Understanding the role of Ubiquitination in the constitutive endocytosis of Group I Metabotropic Glutamate Receptors

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This dissertation is submitted for the degree of

BS-MS

IISER Mohali

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To my teachers, without whose efforts I would not be in this position.

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.

Ritu Roy Chowdhury
(candidate)

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

Dr. Samarjit Bhattacharyya
(supervisor)

Dated: April 22, 2016

Certificate of Examination

This is to certify that the dissertation titled **Understanding the role of Ubiquitination in the constitutive endocytosis of Group I Metabotropic Glutamate Receptors**, submitted by **Ms. Ritu Roy Chowdhury** (Registration Number: MS11025) for the partial fulfilment of the BS-MS dual degree programme of the Indian Institute of Science Education and Research, Mohali, 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. Kavita Babu Dr. Samarjit Bhattacharyya (supervisor)

Dated: April 22, 2016

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Abstract

This study seeks to augment our understanding of how the activity of Group I metabotropic glutamate receptors (mGluRs) is regulated in the absence of glutamate. Trafficking of these receptors after glutamate-binding, has been extensively studied, but not much is known about their ligand-independent endocytic-trafficking. I also investigated if ubiquitination, one of the major post-translational modification, regulates constitutive endocytosis of these receptors. mGluRs are GPCRs that have the capacity to trigger several signal-transduction cascades in a cell and are major targets of GPCR-directed pharmacotherapy. Group I mGluRs are pertinent for neurodevelopment, circuit formation, synaptic plasticity, neurodegeneration and induction of reactive astrocytes. Deficiencies in these diverse functions lead to an array of neurological and neurodegenerative disorders. Compelling studies have shown that exaggerated signalling through mGluR5 lead to cognitive and syndromic features characteristic Fragile X syndrome. In fact, mGluR5 antagonists revert phenotypes of Fragile X syndrome. These receptors play a definite role in maintaining the excitation-inhibition balance in the neural circuits. Consequently, the loss of mGluR signalling can have devastating effects including inefficient synaptic plasticity, culminating in loss of learning and memory.

In this study, I determined if mGluR1 and mGluR5 undergo ligand-independent (constitutive) endocytosis in the heterologous cell line, HEK293 and mouse primary hippocampal neurons. Further, I was interested to see if ubiquitination is involved in the constitutive trafficking of these receptors. My data suggest that both group I mGluRs undergo constitutive endocytosis in primary neurons and this process is regulated by ubiquitination. Upon pharmacologically inhibiting the ubiquitin activating enzyme E1, these receptors remain on the cell surface. I also observed that the mutant form of mGluR1 which lacks a critical Lysine residue at the C-terminal end, undergoes constitutive endocytosis, despite not undergoing internalization upon ligand binding. This suggests that ligand-independent endocytosis perhaps occurs through a pathway, not involved in the ligand-mediated internalisation of

mGluR1. Therefore, my study adds to our understanding of the basal level endocytosis of these receptors, which is crucial for normal homeostatic brain function.

Contents

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Chapter 1

Introduction

1.1 Glutamatergic signalling: The players

L-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. At the synapse, two distinct classes of receptors bind to this neurotransmitter, namely ionotropic (iGlu) and metabotropic Glutamate (mGlu) receptors (see Figure 1.1).

While iGluRs act as glutamate gated ion-channels, acting rapidly upon activation, mGluRs trigger signal transduction cascades that can have long lasting effects on synaptic transmission and cellular signalling and metabolism [1].

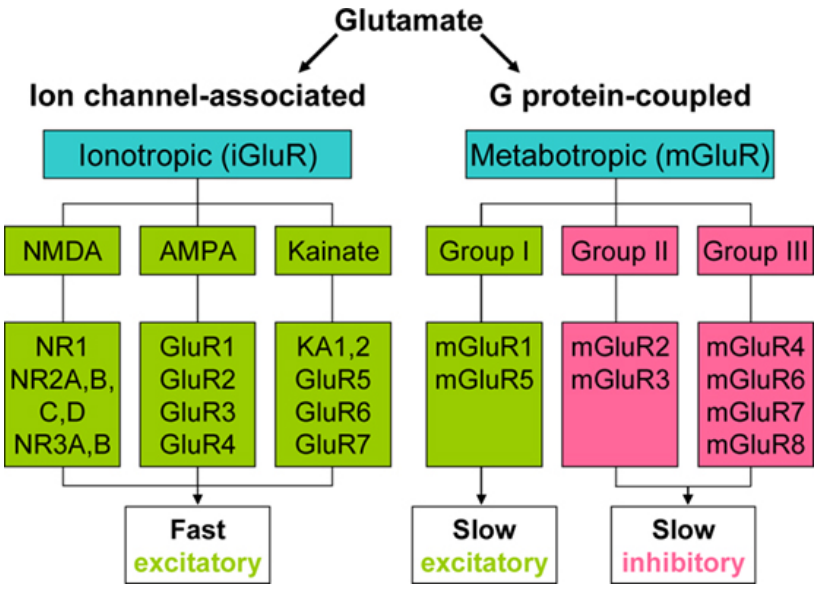


Figure 1.1: Types of Glutamate receptors [1]

1.2 Metabotropic Glutamate receptors

Metabotropic glutamate receptors show a rich diversity in terms of sequence similarity, distribution in the central and peripheral nervous system, cellular localisation and agonist preference, based on which they are divided into three groups (see Figure 1.2). They are G-protein coupled receptors and are bound to different trimeric G-proteins and coupled to accompanying downstream targets [1].

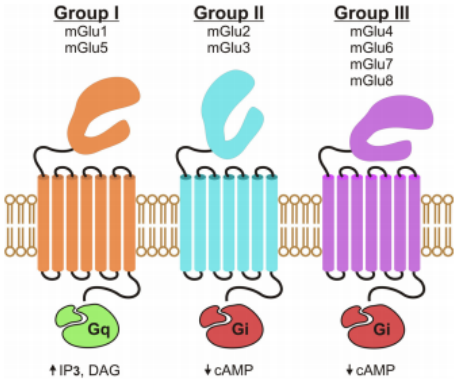


Figure 1.2: mGluR families[1]

1.2.1 Group I metabotropic glutamate receptors

Group I mGluRs are differentially distributed in the central nervous system [8]. They are also widely present in the peripheral nervous system and non-neural tissues. mGluR1 is highly expressed in the CA3 pyramidal neurons of the hippocampus, cerebellum and the olfactory bulb, in contrast mGluR5 is expressed in low levels in the cerebellum, but in high levels in the cortex [9] (see Figure 1.3).

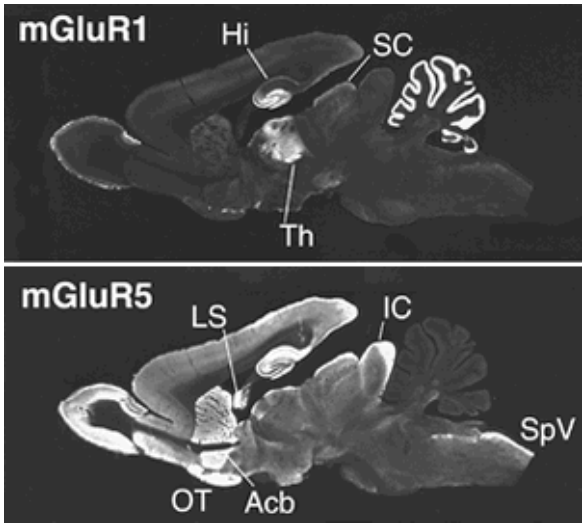


Figure 1.3: Distribution of mGluR1/5 in the rat brain [2]

Group I mGluRs are found in the perisynaptic region of the post-synaptic neuron. They are positively coupled to phospholipase C, and receptor activation triggers phosphoinositide turnover, release of intracellular Ca^{2+}, and activation of Protein Kinase C (PKC). Interestingly, group I mGluRs play different roles in the nervous system despite being greatly similar sequentially and by signalling homology to each other.

1.2.2 Physiological functions and aberrations

Metabotropic glutamate receptors serve important roles in neuronal development, neurodegeneration, induction of reactive astrocytes and synaptic plasticity. Alterations in glutamatergic signalling is the basis of several neurological and neurodevelopmental disorders. For instance, epilepsy, Ischemia, ALS, multiple sclerosis, diabetes, Huntington's disease and Parkinson's disease [9]. Disorders arising out of excitation-inhibition imbalance like autism are also thought to arise due to mGluR dysfunction. mGluR antagonists are the focus of major therapeutic strategies for treating disorders like Fragile-X syndrome [10].

1.2.3 mGluRs in synaptic plasticity

Synaptic plasticity is the long and short term changes in the efficiency of synaptic transmission. LTD and LTP are two forms of synaptic plasticity which are believed to underlie learning and behaviour. Experiments in mGluR1-/- and mGluR5-/- mice have shown that expression of LTD demands selective activation of mGluR1 and LTP requires mGluR1 and

mGluR5 [11, 12]. A proposed mechanism to mediate this effect is thought to be through the internalization of post-synaptic AMPARs following a signalling cascade initiated by the mGluR1/5 [3] (see Figure 1.4).

