Title: Modulation of metabotropic glutamate receptor 1 (mGluR1) intracellular trafficking

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A thesis submitted for the partial fulfillment

of the degree of

Doctor of Philosophy

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March 2017

Declaration

I declare that the thesis entitled “Modulation of metabotropic glutamate receptor 1 (mGluR1) intracellular trafficking” presents the work done by me under the supervision of Dr. Samarjit Bhattacharyya at Indian Institute of Science Education and Research (IISER) Mohali. This work is original and has not been done earlier by anyone else. The results presented in this thesis have not been submitted previously to this or any other University for a Ph.D or any other degree.

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

(Dr. Samarjit Bhattacharyya) **(Saurabh Pandey)**

Place: **Place:**

Date: **Date:**

Certificate

I certify that this thesis entitled “Modulation of metabotropic glutamate receptor 1 (mGluR1) intracellular trafficking” comprises research work carried out by Saurabh Pandey 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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Acknowledgements

Looking back to the journey of my PhD, I have been blessed with many people who helped me directly or indirectly to achieve this endeavor. I would like to acknowledge everyone who helped and supported me during the entire process. Due to page restrictions, I have mentioned some of the names below and would like to apologize to those about whom I forgot to write.

First and foremost I owe my greatest debt and heartfelt gratitude to my PhD supervisor Dr. Samarjit Bhattacharyya, who has been soul behind every effort that I have put forth in this thesis. He taught me every basic thing starting from performing experiments to think scientifically. In spite of my shortcomings, he always continued his support and guidance to me. It was under his tutelage that I developed an understanding of the subject and the research curriculum. I admire and respect him for his loving and caring behavior, valuable feedbacks and amount of freedom he renders to each of his students. He has always been a great mentor and his exceptional dedication, guidance and enthusiasm helped me to expand my knowledge and horizon throughout the journey of my thesis work. Thank you very much sir for everything.

I express my immense gratitude to my doctoral committee members, Dr. Sudip Mandal and Dr. Kavita Babu for their critical comments and timely review of the progress of my work. Their constant motivation and valuable discussions have always been improved the quality of my work. Thank you.

I would like to thank Prof. N. Sathymurthy, Director, IISER Mohali, for giving me the opportunity to work at this premier institute and avail the excellent infrastructure to carry out my research work.

I would like to thank All DBS faculty members for their suggestions and help during the entire period. Special thanks go to Dr.Kausik Chattopadhyay, Dr.Lolitika Mandal, Prof.Anand Bachhawat, Prof. Purnananda Guptasarma, Dr. Arunika Mukhopadhaya, Dr. Mahak Sharma, Dr.Rachna Chaba, Dr.Samrat Mukhopadhyay, Dr.Shravan Mishra, and Dr. Sharvan Sehrawat for their help and support.

I am extremely grateful to have incredible lab members. Their active interest in my work, useful suggestions and constructive criticism improved the quality of my work. Special thanks go to Prabhat Kumar Mahato and Ravinder Gulia for setting up the lab and standardizing every protocol. Rohan Sharma being a joyful person and making the lab fun place to work even in stressful situations. Namrata Ramsakha, my official collaborator and bench partner. I would like to thank her for the help in generation of mutants. Prachi Ojha the junior most graduate member of the lab, bringing the fresh air and style to the lab. All the past and present final year BS-MS students (Rishi, Bia, Kshitij, Ritu and Vibhu). All trainees who worked with me.

I am thankful to my batch mates: Prabhat, Saikat, Nitin, Shruti and Amandeep for their help and criticism.

I would specially like to thank the DAVV gang: Saikat, Ravinder and Prince being great friends since M.Sc. days. Their presence always supported me professionally and personally. Saikat being a great friend and accompanying me the IISER since day 1 and always present here for scientific discussions to jocks. Ravinder Gulia being the best pal and lab mate. He always helped me professionally as well as personally. I would like to thank Neetu bhabhi (Gulia's wife) and their son Shorya for the help and encouragements. Prince for being an awesome sports partner since M.Sc. days and together we won some tournaments of table tennis at IISER mohali.

I consider myself very lucky to have a large friend circle. I would like to thank Nidhi Kundu and Dr. Anand Rai for teaching me the basics of western blotting. Special thanks go to Vina Tikiyani for teaching me the cloning and constantly monitoring my progress. I would like to thank every lab member of DBS for their help and support.

I would like to thank all IISER community members including non teaching staffs; library staffs, hostel staffs specially Brijesh Bajpai, mess members and all the security guards for their help and support.

Words are never enough to describe the gratitude one feels towards their family. As always, I fell short of words to describe what I feel for them. They have been my strongest pillar of support-understanding and encouraging me during all my trials in life. All of my achievements in life till date have been and will always be because of their constant love, prayers, support, encouragement and sacrifices they have made for me. Their immense unwavering faith in me and my abilities, greater than my own has always driven me to aspire for the pinnacle of success and this thesis is dedicated to them. Thank you very much Mummy, Papa, Vina, Monu, Didi, Jijju, Neelu, Ansh and Abhi.

I owe my deepest gratitude to my wife Vina for her constant support and understanding of my goals and aspirations. She has been always here to support me both personally and professionally. Her love, support, care and encouragement have always been my strength. Thank you very much for being with me.

Thank you, All.

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Abbreviations

- **AMPAR:** α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid receptor
- **AP5:** D-(-)-2-Amino-5-phosphonopentanoic acid
- **BSA:** bovine serum albumin
- **CNS:** central nervous system
- **cAMP:** cyclic AMP
- **DHPG:** 2,4- bis dihydroxy phenyl glycine
- **DAG:** diacylglycerol
- **DNQX:** 6,7-Dinitroquinoxaline-2,3-dione disodium salt
- **DMEM:** Dulbecco's modified eagle's medium
- **EDTA:** ethylene diamine tetraacedic acid
- **FBS:** fetal bovine serum
- **GRK:** G protein coupled receptor kinase
- **HEK293 cells:** human embryonic kidney 293 cells
- **IP3**: inositol 1,4,5-triphosphate
- **mGluR:** metabotropic glutamate receptor
- **NMDAR:** N-methyl-D-aspartate receptor
- **PBS:** phosphate buffer saline
- **PKA:** protein kinase A
- **PKC:** protein kinase C
- **PP1:** protein phosphatase 1
- **PP2A:** protein phosphatase 2A
- **PP2B:** protein phosphatase 2B
- **PFA:** paraformaldehyde
- **PCR:** polymerase chain reaction
- **SDS-PAGE:** sodium dodecyl sulfate-polyacrylamide gel electrophoresis
- **DIV:** days *in vitro*

Introduction

To maintain the homeostasis in any living organism, it is essential that the cells have the ability to sense and respond to the external environment and in case of multicellular organisms cells should be able to communicate with each other *via* mediators released into the extracellular milieu. It is also important for the cells to adapt rapidly as a result of change in the extracellular environment. Many important physiological processes are regulated by the coordinated actions of receptor-mediated signaling pathways. In addition to the intracellular receptors that frequently act as transcription modulators, a large variety of cell surface receptors also play an important role in this process by sensing the extracellular cues and initiating the intracellular signaling cascades. Cell surface receptors are membrane bound proteins, that interacts with variety of ligands and initiate various intracellular signaling pathways. These signaling pathways in turn mediate or modulate a wide variety of functions ranging from physiology of the cardiovascular, neuronal and immune system (Heldin, 1995).

1.1. G protein coupled receptors (GPCRs)

G protein coupled receptors (GPCRs) are the largest family of integral membrane proteins. They are composed of seven transmembrane domains with each domain consists of 20-25 amino acids, an extracellular ligand binding domain and an intracellular C-terminal tail (Gether, 2000; Kobilka, 2007) **(Figure 1.1)**. GPCRs are expressed exclusively in the eukaryotes and by sequencing analysis it has been estimated that about 4% of the human and mouse genome codes for GPCRs (Bjarnadóttir et al., 2006). GPCRs have been broadly classified into six groups (class A – class F) on the basis of structural homology and functional similarity (Bjarnadóttir et al., 2006). Class A (Rhodopsin like) is the largest class of GPCR subfamily consists of nearly 85% of GPCR coding genes and activated by a variety of ligands. This family has been further subdivided into 19 subgroups based on phylogenetic analysis (Joost and Methner, 2002). The members of this class include light, hormone and neurotransmitter receptors. Olfactory receptors and light activated rhodopsin receptors constitute over half of the class A members. The class B of the GPCRs (Secretin receptor family) consists of evolutionary related family of approximately 20 seven transmembrane proteins (Harmar, 2001). The receptors belonging to this family are activated by peptides like secretin and glucagon, which bind at the transmembrane and extracellular domains of the receptors. The third family of GPCRs, family C consists of the metabotropic like receptors for glutamate, γ-aminobutyric acid (GABA), pheromones and various taste

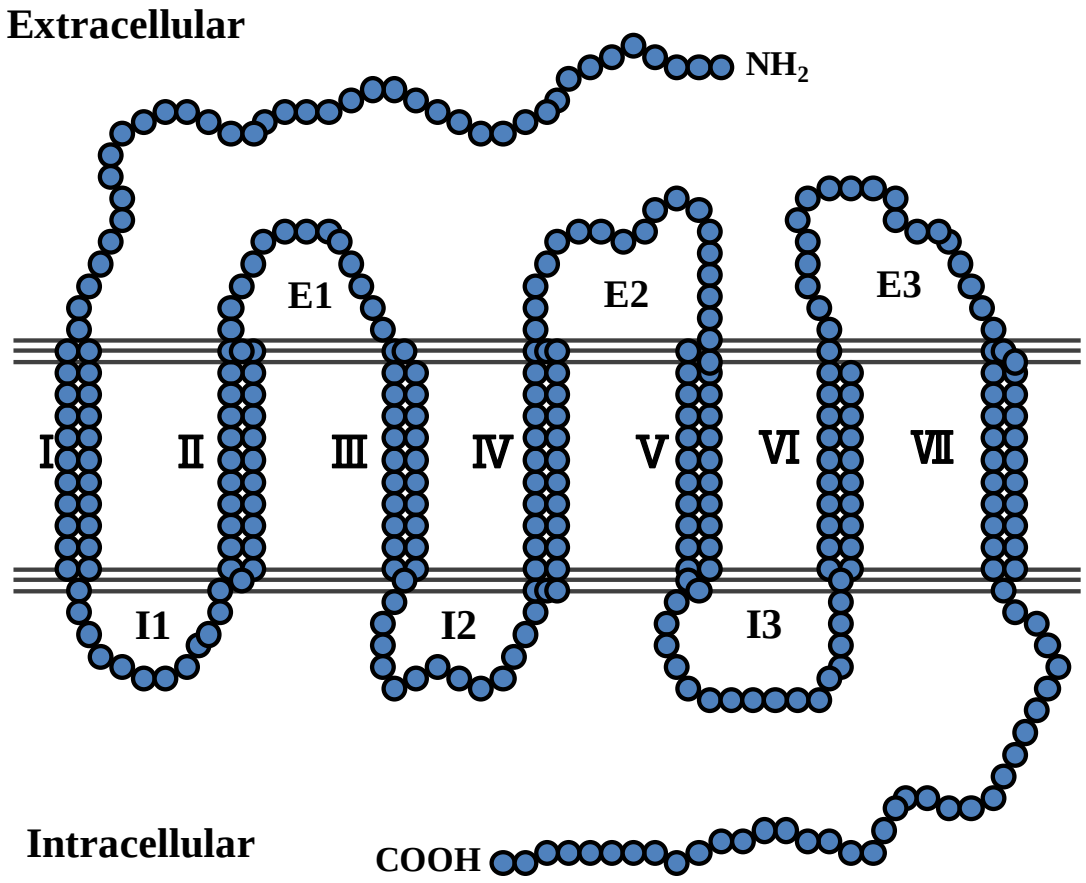


Figure 1.1. Diagrammatic representation of the G protein coupled receptors

G protein coupled receptors (GPCRs) are seven transmembrane receptors with an extracellular amino terminal domain and an intracellular carboxy terminal domain. These receptors have three extracellular loops (E1, E2 and E3), three intracellular loops (I1, I2 and I3) and a carboxy terminus tail.

molecules (Gether, 2000). In these types of receptors the ligand binds exclusively at the amino terminal domain of the receptor.Yeast pheromone receptors STE2 and STE3 constitute the family D of GPCRs (Nakayama et al., 1985; Marsh and Herskowitz, 1988). Class E (Cyclic AMP receptors) consists of a distinct family of G protein coupled receptors of slime molds. Cyclic AMP receptors control development of *Dictyostelium discoideum* by regulating the developmentally regulated genes and also coordinating the aggregation of single cells to form multicellular entity (Klein et al., 1988; Saxe et al., 1993). Class F GPCRs correspond to the frizzled receptors involved in Wnt signaling pathway. They have an extracellular cysteine rich domain where wnt ligand binds. These receptors play an important role in the embryonic development, cell polarity, neuronal synapse formation and many other developmental processes in adults (Dann et al., 2001; Huang and Klein, 2004; Malbon, 2004).

GPCRs respond to a variety of chemical and sensory stimuli such as, hormones, odors, pheromones, tastes, light, neurotransmitters etc (Kolakowski Jr, 1993; Hoon et al., 1999; Filmore, 2004). There are various kinds of ligands which can bind to GPCRs and may or may not activate the downstream signaling pathways. The ligands that activate the receptor are called “agonists”, whereas ligands which do not activate receptors upon binding are called “antagonists”. Ligands that activate the receptor to a lesser extent than the agonists are called “partial agonists”. Many ligands decrease the GPCRs basal level activity upon binding and they are called “inverse agonists”. Around 30 - 40% of the total available drugs in the market target GPCRs, because of their involvement in various physiological processes and diseases (Wise et al., 2002; Trzaskowski et al., 2012). Majority of these drugs act as antagonists or inverse agonists towards the receptor. In recent years, crystal structures of many GPCRs have been reported and these studies along with previous reports have provided important information about the GPCRs activation mechanisms, their structural dynamics and coupling with different G proteins (Palczewski et al., 2000; Edwards et al., 2007; Venkatakrishnan et al., 2013; Ghosh et al., 2015). Recent high resolution crystal structure of many GPCRs have suggested that the receptors can attain several conformational states due to binding with different types of ligands. Depending on the conformational change these receptors can activate a variety of heterotrimeric G protein complexes ($G_{\alpha\beta\gamma}$) , which in turn initiate a variety of signaling cascades (Ghosh et al., 2015). In an inactive stage G_{α} of the G protein is associated with GDP and exists as a complex with $G_{\beta\gamma}$. Activation of a GPCR either by agonists or partial agonists is believed to cause a conformational change of the rceptor.

Subsequently, the receptor acts as GEF (guanine nucleotide exchange factor) and exchange the GDP of G_α for GTP. As a result, activated heterotrimeric G protein dissociates into GTP bound G_α monomer and $G_{\beta\gamma}$ stable dimer subunits (Neer, 1995; Surya et al., 1998; Digby et al., 2006). Both the activated G_α and $G_{\beta\gamma}$ subunits further activate or inhibit the activity of a series of downstream effecters, such as phospholipases, nucleotide cyclases, kinases or ion channels etc. which in turn modulates various cellular processes (Gautam et al., 1998; Hamm, 1998; Digby et al., 2006). Due to its intrinsic GTPase activity, the G_α subsequently hydrolyzes the bound GTP to GDP, resulting in the inactivation of G_α and reassociation with the $G_{\beta\gamma}$ to form an inactive heterotrimeric G protein complex (Tuteja, 2009) **(Figure 1.2)**. The GTP hydrolysis is also regulated by another family of proteins called "regulator of G protein signaling (RGS)" (De Vries et al., 2000). The specificity and complexity in GPCR signaling comes from the presence of diverse array of G proteins subunits. At least 23 types of G_α subunits encoded by 17 genes have been discovered till today. These subunits have been divided mainly into 4 subclasses ($G_{\alpha i/o}$, $G_{\alpha s}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$) depending on the type of signaling cascade initiated by them upon activation (Gautam et al., 1998; Wettschureck and Offermanns, 2005; Hanlon and Andrew, 2015). $G_{\alpha i/o}$ and $G_{\alpha s}$ regulates adenylyl cyc1ase pathway, $G_{\alpha s}$ acts as stimulatory signal whereas $G_{\alpha i/o}$ acts as an inhibitory signal. Adenylyl cyc1ase catalyses the conversion of cytosolic adenosine triphosphate into cyclic-adenosine monophosphate (cAMP). The cAMP levels in cells regulate the activity of protein kinase A (PKA) and also regulate the activity of various ion channels directly or indirectly through various second messenger responses. . On the other hand, upon activation $G_{\alpha q/11}$ activates phospholipase C (PLC), which subsequently cleaves phosphatidylinositol 4, 5-bisphosphate (PIP_2) into two second messengers: inositol 1,4,5-triphosphate (IP_3) and membrane bound diacylglycerol (DAG). IP_3 binds its receptors present on the endoplasmic reticulum (ER) membrane, which results in the release of Ca^{2+} from the ER and thus rises in the intracellular Ca^{2+}. DAG and Ca^{2+} are responsible for the activation of protein kinase C (PKC) which phosphorylates many target proteins leading to various cellular responses. $G_{\alpha 12/13}$ activates Rho GEF, which in turn activates Rho factors which are responsible for cytoskeleton regulation. There are at least 6 different G_β and 12 different G_γ subunits have been discovered and have been shown to play very important roles in the regulation of various ion channels along with activation of some isoforms of PLC and IP_3 (Vanderbeld and Kelly, 2000; Hanlon and Andrew, 2015). In addition to above mentioned signaling processes, it has also been reported that GPCRs in some cases transduce their signaling through G protein independent mechanisms (Heuss and Gerber, 2000; Marinissen and Gutkind, 2001). In comparison to the

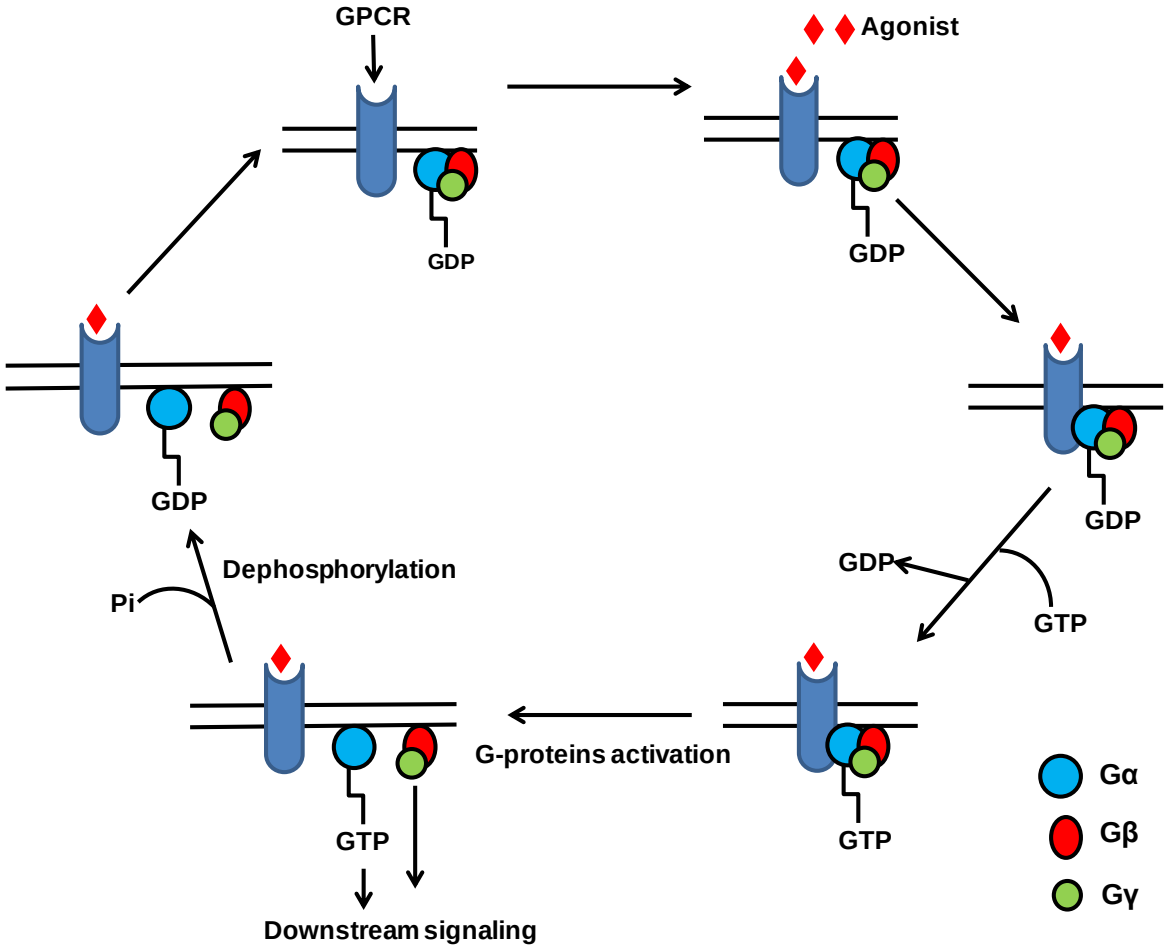


Figure 1.2. The G protein activation mechanism by GPCR

The GPCR becomes active upon agonist/ligand binding and subsequently, the inactive form of the G protein interacts with the ligand-bound receptor. The activated receptor acts as a guanosine nucleotide exchange factor and as a result GDP bound to Gα is replaced by GTP. Subsequently, the α-subunit of the G protein dissociates from the βγ subunit. These dissociated subunits transduce various intracellular signaling. Hydrolysis of GTP by the alpha subunit allows reformation of the inactive G protein complex.

variety of GPCRs and a diverse array of G proteins exist in nature; the numbers of downstream effectors are rather limited. It is likely that a particular receptor may interact independently with many G proteins within the same class or occasionally with the other classes of G proteins depending on their availability in the vicinity of the receptor (Offermanns et al., 1994). The G proteins involve in the coupling of receptor to effector may also play a crucial role in the efficacy and potency of cellular signaling (Offermanns et al., 1994; Wise et al., 1999; Albert and Robillard, 2002; Moreira, 2014; Smrcka, 2015).

1.2. G protein coupled receptor desensitization

An important aspect of GPCR signaling is the memory of any prior activation state and that influence their ability to get stimulated in future. Prolonged or repeated ligand exposure results in decreased sensitivity of the receptor towards the ligand and this phenomenon is called "desensitization" of the receptor. Desensitization of the receptor is believed to be an important physiological feedback mechanism adopted by the cells to prevent themselves from chronic or acute receptor overstimulation. Additionally, GPCR desensitization also filters information from multiple receptor inputs into an integrated and meaningful biological signal (Ferguson, 2001). GPCRs undergo desensitization by various mechanisms or combination of various mechanisms. These mechanisms include: 1) uncoupling of the receptor from G protein involved due to modifications of the receptor such as phosphorylation, ubiquitination etc. 2) sequestration of the receptors in the endocytic compartments 3) down regulation of the pre-existing receptor either by lysosomal degradation or reduced *de novo* synthesis (Bouvier et al., 1988; Hausdorff et al., 1990; Lefkowitz, 1998; Oakley et al., 1999; Kohout and Lefkowitz, 2003; Hanyaloglu and Zastrow, 2008; Jean-Alphonse and Hanyaloglu, 2011; Magalhaes et al., 2012; Black et al., 2016).

The activated GPCRs not only initiate G protein mediated signaling but also serve as a substrate for phosphorylation by various types of protein kinases like, protein kinase A (PKA), protein kinase C (PKC) and G protein coupled receptor kinases (GRKs). Phosphorylation of some GPCRs leads to the binding of β-arrestin which uncouples the receptor from the G protein which results in the desensitization of the receptor (Ferguson, 2001; Gurevich and Gurevich, 2015). This is the most rapid way to desensitize a GPCR (Krupnick and Benovic, 1998; Lefkowitz, 1998; Ferguson, 2001; Penela et al., 2003; Gainetdinov et al., 2004; Lefkowitz and Shenoy, 2005; Premont and Gainetdinov, 2007;

Luttrell, 2008; Tobin et al., 2008; Ritter and Hall, 2009; Magalhaes et al., 2012; Black et al., 2016). In addition to GRK, PKA and PKC, GPCRs could serve as a substrate for other protein kinases as well. For example, casein kinases have been reported to phosphorylate some GPCRs that result in the desensitization of the receptor (Tobin et al., 1997; Pak et al., 1999; Budd et al., 2000).

G protein coupled receptor kinases (GRKs) phosphorylate only the agonist activated receptors. There are seven types of GRKs (GRK1 - GRK7) in mammals and they have been divided into 3 subgroups (GRK1/7, GRK2/3, GRK 4/5/6) based on their sequence and functional similarity (Benovic et al., 1987; Benovic and Gomez, 1993; Lefkowitz, 1998; Penela et al., 2003; Willets et al., 2003; Premont and Gainetdinov, 2007; Magalhaes et al., 2012; Black et al., 2016). GRK1 and GRK7 are found to be expressed predominantly in retinal tissues whereas, GRK4 is primarily localized in the testes (Ferguson, 2001). GRK2/3/5/6 on the other hand, expresses ubiquitously and accounts for the regulation of most of the GPCRs in the system. All GRKs share similar structural organization with an amino terminal domain thought to be important for substrate recognition, a central catalytic protein kinase domain for the phosphorylation of the substrates and a variable carboxyl-terminal domain that is involved in the targeting of the kinase to the plasma membrane. The amino terminal domain of these kinases has been shown to be homologous to RGS proteins (Carman et al., 1999b; Sallese et al., 2000). The specificity in GPCRs recognition by GRKs is largely based on the activation state of the receptors and the relative abundance of the specific GRKs in the given cell type. Although, GRKs show some preference over the phosphorylation sites on the receptors but several studies have also suggested that they may find other sites to phosphorylate in case the preferred site is mutated (Gurevich et al., 2012; Black et al., 2016). Thus, GRKs generally do not show strict consensus sequences for their substrate specificity. The second messenger-dependent protein kinases (PKA, PKC etc.) phosphorylate the activated receptors as a feedback mechanism, but they can also phosphorylate the receptor that has not been exposed to agonist (Hausdorff et al., 1989; Lohse et al., 1990). In addition to the receptors, these kinases also phosphorylate the downstream effectors and thereby regulate GPCR signaling (Hausdorff et al., 1989; Lohse et al., 1990; Kohout and Lefkowitz, 2003).

Phosphorylation of the receptors leads to the recruitment of another class of regulatory proteins called, arrestins. Arrestin 1 or s-antigen was the first arrestin protein identified as a 48 kDa protein bound to the light activated receptor, rhodopsin (Pfister et al., 1985). Till date

four members have been reported which constitute the arrestin family viz., arrestin1/2/3/4. They have been further subdivided into two subfamilies based on their sequence homology, tissue distribution and function. The first subgroup consists of arrestin 1 and arrestin 4 (also called as visual arrestins), predominantly present in the rod cells and cone cells of retinal tissue along with low expression has been seen in the pineal gland. The second group that consists of arrestin 2 (β-arrestin 1) and arrestin 3 (β-arrestin 2), are present throughout the body and are responsible for the desensitization of vast variety of GPCRs (Ferguson, 2001; Gainetdinov et al., 2004; Premont and Gainetdinov, 2007). β-arrestin proteins are widely distributed in the mammalian central nervous system and are concentrated at neuronal synapses along with GRKs to regulate various synaptic processes (Arriza et al., 1992; Attramadal et al., 1992). The solution of the crystal structure of visual arrestin together with mutagenic studies have provided great insights into the structural details of arrestin binding to the light activated rhodopsin receptor. These studies have suggested that arrestin protein could be divided into two regulatory and three functional domains. The regulatory domains comprise of an amino terminal and a carboxyl terminal domain. The functional domains consist of: 1) receptor activation recognition domain 2) secondary receptor binding domain and 3) phosphate sensor domain. The phosphate sensor domain forms a polar core and in basal condition it is embedded between the receptor activation recognition and secondary receptor binding domains to form the "fulcrum" of the arrestin molecule. It is also suggested that the polar core is highly conserved between different arrestin isoforms (Gurevich et al., 1995; Hirsch et al., 1999). The carboxyl terminal regulatory domain is attached to the arrestin molecule through a flexible linker sequence, which gives this domain freedom to interact with the other domains in various ways to regulate the arrestin structure. The carboxyl terminal regulatory domain interacts with the polar core to stabilize the basal inactive state of the arrestin. However the highly charged phosphate residues present in the phosphorylated receptors invade the polar core and disrupt the basal structure by releasing the regulatory carboxyl domain. As a result, the receptor activation recognition and secondary receptor binding domains reorient along the "fulcrum" to form receptor–arrestin complex. The released carboxyl domain further interacts with various molecules to initiate the internalization of the receptors (Gurevich et al., 1995; Granzin et al., 1998; Hirsch et al., 1999; Vishnivetskiy et al., 1999; Xu et al., 2007; Lohse and Hoffmann, 2014).

Many GPCRs have also been reported to desensitize through phosphorylation-independent mechanisms. These mechanisms include phosphorylation-independent desensitization by

GRKs, phosphorylation-independent binding of arrestin and agonist-induced internalization of the receptor (Palczewski et al., 1994; Ferguson et al., 1996; Beaumont et al., 1998; Junjiishida et al., 2000; Sallese et al., 2000; Ferguson, 2001; Dhami et al., 2002). GRKs not only phosphorylate the activated GPCRs but also reported to interact with various molecules such as Gα and $G_{\beta\gamma}$ subunits of the G protein, various cytoskeletal and calcium sensing proteins to regulate the desensitization process of the receptor (Pitcher et al., 1992; Carman et al., 1998; Freeman et al., 1998; Carman et al., 1999a; Junjiishida et al., 2000; Sallese et al., 2000). For example, the RGS domain of GRK2 has been reported to bind exclusively with activated $G_{\alpha q/11}$ and effectively inhibits the downstream signaling. Over-expression of the RGS domain of GRK2 alone has been reported to inhibit the IP_3 production by a number of GPCRs including the thromboxane A_2 receptors, mGluR1 receptors, endothelin A and B receptors, 5-hydroxytryptamine 2C receptors, angiotensin II type 1A receptors and m3 muscarinic acetylcholine receptors (Freedman et al., 1997; Carman et al., 1999b; Sallese et al., 2000; Dhami et al., 2002). Arrestins have been reported to bind many GPCRs in phosphorylation-independent manner and act as an adapter molecule. Binding of arrestin to the receptor leads to the uncoupling of the receptor from the G protein and as a result receptor may get desensitized (Palczewski et al., 1994; Ferguson, 2001; Drake et al., 2006). Additionally, arrestin binding to the receptor promotes the binding of many other proteins which help in receptor internalization. Upon agonist stimulation, many GPCRs have been reported to get internalized which leads to the separation of the receptor from the G protein and thus the receptor gets desensitized. The underlying mechanisms and regulations of GPCR endocytosis has been discussed in detail below.

1.3. G protein coupled receptor endocytosis

Another important aspect of GPCR activity and regulation is the internalization or sequestration of various GPCRs from the cell surface to the endosomal compartments upon agonist stimulation. The concept of GPCR internalization first came from the observation that upon agonist stimulation, β-adrenergic receptor recognition sites internalized from the surface of frog erythrocytes (Chuang and Costa, 1979). Large amount of work has been done since then, and accumulated evidences have enriched our current understanding about the process and the molecular mechanisms that regulate the endocytosis of various GPCRs from the cell surface. There are three principal pathways by which a particular GPCR could be internalized

from the cell surface: 1) arrestin and dynamin-dependent endocytosis, 2) arrestin-independent and dynamin-dependent endocytosis and 3) arrestin and dynamin-independent internalization of GPCRs (Claing et al., 2002; Tan et al., 2004). It has been observed that the mechanism of internalization and the subcellular fate of the receptor subsequent to internalization depend on the type of the receptor, type of the ligand and the cellular background. Initially, arrestin and dynamin-dependent endocytic pathway was considered as a paradigmatic model for agonist-evoked GPCR endocytosis. Arrestin proteins recognize the critical phosphorylated residues of the receptor and uncouple the G protein from the receptor. Arrestin also acts as a scaffold protein and recruits the AP2 adaptor protein and clathrin to form clathrin coated pits. Subsequently, dynamin (Rab GTPase) interacts with the complex and oligomerize to induce neck formation of the coated pits and clathrin-coated vesicles are formed. Subsequently, the coated vesicles then fuse with early endosomes and either recycle back to the cell surface or are targeted to the lysosome for degradation (Giannini and Boulay, 1995; Oakley et al., 1999; Trejo and Coughlin, 1999; Brodsky et al., 2001; Claing et al., 2002; Tian et al., 2014; Gurevich and Gurevich, 2015). On the other hand, some GPCRs have been reported to get internalized *via* arrestin-independent and dynamin-dependent manner. For example, $5HT_{2A}$ receptor (serotonin 2A receptor) endocytosis was inhibited upon over-expression of dominant negative dynamin but not by over-expression of dominant negative arrestin (Bhatnagar et al., 2001). Arrestin and dynamin-independent endocytosis has been reported in the case of angiotensin II AT_{1A} receptor (Zhang et al., 1996). Thus, works done in last few years have suggested that each GPCR is unique and a widely studied GPCR like β-adrenergic receptor may not serve as a model system. If one needs to understand a GPCR, that particular receptor has to be studied. Moreover, a particular receptor can get internalized *via* different mechanisms depending on the phosphorylation status of the receptor and the cellular background. For example, β_1-adrenergic receptor upon GRK mediated phosphorylation internalizes *via* clathrin coated pits but PKA-dependent phosphorylation of the receptor leads to the caveolin-mediated internalization of the same receptor (Rapacciuolo et al., 2003). Internalization of β_2-adrenergic receptor has been shown to be caveolin-dependent in A431 cells, whereas, the internalization of the same receptor occurs *via* the clathrin-dependent pathway in a number of other cell types (Raposo et al., 1989; Couet et al., 1997; Prasad et al., 2002). Phosphorylation of the receptor was considered earlier as an essential mechanism for the receptor endocytosis, but now many GPCRs have been reported to get internalized in phosphorylation-independent manner (Black et al., 2016). These observations have led to the conclusion that G protein coupled receptors can internalize in a variety of pathways and the

pathway a GPCR will choose is dependent on the type of the GPCR, type of the ligand and its cellular background.

Due to the intense research in the past few decades, our understanding of the biological importance of GPCR endocytosis expanded rapidly. Initially, the endocytosis of GPCRs was believed to be the primary mechanism for the desensitization of the receptors, since internalization physically separates the receptors from the G proteins (Sibley and Lefkowitz, 1985; Ferguson, 2001). However, this hypothesis was challenged by the following observations: 1) for many receptors, the receptor desensitization proceeds more rapidly than the endocytosis of the receptor, 2) desensitization profile of the β_2-adrenergic receptor remained unaltered even after blocking the endocytosis by various pharmacological and chemical inhibitors (Yu et al., 1993; Pippig et al., 1995; Ferguson, 2001). These results together suggested that for many receptors endocytosis is not the mechanism to desensitize the receptor. In fact, internalization is necessary for the resensitization and downregulation of many GPCRs. The idea that internalization is necessary for the receptor resensitization came from several observations: 1) β_2-adrenergic receptors isolated from the light endosomal fractions were less phosphorylated than the receptors isolated from the plasma membrane fraction (Sibley and Lefkowitz, 1985), 2) the endosomal fractions were found to be enriched in GPCR specific phosphatases activity (Sibley and Lefkowitz, 1985; Pitcher et al., 1995), 3) internalization defective mutants were not able to resensitize although their signaling and desensitization properties were intact (Barak et al., 1994) and 4) resensitization of the GPCRs was blocked upon inhibition of the endocytosis using pharmacological and chemical inhibitors (Pippig et al., 1995; Garland et al., 1996; Hasbi et al., 2000). It has been reported that for many GPCRs such as, m3AChR, delta opioid receptor, β_2-adrenergic receptors, mu opioid receptor, endothelin A receptor, cholecystokinin receptor, endocytosis is necessary for their resensitization process (Lutz et al., 1993; Giannini and Boulay, 1995; Pippig et al., 1995; Garland et al., 1996; Edwardson and Szekeres, 1999; Wolf et al., 1999; Bremnes et al., 2000; Hasbi et al., 2000). Internalization is also necessary for downregulation of many GPCRs. For example, protease activated receptors and endothelin B receptors, upon internalization, predominantly targeted to lysosomes for degradation (Trejo and Coughlin, 1999; Bremnes et al., 2000). Several studies using chimeric GPCR constructs have revealed that specific determinant in the cytoplasmic tail of many GPCRs play an important role in sorting of the receptor either to the recycling endosome or to the lysosomal compartment (Oakley et al., 1999; Trejo and Coughlin, 1999; Anborgh et al., 2000). **(Figure 1.3)**

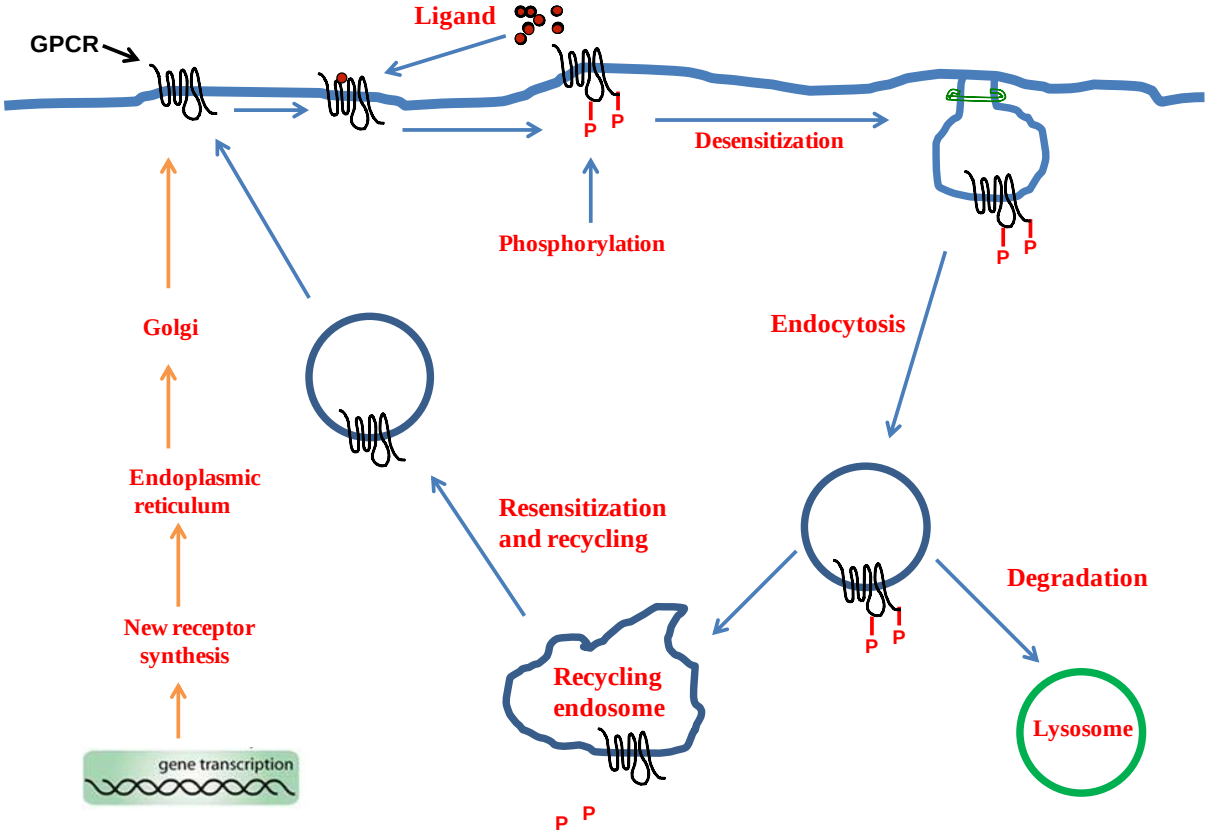


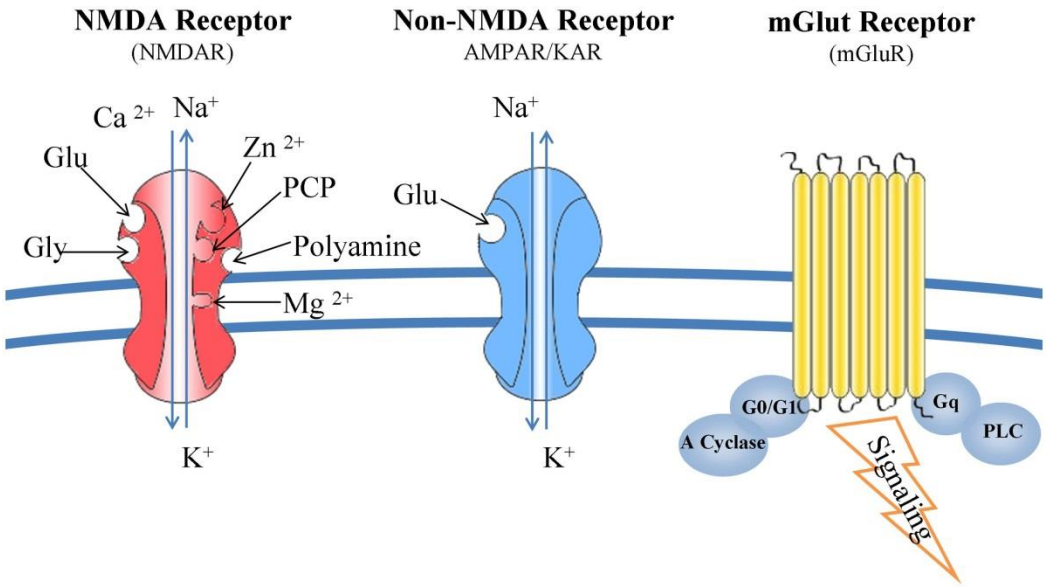
Figure 1.3. Mechanisms involved in the GPCR trafficking

Agonist activation of the GPCR results in the activation of kinases, which phosphorylate the receptor. The phosphorylated receptor is recognized by β-arrestin and as a result the receptor becomes desensitized. The desensitized receptor internalizes *via* dynamin and clathrin-dependent pathway. The internalized receptor subsequently resensitizes due to dephosphorylation and the receptor recycles back to the cell surface. Alternatively, GPCRs that internalize are targeted for degradation in lysosomes.

Yet, other important aspects of GPCR internalization are the alternative signaling pathways triggered by various GPCRs after internalization. For example, many GPCRs have been reported to induce MAP kinase signaling pathway upon internalization (Daaka et al., 1998). Thus, GPCR endocytosis is a necessary mechanism for regulating GPCR turn over and also maintenance of signaling and homeostasis in the cell.

1.4. Glutamate and its receptors

Glutamate is a nonessential amino acid, known to act as a major excitatory neurotransmitter in the central nervous system (CNS). It is the most abundant neurotransmitter in the CNS and accounted to be involved in more than 90% of the excitatory synapses in the human brain. Glutamate has been reported to be synthesized in the CNS through two major pathways: 1) the *de novo* synthesis of glutamate from glucose and amino acid derivatives *via* energy metabolism and 2) synthesis from glutamine as a part of glutamate-glutamine cycle by the activity of principal enzyme glutaminase (Erecińska and Silver, 1990). Despite its ubiquitous presence, the extracellular glutamate level is tightly regulated, thus allowing glutamate to serve as an excitatory neurotransmitter in the CNS. The low extracellular concentration of glutamate, which is necessary for optimal brain function is maintained by the coordinated activity of glutamate transporters, viz., vesicular glutamate transporters (VGLUTs) and excitatory amino acid transporters (EAATs), present in the neurons, astrocytes, and the blood brain barrier (Danbolt, 2001; Shigeri et al., 2004; Hawkins, 2009). VGLUTs are multimeric proton/glutamate antiporters, play an important role in the transport of the cytoplasmic glutamate into the synaptic vesicles (Fremeau et al., 2004; Takamori, 2006; Wallén-Mackenzie et al., 2010). Excitatory amino acid transporters (EAATs) on the other hand, are the sodium-dependent glutamate antiporters, known to transport the extracellular glutamate present in the synaptic clefts and at the extrasynaptic sites actively into the neurons and glial cells, thus protecting the brain from excessive glutamate signaling which causes excitotoxicity (Shigeri et al., 2004; Holmseth et al., 2012; Underhill et al., 2014). Glutamate transduces its signal by binding with receptors present in the synaptic and extrasynaptic region. It activates two types of receptors in the CNS viz., ionotropic glutamate receptors and metabotropic glutamate receptors (Fonnum, 1984; Platt, 2007; Niciu et al., 2012; Bhattacharyya, 2016) **(Figure 1.4)**.



From: Kritis, A.A., *et al.*, *Frontiers in neuroscience* 2015

Figure 1.4. Glutamate receptors

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

1.5. Ionotropic glutamate receptors (iGluRs):

Ionotropic glutamate receptors are ligand gated ion channels, which upon glutamate binding open up and allow the cations to pass through them (Dingledine et al., 1999; Mayer, 2005; Niciu et al., 2012). These receptors mediate fast excitatory neurotransmission in the CNS. In general, all the iGluRs consists of four subunits and each subunit contains well conserved four domains including an extracellular amino terminal domain followed by ligand binding domain, a transmembrane domain spanning the cell membrane four times and an intracellular carboxy-terminal domain. One of the most intriguing features of the iGluRs is their diverse ion channel properties which come from the several permutation combinations of the subunits that make the ion channels. On the basis of pharmacology and sequence similarity these receptors have been further categorized into three groups viz., α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors and Kainate receptors.

1. **AMPA receptors (AMPARs):** AMPA receptors are widely distributed in the mammalian CNS and induce fast excitatory neurotransmission upon glutamate binding. These receptors were initially named as "quisqualate receptor" as their ability to bind quisqualate, a naturally occurring agonist (Honoré et al., 1982; Murphy et al., 1987). Later they have been renamed to AMPA receptors after the development of the selective agonist, α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (Honoré et al., 1982). These receptors consist of four types of subunits; GluA1/2/3/4, which assemble together in different combinations to form tetrameric ion channel (Shi et al., 1999; Song and Huganir, 2002). Majority of the AMPARs are heterotetrameric ion channels, consisting of symmetric "dimer of dimers" having an obligatory GluA2 subunit and either of the GluA1, GluA3 or GluA4 subunits (Shi et al., 1999; Greger et al., 2007). However, they are also present in homo and heterotetrameric forms lacking GluA2 subunit (Mayer, 2005; Greger et al., 2007). The carboxy terminal intercellular domain of each subunit varies greatly and dictates their differential binding with the intracellular proteins. For example, GluA2 subunit has been reported to bind with PICK1, a scaffolding protein through its PDZ domain, whereas GluA1 subunit binds preferentially with SAP91, another scaffolding protein (Leonard et al., 1998; Greger et al., 2002). Phosphorylation of AMPARs by various kinases can regulate their conductance, channel opening probability and localization on the cell surface (Derkach et al., 1999; Banke et al., 2000; Hayashi et al., 2000; Boehm et al., 2006).

Each AMPAR subunit contains agonist binding site and agonist occupancy at two sites is sufficient for channel opening. However the ion conductance increases drastically after all the sites are occupied by the agonists (Rosenmund et al., 1998; Platt, 2007). Open AMPARs close quickly, due to change in their confirmation by various factors such as change in the scaffolding protein interaction, phosphorylation status of the receptor etc. (Platt, 2007). The cation permeability across the channel is governed by the presence of GluA2 subunit. GluA2 lacking receptors allow the calcium entry whereas presence of GluA2 restricts the calcium entry (Hollmann and Heinemann, 1994; Keifer et al., 2003). AMPARs have been known to modulate the synaptic efficacy by either enhancing or decreasing the neurotransmission across the synapse. Increased synaptic strength is known as long term potentiation (LTP), whereas decrease in synaptic strength is called long term depression (LTD). Together these phenomena regulate the plasticity of synapses, which is believed as a cellular correlate for learning and memory formation (Whitlock et al., 2006; Wang, 2008).

2. **NMDA receptors:** NMDA receptors are the second member of the iGluR family, known to play key role in the basal neurotransmission as well as in the regulation of synaptic plasticity (Moriyoshi and Masu, 1991). These receptors are called NMDA receptors because N-methyl-D-aspartate (NMDA), an agonist binds selectively to these receptors and activate them. These receptors are also distributed extensively in the CNS and have highest affinity for glutamate (Vyklicky et al., 2014). NMDA receptors are heterotetrameric cation channels, consists of three types of subunits viz. NR1, NR2A-D and NR3A-B. NR1 is an obligatory subunit and appears to express throughout the brain (Nakanishi, 1992; Goebel and Poosch, 1999). NR2 subunits, on the other hand, are found to be expressed differentially in the CNS. NR2A was observed to express predominantly in the hippocampus and in the neocortex, whereas NR2B expression was observed primarily in the forebrain (Goebel and Poosch, 1999). Importantly, NR2C and NR2D were observed to express prominently in the cerebellum and lower brain stem (Goebel and Poosch, 1999). NR3 subunits were also observed to express differentially like NR2 subunits. NR3A expression was seen predominantly in the neocortex, whereas NR2B mRNA expression was observed in the neurons of brainstem and spinal neurons (Nakanishi, 1992). Opening of NMDA channel is tightly regulated in the CNS. For the activation, NMDA receptors uniquely

require co-agonist glycine, in addition to the agonist glutamate. At the resting membrane potential the channel opening is blocked by the Mg^{2+}, which can be relieved by strong depolarization of the membrane. As a result, NMDA receptors act as a coincident detector and can be activated only upon the simultaneous binding of glutamate to the receptor and depolarization of the membrane (Kleckner and Dingledine, 1988; Dingledine et al., 1999; Liu and Zhang, 2000; Zito and Scheuss, 2009; Vyklicky et al., 2014). NMDA receptors have been reported to play crucial roles in various important physiological processes such as, development of the CNS, generation of rhythms involve in breathing, locomotion and in learning and memory formation (Collingridge, 1987; Greer et al., 1991; Scheetz and Constantine-Paton, 1994).

