA receptors are also glutamate gated cation channels and play a key role in the basal neurotransmission as well as in the modulation of synaptic plasticity (Moriyoshi et al., 1991). These receptors are called NMDA receptors because N-methyl-D-aspartate (NMDA), an agonist, binds selectively to these receptors and activates them. These receptors are also distributed extensively in the CNS and have the highest affinity for glutamate (Vyklicky et al., 2014). These receptors also consist of different subunits; NR1, NR2A-D and NR3A-B and exist as a heteromer. NR1 is an obligatory subunit and appears to express throughout the brain (Nakanishi, 1992; Goebel and Poosch, 1999). NMDARs cannot be activated with glutamate alone; they require a co-agonist for their activation. Glycine acts as a co-agonist for these receptors, although the binding sites of glycine and glutamate are in different subunits (Blanke and Van Dongen, 2009). Glycine binds at the NR1 subunit, whereas, glutamate binds to the NR2A subunit. At resting membrane potential, the NMDARs are blocked by $Mg^{2+}$ which can only be removed by membrane depolarization that results in the restoration of the activity of NMDARs. These receptors have been reported to play crucial roles in various important physiological processes, such as the development of the CNS, generation of rhythms involve in breathing, locomotion and in learning and memory formation (Collingridge, 1987; Greer et al., 1991; Scheetz and Constantine-Paton, 1994).

**3. Kainate receptors (KA):** Kainate receptors are ionotropic glutamate receptors with remarkable structural diversity. These receptors were first identified as distinct receptors from the AMPA and NMDA receptors due to their selective activation by a drug called kainate, isolated from the red algae *Digenea simplex*, (Coyle, 1987). Pharmacologically, they are difficult to distinguish from other types of glutamate receptors, such as AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), but important progress has been made in identifying several relatively selective agonists and antagonists for these receptors. There are five different subunits: GluK1-3, GluK4 and GluK5 that constitute these receptors. They also exist as a heterotetramer; GluK1-3 subunits can combine to form functional heteromeric or homomeric assemblies when they are expressed in cell lines (Gallyas et al., 2003; Pinheiro

and Mulle, 2006). The absence of specific antibodies against different KAR subunits has been a significant limitation in terms of exploring the receptor distribution. Thus, most of the information available regarding their tissue expression comes from *in situ* hybridization studies. Expression of the GluK1-3 subunits is higher in the CA3 region of the hippocampus, striatum and the inner layers of the cortex. In contrast, GluK4 and 5 subunits have a much more restricted distribution, with GluK4 found almost exclusively in the hippocampus (Darstein et al., 2003; Lerma and Marques, 2013). The channel conductance of these receptors is similar to AMPA receptors; however, the rise and decay time of the potential generated by them is slower than that of AMPA receptors (Huettner, 2003).

**Metabotropic glutamate receptors (mGluRs)**: mGluRs are members of the class C G-protein coupled receptor (GPCR) family. They have been subdivided into three groups based on their sequence similarity, pharmacology and the second messenger pathways that they initiate upon activation (Pin and Duvoisin, 1995; Conn and Pin, 1997; Bhattacharyya, 2016). Group I consists of mGluR1 and mGluR5 which are primarily coupled to the $G_{\alpha q/11}$ pathway and activate phospholipase C, group II mGluRs consists of mGluR2 and mGluR3, whereas group III comprises of mGluR4, mGluR6, mGluR7 and mGluR8. Both group II and group III mGluRs are predominantly coupled with the $G_i/G_o$ pathway and negatively regulate the adenylyl cyclase activity upon activation with the ligand (Tanabe et al., 1992; Pin and Duvoisin, 1995; Conn and Pin, 1997; Niswender and Conn, 2010; Bhattacharyya, 2016). Group I mGluRs are primarily localized in the peri-synaptic region of the post-synaptic neurons and group II mGluRs are expressed in both pre and post-synaptic neurons, whereas group III mGluRs are found predominantly in the pre-synaptic neurons. These mGluRs activate a variety of signaling cascades upon ligand binding and mediate slower responses in the glutamatergic system compared to iGluRs, as well as regulating the other pathways within this system (Viaene et al., 2013). These receptors are also present on glia and have been reported to play crucial roles in the glutamate release and uptake from the astrocytes, neuroprotection and communication between neurons and glial cells (Benarroch, 2008; Bélanger and Magistretti, 2009). The mGluRs and their signaling have been reported to be involved in various physiological functions in the brain including regulation of the activity of various ion channels, learning and memory formation and neuropsychiatric disorders (Bordi and Ugolini, 1999; Dölen et al., 2007; Kauer and Malenka,

2007; Benarroch, 2008; Byrnes et al., 2009). Since the objective of this work is to understand the regulation of group I mGluRs, in the subsequent section we have concentrated on the group I mGluRs.

## 1.5. Group I mGluRs: distribution and signaling

Group I mGluRs have been observed to express differentially throughout the CNS. Although, mGluR1 expresses extensively in the olfactory bulb and cerebellar purkinje cells, a strong expression has also been observed in the hippocampus, thalamus, lateral septum, substantia nigra and globus pallidus (Tanabe et al., 1992; Shigemoto et al., 1993; Bordi and Ugolini, 1999). The expression of mGluR5, on the other hand, has been observed in the hippocampus, cerebral cortex, striatum, nucleus accumbens, granule cells of the olfactory bulb and lateral septal nucleus (Bordi and Ugolini, 1999; Sun et al., 2009; Bhattacharyya, 2016). According to some reports, expression of group I mGluRs varies with the development of the brain. A steady increase in the expression of mGluR1 has been observed in the neocortex and hippocampus during development (Catania et al., 1994). Expression of mGluR5a increases gradually during the development of the cortex and reaches a maximum during the second postnatal week in rodents and then decreases gradually. On the other hand, the expression of mGluR5b mRNA increases postnatally and this is the most prominent form of mGluR5 in adults (Catania et al., 1994; Minakami et al., 1995; Romano et al., 1996; Bhattacharyya, 2016). Moreover, group I mGluRs are also observed to be expressed in certain non-neuronal cells like melanocytes, hepatocytes, heart cells, osteoblasts and skin cells (Gill et al., 1999; Gu and Publicover, 2000; Bhave et al., 2001). These receptors are primarily localized at the perisynaptic region of the postsynaptic neurons in the CNS (Lüscher and Huber, 2010). The structure of both mGluR1 and mGluR5 comprises a very large extracellular domain containing characteristic ligand binding grooves (Venus flytrap domain) and a cysteine rich domain, followed by a serpentine transmembrane domain and an intracellular carboxyl-terminal tail. As mentioned earlier, an activated group I mGluR transduces *via* $G_{\alpha q/11}$ signaling pathway. The binding of the ligand introduces a conformational change that activates the G-protein which further activates the phospholipase C (PLC). The PLC, in turn, acts upon phosphatidylinositol 4,5-bisphosphate ($PIP_2$) which gets cleaved into inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ causes the release of $Ca^{2+}$ from the intracellular stores. Both

DAG and $Ca^{2+}$ together activate the protein kinase C (PKC) (Dhami and Ferguson, 2006a; Wang et al., 2007). Subsequently, the activated PKC modulates the activity of various ion channels and various other target substrates in order to regulate several physiological processes (Gereau and Heinemann, 1998a; Dhami and Ferguson, 2006a; Niswender and Conn, 2010; Wang and Zhuo, 2012). These receptors mainly couple with the $G_{\alpha q/11}$ but depending on the circumstances they can also couple to other G-proteins in various cell types (Marinissen and Gutkind, 2001). In majority of the cell types, the activation of group I mGluRs finally activates the MAP kinase pathway (Choe et al., 2002; Berkeley and Levey, 2003; Wang et al., 2007). Some reports have suggested that this mGluR-mediated MAP kinase activation is involved in the mGluR-dependent synaptic plasticity in hippocampal neurons (Choe et al., 2002; Mao and Wang, 2016). Furthermore, activation of group I mGluRs results in the internalization of synaptic AMPARs which is the cellular correlate for the mGluR-mediated synaptic plasticity (Snyder et al., 2001).

## 1.6. Role of group I mGluRs in synaptic plasticity and diseases

Activity-dependent plasticity of pre-existing synaptic connections is an astonishing feature of the adult brain which modulates the properties of neuronal circuits and behavior. Synaptic plasticity is believed to be the direct cellular correlate of learning and memory. Also, work done in the past four decades suggests its importance in the circuit formation during the brain development (Malenka, 1994; Malenka and Bear, 2004; Citri and Malenka, 2008a). Group I mGluRs, in addition to modulating synaptic efficacy by regulating the trafficking of ion channels, also induce the transcription and translation of numerous genes essential for the maintenance of synaptic plasticity (Snyder et al., 2001; Wang and Zhuo, 2012). Long-term increase in the synaptic strength due to enhanced synaptic efficacy is known as long-term potentiation (LTP) (Malenka, 1994). In contrast, long-term decrease in the synaptic strength is known as long-term depression (LTD) (Malenka, 1994). Therefore, the strength of an excitatory synapse can be modulated bi-directionally. LTD is triggered in the CNS by the activation of either NMDARs or mGluRs (Citri and Malenka, 2008b). Although the mechanisms of NMDAR-dependent LTD have been studied extensively, very little is known about the mechanisms underlying mGluR-LTD. Both NMDAR-LTD and mGluR-LTD involve the endocytosis of AMPARs. However, several reports have suggested that the mechanisms of NMDAR-dependent AMPAR endocytosis

and mGluR-dependent AMPAR endocytosis are significantly different as well as cell type specific (Oliet et al., 1997; Snyder et al., 2001; Lüscher and Huber, 2010). Since the involvement of altered mGluR-LTD has been reported in the mouse model of mental retardation, autism and Fragile X syndrome, the understanding of the mechanisms and functions of mGluR-LTD has become a major attractive area of study (Ronesi and Huber, 2008). The major cause of autism spectrum disorders is considered to be Fragile X syndrome which is an inherited intellectual disability (Santoro et al., 2012). Enhanced group I mGluR-LTD is reported in the hippocampus and cerebellum of the mouse model of Fragile X syndrome (Dölen et al., 2007). Importantly, the cognitive and intellectual deficits in the mouse, zebrafish and fruit fly models of Fragile X syndrome were shown to be rescued upon administration of the mGluR5 antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP) (McBride et al., 2005; Yan et al., 2005; Santoro et al., 2012). Moreover, the rescued phenotype of the disease was observed in the Fragile X syndrome mice carrying selective genetic deletions within the mGluR5 gene (Dölen et al., 2007; Bassell and Gross, 2008).

## 1.7. Group I mGluRs: desensitization and trafficking

Prolonged or repeated ligand exposure results in the decreased sensitivity of the receptor towards the ligand and this phenomenon is called “desensitization” of the receptor. Like many other GPCRs, group I mGluRs also get desensitized upon agonist activation and subsequently get internalized (Gereau and Heinemann, 1998b; Francesconi and Duvoisin, 2000b; Dhami and Ferguson, 2006b). Group I mGluRs get phosphorylated by various kinases, viz., GRKs, PKC, PKA resulting in the binding of β-arrestin to the receptor, which in turn leads to the desensitization of the receptor by uncoupling the receptor from the G-protein involved (Catania et al., 1991; Alaluf et al., 1995; Gereau and Heinemann, 1998a; Ciruela et al., 1999; Dale et al., 2000a; Francesconi and Duvoisin, 2000a; Sallese et al., 2000; Iacovelli et al., 2003a; Kim et al., 2005; Dhami and Ferguson, 2006a; Bhattacharyya, 2016). Desensitization is believed to be an important physiological feedback mechanism adopted by cells to protect themselves from chronic or acute receptor overstimulation. Desensitization of group I mGluRs is phosphorylation dependent and very well studied. For example, activation of PKC plays a crucial role in the desensitization and trafficking of group I mGluRs (Francesconi and Duvoisin, 2000a; Dale et al.,

2001; Mundell et al., 2003). PKC-dependent phosphorylation of mGluR1a and mGluR1b leads to the desensitization of the receptor (Mundell et al., 2004; Dhami and Ferguson, 2006a). PKC has also been reported to phosphorylate multiple serine/threonine residues present in the intracellular carboxy-terminal tail of mGluR5 and is also known to initiate the desensitization as well as the internalization processes (Gereau and Heinemann, 1998a).

In addition to the above results, another protein kinase, viz., PKA has been reported to regulate the desensitization of mGluR1. Phosphorylation by PKA results in the uncoupling of the adapter proteins, which in turn inhibits the endocytosis of the receptor (Francesconi and Duvoisin, 2000b). The G-protein coupled receptor kinases (GRKs) also play crucial roles in the desensitization of group I mGluRs. For example, the role of GRK4 has been reported in mGluR1 desensitization in cerebellar Purkinje cells, whereas desensitization of mGluR5 seems to be GRK4-independent (Iacovelli et al., 2003b; Sorensen and Conn, 2003). GRK2 desensitizes mGluR1 as well as mGluR5 in phosphorylation-dependent and phosphorylation-independent manner (Ferguson et al., 1996a; Dale et al., 2000b; Dhami et al., 2002; Ribeiro et al., 2009). The residues phosphorylated by GRKs are recognized by the arrestin group of proteins, which uncouple the G-proteins from the receptor, leading to the desensitization of the receptor (Lefkowitz and Shenoy, 2005). The arrestin group of proteins also recruit other proteins which promote receptor internalization and are believed to be important for GPCR resensitization and downregulation (Mundell et al., 2001). Furthermore, interaction of the group I mGluRs with the Huntingtin binding protein, Optineurin, restrains their coupling with G-proteins in the PLC/$IP_3$ pathway resulting in the phosphorylation-independent desensitization of group I mGluRs (Dhami and Ferguson, 2006b). mGluR activity can also be attenuated at the level of G-proteins by proteins called regulators of G-protein signaling (RGS). RGSs catalyze the hydrolysis of GTP bound to the $G_{\alpha}$ subunit of heterotrimeric G-proteins, leading to their inactivation. Among them, RGS2 and RGS4 associate with $G_{\alpha q/11}$ proteins to attenuate the group I mGluR-mediated PLC/$IP_3$ signaling pathway (Dhami and Ferguson, 2006b).

Like many other GPCRs, group I mGluRs also undergo rapid internalization following ligand exposure (Mundell et al., 2001; Mundell et al., 2003; Mundell et al., 2004). The internalization of group I mGluRs subsequent to the ligand stimulation starts as early as 1 min and maximum

internalization was observed till 30 min post ligand application in both non-neuronal cells as well as in primary hippocampal neurons (Pandey et al., 2014; Mahato et al., 2015). The receptors recycle back to the cell surface in around 2.5 hr - 3 hr in the heterologous cells as well as in primary hippocampal neurons (Pandey et al., 2014; Mahato et al., 2015). Over-expression of the dominant negative form of arrestin and dynamin inhibits the ligand-mediated endocytosis of group I mGluRs, suggesting that internalization of group I mGluRs is arrestin and dynamin dependent (Dale et al., 2001; Mundell et al., 2001). The phosphorylation of group I mGluRs mediated by GRKs and second messenger-dependent kinases regulate the trafficking of these receptors. GRK4 has been found to be involved in the internalization of mGluR1 in heterologous cells. In addition, acute knockdown of GRK4 in cerebellar purkinje cells inhibits ligand-mediated internalization of mGluR1 (Sallese et al., 2000; Iacovelli et al., 2003a). GRK2, on the other hand, modulates the trafficking of group I mGluRs in a different way. It affects the internalization of mGluR1 over a different time course compared to GRK4 (Iacovelli et al., 2003a). Various second messengers have also been reported to regulate the trafficking of group I mGluRs. The internalization of mGluR1 and mGluR5 has been reported to be PKC-dependent (Mundell et al., 2003). In case of mGluR5, the calmodulin protein is associated with the receptor in its basal state. Activation of mGluR5 triggers the increase in the intracellular $Ca^{2+}$ and activation of PKC which in turn phosphorylates S901 at the C-terminus of the receptor (Lee et al., 2008; Ko et al., 2012). This phosphorylation results in the disruption of the binding of calmodulin with the receptor, the end result of which is the increased internalization and decreased surface expression of the receptor (Ko et al., 2012). Group I mGluRs can also undergo internalization in the absence of the ligand, which is termed as “constitutive endocytosis”. mGluR5 is known to enter the recycling compartment subsequent to the constitutive internalization (Trivedi and Bhattacharyya, 2012; Bhattacharyya, 2016). The mechanism of recycling of the constitutively endocytosed receptors is currently unknown.

Subsequent to the internalization, GPCRs can have various subcellular fates. Some GPCRs go to the recycling compartment and recycle back to the cell surface, whereas others land in the lysosome for degradation, like the PAR1 receptor (Trejo and Coughlin, 1999). What route a particular GPCR would take depends on the type of the receptor, type of the ligand and the type of the system (Bhattacharyya, 2016). Group I mGluRs enter the recycling compartment

subsequent to the ligand-mediated internalization and the kinetics of recycling seems to be similar in both non-neuronal and neuronal cells. Interestingly, the exit of the receptor from the recycling compartment is dependent on the pH of the endosomes (Pandey et al., 2014; Mahato et al., 2015). The receptors get trapped in the recycling compartment if the pH gradient is destroyed. More importantly, recycling of mGluR1 depends on the activity of protein phosphatase 2A (PP2A) (Pandey et al., 2014). Recycling of the other member of the group I mGluR family, mGluR5, appears to be completely dependent on the activity of PP2A. Inhibition of protein phosphatase 2B (PP2B) partially affects the recycling of mGluR5 (Mahato et al., 2015). These results together suggest that the recycling of both mGluR1 and mGluR5 is dependent on the dephosphorylation events mediated by PP2A and PP2B. The identity of the substrates for these two phosphatases is currently unknown **(Figure 1.8)**.

## 1.8. Role of ubiquitination in group I mGluR regulation

An important feature of GPCR-linked signaling networks is that they are extensively regulated, particularly at the level of the receptor itself. Ubiquitination is a reversible post-translational modification responsible for regulating trafficking of various GPCRs. Ubiquitination acts as a sorting signal to facilitate the trafficking of many mammalian GPCRs from endosomes to lysosomes for degradation (Marchese et al., 2008). But for a number of GPCRs, ubiquitination plays many other roles as well (Tanowitz and von Zastrow, 2002). In case of yeast GPCR (ste2 receptor), ubiquitination of a lysine residue at the C-terminus of the receptor serves as a signal for the ligand-stimulated endocytosis (Hicke and Riezman, 1996). Many GPCRs have been reported to get ubiquitinated subsequent to the activation by the ligand, like $\beta_2$ -adrenergic receptor, μ-Opioid (MOR) receptor, chemokine receptor, CXCR4, PAR1 receptor etc. (Marchese and Benovic, 2001; Hislop and von Zastrow, 2011; Xiao and Shenoy, 2011). Group I mGluRs get ubiquitinated by Siah-1A. Seven in absentia homolog (Siah-1A) belongs to the RING family of E3 ubiquitin ligases. Siah-1A binds at the C-terminus of both mGluR1 and mGluR5 (Ishikawa et al., 1999; Kammermeier and Ikeda, 2001; Moriyoshi et al., 2004). Siah-1A competes with the $Ca^{2+}$/calmodulin (CaM) for binding with the mGluR1 and mGluR5 in a phosphorylation-dependent manner. In case of mGluR5, phosphorylation at the S901 favours Siah-1A binding by

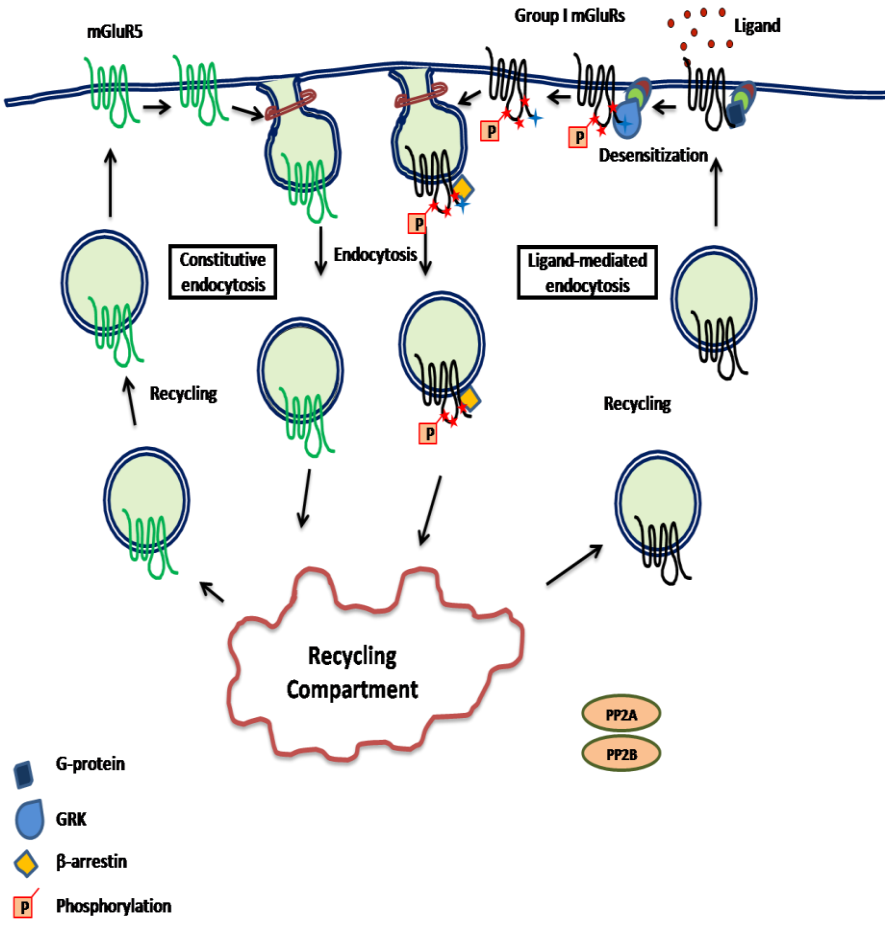


**Figure 1.8. Trafficking of group I mGluRs.** Group I mGluRs internalize constitutively, as well as upon ligand application. Ligand-mediated internalization of group I mGluRs is phosphorylation-dependent. The endocytosed receptors enter the recycling compartment. Majority of the internalized receptors recycle back to the cell surface. PP2A and PP2B play important role in the recycling of group I mGluRs subsequent to the ligand-mediated internalization. Constitutively endocytosed receptors also recycle back to the cell surface.

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displacing CaM (Lee et al., 2008; Ko et al., 2012). Thus CaM regulated Siah-1A binding to mGluR5 dynamically regulates mGluR5 trafficking.

## 1.9. Prelude to the present study

Ubiquitination is an important reversible post-translational modification that has been reported to regulate the function of many GPCRs. Ubiquitination acts as a sorting signal to facilitate the trafficking of many mammalian GPCRs from endosomes to lysosome for degradation. It also serves as a signal for receptor endocytosis. In the present study, we have shown that ubiquitination of group I mGluRs is crucial for the agonist-induced internalization of the receptor. Monoubiquitination is not sufficient to induce the internalization, whereas, K63-linked polyubiquitination is required for the agonist-induced internalization of mGluR1. We have shown that lysine present at the 1112 position in the C-terminus tail of mGluR1 is crucial for the ligand-mediated internalization of mGluR1. Inhibition of ubiquitination using pharmacological inhibitor, as well as knockdown of E3 ligase, Siah-1A using siRNA blocked the ubiquitination of mGluR1 as well as the internalization of the receptor. We have also shown that blocking the ubiquitination and activating the group I mGluRs with agonist, led to the enhanced AMPAR internalization. Work done for various GPCRs have suggested that balance between the ubiquitination and deubiquitination orchestrates cellular physiological response. We also investigated the role of deubiquitinases in the trafficking of mGluR1 in non-neuronal HEK293 cells as well as in primary hippocampal neurons. Our data suggested that the deubiquitinase USP19 played a critical role in the trafficking of mGluR1.

# Materials and Methods

## 2.1. Materials

### 2.1.1. Cell culture reagents

Neurobasal medium, B27 supplement, Dulbecco's Modified Eagle Medium (DMEM), Minimal Essential Medium (MEM), Fetal Bovine Serum (FBS), Glutamax-100, antibiotic-antimycotic mix, Trypsin-EDTA, Lipofectamine 2000 and all other cell culture reagents were purchased from Invitrogen (USA). 5-Fluoro-2'-deoxyuridine (FUDR), poly-D-lysine, Mito + Serum Extender, Uridine, Bovine Serum Albumin (BSA), DNaseI, Polyethylenimine (PEI), FluoromountTM aqueous mounting medium and Paraformaldehyde (PFA) were purchased from Sigma (USA). HEK293 cells and HEK293T cells were purchased from NCCS Pune (India). Alexa-568 labelled Transferrin was purchased from Invitrogen (USA).

### 2.1.2. Molecular biology reagents

Various restriction enzymes, Q5 DNA polymerase, Phusion DNA polymerase, dNTP mixture were purchased from New England Biolabs (USA). Red taq jump start polymerase was bought from Sigma (USA).

### 2.1.3. Plastic wares and chemicals

All plastic wares related to cell culture studies were purchased from BD Falcon (USA). Plastic wares that were used for molecular biological and bacteriological experiments were obtained from Tarsons (India). Fine chemicals were procured from Merck limited (USA) and Life technologies (USA). Bacterial media, agar, salts and buffers were purchased from HiMedia.

### 2.1.4. Antibodies

Anti-myc mouse monoclonal and anti-myc rabbit polyclonal antibodies, anti-LAMP1 rabbit polyclonal antibodies were purchased from Abcam (UK). Anti- HA rat monoclonal antibody was from Roche (USA), anti-GluA1 rabbit polyclonal antibody was purchased from Calbiochem

(USA), anti-Bassoon mouse monoclonal antibody was purchased from Enzo Life Sciences (Switzerland). Anti-β-actin mouse monoclonal antibody, anti-GFP rabbit polyclonal antibody and anti-GAPDH rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology (USA). Anti-FLAG rabbit polyclonal antibody was purchased from Sigma (USA). Goat anti-mouse HRP, goat anti-rabbit HRP and goat anti-rat HRP were purchased from Sigma (USA). All other secondary antibodies were purchased from Invitrogen (USA).

### 2.1.5. Drugs

2,4- bis dihydroxy phenyl glycine (R,S-DHPG), 4-[4-[(5-Nitro-2-furanyl)methylene]-3,5-dioxo-1-pyrazolidinyl]benzoic acid ethyl ester (PYR-41), 6,7-Dinitroquinoxaline-2,3-dione disodium salt (DNQX), D-(-)-2-Amino-5-phosphonopentanoic acid (AP5) were purchased from Tocris (USA). Tetrodotoxin (TTX) was from Adooq bioscience (USA). Dimethyl sulfoxide (DMSO), N-Ethylmaleimide (NEM) were obtained from Sigma (USA).

### 2.1.6. Constructs

The myc-mGluR1 and myc-mGluR5 constructs were generously gifted by Kathrine Roche (National Institute of Health, USA). In this construct, the myc epitope was tagged at the N-terminus of the full-length mGluR1 and mGluR5. The FLAG-mGluR1 construct was a generous gift from Johanna Montgomery (The University of Auckland, New Zealand). The USP19 constructs, viz., wild-type myc-USP19, catalytically inactive myc-USP19 (myc-USP19C506S), wild-type USP19-GFP and catalytically inactive USP19-GFP (USP19C506S-GFP) were obtained from Maria G Masucci (Department of cell and molecular biology, Karolinska Institute Stockholm, Sweden).

We have used various ubiquitin constructs in this study. Ubiquitin constructs were kind gifts from Maddika Subba Reddy (Center for DNA Fingerprinting and Diagnostics, India). All ubiquitin constructs were Hemagglutinin (HA) tagged at the N-terminus of the ubiquitin protein. Ubiquitin is a highly conserved 76 amino acid protein. The HA-tagged wild-type ubiquitin construct (HA-WTUb) contains seven lysine residues. In another construct, HA- K0Ub, all seven

lysine residues were mutated to arginine. Overexpression of this construct will result in monoubiquitination or multi-monoubiquitination of proteins. In HA-K48RUb construct, the lysine residue present at the 48 position was mutated to arginine. Overexpression of this construct leads to the inhibition in the formation of K48-linked polyubiquitin chain. Similarly, to inhibit K63-linked polyubiquitin chain formation, we used a ubiquitin construct (HA-K63RUb), in which, the lysine residue present at the 63 position was mutated to arginine **(Figure 2.1)**.

### 2.1.7. Buffers and media

**Luria-Bertani (LB) medium:** 10 g tryptone, 5 g yeast extract and 10 g NaCl were dissolved in 800 ml of double-distilled water. The pH was adjusted to 7 and the volume was made upto 1 litre in double-distilled water and autoclaved.

**10X phosphate buffered saline (PBS):** 80 g NaCl, 2 g KCl, 14.4 g $Na_2HPO_4$ and 2.4 g $KH_2PO_4$ were dissolved in 800 ml of double-distilled water. The pH was adjusted to 7.4 and the volume was made upto 1 litre with water and sterilized by autoclaving.

**4% paraformaldehyde (PFA):** 4 g paraformaldehyde was dissolved in 70 ml of phosphate buffer saline (pH - 7.4) at 60°C by adding 1 M NaOH dropwise till solution become transparent. The pH of the solution was then adjusted to 7.4 and subsequently final volume was made up to 100 ml. Solution was then aliquoted into 15 ml falcon tubes and stored at -20°C.

**Laemmli sample buffer:** 50 mM Tris-HCl (pH - 6.8), 10% Glycerol, 0.005% Bromophenol blue, 1% β-mercaptoethanol and 2% SDS were made in double-distilled water.

**RIPA lysis buffer:** 50 mM Tris (pH - 8), 150 mM NaCl, 1% Triton-X 100, 0.1% SDS, 2 mM EDTA and 0.5% sodium deoxycholate were made in double-distilled water.

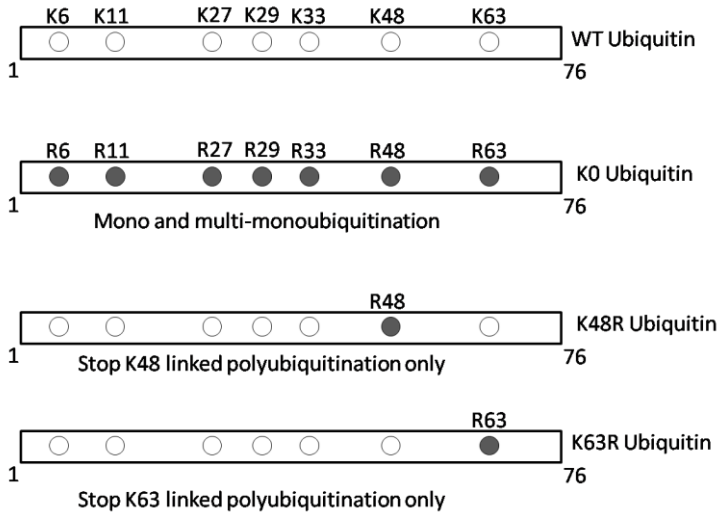



**Figure 2.1. Schematic is showing various ubiquitin constructs used in this study (wild-type Ub, K0-Ub, K48R-Ub, and K63R-Ub).**

**SDS running buffer (1X):** 3 g Tris base, 14.4 g Glycine and 1 g SDS were dissolved in 800 ml of distilled water followed by making up the volume to 1 litre (final pH - 8.3)

**Transfer buffer for western blot (1X):** 14.4 g Glycine, 3 g Tris base, 150 ml Methanol and 850 ml double-distilled autoclaved water were mixed together.

**DMEM:** Dulbecco's Modified Eagle medium (DMEM) powder and 3.7 g sodium bicarbonate were dissolved in 800 ml double-distilled autoclaved water. The pH of the media was adjusted to 7.4 and then the volume was made upto 1 litre with double-distilled autoclaved water. The media was filter-sterilized using 0.22 µm filter.

**10% DMEM:** Fetal bovine serum (FBS) and antibiotic-antimycotic (1X) mixture were added in DMEM such that the DMEM contains 10% FBS.

**Cell freezing mixture:** 10% Dimethyl sulfoxide (DMSO) in 90% fetal bovine serum (FBS).

**2X HEPES buffer saline (HEBS):** 274 mM NaCl, 9.5 mM KCl, 15 mM Glucose, 42 mM HEPES, 1.4 mM $Na_2HPO_4$.

**Poly-D-Lysine sodium borate solution:** 500 ml of 0.1 M sodium borate solution of pH - 8.4 was prepared by dissolving sodium borate in autocleaved double distilled water. Subsequently, 5 mg poly-D-Lysine was added into the 0.1 M sodium borate solution and filter sterilized.

**Dissection solution:** Dissection solution for the dissection of the mouse brain was prepared by mixing 18.8 g NaCl , 0.74 g KCl, 0.26 g $MgSO_4$, 0.86 g $CaCl_2$, 2.4 g HEPES, 2.0 g glucose and 0.004 g phenol red in 2 litre sterile water. pH of the solution was then adjusted to 7.4 and filter sterilized using 0.22 µm filter.

**Enzyme solution:** Enzyme solution was prepared by adding 2 mg L-Cysteine, 100 µl of 50 mM EDTA, 100 µl of 100 mM $CaCl_2$, 30 µl of 1 N NaOH, papain and 100 µl DNase in 10 ml dissection solution.

**Serum media:** Add 25 ml fetal bovine serum, 7.5 ml Hi-Glucose/MEM and 1 ml Mito+ serum extender in 500 ml MEM w/ Earle's salts w/o L-glutamine. Mix well and filter sterile. Make small one time use aliquots in 50 ml falcon.

**Inactivation solution:** Inactivation solution was prepared by mixing 25 mg BSA and 100 µl DNase in 10 ml serum media.

**Polyethylenimine (PEI) solution:** Polyethylenimine is a stable cationic polymer having density close to 1 (Boussif et al., 1995). PEI condenses DNA into positively charged particle and the DNA-PEI complex through endocytosis enters into the cell (Sonawane et al., 2003). 100 mg PEI was dissolved in 10 ml water (DNase free, Sigma). This PEI solution was further diluted to obtain 1 µg / µl solution and pH was adjusted to 7.0. Finally, the solution was filter sterilized.

### 2.1.8. Instruments

**Cell culture related instruments:** Biosafety cabinets for cell culture were from Labconco (USA), Eppendorf 5810R centrifuge was from Eppendorf, tissue culture incubators (Galaxy 170R) were from Eppendorf (Germany), dissection microscope was from Olympus (Japan), fluorescence microscope was from Zeiss (Germany), pipette aids were from Thermo Fisher Scientific (USA), water bath was from Grant (UK) and sesaw rocker was from Stuart (UK).

