Role of Sorting Nexin 1 (SNX1) in the group I metabotropic glutamate receptor trafficking

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Declaration

The work presented in this thesis has been carried out by me under the guidance of Dr. Samarjit Bhattacharyya at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bona fide record of original work done by me and all sources listed within have been detailed in the bibliography.

Rohan Sharma

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

Dr. Samarjit Bhattacharyya

Certificate

I certify that this thesis entitled "Role of Sorting Nexin 1 (SNX1) in the group I metabotropic glutamate receptor trafficking" comprises research work carried out by Rohan Sharma 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
- **APV:** D-(-)-2-Amino-5-phosphonopentanoic acid
- **AB-AM mix:** antibiotic-antimycotic mix
- **BSA:** Bovine serum albumin
- **CNS:** Central nervous system
- **cAMP:** cyclic AMP
- **CaM:** Calmodulin
- **CaMKIIα:** Ca^{2+}/calmodulin-dependent protein kinase IIα
- **CREB:** cAMP-response element-binding protein
- **CBP:** CREB-binding protein
- **CBD:** Clathrin binding domain
- **DIV:** Days *in vitro*
- **DMSO:** Dimethyl sulfoxide
- **DUBs:** Deubiquitinating enzymes
- **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
- **EAATs:** Excitatory amino acid transporters
- **EDTA:** Ethylene diamine tetraacedic acid
- **EGFR:** Epidermal growth factor receptor
- **FUDR:** 5-Fluoro-2'-deoxyuridine (FUDR)
- **FBS:** Fetal bovine serum
- **GRK:** G-protein coupled receptor kinase
- **Hrs:** Hepatocyte growth factor-regulated tyrosine kinase substrate
- **IP_3**: Inositol 1,4,5-triphosphate
- **mGluR:** Metabotropic glutamate receptor
- **NMDAR:** N-methyl-D-aspartate receptor
- **PtdIns:** Phosphatidylinositol's

- **PBS:** Phosphate buffer saline
- **PEI:** Polyethylenimine
- **PIP_2:** Phosphatidylinositol 4,5-bisphosphate
- **PKA:** Protein kinase A
- **PKC:** Protein kinase C
- **PLC:** Phospholipase C
- **PFA:** Paraformaldehyde
- **PCR:** Polymerase chain reaction
- **PP2A:** Protein phosphatase 2A
- **PP2B:** Protein phosphatase 2B
- **PAR1:** Protease-activated receptor-1
- **P2Y1:** Purinergic Receptor
- **SNX1:** Sorting Nexin 1
- **TGN:** Trans-Golgi network
- **LTP:** Long-term potentiation
- **LTD:** Long-term depression
- **NGS:** Normal goat serum
- **TTX:** Tetrodotoxin
- **TMD:** Transmembrane domain motif
- **UIM:** Ubiquitin interacting motif
- **VFD:** Venus Flytrap Domain
- **VGLUTs:** Vesicular glutamate transporters

Chapter 1

Introduction

The communication is a process which organisms use to increase their chance of survival among species, and this process has evolved through the course of evolution from unicellular to multicellular organisms. Multicellular organisms communicate in numerous ways to coordinate their social behavior, like ants communicate with each other in a colony to coordinate their motion while moving collectively in a traffic-like unidirectional way on trails. This kind of collective behavior is common in animal groups like milling of fish schools under water, flocks of birds flying synchronously and swarms of insects which show remarkable coordination achieved by communication. Such kind of communication takes place at the cellular level as well and allows unicellular organisms to coordinate with one another in a population to work as a team to achieve their objective. The mechanisms of cell-cell communication used by these organisms are very similar to the way cells of a multicellular organism communicate with one another. This process takes place as a cell responds to an environmental stimulus by releasing some chemical signal termed as a ligand which further stimulates a target cell. These target cells are characterized by the presence of ligand-specific receptors on the cell membrane which gets activated upon ligand binding and transduce intracellular signaling. In this way, signaling by surface receptors allows the cell to sense the external environment and modulate its ongoing intracellular dynamics to maintain homeostasis. A variety of cell signaling mechanisms have evolved in cells; each of them comprises a cascade of interacting signaling molecules that directs various cellular responses. These cellular responses form the basis of a wide range of vital functions including immunity, development, and regeneration. But improper signaling and inaccurate processing of signals could lead to diseases like cancer, epilepsy, and autoimmunity.

1.1. G protein-coupled receptors (GPCRs)

The G protein-coupled receptors (GPCRs) belong to the largest family of cell surface receptors. They are often referred to as seven transmembrane receptors as they share a common structural design which comprises of an N-terminus of the protein positioned outside of the cell and the C-terminus inside connected by seven transmembrane domains linked by three intracellular loops and three extracellular loops **(Figure 1.1)**. The human genome encodes around 800 GPCRs;

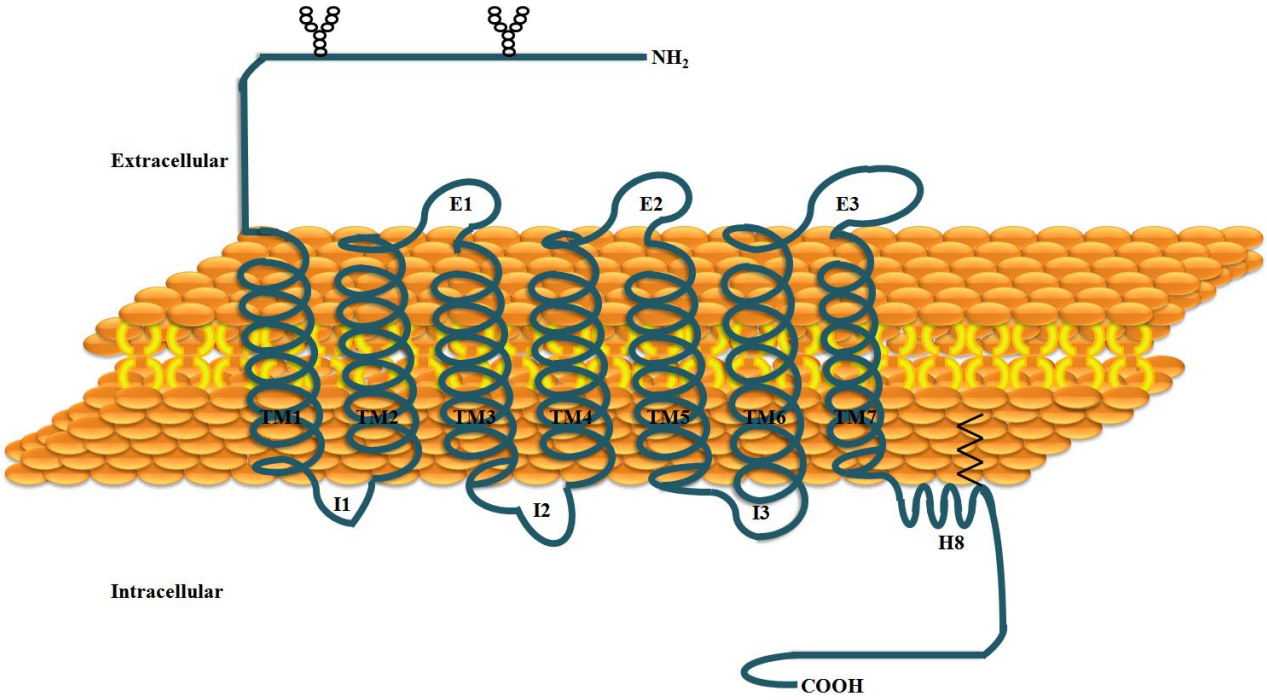


Figure 1.1. Diagrammatic representation of the G-protein coupled receptors. G-protein coupled receptors (GPCRs) are comprised of seven hydrophobic transmembrane domains (TM1-7), with an extracellular N-terminus and an intracellular C-terminus. These seven transmembrane receptors have three extracellular loops (E1, E2 and E3) and three intracellular loops (I1, I2 and I3).

majority of them are involved in sensory functions (Fredriksson and Schiöth 2005; Hanlon and Andrew 2015). The activity of these receptors depends on the binding of their specific ligands although a majority of them have been identified by DNA sequence information, and not the ligand they bind. Among them which remain unmatched to the existing ligands are considered as orphan GPCRs (Civelli 2012). The GPCRs are classified into six classes (Class A- Class F) depending on the sequence homology and functional relevance (Kolakowski 1994). Rhodopsin family (Class A) of receptors is the largest group of G protein-coupled receptors characterized by the presence of short N-terminal domain and binding with a wide range of ligands. This group is comprised of hormone, neurotransmitter and light receptors which respond to the extracellular signal and transduces G protein-mediated signal transduction pathway. On the basis of phylogenetic analysis, they are subdivided into 19 subgroups. The secretin receptor (Class B) family of GPCRs responds to ligands comprised of polypeptide hormones ranging from 27-141 aa residues like secretin and glucagon. This family comprises of approximately 20 receptors and in humans, 15 genes have been reported to express the receptors of this family (Harmar 2001). The glutamate family (Class C) of receptors includes metabotropic glutamate receptors, γ-aminobutyric acid (GABAB) receptors, pheromone receptors and three type1 taste receptors. The long amino-terminal domain of these receptors acts as the endogenous ligand-binding regions (Ryba and Tirindelli 1997; Sullivan, Chateauneuf et al. 2000; Malherbe, Kratochwil et al. 2003; Brauner-Osborne, Wellendorph et al. 2007; Suzuki, Kimura et al. 2007; Fukuda, Suzuki et al. 2009; Kniazeff, Prézeau et al. 2011; Chun, Zhang et al. 2012; Wu, Wang et al. 2014). The fungal mating pheromone receptors STE2 and STE3 of yeast corresponds to the family D of GPCRs (Nakayama, Miyajima et al. 1985; Marsh and Herskowitz 1988). The Class E receptors comprise of cyclic AMP receptors involved in various distinct processes like chemotaxis. These receptors play a key role in regulating the development of *Dictyostelium discoideum* (Klein, Sun et al. 1988; Johnson, Saxe et al. 1993; Saxe, Ginsburg et al. 1993). The Frizzled family (Class F) of receptors is the most unconventional class of GPCRs consisting of Frizzled proteins (FZD) and Smoothened (SMO). FZD receptors have a characteristic cysteine-rich ligand-binding domain and are activated by lipoglycoproteins of WNT family while the family of Hedgehog proteins activates the SMO proteins indirectly. Frizzled proteins play a central role in regulating essential processes like embryogenesis, synaptogenesis and cell polarity (Xu and Nusse 1998; Huang and Klein 2004).

1.2. GPCR Signaling

G protein-coupled receptors (GPCRs) represent a large protein family of receptors that bind to diverse set of ligands like hormones, photons and neurotransmitters to accomplish broad range of functions. The specificity of ligand GPCR interaction is essential for the efficient cellular response, which is crucial for the health of both the cells as well as the organism (Rosenbaum, Rasmussen et al. 2009). The binding of a ligand to the extracellular domain of a GPCR introduces conformational changes in its structure which transduce the information inside the cell. This process involves activation of the heterotrimeric G-proteins induced by the change in the receptor conformation which further activates the downstream signaling pathways (Bockaert and Pin 1999; Deupi and Standfuss 2011; Hanlon and Andrew 2015). These heterotrimeric G-proteins consists of three different subunits, viz., α, β and γ which associate with the membrane by their covalent interaction with lipid molecules. The G_α subunit that is associated with GDP, gets exchanged with GTP after ligand-mediated activation of the receptor. This process activates G_α and results in the dissociation of the heterotrimeric G-protein into G_α subunit and $G_{\beta\gamma}$ complex. Both of them activate distinct downstream effector molecules including adenylyl cyclase, protein kinases, phospholipases which in turn regulate the concentrations of secondary messengers like cAMP, IP_3, DAG (Milligan and Kostenis 2006; Mahoney and Sunahara 2016) **(Figure 1.2)**. The intrinsic GTPase activity of G_α catalyzes the hydrolysis of its bound GTP to GDP leading to its inactivation and reassociation with the $G_{\beta\gamma}$ complex (Milligan and Kostenis 2006; Syrovatkina, Alegre et al. 2016; Spangler and Bruchas 2017).

GPCRs are principally involved in two kinds of signal transduction pathways known as cAMP signal pathway and phosphatidylinositol signal pathway.

1.2.1. cAMP signal pathway

This signaling pathway is also known as adenylyl cyclase pathway. Adenylyl cyclase is a membrane-bound enzyme which catalyzes the conversion of 5'-ATP into 3'5'-cyclic AMP and pyrophosphate (Homcy, Wrenn et al. 1978; Désaubry, Shoshani et al. 1996). The activity of adenylyl cyclase in conjunction with the action of phosphodiesterase regulates the intracellular concentrations of cAMP. The activation of adenylate cyclase is induced by the α subunit of the G_s protein (αs) following the agonist-dependent activation of the receptor. The βγ subunits can

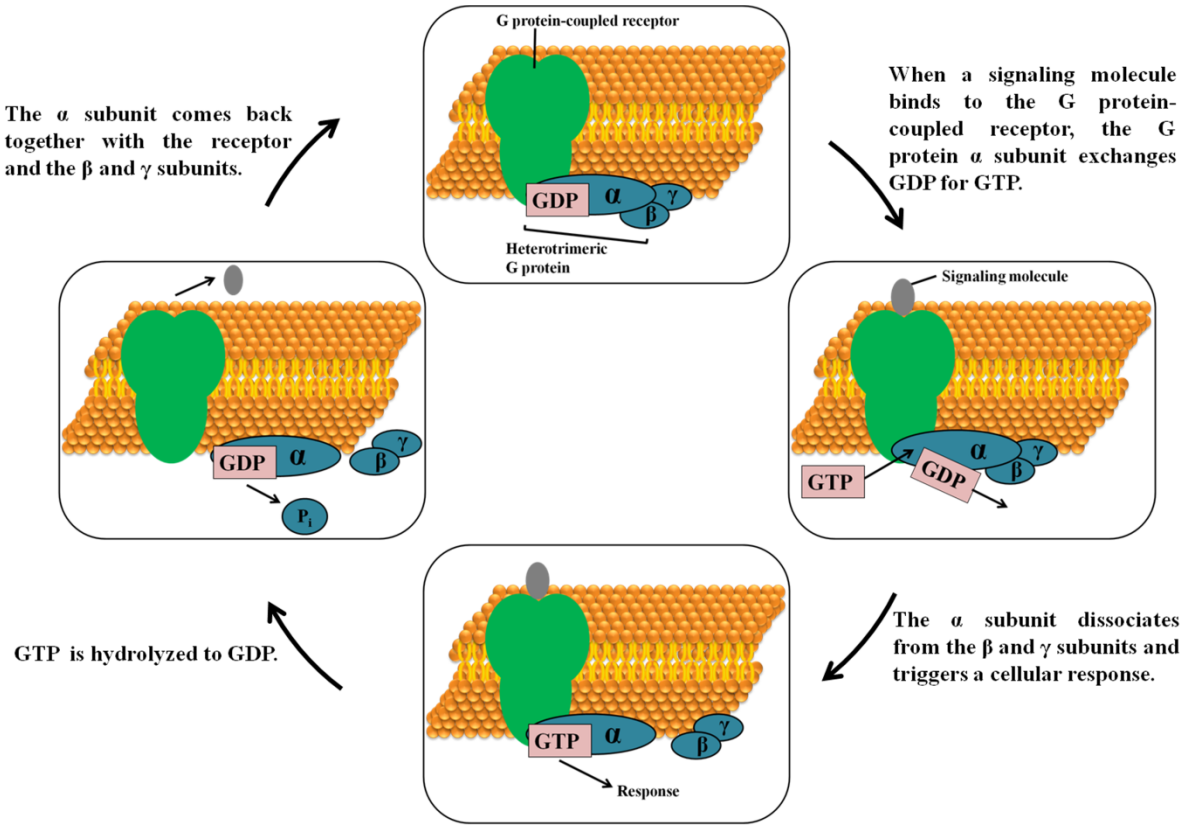


Figure 1.2. The G-protein activation mechanism by GPCR. Heterotrimeric G-proteins comprise of three subunits: α, β, and γ. When a GPCR gets activated upon binding of the agonist/ligand, it interacts with the inactive form of the G-protein. The activated receptor acts as a guanosine nucleotide exchange factor, and as a result, a GDP molecule associated with the α subunit is exchanged for GTP. Subsequently, the β and γ subunits (βγ) dissociate from the α subunit. The activated G_α can then interact with an effector like adenylate cyclase or phospholipase C (PLC), which leads to the initiation of the second-messenger cascade. The βγ subunit also transduces various intracellular signaling. Hydrolysis of GTP to GDP terminates the signal and allows reformation of the inactive G-protein complex.

also activate some isoforms of adenylate cyclase. The active form of adenylate cyclase generates cAMP which further activates the downstream effector molecules including cAMP-dependent protein kinase (PKA) (Thatcher 2010; Sassone-Corsi 2012). PKA acts upon several cytosolic and nuclear proteins as substrates. It plays a crucial role in regulating cellular processes like metabolism, transcription and other signaling pathways by phosphorylating the respective metabolic enzymes, transcription factors and signaling molecules involved (Rogue, Humbert et al. 1998; Fimia and Sassone-Corsi 2001; Yan, Gao et al. 2016; Tillo, Xiong et al. 2017).

Transcriptional regulation by protein kinase A involves phosphorylation of the transcription factors like cAMP-response element-binding protein (CREB) which facilitates the binding of transcriptional coactivators like CREB-binding protein (CBP) to them (Mayr and Montminy 2001; Sassone-Corsi 2012). Studies in rats and mice have shown the involvement of CREB in the formation of long-term memories and promoting the survival of neurons (Morris and Gold 2012; Ortega-Martínez 2015).

1.2.2. Phosphatidylinositol signal pathway

This pathway begins when an extracellular signal stimulates the seven transmembrane receptors, resulting in a conformational change of the receptor which facilitates the exchange of GDP bound to $G_{\alpha q}$ subunit of the heterotrimeric G-protein with GTP. This activated form of $G_{\alpha q}$ dissociates from $G_{\beta\gamma}$ and binds to the membrane-bound phospholipase C. Phospholipase C upon activation by $G_{\alpha q}$ catalyzes the hydrolysis of membrane phospholipid PIP_2 to form inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Cooper 2004; Gamper and Shapiro 2007). DAG remains associated with the membrane. It moves along the membrane and acts upon protein kinase C. On the other hand, IP_3 being water soluble, diffuses through cytosol and activates the ligand-gated calcium channels present on the membrane of smooth endoplasmic reticulum (SER) and mitochondria-associated endoplasmic reticulum. The activation of these channels causes the release of calcium ions into the cytoplasm which essentially regulates various cell functions (Berridge 1984; Berridge 1987; Epand 2017). This intracellular calcium in conjunction with DAG activates protein kinase C (PKC) which further activates its target proteins by phosphorylating their serine and threonine residues (Purves, Augustine et al. 2001). Furthermore, the binding of calcium to calmodulin (CaM) results in the formation of calcium-calmodulin

complex which in turn activates CaM kinaseII, triggering the CaM kinase pathway (Colbran 2004; Stratton, Chao et al. 2013; Berchtold and Villalobo 2014). The activation of all these kinases in a cascade to carry out cellular functions, ultimately results in a particular physiological effect (Berchtold and Villalobo 2014; Epand 2017).

1.3. Mechanisms of GPCR desensitization

GPCRs have an inherent ability to modulate the susceptibility of the receptor to get stimulated according to their previous signaling exposure (Hausdorff, Caron et al. 1990). So, if the prior activation was high, then it will reduce the ability of the receptor to stimulate further in response to the ligand leading to receptor desensitization. On the other hand, a lower activation results in the increased susceptibility of the receptor to get stimulated in future (sensitization) (Schmidt, Frings et al. 2000; Billington and Penn 2003; Ruthirago, Julayanont et al. 2017; Svensson and Sorkin 2017). Desensitization is a very crucial regulatory trait of these receptors adopted by cells as a feedback mechanism to prevent them from chronic or acute receptor overstimulation (Gainetdinov, Premont et al. 2004).

The signaling of these receptors could be regulated at the receptor level by either regulating the number of receptors on the cell surface or by modulating their efficiency to respond to the ligand. The receptors continuously mobilize from the cell surface to the endosomal compartment in a ligand-dependent or independent (constitutive endocytosis) manner. In addition to that, they also undergo a cycle of degradation and synthesis to maintain a constant pool of receptors. The ligand-dependent activation of receptors could be followed by internalization of receptors leading to either lysosomal degradation (downregulation) or recycling back to the surface (resensitization) for further signaling. The different ways by which GPCRs undergo desensitization include: 1) receptor modifications like ubiquitination or phosphorylation which results in the uncoupling of G-protein from the receptor, 2) receptor sequestration in the endosomes, 3) receptor downregulation by either lysosomal degradation of pre-existing receptors or reduction in the biosynthesis of new receptors or both (Bouvier, Hausdorff et al. 1988; Hausdorff, Caron et al. 1990; Lefkowitz 1998; Oakley, Laporte et al. 1999; Kohout and Lefkowitz 2003; Hanyaloglu and Zastrow 2008; Jean-Alphonse and Hanyaloglu 2011; Magalhaes, Dunn et al. 2012; Black, Premont et al. 2016).

Activation-dependent attenuation of GPCR function by receptor modification is called homologous desensitization. More specifically, a class of serine-threonine GPCR kinases is involved in the phosphorylation of these receptors. Whereas, activation-independent desensitization of receptor is carried out by the activity of other receptors which leads to the heterologous desensitization. One such way is the feedback regulation by second messenger kinases such as protein kinase A (PKA) to carry out desensitization as a result of G-protein uncoupling. In the same way, other GPCR activated protein kinases like protein kinase C (PKC), mitogen-activated protein (MAP) kinases, etc. also regulates different types of receptors. Moreover, these kinases can also phosphorylate and regulate the downstream effectors, such as adenylyl cyclase, phospholipase C, and thus regulate the GPCR signaling (FEHMANN and HABENER 1991; Chuang, Iacovelli et al. 1996; Ferguson 2001; Ghadessy and Kelly 2002; Katz, Amit et al. 2007; Kelly, Bailey et al. 2008; Vandamme, Castermans et al. 2012; Yamamoto, Higa-Nakamine et al. 2014; Han, Ma et al. 2016; Gergs, Fritsche et al. 2017; Zhang and Kim 2017).

Phosphorylation of these receptors promotes the binding of another class of proteins called arrestins. The arrestin family of proteins comprises of visual arrestins and β-arrestins. Visual arrestins are expressed mainly in retinal cells and are involved in the regulation of phototransduction. They are further classified into rod arrestin (arrestin-1 or S-antigen) and cone arrestin (arrestin- 4 or X-arrestin) (Sunayashiki-Kusuzaki, Kikuchi et al. 1997). On the other hand, both the non-visual subtypes of arrestins, viz., β arrestin-1 and β arrestin-2 are ubiquitously expressed throughout the body and believed to be involved in the regulation of a majority of GPCRs (Gurevich and Gurevich 2006).

The transmembrane helices of GPCRs arrange themselves in an open cytoplasmic crevice conformation stabilized by the binding of ligand. The binding of a ligand activates the receptors (R*) which stabilizes the arrangement of transmembrane helices in an open cytoplasmic crevice. This conformation allows the binding and activation of specific G-proteins (Gether 2000; Kobilka 2007). The activated GPCR serves as a substrate to specific GPCR kinase which phosphorylates multiple sites on the receptor, present mainly at the C-terminus. The phosphorylation of these residues facilitates the binding of arrestins which offers steric hindrance to further G protein-coupling. Hence, arrestins by virtue of their flexible structure serve

multipurpose functions by interacting with both GPCRs and their binding partners in several different ways (Gurevich and Gurevich 2006; Tobin, Butcher et al. 2008; Rosenbaum, Rasmussen et al. 2009; Tian, Kang et al. 2014; Smith and Rajagopal 2016; Peterson and Luttrell 2017). Recent studies from the crystal structures have provided several significant insights into this mechanism. Many more features need to be explored in future including the role of different receptor states, oligomerization and phosphorylation pattern in modulating the configuration of the arrestin-receptor complex (Scheerer and Sommer 2017; Takenouchi, Yoshimura et al. 2018). Additionally, binding of arrestins also connects receptor to the components of internalization machinery including clathrin and its adaptor protein AP2, which helps in receptor internalization (Shenoy and Lefkowitz 2011; Gurevich and Gurevich 2014; Kang, Tian et al. 2014; Tian, Kang et al. 2014). Several GPCRs get internalized upon ligand stimulation resulting in the dissociation of G-protein from the receptor leading to receptor desensitization. Subsequent to internalization, receptors get transported to internal compartments, called endosomes and mechanisms underlying this phenomenon have been discussed below in detail (Paing, Johnston et al. 2006; Tian, Kang et al. 2014).

1.3.1. GPCR endocytosis

GPCR internalization takes place either *via* clathrin-mediated or caveolae-dependent pathways (Billington and Penn 2003; Gong, Huntsman et al. 2008; Mayor, Parton et al. 2014; Xu, Liu et al. 2017). In clathrin-mediated endocytosis, surface receptors get concentrated in clusters to form clathrin-coated pits. These pits bud off from the plasma membrane and form clathrin-coated vesicles in a dynamin-dependent manner. It is a highly regulated process which involves various adaptor and accessory proteins that function in a stepwise manner to connect the receptor to clathrin and other protein complexes (Oakley, Laporte et al. 1999; Drake, Shenoy et al. 2006; Magalhaes, Dunn et al. 2012).

Caveolar endocytosis is one form of clathrin-independent endocytosis, which is sensitive to cholesterol depletion (Kirkham and Parton 2005; Sandvig, Torgersen et al. 2008; Sandvig, Kavaliauskiene et al. 2018). Caveolae is flask-shaped small invaginations of plasma membranes rich in proteins and lipids. Caveolin family of proteins, viz., caveolin-1, caveolin-2 and

caveolin-3 constitute the main structural proteins of caveolae, whereas its lipid fraction comprises of cholesterol and sphingolipids (Kiss and Botos 2009). The monomers of caveolins oligomerize on lipid rafts leading to the expansion of the plasma membrane invaginations and formation of caveolar endocytic vesicles. GTPase dynamin II drives the fission of these vesicles from the plasma membrane which later fuses with early endosomes or caveosome (Nabi and Le 2003; Parton and Simons 2007; Lajoie and Nabi 2010). A GPCR upon activation can internalize by either of these mechanisms depending on its phosphorylated state and cell type. For example, GRK-mediated phosphorylation of the β_1-adrenergic receptor leads to the clathrin-mediated endocytosis of the receptor, whereas PKA-dependent phosphorylation of the same receptor leads to the caveolin-mediated endocytosis (Rapacciuolo, Suvarna et al. 2003). In the same way, mechanisms of endocytosis of β_1-adrenergic receptor vary in different cell types. In A431 cells, they internalize in a caveolin-dependent manner, while in other cell types they undergo clathrin-mediated endocytosis (Raposo, Dunia et al. 1989; Couet, Li et al. 1997; Prasad, Laporte et al. 2002).

Subsequent to the internalization, receptors can either be degraded following the lysosomal degradation pathway or they can recycle back to the cell surface (Alberts, Johnson et al. 2002; Bareford and Swaan 2007; Gindhart and Weber 2009; Chi, Cao et al. 2011). Apart from this mechanism, some GPCRs like $5HT_{2A}$ receptor (serotonin 2A receptor) are reported to internalize in an arrestin-independent and dynamin-dependent manner (Bhatnagar, Willins et al. 2001). Whereas, the internalization of angiotensin II AT1A receptor is independent of both arrestin and dynamin proteins (Zhang, Ferguson et al. 1996). Moreover, ubiquitination of some GPCRs seems to be critical for their internalization (Hislop and von Zastrow 2011; Piper, Dikic et al. 2014). These reports conclude the existence of several different mechanisms for GPCR internalization depending upon the type of the GPCR, nature of the ligand and its cellular background **(Figure 1.3)**. Thus works done in the last few years suggests that each GPCR is unique and studies from one GPCR cannot be extrapolated to others.

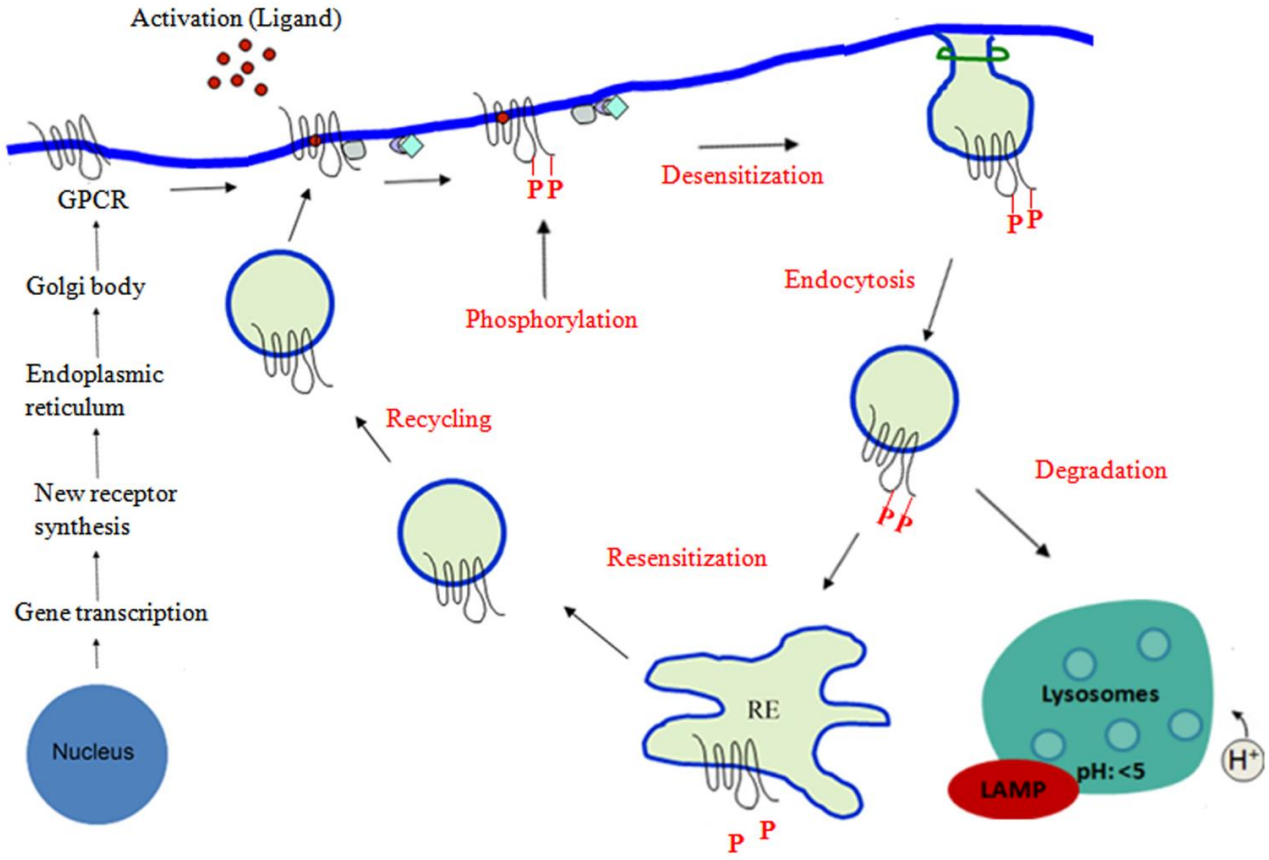


Figure 1.3. Mechanisms involved in the GPCR trafficking. Ligand-induced activation of the GPCR leads to the activation of kinases, which phosphorylate the receptor. The phosphorylation of the receptor facilitates the binding of β-arrestin which results in the desensitization of the receptor. Subsequently, the desensitized receptor internalizes *via* dynamin and clathrin-dependent pathway. After that, the internalized receptor undergoes dephosphorylation and the receptor gets resensitized. The resensitized receptor recycles back to the cell surface. Alternatively, GPCRs that internalize are targeted for degradation in lysosomes.

1.4. Importance of trafficking

Recent advances in the studies of GPCR trafficking have expanded our understanding of the biological significance of GPCR endocytosis. Initially, the trafficking of GPCRs was believed to be a principal mechanism for desensitization of receptors by physically removing them from G-proteins. But following findings challenged this hypothesis: i) most of the receptors get desensitized more rapidly than their endocytosis, ii) despite blocking the endocytosis of β_2-adrenergic receptor by using pharmacological and chemical inhibitors, the desensitization profile of the receptor remains unaltered (Yu, Lefkowitz et al. 1993; Pippig, Andexinger et al. 1995; Ferguson 2001). Hence, for many receptors, endocytosis does not serve as a mechanism for desensitization. While the prime role of trafficking is not merely a mechanism of desensitization, trafficking is also required for resensitization and downregulation of receptors (Camiña, Carreira et al. 2004; Shankaran, Wiley et al. 2007; L Mohan, T Vasudevan et al. 2012; Esseltine and Ferguson 2013; Nooh, Naren et al. 2013).

The essential role of internalization in the resensitization of receptors was evident from the following observations: i) endosomal fractions contain less phosphorylated form of β_2-adrenergic receptors with respect to those present in the plasma membrane fractions (Sibley and Lefkowitz 1985), ii) presence of active GPCR specific phosphatases in endosomal fractions (Sibley and Lefkowitz 1985; Pitcher, Touhara et al. 1995), iii) loss of resensitization potential in internalization defective mutants while their signaling and desensitization properties were intact (Barak, Tiberi et al. 1994), iv) blocking of resensitization of the GPCRs by using pharmacological and chemical inhibitors to inhibit the endocytosis (Pippig, Andexinger et al. 1995; Garland, Grady et al. 1996; Hasbi, Allouche et al. 2000). Studies in many other GPCRs like m3AChR, delta opioid receptor, β_2-adrenergic receptors, mu opioid receptor, endothelin A receptor, cholecystokinin receptor has shown that endocytosis is necessary for their resensitization process (Lutz, Pinon et al. 1993; Giannini and Boulay 1995; Pippig, Andexinger et al. 1995; Garland, Grady et al. 1996; Edwardson and Szekeres 1999; Wolf, Koch et al. 1999; Bremnes, Paasche et al. 2000; Hasbi, Allouche et al. 2000). Apart from its role in resensitization, internalization of receptors is also crucial for downregulation of some GPCRs. Like, in the case of protease-activated receptors and endothelin B receptors, which upon internalization follows the lysosomal degradation pathway (Trejo and Coughlin 1999; Bremnes, Paasche et al. 2000).

Sorting of GPCRs to either recycling endosome or lysosomal compartment is determined by the presence of specific signature sequences in their cytoplasmic tail (Oakley, Laporte et al. 1999; Trejo and Coughlin 1999; Anborgh, Seachrist et al. 2000). Moreover, several GPCRs upon internalization reportedly trigger alternative signaling pathways like the MAP-kinase signaling pathway (Daaka, Luttrell et al. 1998). Thus, GPCR endocytosis is an essential process for regulating GPCR turn over and also maintenance of signaling and homeostasis in the cell.

1.5. Glutamate and its receptors

Glutamate is the anionic form of glutamic acid, which is a nonessential amino acid, abundantly found in the human body (Misner 2010). It plays a key role in cellular metabolism and serves as an excitatory neurotransmitter in the central nervous system (CNS). Most of the excitatory neurons in CNS are glutamatergic and release glutamate from their synapses. Thus, glutamate is required for normal brain functions. The biosynthesis of glutamate in the CNS takes place in two ways: i) *de novo* synthesis of glutamate occurs in astrocytes from the derivatives of glucose and amino acids *via* energy metabolism and ii) phosphate-activated glutaminase (PAG)-mediated hydrolytic deamination of glutamine to form glutamate and ammonia (Erecińska and Silver 1990; Schousboe, Scafidi et al. 2014). Detailed studies of glutamate and glutamine biosynthesis using radio labeled precursors revealed that glutamate exists in separate metabolic pools in the brain referred to as metabolic compartments, viz., neurons and astrocytes (Schousboe, Scafidi et al. 2014). Apart from this compartmentalization, glutamate also exists in extracellular pools where its level is tightly regulated. A lower level of extracellular glutamate is essential for normal brain function. The coordinated activity of glutamate transporters maintains the concentration of glutamate. Vesicular glutamate transporters (VGLUTs) and excitatory amino acid transporters (EAATs), present in neurons allow the movement of glutamate across the membrane (Danbolt 2001; Shigeri, Seal et al. 2004; Hawkins 2009). VGLUTs are multimeric proton/glutamate antiporters which transport glutamate from the cell cytoplasm into synaptic vesicles (Fremeau Jr, Voglmaier et al. 2004; Takamori 2006; Wallén-Mackenzie, Wootz et al. 2010). Whereas, excitatory amino acid transporters (EAATs) are the sodium-dependent glutamate antiporters, known to remove glutamate from the synaptic cleft and extrasynaptic sites by active reuptake of glutamate into neurons and glial cells. Thus, active removal of glutamate

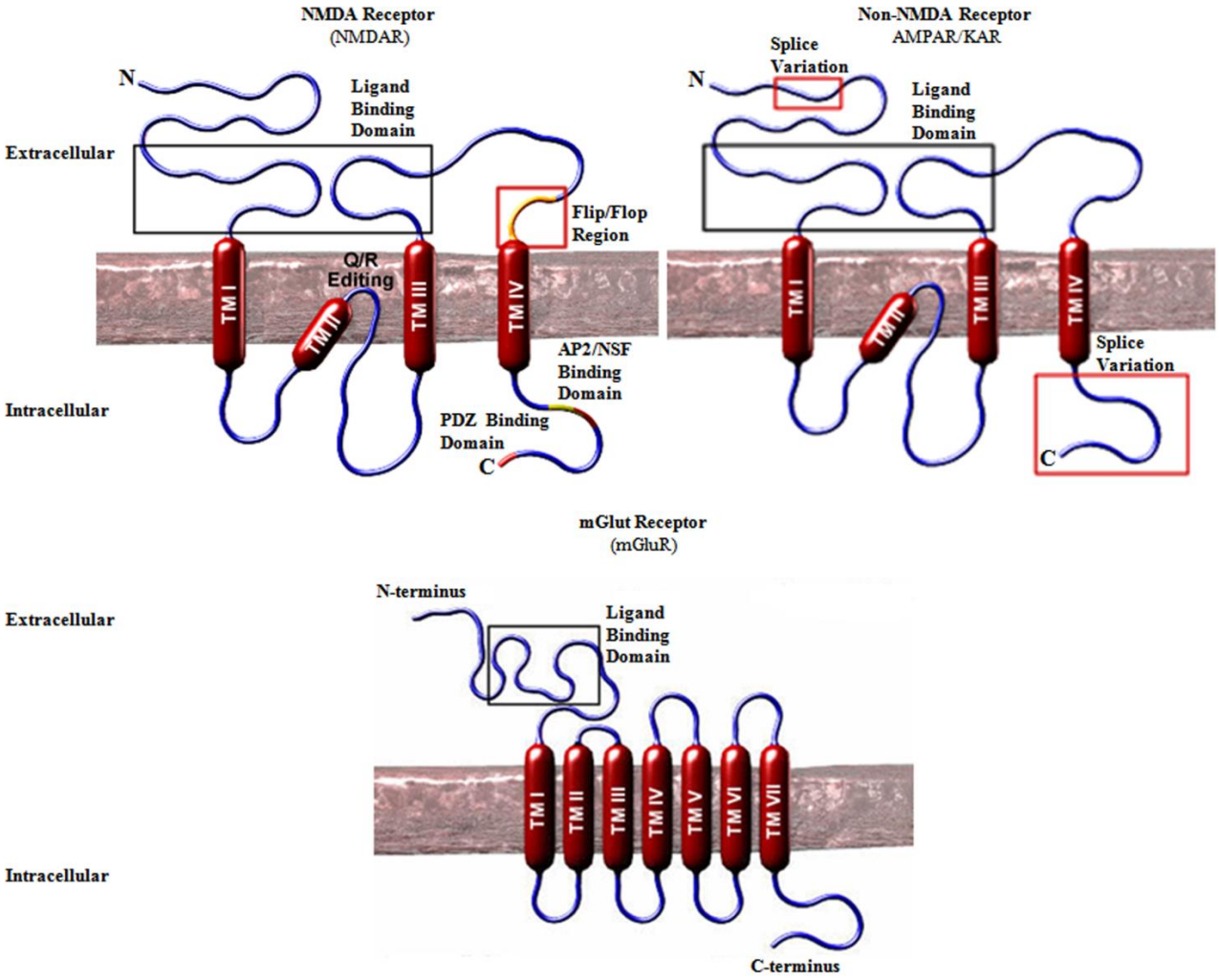
from the extracellular space terminates synaptic transmission, thereby protecting the brain from glutamate excitotoxicity (Shigeri, Seal et al. 2004; Holmseth, Dehnes et al. 2012; Underhill, Wheeler et al. 2014). Glutamate signals by activating receptors localized in the synaptic and extrasynaptic region on the membranes of neuronal cells. It functions in CNS *via* two types of receptors viz., ionotropic glutamate receptors and metabotropic glutamate receptors (Fonnum 1984; Niciu, Kelmendi et al. 2012; Bhattacharyya 2016). The different types of glutamate receptors have been depicted in **(Figure 1.4).**

1.5.1. Ionotropic Glutamate receptors (iGluRs)

These are ion channels which upon ligand stimulation allow the passage of various ions resulting in membrane depolarization and generation of an electric impulse. These receptors are responsible for fast excitatory neurotransmission in the CNS. In general, these receptors exist in multimeric assemblies of four subunits which share a common basic structure. Each of these subunits comprises four domains including; the extracellular N-terminal domain, ligand binding domain, a transmembrane domain spanning the cell membrane through four hydrophobic regions (TMI-TMIV) and an intracellular C-terminal domain (Dingledine, Borges et al. 1999; Mayer 2005; Qiu, Hua et al. 2005; Meyer, Fuchs et al. 2008; Nakagawa 2010; Niciu, Kelmendi et al. 2012). One of the fascinating characteristics of the iGluRs is their distinct channel properties which are generated both before and after gene transcription. These receptors are comprised of subunits transcribed from separate genes and further splice variations in their mRNA can give rise to several permutation combinations of the subunits that make the ion channels. On the basis of pharmacology and sequence similarity, they are subdivided into three groups, viz., α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors and Kainate receptors.

1.5.1.1. AMPA receptors (AMPARs)

AMPA receptors are distributed throughout the CNS where they mediate fast excitatory synaptic transmission and their modulation accounts for the majority of synaptic plasticity resulting from the excitatory transmission in the brain (Dai, Egebjerg et al. 2001). They are composed of four



modified from http://www.bristol.ac.uk/synaptic/receptors/mglur/

Figure 1.4. Glutamate receptors. Above picture shows a schematic representation of ionotropic glutamate receptors (NMDA, AMPA and Kainate receptor) and metabotropic glutamate receptors (mGluRs).

GluA subunits transcribed from separate genes and each of them expressed in two splice variant forms; flip and flop. GluA1-4 subunits form tetramer complexes of AMPARs in homologous or heterologous combinations (Wenthold, Petralia et al. 1996; Mayer 2005; Greger, Ziff et al. 2007; Lu, Shi et al. 2009; Huganir and Nicoll 2013; Sumioka 2013; Hussain, Thomas et al. 2015). The compositions of these subunits determine the electrophysiological properties of the AMPAR channel. For example, the presence of GluA2 subunits in AMPAR channel hampers the calcium entry whereas the absence of GluA2 increases the permeability for calcium ions and gating kinetics to the order of sub-milliseconds (Geiger, Melcher et al. 1995; Derkach, Barria et al. 1999; Banke, Bowie et al. 2000; Mayer 2005; Greger, Ziff et al. 2007; Hideyama and Kwak 2011; Gruszczynska-Biegala, Sladowska et al. 2016). Such GluA2-lacking AMPARs are majorly expressed in neurons which fire high-frequency action potentials. On the other hand, AMPARs dominated by GluA1 subunits are characterized by slow gating kinetics (Rubio, Matsui et al. 2017). Moreover, each of these subunits has differential binding properties to intracellular proteins.

Variations in the carboxy-terminal domains of these subunits confer them differential binding properties to intracellular proteins. For example, GluA2 subunits have the binding sites for proteins like NSF, AP2 and PDZ domain-containing scaffolding proteins GRIP and PICK1, whereas GluA1 subunit binds preferentially with SAP91, another scaffolding protein (Leonard, Davare et al. 1998; Greger, Khatri et al. 2002). These protein-protein interactions regulate the localization of the receptor and drive their intracellular trafficking. Thus, they play crucial roles in AMPAR-mediated synaptic plasticity. Synaptic efficacy depends on the availability of AMPARs in a specific region of the neuron.

AMPARs are critically involved in regulating the synaptic neurotransmission. They modulate the synaptic efficacy by either increasing the synaptic strength known as long term potentiation (LTP) or decreasing the synaptic strength called long term depression (LTD). These processes together form the basis of synaptic plasticity, which is believed as a cellular correlates for learning and memory formation (Whitlock, Heynen et al. 2006; Wang 2008).

The tetramer of GluA2 AMPA receptor was the first iGluR to be crystalized (Armstrong, Sun et al. 1998; Sobolevsky, Rosconi et al. 2009; Yao, Zong et al. 2011). Initially, it was named as "quisqualate receptor" after a naturally occurring agonist quisqualate. Later on, it was renamed as

"AMPA receptor" after the development of selective agonist, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (Honoré, Lauridsen et al. 1982; Purves, Sadava et al. 2001; Watkins and Jane 2006).

Each subunit of these receptors contains agonist binding site and binding of the agonist to two such sites on AMPAR can induce the channel opening. The binding of agonists leading to the occupancy of all these sites results in a drastic increase in ion conductance through these channels (Rosenmund, Stern-Bach et al. 1998; Platt 2007). The AMPARs closes quickly after opening due to changes in conformation which are governed by various factors including their phosphorylation state, protein interactions, etc. (Platt 2007).

1.5.1.2. NMDA receptor

The NMDA receptors are another member of the ionotropic glutamate receptor family which share similar subunit structure as other members. There are three types of subunits viz. NR1, NR2A-D and NR3A-B which assemble to form NMDA receptors. NR1 subunit expresses extensively throughout the brain, whereas NR2 subunits are reported to express differentially in the CNS (Nakanishi 1992; Goebel and Poosch 1999). The NR2A subunits were found to be expressed predominantly in the hippocampus and the neocortex whereas the expression of NR2B subunits was reported primarily in the forebrain. NR2C and NR2D were found to be expressed prominently in the cerebellum and lower brain stem whereas a differential expression pattern was observed for NR3 subunits in the CNS (Goebel and Poosch 1999). The expression of NR3A was found to be predominant in the neocortex region, whereas mRNA expression of NR3B was observed in the spinal neurons and neurons of the brainstem (Nakanishi 1992). These subunits form heterotetrameric cation channels by assembling in different combinations to generate different types of NMDA receptors. The subunit composition varies in different NMDA receptor types which confers diverse pharmacological and biological properties to them. Hence, NMDA receptors expressing in different regions of the brain, or at the different developmental stage, may not function similarly (Newcomer, Farber et al. 2000; Nagy, Kolok et al. 2005).

Glutamate alone is not sufficient to activate NMDARs and require another amino acid, glycine as a co-agonist. Although the binding sites of glutamate and glycine are present on different

subunits, binding of both these neurotransmitters is essential for receptor function (Blanke and Van Dongen 2009; Cummings and Popescu 2015). NMDARs are inactive during resting potential due to the voltage-dependent block by magnesium ion at channel pore which inhibits the flow of ions through it. Only the strong depolarization of the membrane can release the channel inhibition leading to the receptor activation. Thus, NMDA receptor functions as a coincidence detector which can only be activated by simultaneous binding of glutamate to the receptor along with depolarization of the membrane (Kleckner and Dingledine 1988; Dingledine, Borges et al. 1999; Liu and Zhang 2000; Zito, Scheuss et al. 2009; Vyklicky, Korinek et al. 2014). Activation of NMDARs results in a flow of calcium ions into the post-synaptic cells leading to activation of a number of signaling cascades. These NMDAR activation mediated signal transduction pathways are responsible for a wide range of post-synaptic functions involved in various physiological processes like neurogenesis, synaptic plasticity and generation of rhythms involve in breathing and locomotion (Collingridge 1987; Greer, Smith et al. 1991; Scheetz and Constantine-Paton 1994).

1.5.1.3. Kainate receptors

Initially, these receptors were identified as a separate group from the NMDA and AMPA receptors mainly because of its activation by the agonist kainate, a drug obtained from red algae, *Digenea simplex* (Dingledine, Borges et al. 1999; Collingridge, Olsen et al. 2009; Swanson and Sakai 2009; Rogawski 2011). These receptors are distributed in a slightly more limited manner in the brain compared to AMPARs and NMDARs. Kainate receptors, depending on their location can induce either excitatory or inhibitory impulses. For example, presynaptically localized kainate receptors are involved in inhibitory neurotransmission by altering gamma-aminobutyric acid (GABA) release, whereas, postsynaptic kainate receptors are implicated in excitatory neurotransmission (Huettner 2003). Thus, kainate iGluRs play a crucial role in both pre and post-synaptic neurons. These are tetrameric cation channels comprised of five possible subunits viz., KA1, KA2 and GluA5/6/7 (Dingledine, Borges et al. 1999). The composition of these subunits and their own post-translational modifications determine the affinity of these channels for glutamate and are also responsible for the electrophysiological properties like current characteristics and gating properties. Although, the rise and decay time of the potential

generated by these channels is slower than that of AMPA receptors, but their channel conductance is similar to AMPA iGluRs (Huettner 2003). Some reports have suggested the role of kainate iGluRs in seizure induction and epileptic discharges, although very little is known about their role in CNS and needs to be explored (Fritsch, Reis et al. 2014).

1.5.2. Metabotropic glutamate receptors (mGluRs)

Metabotropic glutamate receptors are G-protein coupled receptors (GPCRs) that belong to the class C family of GPCRs (Pin and Duvoisin 1995; Pin, Galvez et al. 2003; Brauner-Osborne, Wellendorph et al. 2007). Structurally, these receptors possess a large extracellular N-terminal domain, a cysteine-rich domain, a 7-transmembrane domain motif (TMD) and an intracellular C-terminal tail. The N-terminal domain contains two hinged globular domains known as the Venus Flytrap Domain (VFD) which forms the ligand binding site. These two domains get closed upon glutamate binding which causes structural changes in the TMD leading to the G-protein activation (Tsuchiya, Kunishima et al. 2002; Jingami, Nakanishi et al. 2003; Mun, Franks et al. 2004; Muto, Tsuchiya et al. 2007; Dobrovetsky, Khutoreskaya et al. 2010). The mGluR family contains eight members, viz., mGluR1 - mGluR8 which have been divided into three subgroups: group I, group II and group III on the basis of sequence similarity, intracellular signaling and pharmacology (Nakanishi 1992; Pin and Duvoisin 1995; Brock, Oueslati et al. 2007).

