Norbin: A critical regulator of group I metabotropic glutamate receptor internalization and synaptic AMPA receptor endocytosis

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Dedicated to my parents for their endless love, support and encouragement.

Declaration

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

Prachi Ojha

Date: 04/07/2022

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

Dr. Samarjit Bhattacharyya

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List of abbreviations

- **AKAP:** A-kinase anchoring protein
- **AMPAR:** α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
- **APV:** 2-Amino-5-phosphonopentanoic acid
- **AB-AM mix:** Antibiotic-antimycotic mix
- **BSA:** Bovine serum albumin
- **CaM:** Calmodulin
- **CaMKII:** $Ca^{2+/}$calmodulin-dependent protein kinase II
- **cAMP:** cyclic AMP
- **CCP:** Clathrin-coated pit
- **CCV:** Clathrin-coated vesicle
- **CHX:** Cycloheximide
- **CIP:** Calf intestinal phosphatase
- **CNS:** Central nervous system
- **DAG:** Diacylglycerol
- **DHPG:** 3,5-dihydroxyphenylglycine
- **DIV:** Days *in vitro*
- **DMEM:** Dulbecco's modified eagle's medium
- **DMSO:** Dimethyl sulfoxide
- **DNQX:** 6,7-dinitroquinoxaline-2,3-dione
- **EAATs:** Excitatory amino acid transporters
- **EDTA:** Ethylenediamine tetraacetic acid
- **EGFP:** Enhanced green fluorescent protein
- **ER:** Endoplasmic reticulum
- **ERK:** Extracellular-signal-regulated kinase
- **FBS:** Fetal bovine serum
- **FMRP:** Fragile X mental retardation protein
- **FUDR:** 5-Fluoro-2'-deoxyuridine
- **GABA:** gamma-Aminobutyric acid
- **GAP:** GTPase-activating protein

- **GDP:** Guanosine 5' diphosphate
- **GEF:** Guanine nucleotide exchange factor
- **GPCR:** G protein-coupled receptor
- **GRK:** G-protein coupled receptor kinase
- **GTP:** Guanosine triphosphate
- **HBSS:** Hank's balanced salt solution
- **HEPES:** 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- **Hrs:** Hepatocyte growth factor-regulated tyrosine kinase substrate
- **IP_3:** Inositol 1,4,5-triphosphate
- **IPSP:** Inhibitory post-synaptic potential
- **IRES:** Internal ribosome entry site
- **KAR:** Kainate receptor
- **LTD:** Long-term depression
- **LTP:** Long-term potentiation
- **MAPK:** Mitogen-activated protein kinase
- **MCHR1:** Melanin-Concentrating Hormone Receptor 1
- **mGluR:** Metabotropic glutamate receptor
- **MPEP:** 2-Methyl-6-(phenylethynyl)pyridine
- **MTEP:** 3-(2-Methyl-4-thiazolyl)ethynyl)pyridine)
- **NCDN:** Neurochondrin
- **NGS:** Normal goat serum
- **NMDAR:** N-methyl-D-aspartate receptor
- **PBS:** Phosphate-buffered saline
- **PCR:** Polymerase chain reaction
- **PDZ:** Post-synaptic density-95 (PSD-95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 (zo-1)
- **PEI:** Polyethylenimine
- **PFA:** Paraformaldehyde
- **PICK1:** Protein interacting with C kinase 1
- **PIP_2:** Phosphatidylinositol 4,5-bisphosphate
- **PIP_3:** Phosphatidylinositol (3,4,5)-trisphosphate

- **PKA:** Protein kinase A
- **PKC:** Protein kinase C
- **PKD:** Protein kinase D
- **PLC:** Phospholipase C
- **PP2A:** Protein phosphatase 2A
- **PP2B:** Protein phosphatase 2B
- **PSD:** Post-synaptic density
- **QUIS:** Quisqualate
- **RGS:** Regulators of G protein signalling
- **RIPA:** Radio-immunoprecipitation assay
- **SNP:** Single nucleotide polymorphism
- **SNX1:** Sorting Nexin 1
- **TEA:** Tetraethylammonium
- **TGN:** trans-Golgi network
- **TMD:** Transmembrane domain
- **TTX:** Tetrodotoxin
- **VFD:** Venus Flytrap Domain
- **VGLUTs:** Vesicular glutamate transporters

Abstract

G protein-coupled receptors (GPCRs) are seven transmembrane receptors that transduce information provided by the extracellular stimuli into intracellular signals via their coupling to G-proteins. Due to the diversity in GPCR regulation, each GPCR is unique and an extensively studied GPCR may not provide all the details about other GPCRs. Glutamate is a major excitatory neurotransmitter in the central nervous system. It activates three types of receptors, viz., NMDARs, AMPARs and metabotropic glutamate receptors (mGluRs). Among them, mGluRs belong to the GPCR family. Group I mGluR family consists of mGluR1 and mGluR5. Group I mGluRs are believed to be involved in multiple forms of experience dependent synaptic plasticity including learning and memory. In addition, they also have been implicated in various neuropsychiatric disorders like Fragile X syndrome, autism etc. Like many other GPCRs, group I mGluRs get desensitized subsequent to the ligand exposure and undergo rapid internalization. These receptors are localized in a protein dense region at the post-synaptic membrane called the post-synaptic density (PSD). The post-synaptic density of excitatory synapses is very complex in composition and dynamic in nature. The involvement of post-synaptic density proteins in the ligand-mediated trafficking of group I mGluRs is not well understood.

Norbin is a neuronal cytoplasmic protein that interacts with group I mGluRs and affects mGluR5 activity. However, the mechanism by which it modulates mGluR function is largely unknown. Using an experimentally tractable system that can closely mimic the in vivo trafficking events, i.e., dissociated primary hippocampal neurons from mice, we show that Norbin is a scaffolding protein that is crucial for the ligand-mediated internalization of group I mGluRs. We report that Norbin, through its N-terminus, associates with protein kinase A (PKA) and anchors mGluR5 through its C-terminus, both of which are necessary for the ligand-mediated mGluR5 endocytosis. We also found a novel role for Norbin in the regulation of mGluR-mediated AMPAR endocytosis. Our results suggest that recruitment of PKA to AMPARs through Norbin upon mGluR activation is an important mechanism specifically for mGluR-triggered AMPAR endocytosis and consequently for mGluR-mediated synaptic plasticity in the hippocampus. Thus, Norbin is crucial for the spatio-temporal regulation of group I mGluRs and it also provides a control over mGluR-mediated AMPAR endocytosis that may be relevant for neuropsychiatric disorders that report a dysregulation of mGluR5 and Norbin.

Chapter 1

Introduction

The neurotransmitters or the chemical messengers of our brain confer some remarkable abilities starting from the perception of self and the surroundings to the conversion of day to day experiences into memory as well as the feeling of reward and pleasure that motivates us to carry out a particular task. Although these activities might seem trivial but they form the basis of our existence. But what happens when one or more of these neurotransmitter pathways get haywire? This can happen at the level of the neurotransmitter itself or it can happen at the level of enzymes that synthesize them or the proteins that are responsible for the reuptake of these neurotransmitters or it can also happen at the level of receptors that respond to these neurotransmitters and maintain proper downstream signalling. The picture is most often not so clear because several neurotransmitter pathways cross-talk within the brain and hence such an aberration often leads to complex brain disorders like autism, Alzheimer's disease, Parkinson's disease etc. All of these neurological disorders involve multiple neurotransmitter signalling pathways, thus emphasizing the need for in-built mechanisms that regulate proper homeostasis and signalling within the neurons. Although we have come a long way in comprehending the complexities and molecular underpinnings of these complex brain disorders, a lot still remains to be understood at the cellular and the molecular level.

1.1. Glutamate and its receptors

L-Glutamate is a non-essential dicarboxylic amino acid. Although people had known about the abundance of this amino acid in the central nervous system as an element of certain metabolic pathways, its role as a neurotransmitter was established much later. Some of the earliest experiments showed its involvement in electrophysiological processes in the brain like induction of convulsions (Hayashi, 1954). The fact that it can cause excitation and depolarization of neurons was shown in the 1960s (Curtis et al., 1960, 1961; Curtis & Watkins, 1960). Electrical conduction of signals from one neuron to the other was well established but the possibility that glutamate and some other amino acids can help in chemical transmission and act as neurotransmitters was suggested by the presence of unique high affinity transporters for the uptake of glutamate in the neurons and glial cells which had already been reported for some catecholamines (Balcar & Johnston, 1975; Danbolt, 2001; Logan & Snyder, 1971, 1972). Later, several transporters like the excitatory amino acid transporters (EAAT1-5) were cloned and characterized (Arriza et al., 1994, 1997; Fairman et al., 1995; Kanai & Hediger, 1992; Pines et al., 1992; Storck et al., 1992).

Glutamate can be synthesized in the brain from glucose or by hydrolytic deamination of glutamine through glutaminase enzyme although, glutamine acts as the key precursor for the releasable pool of glutamate (A. Hamberger et al., 1979; A. C. Hamberger et al., 1979). Thus, glutamate acts as a major excitatory neurotransmitter in the brain and about 90% of the excitatory synapses in the brain are glutamatergic. Apart from the brain, glutamate signalling might play a homeostatic role in other tissues such as bone, testis, pancreas and the adrenal gland. This is suggested by the presence of the molecular machinery required for signal output (VGLUTs or vesicular glutamate transporters), signal termination (EAATs) and signal detection (glutamate receptors) in these tissues (Hinoi et al., 2004). In the brain, glutamate has to be maintained at low extracellular levels in order to protect the cells from "excitotoxicity" or neuronal death. This is ensured by proper reuptake of glutamate from the synapse through sodium-dependent glutamate antiporters or EAATs into neurons or glia as well as by proper signal transduction through various glutamate receptors localized on the synaptic membranes. Broadly, there are two types of glutamate receptors in the brain: ionotropic glutamate receptors or iGluRs and metabotropic glutamate receptors or mGluRs (Meldrum, 2000) **(Figure 1.1)**.

1.1.1. Ionotropic glutamate receptors (iGluRs)

The quest for identifying the glutamate receptors involved looking for amino acid binding sites and identifying if it is present on the cell membrane or intracellular. Curtis and Watkins proposed that the active site of receptor must contain two regions which bear positively charged groups that can interact with α and γ carboxyl groups of glutamate. A third region with a negatively charged group which can interact with the positively charged α amino group of glutamate must also be a component of the receptor active site. The presence of such neuronal receptors that face the extracellular environment was supported by the fact that L-glutamate injected intra-neuronally did not produce any electrical excitation while extracellular application did (Curtis & Watkins, 1960; Michaelis et al., 1981). This led to the identification of N-methyl D-aspartate receptors (NMDARs) and non-NMDA receptors. Ionotropic glutamate receptors are ion channels that open up upon activation and allow cations to pass through them. These are the longest known and best studied glutamate receptors. They have further been characterized into different subtypes based on pharmacological and electrophysiological data as well as their response to selective agonists: NMDA receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors,

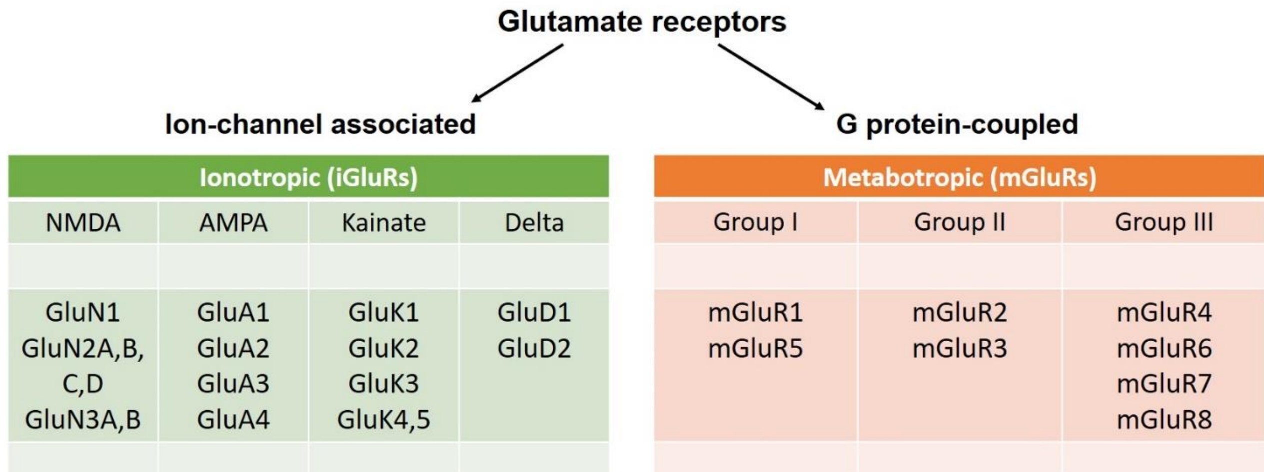


Figure 1.1. Glutamate receptors. Schematic representation of the two types of glutamate receptors in the brain, i.e., ionotropic glutamate receptors or iGluRs and metabotropic glutamate receptors or mGluRs as well as their subtypes.

kainate (KA) receptors and the poorly understood GluD receptors (Dingledine et al., 1999; Hollmann & Heinemann, 1994; Reiner & Levitz, 2018; Michisuke Yuzaki & Aricescu, 2017). These receptors mediate fast excitatory neurotransmission in the central nervous system (CNS). In general, iGluRs consist of four subunits and each subunit contains four well conserved domains including an extracellular amino-terminal domain, an extracellular ligand binding domain, a transmembrane domain spanning the cell membrane four times and an intracellular carboxy-terminal domain.

1.1.1.1. NMDA receptors

NMDA receptors were one of the first identified subfamily of excitatory amino acid receptors (Watkins & Jane, 2006). N-methyl-D-aspartic acid (NMDA), a selective agonist of the NMDA receptor activates voltage-dependent sodium (Na^{+}) and calcium (Ca^{2+}) conductance (Macdermott et al., 1986; Mayer & Westbrook, 1985). Unlike other excitatory amino acid receptors, this class of receptors displays a peculiar voltage sensitivity and are activated only when the membrane is depolarized. This voltage sensitivity in the NMDA response could be correlated with increasing magnesium (Mg^{2+}) sensitivity and was reduced upon removing Mg^{2+} from the extracellular medium. It is now known that the voltage-dependence of NMDA receptor channels is the consequence of a Mg^{2+} blockade that gets removed once the membrane is depolarized (Nowak et al., 1984). Thus, NMDA receptors act as coincidence detectors which means these receptors get activated upon simultaneous activation of both the neurons; one that releases glutamate to bind to the channel and the other that gets depolarized for magnesium to release and unblock the channel (Dingledine et al., 1999). NMDARs have a high glutamate sensitivity, around $10^{-3} – 10^{-2}$ mM [Glu] (Reiner & Levitz, 2018). Another unique property of these receptors is that glycine, which is otherwise an inhibitory neurotransmitter, acts as a co-agonist and potentiates the response of NMDA receptors (J. W. Johnson & Ascher, 1987). This class of receptors consists of three different subunits named GluN1-3. Post-transcriptional processing generates eight different splice variants of GluN1 subunit from a single gene, four GluN2 subunits (GluN2A-D) from four different genes and two GluN3 subunits (GluN3A, B) from two separate genes (Vyklicky et al., 2014). Functional NMDARs in mammalian cells assemble as heterotetramers comprising of two obligatory GluN1 subunits in combination with two GluN2 and/or GluN3 subunits. Glycine binds to GluN1 and GluN3 subunits whereas glutamate binds to GluN2 subunits. The subunits show

differential expression across different brain regions and their assembly is cell type specific. The messenger RNA (mRNA) for NMDA receptors is expressed throughout the brain particularly in the hippocampus, cerebral cortex and cerebellum (Goebel & Poosch, 1999; Moriyoshi et al., 1991; Paoletti & Neyton, 2007; Ulbrich & Isacoff, 2008; Vyklicky et al., 2014). NMDA receptors have been implicated in neuronal excitotoxicity, neural plasticity and several neurological disorders like depression, schizophrenia, epilepsy and other neurodevelopmental disorders (Hansen et al., 2017; Olney et al., 1999).

1.1.1.2. AMPA receptors

AMPA is a structural analogue of L-glutamic acid. Using radioligand binding experiments, AMPA was shown to bind rat brain homogenates, cultured neurons and to the glutamate-sensitive sites in brain sections (Honore et al., 1982; Keinänen et al., 1990; Monaghan et al., 1984; Olsen et al., 1987). They were initially named as “quisqualate receptors” but were later renamed to AMPA receptors due to the higher affinity and specificity of AMPA for ‘QUIS’ sites (Honoré & Nielsen, 1985; Olsen et al., 1987). AMPA receptor channels are permeable to monovalent cations and Ca^{2+} (Hollmann et al., 1991). The AMPA receptor is involved in fast glutamatergic transmission (glutmamate sensitivity between 10^{-2} – 1 mM [Glu]) in the mammalian brain and is composed of four subunits, GluA1-4, each of which exists as two splice variants- ‘flip’ and ‘flop’ (Greger et al., 2017; Wenthold et al., 1996). The CA1/CA2 hippocampal pyramidal neurons express high levels of GluAl-3 in both flip and flop splice variants. In the hippocampal excitatory synapses, heterotetramers of GluA1/GluA2 and GluA2/GluA3 subunits, together with some GluA1 homomers are most commonly represented (Greger & Esteban, 2007; Wenthold et al., 1996). The assembly of these subunits determines the electrophysiological properties of the AMPAR channel. For example, the presence of GluA2 subunit renders the AMPARs impermeable to Ca^{2+} (B. Bettler & Mulle, 1995; Greger et al., 2017). The AMPAR subunits assemble in a cell-type specific manner and even within the same neuron multiple complexes can be assembled and targeted to different synaptic populations thus generating heterogeneity. This is accomplished by the association of AMPARs with different auxiliary subunits like stargazin which belongs to the transmembrane AMPAR regulatory protein (TARP) family (Greger et al., 2017; Kamalova & Nakagawa, 2021; Lambolez et al., 1992; Wenthold et al., 1996).

Yeast two-hybrid screens have shown that many proteins bind to the C-terminal domains of AMPARs in a subunit-specific manner. For example, GluA2 and GluA3 bind to the PDZ domains (named after the first three proteins identified to contain them- post-synaptic density protein (**P**SD-95), Drosophila disc large tumor suppressor (**D**lg-1), and zonula occludens-1 protein (**Z**o-1)) of glutamate receptor-interacting protein 1 and 2 (GRIP1/2) and protein interacting with C-kinase 1 (PICK1). GluA2 also binds to the N-ethylmaleimide-sensitive factor (NSF) protein. GluA1 subunit binds to the PSD-95 family member, SAP97, through its C-terminal PDZ domain (Huganir & Nicoll, 2013). AMPARs have been the key molecular players in various forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD). Together these processes lead to the strengthening or weakening of synapses which is the cellular correlate for learning and memory (Carroll et al., 2001; Huganir & Nicoll, 2013).

1.1.1.3. Kainate receptors

Kainic acid or kainate (KA) is a pyrrolidine derivative isolated from the seaweed *Digenea simplex*. It is a structural homologue of L-glutamic acid that was identified to have excitatory action in spinal interneurons (Johnston et al., 1974; Mcculloch et al., 1974). In vitro autoradiographic techniques have shown the distribution of kainic acid binding sites throughout the gray matter structures of the forebrain including the striatum, cingulate cortex, stratum lucidum of CA3 of the hippocampus, molecular layer of the dentate gyrus and in the cerebellum with little to no expression in the midbrain (Monaghan & Cotman, 1982; Unnerstall & Wamsley, 1983). Five kainate receptor (KAR) subunits have been identified till now, i.e., GluK1-5 (originally named GluR5-7 and KA1-2). GluK1 (or GluR5) was the first mammalian subunit to be cloned followed by the cloning of other subunits that assemble as tetramers *in vivo* to form both homo-oligomeric or hetero-oligomeric kainate receptors (Bernhard Bettler et al., 1990; Carta et al., 2014; Chittajallu et al., 1999; Egebjerg et al., 1991; Hollmann et al., 1989; N. Nakanishi et al., 1990; Werner et al., 1991). Kainate receptors are located both pre-synaptically as well as post-synaptically. Unlike the post-synaptic kainate receptors that mediate excitatory transmission, the pre-synaptic kainate receptors modulate inhibitory responses (Rodríguez-Moreno et al., 1997). This was first evidenced by the depression of glutamate release and glutamate-mediated synaptic transmission in the hippocampus upon application of kainate and its agonists (Clarke et al., 1997). GluR5 containing pre-synaptic kainate receptors reduced the gamma-aminobutyric acid (GABA)-evoked inhibitory

post-synaptic potentials (IPSPs) in the hippocampus which might contribute to the convulsant and neurotoxic effects of kainate (Chittajallu et al., 1996, 1999; Clarke et al., 1997; Frerking et al., 1998; Huettner, 2003; Rodríguez-Moreno et al., 1997). Apart from the canonical ion conductance pathway, KARs also signal through a non-canonical metabotropic mechanism which confers a diverse range of functions to these receptors (Rodríguez-Moreno & Lerma, 1998). Kainate receptors have been implicated in synaptic plasticity and in several brain disorders such as schizophrenia, bipolar disorder, mental retardation, Huntington disease, epilepsy and pain (Bortolotto et al., 1999; Lerma & Marques, 2013).

1.1.1.4. Delta (GluD) receptors

The first subunit of the delta family of receptors was identified in 1992 and was named δ1. Later another subunit was characterized from rat brain and the two subunits, GluD1 and GluD2 completed the iGluR family based on their sequence similarity with the other iGluR subunits (Lomeli et al., 1993; Yamazaki et al., 1992). For the longest time the delta receptors (or GluD receptors) were categorized as 'orphan' receptors since no known agonists could activate them. However recently, two endogenous ligands have been identified to bind to the GluD2 subunit; D-serine and Cbln-1 (Matsuda et al., 2010; Naur et al., 2007). GluD1 is mainly distributed in the telencephalon, whereas GluD2 expression is restricted to parallel fiber Purkinje cells in the cerebellum (Hepp et al., 2015; Konno et al., 2014; Valbuena & Lerma, 2016). The Cbln1-GluD2 complex acts as a bidirectional synaptic organizer, i.e., it directly regulates the differentiation, formation and plasticity of cerebellar synapses (Matsuda et al., 2010). Although the GluD receptor (GluDR) function as a cation channel remains poorly understood, the only known ion-conducting GluD receptor is the constitutively active gain-of-function mutant in '*lurcher*' mice (Michisuke Yuzaki & Aricescu, 2017; Zuo et al., 1997). Application of D-serine caused an inhibition in the ion channel conductance in GluD2 containing the lurcher mutation (Naur et al., 2007). Recently, mGluR1 has been shown to gate the GluD2 receptors and trigger channel opening (Ady et al., 2014). GluD2 associates with mGluR1 through the scaffolding proteins Shank and Homer and also interacts directly with PSD-93, S-SCAM through PDZ-binding (Kato et al., 2012; Roche et al., 1999; Uemura et al., 2004; Yap et al., 2003). These receptor-protein interactions and novel gating mechanisms mediate synaptic plasticity through GluDRs, in particular cerebellar LTD (Hirano et al., 1994; Kakegawa et al., 2011; Valbuena & Lerma, 2016).

1.1.2. Metabotropic glutamate receptors (mGluRs)

The earliest evidence for the presence of a metabotropic type of glutamate receptor came from the studies in xenopus oocytes when injection of rat brain mRNA elicited inositol 1,4,5-triphosphate (IP_3) formation and intracellular calcium influx upon activation with quisqualate as agonist. These responses were blocked by the application of pertussis toxin suggesting that they might be G_i or G_o mediated (Murphy & Miller, 1988; Sugiyama et al., 1987). 'Metabotropic', as the name suggests, are receptors that mediate their actions through several metabolic pathways like intracellular second messengers and thus modulate slow synaptic transmission (Baskys, 1992; Ferraguti & Shigemoto, 2006). mGluRs belong to the class C family of G-protein coupled receptors (GPCRs) (Niswender & Conn, 2010). Like other GPCRs, these receptors possess an extracellular N-terminal domain, a cysteine-rich region, a heptahelical transmembrane domain (TMD) motif and an intracellular C-terminal tail. However, the unique feature of mGluRs is that the extracellular region is large (~600 amino acids) and it contains two hinged globular domains known as the Venus flytrap domain (VFD) which forms the ligand binding site (Niswender & Conn, 2010). These two domains get closed upon glutamate binding which causes structural changes in the TMD leading to the G-protein activation (Jingami et al., 2003; Kunishima et al., 2000; Mun et al., 2004; Muto et al., 2007; S. Nakanishi, 1994; Niswender & Conn, 2010). Till date, eight subtypes of mGluRs have been cloned, i.e., mGluR1-8 that have been numbered in the order in which they were characterized and have been subdivided into three groups based on their sequence similarity, pharmacology and second messenger coupling, viz, group I, group II and group III mGluRs (Abe et al., 1992; Bhattacharyya, 2016; Conn & Pin, 1997; Duvoisin et al., 1995; Masu et al., 1991; Nakajima et al., 1993; Okamoto et al., 1994; Prezeau et al., 1992; Tanabe et al., 1992). Group I comprises of mGluR1 and mGluR5, which are positively coupled to G_q-linked pathway, which activates phospholipase Cβ and generates diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Bhattacharyya, 2016). Group II comprises of mGluR2 and mGluR3 and group III comprises of mGluR4, mGluR6, mGluR7 and mGluR8, all of which are linked with $G_{i/o}$ and are negatively coupled to adenylate cyclase (Bhattacharyya, 2016; Ferraguti & Shigemoto, 2006). Thus, group I mGluRs are generally associated with excitatory responses and group II and group III mGluRs are associated with inhibitory responses. Also, group I mGluRs are primarily expressed at the post-synaptic sites, group II at both pre and post-synaptic sites and group III mGluRs are predominantly localized at the pre-synaptic sites (Bhattacharyya, 2016;

Watkins & Jane, 2006) **(Figure 1.2)**. mGluRs mediate long-term changes in synaptic efficacy through the modulation of ion channels and have been implicated in several neuropsychiatric disorders including schizophrenia, epilepsy, pain, Parkinson's disease, Alzheimer's disease, anxiety and drug addiction (Benarroch, 2008; Conn & Pin, 1997; S. Nakanishi, 1994; Niswender & Conn, 2010; Schoepp & Conn, 1993). Our study focuses on the cellular and molecular mechanisms underlying group I mGluR trafficking and since mGluRs are GPCRs, the subsequent sections discuss GPCRs in general and then we narrow down to a more elaborate discussion of group I mGluRs.

1.2. G protein-coupled receptors (GPCRs)

GPCRs or G protein-coupled receptors are the largest and most diverse family of membrane proteins in the human genome. As the name suggests, they communicate through a repertoire of G proteins that bind to guanosine triphosphate (GTP) upon activation. Over 800 GPCRs have been identified in the human genome (Fredriksson & Schiöth, 2005; Hanlon & Andrew, 2015). Among the GPCRs, one thing that is common is their structure which comprises of seven membrane spanning domains, first found in bacteriorhodopsin, the major light-sensitive protein of *Halobacterium halobium* (Engelman et al., 1980; Lameh et al., 1990; Ross, 1989). Apart from this, these receptors consist of an extracellular N-terminus, an intracellular C-terminus, three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3) linking the hydrophobic transmembrane domains (TM1-7) **(Figure 1.3)**. The first proposed classification system divided the GPCRs into six classes based on sequence homology and functional similarity, viz. A to F system (Kolakowski, 1994). These included: Class A (rhodopsin-like), class B (secretin receptor family), class C (metabotropic glutamate receptors), class D (fungal mating pheromone receptors) (Herskowitz, 1989; Nakayama et al., 1985), class E (cyclic AMP receptors) (Devreotes, 1989; R. L. Johnson et al., 1993; Klein et al., 1988; Saxe et al., 1993) and class F (frizzled/smoothened) (Huang & Klein, 2004; Y. K. Xu & Nusse, 1998). Then came the numbering scheme, i.e, 1 to 5 system which was based on both structural and physiological features (Bockaert & Pin, 1999). But an alternative classification system was needed because the A-F system grouped GPCRs from both vertebrates and invertebrates and some of these classes like D and E are not found in mammals.

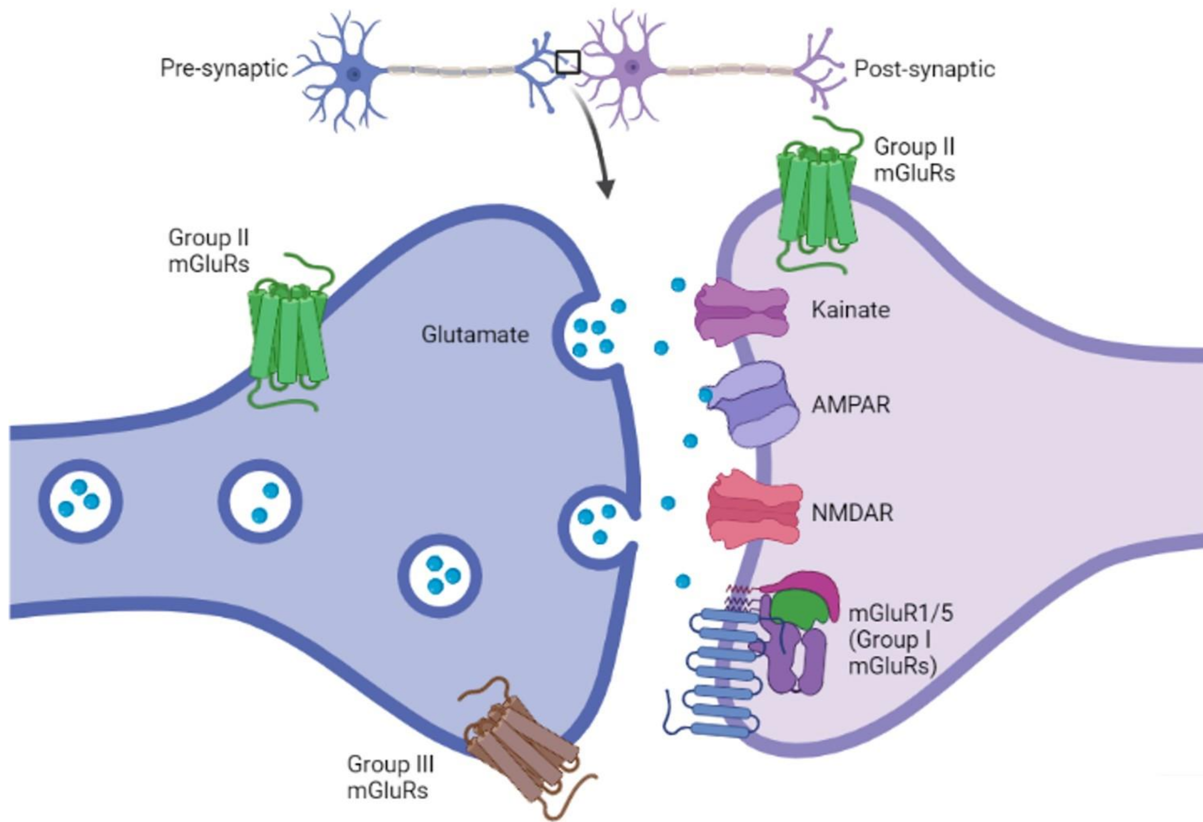


Figure 1.2. Localization of the glutamate receptors at the synapse. Diagrammatic representation of iGluRs and mGluRs at the synapse. Note that group I mGluRs are located at the perisynaptic region of the post-synaptic neuron whereas group II mGluRs are present at both pre and post-synaptic sites and and group III mGluRs are majorly pre-synaptic.

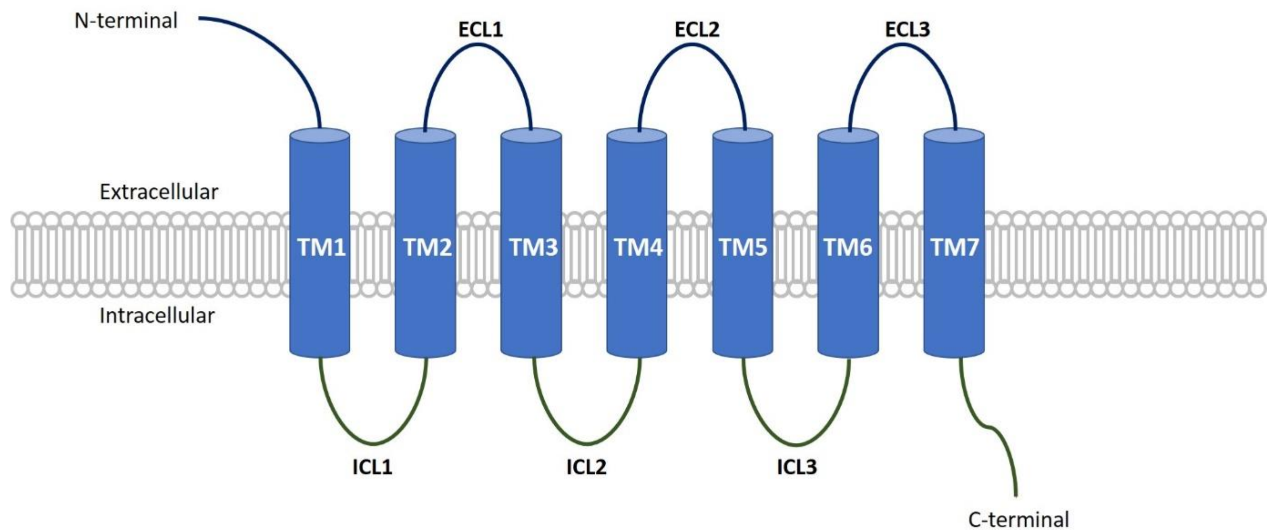


Figure 1.3. Structure of a G protein-coupled receptor (GPCR). GPCRs are seven transmembrane receptors comprising of three extracellular loops (ECL1-ECL3), seven transmembrane domains (TM1-TM7) and three intracellular loops (ICL1-ICL3).

Thus, the GRAFS classification system was proposed comprising of **G**lutamate family (class C) which includes mGluRs, a calcium-sensing receptor, $GABA_B$ receptors, three taste type 1 receptors and a family of pheromone receptors (V2 receptors); **R**hodopsin family (class A) which includes visual pigments, olfactory receptors, receptors for a variety of small molecules, neurotransmitters, peptides and hormones, taste type 2 receptors and five pheromone receptors (V1 receptors) (Attwood & Findlay, 1994; Dixon et al., 1987; Findlay & Pappin, 1986; Hanlon & Andrew, 2015; Mustafi & Palczewski, 2009; Palczewski et al., 2000); **A**dhesion family (Alexander et al., 2017; Hanlon & Andrew, 2015; Harmar, 2001; Scholz et al., 2019); **F**rizzled family which includes 10 Frizzled proteins (FZD(1-10)) and Smoothened (SMO) and lastly, the **S**ecretin family (class B) which includes receptors for polypeptide hormones like secretin, glucagon, glucagon-like peptides (GLP-1,2), glucose-dependent insulinotropic polypeptide (GIP), vasoactive intestinal peptide (VIP), growth-hormone-releasing hormone (GHRH) and pituitary adenylate cyclase-activating polypeptide (PACAP). Apart from these, there are some receptors whose endogenous ligands are not known and they have been named as orphan receptors (Alexander et al., 2017; Civelli, 2012; Fredriksson & Schiöth, 2005; Schiöth & Fredriksson, 2005).

Studies in β-adrenergic receptors have shown that binding of an agonist causes a conformational change in the receptor that promotes coupling of the receptors with their respective G proteins (Ghanouni et al., 2001). The GPCR-G-protein complex was not formed when the binding site was unoccupied or was occupied with antagonists (Ghanouni et al., 2001; R. J. Lefkowitz & Caron, 1988; L. E. Limbird et al., 1980; Weis & Kobilka, 2018). An "agonist" is a ligand which when binds to the receptor induces full signal transduction in the cell. It can either facilitate the endogenous ligand or can act as a substitute for the natural ligand. An "antagonist" on the other hand blocks or reduces the effect of the agonist on the receptor, i.e., no signal transduction occurs. There are some ligands that can act as an agonist but induce a relatively smaller net effect on signal transduction compared to agonists. These are called "partial agonists". In case of antagonists, there can still be basal or constitutive receptor activity because it was found that receptors can be basally active without an activating ligand. Thus, a new class of ligands were discovered called "inverse agonists" that act opposite to the agonist and cause negative effect on signal transduction upon binding with the receptor (Berg & Clarke, 2018; Kenakin, 1987; Kowalski et al., 2017; Rosenbaum et al., 2009; Weis & Kobilka, 2018). GPCRs are involved in

almost every aspect of animal life, from early development to vision, olfaction, taste, heart function, neuronal activity/signal propagation, cell migration etc. Thus, nearly 40-50% of all modern drugs target GPCRs and most of them belong to one of the ligand categories described above (Ford et al., 2017; Fredriksson & Schiöth, 2005; Hanlon & Andrew, 2015; Lagerström & Schiöth, 2008; Lindsley et al., 2016; Rask-Andersen et al., 2011; Rosenbaum et al., 2009; Schlyer & Horuk, 2006; Shenker, 1995).

1.2.1 GPCR signalling

One of the earliest observations of GPCR signalling was an increase in the cyclic adenosine monophosphate (cAMP) levels upon binding of a hormone to its receptor. It was demonstrated that G proteins act as effectors of GPCR activation and regulate the activity of adenylate cyclase enzyme which is responsible for the generation of cyclic AMP in the cell (L. Limbird, 1981). The G-proteins are heterotrimeric, i.e., they contain three subunits G_{α}, G_{β} and G_{γ}. Organisms encode many subtypes of each subunit. For example, in mammals there are 23 genes for G_{α}, 5 genes for G_{β} and 12 genes for G_{γ}. Thus, different combinations of these subunits can assemble into proteins that can in turn activate different signalling pathways. G_{α} proteins can be myristoylated and N-terminal palmitoylated and are typically anchored to the membrane. G_{γ} proteins can be isoprenylated. G_{β} proteins do not have any membrane-anchoring post- translational modifications but they are tightly linked to G_{γ} through hydrophobic interactions (Hanlon & Andrew, 2015; Neer & Clapham, 1988; Ross, 1989). Once activated, GPCRs act as guanine nucleotide exchange factors (GEFs) and exchange GDP for GTP in the G_{α} subunit, which activates the protein. Binding to GTP to G protein causes a change in the conformation of G_{α}, allowing it to separate from the $G_{\beta\gamma}$ dimer (Digby et al., 2006). Activated subunits are then free to interact with the catalytic moieties of downstream targets (Hamm, 1998; Vanderbeld & Kelly, 2000). G_{α} has intrinsic GTPase activating protein (GAP) activity which results in the hydrolysis of bound GTP to GDP causing its inactivation, allowing it to reassociate with $G_{\beta\gamma}$ (Kleuss et al., 1994). This results in the termination of downstream signalling and represents a full GPCR G-protein cycle (Milligan & Kostenis, 2006; Neer, 1995) (**Figure 1.4**). G_{α} proteins are weak GTPases which slow down the signalling cascade. To accelerate GTP hydrolysis, G_{α} proteins are targeted by the regulator of G-protein signalling (RGS) molecules. RGS proteins have been shown to bind to specific G_{β} proteins and prevent re-formation of the heterotrimeric complex (De Vries, Zheng, et al., 2000).

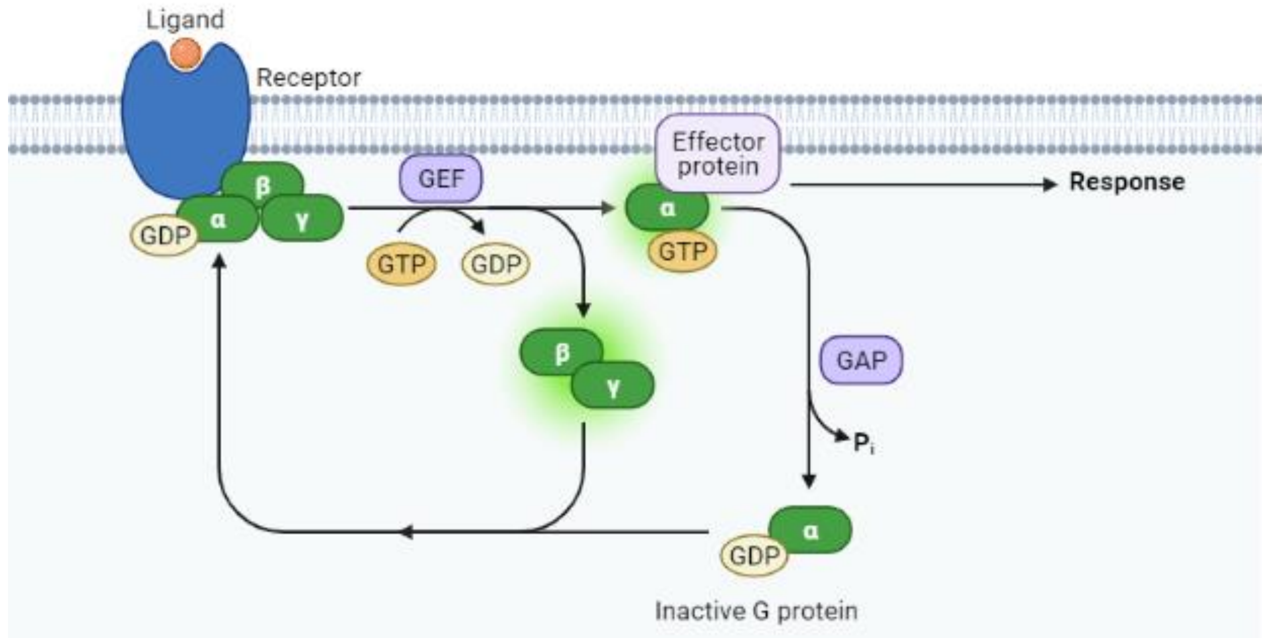


Figure 1.4. The GPCR-G protein cycle. GPCRs couple to heterotrimeric G proteins which comprise of three subunits: α, β, and γ. Agonist binding to the GPCR activates the receptor. The activated receptor acts as a guanosine nucleotide exchange factor (GEF), and as a result, a GDP molecule associated with the α subunit is exchanged for GTP. Subsequently, the β and γ subunits (βγ) dissociate from the α subunit. The activated G_{α} can then interact with an effector like adenylate cyclase or phospholipase C (PLC), which leads to the initiation of the second-messenger cascade. The βγ subunit also transduces various intracellular signalling and recycles back. The intrinsic GTPase activity of the α subunit leads to the hydrolysis of GTP to GDP and terminates the signal and allows reformation of the inactive G-protein complex.

Conversely, there are activators of G-protein signalling (AGS) that can act as GEFs for G_α to prolong signalling (De Vries, Fischer, et al., 2000). Apart from the adenylate cyclase/cyclic AMP pathway, G proteins can target various other signalling cascades. The specificity for a particular pathway is achieved by the class of G_α protein involved. G_α proteins are divided into four subclasses: 1) $G_{\alpha(s)}$, 2) $G_{\alpha(i)}$ and $G_{\alpha(o)}$, 3) $G_{\alpha(q/11)}$ and 4) $G_{\alpha(12/13)}$ (Wettschureck & Offermanns, 2005). $G_{\alpha(s)}$ and $G_{\alpha(i/o)}$ regulate adenylate cyclases where $G_{\alpha(s)}$ is stimulatory whereas $G_{\alpha(i/o)}$ inhibits the adenylate cyclase activity. The cAMP levels in cells regulate the activity of various ion channels and also the activity of protein kinase A (PKA) (Wettschureck & Offermanns, 2005). $G_{\alpha(q/11)}$ activates phospholipase C (PLC) which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and membrane-bound diacylglycerol (DAG) (Gurpreet K. Dhami & Ferguson, 2006). The fourth subclass, $G_{\alpha(12/13)}$ activates Rho GEFs, which in turn activate Rho which is responsible for cytoskeleton regulation (Hanlon & Andrew, 2015). In addition to the above mentioned canonical pathways, it has also been reported that some GPCRs can transduce their signalling through G-protein-independent mechanisms as well as through multiple types of G proteins (Heuss & Gerber, 2000; Offermanns et al., 1994).

1.2.2 GPCR desensitization

The waning of cellular response or deactivation of the receptor even during continuous agonist exposure is termed as "desensitization". Thus, desensitization is a protective mechanism adopted by the cells to prevent chronic overstimulation of the receptors. Several mechanisms exist to attenuate the signalling of GPCRs. This mainly involves three families of regulatory molecules: second messenger-dependent protein kinases e.g., PKA and PKC; G protein-coupled receptor kinases (GRKs) e.g., rhodopsin kinase and β-adrenergic receptor kinase (βARK); and arrestins (visual and non-visual) (J. L. Benovic et al., 1987; Bouvier et al., 1988; Ferguson, 2001; Hausdorff et al., 1990; Robert J Lefkowitz, 1998). Desensitization itself can be studied along several lines: "agonist-specific" or homologous desensitization and "non-agonist-specific" or heterologous desensitization; rapid or short-term desensitization (seconds to minutes) and slow or long-term desensitization (hours to days); and loss of receptor signalling function (uncoupling) or loss of receptor number (downregulation). Homologous desensitization involves loss of GPCR responsiveness due to prolonged activation by their agonists and is often mediated by GRKs and arrestins, whereas heterologous desensitization involves diminished responsiveness to multiple

stimuli due to feedback regulation by the activation of second messenger-regulated kinases downstream of the same or a different GPCR (Kelly et al., 2008). Also, uncoupling of GPCRs from G-proteins is generally a mechanism for rapid desensitization while downregulation or lysosomal degradation is a mechanism for slow desensitization of the receptor (Gainetdinov et al., 2004; Hausdorff et al., 1990; Kelly et al., 2008; Krupnick & Benovic, 1998; Robert J Lefkowitz, 1998).

Agonist-specific desensitization starts with the conformational change in the receptor upon binding with its ligand, that causes the release of the heterotrimeric G protein complex as described earlier. The same conformational change allows access to the GRKs to promote phosphorylation of the receptor (Krupnick & Benovic, 1998). GRKs phosphorylate the receptor at serine/ threonine residues in the carboxyl-terminal tails and/or third intracellular loops. The $G_{\beta\gamma}$ subunit recruits GRKs to GPCRs, hence establishing a negative-feedback loop (Ferguson, 2001). Phosphorylation of the receptor results in β-arrestin binding, which then recruits clathrin and its adaptor AP-2 to cause GPCR internalization (Ferguson et al., 1998; Hanlon & Andrew, 2015). Mammals express seven GRK subtypes (GRK1-GRK7), which are further divided into three subfamilies based on sequence and functional similarity: GRK1/7, GRK2/3 and GRK4/5/6. GRK1 (earlier called rhodopsin kinase) was the first GPCR kinase to be discovered. It specifically binds and phosphorylates light-activated rhodopsin which is necessary to quench rhodopsin signalling (kuhn, 1978; Weller et al., 1975). GRK1/7 family is strictly localized to the visual system, where GRK1 is primarily present in rod cells while GRK7 is found in the cone cells of retina (Weiss et al., 1998). Other GRKs are non-visual. Both GRK2 and GRK3 were discovered *via* their ability to phosphorylate β_2-adrenergic receptor (β_2-AR) and facilitate its desensitization (J. L. Benovic et al., 1987, 1991). They were later shown to have broad specificity for agonist-dependent phosphorylation of many other GPCRs (Jeffrey L. Benovic, 2021; Gainetdinov et al., 2004; E. V. Gurevich & Gurevich, 2021; V. V. Gurevich & Gurevich, 2019; Krupnick & Benovic, 1998; Magalhaes et al., 2012).

Phosphorylation by GRKs alone was not able to cause complete desensitization of some GPCRs. This suggested the presence of an additional protein which enhanced the inactivating effect of GRK-mediated phosphorylation. Thus, a 48 kDa protein was identified that was able to bind to the phosphorylated form of light-activated rhodopsin and turn-off or arrest phototransduction

leading to its current nomenclature, arrestin (J. L. Benovic et al., 1987; Jeffrey L. Benovic, 2021; Weller et al., 1975). There are four arrestin isoforms in mammals: arrestin1 (visual or rod arrestin, previously called 48-kDa protein or S-antigen), arrestin2 (β-arrestin or β-arrestin1), arrestin3 (β-arrestin2) and arrestin4 (cone arrestin or X-arrestin). Thus, there are two visual arrestins (1 and 4) and two non-visual arrestins (2 and 3). Because of their clear preference for β_2-AR, the non-visual arrestins were originally termed as β-arrestins. The β-arrestin C-terminus directly binds clathrin heavy chain and the β_2 adaptin subunit of the adapter protein-2 (AP-2) complex which is important for agonist-induced receptor internalization as discussed in the next section. GRKs and arrestins play a central role in regulating (i) the desensitization (uncoupling) of GPCR/G-protein signalling, (ii) the endocytosis of GPCRs to endosomes to allow GPCR dephosphorylation and resensitization, and (iii) GPCR signalling *via* G-protein-independent mechanisms (Gainetdinov et al., 2004; E. V. Gurevich & Gurevich, 2006; V. V. Gurevich & Gurevich, 2019; Krupnick & Benovic, 1998; Robert J Lefkowitz, 1998; Magalhaes et al., 2012; Pavlos & Friedman, 2017; Peterson & Luttrell, 2017; Smith & Rajagopal, 2016). GRKs can also regulate the phosphorylation-independent GPCR desensitization. This was suggested by the ability of a catalytically inactive form of GRK2 to bind to $G_{\alpha(q/11)}$ and attenuate the downstream signalling (Ferguson, 2007; Pao & Benovic, 2002).

1.2.3 GPCR trafficking: Significance of internalization and recycling

Internalization or sequestration of the receptors into endosomes or intracellular membrane compartments of the cell is an important mechanism to regulate the activity and signalling of GPCRs. The earliest evidence for agonist-mediated endocytosis of GPCRs came from the studies of desensitization of β-adrenergic receptors induced by isoproterenol (an agonist of β-ARs) (Chuang & Costa, 1979; Staehelin & Simons, 1982). As mentioned earlier, β-arrestins confer a steric hindrance to the GRK-phosphorylated GPCRs that uncouples them from their respective G-proteins, following which GPCRs can internalize *via* different endocytic pathways. β-arrestins specifically target GPCRs for endocytosis through clathrin-coated vesicles (Goodman et al., 1996; Laporte et al., 1999; Oakley et al., 1999). Two discoveries led to this hypothesis: 1) β_2-ARs that were deficient in agonist-induced phosphorylation by GRKs failed to recruit β-arrestins and failed to undergo internalization and expression of mutant β-arrestins acted as dominant negatives for receptor internalization (Miller & Lefkowitz, 2001); 2) Chimeras of β-arrestins and visual arrestins

that lacked the binding site to clathrin triskelions failed to promote internalization of the receptor (Goodman et al., 1996). The coat protein clathrin along with its adaptor AP2 or some other adaptors forms clathrin-coated pits (CCPs). Clathrin polymerization then helps in the formation and constriction of vesicle neck. Then, the membrane scission protein dynamin is recruited which is a GTPase, it forms a helical polymer around the constricted neck and upon GTP hydrolysis, mediates fission and formation of a clathrin-coated vesicle (CCV). This is followed by the release of the clathrin basket (Doherty & McMahon, 2009; Guo et al., 2015; Schmid, 1997; Weinberg & Puthenveedu, 2019; Wolfe & Trejo, 2007). The other well-characterized route for GPCR endocytosis is caveolae-dependent pathway. Caveolae are caveolin-1 enriched subdomains of lipid rafts that form smooth invaginations of plasma membrane. Cholesterol depletion flattens caveolae and they require the GTPase activity of dynamin for budding (Anderson, 1998; Guo et al., 2015; Henley et al., 1998; Nabi & Le, 2003; Parton & Simons, 2007; Rothberg et al., 1992; X. Zhang & Kim, 2017).

Not all GPCRs require β-arrestins for internalization. There are three principle ways through which GPCRs can internalize: (1) arrestin and dynamin-dependent, (2) arrestin-independent, dynamin-dependent and (3) arrestin and dynamin-independent endocytosis (Claing et al., 2002; Tan et al., 2004). The route that a particular GPCR takes depends on the type of the receptor, type of the ligand and type of the system (Bhattacharyya, 2016). Many GPCRs like β_2-AR internalize in an arrestin, clathrin and dynamin-dependent manner. However, internalization of 5-Hydroxytryptamine 2A (5-HT2A) receptors, rat Gonadotropin-Releasing Hormone Receptor (GnRH-R) and m1, m3, m4 mAChRs in HEK293-tsA cells is dynamin-dependent and arrestin-independent (Bhatnagar et al., 2001; Heding et al., 2000; Lee et al., 1998). In contrast, internalization of m2 mAChRs in HEK293-tsA cells and the angiotensin II type 1A receptor ($AT_{1A}R$) is independent of the function of both β-arrestin and dynamin (Pals-Rylaarsdam et al., 1997; J. Zhang et al., 1996). 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 internalizes *via* clathrin-coated pits upon GRK-mediated phosphorylation but PKA-dependent phosphorylation of the same receptor directs it to the caveolin-mediated pathway (Rapacciuolo et al., 2003). Internalization of the β-adrenergic receptors occurs *via* non-coated vesicles in A431 cells, whereas, internalization of the same receptor occurs *via* clathrin-coated vesicles in a number of other cell types (Raposo et al., 1989).

Phosphorylation of GPCRs has long been considered to be an indispensable mechanism for influencing receptor trafficking and function. In addition to phosphorylation, ubiquitination is another reversible post-translational modification that is now well-established to regulate the endocytic membrane trafficking of GPCRs (Hislop & Von Zastrow, 2011; Kennedy & Marchese, 2015; Marchese et al., 2003; Marchese & Trejo, 2013; Piper et al., 2014; Shenoy, 2014).

Once internalized, GPCRs can either enter the lysosomal compartment for degradation which leads to the downregulation of the receptor or they can enter specialized intracellular compartments where they are dephosphorylated and recycled back to the cell surface regaining access to the extracellular ligand, a phenomenon known as "resensitization" (Pippig et al., 1995; Pöll et al., 2011; Tsao et al., 2001) **(Figure 1.5)**. Initially, receptor internalization was thought to be an important mechanism only for desensitization because it physically uncouples the receptors from the G proteins. The idea that internalization also plays an important role in GPCR resensitization came from the following observations: (a) receptors from the "sequestered" membrane fraction are phosphorylated to a lesser extent than those from the plasma membrane, (b) pharmacological treatments that block receptor internalization like concanavalin A and hypertonic sucrose block receptor resensitization without affecting its ability to signal and desensitize, (c) some GPCR mutants which have the ability to signal and desensitize, neither internalize nor resensitize, (d) for many GPCRs, desensitization proceeds more rapidly than receptor internalization and lastly, (e) β-arrestin plays an important role in resensitization because expression of a mutant form of β-arrestin not only blocks β_2-AR sequestration but also impairs its ability to dephosphorylate and resensitize (Ferguson, 2001; Ferguson et al., 1998; Krueger et al., 1997; Pippig et al., 1995; Sibley et al., 1986; Staehelin & Simons, 1982; Yu et al., 1993). Association of β-arrestin with GPCRs dictates the profile of receptor resensitization and GPCRs have been divided into two classes based on how strongly they maintain β-arrestin binding: class A and class B (different from the nomenclature used in receptor classification). Class A receptors, such as the β_2-ARs lose β-arrestin at or near the plasma membrane and can be dephosphorylated and recycled back to the cell surface (Hanyaloglu & Zastrow, 2008). Whereas, class B receptors, like the V2 vasopressin receptors maintain β-arrestin binding in the endosomes and can stimulate ubiquitination of the receptor

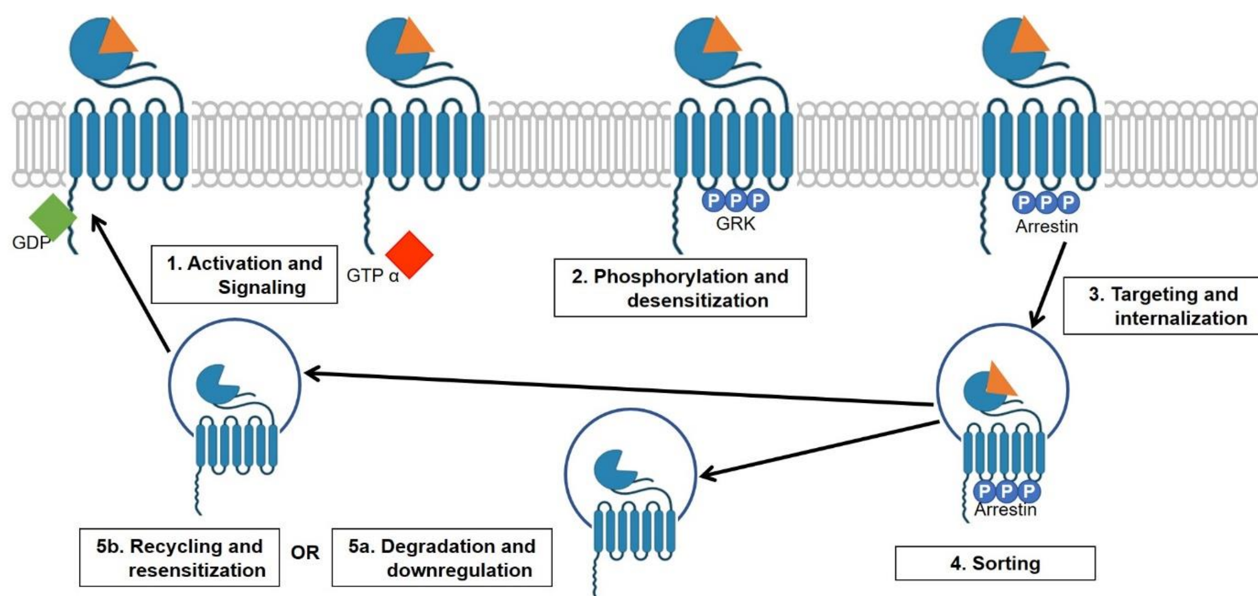


Figure 1.5. Mechanisms of GPCR trafficking. Activation of the GPCR by the agonist results in the dissociation of G proteins into G_{α} and $G_{\beta\gamma}$ subunits followed by phosphorylation of the receptor by the activated kinases. The phosphorylated receptor subsequently binds β-arrestin which leads to the desensitization of the receptor. The desensitized receptor internalizes and enters the sorting endosome. From here, the receptor can enter the recycling endosome to recycle back to the cell surface and resensitize. Alternatively, the internalized receptor can be targeted to the lysosomes for degradation.