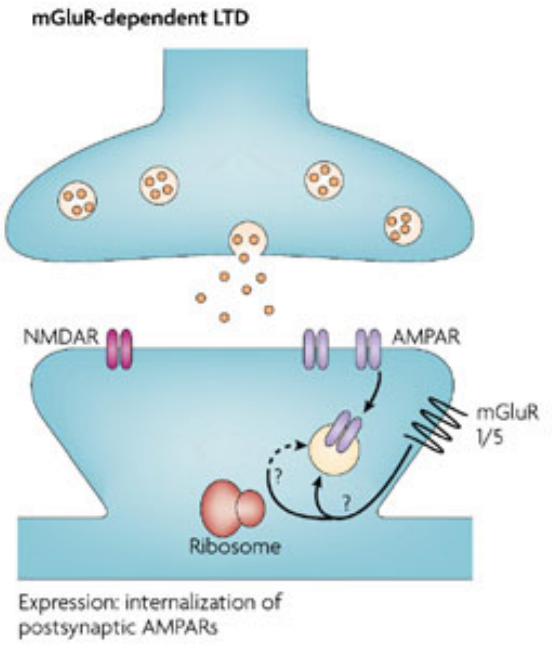


Figure 1.4: mGluR in LTD [3]

1.2.4 Regulation of group I mGluR activity through trafficking: Agonist dependent internalization of mGluRs.

The localisation of group I mGluR is strictly regulated. Also overstimulation of these receptors is prevented by the receptor undergoing desensitisation, followed by internalization and recycling of the receptor back to the membrane for the next round of signalling. β-arrestin, Dynamin, Clatherin dependent mechanism is involved in the agonist dependent mGluR1a internalization [9] (see Figure 1.5). The mechanism of internalization of a receptor is elaborate and specific. It depends on the type of agonist, isoform of the receptor and on the system [4].

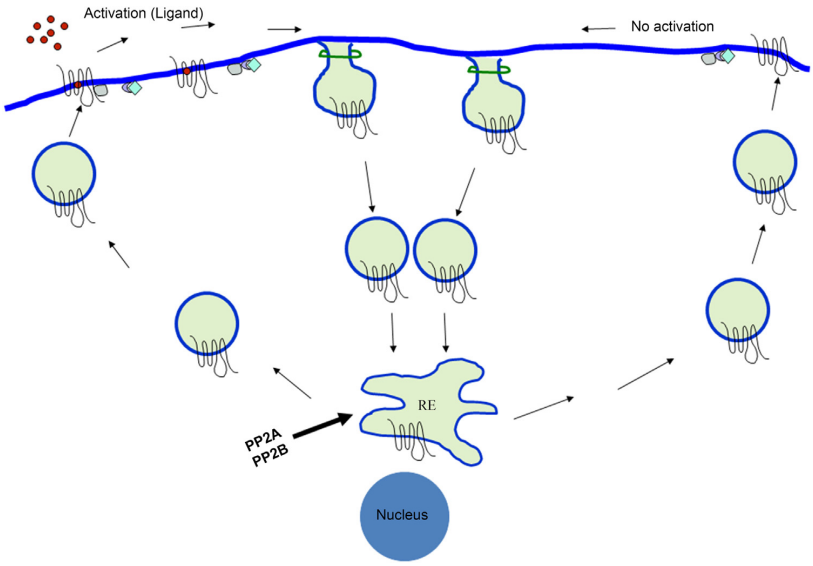


Figure 1.5: Recycling of mGluRs after agonist-dependent and agonist independent internalisation [4].

1.2.5 Constitutive internalization of Group I mGluRs

Constitutive internalization refers to the endocytosis of a receptor in the absence of an agonist (see Figure 1.5). Originally it was thought that constitutive internalization is a result of glutamate being secreted by other cells. But studies done by Dale et. al. [13] and R.R. Trivedi et. al. [14] have shown that ligand-independent internalization is indeed a phenomenon in its own right, as shown in Figure 1.6. This mechanism appeals to the maintenance of receptor homeostasis in the nervous system. In contrast to agonist-mediated internalization, constitutive internalization is thought to be β-arrestin and Dynamin independent, although it also involves the formation of Clathrin-coated pits [9].

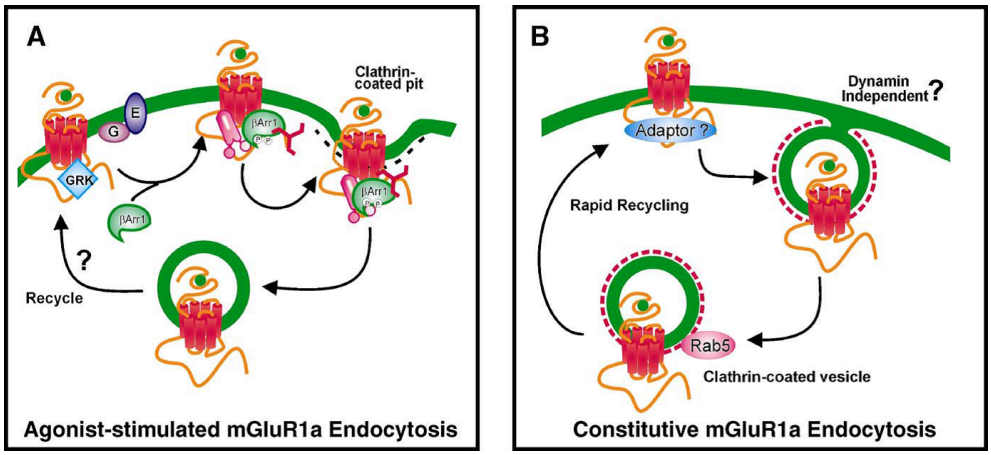


Figure 1.6: Contrast between agonist dependent and independent endocytic pathways

1.3 Objective of the Study

Neurotransmitters and their receptors are crucial for synaptic transmission, plasticity and connectivity. Therefore, their turnover needs to be strictly regulated. Ubiquitination is a unique post-translational modification as it can alter the fate of the substrate by covalently attaching a 76 aa protein to it [6]. The specific fate is determined by exact type of ubiquitination as illustrated in 1.7 and 1.8. Lysosomal sorting of a number of GPCRs, for example, the EGF receptor tyrosine kinase, occurs this way [15].

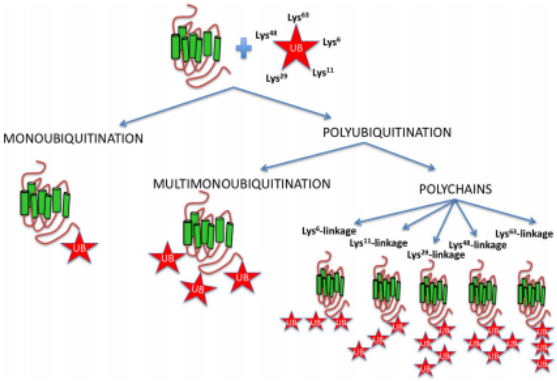


Figure 1.7: Types of ubiquitination [5].

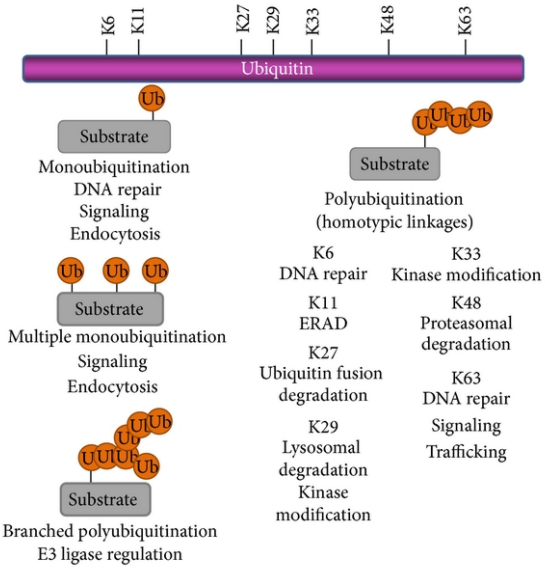


Figure 1.8: Types of ubiquitination and their corresponding functions [6].

Defects in the ubiquitination machinery has been implicated in several neurological and neurodegenerative disorders. Therefore, understanding the function of different types of ubiquitination is crucial. Ubiquitination occurs via three enzymes, namely, Ubiquitin

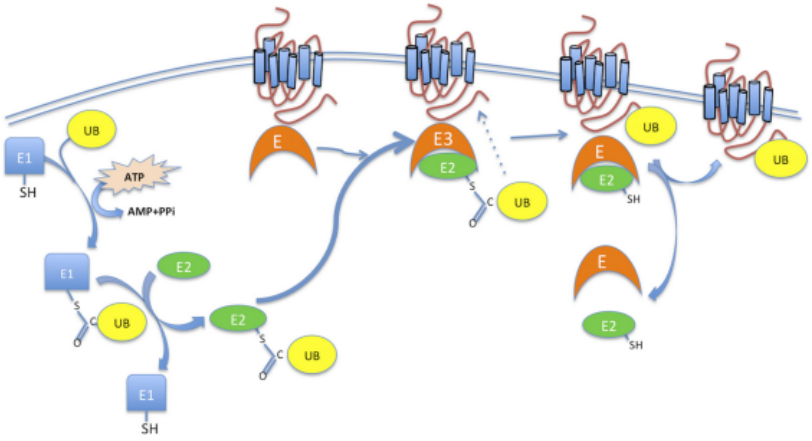


Figure 1.9: Ubiquitination of GPCRs by the sequential action of E1, E2 and E3 enzymes [5].

