Investigation of the cellular dynamics of antagonist-mediated endocytosis of group I metabotropic glutamate receptors

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A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science

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Certificate of Examination

This is to certify that the dissertation titled “Investigations of the cellular dynamics of antagonist-mediated endocytosis of group I metabotropic receptors” submitted by Ms. Manjushree Sarda (Reg.No. MS14123) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr.Kausik Chattopadhyay **Dr. Arunika Mukhopadhaya** **Dr. Samarjit Bhattacharyya (Supervisor)**

Dated: April 26, 2019

Declaration

The work presented in this dissertation 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 bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Manjushree Sarda
(Candidate)

Dated: April 26, 2019

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

Dr. Samarjit Bhattacharyya
(Supervisor)

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I duly acknowledge the efforts put in by the faculty members of IISER Mohali, who have guided and helped me in the course of last five years.

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Abstract

G-protein coupled receptors bind to a variety of ligand molecules. An agonist is a ligand that when binds to the receptor leads to its activation and consequently a biological cascade in the cell. An antagonist however, is known to pharmacologically block the action of the agonist by binding to the receptor and preventing activation of the receptor by blocking receptor-agonist interaction and downstream signaling. Subsequent to the activation of the second messenger pathways, many G-protein coupled receptors (GPCRs) are known to get desensitized and get internalized. Till date, antagonists, which are viewed as pharmacological blockers only, were not known to promote sequestration of receptors upon binding. However, some studies have reported antagonist-induced desensitization of a few GPCRs and uncoupling of the receptor from the G-protein involved. Group I metabotropic glutamate receptors (mGluRs) play crucial roles, especially in inducing different forms of synaptic plasticity which are responsible for learning and memory formation. Group I mGluRs activate the phospholipase C pathway by coupling to the $G_{\alpha q/11}$ pathway.

In this study I determined whether the lesser known concept antagonists-mediated endocytosis is applicable to group I mGluRs in primary hippocampal neurons. It has been reported earlier that group I mGluRs show maximum internalization 30 mins post agonist stimulation. The objective of this study was to check if antagonists induce the internalization of mGluR1 and mGluR5, the two subtypes of the group I mGluRs. Further, I was interested to investigate the kinetics and the fate of the receptor subsequent to the internalization. Our results add to the understanding of the little known concept of antagonist-mediated internalization which is perhaps crucial because these antagonists are widely used in therapeutics. The detailed cellular mechanisms need to be investigated in future

Chapter 1

Introduction

1.1. Glutamatergic signaling

L-Glutamate is the principle neurotransmitter found in majority of the excitatory synapses in the mammalian central nervous system. At the synapse, the cell excitability and synaptic transmission is modulated by two distinct kinds of neuromodulatory glutamate receptors: the ionotropic glutamate receptors (iGluRs) and the metabotropic glutamate receptors (mGluRs).

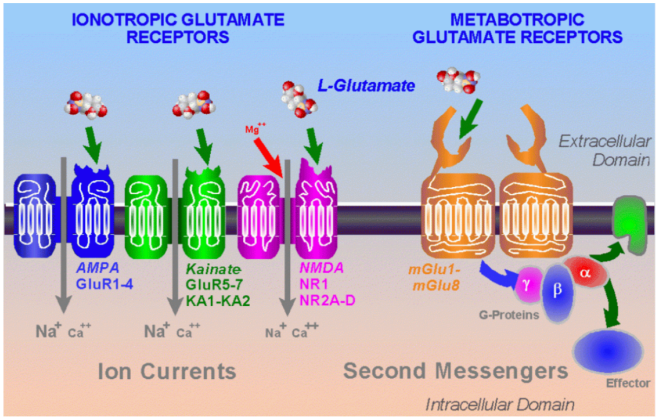

Figure 1: Types of glutamate receptors

(Adapted from 'Searching for the mind', Jon Lieff)

While the iGluRs are ligand gated ion channels responsible for fast excitatory transmission at the synapse, mGluRs participate in modulation of synaptic transmission *via* second messenger signalling pathways. Ubiquitous distribution of mGluRs in the brain, suggest an important neuromodulatory function for these receptors and also make them an ideal therapeutic target in various disorders of the CNS.

1.2. Metabotropic glutamate receptors

mGluRs are members of the G-protein coupled receptor superfamily (GPCR). They couple to a range of second messenger cascades and ion channels through the heterotrimeric G-proteins to mediate slow glutamate responses at the synapses. (Conn and Pin, 1997; Dale et al., 2002; Nakanishi and Masu, 1994).

Based on their pharmacological profiles, sequence similarities and transduction mechanisms, eight subtypes of mGluRs have been identified and further classified into three groups as shown in the figure 2.

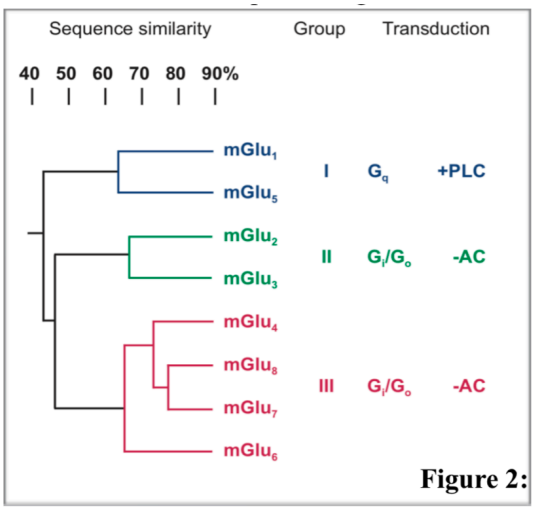

Figure 2: mGluR subtypes *(FRANCINE C ACHER, 2016)*

1.3. Group I metabotropic glutamate receptors

Group I mGluRs are widely distributed in the nervous system and in certain non-neuronal tissues also. In the CNS, they are differentially distributed in that mGluR1 is highly expressed in the CA3 pyramidal neurons of the hippocampus, in the cerebellum and the olfactory bulb, while mGluR5 is abundant in the hippocampus and cerebral cortex but sparsely expressed in the cerebellum (Gurpreet K Dhami, 2006).

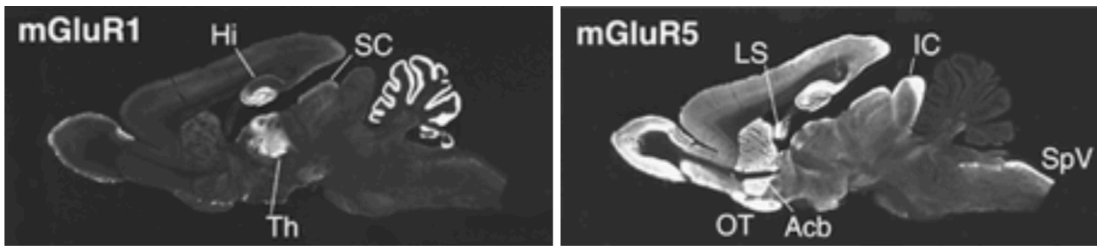

Figure 3: Distribution of mGluR1/5 in the rat brain (Shigemoto, 2006)

mGluR1 is concentrated in the perisynaptic and extra synaptic areas of the post synapse of the excitatory neurons (Nicoletti, 2011), while mGluR5 is known to functionally interact with the NMDARs in the post synaptic density (Collett and Collingridge, 2004). The stimulation of both the receptors triggers the release of calcium from intracellular stores which controls the receptor signaling, turnover and trafficking.