(Hanlon & Andrew, 2015; Hanyaloglu & Zastrow, 2008; Oakley et al., 1999; Smith & Rajagopal, 2016). Ubiquitination leads to the downregulation of many GPCRs. For example, protease-activated receptor 1 and 2 (PAR1, PAR2), chemokine receptor CXCR4 and endothelin-B receptors are predominantly targeted to lysosomes for degradation after internalization (Bremnes et al., 2000; Liebick et al., 2016; Marchese & Trejo, 2013; Trejo & Coughlin, 1999). It has been reported that many GPCRs such as endothelin-A receptors, m3 mAChRs, β_2-adrenergic receptors, delta opioid receptors (DOR) and μ-opioid receptors (MOR) recycle back and regain responsiveness or resensitize (Bremnes et al., 2000; Edwardson & Szekeres, 1999; Hasbi et al., 2000; Pippig et al., 1995; Qiu et al., 2003). Rhodopsin is a GPCR that gets dephosphorylated without internalization (Ferguson, 2001; Palczewski et al., 1989). This suggests that each GPCR is unique and findings from a particular GPCR cannot define the fate of another GPCR. Thus, it is important to study individual GPCR trafficking as it plays a key role in regulating receptor responsiveness and turnover and also in maintaining the signalling and homeostasis within the cell.

1.3. Group I mGluRs

Group I mGluRs comprising of mGluR1 and mGluR5 belong to the class C family of GPCRs and are located at the perisynaptic region of the post-synaptic neuron (Luján et al., 1996; Niswender & Conn, 2010). The glutamate sensitivity of group I mGluRs ranges between 10^{-3} – 1 mM [Glu] (Reiner & Levitz, 2018). Both members of the group I mGluR family show differential expression in the CNS. Also, different splice variants of the same gene show differential cellular localization in the brain. The *Grm1* gene encoding mGluR1 generates four different isoforms through alternative splicing, namely mGluR1α (a), mGluR1β (b), mGluR1γ (d) and mGluR1δ (E55), which differ primarily in their intracellular C-terminal domain (Laurie et al., 1996; Naito et al., 2018; Zhu et al., 1999). In rat brain, mGluR1α, a 142 kDa protein is enriched in the cerebellum, basal ganglia, thalamus, superior colliculus, stratum oriens of CA1 and polymorph layer of dentate gyrus in hippocampus and olfactory bulb. Lower levels are present in neocortex, amygdala, medulla and hypothalamus. The mGluR1β splice variant, a 100 kDa protein is the major form found in the hippocampus while mGluR1α predominates in other brain regions. This distribution is largely similar in rats and humans (Martin et al., 1992; Naito et al., 2018; Shigemoto et al., 1992; Stephan et al., 1996; M. Yuzaki & Mikoshiba, 1992). mGluR5, on the other hand, is encoded by the *Grm5* gene and exists as three splice variants, mGluR5a, mGluR5b and mGluR5d. mGluR5b is the longer

isoform compared to mGluR5a and is the major isoform that predominates in adults (Romano, Van Den Pol, et al., 1996). In contrast, the expression of mGluR5a increases and reaches a peak during second postnatal week and subsequently comes down (Joly et al., 1995; Malherbe et al., 2002; Minakami et al., 1995; Romano, Van Den Pol, et al., 1996). mGluR5 immunoreactivity is seen in the CA1 and CA3 region of the hippocampus, dentate gyrus, cerebral cortex, striatum, olfactory bulb and nucleus accumbens. It is also expressed in the cerebellum, but at a much lower level (Bordi & Ugolini, 1999; Romano et al., 1995; Shigemoto et al., 1993). The two group I mGluRs are differentially distributed during development with mGluR5 binding sites being more than mGluR1 at embryonic day 18 followed by an increase in the expression of mGluR1 during second postnatal week (Catania et al., 1994; Ferraguti & Shigemoto, 2006; López-Bendito et al., 2002). Apart from the central nervous system, glutamate signalling through group I mGluRs is also prevalent in peripheral tissues. For example, group I mGluRs are present in osteoblasts, heart, testis, pancreas, hepatocytes as well as in skin cells where they play a crucial role in pain sensation (Bhave et al., 2001; Gill et al., 1999; Gu & Publicover, 2000; Hinoi et al., 2004). Structurally these receptors contain a large extracellular domain called the Venus flytrap (VFT) which is made up of two opposing lobes separated by a cleft where endogenous ligands bind (Brauner-Osborne et al., 2006; Chun et al., 2012; Kniazeff et al., 2011; Lindsley et al., 2016; Vafabakhsh et al., 2015; Wu et al., 2014). Another characteristic feature is that they form obligatory homodimers or heterodimers providing a unique mode of activation of these receptors (Kunishima et al., 2000; Romano, Yang, et al., 1996; Techlovská et al., 2014). The crystal structures of the ligand binding region as well as the transmembrane domain of both mGluR1 and mGluR5 have been elucidated thus providing an impetus to the discovery of novel therapeutics in the form of agonists, antagonists and allosteric modulators (Brauner-Osborne et al., 2006; Doré et al., 2014; Jingami et al., 2003; Tsuchiya et al., 2002; Wu et al., 2014).

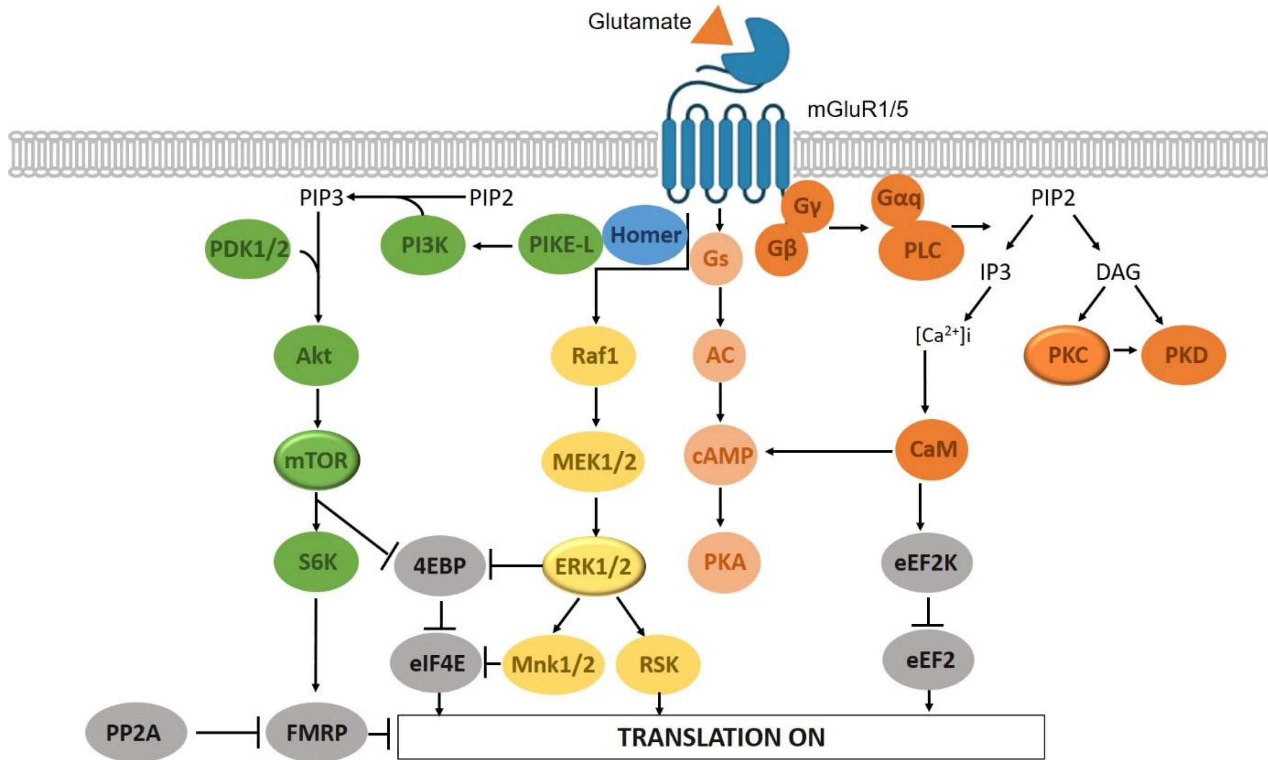
1.3.1. Group I mGluR signalling

As mentioned earlier, group I mGluRs are positively coupled to phospholipase C (PLC) through $G_{\alpha(q/11)}$. PLC leads to the hydrolysis of phosphatidyl inositol-4,5-bisphosphate (PIP_2) which generates two second messengers: inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 being a soluble molecule diffuses through the cytoplasm to open channels present in the endoplasmic reticulum, leading to a rise in cytosolic calcium (Ca^{2+}) levels. DAG, which stays on the membrane,

along with Ca^{2+}, activates protein kinase C (PKC), whose activity has been associated with synaptic plasticity in the hippocampus (Abdul-Ghani et al., 1996; Abe et al., 1992; Maiese et al., 1999; Nicoletti et al., 1986, 1988; Sugiyama et al., 1987). mGluR5 also induces the phosphorylation of protein kinase D (PKD) at the autophosphorylation site Ser-916 which is dependent on the binding of DAG and phosphorylation by PKC. The autophosphorylation of PKD correlates with its catalytic activity and activated PKD plays a role in regulating transcription and potentially in mGluR5-mediated plasticity (Krueger et al., 2010).

Group I mGluRs can also activate other G proteins like $G_{\alpha(s)}$ and $G_{\alpha(i/o)}$ (Aramori & Nakanishi, 1992; Gurpreet K. Dhami & Ferguson, 2006; Francesconi & Duvoisin, 1998). $G_{\alpha(s)}$ stimulates the production of cyclic AMP (cAMP) which regulates the intracellular levels of protein kinase A (PKA). Treatment with pertussis toxin partially inhibited mGluR1α-stimulated phosphatidyl inositol (PI) hydrolysis, enhanced mGluR1-stimulated cAMP formation and completely inhibited the stimulation of arachidonic acid release suggesting that mGluR1 activates multiple signal transduction pathways by coupling with different G proteins (Aramori & Nakanishi, 1992). On the other hand, pertussis toxin did not block glutamate-stimulated PI hydrolysis in cells expressing mGluR5 (Abdul-Ghani et al., 1996; Aramori & Nakanishi, 1992). The selectivity of mGluRs to couple to either G_q or G_s and thus activate the dual signal transduction pathways, viz., PI turnover and adenylyl cyclase/cAMP-dependent protein kinase A comes from specific residues located within the second and third intracellular loops (i2 and i3) of group I mGluRs (Francesconi & Duvoisin, 1998). Recent reports suggest that apart from $G_{\alpha(s)}$, coupling to $G_{\alpha(q)}$ can also activate PKA which is mediated by parallel signalling *via* either PKC or Ca^{2+}-calmodulin (CaM) dependent pathways (Y. Chen et al., 2017; Yang et al., 2016a). While PKC selectively inhibits the agonist-dependent stimulation of IP_3 pathway providing a negative feedback, PKA potentiates the agonist-independent stimulation of IP_3 pathway. Furthermore, PKA modulates mGluR5 function by directly phosphorylating the receptor (Francesconi & Duvoisin, 2000; Uematsu et al., 2015).

In addition to these canonical signalling pathways, group I mGluRs can also activate G-protein-independent signalling leading to the activation of extracellular signal-regulated protein kinase (ERK) as well as mammalian target of rapamycin (mTOR) pathways (Banko et al., 2006; Gerber et al., 2007; Hermans & Challiss, 2001; Heuss et al., 1999; Maiese et al., 2005; Nicodemo et al., 2010; Page et al., 2006) **(Figure 1.6)**.



Adapted from Bhakar et al., 2012, Annu. Rev. Neurosci.

Figure 1.6. Various signalling pathways of group I mGluRs. Agonist binding to group I mGluRs activates three main pathways that couple the receptors to the regulation of translation: (a) the canonical PLC/calcium and PKC pathway, (b) the ERK pathway and (c) the mTOR pathway. In addition, coupling of group I mGluRs with G_s proteins can lead to the activation of a fourth pathway that leads to the activation of PKA. PKA activation can also be triggered through the G_q pathway by the coupling of Ca^{2+} released from the intracellular stores to calmodulin (CaM) that trigger Ca^{2+}-CaM dependent pathways including cAMP-dependent PKA. These multiple signalling pathways ultimately converge to initiate translation downstream of group I mGluRs.

Some studies have suggested that group I mGluRs may couple to the ERK cascade through β-arrestins which can serve to regulate protein synthesis through Mnk1 and eukaryotic translation initiation factor 4E (eIF4E) (DeWire et al., 2008; Eng et al., 2016). Group I mGluR-mediated ERK(1/2) activation can also be PKC-dependent (Ferraguti et al., 1999; Peavy & Conn, 1998). ERK activation is required for enhanced pain sensitivity as well as for mGluR-dependent LTD and mGluR5-mediated activation of fragile X mental retardation protein (FMRP)-regulated mRNA translation (Gallagher et al., 2004; Karim et al., 2001; Li et al., 2011; Osterweil et al., 2010).

To activate the mTOR pathway, mGluR5 couples to a post-synaptic density scaffolding protein, Homer that recruits a GTPase, PIKE-L (PI 3-Kinase Enhancer-L) forming an mGluR-Homer-PIKE complex (Ahn & Ye, 2005). PIKE directly enhances the lipid kinase activity of PI3K (phosphoinositide 3-kinase), leading to the phosphorylation of PIP_2 to form PIP_3. PIP_3 along with PDK (phosphoinositide-dependent kinase) activates the serine/threonine kinase Akt or protein kinase B. Akt can then activate mTOR by direct phosphorylation and indirectly through inhibition of the tumor-suppressor complex, composed of TSC1 and TSC2 (Antion et al., 2008; Banko et al., 2006; Bhakar et al., 2012). Along with other effects in the cell, the PI3K-Akt-mTOR pathway also regulates FMRP phosphorylation and subsequent modulation of target mRNA expression required for protein synthesis-dependent synaptic plasticity (Narayanan et al., 2008).

1.3.2. Group I mGluR desensitization

Desensitization of mGluRs attenuates the responsiveness of these receptors towards the ligand that acts as an important feedback mechanism to protect these receptors from overstimulation. Overstimulation of group I mGluRs can lead to excitotoxicity which induces neuronal cell death and contributes to a variety of neurodegenerative diseases such as amyotropic lateral sclerosis (ALS), Huntington’s disease and Alzheimer’s disease (Bordi & Ugolini, 1999; Lianne B. Dale et al., 2000). In comparison to family A and B GPCRs, the desensitization of family C GPCRs is relatively less understood (Abreu et al., 2021). The earliest evidence for the desensitization of group I mGluRs came from the inhibition of mGluR-mediated phosphoinositide hydrolysis upon prolonged and repeated agonist exposure in non-neuronal cells as well as in rat cerebrocortical nerve terminals (Desai et al., 1996; Herrero et al., 1994). As stated earlier, like other GPCRs, desensitization of mGluRs can also proceed through phosphorylation-dependent mechanisms

involving the action of second messenger-dependent kinases like PKA or PKC and through GRKs that recruit β-arrestins subsequent to receptor phosphorylation which uncouples the receptors from their heterotrimeric G proteins (Krupnick & Benovic, 1998; Prabhat Kumar Mahato et al., 2018; Li-min Mao et al., 2008; Premont et al., 1995). For example, PI hydrolysis leads to a rise in intracellular Ca^{2+} and activation of PKC through DAG, and phosphorylation by PKC has a negative feedback control on mGluR5 activated by glutamate (Gereau IV & Heinemann, 1998; Herrero et al., 1994). PKC-mediated phosphorylation of mGluR5 inhibits the binding of CaM to the receptor and CaM binding in turn inhibits the phosphorylation by PKC. Thus, CaM binding to mGluR5 and its PKC phosphorylation are mutually antagonistic (Minakami et al., 1997). In transfected cells, mGluR1 activation induces a single-peaked Ca^{2+} rise whereas mGluR5 activation specifically triggers high-frequency Ca^{2+} oscillations that are dependent on the PKC phosphorylation of a single serine residue in mGluR5 (Kim et al., 2005, 2008; Li-min Mao et al., 2008). These results provide new leads for approaching the Ca^{2+}-CaM mediated modulation of the mGluRs. In contrast to PKC, another second messenger-dependent protein kinase, PKA seems to have opposite effect on the desensitization of group I mGluRs. PKA inhibits desensitization by potentiating the IP_3 pathway downstream of mGluR1 and induces sustained coupling of mGluR1α to IP_3 pathway even in the absence of agonist (Francesconi and Duvoisin, 2000). PKA activation has been reported to inhibit the agonist-mediated desensitization of mGluR1 by occluding the association of GRK2 and arrestin-2 with mGluR1a and mGluR1b (Mundell, Pula, More, et al., 2004). In addition to the homologous desensitization, PKC also modulates the heterologous desensitization of mGluR1 together with Ca^{2+} calmodulin-dependent kinase II (CaMKII) since, PKC and CaMKII inhibitors blocked the group I mGluR desensitization induced by stimulation of heterologous $G_{\alpha(q/11)}$-coupled m1 mAChRs (Mundell, Pula, McIlhinney, et al., 2004).

Another group of kinases called GRKs also play important roles in the desensitization of group I mGluRs. For example, GRK-mediated phosphorylation of mGluR1a, specifically by GRK2 and GRK5, results in the desensitization of agonist-stimulated mGluR1a responses (Lianne B. Dale et al., 2000, 2002). GRK2 also regulates the phosphorylation of mGluR5 that is dependent, in part, on the presence of Thr^{840} in the carboxyl terminal tail of mGluR5 (Sorensen & Conn, 2003). GRK4 regulates the desensitization of mGluR1 in HEK293 cells and cultured Purkinje cells in an agonist-dependent manner (homologous desensitization) through phosphorylation. Phosphorylation of mGluR1 by GRK4 can also recruit the adaptor protein β-arrestin 1 to plasma membrane that leads

to the internalization of mGluR1 which is also an important mechanism for receptor desensitization (Iacovelli et al., 2003; Sallese et al., 2000).

Group I mGluRs can also be desensitized through phosphorylation-independent mechanisms. The observation that expression of the N-terminal domain of GRK2 that harbours the RGS homology (RH) domain alone is sufficient to fully uncouple mGluR1 from $G_{\alpha q/11}$ suggests that this domain functions to regulate the phosphorylation-independent desensitization of the receptor (Gurpreet Kaur Dhami et al., 2002). GRK2 augments mGluR desensitization but requires intact RH domain interactions with both mGluR1a and $G_{\alpha(q/11)}$ (Gurpreet K. Dhami et al., 2004; Gurpreet Kaur Dhami et al., 2002). GRK2 also regulates phosphorylation-independent desensitization of mGluR5 in striatal neurons using similar mechanisms as observed by the ability of the catalytically inactive GRK2 mutant to still cause receptor desensitization but a mutant devoid in $G_{\alpha(q/11)}$ binding is unable to do so (Ribeiro et al., 2009). Additionally, the interaction of group I mGluRs with Huntington binding protein optineurin, restrains their coupling with G-proteins, resulting in the phosphorylation-independent desensitization of group I mGluRs (Gurpreet K. Dhami & Ferguson, 2006).

1.3.3. Group I mGluR trafficking

Receptor trafficking plays crucial roles in regulating their proper spatio-temporal localization in the neuron and the appropriate delivery of GPCRs to the cell surface is critical for proper activity of these receptors. Thus, inappropriate trafficking of the receptor could result in aberrant signalling with pathological consequences. Despite this obvious significance, the molecular mechanisms governing group I mGluR trafficking have not been explored much compared to their ionotropic counterparts (Beattie et al., 2000; Biou et al., 2008). Like many other GPCRs, group I mGluRs have been shown to undergo rapid internalization following agonist exposure (Mundell et al., 2001, 2003; Mundell, Pula, McIlhinney, et al., 2004). The internalization of group I mGluRs begins as early as 1 min post agonist exposure and majority of the receptors are internalized 30 min post ligand application (P. K. Mahato et al., 2015; Pandey et al., 2014). Initially, receptor internalization was studied as a mechanism for the desensitization of these receptors. Work done in β-adrenergic receptors had shown that GRKs induce phosphorylation of these receptors which acts as a signal for the recruitment of β-arrestins (Claing et al., 2002; Sibley et al., 1986). β-arrestins

uncouple the receptors from their respective G proteins and target them for endocytosis through clathrin-coated vesicles (Claing et al., 2002; Drake et al., 2006; Goodman et al., 1996; Laporte et al., 1999). At first, it was thought that a well-studied GPCR like the β_2-AR could serve as a model system for other GPCRs, but work done by multiple labs on various GPCRs over the last 10-15 years has shown that each GPCR is unique and work done on one receptor like the β_2-AR cannot be extrapolated to other GPCRs as different molecular players may be involved (Bhattacharyya, 2016).

The internalization of group I mGluRs is also phosphorylation-dependent and various second-messenger-dependent kinases and GRKs play a crucial role in this process. For example, GRK4 and β-arrestin are involved in the agonist-mediated internalization of mGluR1 in cerebellar Purkinje cells and co-transfection of GRK4 with mGluR1 in HEK293 cells significantly enhanced agonist-dependent receptor internalization (Iacovelli et al., 2003; Sallese et al., 2000). PKC activation is important for glutamate-induced internalization of the mGluR1a and mGluR1c in HEK293 cells while PKA activation inhibits the agonist-induced internalization of mGluR1a and mGluR1b (Mundell et al., 2003; Mundell, Pula, More, et al., 2004). Activation of PKC as a result of mGluR5-triggered rise in the intracellular Ca^{2+} can lead to the phosphorylation of Ser^{901} at the C-terminus of the receptor which disrupts the binding of CaM, hence resulting in increased receptor internalization (Choi et al., 2011; Jeong et al., 2008). Ubiquitination also plays a critical role in the ligand-mediated internalization of group I mGluRs (Gulia et al., 2017). The ligand-mediated internalization of group I mGluRs is arrestin and dynamin-dependent (L. B. Dale et al., 2001; Mundell et al., 2001). Group I mGluRs can also undergo agonist-independent internalization, which is termed as "constitutive endocytosis" (Trivedi & Bhattacharyya, 2012). Some reports have shown that constitutive endocytosis of mGluR1 and mGluR5 is arrestin-independent and is governed by an alternate pathway comprising of a small GTP-binding protein Ral and phospholipase D2 (Bhattacharya et al., 2004; L. B. Dale et al., 2001). Both agonist-dependent and agonist-independent internalization of group I mGluRs have been shown to be dependent on clathrin (Fourgeaud et al., 2003; Luis Albasanz et al., 2002; Pula et al., 2004). mGluR1 and mGluR5 were seen to be associated with lipid rafts and alteration of membrane cholesterol content or perturbation of lipid rafts regulates agonist-dependent signalling by group I mGluRs (Burgueño et al., 2003; Kumari et al., 2013). A report has suggested that the constitutive

internalization of mGluR1/5 is mediated by caveolin-dependent pathways in heterologous cells as well as in neurons (Francesconi et al., 2009).

Following internalization, group I mGluRs take the recycling route and recycle back to the cell surface in 2.5 to 3.5 h in heterologous cells as well as in primary hippocampal neurons (P. K. Mahato et al., 2015; Pandey et al., 2014; Trivedi & Bhattacharyya, 2012). The recycling of mGluR1 is protein phosphatase 2A (PP2A)-dependent whereas recycling of mGluR5 depends completely on PP2A and partially on protein phosphatase 2B (PP2B) (P. K. Mahato et al., 2015; Pandey et al., 2014). Importantly, their exit from the recycling compartment is dependent on the pH of the endosomes. These results suggest that dephosphorylation is important for the recycling of group I mGluRs. The post-synaptic proteins Sorting Nexin 1 (SNX1) and Hrs regulate the recycling of group I mGluRs and mediate recycling through the slower recycling route which leads to the resensitization of these receptors (Sharma et al., 2018). Group I mGluR signalling depends on the type of cell as well as the type of ligand that activates them. The type of ligand can lead to "ligand bias" or biased agonism where a receptor's signalling can shift from G protein dependence to independence (Sengmany et al., 2017). The type of cell or the particular intracellular membrane from where the receptor signals can lead to "location bias". A recent study has identified the sequences within the C-terminus of mGluR5 that are important for its inner nuclear membrane localization. Nuclear trafficking of mGluR5 suggests that mGluR5 is a membrane-crossing multi-functional receptor that can activate various intracellular signalling pathways (Jong et al., 2014; Sergin et al., 2017; Suh et al., 2018).

1.3.4. Group I mGluRs: Role in synaptic plasticity and neuropsychiatric disorders

An important and intriguing property of the brain is its ability to transform experiences into memory by modification of the activity and organization of neural circuits and systems. Synapses are dynamic formations within the neuronal architecture, they tend to strengthen and weaken depending on various neuronal responses. This activity-dependent modification of the efficacy or strength of synaptic transmission at pre-existing synapses is referred to as synaptic plasticity (Citri & Malenka, 2008). Multiple evidences have accumulated over the past few years highlighting the impairment of synaptic plasticity in many neuropsychiatric disorders (Ebert & Greenberg, 2013; Hasan et al., 2012; Kauer & Malenka, 2007; Yang et al., 2016b; Zhao et al., 2011). Synaptic

plasticity can be defined in temporal terms as either short-term synaptic plasticity (lasting from milliseconds to minutes) or long-term synaptic plasticity (lasting from minutes to hours to days to lifetime). Such long-lasting forms of synaptic plasticity lead to more permanent changes in behavior and because of their nature, they are widely believed to be the cellular correlates of learning and memory (Citri & Malenka, 2008; Malenka, 1994; Malenka & Bear, 2004; Riedel et al., 2003). The earliest experimental evidence for such long-term modifications in synaptic strength came in the early 1970s when Bliss and Lomo demonstrated that repetitive activation of excitatory synapses in the hippocampus caused a long-lasting strengthening of synaptic strength that could last for hours or even days (Bliss & Lømo, 1973). This phenomenon is called "long-term potentiation" or LTP. Likewise, the long-lasting decrease or weakening of synaptic strength is known as "long-term depression" or LTD. Thus, LTP and LTD can modulate the strength of excitatory synapses bidirectionally (Citri & Malenka, 2008; Dudek & Bear, 1993; Malenka, 1994; Malenka & Bear, 2004).

Group I mGluRs are localized post-synaptically in a perisynaptic zone surrounding the iGluRs and are thus well positioned for causing the redistribution of AMPARs and NMDARs. They can induce both LTP and LTD (Gladding et al., 2009; Kopec et al., 2006; Christian Lüscher & Huber, 2010; Palmer et al., 1997). The cellular mechanisms underlying plasticity involve the activation of post-synaptic second messenger systems that usually result in an alteration in the intracellular Ca^{2+} levels in the post-synaptic neurons. Calcium-dependent second messenger systems alter the activity of protein kinases and phosphatases and these alterations in protein phosphorylations mediate the early stages of long-term synaptic plasticity. Long-lasting changes in synaptic strength are brought about by alterations in gene transcription and protein synthesis (Linden, 1996; Lu et al., 2008; Neyman & Manahan-Vaughan, 2008; Snyder et al., 2001). A rise in Ca^{2+} leads to the activation of protein kinases that phosphorylate target proteins and lead to the insertion of new AMPA receptors at the post-synaptic plasma membrane. This can be the cellular mechanism for mGluR-LTP. mGluR-LTD on the other hand, appears to result from a "slow" rise in Ca^{2+} and activation of phosphatases that dephosphorylate these target molecules and result in the internalization of AMPARs into the post-synaptic cell (Beattie et al., 2000; Carroll et al., 2001; Huganir & Nicoll, 2013; Malinow & Malenka, 2002; Schnabel et al., 2001; Snyder et al., 2001). In the hippocampus, two distinct forms of LTD exist. One form depends on the activation of NMDARs, whereas the other form depends on the activation of group I mGluRs (Oliet et al.,

1997). Work done by various groups has suggested that NMDAR-dependent LTD and mGluR-dependent LTD are mechanistically different (Bhattacharyya et al., 2009; Citri et al., 2009; Citri & Malenka, 2008; C Lüscher & Malenka, 2012; Malenka, 2003; Malinow & Malenka, 2002).

In the hippocampus, activation of mGluR triggers protein synthesis and mGluR-LTD has been observed to be dependent on these locally synthesized proteins in the dendrites (Costa-Mattioli et al., 2009; K. Huber et al., 2000; K. M. Huber et al., 2001; Richter & Klann, 2009; Steward & Schuman, 2003). Although the mechanisms of mGluR-LTD are relatively less understood compared to NMDAR-LTD, it 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 (Bear et al., 2004; Dölen et al., 2007; Dölen & Bear, 2008, 2009). Fragile X syndrome is one of the genetic causes of autism (Brown et al., 1982). *Fmr1* is the single gene responsible for Fragile X syndrome because a trinucleotide expansion of this gene (CGG_n) results in a non-functional Fragile X mental retardation protein (FMRP) (Bhakar et al., 2012). Group I mGluR signalling leads to the *de novo* translation of immediate early genes like *Arc* and *MAP1B* that play a role in maintaining LTD (Benoist et al., 2013; Y.-L. Chen & Shen, 2013; Davidkova & Carroll, 2007; Rial Verde et al., 2006; Waung et al., 2008). An unregulated translation of these plasticity related proteins leads to exaggerated mGluR-LTD in mouse models of Fragile X syndrome (Hou et al., 2006; K. M. Huber et al., 2002; Laggerbauer et al., 2001; Osterweil et al., 2010). Prolonged mGluR5 signalling can also lead to inflammatory pain. Proteins like Preso1 that regulate the binding of Homer with mGluR help in downregulation of mGluR signalling (Hu et al., 2017). But in case of knockdown of such proteins, mGluR-mediated pain remains elevated. Thus, mGluR5 antagonists like 2-methyl-6-(phenylethynyl)-pyridine (MPEP) can serve to therapeutically target mGluRs and reverse this phenotype (Hu et al., 2012). Apart from this, mGluR5 has been implicated in the pathology of schizophrenia, and the alterations in mGluR5 trafficking through its endogenous regulators like Norbin and Tamalin might contribute to the cognitive dysfunctions associated with this disorder (Lum et al., 2016; Matosin et al., 2014, 2015, 2017). Group I mGluR signalling is altered in a number of other neurological disorders like Alzheimer’s disease, Parkinson’s disease, epilepsy and addiction (Bordi & Ugolini, 1999).

1.4. Norbin

Ever since the discovery of a G-protein coupled glutamate receptor that could cause mobilization of Ca^{2+} ions using inositol triphosphate as a second messenger, there have been a lot of studies to find out the molecules that interact with these receptors and link them to the second messenger pathways (Brakeman et al., 1997; Houamed et al., 1991; Tanabe et al., 1992). The earliest ones to be identified were proteins that act as scaffold and interact directly with mGluRs. These scaffolding proteins are a part of the electron dense region present on the cytoplasmic face of the synapse called the post-synaptic density (PSD). The scaffolding proteins along with other interacting proteins form multivalent complexes that either cause the coupling of receptors to the intracellular second messengers or facilitate their internalization (Gao et al., 2013; Kaizuka & Takumi, 2018).

Norbin is a neurite-outgrowth promoting protein from rat brain. It was identified as a protein whose expression levels were increased during LTP induction in rat hippocampal slices using tetraethylammonium (TEA), a potassium channel blocker (Shinozaki et al., 1997). Human and mouse homolog of Norbin is called neurochondrin (NCDN) (Mochizuki-Sakisaka et al., 2004; Mochizuki et al., 1999). It is expressed mainly in neurons of brain cortex, cerebellum, hippocampus and spinal cord and is not expressed in glia (Shinozaki et al., 1997). However, the protein has been also detected in non-neuronal cells like chondrocytes, osteoblasts and osteocytes as well as in macrophages and neutrophils (Ishizuka et al., 1999; Pan et al., 2016a).

Norbin staining coincides with staining pattern of MAP2 and spinophilin. Thus, Norbin is distributed in dendrites and soma and is a cytosolic adaptor protein (Shinozaki et al., 1999; Wang et al., 2009b). It is a 75 kDa protein found in both vertebrates and invertebrates. Importantly, Norbin is a highly conserved protein among vertebrates with no known functional domains or homology to other known proteins (Shinozaki et al., 1999; Wang et al., 2010). Norbin mRNA is detectable by E18 and increases until P21. Norbin is crucial in the nervous system because its deletion in mouse is early embryonic lethal (Mochizuki et al., 2003) and forebrain specific Norbin knockouts are defective in synaptic plasticity (Wang et al., 2009b). Hence, it regulates neurite outgrowth and promotes synaptic plasticity in the CNS (Shinozaki et al., 1997, 1999).

It also has other important roles in the CNS. The C-terminus of Norbin (Glu499 to Pro729) specifically interacts with the membrane proximal region of mGluR1a, mGluR5a and mGluR5b

(Wang et al., 2009b). Norbin binding site overlaps with calmodulin binding site and knockdown of the endogenous Norbin decreases the cell surface expression of mGluR5 (Wang et al., 2009b). Status of the downstream effectors of mGluR signalling upon Norbin overexpression suggests that it positively regulates mGluR5 signalling as seen by the increase in the calcium oscillations in mGluR5 expressing HEK293 cells transfected with Norbin (Wang et al., 2009b). In mouse neural stem cells, targeted deletion of Norbin led to an impairment in spatial learning and sensorimotor gating, causing epilepsy (Dateki et al., 2005). Targeted deletion of Norbin in postnatal forebrain impaired hippocampal synaptic plasticity, leading to schizophrenia-like behaviours (Wang et al., 2009b), and deletion in the cortical and hippocampal neurons disrupted adult neurogenesis and caused depression-like behaviours (Wang et al., 2015). These rodent phenotypes may be relevant to humans, as Norbin levels are down-regulated in patients with epilepsy and dysregulated in schizophrenia subjects (Matosin et al., 2015; Y. Xu et al., 2017). Norbin has also been identified as a neuronal target antigen in autoimmune cerebellar degeneration (Miske et al., 2017).

Mechanistically, Norbin binds directly to numerous GPCRs (33 GPCRs out of the 45 tested). For example, C-terminus of Norbin interacts with the C-terminal of melanin-concentrating hormone receptor-1 (MCHR1), a member of the GPCR A superfamily. Overexpression of Norbin affects the G_q and $G_{i/o}$-mediated signal transduction of MCHR1 but does not affect its agonist-mediated internalization (Francke et al., 2006; Ward et al., 2009). Norbin binds through its own C-terminus at the membrane-proximal part of the intracellular C-terminal tail of the family C GPCRs (mGluR1 and mGluR5) (Wang et al., 2009b). It can affect the surface expression of these receptors. Furthermore, Norbin can affect GPCR signalling, resulting in altered intracellular Ca^{2+} levels and ERK activity (Wang et al., 2009b; Ward et al., 2009). Therefore, the regulation of GPCR signalling and surface expression of the receptor by Norbin is context-dependent with regards to the GPCR it couples to. Moreover, it is not possible to predict the effect of Norbin binding on the trafficking and signalling of a GPCR, nor are the mechanisms known through which Norbin binding affects the trafficking and signalling of GPCRs. These reasons prompted us to look at the role of this important protein in agonist-mediated internalization of mGluR5 in detail.

1.5. Prelude to the present study

As more and more advances in techniques for visualizing, characterizing and purifying novel proteins are being made, we are slowly starting to understand the networks that lie hidden within the post-synaptic density and the mechanisms by which they regulate particular neurotransmitter receptors. Trafficking plays a crucial role in targeting these receptors to the right place in the cell at the right time. Since activation of mGluRs can lead to the internalization of its ionotropic counterparts and regulate various forms of synaptic plasticity in the brain, we decided to first understand if Norbin plays any role in the agonist-mediated trafficking of group I mGluRs. We chose Norbin for our study because Norbin knockout mice display a behavioral phenotype associated with schizophrenia which is reminiscent of the mGluR5 knockout phenotype. Also, how Norbin binding affects the agonist-dependent internalization of GPCRs is not yet understood. Chapter 3 discusses the role of Norbin in group I mGluR trafficking and Chapter 4 discusses the detailed molecular mechanisms by which Norbin regulates agonist-mediated mGluR5 internalization. Finally, chapter 5 talks about the role of Norbin in mGluR-mediated AMPAR endocytosis which is believed to be the cellular correlate of mGluR-mediated synaptic plasticity.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Cells and cell lines

Mice were housed in small animal facility for experimentation (SAFE), IISER Mohali with 12 hr light dark cycles and *ad libitum* access to mouse chow and water. Primary hippocampal and cortical neurons were cultured from P0 C57BL/6J mice of both sexes in accordance with the protocols approved by the IAEC. HEK293 and HEK293T cell lines were purchased from NCCS Pune (India).

2.1.2. Cell culture reagents

Cell culture media like Dulbecco's modified Eagle medium (DMEM), neurobasal medium, minimal essential medium (MEM) and other reagents like B27 supplement, antibiotic-antimycotic (AB-AM) mix, fetal bovine serum (FBS), glutamax-100 were purchased from Invitrogen (USA). Penicillin-streptomycin mixture was purchased from Lonza (Switzerland), goat serum from Geneilabs (India) and trypsin-EDTA from HiMedia (India). All salts and fine chemicals like poly-D-lysine, polyethyleneimine (PEI), 5-fluoro-2'-deoxyuridine (FUDR), bovine serum albumin (BSA), L-cysteine, paraformaldehyde (PFA), Triton X-100, papain, deoxyribonuclease I (DNase I), dimethyl sulfoxide (DMSO), Fluoromount™ aqueous mounting medium were purchased from Sigma (USA). MITO+ serum extender was purchased from Corning (USA).

2.1.3. Antibodies

Antibody	Source
Mouse anti-myc monoclonal antibody	Abcam (UK)
Mouse anti-Norbin polyclonal antibody	Abcam (UK)
Rat anti-HA monoclonal antibody	Roche (Switzerland)
Rabbit anti-FLAG polyclonal antibody	Sigma (USA)
Mouse anti-GFP monoclonal antibody	Sigma (USA)
Rabbit anti-GFP polyclonal antibody	Invitrogen (USA)
Rabbit anti-GluA1 polyclonal antibody	Millipore (USA)
Mouse anti-Bassoon monoclonal antibody	Enzo Life Sciences (USA)

Mouse anti-ERK1/2 monoclonal antibody	Cell Signaling Technology (USA)
Rabbit anti-phospho-ERK1/2 monoclonal antibody	Cell Signaling Technology (USA)
Mouse anti-β-actin monoclonal antibody	Santa Cruz Biotechnology (USA)
Mouse anti-PKAα cat monoclonal antibody	Santa Cruz Biotechnology (USA)
Rabbit anti-PKA RIIα monoclonal antibody	R&D Systems (USA)
Rabbit anti-Arc polyclonal antibody	Synaptic Systems (Germany)
Alexa Fluor-conjugated secondary antibodies	Invitrogen (USA)
Horseradish peroxidase-conjugated secondary antibodies	Sigma (USA)

2.1.4. Drugs and neurotransmitters

(*RS*)-3,5-dihydroxyphenylglycine [(*RS*)-3,5-DHPG], *N*-methyl-D-aspartic acid (NMDA), D-(-)-2-amino-5-phosphonopentanoic acid (APV), 6,7-dinitroquinoxaline-2,3-dione disodium salt (DNQX) and KT-5720 were purchased from Tocris (UK). Tetrodotoxin citrate (TTX) was purchased from Abcam (UK). MTEP hydrochloride, cycloheximide and dopamine were purchased from Sigma (USA). Ampicillin and kanamycin were purchased from HiMedia (India).

2.1.5. Plasticwares and glasswares

All the plasticwares for cell biological studies were purchased from BD Falcon (USA). Plasticwares used in molecular biology experiments were purchased from Tarsons (India). Coverslips were purchased from Fisher Scientific (USA).

2.1.6. Molecular biology reagents

Restriction enzymes like EcoRI, BamHI, XhoI, XbaI, BglII and other enzymes like T4 DNA ligase, T4 polynucleotide kinase, Phusion DNA polymerase, calf-intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs (USA). dNTP mixture was purchased from Geneilabs (India). Red Taq jump start polymerase and anti-FLAG M2 affinity beads were purchased from Sigma (USA). Protein G agarose beads were purchased from GenScript (USA) and femtoLUCENT plus-HRP kit was purchased from G-Biosciences (USA). Luria Bertani (LB) broth, agar powder and various buffer related salts were purchased from HiMedia (India).

2.1.7. Constructs

The myc-mGluR1 and myc-mGluR5 constructs were generously gifted by Kathrine Roche (National Institute of Health, USA). The myc epitope was tagged at the N-terminus of the full-length mGluR1 and mGluR5. The FLAG-mGluR5 construct was obtained from Stephen Ferguson's lab (Canada). pCMV3 myc-Neurochondrin construct was gifted by Heidi Welch (Babraham Institute, UK).

2.1.8. Instruments and apparatus

2.1.8.1. Cell biology related: Class II biological safety cabinets were purchased from Labconco (USA), Galaxy 170R CO_2 incubators were from Eppendorf (Germany), 5810R refrigerated centrifuge from Eppendorf (Germany), stereo microscope from Olympus (Japan), inverted compound microscope and fluorescence microscope from Zeiss (Germany), pipette fillers from Thermo Fisher Scientific (USA), hybridization ovens from Major Science (USA) and water bath from Grant (UK).

2.1.8.2. Molecular biology related: Refrigerated microcentrifuge was purchased from LaboGene (Denmark), MiniSpin centrifuge from Eppendorf (Germany), vortex from Stuart (UK), heating block from Techne (UK), thermal cycler from Bio-Rad (USA), spectrophotometer from Jenway (UK), thermomixer from Eppendorf (Germany), see-saw rockers from Tarsons (India), water baths from Memmert (Germany), biosafety cabinet from Esco (Singapore), incubator shaker from Eppendorf (Germany) and Rotospin from Tarsons (India). Vertical and horizontal gel electrophoresis units, electroblotting apparatus, PVDF blotting membrane and PowerPac power supplies were purchased from Bio-Rad (USA).

2.1.8.3. Common instruments: 4˚C chiller (Vestfrost, Denmark), 4˚C fridge (Hitachi, Japan), -20˚C freezer (Vestfrost, Denmark), -80˚C freezer (New Brunswick, Germany), weighing balance (Sartorius, Germany) and pH meter (Mettler Toledo, USA).

2.1.9. Primers

Primer description	Name	Sequence
Oligos for cloning shNor into LRV1 vector	Sense strand shNor	5’TCGAG**AGGCCAAGAATGACAGCGA**TTCAAGAGA**TCGCTGTCATTCTTGGCCT**TTTT 3’
	Antisense strand shNor	5’CTAGAAAA**AGGCCAAGAATGACAGCGA**TCTCTTGAA**TCGCTGTCATTCTTGGCCT**C 3’
For sequencing shNor	H1 promoter forward primer (H1 FP)	5’ TGGCAGGAAGATGGCTGTGAG 3’
	Ubiquitin promoter reverse primer (Ub RP)	5’ GTCACCCAAGTCCCGTCCTAA 3’
For cloning HA-Norbin in LRV1 vector and for introducing silent mutations in the shNor binding region to make shNor:Nor replacement construct	SET 1 Norbin full-length FP	 5’ATAAGATCTATGTATCCATATGATGTTCCAGATTATGCTGCCTCGGATTGCGAGCCAGCTCTG 3’
	Rescue construct RP	5’CAGCAGGGCTGCAAACTGCTC**A**CT**A**TC**G**TT**T**TT**C**GC**T**TCACGGAGGGCTCCCAGGTAGCG 3’
	SET 2 Rescue construct FP	 5’CGCTACCTGGGAGCCCTCCGTGA**A**GC**G**AA**A**AA**C**GA**T**AG**T**GAGCAGTTTGCAGCCCTGCTG 3’
	Norbin full-length RP	5’ATAGAATTCTCAGGGCTCTGACAGGCACTGCTCCAAGGC 3’
For deleting the N-terminal region of Norbin (1-481 amino	Del N-term FP	5’ATAAGATCTATGTATCCATATGATGTTCCAGATTATGCTGAACTCACATCCCCTGGCCACGACAC 3’

acids) to make shNor:NorΔN replacement construct	Norbin full-length RP	5'ATAGAATTCTCAGGGCTCTGACA GGCACTGCTCCAAGGC 3'
For deleting peptide 1 region of Norbin (PEP1- 48 to 67 aa) to make shNor:NorΔPEP1 replacement construct	SET 1 Norbin full-length FP ΔPEP1 RP SET 2 ΔPEP1 FP Norbin full-length RP	 5'ATAAGATCTATGTATCCATATGATG TTCCAGATTATGCTGCCTCGGATTG CGAGCCAGCTCTG 3' 5'AGGAGACGATTGGGTTTGACTGC CTTGGTCACTAGCAGCAGG 3' 5'CCAAGGCAGTCAAACCCAATCGT CTCCTGACCACCAAGGAGG 3' 5'ATAGAATTCTCAGGGCTCTGACA GGCACTGCTCCAAGGC 3'
For deleting peptide 2 region of Norbin (PEP2- 255 to 274 aa) to make shNor:NorΔPEP2 replacement construct	SET 1 Norbin full-length FP ΔPEP2 RP SET 2 ΔPEP2 FP Norbin full-length RP	 5'ATAAGATCTATGTATCCATATGATG TTCCAGATTATGCTGCCTCGGATTG CGAGCCAGCTCTG 3' 5'CAGTCGGAGCCGCAGCTTCCCAG GATGCGTGCCAG 3' 5'GCATCCTGGGAAGCTGCGGCTCC GACTGGATCCCG 3' 5'ATAGAATTCTCAGGGCTCTGACA GGCACTGCTCCAAGGC 3'
For deleting the C-terminal region of Norbin (482-712 amino acids) to make shNor:NorΔC replacement construct	Norbin full-length FP Del C-term RP	5'ATAAGATCTATGTATCCATATGATG TTCCAGATTATGCTGCCTCGGATTG CGAGCCAGCTCTG 3' 5'ATAGAATTCTCACCACTGCTGCAG GAAATACTTGCACAG 3'

2.2. Methods

2.2.1. Buffers and media

2.2.1.1. 10X phosphate buffered saline (PBS): Added 80 g NaCl, 2 g KCl, 2.4 g KH_2PO_4 and 14.4 g Na_2HPO_4 in 800 ml of double-distilled water. The volume was made up to 1 L with water. The pH was adjusted to 7.4 when diluted to 1X PBS. The solution was sterilized by autoclaving.

2.2.1.2. LB and LB agar medium: To make LB media, 12.5 g of LB powder was dissolved in 500 mL double-distilled water followed by autoclaving. For LB agar media, 12.5 g LB powder and 7.5 g (1.5% w/v) bacteriological agar powder were dissolved in double-distilled water. The solution was autoclaved and allowed to cool down for some time before adding the antibiotic and pouring onto petri plates.

2.2.1.3. 10% DMEM: A packet of DMEM powder was dissolved in 800 mL of freshly prepared 3.7 g/L sodium bicarbonate solution. The pH was adjusted to 7.4 and volume was made up to 1 L with double-distilled autoclaved water. The medium was filter sterilized through a 0.22 μm membrane filter. Subsequently, 100 mL (10% v/v) of heat inactivated FBS and 10 mL of penicillin-streptomycin mixture was added to 890 mL medium to make 10% DMEM.

2.2.1.4. B-27 supplemented complete neurobasal medium: Mixed 10 mL B-27 supplement (50X), 5 mL AB-AM mix (100X), 5 mL glutamax (100X) in 480 mL Neurobasal medium.

2.2.1.5. Freezing mixture: Freezing mixture was comprised of 10% (v/v) DMSO and 90% (v/v) FBS.

2.2.1.6. Dissection solution: Mixed 18.8 g NaCl, 0.74 g KCl, 0.26 g $MgSO_4$, 0.86 g $CaCl_2$, 2.4 g HEPES, 2.0 g glucose and 0.004 g phenol red in 1.8 L double-distilled autoclaved water. The pH of the solution was maintained to 7.4 and volume was made up to 2 L. The solution was then filter sterilized through 0.22 μm membrane filter.

2.2.1.7. Serum media: In 467 mL MEM w/ Earle's salts w/o L-glutamine, added 25 mL FBS, 7.5 mL Hi-Glucose/MEM and 0.5 mL Mito+ serum extender. The solution was mixed well and first filter sterilized through 0.45 μm membrane filter followed by 0.22 μm membrane filter.

2.2.1.8. Enzymatic solution: Added 2 mg L-cysteine, 100 µL EDTA (50 mM, pH 8.0), 100 µL $CaCl_2$ (100 mM), 30 µL NaOH (1N), 143 µL (~ 100 units) Papain and 100 µL DNase 1 (2 mg/mL) to 10 mL dissection solution. The solution was filter sterilized before use.

2.2.1.9. Inactivation solution: 25 mg BSA and 100 µL DNase 1 (2 mg/mL) was added to 10 mL of serum media. The solution was sterile filtered before use.

2.2.1.10. Poly-D-lysine sodium borate solution: 0.1 M sodium borate solution was made and pH was adjusted to 8.4. Volume was made up to 500 mL with double-distilled autoclaved water. Subsequently, 25 mL of this solution was added to 5 mg poly-D-lysine bottle to dissolve the powder completely and the dissolved powder was added back to the sodium borate solution hence preparing the poly-D-lysine sodium borate solution. The solution was filter sterilized through 0.22 µm membrane filter.

2.2.1.11. 10X Hank's balanced salt solution with HEPES (HBSS): 1.37 M NaCl, 0.048 M KCl, 0.007 M Na_2HPO_4, 0.075 M D-glucose and 0.21 M HEPES were dissolved in 80 mL double-distilled autoclaved water. pH was adjusted to 7.14 and final volume was made up to 100 mL. The solution was filter sterilized using 0.22 µm syringe filter when diluted to 2X working concentration.

2.2.1.12. Wash buffer: 100 mL 10X HBSS (pH 7.14) and 10 mL 1 M HEPES solution (pH 7.3) were mixed together and volume was made up to 1 L with double-distilled autoclaved water. The final pH was maintained around 6.9. Wash buffer was filter sterilized using 0.22 µm membrane filter.

2.2.1.13. Polyethylenimine (PEI) solution: 100 mg PEI was dissolved in 10 mL water (RNase free, Lonza). This PEI solution was further diluted to obtain 1 µg/µl solution and pH was adjusted to 7.0. Finally, the solution was filter sterilized.

2.2.1.14. FUDR: 250 mg uridine was added to 50 mL MEM. From this, 10 mL of dissolved MEM with uridine was taken and added to the bottle of FUDR. Once FUDR had dissolved, entire solution was transferred to a 50 mL conical tube and the final FUDR solution was sterile-filtered into a new 50 mL conical tube. Following this, aliquots of 400 µL each were made.

2.2.1.15. RIPA lysis buffer: 50 mM Tris (pH 8), 150 mM NaCl, 2 mM EDTA, 1% Triton-X 100, 0.1% SDS and 0.5% sodium deoxycholate were prepared in double-distilled water. Protease inhibitor was added at 1X final concentration before lysing.

2.2.1.16. 5X Laemmli sample buffer: Mixed 250 mM Tris-HCl (pH 6.8), 30% glycerol, 10% SDS and 0.02% bromophenol blue in double-distilled water. 5% β-mercaptoethanol was added before use.

2.2.1.17. 1X SDS running buffer: 3 g Tris base, 14.4 g glycine and 1 g SDS were dissolved in 800 mL double-distilled water and pH was adjusted to 8.3. Final volume was made up to 1 L.

2.2.1.18. 1X transfer buffer: 3 g Tris base, 14.4 g glycine and 150 mL (15% v/v) methanol was added to double-distilled water and volume was made up to 1 L.

2.2.1.19. Stripping buffer: 15 g glycine was added to 800 mL double-distilled water and the pH was adjusted to 2.2. After that, 1 g SDS and 10 mL Tween-20 were added and the final volume was constituted up to 1 L.

2.2.1.20. 4% Paraformaldehyde (PFA): Dissolved 4 g PFA in 100 mL 1X PBS (pH 7.4). The solution was heated at 58°C to 60°C until the powder dissolved. Filtered the solution before using and stored at -20°C.

2.2.2. Competent cells preparation

Frozen glycerol stock of DH5α bacterial cells was streaked onto an LB agar plate. Colonies were allowed to grow overnight at 37°C. Next day, a single colony was picked up and was inoculated into a 10 mL starter culture of LB. The culture was grown at 37°C in incubator shaker overnight. From this primary culture, 100 µL was taken and four secondary cultures of 25 mL each were grown at 37°C until the optical density at 600 nm (OD_{600}) reached 0.4 - 0.6. Once the desired OD was attained, cells were chilled on ice and harvested by centrifugation at 6000 rpm for 15 min at 4°C. The supernatant was discarded and pellet was gently resuspended in 25 mL ice cold 0.05 M $CaCl_2$. The resuspended cells were kept on ice for 45 min. Cells were then harvested by centrifugation at 6000 rpm for 5 min at 4°C. Again, the supernatant was discarded and pellet was resuspended in 12.5 mL ice cold 0.1 M $CaCl_2$. The cells were kept on ice for 5 min. Finally, the

cells were harvested at 2000 rpm for 5 min at 4°C. Supernatant was discarded and cells were resuspended in 2.5 mL ice cold 85% 0.1 M $CaCl_2$, 15% glycerol solution. 50 µL aliquots were made in sterile pre-chilled 1.5 mL microcentrifuge tubes and the aliquots were stored at -80°C.

2.2.3. Transformation

The competent cells were thawed on ice for about 10 min. 100-200 ng of DNA was mixed into 50 µL competent cells by pipetting or gentle tapping. Then cells were kept on ice for 30 min. After that, heat shock was given to the cells at 42°C for 60 sec. The tubes were put back on ice for 2 min. Then, 950 µL of LB media was added to the cells and cells were grown at 37°C for 45-60 min. Subsequently, cells were collected by centrifugation at 7500 rpm for 5 min and were resuspended in 100 µL LB. The entire volume was then plated on an LB agar plate containing the appropriate antibiotic. The plates were incubated at 37°C overnight.

2.2.4. Plasmid DNA isolation

A single colony of the transformed bacteria was picked up and inoculated into 10 mL LB media with antibiotic. The culture was incubated overnight on a shaker at 37°C. Isolation of plasmid DNA was done using Qiagen mini prep DNA isolation kit following the manufacturer's instructions. Isolated plasmid DNA was stored at -20°C.

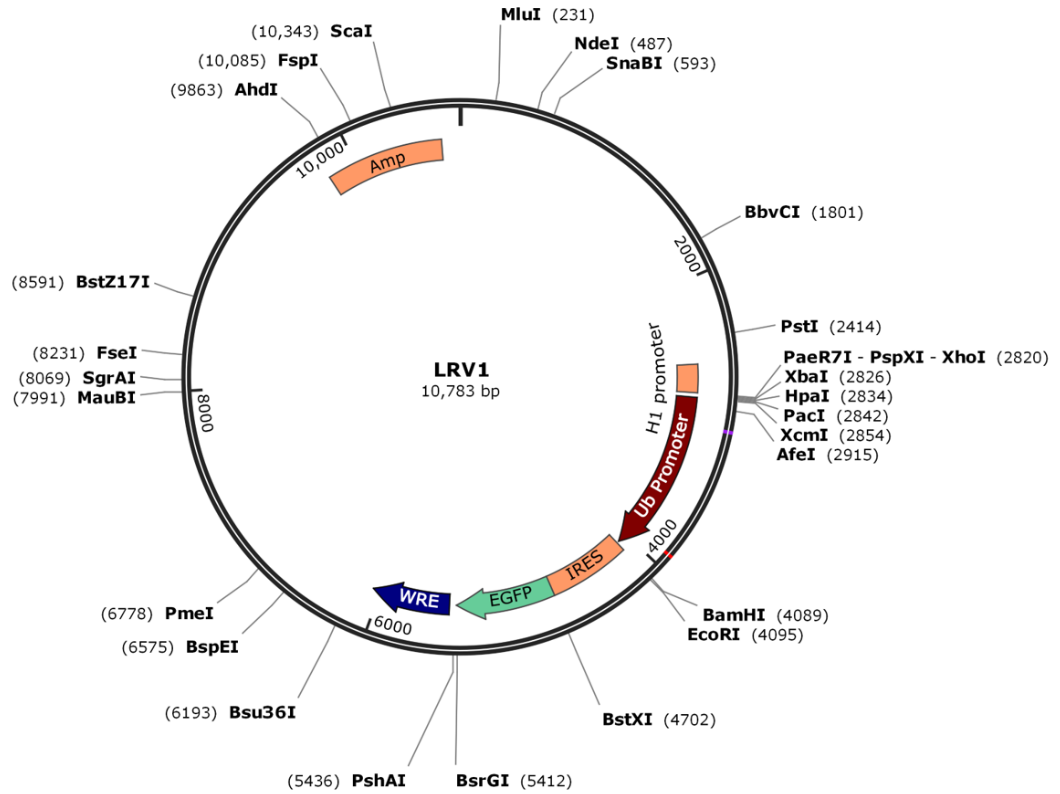
2.2.5. Cloning

All the constructs were cloned in LRV1 vector. LRV1 vector is a multi-promoter vector that contains an H1 promoter and a ubiquitin (Ub) promoter. The H1 promoter drives the expression of the shRNA, while the ubiquitin promoter drives the expression of the wild-type Norbin or various mutants of Norbin and this approach is called the "molecular replacement strategy" **(Figure 2.1A, B)**. There is also an EGFP sequence under the internal ribosome entry site (IRES) which serves as a reporter for all these constructs.

2.2.5.1. Generation of Norbin knockdown constructs

shRNA designing began by searching for siRNAs against Norbin already reported in the literature. Two siRNAs were chosen and shRNA sense and antisense sequences were designed (as listed in **2.1.9.1.**) by adding a loop region in between so that the shRNA forms a hairpin-like structure.

(A)



(B)

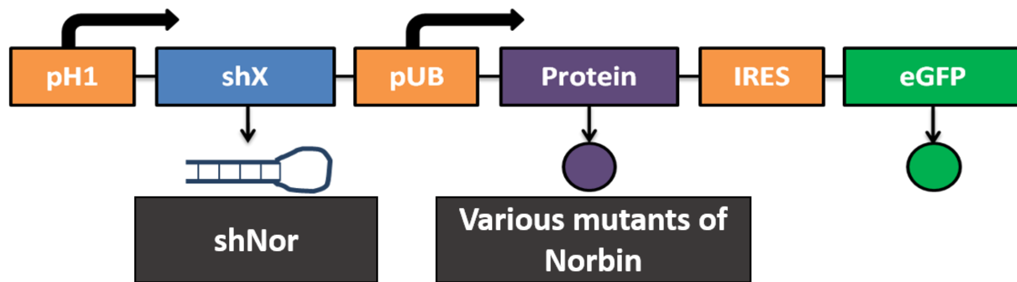


Figure 2.1. The molecular replacement strategy. (A) Vector map of LRV1 plasmid. (B) Schematic of the organization of the promoters and genes in the LRV1 vector.