3. **Kainate receptors:** These receptors were first identified as distinct receptors from the AMPA and NMDA receptors due to their selective activation by a drug kainate, isolated from red algae, *Digenea simplex*. They form tetrameric ion channels consists of five different kinds of subunits viz., KA1, KA2 and GluA5/6/7 (Dingledine et al., 1999). The channel conductance of these receptors is similar to AMPA receptors; however the rise and decay time of the potential generated by them is slower than that of AMPA receptors (Huettner, 2003). The role of Kainate receptors in the CNS is poorly understood, although some studies have suggested their role in seizure induction and epileptic discharges (Fritsch et al., 2014).

1.6. Metabotropic glutamate receptors (mGluRs)

Metabotropic glutamate receptors belong to the class C GPCR family (Pin and Duvoisin, 1995; Pin et al., 2003; Brauner-Osborne et al., 2007). Structurally, these receptors consist of a large extracellular N-terminal domain, a cysteine rich domain, heptahelical transmembrane domain and an intracellular C-terminal tail. mGluR family consists of eight members, viz., mGluR1 - mGluR8. On the basis of sequence similarity, second messenger coupling and pharmacology, these receptors have been divided into three subgroups: group I mGluRs consist of mGluR1 and mGluR5, whereas mGluR2 and mGluR3 belongs to the group II mGluR family and group III mGluR has mGluR4, mGluR6, mGluR7 and mGluR8 as the family members (Nakanishi, 1994; Pin and Duvoisin, 1995). Group I mGluRs are primarily localized in the post-synaptic neurons and group II mGluRs are expressed in both pre and

post-synaptic neurons, whereas group III mGluRs are found predominantly in the pre-synaptic neurons (Benarroch, 2008; Bhattacharyya, 2016). These receptors are also present in the glial cells and have been reported to play crucial roles in glutamate release and uptake from astrocytes, neuroprotection and communication between neurons and glia (Benarroch, 2008). Group I mGluRs are primarily coupled to the $G\alpha_{q/11}$ pathway and activate phospholipase C, whereas both the group II and group III mGluRs are predominantly coupled to the G_i/G_o pathway and negatively regulate adenylyl cyclase activity upon activation (Pin et al., 2003; Kim et al., 2008). The mGluRs perform diverse array of physiological functions in the brain, including regulation of various ion channels activity, pain, anxiety, learning and memory formation etc. (Brauner-Osborne et al., 2007; Benarroch, 2008). Since, the objective of this work is to understand the regulation of group I mGluRs, in the subsequent section we have concentrated on group I mGluRs.

1.7. Group I mGluRs distribution and signaling

Group I mGluRs are expressed differentially throughout the CNS (Ferraguti and Shigemoto, 2006). mGluR1 expresses extensively in the olfactory bulb, cerebellar purkinje cells and strong expression has also been observed in the hippocampus, thalamus, lateral septum, substantia nigra and globus pallidus (Shigemoto et al., 1992; Bordi and Ugolini, 1999). mGluR5 expression on the other hand, has been observed in the hippocampus, cerebral cortex, striatum, nucleus accumbens, granule cells of the olfactory bulb and lateral septal nucleus (Bordi and Ugolini, 1999; Bhattacharyya, 2016). Some reports have suggested that group I mGluRs expression varies with the development of the brain. For example, expression of mGluR1 increases steadily in the hippocampus and neocortex during development (Catania et al., 1994). mGluR5a expression increases gradually in the developing cortex and reaches maximum during the second postnatal week in rodents and then gradually decreases, whereas mGluR5b mRNA expression increases postnatally and in adults this is the most prominent form of mGluR5 (Catania et al., 1994; Minakami et al., 1995; Romano et al., 1996; Bhattacharyya, 2016). In addition to the expression in CNS, group I mGluRs expression is also observed in non-neuronal cells like melanocytes, hepatocytes, heart cells, osteoblast and skin cells (Gill et al., 1999; Frati et al., 2000; Gu and Publicover, 2000; Storto et al., 2000; Bhave et al., 2001; Bhattacharyya, 2016). In the CNS these receptors are predominantly localized at the perisynaptic region of the postsynaptic

neurons (Lüscher and Huber, 2010). Structurally, both mGluR1 and mGluR5 consists of a very large extracellular domain containing ligand binding grooves (Venus flytrap domain) and cystein rich domain, followed by serpentine transmembrane domain and an intracellular carboxyl terminal tail. As mentioned before, group I mGluRs are positively coupled to $G\alpha_{q/11}$. Upon ligand binding the receptor undergoes conformational change, which in turn activates the G protein. Subsequently, the activated G protein activates phospholipase C (PLC). The PLC in turn cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases the Ca^{2+} from the intracellular store. DAG and Ca^{2+} together activate protein kinase C (PKC). PKC subsequently modulates variety of ion channels activity along with phosphorylation of various target substrates to regulate several physiological processes (Niswender and Conn, 2010; Nicoletti et al., 2011; Wang and Zhuo, 2012). Although, the primary coupling of these receptors is with $G_{\alpha q/11}$, under various circumstances these receptors could also couple to other G proteins in various cell types (Gerber et al., 2007). In number of systems, such as, hippocampal cells and cultured cortical glial cells activation of group I mGluRs ultimately activates the MAP kinase pathway (Gerber et al., 2007). This mGluR-mediated MAP kinase activation has been reported to be involved in the mGluR-dependent synaptic plasticity in pyramidal hippocampal neurons (Gerber et al., 2007; Wang and Zhuo, 2012).

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

A remarkable feature of the adult brain is the activity-dependent plasticity of the pre existing synaptic connections, which in turn modulate the properties of the neuronal circuits and behaviour. The plasticity of the synapses is believed to be the cellular correlate of learning and memory. Additionally, work done in past four decades suggested that synaptic plasticity also play important roles in circuit formation in the developing brain (Malenka, 1994; Malenka and Bear, 2004; Citri and Malenka, 2008). Group I mGluRs are known to modulate synaptic efficacy through the regulation of ion channel trafficking and induction of the transcription and translation of various genes required for the maintenance of synaptic plasticity (Wang and Zhuo, 2012). Enhancement in the synaptic efficacy occurs through an increased synaptic strength for long time and the phenomenon is known as long term potentiation (LTP) (Malenka, 1994). Contrary to this, decrease in synaptic strength for long duration is known as long term depression (LTD) (Malenka, 1994). Thus, synaptic strength of an excitatory synapse is bi-directionally modulated. In the CNS, activation of either NMDA

receptor or mGluR could trigger LTD (Citri and Malenka, 2008). Although the mechanisms of NMDAR-dependent LTD has been extensively studied, mechanisms underlying mGluR-LTD has not been investigated in detail. Both NMDAR-LTD and mGluR-LTD involve endocytosis of AMPARs. However, various reports have suggested that the molecular mechanisms of NMDAR-dependent AMPAR endocytosis and mGluR-dependent AMPAR endocytosis is significantly different as well as cell type specific (Oliet et al., 1997; Lüscher and Huber, 2010). Understanding the mechanisms and functions of mGluR-LTD has recently become a major attractive area of study because mGluR-LTD has been reported to be altered in the mouse model of mental retardation, autism and Fragile X syndrome (Ronesi and Huber, 2008). Fragile X syndrome is an inherited intellectual disability and the leading cause of autism spectral disorders (Santoro et al., 2012). Mouse model of Fragile X syndrome showed enhanced group I mGluR-LTD in the hippocampus and cerebellum (Dölen et al., 2007). Interestingly, mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) administration has been shown to rescue the cognitive and intellectual disability deficits in the mouse, zebrafish and fruit fly models of Fragile X syndrome (McBride et al., 2005; Yan et al., 2005; Santoro et al., 2012). In addition, selective genetic deletions in the mGluR5 gene of the Fragile X syndrome mice showed rescued phenotype of the disease (Dölen et al., 2007; Bassell and Gross, 2008).

1.9. Desensitization of group I mGluRs

As stated before, many GPCRs upon agonist stimulation become desensitized subsequent to the generation of the second messenger response in order to protect the cells from acute or chronic overstimulation. Like many other GPCRs, group I mGluRs have also been reported to get desensitized upon agoinst stimulation (Mundell et al., 2004). Phosphorylation by various kinases has been shown to play crucial role in the desensitization of group I mGluRs. For example, activation of PKC plays a crucial role in the desensitization of group I mGluRs (Francesconi and Duvoisin, 2000; Dale et al., 2002). PKC-dependent phosphorylation of mGluR1a leads to the desensitization of the receptor (Mundell et al., 2004). PKC is also reported to desensitize other mGluR1 splice variants. For example; mGluR1b is reported to get desensitized in PKC-dependent manner (Dhami and Ferguson, 2006). PKC has also been reported to phosphorylate multiple serine/threonine residues present in the intracellular carboxyl terminal tail of mGluR5 and initiate the desensitization process (Gereau and

Heinemann, 1998). In contrast to the above results, another protein kinase, viz., PKA has been reported to inhibit the desensitization of mGluR1. Phosphorylation by PKA results in the uncoupling of adapter proteins, which in turn inhibit the internalization of the receptors (Francesconi and Duvoisin, 2000).

In addition to the above mentioned second messenger-dependent protein kinases, the G protein coupled receptor kinases (GRKs) also play crucial roles in the group I mGluR desensitization (Ferguson, 2001; Dhami and Ferguson, 2006). For example, GRK2 has been reported to desensitize both mGluR1 and mGluR5 in phosphorylation dependent as well as in phosphorylation independent manner (Ferguson et al., 1996; Dale et al., 2000; Ferguson, 2001; Dhami et al., 2002; Ribeiro et al., 2009). GRK4 on the other hand, has been implicated in mGluR1 desensitization in cerebellar purkinje cells, whereas mGluR5 desensitization was shown to be GRK4 independent (Iacovelli et al., 2003; Sorensen and Conn, 2003). GRK5 has also been reported to regulate mGluR1 desensitization (De Blasi et al., 2001). The GRK-mediated phosphorylated residues are further recognized by arrestin group of proteins, which uncouple the G proteins from the receptor, leading to the desensitization of the receptor (Lefkowitz and Shenoy, 2005). Arrestins also recruit other proteins which promote receptor endocytosis, believed to be important for GPCR resensitization and downregulation (Mundell et al., 2001).

1.10. Group I mGluR trafficking

As stated before, trafficking regulates the spatiotemporal localization of the receptors on the cell surface, which is critical for the normal ligand/receptor interaction. Since, neuron is a compartmentalized cell, trafficking plays a very important role in the proper localization of the receptor at specific region of the neuron. Improper targeting of the receptor results in aberrant signaling which often have severe pathological consequences. Like many other GPCRs, group I mGluRs have also been reported to get internalized subsequent to desensitization (Mundell et al., 2001; Mundell et al., 2004; Choi et al., 2011). The internalization of these receptors is arrestin and dynamin-dependent (Ferguson, 2001; Mundell et al., 2001). Phosphorylation by GRKs and other second messenger dependent kinases have also been implicated in the endocytosis of group I mGluRs. GRK4 has been shown to induce internalization of mGluR1 in HEK293 cells and acute knockdown of GRK4 in the Cerebellar purkinje cells led to the inhibition of the ligand-mediated internalization of

mGluR1 (Sallese et al., 2000; Iacovelli et al., 2003). GRK2 on the other hand, has been reported to induce mGluR1 internalization in phosphorylation-independent manner (Iacovelli et al., 2003). PKC has also been reported to be involved in the internalization of group I mGluRs (Mundell et al., 2003). The region of the receptor involved in the PKC-dependent internalization is distinct from the GRK-dependent internalization (Mundell et al., 2003). Group I mGluRs have also been reported to get internalized constitutively in an agonist-independent manner (Trivedi and Bhattacharyya, 2012). Some studies have suggested that constitutive endocytosis of mGluR1a is β-arrestin and dynamin-independent whereas others have reported that the constitutive endocytosis of mGluR1a is both β-arrestin and clathrin-dependent (Pula et al., 2004; Francesconi et al., 2009). As stated before, GPCRs upon internalization manifest various subcellular fates. Some receptors, subsequent to endocytosis, resensitizes *via* recycling back to the cell surface whereas, some receptors enter the lysosome for degradation (Hanyaloglu and Zastrow, 2008; Magalhaes et al., 2012). What route a particular GPCR would take depends on various factors viz., type of the receptor, type of the ligand and the system. The post-endocytic fate of the group I mGluRs have not been studied so far.

1.11.Role of Tamalin in Group1 mGluR trafficking:

Scaffold proteins play very important role in the trafficking and signaling of the GPCRs in the central nervous system. Tamalin is a 394 amino acid postsynaptic density scaffolding protein, consists of multiple protein-protein interacting domains (Kitano et al., 2003). The domains comprised of a PDZ domain, a proline-rich region, a leucine-zipper region and a C-terminal PDZ binding motif. The PDZ domain of Tamalin interacts with the C-terminus of group I mGluRs, whereas the leucine-zipper region binds to the guanine nucleotide exchange factor cytohesins (Kitano et al., 2002). Furthermore, Tamalin also interacts with many other important scaffold proteins involved in the postsynaptic organization and protein trafficking in neurons. For example, Tamalin binds to PSD-95 and also interacts with proteins implicated in the trafficking, including MINT2 and GRP-1 (Kitano et al., 2003). Tamalin over-expression has been reported to increase the surface expression of mGluR1 in COS-7 cells and cultured hippocampal neurons (Kitano et al., 2002). These results together suggest that Tamalin might play crucial role(s) in controlling various aspects of group I mGluRs regulation like receptor clustering, trafficking, and intracellular signaling.

1.12. Prelude to the present study

Although some amount of work has been done on the mechanism of desensitization and internalization of mGluR1, almost nothing is known about the post-endocytic fate of the receptor. As stated before, for some receptors the post-endocytic mechanisms could serve as a way to resensitize the receptor and for others it could serve as a route to downregulate those receptors. Works done in the past have suggested that the post-endocytic fate of a GPCR is depended on the type of the receptor, type of the ligand and the system. In the present study, we have studied the agonist-induced endocytosis of mGluR1 and its fate subsequent to the internalization in non-neuronal and neuronal cells. We also investigated the role of a scaffolding protein called Tamalin in the regulation of group I mGluR trafficking. Tamalin interacts with group I mGluRs in the brain and has been shown to influence the cell surface expression of the receptors. The role of Tamalin in group I mGluR trafficking and mGluR-dependent AMPAR endocytosis has been studied.

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, Trypsin-EDTA, Lipofectamine 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). Alexa-568 labelled Transferrin was purchased from Invitrogen (USA).

2.1.2. Molecular biology reagents

Various restriction enzymes like EcoR1, BamH1, Xho1 and Xba1, 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).

2.1.3. Plastic wares and chemicals

All plastic wares related to cell culture studies were purchased from BD Falcon (USA). Plastic wares that were used for molecular biological and bacteriological 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 (France).

2.1.4. Antibodies

Anti-myc mouse monoclonal and rabbit polyclonal antibodies, anti-Rab11 mouse monoclonal, anti-LAMP1 rabbit polyclonal and anti-PP2A mouse monoclonal antibodies were purchased from Abcam (UK). Anti-Tamalin rabbit polyclonal antibody was obtained from Rockland Immunochemicals (USA), Anti- HA rat monoclonal antibody and anti-GluA1 rabbit polyclonal antibodies were purchased from Calbiochem (USA), anti-bassoon rabbit polyclonal antibody was from Synaptic Systems (Germany), anti-β-actin mouse monoclonal antibody, anti-GFP rabbit polyclonal antibody and anti-GAPDH rabbit polyclonal antibodies were purchased from Santa Cruz biotechnology (USA). Anti-FLAG rabbit polyclonal antibody was purchased from Sigma (USA). Goat anti–mouse HRP, goat anti–rabbit HRP

and goat anti-rat HRP were purchased from Sigma (USA). All other secondary antibodies were purchased from Invitrogen (USA)

2.1.5. Drugs

2,4- bis dihydroxy phenyl glycine (DHPG), Tetredotoxin (TTX), 6,7-Dinitroquinoxaline-2,3-dione disodium salt (DNQX), D-(-)-2-Amino-5-phosphonopentanoic acid (AP5) were purchased from Tocris (USA). Endothall was purchased from Calbiochem (USA). Okadaic acid, calyculin A, cyclosporine A and FK-506 were obtained from Sigma (USA).

2.1.6. Constructs

The myc-mGluR1 and myc-mGluR5 constructs were obtained from 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 PP2A-L199P construct was a generous gift from Jürgen Götz (University of Queensland, Australia). In this construct the cDNA of human PP2A Cα L199P that acts as a dominant negative of PP2A was fused to a single Hemagglutinin (HA) epitope located just after the start codon and subcloned into the neuron specific mouse Thy1.2 expression vector. Wild-type Tamalin construct was provided by Shigetada Nakanishi (Osaka Bioscience Institute, Osaka Japan). In this construct wild type mouse cDNA was cloned in pcDNA 3.1(+) inserted between Not1 and Xba1restriction sites.

2.1.7. Buffers and media

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

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

4% paraformaldehyde (PFA): 4 g paraformaldehyde was dissolved in 70 ml of phosphate buffer saline (pH - 7.3) at 60°C by adding 1 M NaOH till solution become transparent. The

pH of the solution was then adjusted to 7.4 and subsequently final volume was made up to 100 ml. Solution was then aliquoted into 15 ml falcon tubes and stored at -20°C.

Laemmli sample buffer (4X): 50 mM Tris-HCl (pH-6.8), 10% Glycerol, 0.005% Bromophenol blue, 1% β-mercaptoethanol and 2% SDS were made in double-distilled water.

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

SDS running buffer (1X): 3 g Tris base, 14.4 g Glycine and 1 g of SDS were dissolved in 1 liter double-distilled autoclaved water (final pH - 8.3).

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

DMEM: Dulbecco's Modified Eagle medium (DMEM) and 3.7 g of sodium bicarbonate were dissolved in 800 ml of double-distilled autoclaved water. The pH of the media was adjusted to 7.4 and then the volume was made upto 1 litre with double-distilled autoclaved water. The medium was filter-sterilized under vacuum through a 0.45 μm filter.

10% DMEM: 10% fetal bovine serum (FBS) and antibiotic-antimycotic (1X) mixture was added in DMEM.

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

HEPES buffer Saline: 274 mM NaCl, 9.5 mM KCl, 15 mM Glucose, 42 mM HEPES, 1.4 mM Na_2HPO_4.

2.1.8. Instruments

Cell culture related instruments: Cell culture hoods 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 Eppendrof (Germany), pipette aids were from Thermo Fisher Scientific (USA), water bath was from Grant (UK), sesaw rocker was from Stuart (UK).

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

2.1.9. Primers

Tamalin shRNA Oligos:

(1) 701 sense strand

5’TCGAG**GCATCTATGACACACTGGAGT**TTCAAGAGA**ACTCCAGTGTGTCATA GATGC**TTTT3’

(2) 701 antisense strand

5’CTAGAAAA**GCATCTATGACACACTGGAGT**TCTCTTGAA**ACTCCAGTGTGTC ATAGATGC**C3’

Forward primer for ShRNA sequencing: 5’ CAGTGTCACTAGGCGGGAACA 3’

Reverse primer for ShRNA sequencing: 5’ TTAAGTAGCTGAAGCTCCG 3’

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

Forward primer:

5'CATGGCCTGGTGGTGAAAGACCCAAGTATATACGATACGCTAGAATCCGTGCG CTCCTGCCTCTACGGC 3'

Reverse primer:

5'GCCGTAGAGGCAGGAGCGCACGGATTCTAGCGTATCGTATATACTTGGGTCTTT CACCACCAGGCCATG 3'

Primers used for cloning of HA-Tamalin in LRV1 vector to make over-expression and replacement constructs:

Forward primer:

5'ATCTGGATCCATGTACCCATACGATGTTCCAGATTACGCTACCCTCCGCCGACT CAGG 3'

Reverse primer:

5' GTTAATTGAATTCCTACAGCTGGCTCTCCTCTTCCTCC 3'

Primers for cloning of N-terminal part of Tamalin (1-209 amino acids) in LRV1 vector to create over-expression and replacement constructs:

Forward primer:

5'ATCTGGATCCATGTACCCATACGATGTTCCAGATTACGCTACCCTCCGCCGACT CAGG 3'

Reverse primer:

5' TTAAGAATTCTTAGGTTTGCTTAAGGTACTGCAGGCG 3'

Primers for cloning of C-terminal segment of Tamalin (173-394 amino acids) in LRV1 vector to make over-expression and replacement constructs:

Forward primer:

5’ATCTGGATCCATGTACCCATACGATGTTCCAGATTACGCTATCATCAAGGCGTC TGGCAACGTTCTC 3’

Reverse primer:

5’ GTTAATTGAATTCCTACAGCTGGCTCTCCTCTTCCTCC 3’

Primers used to make the TamalinΔ8 (Tamalin without the last 8 amino acids at the C-terminus) replacement construct in LRV1 vector:

Forward primer:

5’ATCTGGATCCATGTACCCATACGATGTTCCAGATTACGCTACCCTCCGCCGACT CAGG 3’

Reverse primer:

5’ TTAAGAATTCTTAGGAGCGGTTGAGTCCGGGGATGAAC 3’

2.2. Methods

2.2.1. Preparation of competent cells

A single DH5α bacterial colony from a culture plate was taken and incubated into 10 ml LB broth for overnight at 37°C. From this primary culture 100 µl of culture was taken and incubated into 100 ml LB media till the optical density of the culture reached to 0.4 - 0.6. Subsequently, the culture was centrifuged at 6000 rpm at 4°C for 15 min. Afterwards the supernatant was decanted and pellet was resuspended in 10 ml of 0.1 M ice-cold $CaCl_2$ solution and incubated on ice for 15 min. After that the solution was centrifuged at 6000 rpm at 4°C for 15 min. Supernatant was discarded and then the pellet was resuspended in 5 ml of 0.05 M $CaCl_2$ solution and kept in ice for 45 min. Subsequently, recovery of the cells was done by centrifugation at 2000 rpm at 4°C for 5 min. Finally, the pellet was resuspended in 85% 0.1 M $CaCl_2$ solution and 15% glycerol and aliquoted into 1.5 ml microcentrifuge tubes and stored at -80°C.

2.2.2. Transformation

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

2.2.3. Plasmid isolation

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

2.2.4. Generation of Tamalin knockdown constructs

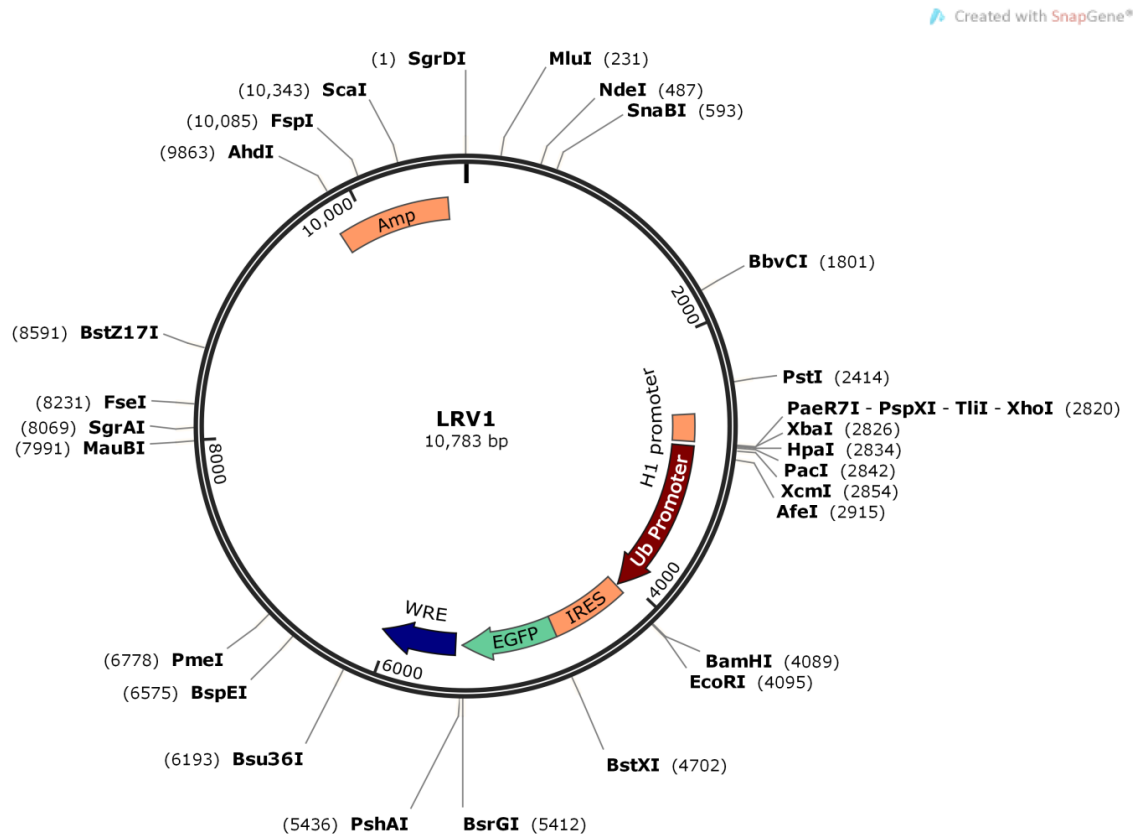
Tamalin knockdown constructs were generated in a two step process: In the first step custom made oligonucleotides were synthesized and in the second step these oligonucleotides were individually cloned into LRV1 vector. LRV1 vector is a multipromoter vector DNA that contains H1 promoter which drives the expression of the shRNA and ubiquitin promoter that drives the expression of wild type Tamalin and various mutants of Tamalin **(Figure 2.1)**.

Briefly, the oligonucleotides (sense and antisense pair) that have the potential to act as shRNA against Tamalin were predicted using Protein Lounge programme. After that oligonucleotides were synthesized and equimolar amount of sense and antisense shRNA oligonucleotide pair was mixed by vortexing in a microcentrifuge tube. Subsequently, they were incubated in water bath for 30 min at 99°C. Water bath was then switched off and temperature was allowed to go down slowly by leaving it overnight so that the oligonucleotide pair can anneal with each other. After annealing, XhoI compatible overhang at the 5' end and XbaI compatible overhang at the 3' end was created in every double stranded oligonucleotide.

Subsequently, the LRV1 vector was digested with XhoI and XbaI restriction enzymes and ligated with each double stranded oligonucleotide to place the shRNAs under the H1 promoter. After cloning of individual shRNAs in LRV1 vector, the screening for the most effective shRNA was performed in primary neurons and HEK293 cells. Briefly, cells were transfected with the shRNA containing LRV1 using the calcium phosphate method as described below. Efficiency of knockdown of respective shRNAs was checked 3-5 days post-transfection by western blot as well as immunocytochemical method. At the end of the screening the following shRNA (shTam) was selected as the most effective in knocking down endogenous Tamalin in primary neurons and have been used for our study:

5'TCGAG**GCATCTATGACACACTGGAGT**TTCAAGAGA**ACTCCAGTGTGTCATA GATGC**TTTT3'

(A)



(B)

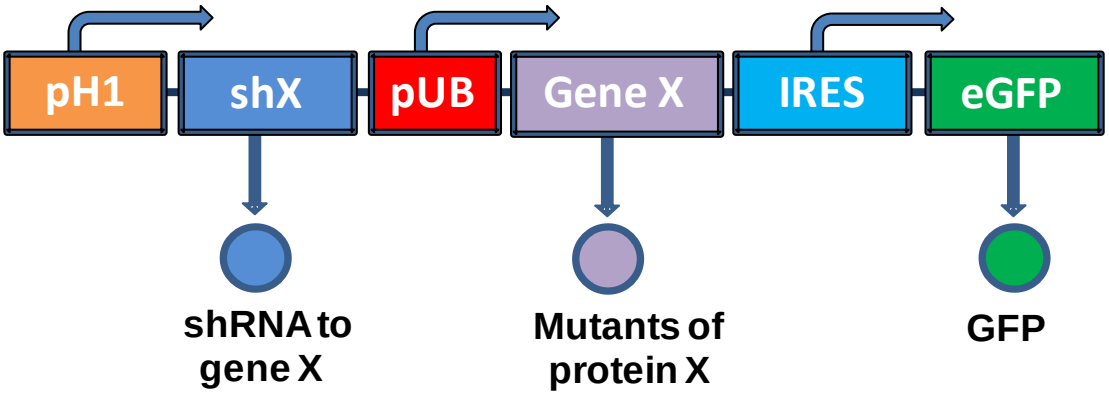
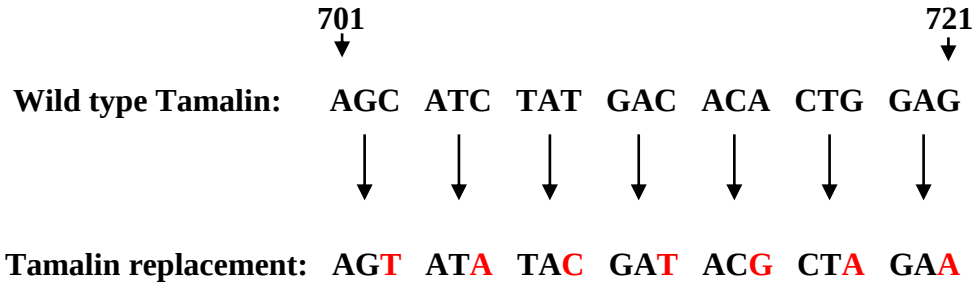


Figure 2.1: The LRV1 vector (A) Vector map of LRV1. (B) Schematic of the organization of the promoters in LRV1.

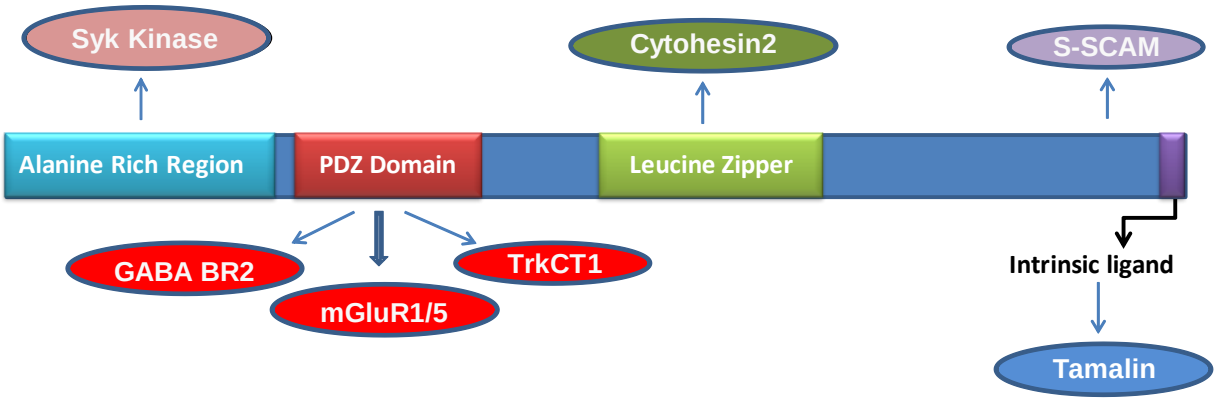
2.2.5. Generation of Tamalin replacement construct

In order to rescue the knockdown effect of Tamalin, the Tamalin replacement construct was generated. Briefly, after finding the suitable and effective shRNA (shTam) through the screening method as described above, few silent mutations were introduced in the wild-type gene of Tamalin at the shRNA binding site through the site directed mutagenesis method. In our case the shTam binding site started at 701th nucleotide of the Tamalin gene. The schematic of the wild type sequence of the shTam binding site and corresponding silent mutations in the replacement construct is shown below:



After introduction of the silent mutations in the wild-type Tamalin gene, it was used as a template to generate the replacement construct. The gene was amplified using forward primer having BamH1 restriction enzyme site and HA sequence and reverse primer having EcoR1 restriction enzyme site. The amplified product was cloned under the ubiquitin promoter using the BamH1 and EcoR1 restriction enzyme sites in the LRV1 vector that also contained shRNA under the H1 promoter. The clone was then confirmed by sequencing. The schematic of the construct is shown in **Figure 2.2**.

(A)



(B)

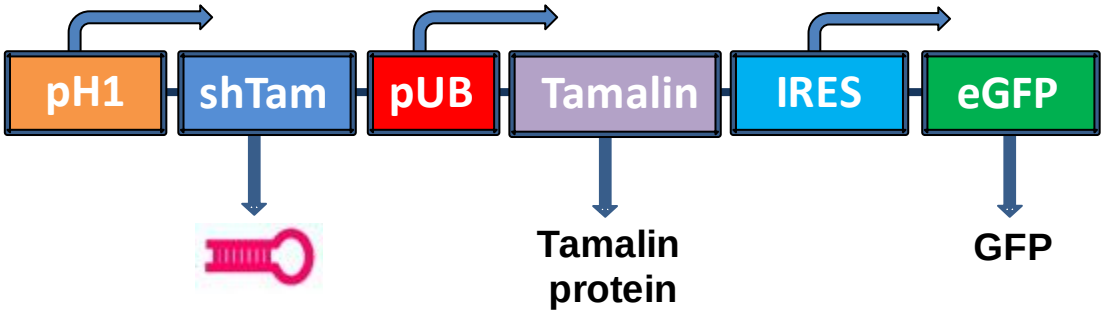


Figure 2.2: Generation of Tamalin replacement construct (A) Schematic of full-length Tamalin and its interaction partners. (B) Schematic of Tamalin replacement construct.

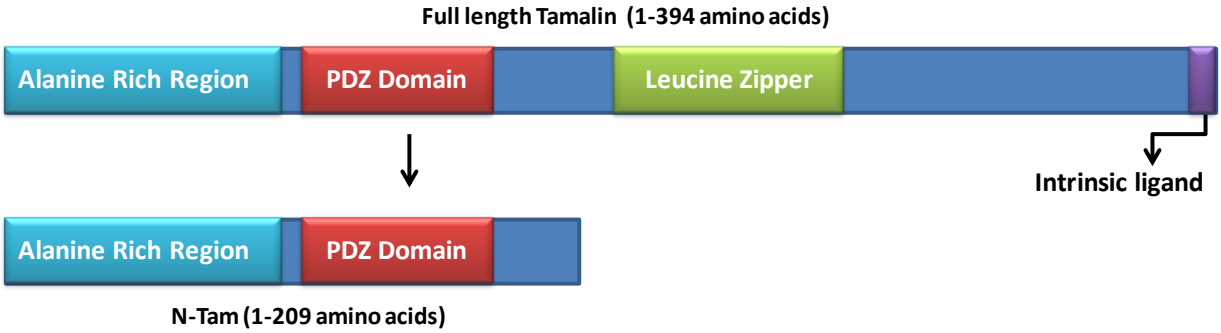
2.2.6. Generation of N-Tam over-expression and N-Tam replacement constructs

In order to investigate the role of N-terminal domain of Tamalin in group I mGluR trafficking N-Tam over-expression and N-Tam replacement constructs were generated by the following methods. Initially PCR was performed taking wild type Tamalin as template to amplify the N-terminal part (1 – 209 amino acids). A forward primer having HA sequence and BamH1 restriction enzyme site and the appropriate reverse primer containing stop codon and EcoR1 restriction enzyme site were used for the PCR reaction. After amplification, the PCR product was digested with BamH1 and EcoR1. The digested product was then ligated in Tamalin knockdown construct (LRV1 having shTam) under the ubiquitin promoter for generation of N-Tam replacement construct. On the other hand, the digested PCR product was also cloned under the ubiquitin promoter in LRV1 that does not have shTam for the generation of N-Tam over-expression construct. **Figure 2.3A** shows the schematic of wild type Tamalin gene and the N-terminal fragment region of Tamalin. **Figure 2.3B** and **2.3C** showing the schematic representation of the N-Tam over-expression and N-Tam replacement constructs respectively.

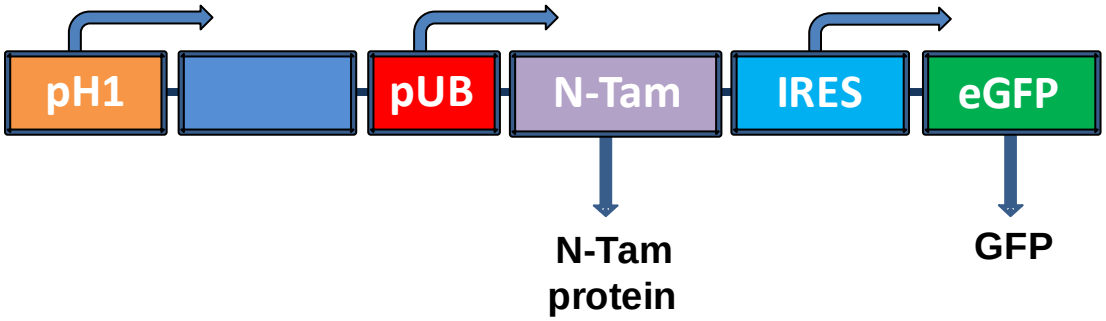
2.2.7. Generation of C-Tam over-expression and C-Tam replacement constructs

In order to investigate the role of C-terminal part of Tamalin in group I mGluR trafficking, the C-Tam over-expression and C-Tam replacement constructs were generated. The strategy of making these constructs were very similar to what has been described for the generation of N-Tam constructs above. Briefly, the C-terminal part of Tamalin was amplified by PCR reaction using the Tamalin DNA that had silent mutations at the shRNA binding site. HA sequence containing forward primer and the appropriate reverse primer having BamH1 and EcoR1 restriction enzyme sites respectively were used for the PCR reaction. Subsequently, restriction enzyme digestion of the insert and the vector (LRV1 without shTam in case of over-expression construct and LRV1 with shTam in case of replacement construct) was done followed by ligation using T4 DNA ligase. The clone was confirmed by sequencing. **Figure 2.4A** shows the C-terminal fragment in Tamalin. The schematic of C-Tam over-expression and C-Tam replacement constructs is shown in **Figure 2.4B** and **2.4C** respectively.

(A)



(B)



(C)

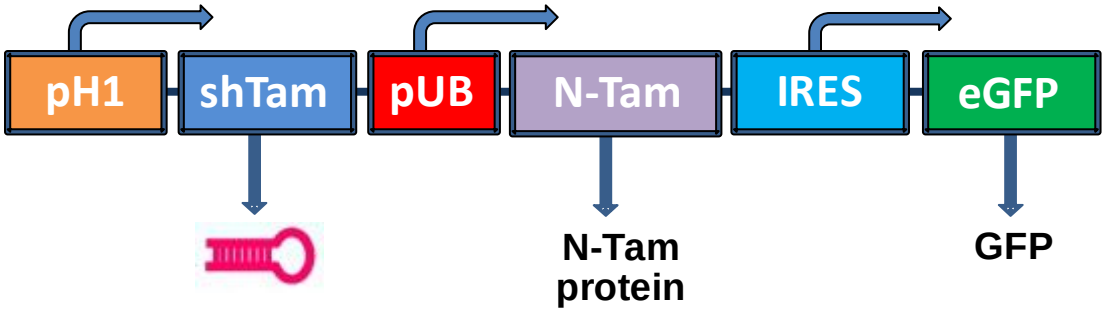
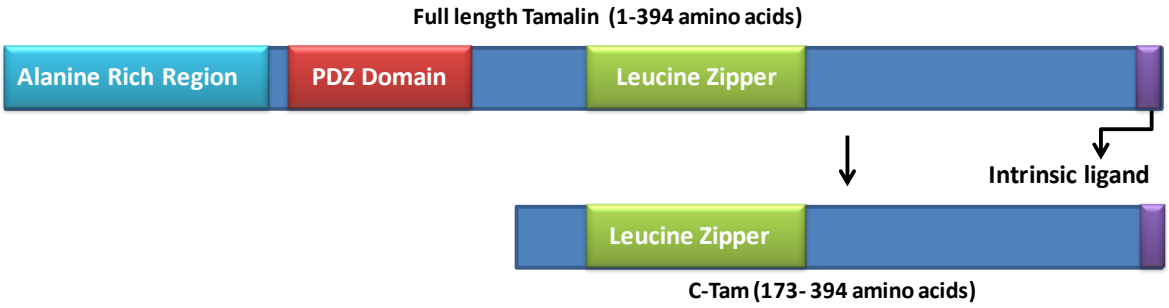
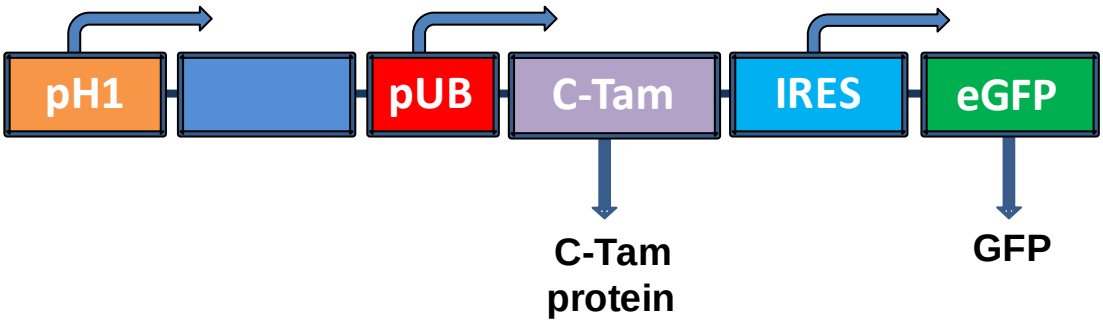


Figure 2.3. Generation of N-Tam over-expression and N-Tam replacement constructs (A) Picture of full-length Tamalin and N-Tam. (B) Schematic of N-Tam over-expression construct. (C) Schematic of N-Tam replacement construct.

(A)



(B)



(C)

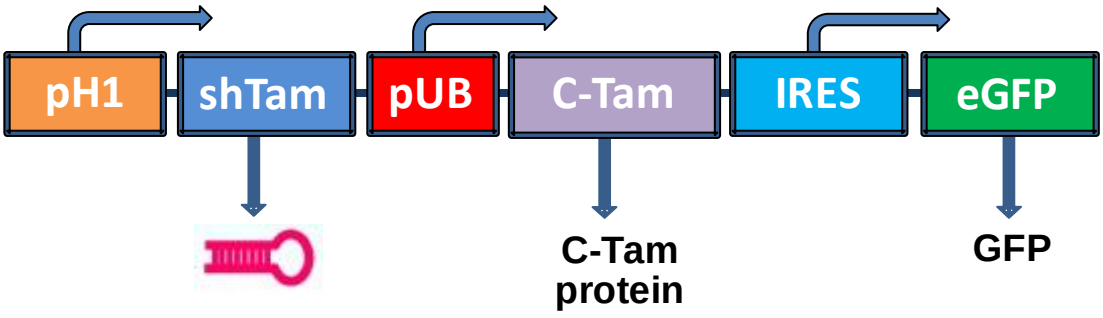


Figure 2.4. Generation of C-Tam over-expression and C-Tam replacement constructs (A) Picture of full-length Tamalin and C-Tam. (B) Schematic of C-Tam over-expression construct. (C) Schematic of C-Tam replacement construct.

2.2.8. Generation of TamΔ8 replacement construct

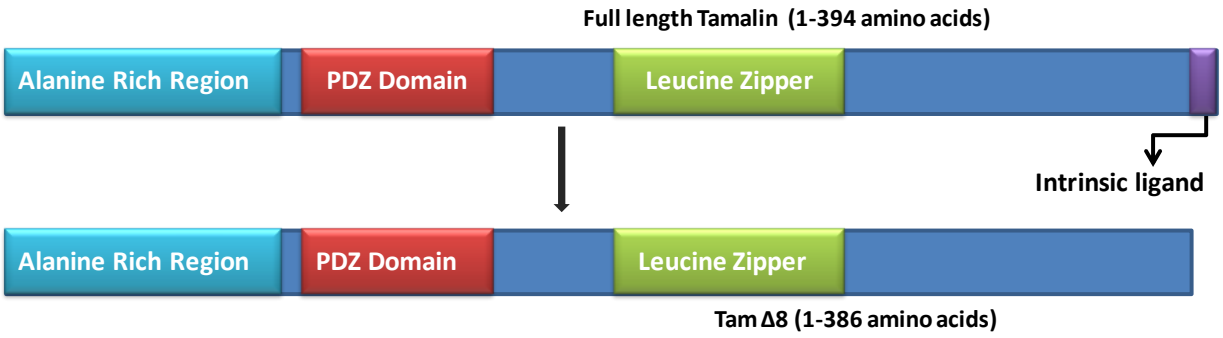
In order to investigate the role of the intrinsic ligand binding domain of Tamalin in group I mGluR trafficking, the Tamalin gene without the sequence for the last 8 amino acids at the C-terminus was amplified through PCR method using appropriate primer sets (Sugi et al., 2007). Similar to the above constructs the forward primer had a HA sequence and BamH1 restriction enzyme site and the reverse primer had EcoR1 restriction enzyme site. The insert and the vector (LRV1 with shTam) were digested with the restriction enzymes followed by ligation using the method as described above. The construct was then confirmed by sequencing. **Figure 2.5 A** and **2.5 B** depicts the pictorial representation of TamΔ8 replacement construct.

2.2.9. Cell culture and transfection

I. HEK293 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 cultured on coverslips, pre coated with poly-D-lysine (50 µg/ml). The cells were transfected with appropriate DNA constructs at 65 – 70% confluency with the help of Lipofectamine2000 reagent according to manufacturer's instructions. Subsequently, the transfection mixture was replaced with fresh 10% DMEM and cells were allowed to express constructs for 24 - 30 hr before performing the experiment.

II. Neuro-2A (N2A) is a mouse neuroblastoma cell line. They were cultured in undifferentiated condition in DMEM containing 10% FBS, 1X AB-AM mix in 5% CO_2 and 95% humidity at 37˚C. Approximately, 70,000 cells were plated per cover slips in a 24 well plate coated with 50 µg/ml poly-D-lysine. Transfection with various cDNA constructs were carried out in undifferentiated cells using Lipofectamine2000 using manufacturer's instructions. The transfection mix was applied for 3.5 hr. Subsequently, cells were incubated further for 24 hr in 10% DMEM for the expression of proteins. Cells were then differentiated by adding fresh DMEM supplemented with 1% FBS. All the experiments were performed in fully differentiated N2A cells.

(A)



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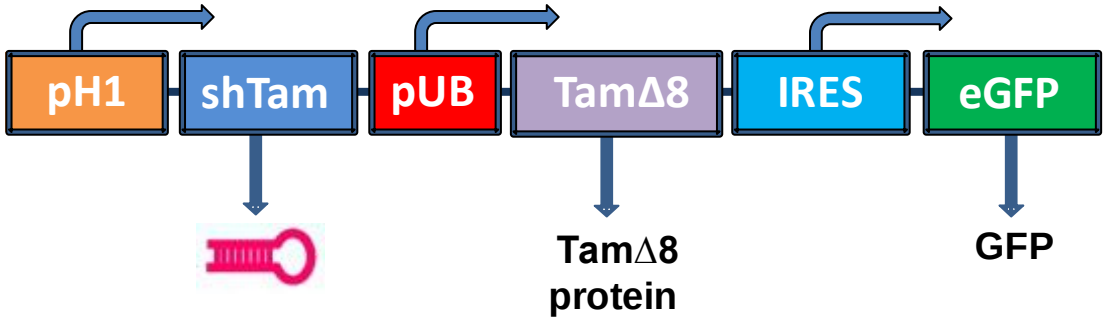

Figure 2.5. Generation of TamΔ8 replacement construct (A) Picture of full-length Tamalin and TamΔ8. (B) Schematic of TamΔ8 replacement construct.

III. **Dissociated primary hippocampal neuron culture –** Primary hippocampal neurons were isolated from P0/P1 C57BL/6 mouse pups. Briefly, pups were first sacrificed and then hippocampi were dissected out. Subsequently, tissues were dissociated by papain treatment for 30 min at 37˚C followed by formation of single cells by trituration with glass pipette. Neurons were then plated on 50 µg/ml poly-D-lysine + 0.1 M sodium borate (pH 8.4) – pre coated cover slips at a density of approximately 100,000 cells for mGluR trafficking experiments and around 80,000 cells for AMPAR trafficking experiments per 12-mm well in a 24 well plate. Cultures were maintained in Neurobasal media with 0.5 mM glutamine and B27 supplement. Glial growth was inhibited by adding FUDR (floxuridine) on 3rd day of culture. Transfection was done on 6-8 day old cultures using calcium phosphate method. Briefly, in calcium phosphate mediated transfection method, old neurobasal-B27 media was replaced by plain Neurobasal media and then 3 µg of DNA constructs for each well were mixed with 250 mM $CaCl_2$ in a microcentrifuge tube and incubated for 5 min, followed by mixing with 2X HEPES buffer and then incubated for 30 min in dark. The mixture was subsequently added in cells and incubated for the time till appearance of sufficient crystals was observed under the bright field microscope. Subsequently the cells were washed with washing buffer and old Neurobasal – B27 media was added. Half feeding was done on next day. All the experiments were performed when the cells were 12-15 days *in vitro.*

2.2.10. Endocytosis assay

Group I mGluR endocytosis assay

HEK293 cells, N2A cells and primary hippocampal neurons were transfected with myc-mGluR1 or myc-mGluR5 cDNA. On the other hand, for studies related to Tamalin, primary hippocampal neurons were co-transfected with myc-mGluR1/myc-mGluR5 and Tamalin constructs using calcium phosphate method as described before. Subsequently, for endocytosis assay, live cells were labelled with anti-myc mouse monoclonal primary antibody (1:500) for 15 min at 37˚C for HEK293 and N2A cells and anti-myc mouse monoclonal primary antibody (1:200) for 30 min at 37˚C for primary hippocampal neurons. Cells were then washed with plain DMEM and treated with agonist DHPG (100 µM) for indicated times or 5 min. Subsequently, cells were chased for indicated times in the absence

of DHPG. Cells were then fixed without permeabilization with ice cold 4% paraformaldehyde (PFA) for 15 min on ice. After fixation, surface receptors were labelled with saturating concentration of goat anti-mouse Alexa-568 (1:100) conjugated secondary antibody for 1 hr at 37˚C. Cells were then permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Subsequently, endocytosed receptors were labelled with second secondary antibody, viz., goat anti-mouse Alexa-647 (1:750) for 1 hr at 37˚C. Cover slips were mounted on glass slides using fluoromount and imaged under the confocal microscope. For experiments that involved drugs, cells were pre incubated with the drug for 30 min before the primary antibody staining and the drug was present throughout the experiment.