**Instruments related to the molecular biology experiments:** The instruments used for the molecular biology experiments and their sources have been given below:
Rocker incubator (MS Major Science, USA), heating block (Eppendorf, Germany), table top centrifuge (Eppendorf, Germany), thermocycler (BioRed, USA), water bath (Memmert, Germany), weighing balance (Sartorius, Germany), pH meter (Sartorius, Germany) and vortex (Stuart, UK), 4˚C chiller (Vestfrost, Denmark), 4˚C fridge (Hitachi, Japan), -20˚C freezer (Vestfrost, Denmark), -80˚C freezer (New Brunswick, Germany).

### 2.1.9. Primers

Site-directed mutagenesis approach was used to generate various mutants of myc-mGluR1. Lysine residue present at the 1193 position of mGluR1 was changed to arginine and named as 1K/RmGluR1. In this 1K/R background, we generated another construct named 2K/RmGluR1 in which lysine residue present at the 1141 position was also converted to arginine. In this 2K/R background another lysine residue at the 1112 position was changed to arginine (3K/RmGluR1). **Figure 2.2** shows the schematic of various mutants of myc-mGluR1.The primers used for these mutagenesis have been listed below. In all primers sequences, position of the mutation is represented in lowercase.

1K/R forward: 5'- CTGAGGGACTACAgGCAAAGCTCTTCC-3'

1K/R reverse: 5'- GGAAGAGCTTTGCcTGTAGTCCCTCAG-3'

2K/R forward: 5'- CCCACAGCCAGCAgGCTGACCCCTG -3'

2K/R reverse: 5'- CAGGGGTCAGCcTGCTGGCTGTGGG- 3'

3K/R forward: 5'- GACAGTGAGAGATTCAgGCTCCTGCAGGA- 3'

3K/R reverse: 5'- TCCTGCAGGAGCcTGAATCTCTCACTGTC 3'

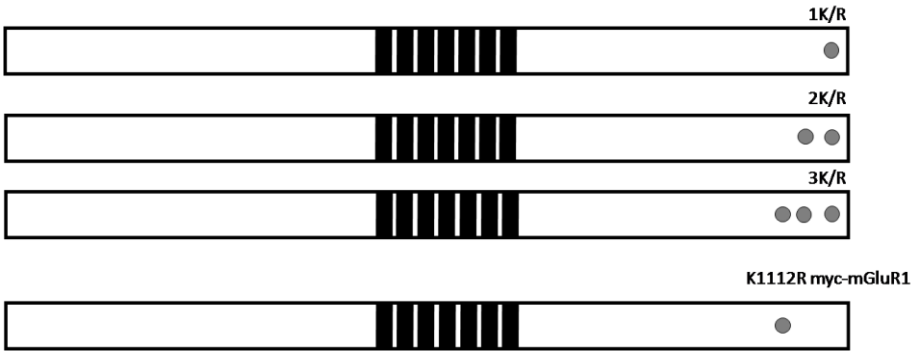



**Figure 2.2. Schematic presentation of variuous mutants of myc-mGluR1.**

## 2.2. Methods

### 2.2.1. Preparation of competent cells

A single TOP10 DH5α bacterial colony from a culture plate was taken and incubated into 10 ml LB broth for overnight at 37°C. From this primary culture, 100 µl was taken and incubated into 100 ml LB media till the optical density of the culture reached at 0.4 - 0.6. Subsequently, the culture was centrifuged at 6000 rpm at 4°C for 15 min. Afterwards, the supernatant was decanted and pellet was resuspended in 10 ml of 0.1 M ice-cold $CaCl_2$ solution and incubated on ice for 15 min. After that, the solution was centrifuged at 6000 rpm at 4°C for 15 min. Supernatant was discarded and then the pellet was resuspended in 5 ml of 0.05 M $CaCl_2$ solution and kept in ice for 45 min. Subsequently, recovery of the cells was done by centrifugation at 5000 rpm at 4°C for 5 min. Finally, the pellet was resuspended in 85% 0.1 M $CaCl_2$ solution and 15% glycerol. The solution was aliquoted into 1.5 ml microcentrifuge tubes and stored at -80°C.

### 2.2.2. Transformation

The TOP10 competent cells were thawed on ice for 10 min. Afterwards, 500 ng DNA was added and incubated for 30 min on ice. Subsequently, heat shock was given for 90 sec at 42°C and after that, cells were kept on ice for 5 min. On completion of the incubation time, 1 ml LB media was added and the culture was placed on water bath for 1 hr at 37°C. Cells were then centrifuged at 5000 rpm for 5 min. Subsequently, the pellet was resuspended in LB media and appropriate amount was plated on antibiotic containing LB agar plates.

### 2.2.3. Plasmid isolation

Isolation of plasmids for transfection purposes in cells was done using Qiagen mini or midi kit following manufacturer's instructions. DNA used for cloning and screening purposes, was isolated by alkaline lysis method.

### 2.2.4. Generation of various mutants of mGluR1

Initially, the PCR amplification was done using appropriate primer set. Subsequently, the PCR amplified product was purified using PCR purification kit (Qiagen, Germany). The purified product was then treated with DpnI restriction enzyme for 1 hr at 37˚C, followed by heat inactivation of DpnI at 80˚C for 20 min. Following that, DpnI digested PCR purified product was transformed into TOP10 bacterial competent cells and plated onto LB agar plates containing 100 µg / ml Ampicillin. Colonies were selected randomly, followed by plasmid isolation and confirmation of mutation by sequencing.

### 2.2.5. Cell culture

**HEK293 and HEK293T cell culture and transfection:** Both, HEK293 cells and HEK293T cells were cultured in DMEM supplemented with 10% FBS, 1X antibiotic-antimycotic (AB-AM) mix in 5% $CO_2$ and 95% humidity at 37˚C. Cells were cultured on 12 mm coverslips placed in 24 well plate, pre-coated with 50 µg / ml poly-D-lysine. The cells were transfected with appropriate plasmid DNA constructs at 65 – 70% confluency with the help of Lipofectamine 2000 reagent by mixing 2 µg cDNA with 10 µg Lipofectamine 2000 in 1 ml OptiMEM. Cells were incubated in the transfection mixture for 6 hr and subsequently, the transfection mixture was replaced with fresh $CO_2$ saturated 10% DMEM and all experiments were done 24 hr post-transfection.

HEK293 cells and HEK293T cells were also transfected using PEI by mixing 2 µg plasmid DNA with 6 µg PEI in 1 ml plain DMEM. Cells were incubated in transfection mix for 6 - 7 hr. Subsequently, the transfection mixture was replaced with fresh 10% DMEM and all experiments were performed 24 hr post-transfection.

**Dissociated primary hippocampal neuron culture:** Primary hippocampal neurons were prepared from P0/P1 C57BL/6 mouse pups. Pups were first sacrificed and hippocampi were dissected out. Subsequently, tissues were dissociated using enzymatic solution containing papain

for 30 min at 37˚C, followed by inactivation of papin with serum media. Following that, trituration step was performed with glass pipette to obtain single cells. Neurons were then plated on pre-coated cover slips (50 µg / ml poly-D-lysine + 0.1 M sodium borate, pH - 8.4) at a density of approximately 120,000 cells for mGluR trafficking experiments and around 80,000 cells for AMPAR trafficking experiments per 12 mm coverslip placed in a 24 well plate. Cultures were maintained in Neurobasal media containing 0.5 mM glutamine and B27 supplement. Glial growth was inhibited by adding FUDR (floxuridine) on 3$^{rd}$ day of culture.

**Transfection in primary hippocampal neurons and knockdown experiments:** Primary hippocampal neurons were transfected with myc-mGluR1 or myc-mGluR5 constructs at 8-9 day *in vitro* using calcium phosphate. Old Neurobasal-B27 media was replaced by plain Neurobasal media. 2.5 µg of DNA constructs for each well were mixed with 250 mM $CaCl_2$ in a microcentrifuge tube and incubated for 5 min, followed by mixing with 2X HEBS buffer. The mixture was then incubated for 30 min in the dark. It was subsequently added in cells and incubated for the time till the appearance of sufficient sand like crystals were observed under the bright field microscope. Subsequently, the cells were washed with washing buffer three times followed by washing with plain Neurobasal media and then old Neurobasal – B27 media was added. Half feeding was done on next day. For knockdown experiments, ON-TARGET plus SMARTpool siRNA against Siah-1A and scrambled siRNA (Thermo Scientific Dharmacon, Lafayette, CO, USA) were either transfected alone (for AMPAR endocytosis experiments) or cotransfected with myc-mGluR1 cDNA (for mGluR1 endocytosis experiments) in primary hippocampal neurons at 8-9 day *in vitro* using Lipofectamine2000 following the manufacturer's instructions and experiments were carried out in cells at 12-14 day *in vitro*.

### 2.2.6. Group I mGluR endocytosis assay

HEK293 cells or primary hippocampal neurons were transfected with either myc-mGluR1 (wild-type or various mutants) or myc-mGluR5 cDNA as described above. Live cells were treated with

mouse anti-myc primary antibody (1:500 for HEK293 cells and 1:200 for primary hippocampal neurons) for 15 min (HEK293 cells) or 20 min (primary hippocampal neurons) at 37˚C. Cells were then washed with plain media (glutamate free) and 100 µM R,S-DHPG was applied for 5 min. Cells were then incubated at 37˚C in plain DMEM / Neurobasal media in the absence of the ligand for various time points. Subsequently, cells were fixed without permeabilization using ice cold 4% paraformaldehyde (PFA) for 15 min on ice. Surface localized receptors were then labelled with saturating concentration of goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) for 1 hr at 37˚C. Cells were then permeabilized with 0.1% Triton X-100 for 30 min in room temperature. The endocytosed receptors were then labelled by the application of goat anti-mouse Alexa-647 conjugated secondary antibody (1:750) for 1 hr at 37˚C. The coverslips were then mounted on glass slides and imaged under the confocal microscope. For experiments involving PYR-41 and NEM, cells were pre-treated with PYR-41 (50 µM) or NEM (5 µM) for 30 min before the application of the primary antibody. Subsequently, the endocytosis assay was performed identically as described above. PYR-41 and NEM was present throughout the experiment. The effect of knockdown of the endogenous Siah-1A on the internalization of myc-mGluR1 in primary hippocampal neurons was investigated by co-transfection of cells with myc-mGluR1 and si-Siah-1A. For all experiments, two coverslips were used for each condition and all experiments were repeated at least three times.

To ensure that the Alexa-647 conjugated secondary antibody did not label any detectable surface receptors in our assays, we performed control experiments to determine the saturating concentration of the Alexa-568 conjugated secondary antibody. When the saturating concentration of Alexa-568 conjugated secondary antibody was applied to visualize the surface receptors, it prevented any further detectable staining of the surface receptor when Alexa-647 conjugated second secondary antibody was applied in non-permeabilized cells. Whereas, upon permeabilization of the cells with 0.1% Triton X-100 for 30 min at room temperature, the internalized receptors were observed on application of Alexa-647 conjugated secondary antibody **(Figure 2.3 A, B)**. The control experiments suggested that in all assays, both in HEK293 cells and primary hippocampal neurons, Alexa-647 conjugated secondary antibody did not label any detectable amount of surface receptors and thus it stains the internalized receptors only.

**A**

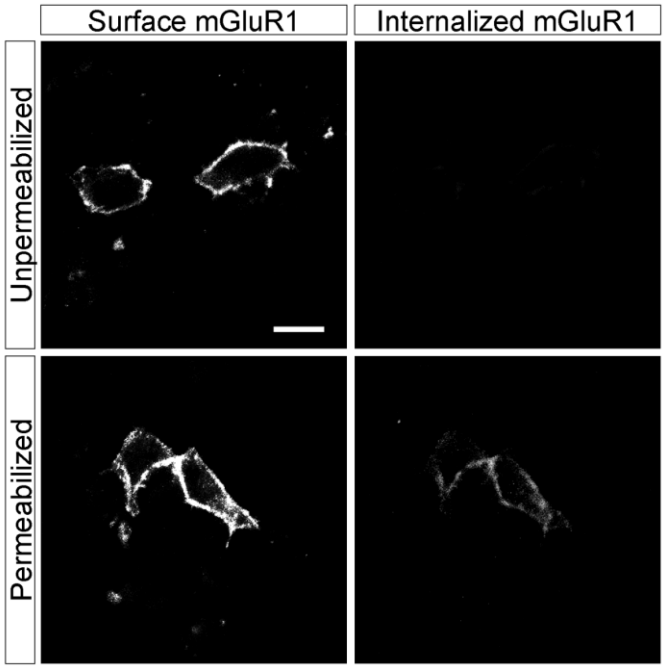



**Figure 2.3A. Standardization of antibody feeding assay in HEK293 cells.** The upper panel shows that saturating concentration of the first secondary antibody (Alexa-568 conjugated) labelled the surface receptors (upper left panel) which prevented any further observable staining of the Alexa-647 conjugated second secondary antibody (upper right panel) in unpermeabilized condition. However, upon permeabilization, the second secondary antibody labelled the internalized myc-mGluR1 (lower right panel). Scale bar = 10 μm.

**B**

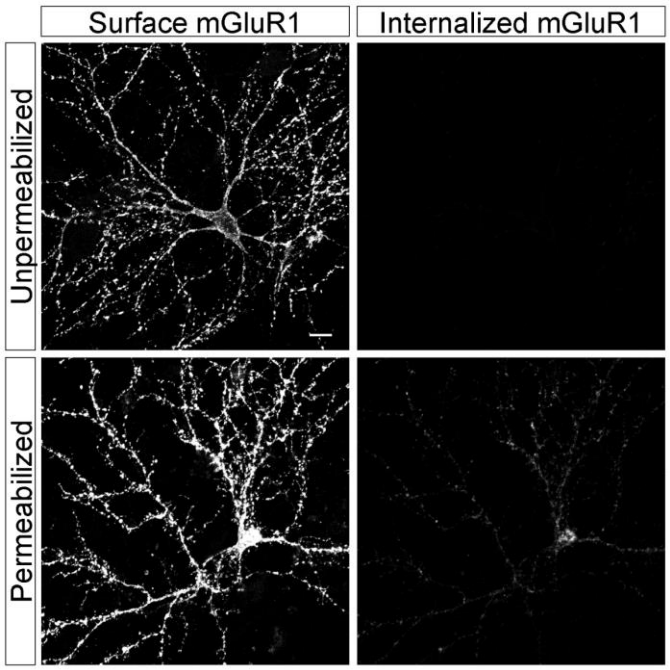



**Figure 2.3B. Standardization of antibody feeding assay in primary hippocampal neurons.** Application of saturating concentration of the Alexa-568 conjugated first secondary antibody stained the surface receptors (upper left panel) and subsequent application of the Alexa-647 conjugated second secondary antibody did not show any detectable staining in unpermeabilized condition (upper right panel). However, when cells were permeabilized, the second secondary antibody labelled the internalized myc-mGluR1 (lower right panel). Scale bar = 10 μm.

### 2.2.7. AMPA receptor endocytosis assay

In order to study the group I mGluR-mediated AMPA receptor (AMPAR) endocytosis, cells were pre-incubated with a mixture of antagonists viz., 1 μM TTX (neurotransmitter release blocker), 20 μM DNQX (AMPAR antagonist) and 50 μM APV (NMDAR antagonist) for 30 min at 37˚C. Subsequently, surface GluA1 containing AMPARs were labelled in live neurons by 15 min incubation at 37ºC with a rabbit polyclonal antibody directed against the N-terminus of the GluA1 subunit (1:20). After washing, cells were treated with 100 μM R,S-DHPG for 5 min. R,S-DHPG was then removed and cells were further incubated in the presence of antagonists for a total of 15 min at 37ºC. Cells were subsequently fixed in 4% PFA for 15 min on ice without permeabilization and surface receptors were visualized by using saturating amount of goat anti-rabbit Alexa-568 conjugated secondary antibody (1:100) for 1 hr at 37˚C. After that, cells were permeabilized with 0.1% Triton X-100 for 30 min at room temperature and staining of internalized receptors with goat anti-rabbit Alexa-647 conjugated secondary antibody (1:750) was performed for 1 hr at 37˚C. Control experiments indicated that application of the first secondary antibody was effective in staining all remaining surface receptors, as application of the second secondary antibody yielded no detectable Alexa-647 signal in non-permeabilized cells (data not shown).

### 2.2.8. Colocalization assay

To investigate whether group I mGluR activation by R,S-DHPG leads to the endocytosis of synaptic AMPARs, synaptic GluA1 puncta were quantified by colocalization of surface GluA1 puncta and presynaptic Bassoon puncta. Subsequent to the staining of the live cells with the anti GluA1 rabbit polyclonal antibody (1:20), cells were either not treated (control) or treated with 100 μM R,S-DHPG using the similar protocol as described for the “AMPAR endocytosis assay”. Cells were then fixed in 4% PFA on ice for 15 min without permeabilization and were incubated with goat anti-rabbit Alexa-568 conjugated secondary antibody for 1 hr at 37ºC. Cells were subsequently permeabilized in 0.1% Triton X-100 for 30 min at room temperature and were incubated with a mouse monoclonal antibody (1:500) against Bassoon (Stressgen) for overnight

at 4ºC. After that, cells were treated with goat anti-mouse Alexa-488 conjugated secondary antibody for Bassoon puncta visualization.

To study the sub-cellular fate of myc-mGluR1 after R,S-DHPG-mediated endocytosis in the presence of NEM (N-Ethylmaleimide), myc-mGluR1 expressing HEK293 cells or primary hippocampal neurons were preincubated with 5 µM NEM and 50 µM MG-132 for 30 min. After that, cells were incubated with anti-myc primary antibody for 20 min at 37˚C followed by the application of 100 µM DHPG for 5 min. Subsequently, cells were chased for longer time point in the absence of the ligand for 30 min and 2.5 hr. On completion of the chase period, cells were surface stripped by treating them with ice cold striping solution (0.5% $CH_3COOH$ + 500 mM NaCl) for 90 seconds on ice to remove the antibodies attached to the surface receptors that did not internalize on application of the ligand. Cells were then fixed by 4% PFA and permeabilized in 0.1% Triton-X 100. Subsequently, internalized receptors were labelled with goat anti-mouse Alexa-568 conjugated secondary antibody. Following that, cells were stained with rabbit polyclonal antibody against LAMP1 (1:500) at 4ºC overnight. To visualise the LAMP1 in cells, secondary antibody tagged with Alexa-488 (1:800) was applied for 1 hr at 37˚C. Finally, cells were mounted on glass slide and observed under the confocal microscope.

### 2.2.9. Recycling assay

HEK293 cells or primary hippocampal neurons were transfected with myc-mGluR1 cDNA. Subsequent to the anti-myc primary antibody staining, 100 µM R,S-DHPG was applied for 5 min at 37˚C. After that, cells were chased for various time periods in plain media in the absence of the ligand. At specific time points, cells were fixed and surface and internalized receptors were labelled by Alexa-568 conjugated and Alexa-647 conjugated secondary antibodies respectively, using the same protocol as described for the endocytosis assay. To study the effect of NEM in presence and absence of MG-132 on the recycling of myc-mGluR1, cells were pre-treated with the drugs for 30 min before the application of the anti-myc primary antibody and recycling assays were performed using the protocol described above. Drugs were present till the cells were fixed in 4% PFA.

### 2.2.10. Transferrin receptor kinetics assay

HEK293 cells were plated on 12 mm coverslips in 24 well plates and were pre incubated in plain DMEM containing either DMSO or 50 µM PYR-41 for 30 min at 37˚C. Subsequently, 20 µg / ml Alexa-568 labelled transferrin was applied for 2 min at 37ºC. Cells were then washed with plain DMEM and chased for various time periods (5 min, 15 min, 30 min and 60 min) at 37ºC (Harding et al., 1983). After the specific time period, cells were stripped for 90 sec with ice cold striping solution (0.5% $CH_3COOH$ + 500 mM NaCl) to remove the remaining transferrin bound at the cell surface. Cells were then washed with PBS and fixed with 4% PFA and mounted on glass slides using Fluoromount mounting media. In the experiments involving PYR-41, drug was present throughout the experiment. Image acquisition was done in Zeiss Axio Observer Z1 fluorescence microscope using 63X oil objective. Quantitation was done using ImageJ (NIH, USA) software (Schneider et al., 2012; Rueden et al., 2017).

### 2.2.11. Immunoprecipitation and western blot analysis

In order to check for the ubiquitination profile of mGluR1 in presence of PYR-41, HEK293T cells were cotransfected with FLAG tagged mGluR1 (FLAG-mGluR1) and HA tagged wild-type ubiquitin using lipofectamine 2000. 28-30 hrs post-transfection, cells were preincubated in 50 µM PYR-41 and 50 µM MG-132 for 30 min followed by the application of 100 µM R,S-DHPG for 5 min. Cells were then placed on ice and lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH-7.4, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 20 mM N-ethylmaleimide, protease inhibitor cocktail). Following lysis of the cells, immunoprecipitation was performed by overnight incubation with anti-FLAG M2 affinity beads. Immunoprecipitates were run in SDS-PAGE, followed by western blot analysis. For immunoblotting, anti-HA antibody was used to check for the ubiquitination profile of the receptor and anti-FLAG antibody was used to check for the pulled down level of the receptor. Immunoblotting was performed using horseradish peroxidase-conjugated secondary antibodies and blots were developed using ECL western detection kit. Image acquisition was done using ImageQuant LAS 4000.

Knockdown of endogenous Siah-1A was studied by transfecting cortical neurons with either siRNA against Siah-1A (ON-TARGET plus SMARTpool) or scrambled siRNA (si-control) (Dharmacon, Thermo Scientific, Lafayette, CO, USA). 72 hr post transfection, neurons were lysed in RIPA lysis buffer having protease inhibitor cocktail. Samples were boiled in Laemmli sample buffer and ran in SDS-PAGE by loading an equal amount of protein in each lane. Subsequently, they were transferred to the PVDF membrane, blocked with 5% skimmed milk for 1 hr at room temperature. Membrane was then incubated with anti-Siah-1A polyclonal antibody (1:800) or anti β-actin (1:800) antibody in 4ºC overnight. Following that, membranes were washed and incubated in horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature. Blots were developed using ECL western detection kit and imaging was performed in ImageQuant LAS 4000.

### 2.2.12. Image acquisition and analysis

Images were obtained in Zeiss LSM 780 confocal laser scanning microscope using a 63X oil immersion objective with 1.4 numerical aperture. 100-120 HEK293 cells and 40-50 primary hippocampal neurons were imaged and every experiment was repeated at least three times. In a particular experiment images from all the conditions were obtained using identical parameters. Subsequently, raw images were used for all the analyses. Quantitation was performed using ImageJ software (NIH, USA) (Schneider et al., 2012; Rueden et al., 2017). All the analyses procedures have been described in our earlier studies (Trivedi and Bhattacharyya, 2012; Pandey et al., 2014; Mahato et al., 2015). Briefly, each raw image was maximally projected and thresholded using identical values for a particular experiment for all conditions. Thresholded areas occupied by the fluorescence of the labelled surface and internalized receptors were measured. The internalization index was then calculated by dividing the value contributed by the internal fluorescence with the value contributed by the total fluorescence (surface + internal). They were then normalized with that of untreated control cells. To measure the surface receptors in all our assays, surface fluorescence was divided by the cell area, which was determined by measuring the background fluorescence using a low threshold level. These values were then normalized to the average surface fluorescence of untreated control cells. In case of primary

hippocampal neurons, the dendritic values were defined by the area that was 10 µm away from the soma. All the quantitation has been represented as combined results for all the repeats of a particular experiment. Raw images were adjusted using equal values of brightness and contrast to obtain the representative images.

### 2.2.13. Statistical analysis

Data are presented as mean ± SEM. As we have mentioned before, 100-120 HEK293 cells and 40-50 primary hippocampal neurons were imaged and each experiment were repeated three times. Experimental group results were compared with each other using student t-test or one-way ANOVA followed by Tukey's post-test. $P > 0.05$ was considered as non-significant.

# Ligand-mediated endocytosis of group I mGluRs is ubiquitination dependent

## 3.1. Introduction

G-protein coupled receptors (GPCRs) are the largest gene family in the human genome and play a critical role in maintaining the homeostasis of the cellular milieu. GPCRs play an important role in signal transduction by detecting extracellular stimuli and activating intracellular downstream pathways by firing a second messenger response (Culhane et al., 2015). Binding of ligand induces receptor coupling to heterotrimeric G-proteins (Dores and Trejo, 2012a). Multiple mechanisms tightly regulate the signaling of these receptors at different levels, and trafficking plays a critical role in the regulation of their signaling. Majority of the receptors, after initiating the second messenger response get desensitized i.e., become unresponsive towards further agonist stimulation. Receptor desensitization is a protective mechanism of the cell to protect it from chronic overstimulation (Lefkowitz, 1998; Magalhaes et al., 2012; Bhattacharyya, 2016). After desensitization, some GPCRs get resensitized on the cell membrane, like rhodopsin receptors (Pippig et al., 1995; Ferguson et al., 1996; Ferguson et al., 1998; Anborgh et al., 2000; Trapaidze et al., 2000; Roosterman et al., 2004; Palczewski, 2006; L Mohan et al., 2012; Azevedo et al., 2015). How rhodopsin receptors get resensitized on the cell membrane is still not fully understood. On the other hand, some GPCRs get endocytosed subsequent to desensitization (Ferguson et al., 1996; Dale et al., 2001; Mundell et al., 2001; Snyder et al., 2001; Bhattacharyya et al., 2002; Mundell et al., 2003; Bhattacharyya et al., 2006; Bhattacharyya, 2016). The subcellular fate of these receptors following endocytosis depends on the type of the receptor, type of the ligand and type of the cellular background (Bhattacharyya, 2016). After endocytosis, some GPCRs go to the lysosome for degradation, which could serve as a mechanism to downregulate those receptors (Marchese et al., 2003; Tan et al., 2004; Hanyaloglu and Zastrow, 2008; Mines et al., 2009; Bhattacharyya, 2016). Few other receptors recycle back to the cell surface subsequent to internalization and that could serve as a mechanism for resensitization of those receptors (Pippig et al., 1995; Trapaidze et al., 2000; Bhattacharyya et al., 2002; Roosterman et al., 2004; Bhattacharyya et al., 2006; Trivedi and Bhattacharyya, 2012; Pandey et al., 2014; Mahato et al., 2015). Subsequent to the activation of the second messenger pathways, GPCRs undergo various post-translational modifications. One such modification is phosphorylation, mediated by G-protein coupled receptor kinases (GRKs), calmodulin kinase, protein kinase C (PKC), protein kinase A (PKA), etc (Ferguson et al., 1996; Ferguson and Caron, 1998; Gereau and Heinemann, 1998; Krupnick and Benovic, 1998; Pitcher et al., 1998; Sallese et

al., 2000; Iacovelli et al., 2003; Kim et al., 2005; Schlyer and Horuk, 2006; Lee et al., 2008; Niswender and Conn, 2010; Ko et al., 2012; Komolov and Benovic, 2017; Mayor Jr et al., 2017). Phosphorylation of GPCRs has long been known to influence receptor function, trafficking and has been extensively studied for various GPCRs including group I mGluRs (Bouvier et al., 1988; Arriza et al., 1992; Alaluf et al., 1995; Gereau and Heinemann, 1998; Ferguson, 2001; Bhattacharyya et al., 2002; Drake et al., 2006; Bhattacharyya, 2016). Group I mGluRs get phosphorylated by GRKs and second messenger-dependent kinases resulting in binding of β-arrestin to the receptor, which in turn leads to the desensitization of the receptor by uncoupling the receptor from the G-protein involved (Alaluf et al., 1995; Gereau and Heinemann, 1998; Ciruela et al., 1999; Dale et al., 2000; Francesconi and Duvoisin, 2000; Sallese et al., 2000; Iacovelli et al., 2003; Kim et al., 2005; Dhami and Ferguson, 2006; Mao et al., 2008; Pandey et al., 2014; Mahato et al., 2015; Bhattacharyya, 2016). Other than phosphorylation, ubiquitination is another important reversible post-translational modification that has been reported to regulate the function of many GPCRs (Hicke and Riezman, 1996; Galan and Haguenauer-Tsapis, 1997; Terrell et al., 1998; Marchese and Benovic, 2001; Shenoy et al., 2001; Tanowitz and von Zastrow, 2002; Haglund et al., 2003; Moriyoshi et al., 2004; Wojcikiewicz, 2004; Platta et al., 2007; Shenoy, 2007; Komander, 2009; Mines et al., 2009; Alonso et al., 2011; Caballero and Marchese, 2011; Hislop and von Zastrow, 2011; Xiao and Shenoy, 2011; Dores and Trejo, 2012b; Alonso and Friedman, 2013; Lahaie et al., 2016; Skieterska et al., 2017). Ubiquitination acts as a sorting signal to facilitate trafficking of many mammalian GPCRs from endosomes to lysosomes for degradation (Marchese et al., 2008). But for number of GPCRs, ubiquitination plays many other roles (Tanowitz and von Zastrow, 2002). In 1996, Hicke and Riezman showed that in case of ste2 receptor (yeast GPCR), ubiquitination of the lysine residue at the C-terminus of the receptor serves as a signal for the ligand-stimulated endocytosis (Hicke and Riezman, 1996). It has been shown that group I mGluRs also get ubiquitinated by the E3 ubiquitin ligase, Siah-1A (Moriyoshi et al., 2004). Like many other GPCRs, subsequent to the desensitization, group I mGluRs internalize in various cell types (Trivedi and Bhattacharyya, 2012; Pandey et al., 2014; Bhattacharyya, 2016). The internalization of these receptors has been reported to be β-arrestin and dynamin dependent (Mundell et al., 2001). We wanted to investigate whether ubiquitination plays any role in the trafficking of group I mGluRs.

## *Chapter 3: Ubiquitination in group I mGluR internalization*

We initially studied the trafficking of group I mGluRs in non-neuronal HEK293 cells which is extensively used as a standard heterologous system since they contain a large repertoire of G-proteins. We have also extended our study in primary hippocampal neurons, which is the most experimentally tractable *in vitro* system that can approximate the *in vivo* situation. We have used myc tagged mGluR1 and mGluR5, where myc was tagged at the N-terminus of the receptor. Previous reports have suggested that this tagged receptor behaves like the native receptor (Choi et al., 2011). Our data suggests that application of the ligand resulted in the endocytosis of group I mGluRs in both non-neuronal and neuronal cells. For both, mGluR1 and mGluR5, the endocytosis was maximum at 30 min post-ligand application. Both receptors recycled back to the cell surface subsequent to the internalization at 2.5 hr in HEK293 cells and primary hippocampal neurons. Thus, the kinetics of internalization and recycling was found to be similar in both HEK293 cells and primary hippocampal neurons. Our data also suggests that upon application of the ligand, mGluR1 got ubiquitinated. Importantly, inhibition of the ubiquitination resulted in the block of the ligand-mediated internalization of group I mGluRs in both non-neuronal HEK293 cells and primary hippocampal neurons.

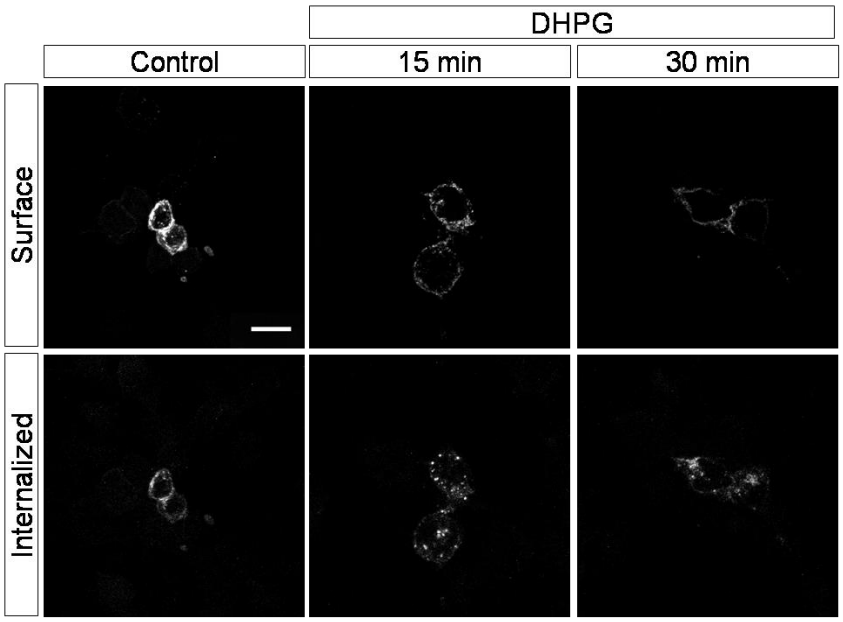
## 3.2. Results

### 3.2.1. Ligand-mediated endocytosis of mGluR1 in HEK293 cells

Previous literatures suggest that upon ligand stimulation, mGluR1 gets endocytosed (Mundell et al., 2003; Dhami and Ferguson, 2006; Pandey et al., 2014; Bhattacharyya, 2016). In order to check whether myc-mGluR1 gets internalized upon ligand binding, we have performed the endocytosis of myc-mGluR1 upon R,S-DHPG application in HEK293 cells. The protocol used to study the R,S-DHPG-mediated internalization of myc-mGluR1 has been described in the "Materials and Methods" chapter. Briefly, myc-mGluR1 cDNA was transfected into HEK293 cells using lipofectamine 2000 (Invitrogen) and subsequently, cells were incubated in 10% DMEM for 24 hr for the expression of myc-mGluR1. myc-mGluR1 expressing HEK293 cells were pre-incubated in plain DMEM (glutamate free) for 30 min followed by live cell staining with anti-myc mouse monoclonal primary antibody (1:500) for 15 min at 37˚C. This was followed by a pulse of 100 µM R,S-DHPG for 5 min. After that, ligand was removed and cells were chased for various time periods (total of 15 min and 30 min) in plain DMEM in the absence of the ligand. Cells were then fixed without permeabilization with ice cold 4% PFA for 15 min on ice. Subsequently, first secondary antibody i.e goat anti-mouse Alexa-568 (1:100) was applied to label the surface receptors. Cells were then permeabilized with 0.1% Triton X-100 and afterwards, second secondary antibody viz., goat-anti mouse Alexa-647 (1:750) was applied to label the internalized receptors. Cells were then mounted on glass slides and scanned under the confocal microscope.