Activating Enzyme (E1), Ubiquitin Conjugating Enzyme (E2) and Ubiquitin ligase (E3). E3 provides substrate specificity (as depicted in 1.9).

Ubiquitination has been reported to regulate the mGluR-LTD as well as mGluR-mediated AMPAR endocytosis in the hippocampal cells [16]. Moreover, ubiquitination seems to regulate the ligand-mediated internalization of both mGluR1 and mGluR5 (unpublished observations). Therefore, the objective of my study was to investigate if constitutive endocytosis of Group-I mGluRs is also mediated through ubiquitination, as ubiquitination is a chief mechanism for maintaining cellular homeostasis. To this end, I determined if group I mGluRs undergo ligand-independent endocytosis using a live cell dual-antibody staining assay in primary hippocampal neurons. Primary hippocampal neurons are tractable systems to obtain physiologically relevant information. Next, I studied if pharmacological inhibition of ubiquitination has any effect on the constitutive endocytosis of these receptors. Further, a mutant form of mGluR1, which does not undergo ligand-mediated internalization was examined for ligand-independent endocytosis.

Chapter 2

Materials and Methods

2.1 Materials

The experiments were performed on mouse primary hippocampal neurons and Human Embryonic Kidney cells (HEK293) post transfection with the relevant DNA. 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 at the N terminus of the full length cDNA.

Dulbecco's modified eagle medium (DMEM), Neurobasal-B27 medium, lipofectamine, penicillin–streptomycin solution, antibiotic–antimycotic mix, Polyethylenimine (PEI), OptiMEM, trypsin–EDTA, fetal bovine serum (FBS), DPBS, distilled water were obtained from Invitrogen (USA). Ampicillin, paraformaldehyde (PFA), poly-D-lysine, Fluoromount™ aqueous mounting medium were purchased from Sigma (USA). Anti-myc mouse monoclonal antibody was obtained from Biorbyt (UK) while the secondary antibodies, namely goat-anti-mouse Alexa-568 and goat-anti-mouse Alexa-647 were purchased from Invitrogen (USA).

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

2.2 Methods

2.2.1 Cell Culture and Transfection

HEK293 cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, antibiotic–antimycotic mix at 37 ˚C, 5% CO_2. Cells were transfected with myc-mGluR5 cDNA at 65–70% confluency on 35 mm coverslips coated with 50 µg/mL poly-D-lysine. Transfection was done by mixing 2 µg of the DNA with 10 µg of lipofectamine in 1 mL OptiMEM. Experiments were performed 24 hours post transfection.

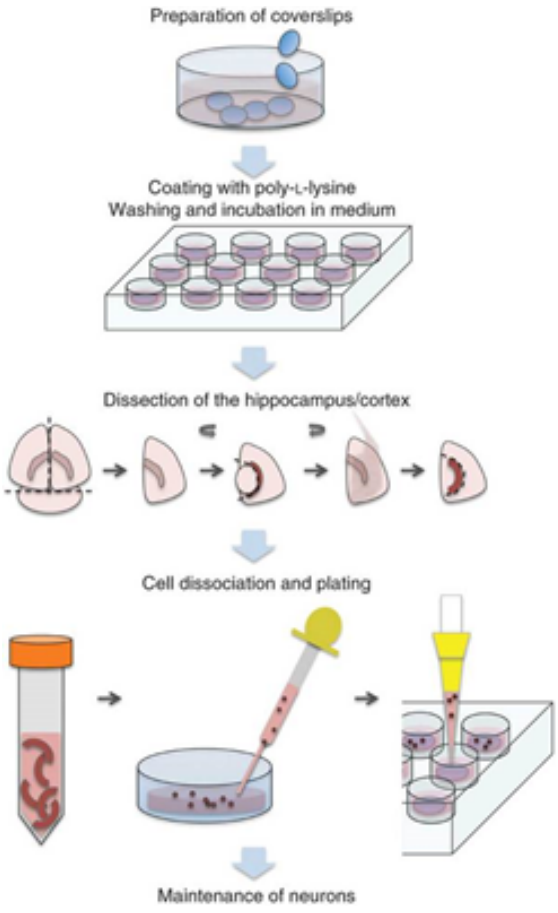


Figure 2.1: Culturing of hippocampal neurons

Primary neurons were cultured (see Figure 2.1) in Neurobasal-B27 medium [17]. Hippocampi were dissected from P0/P1 mouse pups, trypsinised, dissociated and plated in 24 well plates containing Poly-L-lysine Sodium Borate coated cover-slips. The neurons were then maintained in culture at 37˚C, 5% CO_2 for upto 20 days. Fluorodeoxyuridine (FUDR) was added on the fourth day to inhibit growth of glial cells. The neurons were then trans-

fected on the seventh day using the Calcium Phosphate method because of its low toxicity and ease of use [6] as shown in Figure 2.2. For every cover slip, 3 µg of DNA was mixed with $CaCl_2$ (2.5 M) and in MilliQ water, this mixture was mixed drop-by drop with HBS-Phosphate solution. The overall mixture was allowed to incubate for 20-30 minutes and then added to the coverslips immersed in plain Neurobasal media. The neurons were observed every 10 minutes under a compound microscope and the precipitate was washed off when they formed to a sufficient quantity with the washing buffer. The coverslips were restored back to B27 supplemented Neurobasal medium. Experiments were carried out about 5-7 days after transfection.

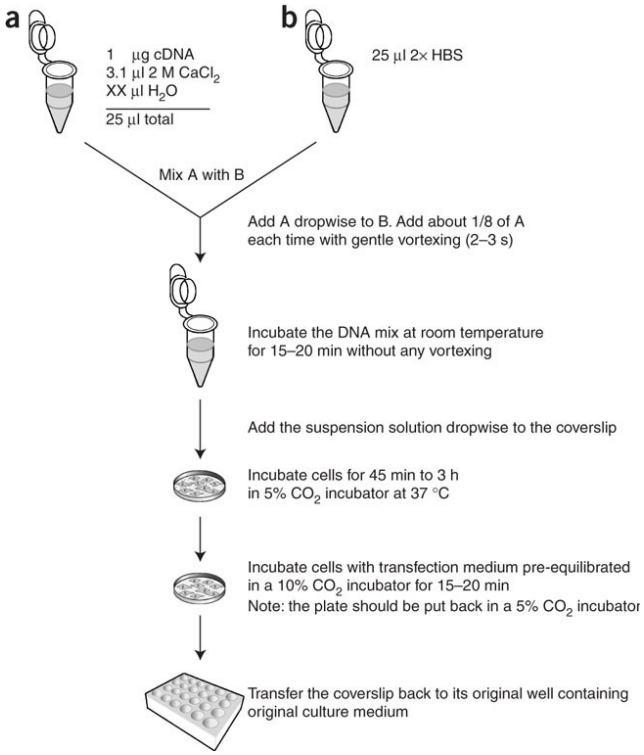


Figure 2.2: Transfection of neurons using Ca^{2+} Phosphate method [7]

2.2.2 Constitutive Endocytosis Assay

Transfected HEK293 cells were taken after 24 hours of transfection. Two coverslips were used for every condition. PYR-41 was used to inhibit the ubiquitin activating enzyme E1 (explained in detail in Section 3.2). Coverslips were incubated in PYR-41 (50 µM) prior to the blocking step. Antibody blocking was applied with 2% Normal Goat Serum for 10 minutes at 37˚C and 5% CO_2. The cells were shifted to ice (4 ˚C) where cellular activity will decline to the minimum. Cells were now incubated in 1:500 anti-myc mouse mono-

clonal antibody for 1 hour on ice. For experiments in primary hippocampal neurons, cells were also treated the same way except for the media being used was Neurobasal instead of DMEM and the concentration of the primary antibody was 1:200. The cells were washed with plain media twice for 5 minutes each and then fixed without permeabilization using ice cold 4 % PFA, this was the 0 minute time point of the experiment. The remaining coverslips were incubated at 37 ˚C for the duration of endocytosis to be observed and then fixed similarly.

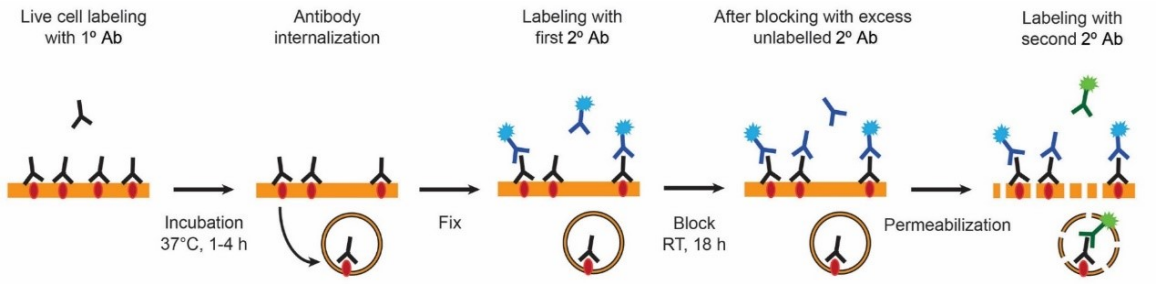


Figure 2.3: Live cell Dual Antibody Feeding Assay (Carrodus, N. L. et. al., J. Vis. Exp. (84), 2014)

The fixed cells were now stained differentially with two different secondary antibodies, which enabled us to distinguish between surface and internalised receptors, as shown in Figure 2.3. The cells were first blocked with 2% NGS and then stained with saturating concentration of first secondary antibody, goat-anti-mouse Alexa 568 (1:100) in 2% NGS for 1 hour at 37 ˚C. This step labelled all the surface receptors. Now the cells were permeabelized with 0.1% Triton X-100 for 30 min at 37 ˚C. PBS (pH 7) was used to wash the coverslips and then after blocking with 2% NGS for 1 hour at 37˚C, the second secondary antibody (goat-anti-mouse Alexa 647) was applied (1:800) in 2% NGS for 1.5 hours at 37 ˚C. The coverslips were then washed and mounted using Fluoromount™ aqueous mounting medium on glass slides.