In order to make sure that the Alexa-647 conjugated secondary antibody that was used to label the endocytosed receptors, did not label any surface receptors, the following control experiment was performed. When the saturating concentration of Alexa-568 conjugated secondary antibody was applied to visualize the surface receptors it prevented any further detectable staining of the surface receptor when Alexa-647 conjugated second secondary antibody was applied in non-permeabilized cells. Whereas, upon permeabilization of the cells with 0.1% Triton X-100 for 30 min at room temperature, the internalized receptors were observed on application of Alexa-647 conjugated secondary antibody **(Figure 2.6)**. These experiments suggest that application of saturating concentration of the first secondary antibody (Alexa-568 conjugated) occupied all the surface receptors and thus the second secondary antibody (Alexa-647 conjugated) did not label any detectable surface receptors (Bhattacharyya et al., 2009; Trivedi and Bhattacharyya, 2012; Pandey et al., 2014; Mahato et al., 2015; Gulia et al., 2016). Similar control experiments were performed in primay hippocampal neurons as well as for experiments related to myc-mGluR5 (data not shown)

AMPA receptor endocytosis assay

In order to study the mGluR-mediated AMPA receptor (AMPAR) endocytosis, primary hippocampal neurons were transfected at 6-7 DIV with various Tamalin over-expression and replacement constructs. Endocytosis assay was performed when cells were 14-15 DIV. Cells were pre-incubated with a mixture of antagonists viz., 1 μM TTX (neurotransmitter release blocker), 20 μM DNQX (AMPAR antagonist) and 50 μM APV (NMDAR antagonist) for 30 min at 37˚C. Subsequently live cells were labelled with anti-GluA1 rabbit polyclonal antibody (1:20) directed against the N-terminus of the endogenous GluA1 subunit for 15 min

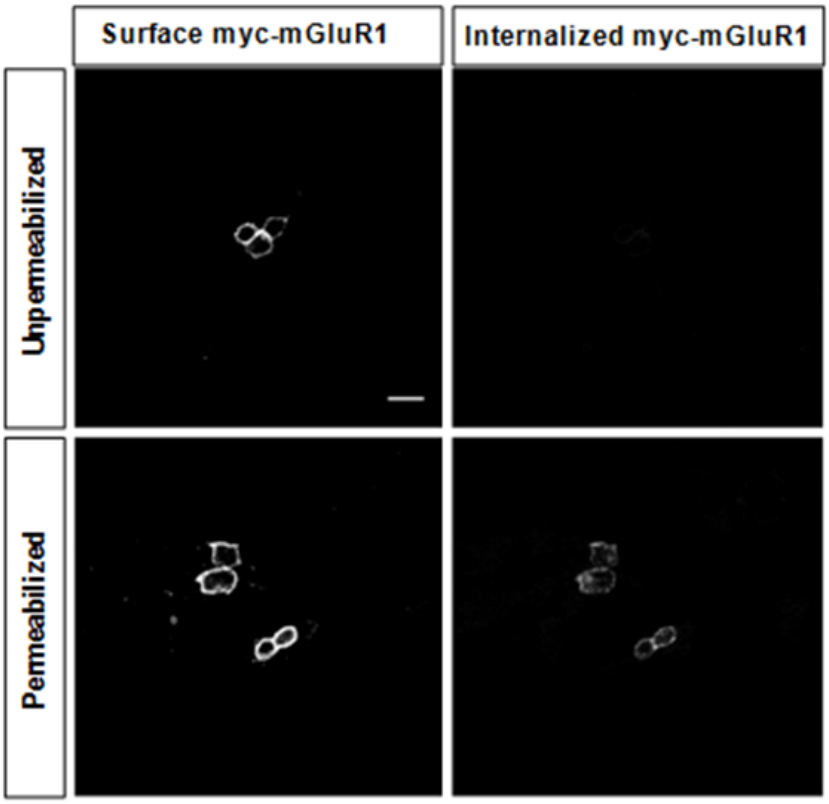

Figure 2.6. Standardization of dual receptor labelling assay: The upper panel shows that saturating amount of the first secondary antibody (Alexa-568) labelled the surface receptors (upper left panel) which prevents any further observable binding of the second secondary antibody which is Alexa-647 labelled (upper right panel) in unpermeabilized condition. However the when cells were permeabilized, the second secondary antibody labelled the internalized myc-mGluR1 (lower right panel). Scale bar = 10 μm.

at 37˚C. Cells were then washed twice with plain neurobasal medium and 100 µM DHPG was applied for 5 min followed by chase for 10 min in plain neurobasal medium having the antagonists. Subsequently, cells were fixed (without permeabilization) with ice cold 4% PFA for 15 min on ice. Surface GluA1 containing receptors were then labelled with saturating concentration of goat anti-rabbit Alexa-568 conjugated secondary antibody (1:100) for 1.5 hr at 37˚C. Cells were then permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Subsequently, endocytosed receptors were labelled with second secondary antibody viz. goat anti-rabbit Alexa-647 (1:750) for 1.5 hr at 37˚C. Cover slips were mounted on glass slides using fluormount and imaged under the confocal microscope. The saturating concentration of the Alexa-568 labelled secondary antibody that was used to label the surface GluA1 containing receptors were determined by performing control experiments similar to the experiments that were discussed above for the group I mGluR endocytosis. These experiments also suggested that the Alexa-647 conjugated secondary antibody that was used to label the internalized receptors didn't label any detectable surface receptors (data not shown) (Bhattacharyya et al., 2009; Gulia et al., 2016).

2.2.11. Recycling assay

HEK293 cells or N2A cells were transfected with myc-mGluR1 cDNA as described above. Subsequent to anti-myc primary antibody labelling, DHPG pulse was given for 5 min to the cells at 37˚C. After that cells were chased for different time periods in plain DMEM in the absence of the ligand. At specific time points, cells were fixed and surface and internalized receptors were labelled by Alexa-568 conjugated and Alexa-647 conjugated secondary antibodies respectively, using the same protocol as described for the endocytosis assay. To study the effect of drugs, cells were pre-treated with the respective drugs for 30 min before the application of the primary antibody and the drug was present till the end of the experiment. The effect of knockdown of the endogenous PP2A on the recycling of mGluR1 in N2A cells was investigated by cotransfection with myc-mGluR1 and si-PP2A.

2.2.12. Co-localization assay

Cells expressing myc-mGluR1 were labelled with anti-myc rabbit polyclonal antibody for the Rab11 (recycling endosome marker) experiments and anti-myc mouse monoclonal antibody

for the LAMP1 (lysosomal marker) colocalization experiments respectively. Subsequent to the endocytosis protocol (as described before), cells were surface stripped by treating them with ice cold 0.5 M NaCl + 0.2 N CH_3COOH for 3 min on ice to remove the antibodies attached to the surface receptors that did not internalize on application of the ligand. Cells were then fixed by 4% PFA and then permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Subsequent to that cells were blocked with 2% NGS for 1 hr at 37˚C. Following that, cells were stained with either mouse monoclonal antibody against Rab11 (1:500) for 4 hr or rabbit polyclonal antibody against Lamp1 (1:750) for 1 hr at 37˚C. After that appropriate secondary antibodies (1:800) were added for 1 hr at 37°C to stain the internalized myc-mGluR1 and Rab11 or LAMP1 respectively. Subsequently, coverslips were mounted on glass slides using fluoromount and observed under the confocal microscope.

In order to investigate whether different mutants of Tamalin are localized at the synapse, the extent of colocalization of the mutants with the presynaptic protein Bassoon were measured. Briefly, hippocampal neurons were transfected with different HA tagged Tamalin mutants using calcium phosphate method as described above on 6 DIV. On 12-14 DIV cells were fixed with ice cold 4% PFA on ice for 15 min. Subsequently, cells were permeabilized by 0.1% Triton X-100 following blocking with 2% NGS for 1 hr at 37˚C. Cells were then stained with rat anti-HA antibody (1:500) and rabbit anti-bassoon antibody (1:500) by incubating overnight at 4˚C. Afterwards, cells were incubated with goat anti-rat Alexa-568 and goat anti-rabbit Alexa-647 for 1.5 hr at 37˚C to visualize the Tamalin constructs and Bassoon respectively. Coverslips were then mounted on glass slides and imaged under the confocal microscope.

2.2.13. Transferrin receptor kinetics assay

HEK293 cells were plated on coverslips in 24 well plates and were incubated in either plain DMEM or 5 nM Okadaic acid for 30 min at 37˚C. Subsequently, 20 µg / ml Alexa-568 labelled transferrin was applied for 2 min at 37ºC. Cells were then washed with plain DMEM twice and chased for various time periods (5 min, 15 min, 30 min and 60 min) at 37ºC (Ciechanover et al., 1983; Harding et al., 1983). After the specific time period, cells were stripped for 2 min with acidic solution (0.5% CH_3COOH + 500 mM NaCl) to remove the remaining transferrin bound at the cell surface. Cells were then fixed with 4% PFA and

mounted on glass slides using Fluoromount mounting media. In the experiments involving Okadaic acid, drug was present throughout the experiment. Image acquisition was done in Zeiss Axio Observer Z1 fluorescence microscope using 63X oil objective. Quantitation was done in ImageJ (NIH, USA) software.

2.2.14. Imaging

Images were obtained in Zeiss LSM 780 confocal laser scanning microscope equipped with 63X oil immersion objective having 1.4 numerical aperture. 100-120 HEK293 cells and 40-50 N2A cells or primary hippocampal neurons were imaged and every experiment was repeated at least three times. Digital gain, digital offset, laser power and all other parameters were constant throughout individual experiment. Alexa-488, Alexa-568 and Alexa-647 exitation/emission were achieved using appropriate filter sets and acquisition was done using identical parameters for a particular experiment.

2.2.15. Image analysis and Statistics

Image analysis

All images were analysed by ImageJ software (NIH, USA). Raw images were used for all analyses and quantitation purposes. For quantitation purpose, images were first maximally projected, and a value of threshold was set for individual fluorophore which remain constant throughout an experiment. Thresholded areas occupied by the fluorescence of the labelled surface and internalized receptors were quantified. The endocytosis index was then calculated by dividing the value contributed by the internal fluorescence with the value contributed by the total fluorescence (surface + internal). They were then normalized with that of untreated control cells.

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

To measure the surface receptors, surface fluorescence was divided by the cell area, which was determined by measuring background fluorescence using a low threshold level. These values were then normalized to the average surface fluorescence of untreated control cells.

For N2A cells and primary hippocampal neurons which are compartmentalized cells, cell body area was calculated by drawing a circle of 20 µM diameter around the cell body and defined the area under the circle as somatic area. The area outside the circle is considered as the dendritic area. In case of primary hippocampal neurons all the data represents dendritic values which was defined by the area that was outside of the 20 µM circle drawn around the cell body. All the quantitation has been represented as combined results for all the repeats of a particular experiment. Raw images were adjusted using equal values of brightness and contrast to obtain the representative images.

Statistics

All data are reported as means ± SEM. As stated before, 100-120 HEK293 cells and 40-50 N2A cells or primary hippocampal neurons were imaged and each experiment were repeated three times. Between groups, statistical comparisons were done by performing either unpaired two tailed Student's t test or one-way ANOVA followed by Tukey's post test. Values of $p<0.05$ were considered significant. Data were analysed and graphs were plotted using Origin software.

2.2.16. Western blot analysis

In order to check for the expression of PP2A dominant negative construct or whether the application of PP2A siRNA resulted in the knockdown of endogenous PP2A, western blotting experiments were performed in N2A cells. Briefly, cells were plated in 60 mm dishes and transfected with either PP2A Cα L199P (PP2A dominant negative) construct or PP2A siRNA smart pool along with scrambled siRNA. After respective incubation times differentiated N2A cells were washed with ice cold PBS twice. Cells were then lysed with the RIPA lysis buffer having protease inhibitor cocktail. Protein concentration was then estimated by Bradford assay. Samples were boiled in Laemmli sample buffer and ran in SDS PAGE by loading equal amount of protein in each lane. Subsequently, they were transferred to a PVDF membrane, blocked with 5% milk for 1 hr at room temperature. Membrane was then incubated with either mouse anti-PP2A (1:1000) or rabbit anti-GAPDH (1:1000)

primary antibodies for 3 hr at room temperature. Subsequently, membranes were washed and incubated in goat anti-mouse HRP (1:5000) or goat anti-rabbit HRP (1:5000) secondary antibodies for 1 hr at room temperature. Blots were developed using ECL western detection kit and image acquisition was done using ImageQuant LAS 4000.

In order to check whether the candidate shRNAs against Tamalin efficiently knocked down the overexpressed Tamalin in HEK293 cells, Tamalin construct along with different shRNA constructs were transfected in HEK293 cells. Cells were then incubated for 3-4 days for the expression of Tamalin protein and effective knockdown of Tamalin by candidate shRNAs, if any. Subsequently, cells were lysed with RIPA buffer having protease inhibitor cocktail. Protein concentration was estimated by Bradford assay. Samples were then subjected to SDS PAGE and western blotting was done subsequently using rabbit anti-Tamalin polyclonal antibody (1:100) using the procedure as described above.

Internalization and recycling of mGluR1 in non-neuronal and neuronal cells

3.1. Introduction

G protein coupled receptors (GPCRs) play crucial roles in maintaining the homeostasis of the cellular milieu. Upon agonist stimulation GPCRs become active and transduce their signal *via* second messenger pathways. Majority of the GPCRs get desensitized or become unresponsive towards further agonist stimulation (Krupnick and Benovic, 1998). The process of desensitization is believed to play a protective role against excessive GPCR signaling. Many GPCRs have been reported to get internalized followed by desensitization (Ferguson, 2001). Subsequent to the internalization some receptors go to the lysosome and get degraded which is the mechanism to "down-regulate" those receptors. Other receptors recycle to the cell surface after they internalize and the recycling seems to be critical to "resensitize" those receptors. Thus, the process of internalization could be important for the downregulation for some receptors or resensitization for others (Kelly et al., 2008; Sorkin and von Zastrow, 2009; Magalhaes et al., 2012). What route a receptor will take subsequent to the internalization depends on the type of the receptor, type of the ligand and type of the system.

Like many other GPCRs, mGluR1 has been reported to get internalized rapidly upon agonist stimulation. The internalization of these receptors has been reported to be β-arrestin and dynamin dependent (Mundell et al., 2004). Over-expression of dominant negative form of β-arrestin (clathrin-binding domain of β-arrestin) and dynamin inhibits the ligand-mediated endocytosis of mGluR1 (Mundell et al., 2001; Mundell et al., 2004). The internalization of these receptors is phosphorylation-dependent and both GRKs and second messenger-dependent kinases have been implicated in this process (Mundell et al., 2001; Dhami and Ferguson, 2006). For example, acute knockdown of GRK4 in Cerebellar Purkinje cells inhibits the ligand-mediated internalization of mGluR1 (Iacovelli et al., 2003). On the other hand GRK4 has been found to be involved in the internalization of mGluR1 in heterologous cells as well (Iacovelli et al., 2003). The second messenger dependent kinase, protein kinase C (PKC) has been implicated in the internalization of mGluR1 (Dhami and Ferguson, 2006). Interestingly, the region of the receptor that is involved in the PKC-dependent internalization and the region that is involved in the GRK-mediated internalization are found to be distinct (Mundell et al., 2003). Although, some amount of work has been done on the cellular and molecular mechanisms regulating the mGluR1 endocytosis, the lack of knowledge about the post-endocytic events of the receptor subsequent to the ligand-mediated internalization is the main motivation of our study.

We have studied the trafficking of mGluR1 in both non-neuronal (HEK293 cells) and neuronal (Neuro2A) cells. HEK293 cells are regularly used as a standard heterologous system to study various GPCRs due to the presence of large repertoire of G proteins in these cells. On the other hand, Neuro2A cells are mouse neuroblastoma cells which upon differentiation gives rise to functional neurons. We have also extended this study in primary hippocampal neurons which till today is most experimentally tractable *in vitro* system that can approximate the *in vivo* situation. We have used myc tagged mGluR1 for our study, where myc has been tagged at the N-terminus of the receptor and previous reports have suggested that this tagged receptor behaves like the native receptor (Choi et al., 2011). Our data suggest that upon agonist stimulation myc-mGluR1 gets internalized in both HEK293 and N2A cell with similar kinetics. In both cell types the internalization of the receptor is rapid and reaches a maximum around 30 min post-ligand application. The endocytosed receptors were seen to colocalize with Rab11 (a recycling endosomal marker) at 30 min post ligand application, suggesting that the internalized receptors enters the recycling compartment subsequent to the ligand-dependent internalization. Furthermore, the internalized receptors did not show any observable colocalization with the lysosomal marker LAMP1. Together these results suggest that majority of the myc-mGluR1 enters the recycling compartment subsequent to the ligand-mediated internalization. Our data also suggest that the internalized receptors recycled back to the cell surface in 2.5 hr in both non-neuronal and neuronal cells when cells were chased for longer time in absence of the ligand. Thus, the kinetics of internalization and recycling was found to be similar in both HEK293 and N2A cells. Finally, our data also suggest that similar to HEK293 cells and N2A cells, myc-mGluR1 internalizes on ligand application in primary hippocampal neurons and recycles back to the cell surface in 2.5 hr as well.

3.2. Results

3.2.1. Endocytosis of mGluR1 upon agonist stimulation in HEK293 and N2A cells

Previous reports have suggested that upon agonist stimulation mGluR1 gets endocytosed. In order to check whether myc-mGluR1 gets internalized upon agonist stimulation we studied the internalization of this receptor on DHPG application in HEK293 and N2A cells. The protocol to study the DHPG-mediated internalization of myc-mGluR1 has been described in the "material and methods" section. Briefly, myc-mGluR1 cDNA was transfected in HEK293 cells and subsequently cells were incubated in 10% DMEM (DMEM + 10% FBS) for 24 hr for the expression of the construct. myc-mGluR1 expressing live HEK293 cells were treated with anti- myc mouse monoclonal primary antibody for 15 min at 37˚C, followed by the application of DHPG (100 µM) for 5 min. After that, DHPG was removed and cells were incubated for 25 more min (total 30 min) in plain DMEM in absence of the ligand. Cells were then fixed without permeabilization with ice cold 4% PFA for 15 min on ice. Subsequently, first secondary antibody viz., goat anti-mouse Alexa-568 (1:100) was applied to label the surface receptors. Cells were then permeabilized with 0.1% Triton X-100 and afterwards second secondary antibody viz., goat-anti mouse Alexa-647 (1:750) was applied to label the internalized receptors. Cells were then mounted on glass slides and imaged under the confocal microscope.

Control cells (DHPG untreated) showed presence of majority of the receptors on the surface of the cell and very little endocytosed receptors were observed **(control = 1 ± 0.06) (Figure 3.1 A, B)**. The little endocytosis that is observed in control cells is due the constitutive endocytosis of the receptor. On the other hand, application of 100 µM DHPG increased the level of the internalized receptors, suggesting that myc-mGluR1 internalized on application of DHPG in HEK293 cells **(30 min = 2.0 ± 0.14)**. In this and subsequent all the experiments related to trafficking of group I mGluRs, endocytosis index was calculated following the method as discussed in the "materials and method" section. Accuracy of the quantitation is governed by the fact that it is necessary to use the saturating concentration of the first secondary antibody which labelled the surface receptors so that the second secondary antibody that is meant to bind the internalized receptors would not be able to label detectable surface receptors. The standardization protocol has been discussed in the method section where we used saturating concentration of the first secondary antibody followed by the

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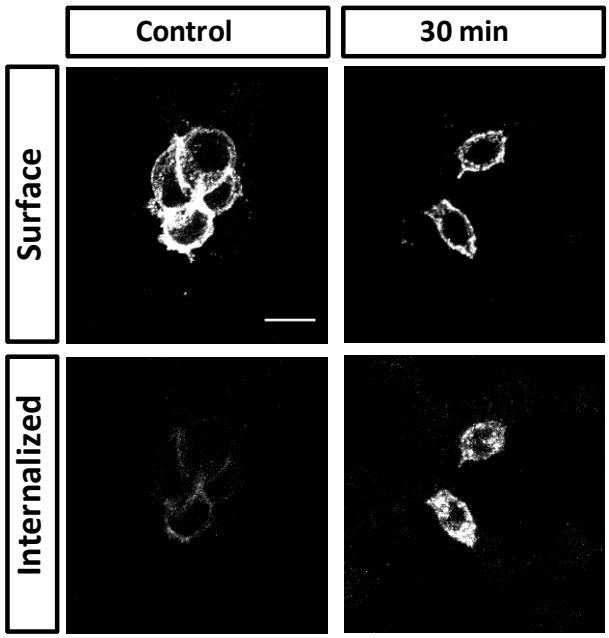

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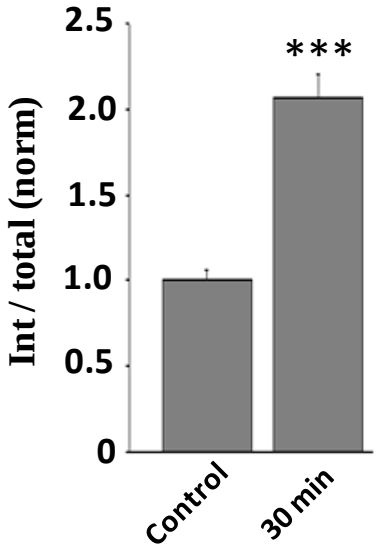
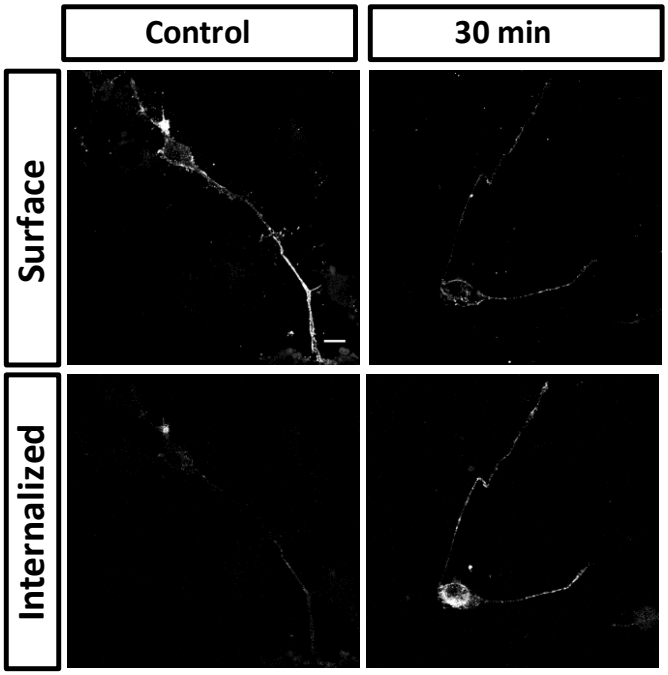


Figure 3.1. Endocytosis of myc-mGluR1 in HEK293 cells (A) Control cells showing very little internal fluorescence and upon 100 μM DHPG application myc-mGluR1 endocytosed in 30 min.(B) Quantitation also showed that myc-mGluR1 endocytosed upon DHPG application in 30 min. Scale bar = 10 μm. ***$p < 0.001$. N=3; where N represents the number of times experiment was repeated.

second secondary antibody staining in both unpermeabilized and permeabilized conditions. These experiments suggested that there was no detectable second secondary antibody staining observed in case of unpermeabilized condition and on the other hand, in permeabilized condition the second secondary antibody stained the constitutively endocytosed receptors.

In order to determine whether myc-mGluR1 internalizes in differentiated N2A cells as well on ligand application, myc-mGluR1 was transiently transfected in undifferentiated N2A cells by the method described in the "materials and methods" section. 24 hr post transfection, 10% DMEM was replaced with differentiation media (1% FBS containing DMEM) and incubated further for another 48 hr for differentiation. Afterwards antibody feeding assay was performed in the similar way as has been described for HEK293 cells. Subsequently goat anti-mouse Alexa-568 and goat anti-mouse Alexa-647 secondary antibody staining were performed to visualize the surface and internalized receptors respectively using the same protocol as described for HEK293 cells. Cells were then mounted on the glass slides and imaged under the confocal microscope. DHPG treated cells showed internalized myc-mGluR1 both in the cell body and in the neurites of the cells. On the other hand, control cells showed presence of majority of the receptors at the surface of the cell body and neurites **(control = 1 ± 0.02, 30 min = 1.99 ± 0.08) (Figure 3.2 A, B)**. The saturating concentration of the first secondary antibody was determined in N2A cells by performing the similar experiments as described for the HEK293 cells above. Taken together, these experiments suggest that myc-mGluR1 internalizes upon agonist exposure, both in non-neuronal and neuronal cells.

(A)



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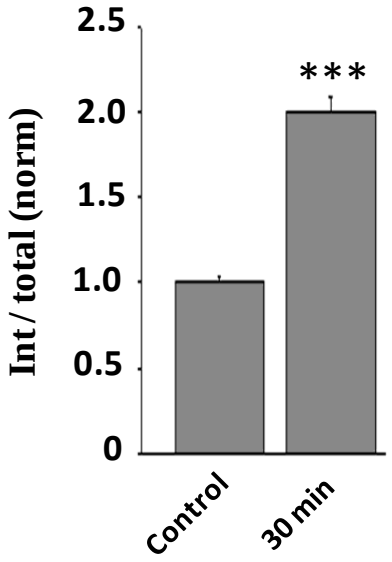


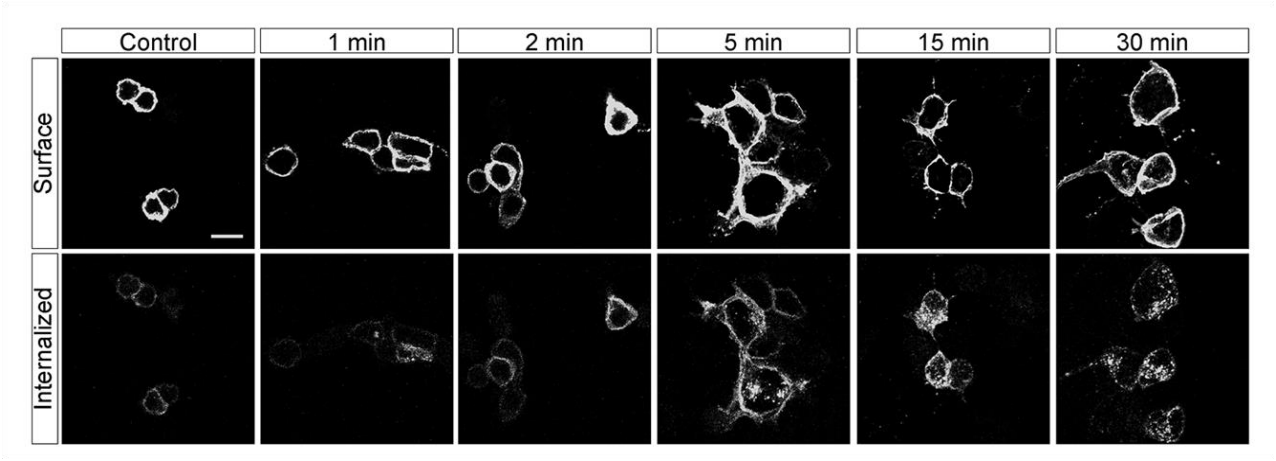
Figure 3.2. Endocytosis of myc-mGluR1 in differentiated N2A Cells (A) Representative images showing myc-mGluR1 endocytosis in differentiated N2A Cells. Control cells showed very little internal fluorescence which increased upon 100 µM DHPG application. (B) Quantitation also showed that upon 100 µM DHPG application myc-mGluR1 got endocytosed in N2A cells. Scale Bar= 10µm. ***$p < 0.001$. N=3; where N represents the number of times experiment was repeated.

3.2.2. Kinetics of myc-mGluR1 internalization in non-neuronal and neuronal cells

In order to study the kinetics of myc-mGluR1 internalization in HEK293 and N2A cells following ligand application, 'pulse chase' experiments were performed. Briefly, HEK293 cells were transfected with myc-mGluR1 as described before. Subsequently, anti–myc primary antibody staining was done in myc-mGluR1 expressing live HEK293 cells. After that 100 μM DHPG was applied for indicated times or for 5 min followed by chase for the indicated time points in absence of the ligand. Cells were then fixed at specific time points with the 4% PFA without permeabilization. Fixed cells were then stained by saturating concentration (1:100) of first secondary antibody (goat anti-mouse Alexa-568) followed by permeabilization with Triton X-100. After that, anti-mouse Alexa-647 labelled second secondary antibody was applied (1:750) to label the internalized receptors. Cells were then mounted on glass slides and imaged under the confocal microscope. In control cells majority of the receptors were seen to be present on the surface **(control = 1 ± 0.05) (Figure 3.3 A, B)**. DHPG-mediated endocytosis of myc-mGluR1 was found to be rapid. The myc-mGluR1 internalized at 1 min post-ligand exposure as observed by an increase in the internal fluorescence **(1 min = 1.26 ± 0.07)**. Furthermore, there was gradual increase in the internal fluorescence and corresponding decrease in the surface fluorescence when cells were chased for longer time periods **(2 min = 1.3 ± 0.07, 5 min =1.45 ± 0.08, 15 min = 1.66 ± 0.08, 30 min = 1.84 ± 0.11)**. The maximum endocytosis of myc-mGluR1 was observed at 30 min after ligand exposure.

Similar experiments were performed in the N2A cells to determine whether ligand-dependent internalization of myc-mGluR1 shows similar kinetics. myc-mGluR1 cDNA was transfected in undifferentiated N2A cells using the procedure described before. Subsequently, cells were differentiated for 48 hr and internalization experiments were performed using the similar protocol as described for HEK293 cells. Similar to in HEK293 cells, the myc-mGluR1 endocytosis was also rapid in the N2A cells. As expected, in control cells majority of the receptors were observed to be present at the cell surface **(control = 1 ± 0.03) (Figure 3.4 A, B)**. Significant amount of myc-mGluR1 endocytosis was noticed after 5 min of DHPG pulse as observed by the increase in the internal fluorescence and decrease in the surface fluorescence **(5 min = 1.33 ± 0.05)**. The extent of the endocytosis increased gradually as cells were incubated for longer period and the maximum endocytosis was observed in

(A)



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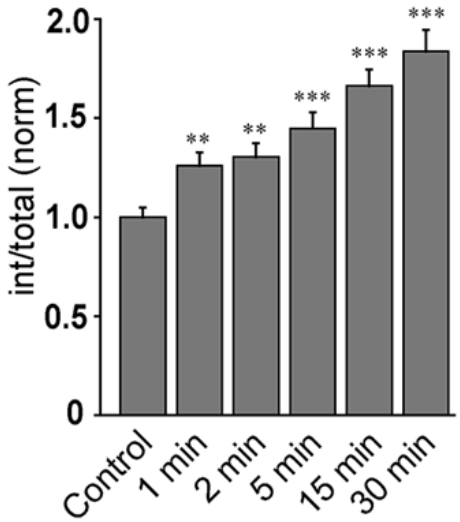


Figure 3.3. Kinetics of DHPG-mediated myc-mGluR1 internalization in HEK293 cells (A) Control cells showed very little internal fluorescence and 1 min after 100 μM DHPG application receptors started getting internalized. The extent of endocytosis increased over subsequent time points (2 min, 5 min, and 15 min) and in 30 min maximum endocytosis was observed. (B) Quantitation of endocytosis also showed gradual increase in myc-mGluR1 endocytosis and at 30 min endocytosis was maximum. Scale bar = 10 μm. **p < 0.01, ***p < 0.001 . N=3; where N represents the number of times experiment was repeated.

30 min **(15 min = 1.51 ± 0.06, 30 min =1.98 ± 0.08)**. Since N2A cells are the compartmentalized cells having cell body and neurites, it is possible that the myc-mGluR1 endocytosis might show differential effect in the cell body and neuritic region of the cell. In order to check for the compartment specific endocytosis of mGluR1, if any, the endocytosis index in the cell body and in the neurites were quantified separately. The results suggest that the myc-mGluR1 endocytosed with similar extent both in the cell body and in the neurites **(soma: control = 1 ± 0.05, 5 min = 1.38 ± 0.07, 15 min = 1.51 ± 0.07, 30 min = 2.13 ± 0.11; neurites: control = 1 ± 0.15, 5 min = 1.17 ± 0.07, 15 min = 1.37 ± 0.07, 30 min = 1.9 ± 0.12)** (**Figur3.4 C, D)**.

Together these results suggest that agonist-induced myc-mGluR1 internalization kinetics is fast in both HEK293 and N2A cells. Furthermore, the kinetics of internalization is similar in both the cell types. Additionally, mGluR1 shows similar endocytosis in both the cell body and the neurites of N2A cells suggesting that on ligand exposure mGluR1 endocytosis happens with similar extent throughout the N2A cells. Since, myc-mGluR1 shows uniform ligand-dependent endocytosis throughout N2A cells, from now onwards in case of N2A cells we will represent the quantitation of the whole cell endocytosis.

3.2.3. mGluR1 enters the recycling compartment after ligand-dependent internalization

Our earlier experiments suggested that myc-mGluR1 internalized upon agonist stimulation and the kinetics of endocytosis was similar in both HEK293 and N2A cells. Further, we wanted to investigate the post-endocytic fate of myc-mGluR1. GPCRs take different routes once they get endocytosed in the cell. Some of them go to the recycling compartment and recycle back to the cell surface which is believed to be important for the resensitization of those receptors. Many GPCRs go to the lysososomal compartment and get degraded which is the mechanism for the downregulation of those receptors (Ferguson, 2001; Drake et al., 2006). Since mGluR1 is a GPCR and we showed earlier that the receptor internalized upon agonist stimulation, we investigated whether the receptor takes the recycling route subsequent to the ligand-dependent internalization or it undergoes down-regulation. In order to check whether the receptor enters th e recycling compartment subsequent to the ligand-mediated internalization, we studied the colocalization of the internalized receptors with

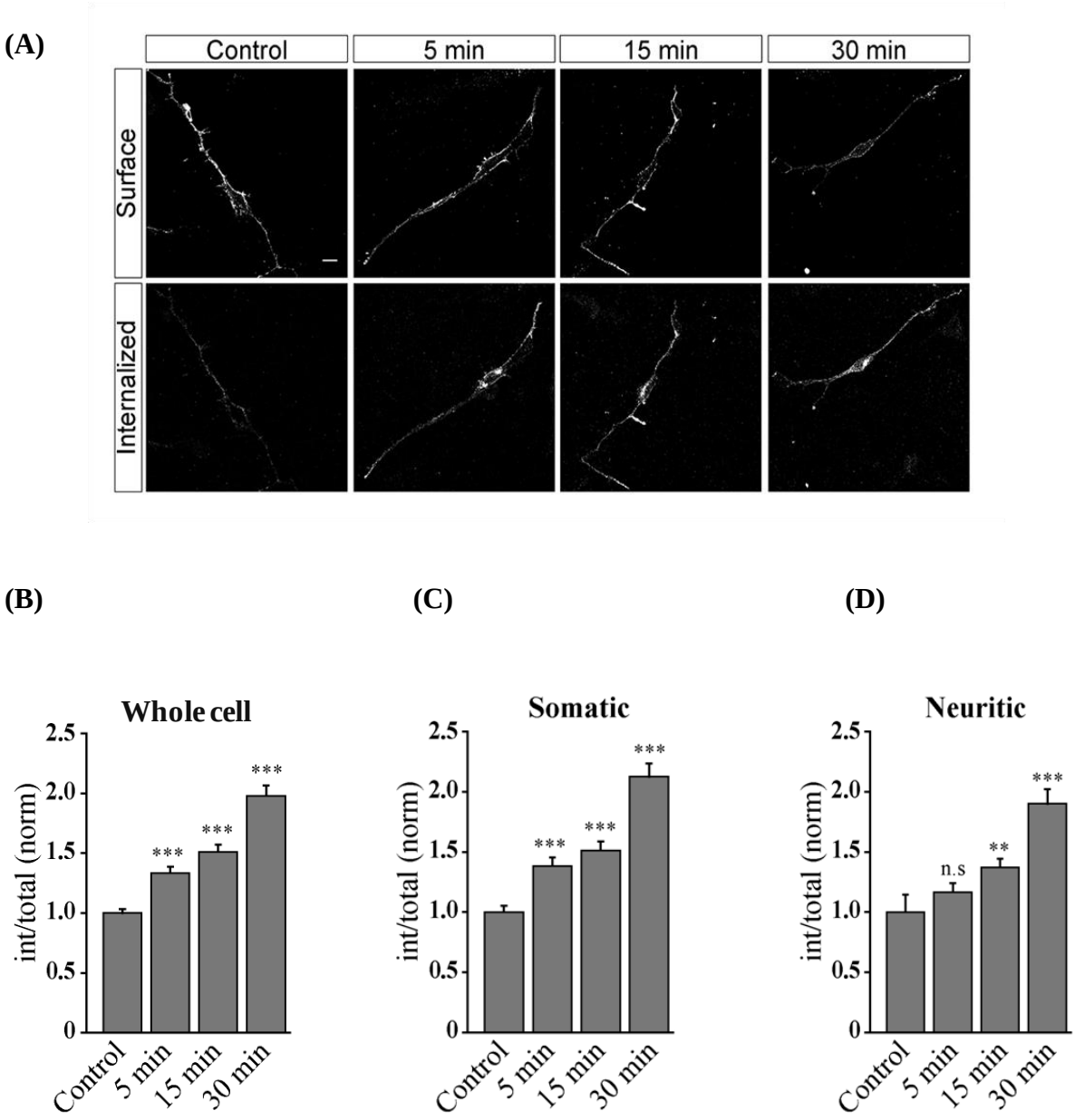
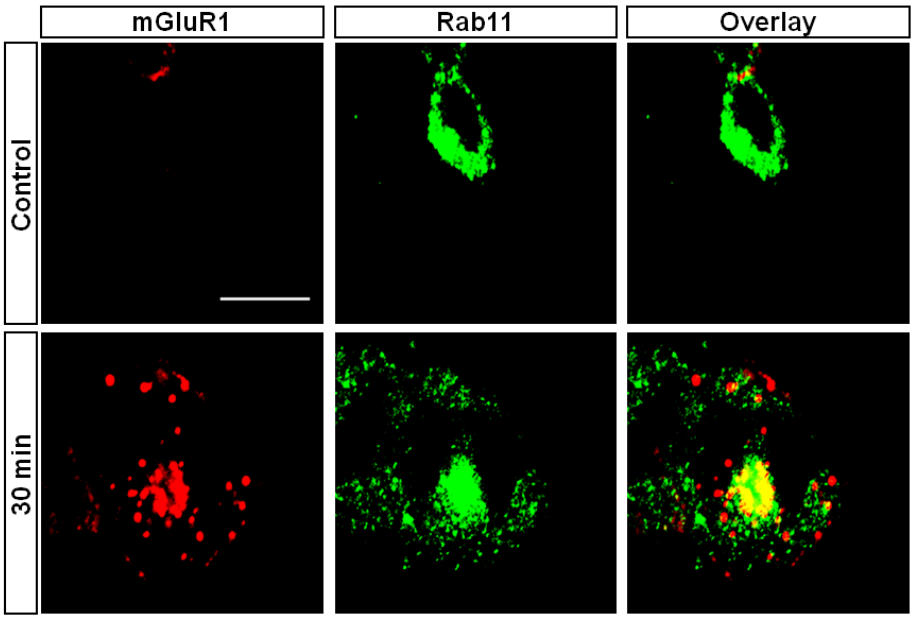


Figure 3.4. Kinetics of DHPG-mediated myc-mGluR1 endocytosis in N2A cells (A) Representative images showing the DHPG-mediated internalization of myc-mGluR1 at different time points. Control cells showed high surface and low internal fluorescence and upon 100 μM DHPG application there was significant endocytosis observed in 5 min. The extent of endocytosis increased further when the chase time extended and in 30 min maximum endocytosis was observed. Quantitation of the internalization in whole cell (B), soma (C) and neurites (D) suggest that the endocytosis of myc-mGluR1 occurs with similar extent throughout the cell. Scale bar = 10 μm. **p < 0.01, ***p < 0.001 and n.s indicates p > 0.05 N=3; where N represents the number of times experiment was repeated.

Rab11, a recycling endosomal marker. Our earlier data suggested that the maximum myc-mGluR1 endocytosis occurred in 30 min post ligand application. Therefore; we chose 30 min time point for the colocalization studies. HEK293 cells were transfected with myc-mGluR1 cDNA and incubated for 24 hr post-transfection to express the protein. Subsequently, endocytosis assay was performed according to the method described before. Briefly, anti-myc rabbit polyclonal primary antibody staining was done in live cells followed by application of 100 µM DHPG for 5 min and subsequent chase for 25 min more in absence of the ligand. Before fixation of the cells with 4% PFA, cells were treated with acid stripping solution (0.5 M NaCl + 0.2 N CH_3COOH) which stripped off the antibodies attached to the remaining surface receptors that did not internalize upon DHPG stimulation. Subsequently, cells were fixed with 4% PFA, followed by permeabilization with 0.1% Trition X-100. Cells were then incubated with mouse anti-Rab11 primary antibody for 4 hr at 37˚C. Subsequently, goat anti-rabbit Alexa-568 and goat anti-mouse Alexa-488 secondary antibodies were applied for 1 hr at 37˚C for myc primary antibody and Rab11 primary antibody respectively. Cells were then mounted on glass slides and observed under the confocal microscope. In control cells very little internalized receptors were observed. Importantly, DHPG application led to the internalization of myc-mGluR1 and majority of the endocytosed receptors colocalized with Rab11 **(control = 1 ± 0.02, 30 min = 3.85 ± 0.05) (Figure 3.5 A, B)**.

In order to investigate whether the receptor also enters the recycling compartment subsequent to the ligand-dependent internalization in N2A cells, similar protocol was used to measure the extent of colocalization of internalized myc-mGluR1 with the recycling compartment marker Rab11, as has been explained for the HEK293 cells. Briefly, myc-mGluR1 was transfected in N2A cells followed by differentiation for 48 hr. Subsequently, endocytosis of the receptor was induced after the anti-myc primary antibody staining. 30 min post ligand application, acid stripping protocol was performed to remove the antibodies bound to the surface receptors that did not internalize on ligand application and then cells were fixed with PFA. Cells were then permeabilized and stained with anti-Rab11 primary antibody followed by staining with secondary antibodies viz., goat anti-rabbit Alexa-568 and goat anti-mouse Alex-488 against anti-myc and anti-Rab11 primary antibodies respectively. **Figure 3.6 A** is showing neuritic area of the representative cells from differentiated N2A cells. The control cells showed very little internalized receptors in the neurites of N2A cells. On the other hand,

(A)



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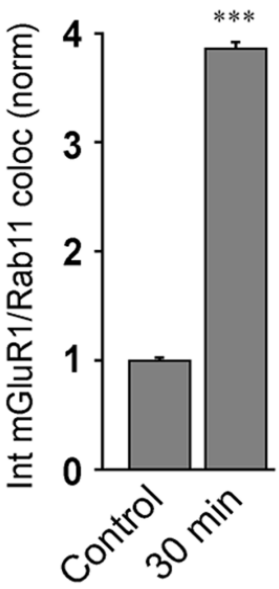
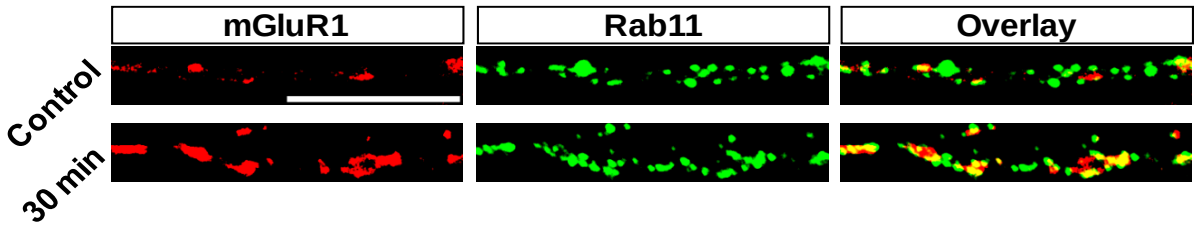


Figure 3.5. myc-mGluR1 enters the recycling compartment subsequent to the ligand-dependent internalization in HEK293 Cells (A) Control cells showed very low internal fluorescence. 30 min after the application of 100 µM DHPG (5 min pulse and 25 min chase) majority of the internalized receptors were observed to colocalize with the recycling endosome marker, Rab11. (B) Quantitation of the colocaliztion of internalized receptors with Rab11 also suggested that majority of the internalized receptors entered the Rab11 positive endosomes. Scale bar = 10 µm. ***$p < 0.001$ N=3; where N represents the number of times experiment was repeated.

(A)



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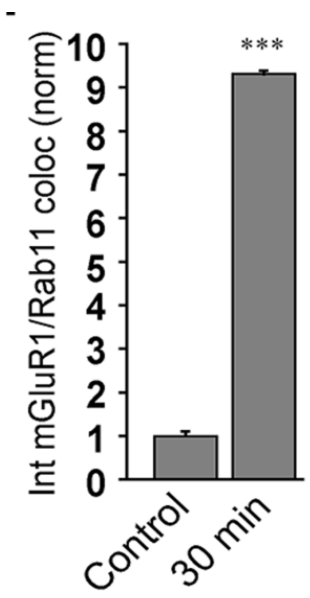


Figure 3.6: Localization of internalized myc-mGluR1 in the recycling compartment in N2A cells (A) Representative images showing colocalization of endocytosed myc-mGluR1 with the recycling endosome marker Rab11 in the neurites of N2A cells. Control cells show very little internal fluorescence. On the other hand 100 µM DHPG induced internalized myc-mGluR1 puncta were observed to colocalize with Rab11 at 30 min. (B) Quantitation of colocalization of the internalized myc-mGluR1 with Rab11 also suggest that compared to control cells there was significant increase in the colocalization of internalized myc-mGluR1 puncta with Rab11. Scale bar = 10 µm. ***$p < 0.001$. N=3; where N represents the number of times experiment was repeated.

in DHPG treated cells majority of the internalized myc–mGluR1 were colocalized with Rab11 **(control = 1 ± 0.01, 30 min = 9.30 ± 0.06) (Figure 3.6 A, B)**.

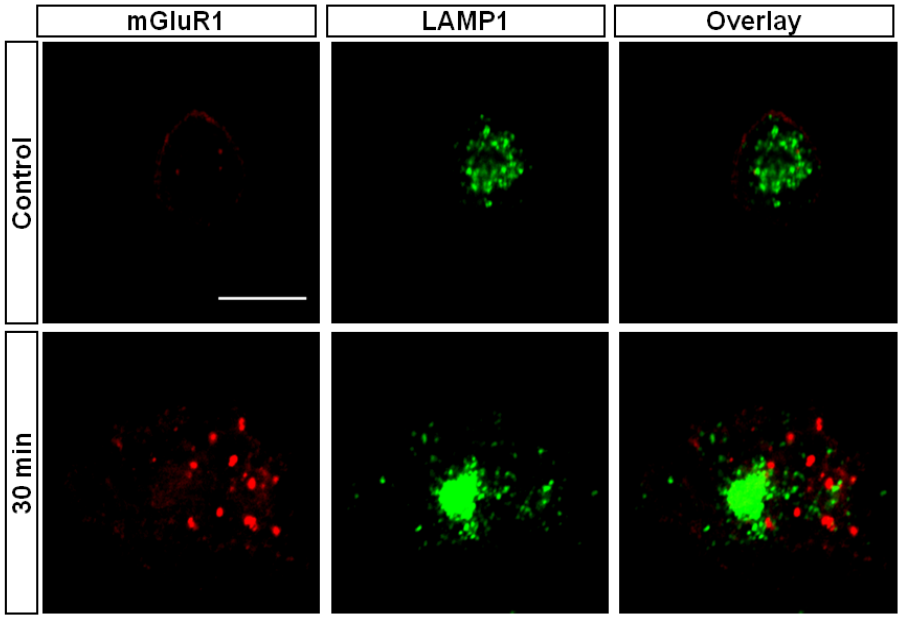
These results together suggest that subsequent to the ligand-dependent internalization myc-mGluR1 enters the recycling compartment in both non-neuronal HEK293 and neuronal N2A cells.

3.2.4. mGluR1 does not enter the lysosomal compartment after ligand-dependent internalization

As discussed earlier that upon agonist-induced internalization some GPCRs go to the recycling compartment and many GPCRs enter the lysosomal compartment for degradation. Our earlier data suggested that majority of the internalized myc-mGluR1 colocalized with Rab11 in both HEK293 and N2A cells, suggesting that myc-mGluR1 enters the recycling compartment subsequent to the ligand-dependent internalization. In order to check whether any population of internalized myc-mGluR1 enters the lysosomal compartment following ligand-induced internalization, we performed colocalization experiments using the lysosomal marker LAMP1 in both HEK293 and N2A cells. HEK293 cells were transfected with myc-mGluR1. Subsequently, anti-myc primary antibody staining was done in myc-mGluR1 expressing live HEK293 cells. Endocytosis of the receptor was induced by the application of 100 µM DHPG using the method described above. At 30 min post-ligand application acid stripping solution was applied to remove the antibody attached to the surface receptors that did not internalize on ligand application, using the similar protocol as described earlier for the Rab11 colocalization experiments. Subsequently, cells were permeabilized by using 0.1% Triton X-100. After that, cells were treated with rabbit anti-LAMP1 primary antibody and subsequently respective secondary antibodies against anti-myc primary and anti-LAMP1 primary antibodies were applied to the cells. Control cells showed very little internalized receptors. On the other hand, cells treated with DHPG showed internalized myc-mGluR1 which did not show significant colocalization with LAMP1, suggesting that no significant population of internalized myc-mGluR1 enters lysosomal compartment subsequent to the ligand-dependent endocytosis. **(control = 1 ± 0.05, 30 min = 1.05 ± 0.04)** (**Figure 3.7 A, B)**.