Control cells (R, S-DHPG untreated) showed the presence of majority of the receptors on the cell surface and very little endocytosed receptors were observed (control = 1 ± 0.1) **(Figure 3.1 A, B)**. myc-mGluR1 endocytosed rapidly upon application of 100 µM R,S-DHPG as observed by an increase in the internal fluorescence in 15 min (15 min = 1.58 ± 0.11). There was a further increase in the internal fluorescence when cells were chased for a longer time point (30 min = 1.71 ± 0.14). The maximum endocytosis of myc-mGluR1 was observed at 30 min post-ligand exposure. In this, and all the subsequent experiments related to trafficking of group I mGluRs, endocytosis index was calculated following the method described in the "Materials and Method" section. The accuracy of quantitation is governed by the fact, that it is necessary to use a saturating concentration of the first secondary antibody which labels almost all the surface

**A**




**B**

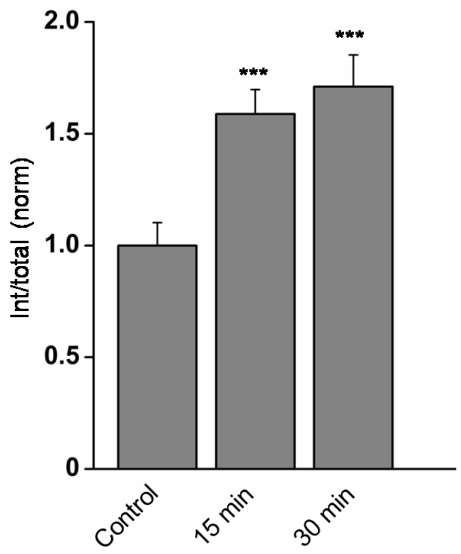



**Figure 3.1. Ligand-mediated internalization of myc-mGluR1 in HEK293 cells.** (A) Representative images showing ligand-mediated endocytosis of myc-mGluR1 in HEK293 cells. (B) Measurement of endocytosis index showing gradual increase in the internalized fraction of myc-mGluR1 in HEK293 cells upon ligand activation. Scale bar = 10 μm. ***, $p < 0.001$.

receptors, so that the second secondary antibody would not be able to label detectable surface receptors and would stain the internalized receptors only. The standardization protocol has been discussed in the "Methods" section. These experiments suggested that there was no detectable second secondary antibody staining observed in case of unpermeabilized condition and on the other hand, in permeabilized condition, the second secondary antibody stained the constitutively internalized receptors.

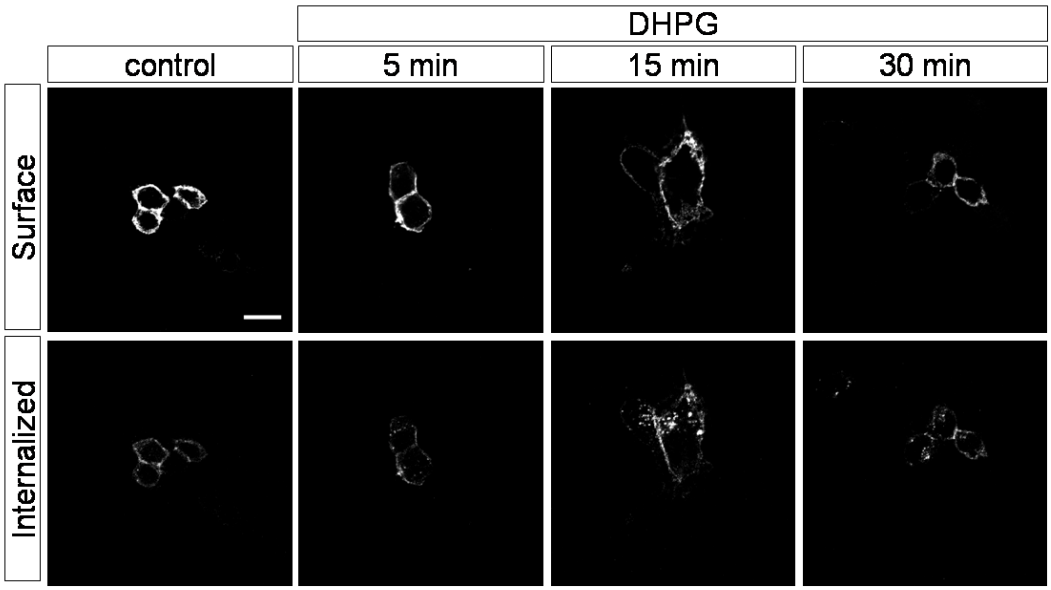
### 3.2.2. Ligand-mediated endocytosis of mGluR5 in HEK293 cells

As stated before, group I mGluRs comprise of two members: mGluR1 and mGluR5. Therefore, we also wanted to investigate the fate of mGluR5 upon ligand-mediated activation. myc-mGluR5 was transiently transfected into HEK293 cells using lipofectamine 2000 by the method described in the "Materials and Methods" section. 24 hr post-transfection, antibody feeding assay was performed in the similar way as has been described for myc-mGluR1. Subsequently, saturating concentration of the goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) was applied for 1 hr followed by permeabilization with 0.1% Triton-X 100 at 37˚C for 30 min. After that, goat anti-mouse Alexa-647 conjugated secondary antibody (1:750) was applied to visualize the internalized receptors using the same protocol as described above. Cells were then mounted on glass slides and imaged under the confocal microscope. Control cells showed presence of majority of the receptors at the cell surface (control = 1 ± 0.09) **(Figure 3.2 A, B)**. myc-mGluR5 internalized upon 100 µM R,S-DHPG application at 5 min, as observed by an increase in the internal fluorescence (5 min=1.26 ± 0.16). There was a gradual increase in the internalized receptors when cells were chased for longer time points (15 min=1.77 ± 0.18) and maximum internalization was observed at 30 min (30 min =2.98 ± 0.18)**.**

### 3.2.3. Ligand-mediated endocytosis of mGluR1 and mGluR5 in primary hippocampal neurons

Our earlier experiments suggested that both myc-mGluR1 and myc-mGluR5 showed ligand-mediated internalization in HEK293 cells. Group I mGluRs are natively present at the

**A**




**B**

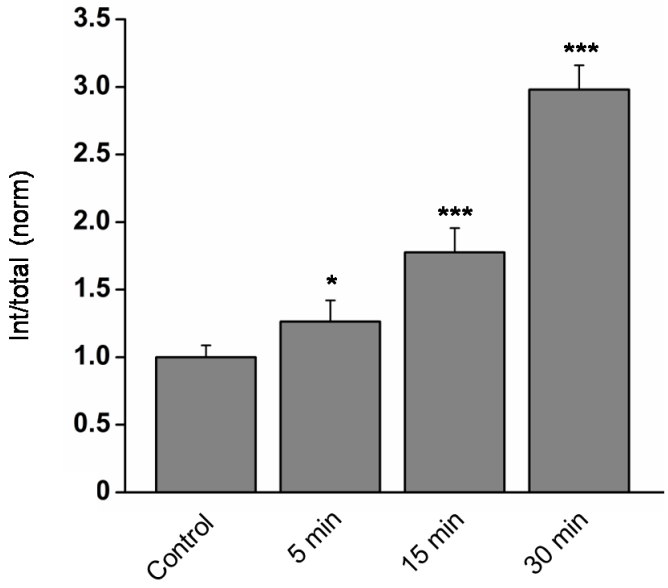


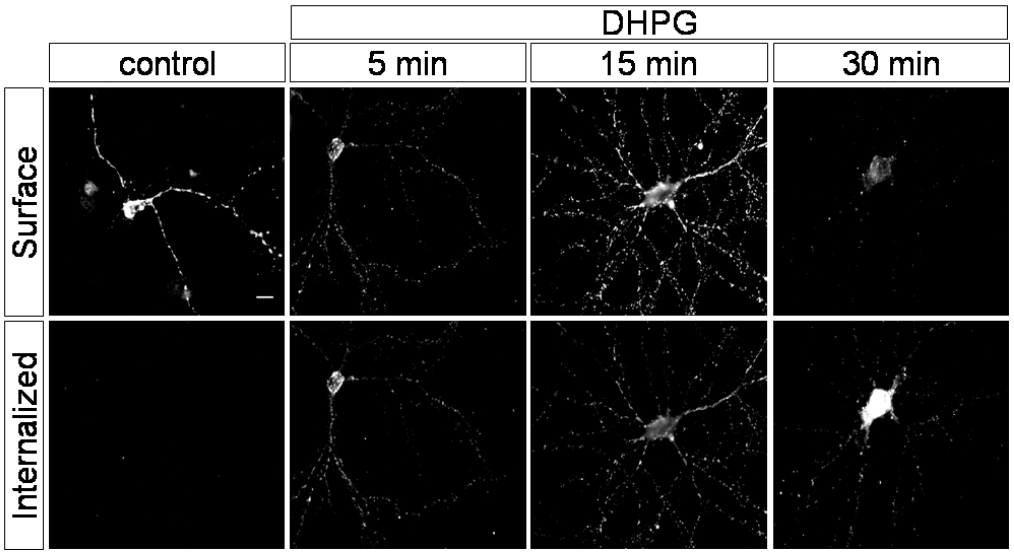

**Figure 3.2. Ligand-mediated internalization of myc-mGluR5 in HEK293 cells.** Representative images (A) and measurement of the endocytosis index (B) shows that upon ligand application, myc-mGluR5 internalized and internalization of the receptor increased gradually over time. Scale bar = 10 µm. ***, $p < 0.001$; *, $p < 0.05$.

perisynaptic region of the post-synaptic neurons. Therefore, we extended our study in primary hippocampal neurons, which are the most experimentally tractable *in vitro* system that can approximate the *in vivo* situation. In order to study the ligand-mediated internalization of group I mGluRs, primary hippocampal neurons were transfected with either myc-mGluR1 or myc-mGluR5 cDNA using calcium phosphate method at 7-8 DIV. Live cells expressing myc-mGluR1/myc-mGluR5 were incubated with mouse anti-myc primary antibody (1:200) for 20 min at 37˚C. Cells were then washed with plain media and 100 µM R,S-DHPG was applied for 5 min. Subsequently, cells were incubated at 37˚C for various time periods in plain neurobasal media in the absence of the ligand. Cells were then fixed without permeabilization using 4% PFA for 15 min on ice. To label the surface localized receptors, cells were incubated with saturating concentration of goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) for 1 hr at 37˚C. After that, cells were permeabilized with 0.1% Triton X-100 for 30 min at 37˚C. The endocytosed receptors were then labelled by the application of goat anti-mouse Alexa-647 conjugated secondary antibody (1:750) for 1 hr at 37˚C. Coverslips were then mounted on glass slides and imaged under the confocal microscope. In case of myc-mGluR1, control cells showed presence of majority of the receptors at the cell surface with a very little internalized receptors (control = 1 ± 0.15) **(Figure 3.3 A, B)**. Application of R,S-DHPG led to the rapid internalization of myc-mGluR1 (5 min = 1.9 ± 0.23). The endocytosis index was increased further as neurons were incubated for longer time periods (15 min = 1.99 ± 0.26, 30 min = 2.25 ± 0.23). We next studied the ligand-dependent internalization of myc-mGluR5 in primary hippocampal neurons using the same method as described above for myc-mGluR1. Application of 100 µM R,S-DHPG led to the robust internalization of myc-mGluR5 compared to control cells (control = 1 ± 0.09, 30 min = 1.65 ± 0.08) **(Figure 3.4 A, B)**.

The above results suggest that upon application of the ligand, both myc-mGluR1 and myc-mGluR5 internalize in HEK293 cells and primary hippocampal neurons.

For all the experiments, two sister coverslips were used for each condition and all experiments were repeated at least three times. To ensure that the Alexa-647 conjugated secondary antibody did not label any detectable surface receptors in our assays, we performed control experiments to determine the saturating concentration of the first secondary antibody similar to what we have

**A**




**B**

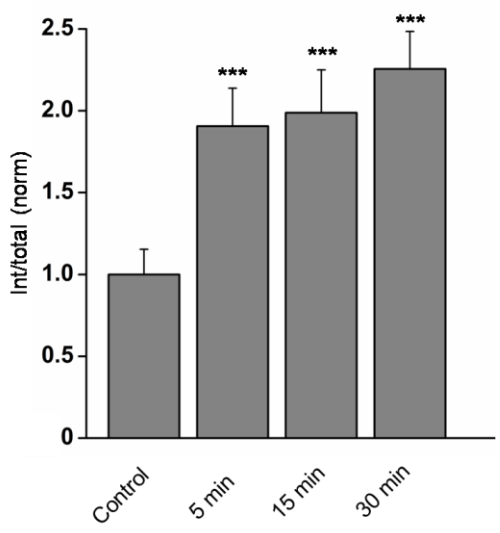
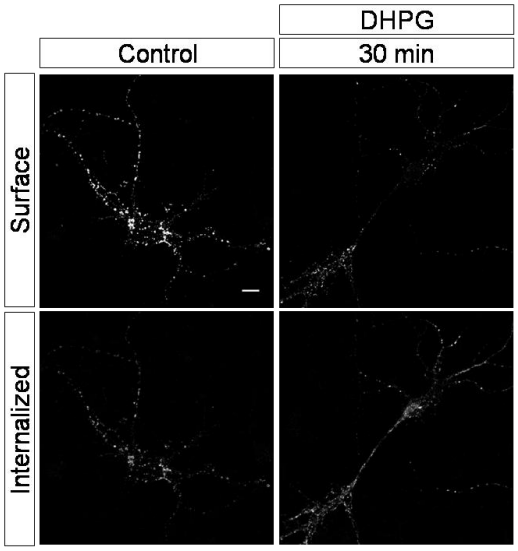



**Figure 3.3. Ligand-stimulated endocytosis of myc-mGluR1 in primary hippocampal neurons.** (A) myc-mGluR1 internalized on 100 µM R,S-DHPG exposure, as shown in the representative images. (B) Dendritic quantitation of the endocytosis index shows endocytosis of myc-mGluR1 upon ligand application in primary hippocampal neurons. Scale bar = 10 µm. ***, $p < 0.001$.

**A**




**B**

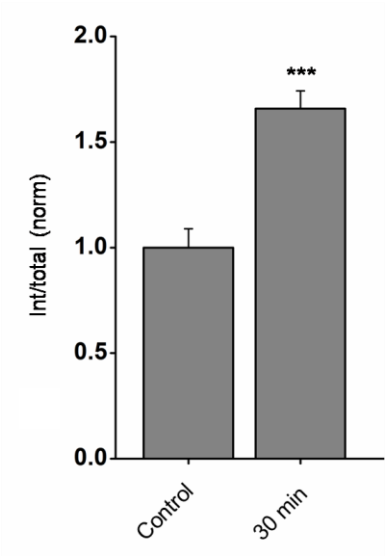


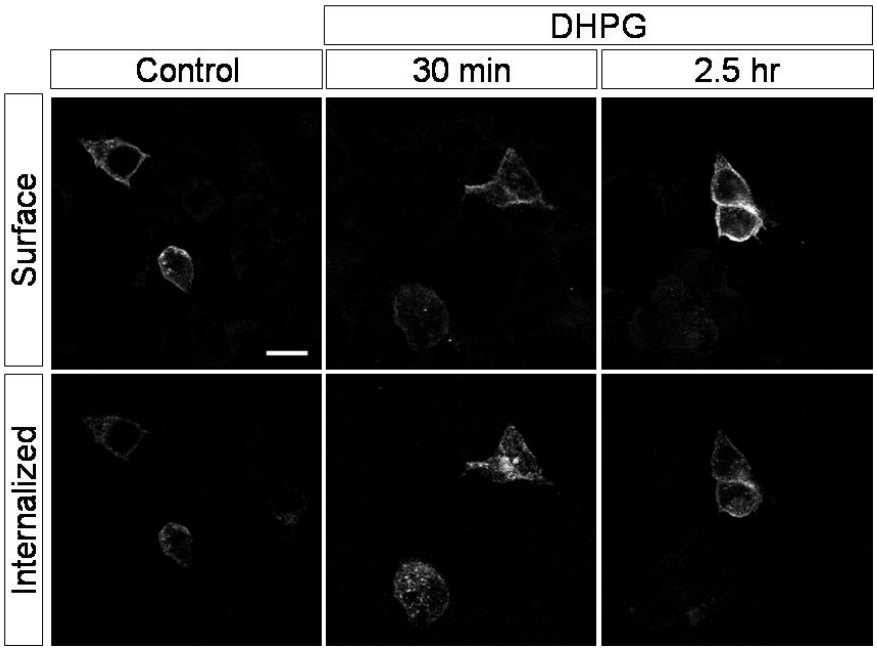

**Figure 3.4. Ligand-mediated endocytosis of myc-mGluR5 in primary hippocampal neurons.** (A) Representative images showing endocytosis of myc-mGluR5 in primary hippocampal neurons upon application of 100 µM R,S-DHPG. (B) Quantitation also shows endocytosis of myc-mGluR5 upon application of 100 µM R,S-DHPG. Scale bar = 10 µm. ***, $p < 0.001$.

described in our earlier studies (Trivedi and Bhattacharyya, 2012; Pandey et al., 2014; Mahato et al., 2015). This control experiment was performed each time whenever we purchased a new batch of first secondary antibody (goat anti-mouse Alexa-568). The control experiments suggested that in all assays, both in HEK293 cells and primary hippocampal neurons, Alexa-647 conjugated secondary antibody did not label any detectable amount of surface receptors and thus it stained the internalized receptors only.

### 3.2.4. Group I mGluRs recycle back to the cell surface subsequent to the ligand-dependent internalization in HEK293 cells

As shown above, upon stimulation with the ligand, group I mGluRs internalized in both non-neuronal HEK293 cells and primary hippocampal neurons. It has been shown by our group that both mGluR1 and mGluR5 enter the recycling compartment subsequent to the ligand-mediated internalization (Pandey et al., 2014; Mahato et al., 2015). In order to investigate whether myc-mGluR1 recycles back to the cell surface after ligand-mediated internalization, we transfected HEK293 cells with myc-mGluR1 cDNA using lipofectamine 2000 according to the protocol discussed in the "Materials and Methods" chapter. 24 hr post-transfection, anti-myc antibody (1:500) was applied for 15 min at 37˚C. After that, 100 µM R,S-DHPG was applied for 5 min followed by chase in the absence of the ligand for 30 min and 2.5 hr. Cells were then fixed in cold 4% PFA on ice for 15 min without permeabilization. Subsequently, the first secondary antibody, i.e., goat anti-mouse Alexa-568 was applied in saturating concentration for 1 hr at 37˚C. Cells were then permeabilized in 0.1% Triton X-100 and the second secondary antibody, i.e. goat anti-mouse Alexa-647 (1:750) was applied for 1 hr at 37˚C to visualize the internalized receptors. Cells were then mounted on glass slides and observed under the confocal microscope. Control cells, expressing myc-mGluR1 showed presence of majority of the receptors at the cell surface, and very little endocytosed receptors was observed (control = 1 ± 0.07). The receptors internalized at 30 min post 100 µM R,S-DHPG application, as observed by the increase in the intracellular fluorescence and decrease in the surface receptors (30 min= 1.67 ± 0.08). At 2.5 hr, majority of receptors recycled back to the cell surface as observed by an increase in the cell surface fluorescence and a decrease in the internalized fraction of the receptor (2.5 hr = 0.96 ± 0.11) **(Figure 3.5 A, B)**.

**A**




**B**

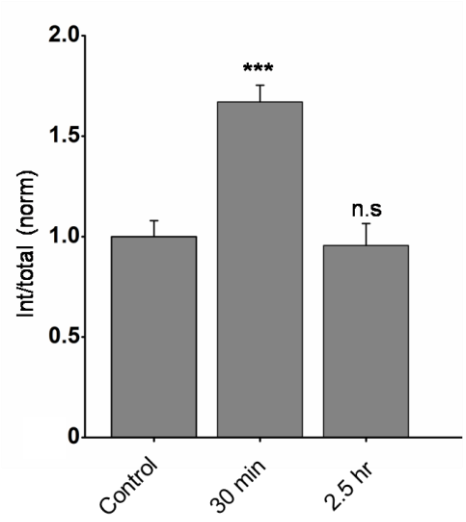


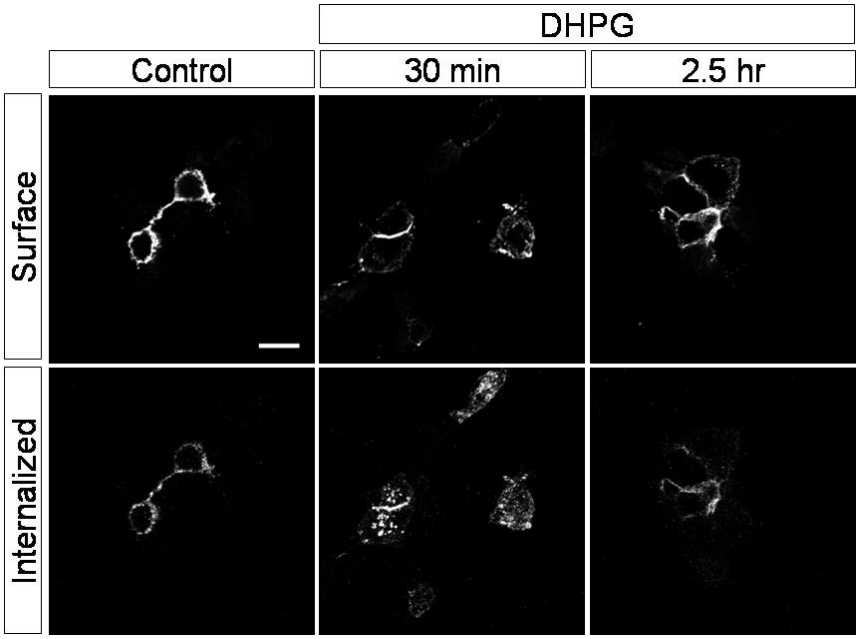

**Figure 3.5. myc-mGluR1 recycles back to the cell surface in HEK293 cells.** Representative images (A) and quantitation (B) show the recycling of myc-mGluR1, subsequent to the ligand-mediated endocytosis, in HEK293 cells. Scale bar = 10 μm. ***, $p < 0.001$; n.s, $p > 0.05$.

In case of myc-mGluR5, a similar result was observed. Measurement of the internalization index showed internalization of the receptor at 30 min post ligand application (control = 1 ± 0.17, 30 min = 3.09 ± 0.27)**.** When cells were chased for longer time points in plain DMEM in absence of the ligand, the receptors recycled back to the cell surface at 2.5 hr (2.5 hr = 0.97 ± 0.22) **( Figure 3.6 A, B)**.

### 3.2.5. mGluR1 recycles back to the cell surface following ligand-dependent internalization in primary hippocampal neurons

To study, whether myc-mGluR1 also recycles back to the cell surface following ligand-dependent internalization in primary hippocampal neurons, cells were transfected with myc-mGluR1 cDNA using calcium phosphate method at 7-8 DIV, and experiments were performed at 12-14 DIV. myc-mGluR1 expressing cells were labelled with anti-myc primary antibody at 37˚C for 20 min. The receptor internalization was stimulated by the application of 100 µM R,S-DHPG for 5 min at 37˚C using the same protocol as discussed in the "Materials and Methods" chapter. Subsequently, cells were chased for a longer time period in plain neurobasal media, in the absence of the ligand. Cells were then fixed, without permeabilization, using 4% PFA on ice for 15 min followed by labelling of the surface receptors with saturating concentration of Alexa-568 conjugated secondary antibody (1:100) for 1 hr at 37˚C. After that, cells were permeabilized in 0.1% Triton X-100 for 30 min at room temperature and then Alexa-647 conjugated second secondary antibody was applied for 1 hr (1:750) to visualise the internalized receptors. Cells were then mounted on glass slides with fluoromount mounting media and scanned under the confocal microscope. The receptors showed internalization at 30 min post 100 µM R,S-DHPG application compared to control cells (control = 1 ± 0.07, 30 min = 2.05 ± 0.10 ). When cells were chased for longer time periods, myc-mGluR1 recycled back to the cell surface at 2.5 hr (2.5 hr = 1.03 ± 0.04) **( Figure 3.7 A, B)**.

**A**

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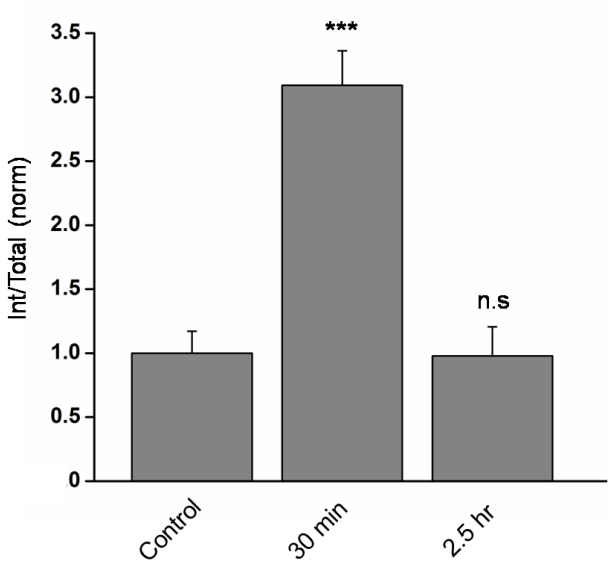
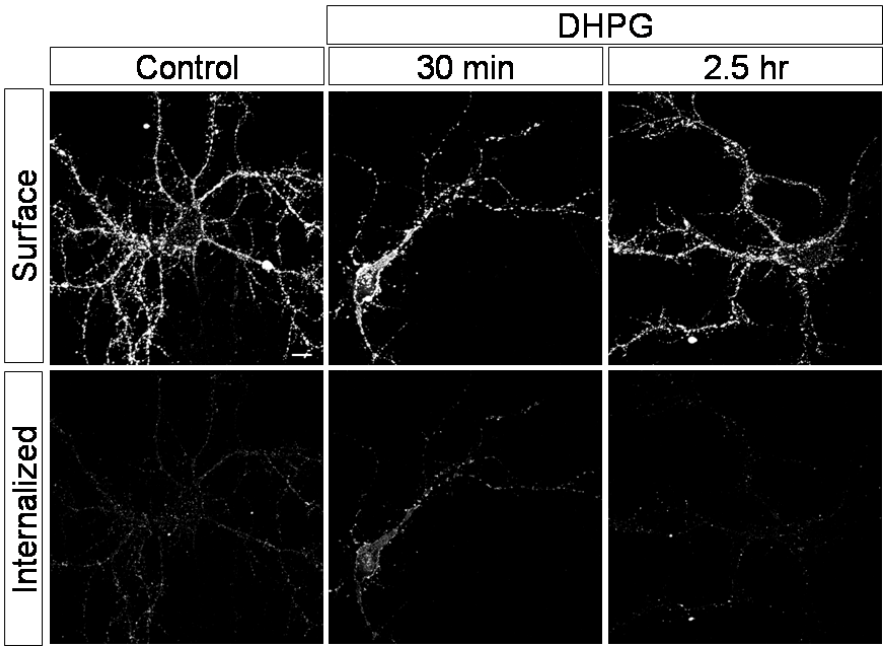



**Figure 3.6. Recycling of myc-mGluR5 in HEK293 cells.** myc-mGluR5 recycled back to the cell surface after 100 µM R,S-DHPG –mediated internalization in HEK293 cells as shown by the representative images (A) and quantitation (B). Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

**A**




**B**

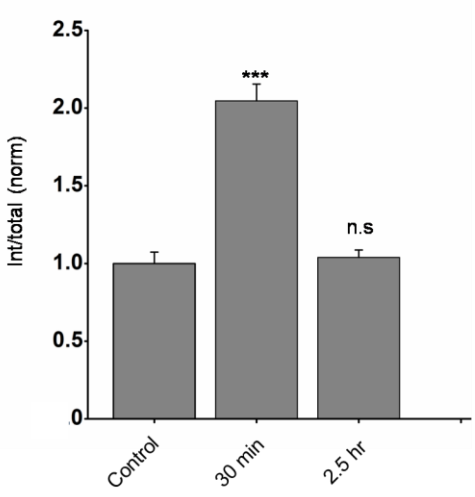


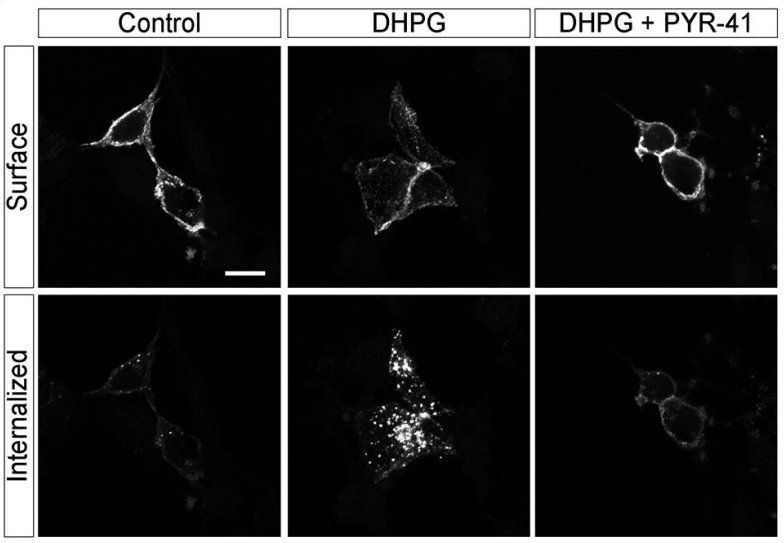

**Figure 3.7. myc-mGluR1 recycles back to the cell surface in primary hippocampal neurons.** Representative images (A) and quantitation of the endocytosis index (B) suggest that, myc-mGluR1 recycled back to the cell surface after 100 µM R,S-DHPG-mediated internalization in primary hippocampal neurons. Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

### 3.2.6. Effect of PYR-41 on the ligand-mediated endocytosis of mGluR1 in HEK293 cells

Various post-translational modifications like phosphorylation, glycosylation, etc. play important role in the trafficking of GPCRs (Alonso and Friedman, 2013). Another post-translational modification, viz., ubiquitination has been reported to play critical role in the internalization of some receptors (Hicke and Riezman, 1996; Terrell et al., 1998; Haglund et al., 2003). Group I mGluRs also get ubiquitinated by the E3 ubiquitin ligase, Siah-1A (Moriyoshi et al., 2004). We, therefore, wanted to check whether ubiquitination plays any role in the trafficking of group I mGluRs. We have used a pharmacological inhibitor 4[4-(5-Nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester (PYR-41) to perform this experiment. PYR-41 is a cell-permeable compound that has been demonstrated to irreversibly inhibit the E1-activating enzyme of the ubiquitin cascade by inhibiting the E1-ubiquitin thioester bond formation (Yang et al., 2007). It has been reported that at 50 µM concentration, PYR-41 inhibits the E1-ubiquitin thioester bond formation by 95% resulting in the inhibition of ubiquitin-dependent intracellular processes (Yang et al., 2007; Dey et al., 2008; Zaarur et al., 2008; Citri et al., 2009; Satheshkumar et al., 2009). To study the role of ubiquitination in the ligand-dependent trafficking of mGluR1, HEK293 cells were transfected with myc-mGluR1 cDNA using lipofectamine 2000. 24 hr post-transfection, cells were treated with 50 µM PYR-41 in plain DMEM for 30 min followed by staining of the cells with anti-myc primary antibody (1:500) for 15 min at 37˚C. Subsequently, internalization of the receptor was induced by the application of 100 µM R,S-DHPG for 5 min and cells were then chased in plain DMEM for a total of 30 min. Application of R,S-DHPG in PYR-41 untreated cells led to the robust internalization of the receptor. On the other hand, PYR-41 treated cells did not show significant internalization of myc-mGluR1 upon 100 µM R,S-DHPG application and most of the receptors were observed to be localized at the cell surface (control = 1 ± 0.1; DHPG = 2.15 ± 0.12; DHPG + PYR-41 = 1.2 ± 0.09) **( Figure 3.8 A, B)**.

As stated before, application of PYR-41 irreversibly inhibits the E1-activating enzyme of the ubiquitin cascade by inhibiting the E1-ubiquitin thioester bond formation which ultimately blocks the ubiquitination of the target protein. In order to investigate whether mGluR1 gets ubiquitinated upon ligand application and also whether PYR-41 inhibits the ligand-mediated ubiquitination of the receptor, HEK293T cells were co-transfected with FLAG tagged mGluR1

**A**




**B**

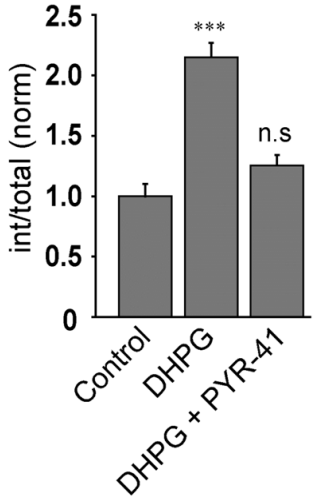



**Figure 3.8. Effect of PYR-41 on the ligand-mediated endocytosis of myc-mGluR1.** (A) HEK293 cells, showing block in the endocytosis of myc-mGluR1 upon application of R,S-DHPG in the presence of PYR-41. (B) Quantitation of the effect of PYR-41 on the internalization of myc-mGluR1 in HEK293 cells. Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

(FLAG-mGluR1) and HA tagged wild-type ubiquitin. Subsequently, cells were treated with 50 µM PYR-41 and 50 µM MG-132 (proteasome inhibitor) for 30 min followed by the application of 100 µM R,S-DHPG for 5 min. Cells were then placed on ice and lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH-7.4, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 20 mM N-ethylmaleimide, protease inhibitor cocktail). The lysate was centrifuged at 15,000 rpm for 25 min. 60 µl of the supernatant was collected after that as input. Following lysis of the cells, immunoprecipitation was performed by overnight incubation with anti-FLAG M2 affinity beads (Sigma Aldrich). Immunoprecipitates were run in SDS-PAGE followed by western blot analysis. For immunoblotting, anti-HA rat monoclonal antibody (Roche) was used to check for the ubiquitination profile of the receptor and anti-FLAG antibody was used to check for the pulled down level of the receptor. Immunoblotting was performed using horseradish peroxidase-conjugated secondary antibodies (1:5000) and blots were developed using ECL western detection kit (Amersham Biosciences). Image acquisition was done using ImageQuant LAS 4000. In control cells, application of 100 µM R,S-DHPG led to the robust ubiquitination of FLAG-mGluR1, whereas PYR-41 inhibited the R,S-DHPG-mediated ubiquitination of FLAG-mGluR1 **(Figure 3.9)**.