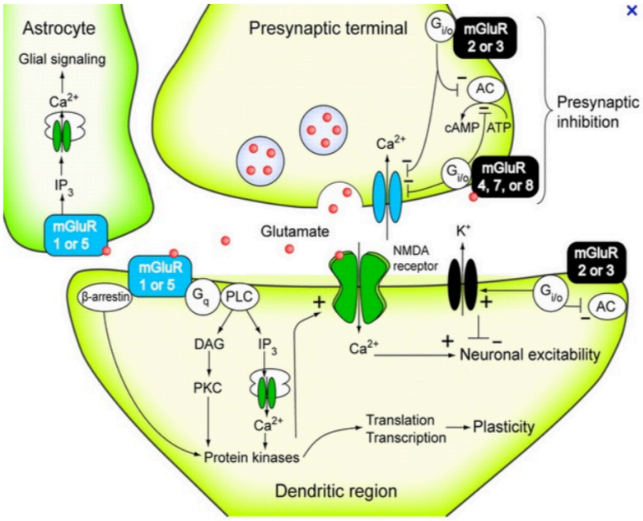

Figure 4: Physiological functions of mGluRs, Benarroch E.E, 2008

1.4. Role of group I mGluRs in synaptic plasticity and their physiological aberrations

Metabotropic glutamate receptors play crucial functions in induction of synaptic plasticity, neuroprotection, neuronal development and neurodegeneration. The two forms of synaptic plasticity: Long term potentiation (LTP) and long term depression (LTD) are the cellular correlates of learning-memory and forgetting respectively. The mechanism believed to be underlying this phenomenon is the trafficking of post synaptic AMPARs subsequent to the signaling cascade initiated by mGluR1/5 (Kauer JA, 2007).

Since metabotropic glutamate receptors serve critical roles, aberrations in their expression, physiology or function are the basis of several neuropsychiatric disorders like Huntington's disease, ALS, MS, Parkinson's disease etc. Furthermore, hyperregulation of mGluRs and imbalance of excitation-inhibition of glutamatergic signaling is implicated in autism spectrum disorders, schizophrenia and fragile X syndrome (Gurpreet K. Dhami, 2006).

1.5. Agonist-mediated trafficking of group I mGluRs

The spatio-temporal localization of GPCRs and its ability to get stimulated in future is critically regulated as a function of its prior activation state. Prolonged or repeated stimulation by an agonist results in reduced sensitivity to its subsequent application by reducing the number of functional receptors at the cell surface. This phenomenon regulating the cell surface receptor turnover and maintaining cellular homeostasis thereof, is known as desensitisation (Hausdorff et al., 1990). Mechanisms that prevent this chronic overstimulation of the receptor include receptor phosphorylation and uncoupling from the G protein involved and internalization into the endocytic compartments and/or lysosomal degradation (Hausdorff et al., 1990, Lefkowitz, 1998). Some GPCRs are known to get desensitized and downregulated by G protein independent mechanisms, such as the application of antagonists (Roettger et al., 1997; Houle et al., 2000; Gray & Roth, 2001). Group I mGluRs, like other GPCRs have been reported to undergo internalization post desensitization upon agonist stimulation (Mundell et al., 2004). Also like some GPCRs, mGluRs recycle back to the cell surface subsequent to the desensitization and internalization. The fate of the receptor upon internalization depends on the type of receptor, the type of ligand and the system. The post endocytic fate of group I mGluRs upon agonist treatment has been studied in some detail as shown in the figure 5 (Mahato et al, 2018).

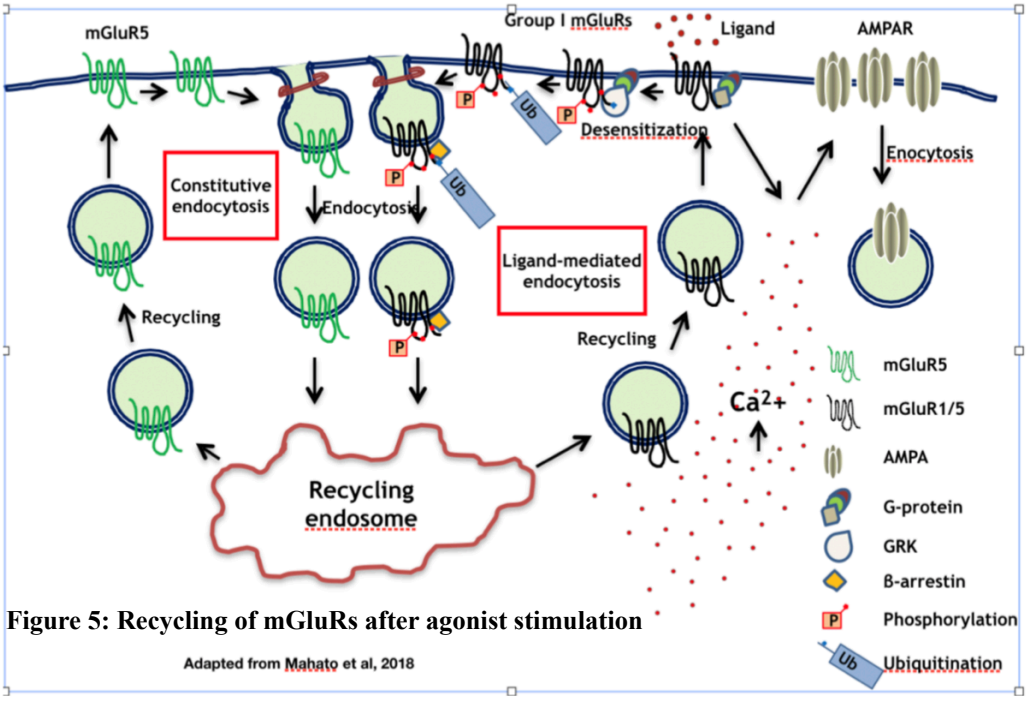

Figure 5: Recycling of mGluRs after agonist stimulation

Adapted from Mahato et al, 2018

1.6. Antagonists

Antagonists have been broadly categorized to be pharmacological blockers of the receptors only and up until recently were not known to promote the sequestration of the receptor. This is primarily because internalization requires activation of the receptor, but antagonists are not known to activate the second messenger pathways. However, some GPCRs have been reported to undergo sequestration upon antagonist binding including cholecystokinin receptors, vasopressin V2, AT1 and bradykinin B2 receptor subtypes (Roettger et al.,1997, Pfeiffer et al., 1998; Hunyady, 1999; Houle et al., 2000). More recently, there are reports of attenuated activity as a result of internalization of beta-adrenergic receptors in response to insulin like growth factor-I, a proposed functional antagonism in action. (Shai Gavi et al, 2007). Furthermore, antagonist induced internalization and the downregulation of the recombinant serotonin transporter expressed in HEK293 is proposed to regulate a complex mechanism in treatment of depression (Katharina Kittler 2010).

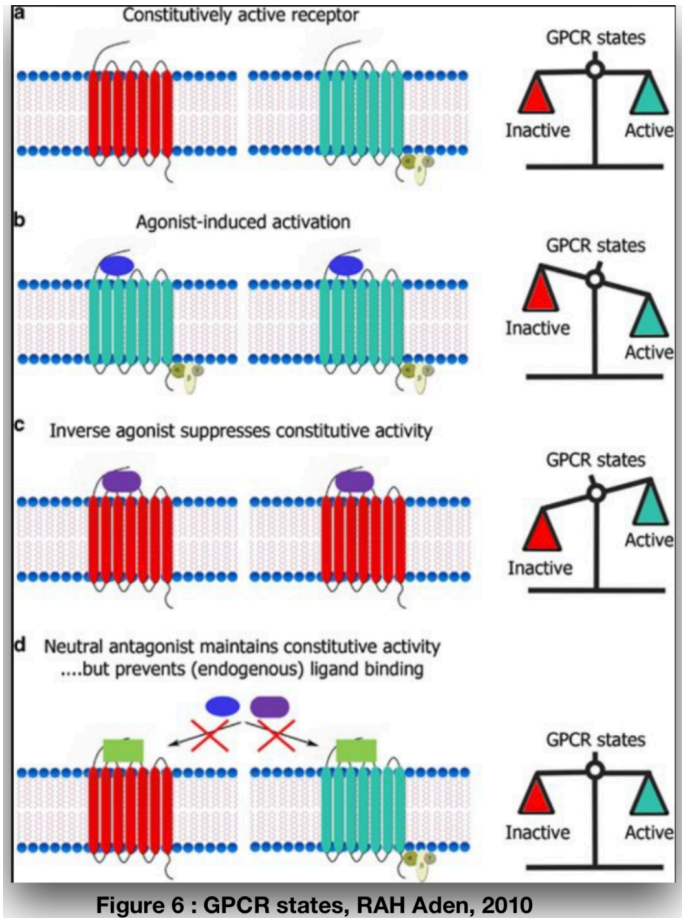

Figure 6 : GPCR states, RAH Aden, 2010

1.7. Antagonists of group I mGluRs

A myriad of studies report favourable outcomes by blocking group I mGluRs to reduce glutamatergic neurotransmission. Studies in a mouse model of fragile X syndrome showed that antagonist of group1 mGluR rescued the LTP facilitation (Xu, 2012). mGluR1 antagonism led to the attenuated cocaine induced conditioned place preference, and cocaine induced lethality (Marcin Bochenski 2009, Q S Liu, 2013). Group I mGluRs antagonism also resulted in the reversal of autistic-like phenotypes caused by deficiency of the eIF4E binding protein 2, a translational repressor (Argel Aguilar-Valles, 2015). Antidepressant effects of mGluR1 antagonists have also been reported in rats and mice. (I.V. Belozertseva, 2006).