We also investigated whether any fraction of endocytosed myc-mGluR1 enters the lysosome in N2A cells by performing identical assay as has been described for the HEK293 cells.

(A)



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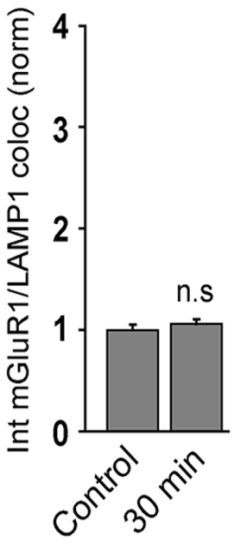


Figure 3.7. myc-mGluR1 does not enter the lysosomal compartment subsequent to the ligand-induced internalization in HEK293 cells (A) Control cells showed very little internalized myc-mGluR1. Application of 100 µM DHPG led to the endocytosis of myc-mGluR1 but the endocytosed receptors did not show significant colocalization with the lysosomal marker, LAMP1, suggesting that DHPG-induced internalized myc-mGluR1 did not enter the lysosomal compartment. (B) Quantitation of the colocalization of internalized myc-mGluR1 with lysosomal marker, LAMP1 before and after DHPG application. Scale bar = 10 µm. n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

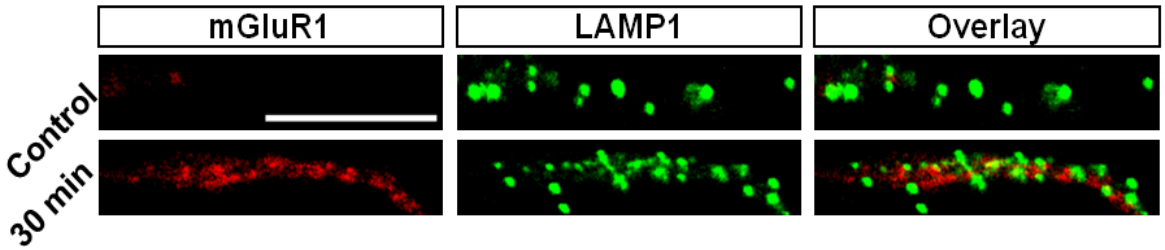
Briefly, myc-mGluR1 cDNA was transfected in undifferentiated N2A cells followed by differentiation for 48 hr. Subsequently, live cells were labelled with anti-myc primary antibody and endocytosis assay was performed. Cells were then surface stripped, fixed and permeabilized. Subsequently, goat anti-mouse Alexa-568 secondary antibodiy against myc primary antibody and goat anti-rabbit Alexa-488 secondary antibody against anti-LAMP1 primary antibody staining was done. Cells were then mounted on glass slides and imaged under the confocal microscope. Similar to in HEK293 cells, the internalized myc-mGluR1 did not manifest any significant colocalization with LAMP1. **(control = 1 ± 0.05, 30 min = 1.02 ± 0.02)** (**Figure 3.8 A, B)**.

Together these results suggest that subsequent to the ligand-dependent internalization majority of myc-mGluR1 enters the recycling compartment and no significant population of the endocytosed receptor enters the lysosomal compartment in both non-neuronal and neuronal cells.

3.2.5: mGluR1 recycles back to the cell surface after ligand-mediated internalization in non-neuronal and neuronal cells

Our previous experiments have suggested that upon agonist stimulation mGluR1 gets internalized and enters the recycling compartment in both HEK293 and N2A cells. In order to investigate whether the myc-mGluR1 recycles back to the cell surface after ligand-mediated internalization, cells were chased for longer time periods (1 hr, 1.5 hr, 2 hr, 2.5 hr) at 37˚C subsequent to endocytosis. In HEK293 cells, majority of the receptors were observed to be present on the cell surface and very little internal fluorescence was observed in control cells **(control = 1 ± 0.09) (Figure 3.9 A, B)**. Upon application of 100 µM DHPG majority of the receptors internalized at 30 min time point **(30 min = 2 ± 0.06)**. When cells were chased for longer time period, there was a decrease in the internal fluorescence and an increase in the surface fluorescence over time was observed. In other words, 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 = 1.72 ± 0.10, 1.5 hr = 1.64 ± 0.09, 2 hr = 1.31 ± 0.08, 2.5 hr = 1.17 ± 0.10)**. Measurement of surface fluorescence alone also showed that the surface expression of the receptor decreased at 30 min subsequent to DHPG application and when cells were chased for longer time period in the absence of DHPG the surface fluorescence recovered at the control level at 2.5 hr time point **(control = 1 ± 0.11, 30 min = 0.64 ± 0.09,**

(A)



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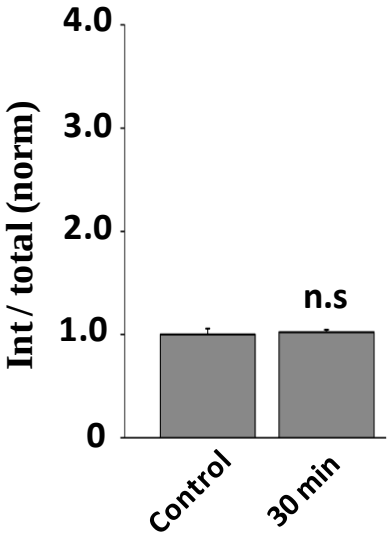
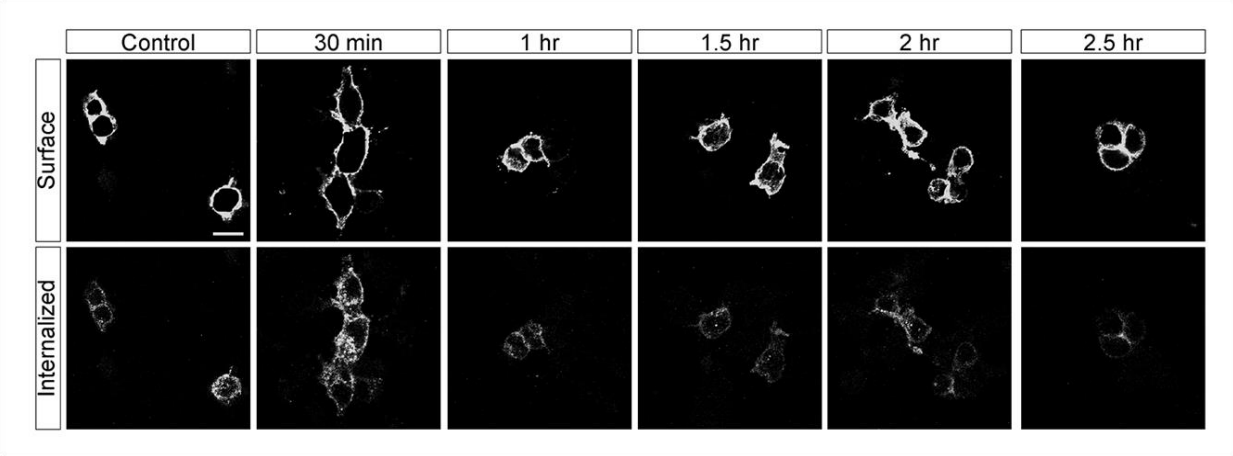


Figure 3.8. Ligand-induced endocytosed myc-mGluR1 does not enter lysosomal compartmet in N2A cells (A) Representative images showing that 100 µM DHPG-induced internalized myc-mGluR1 did not colocalize with the lysosomal marker, LAMP1 in the neurites of N2A cells. As expected, control cells showed very low amount of internalized receptors. (B) Quantitation also suggested that there was no significant colocalization of endocytosed myc-mGluR1 with LAMP1 suggesting that majority of myc-mGluR1 upon intenalization did not go to the degradative pathway. Scale bar = 10 µm. n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

(A)



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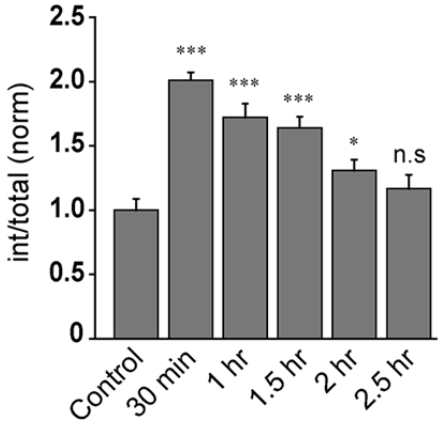


(C)

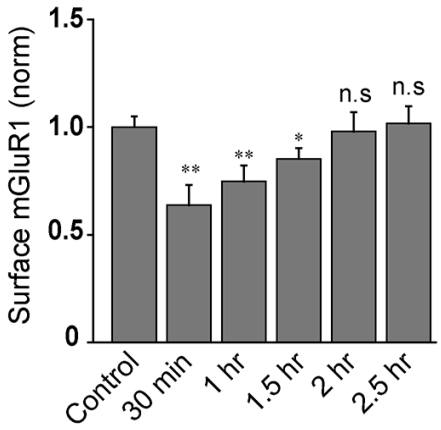


Figure 3.9. DHPG-mediated internalized myc-mGluR1 recycles back to the cell surface in HEK293 cells (A) Control cells showed majority of the receptors at the cell surface and very low internal fluorescence was observed. On application of 100 µM DHPG receptors internalized as observed at 30 min time point. Upon incubation for longer time period (1 hr, 1.5 hr, 2 hr and 2.5 hr) in the absence of the ligand the internalized receptors gradually recycled back to the cell surface and in 2.5 hr majority of the internalized receptors recycled back to the surface as observed by the increase in the surface fluorescence and decrease in the internal fluorescence. (B) Quantitation of endocytosis index at various time points suggested that the recycling of DHPG-induced internalized myc-mGluR1 started at 1 hr and most of the receptor recycled back to the cell surface in 2.5 hr. (C) Surface receptor quantitation also showed decrease in the surface fluorescence at 30 min and recovery of the surface fluorescence at 2.5 hr suggesting that the receptors recycled back to the cell surface in 2.5 hr. Scale bar = 10 µm.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

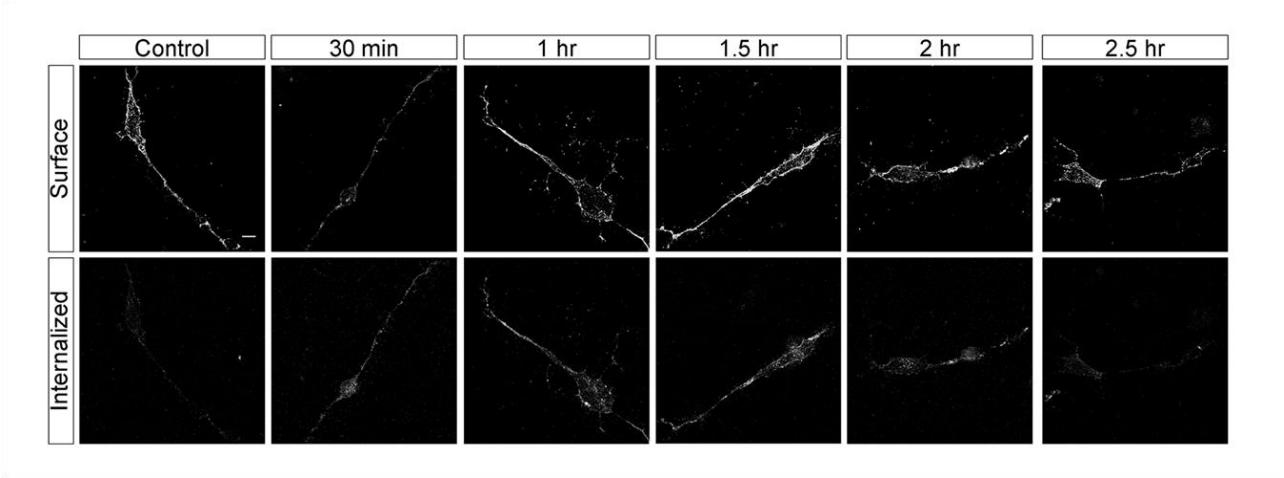
1 hr = 0.74 ± 0.7, 1.5 hr = 0.85 ±_0.14, 2 hr = 0.98 ±_0.09, 2.5 hr = 1 ± 0.14) (Figure 3.9 C).

It is important to mention that the protocol for our recycling assay does not allow us to observe the receptors that have been synthesized after the primary antibody staining. Since, we labelled the receptors with the primary antibody when they were at the surface, this recovery of surface fluorescence is due to the recycling of the same population of receptors that internalized on application of DHPG.

In order to check whether myc-mGluR1 recycles to the cell surface subsequent to the ligand-mediated internalization in neuronal N2A cells, the recycling experiments were performed in N2A cells as well. The recycling experiments in differentiated N2A cells were conducted using the same protocol that had been used in HEK293 cells. In N2A cells also the receptor internalized at 30 min post-DHPG application **(control = 1 ± 0.05, 30 min = 1.9 ± 0.11) (Figure 3.10 A, B)**. When the receptors were chased for longer time they recycled back to the cell surface as observed by the decrease in the intracellular fluorescence and corresponding increase in the surface fluorescence. At 2.5 hr most of the endocytosed myc-mGluR1 recycled back to the cell surface **(1 hr = 1.69 ± 0.09, 1.5 hr = 1.54 ± 0.06, 2 hr = 1.31 ± 0.08, 2.5 hr = 1.13 ± 0.06)**. Measurement of surface myc-mGluR1 in differentiated N2A cells also suggested that upon DHPG application the number of surface myc-mGluR1 decreased at 30 min and subsequent to the chase in the absence of the ligand the internalized receptors came back to the cell surface at 2.5 hr as observed by the complete recovery of the surface fluorescence **(control = 1 ±_0.08, 30 min = 0.45 ±_0.06, 1 hr = 0.59 ±_0.06, 1.5 hr = 0.72 ± 0.07, 2 hr = 0.9 ±_0.07, 2.5 hr = 1 ± 0.07) (Figure 3.10 C)**.

These experiments together suggest that myc-mGluR1 recycles back to the cell surface after ligand-dependent internalization with similar kinetics in both non-neuronal HEK293 and neuronal N2A cells.

(A)



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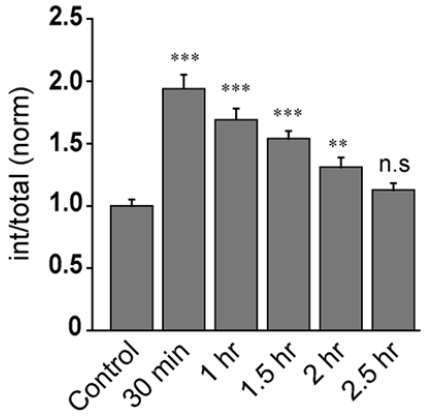


(C)

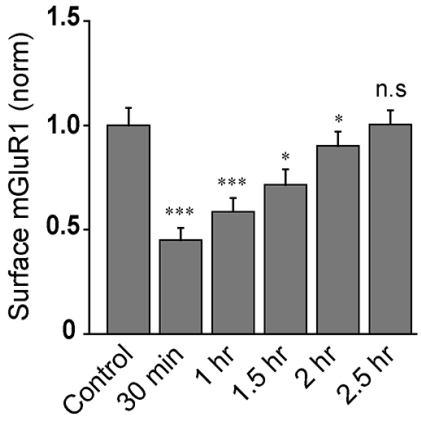


Figure 3.10. DHPG-mediated internalized myc-mGluR1 recycles back to the cell surface in N2A cells (A) Representative images showing the recycling of 100 µM DHPG-mediated internalized myc-mGluR1 in N2A cells. Receptors were seen to internalize in 30 min post DHPG treatment. When cells were chased for longer time (1 hr, 1.5 hr, 2 hr and 2.5 hr) in the absence of the ligand there was gradual decrease in the internal fluorescence and corresponding increase in the surface fluorescence observed and in 2.5 hr majority of the internalized receptors recycled back to the surface. (B) Quantitation of the internalized myc-mGluR1 at different time points also suggested that at 2.5 hr majority of the DHPG-mediated internalized myc-mGluR1 recycled back to the cell surface in N2A cells. (C) Surface quantitation showed that on application of 100 µM DHPG there was a decrease in the surface fluorescence which gradually recovered when cells were chased for longer time in absence of the ligand and majority of the internalized receptors came back to the surface in 2.5 hr. Scale bar = 10 µm.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

3.2.6. Internalization and recycling of mGluR1 in primary hippocampal neurons

Previous experiments suggested that upon agonist stimulation myc-mGluR1 gets internalized in both non-neuronal HEK293 and neuronal N2A cells. The kinetics of internalization was observed to be similar in both the cell lines. The endocytosed receptors go to the recycling compartment and in 2.5 hr majority of the receptors recycle back to the cell surface with similar kinetics in both HEK293 and N2A cells. We next investigated whether similar trafficking events occur in the native environment of the receptor by extending our investigations in primary hippocampal neurons. Primary hippocampal neurons are the most experimentally tractable *in vitro* system that can approximate the *in vivo* situation. The protocol for the preparation of primary hippocampal neurons has been discussed elaborately in the "materials and method" chapter. myc–mGluR1 was transfected using calcium phosphate method on 5-7 DIV as discussed in the "method" section. On 12- 15 DIV neurons were washed with plain neurobasal medium and then anti-myc primary antibody staining was done in live cells for 30 min. Afterwards 100 µM DHPG pulse was given for 5 min followed by chase for 30 min and 2.5 hr. After the respective time periods, cells were fixed with 4% PFA without permeabilization and then first secondary antibody (Alexa-568 tagged) was applied in saturating concentration. Subsequently, permeabilization of the cells with 0.1% Triton X-100 and second secondary antibody (Alexa-647 tagged) staining was done to visualize the internalized receptors. Cells were then mounted on glass slides and observed under the confocal microscope. In control cells majority or the receptors were localized on the cell surface and there were very low internalized receptors observed **(control = 1 ± 0.0**4) (**Figure 3.11 A-D)**. 30 min after 100 µM DHPG application majority of the receptors internalized and amount of surface receptors decreased as compared to control cells **(30 min = 2.00 ± 0.05)**. When cells were chased for longer time period in the absence of the ligand, majority of the receptors recycled back to the cell surface in 2.5 hr as observed by an increase in the surface fluorescence and decrease in the internal fluorescence (**2.5 hr = 1 ± 0.05)**.

The above experiment suggests that myc-mGluR1 upon agonist stimulation internalized in primary hippocampal neurons. Furthermore, the internalized receptors recycled back to the surface in 2.5 hr similar to in HEK293 cells and N2A cells. Since neurons are compartmentalized cells we quantified the endocytosis index in both cell body as well as in dendrites, in order to check whether there is any difference in the extent of internalization in different compartments of the neuron. Our data suggest that myc-mGluR1 endocytosis was

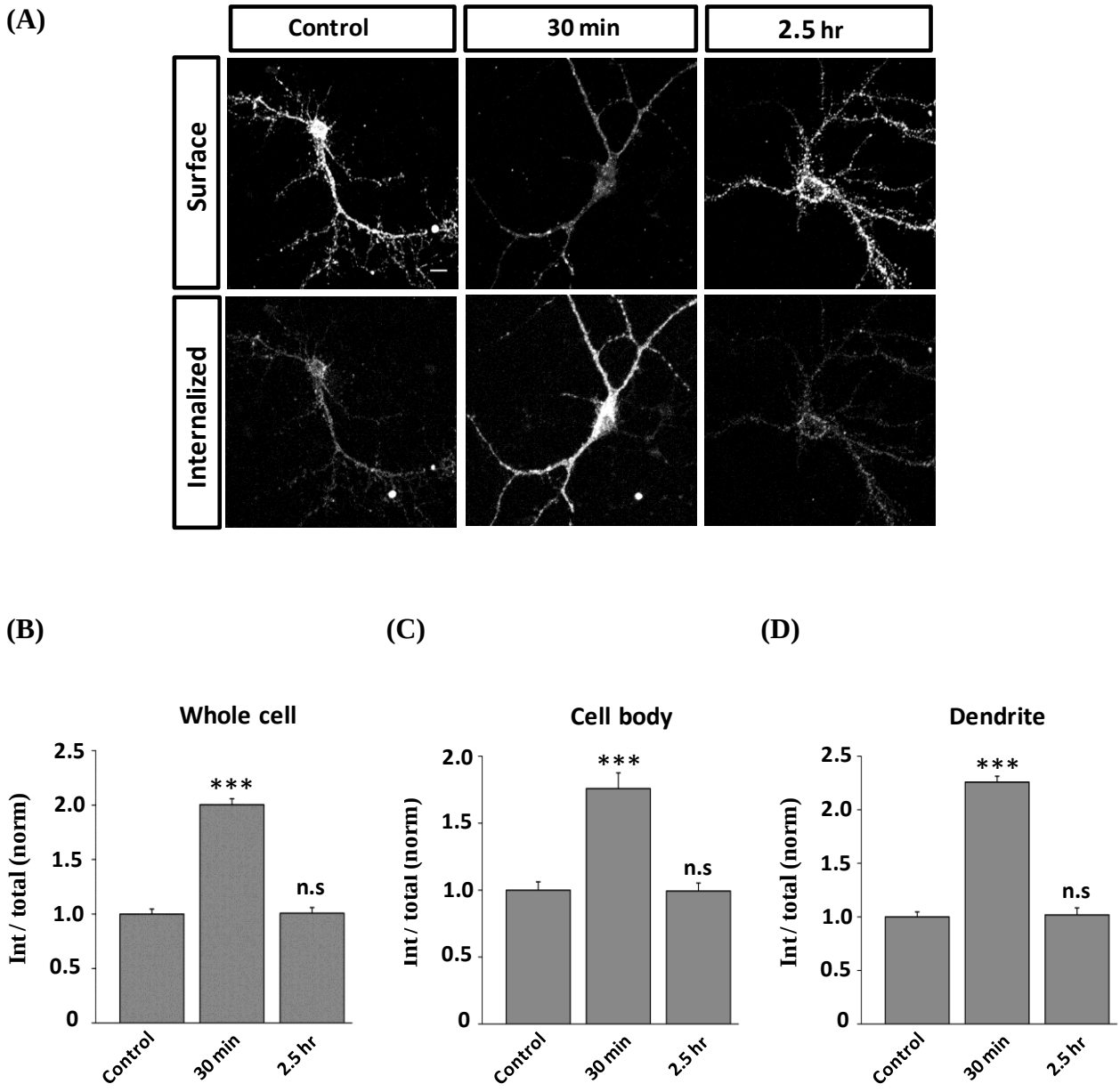


Figure 3.11. Internalization and recycling of myc-mGluR1 in primary hippocampal neurons (A) Representative images showing 100 µM DHPG-mediated trafficking of myc-mGluR1in primary hippocampal neurons. Control cells showed presence of majority of the receptors at the cell surface. Upon DHPG application there was an increase in the internal fluorescence observed at 30 min, suggesting the myc-mGluR1 internalized on ligand application. Incubation of the cells for longer period in the absence of the ligand led to the recycling of the receptors back to the cell surface in 2.5 Hr. (B) (C) (D) Quantitation of the endocytosis index in whole cell, cell body and dendrites respectively, also suggested that myc-mGluR1 recycled back to the cell surface subsequent to the ligand-dependent in internalization at 2.5 hr in primary hippocampal neurons. Scale bar = 10 µm. ***$p < 0.001$ and n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

uniform throughout the neuron. Furthermore, the internalized receptors recycled back to the surface with similar kinetics in both cell body and in the dendrites **(Cell body: control = 1 ± 0.06, 30 min = 1.75 ± 0.11; 2.5 hr = 0.99 ± 0.05; Dendrites: control = 1 ± 0.04, 30 min = 2.25 ± 0.05, 2.5 hr = 1.01 ± 0.06) (Figure 3.11 C, D)**. Since, no significant compartment specific difference in the trafficking of myc-mGluR1 was observed in our assays, in the subsequent chapters we have represented the dendritic internalization in case of experiments related to primary hippocampal neurons.

All these experiments suggest that myc-mGluR1 internalized with similar kinetics and recycled back to the cell surface following endocytosis in similar time in non-neuronal cells, neuronal cells and primary hippocampal neurons.

3.3. Discussion

G protein coupled receptors are the key molecules for intracellular signal transduction and maintaining the cellular homeostasis. The GPCR signal transduction depends on the availability of the receptors at the proper location in the given time frame, their mode of activation and the system. Desensitization and resensitization of GPCRs play a very important role in their regulation and signaling. For accurate signaling, it is necessary that GPCRs should be present at the proper location in both spatial and temporal manner. It has been reported that various GPCRs upon agonist stimulation get desensitized and the process of desensitization acts as a negative feedback regulation which prevents cells from overstimulation. The regulation of GPCRs on the cell surface is regulated by the trafficking machinery of the cell and any alteration in the machinery may cause disease scenario. The mGluR1 is known to get desensitized upon agonist exposure in various cell types. Upon desensitization mGluR1 internalizes in the clathrin-dependent manner (Mundell et al., 2004). The post-endocytic fate of these receptors has not been studied so far.

To study the post-endocytic fate of mGluR1, we used myc-tagged mGluR1, where myc epitope was tagged at the N-terminus of the mouse full length mGluR1. Previous reports have shown that this recombinant gene was behaving like the native receptor. Upon transfection, myc-mGluR1 was targeted properly and inserted into the membrane of various cell types and agonist application causes a rise in the intracellular calcium levels, which suggested that myc-mGluR1 is active like native receptor (Choi et al., 2011). We investigated the kinetics of internalization of myc-mGluR1 upon its interaction with ligand in non-neuronal HEK293 cells, neuronal N2A cells and primary hippocampal neurons. The myc-mGluR1 endocytosis was rapid and in 30 min maximum endocytosis of these receptors was observed in all these cell types. These results suggest that mGluR1 internalization kinetics is similar in various heterologous cell lines as well as in primary hippocampal neurons. The N2A cells and hippocampal neurons are the compartmentalized cells and it has been reported that some processes are compartment specific in neurons. Our data suggest that the endocytosis kinetics was similar in both the cell body and the dendrites of neurons. mGluR1 expresses differentially in the cell body and dendrites of the neurons and it would be interesting to study that although there is a difference between the architecture and molecules present in the cell body and the dendrites, how mGluR1 maintains similar level of endocytosis in cell body and dendrites.

A number of GPCRs manifest various sub-cellular fates upon internalization. In the case of mGluR1, we found that internalized receptors entered the recycling compartment and there was no lysosomal localization observed. Furthermore, in 2.5 hr, majority of the internalized receptors recycled back to the cell surface in HEK293 cells, N2A cells and primary hippocampal neurons. Many GPCRs has been shown to recycle back to the surface upon internalization and the process of recycling has been suggested to be critical for resensitization of those receptors. Thus, our results suggest that the endocytosis of mGluR1 is probably a way to resensitize those receptors.

Role of protein phosphatases in the recycling of mGluR1 in non-neuronal and neuronal cells

4.1. Introduction

A number of GPCRs are known to get internalized upon agonist stimulation and they recycle back to the cell surface subsequent to the internalization. The recycling of these receptors has been suggested to be necessary for the resensitization of the receptors (Sibley and Lefkowitz, 1985). Upon activation, GPCRs initiate second messenger pathways through the activation of G proteins. This in turn activates a number second messenger dependent kinases, G protein coupled receptor kinases (GRKs) and enzymes related to the ubiquitination process (Ferguson, 2001). As stated before, many GPCRs subsequent to the activation of the second messenger pathways activate various kinases which in turn phosphorylate the receptor at specific residues. These phosphorylations lead to the binding of adapter proteins at the site which overlaps with the G protein binding site (Ferguson, 2001). This in turn prevents further coupling of G proteins with the receptor and as a result the receptor becomes desensitized or unresponsive towards further agonist stimulation. Subsequent to desensitization many GPCRs internalize and follow the recycling pathway (Pippig et al., 1995). It has been reported that recycling of many GPCRs depend on the pH of the recycling endosome (Mellman et al., 1986). It has also been postulated that during the recycling process, certain protein phosphatases gets activated in the recycling compartment due to the low pH environment of the compartment and they dephosphorylate the same residues that were phosphorylated during the desensitization process (Cohen, 1989). This process resensitizes the receptor, i.e., converts the desensitized receptor into native receptor and when the resensitized receptor recycles back to the cell surface they are ready to start a new round of signaling.

Various second messenger dependent protein kinases and GRKs have been implicated in the desensitization of mGluR1(Mundell et al., 2003). For example, phosphorylation of mGluR1 by protein kinase C (PKC) leads to the desensitization of the receptor (Dale et al., 2002). Interestingly, PKC seems to affect the mGluR1 coupling with Gq, but does not affect the receptor coupling to the cAMP pathway (Mundell et al., 2003). In addition, G protein coupled receptor kinases (GRKs) also seem to play crucial roles in the desensitization of mGluR1 (Mundell et al., 2003). As stated before, GRK-mediated phosphorylation of specific residues of many GPCRs result in the binding of adapter proteins which uncouple the receptor from the G proteins involved. Various studies have suggested that GRKs regulate mGluR1 desensitization when heterologously expressed in HEK293 cells as well as in primary neurons. For example, GRK2 has been demonstrated to be involved in the desensitization of mGluR1 (Dale et al., 2002). The internalization of mGluR1 is also phosphorylation

dependent and both GRKs and second messenger-dependent kinases have been implicated in this process. For example, GRK4 has been found to be involved in the internalization of mGluR1 and acute knockdown of GRK4 in Cerebellar Purkinje cells inhibits the ligand-mediated internalization of mGluR1 (Dhami and Ferguson, 2006). GRK2 on the other hand also modulates internalization of mGluR1 but the effect of GRK4 on mGluR1 trafficking is kinase-dependent whereas, the GRK2-mediated trafficking of mGluR1 is kinase-independent (Dale et al., 2000; Mundell et al., 2003). Various second messengers also control the endocytosis of mGluR1. The internalization of mGluR1 has been reported to be PKC-dependent (Mundell et al., 2003). Interestingly, the region of the receptor that is involved in the GRK-mediated internalization of the receptor is distinct from the region that is involved in the PKC-dependent internalization (Mundell et al., 2003). Our earlier data suggested that upon internalization, mGluR1 recycled back to the cell surface. Since, it has been reported that recycling of some GPCRs is dependent on the pH of the intracellular compartments; we investigated the effect of pH on the recycling of mGluR1. We also studied the role of endosome localized phosphatases whose activity is dependent on pH in the recycling of mGluR1. Our data suggest that dissipation of the pH of the endosome with NH_4Cl led to the inhibition of the recycling of mGluR1 and the receptors were stuck in the recycling compartment in both non-neuronal and neuronal cells. In addition, our data also suggest that protein phosphatase 2A (PP2A) plays a crucial role in the recycling of mGluR1 in both non-neuronal HEK293 cells and neuronal N2A cells. Other protein phosphatases like protein phosphatase 1 (PP1) and protein phosphatase 2B (PP2B) did not have any effect on the recycling of mGluR1.

4.2. Results

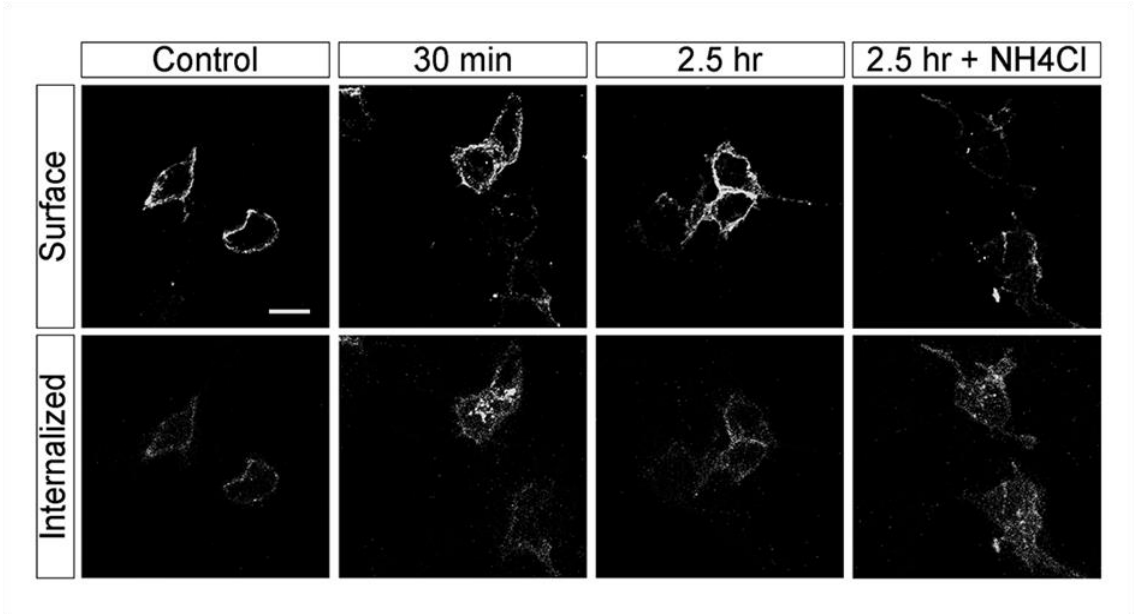
4.2.1. Ammonium chloride inhibits mGluR1 recycling

Our earlier data suggested that upon agonist stimulation, myc-mGluR1 internalized and the kinetics of endocytosis was similar in non-neuronal and neuronal cells. Subsequent to internalization, majority of the receptors entered the recycling compartment and in 2.5 hr the internalized receptors recycled back to the cell surface in both the cell types. Since, the recycling of some GPCRs has been reported to depend on the pH of the recycling compartment; we investigated whether the recycling of mGluR1 is also dependent on the endosomal pH. We treated HEK293 cells and N2A cells with ammonium chloride (NH_4Cl). NH_4Cl is a weak acidotropic agent and upon application it dissipates the transmembrane pH gradient (Raote et al., 2013).

We initially checked the effect of NH_4Cl on the trafficking of myc-mGluR1 in HEK293 cells. myc-mGluR1 was transfected in HEK293 cells using the method as described before. 2 mM NH_4Cl was applied in myc-mGluR1 expressing cells 30 min prior to the application of the ligand and the NH_4Cl was present throughout the assay. Live cells were then labelled with anti-myc primary antibody followed by the application of 100 µM DHPG. Subsequently fixation of the cells was done without permeabilization with 4% PFA for 15 min on ice. Afterwards the surface and internalized receptors were labelled with secondary antibodies as describe before. In control cells, majority of the receptors were observed to be present at the cell surface and very little internal fluorescence was observed **(control = 1 ± 0.04) (Figure 4.1 A, B)**. The internal fluorescence increased in DHPG treated cells, suggesting that myc-mGluR1 internalized and in 2.5 hr majority of the internalized receptors recycled back to the cell surface **(30 min = 1.7 ± 0.06, 2.5 hr = 1.12 ± 0.06)**. On the other hand, in NH_4Cl treated cells, myc-mGluR1 did not recycle back to the cell surface in 2.5 hr and the fluorescence intensity of the internalized receptors at 2.5 hr was comparable to the 30 min condition **(2.5 hr + NH_4Cl = 1.66 ± 0.06),** suggesting that application of NH_4Cl inhibited the recycling of myc-mGluR1.

The effect of NH_4Cl on the recycling of myc-mGluR1 was also studied in N2A cells. The undifferentiated N2A cells were transfected with myc-mGluR1 cDNA and then

(A)



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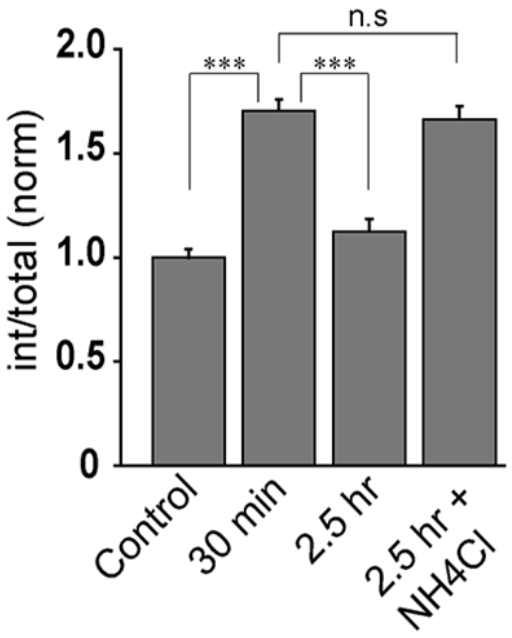


Figure 4.1. Ammonium chloride inhibits the recycling of myc-mGluR1 in HEK293 cells (A) Control cells showed very little internal fluorescence which increased after 100 μM DHPG treatment in 30 min and majority of the internalized receptors recycled back to the cell surface in 2.5 hr. In NH_4Cl treated cells the endocytosed receptors did not recycle back to the cell surface in 2.5 hr. (B) Quantitation also showed that in control cells myc-mGluR1 recycled back to the cell surface in 2.5 hr whereas in NH_4Cl treated cells recycling of myc-mGluR1 was completely blocked. Scale bar= 10 μm. ***$p < 0.001$ and n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

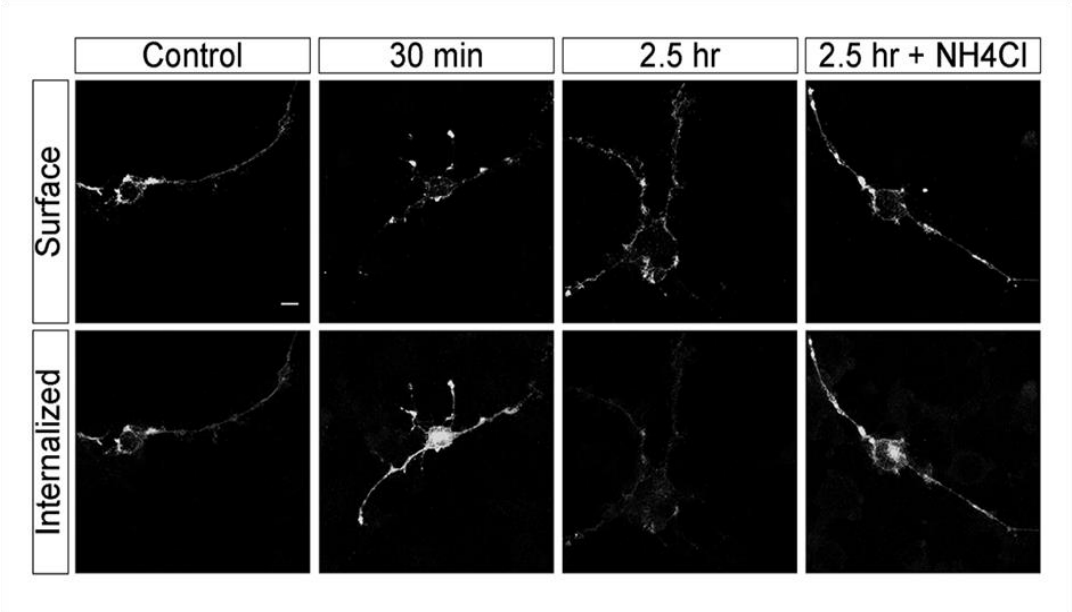
differentiation of the cells was performed using the same protocol as described before. The cells were then pre-incubated with 2 mM NH_4Cl for 30 min before starting the experiment and the NH_4Cl was present throughout the entire experiment. Subsequently, live cells were stained with anti-myc primary antibody and endocytosis assay was performed as described in the methods section. After that, surface and internalized receptors were labelled with secondary antibodies using the method described before. As expected, control cells showed very little internal fluorescence and majority of the receptors were present on the cell surface. Intensity of the internal fluorescence was increased at 30 min post DHPG treatment, suggesting internalization of myc-mGluR1 **(control= 1 ± 0.02, 30 min = 1.69 ± 0.04)** (**Figure 4.2 A, B)**. Majority of the endocytosed receptors recycled back to the cell surface in 2.5 hr in control cells but in NH_4Cl treated cells, receptors were observed to be present inside the cell even at 2.5 hr time point **(2.5 hr = 1.14 ± 0.03, 2.5 hr + NH_4Cl = 1.65 ± 0.03)**. These results suggest that application of NH_4Cl inhibits the recycling of myc-mGluR1 in both HEK293 and N2A cells.

4.2.2. mGluR1 gets trapped in the recycling compartment on NH_4Cl application

Our earlier experiments suggested that the DHPG-mediated endocytosed myc-mGluR1 entered the recycling compartment as observed by the co-localization with the recycling endosome marker Rab11 and there was no significant colocalization observed with the lysosomal marker Lamp1. Our previous experiments also suggested that application of NH_4Cl inhibited the recycling of myc-mGluR1 and the receptors were appeared to be stuck in the endosomal compartment. These observations led us to investigate the identity of the endosomal compartment where internalized myc-mGluR1 got stuck on NH_4Cl application.

Since the ligand-induced internalized receptors enter the recycling compartment, we checked whether in NH_4Cl treated cells the receptors were stuck in the recycling compartment by performing the co-localization experiment using the recycling compartment marker Rab11. The colocalization experiment protocol was similar as described before. Briefly endocytosis assay was performed in myc-mGluR1 expressing HEK293 cells subsequent to the primary antibody staining using the protocol described before. Subsequently, acid stripping was done at respective time points to remove the antibodies attached with the surface receptors that did

(A)



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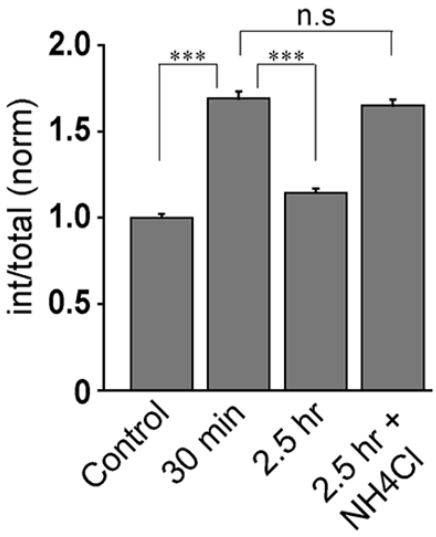


Figure 4.2. Ammonium chloride inhibits the recycling of myc-mGluR1 in N2A cells (A) Representative images showing the effect of NH_4Cl treatment on myc-mGluR1 recycling. In control cells the myc-mGluR1 internalized upon 100 μM DHPG application in 30 min and recycled back to the cell surface in 2.5 hr. On the other hand, in NH_4Cl treated cells the endocytosed receptors did not recycle back to the cell surface in 2.5 hr. (B) Quantitation also showed that in control cells myc-mGluR1 recycled back to the cell surface in 2.5 hr whereas, in NH_4Cl treated cells recycling of myc-mGluR1 was completely blocked. Scale bar= 10 μm. ***$p < 0.001$ and n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

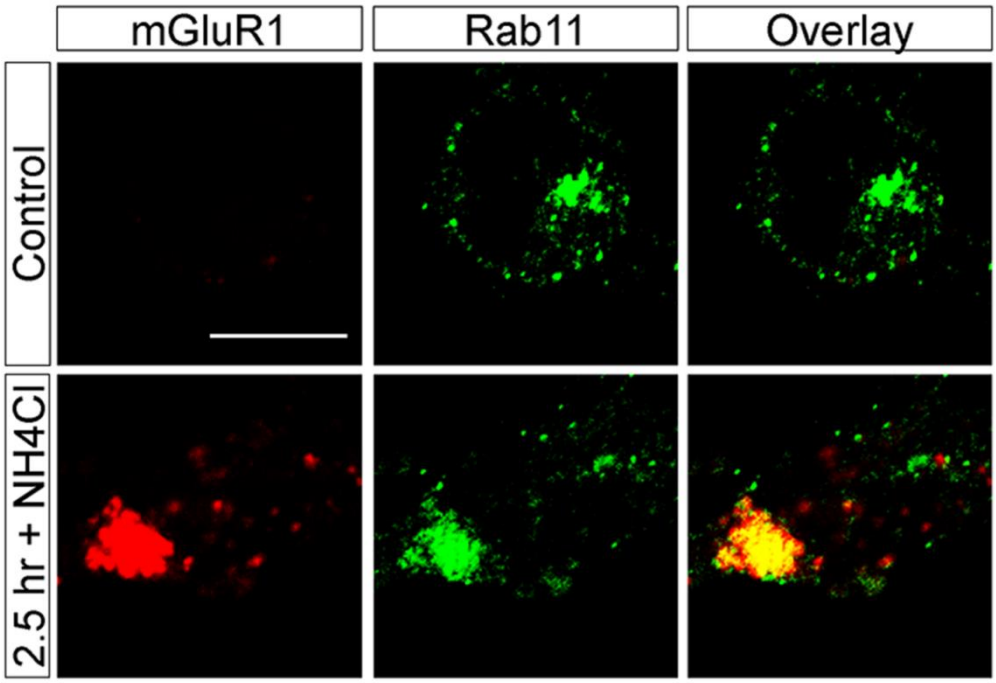
not internalize. Cells were then fixed with 4% PFA and permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Subsequently, primary antibody against Rab11 was applied, followed by the application of secondary antibodies. As expected, in control cells not much internalized receptors were observed, whereas, in NH_4Cl treated cells majority of the receptors were observed to be co-localized with Rab11 at 2.5 hr subsequent to the DHPG-mediated internalization, suggesting that receptors were stuck in the recycling compartment due to the application of NH_4Cl **(Figure 4.3 A)**.

We also investigated the localization of the internalized myc-mGluR1 at 2.5 hr by the co-localization experiments using the recycling endosome marker Rab11 in N2A cells as well. The protocol for the experiment was similar to the one described before. As expected, the control cells showed very low amount of internalized receptors. However, DHPG-mediated internalized receptors were observed to be co-localized with Rab11 at 2.5 hr in NH_4Cl treated cells, suggesting that similar to in HEK293 cells the receptors were stuck in the recycling compartment in N2A cells as well on NH_4Cl treatment (**Figure 4.3 B)**. These experiments suggest that NH_4Cl treatment leads to the inhibition in the recycling of mGluR1 and the receptor gets stuck in the recycling compartment in both non-neuronal HEK293 cells and neuronal N2A cells.

4.2.3. Role of protein phosphatases in the mGluR1 recycling

Our previous experiments suggested that mGluR1 recycling is dependent on the pH of the intracellular compartments and alteration in the pH of these compartments lead to the blocking of the recycling of mGluR1 in both HEK293 and N2A cells. As stated before, it has been reported that mGluR1 endocytosis is phosphorylation dependent. Furthermore, in case of some GPCRs it has been reported that the receptors get dephosphorylated in the recycling compartment and then the receptors recycle back to the cell surface. All these information prompted us to look for phosphatases that might be involved in the mGluR1 dephosphorylation and recycling. We initially screened for phosphatases which have been implicated in synaptic plasticity as well as whose activity has been reported to be dependent on the pH of the intracellular compartments. We studied the role of protein phosphatases viz.; PP1, PP2A, and PP2B on the recycling of mGluR1. Above mentioned protein phosphatases

(A)



(B)

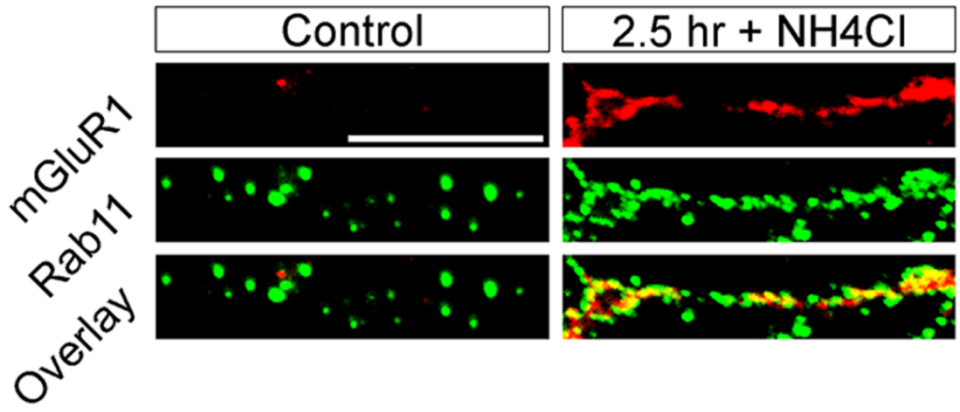
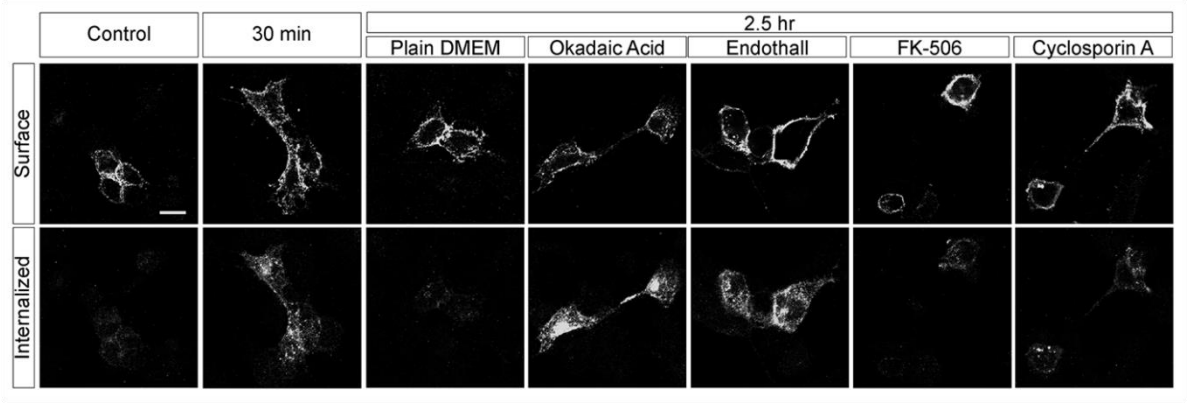


Figure 4.3. Rab11 Localization of internalized myc-mGluR1 in NH_4Cl treated cells (A) myc-mGluR1 gets trapped at the recycling compartment in NH_4Cl treated HEK293 cells. Control cells showing very little internalized receptors whereas, NH_4Cl treated cells showed colocalization of internalized myc-mGluR1 with Rab11 at 2.5 hr (B) Representative images showing colocalization of internalized myc-mGluR1 in NH4Cl treated N2A cells at 2.5 hr. N=3; where N represents the number of times experiment was repeated.

activity has been shown to be pH-dependent (Cohen, 1989). Additionally, these groups of protein phosphatases were reported to be involved in various kinds of synaptic plasticity (Mansuy and Shenolikar, 2006; Mauna et al., 2011).