### 3.2.7. Time course of mGluR1 endocytosis in presence of PYR-41 in HEK293 cells

The above experiments suggested that in PYR-41 treated cells we did not observe much internalized receptors compared to control cells at 30 min post-ligand application. As stated before, normally myc-mGluR1 recycles back to the cell surface in 2.5 hr after R,S-DHPG-mediated endocytosis (Pandey et al., 2014). In order to find out, whether PYR-41 treatment inhibited the endocytosis of the receptor or the receptor internalized and recycled back to the cell surface in 30 min through a faster recycling pathway, we performed a time course experiment of endocytosis. Endocytosis assay was performed in a similar way as discussed in the "Materials and Methods" chapter. myc-mGluR1 expressing HEK293 cells were pre-incubated in presence of 50 µM PYR-41 for 30 min and stained in live condition with anti-myc primary antibody (1:500) for 15 min at 37˚C. After that, a pulse of 100 µM R,S-DHPG was applied for 5 min and cells were chased for various time periods in plain DMEM After completion of the respective time points, cells were fixed in unpermeabilized condition with 4% ice cold PFA.

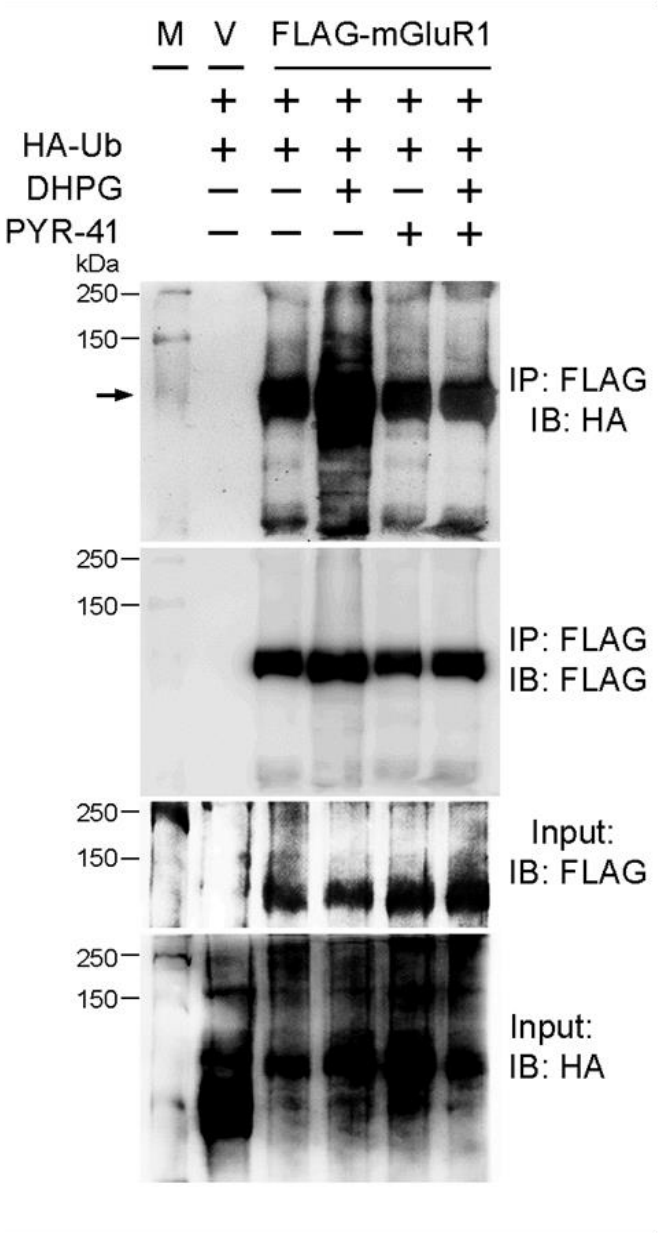



**Figure 3.9.** Co-immunoprecipitation assay showing that application of 50 μM PYR-41 inhibits the ligand-stimulated ubiquitination of FLAG-mGluR1 in HEK293T cells.

Surface localized receptors were then labelled with saturating concentration of goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) for 1 hr at 37˚C. Cells were then permeabilized with 0.1% Triton X-100 for 30 min. The endocytosed receptors were then labelled by the application of goat anti-mouse Alexa-647 conjugated secondary antibody (1:750) for 1 hr at 37˚C. Coverslips were then mounted on glass slides and imaged under the confocal microscope. Application of 100 µM R,S-DHPG resulted in the internalization of myc-mGluR1 in PYR-41 untreated cells (control = 1 ± 0.08; 30 min = 2.27 ± 0.27). On the other hand, the ligand did not induce any significant internalization of the receptor at any time points in PYR-41 treated cells (5 min = 0.9 ± 0.11; 15 min = 1.12 ± 0.09; 30 min = 0.9 ± 0.07) **(Figure 3.10 A, B)**. These results suggest that PYR-41 inhibits the R,S-DHPG-mediated mGluR1 endocytosis.

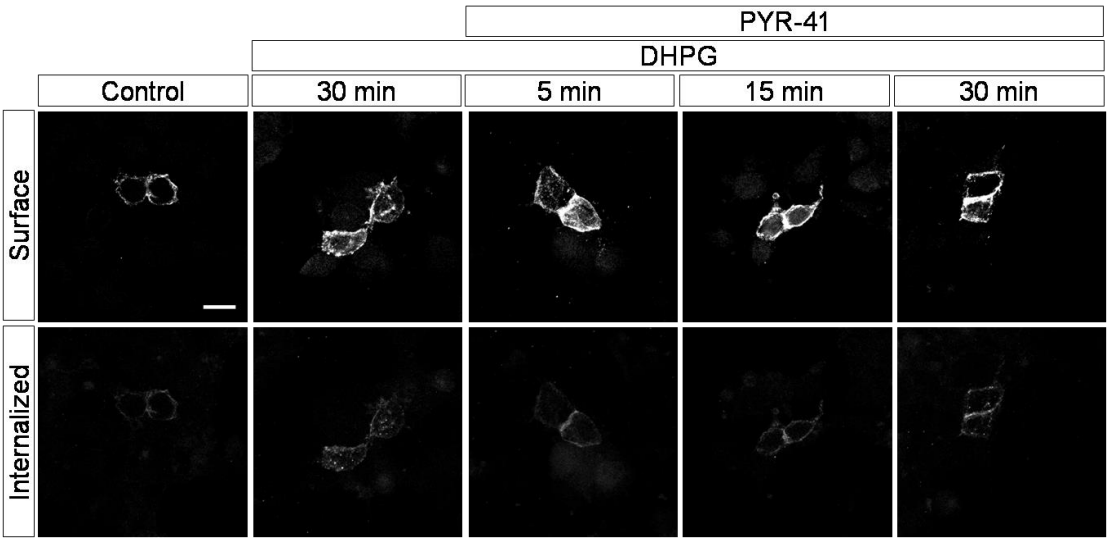
### 3.2.8. Effect of PYR-41 on the ligand-mediated endocytosis of mGluR5 in HEK293 cells

Since, group I mGluRs comprise of two receptors, mGluR1 and mGluR5, and application of 50 µM PYR-41 inhibited the ligand-mediated internalization of mGluR1, we wanted to investigate the effect of PYR-41 on the ligand-mediated internalization of mGluR5. HEK293 cells were transfected with myc-mGluR5 and endocytosis experiment was performed as discussed above. Measurement of endocytosis showed that application of PYR-41 inhibited the ligand-mediated internalization of myc-mGluR5 in HEK293 cells (control = 1 ± 0.06; DHPG = 1.94 ± 0.09; DHPG + PYR-41 = 1 ± 0.04) **(Figure 3.11 A, B)**.

### 3.2.9. Effect of PYR-41 on the R,S-DHPG-mediated endocytosis of group I mGluRs in primary hippocampal neurons

Our earlier experiments suggested that PYR-41 inhibited the ligand-mediated ubiquitination of mGluR1 and also inhibited the ligand-mediated internalization of both mGluR1 and mGluR5 in HEK293 cells. We extended our studies in primary hippocampal neurons, where these receptors are present at the perisynaptic region of the post-synaptic neurons. Cells were transfected with either myc-mGluR1 or myc-mGluR5 cDNA at 7-8 DIV. All experiments were performed when

**A**




**B**

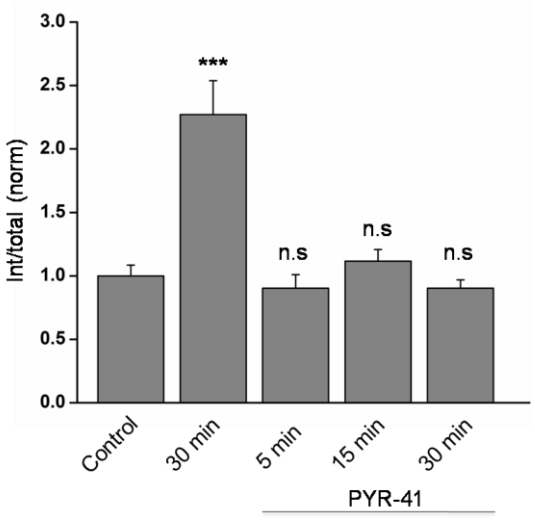
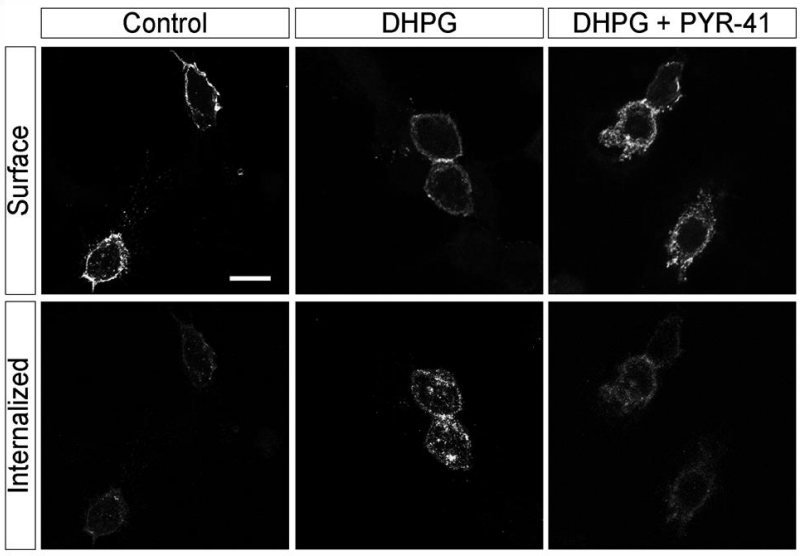



**Figure: 3.10. Time course of myc-mGluR1 endocytosis in presence of PYR-41 in HEK293 cells.** Representative images (A) and quantitation (B) showing block in the ligand-mediated internalization of myc-mGluR1 in presence of PYR-41 in HEK293 cells. Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

**A**

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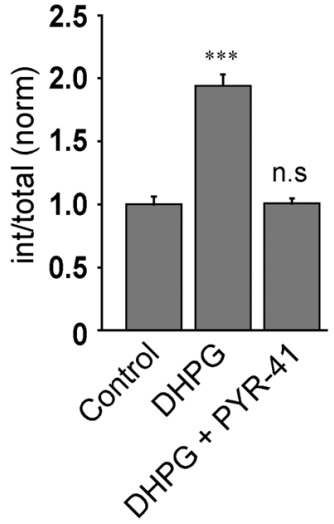


**Figure 3.11. Effect of PYR-41 on the ligand-mediated endocytosis of myc-mGluR5 in HEK293 cells.** Representative images (A) and quantitation (B) showing that application of PYR-41inhibits the ligand-mediated internalization of myc-mGluR5. Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

neurons were 12-13 days in culture. myc-mGluR1 or myc-mGluR5 expressing cells were incubated with the anti-myc primary antibody (1:200) for 20 min at 37˚C. After that, 100 μM R,S-DHPG was applied for 5 min to induce internalization followed by a chase in the absence of the ligand, for a total time of 30 min. In case of PYR-41 treated cells, the drug was applied 30 min before the primary antibody staining and it was present throughout the experiment. After that, cells were fixed without permeabilization, in ice cold 4% PFA for 15 min on ice. Alexa-568 labelled first secondary antibody, against the anti-myc antibody was applied in saturating concentration (1:100) for 1 hr to stain the surface receptors. Subsequently, cells were permeabilized in 0.1% Triton X-100 for 30 min and the second secondary antibody tagged with Alexa-647 was applied (1:750) for 1 hr to visualize the internalized receptors. Neurons were finally mounted on glass slides and imaged under the confocal microscope. Application of 100 μM R,S-DHPG led to the internalization of myc-mGluR1 in PYR-41 untreated cells (control = 1 ± 0.07; DHPG = 1.78±0.18). On the other hand, 50 μM PYR-41 inhibited the R,S-DHPG-mediated myc-mGluR1 endocytosis (DHPG + PYR-41 = 0.9 ± 0.07) **(Figure 3.12 A, B)**.

We obtained similar results when we studied the effect of PYR-41 in the internalization of myc-mGluR5 in primary hippocampal neurons. The receptor internalized in PYR-41 untreated cells, whereas, PYR-41 treated hippocampal neurons showed full block of R,S-DHPG-mediated myc-mGluR5 endocytosis (control = 1 ± 0.09; DHPG = 1.66 ± 0.08; DHPG + PYR-41 = 1 ± 0.08) **( Figure 3.13 A, B)**.

These results suggest that ubiquitination plays critical role in the ligand-mediated internalization of both members of group I mGluRs, viz., mGluR1 and mGluR5 in both non-neuronal HEK293 cells and primary hippocampal neurons.

### 3.2.10. Effect of PYR-41 on the trafficking of transferrin receptors

PYR-41 is a pharmacological inhibitor of the E1 enzyme involved in the process of ubiquitination. In mammalian cells, there are around 10 E1 enzymes, 100s of E2 enzymes and 1000s of the E3 enzymes (Cao and Mao, 2011). In order to investigate whether PYR-41-mediated block in the internalization of group I mGluRs was due to the block in general trafficking machinery in the cell, we studied the trafficking of transferrin receptors in the

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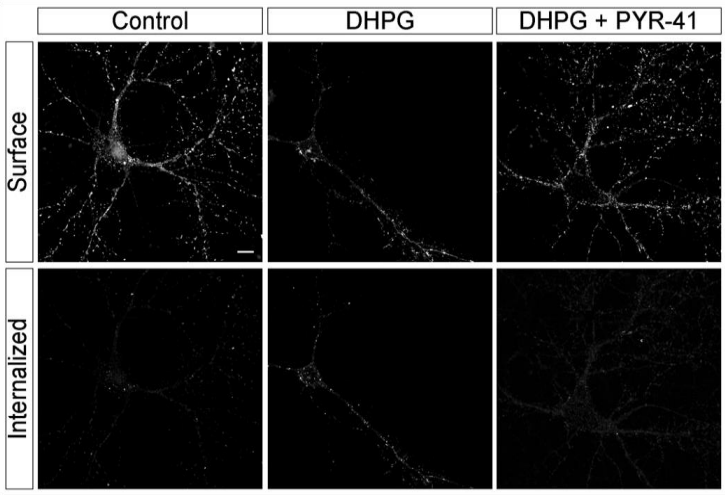



**B**

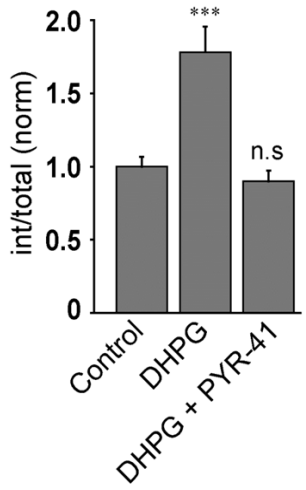
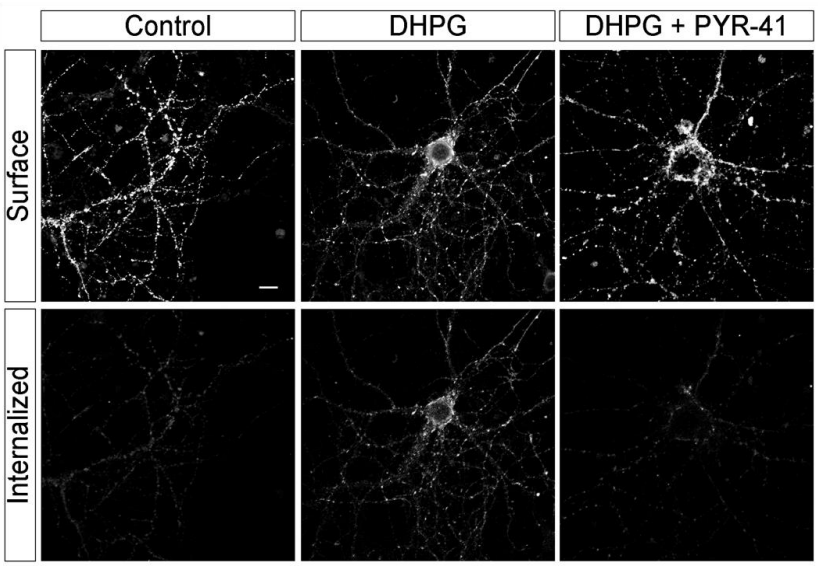



**Figure 3.12. Effect of PYR-41 on the ligand-mediated endocytosis of myc-mGluR1 in primary hippocampal neurons**. (A) Representative images are showing internalization of myc-mGluR1 on 100 µM R,S-DHPG exposure in control cells. Application of PYR-41 completely blocked the R,S-DHPG mediated endocytosis of the receptor in primary hippocampal neurons (B) Quantitation showing the effect of PYR-41 on the R,S-DHPG-mediated endocytosis of myc-mGluR1. Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

**A**

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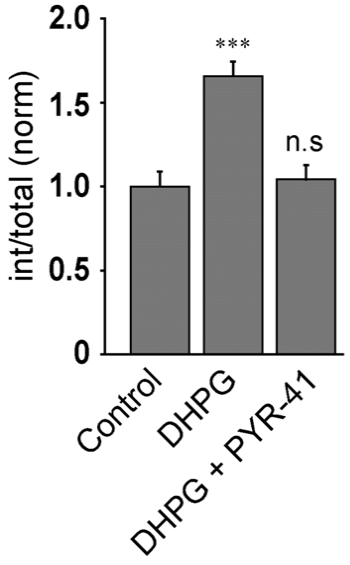
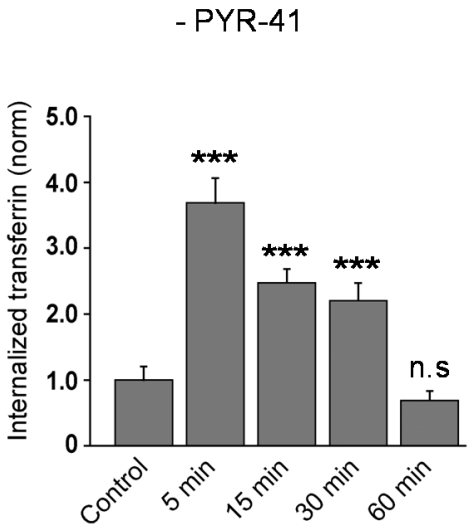



**Figure 3.13. Application of PYR-41 inhibits the ligand-mediated internalization of myc-mGluR5 in primary hippocampal neurons.** (A) Representative images show the myc-mGluR5 endocytosis on application of 100 µM R,S-DHPG in primary hippocampal neurons. Application of PYR-41 completely blocked the R,S-DHPG-mediated endocytosis of the receptor. (B) Quantitation of the effect of PYR-41 on R,S-DHPG-mediated endocytosis of myc-mGluR5. Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

presence of PYR-41 in HEK293 cells. We used Alexa-568 labelled transferrin for these assays and experiments were performed according to the method described in the "Materials and Methods" chapter. In control cells, maximum internalization of Alexa-568 labelled transferrin was observed at 5 min, whereas, at longer chase time points (15 min, 30 min, 60 min), there was a gradual decrease in the internal Alexa-568 labelled transferrin fluorescence, suggesting recycling of transferrin receptors to the cell surface (control = 1 ± 0.33, 5 min = 4.00 ± 0.63, 15 min = 2.57 ± 0.37, 30 min = 2.31 ± 0.38, 60 min = 0.90 ± 0.25) **(Figure 3.14 A**). Importantly, in 50 µM PYR-41 treated cells, a similar kinetics of transferrin receptor trafficking was observed. These cells also showed maximum internalization of Alexa-568 labelled transferrin at 5 min and majority of the receptors recycled back to the cell surface in 60 min (control = 1 ± 0.25, 5 min + PYR-41 = 5.80 ± 0.26, 15 min + PYR-41 = 3.95 ± 0.32, 30 min + PYR-41 = 2.36 ± 0.37, 60 min + PYR-41 = 1.33 ± 0.32) **(Figure 3.14 B)**.These results suggest that PYR-41 does not affect the general trafficking machinery of the cells and thus, the effect of PYR-41 on the ligand-mediated trafficking of group I mGluRs is specific to the receptor.

**A**

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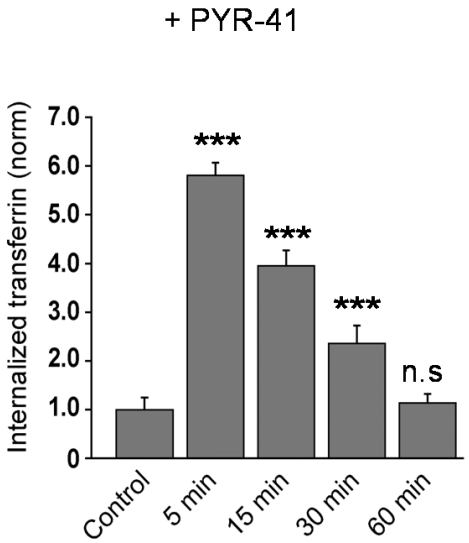


**Figure 3.14. Effect of PYR-41 on the transferrin receptor kinetics in HEK293 cells**. (A) Quantitation showing the endocytosis and recycling of transferrin receptors in control condition. (B) Application of 50 µM PYR-41 does not affect the transferrin receptor internalization and recycling in HEK293 cells. ***, $p < 0.001$; **, $p < 0.01$; n.s, $p > 0.05$.

## 3.3. Discussion

G-protein coupled receptors (GPCRs) are one of the largest families of cell surface receptors that are responsible for sensing the outer environment or signal and transducing this information inside the cells. The signal for these receptors can be a hormone, photon, neurotransmitter, peptide, odorant or could be a taste molecule. By sensing these primary messengers on the cell surface, these receptors fire a secondary messenger response inside the cell. For accurate signaling of these receptors, it is very important that these receptors must be localized at a proper position in the cell. This spatio-temporal localization of these receptors is maintained by trafficking of these receptors. Trafficking also regulates the activity of these receptors and for many GPCRs, it helps in resensitization and down regulation of the receptors. Due to these reasons, understanding the cellular and molecular mechanisms of receptor trafficking has become very important. Like many other GPCRs, group I mGluRs also get desensitized subsequent to the activation of the second messenger pathway, which is an essential feedback mechanism to protect the cells from receptor overstimulation (Francesconi and Duvoisin, 2000; Dhami and Ferguson, 2006). Our lab has earlier shown that, subsequent to the desensitization, group I mGluRs undergo endocytosis and the endocytosed receptors recycle back to the cell surface through a phosphatase-dependent manner (Pandey et al., 2014; Mahato et al., 2015). Other than phosphorylation, ubiquitination is also an important reversible post-translational modification that has been reported to regulate the function of many GPCRs (Hershko and Ciechanover, 1998; Hicke et al., 2005; Dores and Trejo, 2012a; Komander and Rape, 2012; Alonso and Friedman, 2013). Ubiquitination acts as a sorting signal to facilitate trafficking of mammalian GPCRs from endosome to lysosome for degradation (Marchese et al., 2008). For some receptors, ubiquitination also serves as a signal for receptor endocytosis. For example, internalization of ste2 and ste3 receptors (yeast GPCRs) is ubiquitin dependent (Hicke and Riezman, 1996; Terrell et al., 1998).

In order to check whether ubiquitination plays any role in the endocytosis of group I mGluRs, we used a pharmacological inhibitor of the E1 enzyme, viz., PYR-41. PYR-41 is a cell permeable compound, known to irreversibly inhibit the E1-ubiquitin thioester bond formation (Yang et al., 2007; Dey et al., 2008; Zaarur et al., 2008; Satheshkumar et al., 2009; Edelmann et al., 2011). For this study, we used myc-mGluR1 and myc-mGluR5 constructs, where myc was tagged at the

N-terminus of the full-length mGluR1 and mGluR5. Earlier studies have suggested that addition of the myc epitope at the N-terminus of these constructs did not affect the targeting and signaling by these receptors (Choi et al., 2011). Our data suggested that both myc-mGluR1 and myc-mGluR5 internalized rapidly upon application of the ligand, viz.,R,S-DHPG, a specific agonist of group I mGluRs. The maximum internalization was observed at 30 min post-agonist stimulation in both non-neuronal HEK293 cells and primary hippocampal neurons, suggesting that group I mGluR endocytosis kinetics is similar in both non-neuronal cells and primary hippocampal neurons.

Application of 50 µM PYR-41 completely inhibited the internalization of mGluR1 in HEK293 cells as well as in primary hippocampal neurons. Our data also suggested that the endocytosis of mGluR1 was uniform throughout the neuron and PYR-41 had similar effect on the endocytosis of the receptor in both cell body and dendrites. All these data suggest that ubiquitination play critical role in the trafficking of group I mGluRs. The block in the ligand-mediated internalization of mGluR1 could be because of two reasons: (1) PYR-41 inhibited the ubiquitination of group I mGluRs or (2) because of PYR-41 application, some general trafficking machinery was altered. In order to find out which one among the above two possibilities is true, we performed pull down experiments and found that application of 100 µM R,S-DHPG resulted in the robust ubiquitination of mGluR1, whereas, the application of 50 µM PYR-41 inhibited the ligand-mediated ubiquitination of mGluR1. Furthermore, 50 µM PYR-41 did not affect the transferrin receptor kinetics in HEK293 cells, suggesting that it does not have a general effect on the trafficking machinery of the cells. PYR-41 also inhibited the internalization of mGluR5, another member of the group I mGluR family. Together, these results suggest a critical role of ubiquitination in agonist stimulated endocytosis of group I mGluRs in both non-neuronal HEK293 cells and primary hippocampal neurons.

Subsequent to endocytosis, GPCRs can have various sub cellular fates. In case of group I mGluRs, we show here, that internalized receptors recycle back to the cell surface in 2.5 hr post-ligand application in non-neuronal cells and primary hippocampal neurons. Thus, our results suggest that the endocytosis and recycling of group I mGluRs is probably a way to resensitize the receptors.

# Mechanisms of ubiquitination-dependent ligand-mediated endocytosis of mGluR1 and mGluR-mediated AMPAR endocytosis

## 4.1. Introduction

Like many other GPCRs, trafficking of group I mGluRs also plays a critical role in the regulation of the activity of these receptors, as well as it controls the spatio-temporal localization of the receptor at the postsynaptic membrane. Ubiquitination is a post-translational modification that was originally identified as a prerequisite for the degradation of proteins (Schwarz and Patrick, 2012). Ubiquitination of proteins involves sequential action of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin carrying enzyme (E2) and ubiquitin protein ligase (E3) (Tanowitz and von Zastrow, 2002; Ciechanover and Ben-Saadon, 2004; Komander, 2009; Mabb and Ehlers, 2010; Caballero and Marchese, 2011; Dores and Trejo, 2012a; Lin and Man, 2013; Skieterska et al., 2017). In the last few years, it has become clear that the role of ubiquitination is not limited to the degradation of proteins. Ubiquitination also regulates the internalization of several plasma membrane proteins. For example, monoubiquitination is both sufficient and necessary for the constitutive, as well as, agonist-induced internalization of yeast Ste2 and Ste3 receptors (Terrell et al., 1998; Haglund et al., 2003b). On the other hand, number of studies have suggested that for many GPCRs, ubiquitination is not directly involved in the internalization of the receptor (Tanowitz and von Zastrow, 2002). Among the GPCRs, $\beta_2$ adrenergic receptors and C-X-C receptor 4 (CXCR 4) chemochine receptors were the initial mammalian GPCRs studied for their ubiquitination-dependent regulation subsequent to the agonist-stimulation (Marchese and Benovic, 2001; Shenoy et al., 2001a). It has been reported that ubiquitination somehow regulates the mGluR-mediated AMPAR trafficking and mGluR-LTD in hippocampal pyramidal cells (Kauer and Malenka, 2007; Citri et al., 2009). Previous reports have also suggested that group I mGluRs get ubiquitinated upon ligand-stimulation (Moriyoshi et al., 2004a; Ko et al., 2012a). However, the role of ubiquitination in the regulation of ligand-mediated internalization of group I mGluRs, and its physiological significance has not been studied. Since our earlier data suggested that the ligand-dependent internalization of group I mGluRs is ubiquitination dependent, we therefore investigated how ubiquitination regulates the trafficking of group I mGluRs.

In order to investigate whether monoubiquitination or polyubiquitination is involved in the ligand-dependent internalization of group I mGluRs, we used various mutant constructs of ubiquitin (K0Ub, K48RUb, and K63RUb). Our data suggested that the ligand-mediated

internalization of mGluR1 is K63-linked polyubiquitination dependent. Subsequently, we searched for the site of ubiquitination in mGluR1. For that, we targeted the C-terminus tail of mGluR1. The long C-terminus tail of mGluR1 has been reported to regulate the signaling and trafficking of mGluR1 and contains 16 lysine residues (Pin and Duvoisin, 1995; Ciruela et al., 1999; Remelli et al., 2008; Bhattacharyya, 2016). We found that the lysine residue at the 1112 position in the C-terminus tail of mGluR1 is critical for the ligand-mediated internalization of mGluR1. Mutation of this residue to arginine resulted in the inhibition of the ligand-mediated internalization of mGluR1. It has been reported that the E3 ubiquitin ligase, Siah-1A interacts with group I mGluRs and over-expression of Siah-1A results in the degradation of group I mGluRs (Moriyoshi et al., 2004a; Ko et al., 2012a). In our assays, acute knockdown of endogenous Siah-1A resulted in the complete block in the ligand-stimulated internalization of mGluR1. We also show here, that acute knockdown of Siah-1A led to the enhanced mGluR-mediated AMPAR endocytosis which is the cellular correlate of mGluR-LTD.

## 4.2. Results

### 4.2.1. Effects of the over-expression of ubiquitin mutants on the surface expression of mGluR1 in HEK293 cells

Our earlier experiments suggested that ubiquitination plays a critical role in the ligand-dependent endocytosis of group I mGluRs. To further dig deep into the mechanism, we studied how ubiquitination regulates the internalization of group I mGluRs. Various reports have suggested that monoubiquitination is sufficient to induce the internalization of some GPCRs (Hicke and Riezman, 1996; Terrell et al., 1998; Wojcikiewicz, 2004; Hislop and von Zastrow, 2011). When a single moiety of ubiquitin is attached to a target protein, it is known as monoubiquitination and attachment of multiple ubiquitin moieties to a target protein results in multi-monoubiquitination or polyubiquitination (Komander, 2009; Alonso and Friedman, 2013). Ubiquitin protein is a 76 amino acid long peptide which is highly conserved across the phylum. Ubiquitin protein contains 7 lysine (K6, K11, K27, K29, K33, K48, K63) residues and an amino-terminus methionine (Met or M1) that can serve as an acceptor site for additional ubiquitin molecules generating polyubiquitinated proteins (Komander, 2009; Komander and Rape, 2012; Alonso and Friedman, 2013). Thus, ubiquitin chains can be connected to either of the lysines present at position K6, K11, K27, K29, K33, K48, K63 or M1, resulting in the formation of various types of ubiquitin chains. In order to investigate the type of ubiquitination involved in the trafficking of mGluR1, we used various ubiquitin mutant constructs, viz., K0Ub, K48RUb and K63RUb. In order to check for the involvement of monoubiquitination or multi-monoubiquitination in the trafficking of mGluR1, we used K0Ub construct, where all the seven lysine residues were mutated to arginine. Over-expression of this construct would not allow the formation of polyubiquitin chain but will allow the monoubiquitination or multi-monoubiquitination of the receptor. We used another construct, K48RUb, where lysine at the $48^{th}$ position in the ubiquitin protein was mutated to arginine. Expression of this construct, specifically inhibits the formation of K48-linked polyubiquitin chain. Similarly, to inhibit the K63-linked polyubiquitin chain formation, we used a K63RUb construct, where the lysine residue present at the $63^{rd}$ position was mutated to arginine. Diagrammatic representation of various constructs used in this study has been shown in **figure 4.1.**

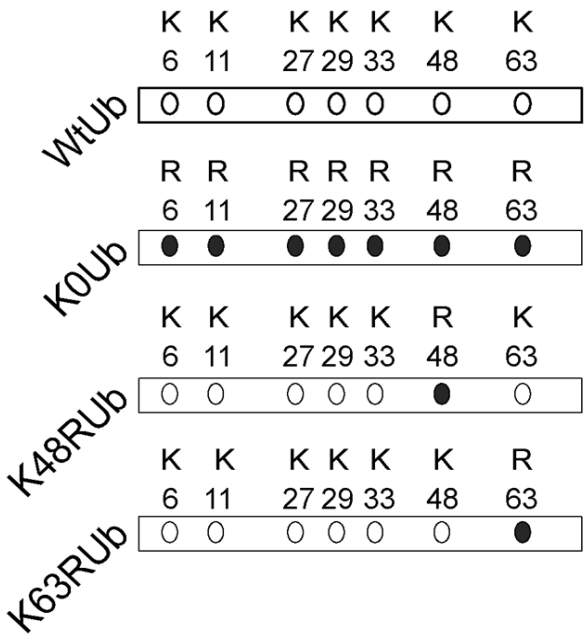


**Figure 4.1. Schematic of various ubiquitin constructs.**

To check for the effect of these ubiquitin mutants on the surface expression of myc-mGluR1, HEK293 cells were cotransfected with myc-mGluR1 cDNA and one of the ubiquitin mutants, using lipofectamine 2000. 24 hr post-transfection, cells were incubated with anti-myc primary antibody (1:500) for 15 min at 37°C and subsequently, they were fixed without permeabilization in cold 4% PFA on ice for 15 min. After that, goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) was applied for 1 hr at 37°C. Following that, cells were permeabilized using 0.1% Triton X-100 for 30 min at room temperature and anti-HA primary antibody (rat monoclonal) (1:500) was applied for overnight at 4˚C to visualize the ubiquitin constructs (all the ubiquitin constructs were tagged with the HA epitope). Subsequently, goat anti-rat Alexa-488 tagged secondary antibody (1:800) was applied for 1 hr at 37°C. Cells were then mounted on glass slides and observed under the confocal microscope. Over-expression of all these mutants (K0Ub, K48RUb and K63RUb) did not have any effect on the surface expression of myc-mGluR1 in HEK293 cells (control: 1 ± 0.04; K0Ub: 1.05 ± 0.09; K48RUb: 1 ± 0.11; K63RUb: 1.05 ± 0.08) **(Figure 4.2 A, B)**.