Group I mGluRs comprises of mGluR1 and mGluR5, whereas mGluR2 and mGluR3 constitute the group II mGluR family and group III mGluR contains mGluR4, mGluR6, mGluR7 and mGluR8 as the family members (Nakanishi 1992; Pin and Duvoisin 1995). Group I mGluRs are found predominantly in the post-synaptic neurons while group II mGluRs are present in both pre-synaptic and post-synaptic neurons. On the other hand, group III mGluRs are primarily localized in the pre-synaptic neurons (Benarroch 2008; Bhattacharyya 2016). Moreover, these receptors are also expressed in glial cells where they are reported to be involved in the process of glutamate uptake and release from astrocytes. Thus, they play crucial roles in neuroprotection and communication between neurons and glia (Benarroch, 2008). mGluRs, upon ligand-mediated stimulation induce G-protein coupled signal transduction pathway. Group I mGluRs are positively coupled with phospholipase C pathway, while group II and group III negatively

induce the adenylate cyclase pathway (Abe, Sugihara et al. 1992; Sharon, Vorobiov et al. 1997; Otani, Auclair et al. 1999; Niswender and Conn 2010). These receptors also modulate the activity of iGluR's. The mGluRs execute a wide range of physiological functions in the brain, including modulation of different ion channels' activity, pain, anxiety, learning and memory formation, etc. (Brauner-Osborne, Wellendorph et al. 2007; Benarroch 2008). Since the objective of this study is to understand the molecular mechanisms of group I mGluR trafficking, in the subsequent section we have concentrated on the group I mGluRs.

1.5.2.1. Group I mGluRs

The two members of the group I mGluR family, mGluR1 and mGluR5 have a widespread but differential expression pattern in the CNS. The mGluR1 expresses extensively in the olfactory bulb, cerebellar Purkinje cells and even more elevated expression levels have been observed in the hippocampus, thalamus, lateral septum, substantia nigra and globus pallidus (Shigemoto, Nakanishi et al. 1992; Bordi and Ugolini 1999). Whereas, mGluR5 are found to express in the hippocampus, cerebral cortex, striatum, nucleus accumbens, superficial cortical layers, granule cells of the olfactory bulb and lateral septal nucleus (Bordi and Ugolini 1999; Bhattacharyya 2016). Observations from some studies have suggested that expression of group I mGluRs vary dynamically during brain development. For example, a steady increase in the mGluR1 expression was observed in the hippocampus and neocortex during development (Catania, Landwehrmeyer et al. 1994). mGluR5a expression, on the other hand, has shown a gradual increase in the developing cortex which reaches a maximum during the second postnatal week in rodents followed by a gradual decrease. While the most prominent form of mGluR5 in adults is mGluR5b, its mRNA expression levels observed to increase postnatally (Catania, Landwehrmeyer et al. 1994; Minakami, Iida et al. 1995; Romano, Van den Pol et al. 1996; Bhattacharyya 2016). These receptors are localized primarily at the perisynaptic region of the post-synaptic neurons (Lüscher and Huber 2010). Apart from the CNS, group I mGluRs are also expressed in non-neuronal cells outside the brain where they play essential roles in various processes. For example, mGluRs present in skin cells play essential roles in pain sensation (Latremoliere and Woolf 2009; Pereira and Goudet 2018). Their expression is also observed in

melanocytes, hepatocytes, osteoblast and heart cells (Shin, Martino et al. 2008; Durand, Carniglia et al. 2011; Teh and Chen 2012; Haas, Pfragner et al. 2013).

After ligand binding, group I mGluRs transduce their signaling *via* $G_{q/11}$, which activates the phospholipase C/inositol 1,4,5-triphosphate (IP_3) pathway, generating Diacylglycerol (DAG) and IP_3 as secondary messengers. IP_3 is soluble and diffuses through the cytoplasm to activate the ligand-gated Ca^{2+} channels, present on the endoplasmic reticulum (ER) membrane, to release Ca^{2+} into the cytoplasm. DAG stays on the membrane and activates PKC in the presence of Ca^{2+} ions. Thus activation of PKC requires both IP_3 and DAG (Gereau IV and Heinemann 1998; Bhattacharyya 2016). PKC then phosphorylates its target substrates and regulates various physiological processes.

1.5.2.1.1. Role of group I mGluRs in synaptic plasticity and neuropsychiatric disorders

Adult brain has an astonishing property to endure activity-dependent plasticity of the pre-existing synaptic connections, which in turn modulate the properties of the neuronal circuits and behavior. This activity-dependent synaptic plasticity is considered to be the cellular correlate of learning and memory formation. Several reports from the past few decades have suggested that synaptic plasticity is essentially involved in the formation of neuronal circuits during the process of brain development (Nakayama, Kiyosue et al. 2005; Colicos and Syed 2006; Tahayori and Koceja 2012; Takeuchi, Duszkiewicz et al. 2014; Chaudhury, Sharma et al. 2016). Reports have shown the role of group I mGluRs in modulating synaptic efficacy by regulating trafficking of ion channels and inducing the transcription and translation of genes essential for the maintenance of synaptic plasticity (Wang and Zhuo 2012). Long term increase in synaptic strength leads to the enhancement of synaptic efficacy and the phenomenon is known as long term potentiation (LTP) (Malenka 1994). In contrast, a decrease in synaptic strength for a long period is known as long term depression (LTD) (Malenka 1994). Thus, the phenomena of LTP and LTD can modulate the synaptic strength of excitatory synapse bi-directionally. Studies have revealed two ways of triggering LTD in the CNS, i.e. activation of either NMDA receptors or mGluRs (Citri and Malenka 2008). Several studies have been conducted to explore the mechanisms of

NMDAR-dependent LTD, but mechanistic insights of mGluR-LTD have not yet been investigated in detail (Bear and Malenka 1994; Thiels, Xie et al. 1996; Nicholls, Alarcon et al. 2008; Lüscher and Malenka 2012; Liu, Gu et al. 2014). Although, NMDAR-dependent LTD and mGluR-dependent LTD are mechanistically different, they both involve AMPARs endocytosis (Bellone, Mameli et al. 2011; Clem and Huganir 2013; Chater and Goda 2014; Loweth, Scheyer et al. 2014; Sanderson, Gorski et al. 2016; Hanley 2018). Due to the reports of altered mGluR-LTD in the mouse model of mental retardation, autism and Fragile X syndrome, the understanding of mechanisms and functions of mGluR-LTD has become a major attractive area of study (Bear, Huber et al. 2004; Ronesi and Huber 2008; Dölen and Bear 2009; Lüscher and Huber 2010; Ronesi, Collins et al. 2012; Aguilar-Valles, Matta-Camacho et al. 2015; Sanderson, Hogg et al. 2016). Fragile X syndrome is an inherited mental disorder which is believed to be the major cause of autism. Mouse model of Fragile X syndrome containing deletion mutation of the gene encoding fragile X mental retardation protein (FMRP) showed enhanced mGluR-LTD in the hippocampus as well as in the cerebellum (Huber, Gallagher et al. 2002; Koekkoek, Yamaguchi et al. 2005). Surprisingly, the administration of mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) rescued the cognitive and intellectual disability deficits in mouse, zebrafish and fruit fly models of Fragile X syndrome (McBride, Choi et al. 2005; Yan, Rammal et al. 2005; Santoro, Bray et al. 2012). Moreover, the disease phenotype of Fragile X syndrome mice was rescued after deleting the mGluR5 gene from the diseased mice (Dölen, Osterweil et al. 2007; Bassell and Gross 2008).

1.5.2.1.2. Desensitization of group I mGluRs

The signaling of group I mGluRs is highly regulated and any overstimulation of the receptor can induce excitotoxicity leading to neuronal cell death, as well as destabilization of the network activity. In order to prevent such condition, the receptor undergoes desensitization, a feedback mechanism characterized by the decrease in agonist-dependent receptor-mediated phosphoinositide hydrolysis (Winder and Conn 1995; Gereau IV and Heinemann 1998; Dale, Bhattacharya et al. 2000; Dhami and Ferguson 2006; Mao, Liu et al. 2008; Jin, Guo et al. 2013). The sequence of events which constitute GPCR desensitization after agonist exposure includes

receptor phosphorylation, arrestin binding, and internalization (Dale, Babwah et al. 2002). But the mechanism of desensitization varies from receptor to receptor and it depends on the type of the receptor, the type of ligand and the type of the system (Bhattacharyya 2016).

The desensitization of group I mGluRs is regulated at different levels, from receptor modification to attenuation of signaling *via* uncoupling of G-proteins. The receptor modification involving phosphorylation is attributed to secondary messenger-dependent protein kinases and GPCR kinases (GRKs) (Dale, Babwah et al. 2002). Among them, PKC-mediated phosphorylation of group I mGluRs results in receptor desensitization (Gereau IV and Heinemann 1998; Peavy, Sorensen et al. 2002; Mao, Liu et al. 2008; Jin, Guo et al. 2013). There are PKC specific phosphorylation sites present in different regions of the intracellular loops and carboxyl-terminal tail of mGluR1 and mGluR5. In mGluR5, these sites include serine residue 613, threonine residue 606 and 665 in the intracellular loops whereas serine residues 881 and 890 in the carboxyl-terminal tail of the receptor (Gereau IV and Heinemann 1998; Jensen 2004; Mao, Liu et al. 2008; Molnár 2008; Mao, Guo et al. 2011). Likewise, threonine residue 695 contributes to PKC-dependent mGluR1a phosphorylation and subsequent desensitization (Gereau IV and Heinemann 1998; Dale, Babwah et al. 2002).

Not every kind of phosphorylation is critical for desensitization of the receptor which is well elucidated by calmodulin (CaM) regulated mGluR5 phosphorylation. CaM binds to mGluR5 and inhibits some phosphorylation of the receptor which is not crucial for receptor desensitization (Kammermeier and Ikeda 2002). Though, CaM doesn't bind to mGluR1 but it is involved in Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα)-mediated agonist-induced desensitization of mGluR1a. CaMKIIα binds at the intracellular C terminus of mGluR1a in a Ca^{2+}-dependent manner and phosphorylates a specific threonine residue (T871) of mGluR1a (Minakami, Jinnai et al. 1997; Jin, Guo et al. 2013; Li-Min, Dao-Zhong et al. 2014). Upon activation, CaMKIIα autophosphorylates the threonine residue (T286) which enables it to function in a Ca^{2+}-independent manner, that is essential for maintaining receptor and kinase interaction (Jin, Guo et al. 2013).

The desensitization of the receptor could also be mediated through phosphorylation-independent manner. The receptor desensitization mediated by GRK2 appears to be phosphorylation-

independent which involves the interaction of GRK2 with receptor by its RGS homology (RH) domain (Dhami, Anborgh et al. 2002; Sterne-Marr, Dhami et al. 2004; Ferguson 2007; Ribas, Penela et al. 2007; Sterne-Marr, Leahey et al. 2009). Although GRK2 mediates the phosphorylation of receptor by its kinase activity, it doesn't seem to affect the signaling of the receptor (Dhami and Ferguson 2006; Bhattacharyya 2016). In addition to interaction with the receptor, GRK2 simultaneously interacts with $G_{\alpha q/11}$, and $G_{\beta\gamma}$ to uncouple them from the receptor, leading to the desensitization of mGluR1 (Dhami and Ferguson 2006). Similarly, the interaction of group I mGluRs with Huntington binding protein optineurin, restrain their coupling with G-proteins, resulting in the phosphorylation-independent desensitization of group I mGluRs (Dhami and Ferguson 2006). Furthermore, the mGluR activity can also be attenuated at the level of G-proteins by the regulators of G-protein signaling (RGS) (Schwendt and McGinty 2007; Ribeiro, Ferreira et al. 2009; Gerber, Squires et al. 2016; Qian and Tang 2016). RGS can cease G-protein signaling following agonist stimulation by acting as GTPase activating proteins (GAPs) and thereby catalyzing the hydrolysis of GTP bound to the G_{α} subunit of heterotrimeric G-proteins, leading to their inactivation (Mukhopadhyay and Ross 1999; Bos, Rehmann et al. 2007; Chuang and Chuang 2012; Sprang 2016). Among them RGS2 and RGS4 associates with $G_{\alpha q/11}$ proteins to attenuate group I mGluR-mediated phospholipase C/inositol 1,4,5-triphosphate (IP_3) signaling pathway (Dhami and Ferguson 2006).

1.5.2.1.3. Group I mGluR trafficking

As stated before, proper signaling by the receptor requires correct localization of the receptor at specific region of the cell, to allow normal ligand / receptor interaction. Receptor trafficking regulates the spatio-temporal localization of the receptors on the cell surface and plays an important role in modulating their activity. Improper positioning of these receptors in the specific region of a cell can affect receptor signaling and any such alteration in receptor signaling could lead to severe pathological consequences. Similar to many other GPCRs, group I mGluRs are also reported to get internalized subsequent to desensitization (Mundell, Matharu et al. 2001; Mundell, Pula et al. 2004; Choi, Chung et al. 2011). These receptors internalize in arrestin and

dynamin-dependent manner (Mundell, Matharu et al. 2001; Mundell, Matharu et al. 2002; Mundell, Pula et al. 2004; Jong, Sergin et al. 2014; Eng, Kelver et al. 2016; Mahato, Ramsakha et al. 2018). Group I mGluR endocytosis reportedly involves phosphorylation by GRKs and other second messenger-dependent kinases. Studies in HEK293 cells have shown GRK4-induced internalization of mGluR1 while in cerebellar purkinje cells, knock down of GRK4 inhibited ligand-mediated internalization of mGluR1 (Sallese, Salvatore et al. 2000; Iacovelli, Salvatore et al. 2003; Perroy, Adam et al. 2003). On the other hand, other studies have reported GRK2-dependent but phosphorylation-independent internalization of mGluR1 (Iacovelli, Salvatore et al. 2003). While group I mGluRs also internalize in a PKC-dependent manner, but a separate region of the receptor is involved in this process as compared to the region that is involved in the GRK-dependent internalization of the receptor (Mundell, Pula et al. 2003). Group I mGluRs also internalize in an agonist-independent (constitutive) manner (Trivedi and Bhattacharyya 2012). Reports from some studies have suggested that β-arrestin and dynamin are not involved in the constitutive endocytosis of mGluR1a, whereas other reports have contradicted and showed that the constitutive endocytosis of mGluR1a is both β-arrestin and clathrin-dependent (Pula, Mundell et al. 2004; Francesconi, Kumari et al. 2009; Mahato, Ramsakha et al. 2018). As already discussed, GPCRs upon internalization can have various subcellular fates. Some GPCRs after internalization follow the recycling route and return back to the cell surface after resensitization, to continue for the next round of signaling. While other receptors, following internalization get sorted to the lysosomal degradation pathway resulting in the downregulation of the receptor. The route taken by a particular GPCR depends on various factors viz., type of the receptor, type of the ligand and the cellular background. In case of group I mGluRs, subsequent to the internalization, both mGluR1 and mGluR5 recycles back to the cell surface. Recent studies have suggested that the recycling of mGluR1 is protein phosphatase 2A (PP2A)-dependent whereas recycling of mGluR5 depends on the activity of both PP2A and PP2B (protein phosphatase 2B) (Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015).

As explained before, trafficking plays a crucial role in controlling proper localization of GPCRs which is required for normal interaction of ligand to the receptor and appropriate signaling through the receptor. Similar to many other GPCRs, trafficking of group I mGluRs also regulates

the signaling through these receptors. Activation of group I mGluRs induce second messenger-dependent protein kinases (Iacovelli, Salvatore et al. 2003; Mundell, Pula et al. 2003; Choi, Chung et al. 2011). Subsequently, second messenger dependent kinase PKC and GRK have been reported to regulate the trafficking of group I mGluRs. It has been observed that in case of mGluR1, the region of the receptor reported to be involved in PKC-dependent internalization is different from the region that is involved in the GRK-mediated internalization of the receptor (Mundell, Pula et al. 2003). Another member of group I mGluR, mGluR5 found to be associated with calmodulin at the cell surface, but ligand stimulation triggers the increase in the intracellular Ca^{2+} and activation of PKC which phosphorylates serine 901 at the C-terminus of the receptor leading to the disruption of the binding of calmodulin with the receptor and subsequent internalization of the receptor (Lee, Lee et al. 2008). Moreover, PKA is another second messenger-dependent kinase that may also regulate mGluR trafficking by controlling the GRK activity (Mundell, Pula et al. 2004).

1.6. Sorting Nexin 1 (SNX1)

Sorting Nexin 1 (SNX1) is the founding member of the Sorting Nexin family of proteins characterized by the presence of the phox homology (PX) domain (Stockinger, Sailler et al. 2002; Zheng, Tang et al. 2006). PX domain contains a phospholipid-binding motif that binds phosphatidylinositol-3-phosphate (PtdIns(3)P) which facilitates the association of SNX1 to the endosomal membrane (Worby and Dixon 2002; Wassmer, Attar et al. 2007). SNX1 also possess a BAR domain at the C-terminus which contains three coiled-coil regions involved in the dimerization of SNX1. The dimerization of SNX1 potentiates the ability of the BAR domain to sense the membrane curvature (Wang, Zhou et al. 2002; Wassmer, Attar et al. 2007). The N-terminus of SNX1 containing PX-domain gets inserted into the endosomal membrane and the BAR domain induces tubulation in the membrane (Pylypenko, Lundmark et al. 2007; Teasdale and Collins 2012; Van Weering, Sessions et al. 2012) **(Figure 1.5 A)**. By virtue of these domains, SNX1 gets associated with early endosomes or sorting endosomes and plays an important role in sorting of surface receptors to their respective trafficking routes. Earlier studies have reported the role of SNX1 as a component of the retromer complex in regulating the

retrograde transport of a large number of cargos from endosome to trans-Golgi network (TGN) (Bujny, Popoff et al. 2007; Popoff, Mardones et al. 2007; Wassmer, Attar et al. 2009; Seaman 2012). However, various reports have shown the involvement of SNX1 in sorting and trafficking of some receptors through other pathways as well. Like in the case of P2Y1 receptor, SNX1 has been reported to modulate their recycling (Nisar, Kelly et al. 2010). On the other hand, SNX1 has been shown to regulate the sorting of the protease-activated receptor-1 (PAR1) and epidermal growth factor receptor (EGFR) towards the lysosomal degradation pathway (Gullapalli, Garrett et al. 2004; Gullapalli, Wolfe et al. 2006). SNX1 has also been reported to interact with the cytoplasmic tails of mGluR1 and mGluR5 (Heydorn, Sondergaard et al. 2004).

1.7. Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs)

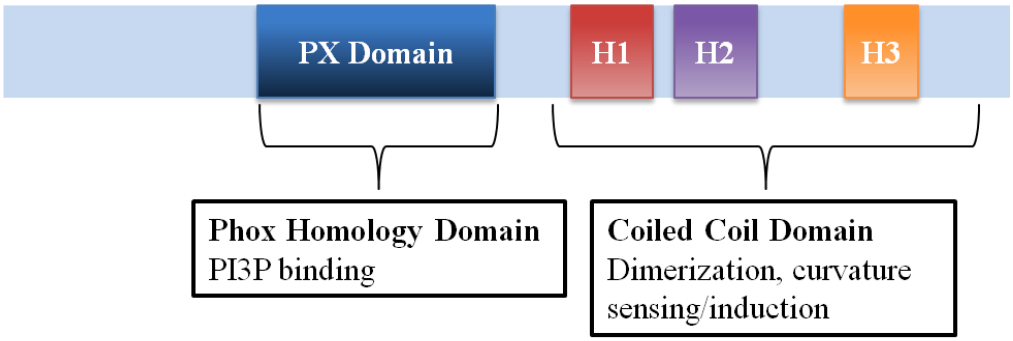
Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) is a 115 kDa protein, primarily localized at the early endosomes and functions as a component of a multiprotein complex that regulates the sorting of ubiquitinated proteins to lysosomes (Chin, Raynor et al. 2001; Hasdemir, Bunnett et al. 2007; Leithe, Sirnes et al. 2012; Mohapatra, Ahmad et al. 2013; MacDonald, Brown et al. 2018). Some reports have also suggested that Hrs plays a crucial role in the trafficking, degradation and recycling of many GPCRs (Hislop, Marley et al. 2004; Hanyaloglu, McCullagh et al. 2005; Hasdemir, Bunnett et al. 2007; Roux, Bauer et al. 2017). The Hrs protein consists of four major domains including an amino-terminal localized VHS domain, a FYVE domain, an Ubiquitin-interacting motif (UIM) and a clathrin binding domain (CBD) at the extreme carboxy-terminus (Chin, Raynor et al. 2001; Hanyaloglu, McCullagh et al. 2005) **(Figure 1.5 B)**. The VHS domain of Hrs is composed of eight α helices forming a right-handed superhelical structure reportedly involved in sequence-directed recycling of. receptors (Misra, Beach et al. 2000; Hanyaloglu, McCullagh et al. 2005). The FYVE domain sits on the top of the base formed by the VHS superhelical structure and plays an important role in endosome association of Hrs (Komada and Soriano 1999; Misra, Beach et al. 2000; Raiborg, Bremnes et al. 2001). Hrs has been reported to interact with SNX1 on the cellular membranes which inhibit the SNX1s association with EGFR. Thereby, Hrs regulates the ligand-induced degradation of EGF receptors (Chin, Raynor et al. 2001). Furthermore, interaction of Hrs with

the signal transducing adaptor molecule (STAM) is believed to play a crucial role in cell growth signaling by cytokines (Asao, Sasaki et al. 1997).

1.8. Prelude to the current study

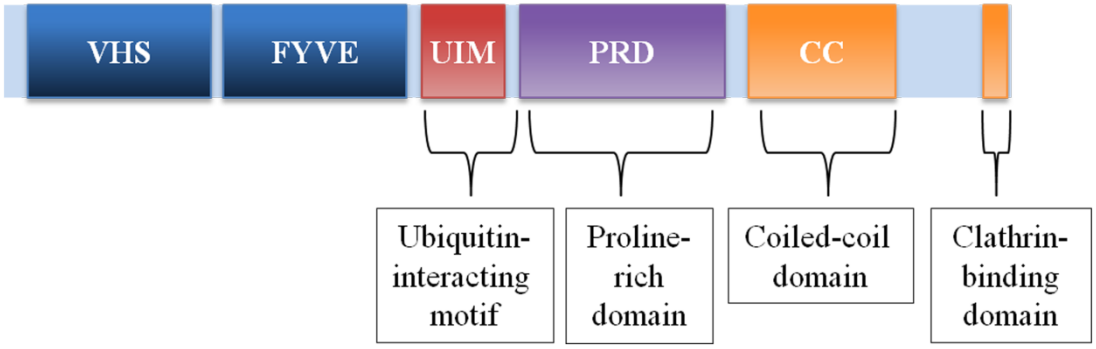
Although lot of work has been done on the mechanism of desensitization of group I mGluRs and some amount of work has also been done on the internalization and post-endocytic fate of these receptors, almost nothing is known about the post-endocytic sorting machinery which decides the fate of these receptors. Since group I mGluRs have been reported to recycle back to the cell surface following ligand-dependent internalization, the recycling is probably critical for the resensitization of these receptors. In the present study, we have investigated the role of a sorting protein called Sorting Nexin 1 (SNX1) in the regulation of group I mGluR trafficking. As stated before, SNX1 co-expresses with group I mGluRs in the hippocampus and has been shown to interact with group I mGluRs. The role of SNX1 in group I mGluR trafficking and mGluR-dependent AMPAR endocytosis has been studied in this work. Apart from SNX1, the role of another sorting protein Hrs which is the interacting partner of SNX1 has been investigated in mGluR1 trafficking.

(A)



Full-length SNX1 (1-521 amino acids)

(B)



Full-length Hrs (1-776 amino acids)

Figure 1.5. (A) Picture of the full-length SNX1 (B) Schematic of the full-length Hrs showing its domain structure.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Cell culture reagents

Dulbecco's Modified Eagle Medium (DMEM), Minimal Essential Medium (MEM), Fetal Bovine Serum (FBS), Neurobasal medium, B27 supplement, antibiotic-antimycotic mix (AB-AM mix), Trypsin-EDTA, Alexa-568 labelled Transferrin, Lipofectamine 2000 and all other cell culture reagents were purchased from Invitrogen (USA). Fluorodeoxyuridine (FUDR), poly-D-lysine, FluoromountTM aqueous mounting medium and Paraformaldehyde (PFA) were purchased from Sigma (USA). HEK293 cells and N2A cells were purchased from NCCS Pune (India).

2.1.2. Molecular biology and biochemistry reagents

A variety of restriction enzymes including EcoRI, BglII, BamHI, XhoI, XbaI, etc. and various other enzymes and reagents like T4 DNA ligase, Q5 DNA polymerase, Phusion DNA polymerase, dNTP mixture, Alkaline phosphatase calf intestinal (CIP) were purchased from New England Biolabs (USA). Red Taq jump start polymerase was bought from Sigma (USA). ECL western blot detection kit was obtained from GE Healthcare (USA). Protein G beads were from GenScript (USA) and anti-FLAG M2 affinity beads were purchased from Sigma (USA).

2.1.3. Plastic wares and chemicals

All plastic wares used in the cell culture studies were purchased from BD Falcon (USA). Plastic wares involved in the molecular biological and bacteriological works 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 (India).

2.1.4. Antibodies

Anti-myc mouse monoclonal and anti-myc rabbit polyclonal antibodies were purchased from Abcam (UK). Anti-FLAG rabbit polyclonal antibody was obtained from Sigma (USA), anti-HA rat monoclonal antibody was from Roche (Switzerland) and anti-GFP rabbit polyclonal antibody was from Life technologies (USA). Anti-Bassoon rabbit polyclonal antibody was purchased from Synaptic System (Germany), anti-Hrs antibody was obtained from Enzo Life Sciences (USA), anti-SNX1 rabbit polyclonal antibody was procured from Proteintech (UK) and anti-β actin antibody was from Santa Cruz (USA). ERK1/2 mouse monoclonal and phospho-ERK rabbit monoclonal antibodies were purchased from Cell Signalling Technologies (USA). Goat anti-mouse HRP, goat anti-rabbit HRP and goat anti-rat HRP were purchased from Sigma (USA). Anti-GluA1 antibody was obtained from Calbiochem (USA). All other secondary antibodies were purchased from Invitrogen (USA).

2.1.5. Drugs

2,4- bis dihydroxy phenyl glycine (R,S-DHPG), D-(-)-2-Amino-5-phosphonopentanoic acid (APV) and 6,7-Dinitroquinoxaline-2,3-dione disodium salt (DNQX) were purchased from Tocris (UK). Tetrodotoxin (TTX) was purchased from Adooq Biosciences (USA). Okadaic acid, FK-506 and Cycloheximide were obtained from Sigma (USA).

2.1.6. Constructs

The myc-mGluR1 and myc-mGluR5 constructs were 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 generously provided by Johanna Montgomery (The University of Auckland, New Zealand). The full-length mouse SNX1 was purchased from Origene (USA) and cloned into pEGFP-C1 vector to obtain GFP-SNX1. Similarly, PCR amplified C-terminal coiled-coil domain of SNX1 (aa 265-521) was also cloned in the pEGFP-C1 vector (SNX1-CC). The FLAG-SNX1 (human) construct was gifted by Johnny K. Ngsee (University of Ottawa, Canada) and it's full-length and the coiled-coil domain were

cloned in pEGP-C1 in a similar way as described above. The full-length Hrs and the deletion mutants of Hrs including ΔVHS and ΔVHS/FYVE were gifted by Mark von Zastrow (University of California, San Francisco, USA). All Hrs constructs were tagged with the myc epitope at the N-terminus of the Hrs protein. ON-TARGET plus SMARTpool siRNA against SNX1 and Hrs were obtained from Thermo Scientific Dharmacon (USA).

2.1.7. Buffers and media

Luria-Bertani (LB) medium: 5 g yeast extract, 10 g tryptone and 10 g NaCl were dissolved in 800 ml of double-distilled water. The pH was adjusted to 7, double-distilled water was added to make the final volume upto 1 litre and autoclaved. Otherwise, 25 g LB powder was dissolved in 1 litre of double-distilled water and autoclaved.

10X phosphate buffered saline (PBS): 2 g KCl, 80 g NaCl, 2.4 g KH_2PO_4 and 14.4 g Na_2HPO_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 - 7.3) at 60°C by adding 1 M NaOH in a dropwise manner till the solution became transparent. Subsequently, the pH of the solution was adjusted to 7.4 and then the final volume was made up to 100 ml. The solution was then aliquoted into 15 ml falcon tubes and stored at -20°C.

Laemmli sample buffer (4X): 40% Glycerol, 4% β-mercaptoethanol, 200 mM Tris-HCl (pH - 6.8), 0.02% Bromophenol blue and 8% SDS were prepared in double-distilled water.

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

SDS running buffer (1X): 14.4 g Glycine, 3 g Tris base and 1 g SDS were dissolved in 1 litre double-distilled autoclaved water (final pH - 8.30).

Transfer buffer for western blot (1X): 14.4 g Glycine, 3 g Tris base, 150 ml Methanol and 850 ml of 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 of double-distilled autoclaved water. Subsequently, 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 medium was filter-sterilized after that under vacuum through a 0.22 µm filter.

10% DMEM: 10% Fetal bovine serum (FBS) and antibiotic-antimycotic (1X) mixture were added to DMEM.

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

2X HEPES buffer saline: 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: Initially, 500 ml of 0.1 M sodium borate solution of pH - 8.4 was prepared by dissolving sodium borate in autoclaved double distilled water. Subsequently, 5 mg poly-D-Lysine was added into the 0.1 M sodium borate solution and filter-sterilized.

Dissection solution: The dissection solution required 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. Subsequently, pH was adjusted to 7.4 and then filter sterilized using a 0.22 µm filter.

Enzyme solution: 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 wax mixed in 10 ml dissection solution.

Serum media: Preparation of the serum media involved the mixing of 25 ml FBS, 7.5 ml Hi-glucose/MEM and 1 ml mito serum extender in 500 ml MEM w/o L-glutamine followed by filter sterilization. The media was aliquoted in 50 ml Falcon tubes.

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

Polyethyleneimine (PEI) solution: Polyethyleneimine (PEI) is a stable cationic polymer having a density close to 1 (Boussif, Lezoualc'h et al. 1995). The characteristically relevant property of PEI is that it condenses DNA into a positively charged particle which enables the entry of the DNA-PEI complex in the cell through endocytosis (Sonawane, Szoka 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. In the end, the solution was filter sterilized.

2.1.8. Instruments

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

Instruments related to the molecular biology experiments: The instruments related to 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 (BioRad 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

Primers for mouse SNX1

Forward (full-length):
5’ ATAAGATCTATGGCGTCAGGAGGTGGTG 3’

Forward (coiled-coil domain):
5’ ATAAGATCTATGGAAAAAGAAGAGCTGCCTCG 3’

Reverse:
5’ ATAGAATTCTCAGGAGATGGCCTTTGC 3’

SNX1 shRNA Oligos:

shSNX1 sense strand:

5’TCGAG**TGAATCATCCCACCATGTT**TTCAAGAGA**AACATGGTGGGATGATTCA**T TTT 3’

shSNX1 antisense strand:

5’CTAGAAAA**TGAATCATCCCACCATGTT**TCTCTTGAA**AACATGGTGGGATGATTC A**C 3’

Forward primer for shRNA sequencing: 5’ CAGTGTCACTATGTTCCCGCC 3’

Reverse primer for shRNA sequencing: 5’ TTAAGTAGCTGAAGCTCCG 3’

Primers used to introduce the silent mutations in the shRNA binding site in wild-type SNX1:

Forward primer:

5’GAAAGGTACCTTCAAAGAATTG**TCAACCACCCAACAATGCTT**CAGGACCCAGATGTCAGG 3’

Reverse primer:

5’CCTGACATCTGGGTCCTG**AAGCATTGTTGGGTGGTTGA**CAATTCTTTGAAGGTACCTTTC 3’

Primers used for cloning of HA-SNX1 in the LRV1 vector to make the shSNX1:HA-SNX1 (full-length SNX1) replacement construct:

Forward primer:

5’ATAAGATCTATGTATCCATATGATGTTCCAGATTATGCTGCGTCAGGAGGTGGTGGCTGTAGCGCT 3’

Reverse primer:
5’ATAGAATTCTCAGGAGATGGCCTTTGC 3’

Primers for the cloning of the C-terminal part of SNX1 (265-521 amino acids) in the LRV1 vector to create shSNX1:HA-SNX1ΔN replacement construct:

Forward primer:

5’ATAAGATCTATGTATCCATATGATGTTCCAGATTATGCTGAAAAAGAAGAGCTGCCTCGAGCTGTGGGC 3’

Reverse primer:
5’ATAGAATTCTCAGGAGATGGCCTTTGC 3’

Primers for the cloning of the N-terminal part of SNX1 (1-271 amino acids) in the LRV1 vector to create shSNX1:HA-SNX1ΔC replacement construct:

Forward primer:

5’ATAAGATCTATGTATCCATATGATGTTCCAGATTATGCTGCGTCAGGAGGTGGTGG CTGTAGCGCT 3’

Reverse primer:

5’ATAGAATTCTCATCGAGGCAGCTCTTCTTT 3’

Primers used to make the shSNX1:HA-SNX1ΔH1 (H1 domain of SNX1, i.e., amino acids 308-341 was deleted) replacement construct in LRV1 vector:

Forward primer:

5’TCAGACATTTGGAACACAGCCCTGTTTGCCAAGAGTCTAG 3’

Reverse primer:

5’CAGGGCTGTGTTCCAAATGTCTGATTCATTCATCTTGATG 3’

Primers used to make the shSNX1:HA-SNX1ΔH2 (H2 domain of SNX1, i.e., amino acids 355-387 was deleted) replacement construct in LRV1 vector:

Forward primer:

5’GCCATGCTTGGGTTCTTCCTCCTTGCTGAACTCCTGAGTG 3’

Reverse primer:

5’CAAGGAGGAAGAACCCAAGCATGGCTAGACTCTTGGCAAA 3’

Primers used to make the shSNX1:HA-SNX1ΔH3 (H3 domain of SNX1, i.e., amino acids 439-475 was deleted) replacement construct in LRV1 vector:

Forward primer:

5’CGGCTGCTGTGGGAAGTGACACGATTTGAGAAAGAGAAGT 3’

Reverse primer:

5’ATCGTGTCACTTCCCACAGCAGCCGTGCCTCAGACTCCCG 3’

2.2. Methods

2.2.1. Preparation of competent cells

A single DH5α bacterial colony was picked up from the culture plate and inoculated in 10 ml LB broth, which was then incubated at 37°C for overnight. Subsequently, 100 µl of culture was taken from that primary culture and incubated in 100 ml of LB media at 37°C until the culture attained an optical density of 0.4 - 0.6. The culture was then centrifuged at 6000 rpm at 4°C for 15 min. After removing the supernatant, the pellet was resuspended in 10 ml of 0.1 M ice-cold $CaCl_2$ solution and then incubated on ice for 15 min. Afterward, the solution was centrifuged at 6000 rpm at 4°C for 15 min. Supernatant was discarded and the remaining pellet was resuspended in 5 ml of 0.05 M $CaCl_2$ solution and incubated on ice for 45 min. Subsequently, cells were recovered by centrifugation at 2000 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 then aliquoted into 1.5 ml microcentrifuge tubes and stored at -80 °C.

2.2.2. Transformation

The competent cells were thawed on ice for 10 min. Then 100-500 ng DNA was added by gentle tapping, followed by 30 min incubation on ice. After that, heat shock was given to the cells for 60 sec at 42°C, followed by 5 min incubation on ice. Subsequently, 1 ml of LB media was added and culture was transferred to the water bath for 1 hr at 37°C. On completion of the incubation time, cells were centrifuged at 5000 rpm for 5 min. Subsequently, the supernatant was discarded and the pellet was resuspended in the LB media. An appropriate amount of cells was plated on antibiotic containing LB agar plates.

2.2.3. Plasmid isolation

Plasmids used for transfection purposes were isolated by Qiagen mini or midi kit following manufacturer's instructions. The isolation of DNA used in cloning and screening purposes was done by the alkaline lysis method.

2.2.4. Generation of the GFP-SNX1 full-length and GFP- SNX1 coiled-coil (SNX1-CC) over-expression constructs

In order to study the effect of full-length SNX1 overexpression in group I mGluR trafficking, GFP-SNX1 full-length over-expression construct was generated by the following method. Initially, PCR was performed taking the wild-type SNX1 as a template to amplify the full-length SNX1. A forward primer having BglII restriction enzyme site and the reverse primer containing a stop codon and EcoR1 restriction enzyme site were used for the PCR reaction. The amplified PCR product was digested with BglII and EcoR1. The digested product was then ligated in pEGFP-C1 vector to generate the GFP-SNX1 full-length over-expression construct **(Figure 2.1 A)**. GFP- SNX1 coiled-coil (SNX1-CC) over-expression construct which has been reported to act in a dominant negative fashion, was prepared to investigate the role of SNX1 in mGluR trafficking (Wang, Zhou et al. 2002) **(Figure 2.1 B)**. In brief, the C-terminal coiled-coil domain (aa 265-521) of SNX1 was amplified by PCR reaction. Subsequently, restriction enzyme digestion of the insert and the vector (pEGFP-C1) was performed followed by ligation using T4 DNA ligase. The constructs were confirmed by sequencing.

2.2.5. Generation of the SNX1 knockdown constructs

Generation of the SNX1 knockdown constructs was carried out in a two-step process: In the first step, custom-made oligonucleotides were synthesized and in the second step they were cloned into the LRV1 vector. LRV1 vector is a multi-promoter vector that contains an H1 promoter and an ubiquitin (Ub) promoter. The H1 promoter drives the expression of the shRNA, while the ubiquitin promoter drives the expression of the wild-type SNX1 or various mutants of SNX1 **(Figure 2.2)**.

In brief, siRNA sequences of the smart pool plus oligonucleotides against the SNX1 (obtained from Dharmacon) were used to generate potential oligonucleotides (sense and antisense pair) that can act as shRNA against the endogenous SNX1. After the synthesis of oligonucleotides, an equimolar amount of sense and antisense oligonucleotide pair was mixed by vortexing in a microcentrifuge tube. Then they were kept in a water bath at 99°C for 30 min. Following that, the water bath was turned off and the temperature was allowed to go down slowly by leaving it overnight so that the oligonucleotide pair can anneal with each other. Each of the double-stranded oligonucleotides formed subsequent to the annealing contained the XhoI compatible overhang at the 5' end and XbaI compatible overhang at the 3' end. Simultaneously, the LRV1 vector was also digested with XhoI and XbaI restriction enzymes and subsequently ligated with each double-stranded oligonucleotide such that the shRNAs were placed under the H1 promoter. Each of these shRNAs were screened for their effectiveness in the knockdown of the endogenous SNX1 in primary neurons. Briefly, cells were transfected at 6-8 DIV with LRV1 containing the shRNA two times on alternate days using the calcium phosphate method (described below).

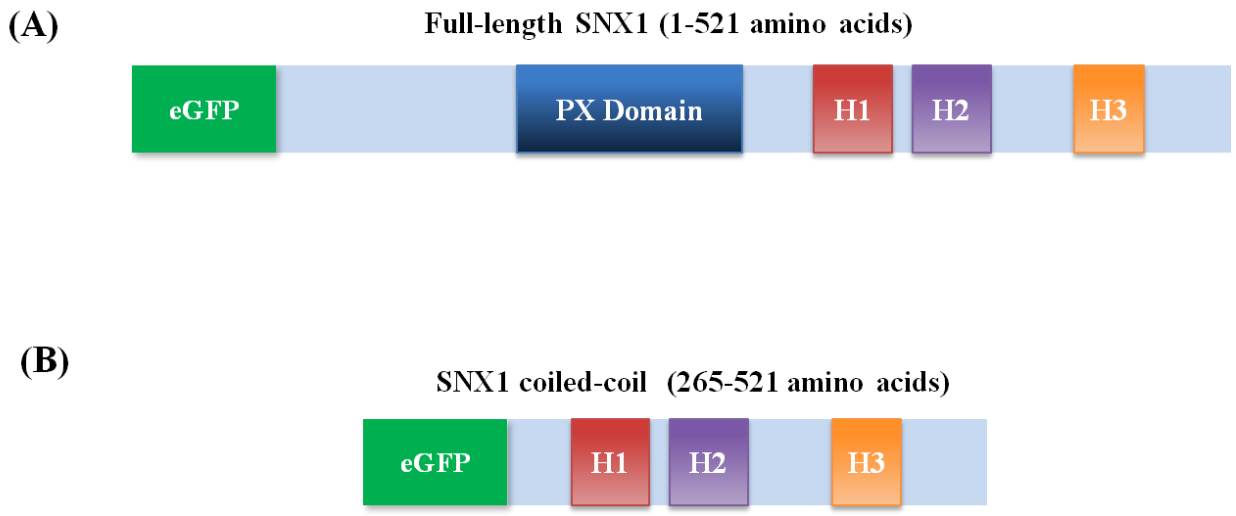


Figure 2.1. Generation of the GFP-SNX1 full-length (A) and GFP-SNX1 coiled-coil (B) over-expression constructs.

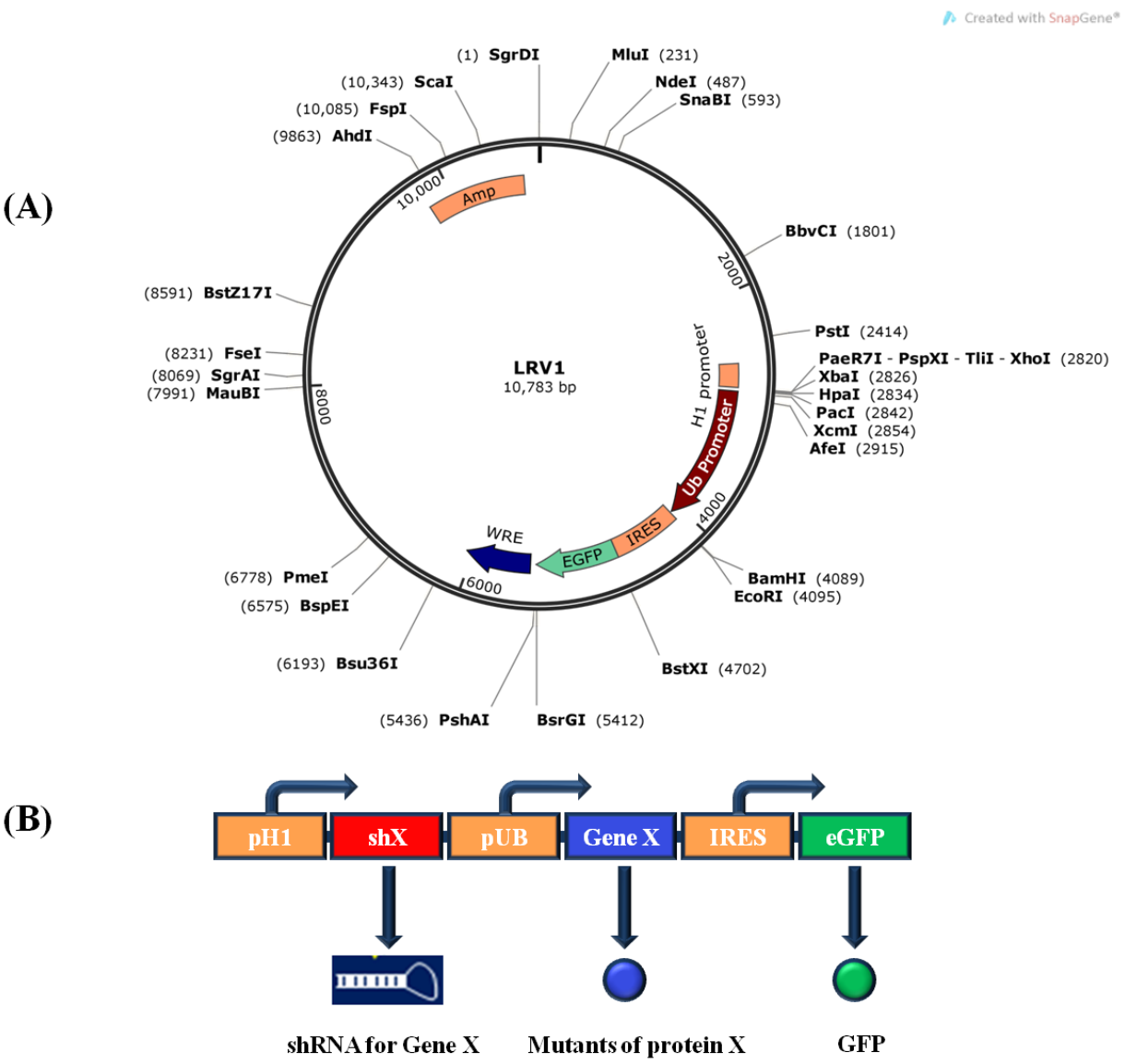


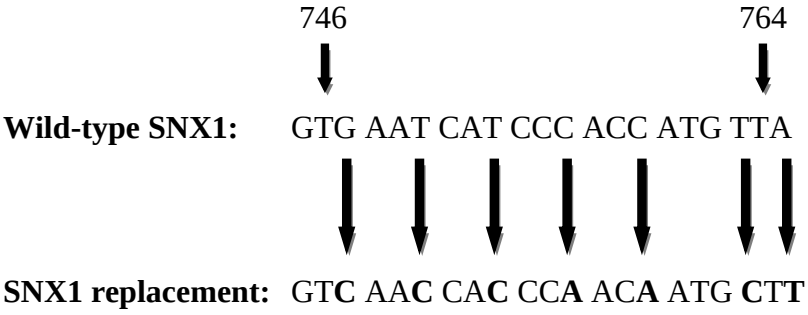
Figure 2.2. (A) Vector map of LRV1 plasmid. (B) Schematic of the organization of the promoters in LRV1 vector.

The efficiency of knockdown of the respective shRNAs were analysed 3 - 5 days post-transfection by western blot as well as by immunocytochemistry. The following shRNA (shSNX1) was selected as the most effective shRNA in knocking down the endogenous SNX1 in primary neurons and used for our study:

5'TCGAG**TGAATCATCCCACCATGTT**TTCAAGAGA**AACATGGTGGGATGATTCA**T TTT 3'

2.2.6. Generation of the full-length SNX1 replacement construct

The full-length SNX1 replacement construct was generated in order to rescue the knockdown effect of shSNX1. Briefly, few silent mutations were introduced in the shSNX1 binding region of the wild-type SNX1. These silent mutations prevented the knockdown of the replacement SNX1 by shSNX1, but simultaneously the endogenous SNX1 was down-regulated. The schematic below depicts the positions of the silent mutations in the replacement construct:



Following the incorporation of the silent mutations in the wild-type SNX1 gene, it was used as a template to generate the replacement construct. The forward primer used to amplify the gene, contained the BglII restriction enzyme site and HA sequence while the reverse primer had the EcoR1 restriction enzyme site. After PCR amplification, the full-length SNX1 replacement construct was cloned under the ubiquitin promoter in the shSNX1 containing LRV1 vector using the BamH1 and EcoR1 restriction enzyme sites. The construct was confirmed by sequencing. The schematic of the full-length SNX1 replacement construct is shown in **Figure 2.3**.

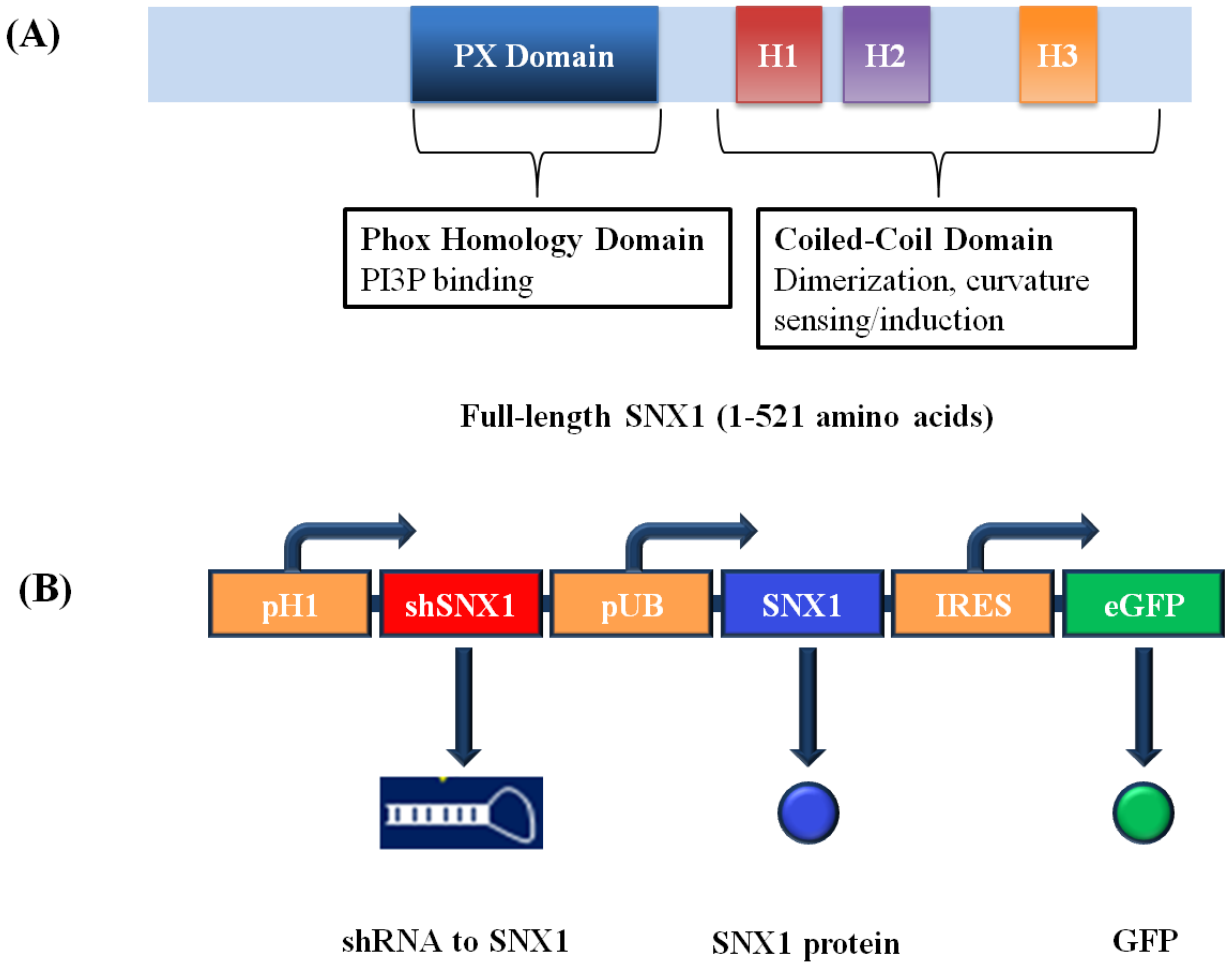


Figure 2.3. Generation of the SNX1 replacement construct. (A) Schematic of the full-length SNX1 showing its domain structure. (B) Schematic of the SNX1 replacement construct.

2.2.7. Generation of the SNX1ΔN replacement construct

The SNX1ΔN (N-terminal deleted) replacement construct was made to examine the role of the N-terminal domain of SNX1 in the group I mGluR trafficking. Following method was used to prepare the SNX1ΔN replacement construct. PCR was performed by taking the full-length SNX1 replacement construct as a template to amplify the C-terminal region (265 – 521 amino acids) of SNX1. A forward primer containing a HA sequence (TATCCATATGATGTTCCAGAT TATGCT) and the BglII restriction enzyme site and the appropriate reverse primer having the EcoR1 restriction enzyme site were used for the PCR reaction. After that, the amplified product was digested with BglII and EcoR1. The digested product was then ligated in the SNX1 knockdown construct (LRV1 having the shSNX1) under the ubiquitin promoter for generation of the SNX1ΔN replacement construct. **Figure 2.4 A** shows the schematic of the wild-type SNX1 and the SNX1ΔN-region of SNX1. **Figure 2.4 B** shows the schematic representation of the SNX1ΔN replacement construct.