The slides were then imaged under a confocal microscope.

2.2.3 Image Acquisition

The glass slides were imaged under the FLUOVIEW FV10i Confocal Microscope Systems from Olympus using a 60X oil immersion objective. The LASER intensity and detector sensitivity values were set with respect to the control set in every experiment. Around 15 cells were imaged for every condition.These parameters were preserved throughout the

experiment (see Figure 2.4).

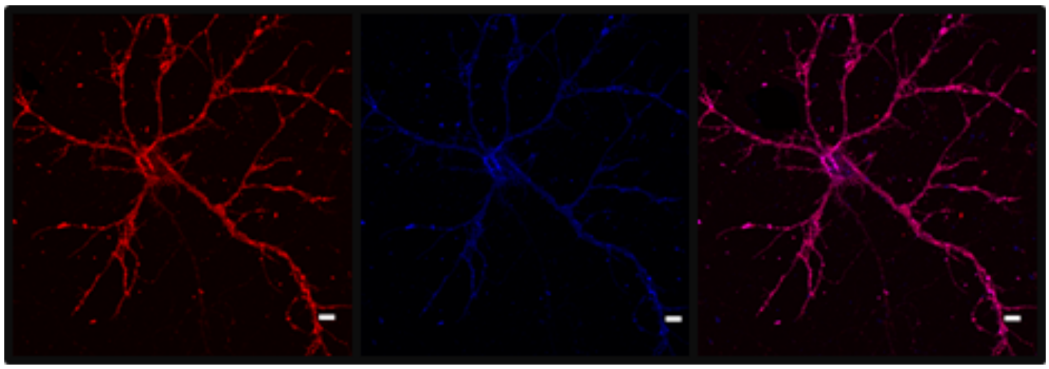


Figure 2.4: Representative images of stained neurons (scale bar = 10 µm)

2.2.4 Image Analysis

The widely used open-source image analysis software ImageJ (NIH, USA/Image Processing and analysis software in Java) was used to quantitate the images. A java macro was written which enabled the batch processing of the images. Every image was converted into 8 bit, all Z planes were projected into one and the channels split. A threshold value was applied to the red and the blue channels individually, maintaining constant throughout the experiment. The total thresholded area of fluorescently labelled surface and internalised receptors was acquired using the software. The following fraction now gives us a metric for endocytosis:

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

This normalisation against the total receptors in the cell accounts for the cell to cell variability in receptor expression levels. This is a major advantage of this technique. (see)

The values were then normalised against the control values of the same experiment and the same condition. This normalised value of the later time-points represent the fold increase in endocytosis index.

2.2.5 Statistical Analysis

The fold change in endocytosis is presented as (Mean ± SEM). The normalised values of endocytosis index in different conditions were checked for statistical significance using the two-tailed t-test. Values of $p > 0.05$ were considered statistically insignificant.

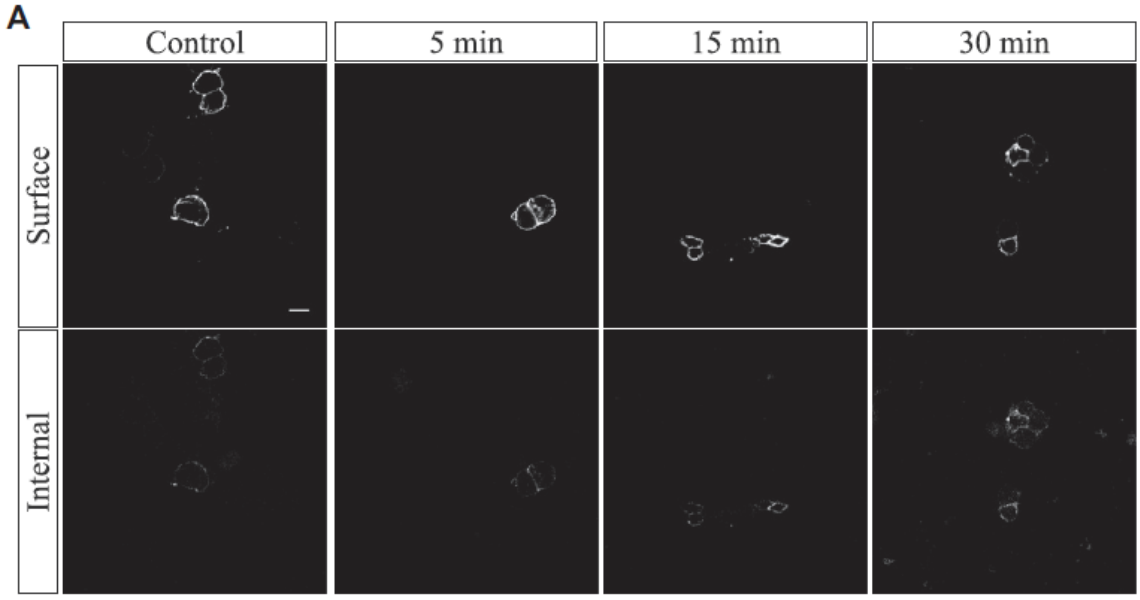
Chapter 3

Results

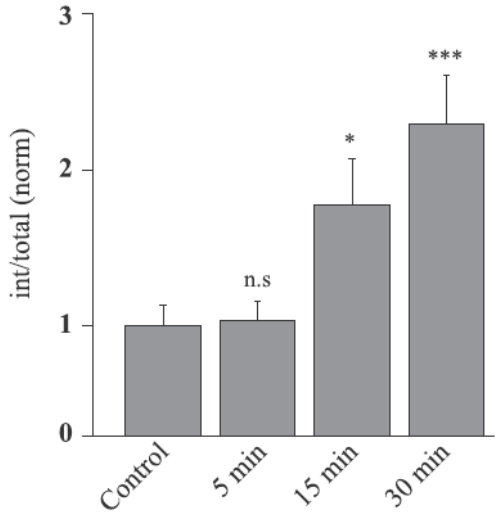
3.1 Group I mGluRs undergo constitutive endocytosis

3.1.1 Kinetics of constitutive endocytosis of myc-mGluR5 in HEK293

The groundwork of this study lies in the finding that myc-mGluR5 undergoes peak constitutive endocytosis at 30 minutes and recycles back to the plasma membrane at 2.5 hours (R.R. Trivedi et. al., 2012, Figure 3.1). The localisation of this receptor at the cell membrane validates that the myc-tagged receptor behaves like the native receptor. Besides, other studies have shown that this construct is functionally identical to the native receptor in its ability to enhance intracellular calcium levels upon activation [18]. R.R. Trivedi et. al., 2012[14] also validated the use of live-cell dual antibody staining assay for studying endocytosis in tissue culture systems. I performed the live cell dual antibody staining assay in mouse primary hippocampal neurons to obtain more physiologically relevant information, as explained in Subsection 2.2.1.



(a) Kinetics of myc-mGluR5 endocytosis in HEK293 cells[14]



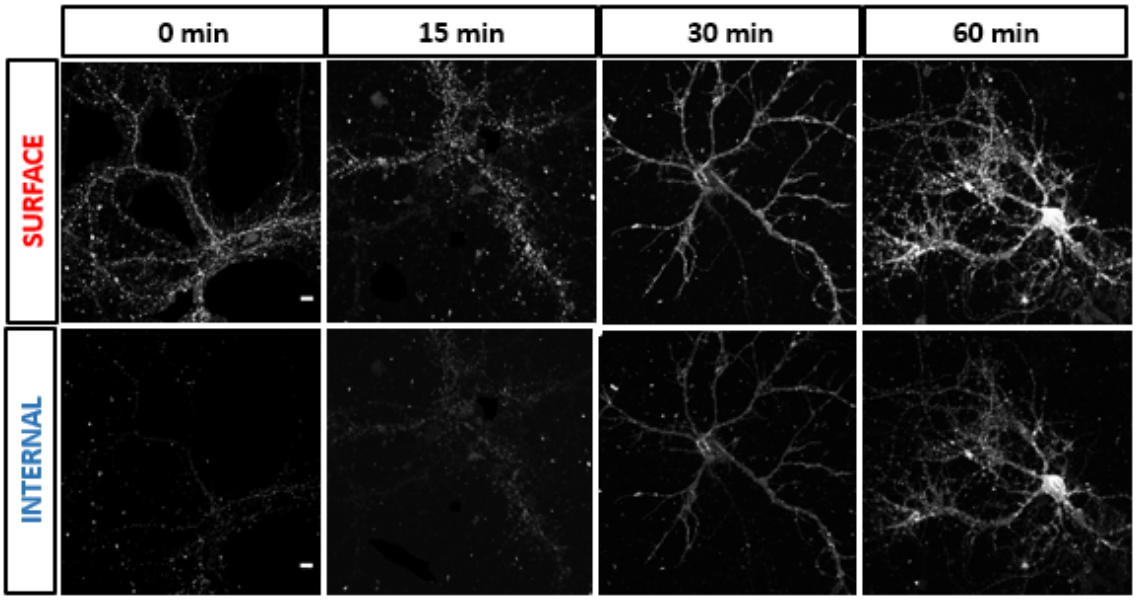
(b) Quantitation shows that endocytosis reaches a maximum at 30 minutes. Scale bar = 10 µm. ** indicates $p < 0.05$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

Figure 3.1: mGluR5 undergoes ligand-independent internalization in HEK293 cells.