Xho1 and Xba1 overhangs were added at the 5' ends of sense and antisense strands respectively. Once the oligos got synthesized, they were treated with T4 polynucleotide kinase (T4 PNK) to phosphorylate the ends. Briefly, 4 µL oligo (100 µM) was mixed with 2 µL 10X ligase buffer in 13 µL double-distilled water. 1 µL T4 PNK was added and reaction was carried out for 1 hr at 37°C. Once prepared, the oligos were pooled in equimolar concentrations and the mix was incubated at 95°C for 10 min to inactivate PNK. The next step was annealing for which, the pooled oligos were kept in a water bath for 30 min at 99°C following which the water bath was switched off and was allowed to cool down slowly to room temperature to facilitate the annealing process.

Simultaneously, 5 µg of LRV1 vector was digested with Xho1 and Xba1 to create 5' and 3' overhangs respectively. Following digestion for about 3 hr at 37°C, the vector was dephosphorylated by adding 1 µL calf intestinal phosphatase (CIP) to the digestion mix. The vector was then gel purified. About 400 ng of purified vector was ligated with different dilutions of the double-stranded oligonucleotide in 10 µL total volume at 16°C for 16 hr. Next day, the different ligation mixtures were transformed and subsequently, the colonies were screened. The efficiency of knockdown of each shRNA was analyzed 3 - 5 days post-transfection by western blot as well as by immunocytochemistry. The following shRNA was selected as the most effective shRNA in knocking down the endogenous Norbin in primary neurons:

5'TCGAG**AGGCCAAGAATGACAGCGA**TTCAAGAGA**TCGCTGTCATTCTTGGCCT**T
TTT 3'

2.2.5.2. Generation of full-length Norbin replacement construct

To generate the Norbin full-length replacement construct, our aim was to introduce silent mutations in the shRNA binding region of Norbin so that these silent mutations would prevent the knockdown of the replacement Norbin by shNor but simultaneously the endogenous Norbin would be down-regulated. We used the degeneracy in amino acid codons to keep the amino acid sequence of the replaced full-length protein same as the endogenous protein. The schematic of the wild-type shNor binding site and corresponding silent mutations in the replacement construct is shown below:

134 152

Wild-type endogenous Norbin: AG GCC AAG AAT GAC AGC GA

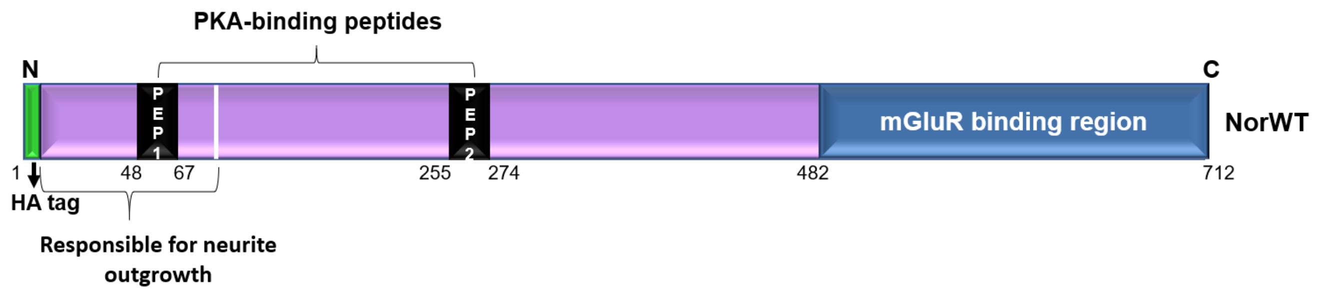
Norbin replacement: A**A** GC**G** AA**A** AA**C** GA**T** AG**T** GA

Subsequent to the incorporation of the silent mutations in the wild-type Norbin gene, it was used as a template to generate the replacement construct. The forward primer used for the amplification of the gene, contained the BglII restriction enzyme site and HA sequence while the reverse primer had the EcoR1 restriction enzyme site **(2.1.9.3)**. The final amplified product was digested with BglII and EcoRI. Simultaneously, the LRV1 vector was digested with BamH1 and EcoRI to generate compatible overhangs. The insert HA-Norbin was cloned under the ubiquitin promoter in LRV1 containing shNor under the H1 promoter to generate full-length Norbin replacement construct **(Figure 2.2A, B)**. The clones were confirmed by sequencing.

2.2.5.3. Generation of NorΔN and NorΔC replacement constructs

In order to examine the role of N-terminal region of Norbin (1-481 aa) in mGluR endocytosis, NorΔN replacement construct was made. Full-length Norbin replacement construct was used as a template and shNor construct was used as the destination vector. The C-terminal region of Norbin was amplified using del N-term forward primer and full-length Norbin reverse primer as stated in **2.1.9.4.** Since the forward primer had BglII restriction site followed by an HA sequence and the reverse primer had EcoRI site, both the vector and insert were digested with appropriate restriction enzymes to generate compatible overhangs and ligation was performed. Positive clones were selected and confirmed by sequencing. In this way shNor:NorΔN replacement construct was made **(Figure 2.3A, B)**.

To check for the role of C-terminal region of Norbin (482-712 aa) in the ligand-mediated endocytosis of group I mGluRs, NorΔC replacement construct was made. N-terminal region of Norbin was amplified using full-length Norbin forward primer and del C-term reverse primer **(2.1.9.7.)**. Insert was digested using BglII and EcoRI and shNor containing LRV1 vector was



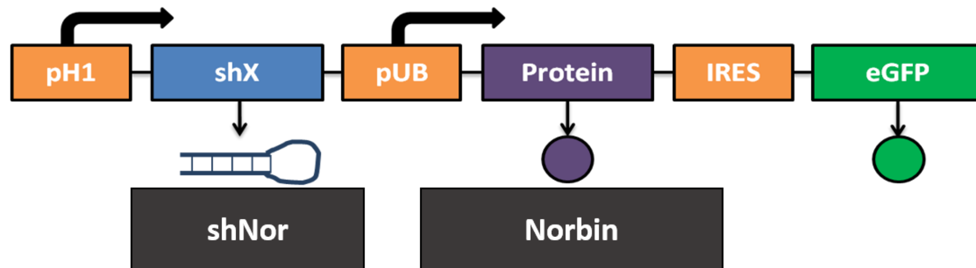


Figure 2.2. Full-length Norbin replacement construct. (A) Schematic of the full-length Norbin showing its domain structure. (B) Schematic of the shNor:Nor replacement construct.

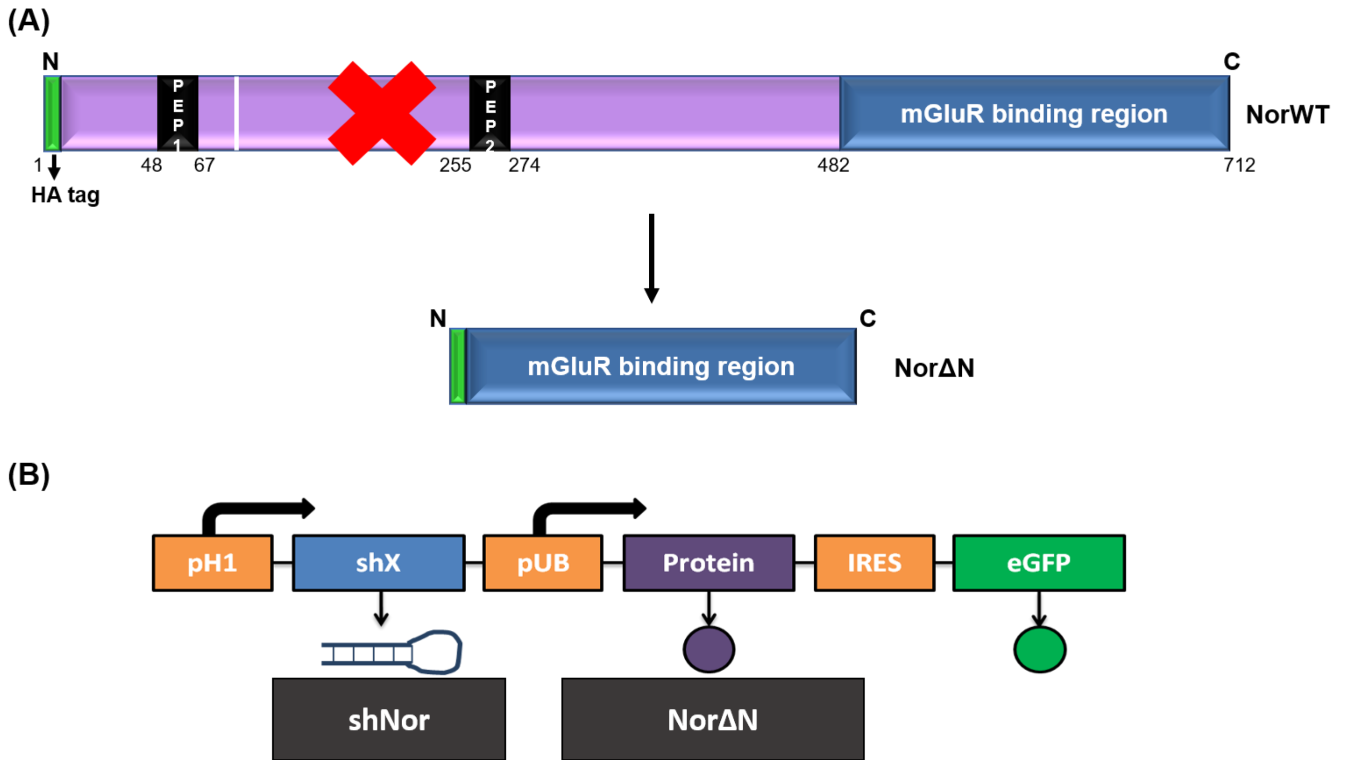


Figure 2.3. Generation of the NorΔN replacement construct. (A) Picture shows deletion of amino acids 1-481 from full-length Norbin to make NorΔN construct. (B) Schematic of the shNor:NorΔN replacement construct.

digested using BamHI and EcoRI. Subsequently, the insert was ligated under the ubiquitin promoter and the ligation mix was transformed. The construct was verified by sequencing. Thus, shNor:NorΔC replacement construct was obtained **(Figure 2.4A, B)**.

2.2.5.4. Generation of NorΔPEP1 and NorΔPEP2 replacement constructs

Two distinct peptides at the N-terminal region of Norbin have been shown to have affinity for binding protein kinase A (PKA) (Hermann et al., 2015). In order to see if PKA binding to Norbin has any role to play in mGluR endocytosis, NorΔPEP1 and NorΔPEP2 replacement constructs were made. Deletion of peptide 1 (48-67 aa) was carried out using PCR. Two sets of primers were used for this purpose as shown in **2.1.9.5.** The final amplified product had BglII and EcoRI restriction enzyme sites at both ends as well as an HA sequence at the N-terminus. The insert was digested and cloned into the vector containing shNor under the ubiquitin promoter to make shNor:NorΔPEP1 replacement construct **(Figure 2.5A, B)**. Similarly, deletion of peptide 2 (255 to 274 aa) in Norbin through PCR was carried out by using the primers mentioned in **2.1.9.6.** The amplified product having the deletion of PEP2 region was cloned into the shNor containing vector to make shNor:NorΔPEP2 replacement construct **(Figure 2.6A, B)**.

2.2.5.5. Generation of NorA687G replacement construct

NorA687G replacement construct was obtained by using full-length Norbin as a template while introducing silent mutations in the shNor binding region. A single residue mutation at the C-terminus of Norbin changed alanine (GCC) at 687 position to glycine (GGC). Amplification was done using Norbin full-length FP and Norbin RP. The amplified product was digested using appropriate restriction enzymes and was cloned into the shNor containing destination vector. In this way, the point mutant shNor:NorA687G construct was made **(Figure 2.7A, B)**.

2.2.6. Cell culture

2.2.6.1. HEK293/293T cell culture and maintenance

HEK293/293T cells were thawed and maintained in 90 mm cell culture dishes in DMEM supplemented with 10% FBS, 1X penicillin-streptomycin mix at 37˚C with 95% humidity and 5% CO_2. When the cells reached around 70% confluency, they were split into two or more plates using

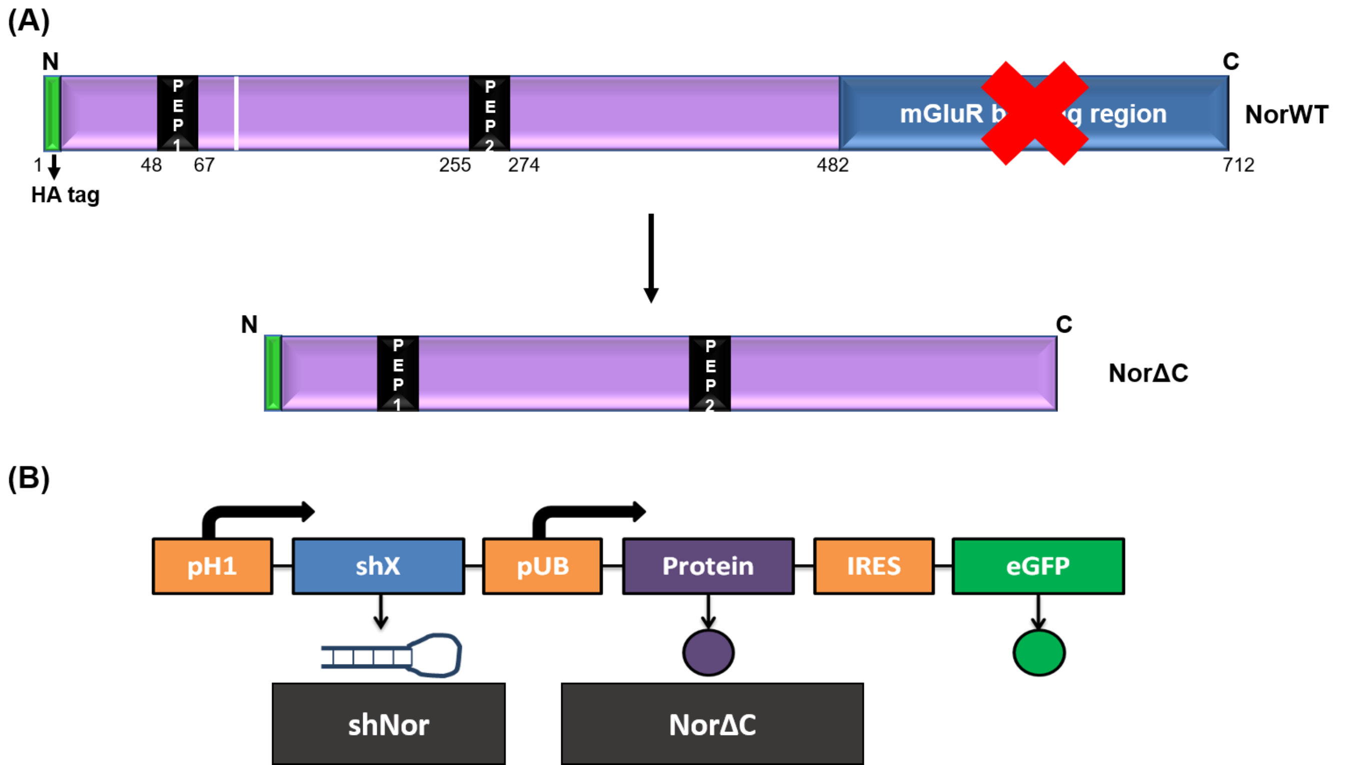


Figure 2.4. Generation of NorΔC replacement construct. (A) Schematic showing deletion of the amino acids 482-712 from full-length Norbin to make NorΔC construct. (B) Pictorial representation of the shNor:NorΔC replacement construct.

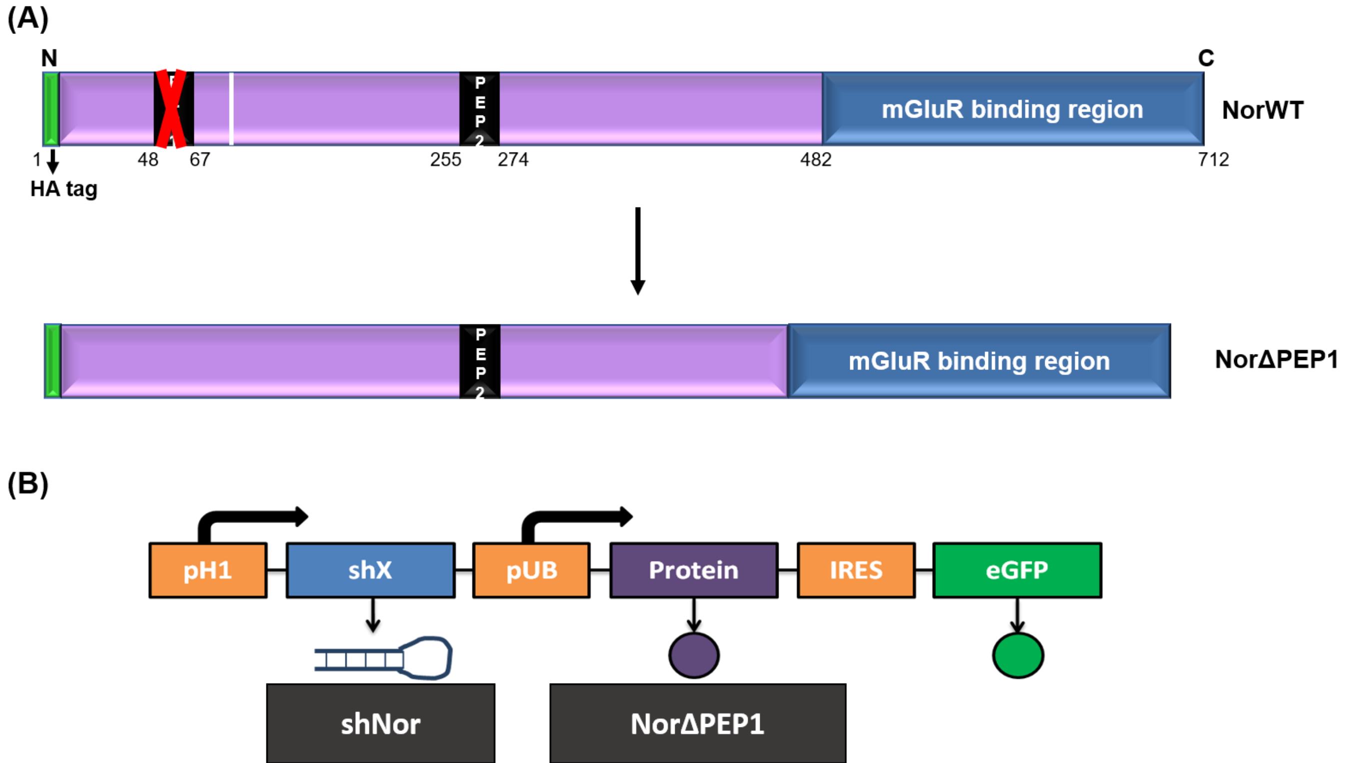


Figure 2.5. Generation of NorΔPEP1 replacement construct. (A) Deletion of amino acids 48 to 67 from full-length Norbin to make NorΔPEP1 construct. (B) Pictorial representation of the shNor:NorΔPEP1 replacement construct.

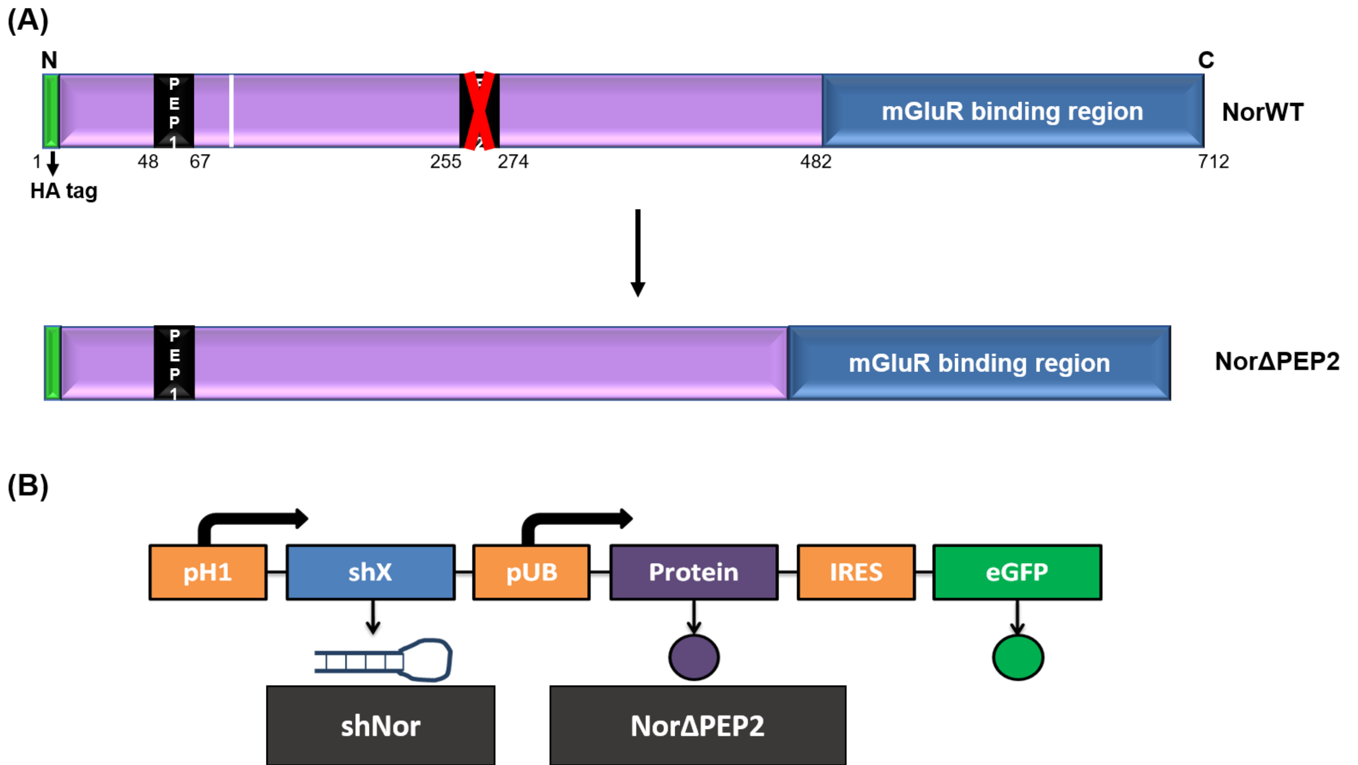


Figure 2.6. Generation of NorΔPEP2 replacement construct. (A) Deletion of amino acids 255 to 274 from full-length Norbin to make NorΔPEP2 construct. (B) Schematic of the shNor:NorΔPEP2 replacement construct.

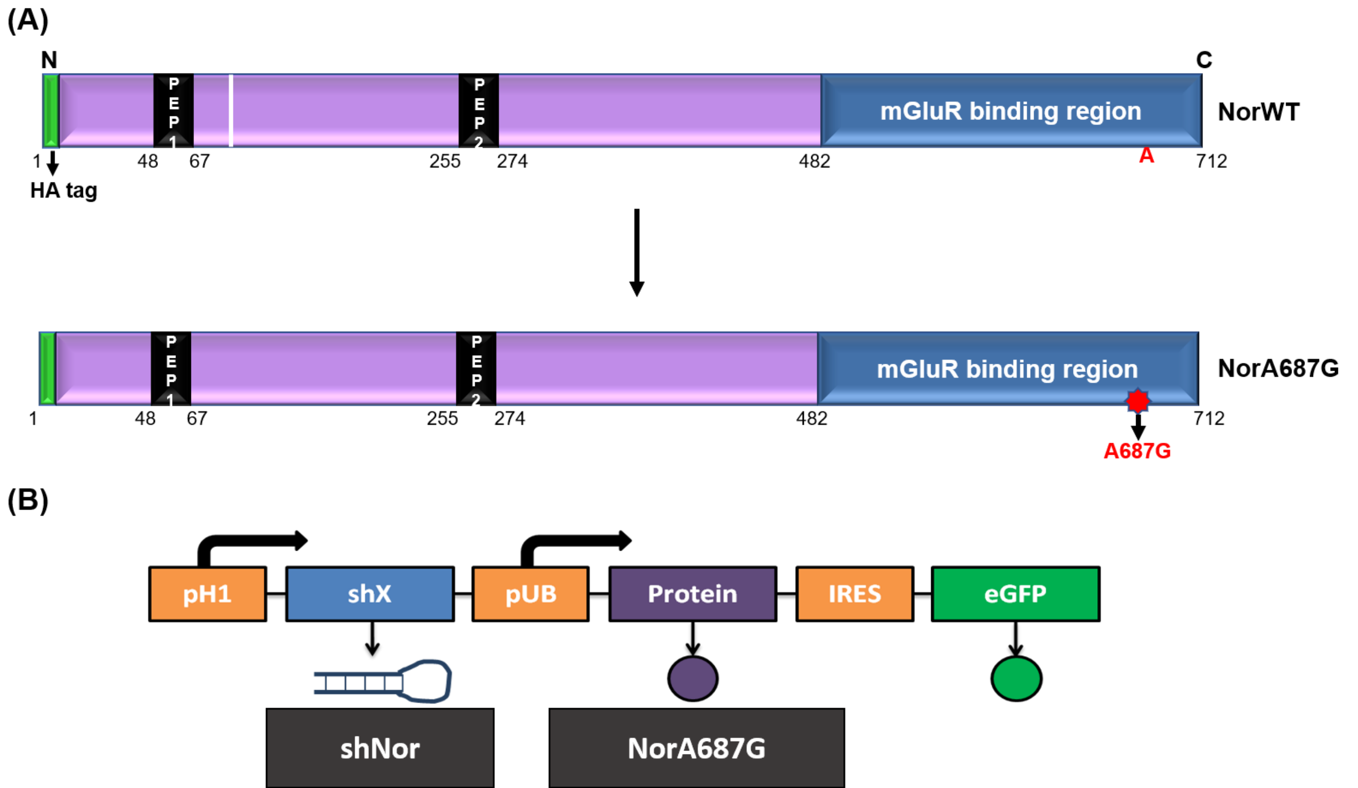


Figure 2.7. Generation of NorA687G replacement construct. (A) A mutation in full-length Norbin changed alanine at 687th position of the wild-type Norbin to glycine. (B) Schematic of the shNor:NorA687G replacement construct.

trypsin-EDTA. For western blotting or co-immunoprecipitation experiments, cells were plated on 60 mm cell culture dishes (4 X 10^5 cells/dish) once they reached 60-70% confluency. For immunocytochemistry experiments, cells were seeded on 12 mm coverslips in a 24-well plate (1.0 X 10^5 cells/well), pre-coated with poly-D-lysine (50 µg/mL). Stocks of these cells were maintained by freezing them at -80°C in the freezing mixture.

2.2.6.2. Dissociated primary neuron culture

Primary neuron cultures were prepared from P0 C57BL/6J mice of both sexes. Pups were first decapitated and hippocampi or cortex were dissected out. The tissues were then digested in the enzymatic solution containing papain for 30 min at 37°C. The enzymes were then inactivated using the inactivation solution. Thereafter, mechanical dissociation was performed by triturating the cells using glass pipettes of different pore diameters to obtain single cells. Neurons were then counted on a hemocytometer and plated on 12 mm coverslips (0.8 X 10^5 cells/well for AMPAR experiments and 1.25 X 10^5 cells/well for mGluR experiments) for immunocytochemistry experiments or 35 mm (8 X 10^5 cells/dish) or 60 mm dishes (22 X 10^5 cells/dish) for western blotting and co-immunoprecipitation experiments. The coverslips and dishes were pre-coated with poly-D-lysine + 0.1 M sodium borate (pH 8.4). Cultures were maintained in neurobasal medium supplemented with B-27 and 0.5 mM glutamine. Growth of glia was inhibited by adding FUDR on the 4th day of culture.

2.2.7. Transfection

2.2.7.1. Transfection in HEK293/293T cells

HEK293/293T cells were transfected using polyethyleneimine (PEI). PEI is a stable cationic polymer having density close to 1 (Boussif et al., 1995). PEI condenses DNA into positively charged particles and the DNA-PEI complex enters into the cell through endocytosis (Sonawane et al., 2003). For a 24-well plate, 1 µg of DNA (in 75 µL plain DMEM) was mixed with 3 µL PEI (in 75 µL plain DMEM) for each well. DNA was added to PEI dropwise to allow sufficient encapsulation. The DNA-PEI mix was incubated for 30 min at room temperature and was then added to cells having 1 mL 10% DMEM. The plate was kept in the incubator for about 6 hr. After

6 hr, the transfection mixture was replaced with fresh 10% DMEM and experiments were carried out 24-72 hr post-transfection. We got up to 90% transfection efficiency using this method.

2.2.7.2. Transfection in primary hippocampal neurons

Primary hippocampal neurons were transfected at 6-7 days *in vitro* (DIV) with the plasmid DNA using calcium phosphate method. Two transfection mixes were prepared- mix A having 3-4 µg DNA with 1.5 µL 2.5 M $CaCl_2$ and DNase free water up to 15 µL and mix B having equal volume of 2X HBSS (pH 7.14). Both the mixes were incubated for 5 min at room temperature. Thereafter, mix B was added to mix A dropwise and the mixture was incubated for 30 min in the dark. Meanwhile, old complete neurobasal media was collected from the cells and was replaced with 500 µL CO_2 saturated plain neurobasal media. After 30 min, the mixture was added to the cells and incubation was carried out at 37°C until the appearance of fine sand-like precipitate on the surface of neurons as observed under the bright-field microscope. Once adequate amount of precipitate had formed, the cells were washed with warm wash buffer until the precipitate got dissolved. Finally, the collected old neurobasal media was added back to the cells. Half-feeding was done 24 hr post-transfection. Endocytosis assay was performed at 12-14 DIV. We got about 60% - 65% transfection efficiency using this method.

2.2.8. Endocytosis assay

2.2.8.1. Group I mGluR endocytosis assay

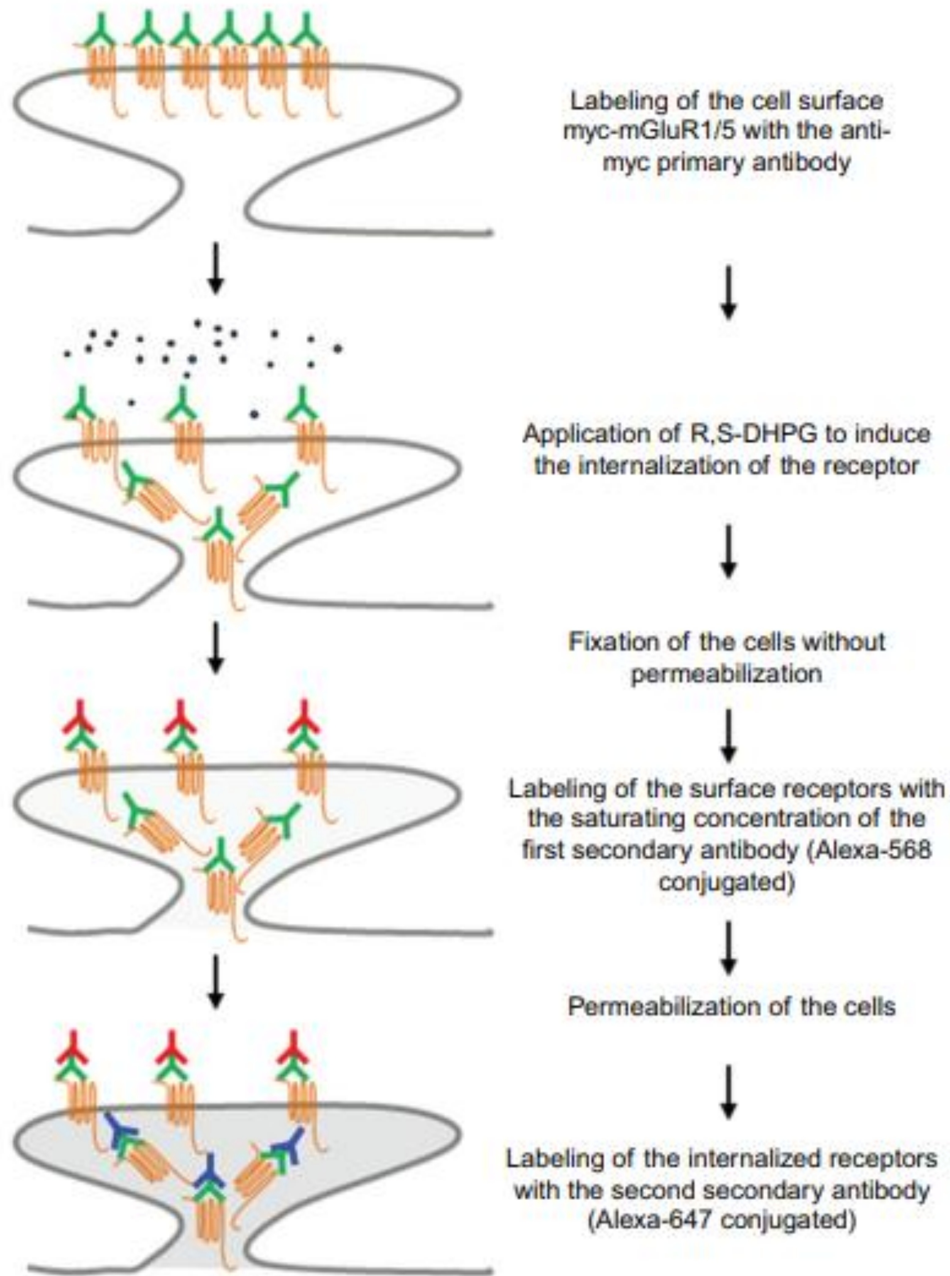
HEK293 cells or primary hippocampal neurons were transfected with either myc-mGluR1/myc-mGluR5 only or co-transfected with the receptor and various constructs of Norbin as described above. Live cells were incubated in plain media for 30 min at 37˚C followed by 5 min incubation in 2% normal goat serum (NGS) for blocking. Cells were then labelled with anti-myc mouse monoclonal primary antibody (1:500 for HEK293 cells and 1:200 for primary hippocampal neurons) for 20 min at 37˚C. Cells were then washed with plain media (glutamate free) and R,S-DHPG (100 µM) or MTEP (100 µM) pulse was applied for 5 min. Ligand was washed away and cells were then chased for different time periods at 37˚C in plain DMEM / neurobasal media in the absence of the ligand. Subsequently, cells were fixed without permeabilization using ice cold 4% paraformaldehyde (PFA) for 15 min on ice. Surface receptors were labelled with a saturating

concentration of goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) for 1 hr at 37˚C. Cells were then permeabilized with 0.1% Triton X-100 for 30 min at room temperature, followed by blocking with 2% NGS for 1 hr at 37˚C. The endocytosed receptors were then labelled by the application of goat anti-mouse Alexa-647 conjugated secondary antibody (1:750) for 1 hr at 37˚C **(Figure 2.8)**. The co-transfected constructs containing GFP and HA-tags were stained with the anti-GFP or anti-HA antibody overnight at 4°C. Next day, they were stained with their respective secondary antibodies. The coverslips were then mounted on glass slides 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. For all experiments, two coverslips were used for each condition.

To ensure that the Alexa-568 conjugated secondary antibody concentration was indeed a saturating concentration, control experiments were performed. Various concentrations of the first secondary antibody were tested and when the saturating concentration of the antibody was applied to visualize the surface receptors, no detectable staining of the Alexa-647 conjugated second secondary antibody was observed 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 stained with Alexa-647 conjugated secondary antibody **(Figure 2.9)**. This experiment suggested two things- first, the concentration of the first secondary antibody that was used (1:100) indeed labelled almost all the surface receptors and second, the second secondary antibody was working properly since it was able to stain the internalized receptors upon permeabilization of the cells (Bhattacharyya et al., 2009; Gulia et al., 2017; Mahato et al., 2015; Pandey et al., 2014; Sharma et al., 2018; Trivedi & Bhattacharyya, 2012).

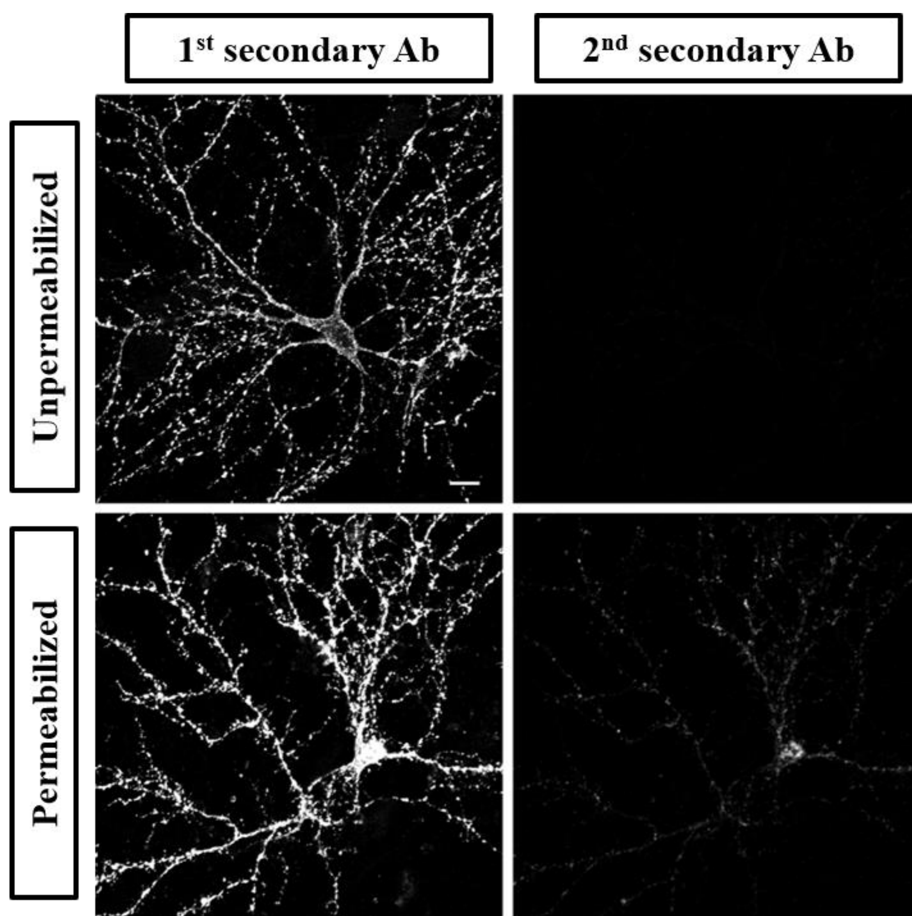
2.2.8.2. AMPA receptor endocytosis assay

AMPA receptor endocytosis assay was performed in primary hippocampal neurons at 14-15 DIV. Cells were first pre-incubated in 1 µM TTX (pre-synaptic release blocker), 20 µM DNQX (AMPAR antagonist) and 50 µM APV (NMDAR antagonist) to study mGluR-mediated AMPAR endocytosis or in 1 µM TTX, 20 µM DNQX to study NMDAR-mediated AMPAR endocytosis for 30 min at 37˚C. This was followed by blocking in 2% NGS for 5 min at 37˚C. Subsequently, AMPA receptors were labelled using anti-GluA1 rabbit polyclonal antibody (1:150) for 15 min at 37˚C.



(Sharma et al., 2019, *Methods in cell biology)*

Figure 2.8. Schematic of the antibody feeding receptor endocytosis assay.



Courtesy- Dr. Ravinder Gulia

Figure 2.9. Standardization of the antibody feeding assay. Application of the first secondary antibody (Alexa-568 conjugated), in a saturating concentration, labelled the surface receptors (upper left panel) which prevented any further observable binding of the second secondary antibody which is Alexa-647 conjugated (upper right panel) in unpermeabilized condition. However, when cells were permeabilized, the second secondary antibody labelled the internalized myc-mGluR1 (lower right panel). Scale bar = 10 µm.

Cells were then washed with plain media and R,S-DHPG (100 μM) or NMDA (100 μM) were applied for 5 min. The agonist was then washed out and cells were allowed to incubate for 10 min at 37˚C in the presence of appropriate antagonists. Subsequently, cells were fixed without permeabilization using ice cold 4% paraformaldehyde (PFA) for 15 min on ice. Surface GluA1-containing receptors were labelled with a saturating concentration of goat anti-rabbit Alexa-568 conjugated secondary antibody (1:100) for 1 hr at 37˚C. Cells were then permeabilized with 0.1% Triton X-100 for 30 min at room temperature, followed by blocking with 2% NGS for 1 hr at 37˚C. The endocytosed receptors were then labelled by the application of goat anti-rabbit Alexa-647 conjugated secondary antibody (1:750) for 1 hr at 37˚C. Staining for GFP was done overnight at 4˚C using mouse anti-GFP primary antibody followed by secondary antibody staining using goat anti-mouse Alexa-488 conjugated secondary antibody. Coverslips were mounted on glass slides using fluoromount and scanned under the confocal microscope.

The saturating concentration of the Alexa-568 conjugated 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 did not label any detectable surface receptors (Bhattacharyya et al., 2009; Citri et al., 2010; Gulia et al., 2017).

2.2.9. Recycling assay

The recycling experiments were performed in a similar way as described above. Briefly, myc-mGluR1/myc-mGluR5 expressing on the surface of the live primary hippocampal neurons were stained with the anti-myc primary antibody. The internalization of the receptors was induced by the application of 100 μM R,S-DHPG for 5 min. Cells were then chased for extended time periods up to 3 hr in the absence of the ligand. Cells were then fixed with ice cold 4% PFA without permeabilization at specified time points and surface and internalized receptors were stained with the Alexa-568 conjugated and Alexa-647 conjugated secondary antibodies respectively, using the same protocol that has been described for the endocytosis assay. Finally, the coverslips were mounted on glass slides and were scanned under the confocal microscope.

2.2.10. Co-localization assay

To check if different mutants of Norbin were localized properly, co-localization of these mutants was observed with a pre-synaptic protein, Bassoon (Tom Dieck et al., 1998). Briefly, different HA-tagged Norbin mutants were transfected in primary hippocampal neurons and co-localization assay was performed on 14-15 DIV. Cells were fixed with ice-cold 4% PFA on ice for 15 min. After that, cells were permeabilized using 0.1% Triton X-100. Then, blocking was done using 2% NGS and cells were stained with the rat anti-HA antibody (1:500) and mouse anti-bassoon antibody (1:500) overnight at 4˚C. Following primary antibody staining, cells were washed and stained with goat anti-rat Alexa-568 and goat anti-mouse Alexa-647 conjugated secondary antibodies for 1 hr at 37˚C to visualize the HA-Norbin constructs and bassoon respectively. Finally, coverslips were mounted on glass slides and scanned under the confocal microscope.

2.2.11. Western blotting

To check the knockdown efficiency of shNor, as well as expression of various mutants of Norbin, primary cortical neurons plated on 35 mm or 60 mm dishes were transfected between 4-6 DIV. 72 hr post-transfection, cells were washed with ice-cold 1X PBS and lysed with RIPA lysis buffer. Thereafter, cells were vortexed for 30 min and supernatant was collected after centrifugation at 15000 rpm for 15-30 min. The protein concentration in the supernatant was determined using Bradford assay. Samples were subsequently boiled in 5X Laemmli sample buffer at 99°C for 10 min. The samples were run on SDS PAGE by loading equal amount of protein in each lane. They were then transferred onto a PVDF membrane and blocked with 5% skimmed milk in 0.05% PBST for 1 hr at room temperature. The membrane was then incubated with primary antibody for overnight at 4°C. After washing, the membrane was incubated with the HRP-conjugated secondary antibody (1:5000) for 45 min at room temperature. Then the blot was developed using femtoLUCENT plus-HRP kit and image was acquired using the ImageQuant LAS 4000 software. For next primary antibody staining, the blot was stripped using stripping buffer and staining was carried out in a similar manner as described above. For example, the membrane was first incubated with either anti-Norbin mouse polyclonal antibody (1:500) or anti-HA rat monoclonal antibody (1:1000) at 4°C overnight followed by its secondary antibody staining and after stripping, β-actin (1:1000) staining was done which served as the loading control.

The ability of mGluRs to upregulate the phosphorylation of ERK1/2 was investigated by transfecting the primary neurons with empty vector or shNor. Both control and shNor transfected cells were pre-incubated with 100 μg/mL cycloheximide for 5 hr to inhibit the synthesis of new receptors. Subsequently, 100 μM R,S-DHPG was applied for 5 min in both control cells and shNor transfected cells to initiate the endocytosis of mGluRs. In both the conditions, one set of cells were fixed after 5 min of application of R,S-DHPG to measure the extent of upregulation of ERK1/2 phosphorylation by the mGluRs, when they were initially present at the cell surface in both control cells and shNor transfected cells. The other set of cells were chased for 2.5 hr, in the absence of the ligand. Subsequently, 100 μM R,S-DHPG was applied again for 5 min, followed by fixation of cells. Both sets of cells were then lysed and samples were run on SDS-PAGE followed by western blotting using the same protocol as described above. The phospho-ERK1/2 and total ERK1/2 immunoblotting was performed using anti-phospho-p44/42 MAPK (ERK1/2) antibody (1:1000) and anti-p44/42 MAPK (ERK1/2) antibody (1:1000) respectively.

2.2.12. Co-immunoprecipitation

Co-immunoprecipitation experiments were done to study the interaction between various constructs of Norbin with mGluR5 and PKA as well as the interaction of AMPA with PKA. Various constructs of Norbin were co-transfected with different proteins in HEK293T cells as well as in primary hippocampal neurons. After sufficient expression of the proteins, cells were lysed gently with rotation on the rotospin for 40 min at 4°C. Supernatant was collected after centrifugation at 15000 rpm for 15-30 min. Immunoprecipitation was performed by incubating the supernatant with protein A/G beads that were prepared by overnight incubation with the anti-HA or anti-FLAG primary antibodies to check for the interaction of Norbin with PKA and mGluR5 respectively, or using anti-GluA1 antibody-bound protein A/G beads to study the effect of Norbin knockdown on the interaction of AMPA with PKA. After 6-8 hr, beads were washed and samples were boiled in 2X Laemmli buffer after elution. Lysate only control was separated from the pull down and was run alongside the immunoprecipitated samples on SDS PAGE. This was followed by western blotting following the protocol as described earlier. For immunoblotting, antibodies against FLAG (1:1000), HA (1:1000), Norbin (1:500), GluA1 (1:1000), PKAα cat (1:100) and PKA RII alpha (1:100) were used.

2.2.13. Arc immunostaining assay

To check whether Norbin plays any role in the group I mGluR-mediated *de novo* translation of Arc protein, primary hippocampal neurons were transfected with either empty vector or shNor or full-length Norbin constructs and experiments were performed on DIV 14. Cells were pre-incubated in either plain media or in plain media with 100 µg/ml cycloheximide for 1 hr at 37˚C. Subsequently, 100 µM R,S-DHPG was applied for 5 min followed by a chase of 10 min in the absence of the ligand. Cells were fixed with 4% PFA on ice for 15 min, followed by permeabilization of the cells using 0.1% Triton X-100 for 30 min at room temperature. Cells were then incubated in anti-Arc rabbit polyclonal antibody (1:500) and anti-GFP mouse monoclonal antibody (1:500) for overnight at 4˚C. Subsequently, cells were stained with goat anti-rabbit Alexa-568 (1:700) and goat anti-mouse Alexa-488 (1:700) conjugated secondary antibodies for 1 hr at 37˚C to observe Arc protein and GFP respectively. Coverslips were then mounted on glass slides and observed under the confocal microscope.

2.2.14. Image acquisition and analysis

Imaging was done for most experiments in Zeiss LSM 780 confocal laser scanning microscope using a 63X oil immersion objective (NA = 1.4) and for some experiments in Olympus Fluoview confocal laser scanning microscope (Model no. FV10i) using a 60X oil immersion objective (NA = 1.35). Each experiment was repeated at least three times and a total of 30-40 primary hippocampal neurons and HEK293 cells were imaged. Images from all the conditions in a particular experiment were obtained using identical parameters.

All analyses were done blind using raw images and quantitation was done using ImageJ software (NIH, USA) (Schneider et al., 2012). Images from each experiment were maximally projected and thresholded using identical values for different experimental conditions and the total thresholded area of fluorescently labeled surface and internalized receptors was measured. Then internalization index for each cell was calculated as follows:

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

Untreated and treated cells from the same condition were always compared with one another. Separate quantitation was done for whole cell, cell body and dendrites. The dendritic values were

defined by the area that was 10 µm away from the soma. To measure the surface receptors in all the assays, surface fluorescence was divided by the cell area, which was determined by measuring the background fluorescence using a low threshold level. These values were then normalized to the average surface fluorescence of untreated control cells. The data quantified in all the experiments have been represented as a combined result of all the three repeats of that particular experiment. For representation, images were processed using Adobe Photoshop software (Adobe Systems) by adjusting brightness and contrast levels to the same degree for all conditions illustrated in each experiment.

The co-localization was quantified at a particular Z section of the image after using identical values of threshold. The magnitude of the co-localization of Norbin puncta with bassoon puncta was measured along 50 µm portions of dendrites in primary hippocampal neurons. All the western blots and immunoprecipitation experiments were also quantified using the ImageJ software.

2.2.15. Statistical analysis

The quantitation of an experiment has been represented as a combined result for all the repeats of that particular experiment. Data are presented as mean ± SEM. Experimental group results were compared with each other using Student's t-test and one-way ANOVA followed by Tukey's post test. $P > 0.05$ was considered as non-significant. Data were analyzed and graphs were plotted using GraphPad Prism 7.

Chapter 3

Norbin: an important regulator of ligand-mediated group I mGluR internalization

3.1. Introduction

GPCRs are the largest family of cell surface receptor proteins. They are important regulators of several physiological processes such as vision, smell, mood and behavior as well as immune surveillance, growth and inflammation (Hanlon & Andrew, 2015; Oakley et al., 1999). Each GPCR is associated with a heterotrimeric G protein in basal conditions, which in response to its activation by the GPCR, binds GTP. When the GPCR is activated by agonist binding on the extracellular side, it undergoes a conformational change that allows it to act as a GEF to facilitate the exchange of GDP for GTP on the G_α subunit. This results in a conformational change in G_α, releasing the $G_{\beta\gamma}$ dimer. The separated G_α subunit and $G_{\beta\gamma}$ dimer are now able to interact with their respective effector proteins, leading to downstream signalling events (Lambright et al., 1996; Neer & Clapham, 1988).

As GPCRs are enormously important for normal cell functions, both their localization in the neurons and their activity must be tightly regulated. Trafficking not only controls the spatio-temporal localization but it also controls the signalling of these receptors. Hence, the regulation of their expression level, their trafficking to and from the cell membrane, and the mechanisms of their inactivation are as important for homeostasis as their activation by agonists (Marchese et al., 2008). Like other transmembrane proteins, GPCRs are synthesized in the ER and are transported constitutively to the plasma membrane through the Golgi and trans-Golgi network (TGN) (Tan et al., 2004). For most GPCRs, their activation and signalling at the plasma membrane is followed by desensitization, which is a negative feedback mechanism that protects the cell from chronic receptor overstimulation (Ferguson, 2001; Gainetdinov et al., 2004). This is followed by recruitment of β-arrestin proteins to the phosphorylated receptor, which sterically impedes the coupling of the GPCR to the heterotrimeric G protein (Shenoy & Lefkowitz, 2011). β-Arrestin proteins have a dual role: on the one hand, they lead to desensitization of the receptor and on the other hand they promote receptor internalization by interacting with clathrin and its adaptor AP2, leading to clathrin-mediated endocytosis of the receptor (Goodman et al., 1996; Krupnick & Benovic, 1998; Laporte et al., 1999). Internalized receptors can follow various trafficking routes (Doherty & McMahon, 2009). Once the GPCR is internalized, it may either be recycled to the plasma membrane or be transported to the lysosome for degradation, leading to receptor

downregulation (Lefkowitz, 1998; Tsao et al., 2001). What route a particular GPCR would take depends on the type of the receptor, type of the ligand and type of the system.

Group I mGluRs, comprising of mGluR1 and mGluR5 belong to the class C GPCR family (Bhattacharyya, 2016; Prabhat Kumar Mahato et al., 2018). The desensitization of group I mGluRs can be phosphorylation-dependent or independent. GRK4 leads to phosphorylation-dependent desensitization of mGluRs, while GRK2 is responsible for phosphorylation-independent desensitization of these receptors (Lianne B. Dale et al., 2002; Gurpreet Kaur Dhami et al., 2002; Iacovelli et al., 2003; Ribeiro et al., 2009; Sorensen & Conn, 2003). Like many other GPCRs, agonist binding to group I mGluRs promotes rapid internalization of these receptors (L. B. Dale et al., 2001; Gurpreet K. Dhami & Ferguson, 2006; P. K. Mahato et al., 2015; Mundell et al., 2001; Suh et al., 2018). Ligand-mediated endocytosis of group I mGluRs involves both clathrin- and caveolin-dependent pathways (Francesconi et al., 2009; Kumari et al., 2013; Luis Albasanz et al., 2002). It is inhibited by the expression of β-arrestin and dynamin dominant-negative mutants suggesting that it is both arrestin and dynamin-dependent (L. B. Dale et al., 2001; Iacovelli et al., 2003; Mundell et al., 2001). Ubiquitination also plays an important role in the ligand-mediated internalization of group I mGluRs (Gulia et al., 2017). These receptors can also internalize in an agonist-independent or constitutive manner. Constitutive endocytosis of mGluR1 occurs in a clathrin-dependent manner whereas constitutive endocytosis of mGluR5 can occur *via* clathrin-independent pathway (Fourgeaud et al., 2003; Pula et al., 2004; Trivedi & Bhattacharyya, 2012). Constitutive endocytosis of group I mGluRs can also occur *via* caveolin and lipid raft-dependent mechanisms (Burgueño et al., 2003; Francesconi et al., 2009).

Although we have a few insights about the cellular and molecular mechanisms underlying group I mGluR trafficking, a lot still remains to be understood. For example, a number of post-synaptic density scaffolding proteins are associated with group I mGluRs that get upregulated or downregulated in various neuropsychiatric disorders caused by glutaminergic dysregulation. One such protein that interacts with group I mGluRs is Norbin or neurite-outgrowth-related protein from rat brain. Both Norbin and mGluR5 levels are altered in the brains of schizophrenia patients and Norbin transiently increases intracellular calcium levels downstream of mGluR5 (Shinozaki et al., 1997, 1999; Wang et al., 2009). Precisely how Norbin binding affects the trafficking and signalling of these receptors is not known. Forebrain specific Norbin knockout mice are defective

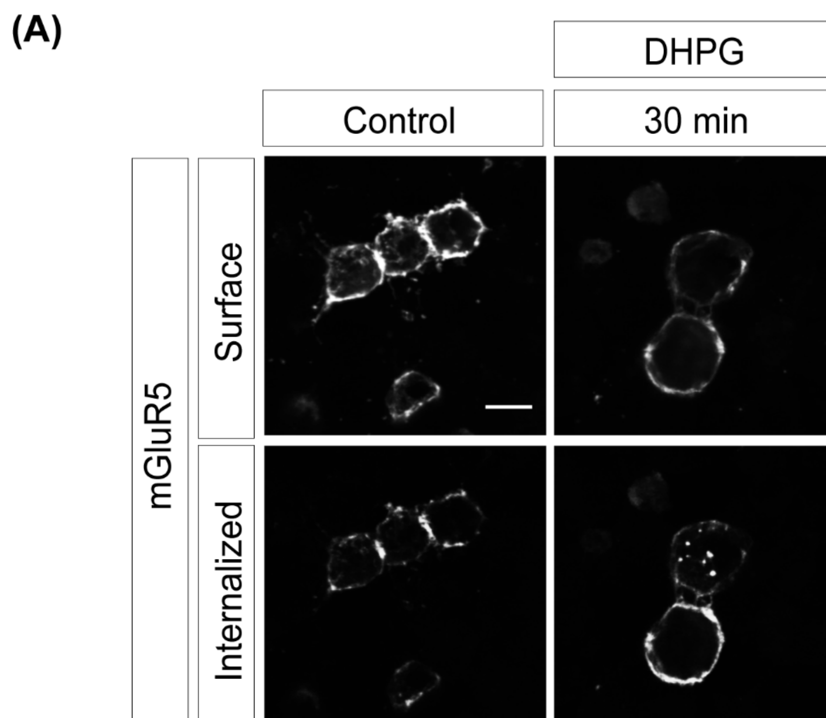
in synaptic plasticity (Wang et al., 2010). In view of these important roles of Norbin in the CNS, we hypothesized that Norbin might be a crucial regulator of group I mGluR trafficking.

We studied the ligand-mediated endocytosis of group I mGluRs using myc-tagged mGluR1 and mGluR5. Myc was tagged at the N-terminus of both the proteins and it has been shown earlier that tagging myc epitope at the N-terminus of these receptors does not affect the functionality of these receptors and these receptors behave like the native receptors (Choi et al., 2011). Initially, we used HEK293 cells which owing to the presence of a large repertoire of G proteins, are the most commonly used heterologous system to study the trafficking of GPCRs. We observed that upon agonist stimulation, myc-mGluR5 and myc-mGluR1 internalized in HEK293 cells. Subsequent to internalization, the receptors recycled back to the plasma membrane. We further extended our studies to primary hippocampal neurons which are the most experimentally tractable *in vitro* system that can approximate the *in vivo* situation. Our data suggested that application of the agonist resulted in the endocytosis of group I mGluRs in primary hippocampal neurons. Similar to HEK293 cells, both members of the group I mGluR family recycled back to the cell surface in primary hippocampal neurons as well. To investigate the role of Norbin in the ligand-mediated group I mGluR endocytosis, we knocked down endogenous Norbin. Our results suggested that acute knockdown of the endogenous Norbin reduced the surface expression of myc-mGluR1 and myc-mGluR5 in primary hippocampal neurons. Knockdown of Norbin also inhibited the agonist-mediated endocytosis of both myc-mGluR1 and myc-mGluR5 in primary hippocampal neurons. We also tested the effect of Norbin on the endocytosis of mGluR5 mediated by a clinically relevant ligand 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP), which is a widely used antagonist of mGluR5. Our results suggested that MTEP was able to cause the internalization of myc-mGluR5 and knockdown of endogenous Norbin inhibited this phenomenon. Thus, taken together our results suggest that Norbin plays an important role in the regulation of the ligand-mediated endocytosis of group I mGluRs.