While the agonist-mediated endocytosis of group I mGluRs have been studied in some detail, the cellular dynamics of antagonist-mediated internalization of group I mGluRs, if any, remains unknown. The possibility of antagonist mediated endocytosis of group I mGluRs was the focus of this study. This was done using the antagonist, JNJ16259685 (3-ethyl-2-methyl-quinolin-6-yl)-(4-methoxy-cyclo- hexyl)-methanone methanesulfonate) for mGluR1, and the mGluR5 antagonist MTEP ([(2- methyl-1,3-thiazol-4-yl)ethynyl]pyridine) following literature review as cited above. Dual antibody labelling and imaging were used to elucidate the fate of the cell surface group I mGluRs upon antagonist stimulation. We also studied the receptor kinetics and dose response.

Chapter 2
Materials and methods

2.1 Materials

2.1.1 Cell culture reagents:

Dulbecco's modified eagle medium (DMEM), Neurobasal medium, B27 supplement, antibiotic–antimycotic mix, Polyethylenimine (PEI), fetal bovine serum (FBS), DPBS, distilled water and most other cell culture reagents were obtained from Invitrogen (USA). Ampicillin, paraformaldehyde (PFA), poly-D-lysine, fluorodeoxyuridine (FUDR), Fluoromount™ aqueous mounting medium were purchased from Sigma (USA). HEK293T cells were purchased from NCCS Pune (India).

2.1.2 Constructs:

The myc-mGluR5b and myc-mGluR1a in pRK plasmids were gifted by Kathrine Roche (National Institute of Health, USA). Both constructs carried a myc epitope on the N terminus of the full length cDNA.

2.1.3 Antibodies:

Anti-myc mouse monoclonal antibody was purchased from AbCam (UK) and the secondary antibodies, namely goat-anti-mouse Alexa Fluor® 568 and goat-anti-mouse Alexa Fluor® 647 were purchased from Life Technologies®, USA.

2.1.4 Drugs:

3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine hydrochloride (MTEP Hydrochloride) was purchased from Sigma®, USA. (3,4-Dihydro-2H-pyrano[2,3-b]quinolin-7-yl)-(cis-4-methoxycyclohexyl)-methanone (JNJ16259685) was purchased from Santa Cruz Biotechnology, USA and 2,4- bis dihydroxy phenyl glycine (DHPG) was purchased from Tocris®, USA.

2.1.5 Plastic wares and chemicals:

Plastic and glass wares for tissue culture studies were purchased from Thermo Scientific®; Falcon®, USA; Tarsons®, India; Fisher Scientific®, USA and Globe

Scientific, USA. Bacterial media, agar, salts and buffers were purchased from HiMedia®, France. Chemicals were procured from Merck limited®, USA.

2.1.6 Cell culture related instruments:

Cell culture hoods were from Labconco (USA), dissection microscope was from Olympus (Japan), tissue culture incubators, Eppendorf 5810R centrifuge was from Eppendorf (Germany), pipette aids were from Thermo Fisher Scientific (USA), water bath was from Grant (UK), seesaw rocker was from Stuart (UK).

2.1.7 Buffers and media:

10X phosphate buffered saline (PBS): 2 g KCl, 80 g NaCl, 2.4 g KH_2PO_4, 14.4 g Na_2HPO_4 were dissolved in 800 ml of MQ water. The pH was adjusted to 7.4, the volume made up to a litre and the buffer was autoclaved.

4% paraformaldehyde (PFA): 4 g paraformaldehyde was dissolved in 70 ml of PBS (1X) at 60°C by adding 1 M NaOH till solution turned transparent. The pH of the solution was adjusted to 7.4 and the volume was made up to 100 ml. Aliquots of 5 ml were made and stored at -20°C.

DMEM: DMEM and 3.7 g of sodium bicarbonate were dissolved in 800 ml of MQ autoclaved water. The pH of the media was adjusted to 7.4, the volume was made up to a litre. The media was sterilized using a 0.45 µm filter at vacuum.

10% DMEM: 10% fetal bovine serum (FBS) and antibiotic-antimycotic (1X) mix were added to the DMEM media.

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

2.2 Methods:

2.2.1 Preparation of competent cells

A single bacterial colony of DH5α was taken from a culture plate and incubated in 10 ml LB media overnight at 37°C.100 µl of this primary culture was taken and incubated in

100 ml LB media till the optical density of the culture reached 0.4 - 0.6. To pellet down the cells, the culture was centrifuged for 15 minutes at 6000 rpm at 4°C. The pellet was resuspended in 10 ml of 0.1 M ice-cold $CaCl_2$ solution and kept on ice for 15 min.

Subsequently the solution was centrifuged at 6000 rpm at 4°C for 15 min. Supernatant was discarded and then the pellet was resuspended in 5 ml of 0.05 M $CaCl_2$ solution and incubated on ice for 45 min. The cell recovery was done by spinning down the solution at 2000 rpm at 4°C for 5 min. The pellet obtained was finally resuspended in 85% 0.1 M $CaCl_2$ solution and 15% glycerol. Aliquots of 100µl were made and stored in -80°C.

2.2.2 Transformation

The competent cells were thawed on ice for 10 minutes. 1 µg DNA was added and incubated on ice for 30 min. Heat shock was given at 42°C for 90 seconds and the cells were transferred back on ice for 5 min. Subsequently, 1 ml LB media was added and the culture was incubated at 37°C for an hour in water bath. Media was then centrifuged at 5000 rpm for 5 min. The pellet was resuspended in 200 µl LB media and plated on antibiotic containing LB agar plates.

2.2.3 Plasmid isolation

Isolation of plasmids for transfection purposes in cells was done using Qiagen® and Fermentas® mini kits according to the instruction manual.

2.2.4 Cell culture and transfection:

A. HEK293 cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with10% fetal bovine serum, antibiotic–antimycotic mix (1X) at 37 °C, 95% humidity and 5% CO_2. Cells were grown to confluence of 65-70% on 35 mm coverslips coated with 50 µg/ml poly-D-lysine in 24 well plates and transfected with myc-mGluR5 or myc-mGluR1. Transfection mix per well contained 1 µg of DNA and PEI in three times concentration of the DNA. Cells were restored back to 10% DMEM media and the constructs were allowed to express for 24-30 hours prior to performing experiments.

B. Hippocampi were dissected from P0/P1 C57BL/6 mouse pups, trypsinised, dissociated and plated on Poly-D-lysine Sodium borate coated cover-slips in 24 well plates. Primary neurons were cultured in Neurobasal-B27 medium (Gerard M J Beaudoin III, 2012). The neurons were maintained in culture at 37˚C, 5% CO_2. FUDR®, Fluorodeoxyuridine was added on DIV4 to inhibit growth of glial cells.The neurons were transfected on DIV 7-8 with plasmid DNA by Calcium Phosphate method. 3 µg of DNA was mixed with $CaCl_2$ (2.5 M) and MilliQ water (per coverslip), incubated for 5 minutes and this mixture was mixed with HEBS (2X) solution, 1/10th volume at a time. The mix was incubated for 30 minutes. 30µl of the transfection mix was added to the wells containing plain Neurobasal media such that it spread uniformly over the surface. In 45-60 minutes, when a sufficient amount of precipitate was formed, it was washed thrice using the wash buffer till the crystals disappeared when observed under the bright field microscope. Cells were restored back to B27 supplemented Neurobasal medium. Experiments on the transfected cells were carried out at DIV 12-15.