In order to investigate the role of protein phosphatases in the mGluR1 recycling, we initially used pharmacological inhibitors against these phosphatases. We used Okadaic acid and Endothall as inhibitors for PP1 and PP2A; FK-506 and Cyclosporin A as blockers for PP2B. The experiments were performed using the similar protocol as has been described for the recycling assay before. Briefly, HEK293 cells were transfected with myc-mGluR1 cDNA. Cells were pre-incubated with 100 nM Okadaic acid, 10 μM Endothall, 1 μM FK-506 and 1 μM Cyclosporin A respectively for 30 min. Subsequently, recycling experiment was performed by the application of 100 μM DHPG for 5 min followed by a chase for the total time of 2.5 hr. In control cells, very little internal fluorescence was observed and majority of the receptors were observed to be present on the cell surface. 30 min post-DHPG application receptors were seen to be internalized and in 2.5 hr majority of the receptors recycled back to the cell surface in control cells **(control = 1 ± 0.06, 30 min =1.6 ± 0.03, 2.5 hr = 1 ± 0.06) (Figure 4.4 A, B)**. Importantly, upon application of Okadaic acid and Endothall, there was a complete block in the recycling of myc-mGluR1 observed **(2.5 hr + OA = 1.7 ± 0.07, 2.5 hr + Endothall= 1.5 ± 0.07)**. On the other hand, in FK-506 and Cyclosporin A treated cells, majority of the receptors recycled back to the cell surface, similar to what was observed in the case of drug untreated cells **(2.5 hr + FK-506 = 1.08 ± 0.07, 2.5 hr + Cyclosporin A = 1 ± 0.06).** Okadaic acid at 100 nM concentration could inhibit both PP1 and PP2A. Thus, the above experiments suggest that either PP1 or PP2A or both are involved in the recycling of mGluR1 and PP2B does not play any role in the recycling of mGluR1.

(A)



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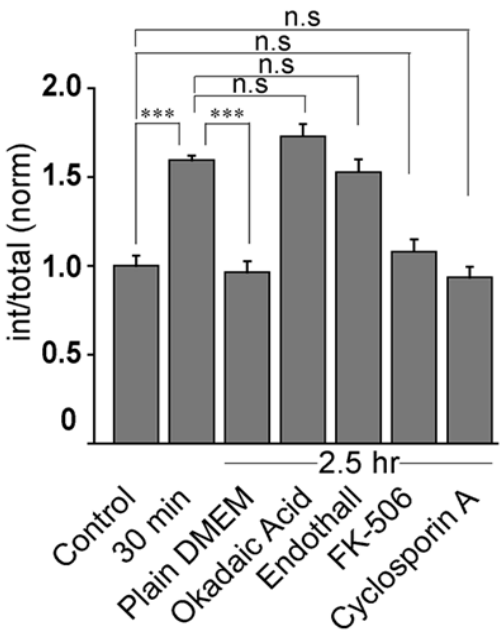


Figure 4.4. Role of protein phosphatases in the recycling of myc-mGluR1 (A) Representative images showing that application of 100 μM DHPG induced the internalization of the receptor at 30 min in HEK293 cells and the receptors were observed to recycle back to the cell surface at 2.5 hr. Treatment of the cells with either Okadaic acid or Endothall inhibited the recycling of myc-mGluR1. On the other hand, FK-506 and Cyclosporin A had no effect on the recycling of the receptor. (B) Quantitation also showed that Okadaic acid and Endothall inhibited the recycling of myc-mGluR1, whereas FK-506 and Cyclosporin A had no effect. Scale bar = 10 μm. ***$p < 0.001$ and n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

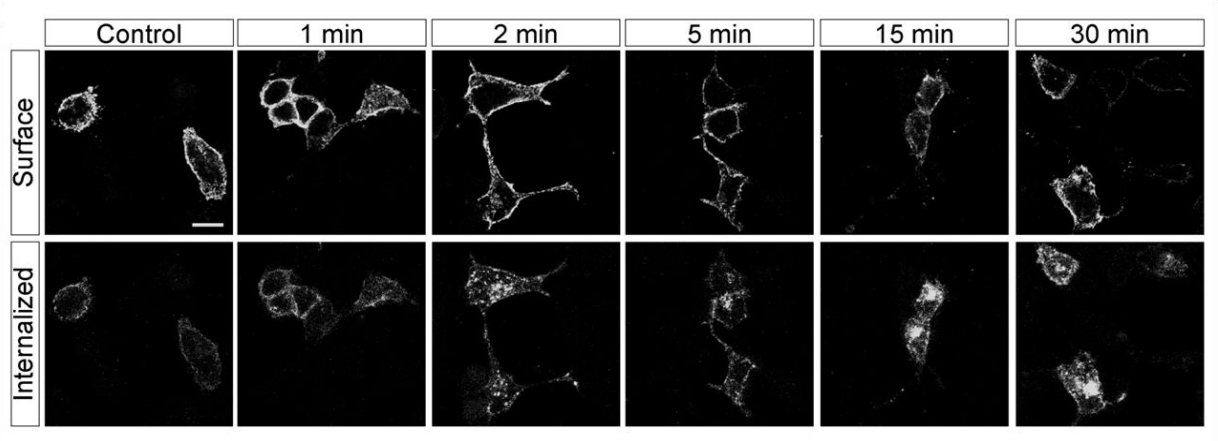
4.2.4. Okadaic acid does not affect the ligand-mediated mGluR1 internalization

In our earlier experiments we observed that although in control cells myc-mGluR1 recycled back to the cell surface at 2.5 hr subsequent to the ligand-mediated internalization, cells treated with 100 nM Okadaic acid showed presence of internalized receptors in the intracellular compartment at 2.5 hr time point. For some GPCRs, it has been reported that protein phosphatases are involved in the endocytosis of the receptor (Hinkle et al., 2012). Hence, it is possible that Okadaic acid might be affecting the forward kinetics of myc-mGluR1 endocytosis instead of its recycling. To rule out this possibility, we studied the kinetics of myc-mGluR1 endocytosis in presence of 100 nM Okadaic acid.

The endocytosis assay was performed using the similar protocol as described in the "method" section. Briefly, HEK293 cells were transfected with myc-mGluR1 cDNA. 24 hr post transfection cells were pre-incubated in 100 nM OA for 30 min. Subsequently, cells were labelled with the anti-myc primary antibody and 100 µM DHPG was applied for various time periods. Cells were then fixed at indicated time points followed by staining with secondary antibodies to visualize the surface and internalized receptors as has been described before. OA was present throughout the experiment. The control cells showed localization of majority of the receptors on the cell surface **(control = 1 ± 0.12)** (**Figure 4.5 A, B)**. Upon 1 min of 100 µM DHPG application receptors started getting internalized, as observed by the increase in the internal fluorescence and the extent of endocytosis increased gradually when the cells were incubated for longer time periods. At 30 min post-DHPG application maximum myc-mGluR1 endocytosis was observed **(1 min = 1.04 ± 0.11, 2 min = 1.54 ± 0.11, 5 min = 1.83 ± 0.08, 15 min = 2.04 ± 0.07, 30 min = 2.37 ± 0.05)**. Thus, the kinetics of DHPG-mediated myc-mGluR1 endocytosis in 100 nM OA treated cells was observed to be similar to that was observed in drug untreated HEK293 cells.

The above experiment suggests that 100 nM OA does not affect the kinetics of ligand-mediated endocytosis of mGluR1, which in turn also suggests that both PP1 and PP2A does not play any role in the mGluR1 endocytosis. Thus, the myc-mGluR1 internal fluorescence that we observed in our earlier recycling experiment in the presence of 100 nM OA at 2.5 hr time point was actually due to the inhibition in the myc-mGluR1 recycling.

(A)



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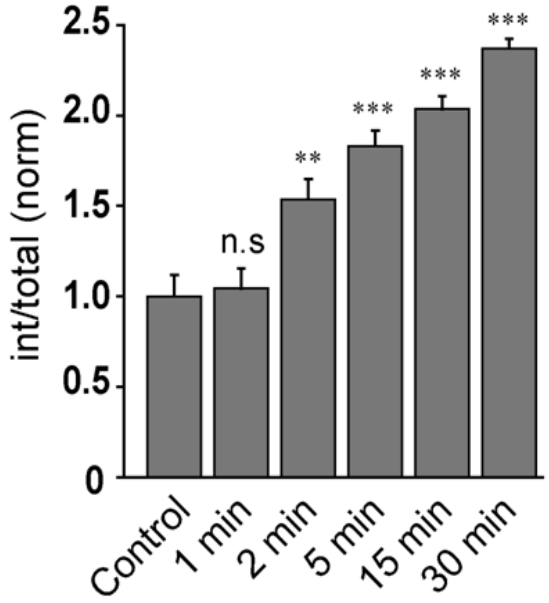


Figure 4.5. Okadaic acid does not inhibit the ligand-mediated myc-mGluR1 internalization (A) Representative images showing the time course of myc-mGluR1 endocytosis in the presence of 100 nM Okadaic acid. Control cells showing very little internal fluorescence and upon 100 μM DHPG application myc-mGluR1 endocytosis increased over time (1 min, 2 min, 5 min, 15 min, 30 min) suggesting that Okadaic acid did not affect myc-mGluR1 endocytosis. (B) Quantitation also showing that Okadaic acid did not inhibit myc-mGluR1 endocytosis. Scale bar = 10 μm.**$p < 0.01$, ***$p < 0.001$ and n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

4.2.5. Activity of protein phosphatase 2A (PP2A) is required for the recycling of mGluR1 in HEK293 cells

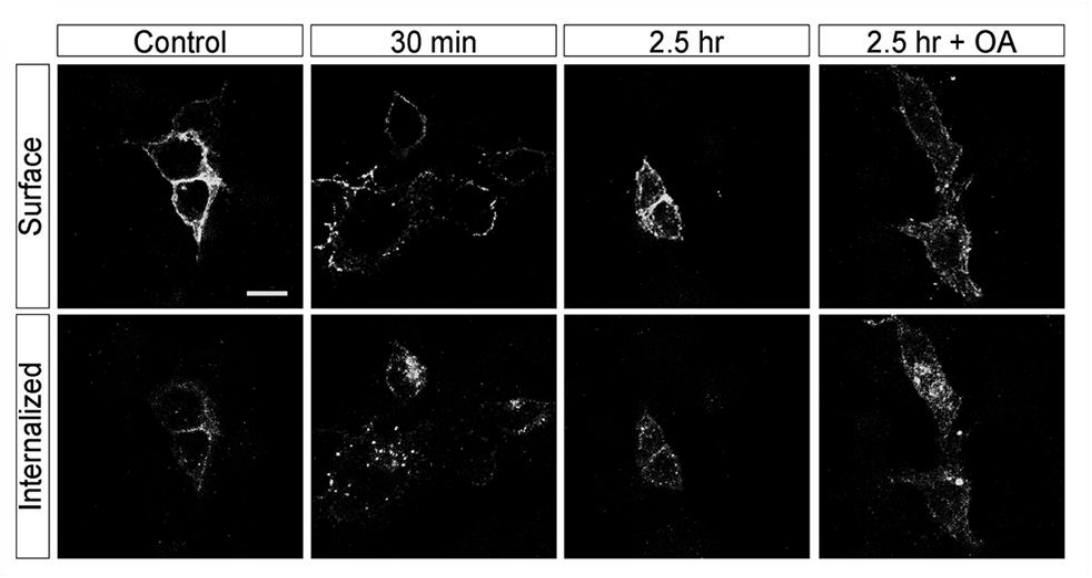
Our earlier experiments suggest that application of 100 nM OA leads to the inhibition in the recycling of myc-mGluR1. As stated before, OA at this concentration blocks the activity of both the PP1 and PP2A. In order to check whether PP1 or PP2A or both the enzymes are involved in the mGluR1 recycling, we investigated the recycling of myc-mGluR1 in presence of 5 nM OA. It has been reported that OA at 5 nM concentration specifically inhibits PP2A and does not inhibit PP1 (Cohen et al., 1990).

The myc-mGluR1 cDNA was transfected in HEK293 cells and after 24 hr cells were pre-incubated in 5 nM OA for 30 min before application of the anti-myc primary antibody. Subsequently, the recycling experiment was performed according to the method described before and the drug was present throughout the experiment. As expected, control cells showed very little internal fluorescence and at 30 min post-DHPG application, receptors were seen to internalize. Similar to the previous observation, DHPG-induced internalized receptors recycled back to the cell surface in 2.5 hr **(control= 1 ± 0.06, 30 min = 2.1 ± 0.05, 2.5 hr = 1.14 ± 0.07)** (**Figure 4.6 A, B)**. Importantly, in 5 nM OA treated cells myc-mGluR1 recycling was completely blocked and the receptors were observed to be localized in the intracellular compartment at 2.5 hr **(2.5 hr + OA = 2.22 ± 0.05)**. This experiment suggests that recycling of mGluR1 is PP2A dependent and PP1 does not play any role in the recycling of the receptor.

4.2.6. Okadaic acid does not affect the Transferrin kinetics in HEK293 cells

Transferrin receptors internalize and recycle back to the cell surface in a phosphatase independent manner (Ciechanover et al., 1983). In order to check whether the inhibition in the recycling of mGluR1 is due to the block in the specific activity of PP2A or due to a general effect in the trafficking machinery of the cell, we studied the trafficking of transferrin receptors in presence of 5 nM OA in HEK293 cells. The experimental protocol has been discussed in the "method" section. Briefly; HEK293 cells were plated on 12 mm coverslips. The cells were pre-incubated with either plain DMEM or plain DMEM containing 5 nM OA for 30 min. Subsequently 20 μg / ml Alexa-568 labelled transferring was applied for 2 min and then cells were washed with plain DMEM followed by a chase in plain DMEM

(A)



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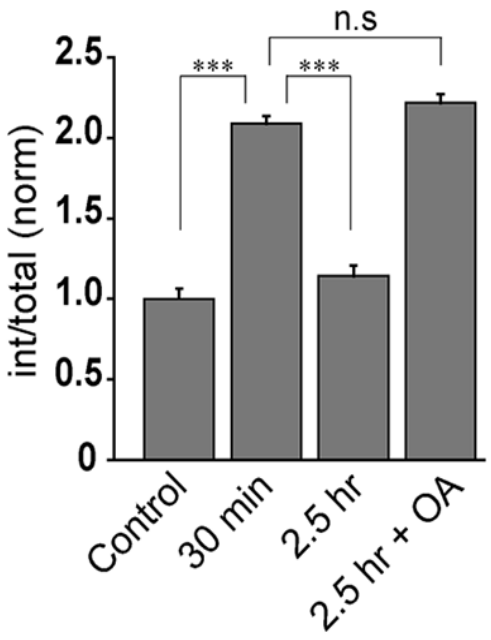


Figure 4.6. Protein phosphatases 2A is involved in myc-mGluR1 recycling in HEK293 cells (A) Control cells showing very little internal fluorescence which increased in 30 min post 100 μM DHPG application and most of the internalized receptors recycled back to the cell surface in 2.5 hr. On the other hand 5 nM Okadaic acid treated cells showed complete block in the myc-mGluR1 recycling at 2.5 hr. (B) Quantitation also showed that 5 nM Okadaic acid inhibited the myc-mGluR1 recycling. Scale bar = 10 μm. ***$p < 0.001$ and n.s indicates $p > 0.05$.N=3; where N represents the number of times experiment was repeated.

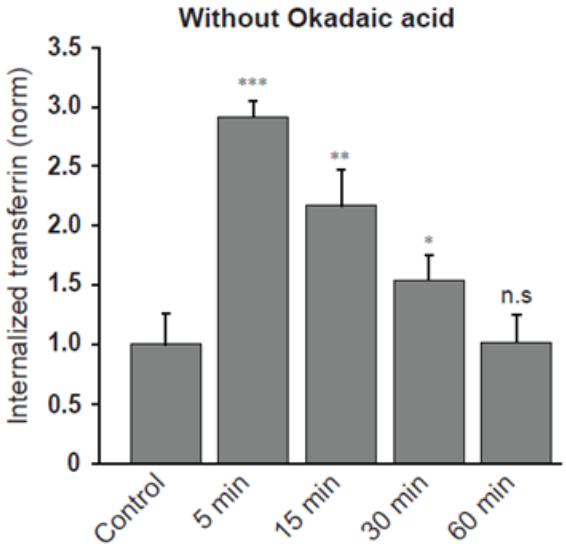
with or without 5 nM OA for the indicated time points. Subsequently, acid stripping was done to remove the remaining transferrin bound at surface receptors followed by fixation with 4% PFA. The cells were then mounted on glass slides and imaged under the fluorescence microscope.

In OA untreated cells, majority of the transferrin receptors internalized at 5 min after labelled transferrin application and as the receptors were chased for longer time periods (15 min, 30 min, 60 min), they recycled back to the cell surface gradually and in 60 min most of the receptors recycled back to the cell surface **(control = 1 ± 0.25, 5 min = 2.91 ± 0.13, 15 min = 2.17 ± 0.29, 30 min = 1.54 ± 0.21, 60 min = 1.02 ± 0.22) (Figure 4.7 A)**. Importantly, in OA treated cells, similar kinetics of transferrin trafficking was observed. In other words, similar to the drug untreated cells, 5 nM OA treated cells also showed maximum internalization of the transferrin receptor at 5 min post transferrin application and majority of the receptors recycled back to the cell surface in 60 min **(control = 1 ± 0.25, 5 min + OA = 2.80 ± 0.16, 15 min + OA = 2.01 ± 0.13, 30 min + OA = 1.54 ± 0.27, 60 min + OA = 1.01 ± 0.23) (Figure 4.7 B)**. These results suggest that OA does not affect the general trafficking machinery of HEK293 cells. Thus, the block in the mGluR1 recycling observed in presence of OA was not due to any alteration in the general trafficking machinery of the HEK293 cells but due to the inhibition of PP2A by OA.

4.2.7. Protein phosphatase 2A (PP2A) activity is required for the recycling of mGluR1 in N2A cells

We next investigated whether PP2A is also involved in the recycling of mGluR1 in neuronal N2A cells by performing the recycling experiment in presence of 5 nM OA using the procedure described in the "methods" section. Briefly, myc-mGluR1 cDNA was transfected in undifferentiated N2A cells and then cells were differentiated. After that, cells were pre-incubated with 5 nM OA for 30 min and recycling experiment was performed in the presence of 5 nM OA. In the drug untreated cells, as expected, the control cells showed very little internal fluorescence and after 30 min post-DHPG application myc-mGluR1 internalized as observed by the increase in the endocytosis index **(control= 1 ± 0.06, 30 min = 2.04 ± 0.05) (Figure 4.8 A, B)**. When cells were chased for longer time majority of the internalized receptors recycled back to the cell surface in 2.5 hr **(2.5 hr = 1.09 ±0.09)**.

(A)



(B)

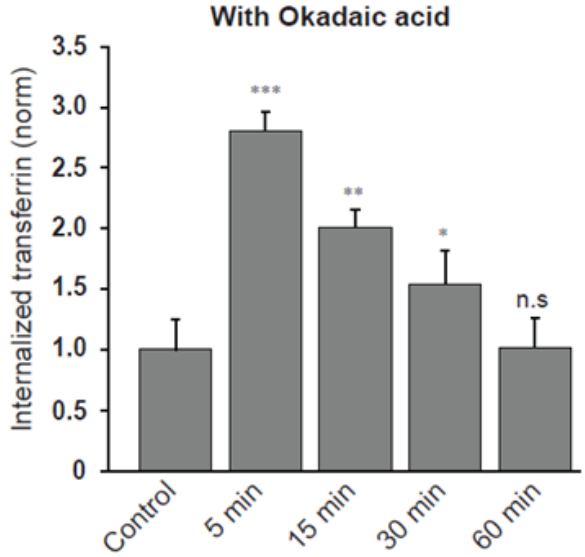
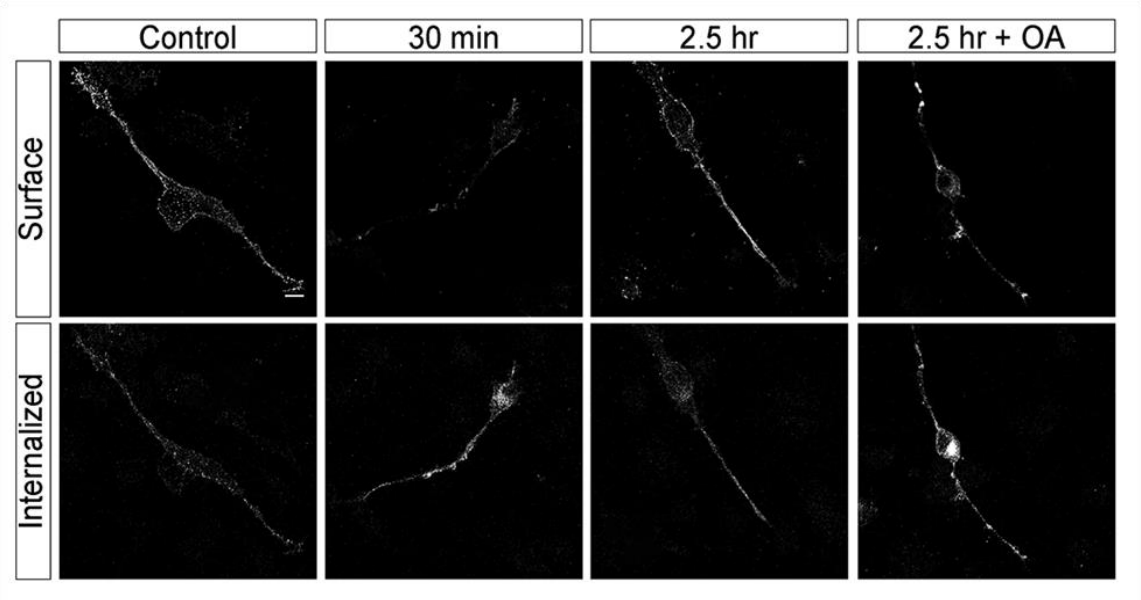


Figure 4.7. Okadaic acid does not affect the transferrin kinetics (A) Quantitation of endocytosis and recycling of the transferrin receptors in control HEK293 cells. (B) Transferrin kinetics quantitation in the presence of 5 nM Okadaic acid suggesting that Okadaic acid does not affect the endocytosis and recycling of the transferrin receptors. *p < 0.05, **p < 0.01, ***p < 0.001 and n.s indicates p > 0.05. N=3; where N represents the number of times experiment was repeated.

(A)



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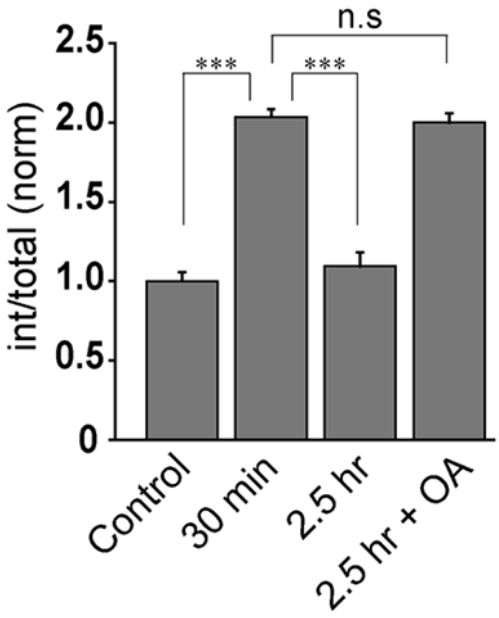


Figure 4.8. Protein phosphatases 2A is involved in the myc-mGluR1 recycling in N2A cells (A) Representative images showing the DHPG-mediated endocytosis of myc-mGluR1 in 30 min whereas the endocytosed receptors recycled back to the cell surface in 2.5 hr. Importantly, in 5 nM Okadaic acid treated cells myc-mGluR1 recycling was completely inhibited. (B) Quantitation also showed that 5 nM Okadaic acid inhibited the recycling of myc-mGluR1 in N2A cells. Scale bar = 10 μm. ***$p < 0.001$ and n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

Importantly, in 5 nM OA treated cells myc-mGluR1 recycling was completely blocked and the receptors were seen to be localized at the intracellular compartment even at 2.5 hr **(2.5 hr + OA = 2 ± 0.06)**.

These experiments suggest that 5 nM OA inhibits the recycling of mGluR1 in both non neuronal HEK293 cells and neuronal N2A cells.

4.2.8. Dominant negative PP2A inhibits the recycling of mGluR1

Our earlier experiments suggest that pharmacologically blocking PP2A leads to the inhibition in the recycling of mGluR1. Since, pharmacological inhibition by drugs might have some non-specific effect, it was important for us to confirm that the inhibition of the recycling of the receptor is due to the inhibition of PP2A only. We first over-expressed the dominant negative form of PP2A (PP2ADN) construct viz., PP2A-L199P (catalytically inactive C subunit of PP2A) in N2A cells and preformed mGluR1 recycling assay (Kins et al., 2001; Kins et al., 2003).

The myc-mGluR1 and PP2A dominant constructs were cotransfected in undifferentiated N2A cells. The cells were then differentiated by incubating them in 1% DMEM for 48 hr. After 48 hr, myc-mGluR1 recycling experiment was performed using the method described earlier. As expected, in control cells majority of the receptors were present at the cell surface and after agonist stimulation, the receptors were seen to internalize in 30 min and upon further incubation in absence of the ligand the internalized receptors recycled back to the cell surface in 2.5 hr **(control = 1 ± 0.04, 30 min = 2.29 ± 0.06, 2.5 hr = 1.06 ± 0.03) (Figure 4.9 A, B)**. Importantly, in PP2A dominant negative expressing cells we observed a complete blockage in the recycling of myc-mGluR1 and the receptors were present at the internal compartment even at 2.5 hr post-ligand application **(2.5 hr + PP2ADN = 2.27 ± 0.04)**. Measurement of the level of surface receptors also suggested that in control cells at 30 min post-DHPG application there was a decrease in the surface fluorescence which recovered at 2.5 hr, suggesting that the receptors internalized at 30 min post-ligand application and subsequently recycled back to the cell surface at 2.5 hr **(control = 1 ± 0.04, 30 min = 0.35 ± 0.03, 2.5 hr = 1.02 ± 0.03) (Figure 4.9 C).** On the other hand, in cells expressing PP2A dominant negative construct there was no recovery in the surface fluorescence observed at 2.5 hr suggesting that

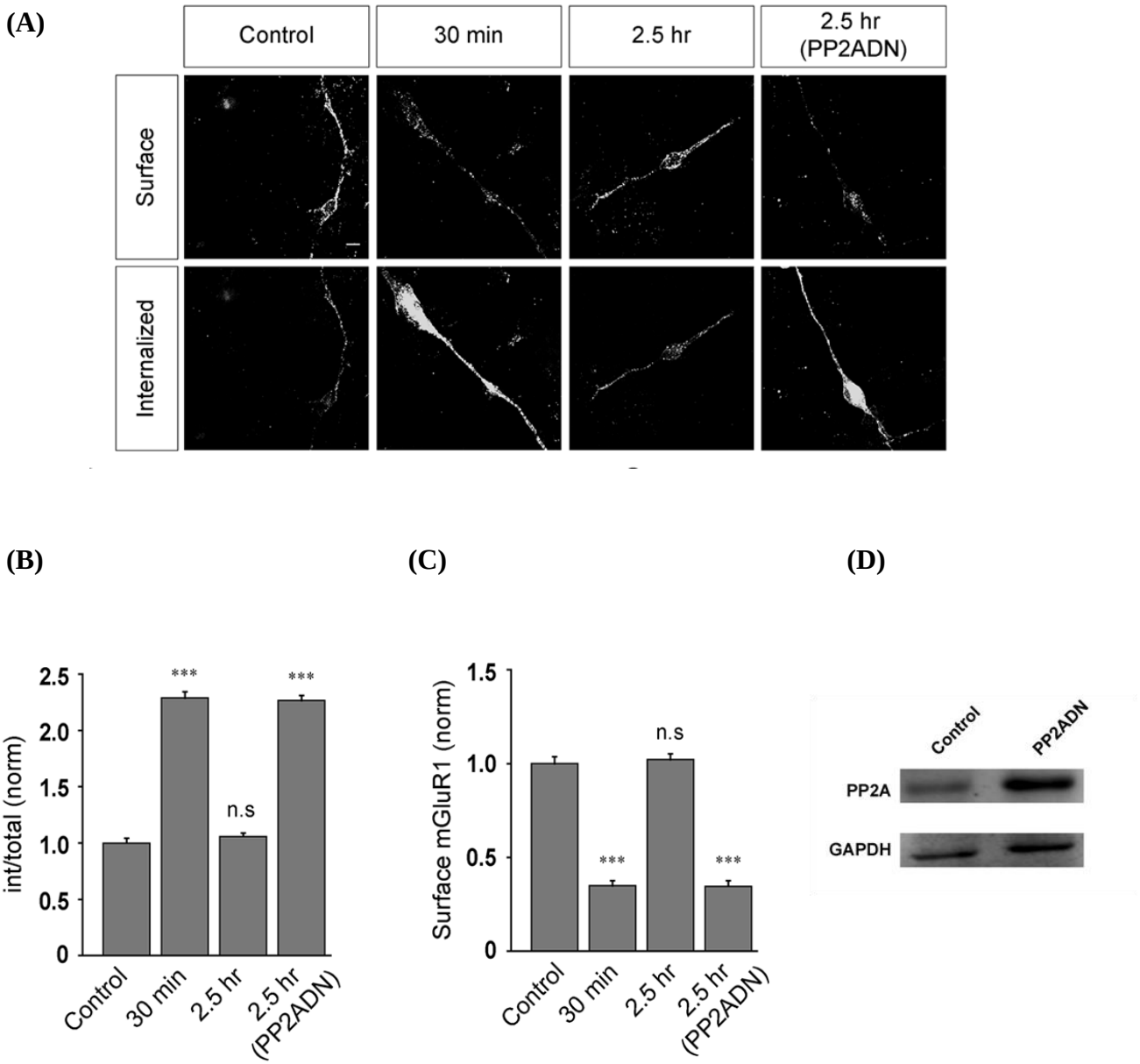


Figure 4.9. Protein phosphatases 2A dominant negative (PP2ADN) over-expression inhibits myc-mGluR1 recycling in N2A cells (A) Control cells showing very little internal fluorescence which increased upon 100 µM DHPG application in 30 min and the majority of the internalized receptors recycled back to the cell surface in 2.5 hr. Importantly in PP2ADN over-expressed cells the recycling of myc-mGluR1 was completely blocked and receptors were seen internalized even at 2.5 hr. (B) Quantitation also suggested that PP2ADN over-expression blocked the myc-mGluR1 recycling. (C) Surface myc-mGluR1 quantitation also showed less surface fluorescence in 30 min whereas in 2.5 hr receptors recycled back to the cell surface as observed by recovery of the surface fluorescence. Importantly, PP2ADN over-expression inhibited the recovery of the surface fluorescence. (D) Western blot showing the over-expression on PP2ADN construct.Scale bar = 10 µm. ***$p < 0.001$ and n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

expression of the PP2A dominant negative construct inhibited the recycling of myc-mGluR1 **(2.5 hr + PP2ADN = 0.35 ± 0.03)**.

4.2.9. Knockdown of endogenous PP2A inhibits the recycling of mGluR1

Our earlier experiments suggest that the mGluR1 recycling is PP2A-dependent and either pharmacologically blocking the activity of PP2A or over-expression of the dominant negative PP2A construct led to the complete blocking of myc-mGluR1 recycling. We further confirmed these results by knocking down the endogenous PP2A and studying its effect on the recycling of mGluR1 in N2A cells.

myc-mGluR1 cDNA along with the siRNA pool against the catalytic C subunit of endogenous PP2A (si-PP2A) and scrambled siRNA (si-control) were transfected in undifferentiated N2A cells according to the manufactures instructions. Subsequently, the N2A cells were differentiated and the recycling experiments were performed in fully differentiated N2A cells. The siRNA oligos were present throughout the experiment. The control cells showed localization of majority of the receptors at the cell surface. The receptors internalized on application of 100 µM DHPG application and they recycled back to the cell surface at 2.5 hr post-DHPG application in control cells **(control = 1 ± 0.05, 30 min = 2.1 ± 0.06, 2.5 hr = 1.03 ± 0.04) (Figure 4.10 A, B)**. Importantly, the myc-mGluR1 recycling was completely blocked upon knocking down of the endogenous PP2A and the receptors were localized at the internal compartment of the cell at 2.5 hr **(2.5 hr + si-PP2A = 2.04 ± 0.05)**. The internalized receptors recycled back to the cell surface in si-control transfected cells in 2.5 hr post-ligand application similar to in control cells **(2.5 hr + si-control = 1.1 ± 0.04)**. As expected, surface receptor quantitation also suggested that in control cells application of 100 µM DHPG led to the decrease in the surface fluorescence at 30 min and in 2.5 hr almost full recovery in the surface fluorescence was observed suggesting recycling of the internalized myc-mGluR1 **(control = 1 ± 0.05, 30 min = 0.28 ± 0.03, 2.5 hr = 1 ± 0.03) (Figure 4.10 C)**. Importantly, although full recovery in the surface fluorescence was observed in si-control transfected cells, we did not observe any surface fluorescence recovery in the si-PP2A transfected cells at 2.5 hr **(2.5 hr + si-control = 1 ± 0.05, 2.5 hr + si-PP2A = 0.3 ± 0.03)**. All these experiments suggest that PP2A plays critical role in the recycling of mGluR1 in both non-neuronal HEK293 cells and neuronal N2A cells.

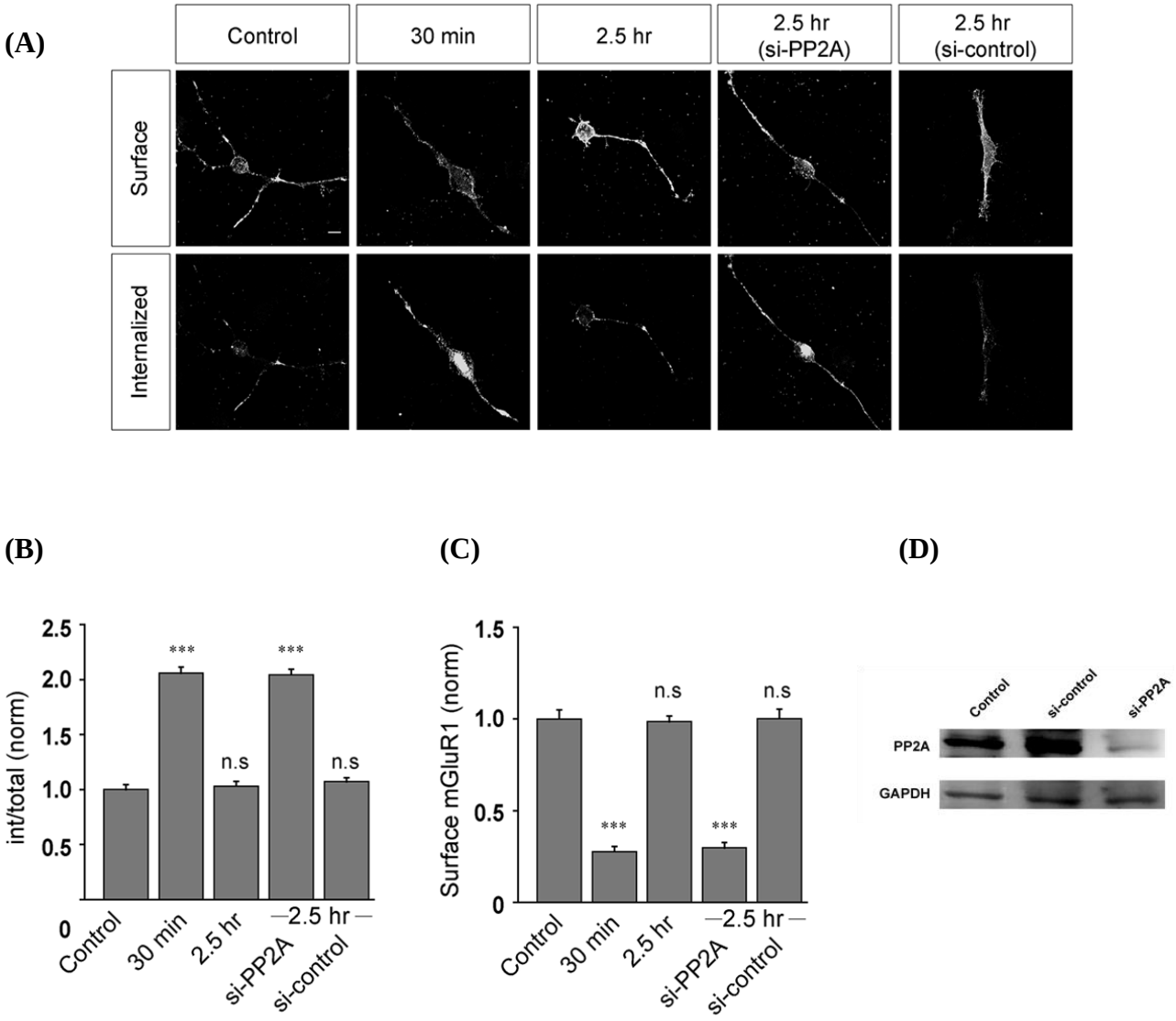


Figure 4.10. Knockdown of endogenous protein phosphatase 2A (PP2A) inhibits the recycling of myc-mGluR1 (A) Representative cells showing normal endocytosis at 30 min upon DHPG application and recycling at 2.5 hr. Importantly in PP2A siRNA treated cells myc-mGluR1 recycling was blocked whereas, si-control transfected cells showed normal recycling in 2.5 hr. (B) Quantitation also showed block in myc-mGluR1 recycling in PP2A siRNA treated cells at 2.5 hr. (C) Surface quantitation also suggested decrease in the surface fluorescence due to endocytosis of the receptor in control cells at 30 min and recovery of the surface fluorescence due to the recycling of the receptors in control and si-control transfected cells at 2.5 hr, whereas in PP2A siRNA treated cells surface fluorescence did not recover. (D) Knockdown of endogenous PP2A in N2A cells. Scale bar = 10 μm. ***$p < 0.001$ and n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

4.3. Discussion

Many GPCRs undergo desensitization and resensitization followed by agonist stimulation and these processes play crucial roles in the regulation of the receptor and its signaling. A number of GPCRs subsequent to desensitization get internalized and recycle back to the cell surface after that *via* various routs such as Rab4-dependent fast recycling pathway or Rab11-dependent slow recycling pathway (Drake et al., 2006). The process of endocytosis followed by recycling has been shown as a necessary step for the "resensitization" of many GPCRs. Previous studies have shown that mGluR1 upon agonist stimulation gets desensitized by GRK and PKC-dependent phosphorylation of the receptor and subsequent to the desensitization mGluR1 gets internalized *via* clathrin and dynamin dependent manner but the detailed molecular mechanisms regulating the resensitization process remains largely unknown (Dale et al., 2000; Dale et al., 2002). In order to investigate the molecular mechanisms regulating the mGluR1 resensitization, we have initially studied the kinetics of mGluR1 internalization and our data suggested that upon agonist stimulation mGluR1 internalized and followed the Rab11-dependent slow recycling route. Our data also suggested that mGluR1 recycling was dependent on the pH of the intracellular compartment. A probable explanation for the dependence of the recycling of mGluR1 on the pH of the intracellular compartment could be that in the recycling compartment due to the acidic environment a conformational change of the receptor occurs and that leads to the interaction of the receptor with the specific phosphatase(s). The phosphatase(s) then dephosphorylates the residue(s) of the receptor that were phosphorylated during the desensitization of the receptor. Furthermore, the dephosphorylation is probably a prerequisite for the exit of the receptor from the recycling compartment. We therefore investigated the phosphatase(s) responsible for the removal of the phosphates which have been shown to be necessary for the desensitization and endocytosis of mGluR1 in earlier studies. We checked for the phosphatases whose activity has been reported to be dependent on pH as well as whose involvement has been reported in synaptic plasticity. Our data suggest that PP2A plays a crucial role in the process of mGluR1 recycling and upon blocking the PP2A activity by various means, mGluR1 recycling was blocked. Since mGluR1 is well documented to be involved in the various forms of synaptic plasticity and PP2A has also been reported to be implicated in synaptic plasticity, so it would be interesting to study the role of PP2A in mGluR1-mediated synaptic plasticity in future studies. Additionally, it would also be important to look up for the critical residues in the third intracellular loop and the C-terminal

tail of mGluR1 that is/are dephosphorylated by PP2A in the recycling compartment. Alternatively, it is also possible that PP2A might be dephosphorylating some other substrates which might be involved in the mGluR1 recycling process. Future studies needs to be directed towards elucidating the above phenomenon.

Role of Tamalin in group I mGluR endocytosis

5.1. Introduction

Group I mGluRs play important roles in synaptic function, plasticity and have been implicated in a variety of brain disorders including Fragile X syndrome, autism and drug addiction. Electron microscopy of the functional excitatory synapses had revealed an electron dense area at the post-synaptic membrane termed as post synaptic density (PSD). PSD contains various molecules such as neurotransmitter receptors, ion channels, cell adhesion molecules and scaffolding proteins (Hall and Lefkowitz, 2002; Sorkin and von Zastrow, 2009). Till date, large number of scaffolding proteins has been discovered and they have been shown to play important roles in various cellular processes at the synapse (Sorkin and von Zastrow, 2009). Similar to many other GPCRs, group I mGluRs are also tightly regulated by a macromolecular protein complex at the post-synaptic membrane, a key component of which is the scaffolding protein Tamalin. Tamalin (also termed GRP1-associated scaffold protein) is a 394 amino acid postsynaptic density scaffolding protein, comprising of multiple protein-protein interaction domains (Kitano et al., 2003). It is comprised of a PDZ domain, a proline-rich region, a leucine-zipper region and a C-terminal PDZ binding motif. The PDZ domain of Tamalin interacts with the C-terminus of group I mGluRs, whereas the leucine-zipper region binds to the guanine nucleotide exchange factor cytohesins (Kitano et al., 2002). Furthermore, Tamalin also interacts with many other important scaffold proteins involved in postsynaptic organization and protein trafficking in neurons. For example, Tamalin binds to PSD-95 and also interacts with proteins implicated in trafficking, including MINT2 and GRP-1 (Kitano et al., 2003). Tamalin over-expression has been reported to increase the surface expression of mGluR1 in COS-7 cells and cultured hippocampal neurons (Kitano et al., 2002). These results together suggest that Tamalin might play crucial role(s) in controlling various aspects of group I mGluRs regulation like receptor clustering, trafficking, and intracellular signaling. Lack of knowledge about how Tamalin regulates the group I mGluR trafficking and also how Tamalin regulates the group I mGluR-mediated AMPAR endocytosis in the central nervous system, which is the cellular correlate for mGluR-LTD is the motivation for this study.

We have studied the role of Tamalin in group I mGluR-trafficking and mGluR-triggered AMPAR trafficking using the “molecular replacement” strategy. This approach allows shRNA-mediated knockdown (KD) of endogenous proteins of interest, as well as simultaneous replacement of the “knocked down” protein(s) with wild type or mutant

recombinant forms of the protein. This approach has two significant advantages. First, compensatory adaptations that may occur during synaptogenesis and synapse maturation due to the loss of the protein of interest are minimized. Second, the function of heterologous constructs can be studied without the necessity of a dominant effect as required by a standard over-expression approach. Our data suggest that acute knockdown of endogenous Tamalin led to the inhibition in the endocytosis of both mGluR1 and mGluR5. Subsequently, the knockdown effect could be rescued for both the receptors by expression of the wild-type Tamalin, suggesting specificity of the knockdown. These results suggest a critical role for Tamalin in group I mGluR endocytosis. As stated before, activation of group I mGluRs leads to the endocytosis of AMPARs which is the cellular correlate for synaptic plasticity. Our studies also suggest that acute knockdown of endogenous Tamalin resulted in the inhibition of the mGluR-mediated AMPAR endocytosis, which could be rescued by expression of wild-type Tamalin. We further show that over-expression of the N terminal domain of Tamalin (N-Tam) that contains the PDZ domain led to the inhibition of the mGluR1 endocytosis. In agreement with this result, expression of the N-Tam replacement construct was not able to rescue the inhibition in the endocytosis of mGluR1 as well as mGluR-mediated AMPAR endocytosis caused by the knockdown of endogenous Tamalin. We also show here that C terminal domain of Tamalin (C-Tam) which contains the PDZ binding autoinhibitory domain, upon over-expression had no effect on the mGluR1 endocytosis and replacement of the wild-type Tamalin with C-Tam did not rescue the inhibition in the mGluR1 endocytosis caused by the knockdown of endogenous Tamalin. Moreover, C-Tam did not show punctate dendritic expression that is typical of wild-type Tamalin. Finally our data also suggest that the last 8 amino acids in the intrinsic ligand domain of Tamalin plays critical role in the ligand-dependent endocytosis of mGluR1.

5.2. Results

5.2.1. shRNA against Tamalin effectively knocks down Tamalin in both HEK293 cells and in primary hippocampal neurons

In order to study the role of Tamalin in group I mGluR trafficking, first we generated an effective shRNA against Tamalin that was efficient in knocking down the endogenous Tamalin in primary hippocampal neurons. The shRNA designing procedure has been described in detail in the "method" section. Briefly, candidate oligos were synthesized and cloned under the H1 promoter in LRV1 vector. Initially, wild type Tamalin construct along with candidate shRNAs were transfected in HEK293 cells using lipofectamine 2000 according to the manufacturer's instructions. 72 hr post-transfection cells were lysed with NP40 lysis buffer and then the cell lysates were ran on SDS-PAGE followed by western blotting. This process helped us in identifying one effective shRNA **(shTam)**, which effectively knocked down the over-expressed Tamalin in HEK293 cells. **(Figure 5.1 A, B)**

Next, we checked whether the shRNA could efficiently knocked down the endogenous Tamalin in primary hippocampal neurons. shTam construct was transfected in the dissociated mouse hippocampal neurons using calcium phosphate method as described in the "method" section. 72 hr post-transfection neurons were fixed with 4% PFA and then permeabilization was done by incubating cells in 0.1% Triton X-100 for 30 min at room temperature. Subsequently, rabbit anti-Tamalin antibody (1:500) staining was done for 1.5 hr at 37˚C followed by the application of goat anti-rabbit Alexa-568 conjugated secondary antibody (1:500) for 1 hr at 37˚C. Cells were then mounted on glass slides and imaged under the confocal microscope. Control cells showed endogenous Tamalin staining in the neurons **(Figure. 5.1 C)**. However, in shTam expressing cells, very little Tamalin staining was observed, suggesting efficient knockdown of the endogenous Tamalin by shTam.