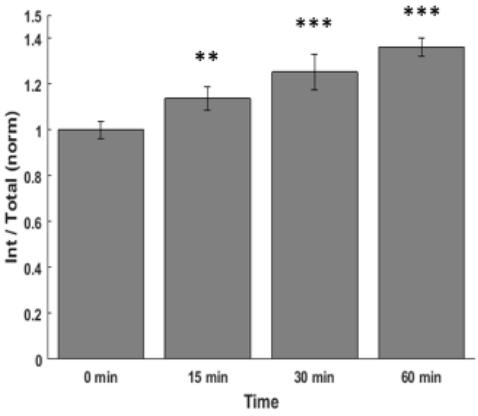
3.1.2 Kinetics of constitutive endocytosis of mGluR1a in primary hippocampal neurons

Initially, I investigated whether group I mGluRs undergo constitutive internalization in primary hippocampal neurons, consistent with the HEK293 cell data. I studied the constitutive endocytosis of myc-mGluR1a in primary hippocampal neurons using the live cell dual antibody staining assay as explained in Subsection 2.2.3. I observed a gradual increase in endocytosis from **0 min (1.00 ± 0.04), 15 min (1.14 ± 0.05), 30 min (1.25 ± 0.07)** and **60 min (1.36 ± 0.04)** (shown in Figure 3.2). The endocytosis index is maximum at 30 and 60 mins.

I was next interested in understanding the role of ubiquitination in the ligand-independent endocytosis of both mGluR1 and mGluR5. As there is substantial endocytosis at 30 mins, I pharmacologically suppressed ubiquitination at the 30 minute time point using PYR-41.



(a) mGluR1 shows maximum internalisation at 30 and 60 minute time-points in primary hippocampal neurons.



(b) Quantitation shows that endocytosis reaches a peak at 30 and 60mins. Scale bar = 10 µm. ** indicates $p < 0.05$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

Figure 3.2: Kinetics of constitutive endocytosis of mGluR1 in primary hippocampal neurons.

3.2 Role of ubiquitination in the constitutive endocytosis of mGluR5

So far, I have elucidated that group I mGluRs undergo constitutive endocytosis in both HEK293 and mouse primary hippocampal neurons. Now in order to see if ubiquitination plays a role in this form of internalisation we used a pharmacological approach (see 3.3). Yili Yang et. al., 2007[19] reported that a pyrazone molecule named PYR-41(4[4-(5-nitro-furan-2-ylmethylene)-3, 5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester) is a cell permeable specific inhibitor of the ubiquitin activating enzyme E1. Inhibiting E1 decreases the amount of active ubiquitin molecules that can attach to their substrates through the sequential ubiquitination cascade as shown in Figure 17.

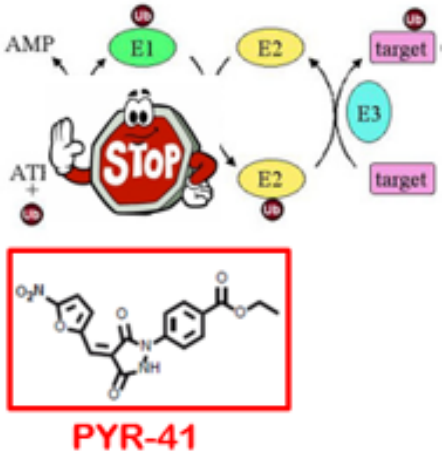
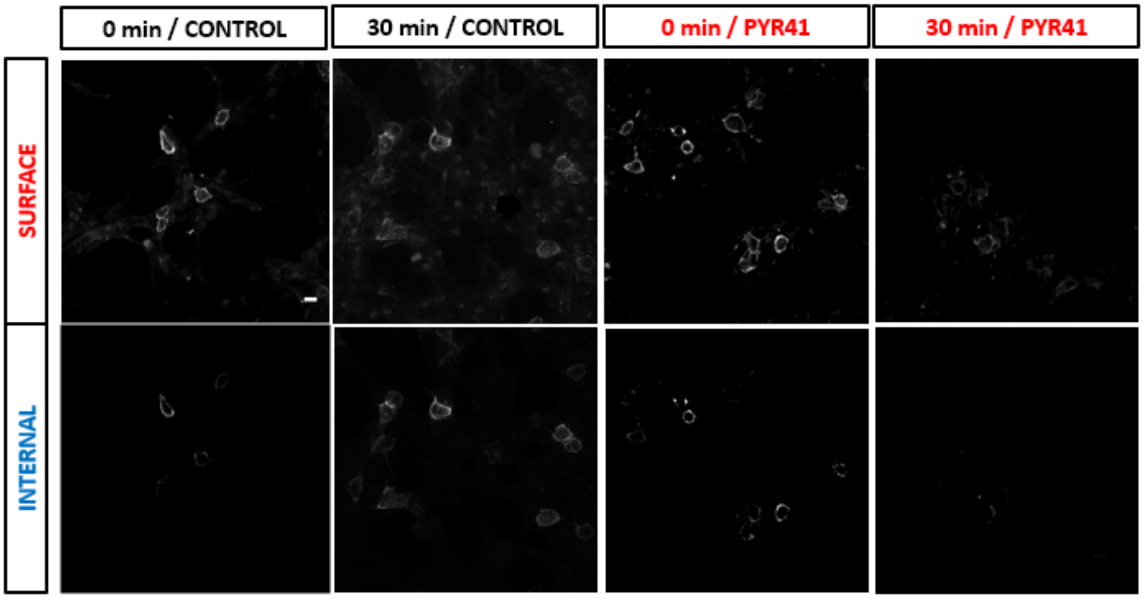


Figure 3.3: Pharmacological intervention in the ubiquitination cascade.

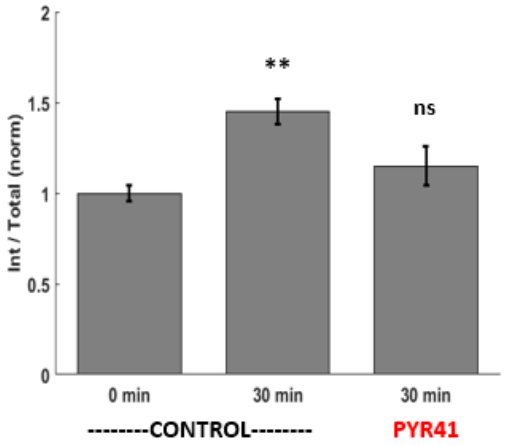
I pre-treated the live-cells (HEK293/neuron) with PYR-41 at 50 µM concentration for 30 minutes at the beginning of the experiments. This caused a temporary block in activation of ubiquitin molecules and subsequent inability for the substrates to be ubiquitinated. I then proceeded with blocking and incubation in the primary antibody for 1 hour on ice. I immediately fixed the cells corresponding to the 0 minute time-point and incubated the rest of the cover-slips for 30 minutes at 37˚C. All the fixed cells were stained with the first secondary antibody followed by permeabilization with Triton and then stained with the second secondary antibody as illustrated in Subsection 2.2.2. The normalised value of internalization was compared between the treated and untreated conditions.

3.2.1 PYR-41 blocks constitutive endocytosis of mGluR5 in HEK293 cells

I preliminarily checked the effect of the drug PYR-41 on the constitutive endocytosis of mGluR5 in HEK293 cells. The PYR-41 treated condition showed a complete block in the constitutive endocytosis of the receptor mGluR5 compared to the untreated coverslips at the 30 minute time-point, **control (1.47 ± 0.08) to PYR-41 (1.18 ± 0.12)** Figure 3.4. I then investigated the same in primary hippocampal neurons.



(a) HEK293 cells treated with PYR-41 relatively show very little endocytosed mGluR5 receptors at 30 mins.