3.2. Results

3.2.1. mGluR5 gets internalized upon agonist stimulation in HEK293 cells

Previous studies have reported that mGluR5 gets endocytosed with and without the agonist application in both non-neuronal and neuronal cells (Fourgeaud et al., 2003; Liu & Kirchgessner, 2000; P. K. Mahato et al., 2015; Trivedi & Bhattacharyya, 2012). In order to confirm if myc-mGluR5 internalizes upon agonist application, we initially studied the internalization of myc-mGluR5 in HEK293 cells using the group I mGluR specific agonist, R,S-DHPG. HEK293 cells were transfected with myc-mGluR5 and R,S-DHPG-mediated internalization of the receptor was studied 24 hr post transfection as described earlier in the "materials and methods" section. Precisely, live cells expressing myc-mGluR5 were stained with anti-myc mouse monoclonal primary antibody (1:500) for 15 min at 37˚C and a pulse of 100 µM R,S-DHPG was given for 5 min to induce endocytosis of the receptor. Subsequently, the agonist was washed and the cells were incubated in plain DMEM, in the absence of the agonist, for a total of 30 min after the initial agonist application. We chose 30 min as the time point since our lab had earlier shown that R-S-DHPG-mediated internalization of myc-mGluR5 reached at maximum level 30 min post agonist application (P. K. Mahato et al., 2015). The cells were then fixed with 4% ice-cold paraformaldehyde (PFA) for 15 min on ice without permeabilization. Thereafter, a saturating concentration of the first secondary antibody, viz., goat anti-mouse Alexa-568 (1:100) was applied for 1.5 hr at 37˚C to label the surface receptors. Then, the cells were permeabilized using 0.1% Triton X-100 for 30 min at room temperature followed by second secondary antibody staining, i.e., goat anti-mouse Alexa-647 (1:800) for 1 hr at 37˚C to label the internalized receptors. Cells were then mounted on glass slides and imaged under the confocal microscope. Endocytosis index for each cell was calculated as the ratio of internalized receptors to the surface plus internalized receptors. In control cells that were not treated with R,S-DHPG, majority of the receptors remained at the cell surface while very little that had constitutively endocytosed were seen in the internalized fraction. Whereas, in case of R,S-DHPG-treated cells, a significant proportion of receptors were observed to be internalized 30 min post agonist application **(control: 1 ± 0.1; DHPG: 1.77 ± 0.24)** **(Figure 3.1A, B)**. Thus, these results suggest that myc-mGluR5 internalizes upon application of the agonist in HEK293 cells.



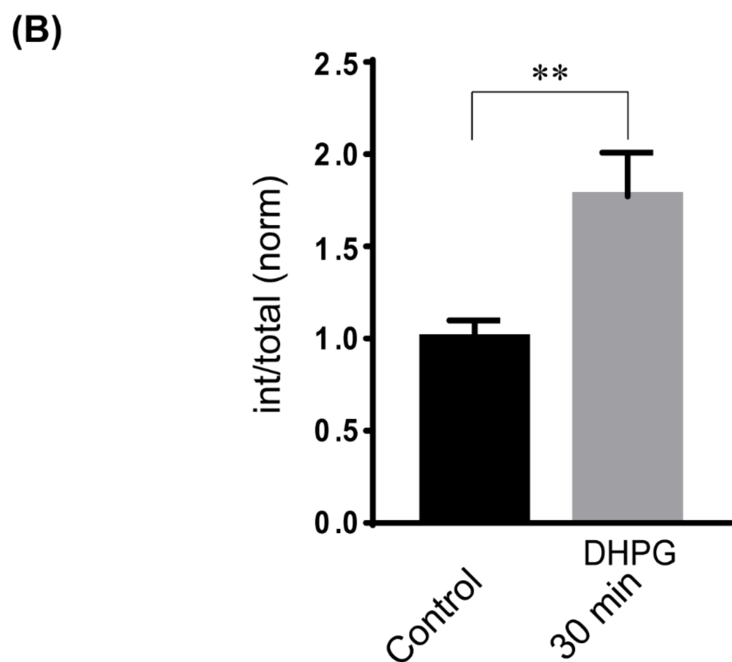


Figure 3.1. mGluR5 gets internalized upon agonist application in HEK293 cells. (A) Control cells showed presence of majority of the receptors at the cell surface and lesser amount of internal fluorescence was observed. In 100 µM R,S-DHPG treated cells, the receptors internalized at 30 min. (B) Quantitation also showed that myc-mGluR5 got internalized upon R,S-DHPG application in 30 min. Scale bar = 10 µm. **, $p < 0.01$.

In this and all the subsequent experiments, the accuracy of quantitation is governed by the fact that we used a saturating concentration of the first secondary antibody that labeled almost all the surface receptors such that the second secondary antibody was not able to bind to a detectable amount of surface receptors and it thus labeled only the internalized receptors upon permeabilization. This standardization protocol has been discussed in detail in the "materials and methods" section.

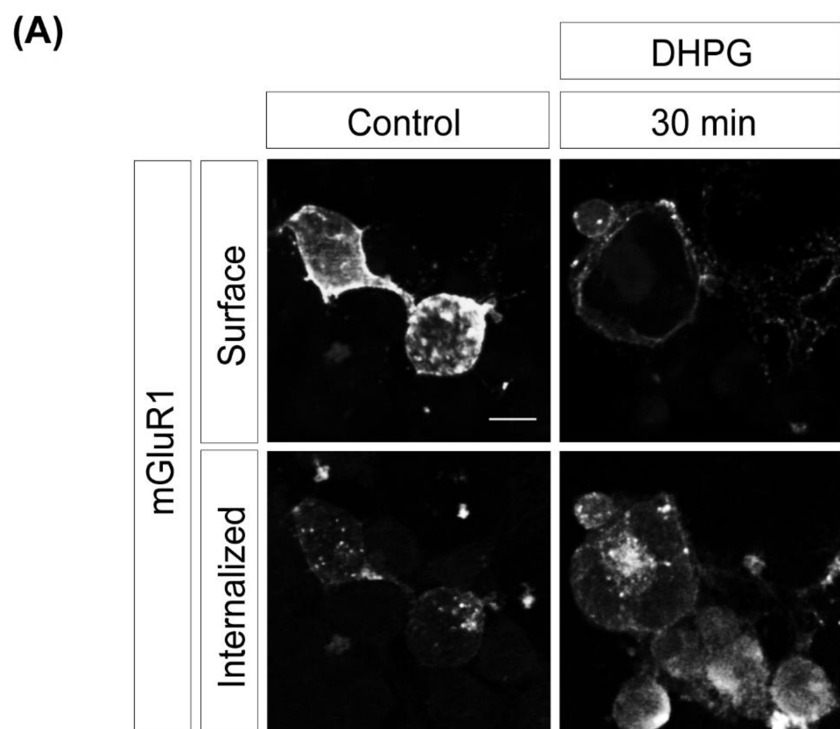
3.2.2. Agonist-mediated endocytosis of mGluR1 in HEK293 cells

We next wanted to investigate if another member of the group I mGluR family, viz., mGluR1 also internalizes upon application of the agonist in HEK293 cells. Like mGluR5, mGluR1 has also been reported to internalize in both agonist-dependent and independent manner (Gulia et al., 2017; Mundell et al., 2001; Pandey et al., 2014; Pula et al., 2004). In order to confirm that mGluR1 internalizes upon application of the agonist in HEK293 cells, we transfected myc-mGluR1 into the cells using PEI as elaborated in the "materials and methods" section. 24 hr after transfection, the endocytosis assay was performed in a similar manner as discussed above for myc-mGluR5. Our data suggested that like myc-mGluR5, cells expressing myc-mGluR1 also showed an increase in the internalized pool of receptors 30 min post R,S-DHPG application, whereas in control cells, majority of the receptors were localized at the cell surface **(control: 1 ± 0.22; DHPG: 2.38 ± 0.38)** **(Figure 3.2A, B)**.

Together, these results suggested that both members of the group I mGluR family; mGluR1 and mGluR5 internalize following application of the agonist in HEK293 cells.

3.2.3. Group I mGluRs recycle back to the cell surface after agonist-mediated internalization in HEK293 cells

Studies from our group have shown that both the members of group I mGluR family viz., mGluR5 and mGluR1 recycle back to the cell surface after ligand-mediated endocytosis and recycling is the mechanism for the resensitization of these receptors (P. K. Mahato et al., 2015; Pandey et al., 2014; Sharma et al., 2018). In order to show that myc-mGluR5 recycles back to the cell surface in HEK293 cells, we chased the cells for longer time periods after endocytosis. Briefly, HEK293 cells were transfected with myc-mGluR5 and the recycling assay was performed 24 hr post-



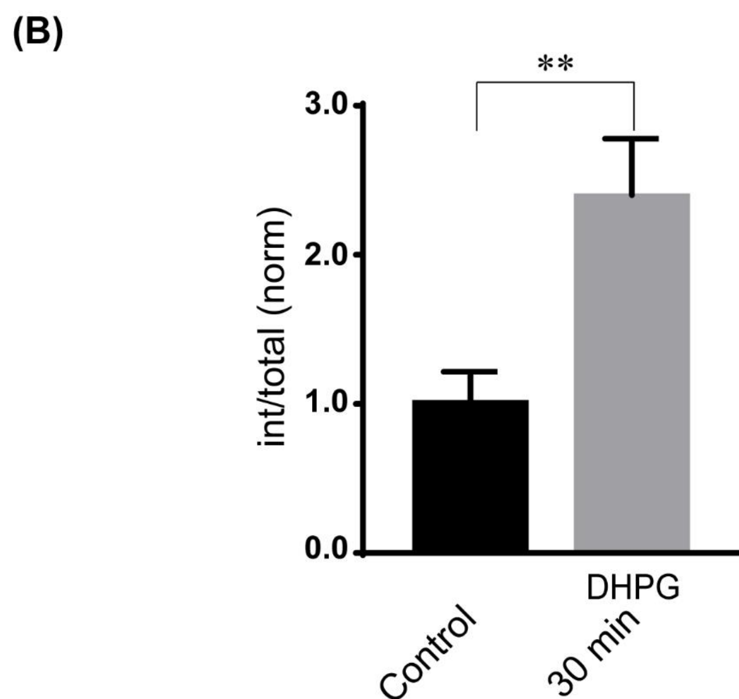


Figure 3.2. mGluR1 internalizes upon application of agonist in HEK293 cells. (A) Control cells showed very little internalized receptors and subsequent to the 100 μM R,S-DHPG application, receptors internalized at 30 min. (B) Quantitation also showed that myc-mGluR1 endocytosed upon R,S-DHPG application in 30 min. Scale bar = 10 μm. **, $p < 0.01$.

transfection. Live cells expressing myc-mGluR5 were stained with anti-myc primary antibody (1:500) for 15 min at 37˚C. 100 µM R,S-DHPG pulse was given for 5 min and after that cells were chased for different time periods (30 min, 2.5 hr) at 37˚C in the absence of the agonist. Then, the cells were fixed with ice-cold 4% PFA on ice for 15 min without permeabilization. Subsequently, cells were stained with a saturating concentration of goat anti-mouse Alexa-568 conjugated secondary antibody (1:100) for 1.5 hr at 37˚C to label the surface receptors followed by permeabilization of the cells with 0.1% Triton X-100 for 30 min at room temperature. After that, the internalized receptors were labelled with goat anti-mouse Alexa-647 conjugated secondary antibody (1:800) for 1 hr at 37˚C. Finally, the cells were mounted on glass slides and observed under the confocal microscope. Control cells had majority of the receptors localized on the cell surface and a few internalized receptors were observed in these cells **(control: 1 ± 0.06) (Figure 3.3A, B)**. Upon application of R,S-DHPG, the receptors got internalized at 30 min post agonist application, as suggested by the decrease in surface fluorescence and a corresponding increase in internal fluorescence **(30 min: 2.07 ± 0.1) (Figure 3.3A, B)**. When chased for an extended time period, most of the receptors recycled back to the cell surface as observed by an increase in the surface fluorescence and decrease in the internalized receptors **(2.5 hr: 0.91 ± 0.06) (Figure 3.3A, B)**.

Similar assay was performed for studying the recycling of the other member of the group I mGluR family, mGluR1. Increase in the number of internalized myc-mGluR1 was observed 30 min post agonist application **(control: 1 ± 0.08; 30 min: 1.56 ± 0.12) (Figure 3.4A, B)**. When chased for longer time period, majority of the receptors recycled back to the cell surface in 2.5 hr **(2.5 hr: 0.95 ± 0.04) (Figure 3.4A, B)**. These results showed that both mGluR1 and mGluR5 recycle back to the cell surface after agonist-mediated endocytosis in 2.5 hr in HEK293 cells.

3.2.4. Internalization of group I mGluRs upon agonist application in primary hippocampal neurons

Our previous results suggested that mGluR5 underwent agonist-mediated internalization in HEK293 cells. We next wanted to investigate whether similar trafficking events occur in the native environment of these receptors. Thus, we studied the agonist-mediated internalization of myc-mGluR5 in primary hippocampal neuron culture. Neurons were transfected with myc-mGluR5 at

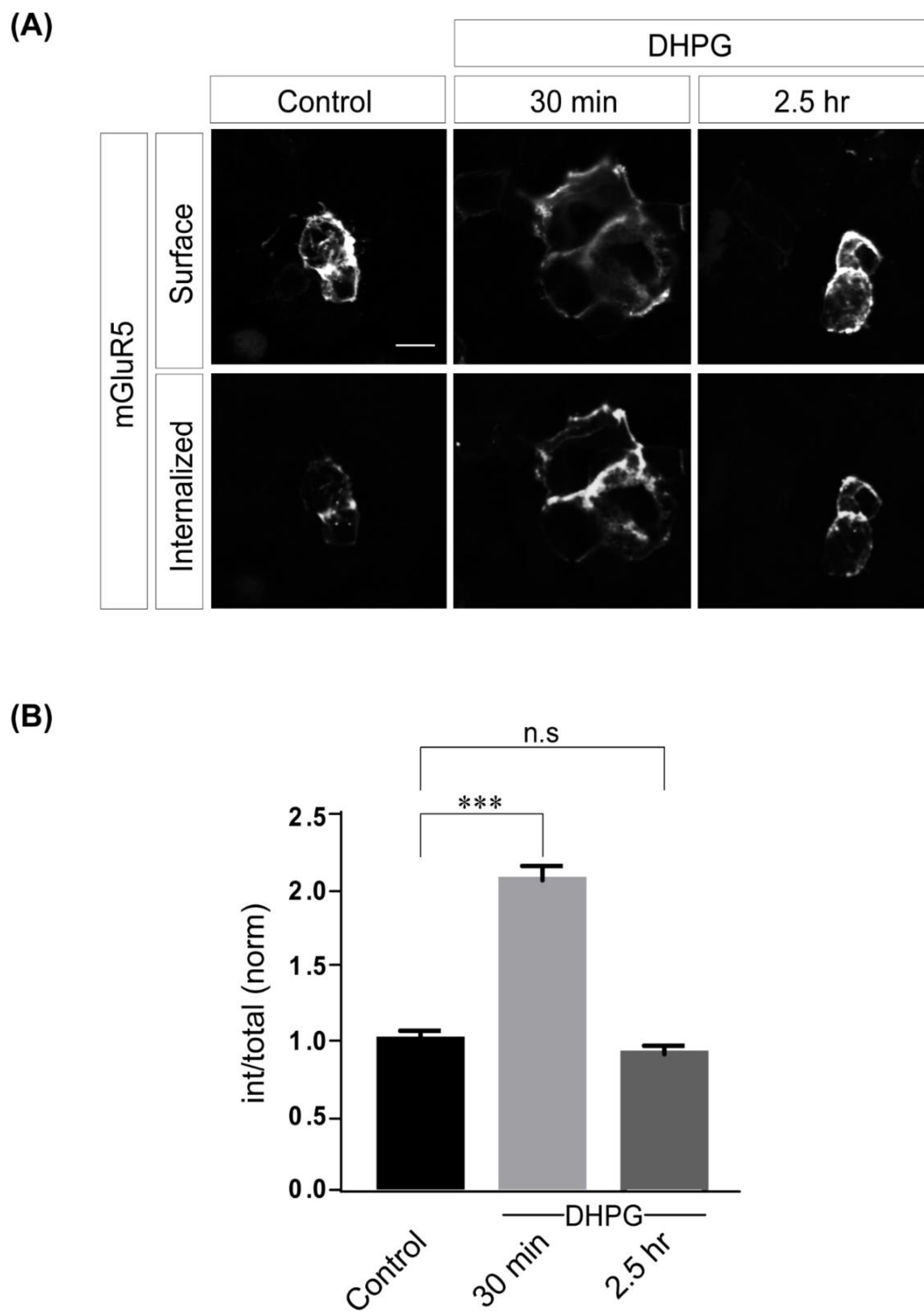
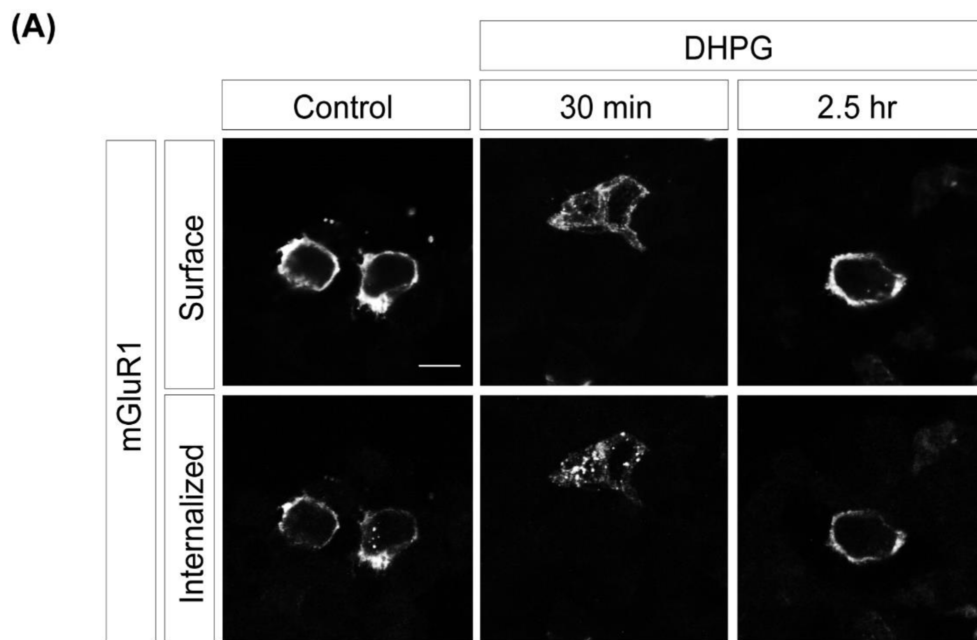


Figure 3.3. mGluR5 recycles back to the cell surface after agonist-mediated internalization in HEK293 cells. (A) Control cells showed surface localization of majority of the myc-mGluR5. Upon 100 µM R,S-DHPG application, majority of the receptors internalized at 30 min. When cells were chased for an extended time period in the absence of the agonist, majority of the internalized receptors recycled back to the cell surface in 2.5 hr. (B) Quantitation also suggested recycling of the agonist-induced internalized myc-mGluR5 in 2.5 hr. Scale bar = 10 µm. ***, $p < 0.001$; n.s., $p > 0.05$.



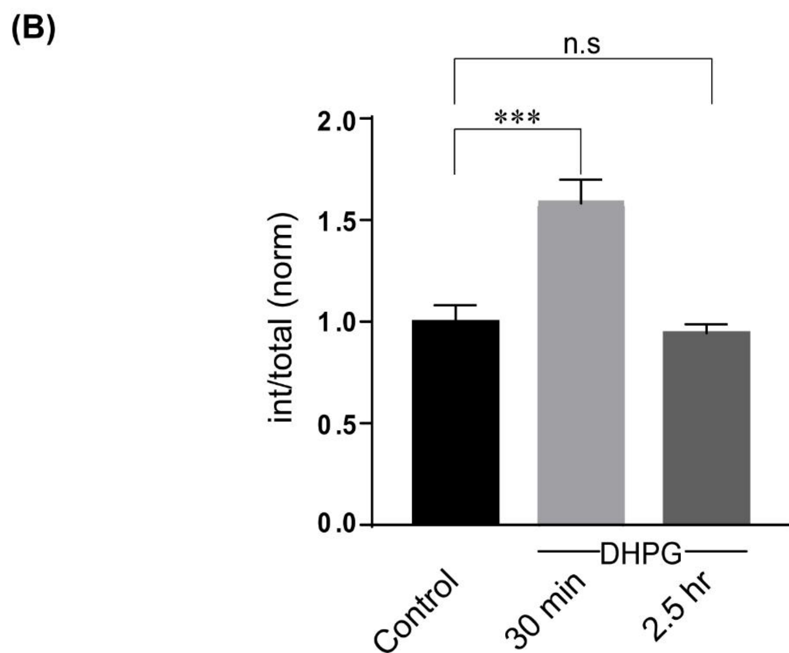
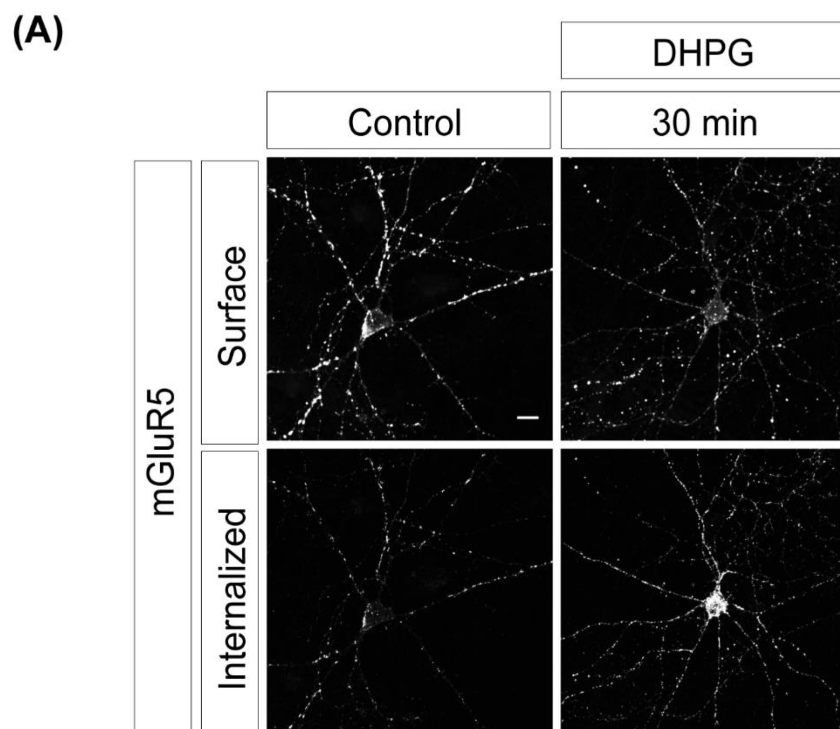


Figure 3.4. mGluR1 recycles back to the cell surface after agonist-mediated internalization in HEK293 cells. (A) Control cells showed localization of majority of the myc-mGluR1 at the cell surface. After 100 μM R,S-DHPG application, majority of the receptors were observed in the internalized compartments at 30 min. When cells were chased for longer time period in the absence of the agonist, majority of the internalized receptors recycled back to the cell surface in 2.5 hr. (B) Quantitation of the endocytosis index also suggested recycling of the internalized myc-mGluR1 in 2.5 hr in HEK293 cells. Scale bar = 10 μm. ***, $p < 0.001$; n.s., $p > 0.05$.

6-7 days *in vitro* (DIV) using calcium phosphate method as described in the "materials and methods" chapter. 5-7 days after transfection, the endocytosis assay was performed. Briefly, live neurons were stained with anti-myc mouse monoclonal primary antibody (1:200) for 20 min at 37˚C. Neurons were given a pulse of 100 µM R,S-DHPG for 5 min to induce endocytosis of the receptor, followed by incubating the cells till 30 min in the absence of the agonist. After 30 min, the cells were fixed without permeabilization with ice-cold 4% PFA on ice for 15 min. Subsequently, surface receptors were labelled with a saturating concentration of goat anti-mouse Alexa-568 conjugated first secondary antibody (1:100) for 1.5 hr at 37˚C. This was followed by permeabilization of the cells by 0.1% Triton X-100 and labelling of internalized receptors with the second secondary antibody, i.e., goat anti-mouse Alexa-647 (1:750) for 1 hr at 37˚C. Neurons were then mounted and observed under the confocal microscope. Similar to HEK293 cells, in control neurons majority of the receptors were present at the cell surface and very few internalized receptors were observed. Subsequent to the 100 µM R,S-DHPG treatment, most of the receptors were observed to be present in the internal compartments of the cells at 30 min and very few receptors were observed on the cell surface compared to control cells **(control: 1 ± 0.12; DHPG: 1.76 ± 0.16) (Figure 3.5A, B)**.

Since neuron is a compartmentalized cell, we did the quantitation separately for whole cell, cell body and dendrites. Since no significant compartment-specific difference was observed in the trafficking of myc-mGluR5 in our assays, in the subsequent results, dendritic endocytosis has been represented in case of experiments related to primary hippocampal neurons.

Subsequently, we wanted to check if mGluR1 also shows similar trafficking kinetics in primary hippocampal neuron culture. For that, we transfected primary neurons with myc-mGluR1 using the calcium phosphate method and performed the endocytosis assay following the same protocol as described above for myc-mGluR5. In case of control neurons expressing myc-mGluR1, most of the receptors were present on the cell surface as suggested by a higher surface fluorescence and lower internalized fluorescence. However, in case of 100 µM R,S-DHPG-treated neurons, majority of the receptors internalized at 30 min **(control: 1 ± 0.07; DHPG: 1.55 ± 0.12) (Figure 3.6A, B)**. These results suggested that both mGluR5 and mGluR1 internalized upon agonist stimulation in primary hippocampal neurons.



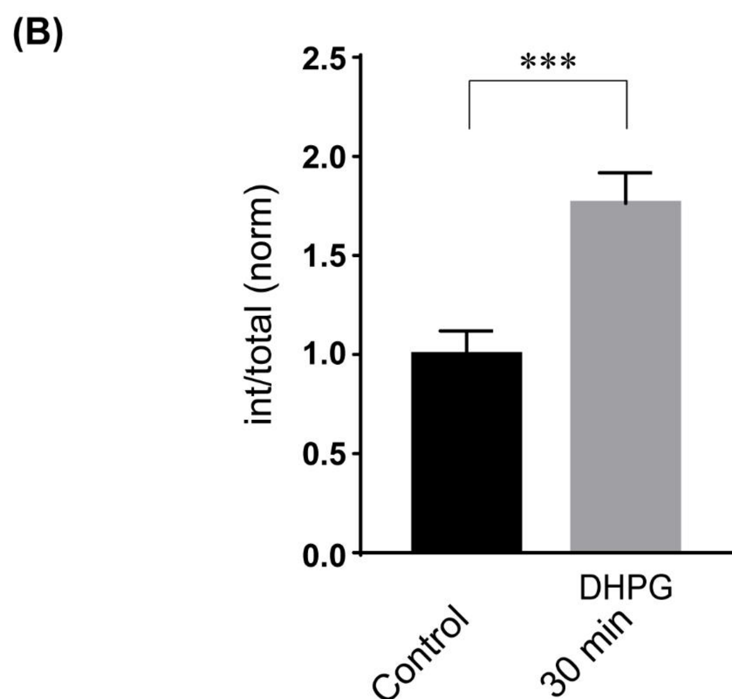
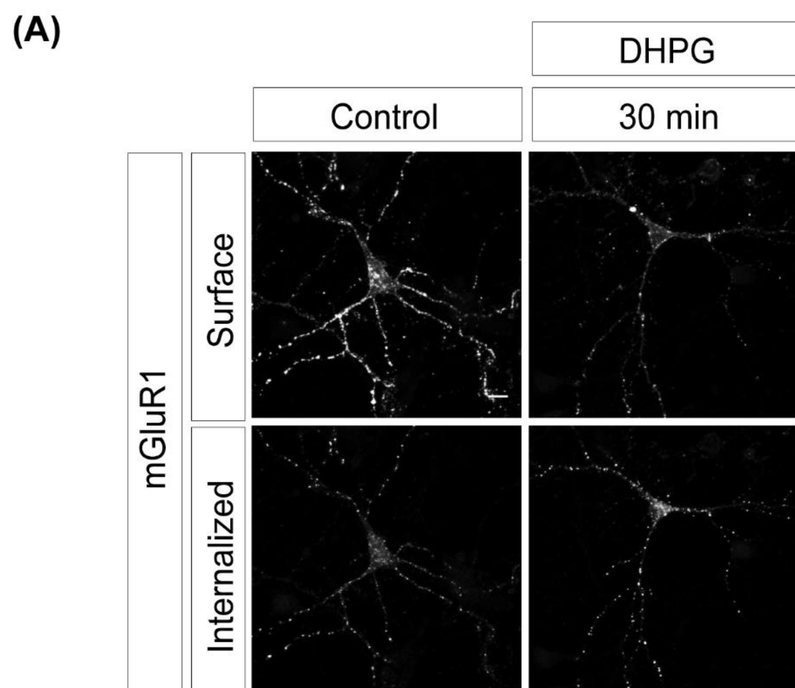


Figure 3.5. Agonist-mediated endocytosis of mGluR5 in primary hippocampal neurons. (A) Control cells showed very little internalized myc-mGluR5 and subsequent to the 100 µM R,S-DHPG application, the receptors internalized at 30 min. (B) Quantitation also showed that myc-mGluR5 endocytosed upon R,S-DHPG application in 30 min. Scale bar = 10 µm. ***, $p < 0.001$.



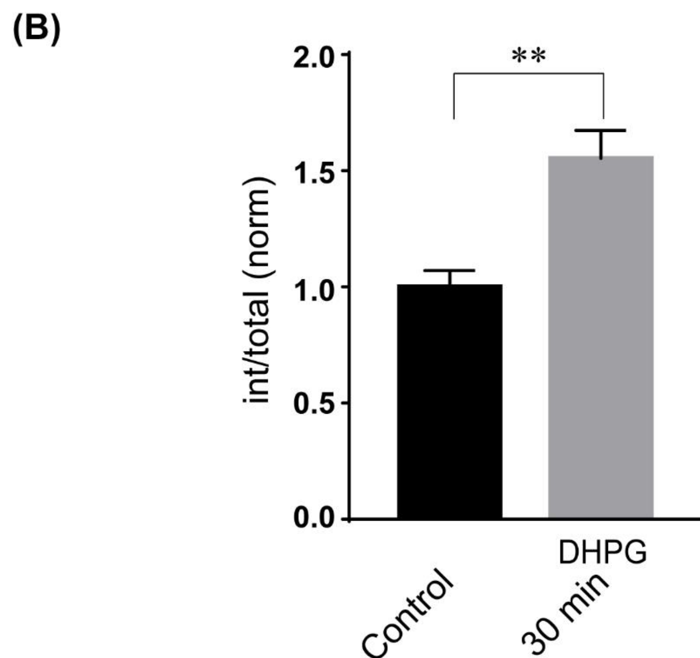
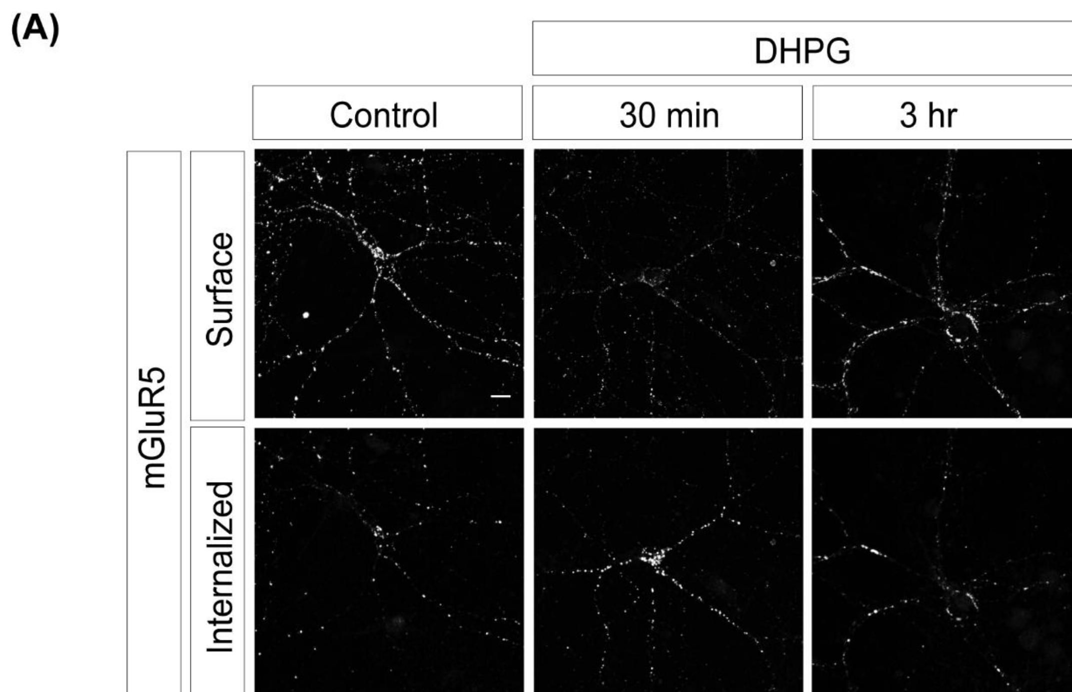


Figure 3.6. mGluR1 internalizes upon application of agonist in primary hippocampal neurons. (A) Control cells showed very little internalized myc-mGluR1 fluorescence and most of the receptors were localized at the cell surface. Subsequent to the 100 μM R,S-DHPG application, majority of the receptors internalized at 30 min. (B) Quantitation also showed that myc-mGluR1 endocytosed upon R,S-DHPG application in 30 min. Scale bar = 10 μm. **, $p < 0.01$.

3.2.5. Group I mGluRs recycle back to the cell surface after agonist-mediated internalization in primary hippocampal neurons

We have shown earlier that group I mGluRs recycle back to the cell membrane subsequent to the agonist-mediated internalization in HEK293 cells. In order to study whether these receptors also recycle to the cell surface following agonist-dependent internalization in primary hippocampal neurons, neurons were transfected with either myc-mGluR5 or myc-mGluR1 plasmid DNA. 5-7 days after transfection, the recycling assay was performed following the protocol described in the "materials and methods" section. Briefly, live neurons expressing myc-mGluR5/myc-mGluR1 were stained with anti-myc primary antibody (1:200) for 20 min at 37˚C followed by application of 100 µM R,S-DHPG pulse for 5 min. After washing out the agonist, cells were chased for an extended time period upto 3 hr in absence of the agonist at 37˚C. Neurons were fixed at different time points (30 min, 2.5 hr, 3 hr) without permeabilization using 4% PFA for 15 min on ice. Subsequently, the surface receptors were stained with a saturating concentration of first secondary antibody, goat anti-mouse Alexa-568 (1:100) for 1.5 hr at 37˚C followed by permeabilization with 0.1% Triton X-100 for 30 min at room temperature. After that, the internalized receptors were labelled with goat anti-mouse Alexa-647 conjugated secondary antibody (1:750) for 1 hr at 37˚C. Finally, the cells were mounted on glass slides and observed under the confocal microscope. In case of myc-mGluR5, control cells showed the presence of majority of the receptors at the cell surface with very little internalized receptors. Application of 100 µM R,S-DHPG led to the maximum internalization of myc-mGluR5 in 30 min **(control: 1 ± 0.12; 30 min: 2.88 ± 0.38) (Figure 3.7A, B)**. When chased for an extended time period, majority of the receptors recycled back to the cell surface in 3 hr post-agonist application **(3 hr: 1.05 ± 0.16) (Figure 3.7A, B)**. In case of myc-mGluR1, application of 100 µM R,S-DHPG led to robust internalization of the receptors in 30 min compared to control cells **(control: 1 ± 0.07; 30 min: 2.39 ± 0.24) (Figure 3.8A, B)**. Subsequent to the endocytosis the receptors recycled to the cell membrane in 2.5 hr **(2.5 hr: 0.81 ± 0.16) (Figure 3.8A, B)**.

The above results suggest that upon application of the agonist, both mGluR5 and mGluR1 internalize in primary hippocampal neurons and recycle back to the cell surface subsequent to that.



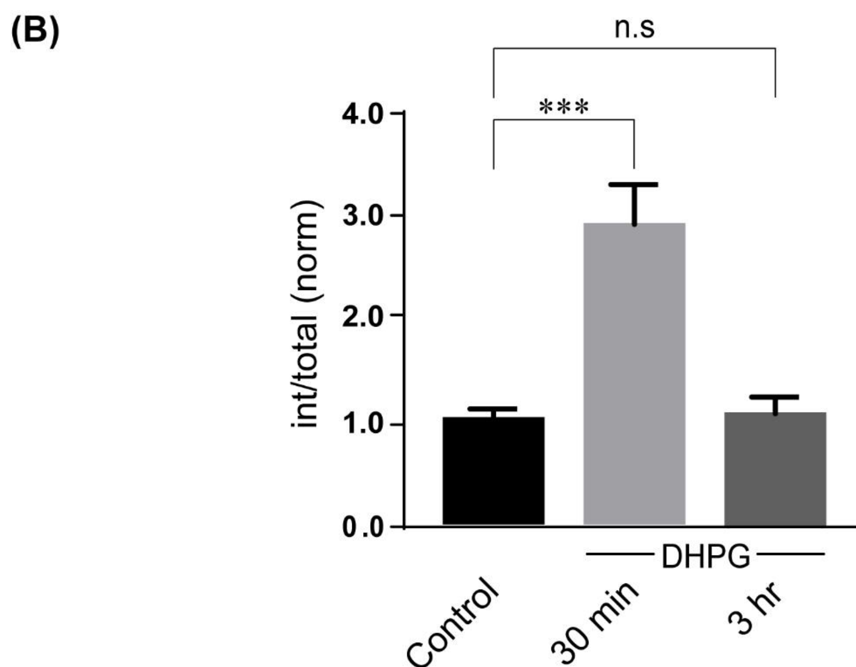
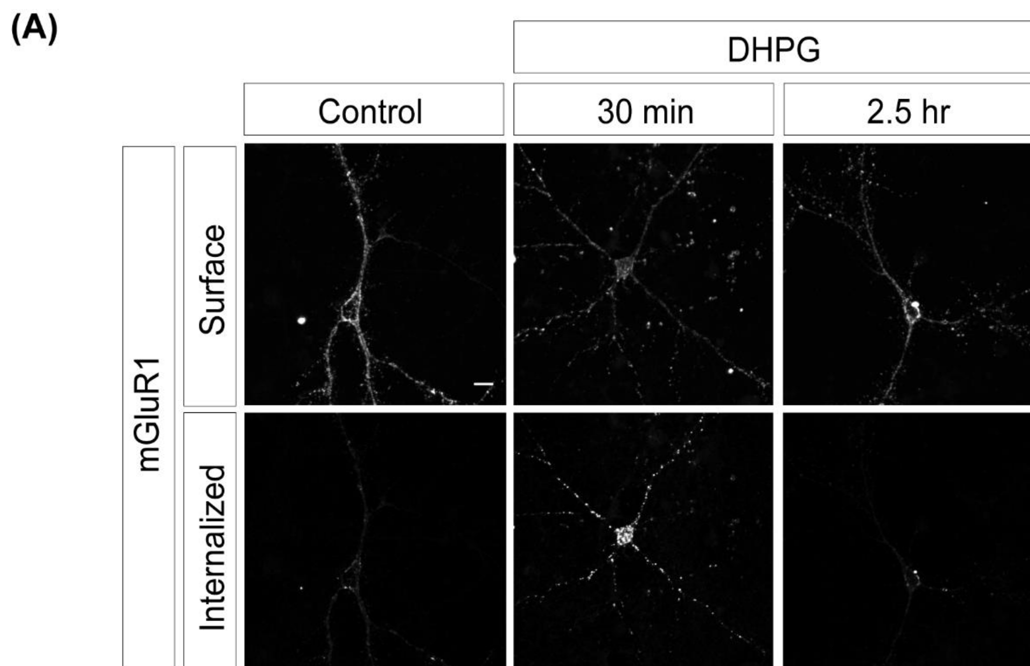


Figure 3.7. mGluR5 recycles back to the cell surface after agonist-mediated internalization in primary hippocampal neurons. (A) Control cells showed surface localization of majority of the receptors. After 100 µM R,S-DHPG application, the receptors internalized at 30 min. When cells were chased for longer time periods, majority of the internalized receptors recycled back to the cell surface in 3 hr. (B) Quantitation also showed recycling of the internalized myc-mGluR5 in 3 hr. Scale bar = 10 µm. ***, $p < 0.001$; n.s., $p > 0.05$.



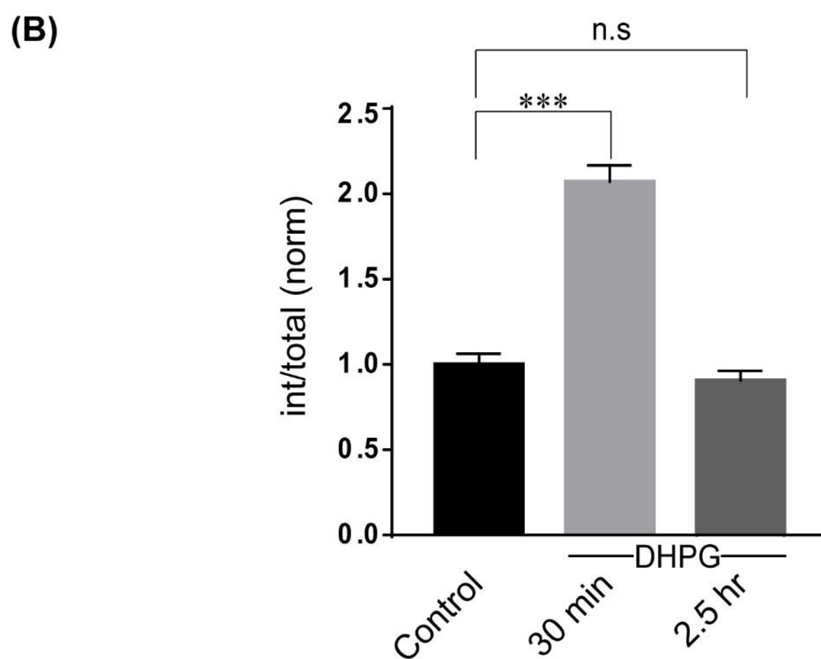


Figure 3.8. Recycling of mGluR1 to the cell surface after agonist-mediated internalization in primary hippocampal neurons. (A) Representative images showing presence of majority of the receptors at the cell surface in control cells. After 100 μM R,S-DHPG application, the receptors internalized at 30 min. When cells were chased for longer time periods, majority of the internalized receptors recycled back to the cell surface in 2.5 hr. (B) Quantitation also showed recycling of the internalized myc-mGluR1 in 2.5 hr. Scale bar = 10 μm. ***, $p < 0.001$; n.s., $p > 0.05$.

3.2.6. Knockdown of endogenous Norbin affects the surface expression and agonist-mediated internalization of mGluR5

Various proteins at the post-synaptic density interact with the group I mGluRs and regulate their localization and signalling (Garner et al., 2000; Scheefhals & MacGillavry, 2018; Tao & Johns, 2006). Norbin is one such protein that has been shown to be associated with mGluR5 in primary hippocampal neurons (Wang et al., 2009a). It also affects prepulse inhibition of startle (PPI), a behavioural phenotype associated with schizophrenia similar to that has been observed in mGluR5 knockout mice (Wang et al., 2009). Due to the above reasons, we were interested in investigating the role of Norbin in mGluR5 trafficking, if any. We first designed various shRNAs against Norbin to acutely knockdown the endogenous Norbin in neurons and screened for one (shNor) which showed robust knockdown of the protein using the method described in the "materials and methods" section. Initially, we checked the efficiency of shNor to knockdown the endogenous Norbin using immunocytochemistry method. Neurons transfected with either shNor or empty vector were fixed 72 hr after transfection with 4% PFA and then permeabilization was done in 0.1% Triton X-100 for 30 min at room temperature. Subsequently, overnight staining was performed to label the endogenous Norbin and EGFP using anti-Norbin mouse polyclonal antibody (1:500) and anti-GFP rabbit polyclonal antibody (1:500) respectively at 4˚C, followed by the respective secondary antibody staining (1:800 each) for 1 hr at 37˚C. Cells were then mounted on glass slides and imaged under the confocal microscope. EGFP was used as the reporter to visualize transfected neurons. In almost all shNor transfected cells there was a significant knockdown of endogenous Norbin as compared to control cells that were transfected with the empty vector **(Figure 3.9A)**. We also checked the efficiency of knockdown of endogenous Norbin by shNor through western blotting. 72 hr post-transfection, primary neurons were lysed in RIPA lysis buffer containing protease inhibitor cocktail and western blotting was performed as described in the "materials and methods" chapter. Control cells showed normal expression of endogenous Norbin and significant knockdown of the endogenous Norbin was observed in the shNor transfected cells **(control: 1 ± 0.08; shNor: 0.33 ± 0.07)** **(Figure 3.9B, C)**.

We subsequently investigated the effect of knockdown of the endogenous Norbin, if any, on the surface expression of mGluR5. Briefly, primary hippocampal neurons were co-transfected with

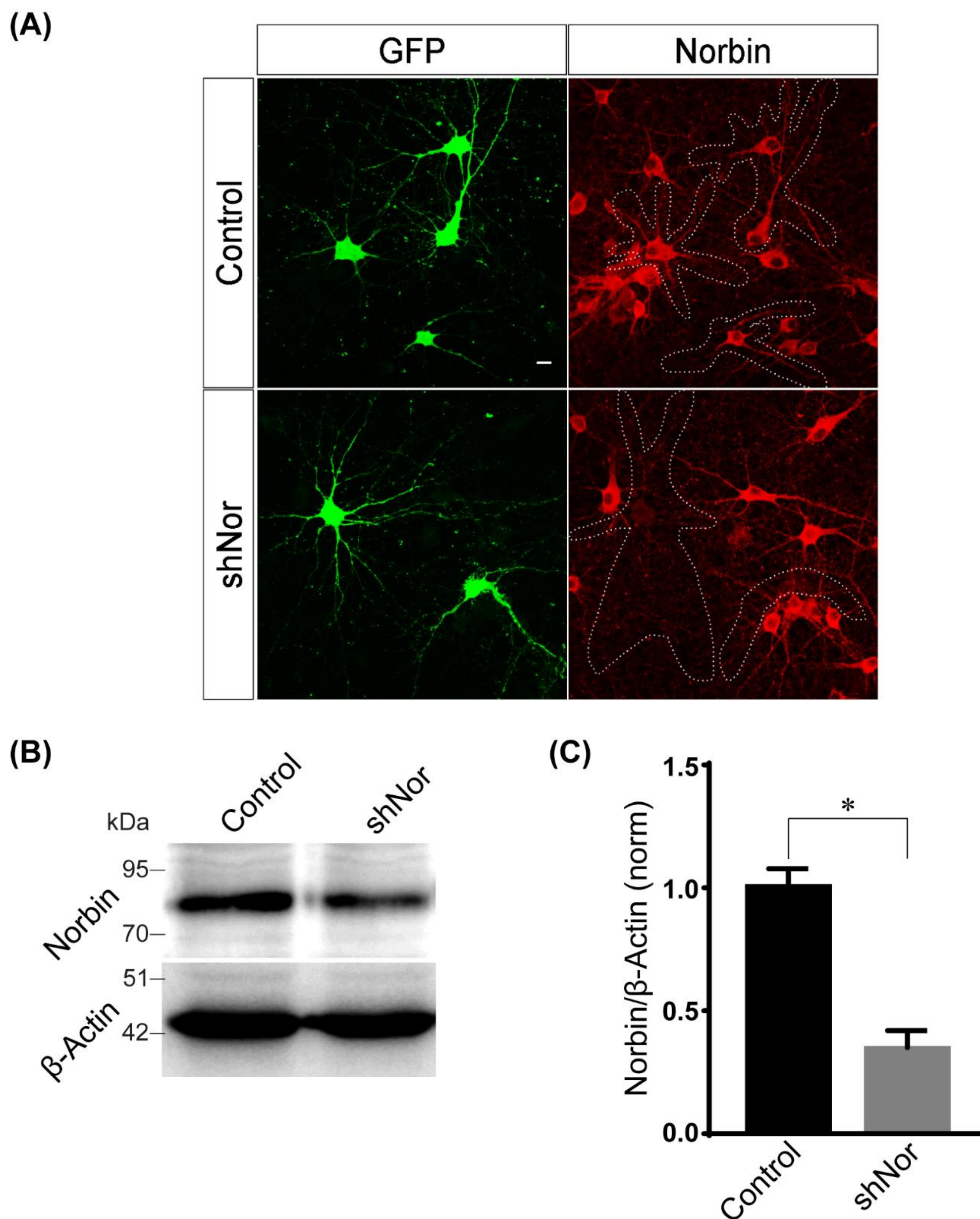


Figure 3.9. Knockdown of endogenous Norbin by shNor in primary hippocampal neurons. (A) Control cells expressing GFP showing the intracellular staining pattern of endogenous Norbin in primary hippocampal neurons (red). In contrast, cells expressing shNor showed almost complete disappearance of endogenous Norbin compared to nearby untransfected cells. (B) Western blot showing efficient knock-down of the endogenous Norbin by shNor. (C) Quantitation also showed that shNor was effectively knocking down endogenous Norbin. Scale bar = 10 µm. *, $p < 0.05$.

myc-mGluR5 and shNor or empty vector. 4-5 days after transfection, surface myc-mGluR5 were labelled with anti-myc primary antibody (1:200) for 20 min at 37˚C, followed by fixation in 4% PFA and secondary antibody staining with goat anti-mouse Alexa-568 (1:100) for 1 hr at 37˚C. Acute knockdown of endogenous Norbin caused a significant decrease in the surface expression of myc-mGluR5 compared to control cells **(control: 1 ± 0.06; shNor: 0.71 ± 0.04) (Figure 3.10A, B)**.

We then wanted to investigate if knockdown of endogenous Norbin had any effect on the agonist-mediated internalization of myc-mGluR5. Our earlier data suggested that upon R,S-DHPG application majority of the myc-mGluR5 internalized at 30 min post agonist application. Therefore, we chose 30 min as the time point for all mGluR5 endocytosis experiments. Primary neurons were co-transfected with myc-mGluR5 and shNor or empty vector and the endocytosis assay was performed using the same protocol as described before. A pulse of 100 µM R,S-DHPG for 5 min caused a significant proportion of surface myc-mGluR5 in control cells to endocytose at 30 min post agonist application, whereas, in shNor transfected cells, the internalization of myc-mGluR5 was inhibited **(control: 1 ± 0.04; control + DHPG: 1.63 ± 0.06; shNor + DHPG: 0.95 ± 0.06) (Figure 3.11A, B)**.

These results suggested that Norbin plays a crucial role in the surface stabilization as well as agonist-mediated internalization of mGluR5. It is important to mention that the decrease in the surface receptor expression upon knockdown of Norbin in case of shNor does not affect the quantitation of endocytosis index in our endocytosis assay since we start with whatever number of receptors are present on the cell surface and calculate the endocytosis index in each cell by measuring the internalized receptors with respect to the total number of receptors (surface + internalized) observed in that cell.

3.2.7. Replacement of endogenous Norbin with wild-type Norbin rescues the knockdown phenotype

Once the effect of knocking down the endogenous Norbin had been established, it was important to confirm that the block in endocytosis and the decrease in surface expression of myc-mGluR5 that we observed were indeed due to the knockdown of endogenous Norbin and not due to some other non-specific effect. For that, we used a "molecular-replacement strategy" wherein full-length

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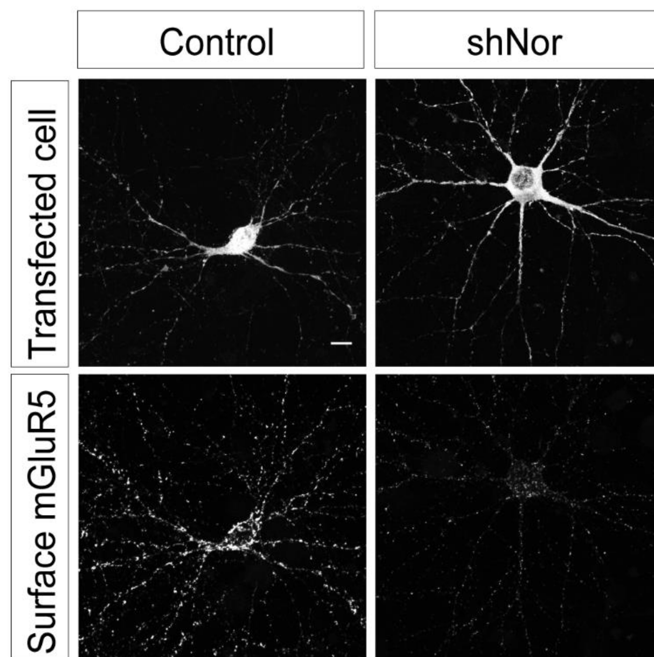

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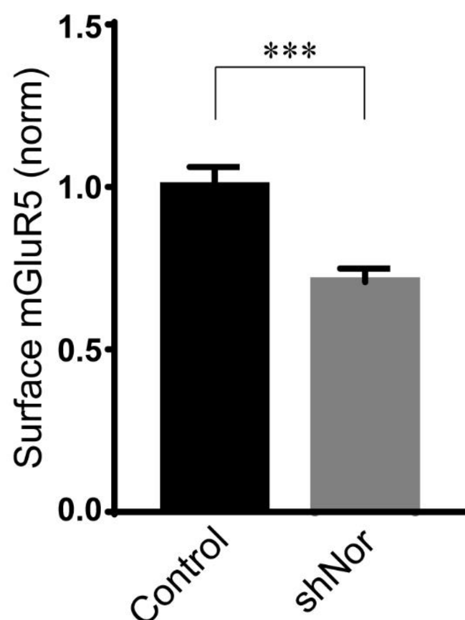
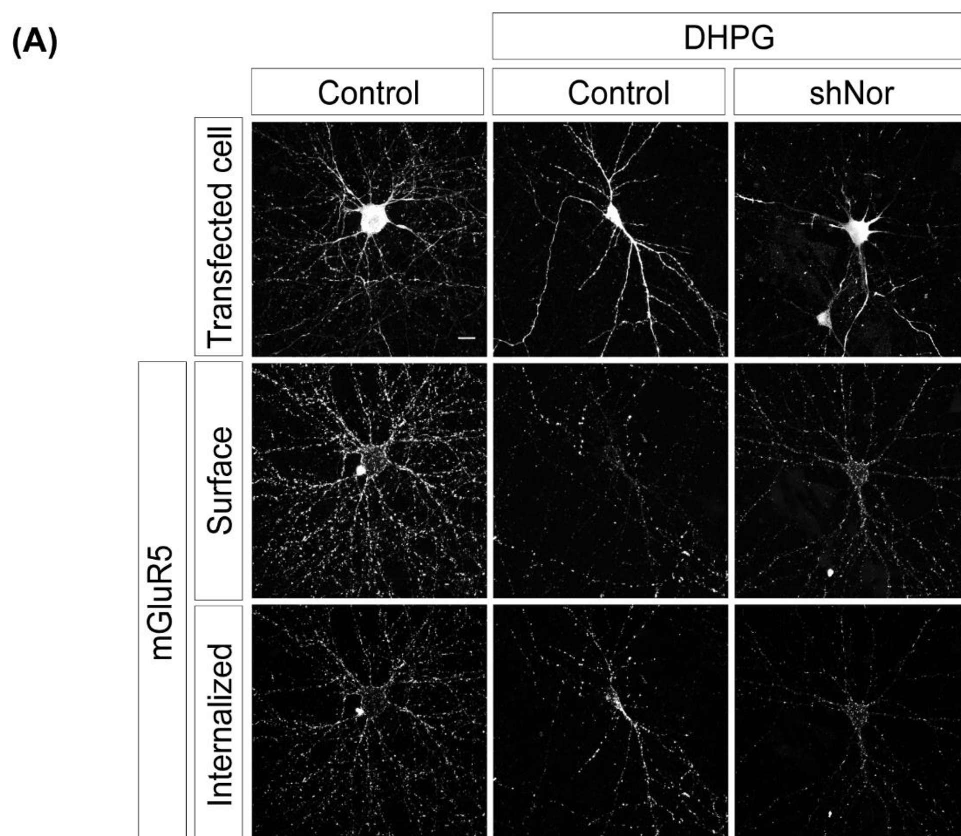


Figure 3.10. Knockdown of endogenous Norbin reduces the surface expression of myc-mGluR5. (A) Representative images showing that knockdown of endogenous Norbin reduced the surface expression of myc-mGluR5. (B) Quantitation of the surface levels of myc-mGluR5 also showed significant decrease in the myc-mGluR5 surface expression in shNor transfected cells as compared to in control cells. Scale bar = 10 μm. ***, $p < 0.001$.

(A)



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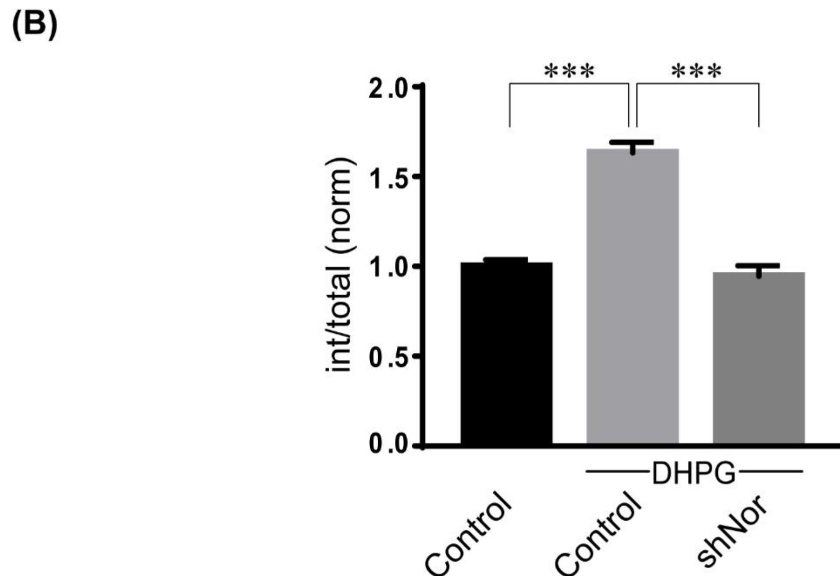


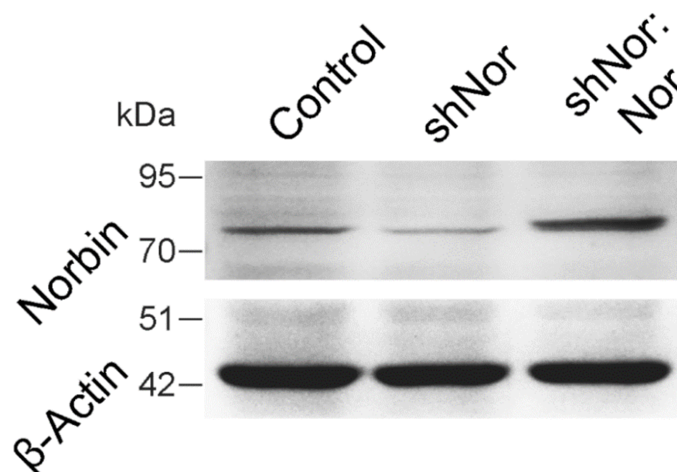
Figure 3.11. Knockdown of endogenous Norbin leads to an inhibition in the agonist-mediated endocytosis of mGluR5. (A) Representative images showing surface and internalized fluorescence of myc-mGluR5 post 100 µM R,S-DHPG application in control cells and shNor expressing cells. (B) Quantitation suggested that knockdown of the endogenous Norbin leads to an inhibition in the R,S-DHPG-mediated endocytosis of myc-mGluR5. Scale bar = 10 µm. ***, $p < 0.001$.