2.2.8. Generation of the SNX1ΔC replacement construct

The SNX1ΔC (C-terminal deleted) replacement construct was generated to explore the function of the C-terminal domain of SNX1 in the group I mGluR trafficking. A similar strategy as described earlier for the generation of the SNX1ΔN construct was used to make this construct. In brief, the N - terminal part of SNX1 was PCR amplified using the forward primer having the HA sequence and the BglII restriction enzyme site and the appropriate reverse primer containing the EcoR1 restriction enzyme site. The SNX1 construct that had silent mutations at the shSNX1 binding site was used as template in the PCR reaction. Subsequently, restriction enzyme digestion of the insert and the vector (LRV1 containing shSNX1) was done followed by ligation using T4 DNA ligase to insert the SNX1ΔC under the ubiquitin promoter. Finally, the construct was confirmed by sequencing. **Figure 2.5 A** shows the N-terminal region of SNX1. The schematic of the SNX1ΔC replacement construct is shown in **Figure 2.5 B**.

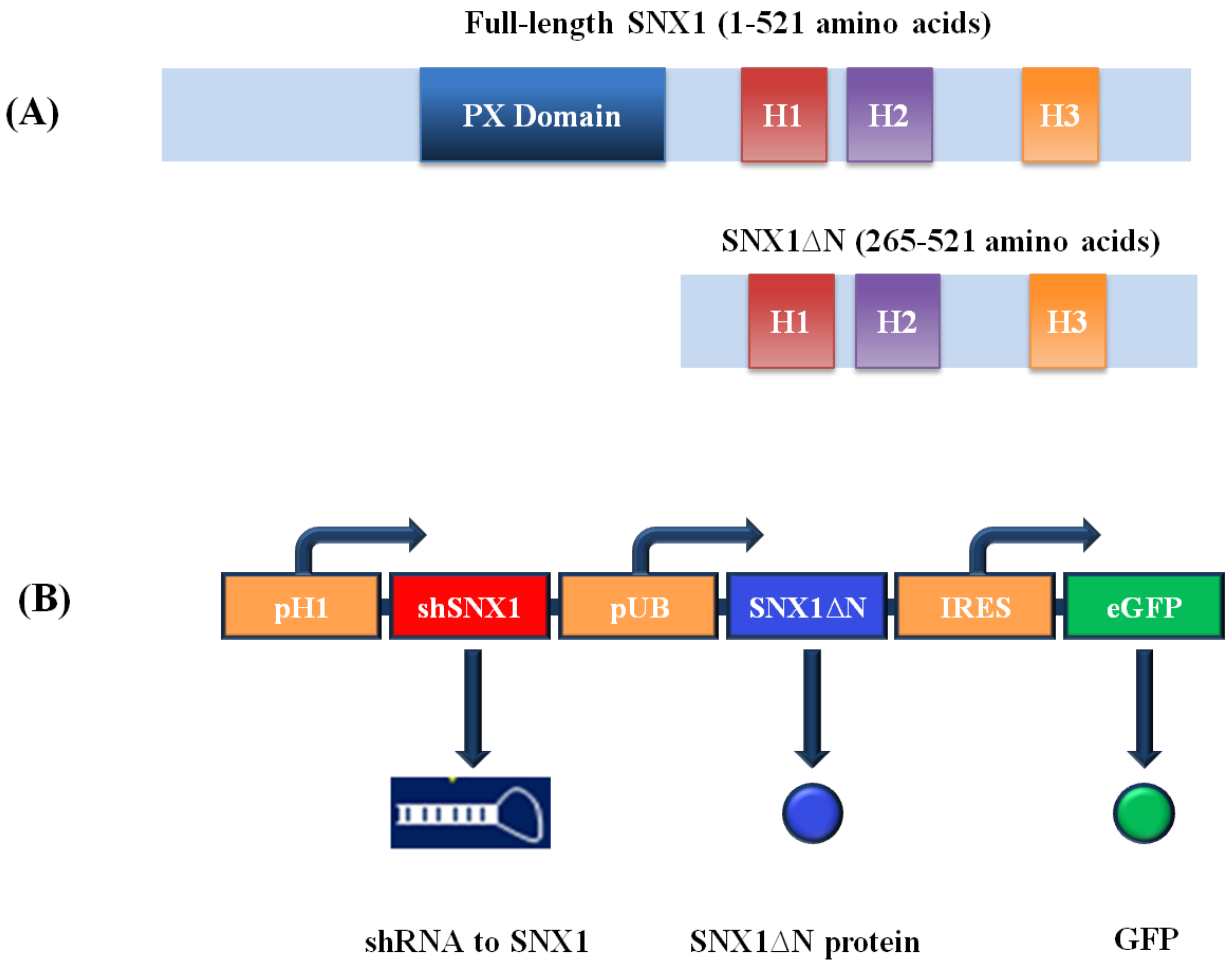
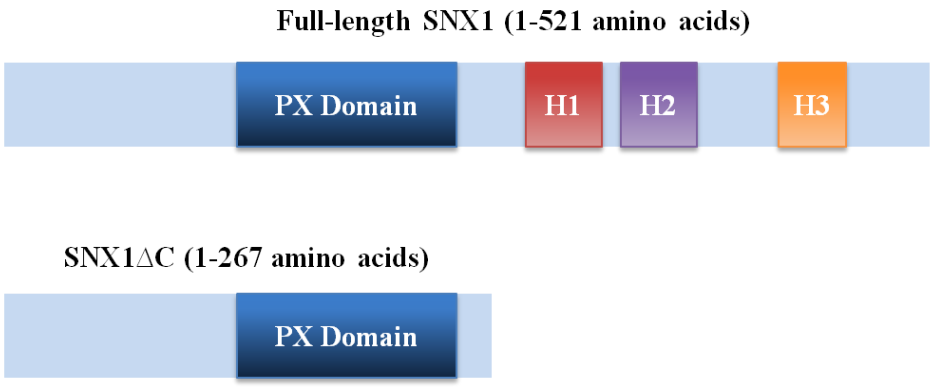


Figure 2.4. Generation of the SNX1ΔN replacement construct. (A) Picture of the full-length SNX1 and SNX1ΔN. (B) Schematic of the SNX1ΔN replacement construct.

(A)



(B)

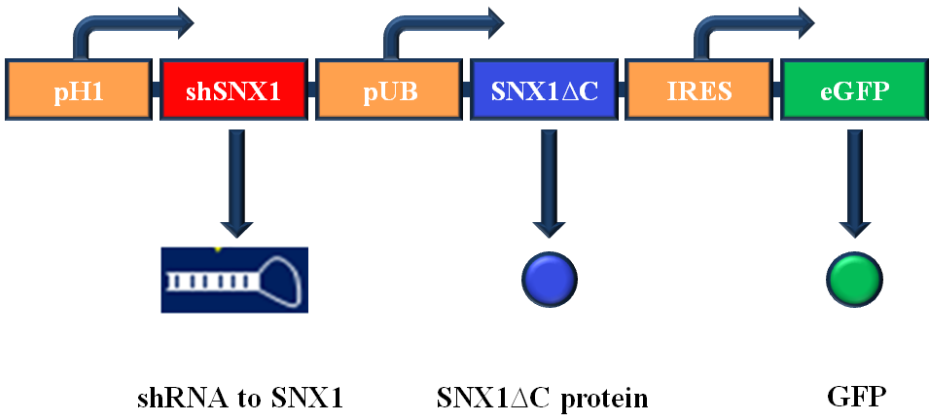


Figure 2.5. Generation of the SNX1ΔC replacement construct. (A) Diagram of the full-length SNX1 and SNX1ΔC. (B) Schematic of the SNX1ΔC replacement construct.

2.2.9. Generation of the SNX1ΔH1, SNX1ΔH2 and SNX1ΔH3 replacement constructs

In order to further investigate the role of the various domains of the C-terminal region of SNX1 in group I mGluR trafficking, following constructs were generated: shSNX1:HA-SNX1ΔH1 (H1 domain of SNX1, i.e., amino acids 308-341 was deleted) **(Figure 2.6)**, shSNX1:HA-SNX1ΔH2 (H2 domain of SNX1, i.e., amino acids 355-387 was deleted) **(Figure 2.7)**, shSNX1:HA-SNX1ΔH3 (H3 domain of SNX1, i.e., amino acids 439-475 was deleted) **(Figure 2.8)**. Each of these deletion mutants were prepared by PCR method using appropriate primers. Subsequently, restriction enzyme digestion of the amplified insert and the vector (LRV1 with shSNX1) was done, followed by the ligation using T4 DNA ligase. Finally, the ligated product was transformed into the TOP10 bacterial competent cells and plated onto the LB agar plates containing 100 µg / ml Ampicillin. Colonies were selected randomly, followed by plasmid isolation and confirmation of the constructs by sequencing.

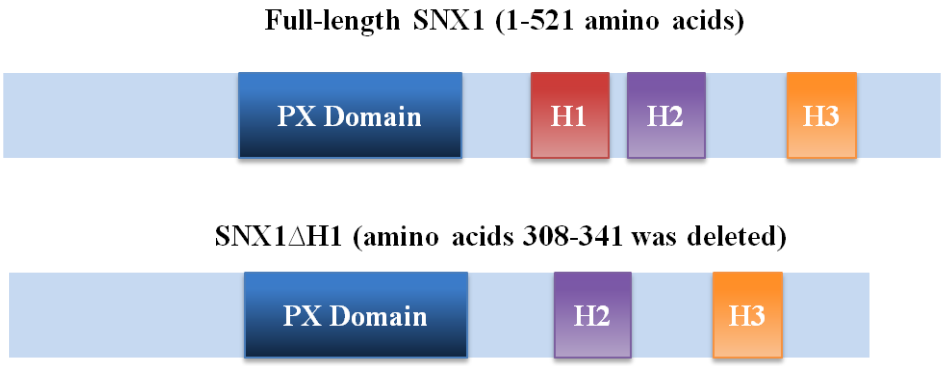
2.2.10. Cell culture and transfection

HEK293 / HEK293T cell culture and transfection

HEK293 cells and HEK293T cells were maintained in DMEM supplemented with 10% FBS, 1X antibiotic-antimycotic (AB-AM) mix in 5% CO_2 and 95% humidity at 37˚C. Cells were seeded on coverslips pre-coated with 50 µg / ml poly-D-lysine. Transfection of the cells was performed at 65 – 70% confluency by mixing 2 µg of DNA with 10 µg of Lipofectamine 2000 in 1 ml of OptiMEM.

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

(A)



(B)

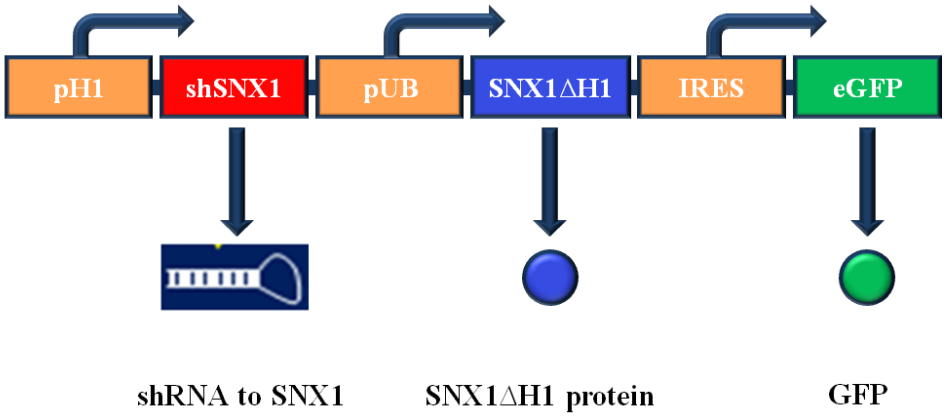
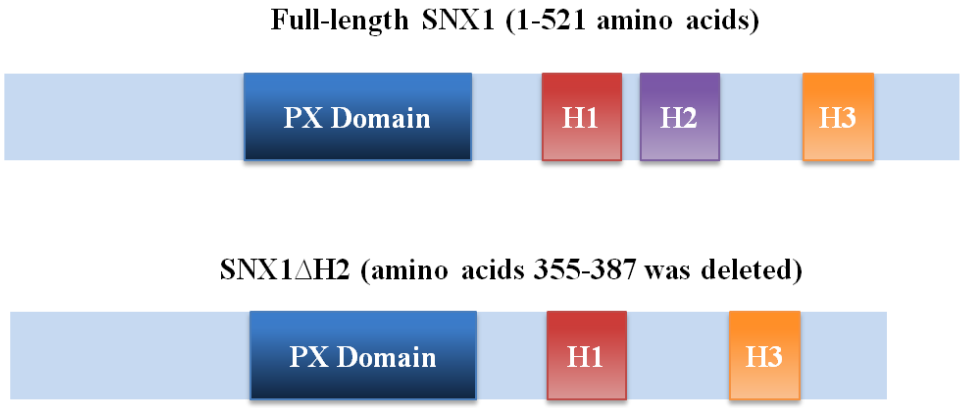


Figure 2.6. Generation of the SNX1ΔH1 replacement construct. (A) Picture of the full-length SNX1 and SNX1ΔH1. (B) Schematic of the SNX1ΔH1 replacement construct.

(A)



(B)

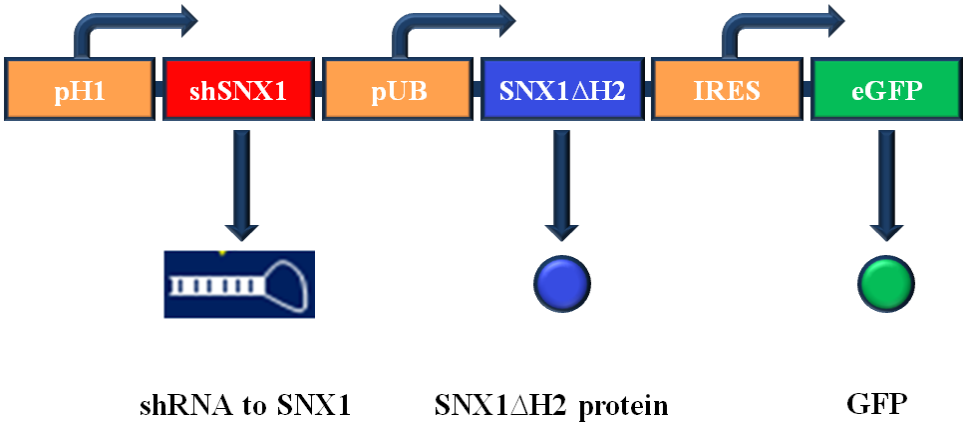
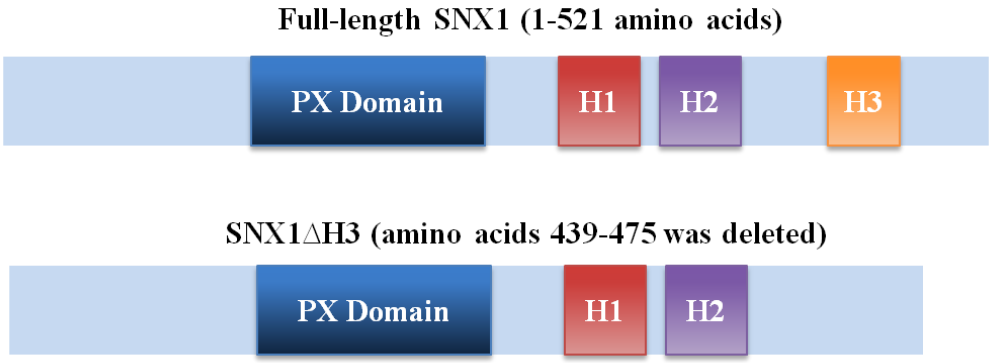


Figure 2.7. Generation of the SNX1ΔH2 replacement construct. (A) Picture of the full-length SNX1 and SNX1ΔH2. (B) Schematic of the SNX1ΔH2 replacement construct.

(A)



(B)

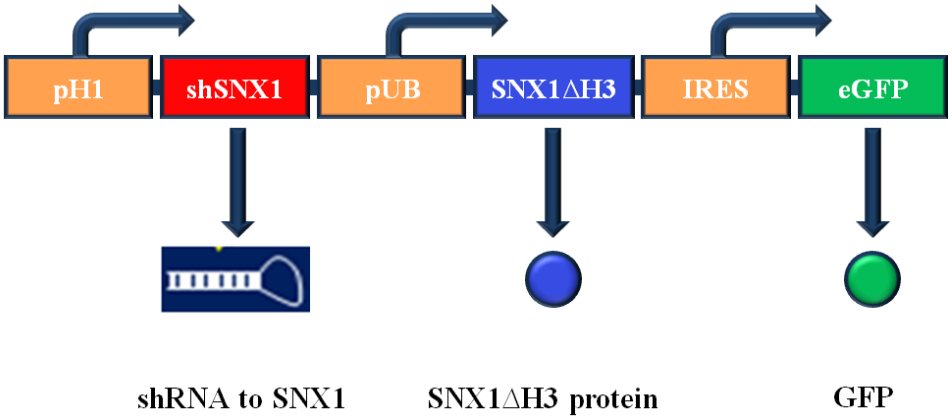


Figure 2.8. Generation of the SNX1ΔH3 replacement construct. (A) Pictorial representation of the full-length SNX1 and SNX1ΔH3. (B) Schematic of the SNX1ΔH3 replacement construct.

Dissociated primary hippocampal neuron culture

Primary hippocampal neuron cultures were prepared from P0 C57BL/6 mouse pups. In brief, after sacrificing the pups, hippocampi were dissected out and subsequently, tissue was dissociated with the papain treatment (30 min at 37˚C). Thereafter, cells were triturated using a glass pipette and single cell suspension was prepared. Neurons were then plated onto the poly-D-lysine in sodium borate coated 12 mm coverslips (50 μg/ml poly-D-lysine + 0.1 M sodium borate) at a density of approximately 150,000 cells for the experiments related to the mGluR trafficking and around 100,000 cells for AMPAR trafficking experiments. Cultures were maintained in neurobasal media with 0.5 mM glutamine and B27 supplement. Glial growth was inhibited by adding FUDR (floxuridine) on the 4^{th} day of the culture.

Transfection in primary hippocampal neurons

Primary hippocampal neurons were transfected with appropriate DNA constructs at 7 - 9 days *in vitro* using calcium phosphate method. Initially, old neurobasal-B27 media was recollected from the cells in culture and replaced with the plain Neurobasal media. After that, 3 μg DNA per well was mixed with 250 mM $CaCl_2$ in a microcentrifuge tube and incubated for 5 min. Subsequently, 2X HEPES buffer was added dropwise and mixed by gentle tapping or mild vortexing and then incubated for 30 min in the dark. Afterward, the mixture was added to the cells for further incubation till the appearance of small sand-like precipitates on the surface of neurons was observed under the bright field microscope. Subsequent to the adequate amount of precipitate formation, cells were washed with the washing buffer and finally the recollected old Neurobasal – B27 media was added. Half feeding was done on next day. All the experiments were performed when the cells were at 12 - 15 days *in vitro*.

2.2.11. Endocytosis assay

Group I mGluR endocytosis assay

HEK293 cells or primary hippocampal neurons were transfected with myc-mGluR1 or myc-mGluR5 cDNA following the method described above. Hrs constructs were co-transfected with FLAG-mGluR1 for Hrs related studies in a similar manner. For endocytosis assay, live cells were first incubated in serum-free media for 30 min followed by 5 min incubation in 2% normal goat serum (NGS) for blocking. Subsequently, live cells expressing myc-mGluR1 or myc-mGluR5 were labelled with anti-myc mouse monoclonal primary antibody (1:500) for 15 min at 37˚C for HEK293 cells and anti-myc mouse monoclonal primary antibody (1:200) for 20 min at 37˚C for primary hippocampal neurons. Similarly, live cells expressing FLAG-mGluR1 were labelled with anti-FLAG rabbit polyclonal primary antibody (1:500) for 15 min at 37˚C for HEK293 cells and anti-FLAG rabbit polyclonal primary antibody (1:200) for 20 min at 37˚C for primary hippocampal neurons.

After that, cells were washed with plain DMEM and treated with 100 μM R,S-DHPG for 5 min. Subsequently, cells were chased for indicated times in the absence of the ligand. Following the completion of the respective chase periods, cells were fixed without permeabilization with ice-cold 4% paraformaldehyde (PFA) for 15 min on ice. Cells were washed with 1X PBS and surface localized receptors were labelled with the saturating concentration of the goat anti-mouse/rabbit Alexa-568 (1:100) conjugated secondary antibody for 1 hr at 37˚C. Thereafter, cells were subjected to permeabilization by 0.1% Triton X-100 treatment for 30 min at room temperature followed by blocking with 2%NGS for 1 hr at 37°C. Internalized receptors were then stained with the second secondary antibody, viz., goat anti-mouse Alexa-647 (1:800) or goat anti-rabbit Alexa-647 (1:800) for 1 hr at 37°C. The co-transfected shRNA construct containing the GFP and HA-tagged replacement constructs were stained with the anti-GFP and anti-HA antibodies respectively for overnight at 4°C. Subsequently, they were stained with their respective secondary antibodies. Finally, the coverslips were mounted on glass slides and scanned under the confocal microscope.

In order to ascertain that the saturating concentration of the Alexa-568 conjugated secondary antibody that was used to label the surface receptors, inhibited any observable binding of the Alexa-647 conjugated secondary antibody with the surface receptors, the following control experiment was performed. Application of the saturating concentration of the Alexa-568 conjugated secondary antibody inhibited any further detectable staining of the surface receptors when Alexa-647 conjugated second secondary antibody was applied in non-permeabilized cells. On the other hand, the internalized receptors were visible subsequent to the application of the Alexa-647 conjugated secondary antibody when cells were permeabilized with the 0.1 % Triton X-100 for 30 min at room temperature **(Figure 2.9 and Figure 2.10)**. Thus, these control experiments suggested that in our assays, Alexa-647 conjugated secondary antibody did not label any detectable amount of surface receptors and thus it stained the internalized receptors only (Bhattacharyya, Biou et al. 2009; Trivedi and Bhattacharyya 2012; Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015; Gulia, Sharma et al. 2017). Similar control experiments were performed in HEK293 cells as well for experiments related to myc-mGluR5 and FLAG-mGluR1 (data not shown).

2.2.12. Recycling assay

The recycling experiments were performed in a similar way as described above. Briefly, myc-mGluR1/FLAG-mGluR1 receptors expressing on the surface of the live primary hippocampal neurons were stained with the anti-myc/anti-FLAG primary antibodies. The internalization of the receptors were induced by the application of 100 μM R,S-DHPG for 5 min. Cells were then chased for specific time periods in the absence of the ligand. Subsequently, cells were fixed with 4% PFA and surface and internalized receptors were stained with the Alexa-568 conjugated and Alexa-647 conjugated secondary antibodies respectively, using the same protocol as has been described for the endocytosis assay. The effect of the overexpression of full-length SNX1 and full-length Hrs on the recycling of mGluR1 was investigated by their co-transfection with the myc-mGluR1 and FlAG-mGluR1 respectively.

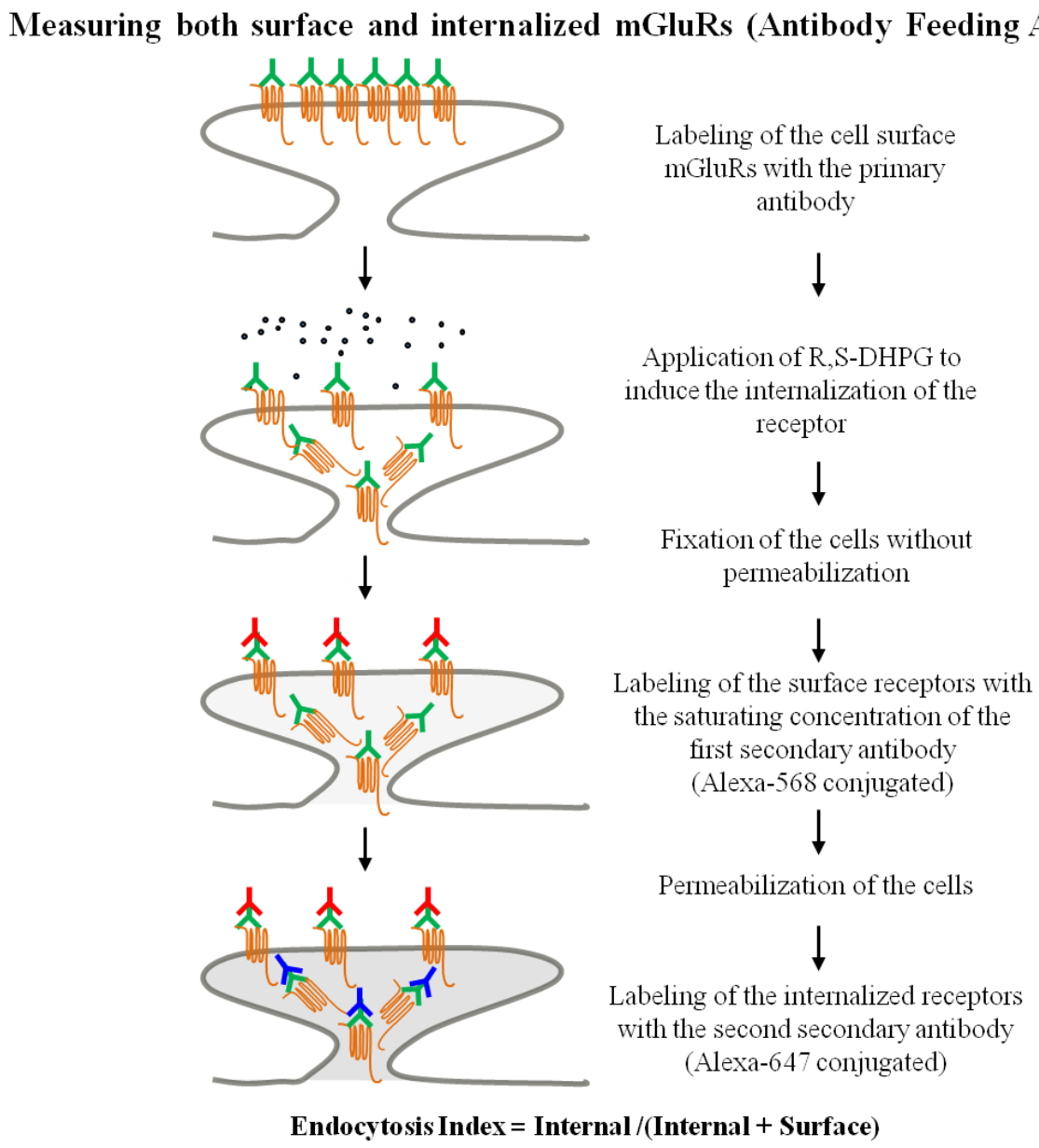


Figure 2.9. Schematic of the antibody feeding receptor endocytosis assay.

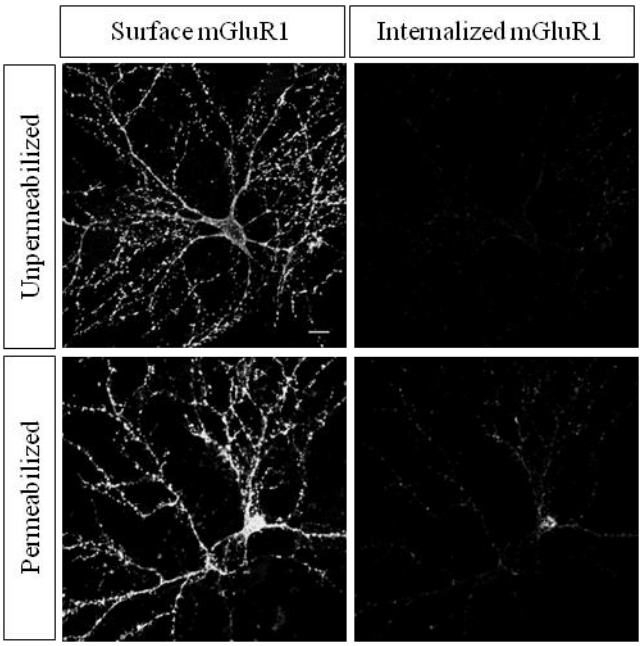


Figure 2.10. Standardization of the antibody feeding assay. Application of the first secondary antibody (Alexa-568 conjugated), in a saturating concentration, labelled the surface receptors (upper left panel) which prevented any further observable binding of the second secondary antibody which is Alexa-647 conjugated (upper right panel) in unpermeabilized condition. However, when cells were permeabilized, the second secondary antibody labelled the internalized mGluR1 (lower right panel). Scale bar = 10 μm.

2.2.13. AMPA receptor endocytosis assay

In order to study the group I mGluR-mediated AMPA receptor (AMPAR) endocytosis, primary hippocampal neurons were transfected at 6-7 DIV with the shSNX1 construct. Endocytosis assay was performed when cells were 14-15 DIV. Both control cells and shSNX1 transfected cells were first treated with appropriate mixtures of antagonists: 1 μM TTX (pre-synaptic release blocker), 20 μM DNQX (antagonist for AMPARs) and 50 μM APV (antagonist for NMDARs) for 30 min at 37˚C. Control cells were then treated with 100 μM R,S-DHPG for 5 min to induce the internalization of group I mGluRs. Subsequently, receptors were allowed to recycle back to the cell surface in 2.5 hr following the method described above. In another set of control cells, the recycling of the receptors was inhibited by the application of 5 nM Okadaic acid and 1 μM FK-506. Likewise, the application of 100 μM R,S-DHPG in shSNX1 transfected cells also induced the internalization of group I mGluRs and receptors were then allowed to recycle back in 30 min following the method described above. In both control cells and shSNX1 transfected cells, subsequent to the respective recycling period of group I mGluRs, 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). To study the AMPAR endocytosis induced by the mGluRs, when they were initially present at the cell surface in both control cells and shSNX1 transfected cells, cells were pre-incubated with antagonists and then GluA1 containing receptors were labelled with the antibody in live cells as described above. In all the conditions, subsequent to the labelling of the GluA1 containing receptors 100 μM R,S-DHPG was applied for 5 min. After removing the agonist, cells were further incubated in the presence of the drugs for a total of 15 min at 37ºC. Subsequently, cells were fixed at the end of the respective time periods in 4% PFA for 15 min on ice without permeabilization. Surface GluA1 containing receptors were stained by using the saturating amount of goat anti-rabbit Alexa-568 conjugated secondary antibody (1:100) for 1.5 hr at 37˚C. Subsequently, the cells were permeabilized with 0.1 % Triton X-100 for 30 min at room temperature. Following that, the internalized GluA1 containing receptors were labelled with the goat anti-rabbit Alexa-647 conjugated secondary antibody (1:750) for 1.5 hr at 37˚C. Again, in order to make sure that the Alexa-647 conjugated secondary antibody did not label any detectable surface receptors in our assays, the saturating concentration of the first secondary antibody was determined through a

control experiment similar to that we have described in our earlier studies (data not shown) (Bhattacharyya, Biou et al. 2009; Citri, Bhattacharyya et al. 2010; Gulia, Sharma et al. 2017).

2.2.14. Transferrin receptor kinetics assay

HEK293 cells were plated on poly-D-lysine-coated coverslips in 24 well plates and transfected with either full-length GFP-SNX1 or GFP-SNX1-CC cDNA using lipofectamine 2000. 24 hrs post transfection, cells were incubated in plain DMEM for 30 min at 37°C. Thereafter, cells were treated with 20 μg / ml Alexa-568 labelled transferrin for 2 min at 37˚C followed by a chase for different time periods (5 min, 15 min, 30 min and 60 min) in plain DMEM without transferrin (Ciechanover, Schwartz et al. 1983; Harding, Heuser et al. 1983). Subsequently, cell surface receptors were stripped-off using stripping solution (0.5% CH_3COOH + 500 mM NaCl) and fixed with 4% PFA at respective time points. Finally, the coverslips were mounted on glass slides using fluoromount. Images were acquired through either Zeiss Axio Observer Z1 fluorescence microscope or LSM 780 Zeiss confocal microscope using 63X oil objective.

2.2.15. Co-localization assay

The synaptic localization of different mutants of SNX1 was investigated by measuring the extent of co-localization of the mutants with the pre-synaptic protein, Bassoon. In brief, different HA-tagged SNX1 mutants were transfected in primary hippocampal neurons using calcium phosphate method (described above) on 6 DIV. Cells were fixed with ice-cold 4% PFA on ice for 15 min on 12-14 DIV. After that, cells were permeabilized by 0.1% Triton X-100. Subsequently, cells were stained with the rat anti-HA antibody (1:500) and rabbit anti-bassoon antibody (1:500) by overnight incubation at 4˚C. Following primary antibody staining, cells were washed and further stained with goat anti-rat Alexa-568 and goat anti-rabbit Alexa-647 for 1 hr at 37˚C to visualize the SNX1 constructs and Bassoon respectively. Finally, coverslips were mounted on glass slides and imaged under the confocal microscope.

2.2.16. Imaging

Imaging of the cells was carried out in Zeiss LSM 780 confocal laser scanning microscope using a 63X oil immersion objective (NA = 1.4). Each experiment was repeated at least three times. 100-120 HEK293 cells and 40-50 primary hippocampal neurons were imaged. All the imaging parameters including the laser power, digital gain and digital offset were kept constant throughout an experiment. Alexa-488, Alexa-568 and Alexa-647 excitation / emission was achieved using appropriate filter sets and images were obtained using identical parameters for all conditions in a particular experiment.

2.2.17. Image analysis and statistics

Image analysis

Raw images were used for all analyses and quantitation was done using ImageJ software (NIH, USA) (Schneider, Rasband et al. 2012). Images were first maximally projected and then threshold values were set and kept constant for a particular experiment. Subsequently, the thresholded areas occupied by the fluorescence of the labelled surface and internalized receptors were quantified. Thereafter, the internalization index was calculated by dividing the value contributed by the internal fluorescence with the value contributed by the total fluorescence (surface + internal). These values were then normalized with respect to their untreated controls.

Endocytosis index = (Internalized receptors) / (Surface receptors + Internalized receptors**)**

In order to quantify 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. The dendritic values were defined by the area that was 10 µm away from the soma.

The co-localization was quantified at a particular Z section of the image after thresholding the cells using identical values. In the dendrites of the primary hippocampal neurons, the magnitude of the co-localization of SNX1 puncta's with bassoon puncta's was measured subsequent to the thresholding using identical values.

The data quantified in all the experiments have been represented as a combined result of all the three repeats of that particular experiment. The representative images were obtained from the processing of raw images in Adobe Photoshop software using identical values of brightness and contrast. All the western blots and immunoprecipitation experiments were also quantified using ImageJ software.

Statistics

As stated earlier, 100-120 HEK293 cells and 40-50 primary hippocampal neurons were imaged and each experiment was repeated three times. Data are presented as mean ± SEM. 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. Data were analyzed and graphs were plotted using Origin software.

2.2.18. Western blot analysis

Primary cortical neurons obtained from mouse P0/P1 pups were cultured and transfected with the siRNA using lipofectamine on 6 DIV. 72 hrs post-transfection, these cells were washed with ice-cold PBS and then lysed using RIPA lysis buffer containing 1X protease inhibitor cocktail. The samples were subsequently boiled in 1X Laemmli sample buffer at 99°C for 10 min. Each of these samples were then run on SDS-PAGE after being loaded equally in each lane respectively. Thereafter, they were transferred to the PVDF membrane and subsequently blocked with 5% skimmed milk in 0.05% PBST for 2 hr at room temperature. The membrane was then kept for overnight incubation in primary antibody (1:1000) at 4°C. After washing, the membrane was incubated with the HRP conjugated secondary antibody (1:5000) for 45 min at room temperature. The blot was developed using ECL western detection kit and image was acquired by ImageQuant LAS 4000. Primarily, the blot was first stained with the anti-SNX1 antibody to confirm the knockdown of endogenous SNX1. Subsequently, the blot was stripped with the stripping buffer and then stained with the anti-β actin antibody, which served as a loading control. For shRNA mediated knockdown and replacement experiments, the western blots were carried out in the similar manner as described above.

2.2.19. Immunoprecipitation

Immunoprecipitation was performed to check for the effect of the deletion of H1, H2 and H3 domains of SNX1 on the interaction of SNX1 with Hrs. To study the interaction of the HA-tagged full-length SNX1 (shSNX1:SNX1) and various mutants of SNX1 (shSNX1:SNX1ΔH1, shSNX1:SNX1ΔH2, shSNX1:SNX1ΔH3) with myc tagged Hrs, the respective cDNAs were co-transfected in HEK293T cells. The protein-G beads were incubated overnight at 4°C with the anti-HA antibody. HA antibody bound protein-G beads were used for the immunoprecipitation. The samples were then subjected to SDS-PAGE electrophoresis followed by western blotting in a similar way described earlier. The immunoblotting of SNX1 mutants and Hrs protein was carried out using anti-SNX1 antibody (1:1000) and anti-myc antibody (1:1000) respectively.

Role of SNX1 in the trafficking of group I mGluRs in non-neuronal cells and primary hippocampal neurons

3.1. Introduction

G-protein coupled receptors (GPCRs) play critical roles in the signaling of numerous biological processes by modulating the phenomenon at the molecular level (Horn, Bettler et al. 2003). Binding of the ligand activates the GPCR, which in turn initiates the signal transduction cascade, thereby regulating a huge variety of effector proteins to modulate various cellular functions (Lefkowitz 1998; Marinissen and Gutkind 2001). Subsequent to the signaling, most of the GPCRs become unresponsive towards further agonist stimulation and this phenomenon is known as the desensitization of the receptor (Krupnick and Benovic 1998; Kelly, Bailey et al. 2008). GPCR desensitization plays a crucial role in preventing the overstimulation of the receptor and excessive signaling.

Subsequent to desensitization, many receptors undergo internalization (Tan, Brady et al. 2004). Trafficking of GPCRs not only regulates the activity of the receptor, it also controls the spatio-temporal localization of the receptor (Marchese, Paing et al. 2008). Following internalization, receptors are sorted to different post-endocytic fates (Hanyaloglu and Zastrow 2008; Sposini, Jean-Alphonse et al. 2017). Divergent sorting of GPCRs to the recycling pathway or degradative/lysosomal pathways is highly regulated and depends on the type of the receptor, type of the ligand and type of the cellular background (Ferguson 2001; Drake, Shenoy et al. 2006). The lysosomal degradation pathway leads to the downregulation of the receptor whereas recycling of the internalized receptor back to the cell surface could serve as a mechanism for the resensitization of the receptor (Tsao, Cao et al. 2001; Von Zastrow 2001; Katzmann, Odorizzi et al. 2002).

Similar to many other GPCRs, group I mGluRs also undergo rapid internalization upon agonist stimulation (Mundell, Pula et al. 2004). Over-expression of the dominant negative form of β-arrestin and dynamin suggested that the ligand-mediated internalization of group I mGluRs is β-arrestin and dynamin-dependent (Mundell, Matharu et al. 2001; Mundell, Pula et al. 2004). Moreover, the K63-linked poly-ubiquitination of mGluR1 plays critical role in the endocytosis of the receptor. The acute knockdown of Siah-1A (E3 ubiquitin ligase), inhibited the ligand-mediated internalization of mGluR1 (Gulia, Sharma et al. 2017). Phosphorylation also plays crucial roles in the endocytosis of group I mGluRs and both GRKs and second messenger

dependent kinases are involved in this process (Alaluf, Mulvihill et al. 1995; Gereau IV and Heinemann 1998; Ciruela, Giacometti et al. 1999; Dale, Bhattacharya et al. 2000; Francesconi and Duvoisin 2000; Sallese, Salvatore et al. 2000; Iacovelli, Salvatore et al. 2003; Kim, Braud et al. 2005; Dhami and Ferguson 2006; Mao, Liu et al. 2008). Subsequent to the ligand-mediated internalization, majority of group I mGluRs recycled back to the cell surface in a pH-dependent manner (Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015; Bhattacharyya 2016; Gulia, Sharma et al. 2017; Mahato, Ramsakha et al. 2018). The recycling of mGluR1 is dependent on the activity of protein phosphatase 2A (PP2A) (Pandey, Mahato et al. 2014). On the other hand, the recycling of mGluR5 completely depends on the activity of PP2A and blocking the activity of PP2B had partial effect on this phenomena (Mahato, Pandey et al. 2015). Although some amount of work has been done on the cellular and molecular mechanisms regulating the endocytosis of group I mGluRs, the lack of knowledge about the mechanisms that control the sorting of the receptor subsequent to the internalization was the main motivation of our study.

Sorting nexins (SNXs) are a group of proteins having phox homology (PX) domain. SNX1, a member of this group, regulates endosome to trans-Golgi network (TGN) retrograde transport of large number of cargos, as well as the trafficking and sorting of some receptors through other pathways (Kurten, Cadena et al. 1996; Trejo and Coughlin 1999; Wang, Zhou et al. 2002; Gullapalli, Garrett et al. 2004; Gullapalli, Wolfe et al. 2006; Wassmer, Attar et al. 2007; Wassmer, Attar et al. 2009; Nisar, Kelly et al. 2010). SNX1 has also been shown to interact *in vitro* with the cytoplasmic tails of mGluR1 and mGluR5 (Heydorn, Sondergaard et al. 2004). Due to the above results, we investigated the role of SNX1, if any, in the trafficking of group I mGluRs. Our data suggested that in HEK293 cells, over-expression of the C-terminal domain of SNX1 (SNX1-CC) that contains the coiled-coil domain, had an effect on the ligand-mediated trafficking of mGluR1. On the other hand, over-expression of the full-length SNX1 resulted in the delayed recycling of the receptor in HEK293 cells. Finally, our data suggested that similar to in HEK293 cells, over-expression of SNX1-CC resulted in the rapid recycling of both mGluR1 and mGluR5 in primary hippocampal neurons. On the other hand, over-expression of the full-length SNX1 delayed the recycling of the receptors in primary hippocampal neurons.

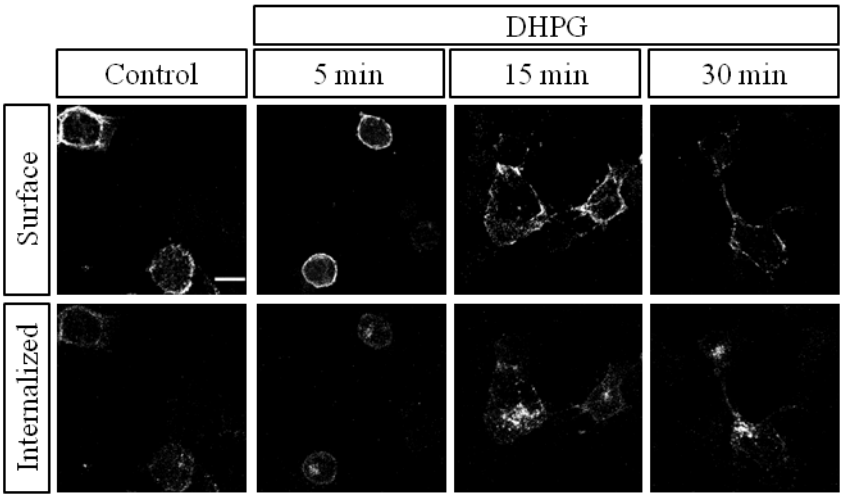
3.2. Results

3.2.1. Endocytosis of group I mGluRs upon agonist-mediated stimulation in HEK293 cells

Previous studies have reported that group I mGluRs get endocytosed upon agonist-mediated stimulation (Dhami and Ferguson 2006; Francesconi, Kumari et al. 2009; Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015; Gulia, Sharma et al. 2017). In order to confirm whether myc-mGluR1 gets internalized upon application of the ligand, we studied the internalization of this receptor on R,S-DHPG application in HEK293 cells. The protocol used for this study has been described in the "material and methods" chapter. In brief, HEK293 cells were first transfected with myc-mGluR1 cDNA and then incubated for 24 hrs in 10% DMEM. Live cells expressing myc-mGluR1were stained with anti-myc mouse monoclonal primary antibody for 15 min at 37˚C, followed by the application of 100 μM R,S-DHPG for 5 min. Subsequently, the ligand was removed and cells were chased for the indicated time points in the absence of the ligand in plain DMEM. After that, cells were fixed at the specific time points with the ice-cold 4% PFA for 15 min on ice without permeabilization. Afterward, surface receptors were labelled using saturating concentration of the first secondary antibody viz., goat anti-mouse Alexa-568 (1:100). Subsequently, cells were permeabilized with 0.1% Triton X-100 followed by the labelling of the internalized receptors with the second secondary antibody viz., goat-anti mouse Alexa-647 (1:750). Finally, cells were mounted on glass slides and imaged under the confocal microscope.

In control cells, majority of the receptors were found to be localized on the cell surface and minimal amount of receptors were internal **(control = 1 ± 0.08) (Figure 3.1 A, B)**. This small amount of endocytosed receptors were due to the constitutive endocytosis of the receptor. On the other hand, R,S-DHPG treated cells showed an increase in the amount of the internalized receptors, suggesting that myc-mGluR1 internalized on the application of R,S-DHPG in HEK293 cells. The myc-mGluR1 internalized at 5 min post-ligand exposure as observed by an increase in the internal fluorescence **(5 min = 1.38 ± 0.1)**. There was further increase in the

(A)



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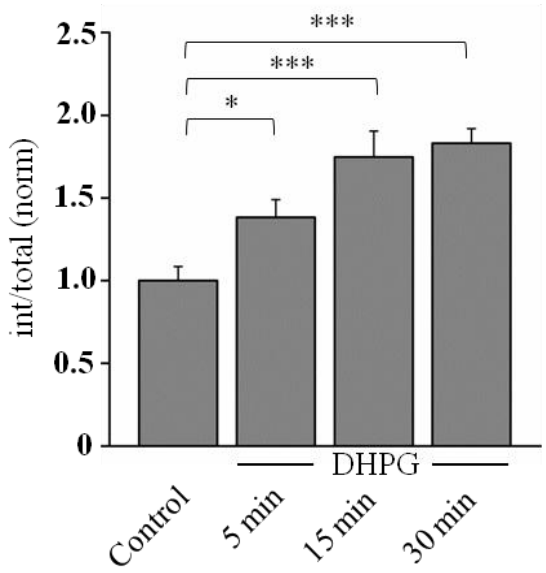


Figure 3.1. DHPG-mediated endocytosis of myc-mGluR1 in HEK293 cells. (A) Control cells showed very little internal fluorescence and subsequent to the 100 μM R,S-DHPG application, receptors internalized. The extent of endocytosis increased over successive time points (5 min, and 15 min) and maximum endocytosis was observed at 30 min. (B) Quantitation of R,S-DHPG mediated myc-mGluR1 internalization also showed gradual increase in the endocytosis index which was maximum at 30 min. Scale bar = 10 μm. * indicates $p < 0.05$ and *** indicates $p < 0.001$.

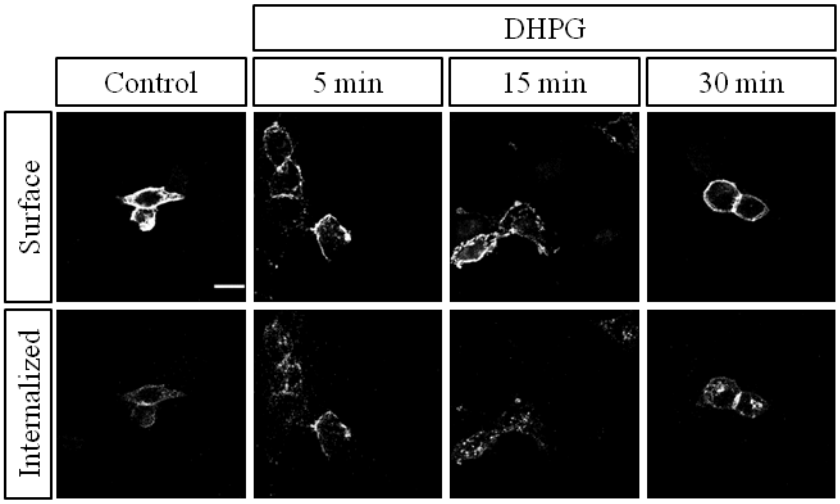
internal fluorescence when cells were chased for longer time periods (**15 min =1.75 ± 0.16, 30 min = 1.83 ± 0.09)**. The endocytosis of myc-mGluR1 was maximum at 30 min post-ligand exposure.

We subsequently studied the ligand-mediated internalization of myc-mGluR5, the other member of the group I mGluR family. In control cells, majority of the receptors were observed to be present at the cell surface **(control = 1 ± 0.15) (Figure 3.2 A, B)**. 100 μM R,S-DHPG application induced myc-mGluR5 internalization at 5 min, as observed by an increase in the internal fluorescence **(5 min = 1.41 ± 0.21)**. A gradual increase in the internalized receptors was observed when cells were chased for longer time points **(15 min = 1.75 ± 0.12)** and maximum internalization was observed at 30 min **(30 min = 2.27 ± 0.16)**.

3.2.2. Recycling of group I mGluRs subsequent to the ligand-dependent internalization in HEK293 cells

As shown above, upon agonist application, group I mGluRs get endocytosed in HEK293 cells. Our group demonstrated that both mGluR1 and mGluR5 entered the recycling compartment subsequent to the ligand-mediated internalization (Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015). In order to study the recycling of myc-mGluR1 after ligand-mediated internalization, HEK293 cells were transfected with myc-mGluR1 cDNA according to the protocol described in the "materials and methods" chapter. Subsequently, live cells expressing myc-mGluR1 were stained with anti-myc mouse monoclonal primary antibody (1:500) for 15 min at 37˚C. 100 μM R,S-DHPG was applied for 5 min. After removing the ligand, cells were chased for extended time periods (1 hr, 1.5 hr, 2 hr, 2.5 hr) at 37˚C 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. After that, saturating concentration of the first secondary antibody, i.e., goat anti-mouse Alexa-568 (1:100) was applied to label the surface receptors and cells were then permeabilized in 0.1% Triton X-100. Subsequently, second secondary antibody, i.e., goat anti-mouse Alexa-647 (1:750) was applied to visualize the internalized receptors. Finally, cells were mounted on glass slides and observed under the confocal microscope.