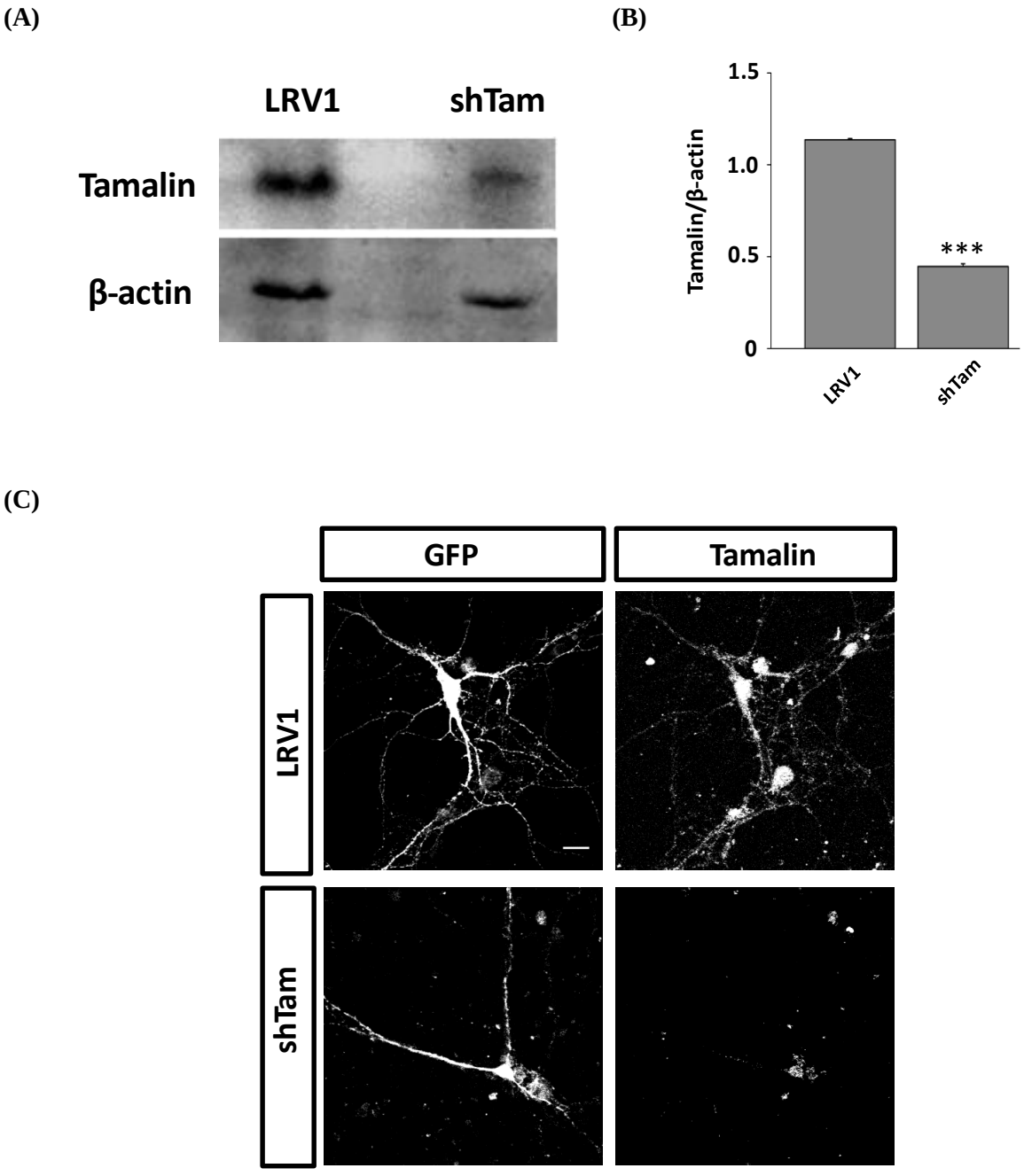


Figure 5.1. Knockdown of Tamalin by shTam (A) Western blot showing the knockdown of over-expressed Tamalin by shTam in HEK293 cells. (B) Quantitation of Tamalin knockdown by shTam. (C) Representative images showing knockdown of endogenous Tamalin by shTam in primary hippocampal neurons. Scale bar = 10 μm ***$p < 0.001$. N=3; where N represents the number of times experiment was repeated.

5.2.2. Knockdown of endogenous Tamalin inhibits the mGluR1 endocytosis

We investigated the effect of knockdown of endogenous Tamalin on the surface expression of mGluR1 in primary hippocampal neurons. The shTam construct along with myc-mGluR1 was transfected in the primary hippocampal neurons using calcium phosphate method on 5-7 DIV as described in the "method" section. On 12-15 DIV, mouse anti-myc primary antibody (1:200) staining was done for 30 min in live cells to label the surface receptors and then cells were 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. Cells were then mounted on glass slides and observed under the confocal microscope. There was no significant difference observed in the myc-mGluR1 surface expression between control cells and cells in which Tamalin was knocked down **(control = 1 ± 0.04, shTam = 0.95 ± 0.05) (Figure. 5.2 A, B)**. Since, neuron is a compartmentalized cell; we also quantified the effect of Tamalin knockdown on the myc-mGluR1 surface expression in the cell body and dendrite separately. Dendritic area was defined by the area that was 10 µm away from soma. Our data suggested that acute knockdown of Tamalin had no effect on the surface expression of myc-mGluR1 in both cell body as well as in the dendrite **(Cell body: - control = 1 ± 0.06, shTam = 0.97 ± 0.06, Dendrite: - control = 1 ± 0.05, shTam = 0.94 ± 0.05) (Figure 5.2 C and D)**.

We next investigated whether knockdown of endogenous Tamalin had any effect on the DHPG-mediated endocytosis of myc-mGluR1. Our earlier data suggested that upon DHPG application majority of the myc-mGluR1 internalized at 30 min. Therefore, we chose 30 min as the time point for the endocytosis experiments. The endocytosis assay was performed using the same protocol as described before. Briefly cells were transfected with shTam and myc-mGluR1 constructs. 4-5 days post-transfection, live cells were stained with mouse anti-myc primary antibody (1:200) for 30 min at 37˚C to label the surface receptors. Subsequently, 100 µM DHPG pulse was given for 5 min followed by chase for 25 min in absence of the ligand. Cells were then fixed without permeabilization in 4% PFA followed by the application of saturating concentration of the goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) to label the surface receptors that did not internalize on ligand application. Subsequently, cells were permeabilized by 0.1% Triton X-100 and then second secondary antibody viz., goat anti mouse Alexa-647 was applied for 1 hr at 37˚C (1:750) to label the internalized receptors.

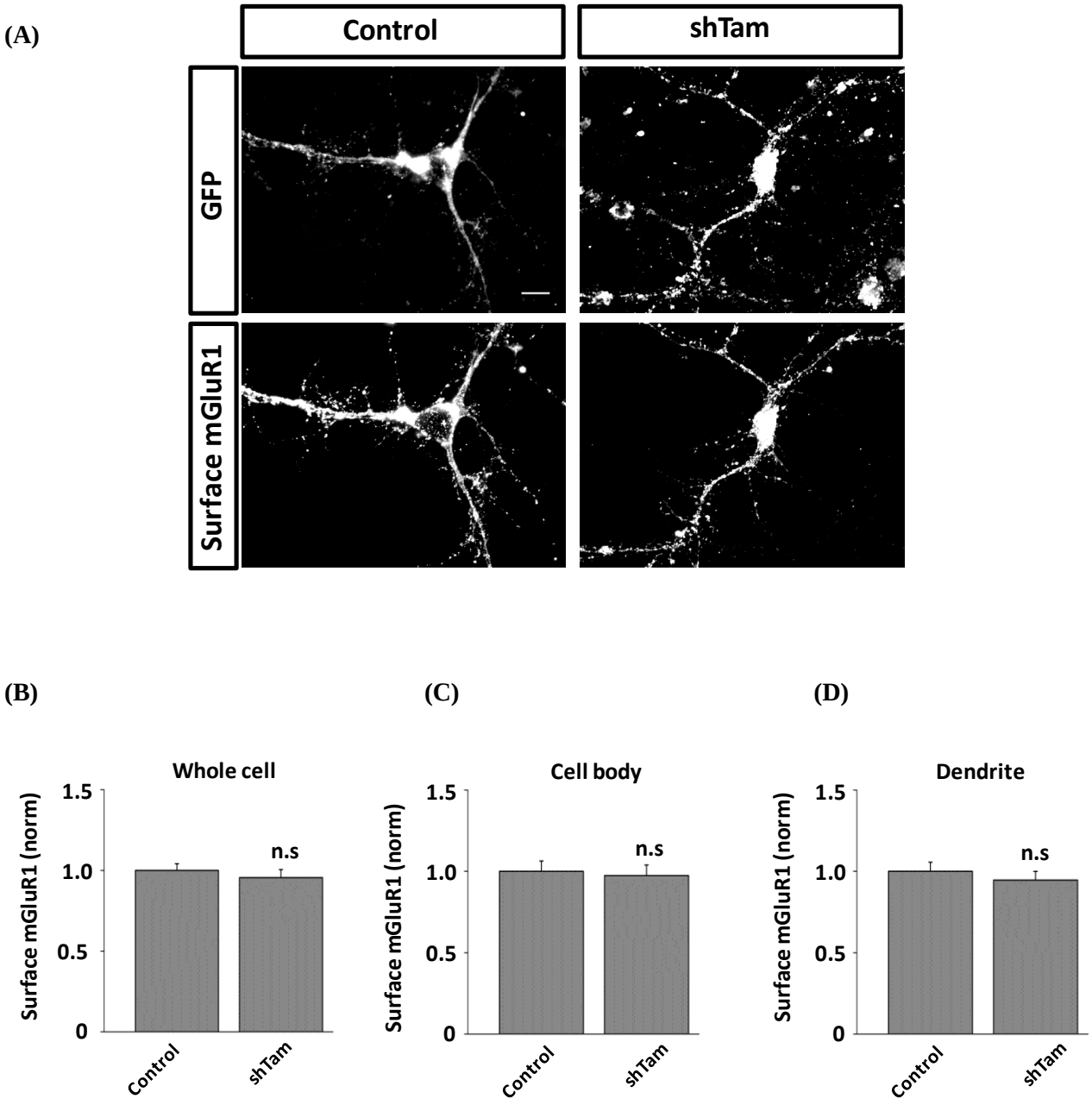


Figure 5.2. Effect of knockdown of Tamalin on the surface expression of mGluR1 (A) Representative images showing that knockdown of endogenous Tamalin had no effect on the surface localization of myc-mGluR1. (B) (C) and (D) Quantitation of surface localization of myc-mGluR1 in whole cell, cell body and dendrite respectively. Scale bar = 10 μm, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

Cells were then mounted on glass slides and observed under the confocal microscope. As expected, control cells showed very little internal fluorescence and significant amount of myc-mGluR1 internalized after DHPG application in 30 min **(control = 1± 0.07, DHPG = 2.07 ± 0.15) (Figure. 5.3 A, B)**. Importantly, Tamalin shRNA expressing cells showed inhibition in the endocytosis of myc-mGluR1 **(shTam + DHPG = 1.09 ± 0.08)**. We also quantified the extent of endocytosis in the cell body vs the dendritic area of the neurons. Our data suggested that the knockdown of endogenous Tamalin inhibited the internalization of the receptor with similar extent in both cell body and dendrites **(Cell body: control = 1 ± 0.08, DHPG. = 1.69 ± 0.13, shTam + DHPG = 1.08 ± 0.08, Dendrite: control = 1 ± 0.09, DHPG. = 2.46 ± 0.20, shTam+ DHPG = 1.16 ± 0.11) (Figure 5.3 C, D)**. These results suggest that Tamalin plays crucial roles in the ligand-mediated internalization of mGluR1.

5.2.3. Time course of mGluR1 endocytosis in presence of Tamalin ShRNA

Our earlier data suggested that primary neurons expressing shTam did not show endocytosis of the receptor at 30 min post-ligand application. This could be either due to inhibition in the endocytosis of the receptor caused by the acute knockdown of endogenous Tamalin, or receptors took a rapid recycling route and came back to the cell surface at 30 min subsequent to the internalization in absence of Tamalin protein. In order to distinguish between these two possibilities, we performed time course of myc-mGluR1 endocytosis in the presence of shTam.

As expected, control cells showed endocytosis of myc-mGluR1 on application of 100 µM DHPG at 30 min time point **(control = 1 ± 0.04, 30 min. = 1.72 ± 0.05) (Figure. 5.4 A, B)**. Importantly, in shTam expressing cells no significant amount of internalization of the receptor was observed on ligand application at any time point **(shTam + 5 min. = 0.97 ± 0.02, shTam + 15 min = 1.06 ± 0.03, shTam + 30 min = 1.01 ± 0.04)**. These results suggest that Tamalin plays a critical role in the ligand-mediated internalization of mGluR1.

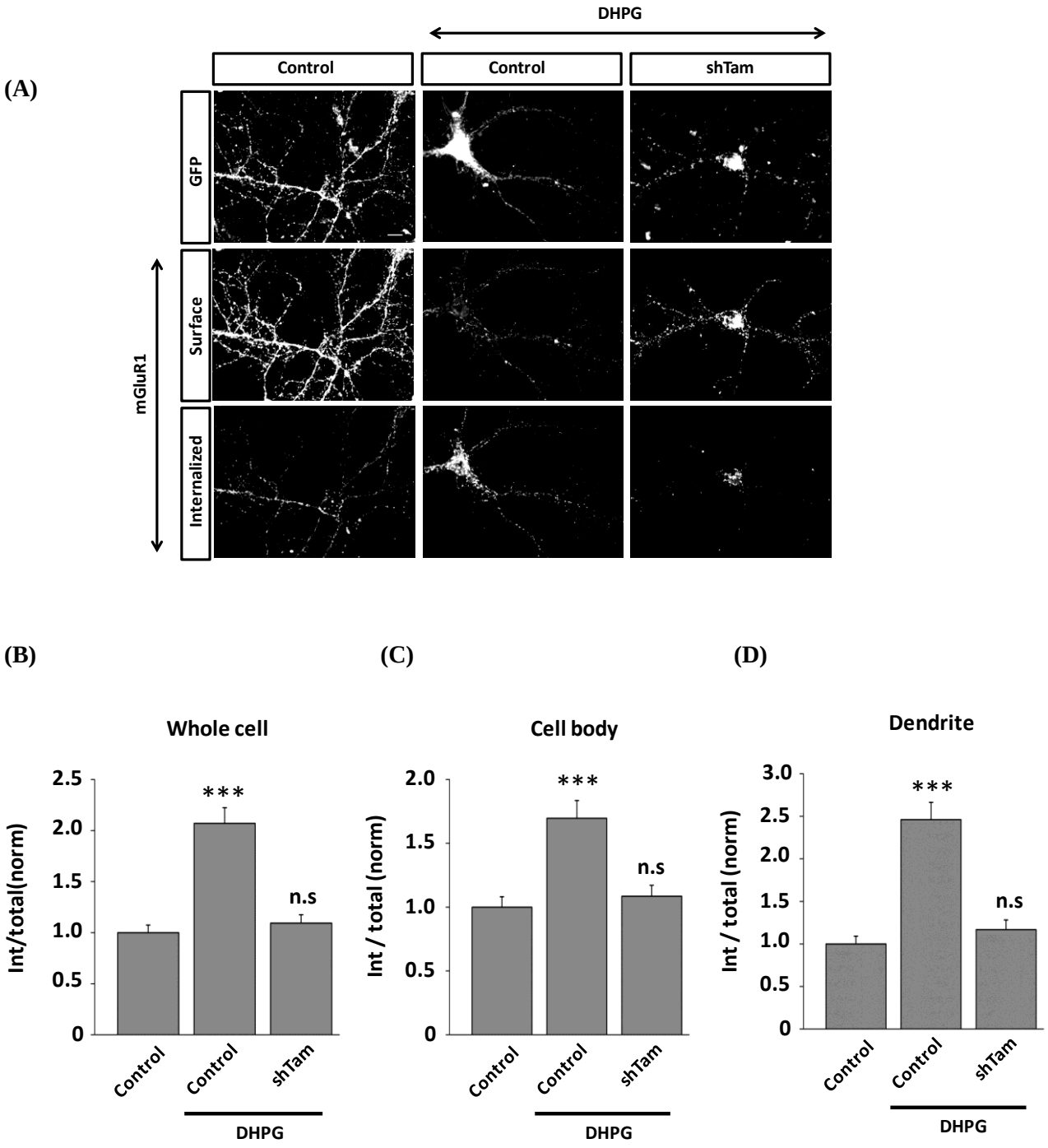
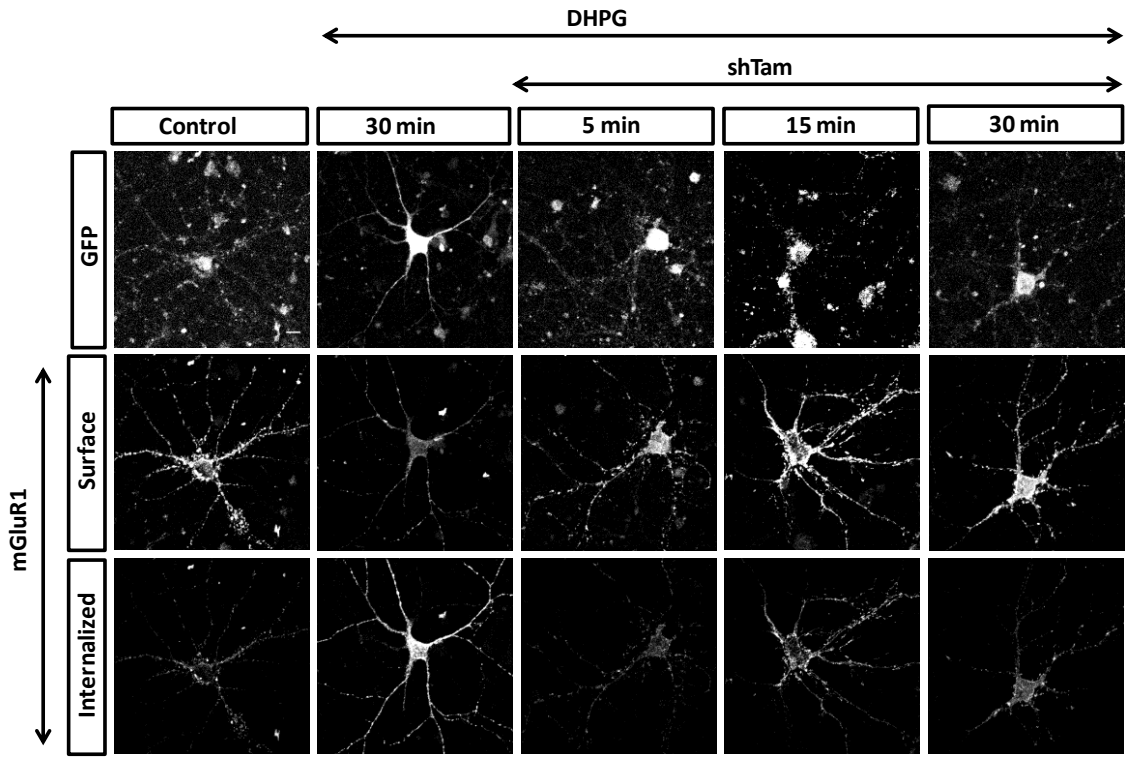


Figure 5.3. Effect of Tamalin knockdown on ligand-mediated endocytosis of mGluR1
(A) Representative images showing the DHPG-mediated endocytosis of myc-mGluR1. Control cells showed very little internal fluorescence which increased upon 100 µM DHPG application. Importantly, shTam transfected cells showed inhibition in the myc-mGluR1 internalization. (B) (C) and (D) Quantitation of DHPG-mediated myc-mGluR1 internalization in whole cell, cell body and dendrites respectively. Quantitation suggested that knockdown of endogenous Tamalin inhibited the ligand-mediated endocytosis of myc-mGluR1 throughout the cell. Scale bar = 10 µm. ***$p < 0.001$, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

(A)



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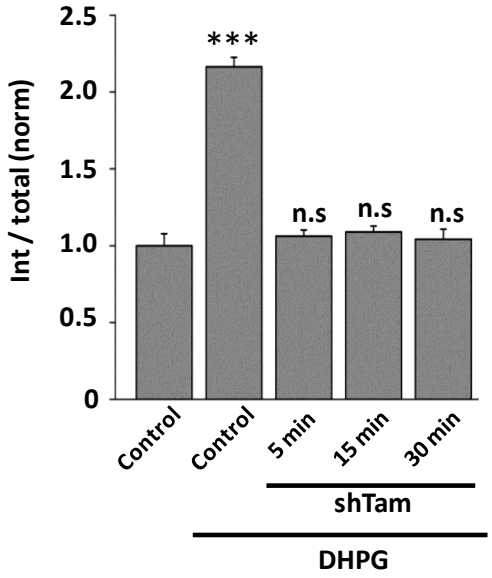


Figure 5.4. Time course of DHPG-mediated mGluR1 endocytosis in presence of Tamalin shRNA (A) Representative images showing DHPG-mediated myc-mGluR1 endocytosis at different time points (5 min, 15 min, and 30 min). Control cells showed increased endocytosis upon 100 µM DHPG application at 30 min. Importantly, in shTam transfected cells there was complete block in the endocytosis observed at all the time points. (B) Quantitation of the DHPG-mediated endocytosis of myc-mGluR1 in absence and presence of Tamalin shRNA. Scale bar = 10 µm. ***$p < 0.001$, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

5.2.4. Rescue of the inhibition of mGluR1 endocytosis caused by the knockdown of endogenous Tamalin

Our earlier experiments suggested that knockdown of endogenous Tamalin led to the inhibition of DHPG-mediated endocytosis of mGluR1. In order to ascertain that the inhibition in the endocytosis of the receptor was due to specific knockdown of Tamalin we checked whether expression of wild-type Tamalin could fully rescue this knockdown effect. For the generation of the Tamalin replacement construct (shTam:HA-Tam), we have utilized the knowledge of the degeneracy in the amino acids coding sequences. We introduced series of silent mutations in the shRNA binding region of the Tamalin gene to inhibit the shRNA binding with this replacement construct. As a result shTam only knocked down the endogenous Tamalin but the over-expressed Tamalin expression was not affected by the presence of shTam. Furthermore, since silent mutations were introduced, amino acids sequence of the over-expressed Tamalin was same as that of wild type protein. Initially we investigated whether the expression of HA-Tam had any effect on the surface expression of the receptor. Cells were transfected with myc-mGluR1 and shTam or vector containing shTam and HA-Tam (shTam:HA-Tam). Subsequently, the staining of the surface myc-mGluR1 was done using the method described before. Our data suggested that there was no significant difference in the surface expression of myc-mGluR1 between control cells, shTam expressing cells and shTam:HA-Tam expressing cells. **(control = 1 ± 0.07, shTam = 1.01 ± 0.04, shTam:HA-Tam = 1.08 ± 0.04) (Figure 5.5 A, B)**. Similar to the previous experiments we also quantified the myc-mGluR1 surface expression in both cell body and dendrites. There was no significant difference observed in myc-mGluR1 surface fluorescence between control, shTam and shTam:HA-Tam expressing cells in both cell body and dendrites **(Cell Body: control = 1 ± 0.08, shTam = 0.99 ± 0.06, shTam:HA-Tam = 1.11 ± 0.05, Dendrite: control= 1 ± 0.09, shTam = 0.99 ± 0.05, shTam:HA-Tam = 0.99 ± 0.04) (Figure 5.5 C, D)**
We next investigated whether expression of HA-Tam could rescue the inhibition of DHPG-mediated endocytosis of myc-mGluR1 caused by the knockdown of endogenous Tamalin. Cells were co-transfected with myc-mGluR1 and shTam or shTam:HA-Tam and ligand-mediated endocytosis assay was performed according to the method described before. As expected control cells showed very little internal fluorescence and upon 100 µM DHPG treatment receptors were seen to internalize in 30 min. shTam transfected cells showed block in the myc-mGluR1 endocytosis **(control = 1 ± 0.04, DHPG = 1.80 ± 0.06, shTam + DHPG**

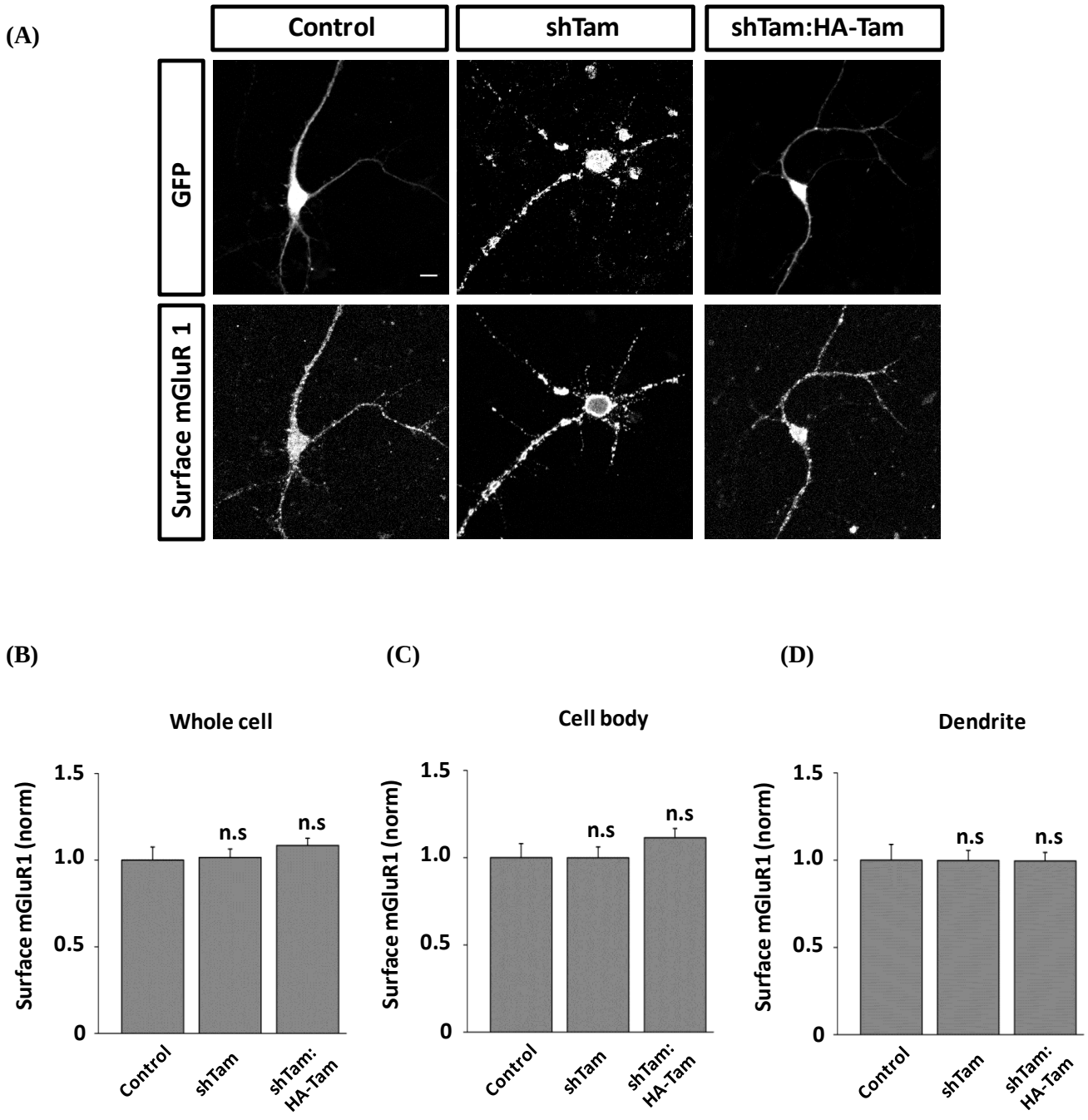


Figure 5.5. Effect of the expression of Tamalin replacement construct on the surface expression of mGluR1 (A) Representative images showing surface expression of myc-mGluR1 in shTam and shTam:HA-Tam transfected cells. There was no significant difference in myc-mGluR1 surface localization. (B), (C) and (D) Quantitation of surface myc-mGluR1 in whole cell, cell body and dendrites respectively suggested that there was no significant difference in myc-mGluR1 surface expression in both Tamalin knockdown cells as well as in cells expressing Tamalin replacement construct as compared to control cells. Scale bar = 10 μm, n.s indicates $p > 0.05$ N=3; where N represents the number of times experiment was repeated.

= 1.07 ± 0.05) (Figure. 5.6 A, B). Importantly, in the cells expressing the HA-Tam replacement construct complete rescue of the myc-mGluR1 endocytosis was observed **(shTam:HA-Tam + DHPG = 1.81 ± 0.09)**. In addition, complete rescue of the DHPG-mediated internalization of myc-mGluR1 was observed in both cell body and dendrites of the cell **(Cell body: control = 1 ± 0.05, DHPG = 1.70 ± 0.07, shTam + DHPG = 1.14 ± 0.04, shTam:HA-Tam + DHPG = 1.80 ± 0.10, Dendrites: control = 1 ± 0.03, DHPG = 1.83 ± 0.05, shTam + DHPG = 1.06 ± 0.06 and shTam:HA-Tam + DHPG = 1.89 ± 0.10)**. **(Figure 5.6 C, D).** Since, in our assays the effect of knockdown and rescue of Tamalin in both surface expression and ligand-mediated endocytosis of the receptor were similar in both cell body and dendrites, in subsequent experiments we have represented the dendritic surface expression as well as dendritic endocytosis of the receptor.

5.2.5. Knockdown of endogenous Tamalin leads to the inhibition of ligand-mediated endocytosis of mGluR5

As stated before, group I mGluRs consists of mGluR1 and mGluR5 and previous reports have suggested interaction of Tamalin with both mGluR1 and mGluR5 (Kitano et al., 2002). Since our earlier experiments suggested that Tamalin plays critical role in the ligand-mediated endocytosis of mGluR1, we investigated whether it also plays any role in the trafficking of mGluR5. We initially checked the effect of knockdown of endogenous Tamalin on the surface expression of myc-mGluR5 in primary hippocampal neurons using the similar protocol as described previously for mGluR1. Similar to myc-mGluR1, knockdown of endogenous Tamalin had no effect on the surface expression of myc-mGluR5 **(control = 1 ± 0.09, shTam = 1.01 ± 0.04) (Figure 5.7 A, B)**.

We also investigated the effect of the knockdown of endogenous Tamalin on ligand-mediated internalization of myc-mGluR5 using the similar protocol as has been described above for the study of myc-mGluR1 internalization. Control cells showed presence of majority of the receptors at the cell surface and upon 100 µM DHPG treatment majority of the receptors internalized in 30 min. Importantly, in shTam transfected cells the DHPG-mediated internalization of myc-mGluR5 was completely inibited **(control = 1 ± 0.09, DHPG. = 2.19 ± 0.07, shTam + DHPG = 1.13 ± 0.05)**. **(Figure 5.8 A, B).** These results suggest that Tamalin plays critical role in the ligand-mediated endocytosis of both members of group I mGluRs, i.e., mGluR1 and mGluR5.

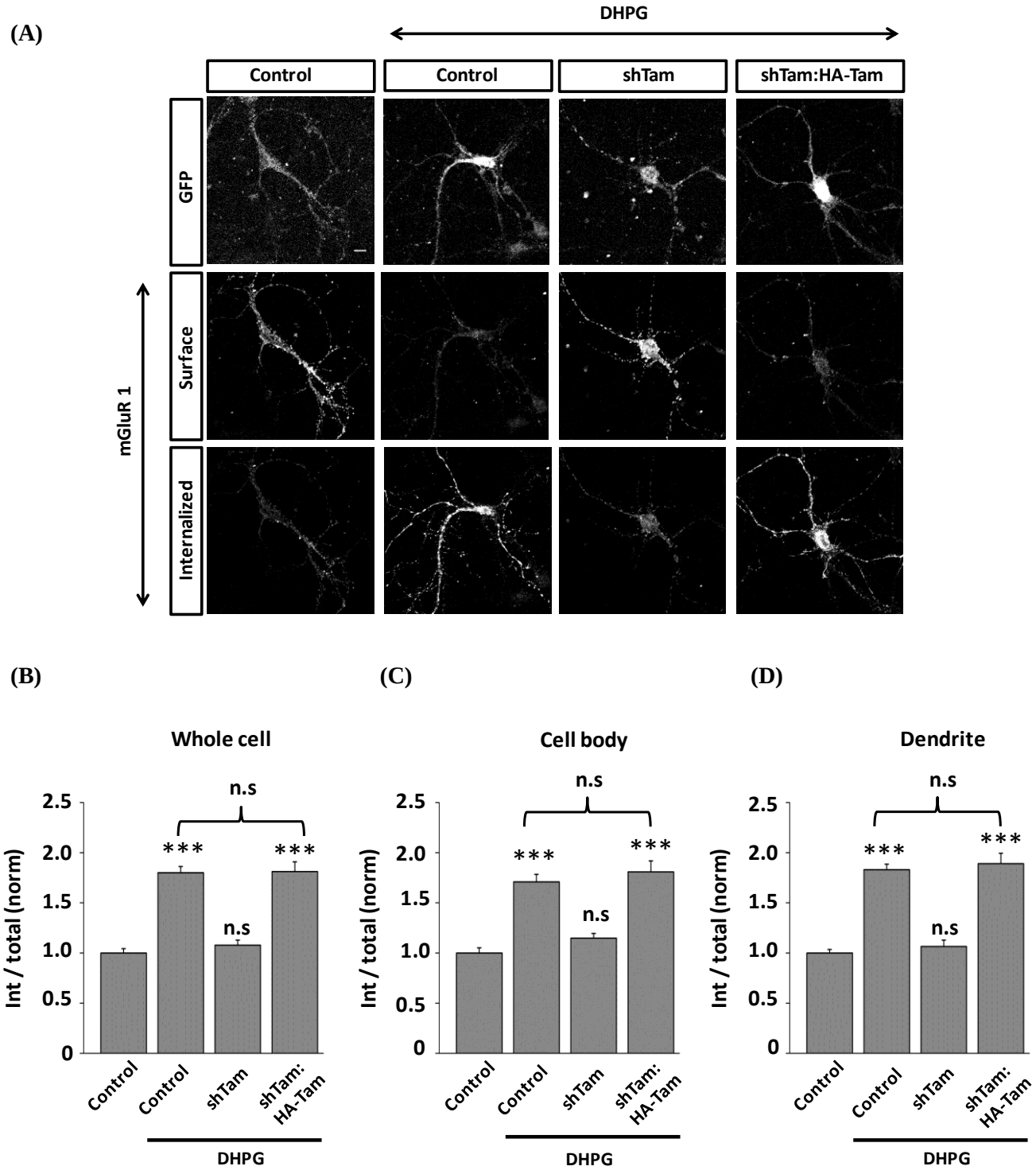
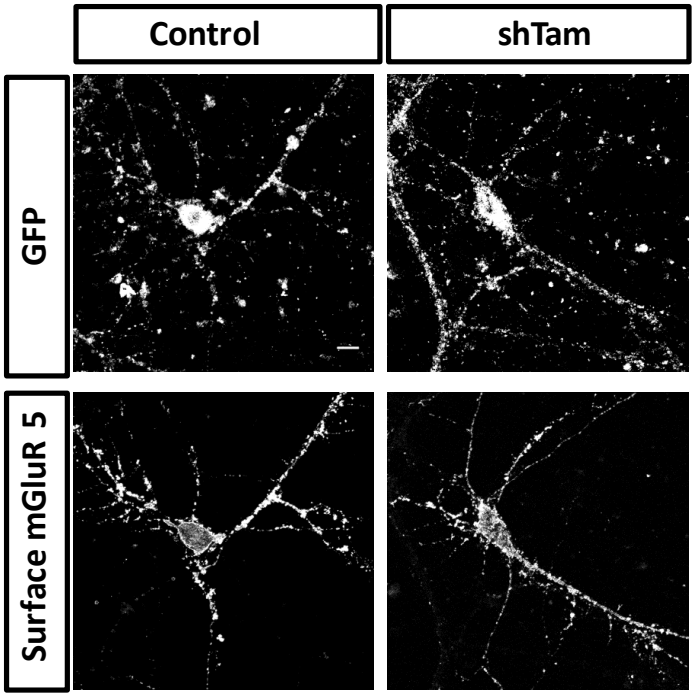


Figure 5.6. Rescue of DHPG-mediated mGluR1 endocytosis by expression of the Tamalin replacement construct (A) Representative images showing the DHPG-mediated myc-mGluR1 endocytosis. Control cells showed endocytosis upon 100 µM DHPG application, whereas shTam transfected cells showed block in the endocytosis. Importantly, expression of the Tamalin replacement construct rescued the myc-mGluR1 endocytosis. (B), (C) and (D) Quantitation of DHPG-mediated endocytosis of myc-mGluR1 in whole cell, cell body and dendrites respectively. Scale bar = 10 µm. ***$p < 0.001$, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

(A)



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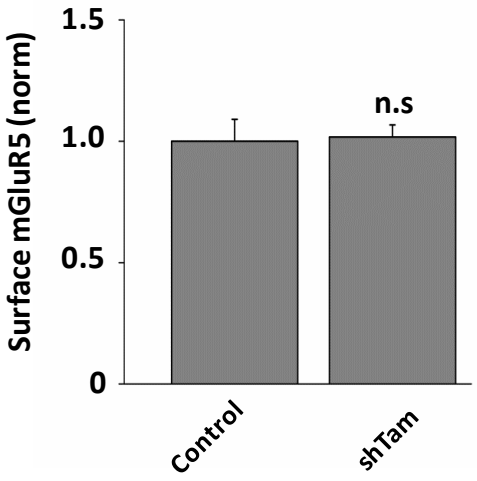
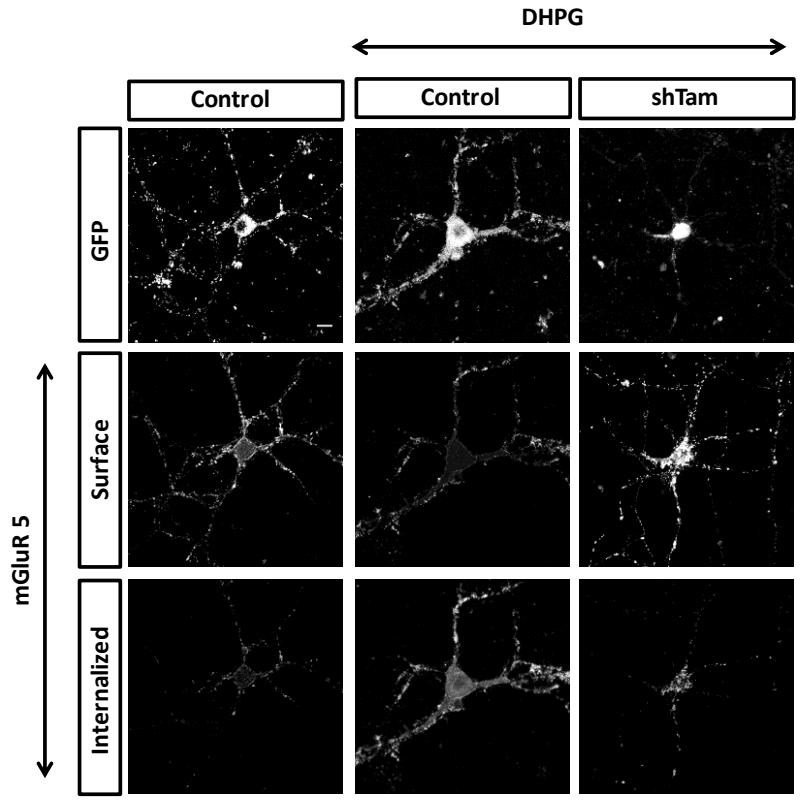


Figure 5.7. Effect of the knockdown of endogenous Tamalin on the surface expression of mGluR5 (A) Representative images showing surface expression of myc-mGluR5 in primary hippocampal neurons. There was no significant difference observed in surface myc-mGluR5 expression in both control cells and shTam expressing cells. (B) Quantitation of myc-mGluR5 surface expression in control and shTam expressing cells also suggested that knockdown of endogenous Tamalin did not affect the surface expression of myc-mGluR5. Scale bar = 10 μm, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

(A)



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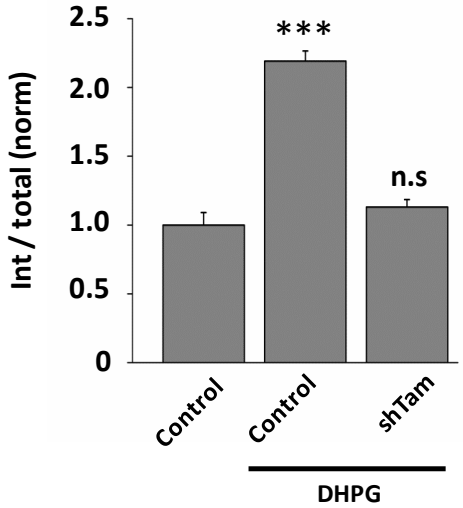


Figure 5.8. Knockdown of endogenous Tamalin leads to the inhibition in the ligand-mediated endocytosis of mGluR5 (A) Representative images showing the inhibition in the DHPG-mediated myc-mGluR5 endocytosis in primary hippocampal neurons due to the knockdown of endogenous Tamalin. Control cells showed very little internal fluorescence which increased upon 100 µM DHPG application. Importantly shTam expressing cells showed inhibition in the endocytosis. (B) Quantitation of this experiment also suggested that knockdown of endogenous Tamalin resulted in the inhibition of the ligand-mediated myc-mGluR5 internalization. Scale bar = 10 µm. ***$p < 0.001$, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated

5.2.6. Expression of wild-type Tamalin rescues the ligand-mediated endocytosis of mGluR5

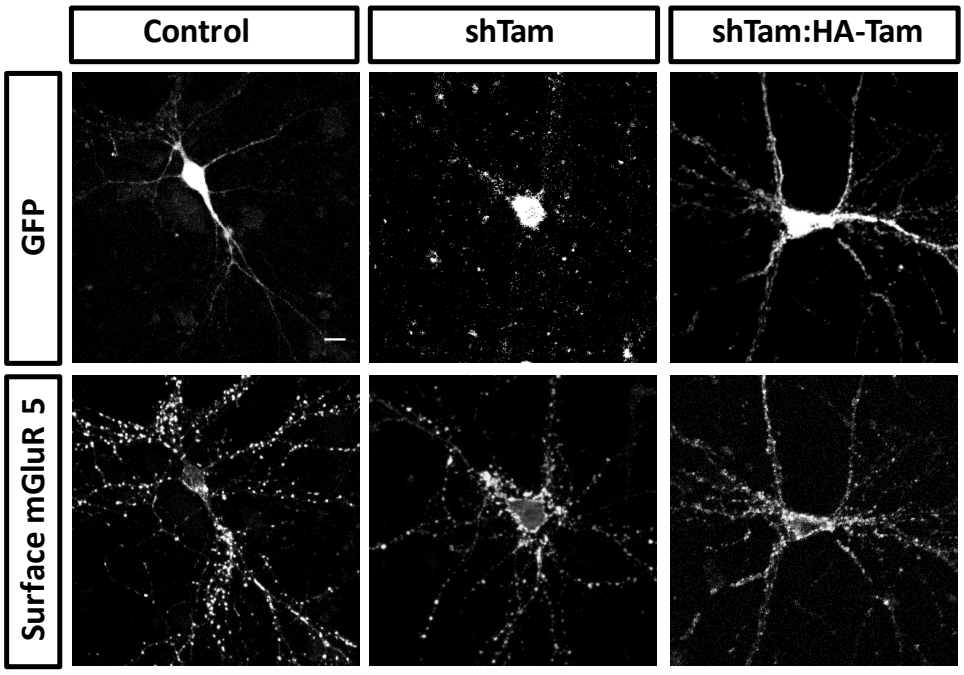
The ability of the wild type Tamalin to rescue the DHPG-mediated endocytosis of myc-mGluR5 was next investigated. Initially we investigated the effect of the expression of wild type Tamalin replacement construct on the surface expression of myc-mGluR5 using the similar protocol that had been used for myc-mGluR1. No significant difference in myc-mGluR5 surface fluorescence was observed in control, shTam and shTam:HA-Tam expressing cells. **(control = 1 ± 0.04, shTam = 0.98 ± 0.04, shTam:HA-Tam = 0.98 ± 0.05) (Figure 5.9 A, B)**.

Subsequently, effect of over-expression of Tamalin replacement construct, i.e., shTam:HA-Tam on the ligand-dependent internalization of myc-mGluR5 was investigated. As expected, in control cells myc-mGluR5 internalized on 100 μM DHPG treatment, whereas, knockdown of endogenous Tamalin inhibited the ligand-dependent internalization of the receptor **(control = 1 ± 0.04, DHPG = 2.00 ± 0.06, shTam + DHPG = 1.02 ± 0.04) (Figure 5.10 A, B)**. Importantly, replacement of endogenous Tamalin with HA-Tam rescued the ligand-dependent internalization of myc-mGluR5 **(shTam:HA-Tam + DHPG = 1.95 ± 0.05)**.

5.2.7. Tamalin plays critical role in the group I mGluR-mediated AMPAR endocytosis

As stated before, rapid endocytosis of surface AMPARs can be triggered in cultured hippocampal neurons by application of various glutamate receptor agonists, including glutamate itself, NMDA, AMPA and group I mGluR agonists (Carroll et al., 2001). mGluR-mediated AMPAR endocytosis is believed to be the cellular correlate for the mGluR-dependent synaptic plasticity (Bhattacharyya, 2016). Our earlier experiments suggested that Tamalin played very important role in the ligand-dependent endocytosis of group I mGluRs. In order to check whether Tamalin plays any role in the mGluR-mediated AMPAR endocytosis, we studied the effect of knockdown of endogenous Tamalin on the mGluR-mediated AMPAR trafficking. The experimental protocol has been described in detail in the "method" section. Briefly, cells were transfected with either shTam or Tamalin replacement construct (shTam:HA-Tam). Initially we checked whether knockdown of endogenous

(A)



(B)

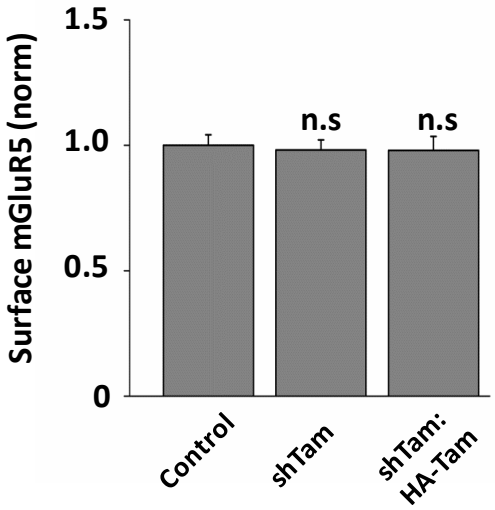


Figure 5.9. Expression of Tamalin replacement construct does not affect the surface localization of mGluR5 (A) Representative images showing surface distribution of myc-mGluR5 in shTam and shTam:HA-Tam expressing cells. (B) Quantitation also suggested that both knockdown of endogenous Tamalin and expression of Tamalin replacement construct had no effect on the surface localization of myc-mGluR5. Scale bar = 10 μm, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

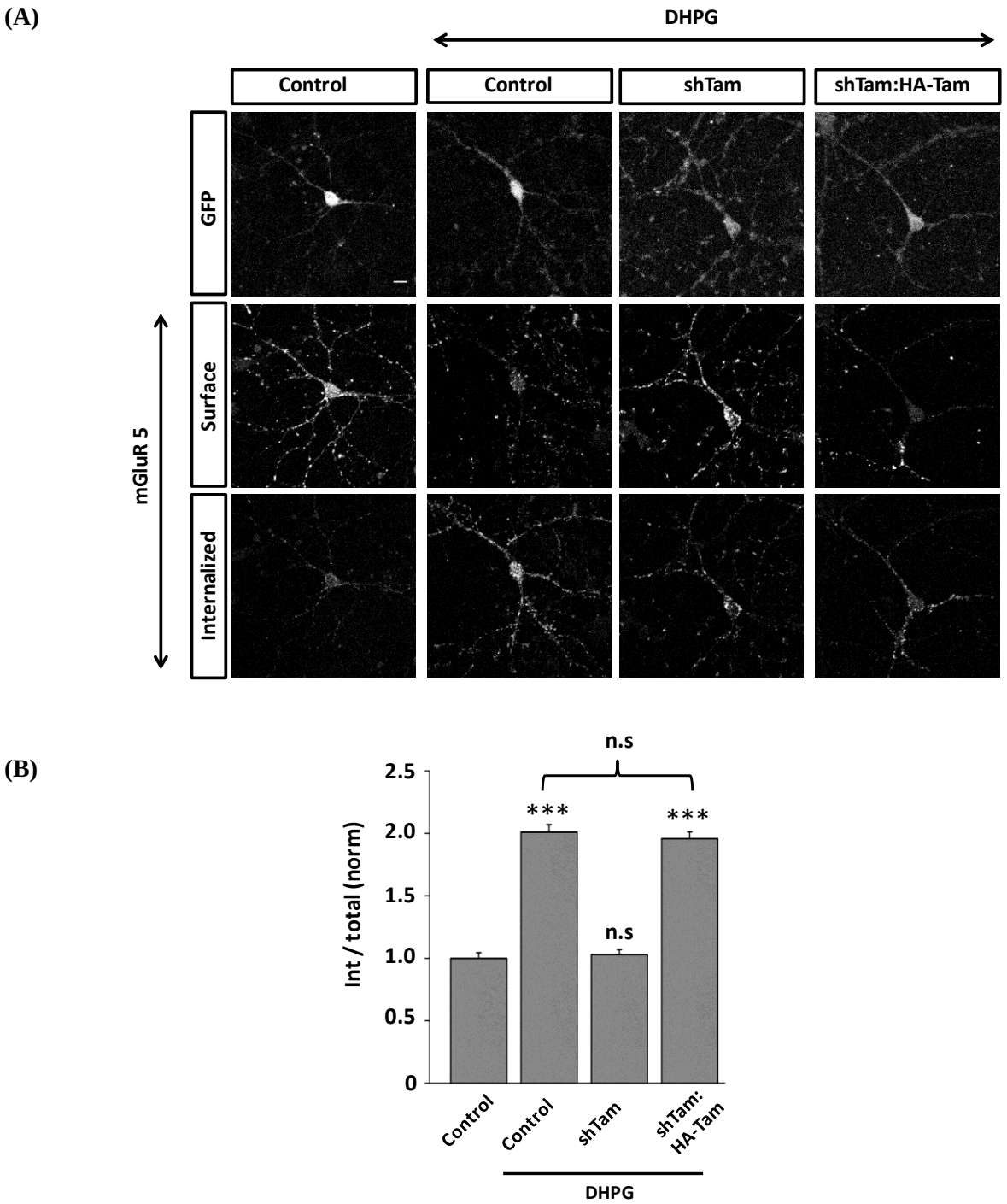


Figure 5.10. Tamalin replacement construct rescues the inhibition in mGluR5 internalization caused by the knockdown of endogenous Tamalin (A) Representative images showing inhibition in the DHPG-mediated myc-mGluR5 endocytosis upon knockdown of endogenous Tamalin and rescue of the endocytosis on expression of Tamalin replacement construct. (B) Quantitation also suggested that knockdown of endogenous Tamalin led to the inhibition in the ligand-mediated endocytosis of myc-mGluR5 which was rescued by the expression of the Tamalin replacement construct. Scale bar = 10 μm. ***$p < 0.001$, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

Tamalin had any effect on the surface expression of GluA1containing receptors. Our data suggested that either knockdown of endogenous Tamalin or replacement of endogenous Tamalin with HA-Tam had no effect on the surface expression of GluA1 containing receptors. **(control = 1 ± 0.04, shTam = 0.98 ± 0.03, shTam:HA-Tam = 1.00 ± 0.04) (Figure 5.11 A, B)**. There was no significant difference in the surface expression of GluA1 containing receptors observed in both cell body and dendrites **(Cell body: control = 1 ± 0.03, shTam = 0.99 ± 0.05, shTam:HA-Tam = 0.99 ± 0.04, Dendrite: control = 1 ± 0.05, shTam = 0.99 ± 0.03, shTam:HA-Tam = 0.99 ± 0.06) (Figure 5.11 C, D)**

Subsequently, the role of Tamalin in the mGluR-mediated AMPAR endocytosis was studied by knocking down the endogenous Tamalin. The mGluR-mediated AMPAR endocytosis assay was performed according to the protocol described in the "method" section. In control cells majority of the GluA1 containing receptors were present at the surface and upon 100 µM DHPG application, receptors were observed to internalize **(control = 1 ± 0.02, DHPG = 1.58 ± 0.02) (Figure 5.12 A, B)**. Importantly, in Tamalin shRNA transfected cells, no significant internalization of the AMPARs was observed and a complete rescue of the mGluR-mediated AMPAR endocytosis was observed by the expression of the Tamalin replacement construct **(shTam + DHPG = 1.02 ± 0.02, shTam:HA-Tam + DHPG = 1.59 ± 0.02)**. Furthermore, in both cell body and dendrites knockdown of endogenous Tamalin resulted in the inhibition of the mGluR-mediated AMPAR endocytosis which was rescued by the expression of Tamalin replacement construct **(Cell body: control = 1 ± 0.02, DHPG = 1.28 ± 0.03, shTam + DHPG = 1.10 ± 0.04, shTam:HA-Tam + DHPG = 1.34 ± 0.05, Dendrite: control = 1 ± 0.04, DHPG = 1.69 ± 0.02, shTam + DHPG = 1.00 ± 0.02 and shTam:HA-Tam + DHPG = 1.68 ± 0.02) (Figure 5.12 C, D)**. These results suggest that Tamalin plays a critical role in the mGluR-mediated AMPAR endocytosis and since its effect is similar throughout the neuron, in the future experiments we will represent the quantitation of dendritic endocytosis only.