Norbin was cloned under the ubiquitin promoter in the shNor containing dual promoter vector. As mentioned before, silent mutations were introduced in the shRNA binding region of Norbin so that once transfected, shNor would knockdown only the endogenous Norbin and simultaneously the full-length Norbin would replace the endogenous Norbin. Moreover, the degeneracy of amino acid codons allowed for the expression of full-length Norbin with the same amino acid sequence as endogenous Norbin. First, we checked the expression of the wild-type replacement Norbin by western blotting. Primary neurons were transfected with either shNor or with the replacement construct containing shNor and wild-type Norbin (shNor:Nor) using calcium phosphate method as described in the "methods" section and western blotting was performed. As seen previously, shNor transfected cells showed knockdown of the endogenous Norbin compared to control cells. On the other hand, shNor:Nor transfected cells showed proper expression of wild-type Norbin **(control: 1 ± 0.07; shNor: 0.39 ± 0.09; shNor:Nor: 1.31 ± 0.01) (Figure 3.12A, B)**.

Initially, we wanted to investigate if expression of wild type Norbin could rescue the decrease in myc-mGluR5 surface expression due to the knockdown of endogenous Norbin. Primary hippocampal neurons were co-transfected with myc-mGluR5 and shNor or shNor:Nor. Subsequently, the staining of surface myc-mGluR5 was done using the method described before. We observed that knockdown of endogenous Norbin caused a decrease in the surface myc-mGluR5 expression as compared to control cells and this decrease was completely rescued when wild-type Norbin was expressed **(control: 1.0 ± 0.03; shNor: 0.8 ± 0.03; shNor:Nor: 1.1 ± 0.06) (Figure 3.13A, B)**.

We subsequently studied whether replacement of endogenous Norbin with wild-type Norbin replacement construct could rescue the inhibition of R,S-DHPG-mediated myc-mGluR5 internalization caused by knockdown of endogenous Norbin. Hippocampal neurons were co-transfected with myc-mGluR5 and shNor or shNor:Nor and endocytosis assay was performed according to the method described before. As expected, control cells showed little internalized receptors without R,S-DHPG treatment and upon application of 100 µM R,S-DHPG, majority of the receptors were seen to internalize in 30 min **(control: 1 ± 0.03; control + DHPG: 1.6 ± 0.06) (Figure 3.14A, B)**. shNor transfected cells showed a block in the myc-mGluR5 endocytosis and this effect was completely rescued upon expression of the shNor:Nor replacement construct, as most of the receptors were observed in the internalized compartments of the cell 30 min post R,S-

(A)



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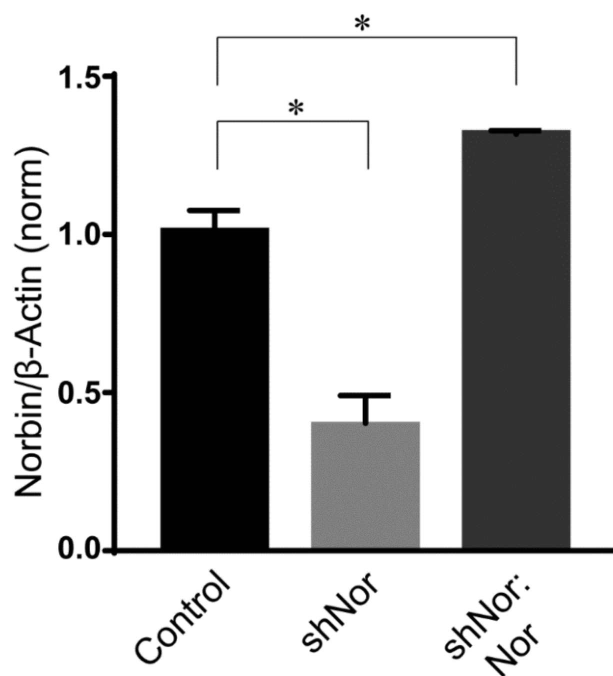
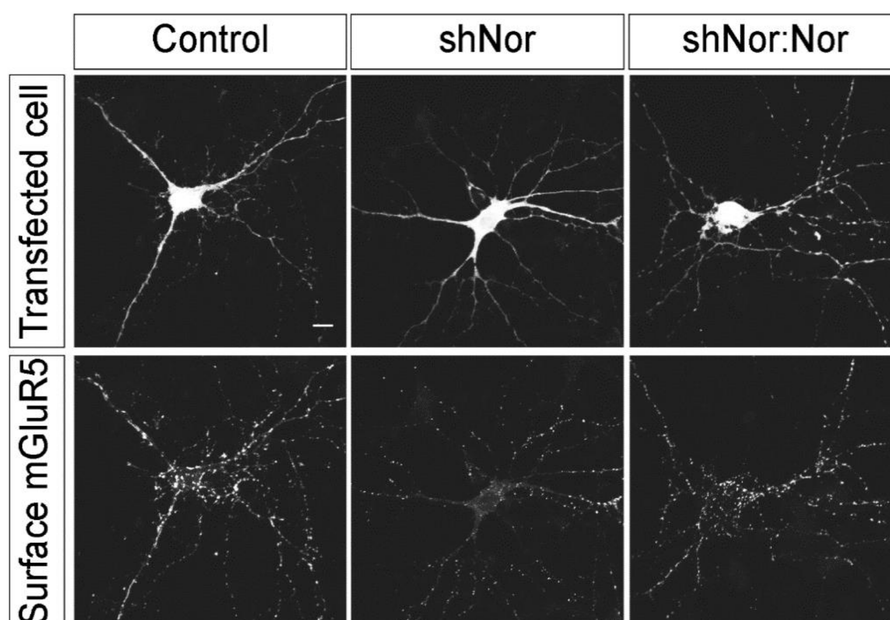


Figure 3.12. Replacement of the endogenous Norbin with full-length Norbin. (A) Representative western blot image showing the knockdown of endogenous Norbin by shNor and replacement of the endogenous protein with full-length Norbin. (B) Quantitation of the western blots also showed effective knockdown of the endogenous Norbin as well as replacement of the endogenous Norbin with the full-length Norbin. *, $p < 0.05$.

(A)



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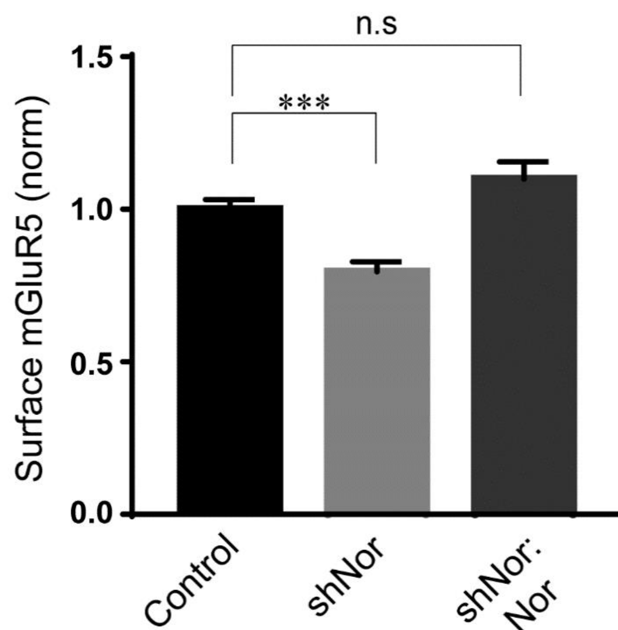


Figure 3.13. Expression of wild-type Norbin rescues the surface expression of mGluR5. (A) Representative neurons showing that knockdown of the endogenous Norbin with shNor decreased the surface expression of myc-mGluR5 and replacement of the endogenous Norbin with the wild-type Norbin replacement construct could rescue the surface myc-mGluR5 levels. (B) Quantitation of the surface myc-mGluR5 also suggested that wild-type Norbin replacement construct rescued the surface expression of myc-mGluR5. Scale bar = 10 μm. ***, $p < 0.001$; n.s., $p > 0.05$.

DHPG application **(shNor + DHPG: 0.94 ± 0.02; shNor:Nor + DHPG: 1.49 ± 0.05) (Figure 3.14A, B)**.

These results showed that replacement of the endogenous Norbin with full-length recombinant Norbin was able to rescue both the decrease in surface receptor expression as well as the block in the endocytosis of myc-mGluR5 and hence confirmed the role of Norbin in agonist-mediated mGluR5 endocytosis.

3.2.8. Time course of mGluR5 endocytosis in Norbin knockdown cells

Our earlier results suggested that knockdown of endogenous Norbin inhibited the agonist-mediated endocytosis of myc-mGluR5, as was measured by the endocytosis index. This no increase in the endocytosis index upon application of 100 µM R,S-DHPG in shNor transfected cells could either be due to an actual block in endocytosis of the receptor or due to rapid recycling of the receptors back to the cell surface in 30 min post agonist application, since mGluRs start internalizing as early as 5 min post R,S-DHPG application (P. K. Mahato et al., 2015; Pandey et al., 2014; Sharma et al., 2018). In order to ascertain the actual role of Norbin in mGluR5 trafficking, we performed time course of myc-mGluR5 endocytosis in Norbin knockdown cells. Live cells expressing myc-mGluR5 were stained with the anti-myc primary antibody. Application of 100 µM R,S-DHPG was able to cause rapid internalization of myc-mGluR5 in control cells and majority of the receptors were seen in the internalized pool at 30 min **(control: 1 ± 0.12; DHPG: 2.55 ± 0.31) (Figure 3.15A, B)**. Importantly, in shNor expressing cells, the block in internalization was consistent at all time points starting from 5 min up to 30 min post agonist application **(shNor:: 5 min: 0.75 ± 0.07; 15 min: 1.07 ± 0.08; 30 min: 0.9 ± 0.1) (Figure 3.15A, B)**. These results suggested that acute knockdown of endogenous Norbin inhibited the agonist-mediated endocytosis of mGluR5 and does not lead to the faster recycling of the receptors.

Our previous data suggested that knockdown of endogenous Norbin decreased the surface expression of myc-mGluR5 in primary hippocampal neurons. The decrease in the myc-mGluR5 surface expression in Norbin knockdown cells could be due to two reasons: (1) reduction in the surface localization of the receptor (2) reduction in the total receptor level. In order to find out the

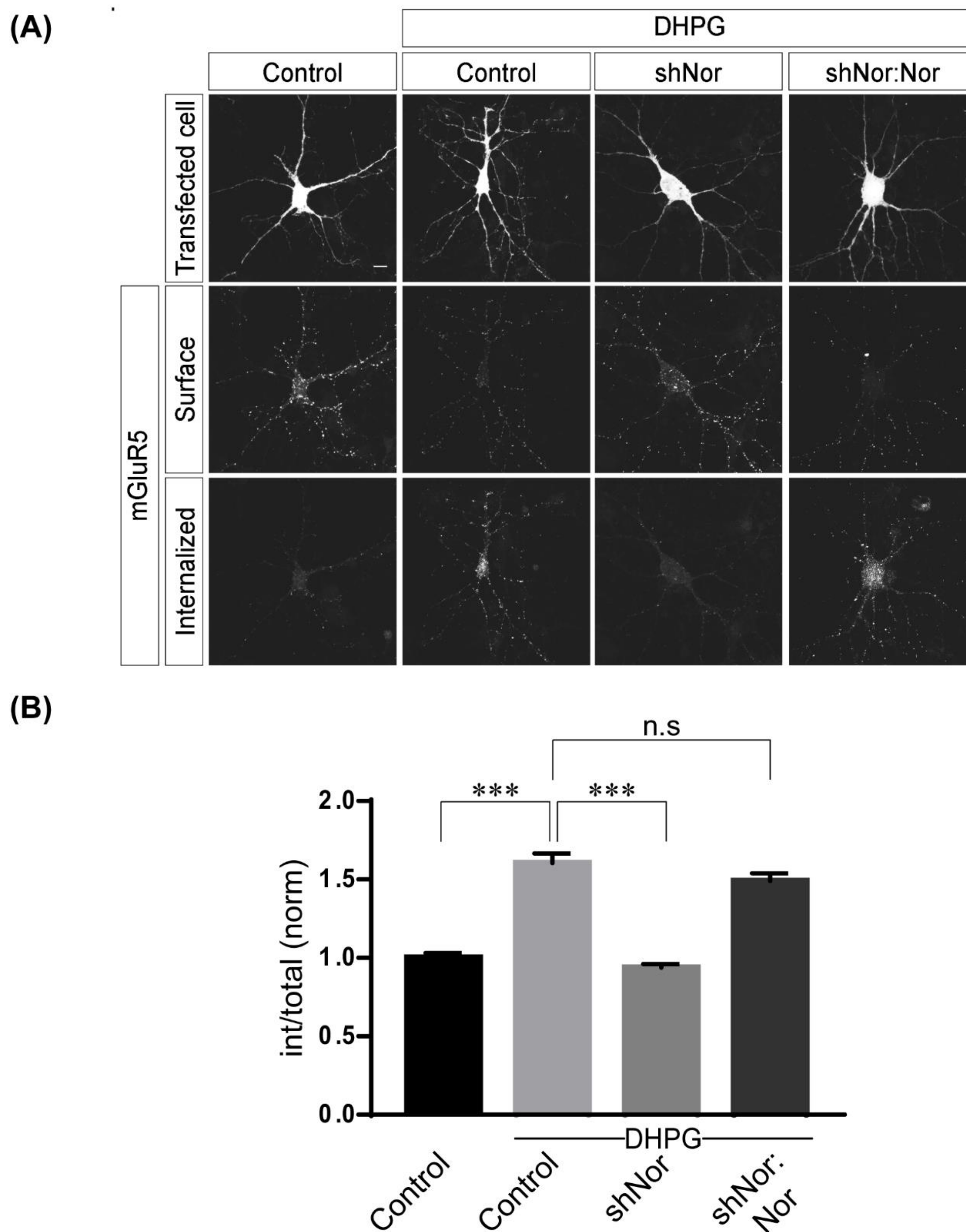


Figure 3.14. Replacement of the endogenous Norbin with wild-type Norbin rescues the agonist-mediated endocytosis of mGluR5. (A) Representative images showing surface and internalized myc-mGluR5 30 min after 100 μM R,S-DHPG application in control cells, shNor expressing cells and wild-type Norbin expressing cells. (B) Quantitation suggested that knockdown of endogenous Norbin inhibited the R,S-DHPG-mediated internalization of myc-mGluR5 and expression of wild-type Norbin replacement construct rescued the normal trafficking of the receptor. Scale bar = 10 μm. ***, $p < 0.001$; n.s, $p > 0.05$.

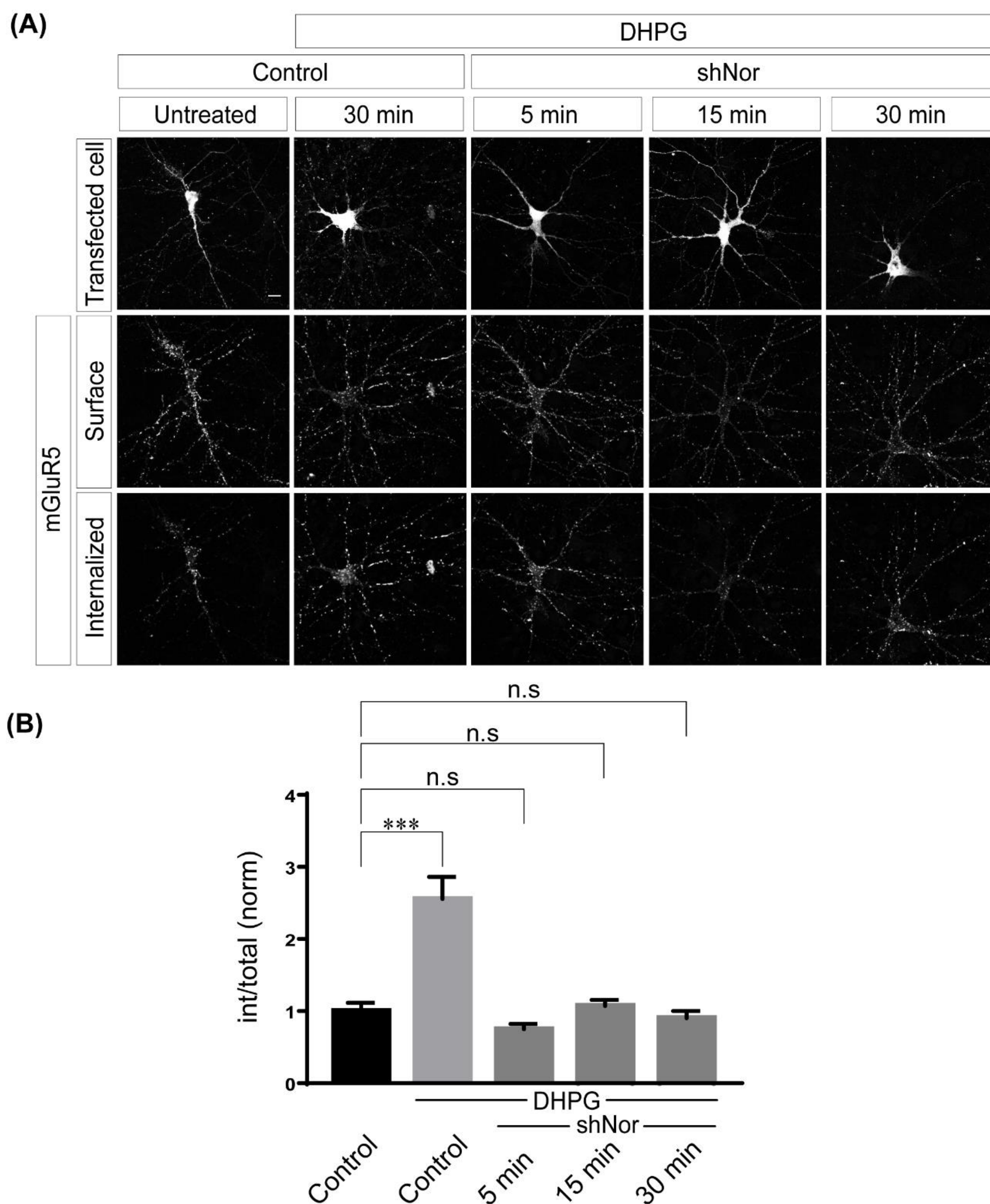


Figure 3.15. Time course of agonist-mediated mGluR5 endocytosis in the presence of shNor. (A) Representative images showing R,S-DHPG-mediated myc-mGluR5 endocytosis at 5 min, 15 min and 30 min post-agonist application in Norbin knockdown cells. Control cells showed increased endocytosis upon 100 µM R,S-DHPG application at 30 min. shNor expressing cells showed a complete block in the endocytosis of the receptor at all the time points. (B) Quantitation also suggested that shNor expressing cells did not show significant amount of internalization of myc-mGluR5 at any time point. Scale bar = 10 µm. ***, $p < 0.001$ and n.s., $p > 0.05$.

reason for the reduced surface expression of the receptor in absence of the endogenous Norbin, primary hippocampal neurons were co-transfected with myc-mGluR5 and shNor or shNor:Nor. Subsequently, cells were lysed in RIPA lysis buffer containing protease inhibitor cocktail and western blots were performed to check for the total myc-mGluR5 level. There was no significant difference observed in the myc-mGluR5 level in control cells, shNor transfected cells and shNor:Nor transfected cells, suggesting that the reduction in the surface expression of myc-mGluR5 was not due to the reduction in the overall level of the myc-mGluR5 in Norbin knocked down cells **(control: 1 ± 0.01; shNor: 1.14 ± 0.05; shNor:Nor: 1.02 ± 0.06) (Figure 3.16A, B)**.

3.2.9. Acute knockdown of endogenous Norbin affects the surface localization as well as agonist-mediated endocytosis of mGluR1

Apart from mGluR5, Norbin has also been shown to interact with the other member of the group I mGluR family, mGluR1 (Wang et al., 2009). But not much is known about the functional consequence of its association with mGluR1. Since we had shown that Norbin is important for the surface stability and agonist-mediated endocytosis of mGluR5, we wanted to check if it has any effect on mGluR1 trafficking. First, we studied the effect of knockdown of the endogenous Norbin on the surface expression of myc-mGluR1 following the same protocol as described for myc-mGluR5. Just like myc-mGluR5, shNor transfected cells showed a decrease in the cell surface expression of myc-mGluR1 as compared to control cells **(control: 1 ± 0.06; shNor: 0.73 ± 0.06) (Figure 3.17A, B)**. This effect was completely rescued when we expressed wild-type Norbin simultaneously in the presence of shNor **(shNor:Nor: 0.89 ± 0.08) (Figure 3.17A, B)**.

We then investigated the effect of the knockdown of endogenous Norbin on agonist-mediated internalization of myc-mGluR1 using the similar protocol as described before. Control cells showed presence of most of the receptors at the cell surface and upon 100 μM R,S-DHPG application majority of the receptors internalized in 30 min. Importantly, in shNor transfected cells, the R,S-DHPG-mediated internalization of myc-mGluR1 was completely inhibited **(control: 1 ± 0.08; control + DHPG: 2.79 ± 0.19; shNor + DHPG: 1 ± 0.09) (Figure 3.18A, B)**. Replacement of the endogenous Norbin with the recombinant wild-type Norbin rescued the normal endocytosis of myc-mGluR1 **(shNor:Nor + DHPG: 2.64 ± 0.18) (Figure 3.18A, B)**.

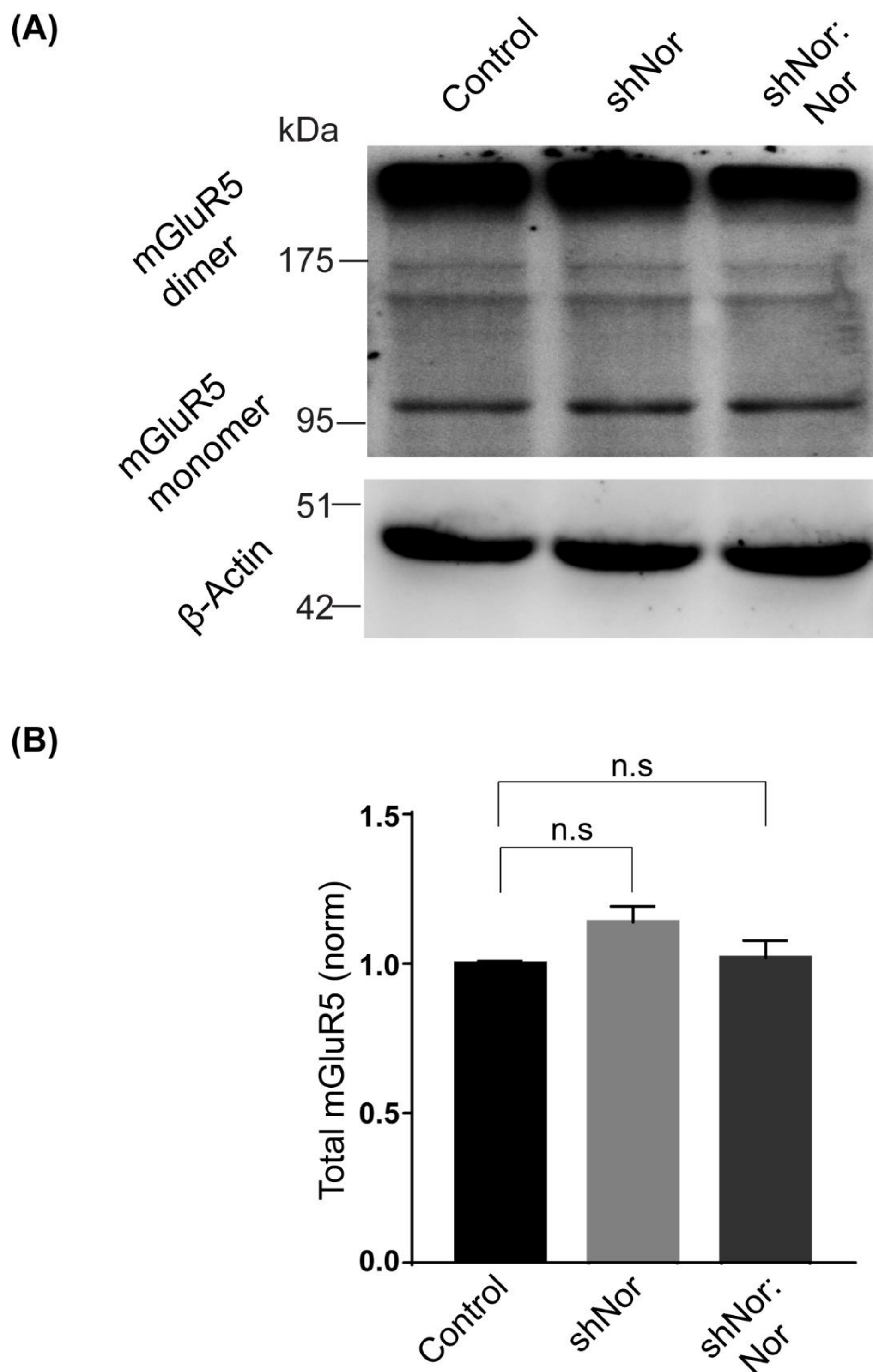
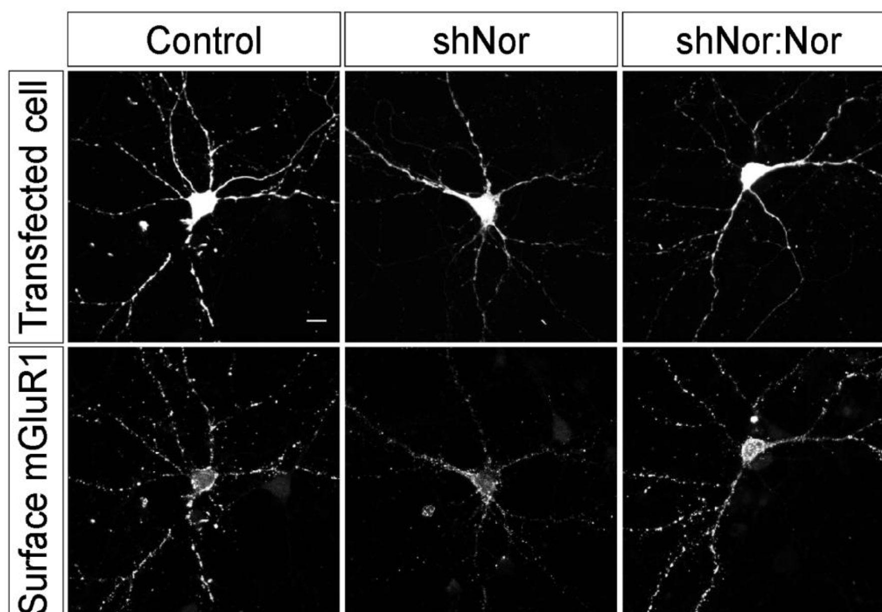


Figure 3.16. The total myc-mGluR5 level does not change in the absence of endogenous Norbin. (A) Representative western blot image showing that knockdown of endogenous Norbin with shNor and replacement with full-length Norbin had no effect on the total myc-mGluR5 level. (B) Quantitation showing that total myc-mGluR5 levels are same in all the three conditions. n.s., $p > 0.05$.

(A)



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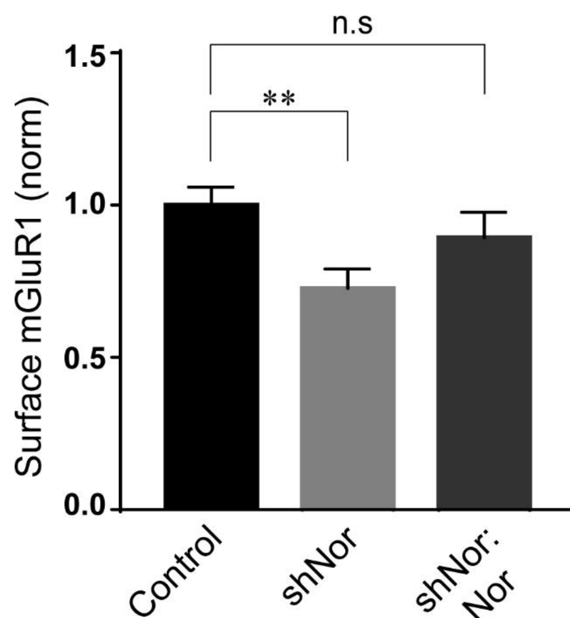


Figure 3.17. Knockdown of endogenous Norbin decreases the surface expression of mGluR1. (A) Representative images showing the surface expression of myc-mGluR1 in control cells, shNor and shNor:Nor expressing cells. (B) Quantitation of the cell surface myc-mGluR1 suggested that following Norbin knockdown, there was a decrease in the surface myc-mGluR1 expression that was rescued upon the replacement of endogenous Norbin with wild-type Norbin. **, $p < 0.01$; n.s., $p > 0.05$.

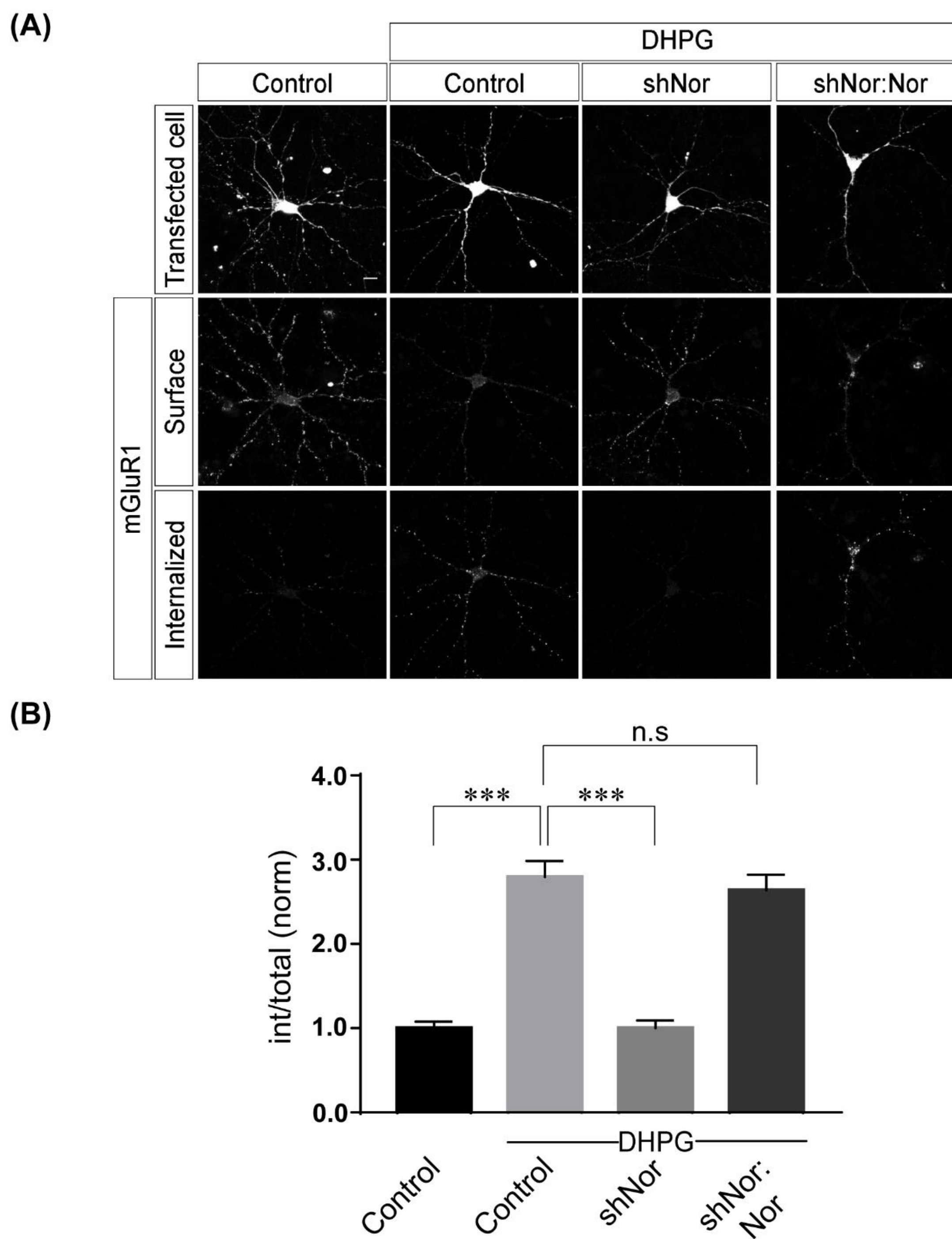


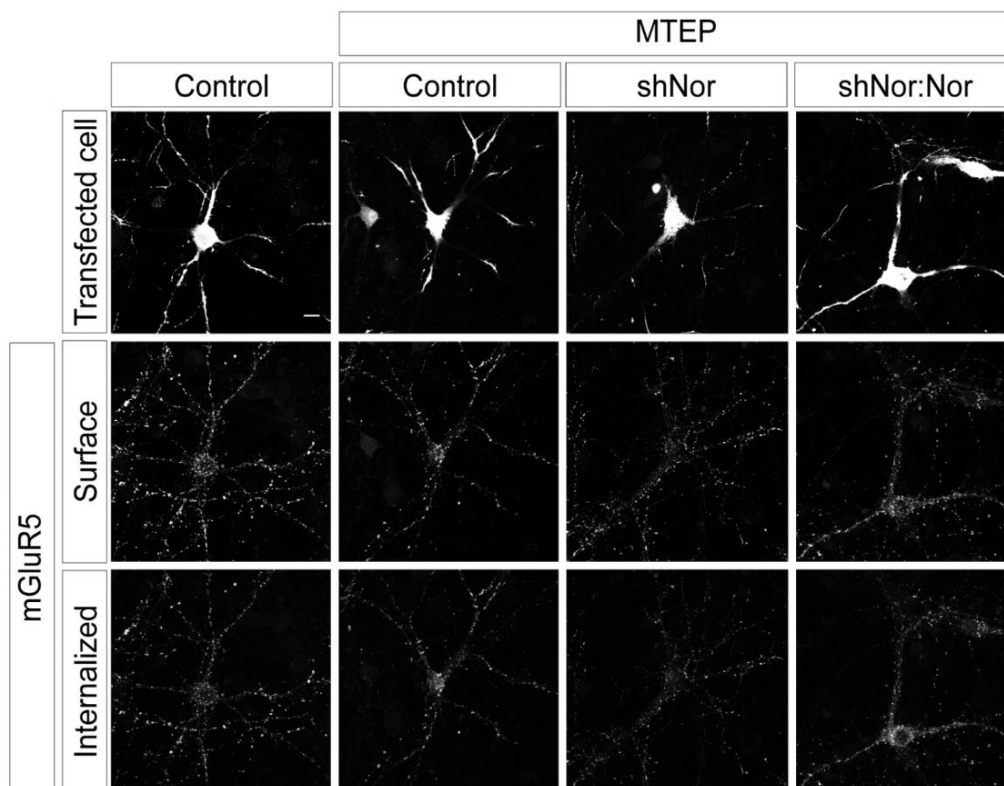
Figure 3.18. Knockdown of endogenous Norbin leads to an inhibition in the agonist-mediated endocytosis of mGluR1. (A) Representative images showing surface and internalized myc-mGluR1 30 min post 100 µM R,S-DHPG treatment in control cells, shNor or shNor and wild-type Norbin expressing cells. (B) Quantitation of the endocytosis index suggested that knockdown of endogenous Norbin inhibited the agonist-mediated endocytosis of myc-mGluR1 and expression of full-length Norbin replacement construct rescued the normal trafficking of the receptor. Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

These results suggested that Norbin plays a critical role in the agonist-mediated internalization of both the members of group I mGluR family, i.e., mGluR1 and mGluR5. Since Norbin and mGluR5 have together been implicated in neuropsychiatric disorders like schizophrenia, we decided to carry out further studies with mGluR5.

3.2.10. Effect of Norbin on antagonist-mediated mGluR5 endocytosis

Antagonists are thought to block the activity of the natural ligand and inhibit the activity of the receptor. But there are several reports where antagonists have been shown to influence the desensitization, internalization and downregulation of GPCRs (Gray & Roth, 2001; Pfeiffer et al., 1998; Von Zastrow & Kobilka, 1994). Considering the widespread use of mGluR5 antagonists for therapeutic interventions in various neurological disorders and their physiological implications in the brain, it becomes important to understand the cellular and molecular events post the binding of antagonist with the receptor as well as the protein machineries that control the antagonist-mediated trafficking events. We checked the effect of mGluR5-specific antagonist MTEP on mGluR5 endocytosis in cells transfected with empty vector, shNor and shNor:Nor to study the role of Norbin in MTEP-mediated mGluR5 endocytosis, if any. The endocytosis assay was performed using the method described in the "materials and methods" section. In control cells, treatment with 100 μM MTEP resulted in the endocytosis of myc-mGluR5 **(control: 1 ± 0.09; control + MTEP: 1.64 ± 0.23) (Figure 3.19A, B)**. Importantly, knockdown of endogenous Norbin with shNor resulted in the inhibition of MTEP-mediated myc-mGluR5 endocytosis and replacement with wild-type Norbin was able to rescue the antagonist-induced internalization of myc-mGluR5 **(shNor + MTEP: 0.72 ± 0.07; shNor:Nor + MTEP: 1.94 ± 0.34) (Figure 3.19A, B)**.

These results suggested that the mGluR5 antagonist MTEP leads to the internalization of these receptors in primary hippocampal neurons and presence of Norbin is crucial for this process. We concentrated on the effect of Norbin in the agonist-mediated internalization of mGluR5 for rest of the study.



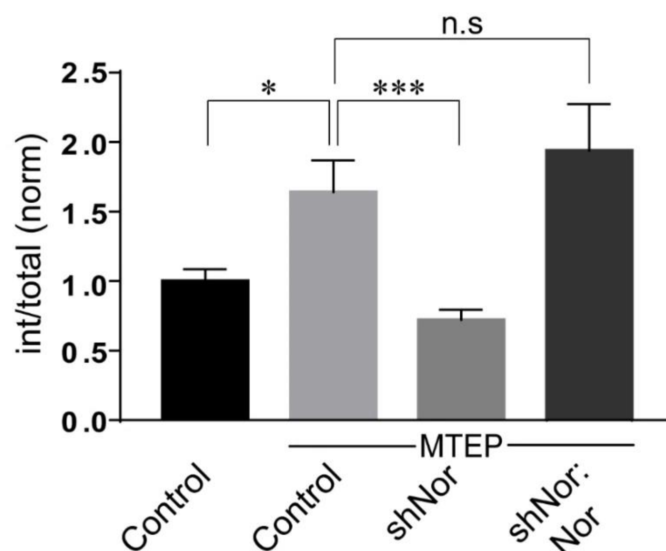


Figure 3.19. Application of mGluR5 antagonist MTEP causes the internalization of mGluR5 which is inhibited by the knockdown of endogenous Norbin. (A) Representative images showing that treatment with 100 µM MTEP caused an increase in the internalization of myc-mGluR5 in control cells and this internalization was blocked upon knockdown of endogenous Norbin. Replacement with wild-type Norbin was able to rescue this phenotype. (B) Quantitation of endocytosis index also suggested that Norbin plays a critical role in the antagonist-mediated myc-mGluR5 endocytosis. Scale bar = 10 µm. ***, $p < 0.001$; *, $p < 0.05$; n.s, $p > 0.05$.

3.3. Discussion

Previous work from our lab has shown that group I mGluRs get endocytosed subsequent to the agonist application in a ubiquitin-dependent manner and recycle back to the cell membrane via a protein phosphatase-dependent mechanism (Gulia et al., 2017; P. K. Mahato et al., 2015; Pandey et al., 2014). A number of proteins present at the post-synaptic density might act as adaptors to facilitate this process. We found Norbin as one of the known interactors of group I mGluRs and considering its important functions in various neuropsychiatric disorders, we decided to investigate the role of Norbin in group I mGluR trafficking, if any. Norbin is a 75 kDa cytosolic adaptor protein that is highly conserved in vertebrates and has no sequence homology to other known proteins. It is also known as Neurochondrin in mouse and humans (Dateki et al., 2004; Mochizuki et al., 1999; Shinozaki et al., 1999). Our study builds on previous literature which had identified Norbin as a direct binding partner and regulator of mGluR5 signalling (Wang et al., 2009). Since receptor trafficking directly modulates the downstream signalling of a receptor, we decided to study Norbin's effect on ligand-mediated group I mGluR internalization in primary hippocampal neurons. We chose hippocampal neurons for our study because it is the primary centre for learning and memory in the brain and the widely distributed group I mGluRs regulate various forms of synaptic plasticity in the hippocampus (Balschun et al., 1999; Lüscher & Huber, 2010).

Our results suggested that both members of the group I mGluR family, viz., mGluR1 and mGluR5 internalized at the same rate in HEK293 cells as well as in primary hippocampal neurons. Internalization can be a mechanism to protect the receptor from overstimulation by the agonist and it can also serve to resensitize the receptors for another round of activation (Ferguson et al., 1998; Krueger et al., 1997; Pippig et al., 1995). In fact, we found that after internalizing, both mGluR1 and mGluR5 recycled back to the cell surface following similar kinetics in non-neuronal cells as well as in primary hippocampal neurons. To study the role of Norbin in the ligand-mediated group I mGluR endocytosis, we designed an shRNA that was able to effectively knockdown endogenous Norbin as observed by immunocytochemistry as well as western blotting. Knock down of endogenous Norbin caused a decrease in the cell surface expression of group I mGluRs in hippocampal neurons which is in concordance with the previous findings (Wang et al., 2009). This suggested that Norbin is important for the surface stability of group I mGluRs. Knockdown of Norbin also caused a block in the agonist-mediated endocytosis of both mGluR1 and mGluR5.

Importantly, this block in endocytosis was rescued upon transfection of a construct where full-length Norbin was expressed simultaneously with shNor, suggesting that the block in endocytosis that was observed was indeed due to the knockdown of endogenous Norbin and not due to some non-specific effect of shNor. Also, the block in endocytosis was consistent at all time points starting from 5 min up to 30 min suggesting that it was not a case of faster recycling of the receptor in 30 min. Another point to be noted is that the decrease in surface receptors that was seen due to the knockdown of endogenous Norbin was not due to the downregulation of the receptors because the total receptor levels in shNor transfected cells were same as control cells. This decrease in surface receptor levels did not affect the quantitation of our endocytosis index because we used an assay that allowed measurement of the proportion of surface receptors that were internalized after ligand application.

Within a clinical context, mGluR5 antagonists like 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and MTEP have been synthesized and have been shown to have a significant impact in reversing the phenotype caused due to increased activation of mGluR5 (Lea IV & Faden, 2006). For example, MPEP has been shown to rescue the exaggerated LTD seen in Fmr1 KO mice (Bear et al., 2004; Dölen et al., 2007). However, a lot of these clinical trials have begun without taking into consideration the effect of these antagonists on the trafficking of mGluRs, if any. Interestingly, Norbin knockdown inhibited the antagonist-induced internalization of mGluR5 suggesting that Norbin is crucial for mGluR5 endocytosis in the presence of MTEP. Taken together, our results suggested that Norbin is crucial for the surface stabilization as well as ligand-mediated endocytosis of group I mGluRs. It would be interesting to investigate if Norbin also regulates other members of the mGluR family like group II and group III mGluRs.

Chapter 4

Investigation of the role of various regions of Norbin in the agonist-mediated internalization of mGluR5

4.1. Introduction

In case of many GPCRs, trafficking and signalling of the receptor in the cell is regulated by a number of scaffold and adaptor proteins present at the post-synaptic membrane (Hall & Lefkowitz, 2002; Sorkin & von Zastrow, 2009). Most of these scaffold proteins organize into specialized structures of the synapse called the post-synaptic density (PSD). The post-synaptic density was identified as an electron-dense thickening of the post-synaptic membrane and it harbors more than thousand proteins including neurotransmitter receptors, scaffold proteins, cell adhesion molecules, signalling enzymes and cytoskeleton proteins (Kaizuka & Takumi, 2018; Sheng & Kim, 2011). The PSD of excitatory synapses which are majorly glutamatergic comprises of iGluRs like NMDARs and AMPARs as well as some adhesion molecules on the exterior side, underneath them lie a number of scaffolding proteins. Group I mGluRs, mGluR1 and mGluR5 are enriched in the perisynaptic domain that surrounds the PSD. Scaffolding proteins such as Homer, Shank and Tamalin link these receptors to the components of the PSD and regulate their signalling (Garner et al., 2000; Scheefhals & MacGillavry, 2018; Tao & Johns, 2006).

Norbin is one such protein that was found to localize at the PSD (Jordan et al., 2004; Wang et al., 2010; Westin et al., 2014). It was identified as one of the proteins that got upregulated upon LTP induction in rats (Shinozaki et al., 1997). It promoted neurite outgrowth in neuron-derived cell lines, hence the name neurite-outgrowth-related protein from rat brain or Norbin (Shinozaki et al., 1997, 1999). Norbin deletion is early embryonic lethal and Norbin ablation results in depressive-like behavior in mice and impaired adult hippocampal neurogenesis (Mochizuki-Sakisaka et al., 2004; Mochizuki et al., 1999, 2003; Wang et al., 2015). Immunocytochemical assays suggested that Norbin colocalized with mGluR5 as well as with a spine marker spinophilin (Westin et al., 2014). But unlike other cytosolic scaffolding proteins that have well known domains, Norbin till date has no known functional domains (Wang et al., 2009a, 2010). In the previous chapter, we reported that knockdown of endogenous Norbin led to a block in the ligand-mediated endocytosis of mGluR5 and reduced the cell surface expression of mGluR5. Replacement with the full-length Norbin construct was able to rescue these phenotypes. Thus, Norbin could potentially play an important role in regulating the spatio-temporal localization as well as the activity of group I mGluRs. In this chapter, we dissected out the function of various regions of Norbin in the agonist-mediated endocytosis of mGluR5 using the "molecular-replacement strategy". We made various

mutants of Norbin and cloned them in a multi-promoter vector which also contained the shNor. Thus, these constructs allowed us to simultaneously knockdown the endogenous Norbin and replace the endogenous Norbin with various mutant/deleted forms of Norbin. This approach has two major advantages: first, the developmental compensatory adaptations that might occur during synaptogenesis and synapse maturation due to the loss of the endogenous protein are minimized, and second, the function of heterologous constructs can be studied without the necessity of a dominant effect.

Our data suggested that both the N-terminal region and the C-terminal region of Norbin are equally necessary for the surface expression as well as agonist-mediated mGluR5 endocytosis because deletion of both of these regions led to a block in the agonist-mediated internalization of mGluR5. The N-terminal region has two distinct peptides that facilitate the interaction of Norbin with the RIIα subunit of PKA (Hermann et al., 2015). We found that presence of both PKA-binding regions in Norbin is crucial for the agonist-mediated mGluR5 endocytosis. We also found a critical alanine residue at the C-terminal region of Norbin which when mutated to glycine inhibited the interaction of Norbin with mGluR5, as well as blocked the agonist-induced mGluR5 endocytosis. Thus, with all the results taken together, we were able to identify some important regions in Norbin that can serve to mechanistically regulate the endocytosis and surface expression of mGluR5.

4.2. Results

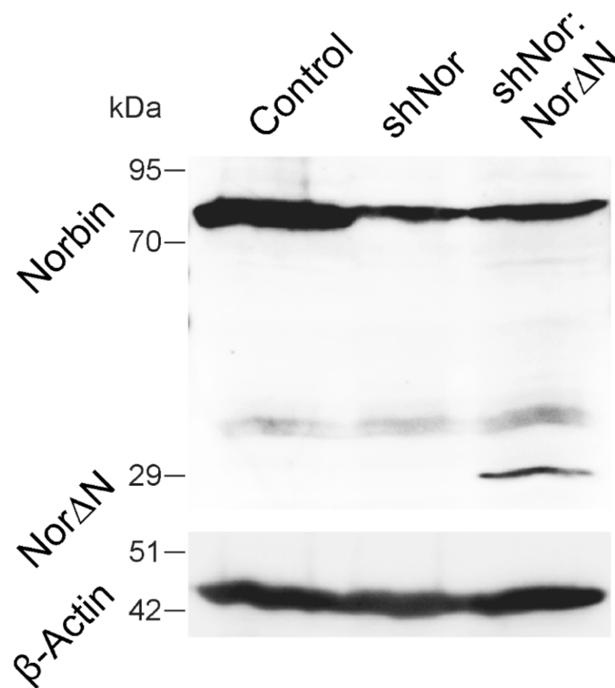
4.2.1. N-terminal region of Norbin is critical for the agonist-mediated internalization of mGluR5

Our earlier results suggested that Norbin plays a specific role in the agonist-mediated endocytosis of myc-mGluR5. Our next aim was to determine the role of various regions of Norbin in the surface expression as well as in the agonist-mediated internalization of the receptor. It has been reported that Norbin interacts with protein kinase A (PKA) through its N-terminal region and thus acts as an A-kinase anchoring protein (AKAP) (Hermann et al., 2015). Furthermore, PKA has been suggested to regulate mGluR5 function by directly phosphorylating the receptor (Uematsu et al., 2015). Therefore, we first deleted the N-terminal region of Norbin and made a Norbin replacement construct lacking the N-terminal region (shNor:NorΔN). The shNor:NorΔN replacement construct was generated by the method elaborated in the "methods" section. Subsequently, the expression level of this construct was analysed by western blotting. Control cells showed normal levels of endogenous Norbin and shNor effectively knocked down endogenous Norbin in primary neurons. Importantly, NorΔN showed expression, albeit at lower levels compared to the endogenous Norbin **(control: 1 ± 0.06; shNor: 0.25 ± 0.03; shNor:NorΔN: 0.13 ± 0.04) (Figure 4.1A, B)**.

We then wanted to investigate if NorΔN replacement construct could rescue the decrease in the surface expression of myc-mGluR5 caused by the knockdown of endogenous Norbin. Primary hippocampal neurons were co-transfected with myc-mGluR5 and shNor or shNor:NorΔN. We observed that knockdown of endogenous Norbin by shNor decreased the surface expression of myc-mGluR5 and replacement of the endogenous Norbin with NorΔN was not able to rescue the surface expression of the receptor **(control: 1 ± 0.03; shNor: 0.89 ± 0.04; shNor:NorΔN: 0.86 ± 0.04) (Figure 4.2A, B)**.

We then went on to investigate the effect of the N-terminal region deletion of Norbin on the agonist-mediated endocytosis of myc-mGluR5. The experiment was performed in primary hippocampal neurons in the similar way as described earlier for the full-length Norbin replacement construct. Control cells showed very little internal fluorescence and upon 100 μM R,S-DHPG application, receptors internalized in 30 min as observed by the increase in the internal fluorescence.

(A)



(B)

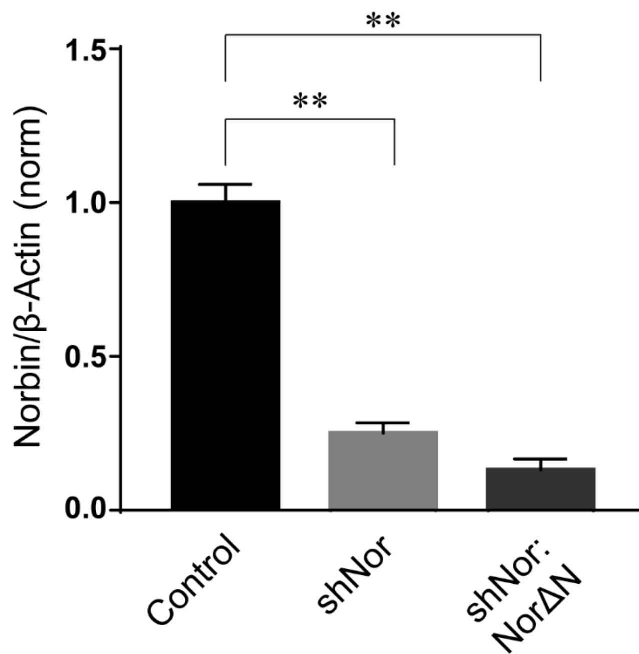
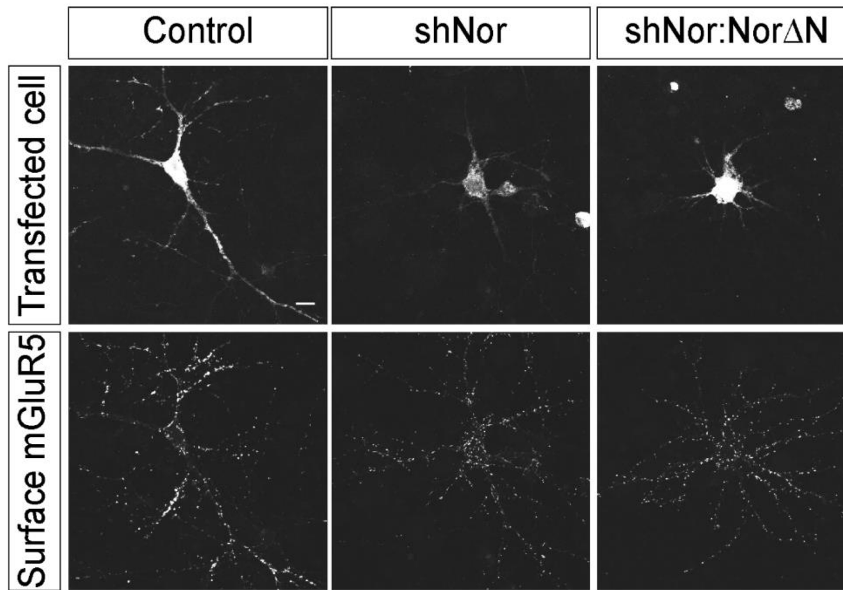


Figure 4.1. Knockdown of endogenous Norbin and replacement with NorΔN. (A) Western blot showing the effective knockdown of the endogenous Norbin by shNor and expression of the NorΔN replacement construct. (B) Quantitation of Norbin knockdown and NorΔN expression. **, $p < 0.01$.

(A)



(B)

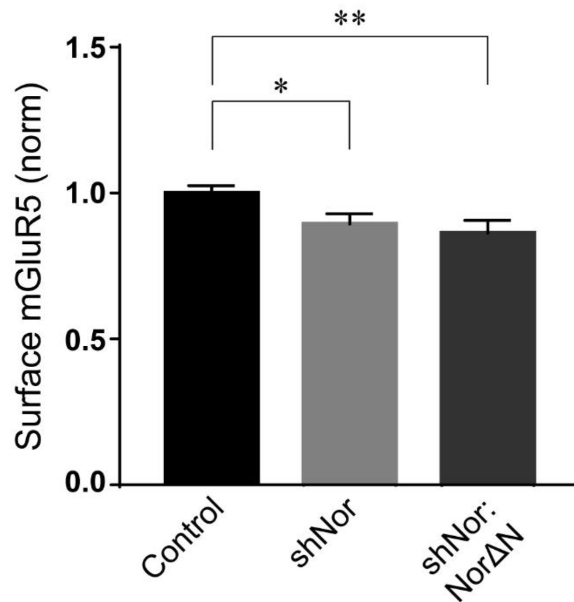


Figure 4.2. N-terminal region of Norbin is important for the surface stabilization of mGluR5. (A) Representative images showing that knockdown of endogenous Norbin with shNor reduced the surface expression of myc-mGluR5 and NorΔN replacement construct did not rescue the surface localization of myc-mGluR5. (B) Quantitation of the surface myc-mGluR5 also suggested that NorΔN replacement construct did not rescue the surface expression of myc-mGluR5. Scale bar = 10 μm. **, $p < 0.01$; *, $p < 0.05$.

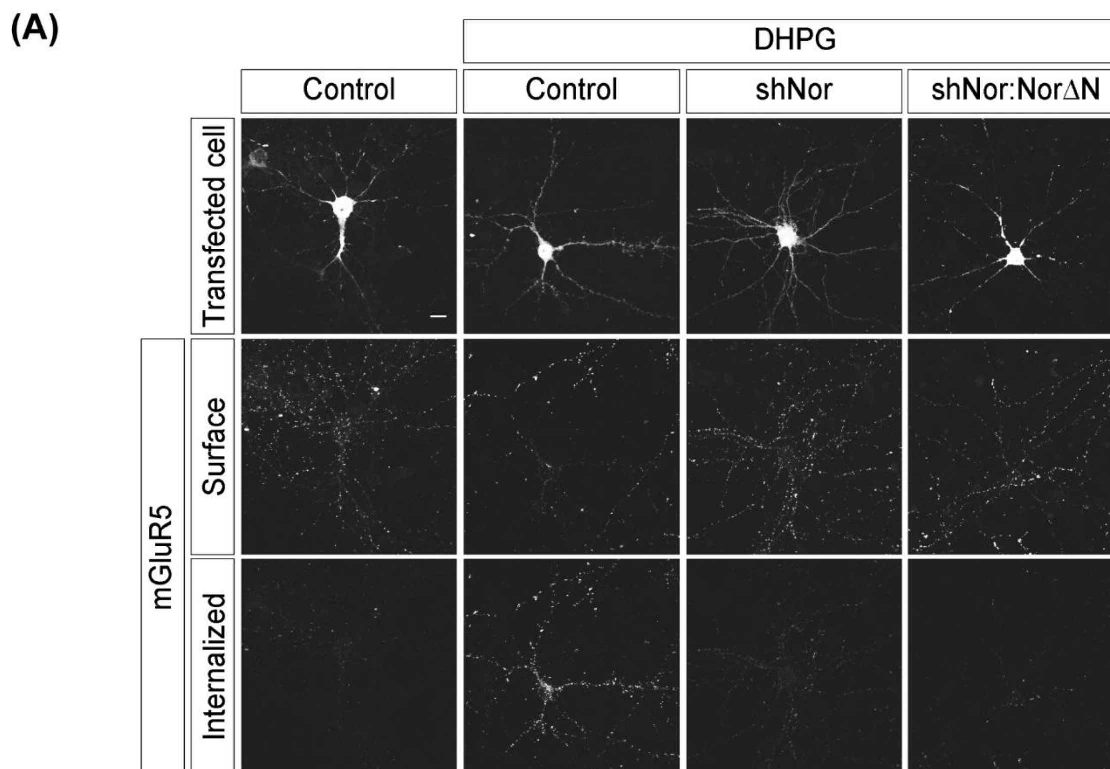
As expected, shNor transfected cells showed complete inhibition in the myc-mGluR5 endocytosis. But unlike full-length Norbin, the NorΔN replacement construct was unable to rescue the inhibition of endocytosis of the receptor as evidenced by the presence of most of the receptors on the cell surface **(control: 1 ± 0.04; control + DHPG: 1.89 ± 0.12; shNor + DHPG: 1.04 ± 0.03; shNor:NorΔN + DHPG: 0.98 ± 0.07) (Figure 4.3A, B)**.