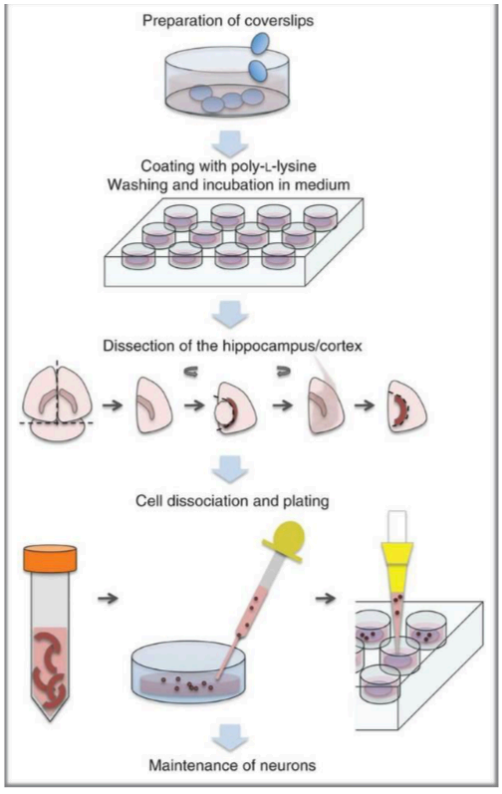

Figure 7: Primary hippocampal neuron culture (Bhattacharyya et al., 2018)

2.2.5 myc-mGluR1 receptor internalization studies

Group I mGluR endocytosis assay

HEK293 cells and primary hippocampal neurons were transfected with myc-mGluR1 or myc-mGluR5 cDNA using calcium phosphate method as described above. For the endocytosis assay, prior to the labelling with primary antibody, blocking was done in the coverslips for 5 minutes using normal goat serum at 37˚C, 5% CO_2. Live cells were labelled with anti-myc mouse monoclonal primary antibody (1:200) for 30 min at 37˚C for primary hippocampal neurons. Cells were then washed with plain Neurobasal media and treated with or without the agonist DHPG (100 μM) for 5 minutes, or the antagonist (MTEP, 100 μM for mGluR5 and JNJ 30μM for mGluR1). Subsequently, cells were chased for 25 minutes in absence of the ligand. HEK cells were treated the same way except the concentration of the primary antibody used was 1:500 for 15 minutes and the media was plain DMEM. Cells were shifted to ice and fixed with ice cold 4% PFA for 15 min without permabilization.The fixed cells were stained differentially with two different secondary antibodies, to differentiate the surface receptors from the internalized pool of receptors. To label almost all the surface receptors, cells were incubated with a saturating concentration of the secondary antibody, goat-anti-mouse Alexa 568 (1:100) in 2% NGS for 1 hour at 37˚C. To label the internalized pool of receptors, cells were then permeabilized with 0.1% Triton X-100 for 30 min at room temperature. The second secondary antibody, viz., goat anti-mouse Alexa-647 (1:800) for 1 hr at 37˚C was applied after blocking with 2% NGS for 1 hour at 37˚C. Coverslips were washed prior to mounting on glass slides using fluoromount.

The coverslips were imaged under the confocal microscope (Zeiss LSM 780).

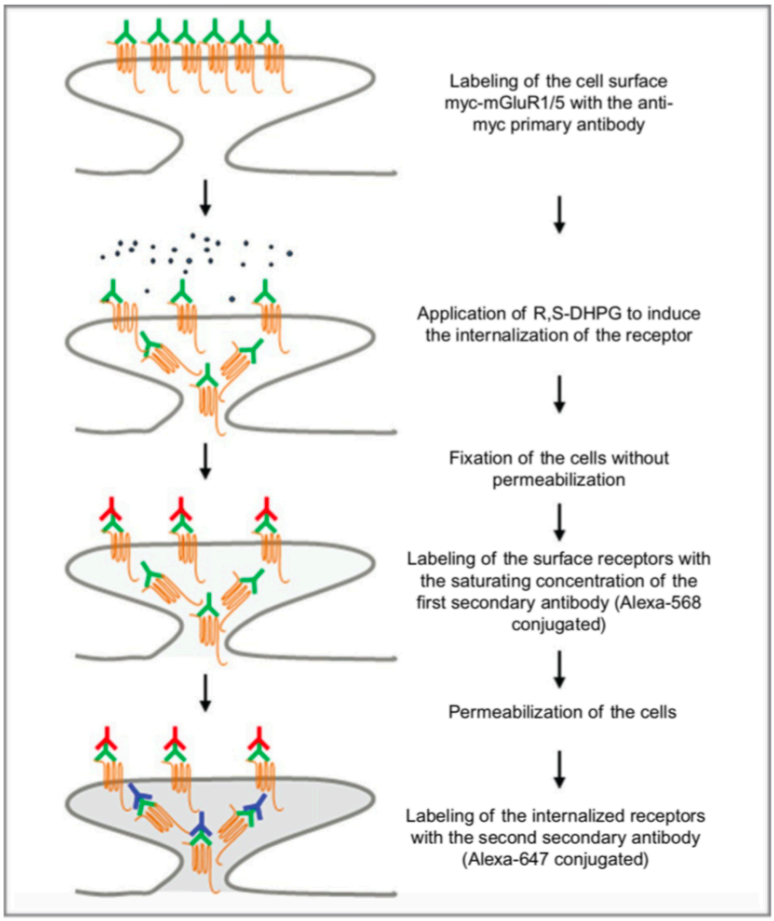

Figure 8: Dual antibody feeding assay in live cells

2.2.6 Receptor recycling assay

Primary hippocampal neurons were transfected with myc-mGluR1 cDNA as described previously. Subsequent to anti-myc primary antibody labelling, cells were given a pulse of 5 min with the antagonist JNJ (30µM) at 37˚C. The cells were subsequently chased in plain Neurobasal media at different time points in the absence of the ligand. Cells were fixed at specific time points and the surface and endocytosed pool of receptors were labelled with Alexa-568 conjugated and Alexa-647 conjugated secondary antibodies respectively using the protocol of endocytosis assay described above.

2.2.7 Time course experiments

Primary hippocampal neurons were transfected with myc-mGluR1 cDNA as described previously. Subsequent to anti-myc primary antibody labelling, cells were given a pulse of 5 min with the antagonist JNJ (30µM) at 37˚C. After washing in plain media, cells were either fixed or chased for various time periods (1 min, 2 min, 5 min, 15 min, 30

min) at 37˚C. The surface and internalized pool of receptors were labelled with Alexa-568 conjugated and Alexa-647 conjugated secondary antibodies respectively using the protocol of endocytosis assay described above.

2.2.8 Dose Response experiments

HEK293T cells and primary hippocampal neurons were transfected with myc-mGluR1 cDNA as described previously. Subsequent to anti-myc primary antibody labelling, cells were given a pulse of 5 min with different concentrations of the antagonist JNJ (10 nM, 100 nM, 1 μM, 10 μM, 30 μM) at 37˚C. Cells were chased in plain DMEM for 25 minutes and fixed on ice. The pool of surface and internalized receptors were differentially stained using the protocol described previously.

2.2.9 Imaging

Images were obtained in Zeiss LSM 780 confocal laser scanning microscope using 63X oil immersion objective. Digital gain, digital offset, laser power and all other parameters were set in accordance with experimental controls and were preserved throughout the experiment.

2.2.10 Analysis and statistics

ImageJ, an open source software (NIH, USA) was used to analyse all images. A threshold value was set for individual fluorophores initially by projecting all the Z planes maximally. These values remained constant throughout the experiment. Using the software, the total thresholded area of fluorescently labelled surface and internalized receptors was acquired and endocytosis index was calculated as the following metric:

$$\textbf{Endocytosis Index} = \frac{\textbf{Internal}}{\textbf{(Surface + Internal)}}$$

The cell-cell variability for the total receptor levels were accounted for, by normalization against control values for the same experiment. Thus, for any experimental set, normalization effectively gave the fold change in the endocytosis index for that particular condition.

For hippocampal neurons, soma was defined by drawing a circle of 20 μm diameter around the cell body and the remaining area of the cell represented the dendrites. Results are shown for whole cell, cell body and dendrites by the analysis described above. Statistical significance was checked using the two-tailed t-test. $P > 0.05$ was considered statistically insignificant.