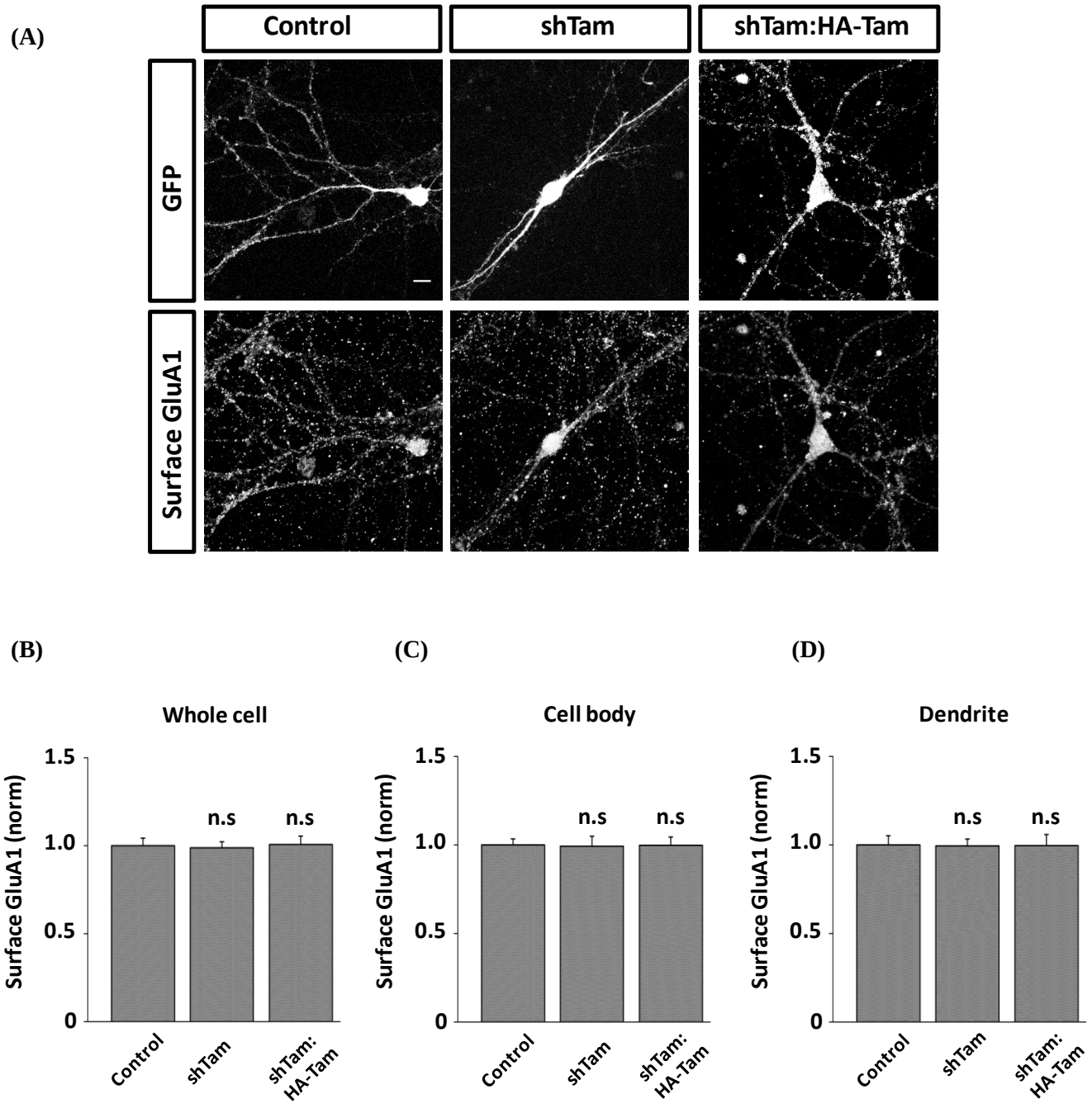


Figure 5.11. Knockdown of endogenous Tamalin does not affect the surface localization of GluA1 containing receptors (A) Representative images showing surface localization of GluA1 containing receptors. No significant difference in surface distribution of GluA1 containing receptors were observed upon knockdown of endogenous Tamalin or expression of the Tamalin replacement construct. (B), (C) and (D) Quantitation of surface GluA1 containing receptors in whole cell, cell body and dendrites respectively. Scale bar = 10 μm, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

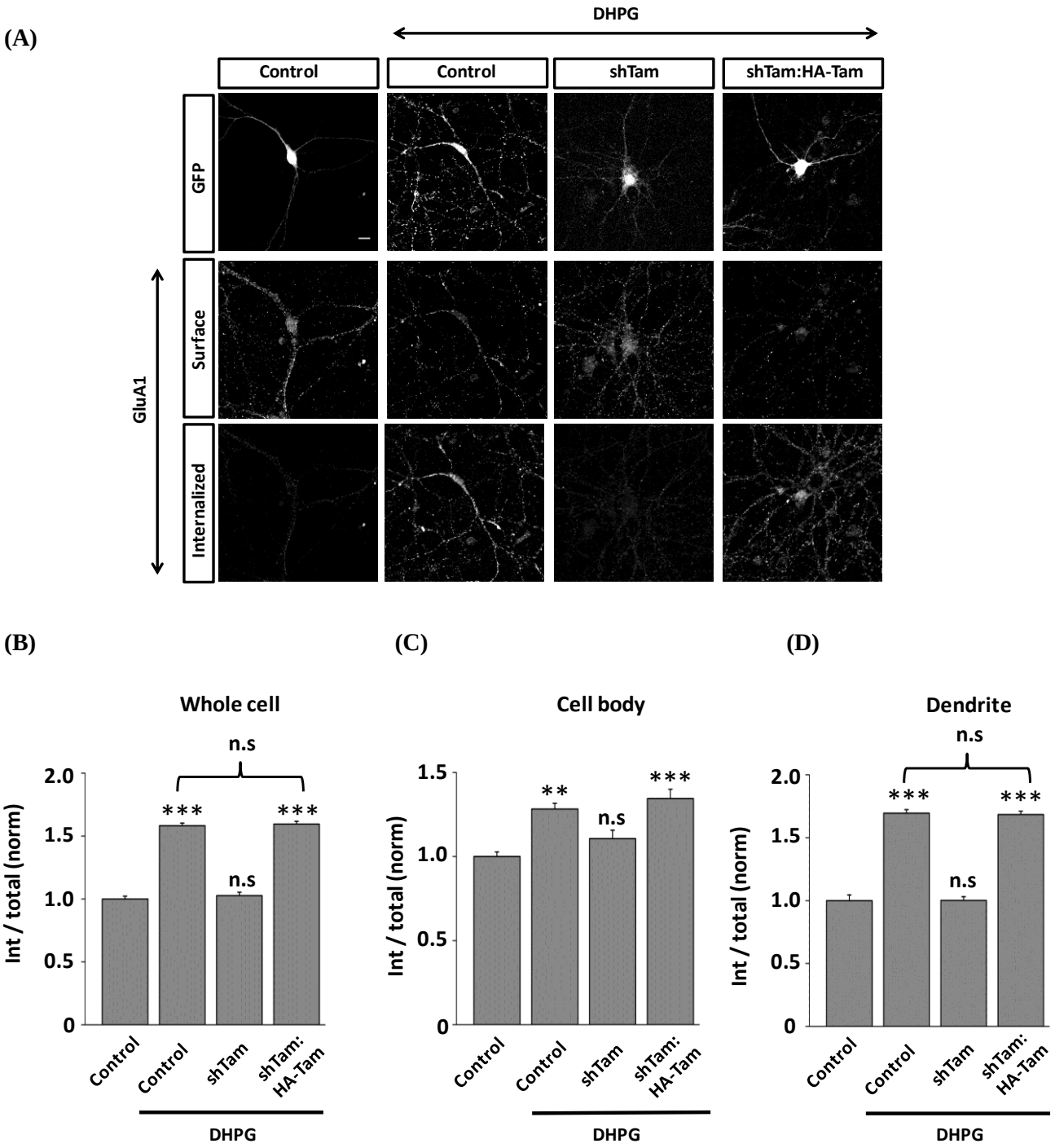


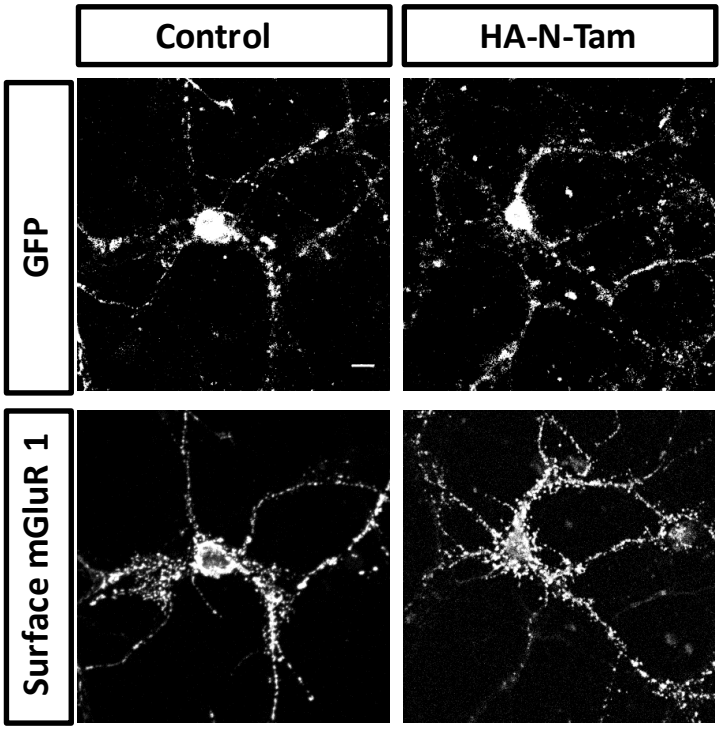
Figure 5.12. Tamalin plays critical role in the mGluR-mediated AMPAR endocytosis (A) Representative images showing DHPG-mediated endocytosis of GluA1 containing receptors. Control cells showed very little internalized receptors and upon DHPG application the receptors internalized. Upon knockdown of endogenous Tamalin endocytosis of GluA1 containing receptors were completely inhibited and which was rescued by the expression of the Tamalin replacement construct. (B), (C) and (D) Quantitation of the effect of Tamalin knockdown and replacement construct on the mGluR-mediated AMPAR endocytosis in whole cell, cell body and dendrites. Scale bar = 10 μm. ***$p < 0.001$, **$p < 0.01$, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

5.2.8. Over-expression of the N-terminal domain of Tamalin (N-Tam) leads to the inhibition in the mGluR1 endocytosis

In order to investigate the domains of Tamalin that are critical for the ligand-dependent endocytosis of mGluR1 we generated various Tamalin mutants. Initially, we made a construct that contains the N-terminal domain of Tamalin (N-Tam). Previous reports have suggested that Tamalin interacts with group I mGluRs through its PDZ domain and the N-Tam (1-209 amino acids) contains the PDZ domain but lacks the C-terminal domain of the protein. Initially, the effect of over-expression of HA-N-Tam on the surface expression of myc-mGluR1 was investigated. Over-expression of N-Tam did not cause any significant change in the myc-mGluR1 surface expression. **(control = 1 ± 0.04, HA-N-Tam = 0.94 ± 0.05) (Figure 5.13 A, B)**.

Subsequently, the over-expression of HA-N-Tam on the ligand-mediated endocytosis of myc-mGluR1 was studied. HA-N-Tam and myc-mGluR1 constructs were co-transfected in the primary hippocampal neurons and endocytosis experiments were performed subsequent to that following the protocol as described before. As expected, control cells showed very low internal fluorescence and majority of the receptors were observed to be present on the cell surface. Receptors were observed to be internalized on 100 µM DHPG application at 30 min **(control = 1 ± 0.05, DHPG = 1.72 ± 0.03) (Figure 5.14 A, B)**. Importantly, In N-Tam overexpressed cells, complete block in the DHPG-mediated endocytosis of the receptor was observed **(HA-N-Tam + DHPG = 1.02 ± 0.05)**. These results suggest that N-Tam might be acting as a dominant negative in the ligand-mediated endocytosis of mGluR1.

(A)



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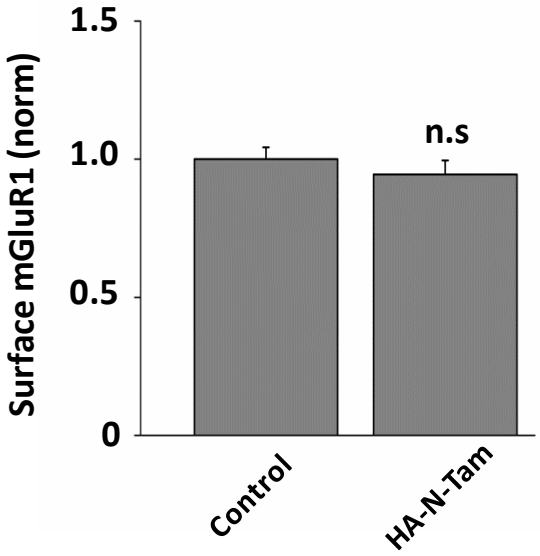


Figure 5.13. Over-expression of N-Tam does not affect the surface expression of mGluR1 (A) Representative images showing surface expression of myc-mGluR1 in control and N-Tam overexpressing cells. No significant change in the surface expression of myc-mGluR1 was observed in N-Tam expressing cells as compared to control cells. (B) Quantitation of the surface expression of myc-mGluR1 in control cells and cells expressing N-Tam. Scale bar = 10 μm, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

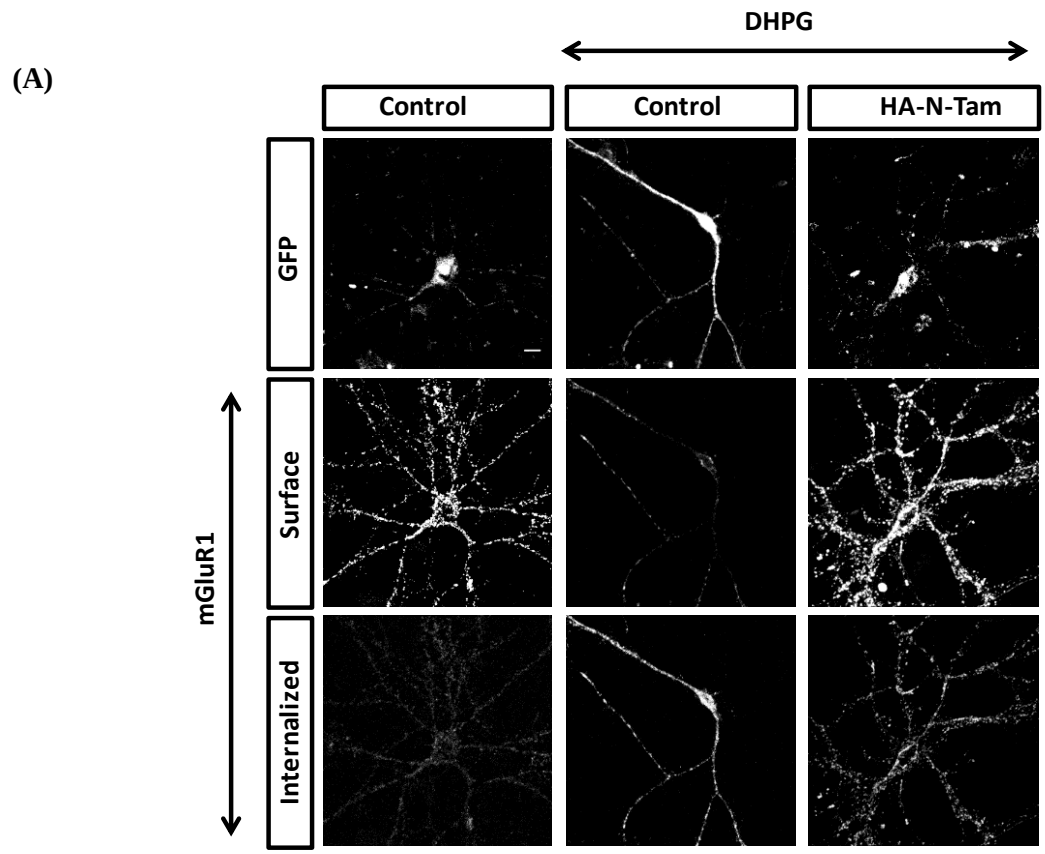


Figure 5.14. Over-expression of N-Tam inhibits the ligand-mediated endocytosis of mGluR1 (A) Control cells showed presence of majority of the receptors at the cell surface and upon 100 µM DHPG application myc-mGluR1 internalized. Importantly, in N-Tam overexpressing cells myc-mGluR1 internalization was completely inhibited. (B) Quantitation also suggested that over-expression of N-Tam inhibited the DHPG-mediated endocytosis of myc-mGluR1. Scale bar = 10 µm. ***$p < 0.001$, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

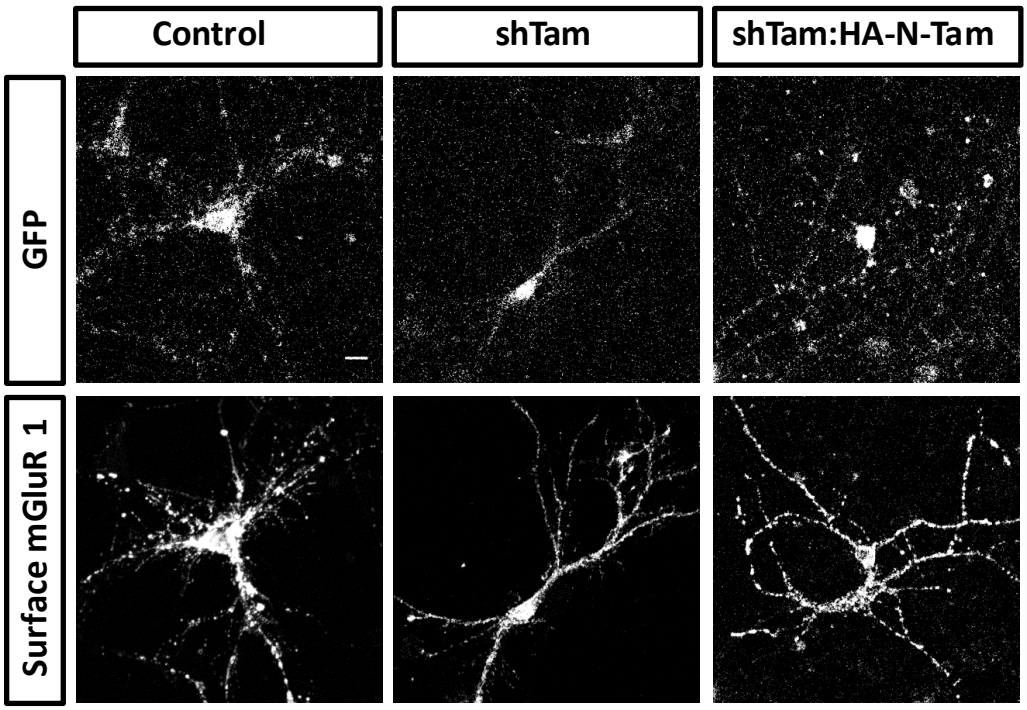
5.2.9. N-Tam replacement construct does not rescue the ligand-mediated internalization of mGluR1

Our previous experiments suggested that knockdown of endogenous Tamalin and over-expression of N-Tam both inhibited the ligand-mediated internalization of myc-mGluR1. In order to check whether expression of N-Tam replacement construct (shTam:HA-N-Tam) could resue the ligand dependent endocytosis of the receptor, the N-Tam replacement construct was generated by the method as described in the "methods" section.

Initially the effect of N-Tam replacement construct on the surface expression of mGluR1 was studied by co-transfection of cells with myc-mGluR1 and either with shTam or shTam:HA-N-Tam. There was no significant difference in myc-mGluR1 surface expression observed between control, shTam and shTam:HA-N-Tam expressing cells. **(control = 1 ± 0.03, shTam = 0.91 ± 0.04, shTam:HA-N-Tam = 0.93 ± 0.04) (Figure 5.15 A, B)**.

Subsequently, we investigated whether N-Tam replacement construct could rescue the inhibition in the ligand-mediated endocytosis of the receptor caused by the knockdown of endogenous Tamalin. Control cells showed very little internal fluorescence and upon 100 µM DHPG application receptors internalized in 30 min as observed by the increase in the internal fluorescence. On the other hand, shTam transfected cells showed complete inhibition in the myc-mGluR1 endocytosis **(control = 1 ± 0.05, DHPG = 1.94 ± 0.03, shTam + DHPG = 1.08 ± 0.05)(Figure 5.16 A, B)**. Importantly, N-Tam replacement construct expressing cells did not show any rescue in the internalization of the receptor, suggesting that N terminal domain of Tamalin is not sufficient for the ligand-mediated endocytosis of myc-mGluR1 **(shTam:HA-N-Tam + DHPG = 1.03 ± 0.04)**.

(A)



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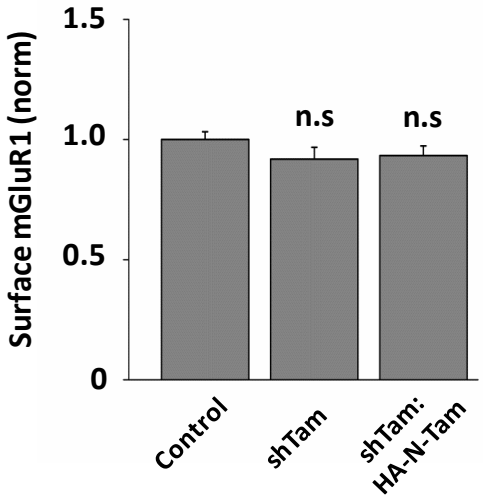


Figure 5.15. Effect of surface expression of mGluR1 upon expression of N-Tam replacement construct (A) Representative images showing similar myc-mGluR1 surface expression in control cells, in cells expressing Tamalin shRNA and in cells expressing Tamalin replacement construct. (B) Quantitation also suggested that both knockdown of endogenous Tamalin and over-expression of Tamalin replacement construct had no effect on the surface localization of myc-mGluR1. Scale bar = 10 μm, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

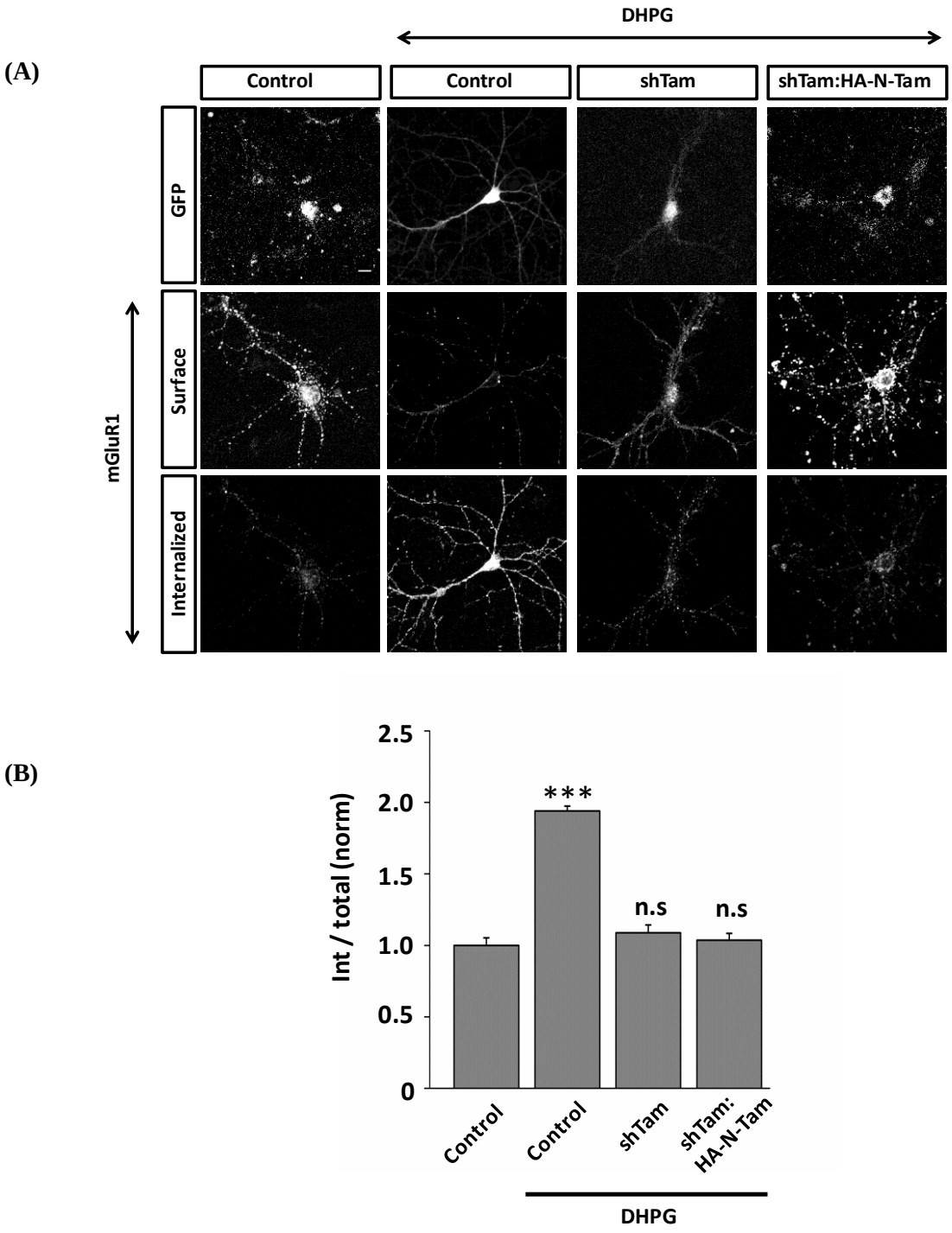


Figure 5.16. N-Tam is not sufficient to rescue the inhibition in the mGluR1 endocytosis caused by the knockdown of endogenous Tamalin (A) Control cells showed very little internalized receptors. The extent of endocytosis increased upon 100 µM DHPG application. The internalization was inhibited upon knocking down the endogenous Tamalin and expression of N-Tam could not rescue this inhibition. (B) Quantitation also suggested that N-Tam replacement construct did not rescue the inhibition in the endocytosis of myc-mGluR1 caused by the knockdown of endogenous Tamalin. Scale bar = 10 µm. ***$p < 0.001$, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

5.2.10. N-Tam replacement construct does not rescue the group I mGluR-mediated AMPAR endocytosis

Our previous experiments suggested that N-Tam replacement construct was not able to rescue the block in the ligand-mediated mGluR1 endocytosis caused by the knockdown of endogenous Tamalin. Subsequently, experiments were performed to investigate whether this construct could rescue the block in the mGluR-mediated AMPAR endocytosis due to knockdown of endogenous Tamalin.

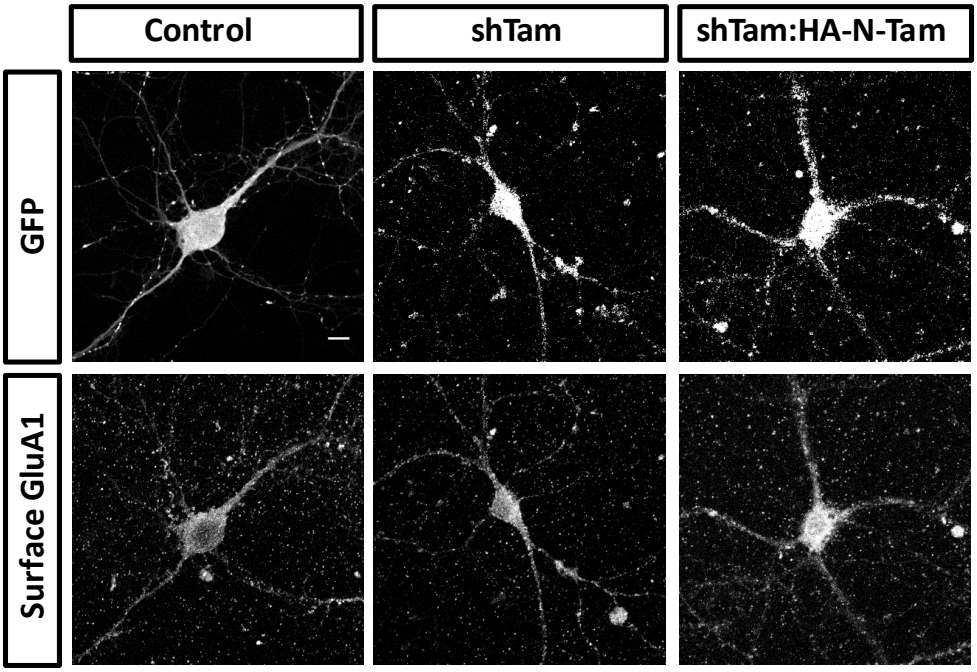
Initially, we studied whether the N-Tam replacement construct affects the surface expression of GluA1 containing receptors in primary hippocampal neurons. Our data showed that both knockdown of endogenous Tamalin and expression of N-Tam replacement construct had no effect on the surface expression of GluA1 containing receptors as compared to control cells **(control = 1 ± 0.03, shTam = 0.96 ± 0.03, shTam:HA-N-Tam = 0.95 ± 0.03) (Figure 5.17 A, B)**.

We next investigated whether N-Tam replacement construct could rescue the Tamalin knockdown effect on the mGluR-mediated AMPAR endocytosis. As expected, control cells showed very little internal fluorescence and after 100 µM DHPG application, AMPARs internalized in control cells at 15 min. Similar to our previous results, knockdown of endogenous Tamalin inhibited the mGluR-mediated AMPAR endocytosis **(control = 1 ± 0.03, DHPG. = 1.76 ± 0.04, shTam + DHPG = 1.13 ± 0.03) (Figure 5.18 A, B)**. Importantly, N-Tam replacement construct did not rescue the mGluR-mediated AMPAR endocytosis **(shTam:HA-N-Tam + DHPG = 0.96 ± 0.05)**. These results together suggest that although expression of N-terminal domain of Tamalin does not affect the surface expression of GluA1 containing receptors, this domain of Tamalin is not sufficient for the mGluR-mediated AMPAR endocytosis.

5.2.11. Role of the C-terminal domain of Tamalin (C-Tam) on the ligand-mediated internalization of mGluR1

Our previous experiments suggested that N-terminal domain of Tamalin alone was not sufficient for the ligand-mediated internalization of mGluR1. These results prompted us to study the role of the C-terminal domain (HA-C-Tam) of Tamalin in the mGluR1 endocytosis. First, the C-Tam over-expression (173 – 394 amino acid) and C-Tam Replacement (shTam:

(A)



(B)

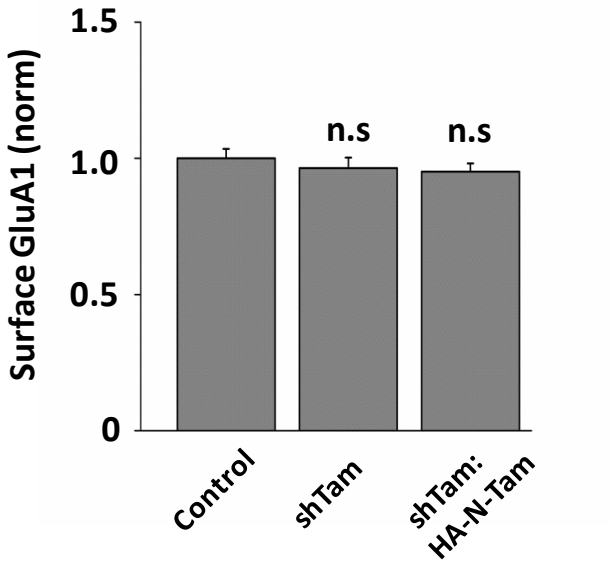
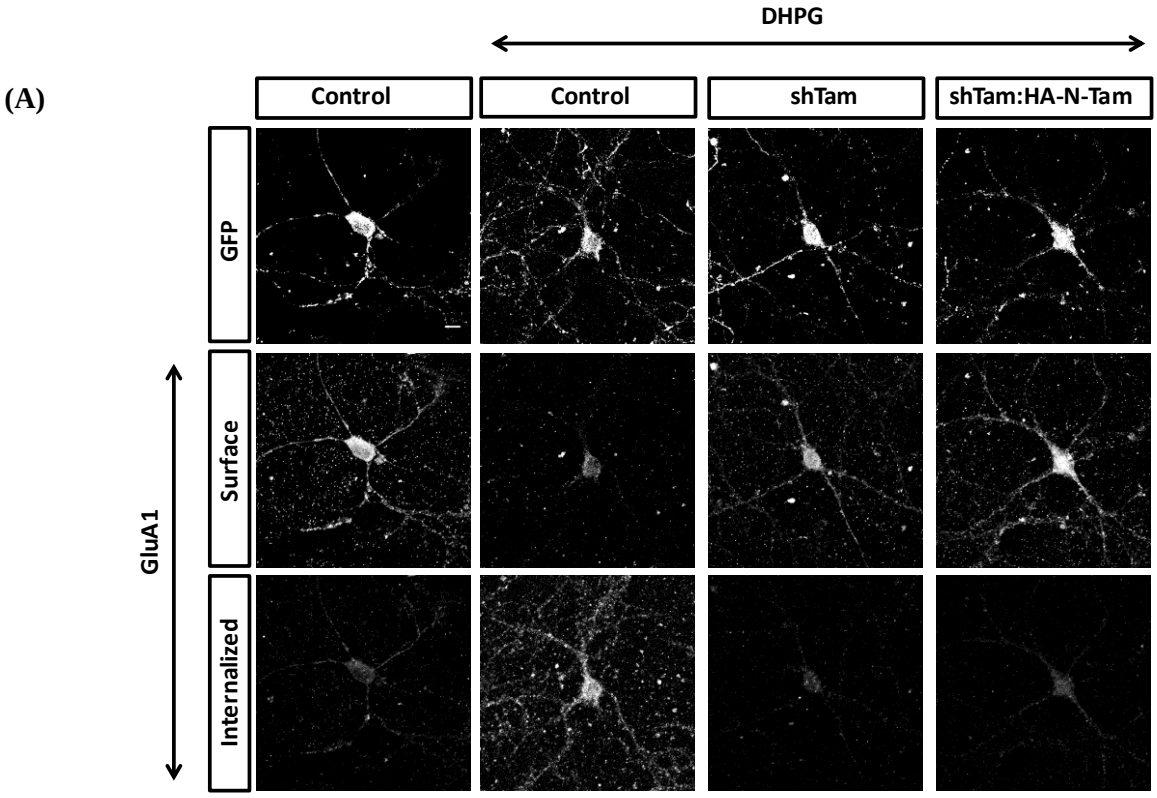


Figure 5.17. N-Tam does not play any role in the surface localization of GluA1 containing receptors (A) Representative images showing surface expression of GluA1 containing receptors in control cells, cells expressing Tamalin shRNA and cells expressing N-Tam replacement construct. (B) Quantitation suggested that knockdown of endogenous Tamalin and expression of N-Tam replacement construct had no effect on the surface expression of the GluA1 containing receptors. Scale bar = 10 μm, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.



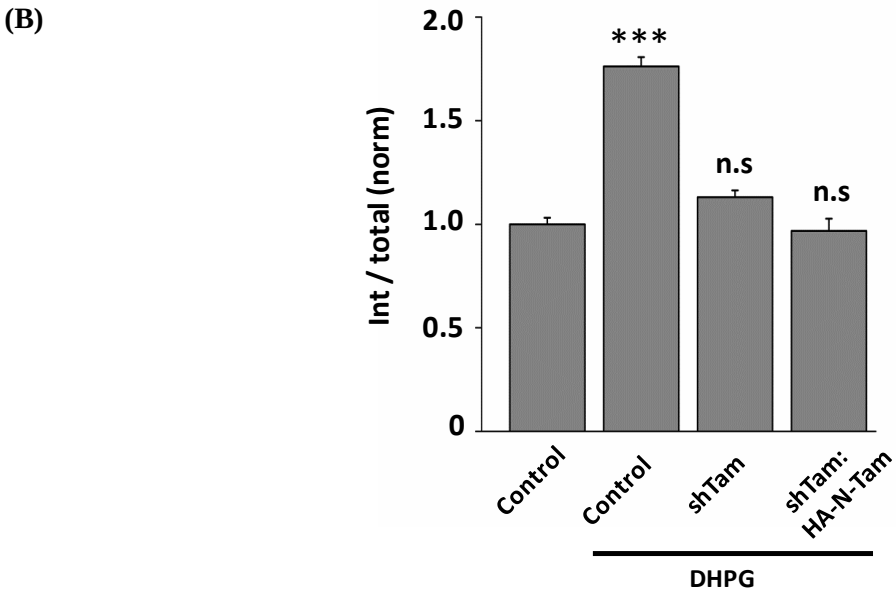


Figure 5.18. Effect of the expression of N-Tam replacement construct on mGluR-mediated AMPAR trafficking (A) Representative images showing mGluR-mediated endocytosis of GluA1 containing receptors in control cells. Knockdown of endogenous Tamalin blocked the endocytosis of these receptors. N-Tam replacement construct did not rescue the mGluR-mediated endocytosis of the GluA1 containing receptors. (B) Quantitation also suggested that N-Tam replacement did not rescue the mGluR-mediated endocytosis of GluA1 containing receptors. Scale bar = 10 μm. ***$p < 0.001$, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

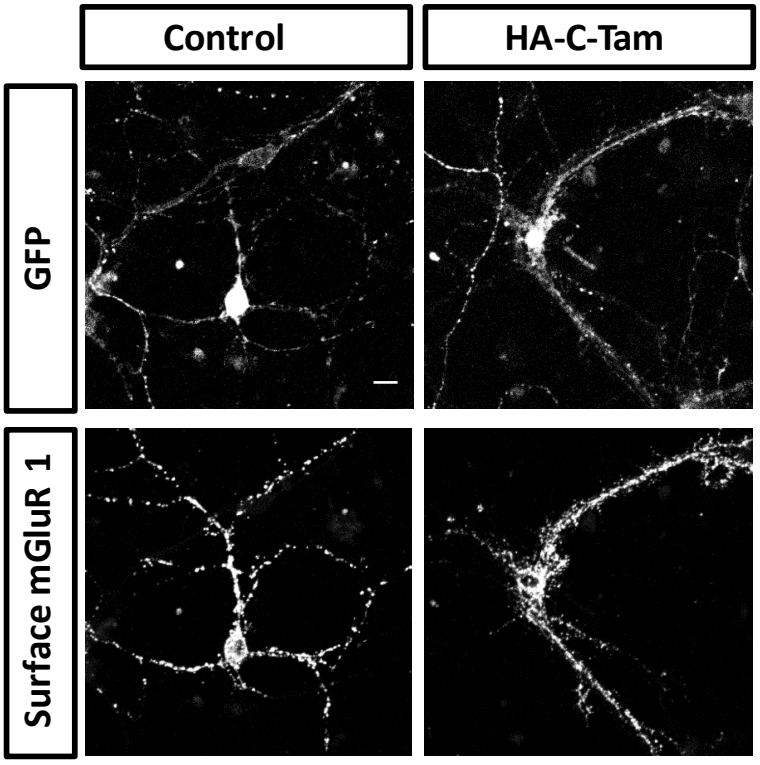
HA-C-Tam) constructs were generated. In order to investigate whether over-expression of HA-C-Tam could affect the surface expression of mGluR1, primary hippocampal neurons were co-transfected with myc-mGluR1 and HA-C-Tam. Subsequently, the myc-mGluR1 present at the cell surface was measured. There was no significant difference in myc-mGluR1 cell surface fluorescence observed upon HA-C-Tam over-expression as compared to control cells **(control = 1 ± 0.03, HA-C-Tam = 1.01 ± 0.03) (Figure 5.19 A, B)**.

Subsequently, we studied the effect of HA-C-Tam over-expression on the ligand-mediated endocytosis of myc-mGluR1. Control cells showed presence of majority of the receptors at the cell surface and receptors were seen to internalize in 30 min upon 100 µM DHPG application **(control = 1 ± 0.02, DHPG = 1.68 ± 0.01) (Figure 5.20 A, B)**. Importantly, in HA-C-Tam over expressed cells, myc-mGluR1 internalized normally similar to control cells, suggesting that HA-C-Tam over expression had no effect on the DHPG-mediated internalization of myc-mGluR1 **(HA-C-Tam + DHPG = 1.69 ± 0.02)**.

Our next sets of experiments were directed towards understanding the effect of C-Tam replacement construct (shTam:HA-C-Tam) on the surface expression and ligand-mediated endocytosis of mGluR1. There was no significant difference in myc-mGluR1 surface fluorescence observed between control cells, cells transfected with shTam and shTam:HA-C-Tam expressing cells **(control = 1 ± 0.03, shTam = 0.92 ± 0.06, shTam:HA-C-Tam = 1.07 ± 0.08) (Figure 5.21 A, B)**.

In order to investigate whether expression of the C-terminal domain of Tamalin could rescue the inhibition in the ligand-mediated endocytosis of mGluR1 caused by the knockdown of endogenous Tamalin, cells were transfected with either shTam or shTam:HA-C-Tam constructs along with myc-mGluR1. Subsequently, DHPG-mediated endocytosis of the receptor was performed using the method described before. Control cells showed very high surface fluorescence and very low internal fluorescence. Upon 100 µM DHPG application significant amount of internalized receptors were observed in control cells whereas in shTam expressing cells the myc-mGluR1 endocytosis was completely inhibited **(control = 1 ± 0.05, DHPG = 1.94 ± 0.03, shTam + DHPG = 1.08 ± 0.05)** (**Figure 5.22 A, B)**. On the other hand, shTam:HA-C-Tam expressing cells did not show significant internalization of the receptor, suggesting that C-terminal domain alone is not sufficient to rescue the ligand-mediated internalization of the receptor **(shTam:HA-C-Tam + DHPG = 0.97 ± 0.02)**.