All these results suggest that the NorΔN replacement construct could not rescue the surface expression and agonist-mediated endocytosis of myc-mGluR5 probably due to its insufficient expression in the neuron.

4.2.2. Pharmacological inhibition of PKA affects the agonist-mediated endocytosis of mGluR5

It has been reported that PKA modulates mGluR5 function by directly phosphorylating the receptor (Uematsu et al., 2015). Surface plasmon resonance experiments had identified that the N-terminal region of Norbin has two distinct peptides which are crucial for its interaction with the RIIα regulatory subunit of protein kinase A (PKA). Thus, Norbin acts as a novel A-kinase anchoring protein (AKAP) (Hermann et al., 2015). First, we wanted to study the role of PKA in mGluR5 trafficking, if any. We used a selective inhibitor of protein kinase A, KT5720 to pharmacologically inhibit PKA in primary hippocampal neurons. We initially wanted to see if PKA plays any role in the surface expression of mGluR5. Our data suggested that inhibition of PKA by 10 μM KT5720 decreased the cell surface levels of myc-mGluR5 **(control: 1 ± 0.06; KT5720: 0.70 ± 0.06) (Figure 4.4A, B)**.

We subsequently studied the role of PKA in the agonist-dependent internalization of myc-mGluR5. Primary hippocampal neurons were transfected with myc-mGluR5 cDNA using the calcium phosphate method. 3-4 days after transfection, cells were treated with 10 μM KT5720 for 30 min in plain neurobasal medium followed by staining of the cells with anti-myc primary antibody (1:200) for 20 min at 37˚C. Subsequently, internalization of the receptor was induced by the application of 100 μM R,S-DHPG for 5 min and cells were then chased in plain media for a total of 30 min. Application of 100 μM R,S-DHPG in control cells (KT5720 untreated cells) led to the robust internalization of the receptor. On the other hand, KT5720 treated cells did not show significant internalization of myc-mGluR5 upon 100 μM R,S-DHPG application and most of the



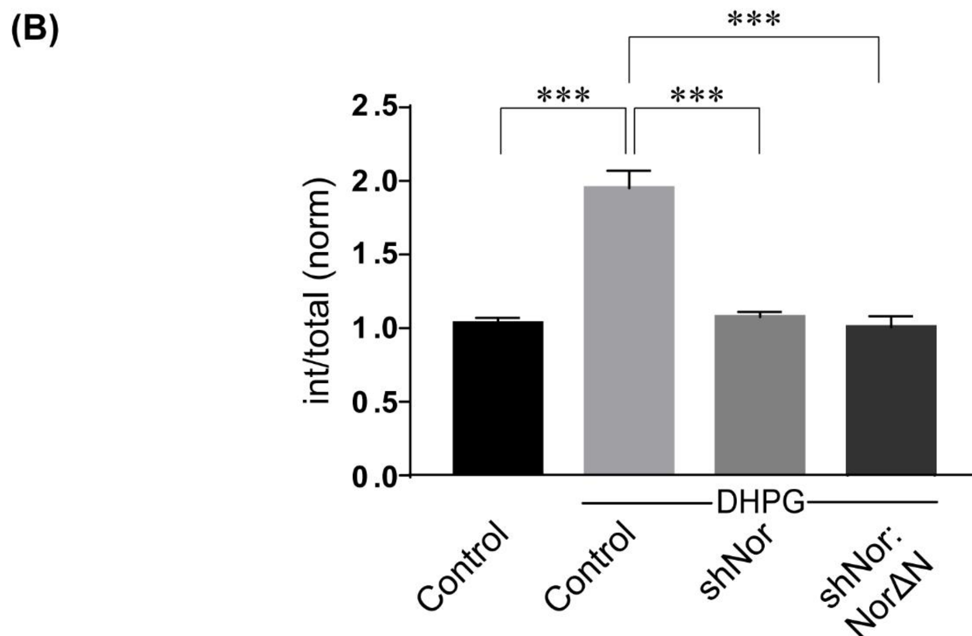
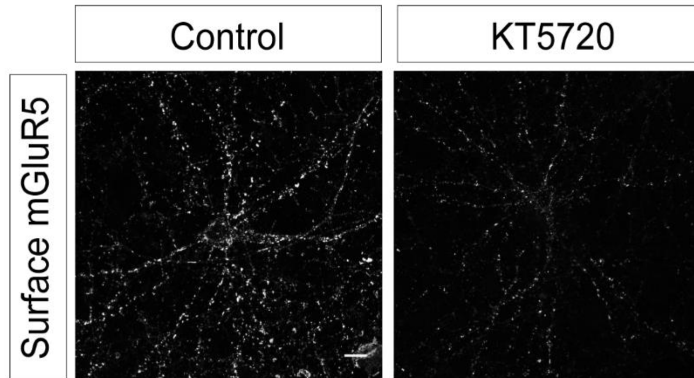


Figure 4.3. N-terminal domain of Norbin is required for the agonist-induced internalization of mGluR5. Representative images (A) and quantitation (B) of the R,S-DHPG-mediated endocytosis of myc-mGluR5 in shNor and shNor:NorΔN transfected cells. Control cells showed endocytosis upon 100 μM R,S-DHPG application, whereas shNor transfected cells showed a block in myc-mGluR5 endocytosis. Importantly, expression of the NorΔN replacement construct did not rescue the normal trafficking of the receptor. Scale bar = 10 μm. ***, $p < 0.001$.

(A)



(B)

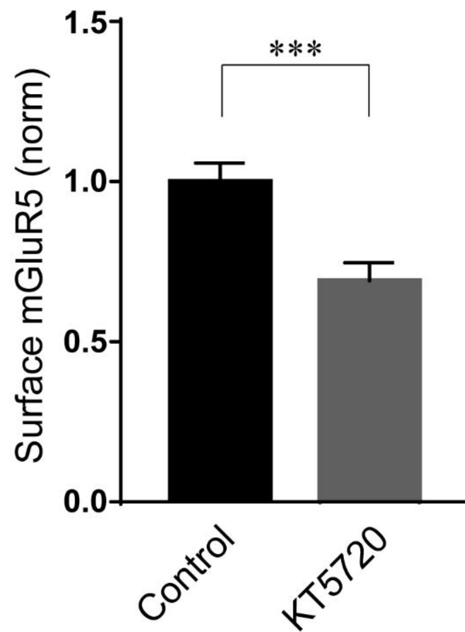


Figure 4.4. Pharmacological inhibition of PKA affects the surface expression of mGluR5. (A) Representative images showing the surface localized myc-mGluR5 in control cells and KT5720-treated cells (B) Quantitation of the surface myc-mGluR5 levels suggested that blocking PKA reduced the surface myc-mGluR5 levels. Scale bar = 10 µm. ***, $p < 0.001$.

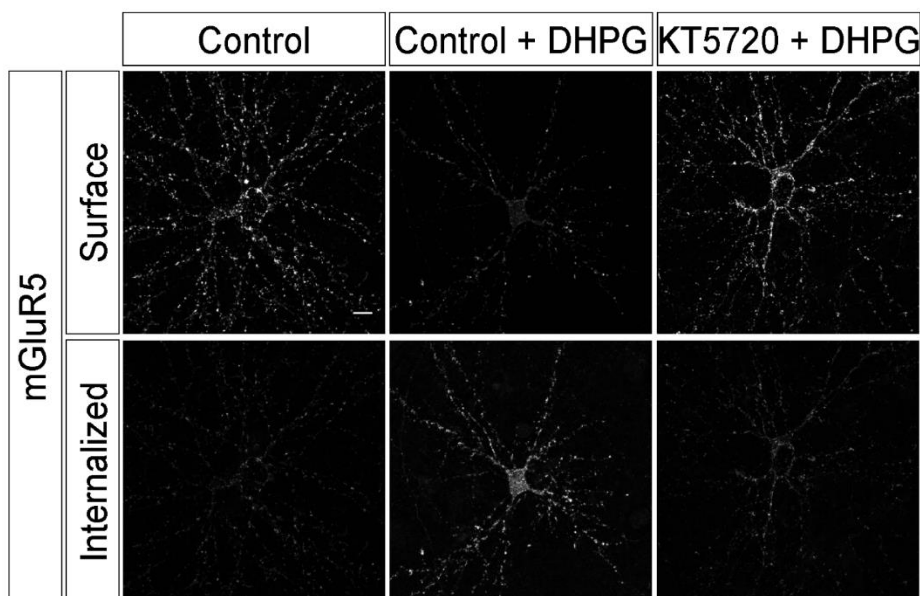
receptors were observed to be localized at the cell surface **(control: 1 ± 0.05; control + DHPG: 1.93 ± 0.15; KT5720 + DHPG: 0.86 ± 0.05) (Figure 4.5A, B)**. These results suggested that PKA plays an important role in the agonist-mediated internalization of mGluR5.

4.2.3. PKA binding at the N-terminal region of Norbin is required for the agonist-mediated internalization of mGluR5

As mentioned earlier, surface plasmon resonance experiments had identified that the N-terminal region of Norbin contains two distinct peptides, PEP1 (48-67 amino acids of Norbin) and PEP2 (255-274 amino acids of Norbin) which are involved in its interaction with the RIIα subunit of PKA (Hermann et al., 2015). Since our earlier experiments suggested that PKA plays an important role in the agonist-mediated internalization of mGluR5, we therefore investigated whether binding of PKA with the N-terminal region of Norbin is important for the agonist-mediated endocytosis of the receptor. For that, we made two replacement constructs of Norbin, viz., NorΔPEP1 and NorΔPEP2, each without one of the PKA binding peptides. We first decided to look at the role of NorΔPEP1 in mGluR5 trafficking. NorΔPEP1 replacement construct expressed properly in primary neurons as observed by western blots **(control: 1 ± 0.04; shNor: 0.37 ± 0.1; shNor:NorΔPEP1: 0.94 ± 0.07) (Figure 4.6A, B)**.

We then checked whether the NorΔPEP1 replacement construct could rescue the decrease in the surface expression of myc-mGluR5 due to the knockdown of endogenous Norbin. As expected, knockdown of the endogenous Norbin decreased the cell surface expression of myc-mGluR5, which was not rescued by the NorΔPEP1 replacement construct **(control: 1 ± 0.02; shNor: 0.82 ± 0.03; shNor:NorΔPEP1: 0.83 ± 0.04) (Fig 4.7A, B)**.

Subsequently, we investigated the effect of NorΔPEP1 replacement construct on the agonist-mediated endocytosis of myc-mGluR5. As expected, in control cells very less internalized receptors were observed which increased on 100 µM R,S-DHPG application at 30 min. Similar to our previous observations, shNor expressing cells showed a block in the myc-mGluR5 endocytosis **(control: 1 ± 0.03; control + DHPG: 1.64 ± 0.08; shNor + DHPG: 1.06 ± 0.04)**. Importantly, no recovery in myc-mGluR5 endocytosis was observed in shNor:NorΔPEP1 expressing cells post agonist application and most of the receptors remained on the cell surface **(shNor:NorΔPEP1 + DHPG: 0.99 ± 0.05) (Fig 4.8A, B)**. These results suggested that PKA binding to Norbin through



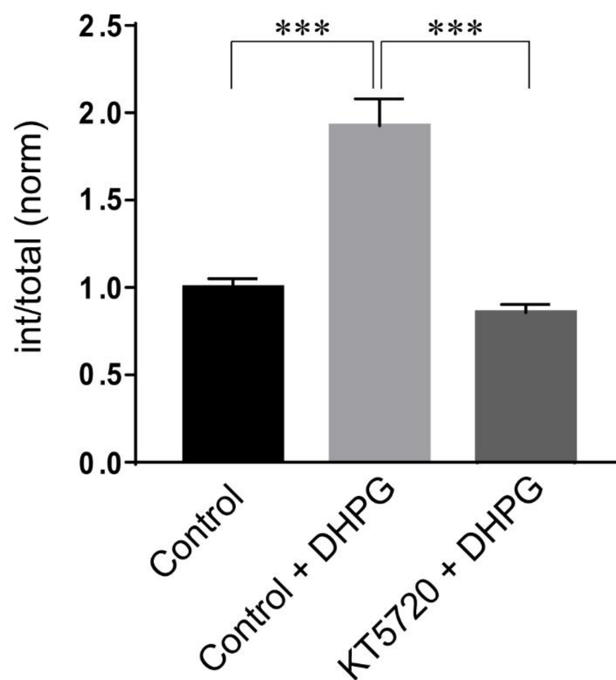
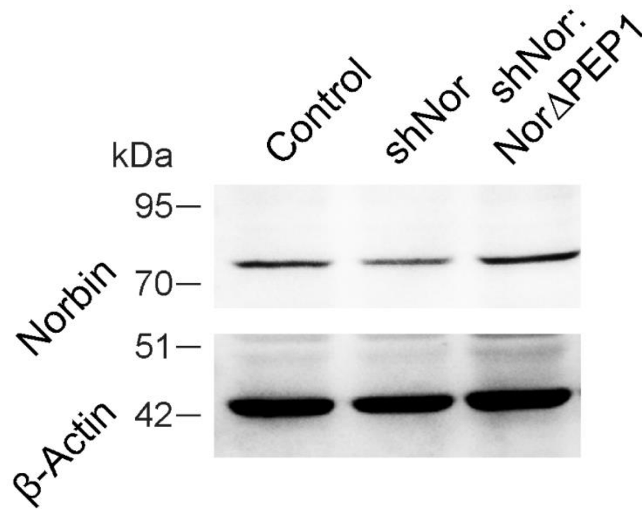


Figure 4.5. Pharmacological inhibition of PKA affects the agonist-mediated mGluR5 endocytosis. (A) Representative images showing the surface and internalized myc-mGluR5 in control cells and KT5720-treated cells (B) Quantitation of the endocytosis index showed that inhibition of PKA led to a block in the R,S-DHPG-mediated myc-mGluR5 endocytosis. Scale bar = 10 µm. ***, $p < 0.001$.



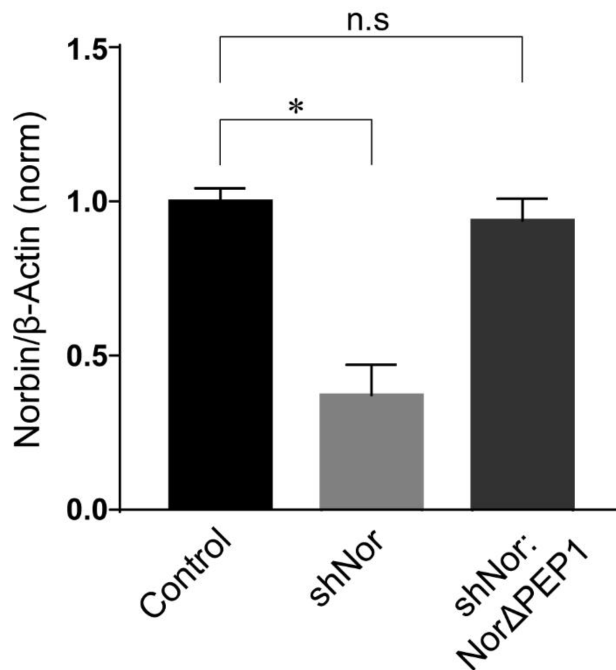
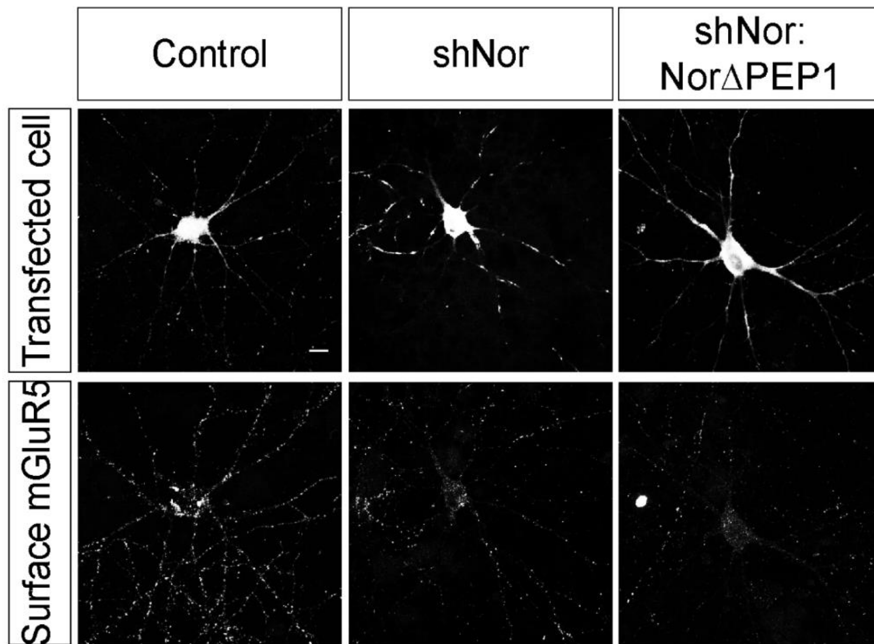


Figure 4.6. Knockdown of the endogenous Norbin and replacement with NorΔPEP1. Acute knockdown of the endogenous Norbin and replacement of the endogenous Norbin with NorΔPEP1 in primary neurons, as shown by the western blot (A) and quantitation of the western blots (B). *, $p < 0.05$; n.s., $p > 0.05$.

(A)



(B)

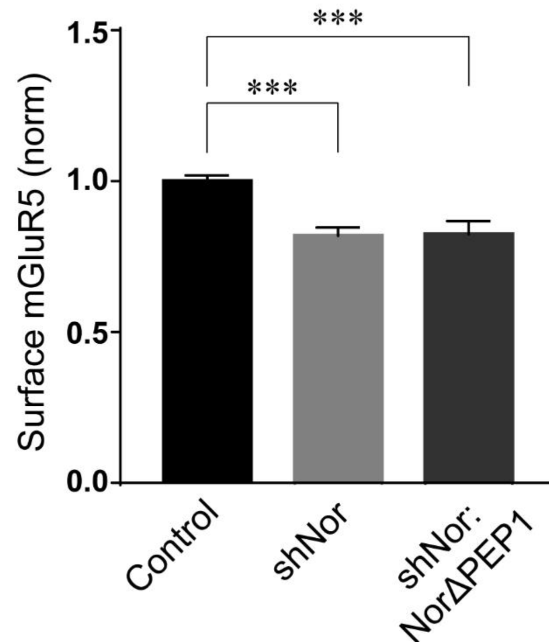


Figure 4.7. PEP1 region of Norbin (48-67 aa) is important for the surface expression of mGluR5. (A) Representative images showing that knockdown of endogenous Norbin with shNor reduced the surface expression of myc-mGluR5 and Nor∆PEP1 replacement construct did not rescue the surface expression of the receptor. (B) Quantitation of the surface myc-mGluR5 also suggested that Nor∆PEP1 replacement construct did not rescue the surface expression of myc-mGluR5. Scale bar = 10 µm. ***, $p < 0.001$.

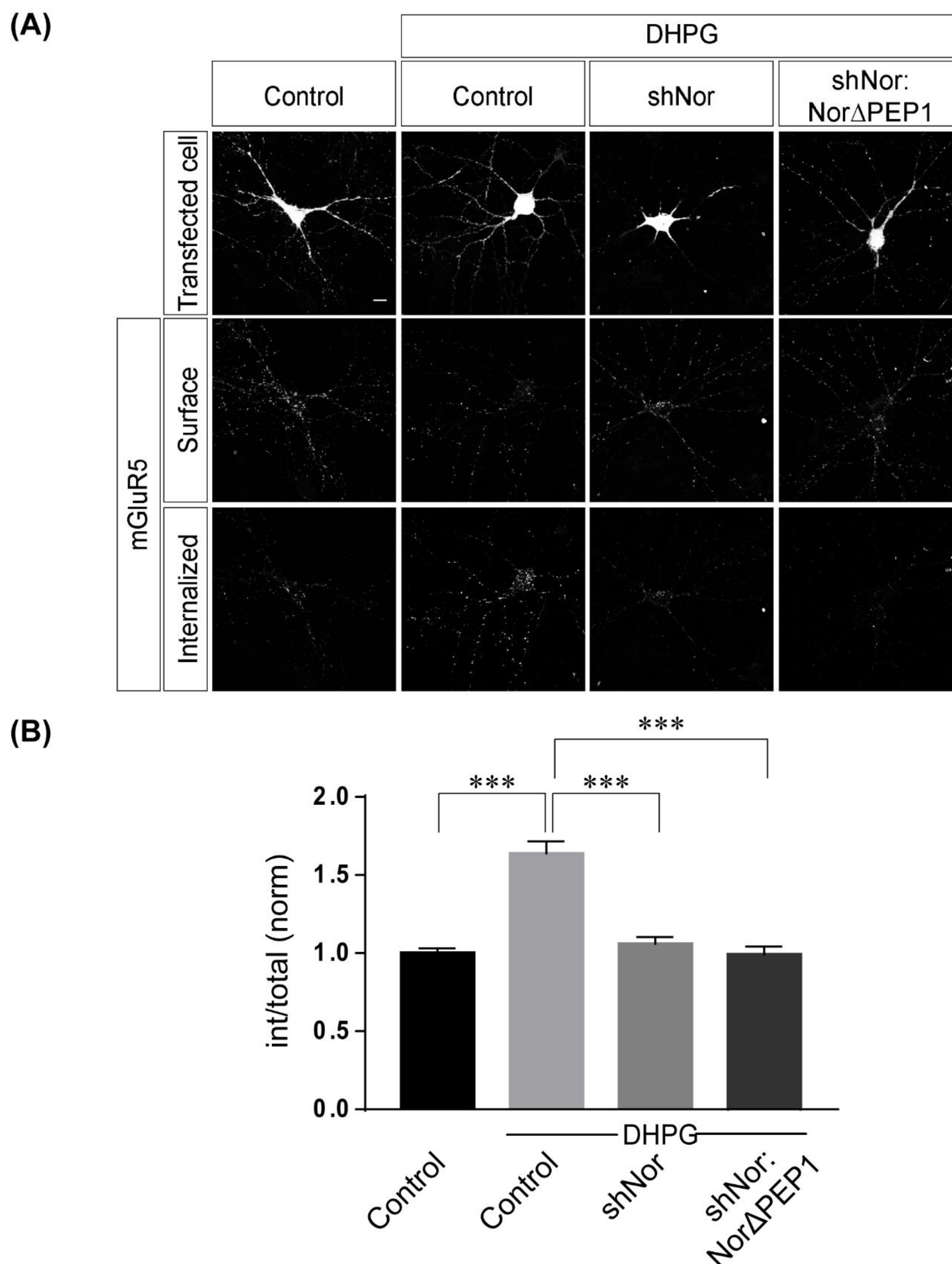


Figure 4.8. PEP1 region of Norbin is crucial for the agonist-mediated mGluR5 endocytosis. Representative images (A) and quantification (B) of the R,S-DHPG-mediated endocytosis of surface myc-mGluR5 in cells expressing GFP, shNor or shNor and NorΔPEP1 showing that NorΔPEP1 could not rescue the R,S-DHPG-mediated internalization of myc-mGluR5. Scale bar = 10 µm. ***, $p < 0.001$.

peptide 1 region is important for it to regulate the surface stabilization as well as agonist-mediated endocytosis of mGluR5.

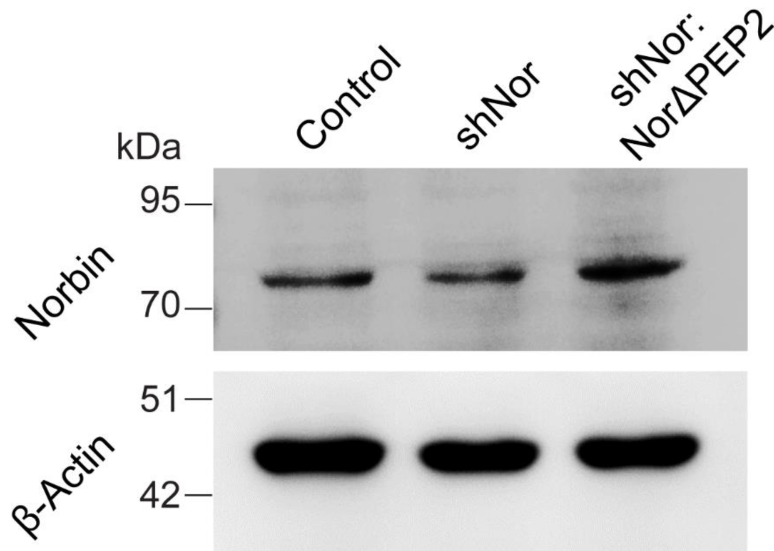
Our next aim was to determine whether the other distinct peptide at the N-terminal region of Norbin, viz., PEP2 also plays crucial role in the agonist-dependent internalization of mGluR5. For that, NorΔPEP2 replacement construct was generated as described before. First, we checked if the construct expressed properly in primary neurons by western blotting. As evident from the blot, NorΔPEP2 expressed properly and the levels were comparable to those of control cells **(control: 1 ± 0.06; shNor: 0.49 ± 0.12; shNor:NorΔPEP2: 1.21 ± 0.14) (Fig 4.9A, B)**.

We then studied whether the NorΔPEP2 replacement construct could rescue the decrease in the surface receptor expression due to the knockdown of endogenous Norbin. As observed earlier, shNor transfected cells showed a decrease in cell surface myc-mGluR5 levels and similar to NorΔPEP1, NorΔPEP2 was also unable to restore the surface myc-mGluR5 expression levels **(control: 1.0 ± 0.02; shNor: 0.87 ± 0.03; shNor:NorΔPEP2: 0.83 ± 0.04) (Fig 4.10A, B)**.

The effect of NorΔPEP2 replacement construct on agonist-mediated mGluR5 endocytosis was subsequently investigated. Endocytosis assay was performed following the method described before. A pulse of 100 µM R,S-DHPG induced internalization of majority of myc-mGluR5 in control cells while, in case of shNor transfected cells, this internalization of the receptor was inhibited and most of the receptors remained at the cell surface **(control: 1 ± 0.04; control + DHPG: 1.62 ± 0.09; shNor + DHPG: 0.96 ± 0.04) (Fig 4.11A, B)**. Interestingly, replacement of the endogenous Norbin with NorΔPEP2 was able to partially rescue the endocytosis of myc-mGluR5 **(shNor:NorΔPEP2 + DHPG: 1.2 ± 0.07) (Fig 4.11A, B)**. These results suggested that PKA binding to Norbin through peptide 2 is also required for the agonist-mediated endocytosis of mGluR5.

It was also important to confirm the effect of deletion of the PEP1 and PEP2 regions of Norbin on the interaction of Norbin with PKA in primary hippocampal neurons. We performed co-immunoprecipitation experiments wherein we pulled down the wild-type Norbin or its mutants, NorΔPEP1 and NorΔPEP2 through anti-HA primary antibody and immunoblotted for PKA. As expected, full-length Norbin interacted with PKA but in case of NorΔPEP1 and NorΔPEP2, the interaction was disrupted **(Norbin + PKA: 1 ± 0.04; NorΔPEP1 + PKA: 0.19 ± 0.05;**

(A)



(B)

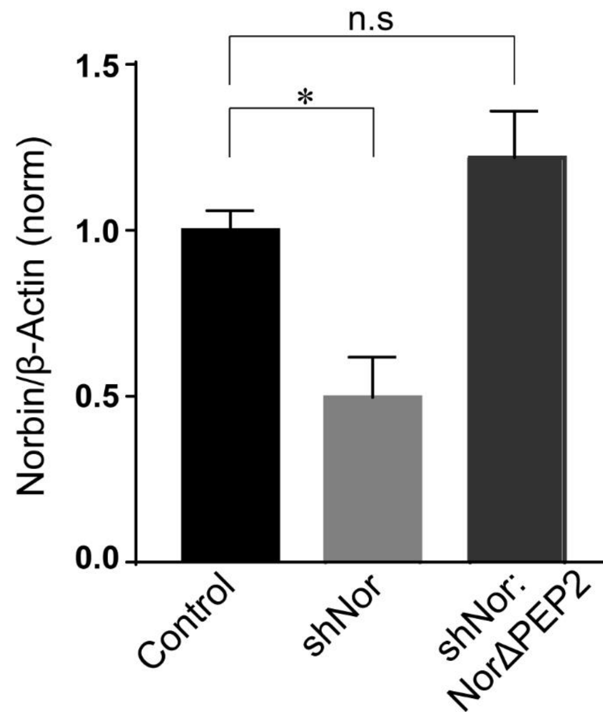
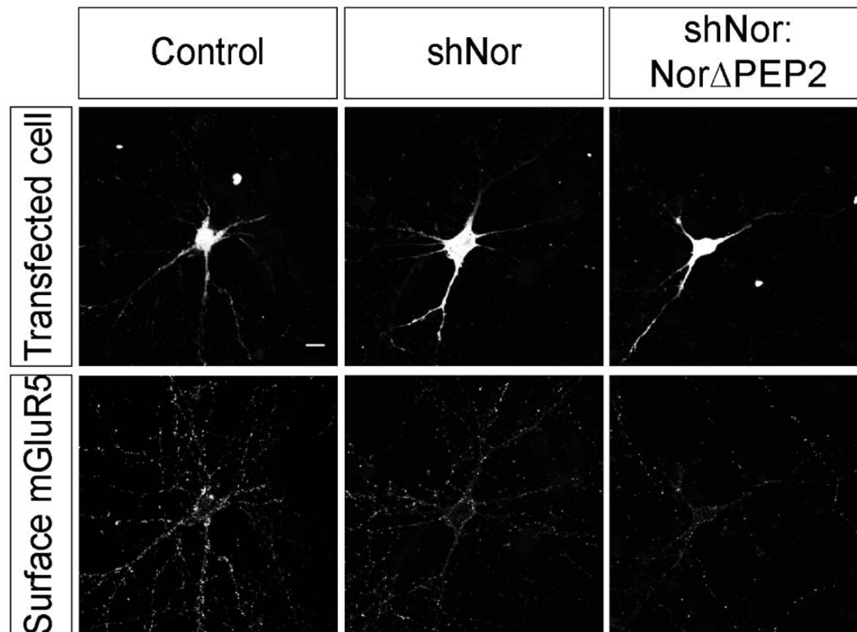


Figure 4.9. Knockdown of the endogenous Norbin and replacement with NorΔPEP2. Western blot (A) and quantitation of the western blots (B), showing the knockdown of the endogenous Norbin and expression of the NorΔPEP2 replacement construct. *, $p < 0.05$; n.s., $p > 0.05$.

(A)



(B)

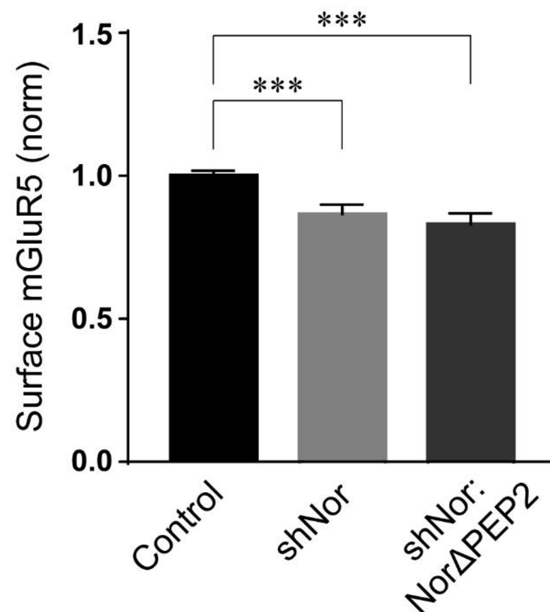


Figure 4.10. PEP2 region of Norbin (255-274 aa) is important for the surface expression of mGluR5. (A) Representative images showing that knockdown of endogenous Norbin with shNor and replacement with the NorΔPEP2 replacement construct reduced the surface expression of myc-mGluR5. (B) Quantitation of the surface myc-mGluR5 also suggested that NorΔPEP2 replacement construct did not rescue the surface expression of myc-mGluR5. Scale bar = 10 μm. ***, $p < 0.001$.

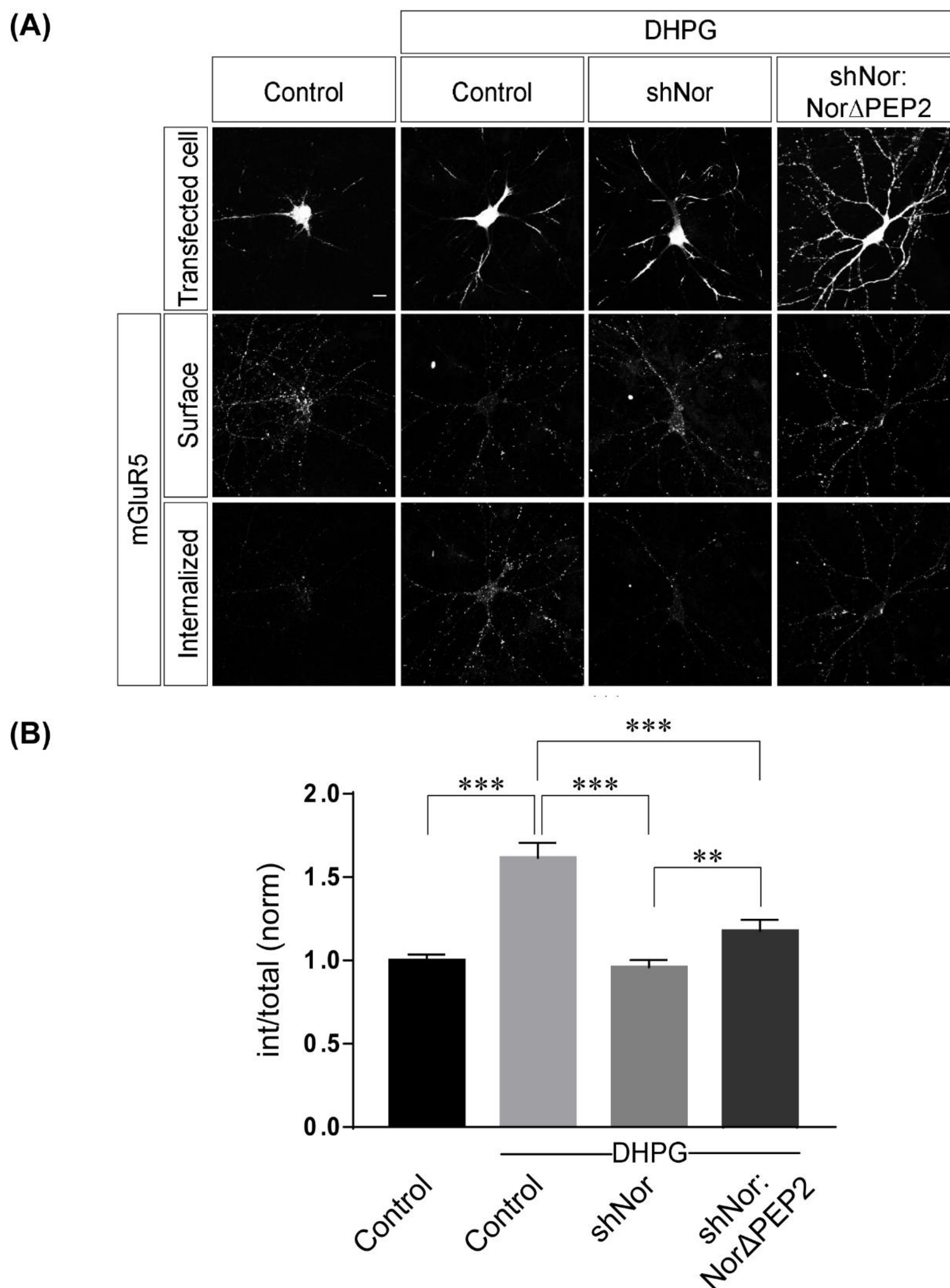


Figure 4.11. PEP2 region of Norbin is crucial for the agonist-mediated mGluR5 endocytosis. (A) Representative images of the R,S-DHPG-mediated myc-mGluR5 endocytosis in cells expressing GFP, shNor or shNor and NorΔPEP2. (B) Quantitation of the endocytosis index suggested that compared to shNor that caused a complete block in myc-mGluR5 endocytosis, the NorΔPEP2 replacement construct could partially rescue the block in myc-mGluR5 endocytosis. Scale bar = 10 μm. ***, $p < 0.001$, **, $p < 0.01$.

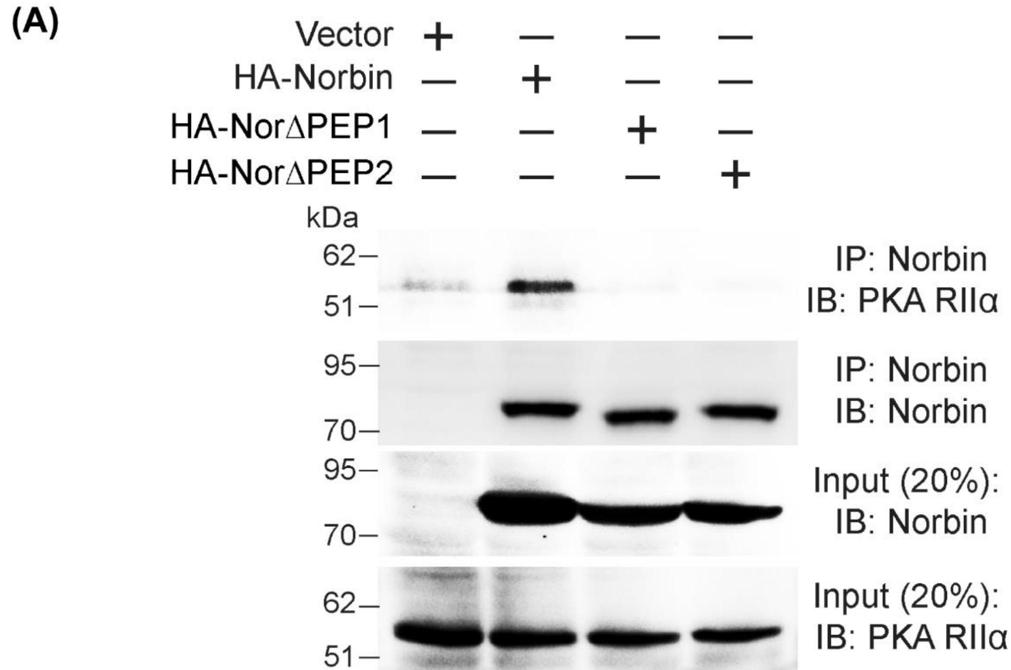
NorΔPEP2 + PKA: 0.15 ± 0.05) (Figure 4.12A, B). Together all these results suggested that PKA binding at the N-terminal region of Norbin is critical for the agonist-mediated internalization of mGluR5.

4.2.4. C-terminal region of Norbin plays important role in the agonist-dependent endocytosis of mGluR5

The C-terminal region of Norbin has been reported to interact with many proteins at the PSD, including mGluR1 and mGluR5 (Wang et al., 2009a). These reports prompted us to study the role of the C-terminal region of Norbin, if any, in the agonist-mediated endocytosis of mGluR5. We made a Norbin replacement construct lacking the C-terminal region (NorΔC) and initially checked its expression in primary neurons by western blotting. We observed that NorΔC expressed properly and it's expression levels were similar to the endogenous Norbin levels **(control: 1 ± 0.05; shNor: 0.43 ± 0.01; shNor:NorΔC: 1.02 ± 0.06) (Fig 4.13A, B)**.

We then checked if NorΔC replacement construct could rescue the decrease in surface myc-mGluR5 expression due to the knockdown of endogenous Norbin. Primary hippocampal neurons were co-transfected with myc-mGluR5 and shNor or shNor:NorΔC. Subsequently, the myc-mGluR5 present at the cell surface was measured using the protocol described in the "methods" section. Our data suggested that similar to NorΔN, NorΔC also could not rescue the cell surface expression of myc-mGluR5 **(control: 1 ± 0.03; shNor: 0.85 ± 0.04; shNor:NorΔC: 0.73 ± 0.03) (Fig 4.14A, B)**.

Subsequently, we studied the role of C-terminal region of Norbin in the agonist-mediated endocytosis of mGluR5. In control cells, minimal amount of internalized receptors were observed which increased upon 100 μM R,S-DHPG application at 30 min **(control: 1 ± 0.04; control + DHPG: 1.62 ± 0.08)**. In shNor transfected cells, this internalization was inhibited **(shNor + DHPG: 1.07 ± 0.03)**. When endogenous Norbin was replaced with NorΔC, the endocytosis of the receptor was not rescued, suggesting that C-terminus of Norbin is important for the internalization of myc-mGluR5 **(shNor:NorΔC + DHPG: 1.04 ± 0.05) (Figure 4.15A, B)**.



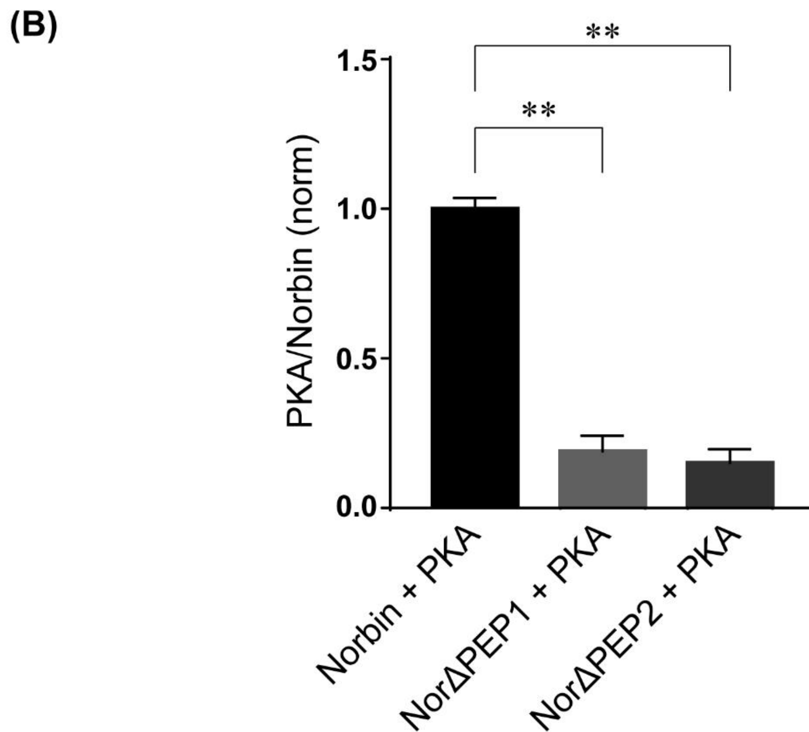
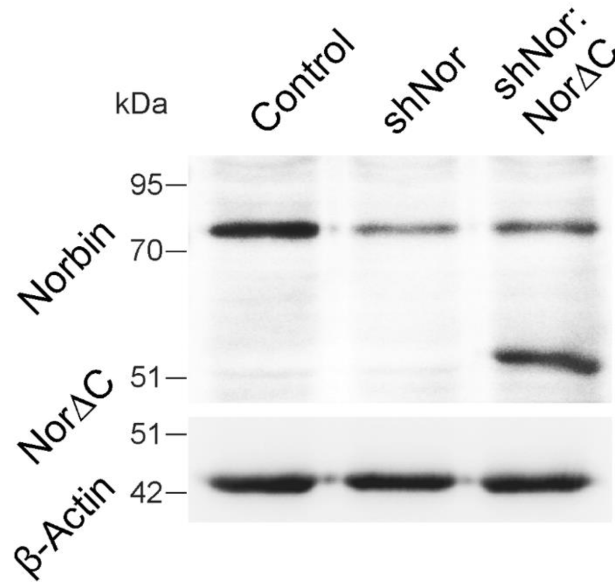


Figure 4.12. Both PEP1 and PEP2 regions of Norbin are crucial for its interaction with PKA. Co-immunoprecipitation assay (A) and quantitation (B) showing that deletion of both PEP1 and PEP2 regions of Norbin disrupted the binding of PKA to Norbin. **, $p < 0.01$.

(A)



(B)

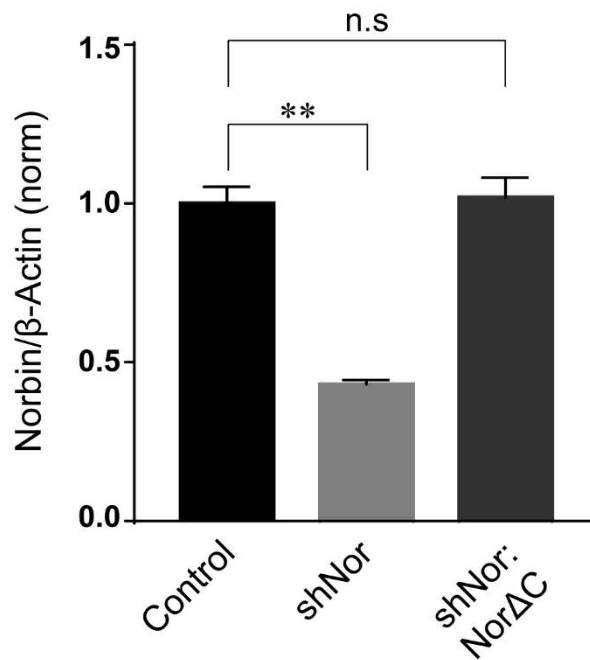
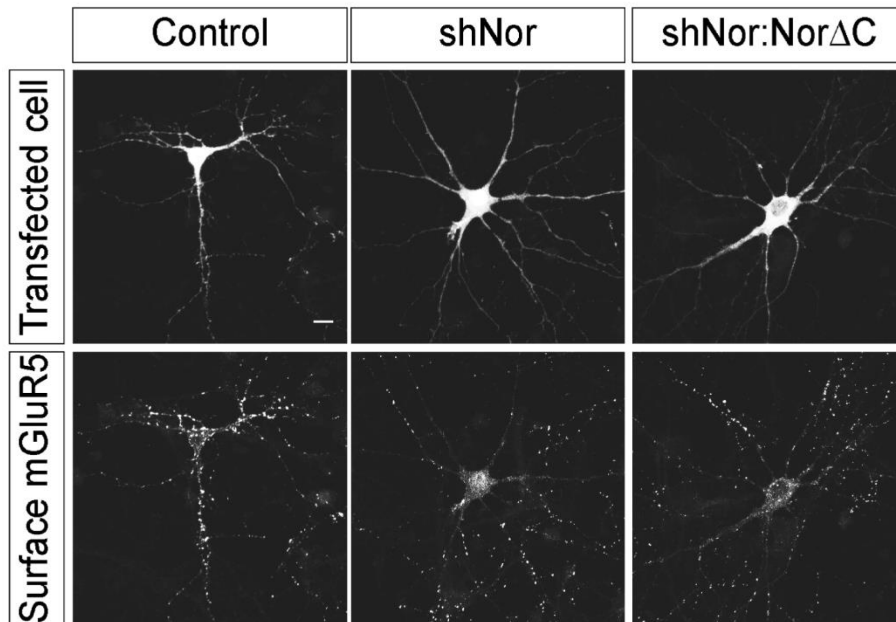


Figure 4.13. Knockdown of the endogenous Norbin and replacement with NorΔC. Western blot (A) and quantitation of the western blots (B), showing the knockdown of the endogenous Norbin and expression of the NorΔC replacement construct. **, $p < 0.01$; n.s., $p > 0.05$.

(A)



(B)

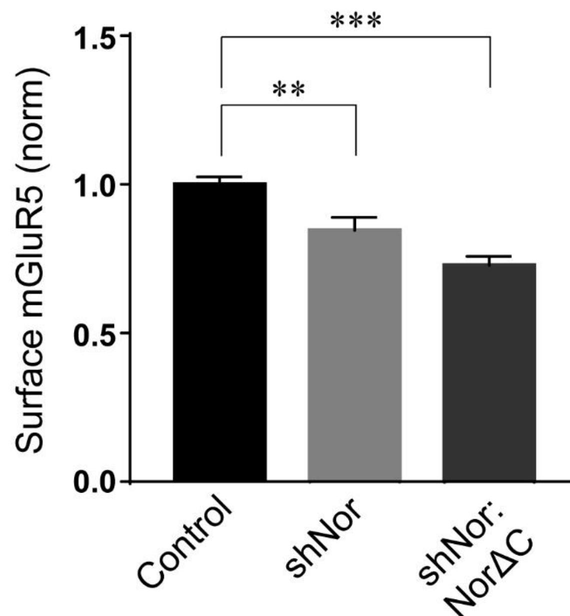
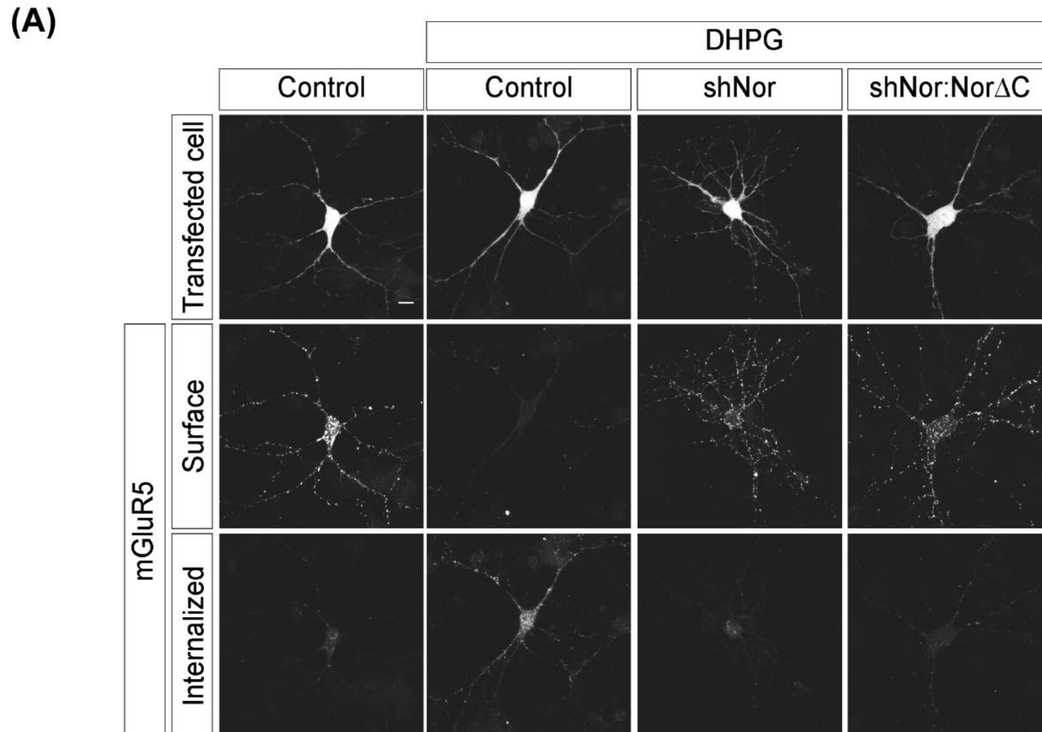


Figure 4.14. C-terminal region of Norbin is important for the surface expression of mGluR5. (A) Representative images showing that knockdown of endogenous Norbin with shNor reduced the surface expression of myc-mGluR5 and NorΔC replacement construct could not rescue the surface localization of the receptor. (B) Quantitation of the surface myc-mGluR5 also suggested that NorΔC replacement construct could not rescue the surface expression of myc-mGluR5. Scale bar = 10 µm. ***, $p < 0.001$; **, $p < 0.01$.

(A)



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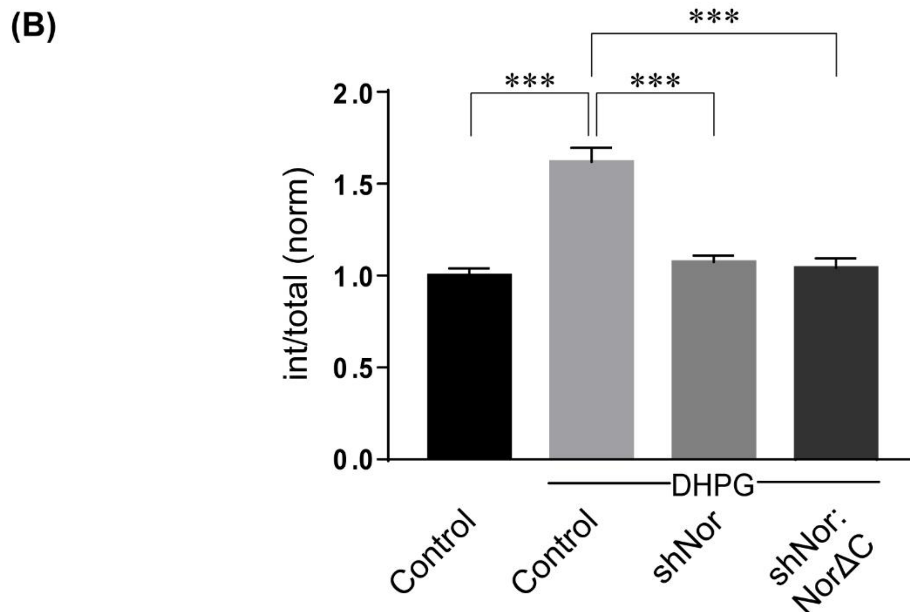

Figure 4.15. C-terminal region of Norbin is required for the agonist-mediated endocytosis of mGluR5. Representative images (A) and quantitation (B) of the R,S-DHPG-mediated myc-mGluR5 endocytosis in GFP, shNor and shNor:NorΔC transfected cells. Control cells showed endocytosis of the receptor upon 100 μM R,S-DHPG application, whereas shNor transfected cells showed a block in myc-mGluR5 endocytosis. Importantly, expression of the NorΔC replacement construct did not rescue the R,S-DHPG-mediated internalization of myc-mGluR5. Scale bar = 10 μm. ***, $p < 0.001$.

Taken together, our results suggested that both the C-terminal as well as N-terminal regions of Norbin are necessary to regulate the surface expression and agonist-mediated internalization of mGluR5 in primary hippocampal neurons.

4.2.5. A point mutation at the C-terminal region of Norbin (NorA687G) inhibits agonist-mediated mGluR5 endocytosis

As mentioned earlier, the C-terminus of Norbin binds many proteins present at the PSD like MCHR1, mGluR1 and mGluR5 (Francke et al., 2006; Wang et al., 2009a). We made a Norbin replacement construct wherein under the ubiquitin promoter we cloned an HA-tagged Norbin mutant which contained a single residue mutation at the 687th position from alanine to glycine. We called this the NorA687G replacement construct. This replacement construct showed expression similar to the endogenous Norbin in western blot **(control: 1 ± 0.07; shNor: 0.43 ± 0.04; shNor:NorA687G: 0.96 ± 0.13) (Figure 4.16A, B)**. We first checked if it had any effect on the interaction of Norbin with mGluR5 by performing co-immunoprecipitation experiments. We pulled down FLAG-mGluR5 and blotted for HA. Interestingly, wild-type Norbin showed prominent interaction with mGluR5, but in case of the point mutant, this interaction was hampered **(mGluR5 + Norbin: 1 ± 0.08; mGluR5 + NorA687G: 0.30 ± 0.06) (Figure 4.17A, B)**. These results suggested that the A687 residue of Norbin is a critical residue for its interaction with mGluR5.

When we expressed this mutant simultaneously with shNor in primary hippocampal neurons, it was unable to rescue the decrease in surface myc-mGluR5 expression due to the knockdown of endogenous Norbin **(control: 1.0 ± 0.04; shNor: 0.85 ± 0.03; shNor:NorA687G: 0.75 ± 0.05) (Figure 4.18A, B)**.

We subsequently investigated the effect of this point mutation in Norbin on the agonist-mediated endocytosis of myc-mGluR5. As expected, knockdown of the endogenous Norbin by shNor inhibited the 100 μM R,S-DHPG-mediated internalization of myc-mGluR5. Importantly, when we replaced endogenous Norbin with NorA687G construct, it was not able to rescue the knockdown phenotype **(control: 1 ± 0.05; control + DHPG: 2.07 ± 0.19; shNor + DHPG: 0.91 ± 0.03; shNor:NorA687G + DHPG: 1.09 ± 0.11) (Figure 4.19A, B)**. These results suggested that the alanine residue at the 687th position in the C-terminus of Norbin plays a critical role in the

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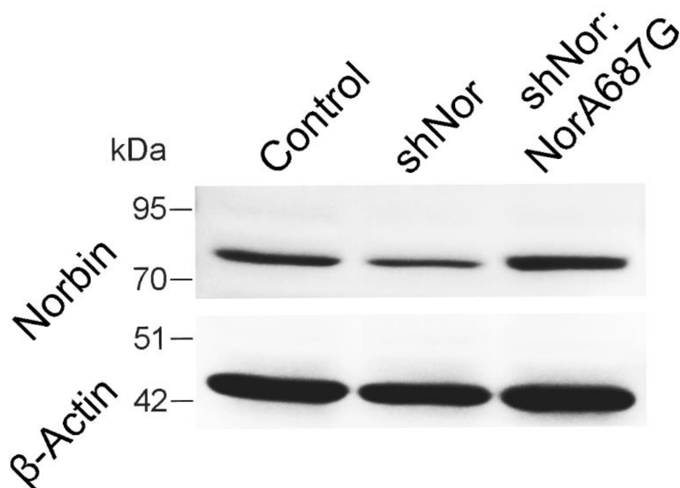

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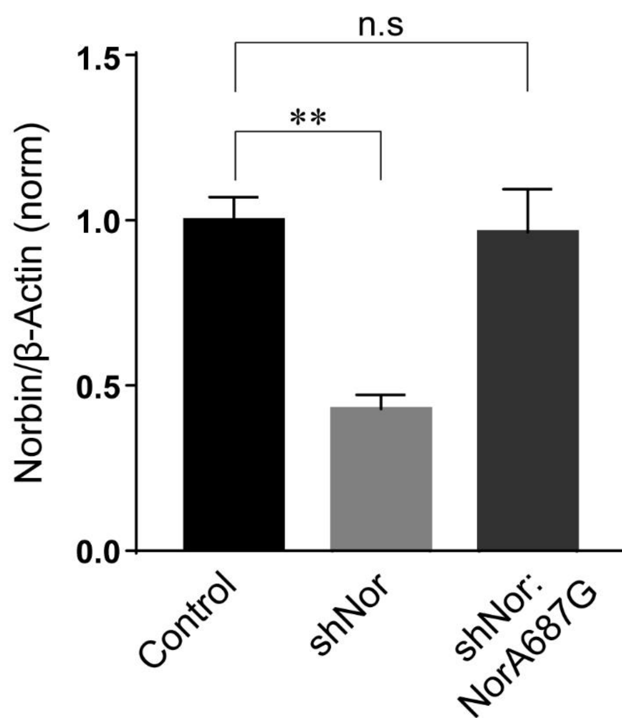
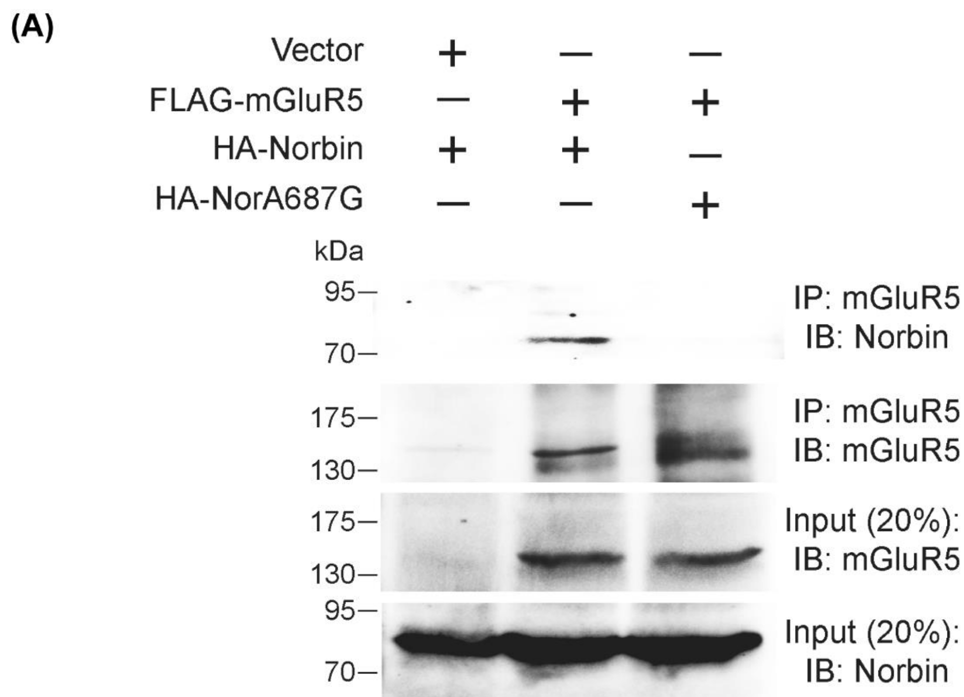


Figure 4.16. Expression of the NorA687G replacement construct. (A) Western blot showing the knockdown of endogenous Norbin with shNor and expression of the NorA687G replacement construct. (B) Quantitation of the western blot. **, $p < 0.01$; n.s., $p > 0.05$.