(A)



(B)

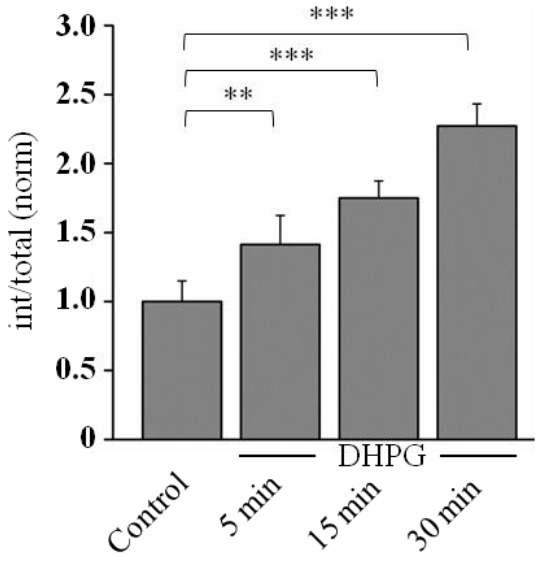


Figure 3.2. Endocytosis of myc-mGluR5 in HEK293 cells. (A) Representative images showing myc-mGluR5 endocytosis at different time points. Control cells showed very little internal fluorescence and upon 100 μM R,S-DHPG application there was significant endocytosis observed in 5 min. The extent of endocytosis increased over subsequent time points and maximum endocytosis was observed at 30 min. (B) Quantitation of the internalization also suggested that the endocytosis of myc-mGluR5 showed gradual increase with maximum endocytosis at 30 min. Scale bar = 10 μm. ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

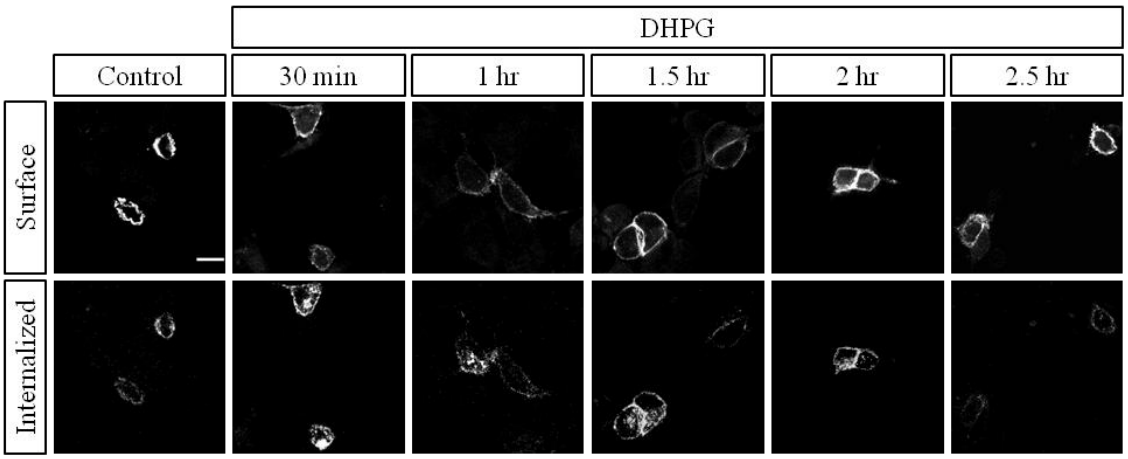
In control cells, majority of the receptors were observed to be present on the cell surface and very little endocytosed receptors were visible **(control = 1 ± 0.10)**. After R,S-DHPG application majority of the receptors internalized at 30 min **(30 min = 2.50 ± 0.16)**. When cells were chased for longer time periods, receptors recycled back to the cell surface at 2.5 hr **(1 hr = 2.13 ± 0.21, 1.5 hr = 2.05 ± 0.18, 2 hr = 1.59 ± 0.16, 2.5 hr = 0.80 ± 0.05) (Figure 3.3 A, B)**.

We further studied the recycling of myc-mGluR5 using similar protocol as described above. Maximum internalization of the receptor was observed at 30 min post ligand application **(control = 1 ± 0.11, 30 min = 2.33 ± 0.18)**. When receptors were chased for longer time periods in the absence of the ligand, most of the receptors recycled back to the cell surface at 2.5 hr **(1 hr = 1.80 ± 0.16, 1.5 hr = 1.34 ± 0.13, 2 hr = 1.25 ± 0.12, 2.5 hr = 0.98 ± 0.13) (Figure 3.4 A, B)**.

3.2.3. Effect of SNX1-CC over-expression on the ligand-mediated trafficking of group I mGluRs

In order to investigate whether SNX1 plays any role in the trafficking of group I mGluRs, experiments were performed to study the ligand-induced trafficking of myc-mGluR1 and myc-mGluR5 in the presence of the C-terminus coiled-coil domain of SNX1 that functions in a dominant-negative manner (Wang, Zhou et al. 2002). SNX1 C-terminus encodes a coiled-coil domain (SNX1-CC) that assembles with the endogenous SNX1 to function as a dominant negative (Wang, Zhou et al. 2002). To study the role of the dominant-negative SNX1 in the ligand-dependent trafficking of myc-mGluR1, HEK293 cells were co-transfected with myc mGluR1 and SNX1-CC constructs. 24 hr post-transfection, live cells expressing myc-mGluR1 were stained with the anti-myc primary antibody (1:500) for 15 min at 37˚C. Subsequent application of 100 μM R,S-DHPG led to the internalization of myc-mGluR1 at 30 min in control cells. On the other hand, SNX1-CC expressing cells did not show significant internalized receptors on 100 μM R,S-DHPG application at 30 min time point and most of the receptors were observed to be localized at the cell surface **(control = 1 ± 0.08; control + DHPG = 2.14 ± 0.21; SNX1-CC = 1 ± 0.08; SNX1-CC + DHPG = 0.94 ± 0.10) (Figure 3.5 A, B)**.

(A)



(B)

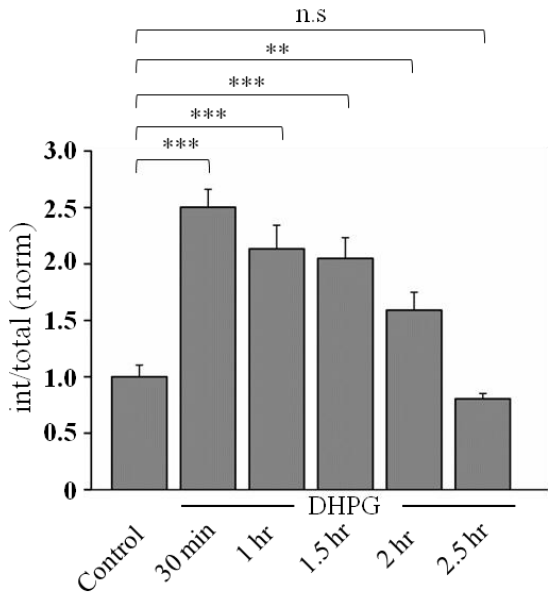
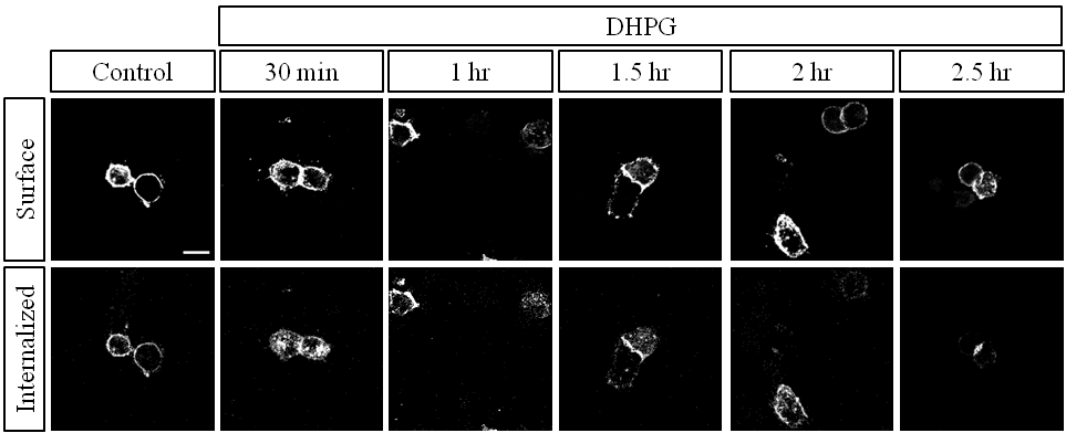


Figure 3.3. myc-mGluR1 recycles back to the cell surface subsequent to the R,S-DHPG-mediated internalization in HEK293 cells. (A) Control cells showed surface localization of majority of the receptors and very low internal fluorescence. After 100 μM R,S-DHPG application, the receptors internalized at 30 min post-ligand application. Chasing the cells for extended time periods (1 hr, 1.5 hr, 2 hr and 2.5 hr) in the absence of the ligand, showed the recycling of the internalized receptors back to the cell surface and majority of the internalized receptors recycled back to the cell surface in 2.5 hr. (B) Quantitation also showed the recycling of ligand-induced internalized myc-mGluR1 in 2.5 hr. Scale bar = 10 μm. ** indicates $p < 0.01$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

(A)



(B)

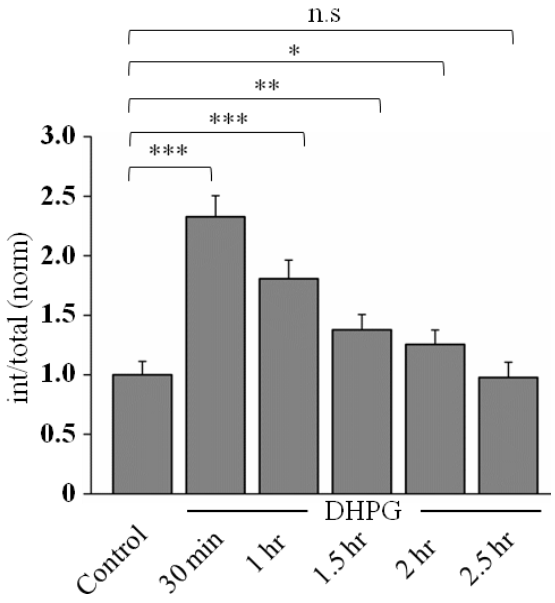
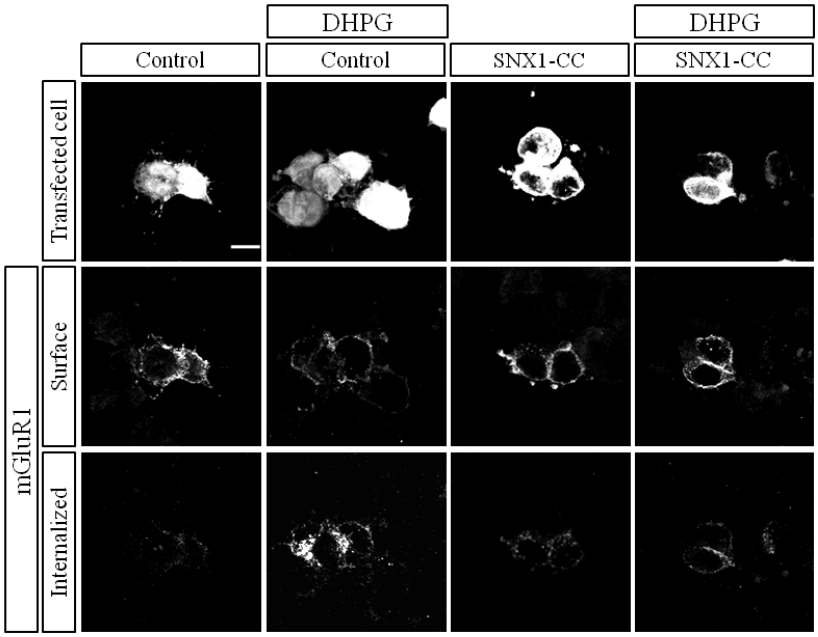


Figure 3.4. Recycling of myc-mGluR5 followed by R,S-DHPG-mediated internalization in HEK293 cells. (A) Representative images showing the recycling of 100 μM R,S-DHPG-mediated internalized myc-mGluR5. In control cells, majority of the receptors were present at the cell surface which internalized in 30 min post-ligand treatment. When cells were chased for longer time periods (1 hr, 1.5hr, 2 hr and 2.5 hr) in the absence of the ligand there was a gradual decrease in the internal fluorescence and a corresponding increase in the surface fluorescence observed and in 2.5 hr majority of the internalized receptors recycled back to the cell surface. (B) Quantitation of the internalized myc-mGluR5 at different time points also suggested that at 2.5 hr majority of the R,S-DHPG-mediated internalized receptors recycled back to the cell surface. Scale bar = 10 μm. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

(A)



(B)

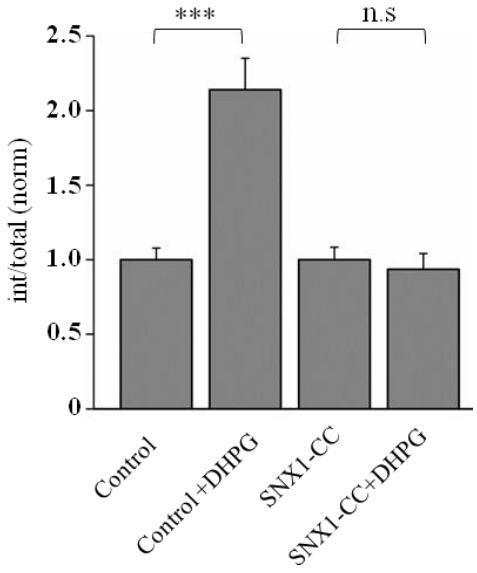
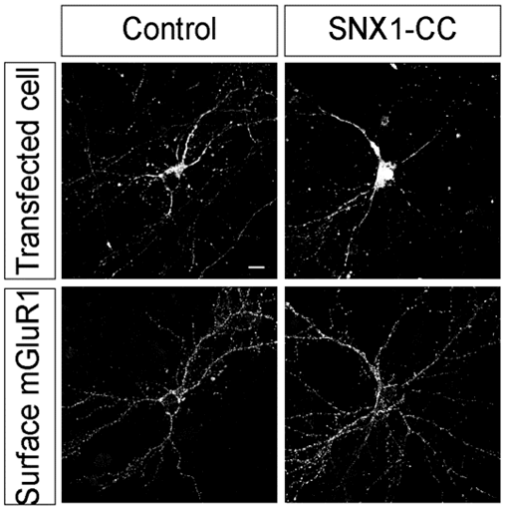


Figure 3.5. Over-expression of the dominant-negative SNX1 (SNX1-CC) affects the ligand-mediated trafficking of myc-mGluR1 in HEK293 cells. (A) Control cells showed presence of majority of the receptors at the cell surface and upon 100 μM R,S-DHPG application myc-mGluR1 internalized. Importantly, in SNX1-CC over-expressing cells myc-mGluR1 trafficking was affected. (B) Quantitation also suggested that over-expression of SNX1-CC altered the R,S-DHPG-mediated trafficking of myc-mGluR1. Scale bar = 10 μm. *** indicates $p < 0.001$ and, n.s indicates $p > 0.05$.

We subsequently studied the effect of SNX1-CC on the ligand-mediated trafficking of group I mGluRs in primary hippocampal neurons. Initially, we investigated the role of SNX1-CC on the surface expression of myc-mGluR1, if any. Primary hippocampal neurons expressing myc-mGluR1 were stained with the mouse anti-myc primary antibody (1:200) to label the surface receptors. Cells were then fixed without permeabilization in 4% PFA on ice for 15 min. Subsequently, cells were incubated in goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) for 1 hr at 37˚C. Finally, cells were mounted on glass slides and observed under the confocal microscope. There was no significant difference observed in the surface myc-mGluR1expression level between control cells and cells in which SNX1-CC was overexpressed. As neuron is a compartmentalized cell, we quantified the effect of SNX1-CC over-expression on the myc-mGluR1 surface expression in the dendrite separately. The dendritic region was defined by the area that was 10 μm away from the soma. Our data suggested that dominant negative mutant of SNX1 (SNX1-CC) had no effect on the dendritic surface expression of myc-mGluR1 **(control = 1 ± 0.04, SNX1-CC = 1 ± 0.05) (Figure 3.6 A, B)**.

We next investigated whether the dominant-negative SNX1 plays any role in the ligand-mediated trafficking of myc-mGluR1 in primary hippocampal neurons. Briefly, primary hippocampal neurons were co-transfected with myc-mGluR1 and SNX1-CC constructs. Live cells expressing myc-mGluR1 were stained with anti-myc primary antibody (1:200). Subsequently, 100 μM R,S-DHPG was applied for 5 min to induce internalization of the receptor, followed by a chase of 30 min in the absence of the ligand. Surface and internalized receptors were then labelled with the secondary antibodies using the protocol described before. In control cells, application of 100 μM R,S-DHPG led to the internalization of myc-mGluR1 at 30 min, whereas cells expressing SNX1-CC did not show significant internalized receptors on 100 μM R,S-DHPG application at 30 min time point and most of the receptors were observed to be localized at the cell surface **(control: 1 ± 0.09; control + DHPG: 2.3 ± 0.16; SNX1-CC: 1 ± 0.07; SNX1-CC + DHPG: 1.1 ± 0.1) (Figure 3.7 A, B)**.

(A)



(B)

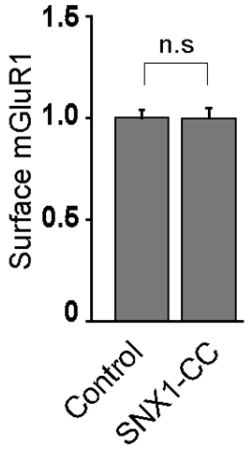
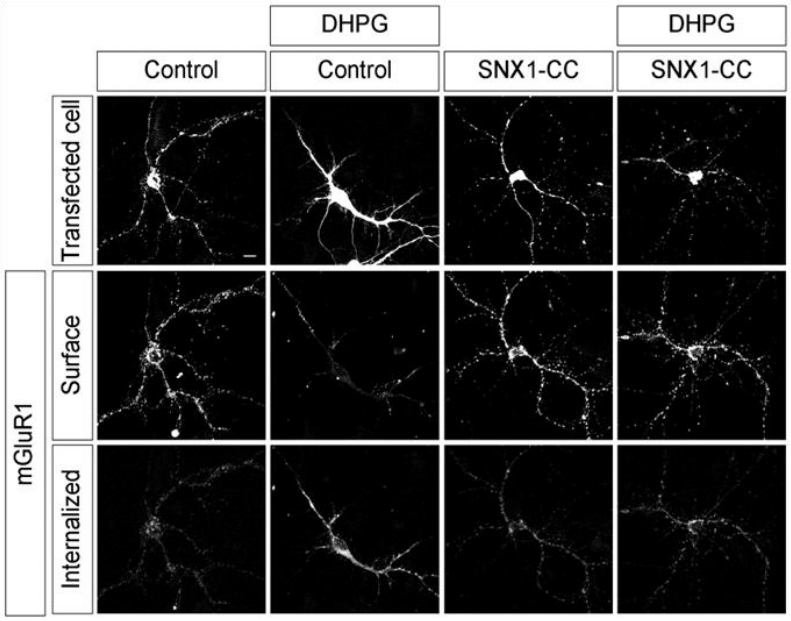


Figure 3.6. Over-expression of the SNX1-CC domain does not affect the surface expression of myc-mGluR1 in primary hippocampal neurons. (A) Representative images showing the surface expression of myc-mGluR1 in control cells and SNX1-CC over-expressing cells. Over-expression of the SNX1-CC domain had no effect on the surface expression of myc-mGluR1. (B) Quantitation of the surface expression of myc-mGluR1 also showed no significant difference between the control cells and SNX1-CC over-expressing cells. Scale bar = 10 μm. n.s indicates $p > 0.05$.

(A)



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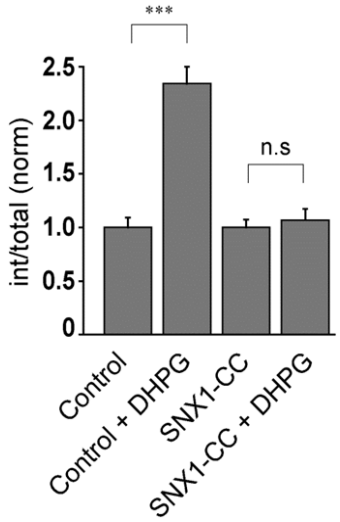


Figure 3.7. Over-expression of the SNX1-CC domain affects the ligand-mediated trafficking of myc-mGluR1 in primary hippocampal neurons. (A) Control cells showed presence of majority of the receptors at the cell surface. The receptors internalized on 100 μM R,S-DHPG application. Over-expression of the SNX1-CC construct affected the ligand-mediated trafficking of myc-mGluR1. (B) Quantitation of the effect of SNX1-CC over-expression on the R,S-DHPG-mediated trafficking of myc-mGluR1. Scale bar = 10 μm. *** indicates $p < 0.001$, n.s indicates $p > 0.05$.

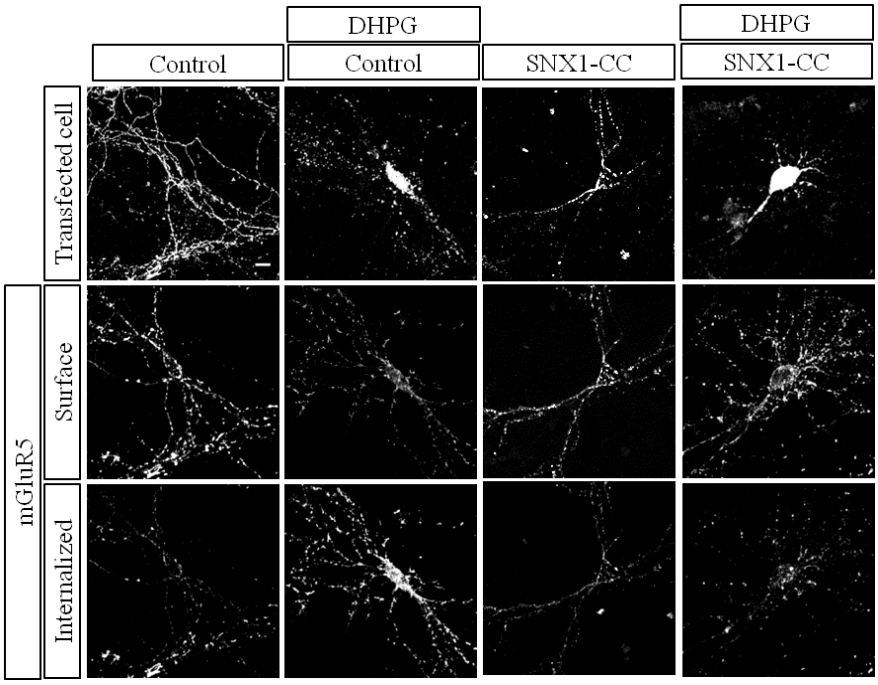
We chose 30 min as the time point since our earlier data suggested that upon R,S-DHPG-application majority of the myc-mGluR1 internalized at 30 min (Pandey et al., 2014). These results suggested that expression of the dominant-negative SNX1 either inhibited the ligand-mediated internalization of myc-mGluR1 or the receptor recycled back faster to the cell surface in 30 min in the presence of SNX1-CC.

Since, group I mGluRs comprise of two receptors, mGluR1 and mGluR5, and over-expression of the dominant-negative SNX1 affected the ligand-mediated trafficking of mGluR1, we also investigated the effect of SNX1-CC on the ligand-mediated trafficking of mGluR5 as well. Primary hippocampal neurons were cotransfected with myc-mGluR5 and SNX1-CC constructs. Subsequently, the endocytosis experiment was performed following the protocol as discussed above. These experiments suggested that similar to myc-mGluR1, SNX1-CC expressing neurons did not show a significant amount of internalized myc-mGluR5 on 100 μM R,S-DHPG application at 30 min post ligand application **(control = 1 ± 0.09; control + DHPG = 1.78 ± 0.10; SNX1-CC = 1 ± 0.15; SNX1-CC + DHPG = 0.99 ±0.06) (Figure 3.8 A, B)**.

3.2.4. Over-expression of the dominant-negative SNX1 (SNX1-CC) resulted in the faster recycling of group I mGluRs

Our earlier data suggested that the dominant-negative SNX1 either blocked the R,S-DHPG-mediated internalization of group I mGluRs or led to the rapid recycling of these receptors. In order to determine whether over-expression of SNX1-CC led to the inhibition in the R,S-DHPG-mediated internalization of myc-mGluR1 or it resulted in the rapid recycling of the receptor to the cell surface, we performed time course of endocytosis experiment in HEK293 cells. myc-mGluR1 expressing cells were stained with the anti-myc primary antibody. Subsequently, application of the 100 μM R,S-DHPG led to the rapid internalization of the receptor. The amount of myc-mGluR1 internalization increased over time till 30 min post-ligand application **(untreated = 1 ± 0.08; 30 min = 2.07 ± 0.18)**. Quantitation of the receptors localized

(A)



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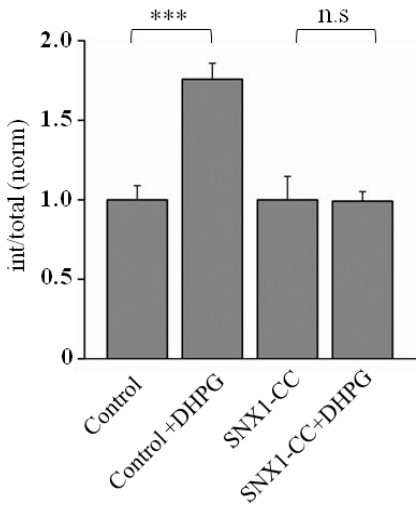


Figure 3.8. Effect of the over-expression of SNX1-CC on the ligand-mediated trafficking of myc-mGluR5 in primary hippocampal neurons. (A) Over-expression of the SNX1-CC domain had an effect on the ligand-mediated trafficking of myc-mGluR5 as shown in the representative images. (B) Quantitation also suggested that the SNX1-CC over-expression led to the altered trafficking of myc-mGluR5. Scale bar = 10 μm. *** indicates $p < 0.001$, n.s indicates $p > 0.05$.

on the cell surface also showed the corresponding decrease in the receptor level from the cell surface on application of the 100 μM R,S-DHPG over time **(untreated = 1 ± 0.06; 30 min = 0.51 ± 0.06)**. Interestingly, in SNX1-CC expressing cells, myc-mGluR1 internalized upon application of 100 μM R,S-DHPG, but it recycled back to the cell surface at 30 min **(untreated = 1 ± 0.11; 5 min = 2.43 ± 0.25; 15 min = 1.84 ± 0.20; 30 min = 0.93 ± 0.10) (Figure 3.9 A, B)**. Furthermore, the measurement of the surface myc-mGluR1 also showed a decrease in the surface localization of the receptor 5 min post-ligand application and subsequent recovery of the surface fluorescence at the untreated level at 30 min **(untreated = 1 ± 0.08; 5 min = 0.54 ± 0.06; 15 min = 0.75 ± 0.07; 30 min = 1.08 ± 0.09) (Figure 3.9 C)**. Previous reports have shown that myc-mGluR1 normally recycles back to the cell surface at 2.5 hr subsequent to the R,S-DHPG-mediated internalization (Pandey et al., 2014). Therefore, the above results suggested that myc-mGluR1 underwent faster recycling in the presence of the dominant-negative SNX1.

Since our earlier experiments suggested that over-expression of the dominant negative SNX1 also affected the trafficking of myc-mGluR5, we therefore investigated whether over-expression of SNX1-CC resulted in the faster recycling of myc-mGluR5 as well. HEK293 cells were transfected with myc-mGluR5 and SNX1-CC constructs, followed by the trafficking experiment in a similar way as described above. In control cells, myc-mGluR5 internalized rapidly upon 100 μM R,S-DHPG application at 5 min and maximum internalization was observed at 30 min time point **(untreated = 1 ± 0.12; 5 min = 1.47 ± 0.16; 30 min = 2.07 ± 0.29)**. The quantitation of myc-mGluR5 present at the cell surface also showed a corresponding decrease in the receptor level from the cell surface on application of 100 μM R,S-DHPG over time **(untreated = 1 ± 0.05; 5 min = 0.86 ± 0.10; 30 min = 0.71 ± 0.05)**. Importantly, in SNX1-CC expressing cells, application of 100 μM R,S-DHPG showed a similar kinetics of myc-mGluR5 trafficking as was observed in the case of myc-mGluR1. Measurement of the endocytosis index showed an increased level of internalized receptors at 5 min that subsequently decreased at 30 min post ligand application **(untreated = 1 ± 0.14; 5 min = 1.88 ± 0.16; 30 min = 0.82 ± 0.11)**.

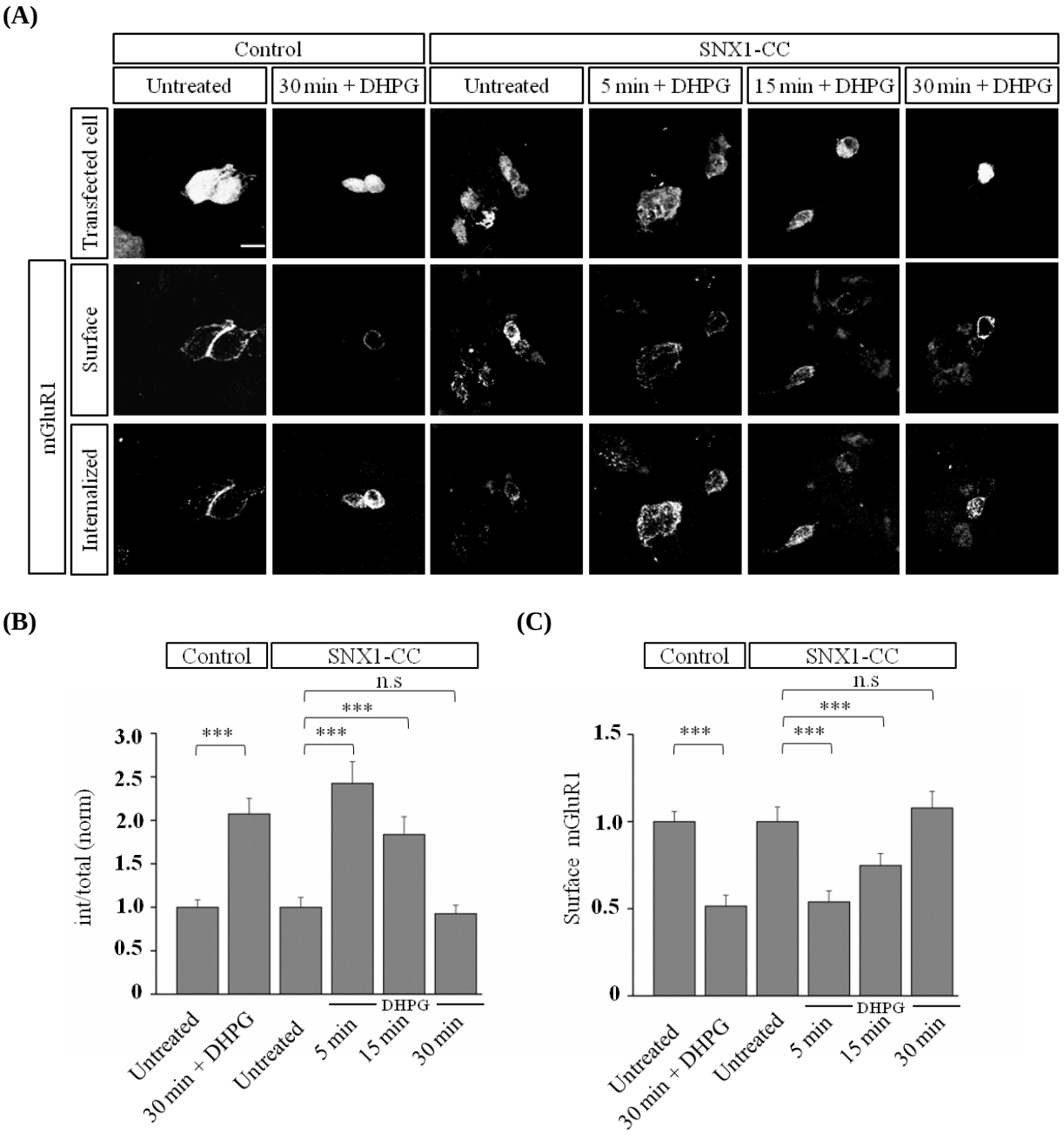


Figure 3.9. Over-expression of the SNX1-CC domain results in the faster recycling of myc-mGluR1 in HEK293 cells. (A) Control cells showing majority of the receptors localized at the cell surface which internalized after 100 μM R,S-DHPG application at 30 min. whereas, in cells over-expressing SNX1-CC, maximum receptor internalization was observed at 5 min after the R,S-DHPG application and the internalized receptors recycled back to the cell surface at 30 min. (B, C) Quantitation of the endocytosis (B) and surface fluorescence (C) also suggested that the cells expressing SNX1-CC showed maximum internalization of myc-mGluR1 at 5 min after 100 μM R,S-DHPG application and receptors recycled back to the cell surface at 30 min. Scale bar = 10 μm. *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

Correspondingly, the quantitation of the surface localized myc-mGluR5 also showed recovery of the surface fluorescence at the untreated level at 30 min post R,S-DHPG-mediated internalization of the receptor **(untreated = 1 ± 0.07; 5 min = 0.67 ± 0.08; 30 min = 1.01 ± 0.07) (Figure 3.10 A- D)**.

We next investigated whether myc-mGluR1 rapidly recycled back to the cell surface in the presence of SNX1-CC in primary hippocampal neurons. myc-mGluR1 and SNX1-CC constructs were co-transfected in primary hippocampal neurons using calcium phosphate method on 6 - 8 DIV and the experiment was performed on 12 - 15 DIV as described in the "method" section. In control cells, myc-mGluR1 internalized normally upon 100 μM R,S-DHPG pulse and endocytosis index showed a gradual increase till 30 min time point **(untreated = 1 ± 0.07; 5 min = 1.55 ± 0.07; 15 min = 1.78 ± 0.09; 30 min = 2.46 ± 0.11) (Figure 3.11 A, B)**. As expected, quantitation of the surface localized myc-mGluR1 also showed a corresponding decrease in the surface receptor level till 30 min post R,S-DHPG application **(untreated = 1 ± 0.05; 5 min = 0.82 ± 0.07; 15 min = 0.59 ± 0.05; 30 min = 0.47 ± 0.05) (Figure 3.11 C)**. On the other hand, measurement of the endocytosis index suggested that in SNX1-CC expressing cells myc-mGluR1 internalized upon ligand application and recycled back to the cell surface 30 min post-ligand application **(untreated = 1 ± 0.07; 5 min = 2.38 ± 0.08; 15 min = 1.93 ± 0.1; 30 min = 1 ± 0.08) (Figure 3.11 D)**. Quantitation of the surface localized myc-mGluR1 also suggested a decrease in the surface fluorescence subsequent to the application of 100 μM R,S-DHPG which was rescued at 30 min post-ligand application **(untreated = 1 ± 0.05; 5 min = 0.61 ± 0.05; 15 min = 0.72 ± 0.06; 30 min = 0.9 ± 0.05) (Figure 3.11 E)**. Earlier reports have shown that myc-mGluR1 normally recycles back to the cell surface at 2.5 hr subsequent to the R,S-DHPG-mediated internalization in primary hippocampal neurons (Pandey et al., 2014). All these results suggested that in the presence of dominant-negative SNX1, group I mGluRs showed faster recycling subsequent to the ligand-dependent internalization as compared to in control cells.

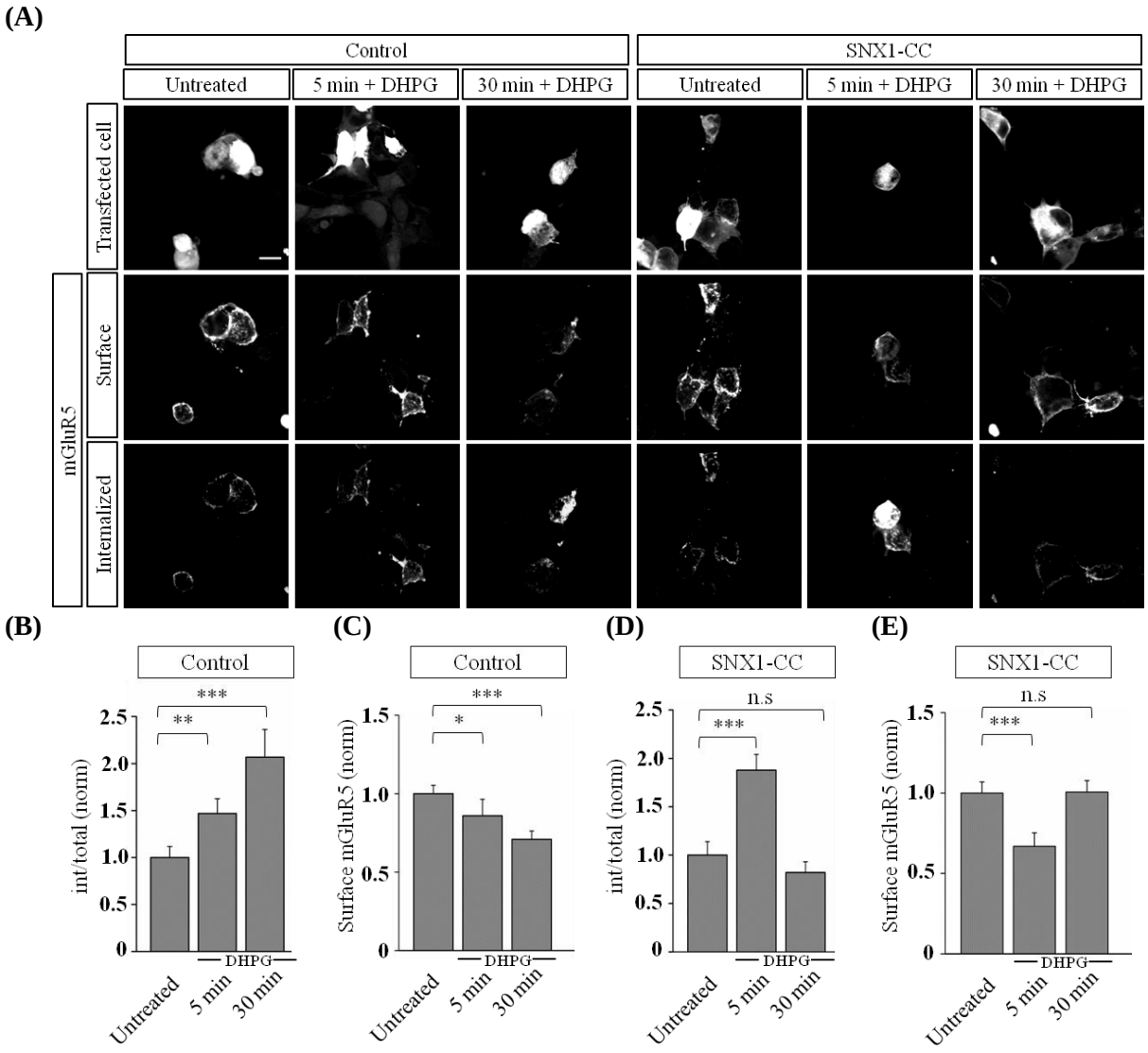


Figure 3.10. SNX1-CC over-expression results in the faster recycling of myc-mGluR5 in HEK293 cells. Representative images showing the R,S-DHPG-mediated internalization of myc-mGluR5 at different time points. Control cells showed high surface and low internal fluorescence and upon 100 μM R,S-DHPG application, there was gradual increase in the endocytosis as observed from 5 min to 30 min. On the other hand, in cells over-expressing SNX1-CC, majority of the receptors internalized at 5 min after 100 μM R,S-DHPG application and recycled back to the cell surface at 30 min. (B - E) Quantitation of the endocytosis index (B) and measurement of the surface localized myc-mGluR5 (C) at various time points also showed that the ligand-induced internalization of myc-mGluR5 started at 5 min which increased to the maximum at 30 min in the control cells. Whereas, in SNX1-CC over-expressing cells, the quantitation of both the internalized (D) as well surface localized (E) receptors showed that the R,S-DHPG-induced endocytosis was maximum at 5 min and majority of the receptors recycled back to the cell surface at 30 min. Scale bar = 10 μm. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

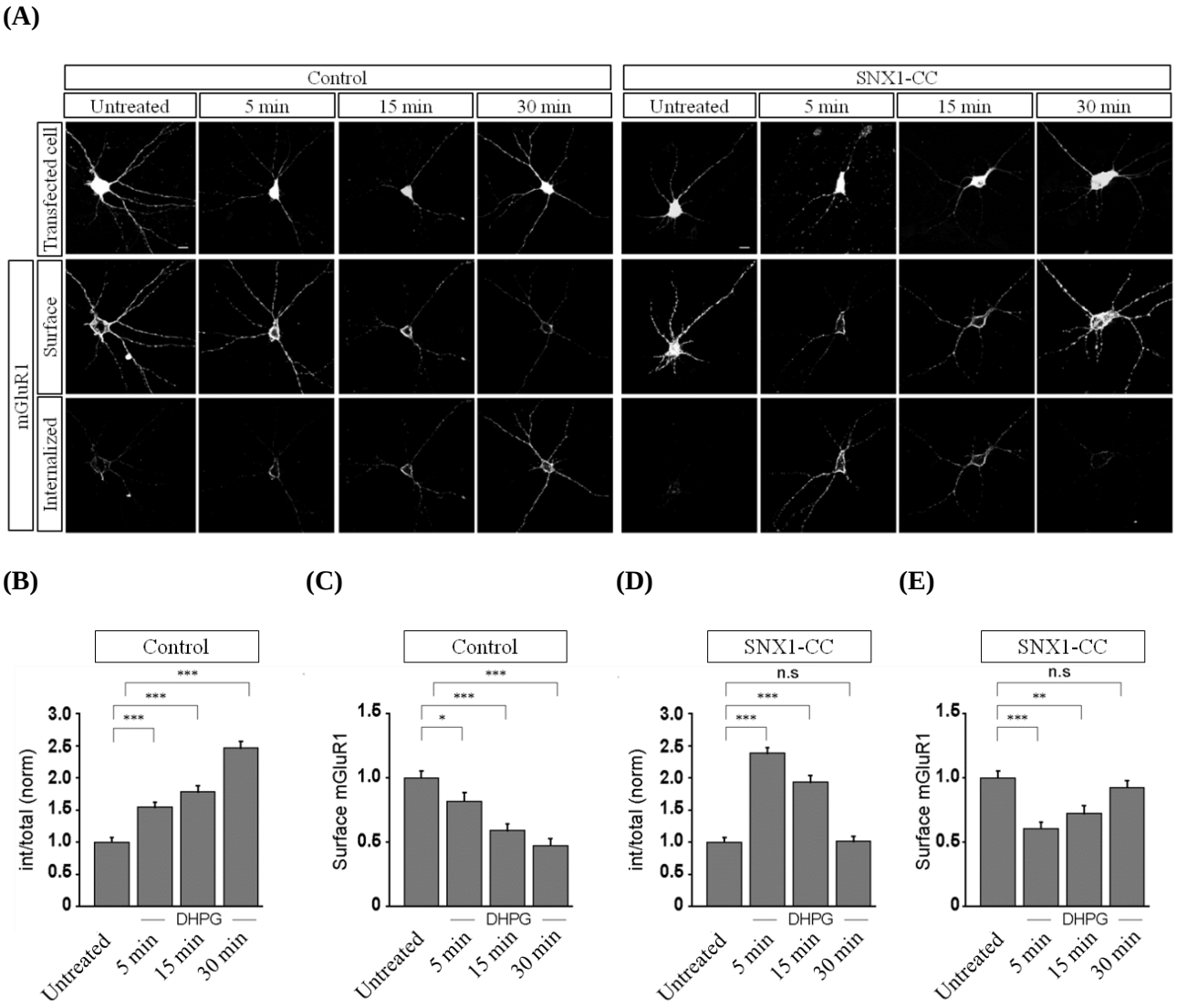


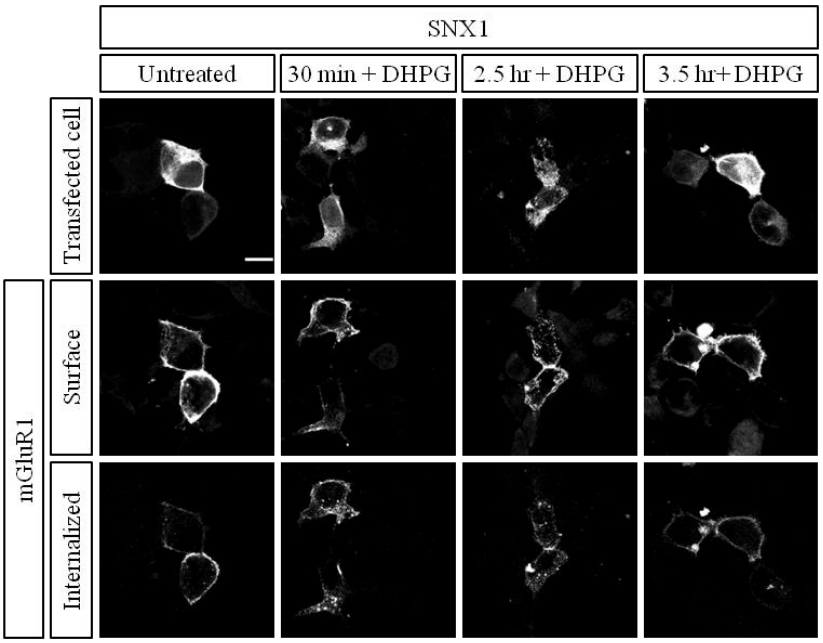
Figure 3.11. Over-expression of the SNX1-CC domain results in the faster recycling of myc-mGluR1 in primary hippocampal neurons. (A) Representative images showing the kinetics of 100 μM R,S-DHPG-mediated trafficking of myc-mGluR1 in control and SNX1-CC over-expressed primary hippocampal neurons. (B, C) In control cells, ligand-induced internalization showed progressive increase in the endocytosis of the receptor till 30 min as shown by the quantitation of the endocytosis (B) as well as measurement of the surface myc-mGluR1 (C). (D, E) On the other hand, in cells overexpressing SNX1-CC, the receptor showed maximum internalization at 5 min after 100 μM R,S-DHPG application and recycled back to the cell surface at 30 min, as shown by the quantitation of the endocytosis index (D) and the quantitation of the surface receptors (E). Scale bar = 10 μm. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

3.2.5. Over-expression of the full-length SNX1 delays the recycling of mGluR1

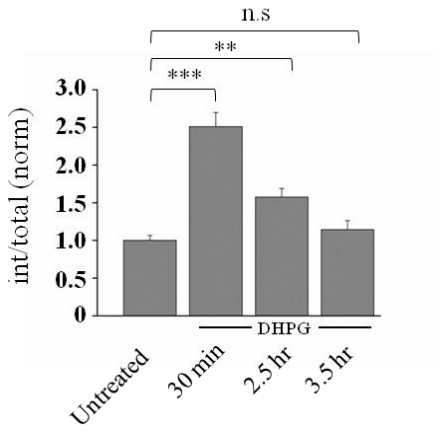
Our earlier experiments suggested that the over-expression of the C-terminal coiled-coil domain of SNX1, which acts as a dominant-negative, resulted in the rapid recycling of group I mGluRs. Therefore, we were interested in examining whether the full-length SNX1 over-expression would have any effect on the recycling of group I mGluRs. The myc-mGluR1 and full-length SNX1 constructs were cotransfected in HEK293 cells. 24 hr post transfection, myc-mGluR1 recycling experiment was performed using the method described earlier. Results from our earlier experiments suggested that in wild-type cells the internalized receptors recycled back to the cell surface in 2.5 hr. Interestingly, over-expression of the full-length SNX1 resulted in a delay in the recycling of myc-mGluR1. The receptors were present at the internal compartment even at 2.5 hr post-ligand application and they recycled back to the cell surface in 3.5 hr post-ligand application **(untreated = 1 ± 0.10; 30 min = 2.42 ± 0.22; 2.5 hr = 1.62 ± 0.14; 3.5 hr = 1.21 ± 0.17) (Figure 3.12 A, B)**. Correspondingly, complete recovery of the surface fluorescence was observed at 3.5 hr, suggesting that over-expression of the full-length SNX1 significantly delayed the recycling of myc-mGluR1 in HEK293 cells **(untreated = 1 ± 0.08; 30 min = 0.52 ± 0.08; 2.5 hr = 0.67 ± 0.06; 3.5 hr = 0.97 ± 0.09) (Figure 3.12 C)**.

Subsequently, we extended our studies in primary hippocampal neurons. Initially, we examined the effect of the full-length SNX1 over-expression on the surface expression of myc-mGluR1 following the method described before. There was no significant difference in the myc-mGluR1 surface expression observed between the control cells and cells in which full-length SNX1 was over-expressed **(control: 1 ± 0.04; SNX1: 1 ± 0.08) (Figure 3.13 A, B)**. We next investigated whether over-expression of the full-length SNX1 affects the recycling of myc-mGluR1 in primary hippocampal neurons. The recycling assay was performed using the protocol described in the "materials and methods" chapter. In control cells, subsequent to the application of 100 µM R,S-DHPG myc-mGluR1 internalized and recycled back to the cell surface

(A)



(B)



(C)

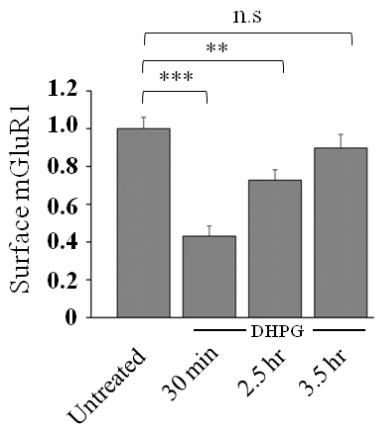
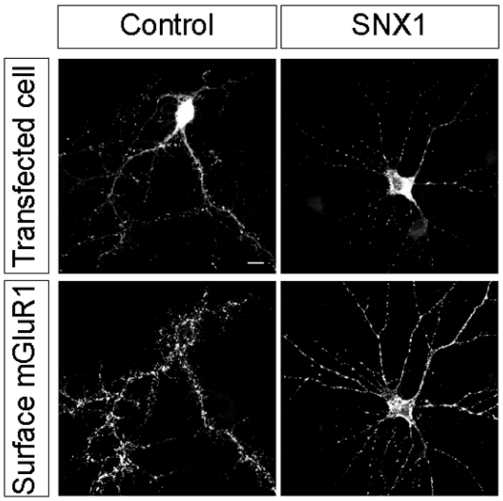


Figure 3.12. Over-expression of the full-length SNX1 delays the recycling of myc-mGluR1 in HEK293 cells. (A) Representative images showing the kinetics of 100 µM R,S-DHPG-mediated internalization and recycling of myc-mGluR1 in HEK293 cells over-expressing full-length SNX1. (B) Quantitation of the endocytosis index showed maximum internalization of the receptor at 30 min post 100 µM R,S-DHPG application and the receptor recycled back to the cell surface in 3.5 hr. (C) Quantitation of the surface receptors also showed decrease in the surface fluorescence at 30 min and recovery of the surface fluorescence at 3.5 hr suggesting that the over-expression of the full-length SNX1 delayed the recycling of myc-mGluR1 and receptors recycled back to the cell surface in 3.5 hr. Scale bar = 10 µm. ** indicates $p < 0.01$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

(A)



(B)

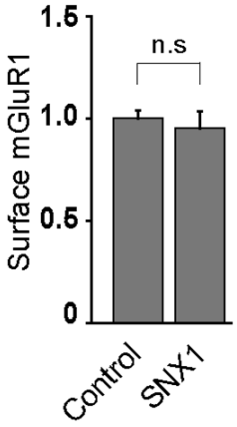


Figure 3.13. Effect of the over-expression of the full-length SNX1 on the surface expression of myc-mGluR1 in primary hippocampal neurons. (A) Representative images showing myc-mGluR1 surface expression in control and full-length SNX1 expressing cells. (B) Quantitation of the surface myc-mGluR1 suggested that over-expression of the full-length SNX1 had no effect on the surface expression of myc-mGluR1. Scale bar = 10 μm. n.s indicates $p > 0.05$.

at 2.5 hr post-ligand application as determined by the quantitation of the endocytosis index **(untreated: 1 ± 0.08; 30 min: 1.93 ± 0.09; 1.5 hr: 1.49 ± 0.09; 2.5 hr: 1.1 ± 0.09) (Figure 3.14 A, B)**, as well as the recovery of the cell surface fluorescence **(untreated: 1 ± 0.12; 30 min: 0.56 ± 0.09; 1.5 hr: 0.71 ± 0.1; 2.5 hr: 1 ± 0.15) (Figure 3.14 C)**. Importantly, over-expression of the full-length SNX1 delayed the recycling of myc-mGluR1 and the receptors recycled back to the cell surface in 4.5 hr post-ligand application **(untreated: 1 ± 0.09; 30 min: 2.26 ± 0.11; 2.5 hr: 1.67 ± 0.13; 4.5 hr: 1.08 ± 0.08) (Figure 3.14 D)**. Measurement of the level of surface receptors also showed that at 30 min post-ligand application there was a decrease in the surface fluorescence which recovered at 4.5 hr, suggesting that myc-mGluR1 recycled back to the cell surface at 4.5 hr in SNX1 over-expressing cells **(untreated: 1± 0.07; 30 min: 0.52 ± 0.04; 2.5 hr: 0.69 ± 0.06; 4.5 hr: 1 ± 0.06) (Figure 3.14 E)**. These results suggested that SNX1 played a critical role in the trafficking of mGluR1.