### 4.2.2. Effects of the over-expression of ubiquitin mutants on the ligand-mediated endocytosis of mGluR1 in HEK293 cells

We next investigated, whether monoubiquitination or polyubiquitination is involved in the ligand-mediated endocytosis of mGluR1, using the above mutants of ubiquitin. HEK293 cells were cotransfected with myc-mGluR1 cDNA and one of the ubiquitin mutants using lipofectamine 2000. 24 hr post-transfection, cells were incubated with anti-myc antibody (1:500) for 15 min at 37°C. Endocytosis of the receptor was then induced by applying 100 µM R,S-DHPG for 5 min followed by chasing the receptor for a total of 30 min in absence of the ligand. After that, cells were fixed without permeabilization in 4% ice cold PFA for 15 min and stained with a goat anti-mouse Alexa-568 secondary antibody (1:100) for 1 hr at 37°C. Subsequently, cells were permeablized and incubated with the rat monoclonal anti-HA primary antibody (1:500) at 4˚C overnight, followed by the application of the secondary antibodies against the myc antibody (goat anti-mouse Alexa-647) and HA antibody (goat anti-rat Alexa-488) for 1 hr at 37°C. Cells were then mounted on glass slides and observed under the confocal microscope. Our data suggested that, cells over-expressing K0Ub did not show any significant endocytosis of the

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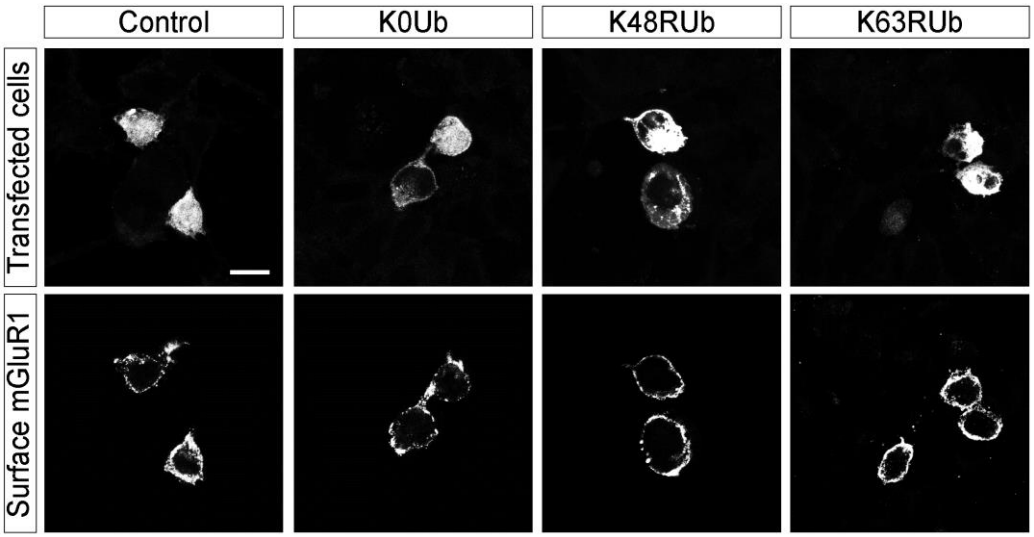


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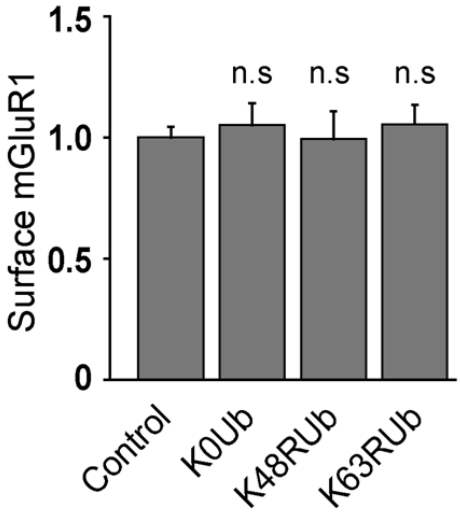
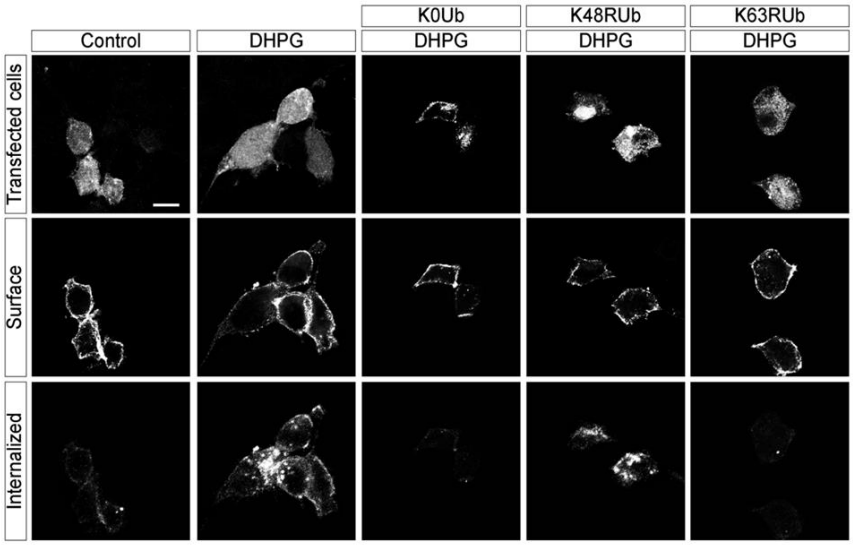



**Figure 4.2. Effect of the over-expression of K0Ub, K48RUb and K63RUb on the surface expression of myc-mGluR1 in HEK293 cells.** Representative images (A) and quantitation (B) of the surface myc-mGluR1 showed that expression of K0Ub, K48RUb and K63RUb had no effect on the surface expression of myc-mGluR1. Scale bar = 10 µm. n.s indicates $p > 0.05$.

receptor as compared to control cells (control: 1 ± 0.06; control + DHPG: 1.75 ± 0.09; K0Ub + DHPG: 0.93 ± 0.07) **(Figure 4.3 A, B)**. These results suggested that monoubiquitination or multi-monoubiquitination was not sufficient for the ligand-mediated endocytosis of mGluR1. To determine, if polyubiquitin chain formation through the K48-linkage is required for the internalization of myc-mGluR1, we studied the internalization of the receptor in cells over-expressing K48RUb. Over-expression of K48RUb did not affect the internalization of myc-mGluR1 in HEK293 cells compared to the control condition (K48RUb + DHPG: 1.77 ± 0.15). The receptor internalized to almost similar extent on R,S-DHPG application in control cells as well as in cells over-expressing K48RUb. Finally, we investigated the role of K63-linked polyubiquitination, if any, on the ligand-mediated internalization of myc-mGluR1 by over-expressing K63RUb. As stated earlier, over-expression of K63RUb specifically inhibits the K63-linked polyubiquitination, leaving all other possibilities of ubiquitination unchanged. Importantly, cells overexpressing K63RUb showed complete inhibition in the 100 µM R,S-DHPG mediated endocytosis of myc-mGluR1 (K63RUb + DHPG: 1.1 ± 0.07). These results suggest that mGluR1 undergoes endocytosis subsequent to the R,S-DHPG application through the K63-linked polyubiquitination chain formation.

In order to ascertain, whether K63RUb was incorporated to the receptor upon application of R,S DHPG, HEK293T cells were co-transfected with FLAG-mGluR1 and either HA-WtUb or HA-K63RUb. Co-immunoprecipitation assays were performed as per the protocol discussed in the "Materials and Methods" chapter. In HA-WtUb expressing cells, application of 100 µM R,S-DHPG resulted in the robust incorporation of HA-WtUb. Similarly in HA-K63RUb expressing cells as well, application of 100 µM R,S-DHPG resulted in the incorporation of HA-K63RUb in FLAG-mGluR1 as compared to R,S-DHPG untreated conditions. These results suggested that HA-K63RUb got incorporated into FLAG-mGluR1 upon ligand-mediated activation **(Figure 4.4)**.

**A**

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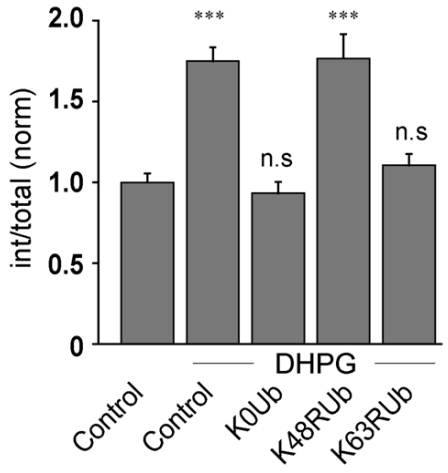


**Figure 4.3. Ligand-mediated endocytosis of myc-mGluR1 is K63-linked poly-ubiquitination dependent in HEK293 cells.** (A) Representative images showing the effect of over-expression of various ubiquitin mutants on the ligand-mediated internalization of myc-mGluR1 in HEK293 cells. (B) Quantitation of the effect of various ubiquitin mutants on the R,S-DHPG-mediated endocytosis of myc-mGluR1. Scale bar = 10 µm. *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

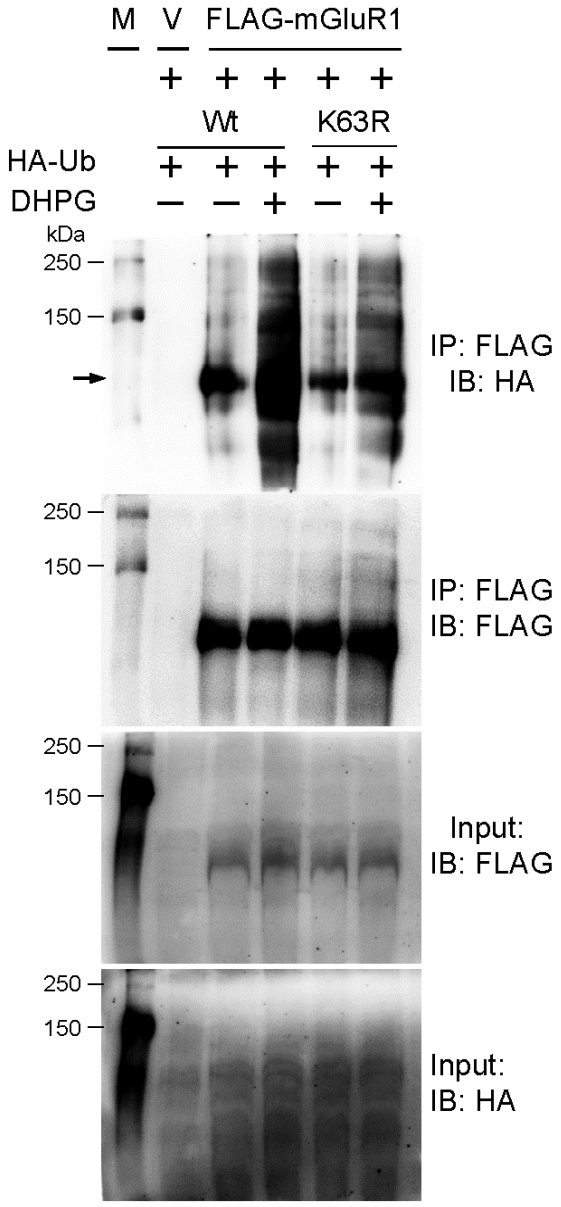


**Figure 4.4. Incorporation of K63RUb in FLAG-mGluR1 upon ligand application.** Co–immunoprecipitation experiment showing K63RUb incorporation in mGluR1 upon application of 100 μM R,S-DHPG.

### 4.2.3. The C-terminal lysine residues of mGluR1 play critical role in the endocytosis of the receptor in HEK293 cells

The G-protein coupled receptor superfamily is characterized by the presence of seven transmembrane domains, 3 cytosolic intracellular loops and a long C-terminal tail. The C-terminal tail of GPCRs is known to regulate the signaling as well as the trafficking of these receptors (Krupnick and Benovic, 1998; Sallese et al., 2000; Marchese et al., 2003; Drake et al., 2006; Shenoy, 2007; Remelli et al., 2008; Azevedo et al., 2015; Bhattacharyya, 2016). Group I mGluRs have a long C-terminal tail which plays a crucial role in their localization at the cell surface, signaling, trafficking, desensitization and resensitization (Ciruela et al., 1999; Stowell and Craig, 1999; Dale et al., 2000; Ferguson, 2001; Marchese et al., 2003; Remelli et al., 2008; Choi et al., 2011; Gulia et al., 2016). To identify the lysine residues present at mGluR1, that play critical role in the ubiquitin-dependent internalization of the receptor, we targeted the C-terminus of the receptor. The C- terminus tail (841-1199 amino acid) of mGluR1 contains 16 lysine residues. We made various mutant constructs of myc-mGluR1, where we mutated the lysine present at the C-terminus to arginine. Lysine residue present at the 1193 position of the receptor was changed to arginine and was named as 1K/R. Application of 100 µM R,S-DHPG resulted in the robust internalization of the 1K/R mGluR1 similar to that was observed in Wt mGluR1 (Wt mGluR1:: control: 1 ± 0.06; DHPG: 1.86 ± 0.08; 1K/R mGluR1:: control: 1 ± 0.1; DHPG: 1.67 ± 0.09) **(Figure 4.5 A, B)**. In this 1K/R background, we generated another construct named, 2K/R mGluR1 in which the lysine residue present at the 1141 position of the receptor was converted to arginine. Interestingly, we found that upon stimulation with 100 µM R,S-DHPG there was a partial block in the internalization of the receptor (2K/R mGluR1: control: 1 ± 0.05; DHPG: 1.3 ± 0.07). However, when another mutation in this 2K/R background at position 1112 (3K/R mGluR1) was done, we observed that application of 100 µM R,S-DHPG was unable to induce internalization of the receptor (3K/R mGluR1: control: 1 ± 0.07; DHPG: 1.02± 0.07).

### 4.2.4. Lysine at the 1112 position of mGluR1 plays critical role in the ligand-mediated endocytosis of mGluR1

Our previous results suggested that the last three lysine residues present at the C-terminal tail of mGluR1 are crucial for the ligand-mediated internalization of the receptor. We wanted to check whether the block in the internalization of myc-mGluR1 was a cumulative effect due to the

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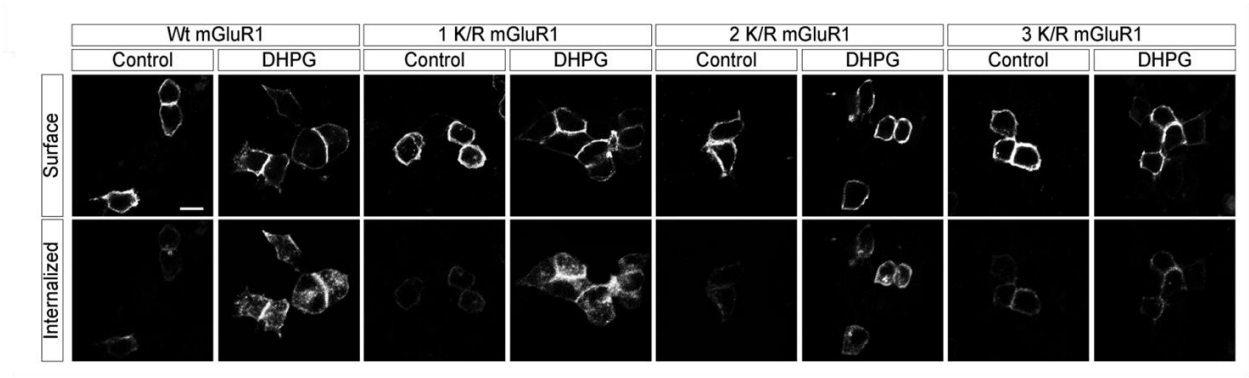


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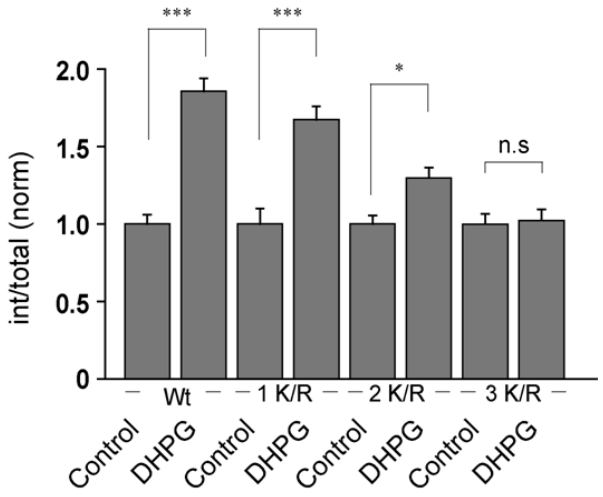


**Figure 4.5. Role of the lysine residues present at the C-terminus of mGluR1 in the ligand-mediated internalization of mGluR1.** (A) Representative images showing endocytosis of wild-type and various mutants (1K/R, 2K/R, and 3K/R) of mGluR1 on 100 µM R,S-DHPG stimulation in HEK293 cells. (B) Measurement of the internalization suggested that wild-type myc-mGluR1 and myc-mGluR1 1K/R showed similar amount endocytosis, whereas myc-mGluR1 2K/R showed lesser endocytosis than Wt upon application of the ligand. In case of myc-mGluR1 3K/R, application of 100 µM R,S-DHPG was unable to induce internalization of the receptor. Scale bar = 10 µm. *** indicates $p < 0.001$; *, $p < 0.05$ and n.s, $p > 0.05$.

mutations of all three lysines or whether the lysine present at the 1112 position played the most critical role. To test this hypothesis, we generated another construct having single point mutation at the 1112 position, resulting in the change of the lysine 1112 to arginine (K1112R myc-mGluR1). Upon application of 100 µM R,S-DHPG, K1112R myc-mGluR1 did not internalize in HEK293 cells (Wt:: control: 1 ± 0.09; DHPG: 1.83 ± 0.12; K1112R:: control: 1 ± 0.09; DHPG: 0.84± 0.08) **(Figure: 4.6 A, B)**.

Subsequently, we investigated the role of the lysine present at the 1112 position in the internalization of mGluR1 in primary hippocampal neurons. Initially, we checked for the effect of mutating the lysine on the surface localization of the receptor. Our data suggested that mutation of the lysine residue at the 1112 position to arginine did not affect the surface expression of the receptor (Wt: 1 ± 0.06, K1112R: 0.95 ± 0.06) **(Fig. 4.7 A, B)**. After that, we studied the ligand-stimulated internalization of the receptor in primary hippocampal neurons and found that similar to in HEK293 cells, the K1112R myc-mGluR1 did not internalize on the application of 100 µM R,S-DHPG, whereas, the wild-type myc-mGluR1 showed normal endocytosis upon application of the ligand in hippocampal neurons (Wt: control: 1 ± 0.07; DHPG: 1.78 ± 0.18; K1112R: control = 1 ± 0.03; DHPG = 0.87 ± 0.06) **(Figure: 4.8 A, B)**. Together these results suggest that the lysine residue present at the 1112 position in mGluR1 plays critical role in the ligand-stimulated internalization of the receptor.

### 4.2.5. Role of Siah-1A in the regulation of ligand-mediated endocytosis of mGluR1 in primary hippocampal neurons

*Drosophila* Sina (seven in absentia) and its mammalian homolog Siah (seven in absentia) are members of the RING finger containing E3 ubiquitin ligase family. In mice, there are three Siah proteins, Siah-1A, Siah-1B, and Siah-2 (Della et al., 1993). Siah-1A has been demonstrated to bind to the carboxyl-terminal domain of group I mGluRs and modulate group I mGluR-mediated signaling (Ishikawa et al., 1999a; Kammermeier and Ikeda, 2001; Moriyoshi et al., 2004b; Ko et al., 2012b).

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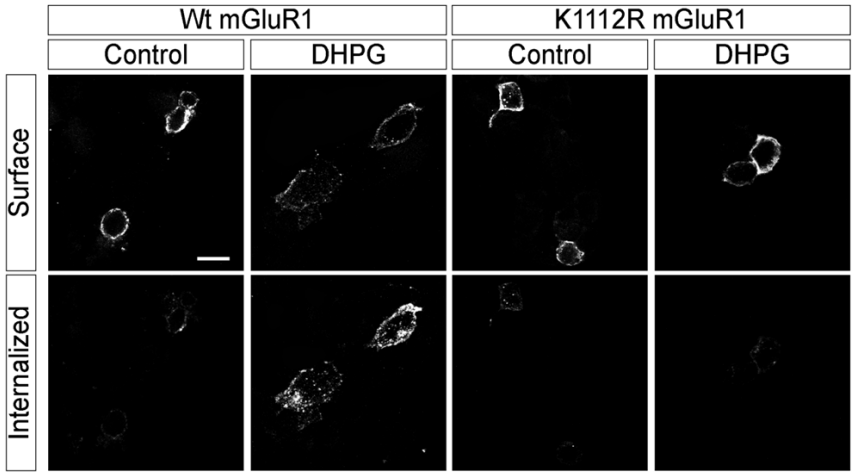


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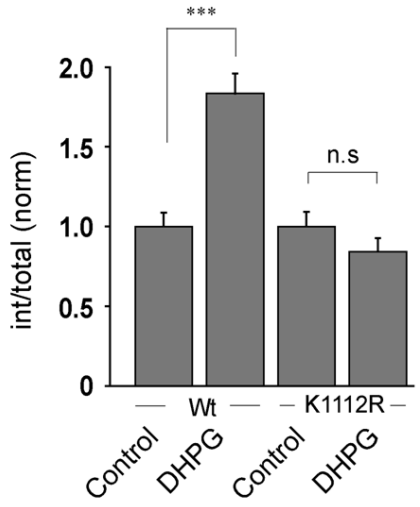


**Figure 4.6. Lysine 1112 residue of mGluR1 plays critical role in the ubiquitin-dependent internalization of the receptor in HEK293 cells.** (A) Representative images of HEK293 cells showing ligand-mediated internalization of wild-type myc-mGluR1 and K1112R myc-mGluR1. (B) Quantitation of the internalization showing that application of 100 µM R,S-DHPG resulted in the robust internalization of the wild-type myc-mGluR1, whereas, application of the ligand was unable to induce the internalization of the K1112R myc-mGluR1 in HEK293 cells. Scale bar = 10 µm. *** indicates $p < 0.001$ and n.s, $p > 0.05$.

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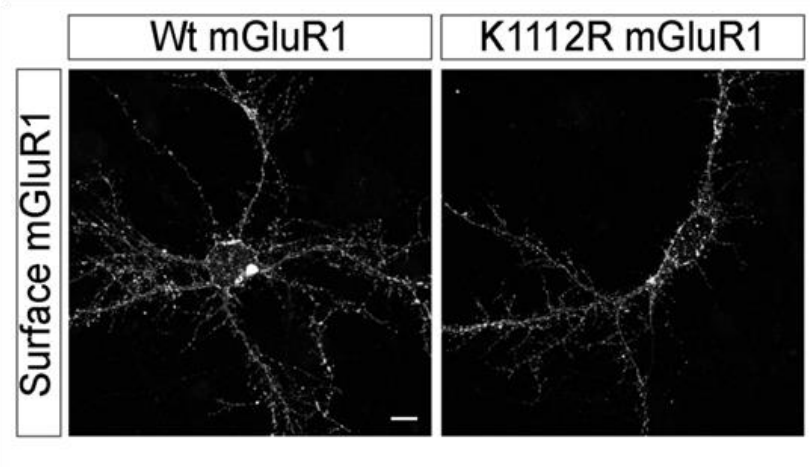


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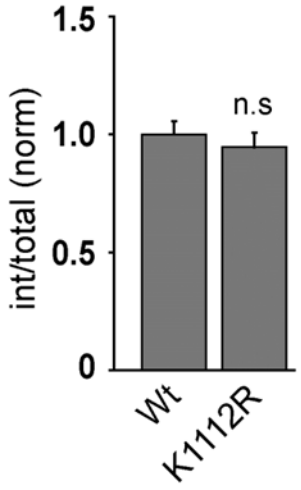


**Figure 4.7. Effect of the mutation of lysine 1112 residue on the surface expression of myc-mGluR1 in primary hippocampal neurons.** (A) Representative images showing the surface expression of wild-type myc-mGluR1 and K1112R myc-mGluR1 in primary hippocampal neurons. (B) Quantitation showing no change in the surface localization of wild-type myc-mGluR1 and K1112R myc-mGluR1 in primary hippocampal neurons. Scale bar = 10 µm. n.s indicates $p > 0.05$.

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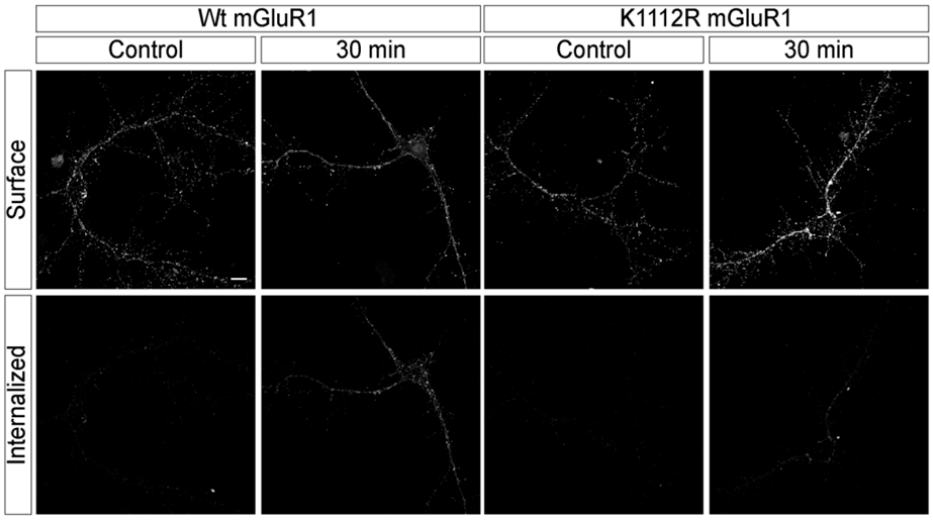


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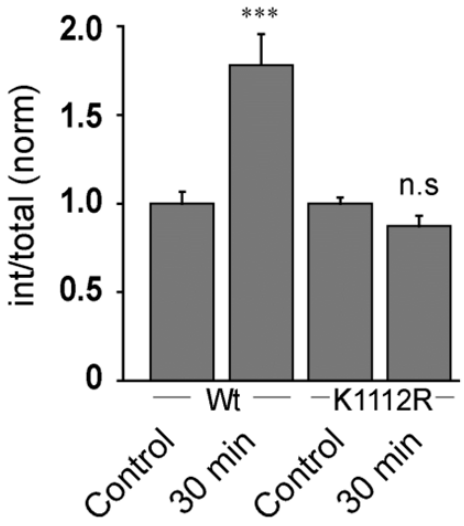



**Figure 4.8. Mutation of the lysine 1112 residue to arginine in mGluR1 blocks the ligand mediated endocytosis of the receptor in primary hippocampal neurons.** Representative images (A) and quantitation (B) showing wild-type myc-mGluR1 internalized upon ligand activation, whereas application of 100 µM R,S-DHPG was unable to induce internalization of K1112R myc-mGluR1 in primary hippocampal neurons. Scale bar = 10 µm. *** indicates $p < 0.001$; and n.s, $p > 0.05$.

In order to investigate whether Siah-1A plays any role in the ligand-mediated internalization of mGluR1, primary neurons were cotransfected with myc-mGluR1 and siRNA against the endogenous Siah-1A (ON-TARGET plus) (si-Siah-1A) or scrambled siRNA (si-control) at 8-9 day *in vitro*. Knockdown of the endogenous Siah-1A was confirmed by western blot. Our data suggested that si-Siah-1A significantly knocked down the endogenous Siah-1A, whereas the si-control did not show any significant knockdown **(Figure 4.9)**. We further studied the ubiquitination status of mGluR1 in Siah-1A knockdown background. FLAG-mGluR1 cDNA and Wt HA-Ub were cotransfected with either si-Siah-1A or si-control in HEK293T cells and pull down experiments were performed 72 hr post-transfection. Interestingly, we found that in control cells as well as in si-control transfected cells, application of 100 µM R,S-DHPG resulted in the robust ubiquitination of FLAG-mGluR1. On the other hand, in si-Siah-1A transfected cells, application of 100 µM R,S-DHPG was unable to induce ubiquitination of FLAG-mGluR1 **(Figure 4.10)**.

Siah-1A has been shown to bind to the Siah-interacting domain (SID) at the carboxy-terminal region of the full-length group I metabotropic glutamate receptors (Ko et al., 2012a; Zhang et al., 2017). We therefore investigated the role of Siah-1A, if any, in the internalization of mGluR1. Primary hippocampal neurons were cotransfected with myc-mGluR1 cDNA and si-Siah-1A or si-control at 8-9 day *in vitro* and experiments were performed when the cells were at 12-13 day *in vitro* according to the method described in the "Materials and Methods" chapter. Initially, we investigated whether acute knockdown of Siah-1A had any effect on the surface expression of myc-mGluR1. Our data suggested that acute knockdown of endogenous Siah-1A did not affect the surface expression of myc-mGluR1 in primary hippocampal neurons (control: 1 ± 0.05; si-Siah-1A: 1.1 ± 0.07; si-control: 0.94 ± 0.11) **(Figure 4.11 A, B)**.

Subsequently, we studied the effect of Siah-1A knockdown on the R,S-DHPG-mediated internalization of myc-mGluR1 in primary hippocampal neurons. We observed, that there was a complete inhibition of myc-mGluR1 endocytosis upon application of 100 µM R,S-DHPG in si-Siah-1A transfected cells, whereas in control cells and si-control transfected cells, myc-mGluR1 endocytosis remained unaffected (control: 1 ± 0.05; control + DHPG: 1.72 ± 0.09; si-Siah-1A + DHPG: 1.1 ± 0.06; si-control + DHPG: 1.84 ± 0.15) **(Figure: 4.12 A, B)**

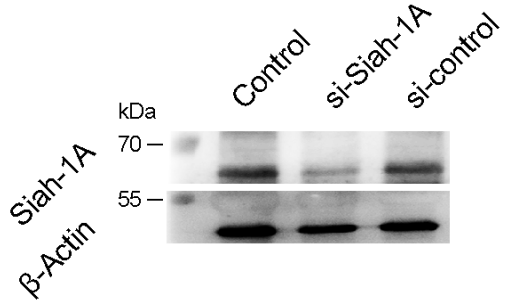



**Figure 4.9. Knockdown of Siah-1A by si-Siah-1A.** Western blot showing significant knockdown of the endogenous Siah-1A by si-Siah-1A in primary neurons.

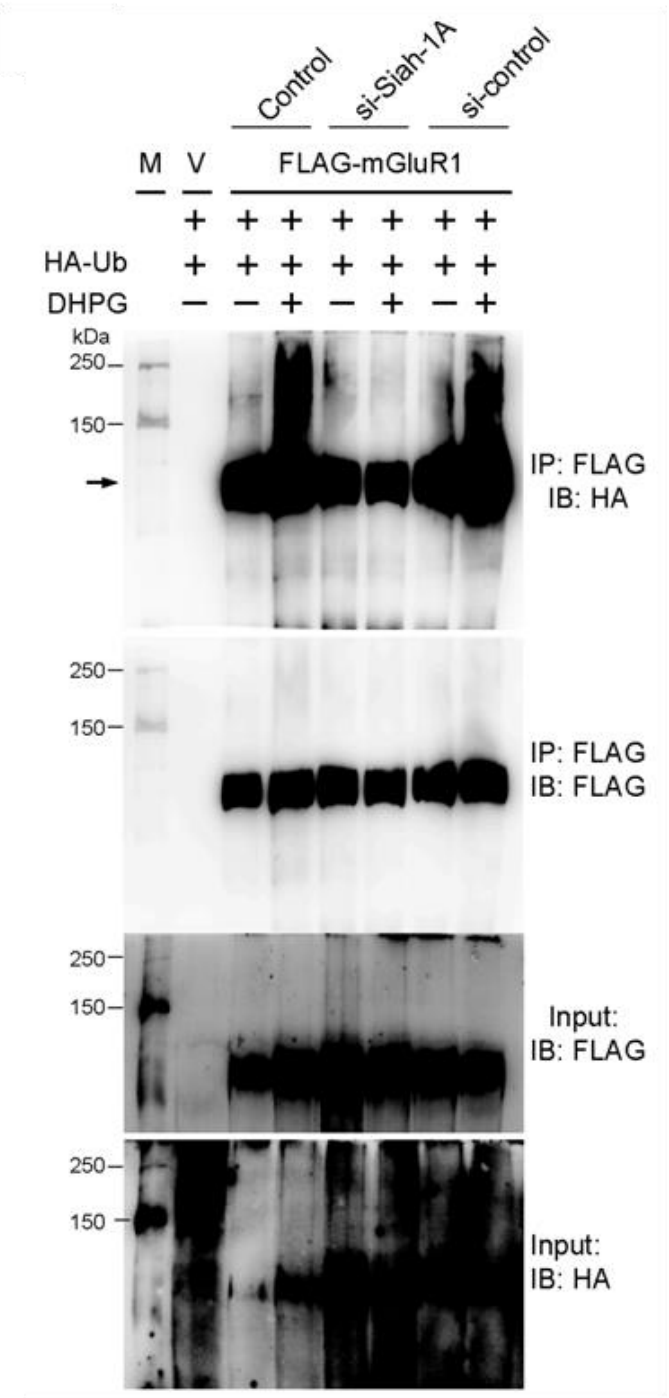
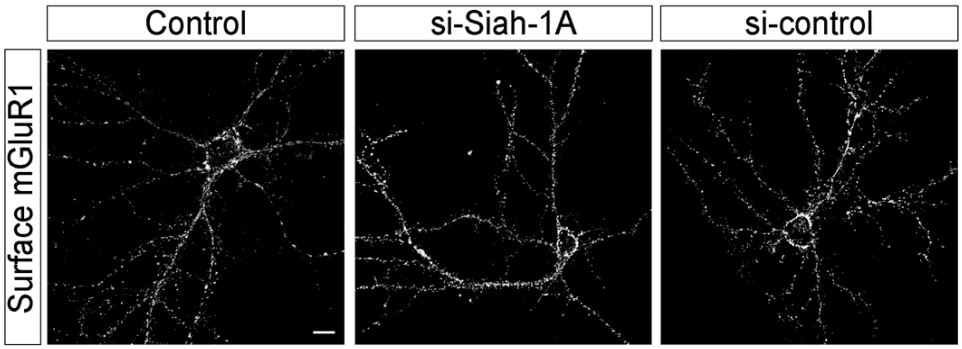



**Figure 4.10. Knockdown of Siah-1A inhibits the ligand-mediated increase in the ubiquitination of mGluR1.** Co-immunoprecipitation assay confirming that acute knockdown of endogenous Siah-1A inhibits the ligand-induced increase in the ubiquitination of myc-mGluR1 in HEK293T cells.