Chapter 3

Results

3.1 Endocytosis of mGluR1 upon agonist stimulation in HEK293 cells and primary hippocampal neurons

In control cells (untreated) a major fraction of the receptors was localized on the surface of the cell **(1.00 ± 0.08)**. Application of the ligand viz.,100 μM DHPG, a specific agonist of the group I mGluR family, caused a significant fraction of the receptors to internalize **(1.85 ± 0.08).** This suggested that myc-mGluR1 internalized on agonist binding in HEK293T cells.

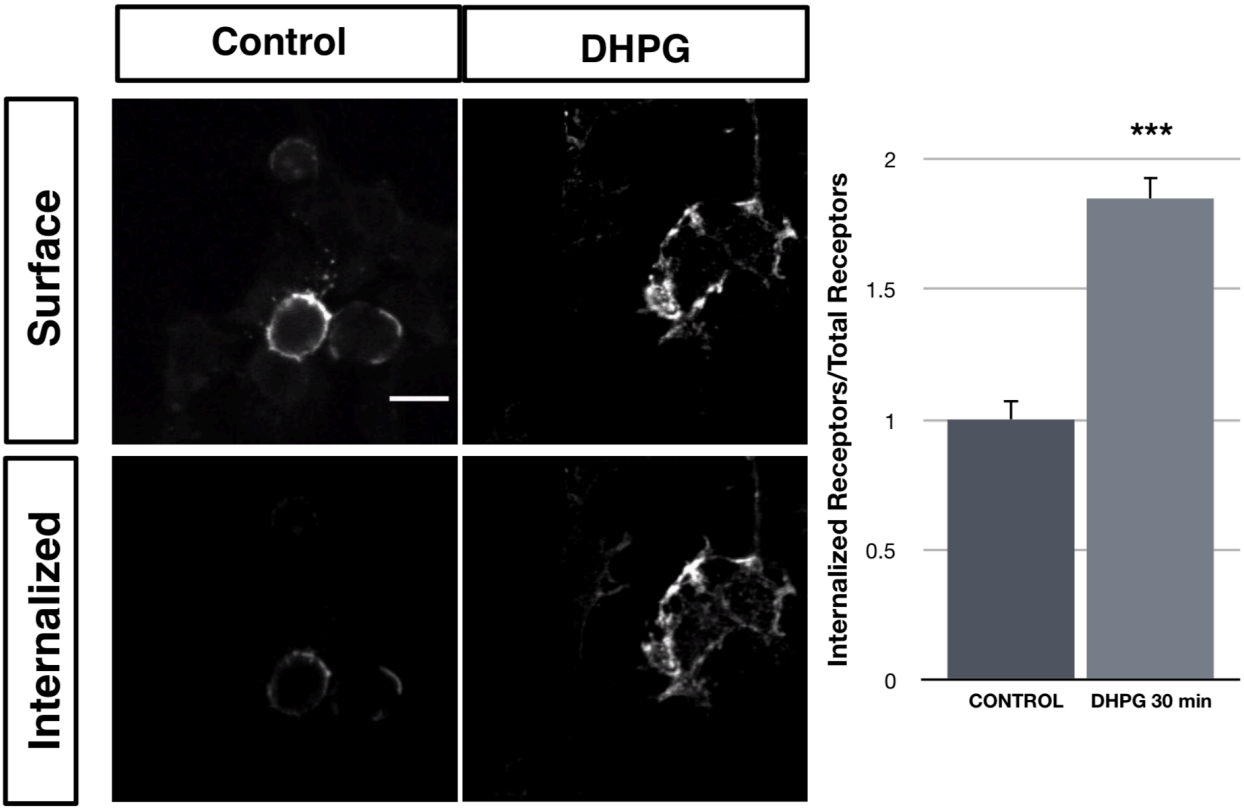

Figure 9 : myc-mGluR1 undergoes endocytosis in 30 mins after agonist (DHPG) treatment in HEK293T cells

The same myc-mGluR1 construct was transfected in primary hippocampal neurons. In control cells, majority of the receptors localized at cell surface **(1.00 ± 0.15)**. Upon agonist application, a significant fraction of receptors endocytosed in primary hippocampal neurons **(1.99 ± 0.22)**.

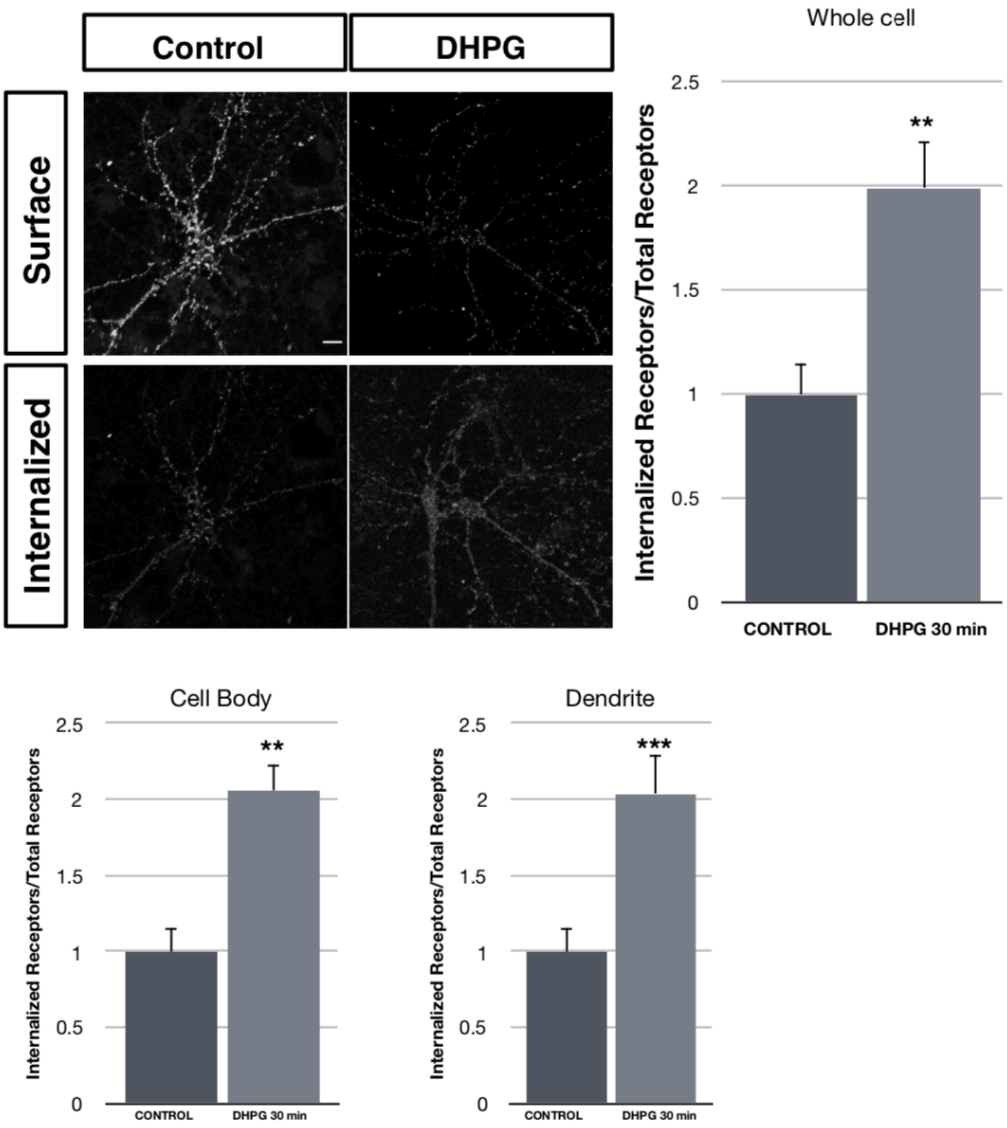

Figure 10 : myc-mGluR1 undergoes endocytosis in 30 mins after agonist (DHPG) treatment in primary hippocampal neurons

3.2 Endocytosis of myc-mGluR1 upon antagonist (JNJ) application in primary hippocampal neurons

We studied endocytosis of myc-mGluR1a in primary hippocampal neurons using the live cell dual antibody staining assay as explained before. We observed that there was comparable endocytosis of myc-mGluR1 at 30 mins **(1.53 ± 0.09**) upon antagonist application as seen in case of agonist-mediated internalization **(1.73 ± 0.26).**