(A)



(B)

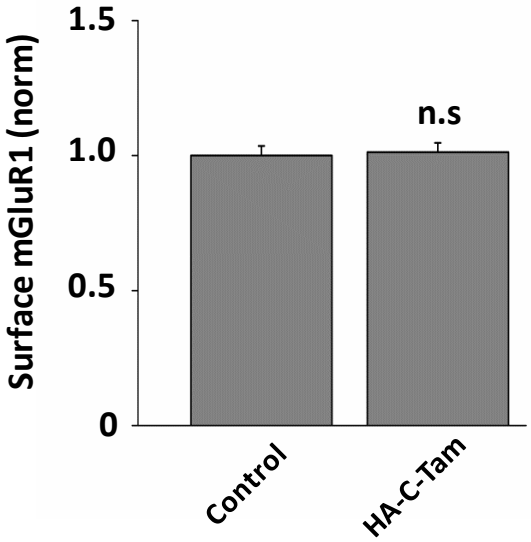


Figure 5.19. Over-expression of C-Tam does not have any effect on the surface expression of myc-mGluR1 (A) Representative images showing surface expression of myc-mGluR1 in control cells and C-Tam overexpressing cells. Over-expression of C-Tam had no effect on the surface expression of myc-mGluR1. (B) Quantitation of surface expression of myc-mGluR1 showed no significant difference between control cells and C-Tam over-expressing cells. Scale bar = 10 μm, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

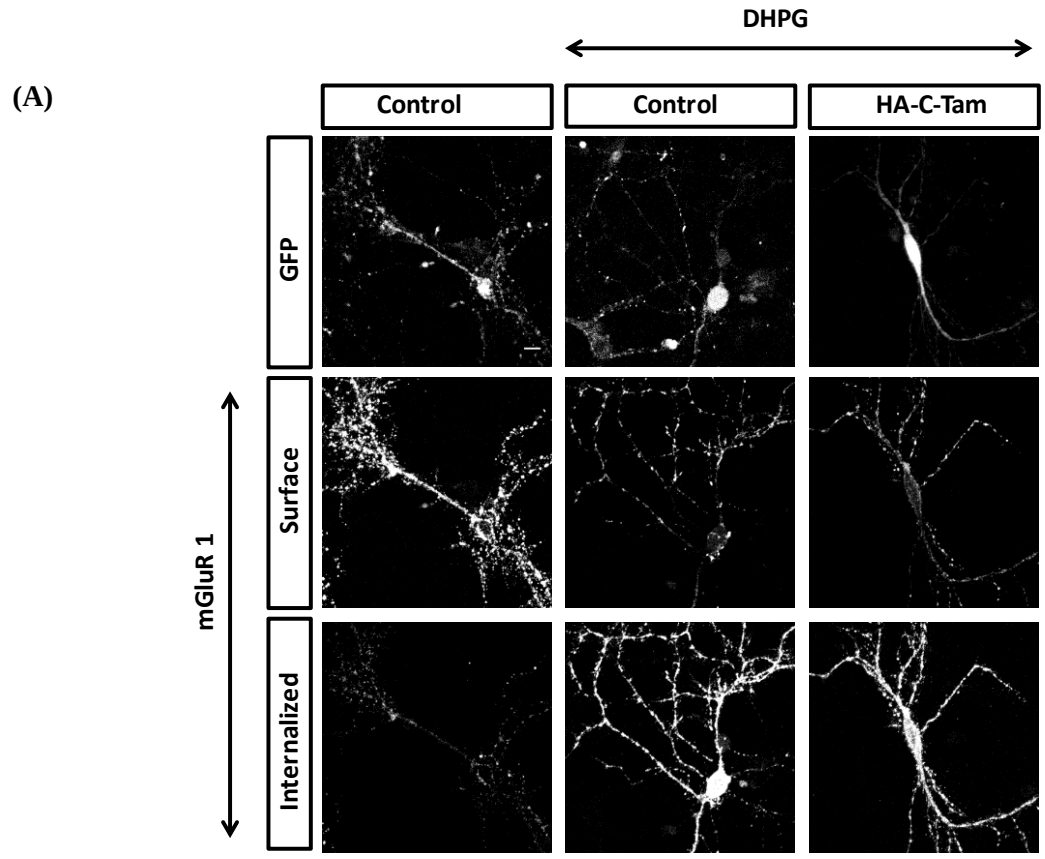
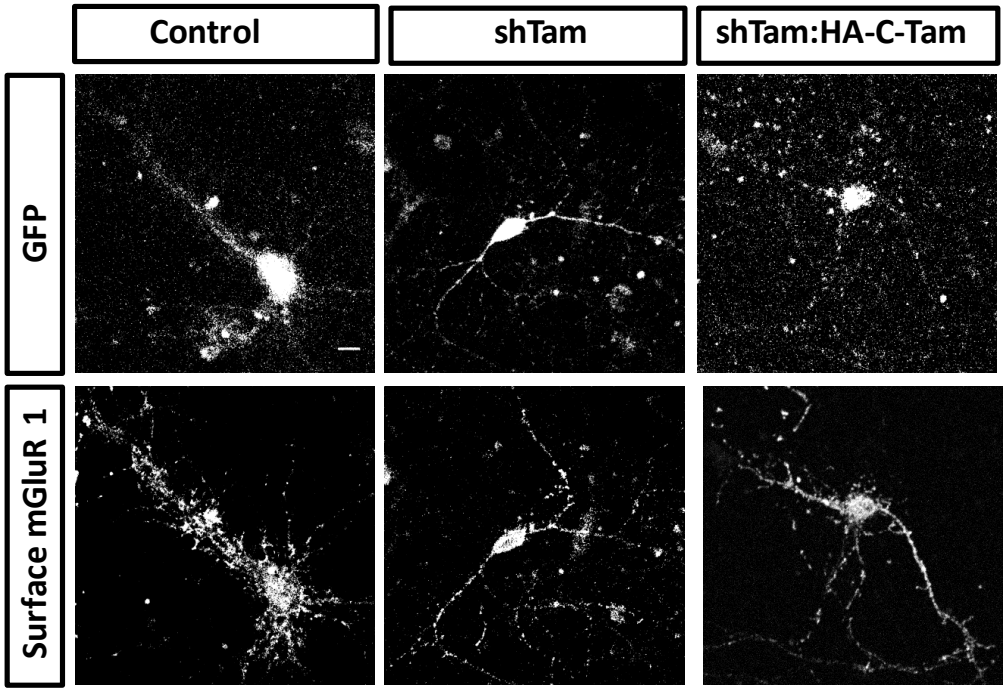


Figure 5.20. Effect of C-Tam over-expression on DHPG-mediated internalization of myc-mGluR1 (A) Control cells showed presence of majority of the receptors at the cell surface. The receptors internalized on 100 µM DHPG application. Over-expression of C-Tam did not affect the DHPG-mediated internalization of myc-mGluR1. (B) Quantitation of the effect of C-Tam on the DHPG-mediated endocytosis of myc-mGluR1. Scale bar = 10 µm. *** indicates $p < 0.001$, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

(A)



(B)

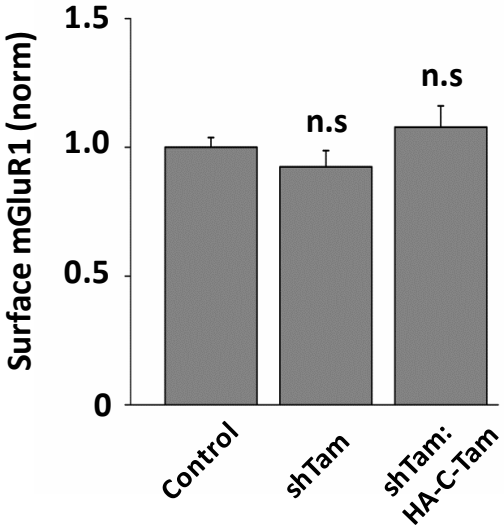
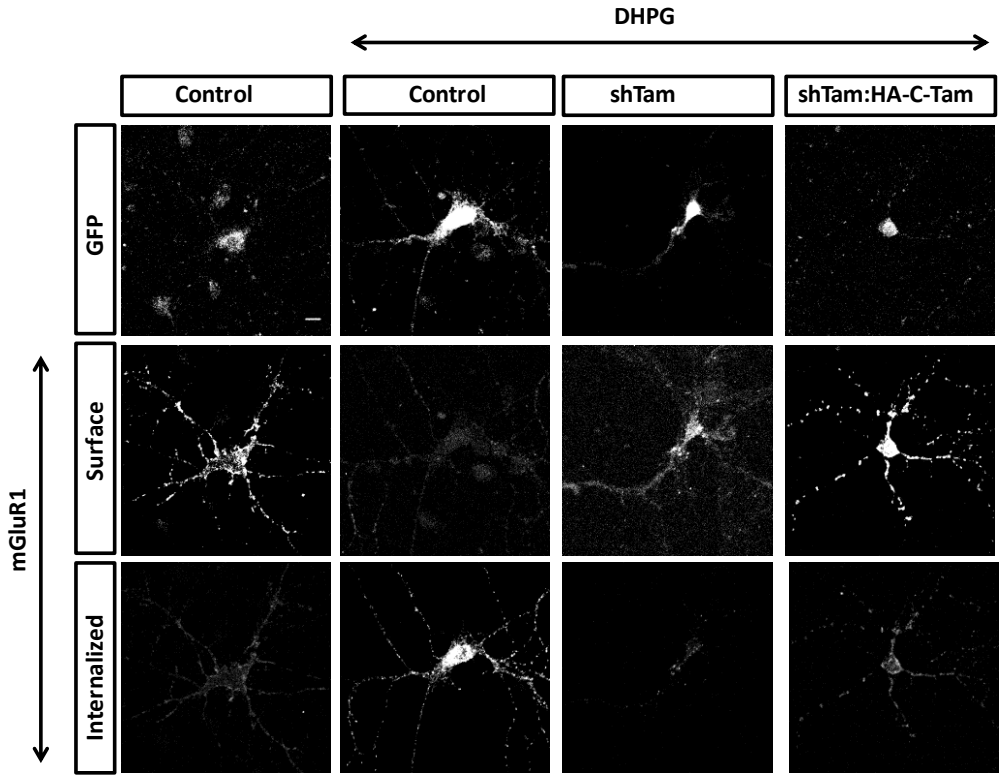


Figure 5.21. C-Tam replacement construct does not affect the surface expression of mGluR1 (A) Representative images showing myc-mGluR1 surface expression in control, shTam and C-Tam replacement construct expressing cells. (B) Quantitation of surface myc-mGluR1 suggested that knockdown of endogenous Tamalin and expression of the C-Tam replacement construct had no effect on the surface expression of myc-mGluR1. Scale bar = 10 μm, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

(A)



(B)

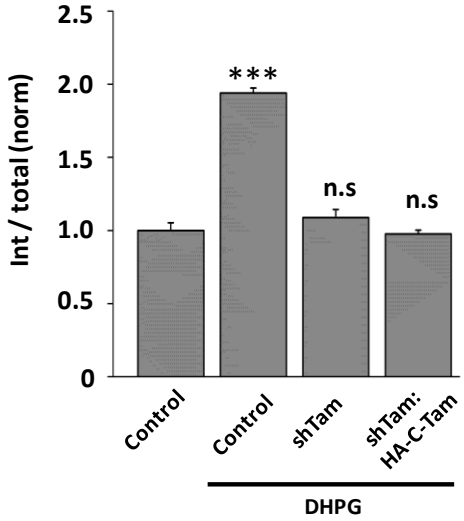


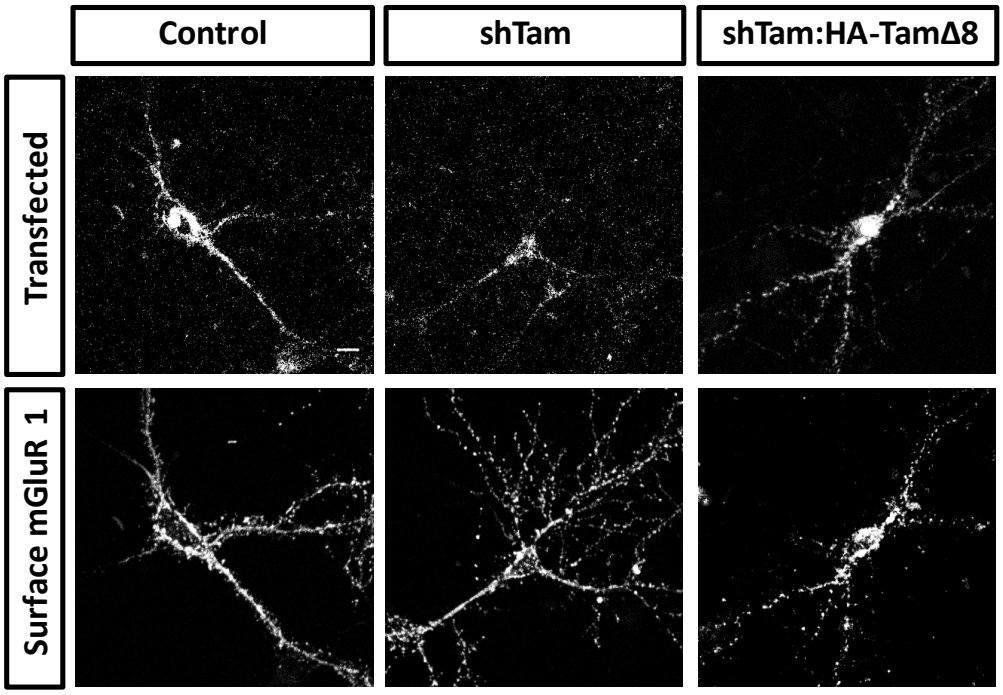
Figure 5.22. C-Tam replacement construct does not rescue the inhibition in the ligand-mediated endocytosis of mGluR1 caused by the knockdown of endogenous Tamalin (A) Representative images showing 100 µM DHPG-mediated myc-mGluR1 endocytosis in control cells which was blocked upon knockdown of endogenous Tamalin. Expression of C-Tam replacement construct did not rescue the endocytosis of the receptor (B) Quantitation of the effect of knockdown of endogenous Tamalin and expression of C-Tam replacement construct on the DHPG-mediated endocytosis of myc-mGluR1. Scale bar = 10 µm. ***$p < 0.001$, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

5.2.12. Last 8 amino acids of Tamalin play critical role in the ligand-mediated endocytosis of mGluR1

As stated before, previous studies have suggested that extreme C-terminal domain of Tamalin acts as autoinhibitory PDZ binding domain and in normal condition this domain forms autoinhibited form of the Tamalin protein in dimeric and tetrameric structures and acts as a reserve pool (Sugi et al., 2007). In order to investigate whether this domain could play a role in the ligand-mediated endocytosis of mGluR1, the last 8 amino acids of the Tamalin protein were deleted (TamΔ8) and a replacement construct was generated **(shTam:HA-TamΔ8)**. Initially, the role of this domain in the surface expression of myc-mGluR1 was studied. The expression of shTam:HA-TamΔ8 had no effect on the surface expression of myc-mGluR1 in primary hippocampal neurons as observed by no significant difference in myc-mGluR1 surface expression between control cells, shTam and shTam:HA-TamΔ8 expressing cells. **(control = 1 ± 0.04, shTam = 0.97 ± 0.06, shTam:HA-TamΔ8= 0.97 ± 0.06) (Figure 5.23 A, B)**.

The effect of TamΔ8 replacement on the mGluR1 endocytosis was next investigated. As expected, in control cells very little internalized receptors were observed which increased on 100 µM DHPG application at 30 min. Similar to our previous observations shTam expressing cells showed block in the myc-mGluR1 endocytosis **(control = 1 ± 0.06, DHPG. = 2.08 ± 0.07, shTam + DHPG = 1.00 ± 0.06) (Figure 5.24 A, B)**. Importantly, no recovery in myc-mGluR1 endocytosis was observed in shTam:HA-TamΔ8 expressing cells, suggesting that the autoinhibitory PDZ binding domain of Tamalin plays a critical role in the ligand-mediated internalization of mGluR1 **(shTam:HA-TamΔ8 + DHPG= 1.00 ± 0.04)**.

(A)



(B)

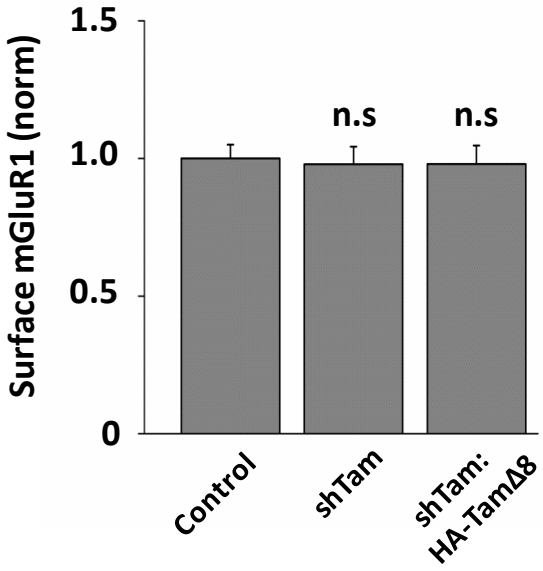


Figure 5.23. Deletion of last 8 amino acids of Tamalin has no effect on the surface expression of mGluR1 (A) Representative images showing surface expression of myc-mGluR1 in neurons where endogenous Tamalin was knocked down and TamΔ8 replacement construct was expressed. (B) Quantitation of surface myc-mGluR1 suggested that there was no significant difference between control cells, shTam and TamΔ8 replacement construct expressing cells. Scale bar = 10 μm, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

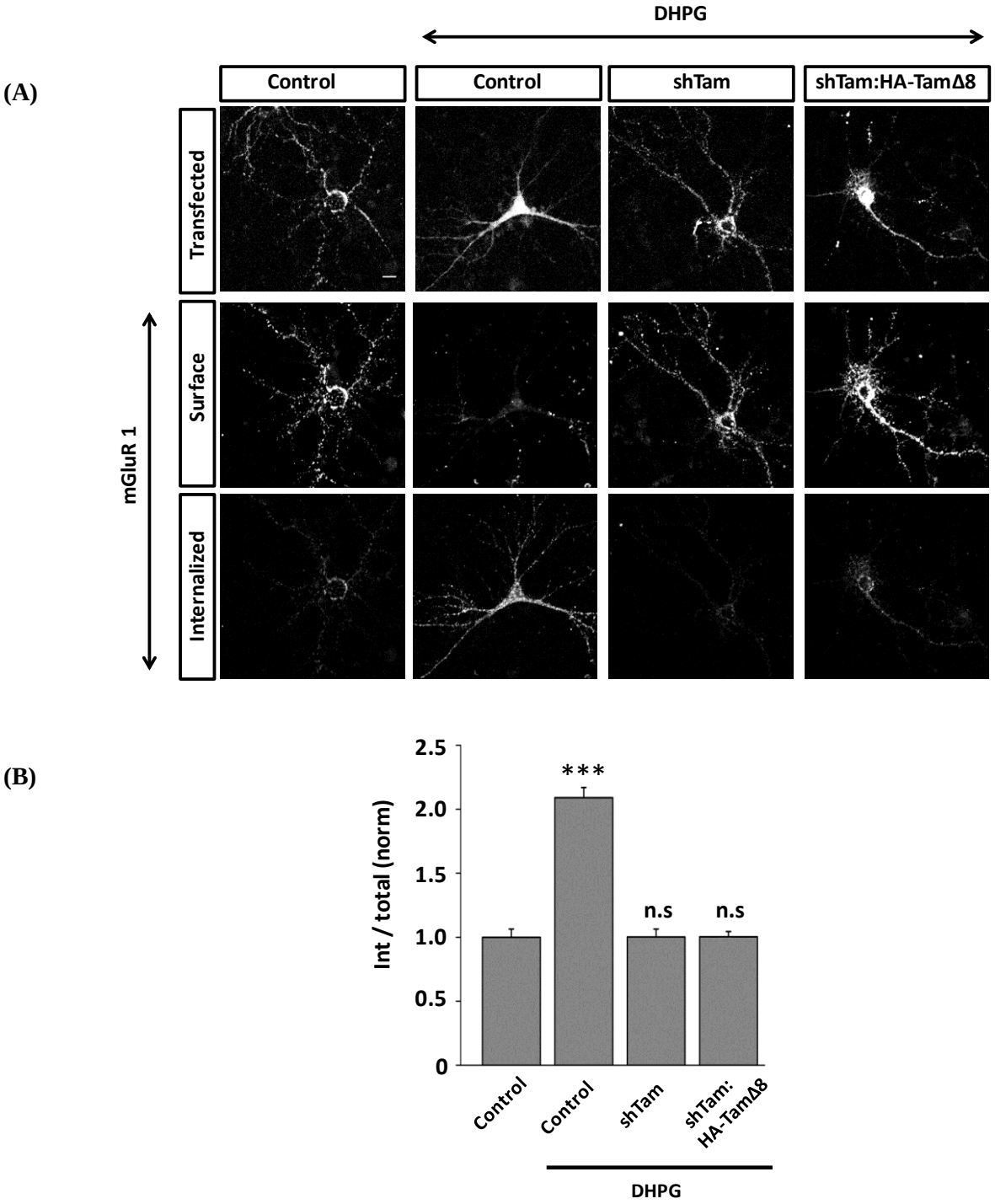


Figure 5.24. Last 8 amino acids of Tamalin is critical for the ligand-mediated internalization of mGluR1 (A) Control cells showed very little internalization of myc-mGluR1 and upon 100 µM DHPG treatment receptors internalized. Knockdown of endogenous Tamalin resulted in the block in myc-mGluR1 internalization. Replacing the endogenous Tamalin with TamΔ8 construct did not rescue the endocytosis of the receptor. (B) Quantitation also suggested that last 8 amino acids of Tamalin played crucial role in the DHPG-mediated internalization of myc-mGluR1 as observed by the inability of TamΔ8 replacement construct to rescue the internalization of myc-mGluR1. Scale bar = 10 µm. ***p < 0.001, n.s indicates p > 0.05. N=3; where N represents the number of times experiment was repeated.

5.2.13. Expression profile and synaptic localization of various mutants of Tamalin in primary hippocampal neurons

In order to check whether the various Tamalin mutants that were used in this study were expressed and targeted properly in neurons, we first checked the expression profile of each of them using immunostaining method.

Each of these constructs was tagged with HA at the N-terminus and as a result upon expression of these constructs, they produce recombinant proteins fused with HA at the N-terminus of the protein. Each of these constructs was transfected in primary hippocampal neurons at 5-7 DIV using calcium phosphate method. When cells were at 12-15 DIV, they were fixed with 4% PFA and subsequently they were permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Cells were then stained with anti-HA rat polyclonal antibody (1:500) followed by the application of goat anti-rat Alexa-568 conjugated secondary antibody (1:500). Our data suggested that the expression of the N-Tam protein and TamΔ8 protein were similar to the expression of the wild-type Tamalin (**Figure 5.25)**. On the other hand, the C-Tam protein did not target properly and they were predominantly localized at the cell body of the neuron. In order to investigate whether the various mutants of Tamalin that were used in this study localize at the synapse, the proportion of synapses containing detectable amount of these variants of Tamalin were quantified by staining for HA containing clusters and counterstaining for Bassoon, a core component of the active zone that is commonly used to identify presynaptic terminals (Dieck et al., 1998). Our data suggested that both the N-Tam and TamΔ8 variants of Tamalin localize at the synapse very similar to the wild-type Tamalin protein **(Tamalin = 80.46 ± 1.7, N-Tam = 80.49 ± 0.92, TamΔ8 = 80.51 ± 1.31)**. (**Figure 5.26 A, B)**. These results together suggested that deletion of the N-terminal domain of Tamalin mislocalized the protein. Furthermore, both the N-terminal domain of Tamalin and Tamalin lacking the last 8 amino acids were targeted properly at the synapse.

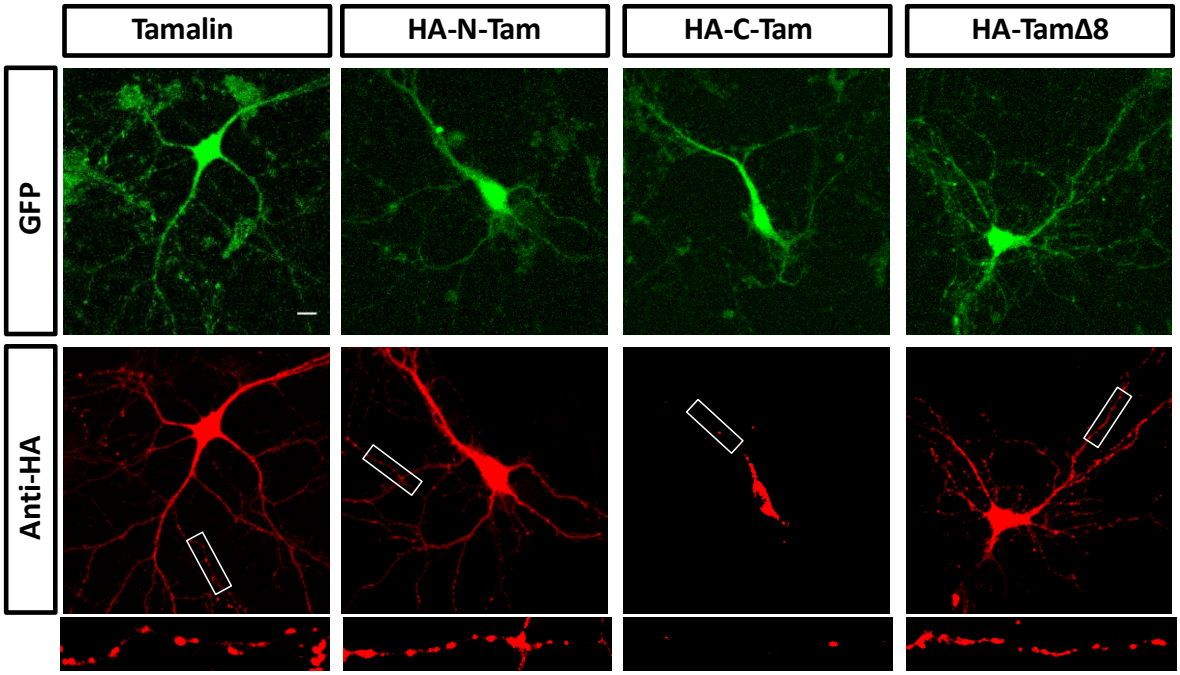
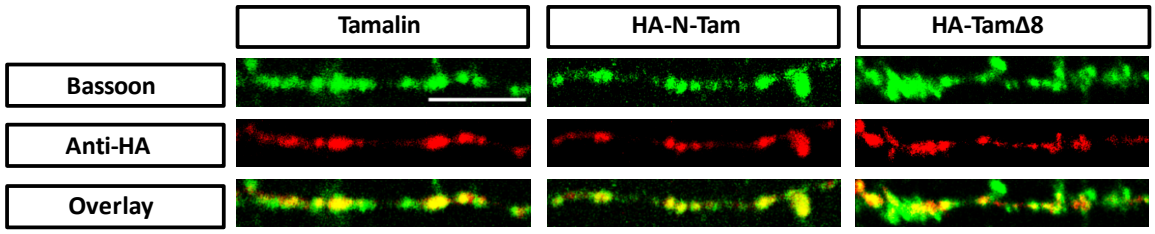


Figure 5.25. Expression of various Tamalin constructs: Wild-type Tamalin is expressed throughout the hippocampal neuron. Inset figure depicts the dendritic distribution of the various forms of Tamalin protein. N-Tam and TamΔ8 expression was also observed throughout the hippocampal neuron and they were seen to be localized in the dendrites, similar to wild-type Tamalin. On the other hand, C-Tam did not target properly to the dendrites of the neuron. Scale bar = 10 μm N=3; where N represents the number of times experiment was repeated.

(A)



(B)

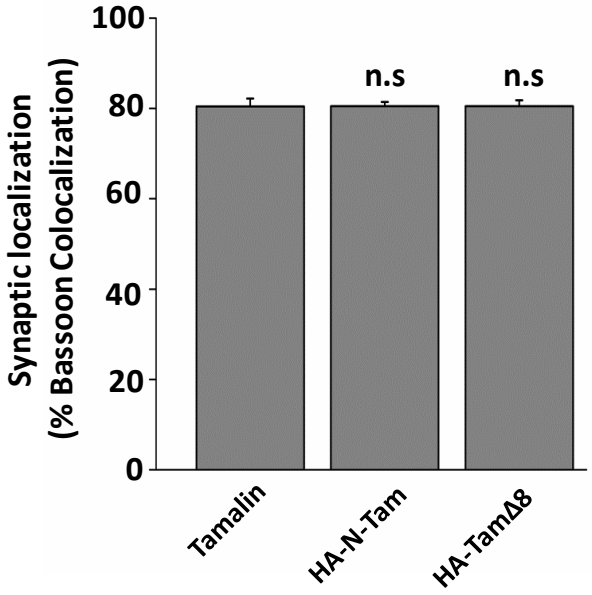


Figure 5.26. Synaptic localization of the Tamalin mutants (A) Representative images showing colocalization of Bassoon, an active zone synaptic marker with various forms of Tamalin (wild-type, N-Tam and TamΔ8). All these constructs were found to colocalize with Basson, suggesting that they were targeted to the synapse. (B) Quantitation also suggested that all the above forms of Tamalin were localized at the synapse with similar extent. Scale bar = 10 µm, n.s indicates $p > 0.05$. N=3; where N represents the number of times experiment was repeated.

5.3. Discussion

Scaffolding proteins present in the post-synaptic density (PSD) at the synapses play a regulatory role in the spatiotemporal localization of various neurotransmitter receptors as well as their signaling and trafficking. Till date, several scaffolding proteins have been identified and many of them have been suggested to play an important role in the regulation of GPCRs that serves as a neurotransmitter receptor in the brain. Tamalin/GRASP, a scaffolding protein present in the PSD has been reported to interact with group I mGluRs and affect their surface stability. Tamalin has also been reported to interact with various synaptic proteins to form macromolecular complexes and regulate the dendritic development and cargo delivery to and from the synapses (Kitano et al., 2003; Mo et al., 2012). In addition, Tamalin has been reported to regulate various subcellular processes required for proper neurotransmission and signaling across the synapse. In the present study, we have tried to unravel the role of Tamalin in group I mGluR trafficking. We used the molecular replacement approach to address the structure-function relationship of Tamalin in group I mGluR trafficking. This technique allowed us to knockdown the endogenous Tamalin protein in the primary hippocampal neurons along with simultaneous replacement with various forms of Tamalin. Our data suggested that knockdown of endogenous Tamalin resulted in the inhibition in the ligand-dependent internalization of group I mGluRs which could be rescued by the expression of wild type Tamalin, suggesting the specificity of the knockdown process. Furthermore, knockdown of endogenous Tamalin also resulted in the inhibition of the mGluR-mediated AMPAR endocytosis, which is believed to be the cellular correlate for mGluR-dependent synaptic plasticity. These results together suggest that Tamalin not only plays a critical role in the ligand-mediated internalization of group I mGluRs, it also affects the mGluR-mediated AMPAR trafficking. Tamalin being a scaffolding protein might acts as a bridge between group I mGluRs and the cellular machinery that regulates mGluR-mediated AMPAR endocytosis. It would be important to investigate how Tamalin regulates the mGluR-mediated AMPAR trafficking. We subsequently investigated various mutant forms of Tamalin to decipher the role of specific domains of Tamalin in group I mGluR trafficking. Over-expression of N-Tam (1-209 amino acids of the N terminal part of Tamalin) inhibited the mGluR1 endocytosis, and replacement of the endogenousTamalin with N-Tam did not rescue the mGluR1 endocytosis. N-Tam might be acting as a dominant negative in overexpressed condition, whereas the rescue experiments suggest that this domain alone is not sufficient to rescue the mGluR1 endocytosis. In addition, N-Tam alone was not sufficient

enough to rescue mGluR-mediated AMPAR endocytosis, suggesting, an important role for the domains present at the C-terminal part of the Tamalin protein in these processes. C-Tam (173 – 394 amino acids) over-expression had no effect on the mGluR1 endocytosis and it was not able to rescue the inhibition in the ligand-mediated mGluR1 endocytosis caused by the knockdown of endogenous Tamalin. This mutant was not able to localize at the synapse, suggesting that the N-terminal domain of Tamalin is critical for the targeting of the protein at proper location in the neuron. Interestingly, our data suggest that the last 8 amino acids of Tamalin could play very important role in the ligand-mediated endocytosis of mGluR1. Previous reports have suggested that last 8 amino acids of Tamalin interact with S-scam (Sugi et al., 2007), another scaffolding protein that regulates cargo delivery by interacting with myosin motors (Sugi et al., 2007). It would be interesting to study how the last 8 amino acids of Tamalin modulate the trafficking of these receptors by interacting with downstream ligands.

Summary

6.1. Aim of the research

G protein coupled receptors (GPCRs) are an intense area of research. GPCRs have been known to regulate variety of important physiological processes and their aberrant signaling leads to various pathological consequences. Approximately 40% of the total therapeutic drugs available in the market have been reported to target GPCRs (Wise et al., 2002; Trzaskowski et al., 2012). Although, our understanding of the mechanisms that regulate the responsiveness of GPCRs has increased considerably due to extensive research done by many labs over many years, but considering the huge 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. In recent years, crystal structures of many GPCRs have been solved and these studies along with previous reports have provided important information about the GPCRs activation mechanisms, their structural dynamics and coupling with different G proteins (Palczewski et al., 2000; Edwards et al., 2007; Venkatakrishnan et al., 2013; Ghosh et al., 2015). These studies also suggested that diversity in the regulation comes at least in part due to variations in the GPCR structure, which in turn leads to binding with different downstream effectors (Venkatakrishnan et al., 2013). Importantly, binding of GPCRs with different types of ligands and coupling with a number of G proteins to initiate a variety of intracellular signaling pathways have established the fact that each GPCR is unique. Hence, it is necessary to study a particular GPCR in detail to understand its signaling and regulations, because no GPCR serves as a model for all the rest. GPCR regulation is an important phenomenon, necessary for proper signal transduction and maintenance of the cellular homeostasis. Some of the major processes involved in the GPCR regulations include, receptor desensitization, receptor endocytosis, resensitization and downregulation of the receptor (Ferguson, 2001). Most of the GPCRs have been reported to get desensitized upon repeated agonist stimulation (Drake et al., 2006). Desensitization is considered as a preventive mechanism developed by the system to protect themselves from excessive signaling (Kelly et al., 2008). Subsequent to desensitization, many GPCRs have been reported to get internalized and manifest various subcellular fates depending upon the type of the receptor, type of the agonist, along with the cellular background (Drake et al., 2006; Hanyaloglu and Zastrow, 2008; Jean-Alphonse and Hanyaloglu, 2011). For many GPCRs, internalization is considered as a necessary step for the resensitization and down regulation of the receptor.

Group I mGluRs which include mGluR1 and mGluR5 have gained immense importance 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). Various antagonists and partial agonists developed against group I mGluRs have emerged as potential therapeutic drugs for various neurological disorders (Ferraguti et al., 2008). Group I mGluRs are class C G protein coupled receptors, primarily coupled to $G\alpha_{q/11}$ and activate phospholipase C-IP_3 pathway in various cell types (Pin et al., 2003; Brauner-Osborne et al., 2007). Like many other GPCRs, group I mGluRs have also been reported to get desensitized upon agonist stimulation and internalized rapidly (Dale et al., 2000; Dale et al., 2002; Dhami and Ferguson, 2006). However, the kinetics of internalization and the fate of the receptor subsequent to internalization have not been explored so far. The lack of knowledge about the subcellular fate of mGluR1 (a member of the group I mGluR family) subsequent to the ligand-mediated internalization was the reason for the initiation of our study.

To study the agonist induced internalization and subsequent subcellular fates of group I mGluRs, we used myc-tagged mGluR1 and myc-tagged mGluR5 constructs. In these constructs, myc epitope was tagged at the amino terminal of the full-length protein. In the past, these constructs have been used by various other groups and they have shown that, these recombinant receptors behave like the native receptor (Choi et al., 2011). We have used both non neuronal and neuronal cell lines to determine whether there was any cell type specific effect. HEK293 cells were used as a non-neuronal heterologous system, since these cells have large repertoire of G proteins. In addition, we have used N2A neuronal cell line as well. These are mouse neuroblastoma cells, which upon differentiation give rise to functional neurons. For the last part of this study, we extended our studies in the dissociated mouse hippocampal primary neurons. Primary hippocampal neurons are most experimentally tractable *in vitro* system till today that can approximate the *in vivo* situation. In the past, a variety of techniques such as surface biotinylation assay and single colour fluorescence measurement assays have been used to quantify the internalization of many GPCRs. However, the variability in the expression of receptors between the cells was not considered in these assays. To overcome this situation, we have used dual antibody staining assay to quantitate the amount of receptors internalized upon agonist stimulation. This method allowed us to normalize the amount of internalized receptors within the cells. Thus, this method has an advantage that it quantitates the proportion of the receptors internalized from

the total number of surface receptors; hence variability in the surface expression of the receptors between cells does not affect the quantitation (Bhattacharyya et al., 2009; Trivedi and Bhattacharyya, 2012).

6.2. Internalization and recycling of the mGluR1 in non-neuronal and neuronal cells

Previous studies have shown that upon agonist stimulation mGluR1 get desensitized in PKC and GRKs-dependent manner and subsequent to desensitization the receptor gets internalized in arrestin and dynamin-dependent manner *via* clathrin coated pits (Ferguson, 2001; Dale et al., 2002; Ferraguti et al., 2008). We observed that the mGluR1 endocytosis was quite fast as seen by the endocytosis of the receptor 1 min post-ligand application. The maximum endocytosis was observed 30 min post-ligand application in all the cell types, viz., HEK293 cells, N2A cells and primary hippocampal neurons. It has been reported that internalization kinetics of various GPCRs is dependent upon the type of GPCR, type of ligand and its cellular background. For example, the A1 adenosine receptor internalizes quite slowly ($t_{1/2}$ = 90 min) as compared to the A3 adenosine receptor ($t_{1/2}$ = 19 min) (Ferguson, 2001). Furthermore, the $5HT_{2A}$ receptors internalizes faster in neurons as compared to in HEK293 cells (Raote et al., 2013). However, in the case of mGluR1 the internalization kinetics was observed to be similar in all the cell types that we have used, suggesting that in all these cell types the receptor internalization probably occurs through similar mechanisms. Additionally, the ligand that we have used for the present study is DHPG, which is a specific agonist for group I mGluRs. Thus, whether other ligands like partial agonist/antagonist/inverse agonist will show the same phenomena with distinct kinetics needs to be checked. Since, N2A cells and hippocampal neurons are compartmentalized cells and it has been shown that some processes are compartment specific, we have checked the mGluR1 endocytosis in both cell body and dendrites. Our data suggested that mGluR1 internalized throughout the cell with similar extent.

As explained earlier, GPCRs manifest various subcellular fates upon internalization. For example, PAR1 receptors and endothelin B receptors predominantly go to the lysosomes after internalization, whereas other GPCRs such as β_2-adrenergic receptors recycle back to the cell surface following internalization (Morrison et al., 1996; Trejo and Coughlin, 1999; Bremnes et al., 2000). Our data showed that majority of the internalized mGluR1 were colocalized with Rab11 (recycling endosome marker) whereas no detectable colocalization was observed

with LAMP1 (lysosomal marker) in HEK293 and N2A cells. These results suggested that mGluR1 has chosen the recycling route upon internalization. We further showed that majority of the internalized mGluR1 recycled back to the cell surface in HEK293 and N2A cells. The internalization followed by recycling for many GPCRs is believed as a process for receptor resensitization (Pippig et al., 1995; Garland et al., 1996; Oakley et al., 1999). Hence, the recycling of mGluR1 subsequent to the ligand-mediated internalization suggests that the internalization might be necessary for the resensitization of these receptors instead of downregulation. Additionally, the recycling process also favours the cells thermodynamically compared to the de novo synthesis of the receptors, which is a huge energy consumption process.

6.3. Role of protein phosphatases in the recycling of mGluR1 in non-neuronal and neuronal cells

Our earlier data suggested that subsequent to the ligand-mediated internalization, mGluR1 recycled back to the cell surface. Recycling of some GPCRs have been reported to be dependent on the pH of the endosomal compartments, especially the recycling endosomes. In order to investigate whether the recycling of mGluR1 is dependent on the pH of the intracellular endosomes, we used ammonium chloride, a weak acidotropic agent which dissipates the transmembrane pH gradient of endosomal compartments (Raote et al., 2013). Our data suggested that mGluR1 recycling was dependent on the acidity of the endosomal compartments and disruption of the pH of the endosomal compartments resulted in the inhibition in the mGluR1 recycling. There could be either of the two possibilities or both, which may explain the pH-dependent recycling of mGluR1: 1) the internalized mGluR1 attains such a favourable conformational change under the acidic pH of the endosomal compartments that the residue(s) which were modified during the desensitization process become accessible to the enzymes present in the endosomes and they remodify the residue(s) of the receptor, resulting in the resensitization of the receptor, 2) activity of the enzymes present in the endosomal compartments is dependent on the specific pH and any alteration in the pH results in the inactivation of the enzymes.

As mentioned earlier, mGluR1 desensitization is dependent on the activity of protein kinase C (PKC) and GRKs, which phosphorylate the critical residues present in the third intracellular loop and carboxy-terminal tail of the receptor to initiate the desensitization

process (Gereau and Heinemann, 1998; Mundell et al., 2004; Mao et al., 2008). Since phosphorylation of some residues is necessary for mGluR1 desensitization, there must be some phosphatase(s) present in the endosomal compartments that would dephosphorylate the internalized mGluR1, in order to resensitize the receptor. This could serve as an exit signal that will allow the receptor to exit the recycling compartment and recycle back to the cell surface to initiate the new round of signaling. We therefore, screened for various group of phosphatases, which might be involved in the dephosphorylation of mGluR1. The phosphatases were selected on the basis of following criteria: they must be present in the endosomal compartments, their activity should be dependent on pH of the intracellular compartments and theirrole has been implicated in synaptic plasticity. Our data suggested that protein phosphatase 2A (PP2A) played crucial role in the recycling of mGluR1. Inhibiting the activity of endogenous PP2A by various means (pharmacologically blocking the activity of PP2A, dominant negative PP2A and siRNA knockdown of PP2A) resulted in the complete inhibition of mGluR1 recycling. It would be important to find out the substrates for PP2A in future. It is possible that PP2A dephosphorylates the phosphorylated residues present at the third intracellular loop and carboxy terminal tail of mGluR1. Alternatively, PP2A might dephosphorylate some other substrate(s) involved in the recycling of mGluR1. mGluR1 has been reported to be involved in various forms of synaptic plasticity and PP2A have also been reported to play important role in synaptic plasticity (Mauna et al., 2011; Liu et al., 2016). Since our data suggests that PP2A is involved in the recycling of mGluR1, it would be interesting to investigate whether PP2A plays any role in mGluR1-mediated synaptic plasticity.

6.4. Role of Tamalin in group I mGluR endocytosis

Scaffolding proteins have been reported to play important roles in the GPCR trafficking and signaling. Tamalin/GRASP is a 394 amino acids scaffolding protein known to regulate surface stability of group I mGluRs (Kitano et al., 2002). Additionally, this protein has been reported to interact with multiple post-synaptic density proteins to form macromolecular complexes. Tamalin interacts with cytoskeleton and motor proteins to regulate the cargo delivery to and from the synapses (Kitano et al., 2002; Kitano et al., 2003). We investigated the role of Tamalin in the ligand-mediated endocytosis of group I mGluRs using molecular replacement strategy (Bhattacharyya et al., 2009). This is a very elegant approach that allows

knockdown of endogenous Tamalin and simultaneous replacement of the endogenous protein with various mutant forms of the Tamalin protein. Interestingly, knockdown of endogenous Tamalin led to the inhibition in the DHPG-mediated endocytosis of both mGluR1 and mGluR5 in primary hippocampal neurons. However whether knockdown of Tamalin leads to the alteration in the receptor function needs to be investigated. Additionally, group I mGluR-mediated AMPAR endocytosis was also inhibited when endogenous Tamalin was knocked down. However we still don't know how knockdown of Tamalin leads to the inhibition of mGluR-mediated AMPAR endocytosis. It is possible that knockdown of Tamalin leads to the abnormal signalling of the receptor which leads to the inhibition in the mGluR-mediated AMPAR endocytosis. Tamalin could modulate the mGluR-mediated AMPAR trafficking by many other ways as well that needs to be studied in future. mGluR-mediated AMPAR endocytosis is the cellular correlate for mGluR-dependent synaptic plasticity. When the endogenous Tamalin was replaced with the full-length Tamalin replacement construct, the endocytosis of group I mGluRs and mGluR-mediated AMPAR endocytosis was rescued. These results suggested an important role of Tamalin in the ligand-mediated endocytosis of group I mGluRs as well as in group I mGluR-mediated AMPAR trafficking. To address the roles of various domains of Tamalin in group I mGluR trafficking, and mGluR-mediated AMPAR endocytosis, we generated over-expression and replacement constructs. The N-Tam (1-209 amino acids of Tamalin) over-expression resulted in the inhibition of mGluR1 endocytosis. The N-Tam part of the Tamalin protein contains PDZ domain and has been shown earlier to interact with group I mGluRs and also with autoinhibitory domain of the same or different Tamalin proteins (Sugi et al., 2007). Upon over-expression, this truncated form of Tamalin may competitively bind with the group I mGluRs which in turn could inhibit the interaction between the receptor and the endogenous Tamalin. Furthermore, this truncated domain was not sufficient to rescue the group I mGluR endocytosis as well as mGluR-mediated AMPAR endocytosis. These results suggested that the other domains are also necessary to mediate the endocytosis of group I mGluRs. We next investigated the role of C-Tam (173 – 394 amino acids of Tamalin) and observed that this domain had no effect on mGluR1 endocytosis and it was also not sufficient to rescue the endocytosis of mGluR1. The expression pattern of the C-Tam suggested that it was not targeted properly at the synapses. Since the signal sequence which is necessary for proper targeting of Tamalin might not be present in C-Tam, probably because of this reason the C-Tam was mislocalized. Our data also suggested that the last 8 amino acids of Tamalin, which constitute the autoinhibitory PDZ binding domain of the protein, played a crucial role in the mGluR1 endocytosis. Deletion of

these residues led to the inhibition in the ligand-mediated internalization of mGluR1. The autoinhibtory domain of Tamalin has also been reported to interact with another scaffolding protein S-Scam, which directly interacts with motor proteins and thus regulates the cargo delivery to and from the membrane (Kneussel, 2005; Sugi et al., 2007). It would be important to explore the mechanisms through which the autoinhibitory domain of Tamalin regulates the ligand-mediated endocytosis of group I mGluRs.

6.5. Summary of the picture that is available till now

The results presented in this thesis provide some advancement in the pre-existing knowledge of group I mGluR trafficking and their regulation. Earlier reports have suggested that mGluR1 gets desensitized upon agonist stimulation in GRKs and PKC-dependent manner. The desensitized receptors internalize rapidly through arrestin and dynamin-dependent pathways. Our data suggest that mGluR1 internalizes within 1 min of agonist stimulation. Most of the receptors internalize in 30 min post-ligand application. Importantly the internalized receptors enter the recycling compartment subsequent to the ligand-mediated internalization. Majority of the internalized receptors recycle back to the cell surface with similar kinetics in all the cell types studied. Our data also suggest that mGluR1 recycling is pH-dependent and protein phosphatase 2A (PP2A) plays crucial role in the mGluR1 recycling. Additionally, we have also shown a novel role for the scaffolding protein Tamalin in the group I mGluR trafficking. Our data suggest that Tamalin plays an important role in mGluR-mediated AMPAR trafficking. The autoinhibitory PDZ domain of Tamalin plays crucial role in the ligand-mediated endocytosis of mGluR1. **(Figure 6.1)**

6.6. Future directions

The studies we have described in this thesis have opened many questions that need to be addressed in the future. We have studied the ligand-induced internalization of mGluR1 in the present work. It has been reported that mGluR1 internalizes in both agonist-dependent and independent manner (Dhami and Ferguson, 2006; Bhattacharyya, 2016). The mechanisms regulating these two distinct processes might be different. Thus, it would be of interest to study the mechanisms regulating the ligand-independent endocytosis of mGluR1 in neuronal system as well. GPCRs manifest various subcellular fates subsequent to agonist-induced

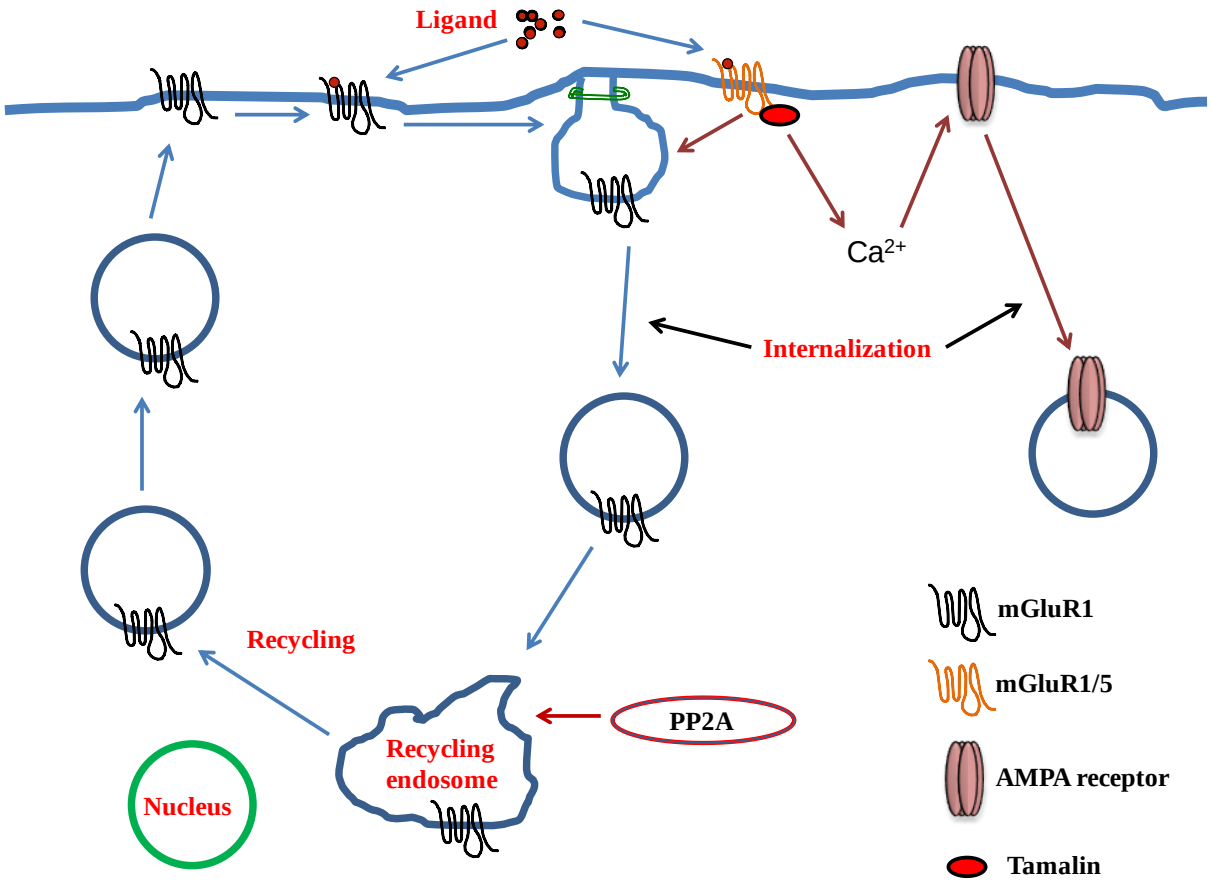


Figure 6.1. Regulation of mGluR1 trafficking and mGluR-dependent AMPAR trafficking: mGluR1 internalizes rapidly upon agonist/ligand application. The endocytosed mGluR1 goes to the recycling compartment. Majority of the internalized mGluR1 recycle back to the cell surface. PP2A plays an important role in the mGluR1 recycling. Tamalin plays a very important role in the group I mGluR (mGluR1 and mGluR5) endocytosis. Upon agonist stimulation, group I mGluRs initiate the AMPAR endocytosis. Tamalin plays critical role in group I mGluR-mediated AMPA receptor endocytosis.

internalization and our data suggest that mGluR1 takes the recycling route following agonist-induced internalization. It will be important to investigate the sorting mechanisms and regulatory molecules that are involved in the targeting of mGluR1 to the recycling compartment. Our data also suggest that PP2A plays a crucial role in the recycling of mGluR1. The exact mechanism(s) through which PP2A regulates the mGluR1 recycling is another important area to be studied in future. It is possible that PP2A dephosphorylates the residues of mGluR1 that got phosphorylated during the desensitization and internalization of the receptor. Alternatively, PP2A might affect other regulatory proteins which are involved in the recycling of mGluR1. Our studies show that Tamalin plays an important role in the ligand-mediated endocytosis of group I mGluRs. Again, it would be interesting to investigate whether the constitutive (ligand-independent) internalization of group I mGluRs is also dependent on Tamalin. Tamlin seems to play critical role in the mGluR-mediated AMPAR trafficking as well, which is the cellular correlate for the mGluR-dependent synaptic plasticity. The mechanisms through which Tamalin modulates the mGluR-mediated AMPAR trafficking would be a very important area to study. Our data suggest that the last 8 amino acids of Tamalin plays critical role in the ligand-dependent internalization of mGluR1. However, the mechanisms through which these residues of Tamalin control mGluR1 trafficking needs to be investigated. Finally, it would be interesting to investigate whether Tamalin plays any role in the NMDA receptor-mediated AMPAR trafficking, which is the cellular correlate for the NMDA receptor-mediated synaptic plasticity or it is specific for the mGluR-dependent synaptic plasticity pathway.

6.7. Final words

G protein coupled receptors are the major players in regulating various physiological processes in response to external stimuli. As the present study and other reports are testaments, there is huge diversity observed in the GPCR specific signaling, desensitization, internalization, resensitization and downregulation processes. It is not surprising, since variety of GPCRs are co-expressed in cells, the system have evolved numerous ways to regulate these receptors differently, in order to maintain synchrony. The diversity in the GPCR responsiveness is likely to be modulated by different ligands binding to the receptors and also structural differences among the members of GPCR family. Additionally, different subsets of interacting regulatory proteins and the effecter molecules might add further

complexity in the signaling and regulations of GPCRs. Although, we have gained much information about the GPCR signaling and regulations in the last few decades but considering the huge diversity it appears that we have just begin to understand the biological complexity that exists in the GPCR signaling and regulation. Thus, continued understanding of the GPCR signaling, regulation and trafficking will provide us important insights to unravel the complexity that exists in nature and also would help usto develop the novel therapeutic strategies, in order to cure various diseases arise due to aberrant GPCR signaling and regulations.

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List of Publications

- **Pandey, S.,** Mahato, P.K., Bhattacharyya, S. (2014) Metabotropic glutamate receptor 1 recycles to the cell surface in protein phosphatase 2A-dependent manner in non-neuronal and neuronal cell lines. ***Journal of Neurochemistry***, 131(5), 602-614.

- Mahato, P.K., **Pandey, S.,** Bhattacharyya, S. (2015) Differential Effects of Protein Phosphatases in the Recycling of Metabotropic Glutamate Receptor 5 (mGluR5). ***Neuroscience***. 306:138-150.

- **Pandey, S.,** Bhattacharyya, S. A critical role for Tamalin in Glutamate receptor trafficking. (Manuscript under Preparation).