**A**




**B**

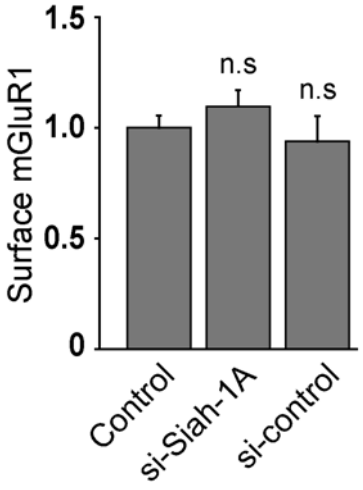
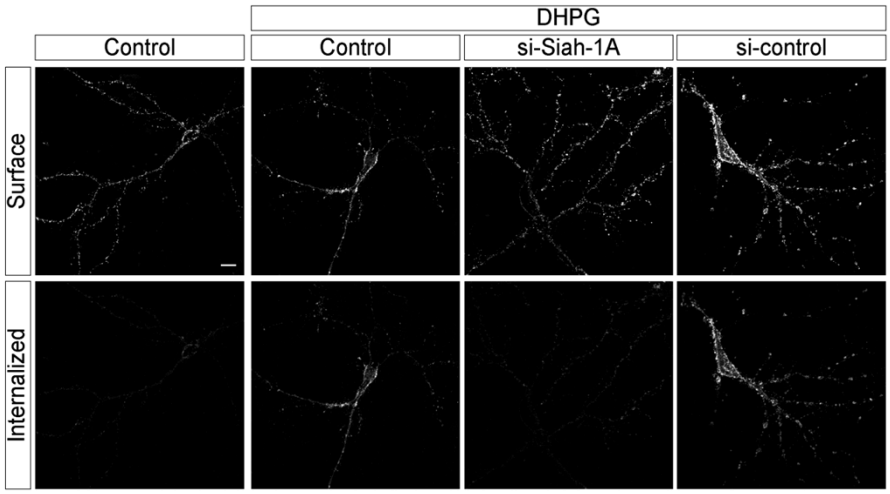



**Figure 4.11. Effect of the knockdown of Siah-1A on the surface expression of mGluR1.** Representative images (A) and quantitation (B) showing that knockdown of endogenous Siah-1A did not affect the surface expression of myc-mGluR1 in primary hippocampal neurons. Scale bar = 10 µm. n.s indicates $p > 0.05$.

**A**




**B**

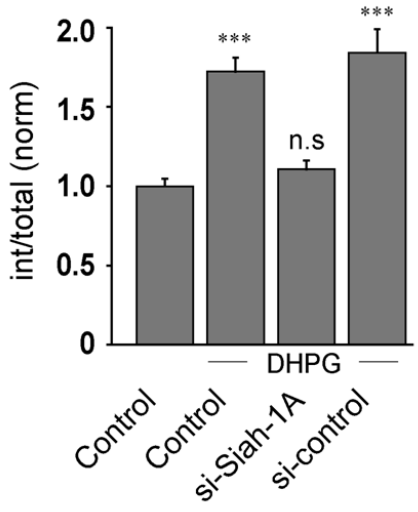



**Figure 4.12. Acute knockdown of Siah-1A results in the inhibition in the ligand-mediated internalization of mGluR1.** (A) Representative images showing acute knockdown of endogenous Siah-1A inhibited the ligand-mediated internalization of myc-mGluR1 in primary hippocampal neurons. (B) Measurement of the quantitation of the endocytosis index also confirmed the inhibition of the ligand-mediated internalization of myc-mGluR1 in Siah-1A knockdown cells. Scale bar = 10 µm. *** indicates $p < 0.001$ and n.s, $p > 0.05$.

All these data suggest that Siah-1A is involved in the ubiquitination of mGluR1 and it also plays critical role in the ligand-mediated internalization of the receptor.

### 4.2.6. Group I mGluR-mediated AMPAR internalization

Trafficking of α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors (AMPARs) inside and outside of the post-synaptic plasma membrane has emerged as an important mechanism in the regulation of synaptic function (Beattie et al., 2000; Carroll et al., 2001; Xiao et al., 2006). Insertion of AMPA receptors at the plasma membrane leads to the long term potentiation (LTP), whereas internalization of AMPARs from the cell surface is believed to be the cellular mechanisms for long term depression (LTD) (Kauer and Malenka, 2007). Rapid internalization of surface AMPA receptors can be induced in cultured hippocampal neurons by the application of various glutamate receptor agonists, including glutamate itself, NMDA, AMPA, and group I mGluR agonists (Biou et al., 2008; Bhattacharyya et al., 2009; Citri et al., 2009; Citri et al., 2010). Activation of group I mGluRs with the agonist, R,S-DHPG, induces the AMPAR endocytosis, which is prerequisite for the induction of mGluR-LTD (Zho et al., 2002; Zhang et al., 2008). It has been reported that in presence of UBEI-41/PYR-41 (an inhibitor of E1-activating enzyme), activation of group I mGluRs results in enhanced mGluR-LTD in the hippocampus (Citri et al., 2009). We therefore wanted to investigate whether inhibition of the ubiquitin pathway had any effect on the group I mGluR-mediated AMPAR endocytosis. Initially, we standardized an assay protocol that enabled us to induce synaptic AMPAR endocytosis upon activation of group I mGluRs. For this purpose, primary hippocampal neurons were pre-incubated with 50 µM AP5 (blocker of NMDAR), 20 µM DNQX (blocker of AMPAR) and TTX (blocker of the pre-synaptic release) for 30 min prior to the start of the experiment. Subsequently, cells were incubated with anti-GluA1 antibody (1:20) for 15 min at 37˚C. After that, group I mGluRs were stimulated with the application of 100 µM R,S-DHPG for 5 min, followed by chasing the cells for 10 min in absence of the ligand. The blockers were present throughout the experiment. Cells were then fixed in 4% cold PFA for 15 min on ice without permeabilization. To visualize the surface AMPA receptors, Alexa-568 tagged secondary antibody was applied in saturating concentration (1:100) for 1 hr at 37˚C. After that, cells were permeabilized in 0.1% Triton-X-100 for 30 min at room temperature and subsequently,

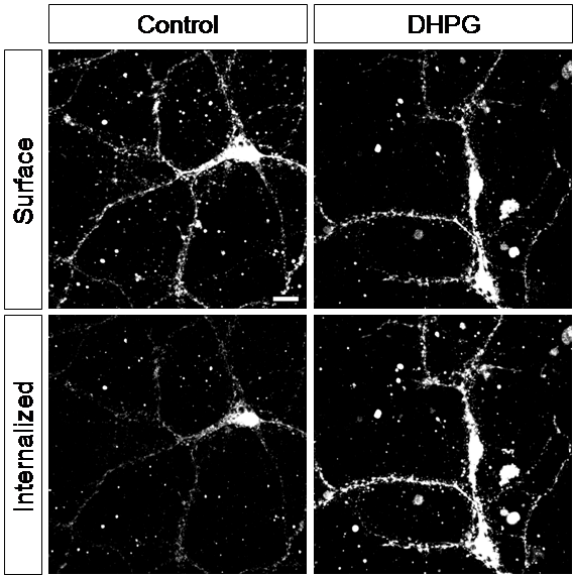
incubated with the second secondary antibody tagged with Alexa-647 for 1 hr at 37˚C to visualize the internalized AMPA receptors. Measurement of the endocytosis index suggested that activation of group I mGluRs with R,S-DHPG resulted in the internalization of GluA1 containing receptors (control: 1 ± 0.05; DHPG: 1.4 ± 0.05) **(Figure 4.13 A, B)**.

We next wanted to investigate whether activation of group I mGluRs resulted in the endocytosis of "synaptic" AMPA receptors. We quantified the proportion of synapses containing detectable level of surface AMPARs by staining for surface GluA1 containing clusters and counterstaining for Bassoon, a core component of the active zone that is commonly used to identify presynaptic terminals (Regalado et al., 2006). Our data suggested that in control condition (without ligand stimulation), significant (around 80%) amount of GluA1 containing puncta co-localized with the bassoon puncta and upon application of 100 µM R,S-DHPG, the amount of surface GluA1 puncta colocalized with Bassoon was significantly reduced. (control: 79.15 ± 2.1%, DHPG: 36.61 ± 2.6%) **(Figure 4.14)**. These results confirmed that in our assay, brief application of the ligand caused internalization of synaptic AMPA receptors.

In order to check whether Siah-1A plays any role in the group I mGluR-mediated AMPAR trafficking, primary hippocampal neurons were transfected with si-Siah-1A or si-control at 8-9 day *in vitro* and experiments were performed 72 hr post transfection using the similar method as described above. First, we studied the effect of acute knockdown of Siah-1A on the surface expression of AMPARs. Our data suggested that blocking ubiquitination of group I mGluRs by acute knockdown of Siah-1A did not affect the surface expression of GluA1 containing receptors (control: 1 ± 0.06; si-Siah-1A: 1.08 ± 0.05; si-control: 1.05 ± 0.04) **(Figure 4.15 A, B)**. Interestingly, knockdown of endogenous Siah-1A caused an enhancement of the dendritic AMPAR internalization triggered by the 100 µM R,S-DHPG as compared to control cells (control: 1 ± 0.05; control + DHPG: 1.62 ± 0.09; si-Siah-1A + DHPG: 2.08 ± 0.12; si-control + DHPG: 1.53 ± 0.1) **(Figure: 4.16 A, B)**.

These results suggest that stimulation of group I mGluRs with 100 µM R,S-DHPG triggers synaptic AMPAR endocytosis and knockdown of endogenous Siah-1A enhances mGluR-mediated AMPAR internalization.

**A**




**B**

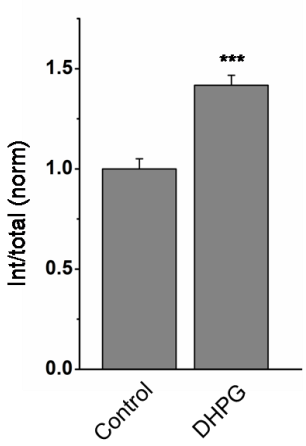
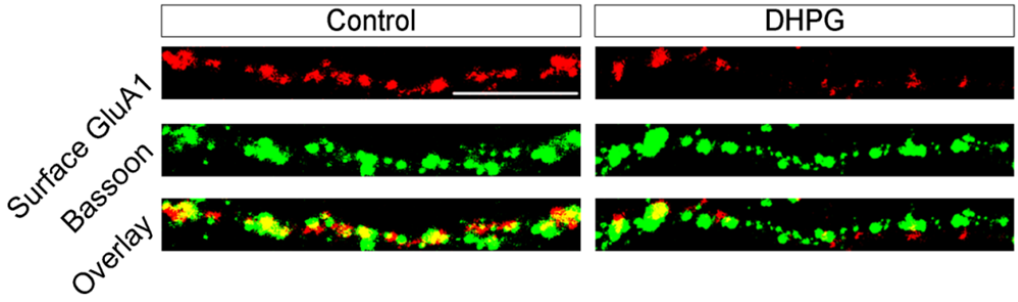



**Figure 4.13. Group I mGluR-mediated AMPAR internalization.** Representative images (A) and quantitation (B) showing that activation of group I mGluRs with 100 µM R,S-DHPG led to the internalization of AMPARs in primary hippocampal neurons. Scale bar = 10 µm. *** indicates $p < 0.001$.

**A**




**B**

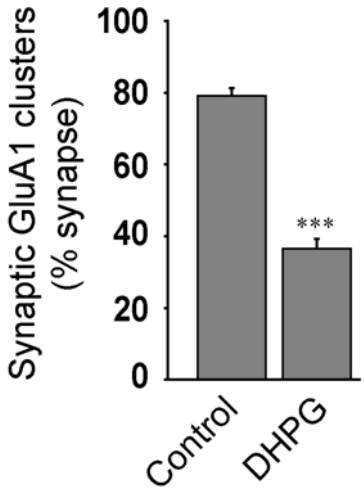
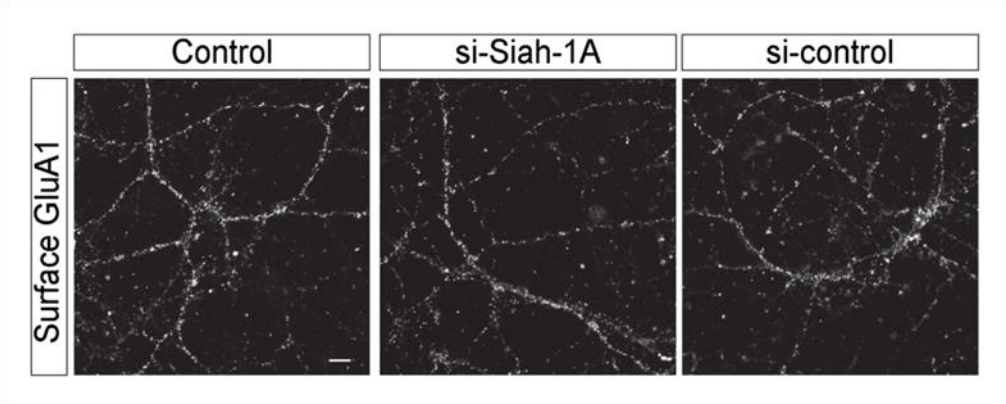



**Figure 4.14. Internalization of synaptic AMPARs upon activation of group I mGluRs.** (A) Example of the dendritic staining for surface GluA1 (red) and pre-synaptic marker Bassoon (green) after 100 µM R,S-DHPG treatment. (B) Measurement of the quantitation showing the percentage of synapses (defined by bassoon staining in green) that contain detectable surface GluA1 puncta in control cells and R,S-DHPG-treated cells. Scale bar = 10 µm. *** indicates $p < 0.001$.

**A**




**B**

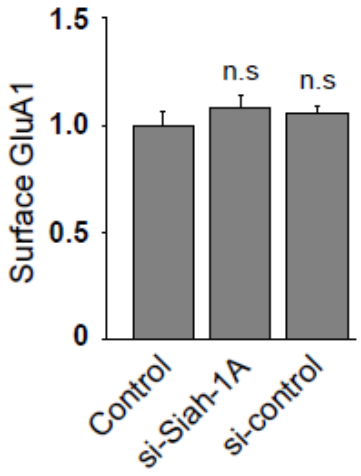
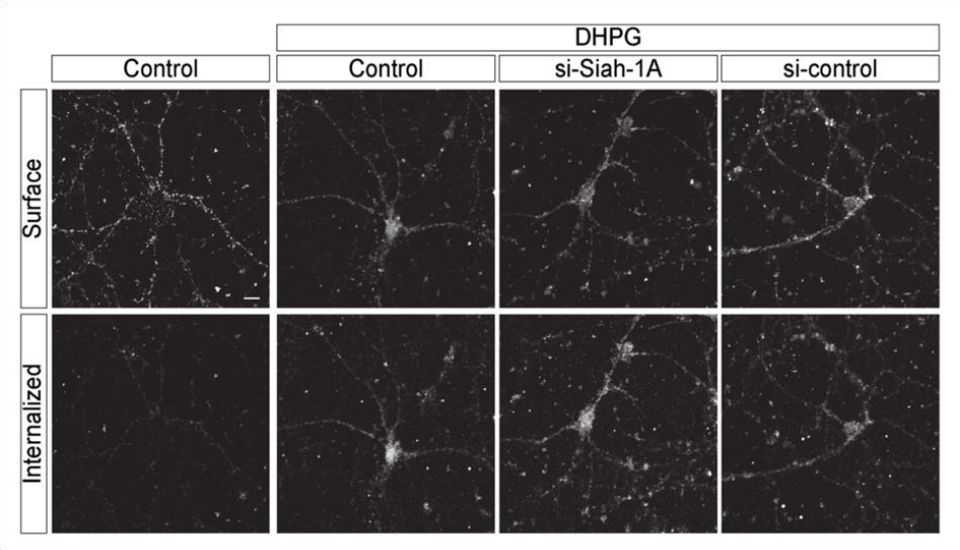



**Figure 4.15. Effect of the acute knockdown of Siah-1A on the surface expression of AMPARs.** Representative images (A) and quantitation (B) suggested that acute knockdown of endogenous Siah-1A had no effect on the surface expression of GluA1 containing receptors. Scale bar = 10 µm. n.s indicates $p > 0.05$.

**A**




**B**

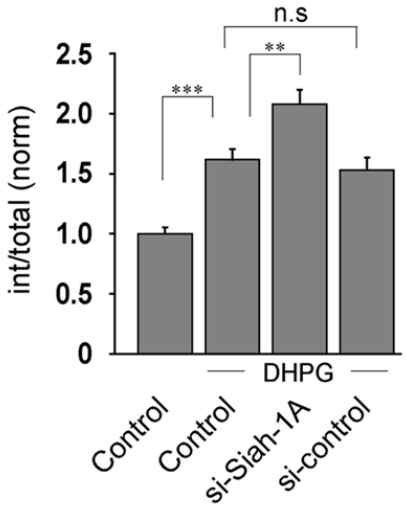



**Figure 4.16. Ubiquitination regulates the mGluR-mediated AMPAR trafficking**. Acute knockdown of Siah-1A resulted in the enhanced mGluR-mediated AMPAR internalization compared to control cells upon application of 100 μM R,S-DHPG as shown in representative images (A) and quantitation (B). Scale bar = 10 μm. *** indicates $p < 0.001$; **, $p < 0.01$; and n.s, $p > 0.05$.

## 4.3. Discussion

Ubiquitination, a reversible post-translational modification, is well studied mainly with respect to the degradation of proteins in the 26S proteasome. In the past few years, emphasis has been given on other functions of ubiquitination apart from degradation and one such process is endocytosis. In the case of β-adrenergic receptor (G-protein coupled receptor), signaling is regulated indirectly by ubiquitination (Shenoy et al., 2001b), whereas for some other GPCRs e.g., CXCR4, ubiquitination directly plays a critical role in the regulation of their signaling (Marchese and Benovic, 2001; Caballero and Marchese, 2011). Signaling of GPCRs is very tightly regulated and GPCR internalization is the key mechanism that helps in maintaining normal cellular homeostasis. Role of ubiquitination in the signaling and regulation of various GPCRs, including group I mGluRs, have been studied (Moriyoshi et al., 2004a; Hislop and von Zastrow, 2011; Dores and Trejo, 2012b; Ko et al., 2012a; Alonso and Friedman, 2013; Lin and Man, 2013; Gulia et al., 2016). Our data suggest that internalization of group I mGluRs is ubiquitination-dependent. It has become clear that different types of ubiquitin conjugates are involved in the regulation of different cellular processes. Literature suggests that mono-ubiquitination is sufficient for the internalization of some GPCRs (Terrell et al., 1998; Haglund et al., 2003a). Our data suggest that monoubiquitination or multi-monoubiquitination is not sufficient to induce the internalization of mGluR1. Interestingly, attachment of the K63-linked polyubiquitin chain is essential for the ligand stimulated internalization of mGluR1. How K63-linked polyubiquitination regulates the internalization and recycling of mGluR1 needs to be investigated in future. Whether the internalization of mGluR5 (another member of group I mGluR family) also depends on the K63-linked polyubiquitination or not, would be another interesting aspect to study.

The results from pharmacologically blocking the ubiquitination of group I mGluRs suggest a critical role of ubiquitination in the ligand-mediated internalization of the receptor. The results from this pharmacological study were further strengthened by the observation that mutation of a lysine residue at the C-terminus of mGluR1 (K1112) to arginine completely inhibited R,S-DHPG-mediated internalization of the receptor, suggesting that ubiquitination of the lysine at the 1112 position could be critical for the endocytosis of the receptor. It needs to be investigated in future, how the K1112 residue plays critical role in the trafficking of mGluR1. It has been

published from our lab, that group I mGluRs get internalized subsequent to the ligand-mediated activation and recycled back to the cell surface in non-neuronal cells as well as primary hippocampal neurons (Pandey et al., 2014; Mahato et al., 2015). Our data suggest that subsequent to the ligand-mediated stimulation, group I mGluRs get ubiquitinated. Since ubiquitination is a reversible process, these ubiquitinated receptors might get deubiquitinated subsequent to the endocytosis.

In a eukaryotic cell, there are approximately 10s of E1 enzymes, 100s of E2 enzymes and 1000s of E3 ligases. In our earlier experiments, we blocked the ubiquitination using a pharmacological inhibitor, PYR-41, which is an irreversible inhibitor of the E1 enzymes. This manipulation might be having non-specific effects. On the other hand, E3 ligases are a diverse family of proteins and are very specific towards its substrate performing a specific function (Glickman and Ciechanover, 2002). To further consolidate our results, we selected an E3 ubiquitin ligase, viz., Siah-1A from the literature, that ubiquitinates group I mGluRs (Ishikawa et al., 1999b; Moriyoshi et al., 2004a). Our data suggest that acute knockdown of endogenous Siah-1A does not affect the surface expression of myc-mGluR1 and it completely inhibits the ligand-mediated mGluR1 internalization in primary hippocampal neurons. At the end, we studied the role of the ubiquitination on the mGluR-mediated internalization of synaptic AMPARs, which is the cellular correlate for mGluR-mediated synaptic plasticity. It has been reported that blocking ubiquitination by pharmacological inhibitor UBE-41/PYR-41 and activating group I mGluRs with a specific agonist results in the enhanced mGluR-mediated AMPAR endocytosis as well as mGluR-LTD in hippocampal cells (Citri et al., 2009). Our data also suggests that in Siah-1A knockdown cells, activation of group I mGluRs results in the enhanced AMPAR endocytosis. The observation that mGluR-mediated AMPAR endocytosis was enhanced in Siah-1A knockdown cells suggests that ubiquitination might function as a negative feedback to inhibit the activity of group I mGluRs. It has been reported that activation of group I mGluRs does not increase the ubiquitination of AMPA receptors (Widagdo et al., 2015). Thus, all these together point towards the fact that a ubiquitin-independent mGluR-mediated AMPAR endocytic pathway probably exists.

# Role of deubiquitination in the mGluR1 trafficking

## 5.1. Introduction

Ubiquitin is a 76 amino acid peptide, highly conserved across all the phyla. It binds covalently to the lysine residues in target proteins through a sequential three-step process involving the ubiquitin activating enzyme E1, the ubiquitin-conjugating enzyme E2 and ubiquitin ligase enzymes E3. Ubiquitin is attached *via* the C-terminal glycine residue to the ε-$NH_2$ lysine side chain of target proteins through isopeptide bond or through the α-$NH_2$ group, forming peptide bond (Hershko and Ciechanover, 1998; Haglund and Dikic, 2005; Komander, 2009). Other than lysine, ubiquitin can also be conjugated to cysteine, serine, threonine, and N-terminal methionine residues as well (Ciechanover and Ben-Saadon, 2004; Cadwell and Coscoy, 2005). Ubiquitination controls the most complex aspects of cell physiology and is reversed by the action of a large family of deubiquitinating enzymes (Song and Rape, 2008; Reyes-Turcu et al., 2009). The deubiquitinating enzymes are emerging as an attractive possible therapeutic target for a number of disease conditions.

To maintain normal balance inside the cell, ubiquitination and deubiquitination processes are choreographed by adaptors that bind to both these enzymes i.e. E3 ubiquitin ligase and deubiquitinase. In the human genome, around 100 genes encode for deubiquitinases (DUBs), which mediate reversal of the ubiquitination process but the functional importance of these DUBs is least studied. For many GPCRs, ubiquitination plays a major role in the trafficking and sorting of the receptor to the lysosomes (Tanowitz and von Zastrow, 2002). However, literatures suggest that although internalization of some GPCRs is ubiquitin-dependent, subsequent to internalization, they recycle back to the cell surface. For example, in case of parathyroid hormone receptor (PTHR), it gets internalized subsequent to the activation and recycles back in 2 hr. It has been reported that activation of PTHR up-regulates the level of USP2 (Ubiquitin-specific protease 2), favoring the balance towards the rapid deubiquitination and recycling of PTHR (Alonso et al., 2011). Agonist stimulation of the $\beta_2$-adrenergic receptor leads to the ubiquitination and lysosomal degradation of the receptor, but over-expression of USP33 and USP20 counteracts these effects and promotes receptor recycling and resensitization (Berthouze et al., 2009). Additionally, knockdown of both USPs 33 and 20 abolishes receptor recycling and resensitization but enhances ubiquitination as well as lysosomal degradation (Berthouze et al., 2011). For metabotropic γ-aminobutyric acid receptor (GABAB), USP14 is responsible for the

deubiquitination of receptors and dictates the post-endocytic fate of the receptor (Lahaie et al., 2016). In case of group I mGluRs, application of the ligand results in the internalization of both mGluR1 and mGluR5. Subsequent to the internalization, these receptors recycle back to the cell surface in a phosphatase-dependent manner (Pandey et al., 2014; Mahato et al., 2015). Our earlier data suggested that ubiquitination plays critical role in the internalization of group I mGluRs and this internalization is K63-linked polyubiquitination-dependent. Our experiments also suggested that Siah-1A is the E3 ubiquitin ligase required for the ubiquitination of group I mGluRs. All the above results led us to hypothesize that the receptor might be getting deubiquitinated following ligand-dependent endocytosis and subsequently, recycle back to the cell surface.

In order to investigate the role of deubiquitination in the trafficking of group I mGluRs, we used a pharmacological broad spectrum deubiquitinase inhibitor viz., N-Ethylmaleimide (NEM). NEM is an irreversible inhibitor of all cysteine peptidases, with alkylation occurring at the active site thiol group (Ruckdeschel et al., 2006; Qiu et al., 2017). Our data suggested that application of NEM inhibited the recycling of myc-mGluR1 in HEK293 cells, as well as in primary hippocampal neurons. Interestingly, application of NEM also resulted in the targeting of some of the internalized receptors to the lysosome. Furthermore, when proteasomal inhibitor, viz., MG-132 was not applied, the total amount of internalized myc-mGluR1 decreased in NEM treated cells, suggesting that receptors that entered the lysosomal compartment underwent degradation. These results suggest that blocking the deubiquitination leads to the inhibition in the recycling of the ligand-induced internalized receptors and a population of the receptor changes the route and enters the lysosome. We next searched for the specific deubiquitinase that might be involved in the deubiquitination process during the trafficking of mGluR1. We chose ubiquitin-specific protease 19 (USP19) as a potential candidate based on the following information from the literature: 1) it is the only USP with a Siah-interacting motif (Zhang et al., 2017), 2) USP19 specifically cleaves the K63-linked polyubiquitinated chains (Wu et al., 2017). We used GFP-tagged USP19 constructs for this study. In all these constructs GFP was tagged at the C-terminus of the protein. Over-expression of the wild-type USP19-GFP did not have any effect on the surface expression or ligand-induced trafficking of myc-mGluR1. We subsequently used a catalytically inactive form of USP19 viz., USP19C506S-GFP, where the cysteine present at the active site was converted to serine. It has been reported, that this conversion of cysteine to serine

at 506 position of USP19 did not affect the binding of USP19 with the substrate but it inhibited its catalytic activity (Altun et al., 2012). Our data suggested that over-expression of USP19C506S-GFP did not affect the surface expression of myc-mGluR1 in primary hippocampal neurons but it inhibited the recycling of myc-mGluR1. These results suggest that USP19 might play a critical role in the deubiquitination process during the trafficking of mGluR1.

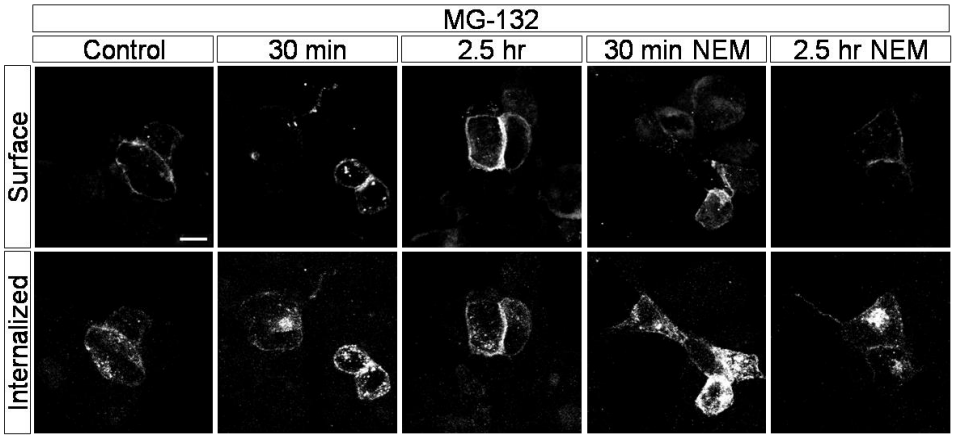
## 5.2. Results

### 5.2.1. Effect of NEM, in the presence of MG-132, on the trafficking of mGluR1 in HEK293 cells

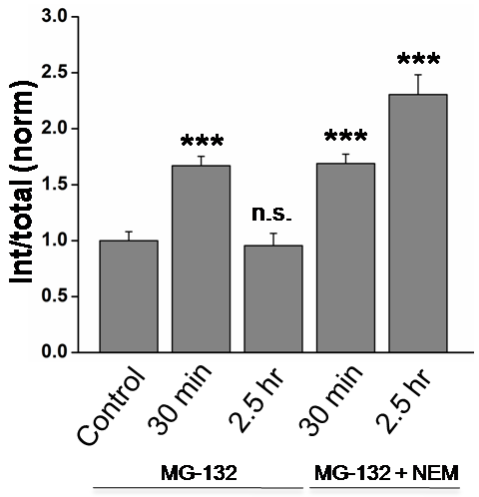
In order to check for the role of deubiquitination in the trafficking of mGluR1, HEK293 cells were transfected with myc-mGluR1 cDNA. 24 hr post transfection, cells were pre-incubated with 5 µM NEM and 50 µM MG-132 for a period of 30 min. After that, cells were treated with anti-myc primary antibody (1:500) for 15 min at 37˚C. Internalization was induced by applying 100 µM R,S-DHPG for 5 min and subsequently, cells were chased for a total of 30 min and 2.5 hr in the absence of the ligand. After that, cells were fixed in 4% PFA on ice for 15 min without permeabilization. To label the surface-localized receptors, cells were incubated with saturating concentration of the goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) for 1 hr at 37˚C. Cells were then permeabilized with 0.1% Triton X-100 for 30 min at room temperature. The endocytosed receptors were then labelled by the application of goat anti-mouse Alexa-647 conjugated secondary antibody (1:750) for 1 hr at 37˚C. Coverslips were then mounted on glass slides and imaged under the confocal microscope. Quantitation of the endocytosis index suggested that in NEM untreated cells, subsequent to the ligand application, myc-mGluR1internalized and recycled back to the cell surface at 2.5 hr (control: 1 ± 0.07; 30 min: 1.67 ± 0.08; 2.5 hr: 0.95 ± 0.11). On the other hand, in NEM-treated condition, subsequent to the ligand treatment, myc-mGluR1 endocytosed but were unable to recycle back to the cell surface at 2.5 hr (30 min: 1.68 ± 0.08; 2.5 hr: 2.30 ± 0.17) **(Figure 5.1 A, B)**. Measurement of the surface localized myc-mGluR1 also suggested that, in NEM-untreated cells, the surface expression of the receptor decreased at 30 min subsequent to the R,S-DHPG application and when cells were chased for a longer time period in the absence of ligand, the surface fluorescence recovered to the control level at 2.5 hr (control: 1 ± 0.09; 30 min: 0.56 ± 0.08; 2.5 hr: 0.92 ± 0.14). On the other hand, in the presence of 5 µM NEM, amount of the surface localized myc-mGluR1 decreased at 30 min post ligand application but the recovery of the surface fluorescence was not observed at 2.5 hr (30 min: 0.62 ± 0.11; 2.5 hr: 0.56 ± 0.09) **(Figure 5.1 C)**.

We next measured the total myc-mGluR1 level in NEM-treated cells, subsequent to the

**A**




**B**




**C**

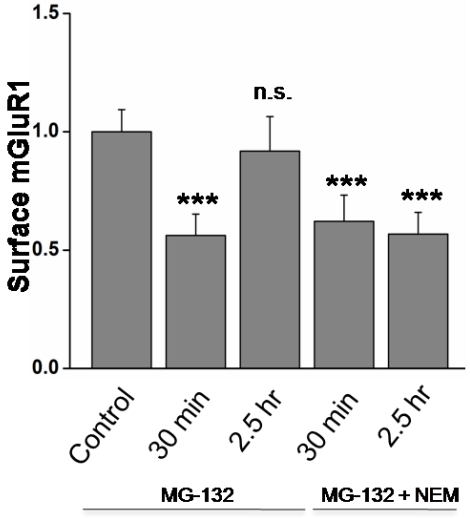


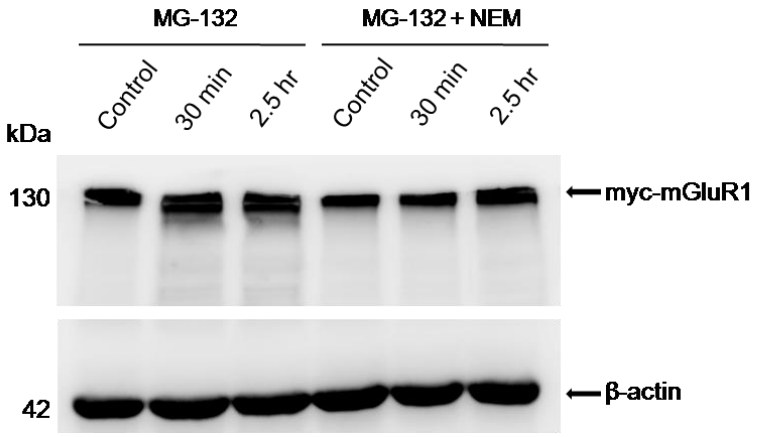

**Figure 5.1. NEM blocks the recycling of myc-mGluR1 in HEK293 cells.** myc-mGluR1 internalized on 100 µM R,S-DHPG exposure in the presence of 5 µM NEM but was unable to recycle back to the cell surface as shown in the representative images (A), quantitation of the endocytosis index (B) and surface myc-mGluR1 quantitation (C). Scale bar = 10 µm. *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

ligand-mediated internalization of the receptor in the presence of the proteasomal inhibitor, MG-132. HEK293 cells were transfected with myc-mGluR1. 24 hr post transfection, control cells were incubated with 50 µM MG-132 and other cells were incubated with 5 µM NEM + 50 µM MG-132 for 30 min. After that, 100 µM R,S-DHPG was applied for 5 min and subsequently, cells were chased for a total of 30 min and 2.5 hr in plain DMEM in the presence of the drugs. After that, cells were lysed in the RIPA buffer having protease inhibitor cocktail. Samples were boiled in Laemmli sample buffer and run in SDS-PAGE by loading an equal amount of protein in each lane. Subsequently, they were transferred to the PVDF membrane, blocked with 5% skimmed milk for 1 hr at room temperature. The membrane was then incubated with anti-myc antibody (1:1000) or anti β-actin (1:1000) antibody at 4ºC overnight. Following that, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature. Blots were developed using ECL western detection kit and imaging was performed in ImageQuant LAS 4000. Western blots were quantified using ImageJ software. Total myc-mGluR1 level in different conditions was normalized with its respective β-actin level that served as the loading control. Our data suggested that following ligand application, in both control cells as well as in NEM-treated cells, total receptor level remains unchanged at 30 min and 2.5 hr time point (MG-132::control: 1 ± 0.09; 30 min: 1.20 ± 0.22; 2.5 hr: 1.29 ± 0.27; NEM + MG-132::control: 1 ± 0.04; 30 min: 0.92 ± 0.10; 2.5 hr: 0.98 ± 0.10) **(Figure 5.2 A, B)**.