3.2.6. SNX1 does not affect the recycling of transferrin receptors in HEK293 cells

In order to investigate whether the effect of the dominant-negative SNX1 on the recycling of mGluR1 is specific for this receptor or due to a general effect in the trafficking machinery of the cell, we studied the trafficking of transferrin receptors in presence of SNX1-CC in HEK293 cells. The experimental protocol to study the trafficking of transferrin receptors has been discussed in the "methods" section. In brief, HEK293 cells were transfected with the SNX1-CC construct using Lipofectamine 2000 as described before. 24 hrs post transfection, cells were pre-incubated in plain DMEM for 30 min and then Alexa-568 labelled transferrin was applied for 2 min. Subsequently, cells were washed with plain DMEM followed by a chase for the indicated time points in plain DMEM. After that, cells were treated with the acid stripping buffer to remove the remaining transferrin bound at the surface receptors. Following that, cells were fixed with 4% PFA. Cells were then mounted on glass slides and imaged under the fluorescence microscope.

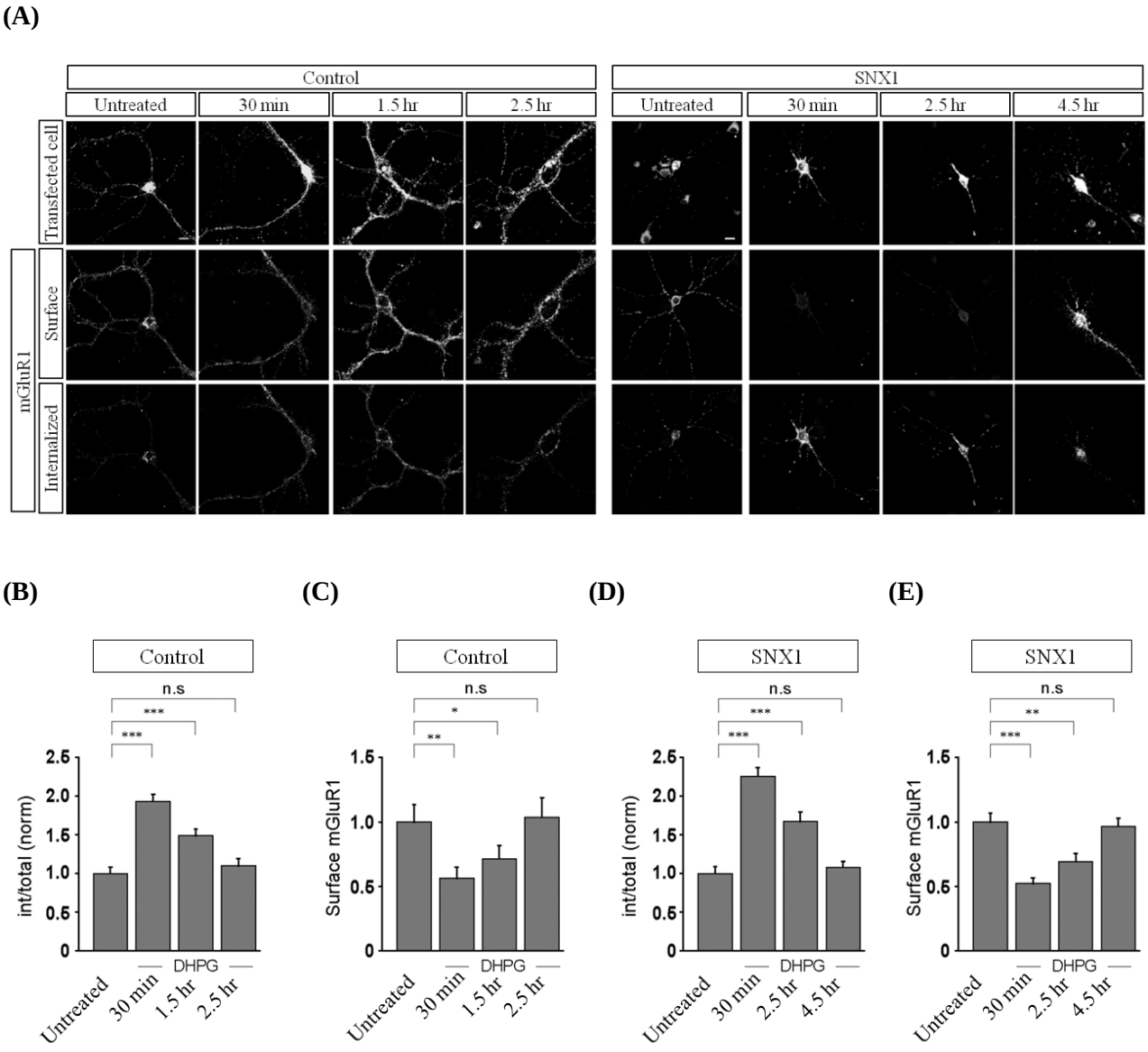


Figure 3.14. Over-expression of the full-length SNX1 delays the recycling of myc-mGluR1 in primary hippocampal neurons. (A) Representative images showing the time course kinetics of ligand-mediated internalization and recycling of myc-mGluR1 in control cells as well as in cells over-expressing full-length SNX1. (B, C) Quantitation of the endocytosis (B) as well as measurement of the surface myc-mGluR1 (C) showed that in control cells 100 µM R,S-DHPG application induced maximum internalization of myc-mGluR1 at 30 min and then receptor recycled back to the cell surface in 2.5 hr. (D, E) On the other hand, over-expression of the full-length SNX1 delayed the recycling of the receptor and myc-mGluR1 recycled back to the cell surface at 4.5 hr post-ligand application as shown by the quantitation of endocytosis (D) and measurement of the surface receptors (E). Scale bar = 10 µm. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

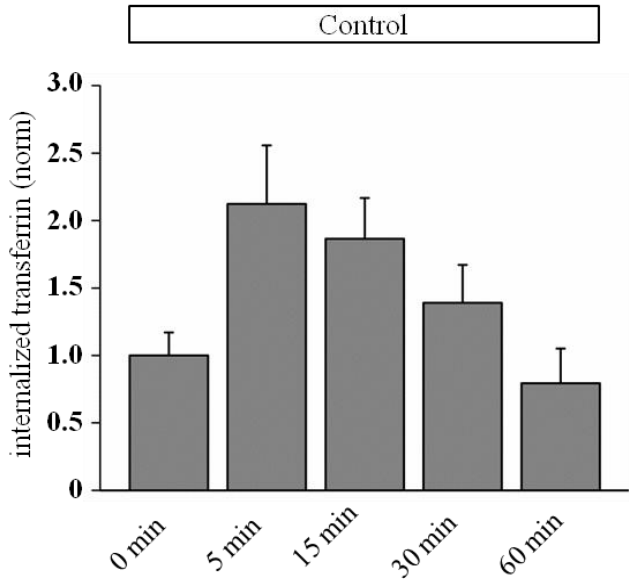
In control cells, majority of the transferrin receptors internalized at 5 min after the application the Alexa-568 labelled transferrin. The receptors that were chased for more extended time periods (15 min, 30 min, 60 min), gradually recycled back to the cell surface and in 60 min most of the receptors recycled back to the cell surface **(control = 1 ± 0.09, 5 min = 2.78 ± 0.25, 15 min = 1.72 ± 0.16, 30 min = 1.35 ± 0.14, 60 min = 0.68 ± 0.14) (Figure 3.15 A)**.

Importantly, in SNX1-CC expressing cells, the kinetics of the transferrin receptor trafficking was similar to that in control cells. The maximum internalization of the transferrin receptor was observed at 5 min post transferrin application and majority of the receptors recycled back to the cell surface in 60 min **(control = 1 ± 0.10, 5 min = 2.79 ± 0.23, 15 min = 1.90 ± 0.26, 30 min = 1.32 ± 0.27, 60 min =0.61 ± 0.15) (Figure 3.15 B)**. These results suggested that SNX1-CC did not affect the general trafficking machinery of the cells and thus, the effect of SNX1-CC on the ligand-mediated trafficking of group I mGluRs was specific to the receptors.

In order to investigate whether over-expression of the full-length SNX1 specifically delayed the recycling of group I mGluRs or it was due to the effect in the general trafficking machinery of the cell, we studied the trafficking of transferrin receptors in the presence of the full-length SNX1 in HEK293 cells. The full-length SNX1 construct was transfected in HEK293 cells and the experiment was performed in a similar way as described above. As expected, control cells showed maximum internalization of Alexa-568 labelled transferrin at 5 min, followed by the recycling of the transferrin receptors to the cell surface **(control = 1 ± 0.16, 5 min = 2.12 ± 0.43, 15 min = 1.86 ± 0.3, 30 min = 1.39 ± 0.28, 60 min = 0.79 ± 0.26) (Figure 3.16 A)**. Similarly, in full-length SNX1 expressing cells, maximum internalization of the transferrin receptor was observed at 5 min post transferrin application and majority of the receptors recycled back to the cell surface at 60 min time point **(control = 1 ± 0.14, 5 min = 2.08 ± 0.43, 15 min = 1.55 ± 0.51, 30 min = 1.36 ± 0.33, 60 min = 0.80 ± 0.25) (Figure 3.16 B)**.

Above results suggested that over-expression the full-length SNX1 specifically delayed the recycling of mGluR1 and it was not due to the effect in the general trafficking machinery in the cell.

(A)



(B)

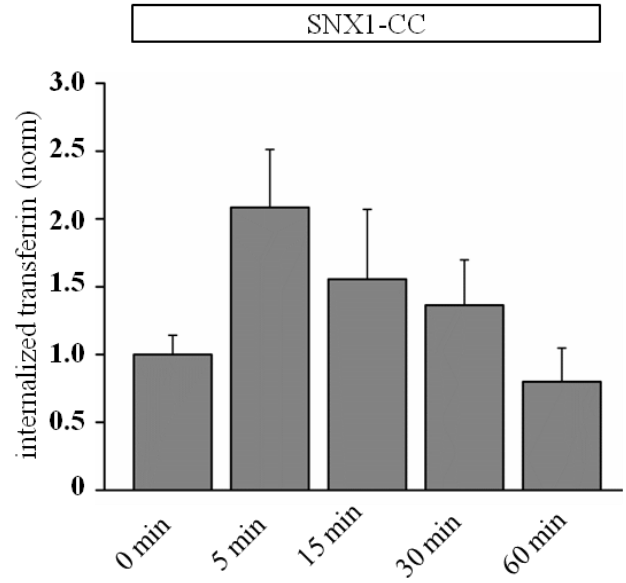
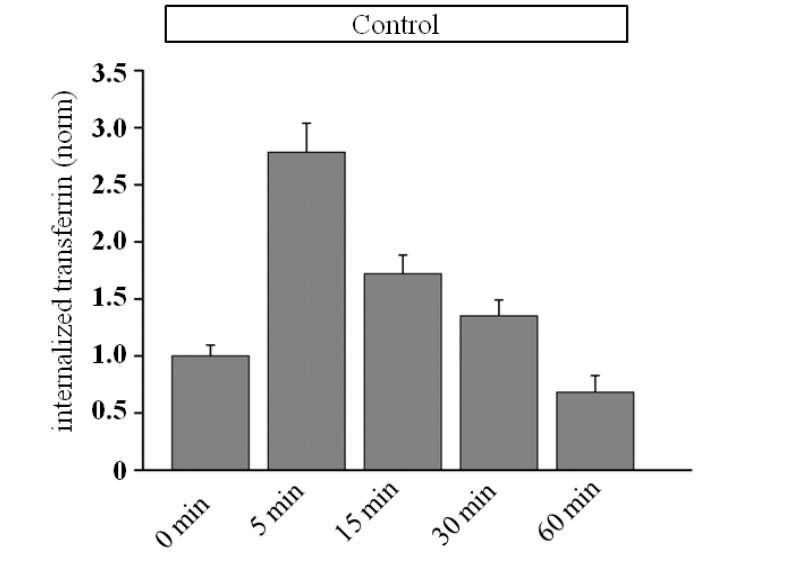


Figure 3.15. Over-expression of the SNX1-CC does not affect the transferrin receptor kinetics. (A) Quantitation of the transferrin receptor internalization and recycling in control HEK293 cells. (B)) Quantitation of the transferrin receptor trafficking kinetics in the SNX1-CC expressing cells showed that in the presence of SNX1-CC the endocytosis and recycling of the transferrin receptors was not affected.

(A)



(B)

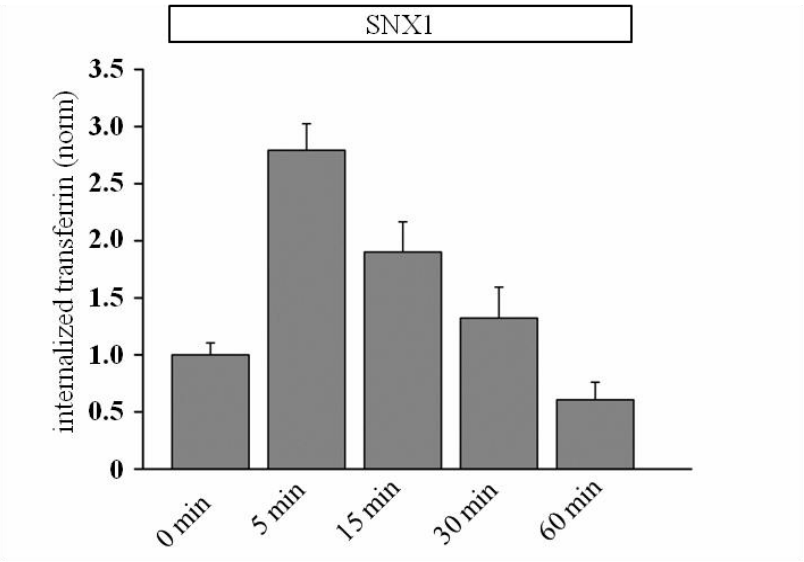


Figure 3.16. Over-expression of the full-length SNX1 does not affect the transferrin receptor kinetics. (A) Quantitation of the endocytosis and recycling of the transferrin receptors in control HEK293 cells. (B) Transferrin kinetics quantitation in the full-length SNX1 over-expressing cells suggesting that full-length SNX1 over-expression did not affect the endocytosis and recycling of the transferrin receptors.

3.3. Discussion

Our lab has earlier shown that, after R,S-DHPG application, group I mGluRs get endocytosed in a ubiquitin-dependent manner and the endocytosed receptors recycle back to the cell surface through a phosphatase-dependent manner (Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015). Relatively less is known about the molecular mechanisms that control the post-endocytic fate of these receptors. Number of proteins that are not directly involved in the signal transduction cascade have been identified which regulate the intracellular sorting of various GPCRs. Sorting nexins are one such group of proteins involved in the intracellular sorting of receptors. Among them, SNX1 regulates the endosome to trans-Golgi network (TGN) retrograde transport of large number of cargos (Worby and Dixon 2002; Wassmer, Attar et al. 2007; Wassmer, Attar et al. 2009). Various other reports have suggested the role of SNX1 in the trafficking and sorting of some receptors through other pathways (Worby and Dixon 2002). For example, SNX1 has been shown to regulate the lysosomal degradation of the protease activated receptor (PAR1), epidermal growth factor receptor (EGFR) and recycling of P2Y1 receptor (Kurten, Cadena et al. 1996; Wang, Zhou et al. 2002; Gullapalli, Wolfe et al. 2006; Nisar, Kelly et al. 2010). Importantly, SNX1 has also been reported to interact *in vitro* with the cytoplasmic tails of mGluR1 and mGluR5 (Heydorn, Sondergaard et al. 2004). In view of the above findings, we investigated the role of SNX1, if any, in the trafficking of group I mGluRs. The results suggested that both myc-mGluR1 and myc-mGluR5 internalized rapidly upon R,S-DHPG application with maximum endocytosis at 30 min and subsequently recycled back to the cell surface in 2.5 hr post-ligand application. In presence of dominant-negative SNX1 both these receptors rapidly recycled back to the cell surface in 30 min. Our data also suggested that over-expression of the full-length SNX1 delayed the recycling of myc-mGluR1. Normally the receptor recycles back in 2.5 hr post-ligand application in both HEK293 cells and primary hippocampal neurons. When full-length SNX1 was over-expressed in HEK293 cells, myc-mGluR1 recycled back in 3.5 hr post-ligand application. On the other hand, over-expression of the full-length SNX1 in primary hippocampal neurons resulted in the recycling of myc-mGluR1 in 4.5 hr post-ligand application. Thus, SNX1 plays crucial role in regulating the post-endocytic sorting of group I mGluRs. Our data ruled out the possibility of alteration in the general trafficking machinery of the cell by SNX1 with the observation that the transferrin receptor

trafficking kinetics was unaltered in the presence of SNX1-CC and full-length SNX1. Together, these results suggest a critical role for SNX1 in the recycling of group I mGluRs in both non-neuronal HEK293 cells and primary hippocampal neurons.

Structure-function analysis of SNX1 in the ligand-mediated trafficking of group I mGluRs

4.1. Introduction

Trafficking of GPCRs plays critical role in the temporal and spatial control of G-protein signaling. The activity of a particular GPCR is also regulated by the trafficking pathway (Rosciglione, Thériault et al. 2014). Subsequent to the desensitization, group I mGluRs undergo internalization and the endocytosed receptors recycle back to the cell surface following that (Mundell, Matharu et al. 2001; Mundell, Pula et al. 2003; Mundell, Pula et al. 2004; Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015; Mahato, Ramsakha et al. 2018). Similar to many other GPCRs, the recycling of group I mGluRs could be important for the resensitization of the receptor (Mundell, Matharu et al. 2001; Mundell, Pula et al. 2004; Choi, Chung et al. 2011; Bhattacharyya 2016). A highly conserved mechanism that governs the sorting of GPCRs, involves the recognition by specific proteins that are part of a particular sorting complex. The understanding of the cellular and molecular mechanisms behind the endosomal sorting of GPCRs is only beginning to emerge. Multiple proteins have been identified that regulate the GPCR endosomal sorting (Drake, Shenoy et al. 2006). One such protein is Sorting Nexin 1 (SNX1) that contains a PX domain which binds to the phosphoinositol 3 phosphate and a bar domain which is involved in the dimerization and binding to highly curved membrane (Kurten, Cadena et al. 1996; Haft, de la Luz Sierra et al. 1998; Kurten, Eddington et al. 2001; Cozier, Carlton et al. 2002). By virtue of its domains, SNX1 is associated with early endosomes and sorting endosomes and plays an important role in directing the surface receptors to their respective fates (Mendrola, Berger et al. 2002; Seaman and Williams 2002; Zhong, Lazar et al. 2002). On the basis of its role in the trafficking of various GPCRs, its expression pattern and its reported interaction with the group I mGluRs, we hypothesized that SNX1 could play an important role in the trafficking of group I mGluRs. Our earlier data suggested that over-expression of the full-length SNX1 resulted in the delayed recycling of group I mGluRs. On the other hand, over-expression of the dominant-negative SNX1 led to the rapid recycling of the receptors. We also studied the structure-function relationship of SNX1 in the group I mGluR-trafficking by knocking down the endogenous SNX1 in primary hippocampal neurons.

Using the "molecular replacement" strategy, we dissected out the domains of SNX1 that play critical role in the recycling of group I mGluRs. Molecular replacement approach employs a multi-promoter vector that allows knockdown of the endogenous protein and at the same time replacing the "knocked down" protein with the wild-type or mutant forms of that protein. This approach has primarily two advantages. First, it minimizes the possibility of any compensatory adaptation during synaptogenesis and synapse maturation due to the absence of the protein of interest. Second, function of the heterologous constructs can be studied without having a dominant effect as required by a standard over-expression approach. Our data suggested that acute knockdown of the endogenous SNX1 led to the rapid recycling of both mGluR1 and mGluR5. Subsequently, the knockdown effect could be rescued for both the receptors by expression of the wild-type SNX1 (replacement SNX1), suggesting specificity of the knockdown. These results suggested a critical role for the SNX1 in the recycling of group I mGluRs. We further showed that the expression of the SNX1ΔN (N-terminal deleted SNX1) replacement construct was not able to rescue the normal recycling of myc-mGluR1 and the receptor still showed rapid recycling caused by the knockdown of the endogenous SNX1. In addition, expression of the SNX1ΔC (C-terminal deleted SNX1) replacement construct also did not rescue the normal recycling of myc-mGluR1. Finally, our data also suggested that the H2 and H3 domains in the C-terminal region of SNX1 played a critical role in the ligand-mediated trafficking of myc-mGluR1.

4.2. Results

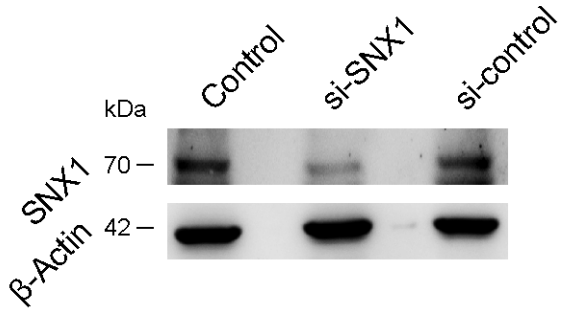
4.2.1. Knockdown of SNX1 results in the faster recycling of mGluR1

Our earlier experiments suggested that over-expression of the dominant-negative SNX1 altered the trafficking of group I mGluRs in both HEK293 cells and primary hippocampal neurons. We further confirmed the role of SNX1 in the trafficking of mGluR1 by acute knockdown of the endogenous SNX1 in primary hippocampal neurons. Primary hippocampal neurons were co-transfected with myc-mGluR1 cDNA and siRNA against the endogenous SNX1 (si-SNX1) (ON-TARGET plus) or scrambled siRNA (si-control) at 8-9 days *in vitro* according to the method described under "materials and methods" chapter. 72 hr post-transfection, primary neurons were lysed in RIPA lysis buffer containing protease inhibitor cocktail and western blots were performed to check for the knockdown of the endogenous SNX1. The control cells showed normal expression of the endogenous SNX1 which was reduced in the si-SNX1 treated cells. si-control treated cells did not show any knockdown of the endogenous SNX1 **(control: 1 ± 0.1; si-SNX1: 0.55 ± 0.16; si-control: 1.06 ± 0.1) (Figure 4.1 A, B)**. This observation suggested that si-SNX1 efficiently knocked down the endogenous SNX1. β-actin served as a loading control.

After confirming the effectiveness of the si-SNX1 on the knockdown of the endogenous SNX1, we investigated the effect of the SNX1 knockdown on the surface expression of myc-mGluR1. myc-mGluR1 cDNA along with the si-SNX1 and si-control were transfected in the primary hippocampal neurons. 4-5 days post-transfection, surface localized myc-mGluR1 were labelled with the anti-myc primary antibody (1:200) and anti-mouse Alexa-568 conjugated secondary antibody (1:100) following the method described in the "methods" section. There was no significant difference observed in the surface expression of myc-mGluR1 between control cells, si-SNX1 and si-control transfected cells **(control: 1 ± 0.05; si-SNX1: 0.9 ± 0.06; si-control: 1 ± 0.08) (Figure 4.2 A, B)**.Thus, our data suggested that acute knockdown of the endogenous SNX1 did not affect the surface expression of myc-mGluR1 in primary hippocampal neurons

We next investigated whether knockdown of the endogenous SNX1 had any effect on the trafficking of myc-mGluR1. Our earlier results suggested that over-expression of the dominant negative SNX1 resulted in the rapid recycling of the receptor. We, therefore, studied the time

(A)



(B)

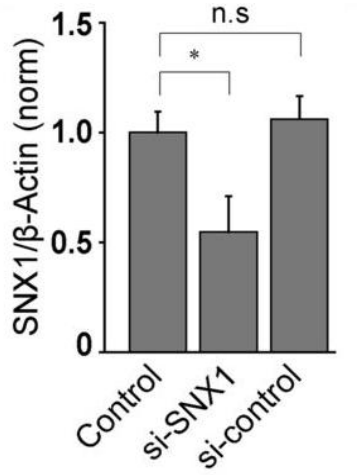
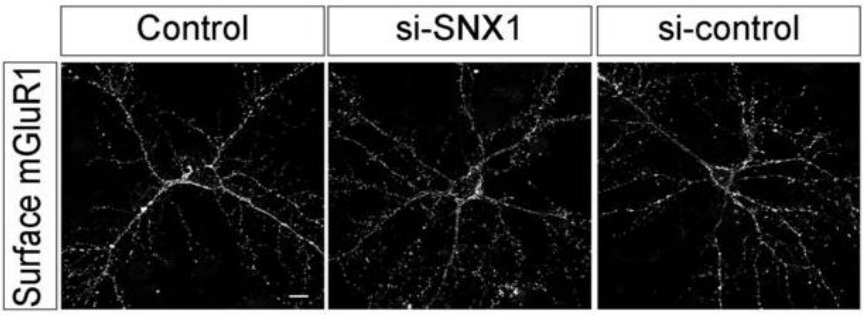


Figure 4.1. Knockdown of the endogenous SNX1 using siRNA against SNX1 (si-SNX1). (A) Western blot image showing knockdown of the endogenous SNX1 by si-SNX1 whereas, si-control had no effect on the SNX1 expression. (B) Quantitation of the western blots confirmed that the endogenous SNX1 was efficiently knocked down by si-SNX1 in primary neurons. *$p < 0.05$, and n.s indicates $p > 0.05$.

(A)



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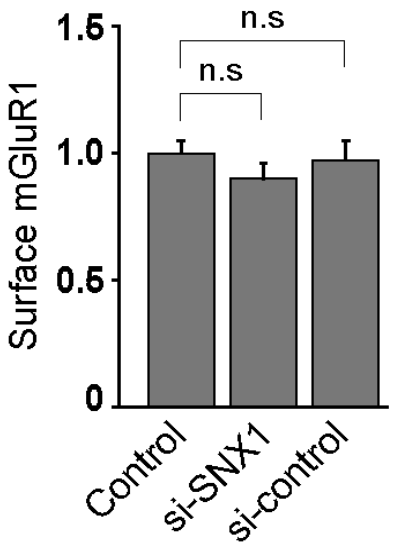


Figure 4.2. Effect of the knockdown of endogenous SNX1 on the surface expression of mGluR1. (A) Representative images showed that acute knockdown of the endogenous SNX1 had no effect on the surface expression of myc-mGluR1. (B) Quantitation of the effect of SNX1 knockdown on the surface expression of myc-mGluR1. Scale bar = 10 μm. n.s indicates $p > 0.05$.

course kinetics of myc-mGluR1 trafficking in primary hippocampal neurons in which SNX1 was knocked down. The endocytosis assay was performed using the same protocol as described before in the "materials and methods" section. Control cells showed normal internalization of the receptor upon application of 100 μM R,S-DHPG and the extent of internalization increased gradually over time till 30 min. Similar time course kinetics of myc-mGluR1 trafficking was observed in si-control transfected cells as well. On the other hand, in si-SNX1 transfected cells the receptor recycled back faster, i.e., in 30 min post 100 μM R,S-DHPG application **(control:: untreated 1 ± 0.06; 5 min: 1.28 ± 0.08; 15 min: 1.77 ± 0.11; 30 min: 1.99 ± 0.08; si-SNX1:: untreated: 1 ± 0.09; 5 min: 2.08 ± 0.09; 15 min: 1.76 ± 0.09; 30 min: 0.92 ± 0.06; si-control:: untreated: 1 ± 0.09; 5 min: 1.46 ± 0.1; 15 min: 1.83 ± 0.11; 30 min: 2.28 ± 0.21) (Figure 4.3 A, B1, C1, D1)**. Correspondingly, the measurement of the surface localized receptors also showed gradual decrease of the receptors from the cell surface subsequent to the application of 100 μM R,S-DHPG till 30 min in both control cells and si-control transfected cells, whereas, in si-SNX1 transfected cells maximum decrease of the surface receptors occurred at 5 min post-ligand application and the recovery of the surface fluorescence was observed at 30 min post-ligand application **(control:: untreated: 1 ± 0.05; 5 min: 0.83 ± 0.07; 15 min: 0.63 ± 0.07; 30 min: 0.5 ± 0.04; si-SNX1:: untreated: 1 ± 0.07; 5 min: 0.64 ± 0.06; 15 min: 0.78 ± 0.06; 30 min: 1 ± 0.06; si-control:: untreated: 1 ± 0.08; 5 min: 0.75 ± 0.09; 15 min: 0.63 ± 0.07; 30 min: 0.43 ± 0.05) (Figure 4.3 B2, C2, D2)**. Thus, knockdown of the endogenous SNX1 in the primary hippocampal neurons resulted in the rapid recycling of myc-mGluR1 subsequent to the 100 μM R,S-DHPG-mediated internalization of the receptor. These results suggested a critical role of SNX1 in the ligand-mediated trafficking of group I mGluRs.

4.2.2. Replacement of the full-length SNX1 rescues the normal recycling of mGluR1

Our previous data suggested that knockdown of the endogenous SNX1 led to the rapid recycling of group I mGluRs subsequent to the R,S-DHPG-mediated endocytosis. In order to determine whether the rapid recycling of the receptor was specifically due to the knockdown of the endogenous SNX1, we investigated the ability of the recombinant wild-type SNX1 to fully rescue the knockdown effect. In order to generate SNX1 replacement construct (shSNX1:SNX1), the degeneracy in the amino acids coding sequences was taken into account. Thereby, few silent

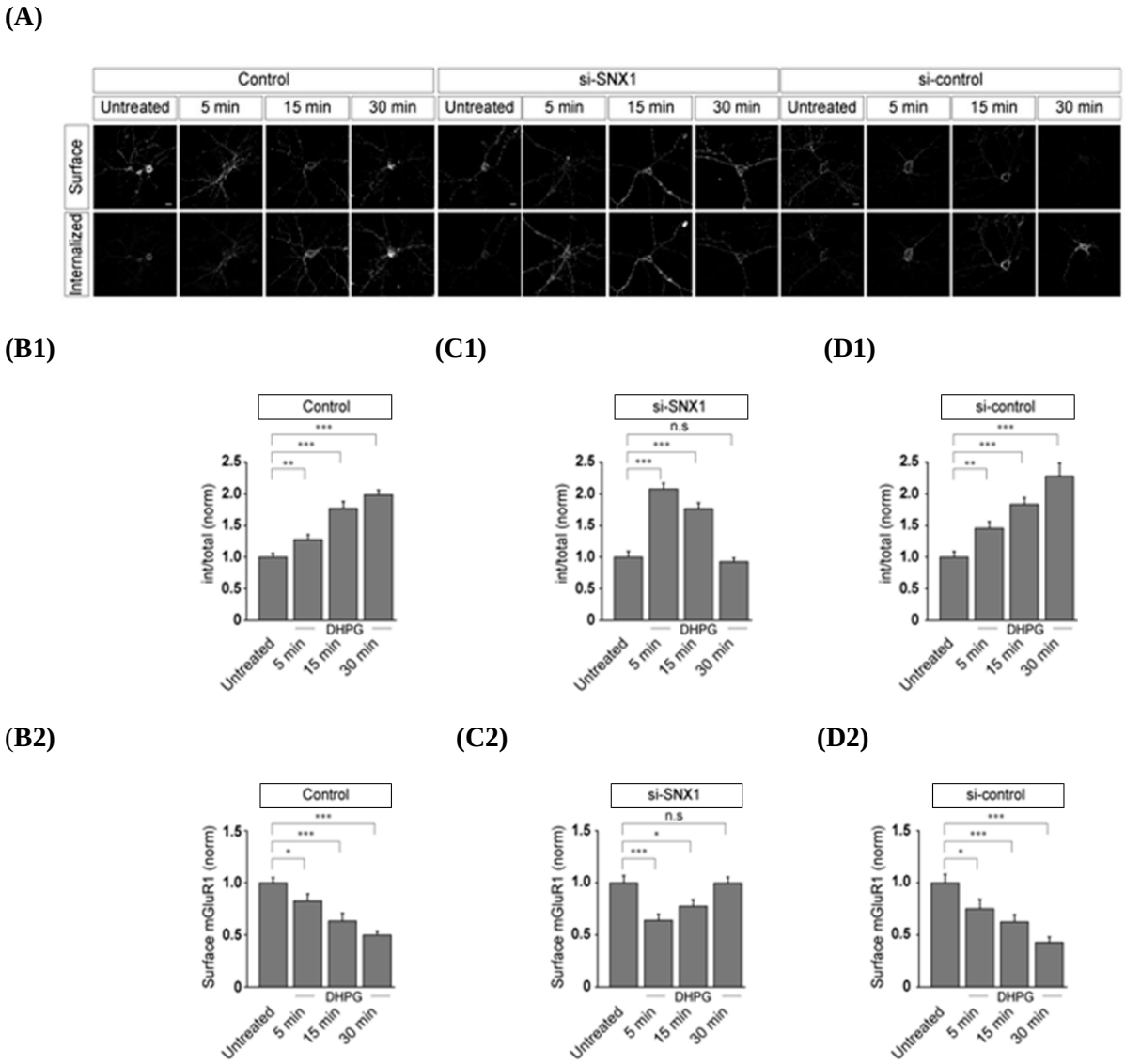


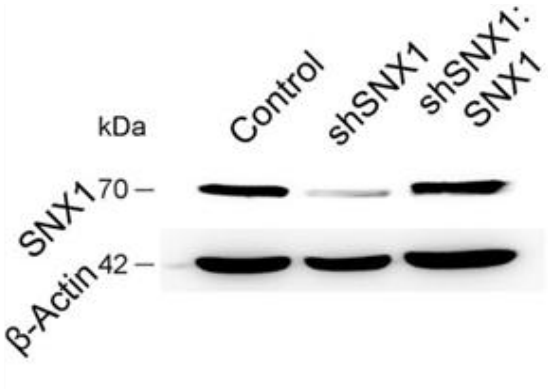
Figure 4.3. Knockdown of the endogenous SNX1 results in the faster recycling of mGluR1. (A) Representative images showing that knockdown of the endogenous SNX1 resulted in the recycling of myc-mGluR1 within 30 min after 100 μM R,S-DHPG application, whereas in control cells and si-control treated cells the receptor showed progressive increase in the internalization till 30 min post-ligand application. (B1, B2) Quantitation of the endocytosis (B1) and surface myc-mGluR1 (B2) also suggested that in control cells the receptor showed maximum endocytosis at 30 min post-ligand application. (C1, C2) Importantly, acute knockdown of endogenous SNX1 resulted in the recycling of the receptor to the cell surface within 30 min after 100 μM R,S-DHPG application as shown by the quantitation of the endocytosis (C1) and measurement of the surface localized myc-mGluR1 (C2). (D1, D2) Measurement of the endocytosis (D1) and surface myc-mGluR1 (D2) suggested that 100 μM R,S-DHPG induced trafficking of myc-mGluR1 in si-control treated cells was similar to the control cells. Scale bar = 10 μm, ***p < 0.001, ** p < 0.01, *p < 0.05; n.s., p > 0.05.

mutations were introduced in the shRNA binding region of the SNX1 gene to prevent the binding of the shSNX1 with this replacement construct. Consequently, shSNX1 knocked down the endogenous SNX1 but the over-expressed SNX1 expression was not affected by the presence of shSNX1. As silent mutations were incorporated in the over-expressed SNX1, the amino acid sequence remained same as that of the wild-type protein. The expression of the full-length SNX1 replacement construct (shSNX1:SNX1) was studied by western blotting. On 8-9 DIV, primary neurons were transfected with either shSNX1 or vector containing shSNX1 and HA-SNX1 (shSNX1:SNX1) using calcium phosphate method as described in the "method" section. 4-5 days post-transfection, cells were lysed with RIPA lysis buffer and western blotting was performed using the procedure described before. The expression of the endogenous SNX1 was normal in control cells, while shSNX1 transfected cells showed significant knockdown of the endogenous SNX1. On the other hand, shSNX1:SNX1 transfected cells showed expression of the HA-SNX1 **(control: 1 ± 0.13; shSNX1: 0.25 ± 0.01; shSNX1:SNX1: 1.08 ± 0.16) (Figure 4.4 A, B)**. These data suggested that shSNX1 construct was highly effective in knocking down the endogenous SNX1 and shSNX1:SNX1 construct could effectively replace the endogenous SNX1 with the over-expressed HA-SNX1.

After confirming the efficacy of shSNX1 on the knockdown of the endogenous SNX1, we investigated whether the knockdown of the endogenous SNX1 had any effect on the surface expression of the receptor. Primary hippocampal neurons were transfected with myc-mGluR1 and shSNX1 or vector containing shSNX1 and HA-SNX1 (shSNX1:SNX1). 3 - 4 days post-transfection, surface myc-mGluR1 was stained with the primary and secondary antibodies according to the protocol described before. Our data suggested that the expression of shSNX1 did not have any effect on the surface expression of myc-mGluR1. Moreover, simultaneous expression of both shSNX1 and HA-SNX1 also had no effect on the surface expression of myc-mGluR1 **(control: 1.0 ± 0.09; shSNX1: 1 ± 0.08; shSNX1:SNX1: 0.9 ± 0.06) (Figure 4.5 A, B**).

We next investigated whether expression of HA-SNX1 could rescue the normal recycling of myc-mGluR1 altered by the knockdown of endogenous SNX1. Our earlier experiments suggested that majority of the myc-mGluR1 internalized at 30 min post R,S-DHPG application in control cells. Moreover, knockdown of the endogenous SNX1 resulted in the rapid recycling

(A)



(B)

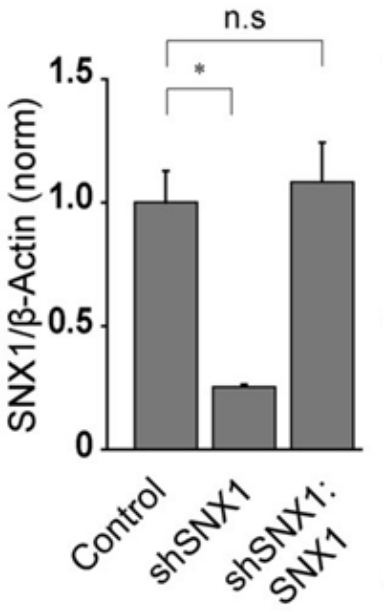
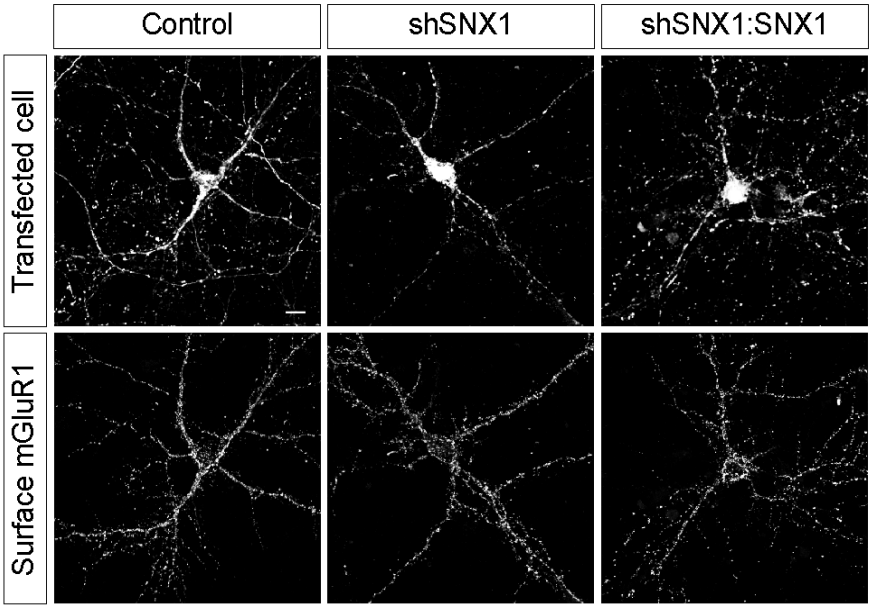


Figure 4.4. Replacement of the endogenous SNX1 with full-length SNX1. (A) Representative western blot image showing the knock-down of the endogenous SNX1 by shSNX1 and replacement of the endogenous SNX1 with full-length SNX1. (B) Quantitation of the western blots also showed effective knock-down of the endogenous SNX1 as well as replacement of the endogenous SNX1 with the full-length SNX1. *p < 0.05; n.s p > 0.05.

(A)



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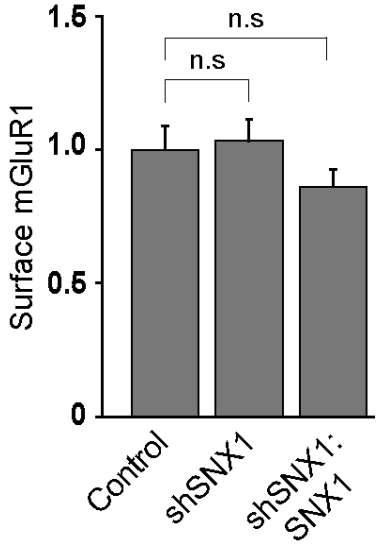
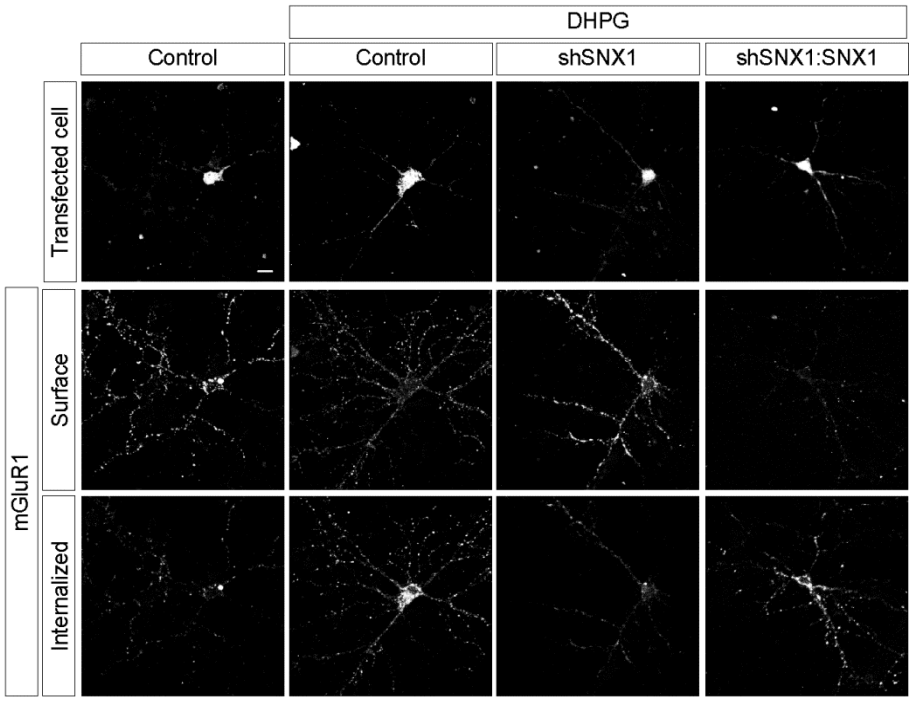


Figure 4.5. Acute knockdown of the endogenous SNX1 does not affect the surface localization of mGluR1. (A) Representative neurons showing that knockdown of the endogenous SNX1 with shSNX1 and expression of the wild-type SNX1 replacement construct had no effect on the surface expression of myc-mGluR1. (B) Quantitation of the surface myc-mGluR1 following knockdown and replacement of the endogenous SNX1 also suggested that expression of the shSNX1 and wild-type SNX1 had no effect on the surface expression of myc-mGluR1. Scale bar = 10 μm. n.s indicates $p > 0.05$.

of myc-mGluR1 to the cell surface in 30 min post-R,S-DHPG pulse. Therefore, we chose 30 min as the time point for the subsequent experiments. The endocytosis assay was performed following the same protocol as described earlier. Briefly, neurons were co-transfected with myc-mGluR1 and shSNX1 or shSNX1:SNX1. 4-5 days post-transfection, myc-mGluR1 expressing on the surface of the live neurons were labelled with mouse anti-myc primary antibody (1:200) for 30 min at 37˚C. After that, 100 μM R,S-DHPG pulse was given for 5 min followed by a chase for 25 min in the absence of the ligand. Subsequently, cells were fixed without permeabilization in 4% PFA for 15 min on ice. Then the goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) was applied in saturating concentration to label the surface receptors that did not internalize on ligand application. Subsequently, cells were permeabilized by 0.1% Triton X-100 followed by the application of the second secondary antibody viz., goat anti-mouse Alexa-647 for 1 hr at 37˚C (1:800) to label the internalized receptors. Finally, cells were mounted on glass slides and observed under the confocal microscope. As expected, minimal amount of internal fluorescence was observed in control cells and after R,S-DHPG application significant amount of myc-mGluR1 were seen to internalize in 30 min **(control: 1 ± 0.08; control + DHPG: 1.8 ± 0.1) (Figure 4.6 A, B)**. shSNX1 expressing cells showed rapid recycling of myc-mGluR1 and the receptors recycled back to the cell surface in 30 min post R,S-DHPG induced internalization **(shSNX1 + DHPG: 1 ± 0.05)**. Importantly, replacement of the endogenous SNX1 with wild-type SNX1 rescued the normal trafficking of myc-mGluR1, as observed by the presence of the endocytosed myc-mGluR1 in the intracellular compartments at 30 min post R,S-DHPG application **(shSNX1:SNX1 + DHPG: 1.9 ± 0.1)**.

As stated before, group I mGluRs comprises of mGluR1 and mGluR5. It has been reported that SNX1 interacts with both mGluR1 and mGluR5 (Heydorn, Sondergaard et al. 2004). Our earlier experiments suggested that both over-expression of the dominant-negative SNX1 and knockdown of the endogenous SNX1 altered the trafficking of mGluR1. Therefore, we investigated whether knockdown of the endogenous SNX1 had similar effect in the trafficking of the other member of the group I mGluR family, viz., mGluR5. We initially studied the effect of acute knockdown of the endogenous SNX1 on the surface expression of myc-mGluR5 in primary hippocampal neurons using the similar protocol as described previously for mGluR1.

(A)



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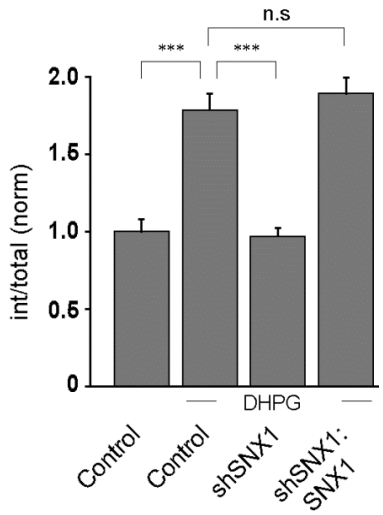


Figure 4.6. Replacement of the endogenous SNX1 with the wild-type SNX1 rescues normal trafficking of mGluR1. (A) Representative images showing surface and internal fluorescence of myc-mGluR1, 30 min after 100 μM R,S-DHPG application in control cells, shSNX1-expressing cells, shSNX1 and wild-type SNX1-expressing cells. (B) Quantitation suggested that knockdown of the endogenous SNX1 resulted in the rapid recycling of myc-mGluR1 and expression of the wild-type SNX1 rescued the normal trafficking of the receptor. Scale bar = 10 μm. ***$p < 0.001$; n.s. indicates $p > 0.05$.

Similar to myc-mGluR1, no significant difference in the surface expression of myc-mGluR5 was observed in control, shSNX1 and shSNX1:SNX1 expressing cells **(control: 1 ± 0.05, shSNX1: 0.94 ± 0.05, shSNX1:SNX1: 0.90 ± 0.06) (Figure 4.7 A, B)**.

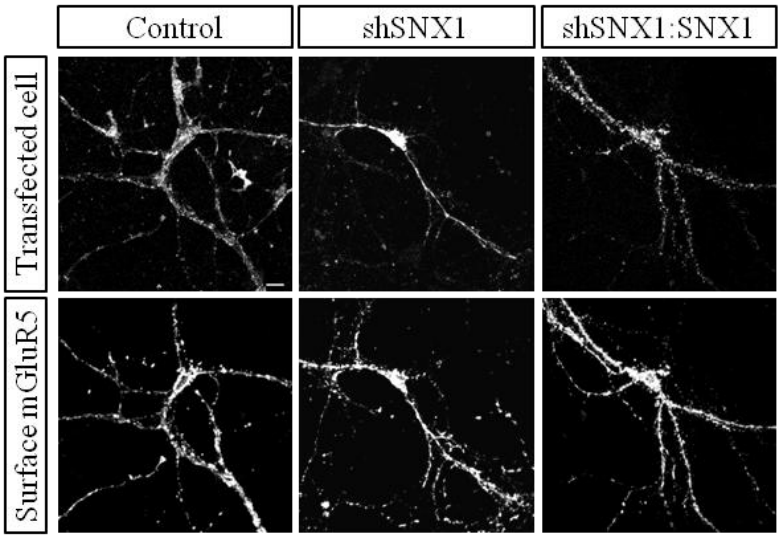
We next investigated the effect of knockdown of the endogenous SNX1 on the ligand-dependent trafficking of myc-mGluR5. We found that knockdown of the endogenous SNX1 resulted in the rapid recycling of the receptor **(control: 1 ± 0.07; control + DHPG: 2.11 ± 0.22; shSNX1 + DHPG: 0.96 ± 0.08) (Figure 4.8 A, B)**. Importantly, replacement of the endogenous SNX1 with the wild-type SNX1 rescued the normal trafficking of myc-mGluR5 **(shSNX1:SNX1 + DHPG: 2.47 ± 0.24)**. These results suggested that SNX1 plays a critical role in the ligand-mediated trafficking of both the members of group I mGluRs, i.e., mGluR1 and mGluR5.