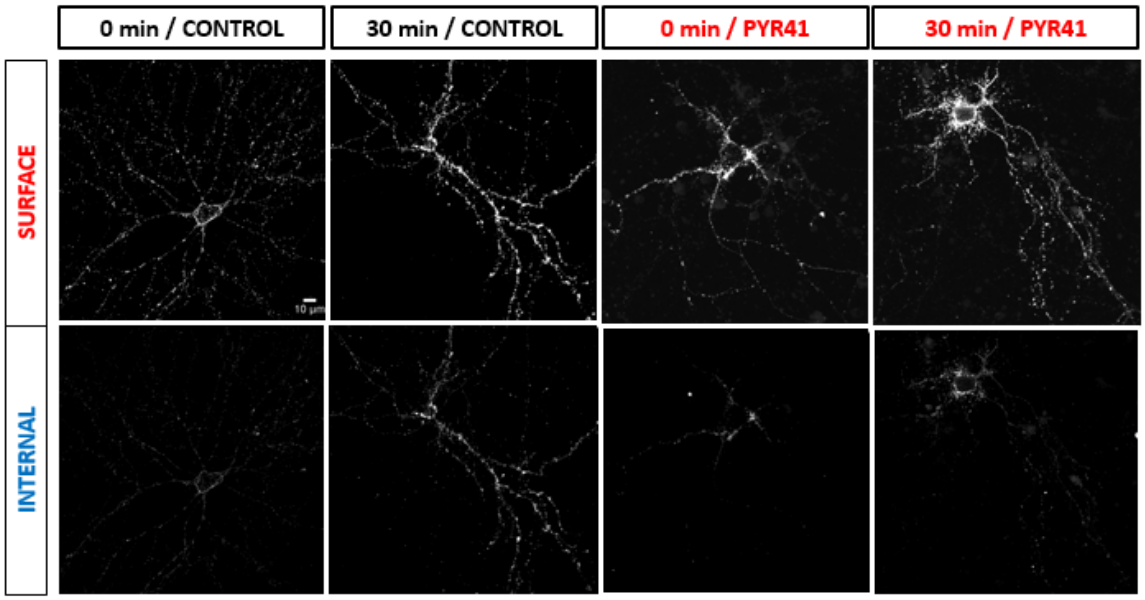


(b) Quantitation of the normalised endocytosis values show a block in ligand-independent endocytosis of mGluR5 on PYR-41 treatment at 30 mins. Scale bar = 10 µm. ** indicates $p < 0.05$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$.

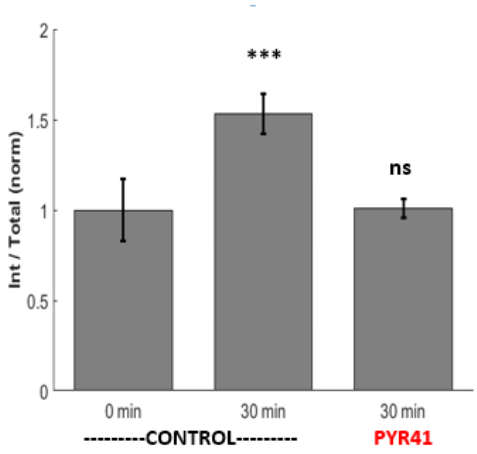
Figure 3.4: Inhibiting ubiquitination blocks constitutive endocytosis of myc-mGluR5 in HEK293

3.2.2 PYR-41 blocks constitutive endocytosis of mGluR5 in primary hippocampal neurons

I next investigated the effect of the application of PYR-41 on mGluR5 constitutive internalization in primary hippocampal neurons. I observed that similar to HEK293 cells, application of PYR-41 led to complete inhibition in the constitutive endocytosis of mGluR5 in primary hippocampal neurons (**control (1.45 ± 0.15) to PYR-41 (1.00 ± 0.04)**, Figure 3.5).



(a) PYR41 mediated ubiquitin E1 inhibition leads to block in endocytosis of mGluR5 receptors at 30 mins.



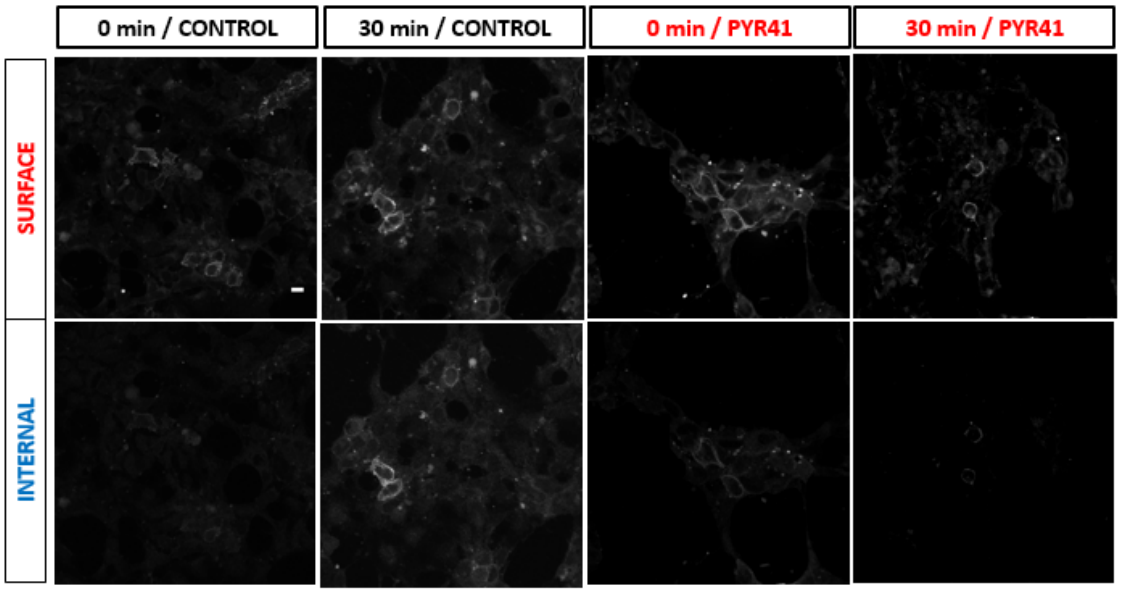
(b) Quantitation of the normalised endocytosis values show a block in endocytosis of mGluR5 on PYR41 treatment at 30 mins. Scale bar = 10 µm. ** indicates $p < 0.05$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$

Figure 3.5: Inhibiting ubiquitination blocks constitutive endocytosis of mGluR5 in primary hippocampal neurons.

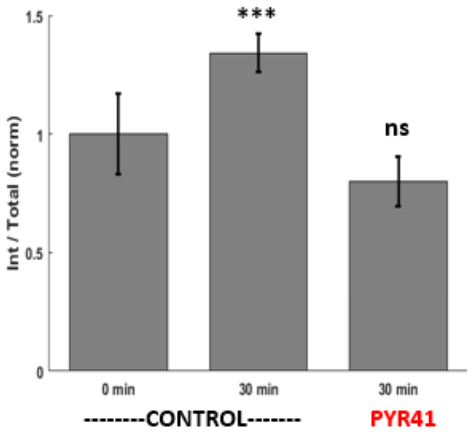
3.3 Role of ubiquitination in constitutive endocytosis of mGluR1.

3.3.1 PYR-41 blocks constitutive endocytosis of mGluR1 in HEK293.

I was next interested to see if the other member of the Group 1 mGluR family, mGluR1 also responds similarly to PYR-41. I first investigated this in HEK293 cells and saw a clear suppression of constitutive endocytosis of mGluR1 at 30 minutes. The internalization index decreased from **control (1.33 ± 0.09) to PYR-41 (0.8 ± 0.13)**, Figure 3.6.



(a) PYR41 mediated ubiquitin E1 inhibition leads to block in endocytosis of mGluR1 receptors at 30 mins.

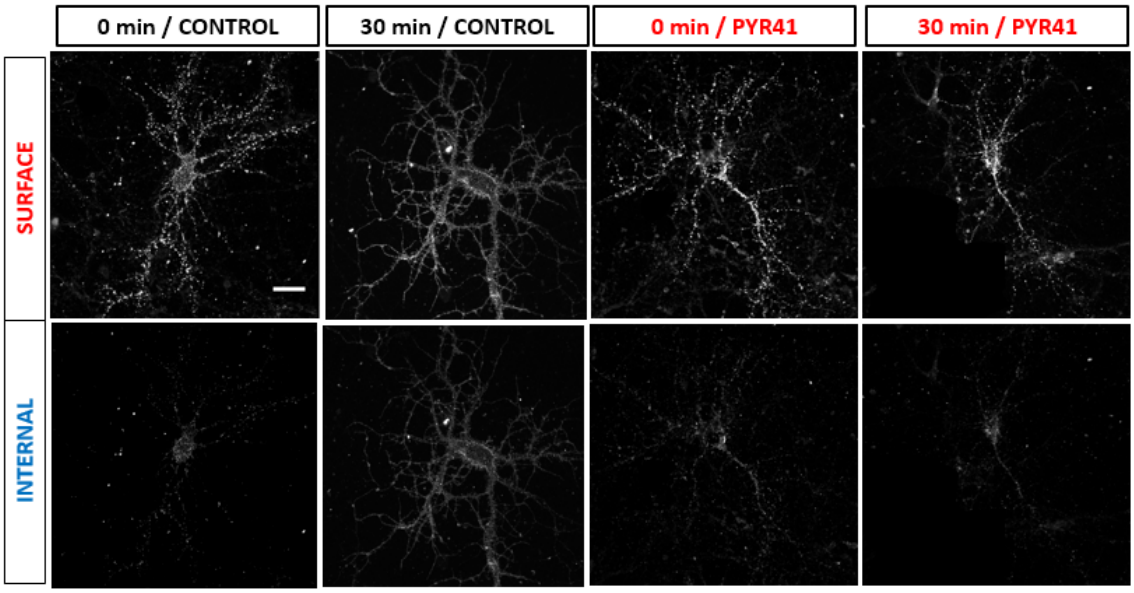


(b) Quantitation of the normalised endocytosis values show a block in the endocytosis on PYR-41 treatment. Scale bar = 10 µm. ** indicates $p < 0.05$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$

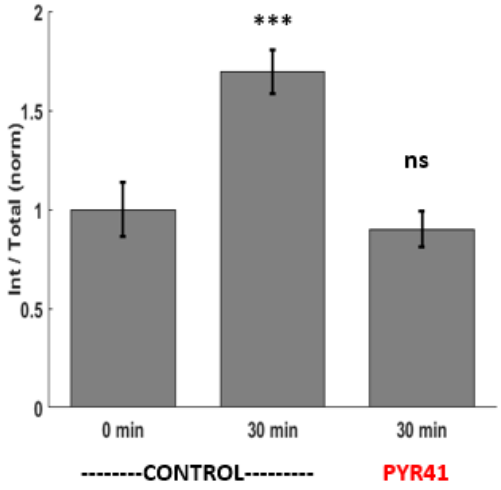
Figure 3.6: Inhibiting ubiquitination leads to block in constitutive endocytosis of mGluR1 in HEK293 cells.