### 5.2.2. Effect of NEM (in the presence of MG-132) on the trafficking of mGluR1 in primary hippocampal neurons

We next wanted to check the effect of 5 µM NEM on the recycling of myc-mGluR1 in primary hippocampal neurons. myc-mGluR1 expressing matured primary hippocampal neurons were treated with 5 µM NEM and 50 µM MG-132 30 min prior to the application of anti-myc primary antibody (1:200). Subsequent to the primary antibody labelling, internalization was triggered by applying 100 µM R,S-DHPG for 5 min followed by chasing the cells for a total of 30 min and 2.5 hr in the absence of the ligand. Cells were then fixed without permeabilization in 4% PFA on ice for 15 min. Surface receptors were visualized by incubating the cells with goat anti-mouse Alexa-568 (1:100) conjugated secondary antibody for 1 hr at 37˚C. Cells were permeablized after that with 0.1% Triton-X 100 for 30 min and second secondary antibody viz., goat anti-

**A**




**B**

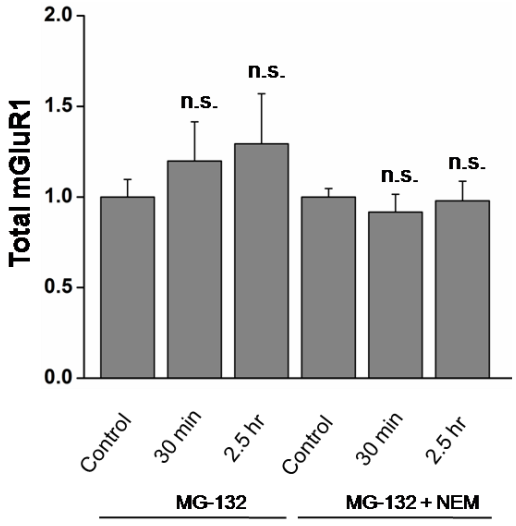



**Figure 5.2. Total myc-mGluR1 level remains unchanged in HEK293 cells in presence of NEM and MG-132.** Western blot image (A) showing the level of myc-mGluR1 at various time points, subsequent to the 100 µM R,S-DHPG application, in the presence of MG-132 as well as in the presence of MG-132 and NEM. (B) Quantitation of the western blots suggesting that the level of myc-mGluR1 remained unchanged over time. n.s indicates $p > 0.05$.

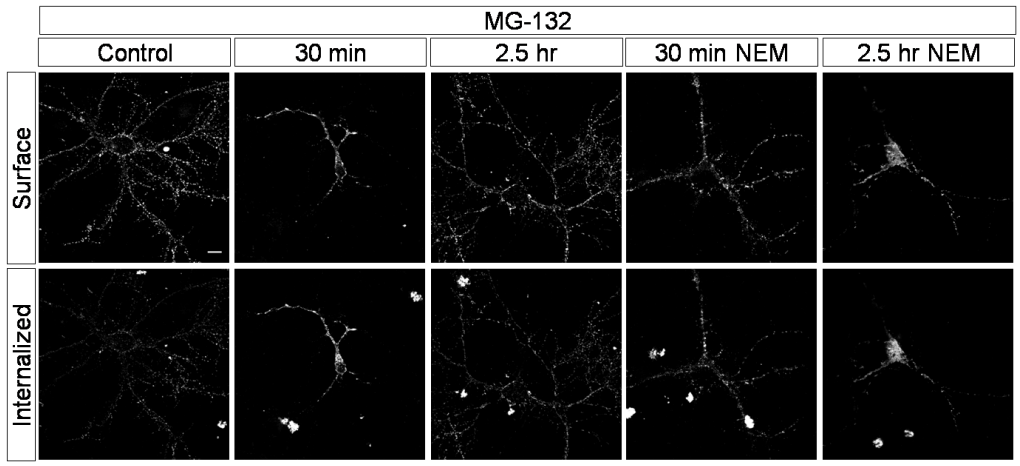
mouse Alexa-647 (1:750) was applied for 1 hr at 37˚C. Cells were mounted on glass slides and observed under the confocal microscope. In control cells, which were treated only with 50 µM MG-132, myc-mGluR1 receptors showed internalization at 30 min and recycling at 2.5 hr time point (control: 1 ± 0.07; 30 min: 1.58 ± 0.09; 2.5 hr: 1.19 ± 0.09). On the other hand, NEM treated cells showed normal endocytosis of myc-mGluR1 upon ligand application but inhibition in the recycling of the receptors back to the cell surface (30 min: 1.79 ± 0.12; 2.5 hr: 1.68 ± 0.13) **(Figure 5.3 A, B)**.

Measurement of the surface localized receptors also suggested that in the control cells, a decrease in the surface myc-mGluR1 at 30 min post ligand application and recovery of the surface myc-mGluR1 at 2.5 hr time point was observed (control: 1 ± 0.07; 30 min: 0.75 ± 0.07; 2.5 hr: 1.02 ± 0.09). On the other hand, cells treated with NEM showed a decrease of the surface myc-mGluR1 upon ligand application but there was no recovery of the surface myc-mGluR1 observed at 2.5 hr (30 min: 0.66 ± 0.09; 2.5 hr: 0.8 ± 0.09) **(Figure 5.3 C)**. These results suggested that inhibition of the deubiquitination resulted in the blocking of the recycling of myc-mGluR1 the in primary hippocampal neurons.

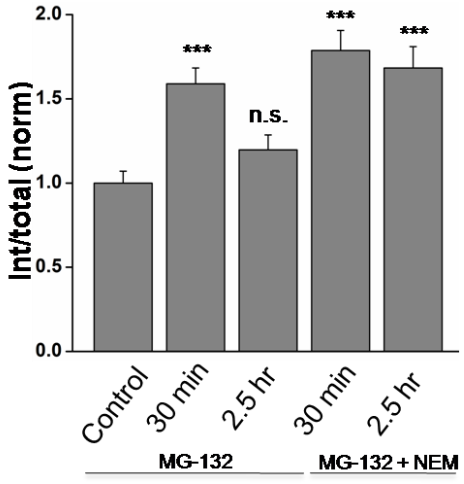
### 5.2.3. Localization of a fraction of the ligand-mediated internalized mGluR1 in the lysosome upon inhibition of deubiquitination

Our earlier data suggested that in the presence of NEM, myc-mGluR1 did not recycle back to the cell surface subsequent to the R,S-DHPG-mediated internalization. Normally, subsequent to the ligand-mediated internalization, majority of the internalized mGluR1 enters the recycling compartment as observed by the co-localization with Rab11 (Pandey et al., 2014). We next investigated whether in NEM treated cells the receptors change the route and enter the lysosomal compartment by performing the co-localization experiment using the lysosomal marker LAMP1. myc-mGluR1 expressing primary hippocampal neurons were pre-incubated with 5 µM NEM and 50 µM MG-132 for 30 min. After that, cells were incubated with anti-myc primary antibody for 20 min at 37˚C, followed by the application of 100 µM R,S-DHPG for 5 min. Subsequently, cells were chased for longer time points in the absence of the ligand for 30 min and 2.5 hr. On completion of the chase period, cells were surface-stripped by treating them with ice cold

**A**




**B**




**C**

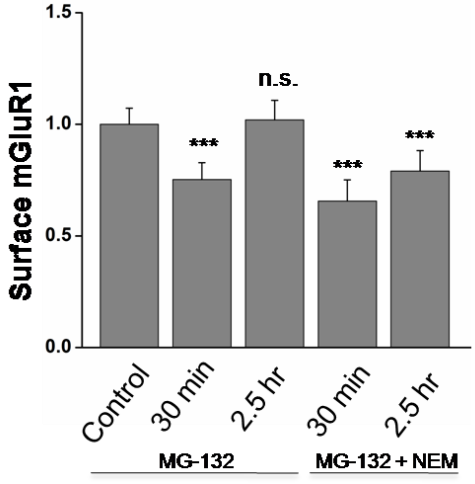


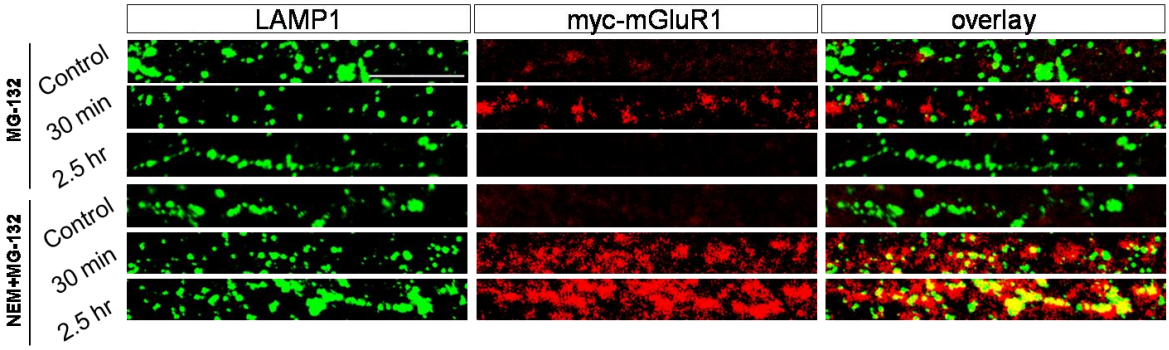

**Figure 5.3. Effect of NEM on the ligand-mediated trafficking of myc-mGluR1 in primary hippocampal neurons.** Representative images (A), measurement of the endocytosis index (B) and surface receptor quantitation (C) showing that upon ligand-mediated activation, myc-mGluR1 internalized but was unable to recycle back to cell surface in the presence of NEM. Scale bar = 10 µm. *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

stripping solution (0.5% $CH_3COOH$ + 500 mM NaCl) for 90 seconds on ice to remove the antibodies attached with the surface receptors that did not internalize. Cells were then fixed in 4% PFA and permeabilized in 0.1% Triton-X 100. Subsequently, internalized receptors were labelled with the goat anti-mouse Alexa-568 conjugated secondary antibody. Following that, cells were stained with anti-LAMP1 primary antibody (rabbit polyclonal) (1:500) at 4˚C overnight. Subsequently, secondary antibody (goat anti-rabbit) tagged with Alexa-488 (1:800) was applied for 1 hr at 37˚C to visualize LAMP1. Quantitation suggested that, as expected, in control cells, co-localization of myc-mGluR1 with LAMP1 remained unchanged over the time (control: 1 ± 0.13; 30 min: 0.82 ± 0.10; 2.5 hr: 0.97 ± 0.16). On the other hand, in NEM-treated condition, there was significant increase in the myc-mGluR1 co-localization with LAMP1 at 2.5 hr time point (control: 1 ± 0.17; 30 min: 1.23 ± 0.10; 2.5 hr: 1.62 ± 0.23) **(Figure 5.4 A, B)**. These results suggested that in NEM-treated cells some amount of the internalized myc-mGluR1 entered the lysosomal compartment at 2.5 hr.

### 5.2.4. Effect of NEM, in the absence of MG-132, on the trafficking of mGluR1 in HEK293 cells

Our earlier experiments suggested that in presence of NEM and MG-132 myc-mGluR1 recycling was inhibited and a fraction of the receptors entered the lysosomal compartment. We subsequently wanted to investigate the effect of NEM, in the absence of MG-132 on the trafficking of myc-mGluR1. Briefly, myc-mGluR1 expressing HEK293 cells were pre-treated with 5 µM NEM. After that, a primary antibody against the myc tag (1:500) was applied for 15 min at 37˚C. Subsequently, internalization was induced by the application of 100 µM R,S-DHPG for 5 min and cells were chased for a total of 30 min and 2.5 hr in plain DMEM. After that, cells were fixed without permeabilization in 4% PFA for 15 min on ice and surface receptors were labelled with saturating concentration (1:100) of goat anti-mouse Alexa-568 conjugated secondary antibody. To visualize the internalized receptors, cells were permeabilized with 0.1% Triton-X 100 for 30 min and internalized receptors were labelled with another secondary antibody, viz., goat anti-mouse Alexa-647. Measurement of the endocytosis index in control cells showed internalization of myc-mGluR1 at 30 min post-ligand application and recycling of the receptors at 2.5 hr time point (control: 1 ± 0.04; 30 min: 1.50 ± 0.07; 2.5 hr: 1.06 ± 0.04). On the

**A**




**B**

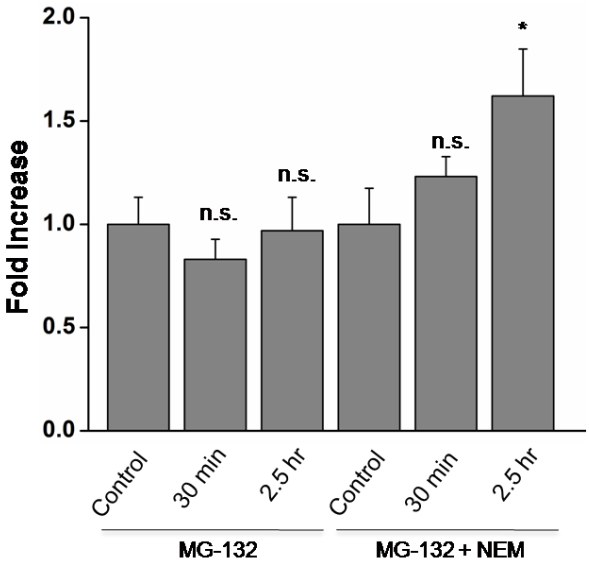


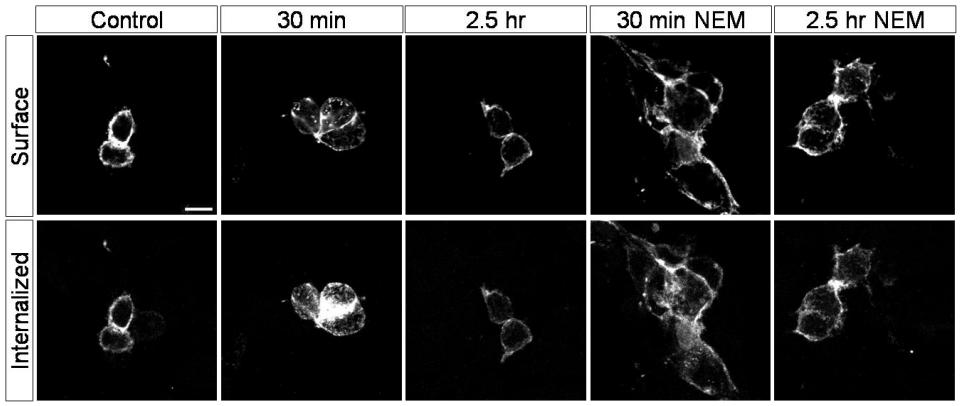

**Figure 5.4. Co-localization of the internalized myc-mGluR1 with LAMP1 in primary hippocampal neurons in presence of NEM and MG-132.** Representative images (A) and quantitation of the co-localization (B) showing that in NEM-treated cells, upon ligand application, the co-localization of the internalized myc-mGluR1 with LAMP1 increased at 2.5 hr. Scale bar = 10 µm. * indicates $p < 0.05$ and n.s indicates $p > 0.05$.

other hand, cells treated with NEM showed normal endocytosis of myc-mGluR1 upon ligand application, but the recycling of the receptors back to the cell surface was inhibited (30 min: 1.63 ± 0.06; 2.5 hr: 1.32 ± 0.06) **(Figure 5.5 A, B)**.

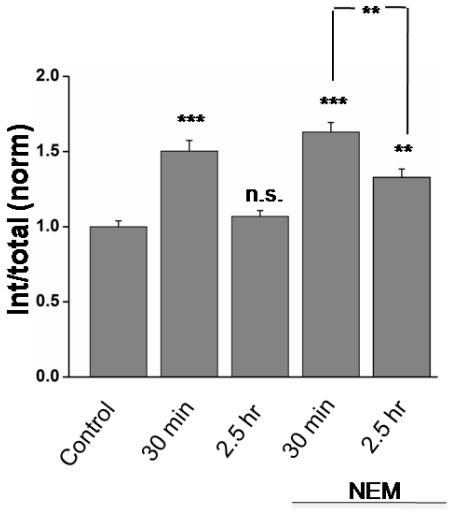
Measurement of surface receptors also suggested that control cells showed a decrease of surface myc-mGluR1 at 30 min post-ligand application and recovery of the surface myc-mGluR1 at 2.5 hr time point (control: 1 ± 0.04; 30 min: 0.49 ± 0.04; 2.5 hr: 0.94 ± 0.04). Cells treated with NEM showed a decrease of surface myc-mGluR1 upon ligand application, but there was no recovery of the surface myc-mGluR1 till 2.5 hr (30 min: 0.58 ± 0.04; 2.5 hr: 0.60 ± 0.05) **(Figure 5.5 C)**.

Our earlier results suggested that in presence of NEM a fraction of the internalized myc-mGluR1 entered the lysosomal compartment. Interestingly, in our previous experiment (Figure 5.5 B) the endocytosis index of myc-mGluR1 in NEM-treated cells at 2.5 hr was lesser than that of 30 min, suggesting probable degradation of some on the internalized receptors in presence of NEM. In order to investigate whether some of the internalized myc-mGluR1 gets degraded in NEM-treated cells, HEK293 cells were transfected with myc-mGluR1. After 24 hr of transfection, 100 µM R,S-DHPG was applied for 5 min and subsequently, cells were chased for a total of 30 min and 2.5 hr in plain DMEM. Subsequently, cells were lysed in RIPA buffer having protease inhibitor cocktail. In NEM treated condition, cells were pre-incubated in 5 µM NEM and the drug was present throughout the experiment. Samples were boiled in Laemmli sample buffer and ran in SDS-PAGE by loading an equal amount of protein in each lane. Subsequently, they were transferred to PVDF membrane and blocked with 5% skimmed milk for 1 hr at room temperature. The membrane was then incubated with anti-myc antibody (1:1000) or anti β-actin antibody (1:1000) at 4ºC overnight. Following that, membranes were washed and incubated in horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature. Blots were developed using ECL western detection kit and imaging was performed in ImageQuant LAS 4000. The western blots were quantified using ImageJ software. myc-mGluR1 amount at different conditions of the experiment was normalized with respective β-actin amount, that served as the loading control. This experiment suggested that in control cells, total receptor levels remain unchanged throughout the experiment (control: 1± 0.28; 30 min: 1.0 ± 0.21; 2.5 hr:

**A**




**B**




**C**

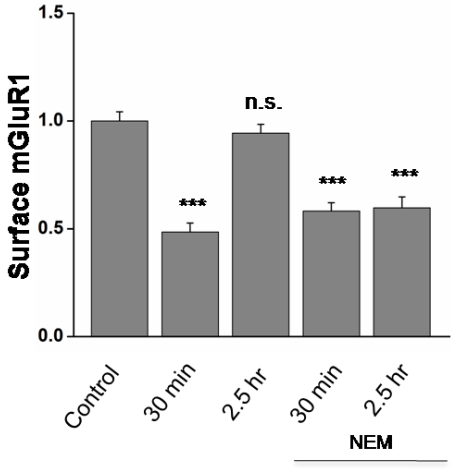



**Figure 5.5. NEM blocks the recycling of myc-mGluR1 in HEK293 cells.** myc-mGluR1 internalized on 100 µM R,S-DHPG exposure in presence of 5 µM NEM but was unable to recycle back to the cell surface as shown in the representative images (A), quantitation of the endocytosis index (B) and measurement of the surface myc-mGluR1 (C). Scale bar = 10 µm. *** indicates $p < 0.001$, ** indicates $p < 0.01$ and n.s indicates $p > 0.05$.

0.84 ± 0.13), whereas, in NEM-treated cells there was a decrease in the total myc-mGluR1 level at 2.5 hr post-ligand application (control: 1 ± 0.13; 30 min: 1.07 ± 0.33; 2.5 hr: 0.48 ± 0.15) suggesting degradation of some of the internalized receptors **(Figure 5.6 A, B)**.

### 5.2.5. Effect of NEM, in the absence of MG-132, on the mGluR1 trafficking in primary hippocampal neurons

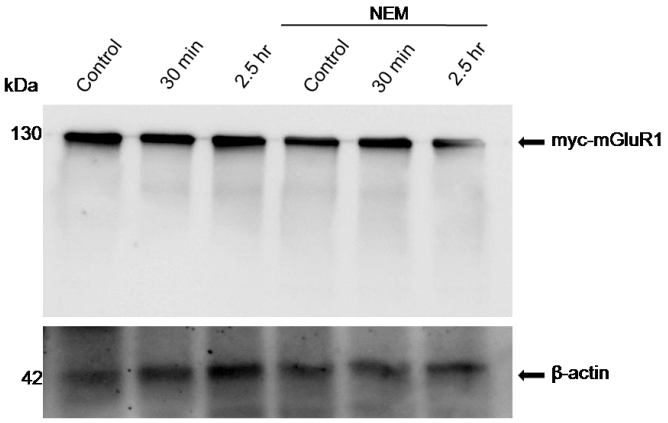
We subsequently investigated the effect of NEM, in the absence of MG-132, on the trafficking of myc-mGluR1 in primary hippocampal neurons, using the method described above. In control cells, application of 100 µM R,S-DHPG resulted in the internalization of myc-mGluR1 at 30 min and recycling of the receptor at 2.5 hr (control: 1 ± 0.07; 30 min: 2.04 ± 0.1; 2.5 hr: 1.03 ± 0.05). Importantly, cells treated with NEM showed normal endocytosis of myc-mGluR1 but the recycling of the receptors was inhibited (30 min: 2.25 ± 0.17; 2.5 hr: 1.67 ± 0.1) **(Figure 5.7 A, B)**.

Quantitation of the surface receptors also suggested that in control condition, cells showed a decrease of myc-mGluR1 from the cell surface at 30 min post-ligand application and recovery of the surface fluorescence at 2.5 hr (control: 1 ± 0.09; 30 min: 0.52 ± 0.06; 2.5 hr: 0.93 ± 0.07). Cells treated with NEM showed a decrease of surface myc-mGluR1 upon application of 100 µM R,S-DHPG but were unable to recover the cell surface myc-mGluR1 at 2.5 hr (30 min: 0.48 ± 0.01; 2.5 hr: 0.55 ± 0.07) **(Figure 5.7 C)**.

### 5.2.6. Effect of the over-expression of the catalytically inactive USP19 on the surface expression and trafficking of mGluR1 in primary hippocampal neurons

Our previous results suggested that ligand-dependent ubiquitination of mGluR1 is Siah-1A dependent and K63-linked polyubiquitination is essential for the internalization of the receptor. We also showed that, blocking of deubiquitination by the application of NEM led to the inhibition in the recycling of myc-mGluR1. In order to find out the deubiquitinase that might be responsible in the ligand-mediated trafficking of myc-mGluR1, we selected one candidate:

**A**




**B**

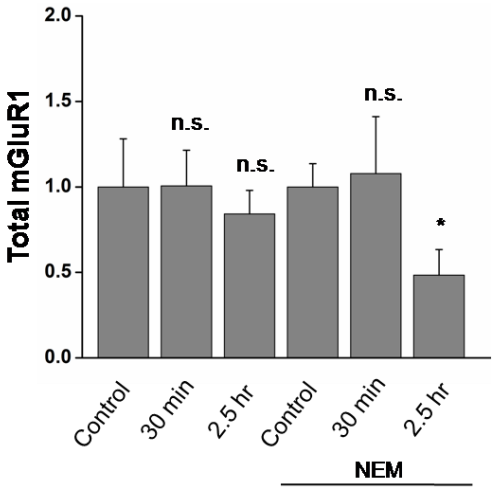
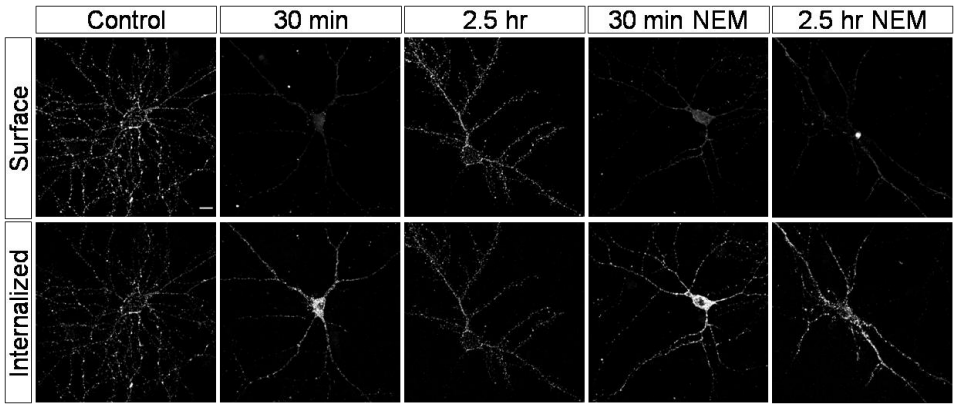
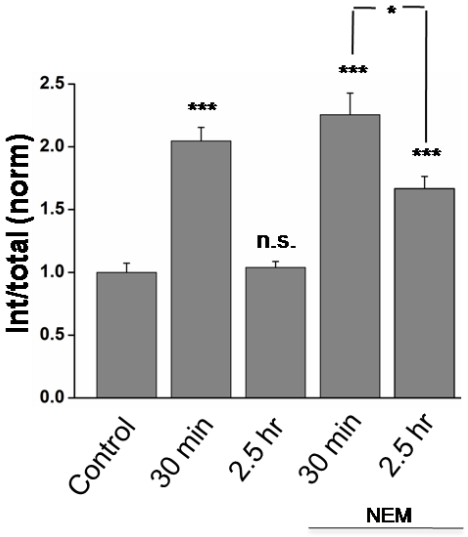



**Figure 5.6. In the presence of NEM, total myc-mGluR1 level decreases in HEK293 cells upon ligand-mediated internalization.** Western blot image (A) showing the level of myc-mGluR1 at various time points in the presence of NEM. Quantitation of the western blots suggested that the level of myc-mGluR1 decreased at 2.5 hr post-ligand application in NEM treated cells (B). * indicates $p < 0.05$, n.s indicates $p > 0.05$.

**A**




**B**




**C**

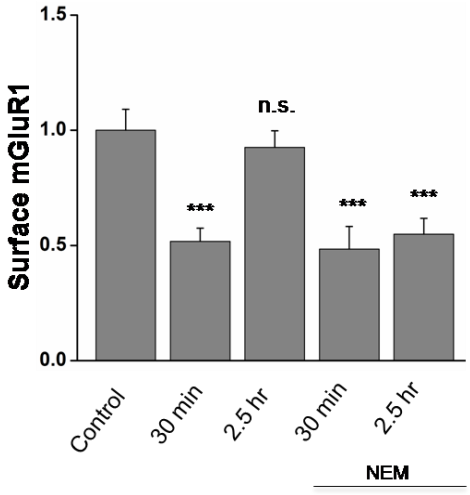
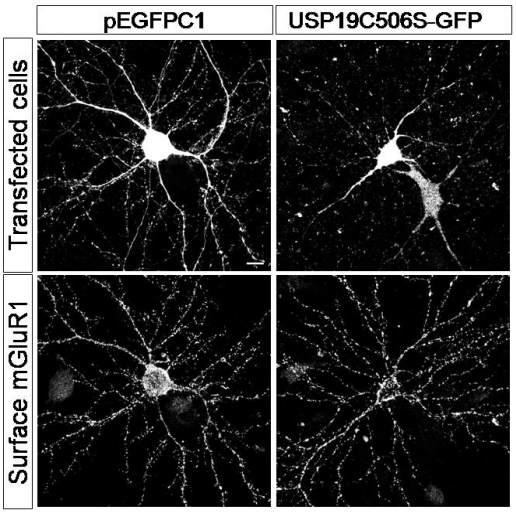



**Figure 5.7. Effect of NEM, in the absence of MG-132, on the ligand-mediated trafficking of myc-mGluR1 in primary hippocampal neurons.** In the absence of MG-132, treatment of the primary hippocampal neurons with NEM inhibited the recycling of myc-mGluR1, subsequent to the ligand-mediated internalization, as evident from the representative images (A). Measurement of the endocytosis index (B) and surface myc-mGluR1 quantitation (C) also showing that upon ligand application, myc-mGluR1 internalized but did not recycle back to the cell surface in presence of NEM. Scale bar = 10 µm. *** indicates $p < 0.001$, * indicates $p < 0.05$ and n.s indicates $p > 0.05$.

ubiquitin-specific protease 19 (USP19) based on the following information: 1) it is the only USP having a Siah-interacting motif (Zhang et al., 2017), 2) USP19 specifically cleaves the K63-linked polyubiquitin chains (Wu et al., 2017). Due to the above reasons, we wanted to check the role of USP19, if any, in the trafficking of mGluR1. For that, we used a catalytically inactive USP-19, viz., USP19C506S-GFP, where GFP was tagged at the C-terminus of USP19. In order to investigate the effect of the over-expression of USP19C506S-GFP on the surface expression of myc-mGluR1, primary hippocampal neurons were co-transfected with myc-mGluR1 cDNA and USP19C506S-GFP or pEGFPC1 using calcium phosphate transfection method on DIV 7-8 and experiments were performed on DIV 11-12. Measurement of the surface receptors showed that over-expression of USP19C506S-GFP did not affect the surface localization of myc-mGluR1 in primary hippocampal neurons (pEGFPC1: 1 ± 0.1; USP19C506S-GFP: 1.0 ± 0.13) **(Figure 5.8 A, B)**.

Subsequently, the effect of the over-expression of USP19C506S-GFP on the ligand-mediated trafficking of myc-mGluR1 was studied. myc-mGluR1 cDNA and USP19C506S-GFP or pEGFPC1 constructs were co-transfected in primary hippocampal neurons and endocytosis experiments were performed following the same protocol as described before. Control cells (pEGFPC1 transfected cells) showed very low internal fluorescence and majority of the receptors were observed to be present on the cell surface. Receptors internalized on 100 µM R,S-DHPG application and they recycled back to the cell surface at 2.5 hr post-ligand application (control: 1 ± 0.04; 30 min: 2.4 ± 0.1; 2.5 hr: 1.05 ± 0.05). On the other hand, in USP19C506S-GFP over-expressed cells, application of 100 µM R,S-DHPG resulted in the internalization of myc-mGluR1 at 30 min time point, but the internalized receptors were unable to recycle back to the cell surface at 2.5 hr time point (contorl = 1 ± 0.08; 30 min: 2.1 ± 0.12; 2.5 hr: 1.93 ± 0.15). **(Figure 5.9 A, B)**.

**A**




**B**

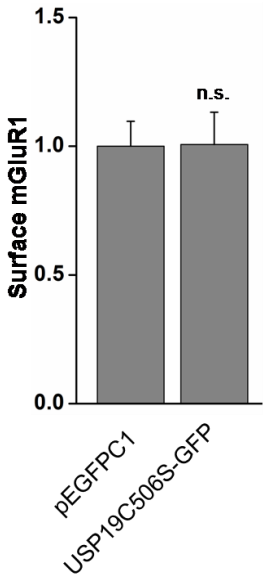
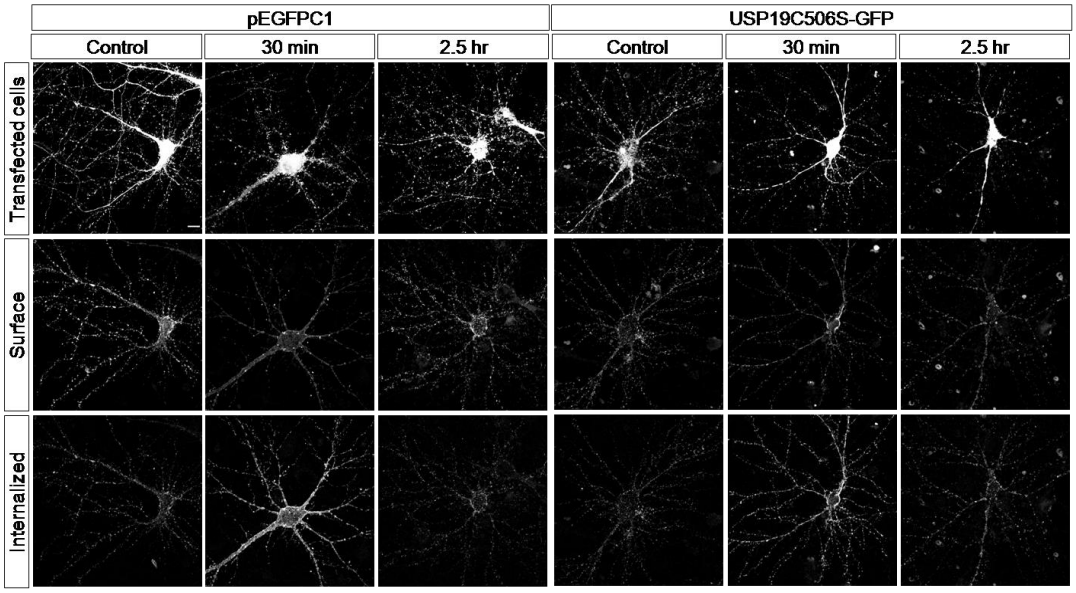



**Figure 5.8. Over-expression of USP19C506S-GFP does not affect the myc-mGluR1 surface expression in primary hippocampal neurons.** Representative images (A) and surface receptor quantitation (B) showing that over-expression of USP19C506S-GFP did not affect the surface expression of myc-mGluR1. Scale bar = 10 μm. n.s indicates $p > 0.05$.