4.2.3. SNX1ΔN replacement construct does not rescue the normal recycling of mGluR1

Our previous experiments suggested that knockdown of the endogenous SNX1 led to the rapid recycling of myc-mGluR1. In order to check whether expression of SNX1ΔN replacement construct (shSNX1:SNX1ΔN) could rescue the normal recycling of the receptor, the SNX1ΔN replacement construct was generated by the method as described in the "methods" section. Subsequently, the expression level of SNX1ΔN was examined in the primary neurons. Initially, shSNX1 or vector containing shSNX1 and SNX1ΔN (shSNX1:SNX1ΔN) were transfected in primary neurons on 8-9 DIV. 4-5 days post transfection, cells were lysed with RIPA lysis buffer and then the cell lysates were run on SDS-PAGE followed by western blotting. As expected, control cells showed normal expression of SNX1 and shSNX1 effectively knocked down the endogenous SNX1. Importantly, SNX1ΔN was observed to have normal expression levels in primary neurons **(control: 1 ± 0.14; shSNX1: 0.23 ± 0.003; shSNX1:SNX1ΔN: 1 ± 0.09) (Figure 4.9 A, B)**.

In order to investigate whether the expression of SNX1ΔN replacement construct could affect the surface expression of mGluR1, primary hippocampal neurons were co-transfected with myc-mGluR1 and shSNX1 or shSNX1:SNX1ΔN. The measurement of myc-mGluR1 present at the cell surface showed that the knockdown of endogenous SNX1 and expression of

(A)



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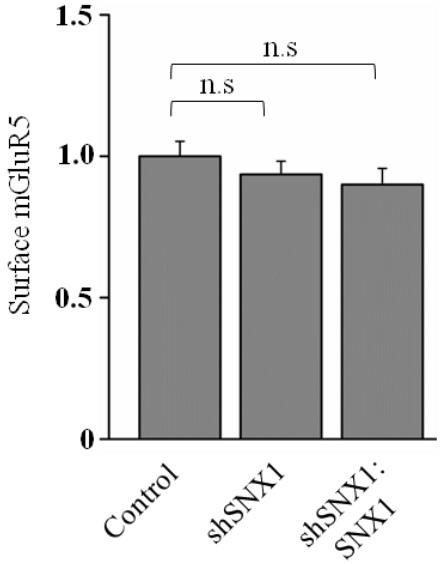
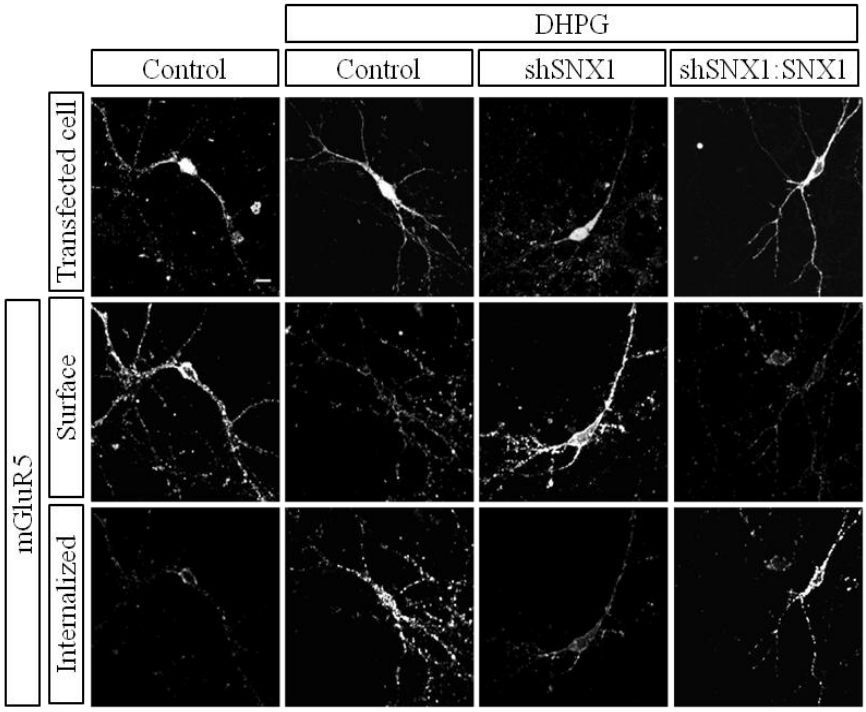


Figure 4.7. Acute knockdown of the endogenous SNX1 does not affect the surface localization of mGluR5. (A) Representative images showing the surface localization of myc-mGluR5 in shSNX1 and shSNX1;SNX1 expressing cells. (B) Quantitation also suggested that both knockdown of the endogenous SNX1 and expression of the wild-type SNX1 replacement construct had no effect on the surface localization of myc-mGluR5. Scale bar = 10 μm. n.s indicates $p > 0.05$.

(A)



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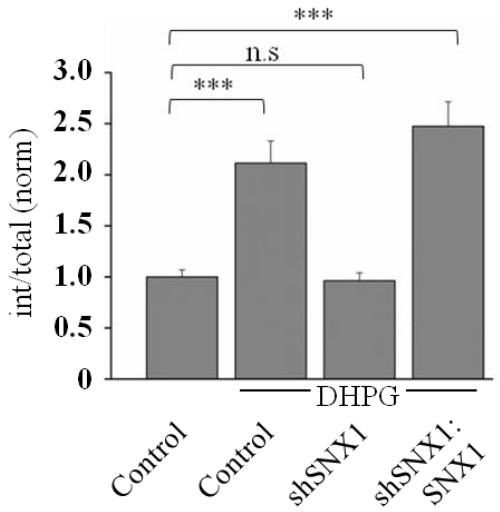
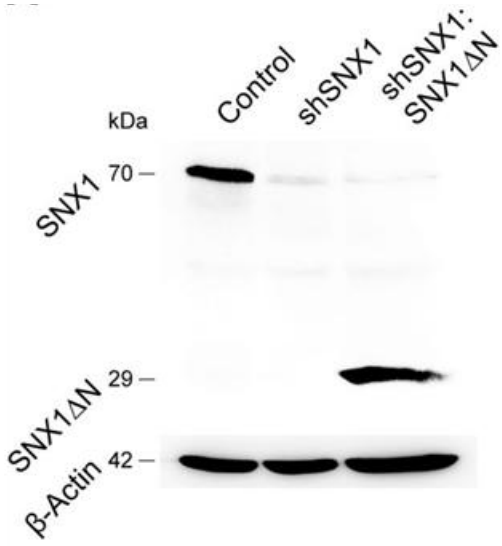


Figure 4.8. Replacement of the endogenous SNX1 with the wild-type SNX1 rescues the ligand-mediated trafficking of mGluR5. (A) Representative images showing alteration in the R,S-DHPG-mediated myc-mGluR5 trafficking upon knockdown of the endogenous SNX1 and rescue of the normal trafficking upon expression of the wild-type SNX1 replacement construct. (B) Quantitation also suggested that knockdown of the endogenous SNX1 affected the ligand-mediated trafficking of myc-mGluR5 which was rescued by the expression of the wild-type SNX1 replacement construct. Scale bar = 10 μm. ***$p < 0.001$, n.s indicates $p > 0.05$.

(A)



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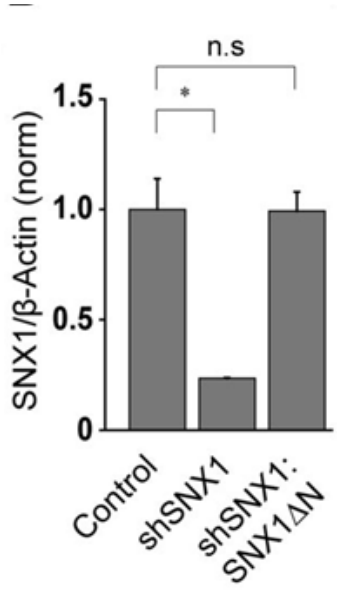


Figure 4.9. Knockdown of the endogenous SNX1 and replacement with SNX1ΔN. (A) Western blot showing the effective knockdown of the endogenous SNX1 by shSNX1 and expression of the SNX1ΔN replacement construct. (B) Quantitation of the SNX1 knockdown and SNX1ΔN expression. *$p < 0.05$; n.s. indicates $p > 0.05$.

shSNX1:SNX1ΔN did not have any effect on the surface expression of myc-mGluR1 **(control: 1.0 ± 0.04; shSNX1: 1 ± 0.07; shSNX1:SNX1ΔN: 0.9 ± 0.05) (Figure 4.10 A, B**

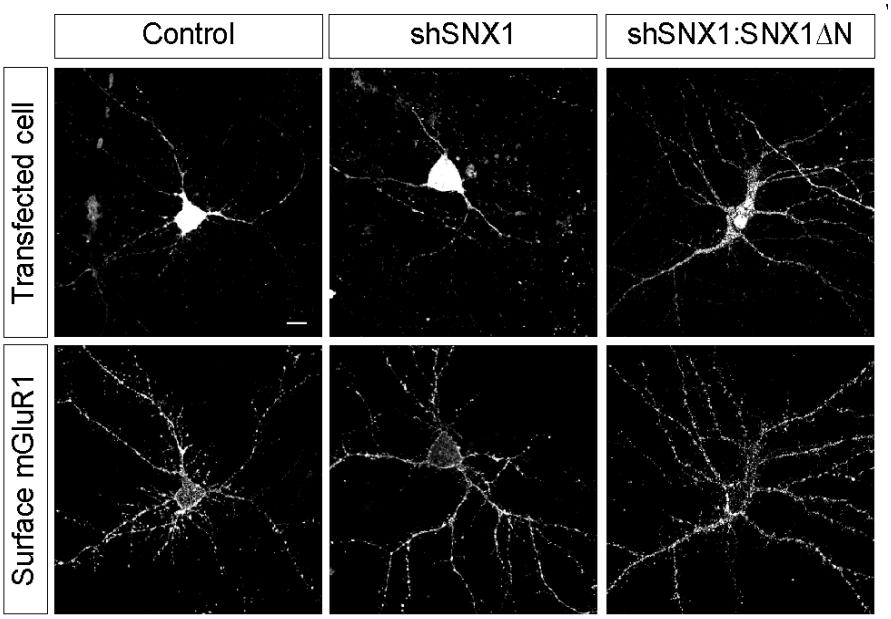
We next investigated whether SNX1ΔN replacement construct could rescue the SNX1 knockdown effect on the ligand-mediated mGluR1 trafficking. The experiment was performed in the primary hippocampal neurons in the similar way as described earlier for the full-length SNX1 replacement construct. Control cells showed minimal internal fluorescence and receptors internalized at 30 min post 100 μM R,S-DHPG application as observed by the increase in the internal fluorescence. On the other hand, unlike full-length SNX1, SNX1ΔN did not rescue the normal recycling of myc-mGluR1 and the receptor still showed rapid recycling in shSNX1:SNX1ΔN expressing cells similar to what was observed in cells where endogenous SNX1 was knocked down **(control: 1 ± 0.04; control + DHPG: 2 ± 0.09; shSNX1 + DHPG: 1 ± 0.06; shSNX1:SNX1ΔN + DHPG: 0.9 ± 0.04) (Figure 4.11 A, B)**. These results suggested that the N-terminal region of SNX1 plays critical role in the normal recycling of mGluR1.

4.2.4. SNX1ΔC replacement construct does not rescue the normal recycling of mGluR1

Our previous experiments suggested that the absence of the N-terminal region of SNX1 prevented the normal recycling of myc-mGluR1. We subsequently studied the role of the C-terminal region of SNX1 in the ligand-mediated mGluR1 trafficking. SNX1ΔC replacement construct was generated to study the role of the C-terminal region of SNX1 in the trafficking of mGluR1. Subsequently, the expression of SNX1ΔC was verified by the western blotting method in a similar way as described previously for the other constructs of SNX1. The expression levels of SNX1ΔC appeared to be normal with respect to the endogenous SNX1 in control cells as observed by the western blot **(control: 1 ± 0.05; shSNX1: 0.38 ± 0.09; shSNX1: SNX1ΔC: 0.95 ± 0.11) (Figure 4.12 A, B)**.

We next studied whether the SNX1ΔC replacement construct affects the surface expression of myc-mGluR1 in primary hippocampal neurons. Our data showed that both knockdown of the endogenous SNX1 and expression of the SNX1ΔC replacement construct had no effect on the surface expression of myc-mGluR1 **(control: 1.0 ± 0.03; shSNX1: 1 ± 0.04; shSNX1:SNX1ΔC: 1 ± 0.05) (Figure 4.13 A, B).**

(A)



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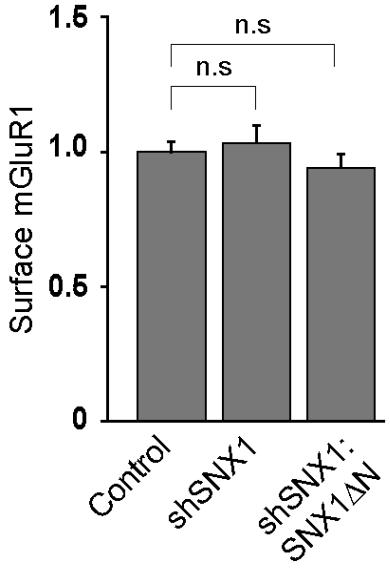
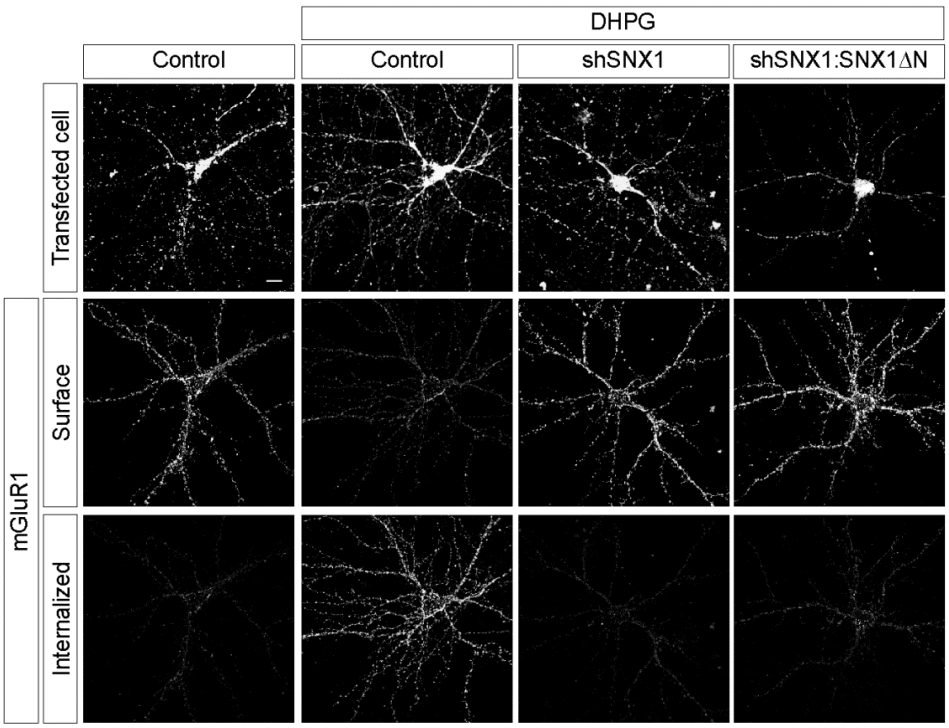


Figure 4.10. Effect of the expression of SNX1ΔN replacement construct on the surface expression of mGluR1. (A) Representative images showing surface expression of myc-mGluR1 in shSNX1 and shSNX1:SNX1ΔN transfected cells. There was no significant difference in myc-mGluR1 surface localization. (B) Quantitation of the surface myc-mGluR1 suggested that there was no significant difference in myc-mGluR1 surface expression in both SNX1 knockdown cells as well as in cells expressing SNX1ΔN replacement construct as compared to control cells. Scale bar = 10 μm. n.s indicates $p > 0.05$.

(A)



(B)

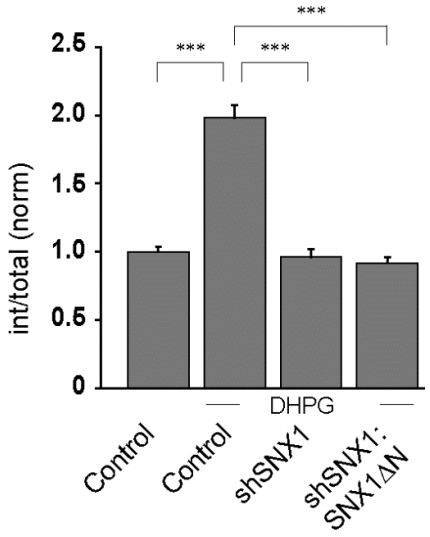
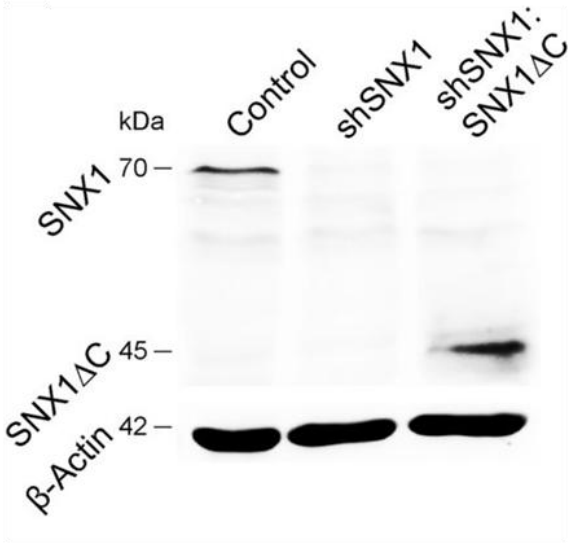


Figure 4.11. N-terminal domain of SNX1 is required for the normal trafficking of mGluR1. Representative images (A) and quantitation (B) of the R,S-DHPG-mediated myc-mGluR1 trafficking in shSNX1 and shSNX1:SNX1ΔN transfected cells. Control cells showed endocytosis upon 100 μM R,S-DHPG application, whereas shSNX1 transfected cells showed the rapid recycling of myc-mGluR1 in 30 min. Importantly, expression of the SNX1ΔN replacement construct did not rescue the normal trafficking of the receptor. Scale bar = 10 μm. ***$p < 0.001$.

(A)



(B)

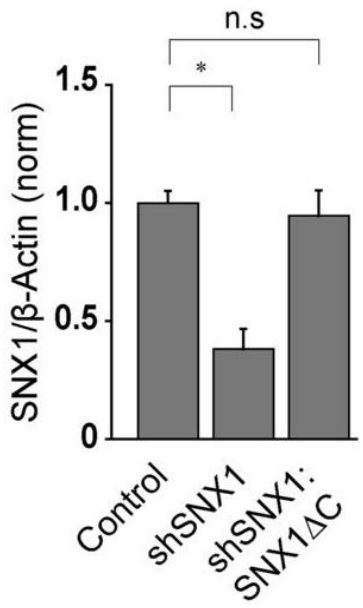
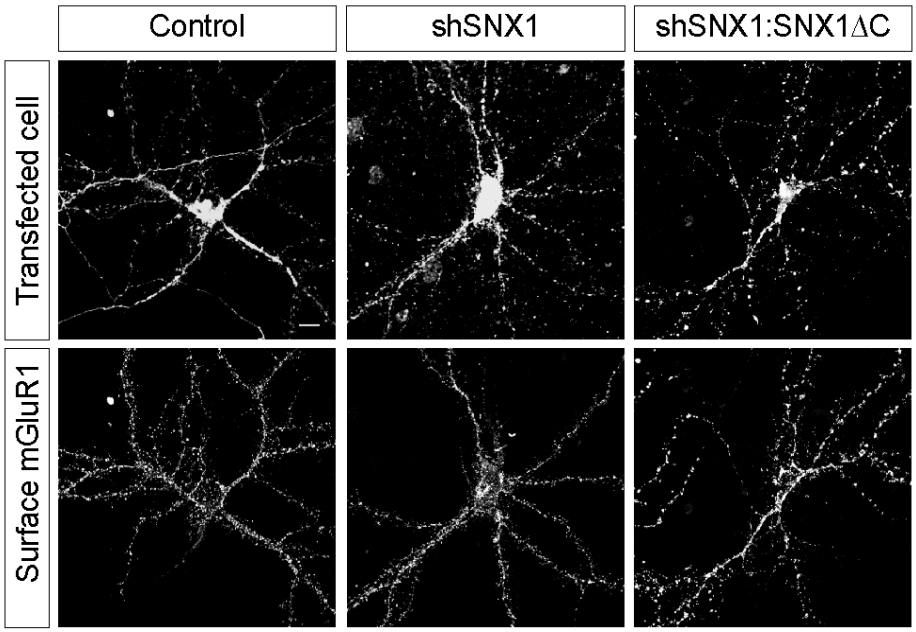


Figure 4.12. Knockdown of the endogenous SNX1 and replacement with SNX1ΔC. Acute knockdown of the endogenous SNX1 and replacement of the endogenous SNX1 with SNX1ΔC in primary neurons, as shown by the western blot (A) and quantitation of the western blots (B). *$p < 0.05$; n.s. indicates $p > 0.05$.

(A)



(B)

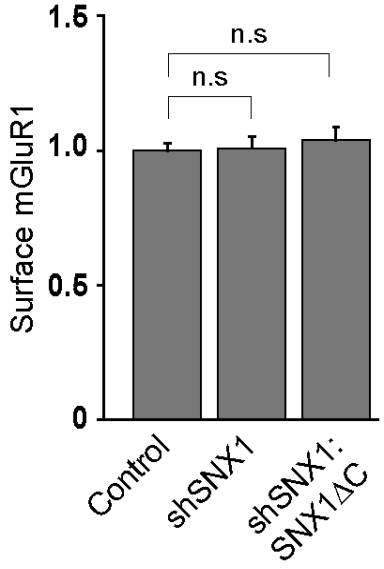


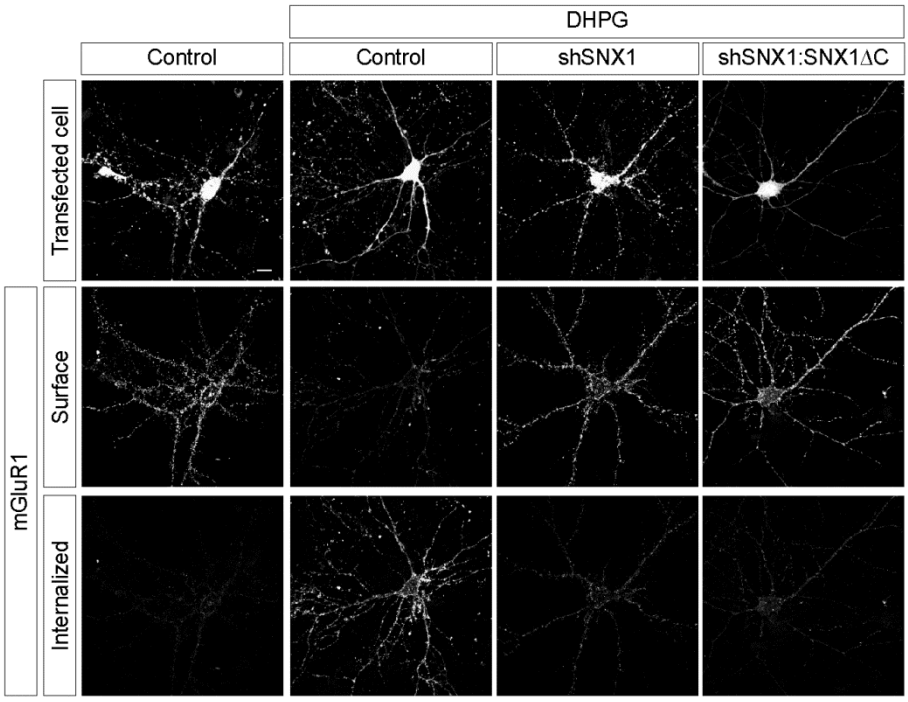
Figure 4.13. Effect of the expression of SNX1ΔC replacement construct on the surface expression of mGluR1. (A) Representative images showing similar myc-mGluR1 surface expression in control cells, in cells expressing shSNX1 and in cells expressing SNX1ΔC replacement construct. (B) Quantitation also suggested that both knockdown of the endogenous SNX1 and over-expression of the SNX1ΔC replacement construct had no effect on the surface localization of myc-mGluR1. Scale bar = 10 μm. n.s indicates $p > 0.05$.

In order to investigate whether SNX1ΔC replacement construct could rescue the normal recycling of mGluR1, primary hippocampal neurons were co-transfected with myc-mGluR1 and shSNX1 or shSNX1:SNX1ΔC. Subsequently, the ligand-mediated trafficking assay was performed according to the method described before. In control cells, minimal amount of internalized receptors were observed which increased on 100 μM R,S-DHPG application at 30 min **(control: 1 ± 0.04; control + DHPG: 1.9 ± 0.07) (Figure 4.14 A, B)**. In shSNX1 expressing cells, the myc-mGluR1 rapidly recycled back to the cell surface after 30 min of R,S-DHPG pulse (**shSNX1 + DHPG: 1 ± 0.04)**. Importantly, SNX1ΔC did not rescue the normal recycling of myc-mGluR1 and the receptor recycled back in 30 min post-ligand application **(shSNX1:SNX1ΔC + DHPG: 1.1 ± 0.05)**. These results suggested that the C-terminal region of SNX1 is crucial for the normal trafficking of mGluR1.

4.2.5. H2 and H3 sub domains of the C-terminal region of SNX1 play a critical role in the ligand-mediated trafficking of mGluR1

Our earlier data suggested that the C-terminal region of SNX1 plays a critical role in the trafficking of mGluR1. Moreover, the C-terminal region of SNX1 contains three putative SH3 domain binding motifs (H1, H2 and H3) which could be potentially involved in interacting with multiple proteins and forming multi-protein complexes *via* various kinds of interactions (Burkhard, Stetefeld et al. 2001; Wang, Zhou et al. 2002). In order to investigate whether these domains could play a role in the trafficking of group I mGluRs, we made SNX1 replacement constructs viz., shSNX1:SNX1ΔH1, shSNX1:SNX1ΔH2 and shSNX1:SNX1ΔH3 lacking the H1, H2 and H3 domains respectively and studied their effect on the trafficking of mGluR1. We initially checked for the expression levels of these constructs in primary neurons by western blotting. All these replacement constructs appeared to express properly as observed by the western blot **(control: 1 ± 0.09; shSNX1: 0.39 ± 0.05; shSNX1:SNX1ΔH1: 1.1 ± 0.06; shSNX1:SNX1ΔH2: 1 ± 0.01; shSNX1:SNX1ΔH3: 0.96 ± 0.09) (Figure 4.15 A, B)**.

(A)



(B)

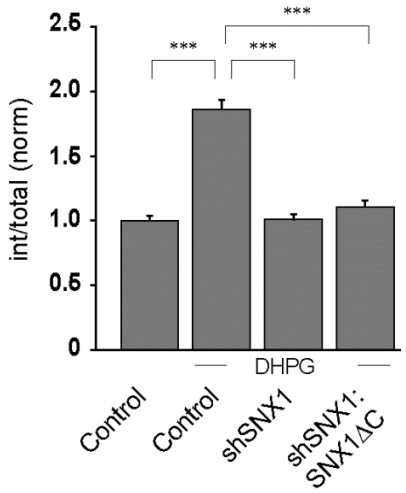
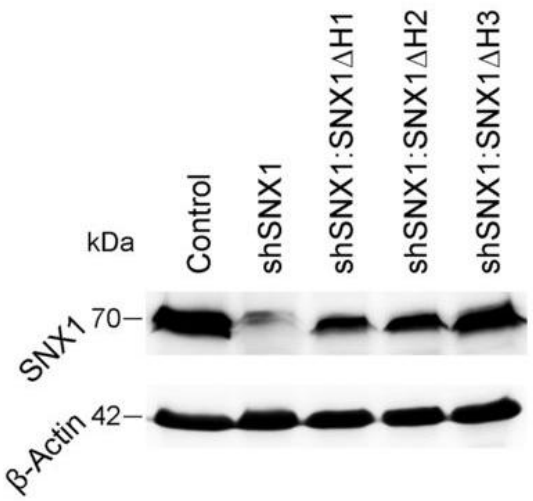


Figure 4.14. C-terminal domain of SNX1 plays critical role in the normal trafficking of mGluR1. (A) Control cells showed very little internalized receptors. The myc-mGluR1 internalized upon 100 μM R,S-DHPG application in control cells. The receptor recycled back to the cell surface faster upon knocking down the endogenous SNX1 and expression of the SNX1ΔC could not rescue this effect. (B) Quantitation also suggested that knockdown of the endogenous SNX1 resulted in the rapid recycling of myc-mGluR1 in 30 min post-ligand application, and replacement of the endogenous SNX1 with SNX1ΔC failed to rescue the normal trafficking of the receptor. Scale bar = 10 μm. ***$p < 0.001$.

(A)



(B)

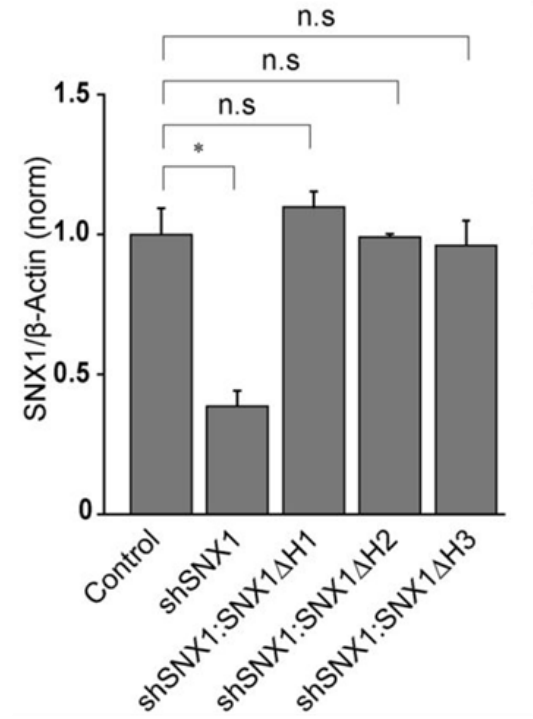


Figure 4.15. Expression of SNX1ΔH1, SNX1ΔH2 and SNX1ΔH3 constructs. Western blot (A) and quantitation of the western blots (B), showing the knockdown of the endogenous SNX1 and expression of SNX1ΔH1, SNX1ΔH2 and SNX1ΔH3 constructs. *$p < 0.05$; n.s. indicates $p > 0.05$.

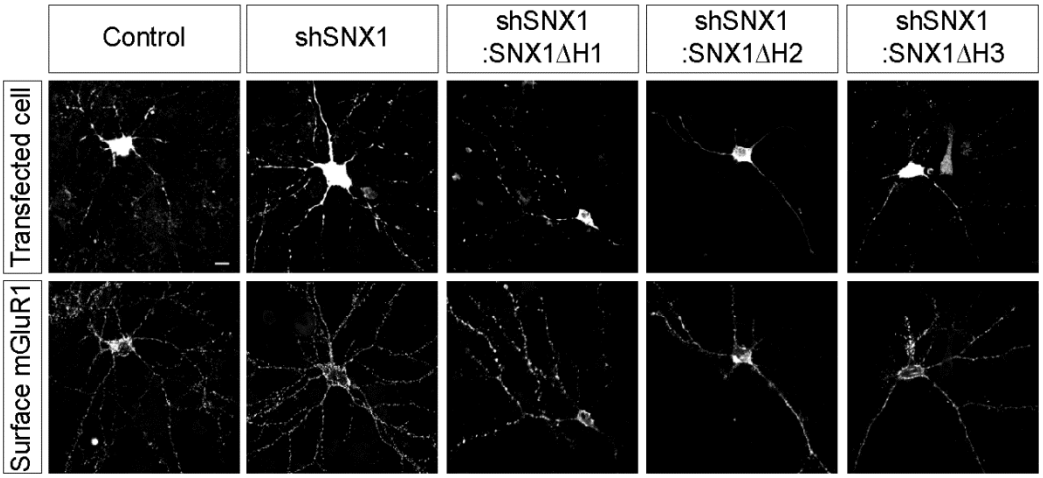
We next studied whether the expression of these replacement constructs could affect the surface localization of mGluR1 in primary hippocampal neurons. The expression of each of these SNX1 replacement constructs (shSNX1:SNX1ΔH1, shSNX1:SNX1ΔH2 and shSNX1:SNX1ΔH3) had no effect on the surface expression of myc-mGluR1 **(control: 1.0 ± 0.04; shSNX1: 0.94 ± 0.04; shSNX1:SNX1ΔH1: 1 ± 0.05; shSNX1:SNX1ΔH2: 1 ± 0.06; shSNX1:SNX1ΔH3: 1 ± 0.07) (Figure 4.16 A, B)**.

Subsequently, we investigated the effect of these replacement constructs on the trafficking of mGluR1. As expected, control cells showed very little amount of internal fluorescence which increased on 100 μM R,S-DHPG application at 30 min. Similar to our previous observations, shSNX1 expressing cells showed rapid recycling of myc-mGluR1 in 30 min post 100 μM R,S-DHPG application **(control: 1 ± 0.04; control + DHPG: 2.28 ± 0.09; shSNX1 + DHPG: 1 ± 0.05)**. Importantly, in SNX1ΔH2 or SNX1ΔH3 expressing cells, normal recycling of myc-mGluR1was not rescued and the receptors recycled back to the cell surface in 30 min post-ligand application. On the other hand, SNX1ΔH1 replacement construct rescued the normal trafficking of myc-mGluR1 as observed by the presence of the internalized receptors at 30 min post-ligand-application **(shSNX1:SNX1ΔH1 + DHPG: 2.26 ± 0.13; shSNX1:SNX1ΔH2 + DHPG: 1 ± 0.05; shSNX1:SNX1ΔH3 + DHPG: 1 ± 0.08) (Figure 4.17 A, B)**. These results suggested that the H2 and H3 domains in the C-terminal region of SNX1 play a critical role in the ligand-mediated trafficking of mGluR1.

4.2.6. H2 and H3 domains of SNX1 is required to bind Hrs

Our earlier results suggested that the H2 and H3 domains in the C-terminal region of SNX1 play a critical role in the ligand-mediated trafficking of mGluR1. Moreover, it has been reported that SNX1 interacts with Hrs through its C-terminal region and Hrs has been implicated in vesicular trafficking (Asao, Sasaki et al. 1997; Chin, Raynor et al. 2001; Hanyaloglu, McCullagh et al. 2005; Gullapalli, Wolfe et al. 2006). Therefore, we wanted to investigate whether binding of SNX1 and Hrs is important for the ligand-mediated trafficking of mGluR1.

(A)



(B)

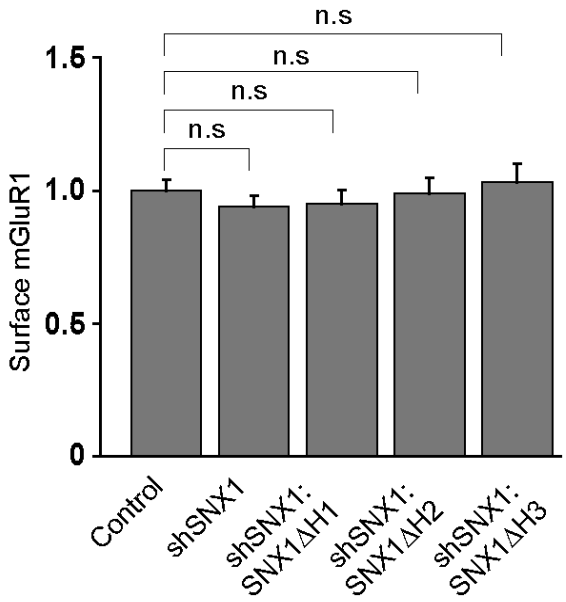
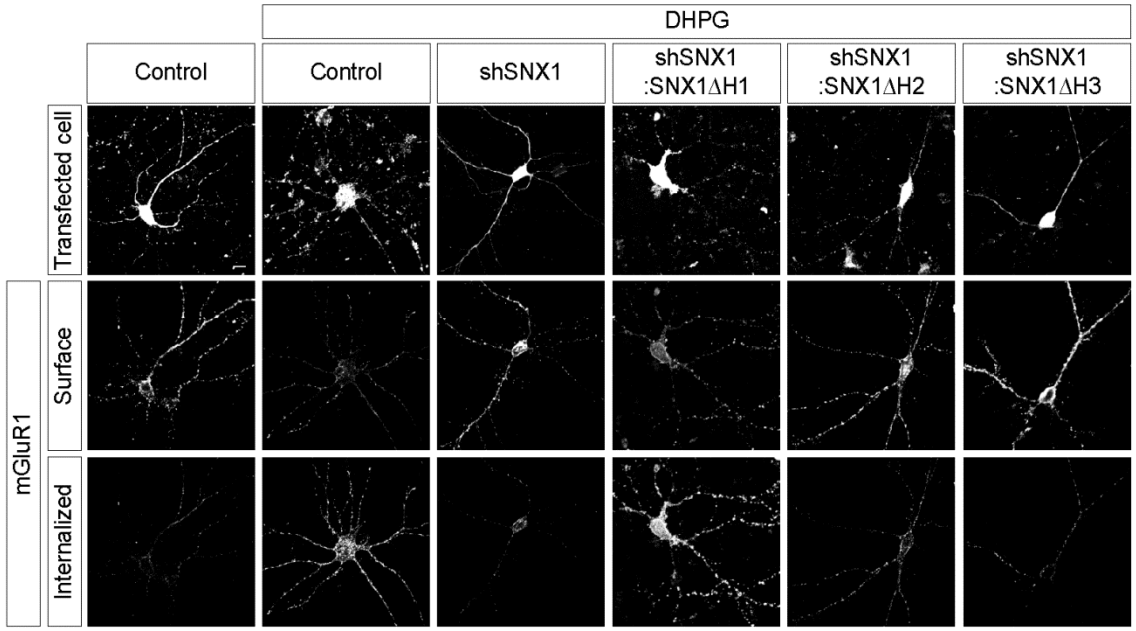


Figure 4.16. Deletion of the H1, H2 and H3 domains of SNX1 has no effect on the surface expression of mGluR1. (A) Representative images showing that knockdown of the endogenous SNX1 and replacement of the endogenous SNX1 with SNX1ΔH1, SNX1ΔH2, SNX1ΔH3 did not have any effect on the surface expression of myc-mGluR1 in neurons. (B) Quantitation of the surface myc-mGluR1 suggested that knockdown of the endogenous SNX1 and expression of the SNX1ΔH1, SNX1ΔH2, SNX1ΔH3 replacement constructs had no effect on the surface expression of myc-mGluR1. Scale bar = 10 μm. n.s indicates $p > 0.05$.

(A)



(B)

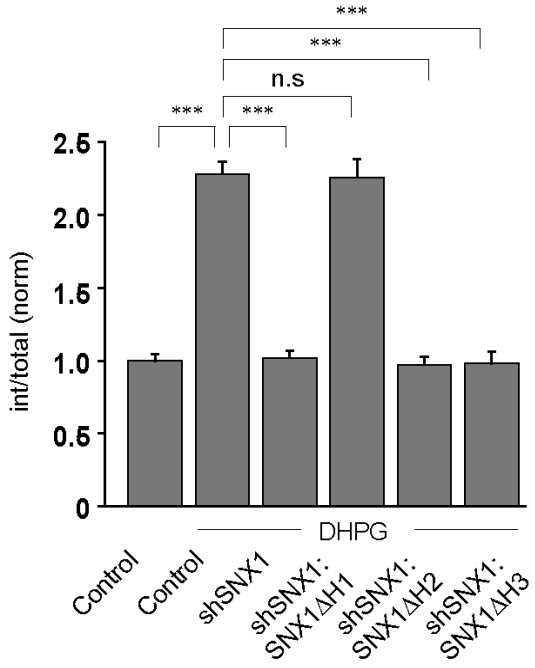


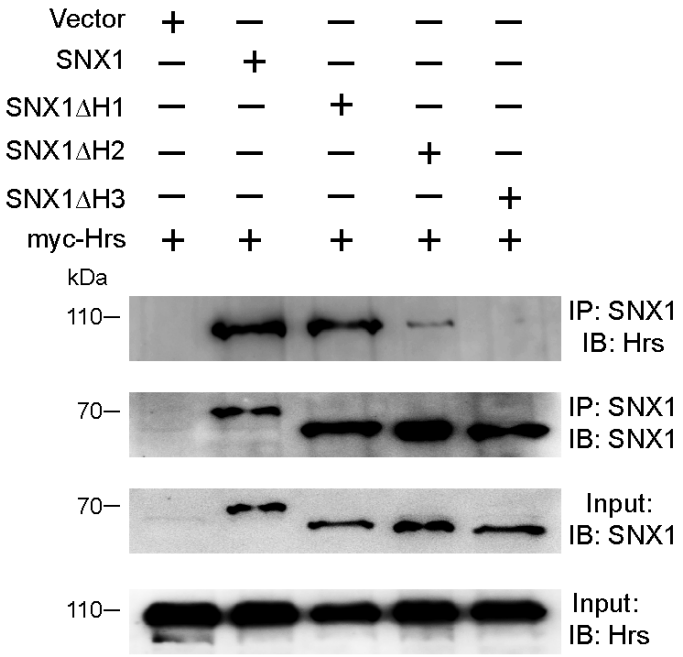
Figure 4.17. H2 and H3 domains of SNX1 are critical for the normal trafficking of mGluR1. Representative cells (A) and quantitation (B) of the R,S-DHPG-mediated trafficking of myc-mGluR1 suggested that, as expected, acute knockdown of the endogenous SNX1 led to the rapid recycling of myc-mGluR1 in 30 min post-ligand application. Replacement of the endogenous SNX1 with SNX1ΔH1 rescued the normal trafficking of the receptor, but replacement with SNX1ΔH2 or SNX1ΔH3 failed to rescue the normal trafficking of the receptor. Scale bar = 10 μm. ***$p < 0.001$; n.s. indicates $p > 0.05$.

In this respect, we examined the impact of deleting H1, H2 and H3 domains of SNX1 individually, on its interaction with Hrs. We cotransfected myc-Hrs with HA-SNX1ΔH1 or HA-SNX1ΔH2 or HA-SNX1ΔH3 in HEK293T cells. 24 hrs post-transfection, cells were gently washed on ice with ice-cold PBS and then lysed in lysis buffer. The lysate was then centrifuged at 15,000 rpm for 25 min. After collecting the input separately, rest of the supernatant was subjected to immunoprecipitation by overnight incubation with anti-HA tagged protein-G beads. Immunoprecipitates were run in SDS-PAGE followed by western blot analysis. For immunoblotting, anti-myc mouse monoclonal antibody (1:1000) was used to check for the interaction profile of Hrs with the respective SH3 domain deletion mutants of SNX1. The anti-SNX1 antibody (1:1000) was used to check for the pulled down level of the HA-SNX1. Our data suggested that deletion of the H2 and H3 domains of SNX1 also disrupted binding of Hrs to SNX1, suggesting that the interaction of SNX1 with Hrs is critical for the trafficking of mGluR1 **(Hrs + SNX1: 1 ± 0.06; Hrs + SNX1ΔH1: 1 ± 0.13; Hrs + SNX1ΔH2: 0.43 ± 0.13; Hrs + SNX1ΔH3: 0.12 ± 0.01) (Figure 4.18 A, B)**.

4.2.7. Synaptic localization of various mutants of SNX1 in primary hippocampal neurons

In order to investigate whether the various SNX1 constructs are targeted to the synapse, we examined the synaptic localization of these constructs by counterstaining with an antibody to the pre-synaptic marker protein Bassoon (Dieck, Sanmartí-Vila et al. 1998). Primary hippocampal neurons were transfected with each of these constructs at 6-8 DIV using calcium phosphate method. Neurons were fixed 4-5 days post-transfection with 4% PFA on the ice and subsequently, they were permeabilized with 0.1% Triton X-100 at room temperature for 30 min. After that, cells were stained with anti-HA rat polyclonal antibody (1:500) to label the mutant forms of SNX1 (all the mutants were tagged with the HA epitope) and visualized by the application of goat anti-rat Alexa-568 conjugated secondary antibody (1:500). We observed that the expression of each of these mutant forms of SNX1 was similar to the expression of the wild-type SNX1. To further investigate whether the various mutants of SNX1 that were used in this study localized properly at the synapse, we estimated the fraction of the constructs that were targeted to the synapse which were measured by staining for the HA-containing clusters and

(A)



(B)

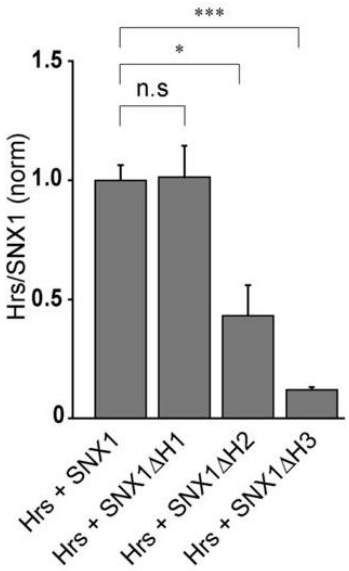
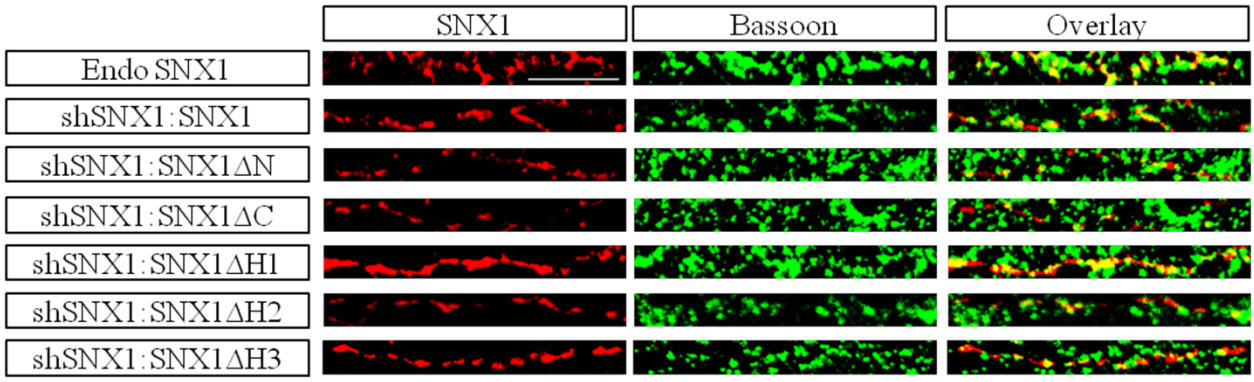


Figure 4.18. H2 and H3 domains of SNX1 are crucial for its interaction with Hrs. (A) Co-immunoprecipitation assays demonstrated that deletion of the H2 or H3 domain of SNX1 disrupted the binding of Hrs to SNX1. (B) Quantitation of the co-immunoprecipitation assays.***$p < 0.001$, *$p < 0.05$; n.s. indicates $p > 0.05$.

counterstaining for Bassoon, a pre-synaptic active zone marker (Dieck, Sanmartí-Vila et al. 1998). Our data suggested that both the N-terminal and C-terminal deletion mutants of SNX1 showed lesser but significant amount of colocalization with Bassoon as compared to the endogenous SNX1 (**Endo SNX1: 64.84 ± 5.46, SNX1ΔN = 49.98 ± 2.42, SNX1ΔC: 50.11 ± 2.35)**. Whereas, other variants of SNX1 localized at the synapse very similar to the endogenous SNX1 (Endo SNX1) protein **(Replacement SNX1: 61.51 ± 1.88, SNX1ΔH1: 61.8 ± 2.12, SNX1ΔH2: 61.0 ± 2.04, SNX1ΔH3: 64.34 ± 3.46**) (**Figure 4.19 A, B)**.

(A)



(B)

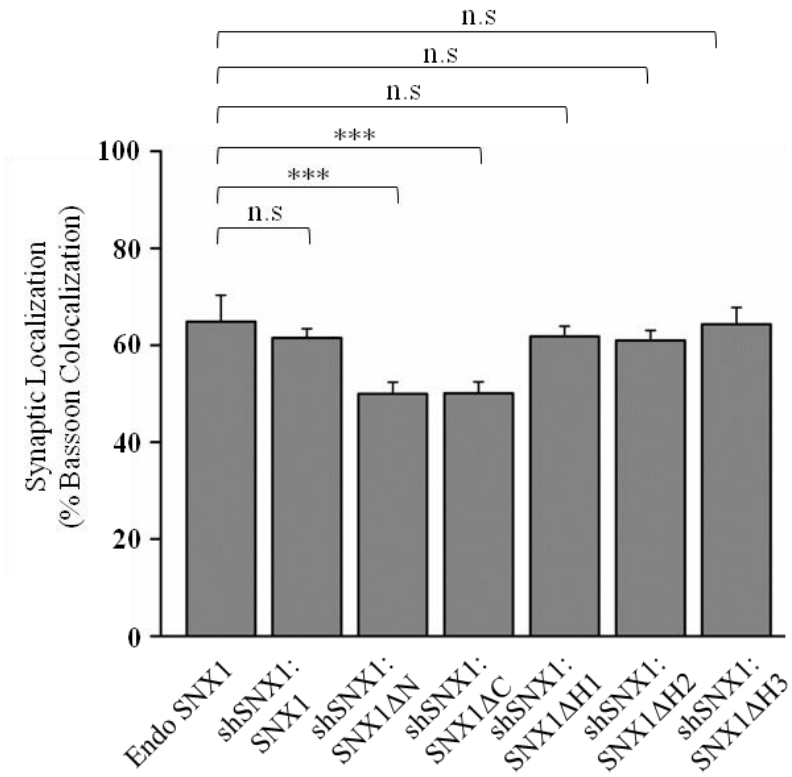


Figure 4.19. Synaptic localization of the SNX1 mutants. (A) Representative images showing colocalization of Bassoon, an active zone synaptic marker with various forms of SNX1 (Replacement SNX1, SNX1ΔN, SNX1ΔC, SNX1ΔH1, SNX1ΔH2 and SNX1ΔH3). (B) Quantitation of the colocalization of the SNX1 constructs. Scale bar = 10 μm. ***$p < 0.001$ n.s indicates $p > 0.05$.