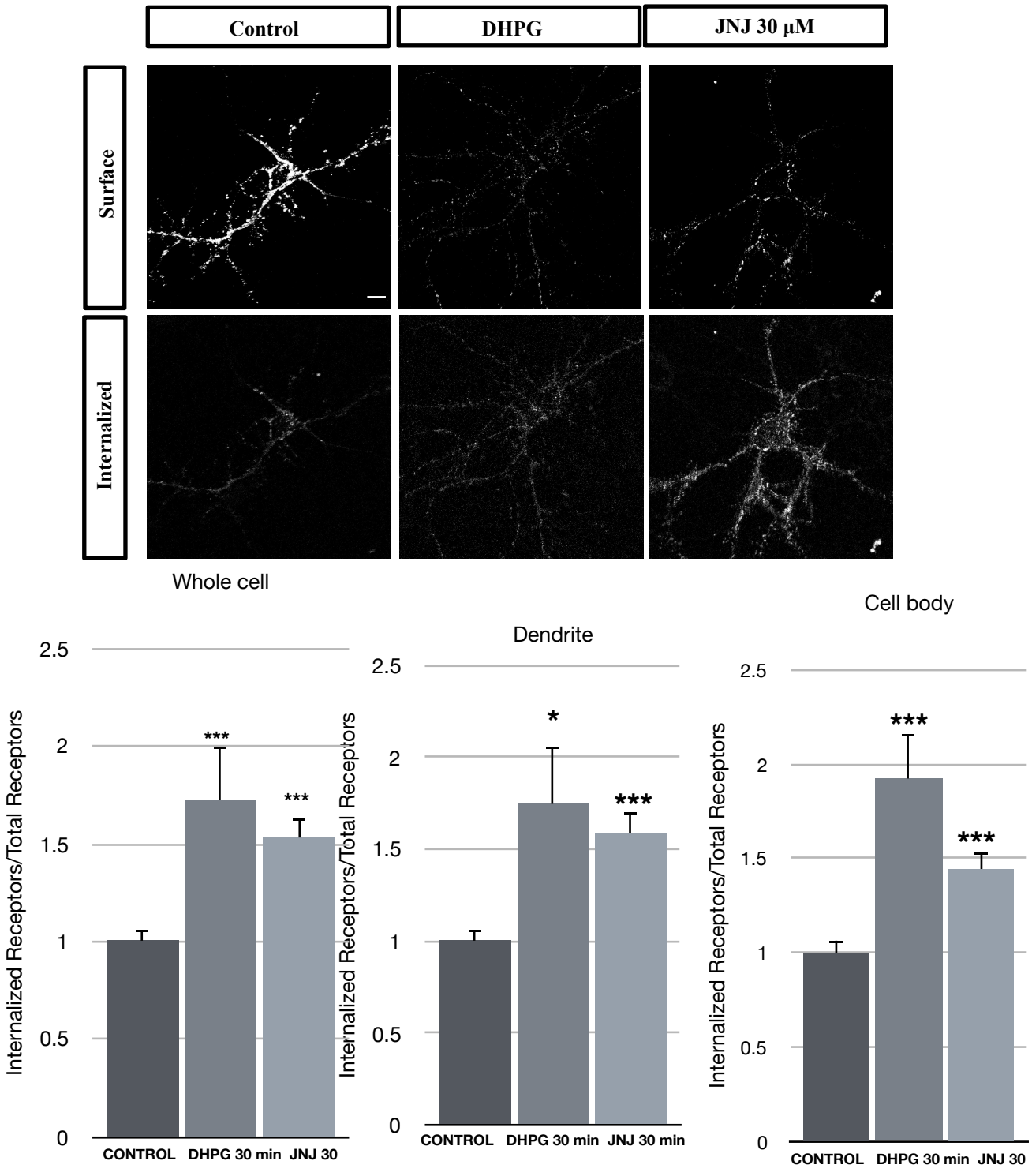

Figure 11 : myc-mGluR1 undergoes endocytosis in 30 mins after antagonist (JNJ) treatment in hippocampal primary neurons.

3.3 Endocytosis of myc-mGluR5 upon antagonist (MTEP) application in HEK293T cells.

To validate the antagonist-mediated internalization of group I mGluRs, we studied the internalization of mGluR5 upon binding of the non-competitive, specific antagonist MTEP. We observed that the internalization index was substantially higher in antagonist-treated cells **(1.55 ± 0.13)** as compared to control cells **(1.00 ± 0.05)**, although slightly lesser than agonist-treated cells **(1.80 ± 0.23)**.

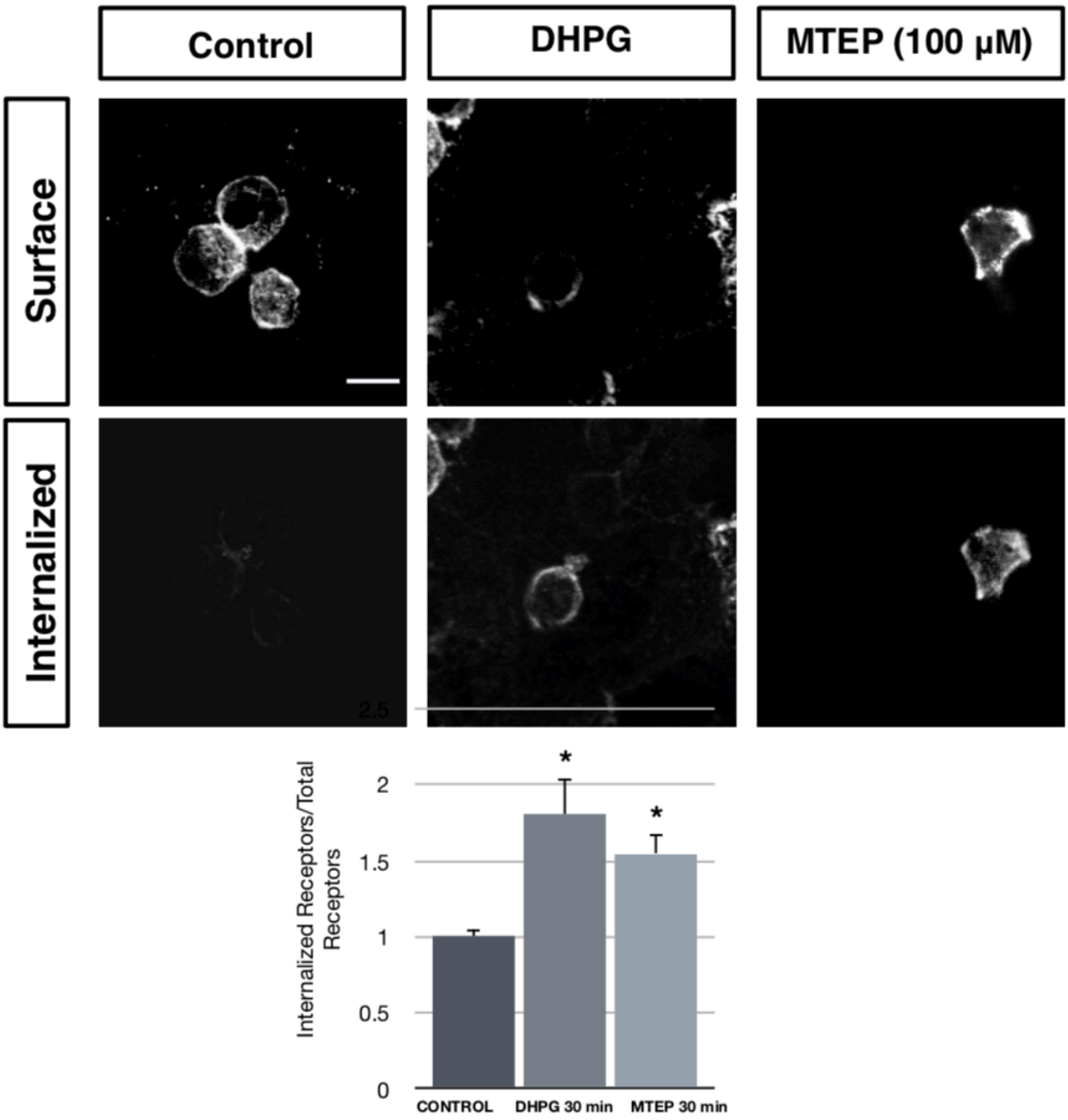

Figure 12 : myc-mGluR5 undergoes endocytosis in 30 mins after antagonist (MTEP) treatment in HEK293T cells.