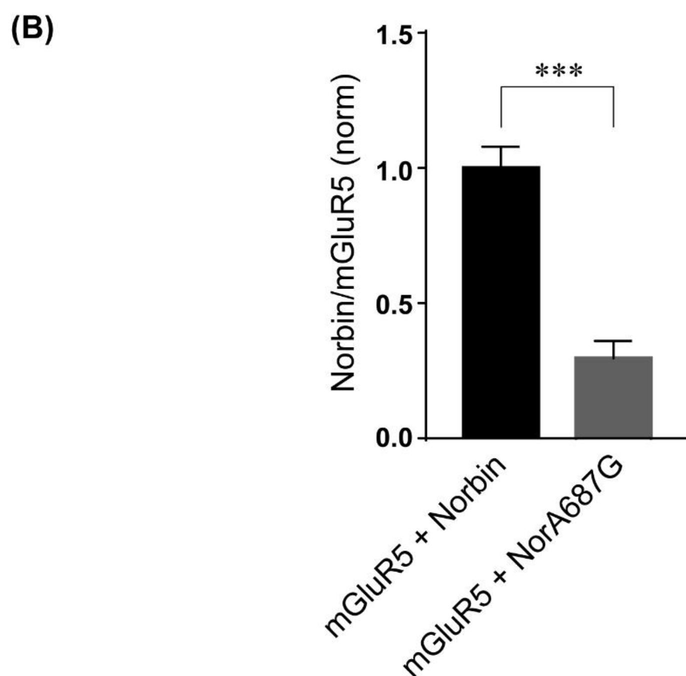
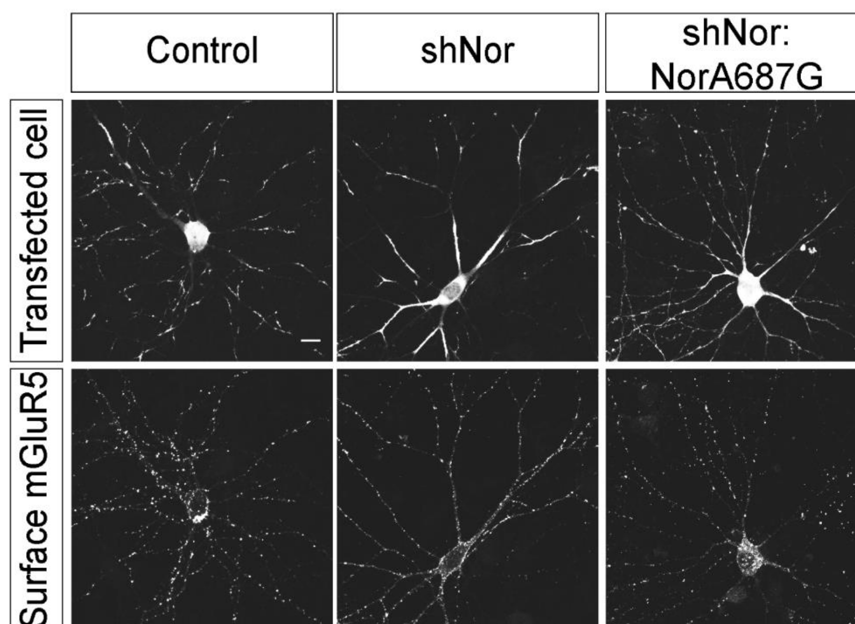


Figure 4.17. Alanine residue at the 687th position of Norbin is critical for its interaction with mGluR5. (A) Co-immunoprecipitation assay demonstrating that full-length Norbin interacted with mGluR5 and a point mutation from alanine 687 to glycine in full-length Norbin disrupted the binding of Norbin to mGluR5. (B) Quantitation of the co-immunoprecipitation assay. ***, $p < 0.001$.

(A)



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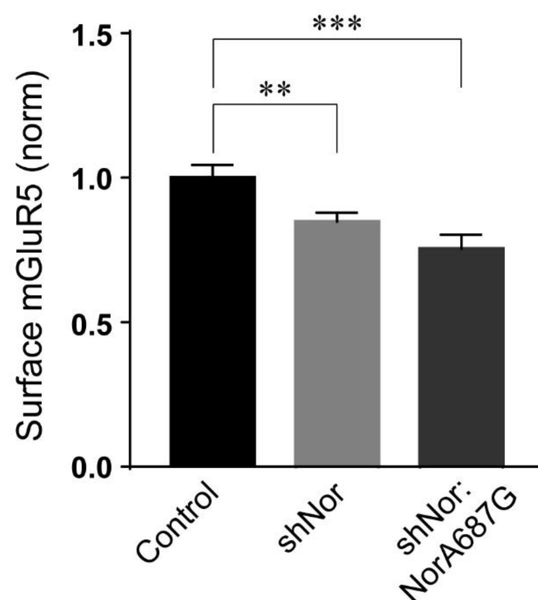


Figure 4.18. Interaction of Norbin with mGluR5 through its A687 residue is important for the surface expression of the receptor. (A) Representative images showing that knockdown of endogenous Norbin with shNor reduced the surface expression of myc-mGluR5 and NorA687G replacement construct could not rescue the surface expression of the receptor. (B) Quantitation of the surface myc-mGluR5 expression also suggested that NorA687G replacement construct did not rescue the surface expression of myc-mGluR5. Scale bar = 10 µm. ***, $p < 0.001$; **, $p < 0.01$.

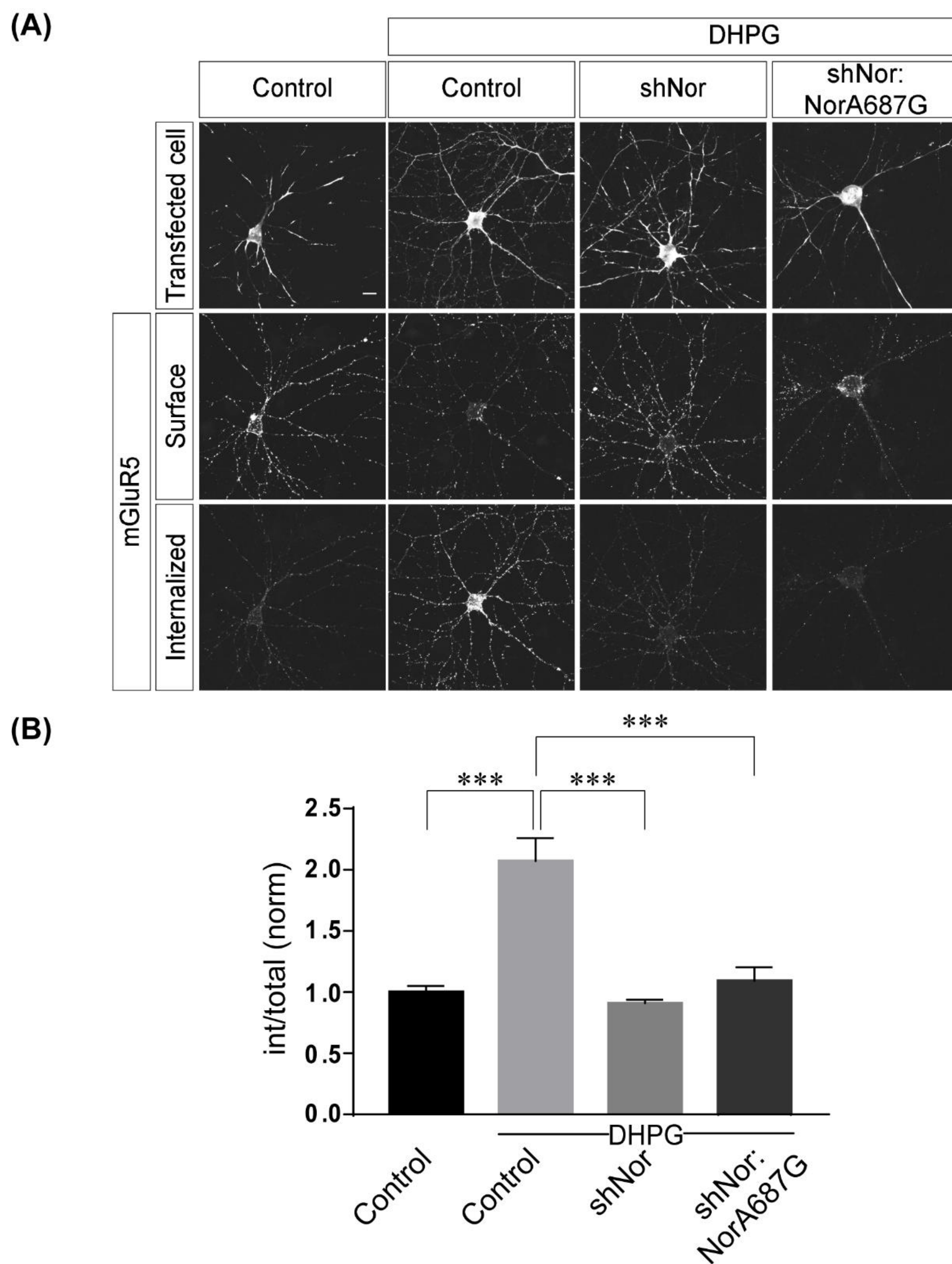


Figure 4.19. Interaction of Norbin with mGluR5 through its C-terminus is important for the agonist-mediated internalization of mGluR5. (A) Representative images showing that knockdown of endogenous Norbin with shNor inhibited the R,S-DHPG-mediated endocytosis of myc-mGluR5 and NorA687G replacement construct was unable to rescue the block in myc-mGluR5 endocytosis. (B) Quantitation of the endocytosis index also suggested that NorA687G replacement construct could not rescue the R,S-DHPG-mediated endocytosis of myc-mGluR5. Scale bar = 10 μm. ***, $p < 0.001$.

interaction of Norbin with mGluR5 and this interaction is required to maintain the surface mGluR5 expression and to carry out normal agonist-mediated internalization of mGluR5.

4.2.6. Targeting and synaptic localization of various mutants of Norbin in primary hippocampal neurons

In order to ensure that all the mutants of Norbin that we made targeted properly in primary hippocampal neurons, we checked the expression profile of each one of them by immunostaining. Since all of these mutant versions of Norbin were tagged with HA at the N-terminus, they expressed as recombinant proteins fused with HA upon transfection. The constructs were transfected in primary hippocampal neurons at 6-7 DIV by calcium phosphate method. When cells were at 12-15 DIV, they were fixed with 4% PFA and were subsequently permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Cells were then stained overnight with anti-HA rat polyclonal antibody (1:500) and anti-GFP rabbit polyclonal antibody (1:500), since GFP was the reporter present in the vector backbone. This was followed by the application of the respective secondary antibodies. Our data suggested that the expression of all the mutant proteins was similar to the expression of the wild-type Norbin except HA-NorΔN. NorΔN construct did not target properly and was predominantly localized at the cell body of the neuron **(Figure 4.20)**.

In order to investigate whether these various mutants of Norbin were localized at the synapse, the proportion of synapses containing detectable amount of these variants of Norbin 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 pre-synaptic terminals (Dieck et al., 1998). Our data suggested that the NorΔPEP1, NorΔPEP2, NorΔC and NorA687G proteins localized at the synapse very similar to the wild-type Norbin protein. On the other hand, the NorΔN protein which did not target properly at the dendritic region of the neuron, did not show much colocalization with Bassoon **(wt-Norbin: 89.72 ± 1.89; NorΔN: 16.93 ± 4.52; NorΔPEP1: 89.23 ± 2.05; NorΔPEP2: 83.49 ± 2.9; NorΔC: 84.46 ± 2.01; NorA687G: 88.83 ± 2.21) (Figure 4.21A, B)**. Thus, all the constructs of Norbin except NorΔN targeted properly at the synapse.

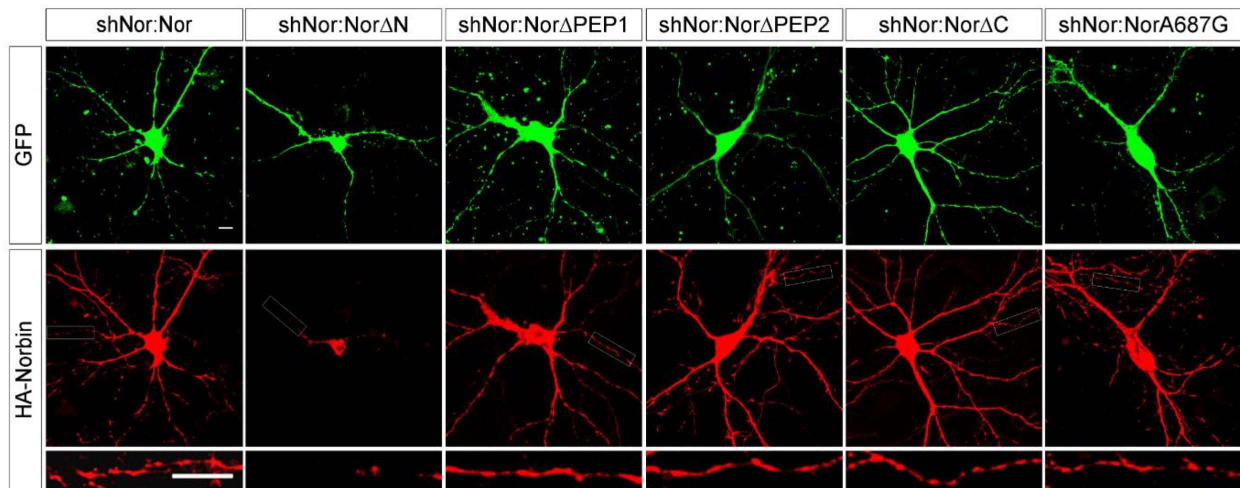


Figure 4.20. Expression and targeting of Norbin constructs in primary hippocampal neurons. Representative images showing that wild-type Norbin is expressed throughout the hippocampal neuron and is targeted to the dendrites. NorΔPEP1, NorΔPEP2, NorΔC and NorA687G showed expression pattern similar to that of the wild-type Norbin replacement protein and they were also seen to be localized at the dendrites, similar to the wild-type Norbin. In contrast, NorΔN did not target properly to the dendrites of the neuron and mostly localized at the cell body.

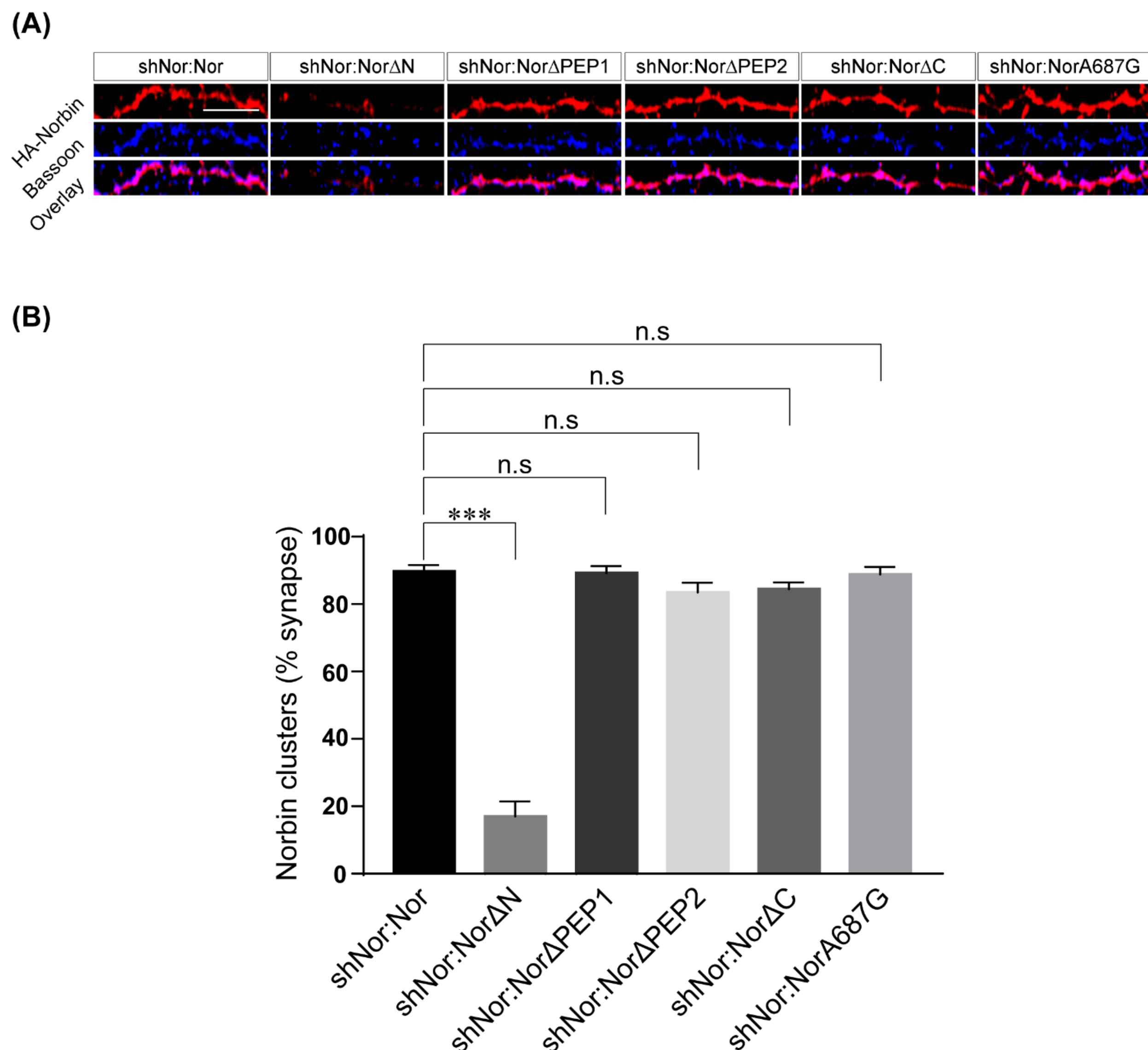


Figure 4.21. Synaptic localization of Norbin constructs in primary hippocampal neurons. (A) Representative images showing the colocalization of various forms of Norbin (wild-type Norbin, NorΔN, NorΔPEP1, NorΔPEP2, NorΔC and NorA687G) with Bassoon, an active zone synaptic marker. (B) Quantitation of the synaptic localization of the Norbin constructs. Note that all the constructs of Norbin were localized at the synapse to the similar extent, except NorΔN. Scale bar = 10 µm. ***$p < 0.001$; n.s, $p > 0.05$.

4.3. Discussion

It has been reported that Norbin directly interacts with numerous GPCRs (Ward et al., 2009). This includes GPCRs from the GPCR A superfamily such as the melanin-concentrating hormone receptor-1 (MCHR1), and from the GPCR C superfamily (mGluR1 and mGluR5). Norbin, through its C-terminus binds at the membrane-proximal region of the intracellular C-terminal tail of mGluR5 (Francke et al., 2006; Pan et al., 2016b; Wang et al., 2009a; Ward et al., 2009). Furthermore, Norbin has been shown to affect mGluR5 signalling, resulting in altered intracellular Ca^{2+} oscillations and ERK activity (Wang et al., 2009a). Till now, Norbin is not known to regulate the agonist-mediated internalization of any GPCR. For example, the interaction of Norbin with melanin concentrating hormone receptor-1 (MCHR1) was shown to inhibit the $G_{\alpha i/o}$ and $G_{\alpha q}$-dependent downstream signalling, but had no effect on the agonist-dependent internalization of the receptor (Francke et al., 2006). In contrast, co-expression of mGluR5 with Norbin had positive effects on the downstream signalling as well as on the steady-state expression of the receptor on the neuronal cell surface (Wang et al., 2009a). Furthermore, dysregulation of Norbin and mGluR5 levels in schizophrenia suggests that Norbin might play an important role in regulating the proper spatio-temporal localization of these receptors under normal physiological conditions. Thus, we investigated the role of Norbin in the agonist-mediated internalization of mGluR5.

We observed that, unlike the full-length Norbin replacement construct, NorΔN construct that did not contain the N-terminal region of Norbin was unable to rescue the decrease in the surface mGluR5 expression as well as the agonist-mediated endocytosis of mGluR5. Its expression level was very low in primary hippocampal neurons compared to the endogenous Norbin and it did not target properly to the dendrites nor could it localize at the synapse. Thus, we found that the N-terminal region of Norbin is crucial for the targeting of this protein to the dendritic regions of the neuron and for its localization at the synapse. As mentioned earlier, N-terminal region of Norbin harbors two distinct peptides; PEP1 and PEP2 through which PKA interacts with Norbin (Hermann et al., 2015). We observed that both PEP1 and PEP2 regions of Norbin are important for the normal surface expression as well as for agonist-mediated endocytosis of mGluR5. However, it is noteworthy that NorΔPEP2 could partially rescue the endocytosis of mGluR5 indicating that Norbin might have differential binding affinities for PKA through each of these peptides and the partial rescue in case of NorΔPEP2 could be a result of stronger affinity of PKA for PEP1 region

compared to PEP2 region of Norbin. Through our co-immunoprecipitation assay, we observed that PKA binding to Norbin was affected in both NorΔPEP1 and NorΔPEP2 constructs. We also saw that inhibition of PKA pharmacologically reduced the surface expression of mGluR5 and also inhibited the agonist-induced mGluR5 endocytosis. Hence, the decrease in surface levels of mGluR5 and inhibition in the agonist-dependent endocytosis of the receptor in case of NorΔPEP1 and NorΔPEP2 expression is probably due to the reduction of PKA available for mGluR5 trafficking. Thus, Norbin being an A-kinase anchoring protein might mediate this process by bringing PKA to mGluR5. This can be evaluated in future using more specific and robust measurements of the binding affinities of these two mutant forms of Norbin for PKA and by checking if mGluR5 interacts with PKA in the presence and absence of Norbin.

The NorΔC replacement construct or N-terminal region of Norbin alone was also unable to rescue the decrease in the surface expression as well as block in the agonist-mediated endocytosis of mGluR5. Importantly, we found a critical alanine residue at the 687th position in the C-terminal region of Norbin, which when mutated to glycine, led to a strong impairment of Norbin's interaction with mGluR5. Interestingly, this Norbin construct having mutation A687G was unable to rescue the decrease in the surface expression of mGluR5 due to the knockdown of the endogenous Norbin and it could not reverse the effect of Norbin knockdown on agonist-mediated mGluR5 endocytosis. Another crucial evidence for the functional relevance of this residue is that the "CREAMRL" region of Norbin harboring A687 is highly conserved in vertebrates as well as invertebrates. Thus far, our results suggest that both the N and C-terminal regions of Norbin together coordinate the Norbin-mediated regulation of mGluR5 trafficking. The simplest model to explain our results is that C-terminal of Norbin positions it in the vicinity of mGluR5 and N-terminal interacts with PKA to assist in mGluR5 internalization.

Chapter 5

Role of Norbin in mGluR-mediated AMPAR endocytosis

5.1. Introduction

Group I mGluRs have been implicated in several cellular mechanisms ranging from neural development to the processing of cognitive, sensory and motor information (Piers et al., 2012). Targeting of these receptors to precise subcellular compartments and the kinetics of their internalization and recycling directly determine their signalling and functional selectivity. Synaptic activity in the brain can trigger long-term changes in synaptic strength called long-term potentiation (LTP) or strengthening of synapses and long-term depression (LTD) or weakening of synapses. Group I mGluRs have been shown to regulate both forms of synaptic plasticity in the hippocampus, although the cellular and molecular mechanisms underlying mGluR-LTP and mGluR-LTD have not yet been fully elucidated (Gladding et al., 2009; Lüscher & Huber, 2010; Nakanishi, 1994; Neyman & Manahan-Vaughan, 2008; Zheng & Gallagher, 1992). Trafficking of AMPA receptors into and out of the synaptic plasma membrane has emerged as an important mechanism in the regulation of synaptic strength (Beattie et al., 2000; Carroll et al., 2001). Insertion of AMPA receptors into the plasma membrane leads to LTP, whereas internalization of AMPARs from the cell surface is believed to be the cellular correlate for LTD (Malinow & Malenka, 2002). Rapid internalization of surface AMPA receptors can be induced in cultured hippocampal neurons by the application of various glutamate receptor agonists, including glutamate itself, NMDA, AMPA, and group I mGluR agonists (S Bhattacharyya et al., 2009; Biou et al., 2008; Citri et al., 2010; Citri & Malenka, 2008). Activation of group I mGluRs with the agonist, R,S-DHPG, induces the AMPAR endocytosis, which is prerequisite for the induction of mGluR-LTD (Zho et al., 2002). Additionally, mGluR-LTP and LTD are the most commonly used models for delineating the cellular mechanisms underlying learning and memory formation (Riedel et al., 2003).

By virtue of their diversity and subtype-specific roles in modulating synaptic plasticity in different regions of the brain, group I mGluRs, particularly mGluR5, have become a major focus of investigation in numerous neurological disorders like Alzheimer’s disease, Parkinson’s disease, schizophrenia, fragile X syndrome, stress, pain, addiction and epilepsy (Mark F. Bear et al., 2004; Bordi & Ugolini, 1999; Crupi et al., 2019; Dölen & Bear, 2008; Huber et al., 2002; Matosin et al., 2017). On the other hand, Norbin deletion is early embryonic lethal (Mochizuki et al., 2003; Hong Wang et al., 2010), although targeted deletion in mouse neural stem cells revealed impairment in

spatial learning and sensorimotor gating, causing epilepsy (Dateki et al., 2005; Hong Wang et al., 2009). Targeted deletion of Norbin in postnatal forebrain impairs mGluR5-dependent stable changes in hippocampal plasticity, leading to schizophrenia-like behaviours and deletion of Norbin in cortical and hippocampal neurons disrupts adult neurogenesis and causes depression-like behaviours (Hong Wang et al., 2009, 2010). These rodent phenotypes may be relevant to humans, as Norbin levels are down-regulated in patients with epilepsy and dysregulated in schizophrenia which is reminiscent of mGluR5 (Matosin et al., 2015; Xu et al., 2017). Therefore, we hypothesized that since Norbin binding to group I mGluRs plays a crucial role in the agonist-mediated group I mGluR trafficking, it can also affect mGluR-mediated AMPAR endocytosis.

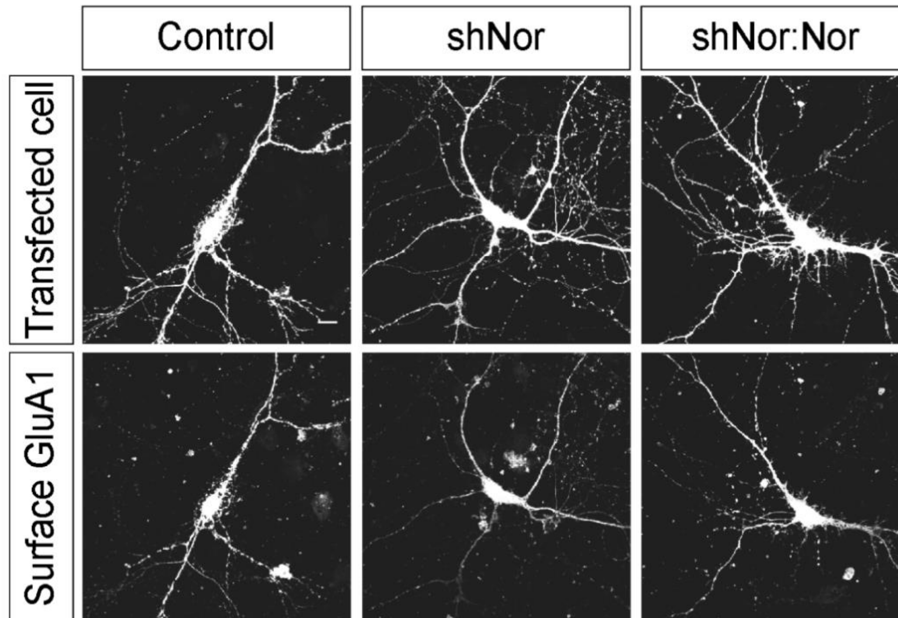
We observed that Norbin regulates mGluR-mediated AMPAR endocytosis only but it plays no role in the NMDA receptor-triggered AMPAR endocytosis. Norbin does not affect the mGluR-mediated ERK signalling and upon knockdown of endogenous Norbin, no change was observed in the mGluR-mediated immediate early gene expression. Although Norbin does not affect mGluR-dependent ERK signalling, activation of mGluRs leads to an increased interaction of PKA with AMPARs in the presence of Norbin and deletion of the PKA-binding regions of Norbin inhibits this increased interaction of PKA with AMPARs upon activation of group I mGluRs. Furthermore, deletion of the PKA binding regions of Norbin leads to impairment in the mGluR-mediated AMPAR endocytosis. Thus, our study provides novel insights into the regulation of mGluR-AMPAR axis through Norbin and PKA.

5.2. Results

5.2.1. Norbin plays a critical role in group I mGluR-mediated AMPAR endocytosis

Trafficking of AMPA receptors in and out of the synaptic membrane is of prime importance because it is involved in various forms of activity-dependent synaptic plasticity (Bredt & Nicoll, 2003; Collingridge et al., 2004.; Malenka & Bear, 2004). AMPA receptors get endocytosed upon activation of group I mGluRs and this is believed to be the cellular correlate for mGluR-dependent synaptic plasticity or mGluR-LTD (Samarjit Bhattacharyya, 2016; Gladding et al., 2009). We have shown that Norbin plays a crucial role in the agonist-mediated endocytosis of group I mGluRs. We subsequently wanted to understand the physiological consequence of Norbin's interaction with mGluR and regulation of mGluR trafficking. Initially we checked if knockdown of Norbin had any effect on the surface expression of AMPARs. Primary hippocampal neurons were transfected with either shNor or shNor:Nor replacement construct. 6-7 days post transfection, live cells were stained with anti-GluA1 primary antibody (1:150) for 15 min at 37˚C to label the GluA1-containing AMPARs. Subsequently, cells were fixed and saturating concentration of the goat anti-rabbit Alexa-568 conjugated secondary antibody was applied to label the surface receptors. Our data suggested that neither knockdown of endogenous Norbin nor replacement of endogenous Norbin with recombinant wild-type Norbin had any significant effect on the surface expression of GluA1-containing receptors **(control: 1.0 ± 0.02; shNor: 0.97 ± 0.03; shNor:Nor: 1.0 ± 0.03) (Figure 5.1A, B)**.

In order to investigate if Norbin plays any role in the mGluR-mediated AMPAR endocytosis, we knocked down endogenous Norbin and studied its effect on AMPAR endocytosis upon application of group I mGluR specific agonist, R,S-DHPG. The protocol has been discussed in detail in the "methods" section. In case of control cells, application of 100 μM R,S-DHPG led to internalization of AMPARs in 15 min but knockdown of endogenous Norbin caused a complete inhibition of mGluR-mediated AMPAR internalization. Importantly, this block in the endocytosis was completely rescued when we replaced endogenous Norbin with full-length Norbin **(control: 1 ± 0.03; control + DHPG: 1.65 ± 0.08; shNor + DHPG: 1.05 ± 0.06; shNor:Nor + DHPG: 1.82 ± 0.11) (Figure 5.2A, B)**. These results suggested that Norbin plays a crucial role in the mGluR-mediated AMPAR endocytosis.



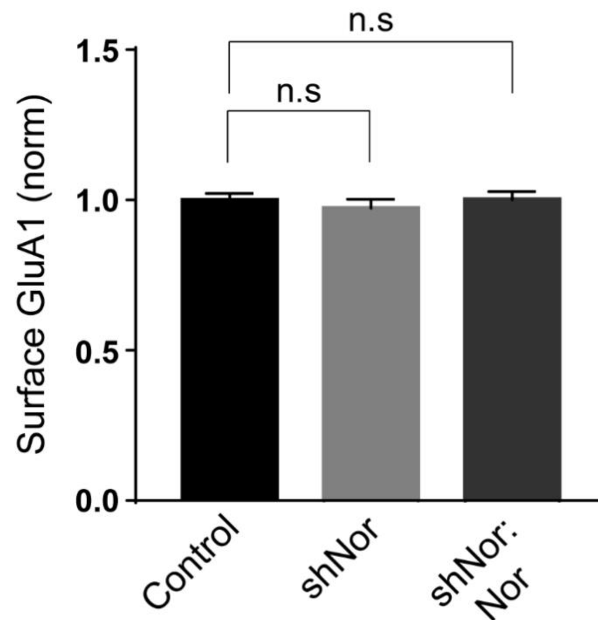


Figure 5.1. Norbin does not affect the surface localization of GluA1-containing AMPA receptors. (A) Representative images showing surface AMPAR expression in cells transfected with GFP, shNor or shNor:Nor. (B) Quantitation of surface levels of GluA1-containing AMPARs showed that knockdown of endogenous Norbin and replacement with wild-type Norbin had no effect on the surface expression of AMPARs. Scale bar = 10 µm. n.s, $p > 0.05$.

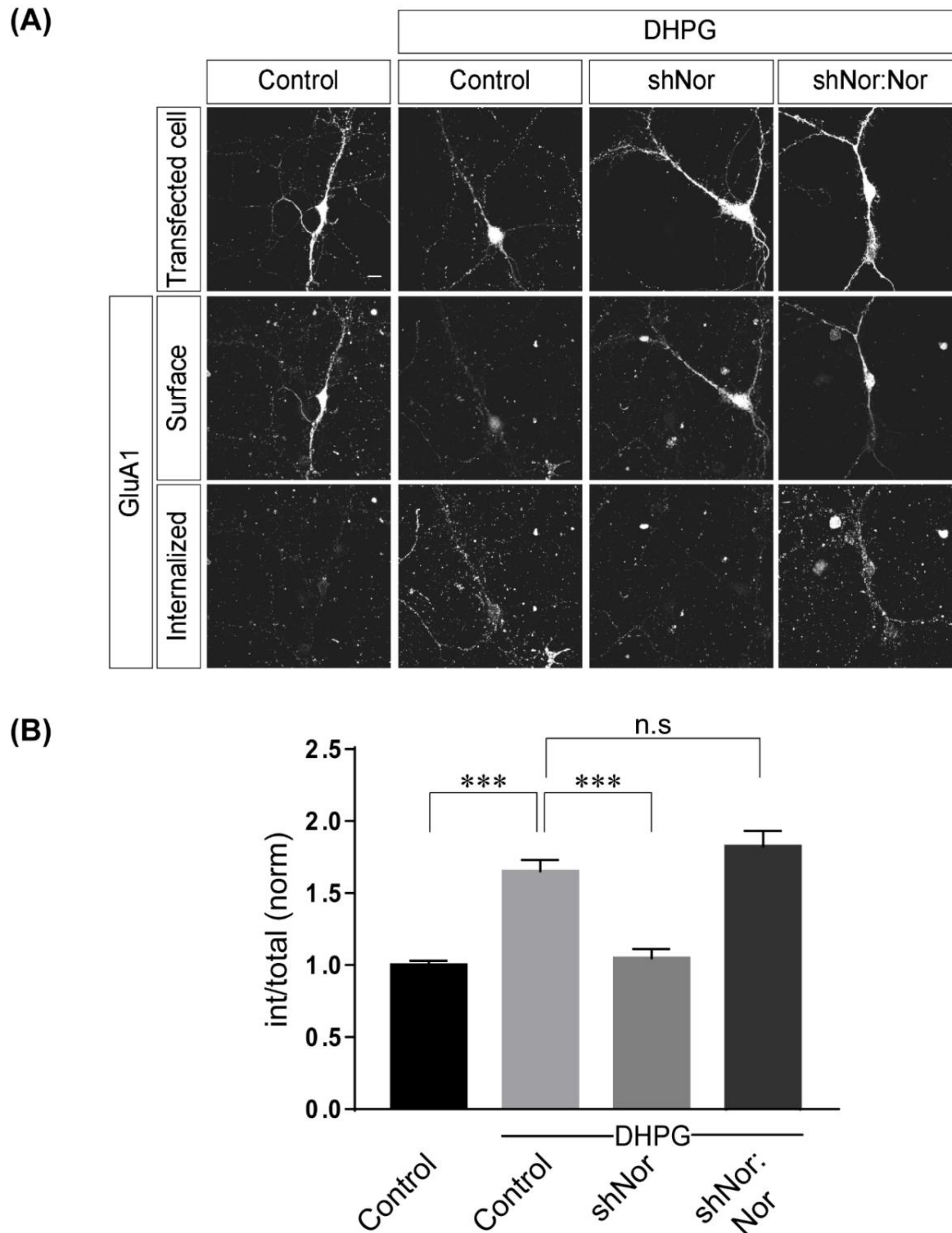


Figure 5.2. Norbin is important for the regulation of mGluR-mediated AMPAR endocytosis. (A) Representative images showing mGluR-mediated endocytosis of GluA1-containing receptors. Control cells showed very few internalized receptors and upon R,S-DHPG application, the receptors internalized. Knockdown of endogenous Norbin led to the inhibition of R,S-DHPG-induced AMPAR endocytosis, which was rescued by the expression of the wild-type Norbin replacement construct. (B) Quantitation of the mGluR-mediated AMPAR endocytosis showed that knockdown of endogenous Norbin blocked the endocytosis of GluA1-containing receptors and replacement with wild-type Norbin was able to rescue this phenotype. Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

5.2.2. Norbin has no effect on NMDAR-mediated AMPAR endocytosis

Apart from mGluR-LTD, another form of LTD that is prevalent in the brain is NMDAR-LTD. Endocytosis of AMPA receptors in response to the activation of NMDA receptors is believed to be the cellular correlate of NMDAR-LTD (Samarjit Bhattacharyya et al., 2009). We therefore investigated if knockdown of endogenous Norbin had any effect on the NMDA receptor-dependent AMPAR endocytosis. For that, primary hippocampal neurons were transfected with empty vector or shNor or shNor:Nor replacement constructs. 6-7 days post transfection, NMDA receptor-mediated AMPAR endocytosis assay was performed according to the procedure described in the "methods" section. Interestingly, application of 100 μM NMDA resulted in the endocytosis of AMPARs in both control cells, shNor transfected cells and shNor:Nor transfected cells **(control: 1 ± 0.07; control + NMDA: 1.73 ± 0.09; shNor + NMDA: 2.03 ± 0.16; shNor:Nor + NMDA: 2.04 ± 0.19) (Figure 5.3A, B)**. Thus, in contrast to the block in mGluR-triggered AMPAR endocytosis, knockdown of endogenous Norbin had no effect on NMDAR-mediated AMPAR endocytosis.

These results suggested that Norbin is specifically required for the mGluR-mediated AMPAR endocytosis and not for the NMDAR-mediated AMPAR internalization.

5.2.3. Norbin does not control the mGluR-induced second messenger responses

Activation of group I mGluRs leads to the stimulation of several second messenger responses that induce the endocytosis of surface AMPARs. Once the role of Norbin in mGluR-mediated AMPAR endocytosis had been established, it was important to identify the key molecular players involved in this process. One of the key signalling molecules that has been shown to be important for DHPG-induced LTD is extracellular signal regulated kinase or ERK. Downregulation of ERK phosphorylation with U0126 results in the inhibition of DHPG-induced LTD (Gallagher et al., 2004). It has also been reported that activation of group I mGluRs leads to the phosphorylation of ERK1/2 (Ferraguti et al., 1999; Limin Mao et al., 2005; Peavy & Conn, 1998). In order to check whether group I mGluR-mediated phosphorylation of ERK1/2 is dependent on Norbin, both control and shNor expressing cells were treated with cycloheximide for 5 hrs to inhibit new receptor synthesis and the drug was present throughout the experiment. In control cells, activation

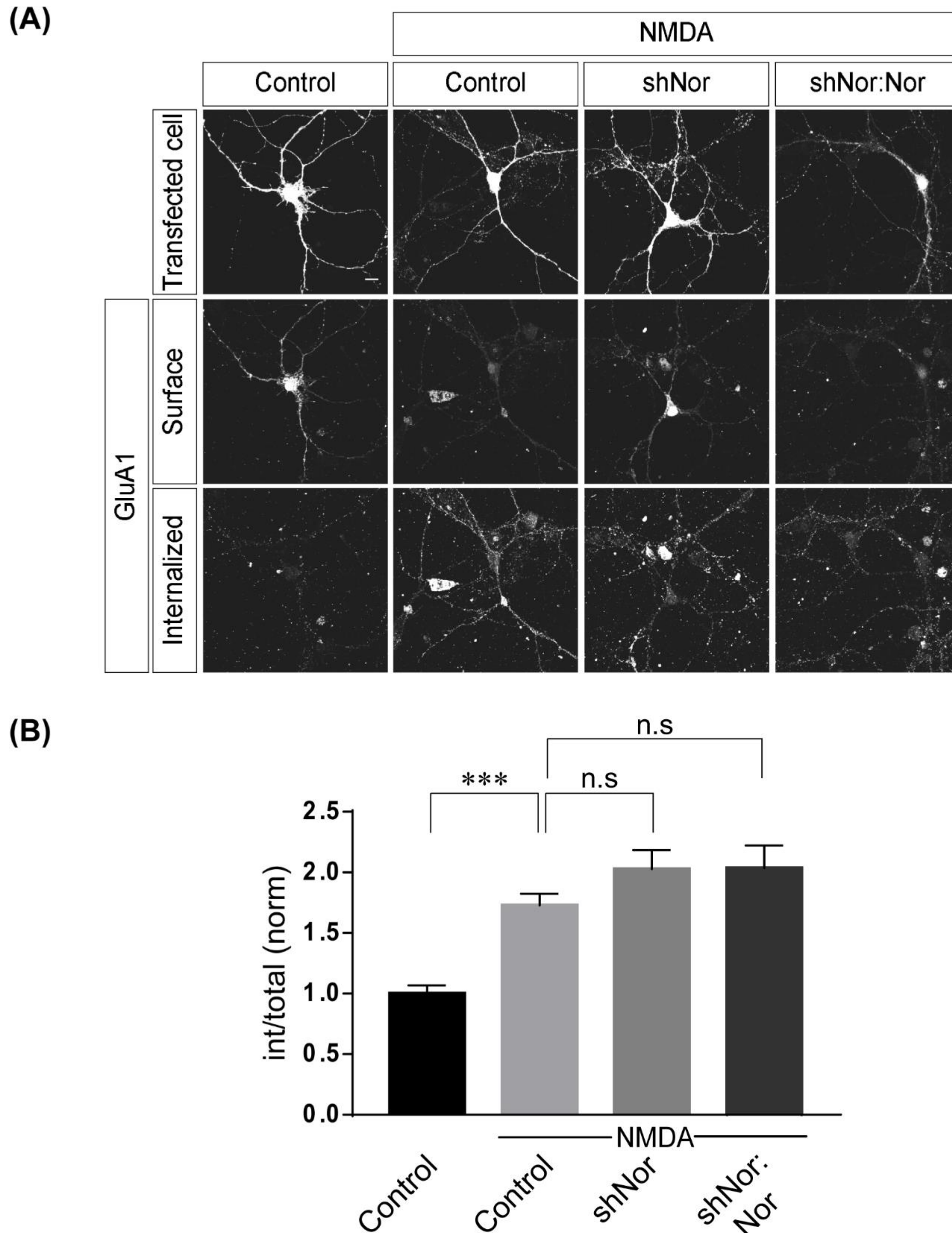


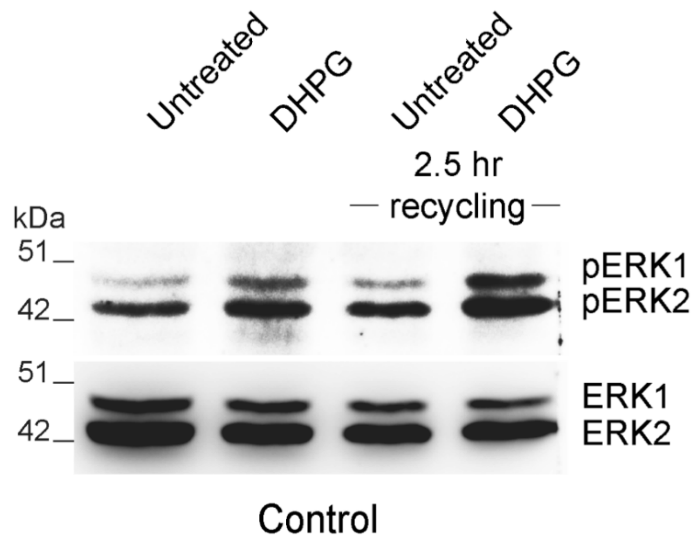
Figure 5.3. Norbin does not regulate NMDAR-mediated AMPAR endocytosis in primary hippocampal neurons. Representative images (A) and quantitation (B) of NMDAR-mediated AMPAR endocytosis suggested that application of 100 µM NMDA for 5 min triggered the internalization of GluA1-containing AMPARs in control cells and this internalization was not affected by knockdown of endogenous Norbin as well as by replacement of endogenous Norbin with full-length Norbin. Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

of mGluRs by R,S-DHPG resulted in the increase in phosphorylation of ERK1/2. When the receptors recycled back after 2.5 hrs, a second pulse of R,S-DHPG again resulted in the upregulation of phosphorylation of ERK1/2 suggesting that the recycled receptors got resensitized **(control:: untreated: 1 ± 0.3; DHPG: 2.39 ± 0.2; 2.5 hr recycling:: untreated: 1.39 ± 0.06; DHPG: 3.38 ± 0.45) (Figure 5.4A, B)** (Sharma et al., 2018). In shNor transfected cells, initial application of R,S-DHPG resulted in the increase in phosphorylation of ERK1/2. Surprisingly, the second pulse of R,S-DHPG after 2.5 hrs also resulted in the upregulation of ERK1/2 phosphorylation which was comparable to the control cells **(shNor:: untreated: 1 ± 0.04; DHPG: 1.71 ± 0.05; 2.5 hr:: untreated: 1.02 ± 0.12; DHPG: 2.03 ± 0.21) (Figure 5.5A, B)**.

These results suggested that Norbin does not affect mGluR-induced pERK1/2 upregulation and something else was affected downstream of Norbin in Norbin knockdown cells that led to the inhibition of mGluR-mediated AMPAR endocytosis.

5.2.4. Norbin does not affect group I mGluR-mediated Arc protein synthesis

It has been reported that mGluRs mediate AMPA receptor endocytosis through Arc protein present in the neuron and stimulate the local synthesis of Arc that results in the long-term increase in the AMPA receptor endocytosis rate (Rial Verde et al., 2006; H. Wang et al., 2016; Waung et al., 2008). In order to investigate whether Norbin controls the group I mGluR-mediated local protein synthesis, we studied the effect of endogenous Norbin knockdown on the mGluR-mediated Arc protein synthesis in primary hippocampal neurons. In control cells, application of 100 µM R,S-DHPG for 5 min led to the increase in the Arc protein expression compared to untreated cells. Whereas, pre-incubation of cells with cycloheximide inhibited the increase in the Arc protein expression upon application of 100 µM R,S-DHPG **(control:: untreated: 1 ± 0.07; DHPG: 1.62 ± 0.12; DHPG + CHX: 0.94 ± 0.08) (Figure 5.6A, B)**. Importantly, in shNor transfected cells, application of 100 µM R,S-DHPG for 5 min also increased the expression of Arc protein which was inhibited in cells pre-incubated with cycloheximide **(shNor:: untreated: 1 ± 0.06; DHPG: 1.69 ± 0.13; DHPG + CHX: 1.12 ± 0.11) (Figure 5.6A, C)**. Furthermore, shNor:Nor transfected cells also showed the increase in Arc protein expression on application of 100 µM R,S-DHPG for 5 min and cells pre-incubated with cycloheximide did not show that increase **(shNor:Nor:: untreated: 1 ± 0.1; DHPG: 1.52 ± 0.09; DHPG + CHX: 0.95 ± 0.1) (Figure 5.6A, D)**.



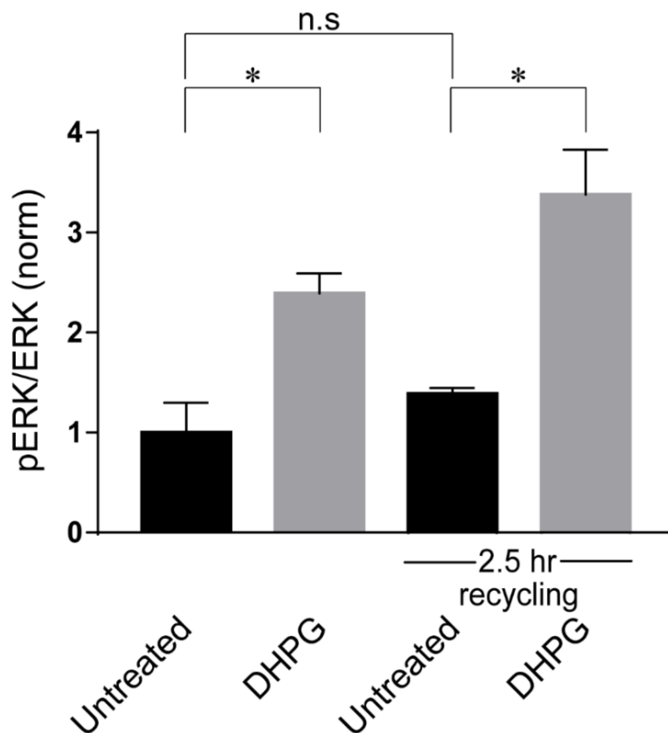
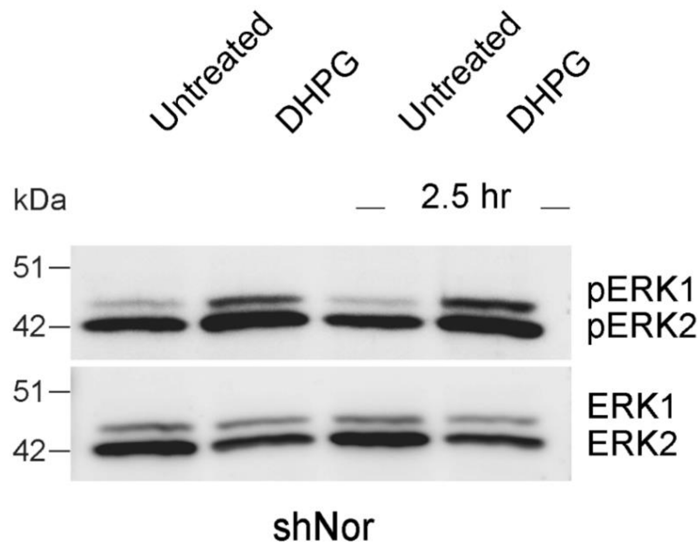


Figure 5.4. Intracellular signalling by group I mGluRs. Western blot (A) and quantitation of the western blots (B) showing that application of 100 μM R,S-DHPG for 5 min led to an upregulation in the phosphorylation of MAP kinases. Furthermore, the receptors that recycled to the cell surface following normal recycling route in 2.5 hr, also showed the ability to induce phosphorylation of MAP kinases upon application of 100 μM R,S-DHPG for 5 min. *, $p < 0.05$; n.s, $p > 0.05$.



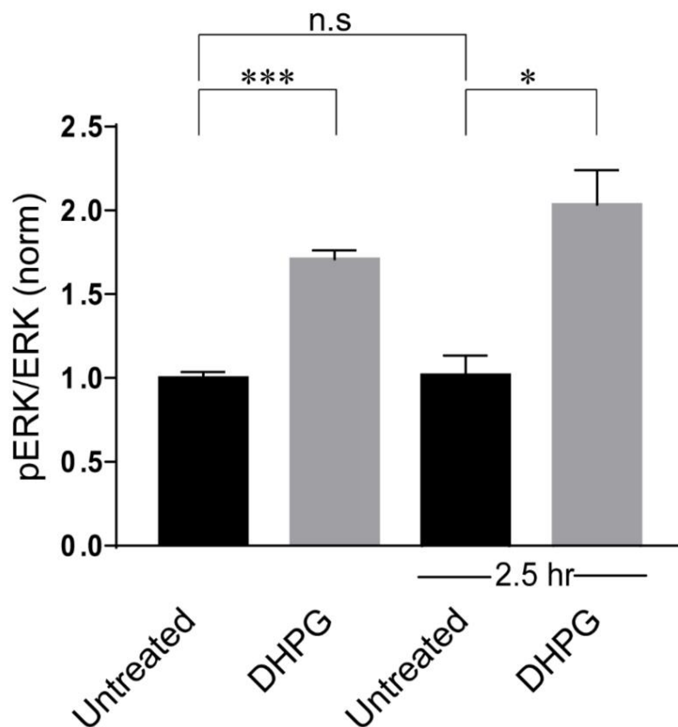


Figure 5.5. Knockdown of endogenous Norbin does not affect mGluR-mediated ERK signalling. Western blot (A) and quantitation of the western blots (B) showing that initial application of 100 µM R,S-DHPG for 5 min led to an upregulation in the phosphorylation of MAP kinases in shNor transfected cells but the receptors that could not internalize due to the knockdown of endogenous Norbin, were still able to induce the phosphorylation of MAP kinases on application of 100 µM R,S-DHPG in 2.5 hr. ***, $p < 0.001$; *, $p < 0.05$; n.s, $p > 0.05$.

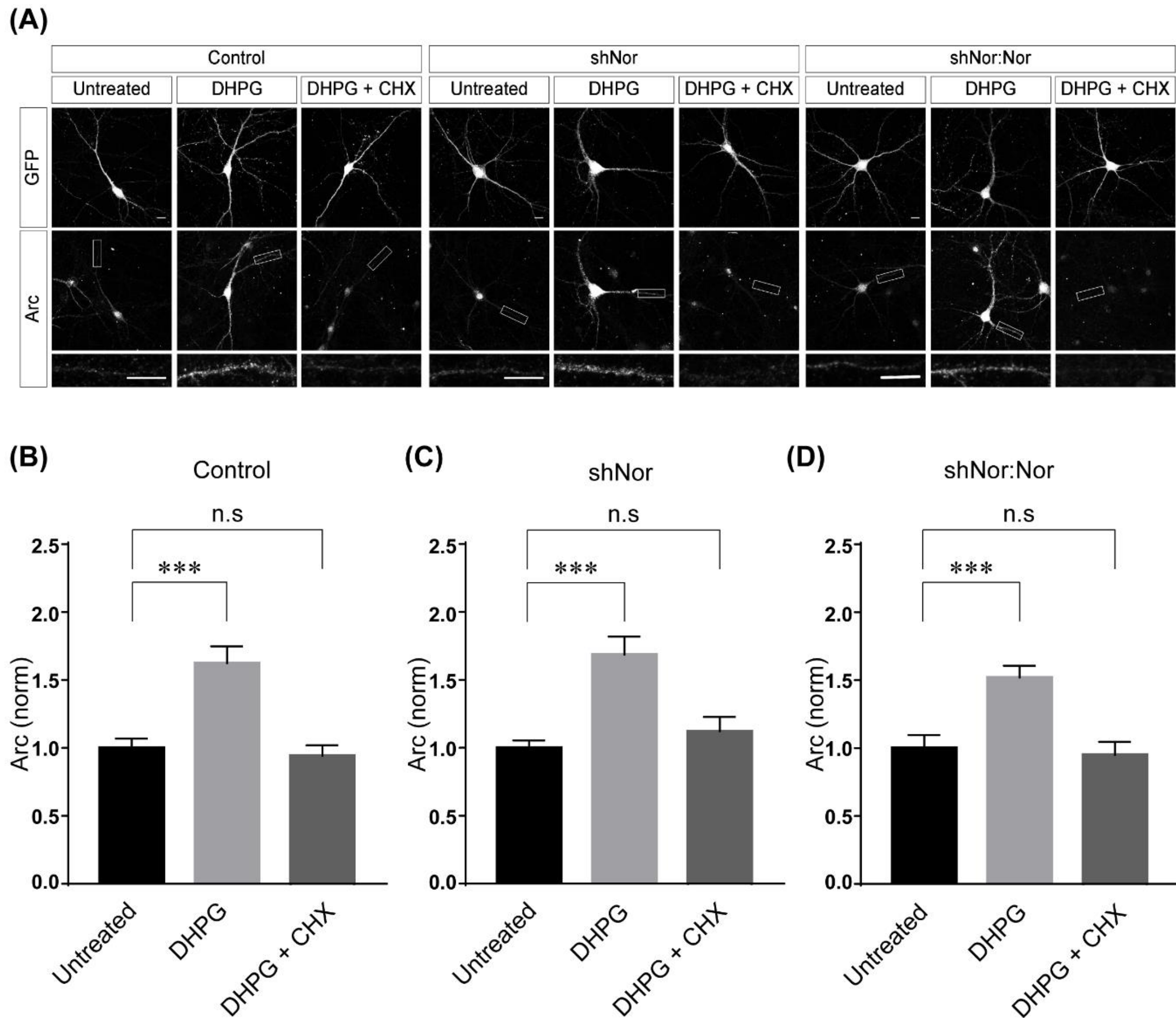


Figure 5.6. Norbin does not play any role in the mGluR-mediated *de novo* Arc protein synthesis. (A) Representative images showing the Arc protein expression in cells expressing GFP, shNor and shNor:Nor upon R,S-DHPG application both in the presence and absence of cycloheximide. (B-D) Quantitation of the Arc protein expression in control cells (B), shNor expressing cells (C) and shNor:Nor expressing cells (D) showed that in all these cells, Arc protein synthesis increased upon application of 100 µM R,S-DHPG and preincubation of these cells with cycloheximide inhibited this increase suggesting that Norbin does not affect mGluR-mediated mRNA translation or de-novo protein synthesis. Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

Thus, these experiments suggested that Norbin does not play any role the mGluR-mediated Arc protein synthesis.