4.3. Discussion

Endosomal sorting of GPCRs is a highly regulated process which involves several proteins. The kinetics of GPCR trafficking from internalization to recycling or lysosomal sorting varies broadly among receptors, suggesting that multiple complex mechanisms exists to control sorting of distinct GPCRs. The divergent sorting of GPCRs to either recycling or lysosomal pathways can be assisted by interaction with an array of adaptor proteins. One of these proteins is the SNX1, which has been reported to direct various GPCRs to their respective sorting pathways. Furthermore, structural domains of SNX1 and their ability to associate with the endosomal membrane components have been broadly investigated (Haft, de la Luz Sierra et al. 1998; Kurten, Eddington et al. 2001; Gullapalli, Garrett et al. 2004). The N-terminal region of SNX1 contains a PX-domain which interacts with phosphoinositol 3 phosphate and the C-terminal region contains a bar domain comprised of three coiled-coil regions which is involved in the dimerization and binding to highly curved membranes (Kurten, Cadena et al. 1996; Haft, de la Luz Sierra et al. 1998; Kurten, Eddington et al. 2001; Cozier, Carlton et al. 2002; Worby and Dixon 2002). Together these domains enable SNX1 to interact with the receptor as well as the endosomal membrane and recruits different proteins to traffic its interacting receptors to their respective sorting pathways. Importantly, SNX1 has also been reported to interact with group I mGluRs (Heydorn, Sondergaard et al. 2004). In the present study, we have intended to explore the role of SNX1 in group I mGluR trafficking. We have used the molecular replacement approach to study the role of various domains and regions of SNX1 in the group I mGluR trafficking. This strategy has allowed the simultaneous shRNA mediated acute knockdown of the endogenous SNX1 and expression of various mutant forms of recombinant SNX1 in primary hippocampal neurons. Our data suggested that SNX1 plays critical role in regulating the recycling of group I mGluRs. Consequently, upon endogenous SNX1 knockdown, both mGluR1 and mGluR5 were re-sorted *via* the faster recycling route and these receptors recycled back to the cell surface at 30 min post-ligand application. Thus, knockdown of the endogenous SNX1 resulted in the rapid recycling of group I mGluRs, which could be rescued by the expression of the wild-type SNX1, suggesting the specificity of the knockdown process. We subsequently investigated the role of specific domains of SNX1 in the group I mGluR trafficking.

The replacement of the endogenous SNX1 with SNX1ΔN did not rescue the normal recycling of mGluR1 suggesting that the N-terminal domain of SNX1is critical for the normal recycling of mGluR1. Similarly, the rescue experiments with SNX1ΔC replacement construct also did not show the rescue of the normal recycling of mGluR1. The receptor still took the rapid recycling route when the endogenous SNX1 was replaced with SNX1ΔC, i.e., SNX1 lacking the C-terminal region. Furthermore, significant amount of both these N-terminal and C-terminal deletion mutants of SNX1 were able to localize at the synapse. These results suggest that both the N-terminal and C-terminal domains of SNX1 are important for the normal trafficking of mGluR1. Importantly, the N-terminal region of SNX1 contains the PX domain that interacts with phosphatidylinositol's (PtdIns) (Cozier, Carlton et al. 2002; Cullen 2008) whereas, the C-terminal region of SNX1 is involved in the protein-protein interactions (Chin, Raynor et al. 2001; Zhong, Lazar et al. 2002; Cullen 2008). Since, our experiments were directed towards understanding the mechanisms through which SNX1 along with its downstream binding partners modulate the trafficking of mGluR1; therefore we focused our study to investigate how the C-terminal region of SNX1 plays a critical role in the trafficking of mGluR1. Interestingly, our data suggested that the H2 and H3 domains of SNX1 are essential for the normal recycling of mGluR1. Our data also suggested that H2 and H3 domains of SNX1 are required for the interaction of SNX1 with Hrs, another accessory protein that has been implicated in both signaling and vesicular trafficking (Komada and Kitamura 1995; Komada, Masaki et al. 1997; Kwong, Roundabush et al. 2000). In view of the above results, we propose that interaction of Hrs to SNX1 is critical for the normal trafficking of mGluR1.

Role of Hrs in the trafficking of group I mGluRs

5.1. Introduction

Many GPCRs have been reported to get internalized subsequent to desensitization and following internalization, some of them recycle back to the cell surface (Ferguson 2001; Tan, Brady et al. 2004; Drake, Shenoy et al. 2006). The phenomena of recycling of these receptors have been considered as an essential process for the resensitization of these receptors (Sibley and Lefkowitz 1985). During the course of recycling, it has been suggested that the modifications of the receptor that leads to its desensitization get remodified resulting in the conversion of the inactive receptor to its native form (Tsao, Cao et al. 2001; Von Zastrow 2001; Katzmann, Odorizzi et al. 2002).

Moreover, the route chosen by a particular receptor subsequent to the internalization depends upon the type of the receptor, type of the ligand and type of the system (Mahato, Ramsakha et al. 2018). The recycling pathway is characterized by the recycling kinetics of that particular receptor. Therefore, recycling routes with differing kinetics categorized as fast ($t_{1/2}$=5 min) and slow ($t_{1/2}$=15-30 min) recycling pathways (Hopkins and Trowbridge 1983). The fast recycling pathway involves the recycling of surface receptors directly from the early endosomes to the plasma membrane. On the other hand, receptors following slow recycling pathway choose an indirect route *via* recycling endosomes for recycling back to the cell surface (Jovic, Sharma et al. 2010; Taguchi 2013). Recycling of receptors from these divergent recycling pathways is a highly regulated process and any alteration in their sorting could essentially lead to the adverse effects on the cell-surface receptor signaling leading to pathophysiological conditions and disease (Barak, Oakley et al. 2001; Hanyaloglu and Zastrow 2008; Sobolik, Su et al. 2014; Sposini, Jean-Alphonse et al. 2017).

Similar to other GPCRs, group I mGluRs subsequent to agonist-induced signaling, undergo desensitization followed by rapid internalization (Dale, Babwah et al. 2002; Mundell, Pula et al. 2003). Subsequent to the internalization, group I mGluRs follow the slow recycling route *via* recycling endosome to recycle back to the cell surface (Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015; Gulia, Sharma et al. 2017). It has been suggested that recycling of these receptors through slow recycling pathway *via* recycling endosome is essential for their resensitization. Our earlier results suggested that the H2 and H3 domains in the C-terminal region of SNX1 play a critical role in the normal recycling of mGluR1. Moreover, it has been

reported that SNX1 interacts with Hrs through its C-terminal region and Hrs has been implicated in vesicular trafficking. We show here, that knockdown of Hrs also resulted in the rapid recycling of mGluR1, whereas over-expression of the full-length Hrs resulted in the delayed recycling of the receptor. Our data also suggest that the N-terminal region of Hrs is essential for the normal trafficking of the receptor. Finally, we show here that the rapid recycling of mGluR1 in the absence of SNX1 led to the recycling of inactive receptors on the cell surface which were unable to initiate the G-protein signaling and also failed to induce the mGluR mediated AMPAR endocytosis.

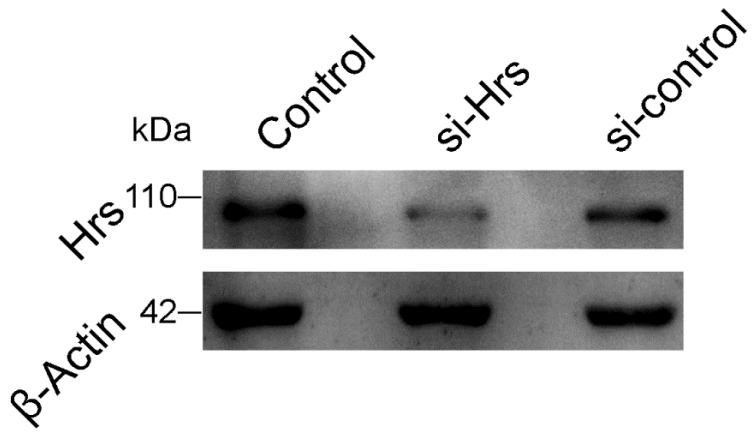
5.2. Results

5.2.1. Knockdown of Hrs results in the faster recycling of mGluR1

Our earlier experiments have shown that knockdown of SNX1 resulted in the rapid recycling of mGluR1 and expression of the wild-type SNX1 could rescue the normal recycling of the receptor. Our data also suggested that deletion of either the H2 or H3 domain of SNX1 inhibited the interaction of SNX1 with Hrs. Furthermore, the H2 and H3 domains of SNX1 seemed to play critical roles in the normal recycling of mGluR1. These results tempted us to investigate the role of Hrs in mGluR trafficking, if any. To explore the role of Hrs in mGluR1 trafficking, we knocked down the endogenous Hrs in primary hippocampal neurons. myc-mGluR1 cDNA and siRNA against endogenous Hrs (si-Hrs) (ON-TARGET plus) or scrambled siRNA were transfected in primary hippocampal neurons at 8-9 DIV according to the method described in the "materials and methods" chapter. For the western blotting experiment, primary neurons were transfected twice with either siRNA against Hrs (ON-TARGET plus SMARTpool) or scrambled siRNA (si-control). Subsequently, the primary neurons were lysed 72 hr post-transfection and samples obtained were subjected to western blot analysis as described earlier. The endogenous Hrs on the blot was stained with the mouse Hrs antibody (1:1000) and visualized by anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5000). Finally, the blot was developed using ECL prime and images were acquired in ImageQuant LAS 4000. In control cells and si-control treated cells, normal expression of the endogenous Hrs was observed whereas, in si-Hrs treated cells, the level of endogenous Hrs was significantly reduced. These observations suggested that si-Hrs was effective in efficiently knocking down the endogenous Hrs **(control: 1 ± 0.03; si-Hrs: 0.42 ± 0.07; si-control: 1.08 ± 0.15) (Figure 5.1 A, B)**.

Subsequently, we checked the effect of Hrs knockdown on the surface expression of myc-mGluR1. Primary hippocampal neurons were co-transfected with myc-mGluR1 cDNA and si-Hrs or si-control at 8-9 DIV. 72 hr post-transfection, myc-mGluR1 expressing live neurons were stained with the anti-myc primary antibody (1:200). After that, the cells were fixed with 4% PFA without permeabilization, followed by the application of goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) for 1 hr to label the surface receptors. The level of surface expression of myc-mGluR1 between si-control treated cells and cells in which si-Hrs pool was transfected

(A)



(B)

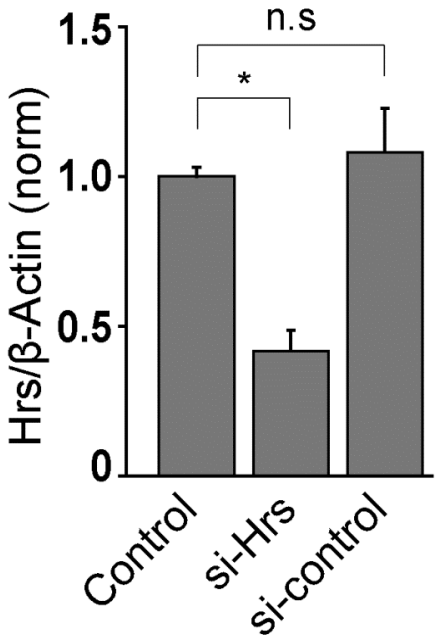


Figure 5.1. Knockdown of endogenous Hrs by si-Hrs. (A) Western blot showing the knockdown of the endogenous Hrs by si-Hrs in primary neurons. (B) Quantitative analyses of western blots showing the efficacy of si-Hrs in knocking down the endogenous Hrs. * indicates $p < 0.05$ and n.s indicates $p > 0.05$.

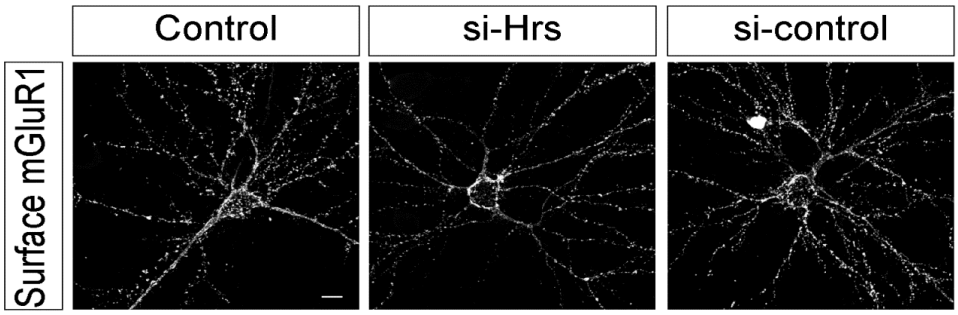
showed no significant difference. This observation suggested that the acute knockdown of Hrs did not have any effect on the surface expression of myc-mGluR1 **(control: 1 ± 0.04; si-Hrs: 1 ± 0.03; si-control: 0.98 ± 0.06) (Figure 5.2 A, B)**.

We next investigated whether knockdown of the endogenous Hrs had any effect on the trafficking of myc-mGluR1. Primary hippocampal neurons were co-transfected with myc-mGluR1 cDNA and si-Hrs or si-control on 8-9 DIV. Subsequently, experiments were conducted when the cells were at 12-13 DIV. The endocytosis assay was performed using the same protocol as described before. As expected, application of 100 μM R,S-DHPG showed normal endocytosis of myc-mGluR1 in control cells which increased gradually over time till 30 min **(control::untreated: 1 ± 0.05; 5 min: 1.48 ± 0.07; 15 min: 2.04 ± 0.09; 30 min: 2.5 ± 0.08) (Figure 5.3 A, B1)**. Similarly, in si-control transfected cells myc-mGluR1 endocytosis was observed to be increased till 30 min post-ligand-application **(si-control:: untreated: 1± 0.07; 5 min: 1.61 ± 0.1; 15 min: 2.11 ± 0.11; 30 min: 2.54 ± 0.17) (Figure 5.3 D1)**. On the other hand, the receptor recycled back faster i.e., in 30 min post 100 μM R,S-DHPG application in si-Hrs transfected cells **(si-Hrs:: untreated: 1 ± 0.04; 5 min: 2.54 ± 0.06; 15 min: 1.68 ± 0.07; 30 min: 1.07 ± 0.04) (Figure 5.3 C1)**. Correspondingly, the measurement of the surface receptors also showed gradual decrease of the receptors from the cell surface on application of 100 μM R,S-DHPG till 30 min in both control cells **(control:: untreated: 1 ± 0.04; 5 min: 0.79 ± 0.04; 15 min: 0.61 ± 0.04; 30 min: 0.44 ± 0.03) (Figure 5.3 B2)** and si-control transfected cells **(si-control:: untreated: 1 ± 0.06; 5 min: 0.73 ± 0.07; 15 min: 0.56 ± 0.05; 30 min: 0.4 ± 0.04) (Figure 5.3 D2)**, whereas in si-Hrs transfected cells the surface fluorescence recovered at 30 min **(si-Hrs:: untreated: 1 ± 0.03; 5 min: 0.47 ± 0.03; 15 min: 0.68 ± 0.03; 30 min: 0.94 ± 0.03) (Figure 5.3 C2)**.

5.2.2. Over-expression of full-length Hrs delays the recycling of mGluR1

Our earlier experiments suggested that the over-expression of full-length SNX1 resulted in the delayed recycling of group I mGluRs. Therefore, we were interested in examining whether full-length Hrs over-expression had any effect on the recycling of group I mGluRs. We first over-expressed the full-length Hrs in HEK293 cells and checked its effect on the surface expression of

(A)



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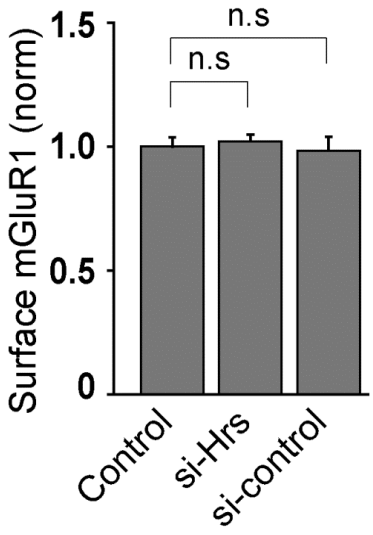


Figure 5.2. Effect of the knockdown of Hrs on the surface expression of mGluR1. (A) Representative images showing that knockdown of the endogenous Hrs had no effect on the surface localization of myc-mGluR1. (B) Quantitation of surface localization of myc-mGluR1. Scale bar = 10 μm. n.s indicates $p > 0.05$.

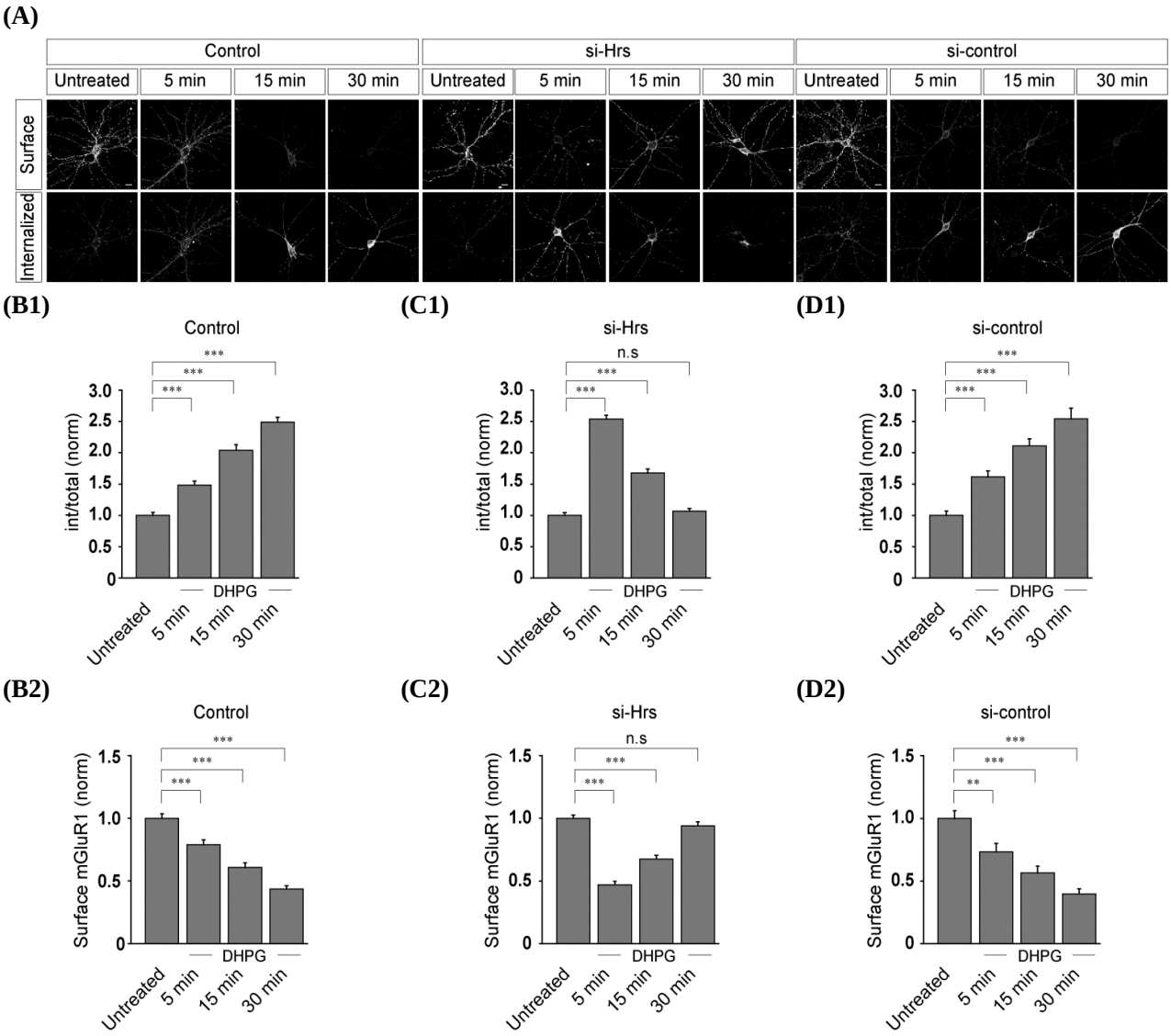


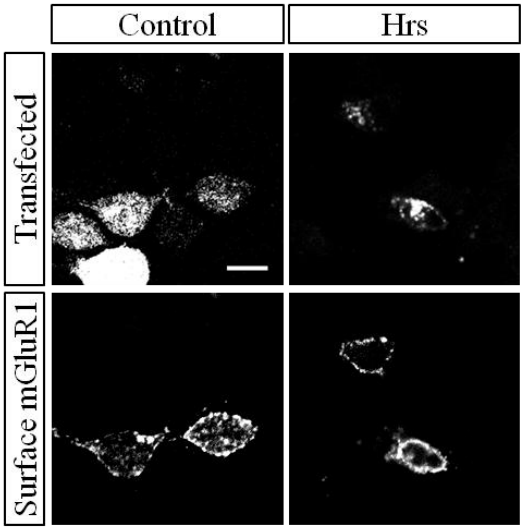
Figure 5.3. Knockdown of Hrs results in the faster recycling of mGluR1. (A) Representative cells showing that knockdown of endogenous Hrs led to the recycling of myc-mGluR1 within 30 min after 100 µM R,S-DHPG application, whereas in control cells and si-control treated cells the receptor showed progressive increase in the internalization till 30 min post-ligand application. (B1, B2) Measurement of the endocytosis (B1) and surface myc-mGluR1 (B2) also suggested that in control cells myc-mGluR1 showed maximum endocytosis at 30 min after R,S-DHPG application. (C1, C2) On the other hand, acute knockdown of the endogenous Hrs resulted in the recycling of the receptor within 30 min after 100 µM R,S-DHPG application as evident by the quantitation of the endocytosis (C1) and measurement of myc-mGluR1 present at the cell surface (C2). (D1, D2) Quantitation of the endocytosis (D1) and surface myc-mGluR1 (D2) suggested that R,S-DHPG-induced trafficking of myc-mGluR1 in si-control treated cells was similar to the control cells. Scale bar = 10 µm. *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

mGluR1. Initially, the cDNA of both FLAG-mGluR1 and full-length myc-Hrs were co-transfected in HEK293 cells. After that, the cells were incubated in 10% DMEM for 24 hr. Live cells expressing FLAG-mGluR1 were stained with the rabbit polyclonal anti-FLAG antibody (1:500) for 15 min at 37°C. Subsequently, cells were fixed with 4% PFA on ice for 15 min, followed by the application of goat anti-rabbit Alexa-568 (1:100) for 1 hr to label the surface-localized receptors. After that, cells were permeabilized by 0.1% Triton-X 100 for 30 min. Subsequently, cells were incubated overnight in the mouse monoclonal anti-myc antibody (1:500) at 4°C to stain the over-expressed myc-Hrs. There was no significant difference observed in the FLAG-mGluR1 surface expression between control cells and cells in which myc-Hrs was over-expressed **(control: 1 ± 0.09; myc-Hrs: 1.09 ± 0.14) (Figure. 5.4 A, B)**.

In order to investigate the effect of Hrs over-expression on the mGluR1 trafficking, FLAG-mGluR1 and full-length myc-Hrs constructs were co-transfected in HEK293 cells and then recycling experiment was performed 24 hr post-transfection using the protocol described in the "materials and methods" section. Control cells showed presence of majority of the receptors at the cell surface and upon 100 µM R,S-DHPG application, most of them internalized in 30 min. Further chase of these receptors for extended periods, in the absence of the ligand, led to the recycling of these receptors at 2.5 hr **(control: untreated: 1 ± 0.05; 30 min: 1.88 ± 0.05; 2.5 hr: 1.07 ± 0.10)**. Importantly, the cells transfected with full-length myc-Hrs showed the endosomal retention of FLAG-mGluR1. Unlike in control cells, the internalized receptors didn't recycle back completely at 2.5 hr post-ligand application and were still present in the endosomal compartment at 2.5 hr time point in the presence of full-length myc-Hrs **(myc-Hrs: untreated: 1 ± 0.10; 30 min: 1.80 ± 0.03; 2.5 hr: 1.52 ± 0.08) (Figure. 5.5 A, B)**. These observations suggested that the expression of the full-length myc-Hrs significantly impedes the recycling of FLAG-mGluR1 in HEK293 cells.

Subsequently, we investigated the effect of the full-length Hrs expression on the trafficking of FLAG-mGluR1 in primary hippocampal neurons. Cells were transfected with FLAG-mGluR1 and myc-Hrs cDNA using calcium phosphate method on 6-7 DIV as described in the "method" section. On 12-15 DIV, live neurons were stained with rabbit anti-FLAG primary antibody

(A)



(B)

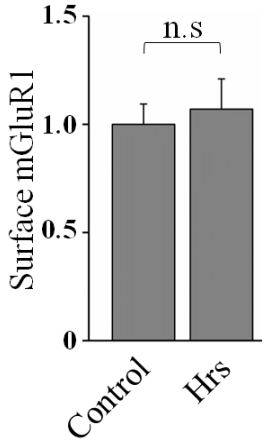
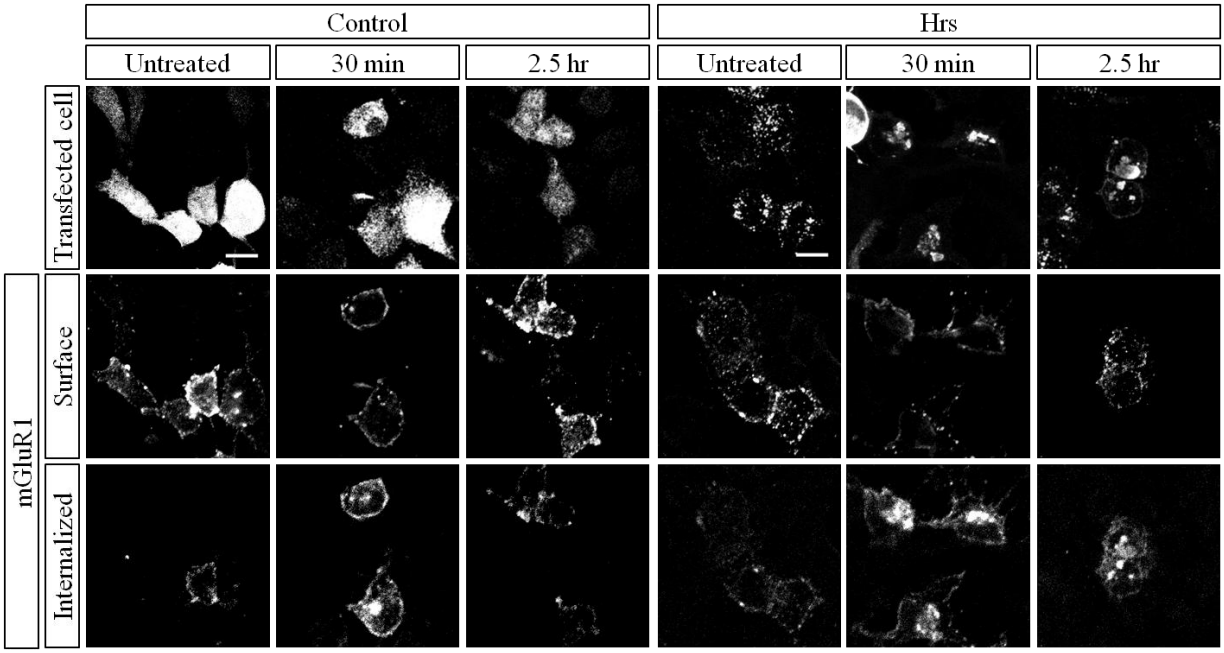


Figure 5.4. Effect of the expression of full-length myc-Hrs construct on the surface expression of mGluR1 in HEK293 cells. (A) Representative images showing surface localization of FLAG-mGluR1 in myc-Hrs transfected cells. (B) Quantitation of the surface FLAG-mGluR1 in HEK293 cells suggested that there was no significant difference in FLAG-mGluR1 surface expression in cells expressing myc-Hrs as compared to control cells. Scale bar = 10 μm. n.s indicates $p > 0.05$

(A)



(B)

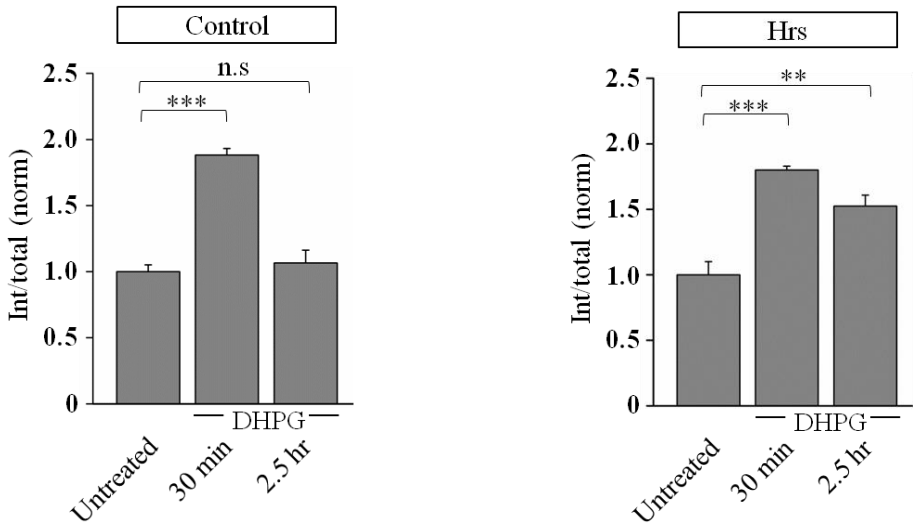


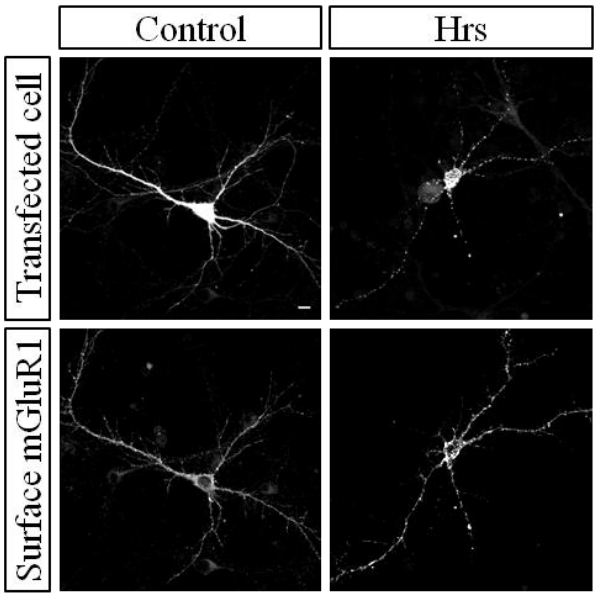
Figure 5.5. Over-expression of myc-Hrs interrupts the recycling of mGluR1 in HEK293 cells. (A) Representative images showing that application of 100 μM R,S-DHPG induced the internalization of the receptor at 30 min in HEK293 cells and the receptors were observed to recycle back to the cell surface at 2.5 hr. Importantly in myc-Hrs over-expressed cells the recycling of FLAG-mGluR1 was partially inhibited and receptors were seen to be in the internal compartments even at 2.5 hr. (B) Quantitation also suggested that myc-Hrs over-expression interrupted the FLAG-mGluR1 recycling in HEK293 cells. Scale bar = 10 μm. *** indicates $p < 0.001$, ** indicates $p < 0.01$ and n.s indicates $p > 0.05$.

(1:200) for 20 min to label the surface receptors and then cells were fixed without permeabilization in ice-cold 4% PFA on ice for 15 min. Subsequently, cells were incubated in goat anti-rabbit Alexa-568 conjugated secondary antibody (1:100) for 1 hr at 37˚C. Finally, cells were mounted on glass slides and observed under the confocal microscope. There was no significant difference observed in the surface expression of FLAG-mGluR1 between control cells and cells in which full-length myc-Hrs was over-expressed **(control: 1 ± 0.07; myc-Hrs: 0.92 ± 0.08) (Figure 5.6 A, B)**.

In order to investigate whether the Hrs over-expression affects the recycling of FLAG-mGluR1 in primary hippocampal neurons as well, we performed recycling assay in the presence of myc-Hrs using the protocol described in the "material and methods" chapter. In brief, neurons were co-transfected with full-length myc-Hrs and FLAG-mGluR1 constructs. On DIV 12-13, rabbit anti-FLAG primary antibody (1:200) was applied for 20 min in live neurons to label the surface-localized receptors. After that, 100 μM R,S-DHPG pulse was given for 5 min followed by chase for more extended periods in the absence of the ligand. Subsequently, the surface localized receptors and internalized receptors were labelled using the goat anti-rabbit Alexa-568 conjugated secondary antibody and goat anti-rabbit Alexa-647 conjugated secondary antibody respectively using the protocol described before. Cells were then mounted on glass slides and observed under the confocal microscope.

As expected, in control cells, upon application of 100 μM R,S-DHPG, FLAG-mGluR1 internalized maximally after 30 min and recycled back to the cell surface at 2.5 hr post-ligand application as represented by the quantitation of the endocytosis index **(control: 1 ± 0.27 ; 30 min: 2.27 ± 0.31; 1.5 hr: 1.72 ± 0.20 ; 2.5 hr: 077 ± 0.24) (Figure 5.7 A, B)** and the recovery of the cell surface fluorescence also suggested the same **(control: 1 ± 0.11; 30 min: 0.50 ± 0.08; 1.5 hr: 0.55 ± 0.06; 2.5 hr: 1.16 ± 0.13) (Figure 5.7 C)**. Importantly, the recycling of FLAG-mGluR1 was delayed in the presence of full-length myc-Hrs as these receptors recycled back to the cell surface in 4.5 hr post-ligand application **(control: 1 ± 0.14; 30 min: 2.83 ± 0.24; 2.5 hr: 2.47 ± 0.13; 4.5 hr: 0.94 ± 0.16) (Figure 5.7 D)**. Moreover, the quantitation of surface receptors also showed a decrease in the surface fluorescence at 30 min post-R,S-DHPG application which was recovered at 4.5 hr, suggesting that FLAG-mGluR1 recycled back to the

(A)



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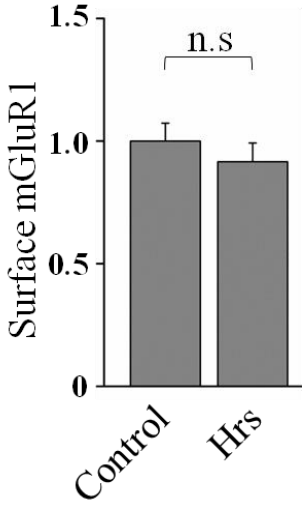


Figure 5.6. Over-expression of myc-Hrs does not affect the surface expression of mGluR1 in primary hippocampal neurons. (A) Representative images showing surface expression of FLAG-mGluR1 in control and myc-Hrs over-expressing cells. No significant change in the surface expression of FLAG-mGluR1 was observed in myc-Hrs expressing cells as compared to control cells. (B) Quantitation of the surface expression of FLAG-mGluR1 in control cells and cells expressing myc-Hrs. Scale bar = 10 μm. n.s indicates $p > 0.05$.

cell surface at 4.5 hr in myc-Hrs over-expressing cells **(control: 1 ± 0.07; 30 min: 0.54 ± 0.08; 2.5 hr: 0.67 ± 0.07; 4.5 hr: 1.09 ± 0.10) (Figure 5.7 E)**. These results also suggested that Hrs plays a critical role in the trafficking of mGluR1.

5.2.3. Over-expression of the ΔVHS/FYVE and ΔVHS mutants of Hrs resulted in the faster recycling of group I mGluRs in the primary hippocampal neurons

As stated before, previous studies have suggested that Hrs, lacking the VHS domain (myc-HrsΔVHS) or VHS/FYVE (myc-HrsΔVHS/FYVE) domains acts in a dominant-negative manner to inhibit the trafficking of EGF receptor when over-expressed in HeLa cells (Chin, Raynor et al. 2001; Morino, Kato et al. 2004; Hanyaloglu, McCullagh et al. 2005). In order to investigate whether these mutants could play a role in the ligand-mediated trafficking of mGluR1, we performed endocytosis experiment in the presence of myc-HrsΔVHS or myc-HrsΔVHS/FYVE using the protocol described in the "material and methods" chapter. Initially, the effect of these mutants in the surface expression of FLAG-mGluR1 was studied. The expression of myc-HrsΔVHS/FYVE or myc-HrsΔVHS did not affect the surface expression of FLAG-mGluR1 in primary hippocampal neurons as observed by no significant difference in FLAG-mGluR1 surface expression between control cells, myc-HrsΔVHS/FYVE and myc-HrsΔVHS expressing cells **(control: 1 ± 0.05; myc-HrsΔVHS/FYVE: 1.08 ± 0.06; myc-HrsΔVHS: 0.98 ± 0.04) (Figure 5.8 A, B)**.

The effect of myc-HrsΔVHS/FYVE and myc-HrsΔVHS over-expression on the mGluR1 trafficking was next investigated. As expected, in control cells very less internalized receptors were observed which increased on 100 μM R,S-DHPG application at 30 min. Importantly, there was no significant amount of endocytosed FLAG-mGluR1 observed in both myc-HrsΔVHS/FYVE and myc-HrsΔVHS expressing cells at 30 min time point, suggesting that both VHS and FYVE domain of Hrs plays a critical role in the ligand-mediated trafficking of mGluR1 **(control:: untreated: 1 ± 0.06; 30 min: 1.67 ± 0.10; myc-HrsΔVHS/FYVE:: untreated: 1 ± 0.09; 30 min: 1.10 ± 0.04; myc-HrsΔVHS:: untreated: 1 ± 0.08; 30 min: 1.13 ± 0.10) (Figure 5.9 A, B)**.

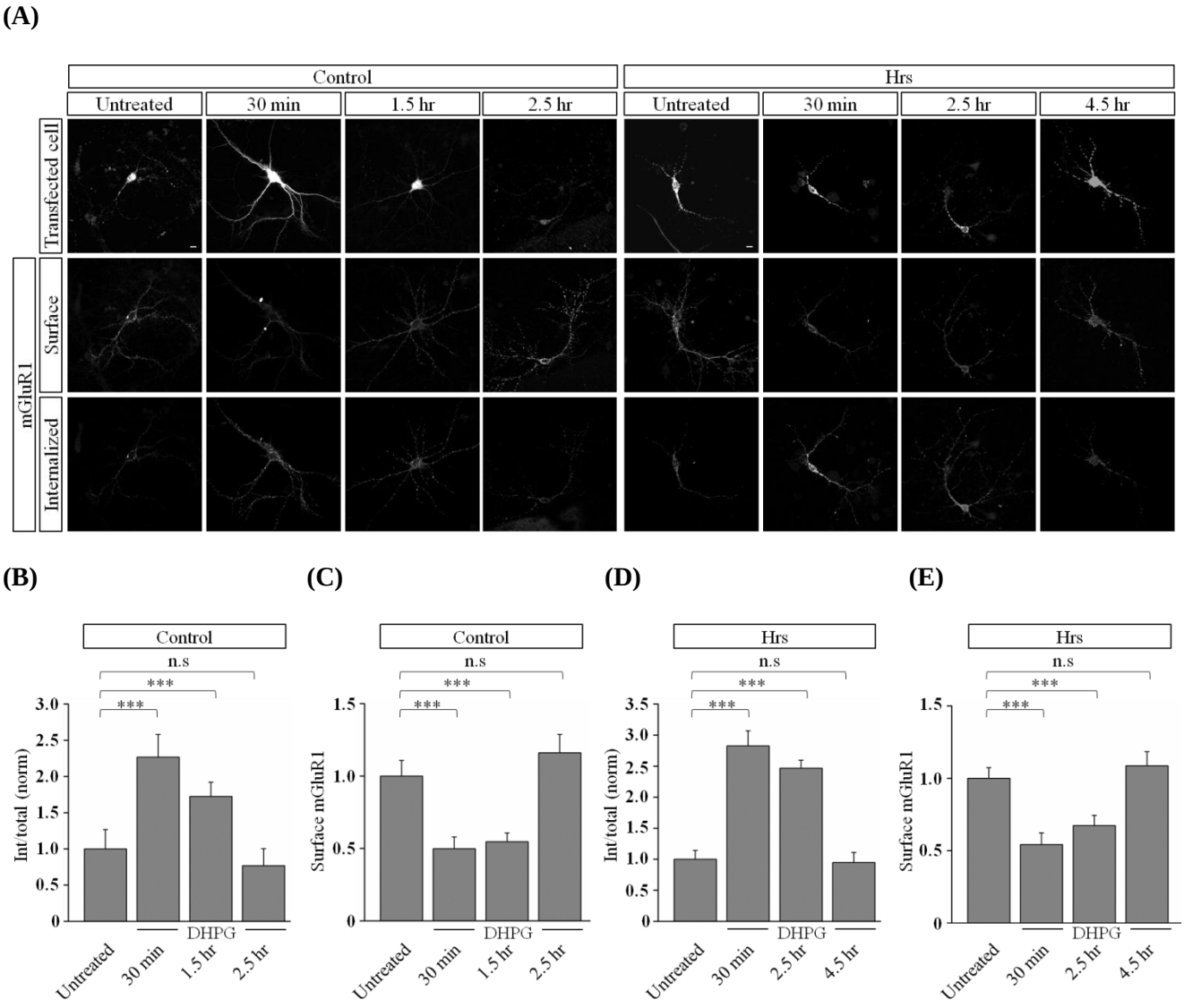
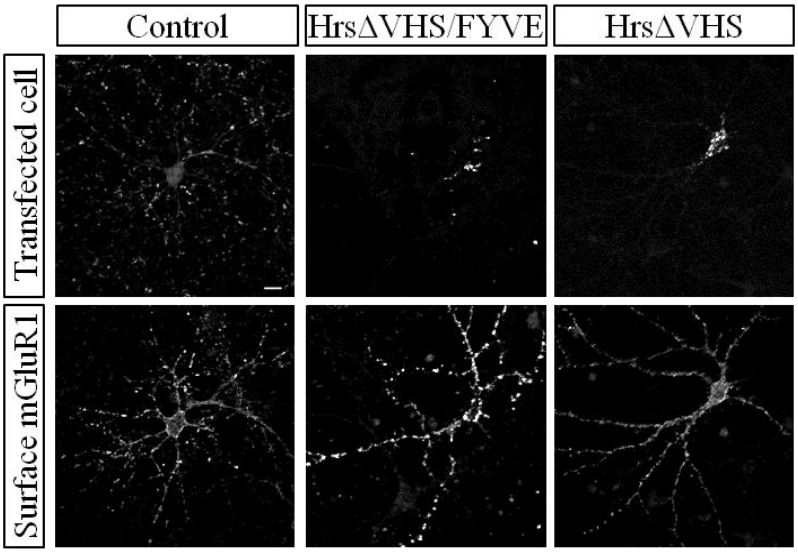


Figure 5.7. myc-Hrs over-expression delays the mGluR1 recycling in primary hippocampal neurons. (A) Control cells showing very less internal fluorescence which increased upon 100 μM R,S-DHPG application in 30 min and the majority of the internalized receptors recycled back to the cell surface in 2.5 hr. Whereas, neurons over-expressing myc-Hrs showed delayed recycling of FLAG-mGluR1 and receptors recycled back at 4.5 hr. (B, C) Quantitation of the receptor internalization (B) as well as measurement of the surface FLAG-mGluR1 (C) showed that in control cells 100 μM R,S-DHPG application induced maximum endocytosis of FLAG-mGluR1 at 30 min and then the receptor recycled back to the cell surface in 2.5 hr. (D, E) On the other hand, over-expression of the myc-Hrs delayed the recycling of the receptor and FLAG-mGluR1 recycled back to the cell surface at 4.5 hr post-ligand application as shown by the quantitation of endocytosis (D) and measurement of the surface receptors (E). Scale bar = 10 μm. ***$p < 0.001$ and n.s indicates $p > 0.05$.

(A)



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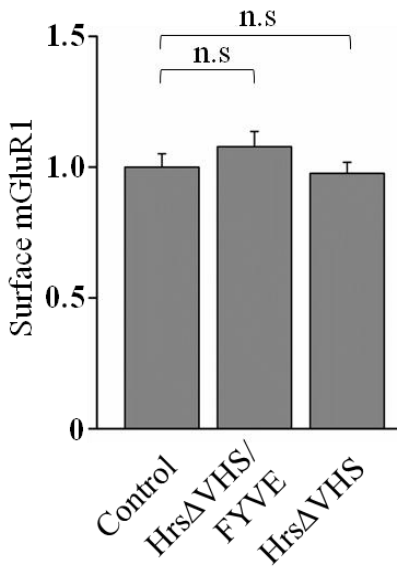
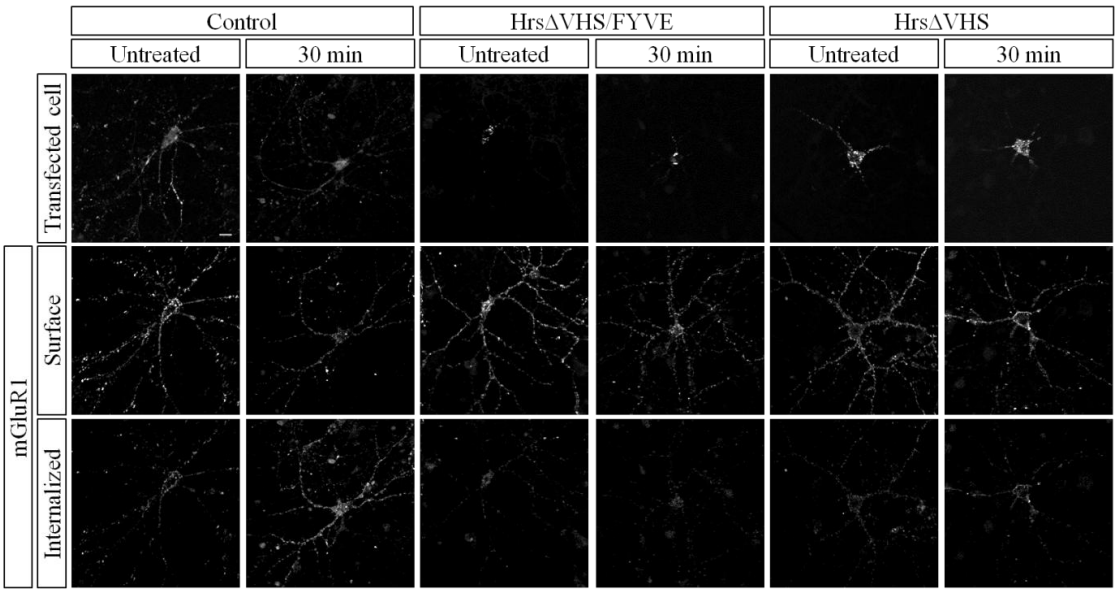


Figure 5.8. Over-expression of myc-HrsΔVHS/FYVE and myc-HrsΔVHS does not have any effect on the surface expression of mGluR1 in primary hippocampal neurons. (A) Representative images showing surface expression of FLAG-mGluR1 in control cells, myc-HrsΔVHS/FYVE and myc-HrsΔVHS overexpressing cells. (B) Quantitation of the surface expression of FLAG-mGluR1 showed no significant difference between control cells, myc-HrsΔVHS/FYVE and myc-HrsΔVHS over-expressing cells. Scale bar = 10 μm. n.s indicates $p > 0.05$.

(A)



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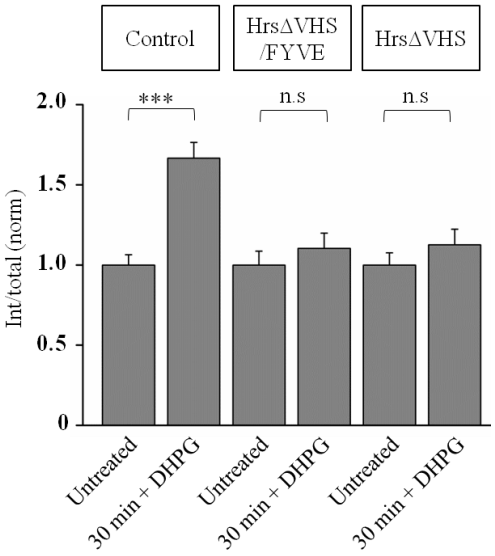


Figure 5.9. Over-expression of the myc-HrsΔVHS/FYVE and myc-HrsΔVHS constructs affects the ligand-mediated trafficking of mGluR1 in primary hippocampal neurons. (A) Control cells showed presence of majority of the receptors at the cell surface. The receptors internalized on 100 μM R,S-DHPG application. Over-expression of both the myc-HrsΔVHS/FYVE and myc-HrsΔVHS constructs affected the ligand-mediated trafficking of FLAG-mGluR1. (B) Quantitation of the effect of myc-HrsΔVHS/FYVE and myc-HrsΔVHS over-expression on the R,S-DHPG-mediated trafficking of FLAG-mGluR1 in primary hippocampal neurons. Scale bar = 10 μm. ***$p < 0.001$ and n.s indicates $p > 0.05$.

Since both the mutants show a similar phenotype and previous reports suggested that particularly VHS domain is involved in the recycling of some surface receptors (Hanyaloglu, McCullagh et al. 2005), therefore we performed time course of trafficking experiment in myc-HrsΔVHS expressing primary hippocampal neurons. FLAG-mGluR1 expressing control cells were stained with the anti-FLAG primary antibody. Subsequently, application of 100 μM R,S-DHPG led to the rapid internalization of the receptor. The amount of FLAG-mGluR1 internalization increased over time till 30 min post-ligand application **(control::untreated: 1 ± 0.15; 5 min: 1.48 ± 0.23; 15 min: 1.58 ± 0.14; 30 min: 2.04 ± 0.11) (Figure 5.10 A, B)**. Quantitation of the surface localized receptors also showed the corresponding decrease in the receptor level from the cell surface on application of 100 μM R,S-DHPG over time **(control::untreated: 1 ± 0.07; 5 min: 0.78 ± 0.10; 15 min: 0.72 ± 0.08; 30 min: 0.60 ± 0.06) (Figure 5.10 C)**. Importantly, FLAG- mGluR1 in myc-HrsΔVHS expressing cells internalized upon application of 100 μM R,S-DHPG, but it recycled back to the cell surface at 30 min post-ligand application **(myc-HrsΔVHS::untreated: 1 ± 0.04; 5 min: 1.92 ± 0.14; 15 min: 1.77 ± 0.18; 30 min: 1.14 ± 0.06) (Figure 5.10 D)**. Moreover, the measurement of the surface FLAG-mGluR1 at 30 min post-R,S-DHPG-mediated internalization of the receptor also showed recovery of the surface fluorescence at the untreated level. **(myc-HrsΔVHS::untreated: 1 ± 0.05; 5 min: 0.56 ± 0.04; 15 min: 0.61 ± 0.06; 30 min: 1.04 ± 0.09) (Figure 5.10 E)**. These results suggest that FLAG-mGluR1 undergoes faster recycling in the presence of myc-HrsΔVHS as compared to control cells.