3.4 Time course of antagonist-mediated myc-mGluR1 internalization in HEK293T cells

We studied the time course of endocytosis of myc-mGluR1a in HEK293T cells upon application of the antagonist using the live cell dual antibody staining assay as explained before. We observed a gradual increase in the endocytosis from **1 min (1.17 ± 0.17), 2 min (1.31 ± 0.18), 5 min (1.37 ± 0.09), 15 min (1.63 ± 0.21) and 30 min (1.66 ± 0.27).** The endocytosis index reached a maximum at 15 mins and 30 mins post antagonist application.

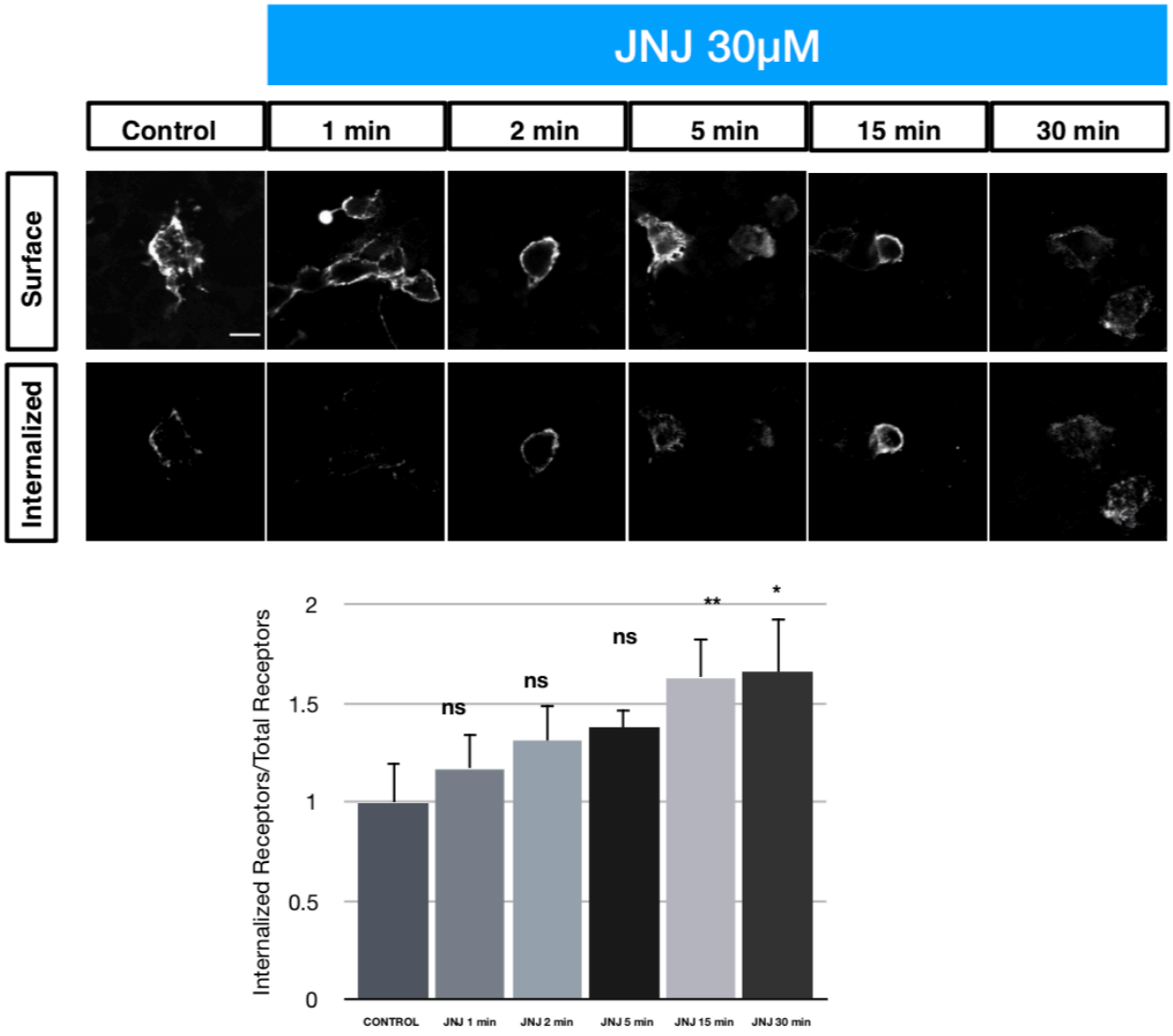

Figure 13 :Time course of myc-mGluR1 internalisation upon antagonist treatment in HEK293T cells

3.5 Dose response of internalization of myc-mGluR1 upon antagonist treatment in primary hippocampal neurons

The antagonist-mediated endocytosis of myc-mGluR1 in primary hippocampal neurons was seen to be dose dependent with negligible internalization observed at **10 nM (1.11 ± 0.04), 100 nM (1.09 ± 0.06), 1 µM (1.04 ± 0.07)** and gradually increased from 10 µM (1.23 ± 0.03) to reach a maximum at **30 µM (1.51 ± 0.11)** concentration of the antagonist, JNJ16259685.