3.3.2 PYR-41 blocks constitutive endocytosis of mGluR1 in primary hippocampal neurons

Following the finding that mGluR1 does not undergo endocytosis upon PYR-41 based inhibition of ubiquitination in HEK-293 cells, we proceeded to verify this in primary hippocampal neurons. Consistent with the above results, I also see a block in endocytosis at 30 mins in primary neurons (**control (1.65 ± 0.11) to PYR-41 (0.86 ± 0.18)**, Figure 3.7).



(a) Primary neurons show an increase in internalised receptors at 30 minutes compared to the PYR-41 treated condition, showing block in endocytosis.

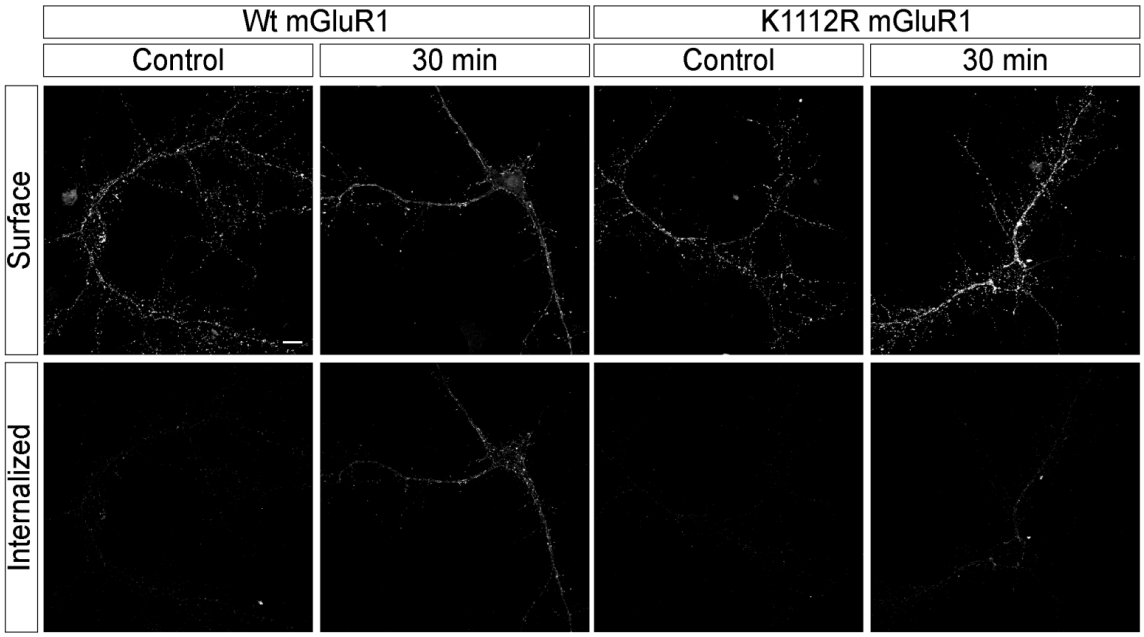


(b) Quantitation of the normalised endocytosis values show a block in the endocytosis on PYR-41 treatment. Scale bar = 10 µm. ** indicates $p < 0.05$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$

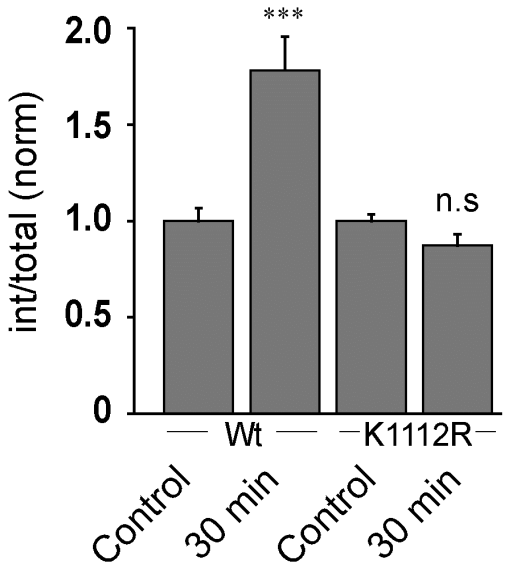
Figure 3.7: Inhibiting ubiquitination leads to block in constitutive endocytosis of mGluR1 in primary neurons.

3.4 Comparison between the ligand-mediated and constitutive endocytosis of myc-mGluR1

Studies performed by Mr. Ravinder Gulia in the lab (see Figure 3.8 and 3.9), has established K1112 as the critical residue that potentially gets K-63 linked polyubiquitinated for the occurrence ligand mediated endocytosis of mGluR1. But, interestingly I observed that the same mutant receptor undergoes constitutive endocytosis. The mGluR1-WT undergoes a fold change in endocytosis of **1.25 ± 0.28 while the mGluR1-sm shows a fold change of 1.26 ± 0.17.** This finding perhaps indicates that constitutive and ligand mediated endocytosis are differently regulated by Ubiquitination. The pathway of endocytic trafficking could be different for both these cases.

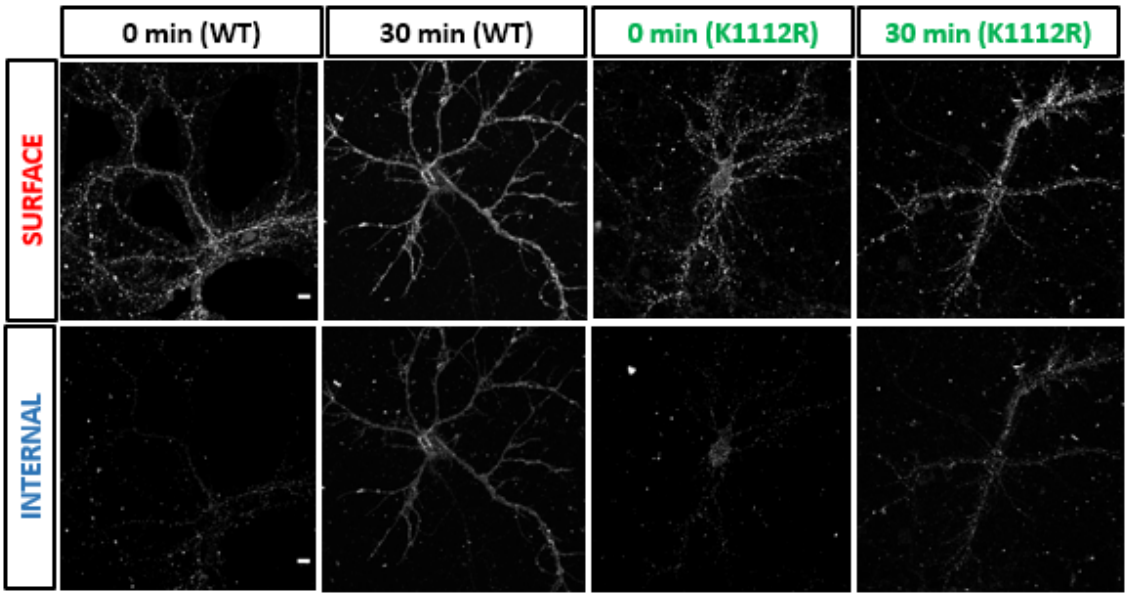


(a) Primary neurons expressing the WT mGluR1 receptor show normal constitutive endocytosis at 30 mins, while the ones expressing K1112R mGluR1 does not show endocytosis at 30 mins. Scale bar = 10 μm. ** indicates $p < 0.05$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$

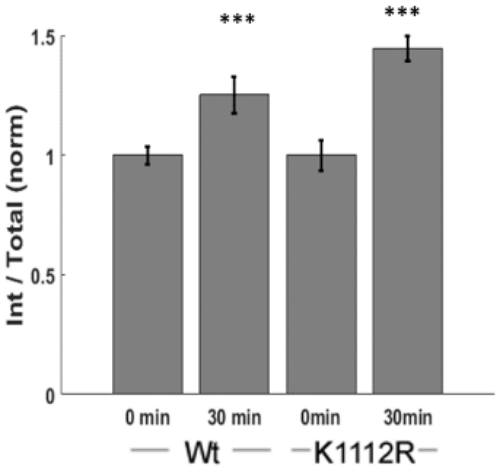


(b) Quantitation of the normalised endocytosis values show that the mutant form of mGluR1 does not undergo endocytosis.