5.2.4. SNX1-mediated recycling of mGluR1 is critical for the resensitization of the receptor

Subsequent to the ligand-induced activation, group I mGluRs undergo desensitization and rapid internalization following which they recycle back to the cell surface in 2.5 hr (Dhami and Ferguson 2006; Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015). The recycling of receptors is considered as a mechanism of resensitization for several receptors leading to their functional recovery while regaining full responsiveness towards agonist (Ferguson 2001; Drake, Shenoy et al. 2006; Sorkin and Von Zastrow 2009). Thus, the route followed by a particular receptor to recycle back to the plasma membrane is crucial for its proper resensitization. To study the resensitization of group I mGluRs, we examined the ability of the recycled receptor to

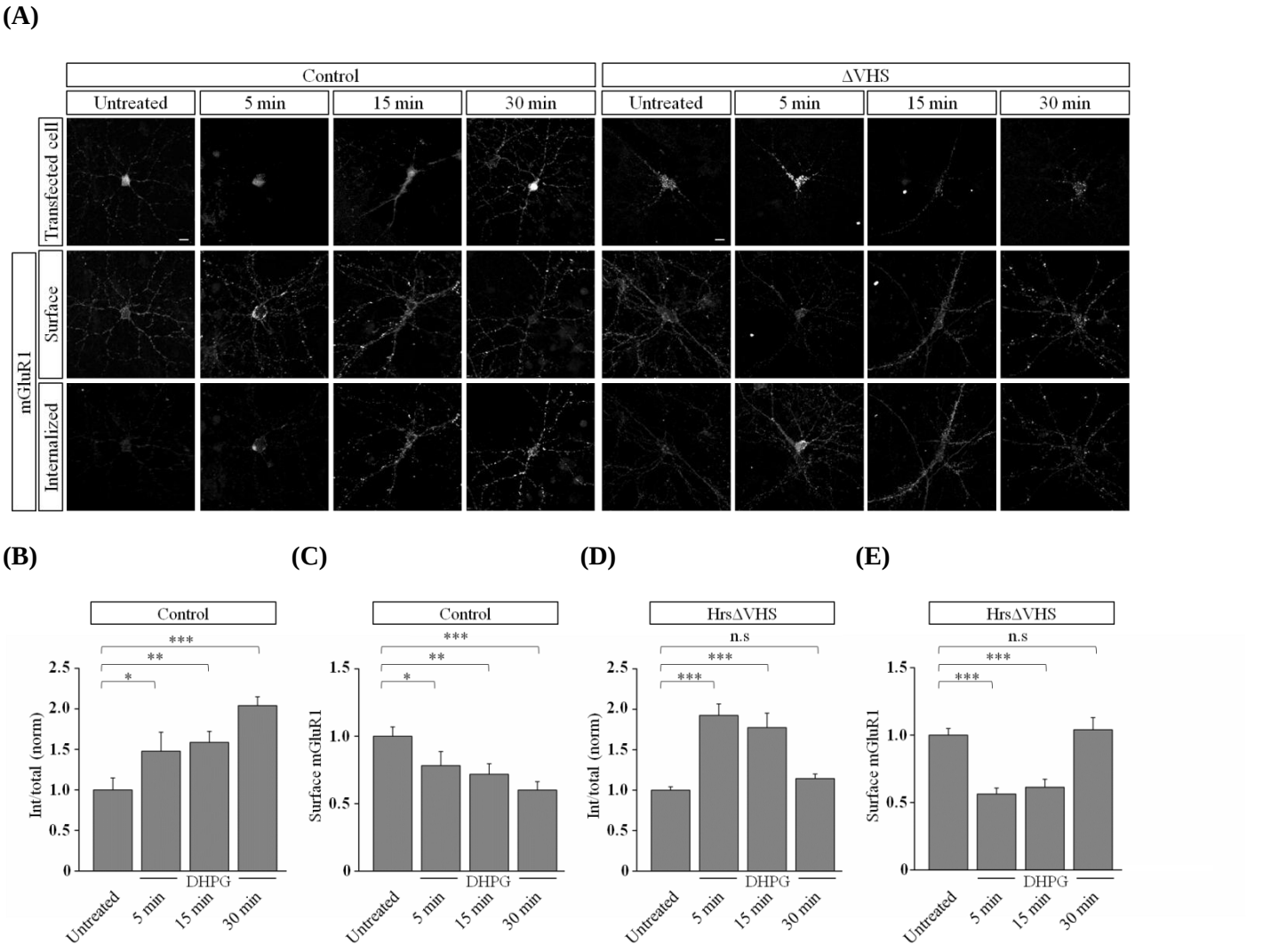


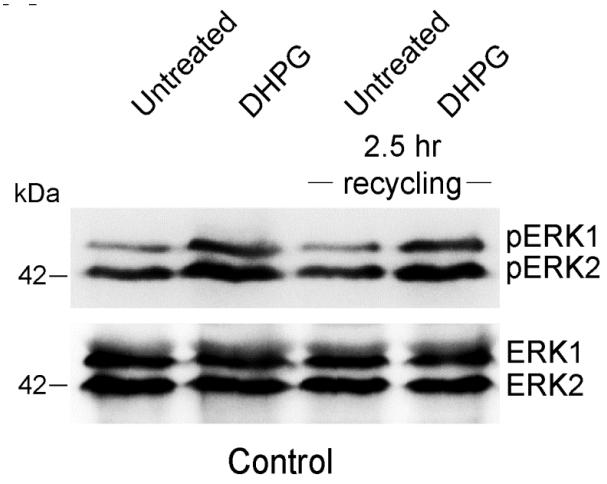
Figure 5.10. Over-expression of the myc-HrsΔVHS mutant results in the faster recycling of mGluR1 in primary hippocampal neurons. (A) Representative images showing the R,S-DHPG-mediated internalization of FLAG-mGluR1 at different time points in control and myc-HrsΔVHS over-expressed primary hippocampal neurons. (B, C) Control cells, upon 100 μM R,S-DHPG application, showed a progressive increase in the endocytosis from 5 min to 30 min as shown by the quantitation of the endocytosis (B) as well as measurement of the surface FLAG-mGluR1 (C). (D, E) Importantly, in myc-HrsΔVHS over-expressed cells, the receptor showed maximum internalization at 5 min after 100 μM R,S-DHPG application and recycled back to the cell surface at 30 min, as shown by the quantitation of the endocytosis index (D) and quantitation of the surface receptors (E). Scale bar = 10 μm. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

induce the second messenger responses. As stated before, activation of group I mGluRs transduces MAPK signaling pathway leading to the phosphorylation of MAPK/ERK1/2 (Kim, Lee et al. 2008; Gladding, Fitzjohn et al. 2009). The resensitization status of the recycled receptors was estimated by their ability to upregulate the phosphorylation of ERK1/2. Initially, cells were treated with 100 μg/ml cycloheximide for 5 hr to inhibit the new receptor synthesis and the drug was present throughout the experiment. Subsequently, 100 μM R,S-DHPG was applied for 5 min to initiate the endocytosis of mGluRs and they were allowed to recycle back to the cell surface, in the absence of the ligand, in 2.5 hr. After that, 100 μM R,S-DHPG was again applied for 5 min. Subsequently, cells were lysed and samples were run in SDS-PAGE followed by western blotting following the similar protocol as described in the "methods" section. The western blotting was performed to measure the extent of upregulation of ERK1/2 phosphorylation by the mGluRs upon ligand stimulation. The immunoblotting of phospho-ERK1/2 and total ERK1/2 was performed using anti-phospho-p44/42 MAPK (ERK1/2) antibody (1:1000) and anti-p44/42 MAPK (ERK1/2) antibody (1:1000) respectively.

Application of 100 μM R,S-DHPG, when the group I mGluRs were initially present at the cell surface, increased the phosphorylation of ERK1/2. Importantly, the activation of the receptors that recycled back to the cell surface in 2.5 hr by the application of 100 μM R,S-DHPG, again led to the increase in the phosphorylation of ERK1/2 **(control:: untreated: 1 ± 0.07; DHPG: 1.94 ± 0.1; 2.5 hr recycling untreated: 1.03 ± 0.07; 2.5 hr recycling DHPG: 1.84 ± 0.03) (Figure 5.11 A, B)**. These results suggested that the receptors that came back to the cell surface through normal recycling route were resensitized.

We next investigated the effect of SNX1 knockdown on the resensitization of group I mGluRs. Primary neurons were transfected with shSNX1 two times alternatively on DIV 5 and DIV 7 while the experiment was performed on DIV 12-13. Western blotting experiment to examine the ability of the recycled receptors to upregulate ERK1/2 phosphorylation in SNX1 knocked down cells was performed in a similar manner as described above. Briefly, cells were first treated with 100 μg/ml cycloheximide for 5 hr followed by the application of 100 μM R,S DHPG for 5 min to induce the internalization of the receptors. Subsequently, neurons were incubated further in the

(A)



(B)

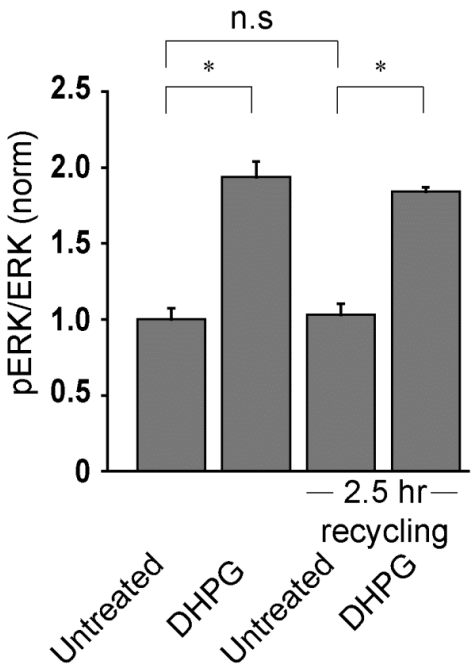


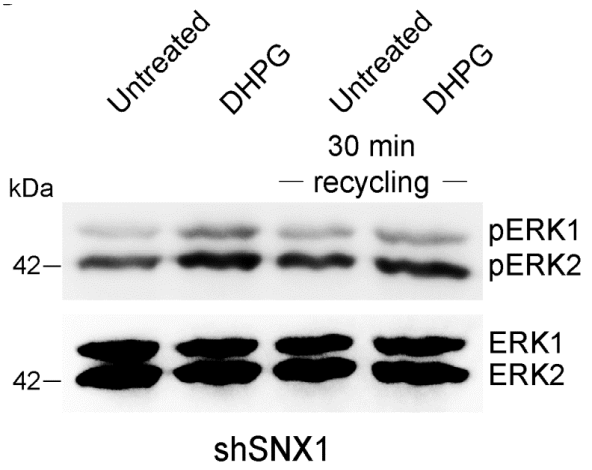
Figure 5.11. Recycled group I mGluRs can induce the second messenger responses. (A, B) Western blot (A) and quantitation of the Western blots (B) showing that application of 100 μM R,S-DHPG for 5 min led to the phosphorylation of MAP kinases. Furthermore, the receptors recycled to the cell surface following normal recycling route in 2.5 hrs, also showed the ability to induce phosphorylation of MAP kinases on application of 100 μM R,S-DHPG for 5 min. * indicates $p < 0.05$ and n.s indicates $p > 0.05$.

absence of the ligand and receptors were allowed to recycle back to the cell surface in 30 min. After that, 100 μM R,S-DHPG was applied again for 5 min. Cells were then lysed and samples were subjected to western blot analysis similarly as described above. The initial application of 100 μM R,S-DHPG augmented the phosphorylation of ERK1/2 in shSNX1 transfected cells as well. Interestingly, the receptors that recycled back to the cell surface in 30 min following rapid recycling route were unable to raise the phosphorylation of ERK1/2, upon activation with 100 μM R,S-DHPG **(shSNX1:: untreated: 1 ± 0.01; DHPG: 1.79 ± 0.01; 30 min recycling untreated: 1.14 ± 0.04; 30 min recycling DHPG: 1.24 ± 0.03) (Figure 5.12 A, B)**. These results suggested that SNX1 plays a crucial role in targeting receptor to the normal recycling route, which allows proper resensitization of the receptor and successful transition to its native state.

As explained before, trafficking of AMPA receptors into and out of the synaptic plasma membrane is believed to be crucial in the expression of long-term potentiation (LTP) and long-term depression (LTD) respectively (Davidkova and Carroll 2007). AMPARs present on the surface of cultured hippocampal neurons undergoes rapid internalization upon activation by the various glutamate receptor agonists, including glutamate itself, NMDA, AMPA and group I mGluR agonists (Carroll, Beattie et al. 2001). The mGluR-mediated endocytosis of AMPARs is thought to be the cellular correlate for the mGluR-dependent synaptic plasticity (Bhattacharyya 2016). The R,S-DHPG-induced endocytosis of AMPARs also indicates the proper functioning of the mGluRs. We have already shown that after ligand-mediated internalization, group I mGluRs recycled back to the surface in 2.5 hrs. The recycling of these receptors believed to be a mechanism for their resensitization. A resensitized receptor is supposed to be fully functional and should behave like a native receptor. Therefore, we performed mGluR-dependent AMPAR endocytosis experiment in cultured primary hippocampal neurons to determine the ability of normally recycled mGluRs to trigger the R,S-DHPG-induced endocytosis of AMPARs.

The experiment was performed in primary hippocampal neurons on DIV 14-15 following the protocol that has been described in detail in the "method" section. Briefly, cells were pre-treated with appropriate mixtures of antagonists: 1 μM TTX, 20 μM DNQX and 50 μM APV for 30 min. Subsequently, first ligand pulse (100 μM R,S-DHPG for 5 min) was given to induce mGluR internalization followed by incubation for 2.5 hr in the absence of the ligand to allow the

(A)



(B)

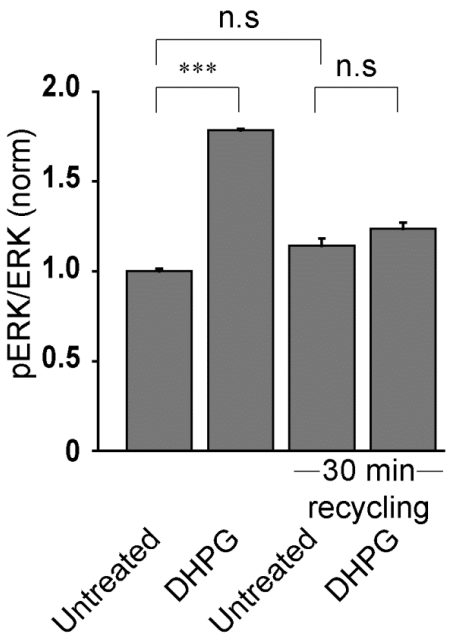


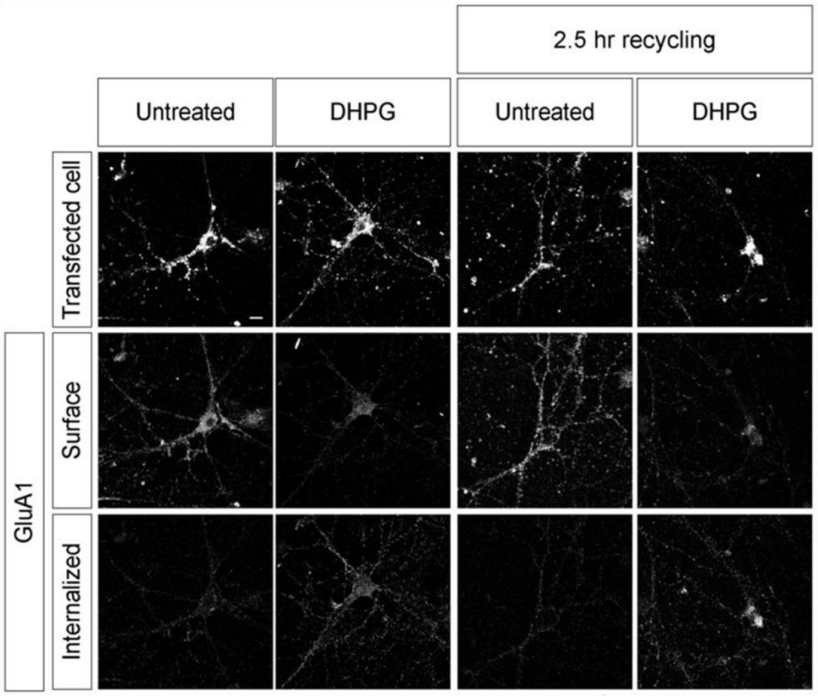
Figure 5.12. Rapidly recycled group I mGluRs in SNX1 knockdown primary neurons are unable to induce the second messenger responses. (A, B) Western blot (A) and quantitation (B) suggested that although in SNX1 knockdown cells, initial application of 100 µM R,S-DHPG for 5 min led to the phosphorylation of MAP kinases, but the receptors that recycled back in 30 min post-ligand application, following the rapid recycling route, were unable to induce the phosphorylation of MAP kinases on application of 100 µM R,S-DHPG for 5 min. *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

recycling of the receptor. After that, rabbit polyclonal anti-GluA1 antibody (1:20) was applied for 15 min to label the AMPARs in live neurons. Subsequently, the second ligand pulse was given for 5 min followed by 10 min chase without the ligand, in the presence of drugs at 37°C.

To study the endocytosis of AMPARs after first ligand exposure, the neurons were stained with the anti-GluA1 antibody (1:20) immediately after the pre-treatment with drugs. After R,S-DHPG pulse, the ligand was removed and cells were incubated in the presence of the drugs for a total of 15 min at 37ºC. Subsequently, cells were fixed with 4% PFA for 15 min on ice without permeabilization. After that, goat anti-rabbit Alexa-568 conjugated secondary antibody was applied in saturating concentration (1:100) for 1.5 hr at 37˚C to stain the surface GluA1 containing receptors. Afterwards, cells were permeabilized using 0.1% Triton X-100 for 30 min and then goat anti-rabbit Alexa-647 conjugated secondary antibody (1:750) was applied for 1.5 hr at 37˚C to visualize the internalized GluA1 containing receptors. As expected, the first round of R,S-DHPG pulse showed rapid internalization of AMPARs **(control:: untreated: 1 ± 0.04; DHPG: 2.02 ± 0.05)**. Moreover, the mGluRs that recycled *via* the normal recycling route in 2.5 hr also showed AMPAR endocytosis upon second R,S-DHPG pulse **(control:: 2.5 hr recycling untreated: 1 ± 0.04; 2.5 hr recycling DHPG: 1.97 ± 0.07) (Figure 5.13 A, B)**. These results suggested that mGluRs recycling *via* the normal recycling route resumed their responsiveness as well as their ability to induce the AMPAR endocytosis when stimulated again with R,S-DHPG.

In order to examine whether the newly synthesized receptors generated during the recycling period of 2.5 hr has contributed to the above results, we performed a control experiment in another set of cells. Earlier reports have suggested that the recycling of group I mGluRs is PP2A and PP2B-dependent (Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015). Therefore, cells were first pre-treated with 5 nM Okadaic acid (PP2A blocker) and 1 μM FK-506 (PP2B blocker) along with the mixtures of antagonists. 5 nM Okadaic acid and 1 μM FK-506 was present throughout the experiment to inhibit the recycling of the mGluRs. Rests of the steps were performed in a similar way as described above. In R,S-DHPG untreated cells majority of the GluA1 containing receptors were present at the cell surface, whereas, the second pulse of R,S-DHPG did not cause endocytosis of GluA1 containing receptors when the mGluR recycling was inhibited with Okadaic acid and FK-506 **(control:: 2.5 hr no recycling untreated: 1 ± 0.04; 2.5 hr no recycling DHPG: 1 ± 0.05) (Figure 5.14 A, B)**.

(A)



(B)

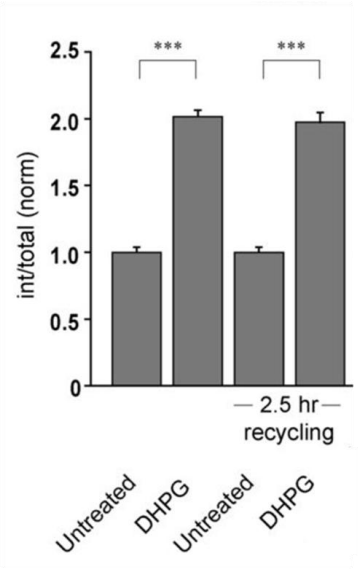
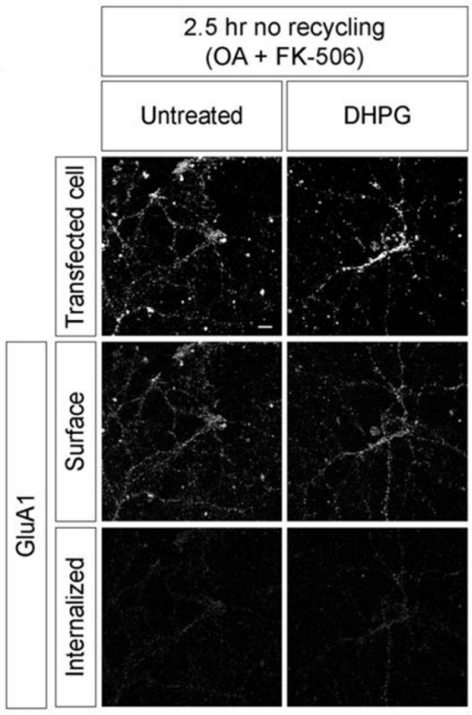


Figure 5.13. Recycled group I mGluRs can induce the DHPG-mediated AMPAR endocytosis. (A, B) Representative images (A) and quantitation of the mGluR-mediated AMPAR endocytosis (B) suggested that in control cells, the receptors that recycled back to the cell surface following normal recycling route in 2.5 hr, were able to induce the endocytosis of GluA1 containing receptors when they were stimulated with 100 µM R,S-DHPG for 5 min. Scale bar = 10 µm. *** indicates $p < 0.001$.

(A)



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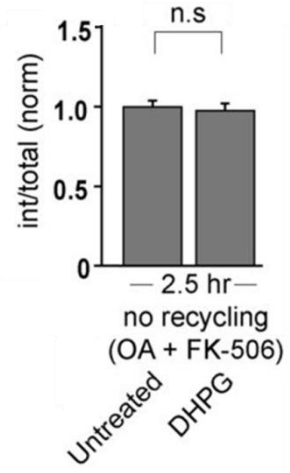
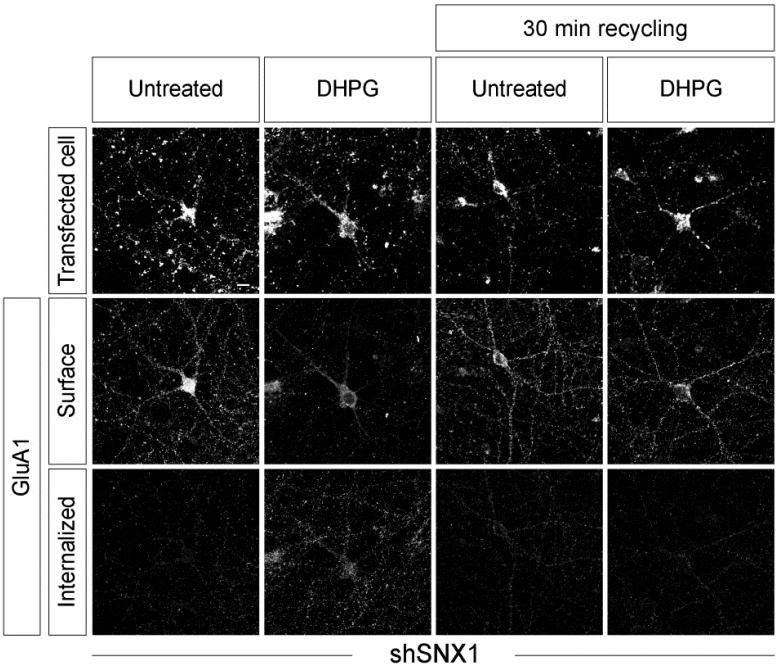


Figure 5.14. Second DHPG pulse induced AMPAR endocytosis contributed exclusively by recycled receptors. (A, B) Representative images (A) and quantitation (B) of the mGluR-mediated AMPAR endocytosis showing that 100 μM R,S-DHPG application did not cause endocytosis of GluA1-containing receptors when the mGluR recycling was inhibited with Okadaic acid and FK-506. Scale bar = 10 μm. n.s indicates $p > 0.05$.

Our earlier experiments suggested that in SNX1 knocked down cells, group I mGluRs recycled rapidly subsequent to the ligand-mediated internalization. In order to check whether rapidly recycled mGluRs in the absence of SNX1 could induce mGluR-mediated AMPAR endocytosis, we performed the above experiments in SNX1 knocked down cells. The detailed protocol has been described in the "method" section. Briefly, cells were transfected with shSNX1 and experiment was performed on 12-13 DIV. In shSNX1 transfected cells, application of the first pulse of R,S-DHPG induced AMPAR endocytosis **(shSNX1:: untreated: 1 ± 0.05; DHPG: 1.9 ± 0.05)**. Subsequently, the mGluRs were allowed to recycle back to the cell surface *via* a rapid recycling route in 30 min. After that, when second R,S-DHPG pulse was given, they were not able to induce the endocytosis of AMPARs **(shSNX1:: 30 min recycling untreated: 1 ± 0.03; 30 min recycling DHPG: 0.93 ± 0.04) (Figure 5.15 A, B)**. These results suggested that the receptors that recycled back *via* the rapid recycling route in the absence of SNX1 were inactive and unable to participate in mGluR-LTD.

(A)



(B)

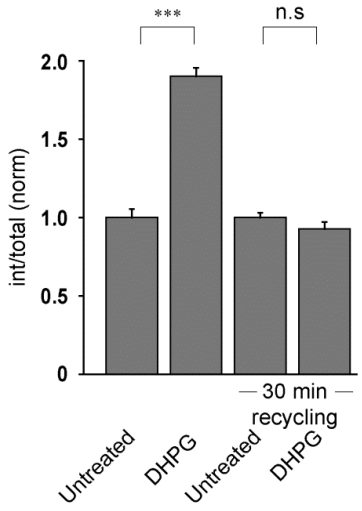


Figure 5.15. Rapidly recycled group I mGluRs in SNX1 knockdown cells were unable to induce the AMPAR endocytosis. (A, B) Representative images (A) and quantitation of the mGluR-mediated AMPAR endocytosis (B) showed that in shSNX1 transfected cells, although initial application of 100 µM R,S-DHPG for 5 min led to the endocytosis of GluA1 containing receptors, but the receptors that recycled back to the cell surface through rapid recycling route in 30 min post-ligand application, did not have the ability to induce the internalization of GluA1-containing receptors. Scale bar = 10 µm. *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

5.3. Discussion

Neurons have a complex morphology characterized by molecularly and functionally distinct domains known as axons and dendrites. Neuronal function essentially depends upon the precise localization and targeting of proteins within these domains (Burack, Silverman et al. 2000). Several post-synaptic density proteins (PSD) play a critical role in the spatiotemporal localization and selective trafficking of neurotransmitter receptors. Trafficking involves the endocytosis of the receptors from the plasma membrane followed by either recycling of the receptor back to the cell surface or targeting for degradation in the lysosomes (Verges 2016). The specific sorting proteins guide post-endocytic targeting of these receptors to a particular pathway. Our data suggest that the correct sorting of group I mGluRs depends on the sorting protein, SNX1. In addition, SNX1 has been reported to interact with various other sorting proteins to form macromolecular complexes and regulate the sorting of various surface receptors (Haft, de la Luz Sierra et al. 1998; Mendrola, Berger et al. 2002; Seaman and Williams 2002; Zhong, Lazar et al. 2002). Among them, Hrs is one such interacting partner of SNX1 which has been reported in the recycling of several other surface receptors and has a similar expression pattern as that of SNX1 in the hippocampus (Komada and Kitamura 1995; Komada, Masaki et al. 1997; Kwong, Roundabush et al. 2000; Chin, Raynor et al. 2001; Hanyaloglu, McCullagh et al. 2005). Moreover, our results have shown that Hrs interacting domains of SNX1 are critical for the trafficking of group I mGluRs. Therefore, we hypothesize Hrs as most likely partner of SNX1 in sorting mGluRs towards recycling endosomes.

In the present study, we tried to explore the role of Hrs in group I mGluR trafficking. We have used siRNA pool against Hrs to study its effect on mGluR1 trafficking. We have also used full-length Hrs and its various mutant forms and studied the effect of their over-expression on the trafficking of mGluR1. Our data suggested that knockdown of the endogenous Hrs resulted in the rapid recycling of mGluR1. Furthermore, over-expression of the full-length Hrs resulted in the delayed recycling of mGluR1. On the other hand, over-expression of the N-terminal truncation mutants of Hrs, viz., myc-HrsΔVHS/FYVE and myc-HrsΔVHS led to the faster recycling of mGluR1. Both these mutants lack VHS domain of Hrs and previous reports suggested that particularly the VHS domain is critically involved in sequence-directed recycling

(Hanyaloglu, McCullagh et al. 2005). The FYVE domain is involved in binding to the inositol lipid PtdIns3P which is required for the recruitment of Hrs to endosomes (MacDonald, Brown et al. 2018). The effect of myc-HrsΔVHS/FYVE mutant which lacks both VHS and FYVE domains on the recycling of mGluR1 could be due to the secondary consequence of reduced association of the mutant on endosomal membranes. Together, these results suggest that specifically, the VHS domain of Hrs play critical role in the normal trafficking of mGluR1.

We subsequently investigated that whether the rapidly recycled receptors in SNX1 knocked down cells can initiate second messenger response after re-stimulation. We have shown that the mGluRs recycling *via* the normal recycling route was able to induce second messenger response upon ligand stimulation. Whereas, the mGluRs that came back to the cell surface *via* faster recycling route, in the absence of SNX1, were unable to induce the second messenger responses as observed by their inability to upregulate the phosphorylation of MAP kinases. These results suggested that mGluRs, recycling *via* the Rab11 positive recycling endosomes, get resensitized during the course of recycling in the wild-type cells. On the other hand, the rapidly recycled receptors in the SNX1 knockdown cells were still inactive as observed by their inability to initiate consecutive round of signaling. Moreover, we also investigated the ability of the recycled mGluRs to induce the AMPAR endocytosis, which is a prerequisite for the mGluR-mediated synaptic plasticity. As expected, when the receptors were allowed to recycle back to the cell surface *via* the slower recycling route in wild-type cells, the recycled receptors were able to induce the AMPAR endocytosis. Whereas, in shSNX1 transfected cells, those receptors that recycled back rapidly were unable to induce AMPAR endocytosis upon successive ligand pulse. These results indicate that SNX1-mediated recycling of these receptors *via* the slower recycling route is critical for the resensitization of these receptors.

Summary

6.1. Aim of the research

The G-protein-coupled receptors (GPCRs) family is coded by more than 1% of the entire human genome. These receptors respond to various signals responsible for the recognition of light, taste, pain, odours, etc. Majority of the essential physiological processes are controlled by GPCR signaling and any aberration in signaling could lead to various pathological consequences (Rosenbaum, Rasmussen et al. 2009; Chen and Palczewski 2016; Leysen, van Gastel et al. 2018; Liauchonak, Dawoud et al. 2018). Recent market analyses show that approximately 40% of the total therapeutic drugs target GPCRs, although only 10% of GPCRs are known drug targets (Wise, Gearing et al. 2002; Vassilatis, Hohmann et al. 2003; Thomsen and Behan 2007; Trzaskowski, Latek et al. 2012). As our understanding of the mechanisms that regulate the responsiveness of GPCRs has increased in the last decade and also more and more orphan receptors are expected to be characterized in the coming years, the number of drugs that target GPCRs can only be expected to increase.

Due to the enormous diversity in the GPCR types and their regulatory mechanisms, it is likely that we have just started to unravel the multiple signal transduction pathways regulated by them along with the variety of mechanisms which regulate their activity. Recent advances in the structural biology and pharmacology have opened up new GPCR research fields. All these studies, along with previous reports, have provided valuable information about the GPCR activation mechanisms, their structural dynamics, signaling and interaction with various effector molecules (Ferguson, Barak et al. 1996; Ferguson, Zhang et al. 1998; Gether 2000; Kelly, Bailey et al. 2008). Importantly, the properties of GPCRs, which allows them to bind different types of ligands and interact with several G-proteins to initiate a variety of intracellular signaling pathways, have established the fact that each GPCR is unique. Thus, there is no GPCR which can serve as a model for the rest. Hence, a particular GPCR is required to be studied in detail to understand its function and regulation. Regulation of activated GPCRs is essential for proper signal transduction and maintenance of the cellular homeostasis (Bhattacharyya 2016). The activity of GPCRs is majorly regulated by the processes of receptor desensitization, internalization, resensitization and downregulation. Several reports have shown that subsequent to the ligand-induced signaling, most of the GPCRs get desensitized

(Ferguson, Zhang et al. 1998; Woolf and Linderman 2003; Kelly, Bailey et al. 2008). Receptor desensitization serves as a protective mechanism adopted by a cell to prevent overstimulation of the receptor and excessive signaling. Many GPCRs have been reported to undergo rapid internalization subsequent to the desensitization (Kelly, Bailey et al. 2008; Klaasse, IJzerman et al. 2008; Hinkle, Gehret et al. 2012; Pope, Tilve et al. 2016). Upon internalization, GPCRs manifest various subcellular fates depending upon the type of the receptor, type of the agonist, along with the cellular background. An increasing number of studies have shown that for many GPCRs internalization is considered as a necessary step for the resensitization and down-regulation of the receptor (Trapaidze, Gomes et al. 2000; Roosterman, Cottrell et al. 2004; Luttrell and Gesty-Palmer 2010).

Regulation of group I mGluRs has become the subject of intense investigation over the past few years because of their involvement in various kinds of synaptic plasticity as well as their putative roles in various neuropsychiatric disorders, such as Fragile X syndrome, autism etc. (Lüscher and Huber 2010; Wang and Zhuo 2012). The widespread expression of these receptors in the central nervous system makes them attractive drug targets, and recent studies have validated various group I mGluR ligands for their therapeutic utility in neurological and psychiatric disorders such as anxiety, depression, autism and schizophrenia (Niswender and Conn 2010). Group I mGluRs are class C G-protein-coupled receptors which upon ligand stimulation induce $G_{\alpha q/11}$ coupled signal transduction pathway and activates phospholipase C-IP_3 pathway in various cell types. Although, group I mGluRs play essential roles in the brain but excessive signaling from these receptors could lead to pathophysiological consequences. Studies have shown that similar to many other GPCRs, group I mGluRs also get desensitized upon agonist stimulation and internalized rapidly (Dale, Bhattacharya et al. 2000; Dale, Babwah et al. 2002; Dhami and Ferguson 2006). Recent studies have reported that subsequent to internalization, group I mGluRs enter the recycling compartment and recycle back to the cell surface *via* slow recycling pathway, suggesting a mechanism for "resensitization" of these receptors (Mundell, Matharu et al. 2001; Mundell, Pula et al. 2004; Choi, Chung et al. 2011; Trivedi and Bhattacharyya 2012; Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015; Mahato, Ramsakha et al. 2018). However, the cellular machineries that control the recycling of the group I mGluRs and its physiological implication have not been studied so far. The lack of knowledge about the role of various cellular

machineries in regulating the trafficking of group I mGluRs and their 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 / mGluR5 and FLAG-tagged mGluR1 / mGluR5 constructs. In these constructs, the myc epitope or the FLAG epitope was tagged at the N-terminus of the full-length protein. Previous reports have already demonstrated that these recombinant receptors behave like the native receptor (Choi, Chung et al. 2011; Trivedi and Bhattacharyya 2012; Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015). We initially used non-neuronal HEK293 cells for our preliminary studies. HEK293 cells are used as a heterologous system, since these cells contain extensive repertoire of G-proteins. We have majorly used dissociated mouse hippocampal primary neurons for our further studies. Primary hippocampal neurons are the most experimentally tractable *in vitro* system that can approximate the *in vivo* situation. There are several techniques like surface biotinylation assay and single colour fluorescence measurement assays that have been used previously 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 labelling assay for differential labelling of the surface localized and internalized receptors by dual antibody staining. This method allowed us to normalize the amount of the internalized receptors within cells. In this way, 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, Biou et al. 2009; Trivedi and Bhattacharyya 2012; Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015).

6.2. Role of SNX1 in the ligand-mediated trafficking of group I mGluRs

As stated before, similar to many other GPCRs, group I mGluRs also reported to undergo desensitization upon agonist stimulation. These receptors get desensitized in PKC and GRK-dependent manner (Dale, Bhattacharya et al. 2000; Dale, Babwah et al. 2002; Mundell, Pula et al. 2004; Mao, Liu et al. 2008). Following desensitization, the receptors get internalized in

arrestin and dynamin-dependent pathway *via* clathrin-coated pits (Ferguson 2001; Dale, Babwah et al. 2002; Ferraguti, Crepaldi et al. 2008). Recent studies from our lab have shown that subsequent to the desensitization, group I mGluRs undergo ubiquitin-dependent internalization and the endocytosed receptors recycle back to the cell surface at 2.5 hr post-ligand application in a phosphatase-dependent manner (Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015; Gulia, Sharma et al. 2017). Our data also suggested that both myc-mGluR1 and myc-mGluR5 undergoes maximum internalization at 30 min post-ligand application and recycles back to the cell surface at 2.5 hr in both HEK293 cells and primary hippocampal neurons. The above observations suggested that the trafficking of group I mGluRs in both these cell types probably occurred through similar mechanisms.

GPCRs manifest various subcellular fates upon internalization and kinetics of their intracellular trafficking is dependent on the type of the receptor, and its cellular background (Drake, Shenoy et al. 2006; Hanyaloglu and Zastrow 2008). The recycling of GPCRs is believed to be a critical step to "resensitize" these receptors. Thus, trafficking of receptors controls their activity and function. Hence, it is critical to study the mechanism of trafficking of these receptors to understand their role in the central nervous system. SNXs are a large group of proteins involved in the trafficking and intracellular sorting of several cellular proteins. SNX1 is the member of this family which has been reported to play an important role in controlling the trafficking and sorting of some receptors (Worby and Dixon 2002; Wassmer, Attar et al. 2007; Wassmer, Attar et al. 2009).

In the case of group I mGluRs, we have observed that the expression of the dominant-negative SNX1 viz. the coiled-coiled SNX1 affected the trafficking of the receptor in non-neuronal HEK293 cells as well as in primary hippocampal neurons. The C-terminal coiled-coil domain of SNX1 has been shown earlier to assemble with the full-length SNX1 (Wang, Zhou et al. 2002). Upon over-expression, this coiled-coil domain may bind with the endogenous SNX1 which in turn could inhibit the interaction between SNX1 and the membrane-associated protein involved in the sorting or endosomal localization leading to dominant-negative effect. Furthermore, the time course study of group I mGluR trafficking in the presence of the coiled-coiled SNX1 showed that the receptors recycled back to the cell surface rapidly at 30 min post-ligand

application in both HEK293 cells and primary hippocampal neurons. Our data also showed that over-expression of the full-length SNX1 delayed the recycling of myc-mGluR1 in both HEK293 cells and primary hippocampal neurons. In order to check for any possible effects of over-expressing full-length SNX1 and coiled-coil SNX1 on the general trafficking machinery of the cells, we studied the trafficking of transferrin receptor. Transferrin receptor showed normal kinetics upon expression of either full-length SNX1 or coiled-coil SNX1, suggesting that SNX1 did not affect the general trafficking machinery of the cell.

6.3. Structure and functional characterization of SNX1 in regulating ligand-mediated trafficking of group I mGluRs

SNX1 has been reported to play essential roles in the trafficking of various GPCRs (Kurten, Cadena et al. 1996; Wang, Zhou et al. 2002; Gullapalli, Garrett et al. 2004; Gullapalli, Wolfe et al. 2006; Nisar, Kelly et al. 2010). Our earlier data suggested that the expression of the dominant-negative mutant of SNX1 resulted in the rapid recycling of group I mGluRs. We further investigated the role of SNX1 in the trafficking of mGluR1 by siRNA-mediated knockdown of the endogenous SNX1 in primary hippocampal neurons. Our data suggested that knocking down of the endogenous SNX1 resulted in the faster recycling of myc-mGluR1. Above findings suggested that SNX1 plays a critical role in regulating the recycling of mGluR1. Furthermore, we examined the role of SNX1 domains in the ligand-mediated trafficking of group I mGluRs using "molecular replacement" strategy (Bhattacharyya et al., 2009). This technique is an exquisite approach that allows knockdown of the endogenous SNX1 and simultaneous replacement of the endogenous protein with various mutant forms of the SNX1 protein. Interestingly, knockdown of the endogenous SNX1 led to the faster recycling of both mGluR1 and mGluR5 in primary hippocampal neurons. When the endogenous SNX1 was replaced with the full-length SNX1, the normal recycling of group I mGluRs was rescued. These results suggested an essential role of SNX1 in the normal trafficking of group I mGluRs.

Chapter 6: Summary

To address the functions of various domains of SNX1 in group I mGluR trafficking, we generated replacement constructs. The replacement of the SNX1ΔN (N-terminal deleted SNX1) was not sufficient to rescue the normal recycling of myc-mGluR1 and the receptor still showed rapid recycling caused by the knockdown of the endogenous SNX1. The N-terminal part of the SNX1 protein contains PX domain which has been reported to bind PI3P (a component of the endosomal membrane) (Worby and Dixon 2002; Wassmer, Attar et al. 2007). Probably, the absence of the PX domain could result in the deficient endosomal association of SNX1ΔN which might lead to the failure of SNX1ΔN to rescue the normal recycling of myc-mGluR1. Furthermore, the replacement of the SNX1ΔC (C-terminal deleted SNX1) was also not sufficient to rescue the normal recycling of myc-mGluR1. The C-terminal domain of SNX1 contains coiled-coil domain which is involved in the dimerization of SNX1 and mediating the interaction of SNX1 with other sorting proteins (Burkhard, Stetefeld et al. 2001; Wang, Zhou et al. 2002). The failure of SNX1ΔC to rescue the normal recycling of myc-mGluR1 could be due to its inability to form dimer and loss of interaction with other vital sorting proteins. These results suggested that both the N-terminal and C-terminal domains of SNX1 are necessary to mediate the normal trafficking of group I mGluRs. As our data suggested the importance of the C-terminal region of SNX1 in the recycling of group I mGluRs, we next investigated the role of three putative SH3 domain-binding motifs (H1, H2, and H3) at the C-terminal region in this process. We studied the role of these domains in the trafficking of group I mGluRs using the following SNX1 replacement constructs: shSNX1:SNX1ΔH1, shSNX1:SNX1ΔH2, and shSNX1:SNX1ΔH3 lacking the H1, H2, and H3 domains, respectively.

Importantly, SNX1ΔH2 and SNX1ΔH3 replacement constructs did not rescue the normal recycling of myc-mGluR1, whereas replacement of the endogenous SNX1 with SNX1ΔH1 rescued the normal trafficking of the receptor. These results suggested that the H2 and H3 domains of SNX1 play a crucial role in regulating the ligand-mediated trafficking of mGluR1. Moreover, our data also indicated that both H2 and H3 domains of SNX1 are required for the interaction of SNX1 with Hrs. We, therefore, propose that interaction of Hrs to SNX1 is critical for the normal trafficking of mGluR1.

6.4. Role of Hrs in group I mGluR trafficking

Our earlier data suggested that interaction of SNX1 with Hrs is critical for the normal ligand-mediated trafficking of mGluR1. In order to investigate whether Hrs plays any role in the trafficking of mGluR1, we knocked down the endogenous Hrs in primary hippocampal neurons. We observed that acute knockdown of the endogenous Hrs also resulted in the rapid recycling of the receptor. Furthermore, our data suggested that over-expression of the full-length Hrs delayed the recycling of mGluR1. Together, these results suggested that both SNX1 and Hrs regulates the normal recycling of group I mGluRs probably through interaction with each other. We next investigated whether over-expression of the N-terminal truncation mutants of Hrs, viz., myc-HrsΔVHS/FYVE and myc-HrsΔVHS have any effect on mGluR1 trafficking. Upon over-expression, both these truncated forms of Hrs led to the faster recycling of mGluR1. Moreover, the VHS domain was missing in both the mutants and previous reports suggested the role of the VHS domain in sequence-directed recycling (Hanyaloglu, McCullagh et al. 2005). The above shreds of evidence suggested that the VHS domain of Hrs play a critical role in regulating the recycling of group I mGluRs.

As mentioned earlier, recycling of GPCRs is believed to be the mechanism for the resensitization of the receptor. Since our earlier data suggested that SNX1 mediated the normal trafficking of group I mGluRs through slower recycling route, this could serve as a route to resensitize these receptors as well. We showed here, that mGluRs recycling *via* the slower recycling route in wild-type cells, were able to upregulate the phosphorylation of MAP kinases. Furthermore, they were also able to induce the AMPAR endocytosis, which is the cellular correlate for the mGluR-mediated synaptic plasticity (Snyder, Philpot et al. 2001). On the other hand, we observed that mGluRs that came back to the cell surface *via* a faster recycling route, in the absence of SNX1, were inactive. These receptors failed to elicit the second messenger responses as observed by their inability to upregulate the phosphorylation of MAP kinases. Secondly, they were also unable to induce the AMPAR endocytosis. These results conclusively suggested that SNX1-mediated recycling of group I mGluRs *via* the slower recycling route is critical for the resensitization of these receptors.

6.5. Summary of the picture that is available till now

The results presented in this thesis has expanded the pre-existing knowledge of group I mGluR trafficking and their regulation. Earlier reports have suggested that group I mGluRs, subsequent to agonist stimulation undergoes desensitization which involves GRKs and PKC-dependent phosphorylation and Siah-1A mediated ubiquitination (Iacovelli, Salvatore et al. 2003; Mundell, Pula et al. 2003; Gulia, Sharma et al. 2017). After that, they internalize in a ubiquitin-dependent manner and enter the Rab11 positive recycling compartment. Their exit from this compartment is pH-dependent. These receptors recycle back to the cell surface in PP2A and PP2B-dependent manner (Pandey, Mahato et al. 2014; Mahato, Pandey et al. 2015; Bhattacharyya 2016). Our data suggested that SNX1 mediates the normal recycling of group I mGluRs through its interaction with Hrs. SNX1 appears to direct group I mGluRs through a slow recycling pathway. In the absence of SNX1, these receptors traffic *via* a faster recycling pathway. The H2 and H3 domains present in the C-terminal region of SNX1 seems to be critical for the normal trafficking of group I mGluRs *via* interaction with Hrs. The N-terminal region of Hrs, primarily the VHS domain in the N-terminal region of Hrs, plays a critical role in regulating the normal recycling of mGluR1. The SNX1-mediated slow recycling of mGluRs seems to be necessary for the resensitization of these receptors (**Figure 6.1)**.

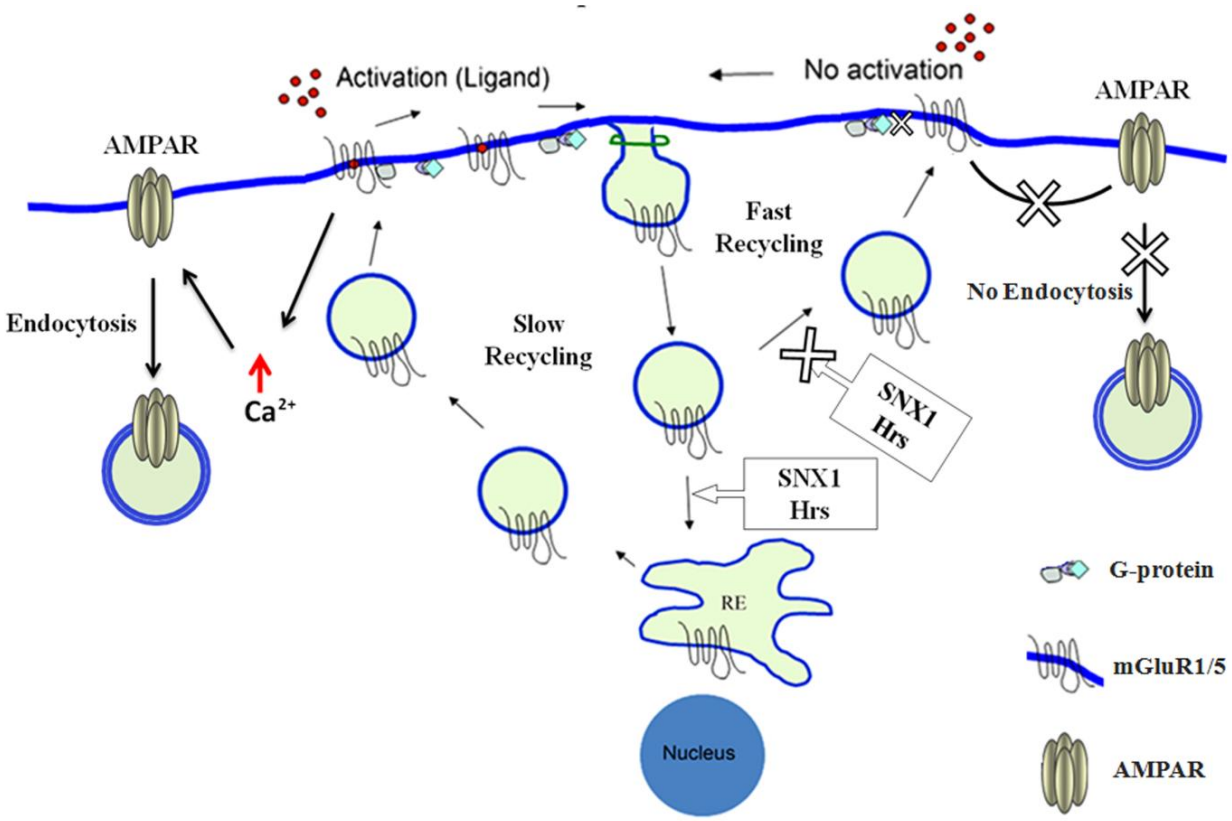


Figure 6.1. Regulation of group I mGluR trafficking and mGluR-dependent AMPAR endocytosis. Ligand-mediated activation of group I mGluRs initiates G-protein mediated signaling and results in the desensitization of the receptors. Subsequently, the desensitized receptors internalize and follow a slower recycling route *via* recycling endosomes to get back to the cell surface. SNX1 and Hrs play a critical role in sorting them towards slower recycling route leading to the resensitization of the receptors. The recycled group I mGluRs, upon restimulation by the ligand, transduces G-protein signaling and also induces AMPAR endocytosis. On the other hand, in the absence of SNX1/Hrs, group I mGluRs recycle back to the cell surface *via* faster recycling route. These recycled receptors, upon ligand stimulation, failed to generate secondary messenger responses and unable to induce the AMPAR endocytosis.

6.6. Future directions

The studies that we have conducted in this thesis have opened several questions that need to be addressed in the future. We have studied the role of SNX1 in the ligand-mediated trafficking of group I mGluRs in this present work. Previous studies have reported that apart from agonist-induced internalization, group I mGluRs also internalizes in an agonist-independent manner, termed as constitutive endocytosis (Dhami and Ferguson 2006; Bhattacharyya 2016). The mechanisms regulating these two distinct processes might be different. It would be important to investigate the role of SNX1, if any, in the constitutive endocytosis of the receptor as well. We studied the role of the C-terminal region of SNX1 in detail, but we have also shown that the N-terminal region of SNX1 plays a critical role in the trafficking of mGluR1 as well. Thus, the mechanism by which N-terminal region of SNX1 regulates the trafficking of group I mGluRs needs to be investigated in the future. As mentioned earlier, SNX1 has also been reported to regulate the lysosomal degradation of some GPCRs and recycling of other GPCRs. It would be a challenging task in the future to elucidate how SNX1 can mediate these differential sorting decisions. In the long run, the role of SNX1 in various forms of synaptic plasticity in normal and diseased brain needs to be investigated.

6.7. Final words

As explained before, each GPCR is unique, and findings from one GPCR cannot be extrapolated to others. In agreement with the previous reports, the present study also substantiates the vast diversity observed in GPCR specific signaling, desensitization, internalization, resensitization and downregulation processes. Since, varieties of GPCRs are co-expressed in cells; it's more likely that the system has evolved numerous ways to regulate these receptors differently, in order to maintain synchrony. As we know, that GPCRs are responsible for sensing the external environment or signal (ligand) and transducing this information inside the cells to regulate various physiological processes. Thus, 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 the GPCR family. Moreover, various combinations 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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