5.2.5. Interaction of PKA with AMPARs through Norbin is essential for the mGluR-mediated AMPAR endocytosis

Since knockdown of the endogenous Norbin did not affect the MAP-kinase signalling by group I mGluRs or mGluR-mediated Arc protein synthesis, it was important to investigate why knockdown of Norbin inhibited the mGluR-mediated AMPAR endocytosis. mGluR activation is known to upregulate PKA signalling in neurons and group I mGluRs promote AMPAR phosphorylation through the activation of PKA (Dell'Anno et al., 2013; Kim et al., 2008). It has been reported that AMPAR endocytosis is dependent on the phosphorylation of AMPAR by PKA (Lee, 2006; Roche et al., 1996). Since Norbin is an AKAP that binds to the RIIα regulatory subunit of PKA, we wanted to see if Norbin plays any role in the association of PKA with AMPARs. In control cells, GluA1-containing receptors showed basal interaction with PKA, which increased significantly upon application of 100 µM R,S-DHPG. Importantly, in shNor transfected cells application of 100 µM R,S-DHPG did not increase the interaction of PKA with GluA1-containing receptors. The increased interaction of PKA with GluA1-containing receptors subsequent to the application of 100 µM R,S-DHPG was restored upon expression of the wild-type Norbin replacement construct. On the other hand, cells transfected with NorΔPEP1 or NorΔPEP2 replacement constructs did not show increased interaction of PKA with GluA1-containing receptors upon application of 100 µM R,S-DHPG **(control: 1 ± 0.11; control + DHPG: 1.64 ± 0.05; shNor: 0.89 ± 0.04; shNor + DHPG: 0.8 ± 0.06; shNor:Nor: 0.93 ± 0.05; shNor:Nor + DHPG: 1.88 ± 0.12; shNor:NorΔPEP1: 0.92 ± 0.09; shNor:NorΔPEP1 + DHPG: 1.06 ± 0.03; shNor:NorΔPEP2: 0.91 ± 0.03; shNor:NorΔPEP2 + DHPG: 0.87 ± 0.04) (Figure 5.7A, B)**. These results suggest that activation of group I mGluRs results in the increased interaction of PKA with AMPARs and Norbin acts as a critical mediator in this process.

We subsequently investigated whether binding of Norbin with PKA is important for the mGluR-mediated AMPAR endocytosis. Application of 100 µM R,S-DHPG led to the internalization of GluA1-containing receptors in control cells. Knockdown of the endogenous Norbin inhibited the AMPAR endocytosis and replacement of endogenous Norbin with NorΔPEP1 could not rescue

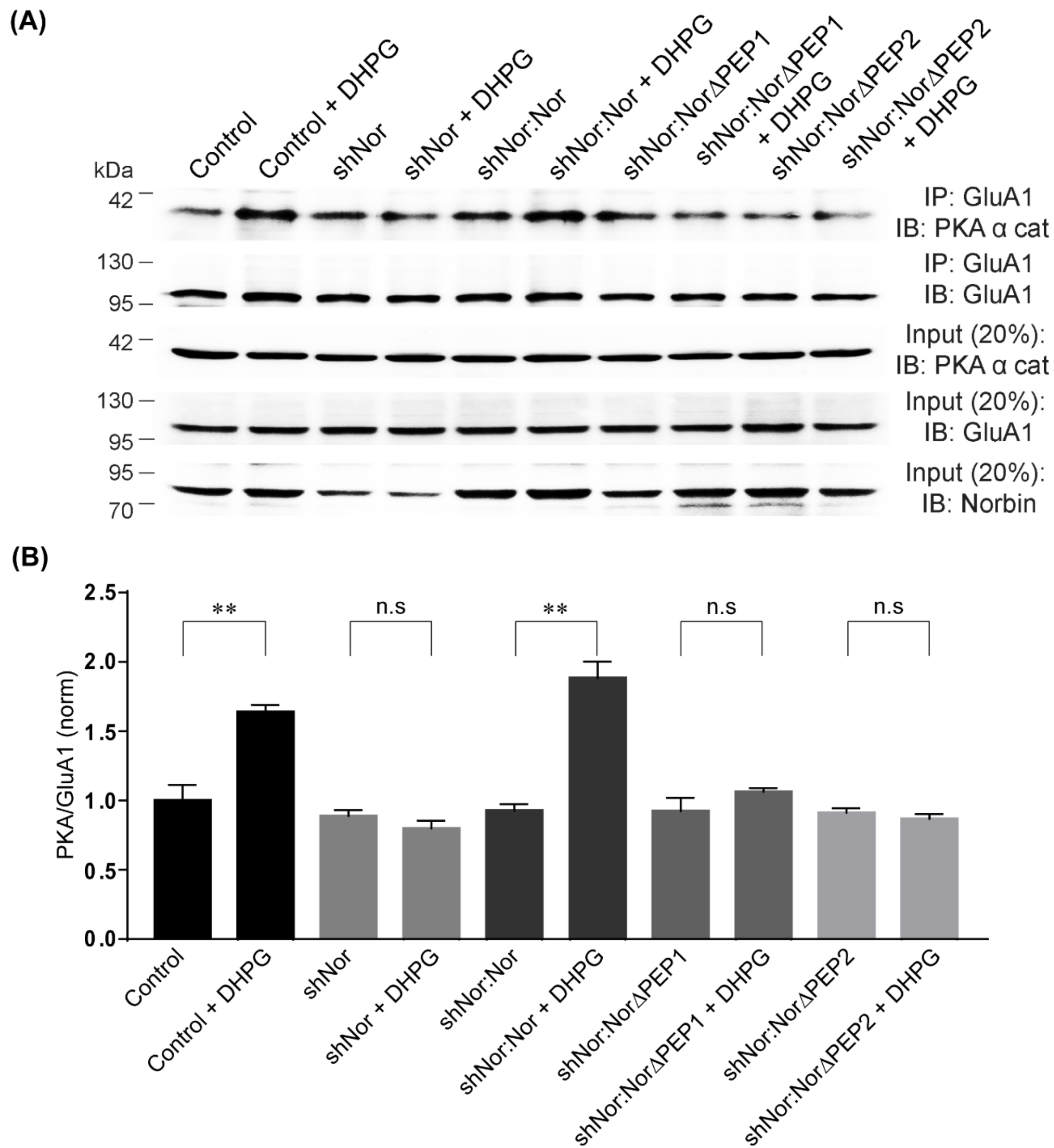


Figure 5.7. Norbin recruits PKA to AMPARs upon activation of group I mGluRs. Co-immunoprecipitation assay (A) and quantitation of the co-immunoprecipitation assays (B) showing the basal interaction of PKA catalytic subunit with AMPARs in primary hippocampal neurons which got increased upon activation of group I mGluRs by treatment with 100 μM R,S-DHPG for 5 min. In contrast, upon Norbin knockdown, application of 100 μM R,S-DHPG was unable to recruit PKA to AMPARs. This increased interaction of PKA with AMPARs upon R,S-DHPG application was rescued when endogenous Norbin was replaced with full-length Norbin but not with NorΔPEP1 and NorΔPEP2. **, $p < 0.01$; n.s, $p > 0.05$.

the mGluR-mediated AMPAR endocytosis **(control: 1 ± 0.05; control + DHPG: 1.86 ± 0.16; shNor + DHPG: 0.89 ± 0.07; shNor:NorΔPEP1 + DHPG: 1.07 ± 0.06) (Figure 5.8A, B)**. Similar to NorΔPEP1, NorΔPEP2 also did not rescue the R,S-DHPG-mediated endocytosis of GluA1-containing receptors **(control: 1 ± 0.06; control + DHPG: 1.53 ± 0.13; shNor + DHPG: 1.02 ± 0.07; shNor:NorΔPEP2 + DHPG: 0.97 ± 0.06) (Figure 5.9A, B)**. Together, these results suggest that activation of group I mGluRs leads to enhanced interaction of PKA with the AMPARs, which is essential for the mGluR-mediated AMPAR endocytosis and Norbin acts as a scaffolding protein which recruits PKA to the AMPARs subsequent to the activation of group I mGluRs.

5.2.6. Interaction of Norbin with mGluR5 is not necessary for the mGluR-mediated AMPAR endocytosis

Our earlier data suggested that Norbin regulates mGluR-mediated AMPAR endocytosis through PKA. We subsequently investigated whether interaction of Norbin with mGluR5 is necessary for the mGluR-mediated AMPAR internalization using the NorA687G replacement construct. We have shown before that mutation of alanine 687 to glycine in Norbin resulted in the inhibition of the interaction of Norbin with mGluR5 and it also inhibited the agonist-mediated endocytosis of mGluR5. We first checked the interaction of NorA687G with PKA by doing a co-immunoprecipitation experiment in primary hippocampal neurons. The neurons were transfected with either wild-type full-length Norbin or NorA687G and the experiment was performed 4-5 days after transfection. We pulled down the constructs using anti-HA primary antibody (1:500) and blotted for PKA using anti-PKA RIIα primary antibody (1:100). Our data suggested that NorA687G construct interacted with PKA similar to wild-type Norbin **(Norbin + PKA: 1 ± 0.01; NorA687G + PKA: 0.99 ± 0.02) (Figure 5.10A, B)**. Thus, this result suggested that mutation of alanine 687 to glycine at the C-terminus of Norbin does not affect the interaction of Norbin with PKA.

We subsequently investigated if A687G mutation in Norbin had any effect on mGluR-mediated AMPAR endocytosis. As expected, application of 100 µM R,S-DHPG led to the internalization of GluA1-containing receptors and in shNor transfected cells this internalization was inhibited. Interestingly, replacement of the endogenous Norbin with NorA687G construct was able to rescue

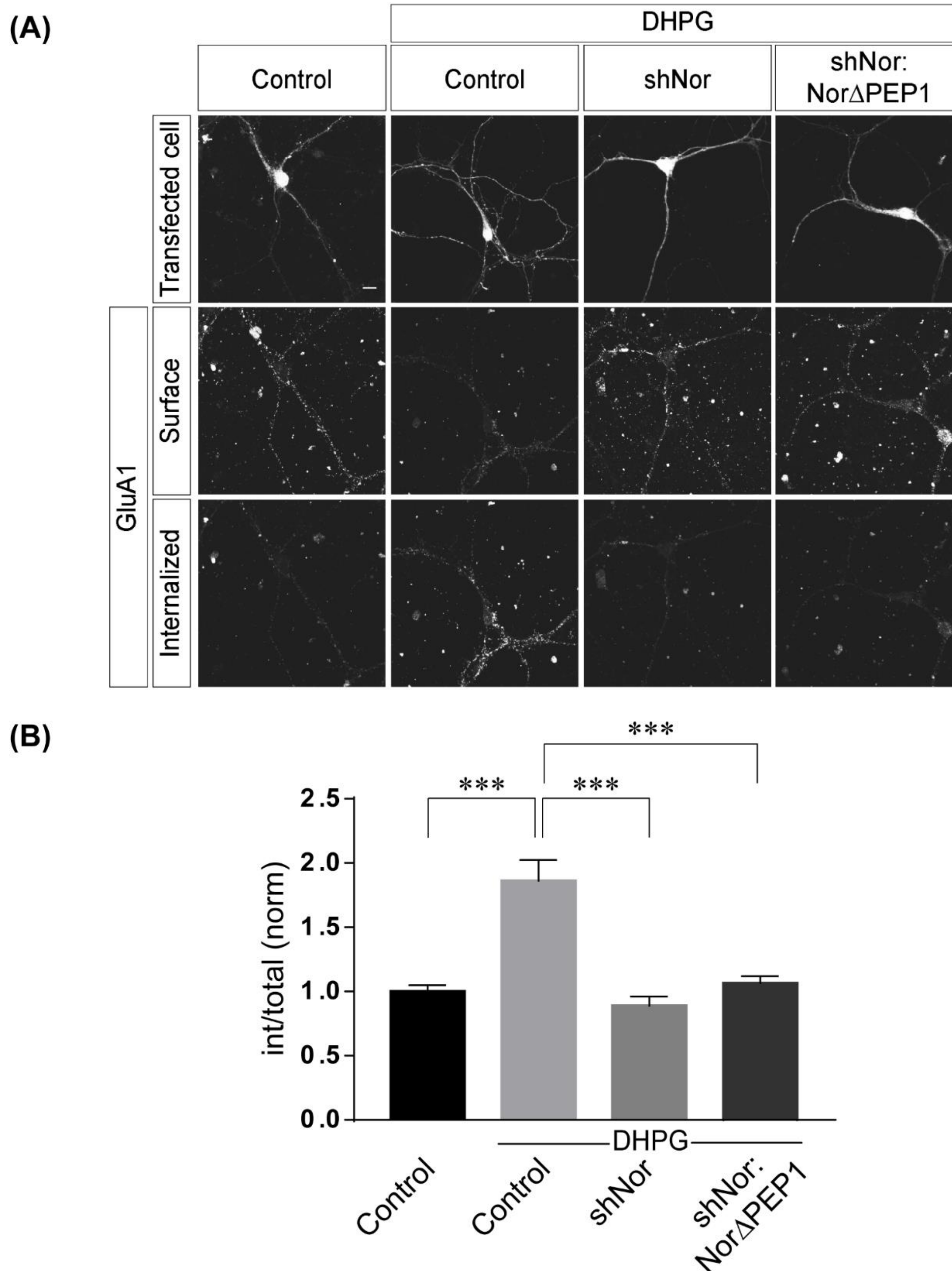


Figure 5.8. Deletion of PKA-binding peptide 1 or PEP1 region of Norbin renders it incapable to rescue the mGluR-mediated AMPAR endocytosis. (A) Representative images showing the mGluR-mediated AMPAR endocytosis in cells expressing GFP, shNor and shNor:NorΔPEP1. (B) Quantitation of the endocytosis index suggested that the inhibition of the mGluR-mediated AMPAR endocytosis due to the knockdown of the endogenous Norbin was not rescued by replacing the endogenous Norbin with NorΔPEP1. Scale bar = 10 μm. ***, $p < 0.001$.

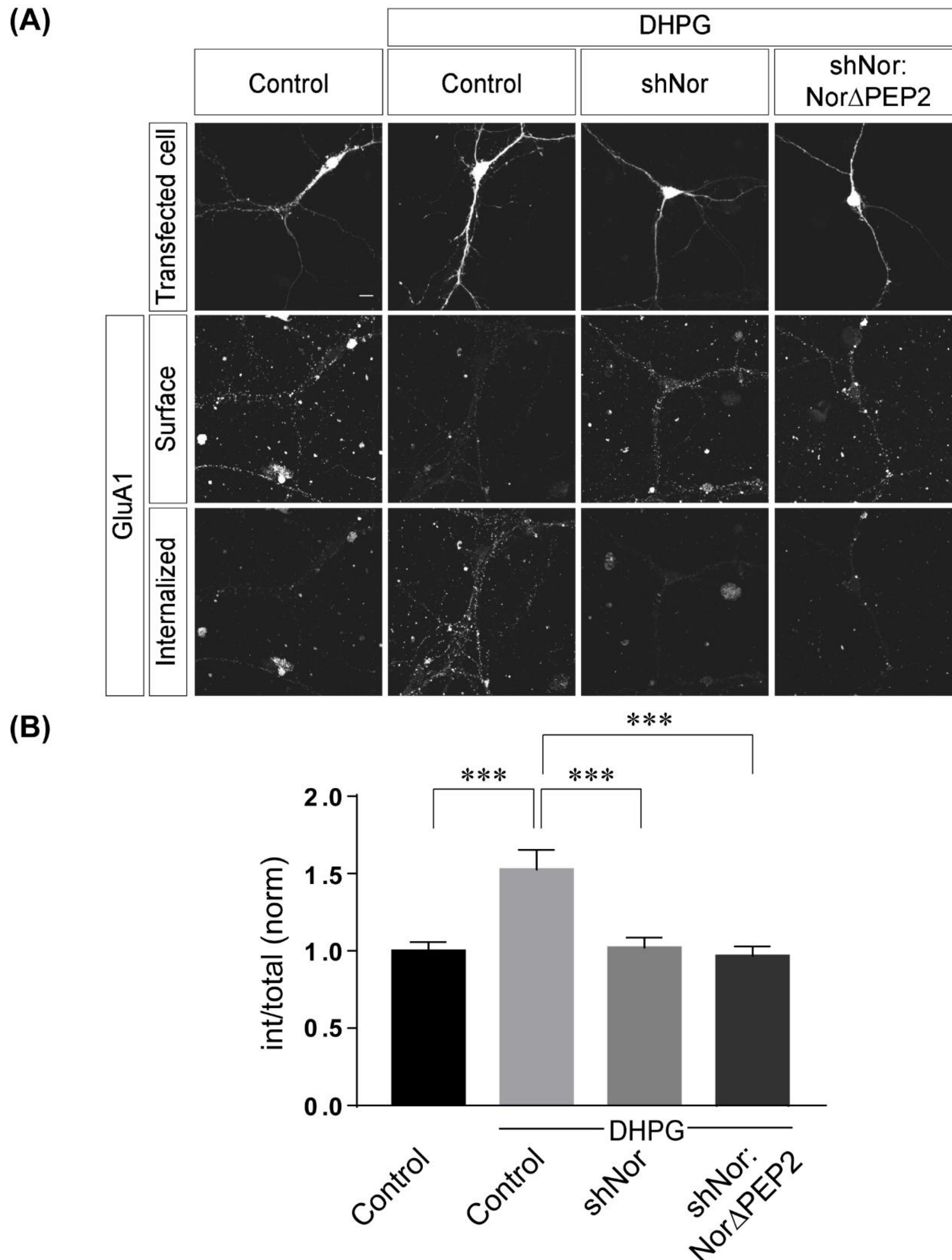
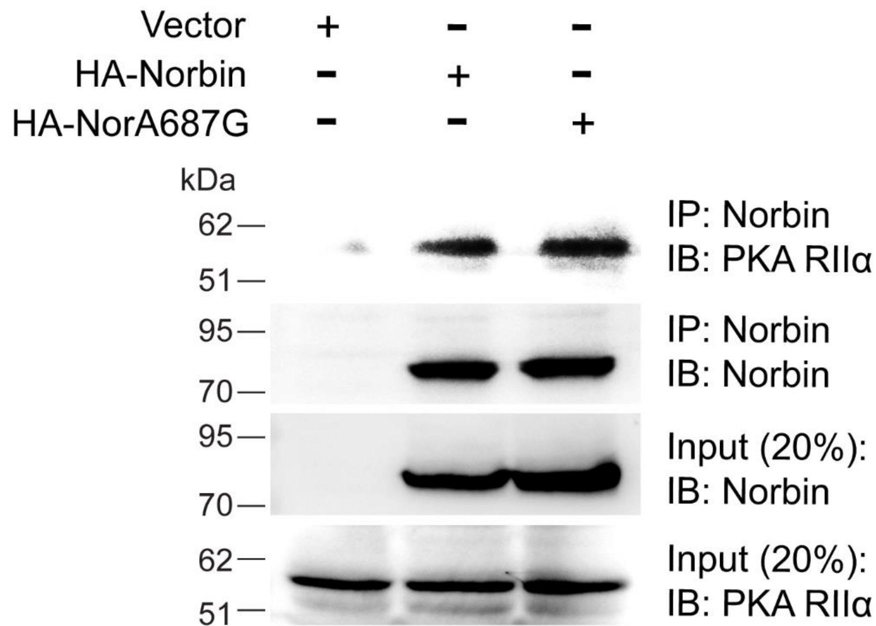


Figure 5.9. Norbin lacking the PKA-binding peptide 2 or PEP2 region is unable to rescue the mGluR-mediated AMPAR endocytosis. (A) Representative images showing the mGluR-mediated AMPAR endocytosis in cells expressing GFP, shNor and shNor:NorΔPEP2. (B) Quantitation of the endocytosis index suggested that the inhibition of the mGluR-mediated AMPAR endocytosis due to the knockdown of the endogenous Norbin was not rescued upon replacement of endogenous Norbin with NorΔPEP2. Scale bar = 10 µm. ***, $p < 0.001$.



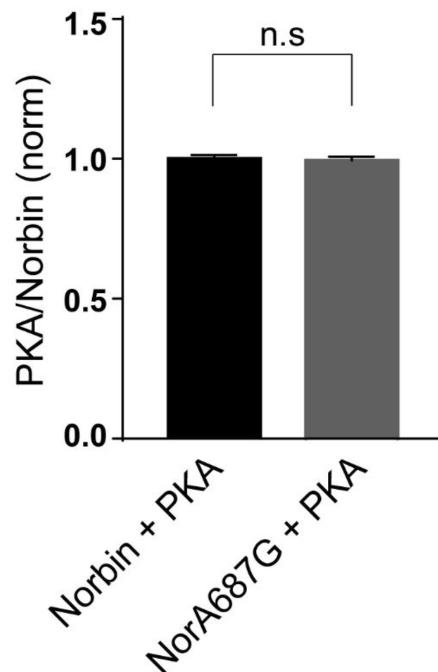


Figure 5.10. NorA687G interacts with PKA. Co-immunoprecipitation assay (A) and quantitation of the co-immunoprecipitation assays (B) showing the interaction of PKA RIIα regulatory subunit with the point mutant NorA687G. The interaction of NorA687G with PKA was comparable to wild-type Norbin. n.s, $p > 0.05$.

the mGluR-mediated AMPAR endocytosis **(control: 1 ± 0.07; control + DHPG: 1.94 ± 0.17; shNor + DHPG: 0.77 ± 0.05; shNor:NorA687G + DHPG: 2.26 ± 0.23) (Figure 5.11A, B)**. These results suggested that inhibition of mGluR-triggered AMPAR endocytosis in case of Norbin knockdown cells is due to its loss of binding with PKA and although the interaction of Norbin with mGluR5 is essential for agonist-mediated mGluR5 endocytosis, it is not necessary for the mGluR-mediated AMPAR endocytosis.

5.3. Discussion

Group I mGluRs have been implicated in various forms of activity-dependent synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) (Collingridge et al., 2004; Malenka & Bear, 2004). The activity-dependent synaptic plasticity is considered to be a cellular correlate for learning and memory formation. There are two forms of synaptic plasticity in the brain- one that is dependent on the activation of mGluRs and subsequent internalization of the ionotropic AMPARs and the other that is dependent on the internalization of AMPARs caused by the activation of NMDARs (Beattie et al., 2000; Samarjit Bhattacharyya et al., 2009; Citri & Malenka, 2008; Snyder et al., 2001). Studies investigating the molecular mechanisms of mGluR-LTD have found links to several neurodegenerative disorders like autism, Fragile X syndrome, schizophrenia and addiction. More recent studies have directed their therapeutic interventions towards one member of the group I mGluR family, mGluR5, because of its central role in the pathophysiology of these disorders (M. F. Bear, 2005; Dölen & Bear, 2009; Gladding et al., 2009; Matosin et al., 2015). Our data suggested that knockdown of endogenous Norbin affected only mGluR-mediated AMPAR endocytosis but did not influence NMDAR-triggered AMPAR endocytosis. This observation is not surprising since, mGluR-mediated LTD involves signalling cascades that are distinct from those underlying NMDAR-triggered LTD (Samarjit Bhattacharyya, 2016; Samarjit Bhattacharyya et al., 2009; Citri et al., 2009; Citri & Malenka, 2008). Our data also suggested that unlike its effect on mGluR5 surface expression, knockdown of Norbin had no effect on the surface expression of AMPARs, rather its effect on surface stability was specific for group I mGluRs.

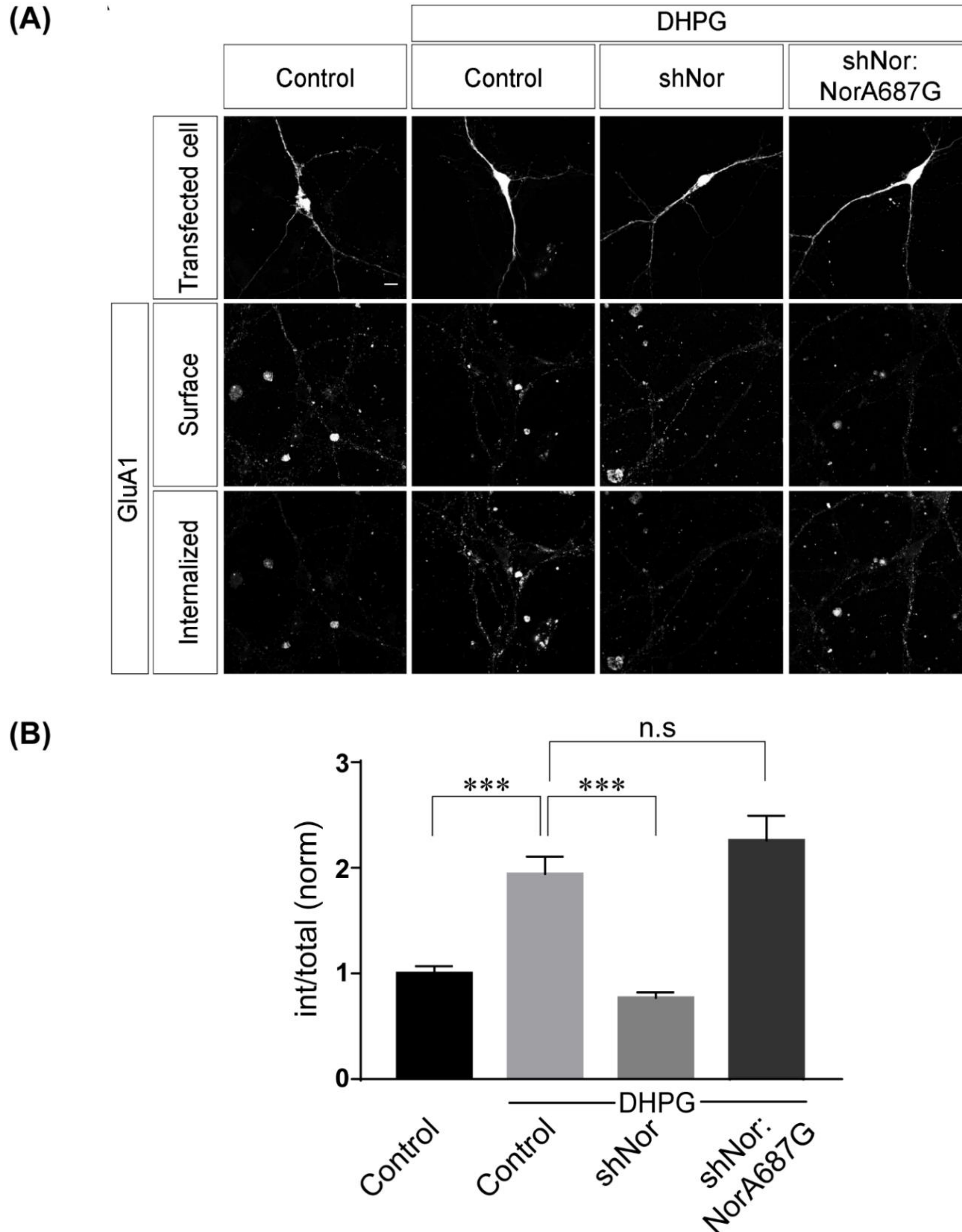


Figure 5.11. Replacement of endogenous Norbin with NorA687G rescues the mGluR-mediated AMPAR endocytosis. (A) Representative images showing the mGluR-mediated AMPAR endocytosis in cells expressing GFP, shNor and shNor:NorA687G. (B) Quantitation of the endocytosis index suggested that the inhibition of the mGluR-mediated AMPAR endocytosis due to the knockdown of the endogenous Norbin was rescued upon replacement of the endogenous Norbin with NorA687G. Scale bar = 10 µm. ***, $p < 0.001$; n.s, $p > 0.05$.

Till date, numerous mechanisms underlying mGluR-LTD have emerged that highlight the role of calcium, extracellular signal regulated kinases (ERKs), phosphatases and protein kinases in mGluR-dependent internalization of AMPARs (Gallagher et al., 2004; Purgert et al., 2014; Schnabel et al., 2001). One of the signalling cascades that we tested was the ERK signalling cascade. Contrary to the previous report by Wang et al (Wang et al., 2009a), where they showed an upregulation in the ERK signalling upon co-expression of Norbin with mGluR5 in HEK293 cells, we found that the receptors that could not internalize due to the knockdown of endogenous Norbin were still able to induce the phosphorylation of MAP kinases on application of R,S-DHPG. Thus, while they saw increased ERK signalling upon overexpression of Norbin in HEK293 cells, we saw increased ERK activity upon activation of group I mGluRs in the absence of Norbin in primary hippocampal neurons. This discrepancy in results can be attributed to the fact that regulation of GPCR surface expression and signalling by Norbin is context-dependent with regards to the cell line used or it could also be due to different approaches used to manipulate Norbin, i.e., overexpression vs acute knockdown. mGluR-mediated local protein synthesis has been reported to be critical for the long-term increase in the AMPAR endocytosis rate (Benoist et al., 2013; Chen & Shen, 2013; Davidkova & Carroll, 2007; Waung et al., 2008). Our data suggested that Norbin did not play any role in controlling the synthesis of Arc protein by mGluRs. Therefore, it was very interesting to find that although mGluR-mediated signalling and mGluR-mediated Arc protein synthesis were normal in Norbin knockdown cells, still mGluR-mediated AMPAR endocytosis was inhibited in these cells.

Phosphorylation of AMPARs by protein kinase A (PKA) has also been implicated in the regulation of LTD, though the role of PKA in mGluR-LTD is quite contentious (Esteban et al., 2003). While, some literatures show that PKA is important for NMDAR-dependent LTD but not for mGluR-dependent LTD, some other reports show that activation of mGluRs can lead to PKA-dependent phosphorylation of AMPARs and this process is critical for mGluR-LTD, whereas others point towards a form of LTD driven by PKA that is neither mGluR nor NMDAR-dependent (Citri & Malenka, 2008; Esteban et al., 2003; Lüscher & Malenka, 2012; Maiese et al., 2005; Sanderson et al., 2016; Teresa et al., 2013). In this study we have proposed that Norbin acts as a central regulator of mGluR-mediated AMPAR endocytosis by recruiting PKA to AMPARs upon mGluR activation. This is supported by the observation that interaction of PKA with GluA1-containing AMPARs is enhanced upon activation of group I mGluRs and the presence of Norbin, that has the PKA binding

sites is essential for this process. The increased interaction of PKA with GluA1-containing receptors upon activation of group I mGluRs probably results in the phosphorylation of critical residue(s) of the AMPARs which is essential for the endocytosis of the receptor. But this hypothesis needs to be investigated in future. In addition, our data suggest that mGluR-mediated AMPAR endocytosis is inhibited in the presence of Norbin that lacks PKA binding regions, PEP1 and PEP2. However, interaction of Norbin with mGluR5 does not seem to be necessary for the mGluR-mediated AMPAR endocytosis. Taken together, these results suggest a novel mechanism for the regulation of mGluR-mediated AMPAR endocytosis that involves recruitment of PKA to the GluA1-containing receptors by Norbin.

Chapter 6

Summary

6.1. Aim of the research

Glutamate is the major excitatory neurotransmitter in the brain. Vast majority of the synapses in the brain utilize glutamate (Andersen et al., 2021). Excessive glutamate signalling can lead to excitotoxicity hence, glutamate has to be maintained at low extracellular levels and this is ensured by the active transport of glutamate from the synaptic cleft into neurons and glia through excitatory amino acid transprters (EAATs) (Meldrum, 2000). Apart from signal termination through the transporters present on neurons and glia, signal detection through glutamate receptors also plays a key role in maintaining glutamate homeostasis within the cell. Glutamate receptors are broadly divided into two categories: ionotropic glutamate receptors or iGluRs and metabotropic glutamate receptors or mGluRs. Ionotropic glutamate receptors are ion channels that allow cations like Na^+ and Ca^{2+} to pass through them. They comprise of NMDA, AMPA, kainate and the lesser known GluD receptors (Hollmann & Heinemann, 1994). mGluRs are G protein-coupled receptors or GPCRs. They have further been divided into group I, group II and group III mGluRs (Ferraguti & Shigemoto, 2006). mGluRs are seven transmembrane receptors that belong to the class C family of GPCRs. GPCRs are the largest family of cell surface protein receptors that transduce their signals by coupling to a repertoire of G proteins and can thus activate multiple signalling pathways (Fredriksson & Schiöth, 2005). They can also transduce the signals mediated by diverse signalling molecules such as ions, peptides, lipids and photons to induce different intracellular functions. The structural and functional diversity of GPCRs has made them an attractive target for nearly 50% of all the modern drugs (Lagerström & Schiöth, 2008). Translocation of membrane proteins between the plasma membrane and intracellular storage compartments is an important in-built mechanism that is used by the cells to ensure proper signalling and turnover. Trafficking also controls the precise spatio-temporal localization of the receptors in the neuron. Initially agonist-induced GPCR internalization or sequestration was perceived as a mechanism for desensitization. It is now known that internalization is a mechanism for the resensitization of many receptors which helps them to regain responsiveness (Ferguson, 2001; Ferguson et al., 1998). But not all GPCRs internalize, some desensitize and get resensitized on the membrane itself; some internalize but instead of recycling back to the cell surface and getting resensitized, they undergo degradation *via* lysosomes (Ferguson, 2001; Marchese & Trejo, 2013; Palczewski et al., 1989). Thus, each GPCR is unique and trafficking of a particular GPCR depends on the type of system, the type of ligand and the type of receptor that is being studied (Bhattacharyya, 2016).

Group I mGluRs comprising of mGluR1 and mGluR5 are located at the perisynaptic region of the post-synaptic neuron and are positively coupled to PLC *via* $G_{\alpha(q/11)}$ (Hermans & Challiss, 2001; Luján et al., 1996). They regulate the internalization of ionotropic glutamate receptors and modulate various forms of synaptic plasticity in the brain (Eng et al., 2016). They have also been implicated in various neuropsychiatric disorders like schizophrenia, Fragile X syndrome, autism etc. (Bordi & Ugolini, 1999). Due to their increasing roles in various neuropsychiatric disorders, they have become important targets for drug discovery (Brauner-Osborne et al., 2006). Like other GPCRs, group I mGluRs also undergo desensitization and internalization (Mundell et al., 2001, 2004). But compared to their ionotropic counterparts, the cellular and molecular mechanisms underlying the trafficking of group I mGluRs still remain poorly understood. So, the broad aim of our study was to identify the cellular and molecular mechanisms responsible for regulating the agonist-mediated group I mGluR trafficking. Group I mGluRs are known to interact with several proteins in the post-synaptic density that act as scaffolds for the proper spatio-temporal localization of these receptors. Targeting of mGluRs to appropriate subcellular locations helps in regulating the signalling of these receptors. Aberrant signalling has been shown to affect mGluR-mediated synaptic plasticity which in turn might lead to various neuropsychiatric disorders (Lüscher & Huber, 2010). Thus, mGluR signalling has to be tightly regulated by various adaptor proteins. Norbin is one such protein that is known to interact with group I mGluRs and positively regulate mGluR5 signalling (Wang et al., 2009a). Furthermore, both Norbin and mGluR5 have been implicated in disorders like schizophrenia (Wang et al., 2009). However, how Norbin controls the trafficking and signalling of these receptors as well as its effect on mGluR-mediated AMPA receptor (AMPAR) endocytosis remains elusive. In view of the important roles of Norbin in the CNS, our specific aim was to check if Norbin has any effect on group I mGluR trafficking and in mGluR-mediated AMPAR trafficking as well as to identify the molecular basis for its effects.

We used primary hippocampal neurons for our study because hippocampus is a major processing centre for many types of learning and memory in the brain and group I mGluRs regulate various forms of synaptic plasticity in the hippocampus (Balschun et al., 1999; Lüscher & Huber, 2010b). Also, they are the most experimentally tractable *in vitro* system that can approximate the *in vivo* trafficking events. To study the agonist-induced internalization and subsequent subcellular fate of the group I mGluRs, we transfected the cells using myc-tagged mGluR1 and mGluR5. In these constructs, the myc epitope was tagged at the N-terminus of the full-length protein. Previous

reports have already demonstrated that these recombinant receptors behave like the native receptor (Choi et al., 2011; Mahato et al., 2015; Pandey et al., 2014; Trivedi & Bhattacharyya, 2012). To quantify receptor internalization, several techniques like surface biotinylation assay and single colour fluorescence measurement assays have been used previously. However, the variability in the expression of the receptors between different cells was not considered in these assays. To overcome this situation, we have used an elegant technique, viz., dual antibody labelling assay to differentially label the surface and internalized receptors using two separate fluorophores. This method allowed us to normalize the amount of the internalized receptors within each cell. These values were then normalized to the average endocytosis index of untreated control cells from the same experiment. In this way, this method confers an advantage that it quantitates the proportion of the internalized receptors within a cell and hence, the variability in the surface expression of the receptors between cells does not affect the quantitation (Bhattacharyya et al., 2009; Mahato et al., 2015; Pandey et al., 2014; Trivedi & Bhattacharyya, 2012).

6.2. Role of Norbin in the ligand-mediated endocytosis of group I mGluRs

Norbin is a 75 kDa neurite outgrowth-promoting protein that is present throughout the cytoplasm of dendrites and soma (Shinozaki et al., 1999; Wang et al., 2009, 2010). It is majorly neuronal and is distributed in different regions of the brain like cortex, cerebellum and hippocampus (Shinozaki et al., 1997). Norbin is crucial in the nervous system because its deletion in mouse is early embryonic lethal (Mochizuki et al., 2003). It has also been implicated in various neuropsychiatric disorders like schizophrenia, depression, epilepsy and its ablation leads to defects in adult hippocampal neurogenesis (Matosin et al., 2015; Wang et al., 2009, 2015; Xu et al., 2017). The C-terminus of Norbin interacts with the membrane proximal region of group I mGluRs including mGluR1a, mGluR5a and mGluR5b (Wang et al., 2009). Previous literature has shown that Norbin can regulate the steady-state surface levels of some GPCRs. For example, co-expression of mGluR5 with Norbin in neuro2A cells led to the constitutively increased levels of the receptor on the neuronal surface, whereas downregulation of Norbin in primary cortical neurons reduced mGluR5 levels on the cell surface. The same study also showed less mGluR5 on the cell surface in primary cortical neurons of mice with conditional knockout of Norbin in the forebrain (Wang et al., 2009). Similarly, co-expression of Norbin with melanin-concentrating hormone receptor-1 (MCHR1) in HEK293 cells was shown to inhibit $G_{\alpha(i/o)}$ and $G_{\alpha(q)}$ dependent Ca^{2+} signalling, but

increased the steady-state cell surface levels of the receptor, without affecting the MCH stimulation-dependent internalization of the receptor (Francke et al., 2006). Although, a more recent study reported normal levels of MCHR1 in HEK293 cells with inducible overexpression of Norbin (Francke et al., 2006; Ward et al., 2009). The mechanisms through which Norbin regulates this steady-state surface expression of GPCRs has not yet been elucidated and Norbin had not been shown to affect the agonist-dependent internalization of any GPCR.

To study the role of Norbin in group I mGluR endocytosis, we designed an shRNA against Norbin which was able to effectively knockdown the endogenous Norbin in primary neurons. In our assay, knockdown of endogenous Norbin decreased the surface expression of group I mGluRs which is consistent with the earlier report on mGluR5 (Wang et al., 2009). Importantly, knockdown of endogenous Norbin led to an inhibition in the agonist-mediated endocytosis of group I mGluRs suggesting that Norbin is crucial for regulating the surface expression as well as the agonist-mediated internalization of group I mGluRs. To check if the effect that we observed was indeed due to the knockdown of endogenous Norbin and not due to some non-specific effect of shNor, we decided to use a "molecular replacement" approach (Bhattacharyya et al., 2009). This is an elegant technique that allowed us to simultaneously knockdown the endogenous Norbin and replace it with full-length Norbin in the same cell using a dual promoter vector. Replacement with full-length Norbin was able to rescue the surface expression and agonist-mediated internalization of group I mGluRs, thus reinstating that Norbin is indeed crucial for group I mGluR endocytosis. Since Norbin and mGluR5 have together been implicated in various neuropsychiatric disorders like schizophrenia, we decided to carry out our further studies with this particular subtype of group I mGluRs. As mentioned earlier, the subcellular fate of a GPCR not only depends on the receptor and the system that we are studying but it also depends on the type of the ligand. So, we also looked at the role of a widely used mGluR5 antagonist MTEP in mGluR5 endocytosis and checked the effect of Norbin in the antagonist-mediated mGluR5 endocytosis. We observed that just like the agonist R,S-DHPG, a pulse of the antagonist was also able to cause the maximum internalization of mGluR5 in 30 mins and interestingly, this antagonist-mediated internalization of the receptor was blocked upon knockdown of the endogenous Norbin. Thus, our study provided new insights into the importance of Norbin in both agonist and antagonist-mediated endocytosis of mGluR5.

Till date there are no known domains or functional motifs in Norbin (Hong Wang et al., 2010). In order to dissect out and characterize the regions responsible for the Norbin-mediated regulation of mGluR5 endocytosis, we made a series of deletions. Starting from the N-terminal region which is responsible for the neurite-outgrowth function of Norbin (Schwaibold & Brandt, 2008), we saw that replacement with NorΔN construct was unable to rescue the surface expression and agonist-mediated internalization of mGluR5 suggesting that N-terminal region of Norbin is crucial for mGluR5 endocytosis. Moreover, we found that the N-terminal region of Norbin is important for targeting this protein to the dendrites and for its localization at the synapse. N-terminal region of Norbin harbours two distinct peptides, PEP1 and PEP2 which allow the binding of PKA to Norbin (Hermann et al., 2015). In contrast to wild type Norbin, replacement of endogenous Norbin with NorΔPEP1 and NorΔPEP2 could not completely rescue the block in mGluR5 endocytosis caused by shNor suggesting that PKA binding to Norbin is important for agonist-mediated mGluR5 endocytosis. However, it is important to note that NorΔPEP2 could partially rescue this block in the endocytosis of the receptor indicating that Norbin might have differential binding affinities for PKA through each of these peptides and the partial rescue in case of NorΔPEP2 could be a result of stronger affinity of PEP1 for PKA over PEP2. Although our co-immunoprecipitation assay showed disruption of PKA binding to both NorΔPEP1 and NorΔPEP2 when compared to wild-type Norbin, this is something that can be evaluated in the future using more specific and robust measurements of binding affinities for these two mutant forms of Norbin.

The C-terminal region of Norbin has been shown to be important for its interaction with various GPCRs including mGluR5 (Francke et al., 2006; Wang et al., 2009). Consequently, deletion of the C-terminal region of Norbin could not rescue the decrease in the surface expression as well as the block in agonist-mediated mGluR5 endocytosis. We decided to narrow down our study further and we found a critical residue at the C-terminus of Norbin that when mutated (A687G), led to a strong impairment of Norbin's interaction with mGluR5. Interestingly, this non-synonymous single residue mutation at the C-terminus of Norbin from alanine to glycine was unable to rescue the decrease in surface expression of mGluR5 when expressed simultaneously with shNor and it could not reverse the effect of Norbin knockdown on agonist-mediated mGluR5 endocytosis. Overall, the simplest model to explain our results is that the C-terminal of Norbin interacts with mGluR5 and N-terminal interacts with PKA and both these regions of Norbin coordinate together to facilitate mGluR5 internalization.

6.3. Role of Norbin in mGluR-mediated AMPAR endocytosis

Group I mGluRs have been shown to be involved in various forms of experience-dependent synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) (Gladding et al., 2009; Lüscher & Huber, 2010; H. Wang et al., 2016). Activation of group I mGluRs with an agonist like R,S-DHPG leads to the internalization of ionotropic AMPA receptors which is the cellular correlate for mGluR-dependent long-term depression or mGluR-LTD (Collingridge et al., 2004; Palmer et al., 1997). Targeted deletion of Norbin in the mouse forebrain has been shown to affect both forms of synaptic plasticity. Depletion of Norbin in the CNS causes a reduction in the DHPG-induced LTD and causes impaired LTP leading to schizophrenia-like behaviours (Wang et al., 2009). Norbin has also been shown to elicit GPCR-dependent intracellular Ca^{2+} transients upon overexpression in HEK293 cells (Francke et al., 2006; Wang et al., 2009). This makes Norbin an interesting candidate that can regulate the mGluR-triggered endocytosis of AMPARs.

To begin with, we saw that unlike its effect on mGluR5 surface expression, knockdown of Norbin had no effect on the surface expression of AMPARs, rather its effect was specific for group I mGluRs. Knockdown of Norbin led to the inhibition in the mGluR-mediated AMPAR endocytosis in dendrites suggesting that Norbin is crucial for the mGluR-mediated endocytosis of AMPARs. The role of Norbin in the endocytosis of synaptic AMPARs is highly specific because Norbin did not affect the endocytosis of AMPARs triggered by the activation of another type of ionotropic glutamate receptors, NMDARs. This observation is not surprising since mGluR-mediated LTD and AMPAR endocytosis involves signalling cascades that are distinct from those underlying NMDAR-triggered LTD (Bhattacharyya et al., 2009; Gallagher et al., 2004; Snyder et al., 2001). One of the signalling cascades that we tested was the ERK signalling cascade. Contrary to the previous report by Wang et al, where they showed an upregulation in the ERK signalling upon co-expression of Norbin with mGluR5 in HEK293 cells, we found that the receptors that could not internalize due to the knockdown of endogenous Norbin were still able to induce the phosphorylation of MAP kinases on application of R,S-DHPG. Thus, while they saw increased ERK signalling upon overexpression of Norbin in HEK293 cells, we saw increased ERK activity upon knockdown of Norbin in primary hippocampal neurons. This discrepancy in results can be attributed to the fact that regulation of GPCR surface expression and signalling by Norbin is

context-dependent with regards to the cell line and approaches used and remains to be further elucidated. mGluR-mediated AMPAR endocytosis is also dependent on the *de novo* translation of immediate early genes like *Arc* and *MAP1B* (Benoist et al., 2013; Chen & Shen, 2013; Davidkova & Carroll, 2007; Rial Verde et al., 2006; Waung et al., 2008). Knockdown of Norbin had no effect on the synthesis of Arc protein downstream of mGluRs. Apart from ERK signalling, PKA phosphorylation of AMPARs has also been implicated in the regulation of LTD (Esteban et al., 2003). We observed that the presence of Norbin led to enhanced interaction of the catalytic subunit of PKA with GluA1-containing AMPARs upon activation of mGluR5 and deletion of the PKA-binding regions of Norbin inhibited this increased interaction. In addition, mGluR-mediated AMPAR endocytosis was also inhibited in the presence of NorΔPEP1 and NorΔPEP2. Here we propose that Norbin acts as a central regulator of mGluR-mediated AMPAR endocytosis by recruiting PKA to AMPARs upon mGluR activation. Thus, our study provides a novel mechanism for the regulation of mGluR-mediated AMPAR endocytosis.

6.4. Summary of the picture available till now

The results presented in this thesis provide some important advancements in our existing knowledge of group I mGluR trafficking and signalling as well as the regulation of AMPAR internalization by group I mGluRs. Earlier reports have suggested that group I mGluRs undergo phosphorylation-dependent desensitization and internalization which is mediated by GRKs and PKC and is dependent on arrestin (Iacovelli et al., 2003; Mundell et al., 2003). Ubiquitination of group I mGluRs through Siah1A is also necessary for their agonist-mediated internalization (Gulia et al., 2017). Subsequent to internalization, group I mGluRs enter the recycling compartment. The exit of these receptors from the recycling compartment is pH dependent and protein phosphatases PP2A and PP2B play an important role in the recycling of group I mGluRs (Mahato et al., 2015; Pandey et al., 2014). SNX1, together with Hrs, plays a crucial role in the recycling of group I mGluRs through the "slow" recycling route which is important for the "resensitization" of these receptors (Sharma et al., 2018). Agonist-mediated internalization of group I mGluRs can be regulated by various scaffolding proteins that form a dense network within the post-synaptic density. For example, Tamalin is a scaffolding protein that regulates the trafficking of both the members of group I mGluR family through its interaction with S-SCAM (Pandey et al., 2020). We have shown that Norbin acts as a dual regulator of both mGluR trafficking as well as mGluR-

mediated AMPAR endocytosis through its interaction with PKA in primary hippocampal neurons. Norbin is necessary for the agonist-mediated internalization of group I mGluRs as its absence completely inhibits this phenomenon. Norbin also affects the surface stability of group I mGluRs independent of its effects on agonist-mediated group I mGluR endocytosis. Both PKA binding to Norbin through its N-terminal region and interaction of Norbin with mGluR5 through its C terminal region are important for it to regulate mGluR endocytosis. On the other hand, Norbin plays a critical role in the mGluR-mediated AMPAR endocytosis in a PKA-dependent manner. In the absence of Norbin, AMPARs are unable to bind PKA even after the activation of group I mGluRs suggesting that PKA recruitment to AMPARs through Norbin is important for mGluR-mediated synaptic plasticity (**Figure 6.1**).

6.5. Future directions

Although our study provides a comprehensive picture of the role of Norbin in group I mGluR endocytosis as well as in mGluR-mediated AMPAR endocytosis, it opens several questions that can be addressed in the future. We have shown the role of Norbin in the agonist-mediated internalization of group I mGluRs. Previous studies have shown that group I mGluRs also internalize in an agonist-independent manner (Dhami & Ferguson, 2006; Trivedi & Bhattacharyya, 2012). The mechanisms underlying these two forms of endocytosis might be distinct. Thus, it would be interesting to see if Norbin plays any role in the constitutive or agonist-independent endocytosis of group I mGluRs. Previous literature has suggested that the sequence of a GPCR cannot predict whether Norbin will interact with that GPCR and since Norbin has differential effects on different GPCRs, the role of Norbin in group II and group III mGluR trafficking as well as its interaction with these glutamate receptors can be investigated in the future. Norbin acts as an A-kinase anchoring protein (AKAP) through its N-terminal region and interacts with mGluR5 through its C-terminal region (Hermann et al., 2015; Wang et al., 2009). Moreover, pharmacological inhibition of PKA affects the surface expression and agonist-mediated internalization of mGluR5. This suggests that PKA, Norbin and mGluR5 can exist as a complex within neurons and this can be checked by the co-immunoprecipitation of PKA with mGluR5 in the presence and absence of Norbin.

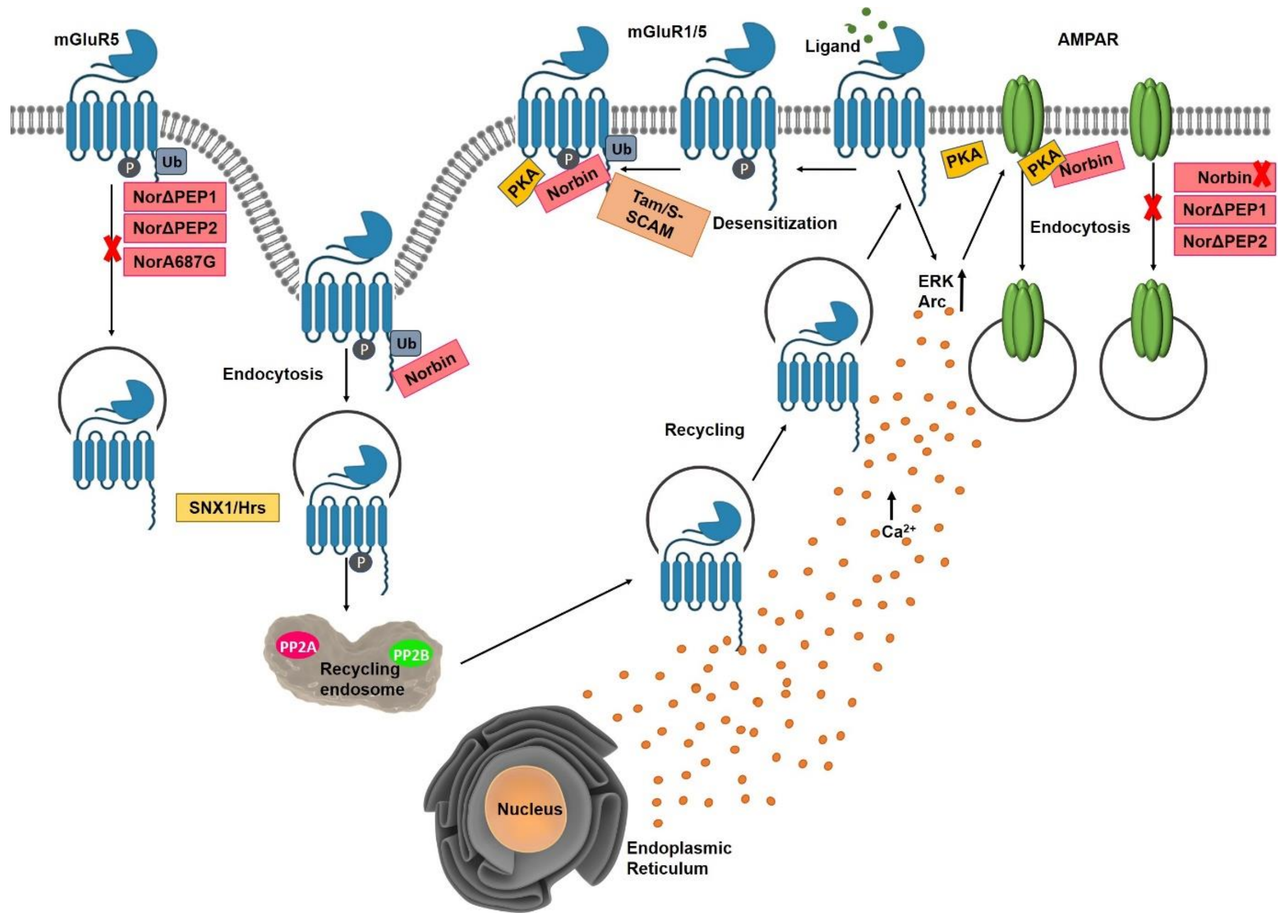


Figure 6.1. Working model of the mechanisms of group I mGluR trafficking and mGluR-mediated AMPAR endocytosis. Ligand-mediated activation of group I mGluRs initiates G-protein mediated signalling and results in the desensitization of the receptors. Subsequently, the desensitized receptors internalize upon ubiquitination and recycle back to the cell surface in a PP2A and PP2B-dependent manner. Norbin plays a crucial role in the surface expression and ligand-mediated internalization of group I mGluRs. In the absence of interaction of Norbin with mGluR5 as well as upon deletion of the PKA-binding regions of Norbin, mGluR5 is unable to internalize. Upon activation of group I mGluRs, Norbin leads to the recruitment of PKA to AMPARs which is essential for the mGluR-mediated AMPAR endocytosis. Absence of Norbin and deletion of the PKA-binding regions of Norbin leads to a block in the mGluR-mediated AMPAR endocytosis even in the presence of upregulated ERK signalling and Arc expression.

Since a single residue mutation at the C-terminus of Norbin has a drastic effect on mGluR5 trafficking, it would be very interesting and physiologically relevant to find out single nucleotide polymorphisms (SNPs) in the C-terminus of Norbin and check if they are associated with neuropsychiatric disorders. Phosphorylation and dephosphorylation of target proteins is important for the insertion and internalization of AMPARs to and from the synapse, respectively (Beattie et al., 2000; Huganir & Nicoll, 2013; Malinow & Malenka, 2002). Therefore, it would be important to find out the substrates of PKA and the mechanism by which PKA regulates mGluR-mediated AMPAR trafficking. PKA is known to phosphorylate AMPARs at the Ser845 residue (Dell'Anno et al., 2013). Thus, the phosphorylation status of AMPARs at the PKA-phosphorylation site can be checked in the presence and absence of R,S-DHPG and Norbin. It can also be tested if blocking GluA1 phosphorylation at the PKA site has the same effect on AMPAR endocytosis as Norbin knockdown. As Norbin is present in the post-synaptic density with many other proteins, it will be very interesting to find new interacting partners of Norbin and to check its interaction with proteins like Tamalin, SNX1 etc., that have already been implicated in group I mGluR trafficking. Several antagonists of group I mGluRs have been synthesized and have been shown to have dramatic effects in mouse models of various neuropsychiatric disorders like Fragile X-syndrome, autism etc. However, the effect of these antagonists on the trafficking and regulation of group I mGluRs, if any, have not been investigated and taken into consideration. Our finding that Norbin affects the antagonist-mediated internalization of mGluR5 has opened the possibility of a completely different mechanism by which antagonist-mediated endocytosis of group I mGluRs might be regulated and this needs to be investigated in the future. Similarly, Norbin might or might not regulate the internalization of mGluRs caused by other non-canonical ligands such as heterologous neurotransmitters. This is another area that can be investigated in the future.

6.6. Final words

GPCR signalling and trafficking are mechanisms adopted by the cells to maintain homeostasis and appropriate receptor number within the cell. The diversity in GPCR responsiveness can be attributed to the variety of different ligands binding to these receptors and also to structural differences among the members of GPCR family. GPCRs have the ability to sense and respond to the external environment or stimuli (ligand) and transduce this information inside the cells to regulate various physiological processes. In case of group I mGluRs, this endogenous ligand is

glutamate. Aberrant glutamatergic signalling can lead to various pathological consequences and cell death. Thus, mGluRs have to be tightly regulated by post-synaptic density proteins like Norbin. Our study has demonstrated that a well-orchestrated relationship between group I mGluRs, Norbin, PKA and AMPARs is integral for the normal functioning of the brain and it may have clinical relevance in targeting several neuropsychiatric disorders which report alterations in mGluR/Norbin interaction or mGluR-dependent synaptic plasticity. Although we have gained much information about group I mGluR trafficking in the last few years, it appears that we have just begun to understand the huge complexity that exists in group I mGluR signalling and regulation. Further research on group I mGluR signalling, regulation and trafficking will provide us important insights to unravel the complexity that exists in nature and to develop novel therapeutic strategies to cure various neurological disorders that arise due to aberrant GPCR signalling and regulation.

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