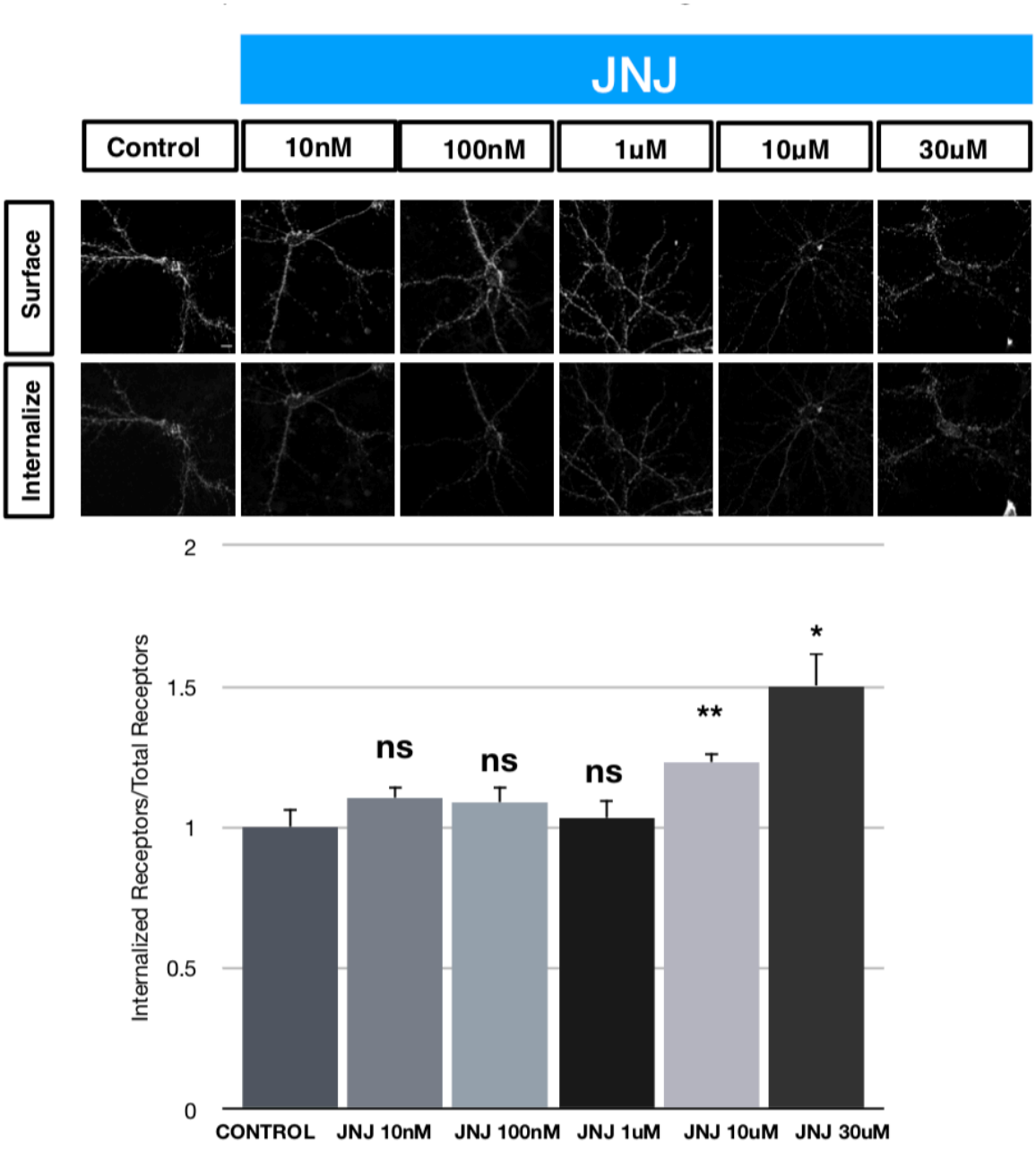

Figure 14 : myc-mGluR1 undergoes maximum endocytosis at 30µM concentration of antagonist treatment in primary hippocampal neurons

3.6 myc-mGluR1 recycles back to the cell surface subsequent to the antagonist-mediated internalization in primary hippocampal neurons

Colocalization studies performed by Ms. Mekhla Rudra in the lab established that a majority of the antagonist-mediated internalized receptors were colocalized with Rab11, the recycling endosome marker and entered the recycling route post internalization (data not shown). To investigate the time frame of the receptor recycling, we performed the recycling assay in primary hippocampal neurons as described before . We observed that there was a gradual increase in the endocytosis index from **control (1.00 ± 0.08) to 4.5 hr (1.71 ± 0.05)**, and at **7.5 hr the receptor recycled back to the cell surface (1.02±0.05).**

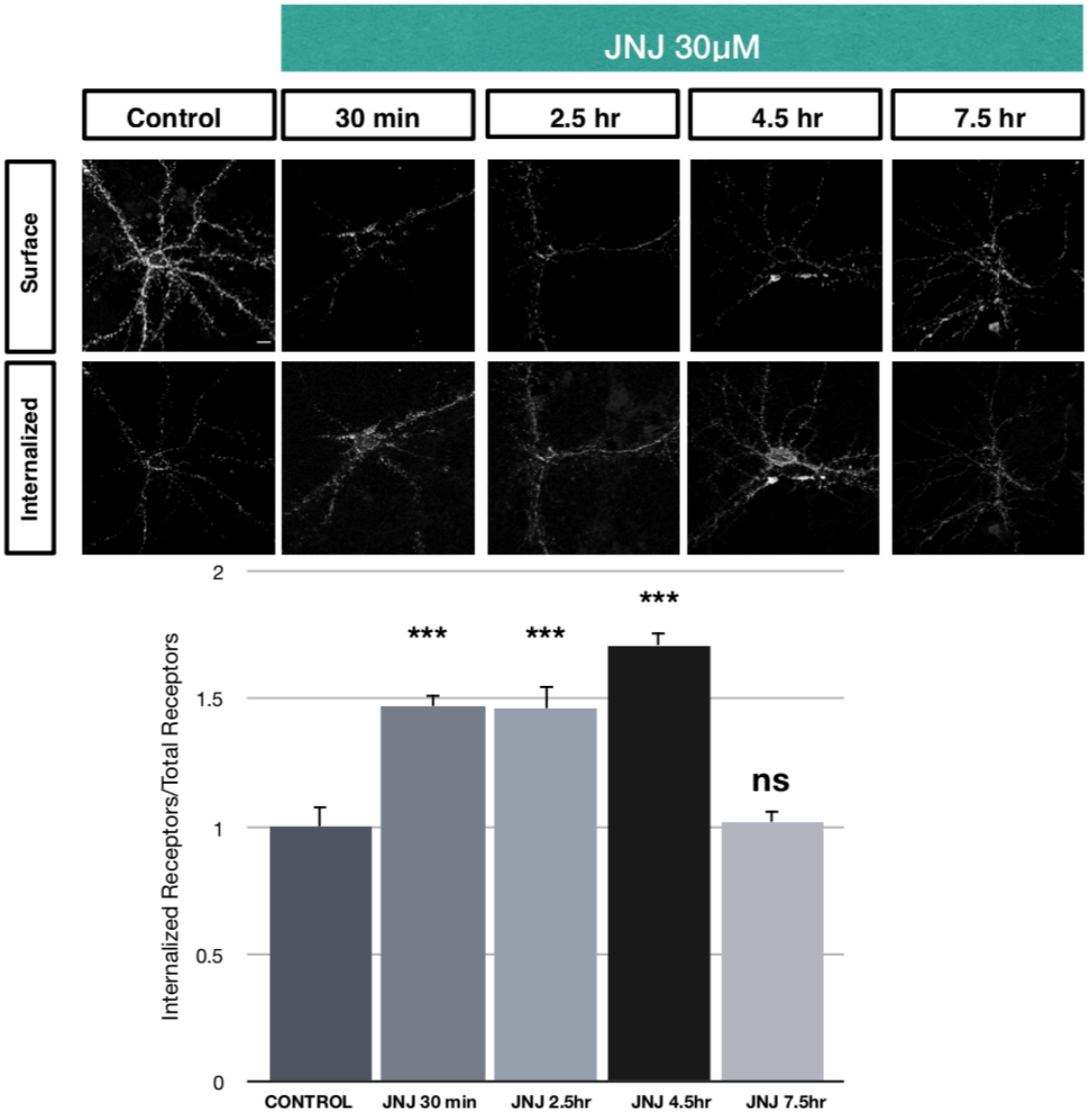

Figure 15 : myc-mGluR1 recycles back to the cell surface by 7.5 hrs post antagonist treatment in primary hippocampal neurons

Discussions :

Group I mGluRs are known to play crucial roles in physiological processes including neurodevelopment and regulation of various kinds of synaptic plasticity. Their aberrant signaling has been therefore implicated in neuropsychiatric disorders such as Fragile X syndrome, autism, schizophrenia etc. Various antagonists developed against group I mGluRs have emerged as potential therapeutic drugs for these neurological conditions (Ferraguti et al., 2008). Desensitization and internalization are considered to be protective mechanisms to prevent hyper-regulation and excessive signalling through the system. Furthermore, internalization is crucial for downregulation of some receptors and resensitization for some other receptors. While agonists are known to cause the desensitization, internalization and further resensitization, thus regulating the receptor turnover at the cell surface, the role of the antagonists of group I mGluRs in internalization and consequently elucidating the fate of the receptor at the cell surface was the primary focus of this study.

The major findings of this study are as follows:

- myc-mGluR1 is endocytosed in 30 min upon agonist stimulation (DHPG, 100 μM) in both HEK293T cells and primary hippocampal neurons.
- mGluR1 antagonist JNJ16259685 causes endocytosis of myc-mGluR1 at 30 min post antagonist treatment in primary hippocampal neurons.
- Maximum internalization of myc-mGluR1 is at 30 μM concentration of the antagonist JNJ16259685 in primary hippocampal neurons.
- Maximum receptor sequestration is seen at 15 min post antagonist treatment in HEK293T cells.
- myc-mGluR1 recycles back to the cell surface between 4.5 hrs and 7.5 hrs post antagonist treatment in primary hippocampal neurons.
- mGluR5 antagonist MTEP causes endocytosis of myc-mGluR5 at 30 min post antagonist treatment in HEK293T cells.

Future directions:

1. Studying the mechanisms of the sequestration processes in antagonist-mediated internalization
2. Investigating the role of kinases in antagonist-meditated internalization of group I mGluRs.
3. Investigating the role of adaptor proteins such as Homer, Tamalin, PICK1 etc. if any, in antagonist-mediated endocytosis of group I mGluRs.
4. Investigating the role of phosphatases in the recycling of the receptors.
5. Investigating if the recycled pool of receptors can activate the second messenger response upon encounter with the agonist .
6. To check if the agonist and antagonist of group I mGluRs target the same pool of the receptor.

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