Figure 3.8: Ligand mediated endocytosis of mGluR1: Lysine at position 1112 is the critical residue where ubiquitination takes place. (Unpublished data from Mr. Ravinder Gulia)



(a) Primary neurons expressing the mutant version of mGluR1, K1112R mGluR1 undergo endocytosis similar to the wild type receptor.



(b) Quantitation of the normalised receptor endocytosis fold change shows that the K1112R mGluR1 is capable of constitutive endocytosis. Scale bar = 10 µm. ** indicates $p < 0.05$, *** indicates $p < 0.001$ and n.s indicates $p > 0.05$

Figure 3.9: Constitutive endocytosis of mGluR1: Lysine K1112 is not the critical residue

Chapter 4

Discussion

Group I mGluRs are glutamate receptors that trigger signal transduction pathways in the post-synaptic neuron upon activation. They play indispensable roles in synaptic plasticity, neurodevelopment, functioning of glia, to name a few. Moreover, their inappropriate activation is believed to lead to neurodevelopmental and cognitive disorders like Autism and Fragile-X syndrome [10]. Therefore, there is an urgent need to understand the molecular mechanisms behind the diverse functions these receptors perform, which would lead to specific therapeutic solutions to the many diseases they are implicated in.

The intracellular trafficking machinery is thought to regulate the proper localisation of these receptors through receptor desensitisation upon ligand binding, resensitisation, down-regulation and recycling back to the cell surface [4]. In this study, we have shown that group I mGluRs undergo endocytosis even in the absence of a bound ligand in mouse primary hippocampal neurons. This mechanism possibly helps in the maintenance of cellular homeostasis and normal brain function. Unpublished data from our laboratory illustrate regulation of endocytosis of group I mGluRs by ubiquitination, after ligand binding. I was to tempted ask if a similar mechanism exists even in the case of ligand-independent internalization of the receptors. My data suggest that constitutive endocytosis does occur for group I mGluRs.

Ubiquitin carries out its functions through its covalent attachment to other cellular proteins, thereby changing the stability, localization, or activity of the target protein[20]. Depending on the modification, whether monoubiquitination, polyubiquitination or multi-monoubiquitination, the substrates serve different regulatory functions like DNA repair, proteosomal degradation, endocytosis etc. Polyubiquitination and monoubiquitination have

emerged as important signalling mechanisms that control diverse physiological and pathological processes [21].

My results raise the possibility that the receptor trafficking machinery works in conjunction with the Ubiquitin machinery to maintain proper receptor localisation at the normal synapse. It further needs to be seen, if the substrate of ubiquitination is the receptor itself or other adapter proteins. In the case of ligand-mediated endocytosis, it is thought that the ligand binding induces conformational changes in the receptor which initiates ubiquitination of the receptor, but it is not clear what initiates constitutive internalization of the same receptor.

The critical residue identified for the ligand-mediated internalization of mGluR1 in earlier studies (unpublished data) showed no evident role in the constitutive endocytosis of mGluR1 in my study. The mutant form of mGluR1, with the lysine residue replaced with arginine at position 1112 at the C terminal of the receptor, showed no block in constitutive endocytosis, although it failed to show endocytosis upon ligand binding. This might mean that the ligand dependent and independent endocytosis take place through distinct pathways. How these pathways crosstalk and switch from one to another upon ligand binding would be intriguing to find out. Although, I cannot make similar comments for mGluR5, because such mutagenesis experiments to identify the critical residue responsible for internalization, have not yet been performed.

Also, R.R. Trivedi et. al., 2012[14], had shown that mGluR5 recycles back to the membrane after constitutive endocytosis after 2.5 hours. This points to the role of deubiquitinases in the sorting of these receptors to the recycling compartment or to the lysosome for degradation.

4.1 Future Perspectives

Understanding ligand-dependent and ligand-independent endocytosis of Group I mGluRs is of chief importance to resolve physiological brain related-disorders. Ubiquitination seems to play an integral role in regulating trafficking of group I receptors in both of these scenarios. More work needs to be done to determine the kinetics of receptor recycling in primary hippocampal neurons. The critical residue(s) for ligand-dependent and independent-endocytosis of mGluR5 need to be identified. This would help in the com-

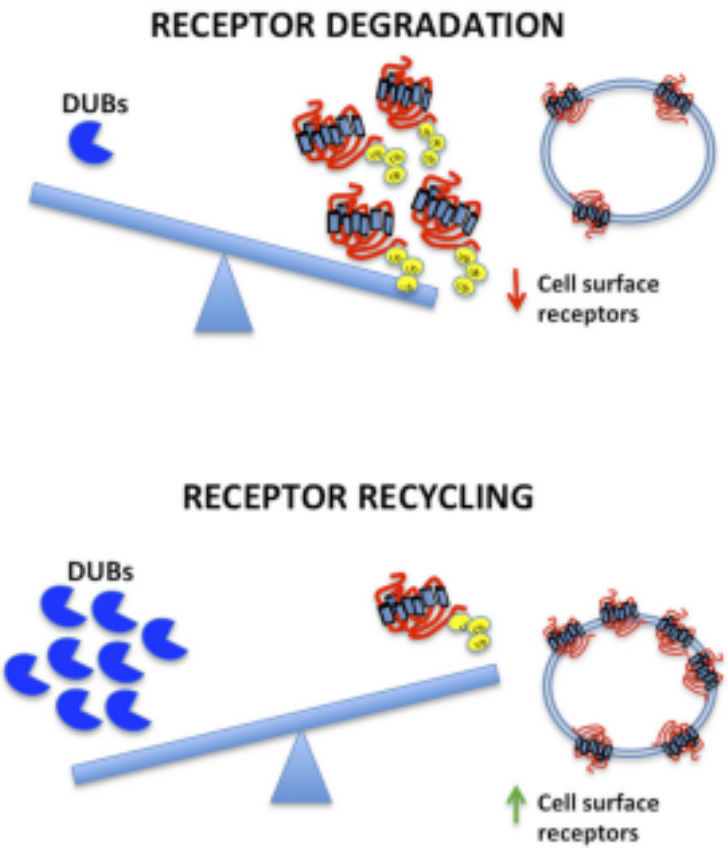


Figure 4.1: Fate of receptor, depending on concentration of DUBs

parison of the kinds of modifications mGluR1 and mGluR5 undergo, and also explain how ligand-dependent endocytosis is different from ligand-independent endocytosis.

The role of deubiquitinases (DUBs) in constitutive recycling of these receptors needs to be investigated. As ubiquitination is pertinent for receptor endocytosis, deubiquitination is likely to be involved in the recycling the receptor back to the plasma membrane. R.R. Trivedi et. al., 2012[14] had shown sorting of the endocytosed mGluR5 receptors into the lysosome and the recycling endosome. DUBs could have potential roles in this type of sorting, as also seen for other GPCRs (as shown in Figure 4.1).

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Appendix A

Macro for Batch Processing of Images

Listing A.1: "Macro for Batch Processing of Images written in Java."

```
/*
This macro allows you to batch process images!
Written by Ritu Roy Chowdhury
For queries:
Contact rituroych@gmail.com
*/
run("Close All");

waitForUser("WELCOME", "Are you ready to get started?");
beep();

input = getDirectory("Input directory");
output = getDirectory("Output directory");

Dialog.create("File type");
Dialog.addString("File suffix: ", ".oif", 5);
Dialog.show();
suffix = Dialog.getString();

processFolder(input);
```

```
function processFolder(input)
{
        list = getFileList(input);
        for (i = 0; i < list.length; i++)
        {
                /*if(File.isDirectory(list[i]))
                        processFolder("" + input + list[i
                           ]);
                        "In the loop";*/
                if(endsWith(list[i], suffix))
                        processFile(input, output, list[i
                           ]);
                        "Begin␣processing:␣";
        }

Dialog.create("Finish");
beep();
Dialog.show();

}


function processFile(input, output, file)

{
        //run("Close All");
        "Inside␣the␣function";
        open(input + file);
        imgName=getTitle();
        run("8-bit");
        lowerRED=50;
```

```
lowerBLUE=35; //Set threshold values here!
                                //Set threshold values
   here!
upper=255;

run("Z␣Project...", "projection=[Max␣Intensity]");
run("Split␣Channels");
"I␣am" + imgName;

selectWindow("C1-MAX_" + imgName); //RED channel
run("Grays");
setTool("freehand");

/*if (selectionType() == 0)
exit("Sorry, no ROI");*/
waitForUser("Set␣ROI␣for␣red_C1-MAX_" + imgName, "
   Select␣your␣region␣of␣interest.␣Then␣press␣OK.")
   ;
setThreshold(lowerRED, upper);

run("Measure");

//close();

selectWindow("C2-MAX_" + imgName); //BLUE channel
run("Grays");
setTool("freehand");

/*if (selectionType() == 0)
                                    //make sure we have
   got a rectangular selection
exit("Sorry, no ROI");*/
```

```
        run("Restore␣Selection");
        //waitForUser("Set ROI for blue_C2-MAX_" + imgName
           , "Select your region of interest. Then press OK
           .");
        setThreshold(lowerBLUE, upper);

        run("Measure");

        //close();

        selectWindow(file);
        close();
        //saveAs("Results", output);
        //close();
}
```