**A**




**B**

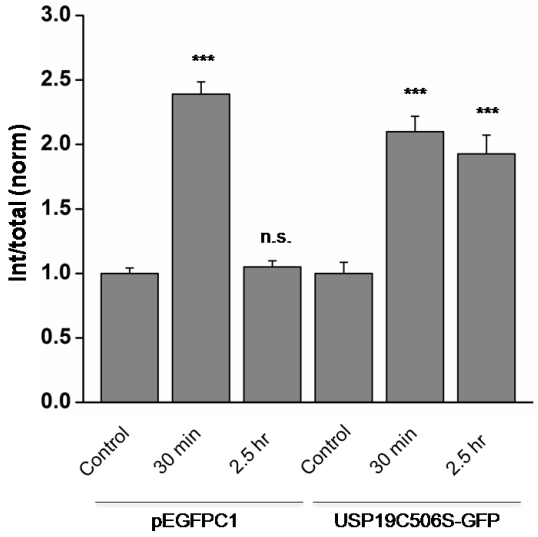


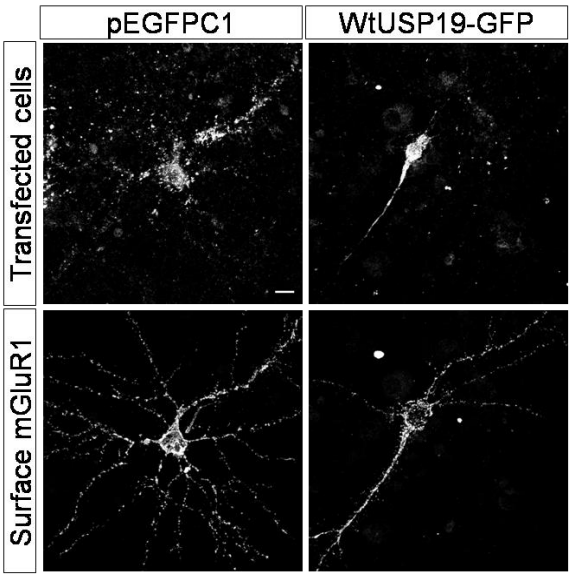

**Figure 5.9. Effect of the USP19C506S-GFP over-expression on the ligand-mediated trafficking of myc-mGluR1 in primary hippocampal neurons.** Over-expression of the USP19C506S-GFP inhibited the recycling of myc-mGluR1 as evident from the representative images (A) and measurement of the endocytosis index (B). Scale bar = 10 µm. *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

### 5.2.7. Effect of the over-expression of WtUSP19-GFP on the surface expression and trafficking of mGluR1 in primary hippocampal neurons

Over-expression of the catalytically inactive USP19 did not affect the surface expression of myc-mGluR1 and inhibited the recycling of the receptor in primary hippocampal neurons. We subsequently investigated the effect of the over-expression of WtUSP19-GFP on the surface expression of myc-mGluR1 in primary hippocampal neurons. Primary hippocampal neurons were co-transfected with myc-mGluR1 and WtUSP19-GFP or pEGFPC1 using calcium phosphate transfection method on DIV 7-8 and experiments were performed on DIV 11-12 according to the method described in the "Materials and Methods" chapter. Over-expression of the WtUSP19-GFP did not affect the surface localization of myc-mGluR1 in primary hippocampal neurons (pEGFPC1: 1 ± 0.08; WtUSP19-GFP: 1.04 ± 0.17) **(Figure 5.10 A, B)**.

Subsequently, the effect of the over-expression of WtUSP19-GFP on the ligand-mediated trafficking of myc-mGluR1 was studied. myc-mGluR1 cDNA and WtUSP19-GFP or pEGFPC1 constructs were co-transfected in the primary hippocampal neurons and trafficking experiments were performed subsequent to that, following the same protocol as described before. Control cells showed very low internal fluorescence and majority of the receptors were observed to be present on the cell surface. As expected, myc-mGluR1 internalized on 100 µM R,S-DHPG application and recycled back to the cell membrane at 2.5 hr (control: 1 ± 0.06; 30 min: 2.10 ± 0.07; 2.5 hr: 0.91 ± 0.08). In WtUSP19-GFP over-expressing cells, application of 100 µM R,S-DHPG resulted in the internalization and recycling of myc-mGluR1 at 2.5 hr (control = 1 ± 0.07; 30 min: 1.62 ± 0.06; 2.5 hr: 1.12 ± 0.06) **(Figure 5.11 A, B)**. These results suggested that over-expression of the wild-type USP19 did not affect the surface expression and ligand-mediated trafficking of myc-mGluR1.

**A**




**B**

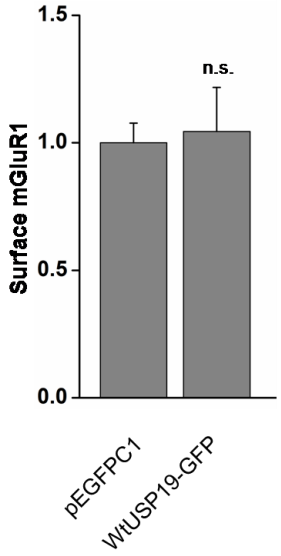
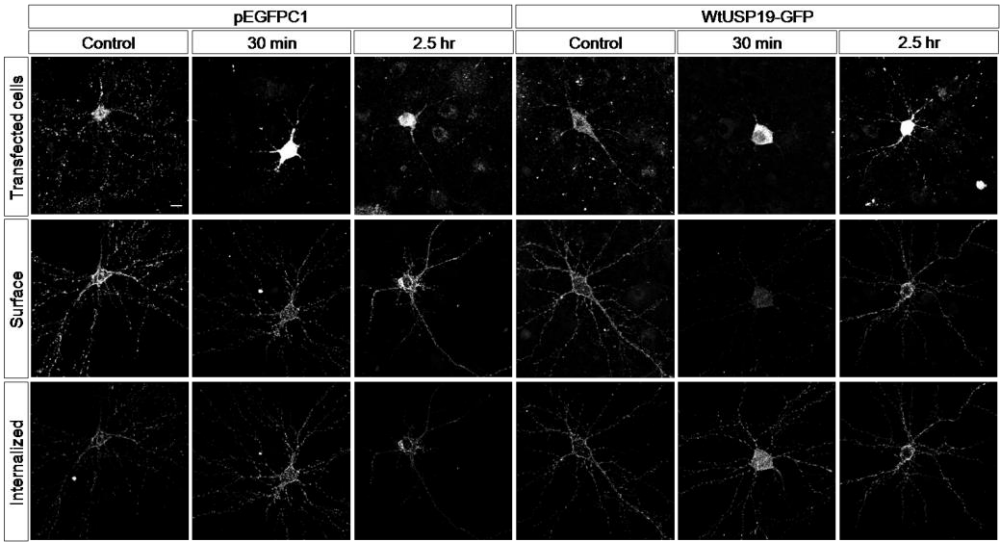



**Figure 5.10. Over-expression of the wild-type USP19-GFP does not affect the surface expression of myc-mGluR1 in primary hippocampal neurons.** Surface expression of myc-mGluR1 remained unchanged upon over-expression of the wild-type USP19-GFP in primary hippocampal neurons as shown in the representative images (A) and quantitation of the surface myc-mGluR1 (B). Scale bar = 10 μm. n.s indicates $p > 0.05$.

**A**




**B**

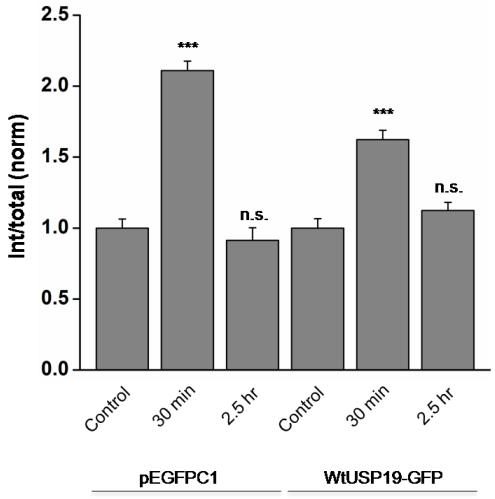



**Figure 5.11. Effect of the wild-type USP19-GFP over-expression on the ligand-mediated trafficking of myc-mGluR1 in primary hippocampal neurons.** In the wild-type USP19-GFP over-expressing primary hippocampal neurons, ligand-mediated trafficking of myc-mGluR1 remained unchanged as shown in the representative images (A) and endocytosis index (B). Scale bar = 10 µm. *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

## 5.3. Discussion

Activation of the G-protein coupled receptors with the ligand results in the activation of the second messenger responses. This subsequently leads to various post-translational modifications like phosphorylation and ubiquitination of the receptor. Ubiquitination plays an important role in regulating the post-endocytic fate of many GPCRs. Group I mGluRs also reported to get ubiquitinated subsequent to the ligand-mediated activation (Marchese and Benovic, 2001; Shenoy et al., 2001; Moriyoshi et al., 2004; Citri and Malenka, 2008; Bhandari et al., 2009; Hasdemir et al., 2009; Alonso et al., 2011; Ko et al., 2012; Lahaie et al., 2016). Ubiquitination is a reversible process and is required for the internalization of some GPCRs including group I mGluRs. Since group I mGluRs, subsequent to the internalization, recycle back to the cell surface, removal of the ubiquitin might be equally important for the recycling of the receptor. It is believed that ubiquitination and deubiquitination processes help in maintaining the normal homeostasis of the cellular milieu. The human genome encodes for nearly 100 deubiquitinases responsible for the reversal of ubiquitination but the functional importance of these deubiquitinases is least studied. For some GPCRs, alternation in the ubiquitination-deubiquitination process results in a 'trip switch' between the recycling and degradation pathways (Berthouze et al., 2009). In order to understand the role of deubiquitination in the ligand-mediated trafficking of mGluR1, we used a broad spectrum deubiquitinase inhibitor, viz., N-Ethylmaleimide (NEM). Our data suggested that in the presence of NEM, application of 100 µM R,S-DHPG resulted in the internalization of myc-mGluR1, but internalized receptors did not recycle back to the cell surface in both HEK293 cells as well as in primary hippocampal neurons. Moreover, we found that in the presence of NEM, some amount of the internalized receptors entered the lysosomal compartment. Interestingly, in the absence of MG-132, a proteasomal inhibitor, some of the internalized receptors got degraded. Together these results suggested that NEM led to the blocking of the recycling of myc-mGluR1 and diverted the route of some of the internalized receptors to the degradative pathway. Subsequently, we found one deubiquitinase from the literature, viz., ubiquitin-specific protease 19 (USP19) which has a Siah-interacting motif. Over-expression of the catalytically inactive form of USP19, where the active site cysteine present at the position 506 of USP19 was mutated to serine, did not affect the surface expression of myc-mGluR1 in primary hippocampal neurons. On the other hand, it inhibited the recycling of myc-mGluR1. Over-expression of the wild-type USP19 did not affect the surface expression, as

well as the normal trafficking of myc-mGluR1. These data suggest that deubiquitination, recycling and resensitization are inter-linked and USP19 plays a key role in the recycling and resensitization of mGluR1. Experiments need to be performed in order to check whether catalytically inactive USP19 affects the recycling and resensitization of the other member of the group I mGluR family, mGluR5.

# Summary

## 6.1. Aim of the research

G-protein coupled receptors (GPCRs) are remarkably versatile signaling molecules. GPCRs have been known to regulate a variety of important physiological processes and their aberrant signaling leads to various pathological consequences (Kolakowski, 1994; Joost and Methner, 2002). GPCRs respond to a variety of signaling molecules ranging from hormones and neurotransmitters to photons (Rosenbaum et al., 2009). Approximately 40% of the therapeutic drugs available in the market have been reported to target GPCRs (Wise et al., 2002; Trzaskowski et al., 2012). In the last three decades, much information has been accumulated towards the understanding of the mechanisms that regulate the responsiveness of GPCRs (Ferguson and Caron, 1998; Ferguson et al., 1998; Gether, 2000; Kelly et al., 2008). On the other side, considering the huge diversity of GPCR types and their differences in terms of their regulatory mechanisms, it can be assumed that we have just started to unravel the multiple signal transduction pathways regulated by them and the mechanisms which regulate their activity. The crystal structures of many GPCRs have been solved and these studies, along with previous reports, have provided important information about the GPCR activation mechanisms, their structural dynamics, signaling and interaction with various effector molecules. Importantly, binding of GPCRs with different types of ligands and coupling with a number of G-proteins to initiate a variety of intracellular signaling pathways has established the fact that no two GPCRs are similar. Each GPCR is unique, and there is no model GPCR. Hence, it is necessary to study an individual GPCR in details to understand its signaling and regulation. For the maintenance of cellular homeostasis and proper signal transduction, the major processes involved in the GPCR regulation are receptor desensitization, internalization, resensitization and downregulation. Most of the GPCRs, subsequent to the activation by the ligand, get desensitized. Desensitization is a protective mechanism adopted by the cells to protect themselves from chronic receptor activation and excessive signaling. Subsequent to desensitization, many GPCRs have been reported to internalize and manifest various subcellular fates depending upon the type of the receptor, the type of the agonist, and the system used in the study (Bhattacharyya, 2016). For many GPCRs, internalization is considered to be a necessary step for the resensitization and downregulation of the receptor (Trapaidze et al., 2000; Roosterman et al., 2004; L Mohan et al., 2012; Gupta et al., 2018).

## *Chapter 6: Summary*

Various post-translational modifications such as phosphorylation and ubiquitination play a crucial role in the modulation of signaling and trafficking of GPCRs (Alaluf et al., 1995; Gereau and Heinemann, 1998; Mao et al., 2008; Ribeiro et al., 2009; Ko et al., 2012; Pandey et al., 2014; Mahato et al., 2015). The role of phosphorylation in the trafficking of group I mGluRs has been studied extensively, but the role of ubiquitination in this process has not been studied in detail. Ubiquitination acts as a sorting signal to facilitate the trafficking of many mammalian GPCRs from endosomes to lysosomes for the degradation (Marchese et al., 2008). But for a number of GPCRs, ubiquitination plays many other roles as well (Tanowitz and von Zastrow, 2002). One of the earliest reports in 1996 showed that in case of the ste2 receptor (yeast GPCR), ubiquitination of a lysine residue at the C-terminus of the receptor serves as a signal for the ligand-stimulated endocytosis (Hicke and Riezman, 1996; Terrell et al., 1998; Haglund et al., 2003). The balance between the ubiquitination and the deubiquitination dictates the trafficking of GPCRs (Song and Rape, 2008; Clague et al., 2013; Coyne and Wing, 2016; Lahaie et al., 2016; Clague and Urbé, 2017). For example, the parathyroid hormone receptor (PTHR) gets internalized subsequent to the activation and recycles back to the cell surface in 2 hr. It has been reported that activation of PTHR upregulates the level of USP2 (Ubiquitin-specific protease 2), favoring the balance towards the rapid deubiquitination and the recycling of PTHR (Alonso et al., 2011). The balance between the E3 ligase and the deubiquitinase plays a crucial role in maintaining the normal cellular processes (Alonso and Friedman, 2013).

Group I mGluRs are class C G-protein coupled receptors, primarily coupled to the $G_{\alpha q/11}$ protein, and activate the phospholipase C - $IP_3$ pathway in various cell types. This family of mGluRs comprises of two receptors: mGluR1 and mGluR5 and have gained immense importance because of their involvement in various kinds of synaptic plasticity as well as in various neuropsychiatric disorders such as fragile X syndrome, autism etc (Bordi and Ugolini, 1999; Xu et al., 2012; Bhattacharyya, 2016). Various antagonists and partial agonists developed against the group I mGluRs have emerged as potential therapeutic drugs for various neurological disorders. Like many other GPCRs, group I mGluRs also get ubiquitinated by the E3 ubiquitin ligase, Siah-1A (Moriyoshi et al., 2004). However, the role of ubiquitination in the internalization of the group I mGluRs and its effect on the group I mGluR-mediated AMPAR internalization has not been

studied so far. The lack of knowledge about the role of ubiquitination and deubiquitination in the trafficking of group I mGluRs and its effect on the mGluR-mediated AMPAR endocytosis was the reason for the initiation of our study. To study the agonist-induced internalization and subsequent subcellular fate of the group I mGluRs, we used myc-tagged mGluR1 and myc-tagged mGluR5 constructs. In these constructs, myc epitope was tagged at the N-terminus of the full-length protein. In the past, these recombinant receptors have been shown to behave like the native receptor (Choi et al., 2011; Trivedi and Bhattacharyya, 2012; Pandey et al., 2014; Mahato et al., 2015). We have used both non-neuronal HEK293 cells and primary hippocampal neurons for our study. HEK293 cells were used as a heterologous system, since these cells have a large repertoire of G proteins. On the other hand, primary hippocampal neurons are the most experimentally tractable *in vitro* system that can approximate the *in vivo* situation. In the past, a variety of techniques such as surface biotinylation assay and single colour fluorescence measurement assays have been used to quantify the internalization of many GPCRs. However, the variability in the expression of the receptors between the cells was not considered in these assays. To overcome this situation, we have used an elegant technique, viz., dual antibody staining assay or antibody feeding assay to measure the amount of receptors internalized upon agonist stimulation. This method allowed us to normalize the amount of the internalized receptors within cells. Thus, this method has an advantage that it quantitates the proportion of the internalized receptors within a cell and hence, the variability in the surface expression of the receptors between cells does not affect the quantitation (Bhattacharyya et al., 2009; Trivedi and Bhattacharyya, 2012; Pandey et al., 2014; Mahato et al., 2015).

## 6.2. Role of ubiquitination in the ligand-mediated internalization of group I mGluRs

Group I mGluRs get desensitized in PKC and GRK-dependent manner and subsequent to the desensitization, the receptors get internalized in arrestin and dynamin-dependent manner *via* clathrin coated pits (Ferguson, 2001; Dale et al., 2002; Ferraguti et al., 2008). Our lab has earlier shown that subsequent to the desensitization, group I mGluRs undergo endocytosis and the endocytosed receptors recycle back to the cell surface in a phosphatase-dependent manner (Pandey et al., 2014; Mahato et al., 2015). Furthermore, for both myc-mGluR1 and myc-mGluR5, the internalization kinetics was observed to be similar in both HEK293 cells as well as

in primary hippocampal neurons, suggesting that in all these cell types the receptor internalization probably occurred through similar mechanisms. Since hippocampal neurons are compartmentalized cells and it has been shown that some processes are compartment specific, we checked the myc-mGluR1 endocytosis in both cell body and dendrites. Our data suggested that myc-mGluR1 internalized throughout the cell to a similar extent. In agreement with the earlier reports, subsequent to the internalization, group I mGluRs were observed to recycle back to the cell surface.

Ubiquitination is an important reversible post-translational modification that has been reported to regulate the function of many GPCRs (Hershko and Ciechanover, 1998; Hicke et al., 2005; Dores and Trejo, 2012; Komander and Rape, 2012; Alonso and Friedman, 2013). For some receptors, ubiquitination also serves as a signal for the receptor endocytosis. For example, internalization of ste2 and ste3 receptors (yeast GPCRs) is ubiquitin-dependent (Hicke and Riezman, 1996; Terrell et al., 1998). In the case of group I mGluRs, we have observed robust ubiquitination of mGluR1 upon stimulation of the receptor by the ligand. Our data also showed that blocking the ubiquitination resulted in a block in the endocytosis of both members of group I mGluRs in non-neuronal HEK293 cells as well as in primary hippocampal neurons. In order to check for any possible effects of inhibiting ubiquitination on the general trafficking machinery of the cells, we studied the trafficking of transferrin receptor. Transferrin receptor showed normal kinetics upon inhibition of ubiquitination, suggesting that blocking ubiquitination did not affect the general trafficking machinery of the cell.

## 6.3. Mechanisms of ubiquitination-dependent group I mGluR internalization and group I mGluR-mediated AMPAR trafficking

In the last few years, it has become clear that the role of ubiquitination is not limited to the degradation of proteins. Ubiquitination also regulates the internalization of several plasma membrane proteins. Ubiquitination either directly modulates the biophysical properties of the substrate or serves as a signal that is recognized by other proteins which orchestrate the cellular events. Therefore, the type of the modification and the site of the modification can give an insight into the understanding of the molecular mechanisms controlling the ligand-mediated

trafficking of group I mGluRs. Our previous data suggested that internalization of group I mGluRs was ubiquitination-dependent. We have shown here that monoubiquitination is not sufficient to trigger the ligand-mediated internalization of myc-mGluR1. Our data suggested that K63-linked polyubiquitination was essential for the internalization of myc-mGluR1. Subsequently, we searched for the site of ubiquitination in mGluR1. For that, we targeted the C-terminus tail of mGluR1. The long C-terminus tail of mGluR1 has been reported to regulate the signaling and trafficking of mGluR1 and contains 16 lysine residues (Pin and Duvoisin, 1995; Ciruela et al., 1999; Remelli et al., 2008; Bhattacharyya, 2016). We changed each lysine residue to arginine through site-directed mutagenesis and found that the lysine residue at the 1112 position in the C-terminus tail of mGluR1 is critical for the ligand-mediated internalization of the receptor. We also inhibited the ubiquitination by specifically knocking down the E3 ligase, Siah-1A, using siRNA. Siah-1A is an E3 ligase that directly interacts with the group I mGluRs and results in the ubiquitination of the receptors (Moriyoshi et al., 2004; Ko et al., 2012). Our data suggested that acute knockdown of endogenous Siah-1A resulted in a complete block in the ligand-stimulated internalization of myc-mGluR1 as well as in the inhibition of the ubiquitination of the receptor. Activation of group I mGluRs with the agonist R,S-DHPG induces AMPAR endocytosis, which is the cellular mechanism for the induction of mGluR-LTD (Zho et al., 2002; Zhang et al., 2008). It has been reported that in the presence of UBEI-41/PYR-41 (an inhibitor of E1-activating enzyme), activation of group I mGluRs results in the enhanced signaling and enhanced mGluR-LTD in the hippocampus (Citri et al., 2009). We also showed here that acute knockdown of Siah-1A led to an enhanced mGluR-mediated AMPAR endocytosis. Altogether these results not only suggest the existence of an ubiquitin-independent mGluR-mediated AMPAR endocytosis pathway, they also imply that the ubiquitin system limits a step in the multistep process leading from mGluR activation to AMPAR endocytosis.

### 6.4. Role of deubiquitination in the mGluR1 trafficking

Ubiquitination and deubiquitination processes help in maintaining the normal homeostasis of the cellular milieu. The human genome encodes for nearly 100 deubiquitinases responsible for the reversal of ubiquitination but the functional importance of these deubiquitinases has not been studied in detail. For some GPCRs, alternation in the ubiquitination-deubiquitination process

results in a 'trip switch' between the recycling and degradation pathways (Berthouze et al., 2009). Our data suggested that, pharmacologically blocking the deubiquitinases inhibited the recycling of myc-mGluR1 in HEK293 cells as well as in primary hippocampal neurons. Interestingly, blocking the deubiquitination pharmacologically resulted in the targeting of some of the internalized myc-mGluR1 to the lysosomes. Furthermore, when the proteasomal inhibitor MG-132 was not applied, in the presence of the deubiquitinase inhibitor, the total amount of the internalized myc-mGluR1 decreased, suggesting that the receptors underwent proteasomal degradation. These results suggested that blocking the deubiquitination led to the inhibition in the recycling of the ligand-induced internalized receptors and a population of the receptor changed the route and entered the lysosome. We extended our study in search for a specific deubiquitinase involved in the deubiquitination process during the trafficking of mGluR1. We chose ubiquitin-specific protease 19 (USP19) as a potential candidate based on the following information from the literature: 1) It is the only USP with a Siah-interacting motif (Zhang et al., 2017), 2) USP19 specifically cleaves the K63-linked polyubiquitinated chains (Wu et al., 2017). Over-expression of the wild-type USP19-GFP did not have any effect on the surface expression or in the ligand-induced trafficking of myc-mGluR1. We subsequently used a catalytically inactive form of USP19, viz., USP19C506S-GFP, where the cysteine present at the active site was converted to serine. It has been reported that this conversion of cysteine to serine at 506 position of USP19 did not affect the binding of USP19 with its substrate but it inhibited its catalytic activity (Altun et al., 2012). Our data suggested that over-expression of the USP19C506S-GFP did not affect the surface expression of myc-mGluR1 in primary hippocampal neurons but it inhibited the recycling of myc-mGluR1. These results suggested that USP19 might play a critical role in the deubiquitination process during the trafficking of mGluR1.

### 6.5. Summary of the picture that is available till now

The results presented in this thesis provide some advancement in the pre-existing knowledge of group I mGluR trafficking and their regulation. Earlier reports have suggested that group I mGluRs, subsequent to the stimulation by the ligand get internalized in a phosphorylation-dependent manner. Our data suggest that group I mGluRs get ubiquitinated subsequent to the

activation by the ligand. Pharmacological inhibition of the ubiquitination completely inhibits theligand-mediated endocytosis of mGluR1 and mGluR5 in non-neuronal cells and primary hippocampal neurons. Monoubiquitination is not sufficient to trigger the internalization of mGluR1. The ligand-mediated internalization of mGluR1 is K63-linked polyubiquitination-dependent. We have observed that the lysine present at the 1112 position in the C-terminus tail of mGluR1 plays critical role in the ligand-mediated internalization of the receptor. Our data suggests that ubiquitination of mGluR1 is Siah-1A dependent. Acute knockdown of Siah-1A completely inhibits the ligand-mediated endocytosis of mGluR1. We have also shown that activation of group I mGluRs leads to the internalization of synaptic AMPARs, which is the cellular correlate for mGluR-LTD. Interestingly, in Siah-1A knockdown background, activation of group I mGluRs led to the enhanced AMPAR endocytosis. Deubiquitinases also play a key role in the regulation of mGluR1 trafficking. Pharmacological inhibition of the cysteine peptidases results in the block in the recycling of mGluR1 in non-neuronal cells as well as in primary hippocampal neurons. USP19 plays a key role in the ligand-stimulated trafficking of mGluR1. Over-expression of the catalytically inactive form of USP19 inhibits the recycling of mGluR1 in primary hippocampal neurons **(Figure 6.1).**

## 6.6. Future directions

The studies described in this thesis have opened many questions that need to be addressed in the future. We have studied the role of ubiquitination in the ligand-induced trafficking of group I mGluRs. It has been reported that mGluR1 and mGluR5 internalize in both agonist-dependent and agonist-independent manner (Dhami and Ferguson, 2006; Trivedi and Bhattacharyya, 2012; Bhattacharyya, 2016). The mechanisms regulating these two distinct processes might be different. In this study, we have shown ubiquitination to be a critical regulator of the ligand-mediated internalization of mGluR1 and mGluR5 in both non-neuronal HEK293 cells as well as in primary hippocampal neurons. It would be important to investigate the role of ubiquitination, if any, in the constitutive endocytosis of the receptor as well. Literature suggests that ubiquitination and phosphorylation are coupled phenomena; it would also be interesting to check how impairment of one modification would affect the other. Our data suggest that

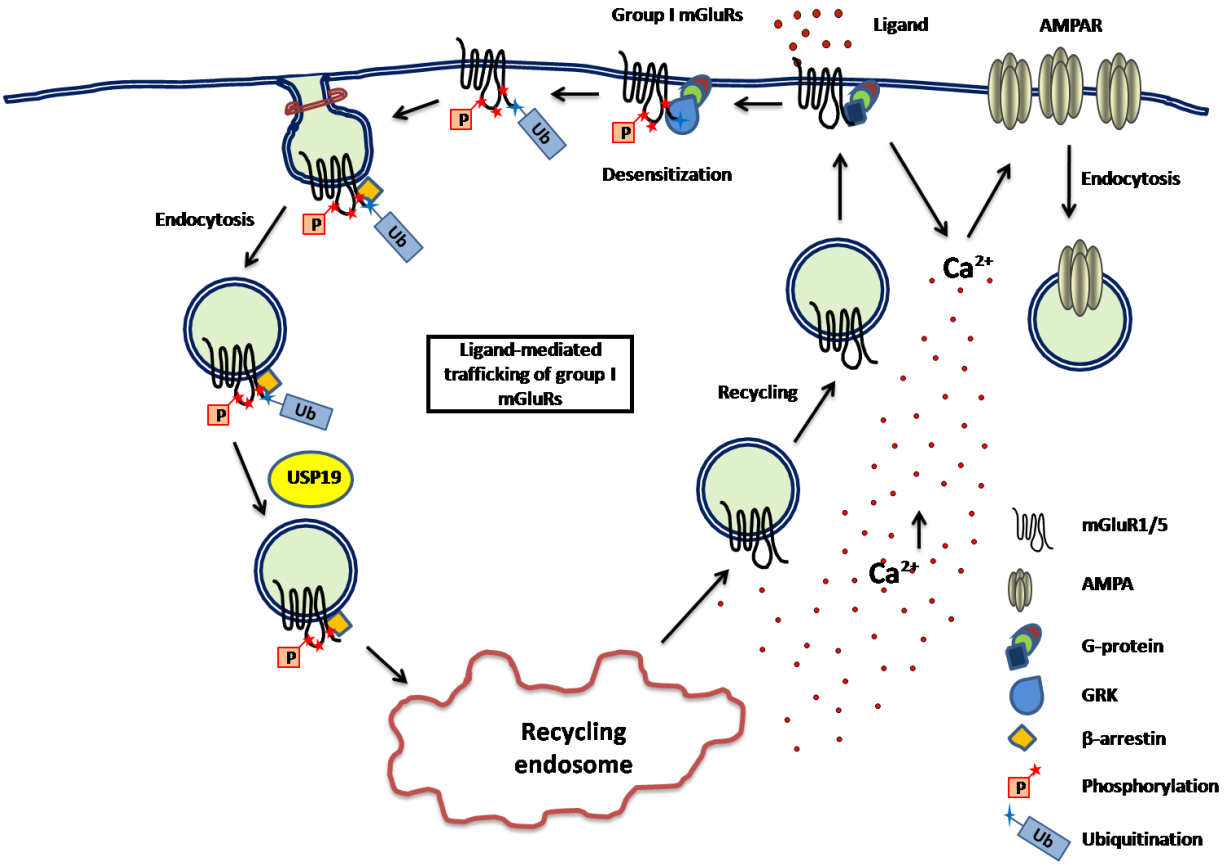



**Figure 6.1. Regulation of group I mGluR trafficking and mGluR-mediated AMPAR internalization.** Ligand-mediated activation of group I mGluRs result in the internalization of the receptors. Internalization of group I mGluRs is K63-linked polyubiquitination dependent. The endocytosed mGluR1 goes to the recycling compartment. Majority of the internalized receptors recycle back to the cell surface. USP19 plays an important role in the mGluR1 recycling. Upon agonist stimulation, group I mGluRs initiate the AMPAR endocytosis.

monoubiquitination is not sufficient to stimulate the ligand-mediated internalization of mGluR1 but the internalization is K63-linked polyubiquitination-dependent. Whether similar mechanisms regulate the ligand-mediated trafficking of the other member of the group I mGluR family, i.e., mGluR5 would be another important area to study in future. We have shown that the lysine residue present at the 1112 position in the carboxy-terminal tail of mGluR1 is critical for the ligand-mediated internalization of the receptor and the internalization of the receptor is E3 ligase, Siah-1A-dependent. Whether the 3$^{rd}$ lysine from the extreme C terminus of mGluR5 also plays a critical role in the ligand-mediated internalization of the receptor and whether the ligand-mediated endocytosis of mGluR5 is Siah-1A dependent, needs to be investigated. Some GPCRs, subsequent to the internalization, go to the recycling compartment and recycle back to the cell surface. Group I mGluRs also recycle back to the cell surface subsequent to the ligand-mediated internalization (Pandey et al., 2014; Mahato et al., 2015; Bhattacharyya, 2016). Deubiquitinases are proteins, known to reverse the ubiquitination of the substrates. The balance between ubiquitin ligase and deubiquitinase drives the cycling of GPCRs (Song and Rape, 2008; Clague et al., 2013; Coyne and Wing, 2016; Lahaie et al., 2016; Clague and Urbé, 2017). Our data suggests that USP19 plays a key role in the ligand-mediated trafficking of mGluR1. It would be critical to identify the substrate(s) of USP19. The substrate(s) could be the ubiquitinated receptor and/or some other protein involved in the trafficking of the receptor. Another important aspect to investigate would be to determine the intracellular compartment at which the deubiquitination takes place. The molecular mechanisms of how the deubiquitination regulates the recycling/resensitization of group I mGluRs need to be investigated in future.

## 6.7. Final words

G-protein coupled receptors are the major players in regulating various physiological processes and respond to various stimuli. As the present study and other reports are testaments, there is a huge diversity observed in GPCR specific signaling, desensitization, internalization, resensitization and downregulation processes. It is not surprising since varieties of GPCRs are co-expressed in cells; the system has evolved numerous ways to regulate these receptors differently, in order to maintain synchrony. The diversity in GPCR responsiveness is likely to be modulated by different ligands binding to the receptors and also structural differences among the

members of GPCR family. Additionally, different subsets of interacting regulatory proteins and the effector molecules might add further complexity in the signaling and regulation of GPCRs. Although we have gained much information about GPCR signaling and regulation in the last few decades, considering the huge diversity, it appears that we have just begun to understand the biological complexity that exists in GPCR signaling and regulation. Thus, continued understanding of GPCR signaling, regulation and trafficking will provide us important insights to unravel the complexity that exists in nature and also to develop novel therapeutic strategies to cure various diseases that arise due to aberrant GPCR signaling and regulation.

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# Publications:

- **Ravinder Gulia**, Rohan Sharma, and Samarjit Bhattacharyya (2017) A critical role for ubiquitination in the endocytosis of glutamate receptors. ***Journal of Biological Chemistry,*** 292:1426-1437.

- Prabhat Kumar Mahato, Namrata Ramsakha, Prachi Ojha, **Ravinder Gulia**, Rohan Sharma and Samarjit Bhattacharyya (2018) Group I Metabotropic Glutamate Receptors (mGluRs): Ins and Outs, ***Biochemical and Biophysical Roles of Cell Surface Molecules,*** Springer publications **(equal first author)**.

- Rohan Sharma, **Ravinder Gulia** and Samarjit Bhattacharyya (2018) A critical role for Sorting Nexin 1 in the trafficking of metabotropic glutamate receptors: ***Journal of Neuroscience.*** 38(40):8605-8620

- Rohan Sharma, **Ravinder Gulia** and Samarjit Bhattacharyya (2018) Analysis of ubiquitination and ligand-dependent trafficking of group I mGluRs ***Methods in cell biology,*** Volume 149, ISSN 0091-679X, Elsevier Publication. (**equal first author**)

- **Ravinder Gulia** and Samarjit Bhattacharyya (2019) A critical role for deubiquitination in the trafficking of group I mGluRs (manuscript under preparation).