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의학석사 학위논문

Interplay of β-arrestins and Nedd4 E3 ligase regulates ubiquitination and trafficking of mGluR7

베타-어레스틴 단백질과 유비퀴틴화 효소 네드 4 의 상호작용에 의한 글루타메이트 수용체 7 의 유비퀴틴화와 트레피킹 조절

2018 년 2 월

서울대학교 대학원

의학과 의과학 전공

이 효 진

A thesis of the Degree of Master of Science

베타-어레스틴 단백질과 유비퀴틴화 효소 네드 4 의 상호작용에 의한 글루타메이트 수용체 7 의 유비퀴틴화와 트레피킹 조절

Interplay of β-arrestins and Nedd4 E3 ligase regulates ubiquitination and trafficking of mGluR7

February 2018

The Department of Biomedical Science,

Seoul National University

College of Medicine

Hyo Jin Lee

Interplay of β-arrestins and Nedd4 E3 ligase regulates ubiquitination and trafficking of mGluR7

by

Hyo Jin Lee

(Directed by Prof. Young Ho Suh)

A thesis submitted to the Department of Biomedical Science in partial fulfillment of the requirements for the Degree of Master of Science in Medicine at Seoul National University College of Medicine

February 2018

Approved by Thesis Committee:

Professor Yong Tae Kwon Chairman

Professor Young Ho Suh Vice chairman

Professor Myung-Hwan Kim

베타-어레스틴 단백질과 유비퀴틴화 효소 네드 4 의 상호작용에 의한 글루타메이트 수용체 7 의 유비퀴틴화와 트레피킹 조절

지도 교수 서 영 호

이 논문을 의학석사 학위논문으로 제출함

2018년 2월

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이 효 진

이효진의 의학석사 학위논문을 인준함

2018년 2월

위 원 장 권 용 태 (인)

부위원장 서 영 호 (인)

위 원 김 명 한 (인)

ABSTRACT

Metabotropic glutamate receptor 7A belongs to group III of metabotropic glutamate receptors as a G protein-coupled receptor. GPCRs undergo a number of post-translational modifications, which regulate receptor trafficking and function. These modifications of GPCR have been known as important role for mental disorders such as neurodegenerative diseases.

β-arrestins are well known as adaptor protein of seven-transmembrane receptors. And β-arrestins mediate trafficking of many cell-surface receptors. In many previous reports, it have been revealed that β-arrestins-dependent ubiquitinylation plays a role in protein turn-over and receptor trafficking. Although mGluR7A can be an attractive drug target due to the GPCR properties, whether mGluR7A is a target of ubiquitin conjugation has not been identified yet.

For the first time I investigated whether mGluR7A is ubiquitinylated in HEK293T cells and Neuronal cultured cells by immunoprecipitation. These experiments show that mGluR7A is ubiquitinylated by agonist. And also ubiquitinylation of mGluR7A is happened at C-terminus and Loop2 regions of mGluR7A.

In this study I found that β-arrestin1 binds strongly to mGluR7A but not beta-arrestin2 in HEK293T cells using immunoprecipitation method. And this binding is regulated with metabotropic glutamate receptor group III specific agonist (L-AP4) dependent manner. In addition GST-pull down assay results show that β-arrestin1 binds to C-terminus and Loop2 of mGluR7A. Furthermore E3 ligase, Nedd4 also binds to mGluR7A and beta-

arrestin1. I identified that this triplet-complex-form regulates mGluR7A ubiquitinylation in HEK293T cells and neuronal cultured cells.

Also my confocal microscopy imaging results represent that surface stability of mGluR7A is regulated with the forming complex of mGluR7A, beta-arrestin1 and Nedd4 in neuronal cell. So it is reasonable to assume that beta-arrestin1 and Nedd4 interact with mGluR7A and make a form of multi-complex to regulate mGluR7A ubiquitinylation and cell surface stability.

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Keywords : GPCR, mGluR7A, beta-arrestin, Nedd4, ubiquitination, trafficking, adaptor protein

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LIST OF ABBREVIATIONS

mGluR7 : Metabotropic glutamatergic receptor 7

Nedd4 : Neural precursor cell expressed developmentally down-regulated protein 4

barr : beta-arrestin

DN : Dominant negative

L-Glu : L-Glutamate

L-AP4 : L-2-amino-4-phosphonobutyric

PBS : Phosphate buffer saline

FBS : Fetal bovine serum

CTL : Control

DMEM : Dimethyl sulfoxide

NS : Not significant

INTRODUCTION

Metabotropic glutamate receptor 7 belongs to the metabotropic glutamate receptors (mGluRs) are ubiquitously expressed in Central Nervous Systems and known to modulate synaptic transmission and synaptic scaling. These mGluRs have three groups and are involved in G-protein coupled receptor family which have seven-transmembrane. When they have agonist stimulations, GPCRs change their conformation and react to various intracellular signaling and physiological neural activations (1).

mGluR7 belongs to pre-synaptic GPCRs and have been studied for function of neurotransmitter inhibition. mGluR7 have five-alternative slicing forms, mGluR7 (a, b, c, d, e), these forms have different c-terminus sequences and full-length.

In this study, I focused mGluR7A form because mGluR7A is more abundantly expressed in all over hippocampus comparing with mGluR7B (2). In recent years it comes before that mGluR7A can act as important mediator of diverse pre-synaptic signaling and neurological disease.

In several studies, it was revealed that C-terminus of mGluR7A interacting with various proteins such as PICK1 and SUMO (3, 4). In study of using mGluR7A which have mutated C-terminus in mouse and rat, that mutated receptors and PICK1 complex did not interact with each other and that leads to absence epilepsy (5). Moreover mGluR7 knockout mice showed deficit in fear response and conditioned taste aversion (6). At this study, they suggest that impairment of mGluR7A causes hindrance to amygdala -

dependent behaviors.

By these clues, studies have expanded to correlation of short-term memory, modulation of theta rhythm and antidepressant and anxiolytic effect of mGluR7A (7, 8, 9, 10). These results show insights of the very novel possibilities in mGluR7A as drug target to psychiatric disorders.

How to control mGluR7A in cells is my pure curiosity in this study. To unearth the precise mechanism of mGluR7A trafficking I concerned to post-translational modification of receptors. GPCRs undergo a number of post-translational modifications (PTM), which regulate receptor trafficking and function. In addition, there are many studies on PTM of other G-protein coupled receptors. (30, 32)

In the preceding study, it was revealed that SUMOylation of mGluR7A regulates mGluR7A trafficking (11). In that previous study I found out such a PTM, SUMOylation, can modulate mGluR7A's surface stability in neurons. SUMO is small protein which have 97 amino acids and this small protein form an iso-peptide bond with lysine residues. SUMOylation needs E1, E2, E3 ligase to deliver SUMO protein to lysine residues. This process is very similar to that of ubiquitinylation process. Ubiquitin protein has 76 amino acids and covalently binds to lysine residues of target substrate protein. This ubiquitinylation pathway go along with E1, E2, E3 ligase. At first E1 activates ubiquitin and this process is ATP-dependent. E2 move activated ubiquitin to active site and then finally E3 ligase promote ubiquitin to bind to target substrate protein.

One of the receptors that is widely known about receptor's undergoing ubiquitinylation pathway is β-2-adrenergic receptor. Especially, β-2-adrenergic receptor have become a

lot of research to act with β-arrestins and undergo ubiquitinylation pathway (12).

β-arrestin proteins are known as adaptor protein of GPCRs and can control receptor trafficking and receptor downstream signaling. (33)

Classification of β-arrestin protein family is based on the sequence of protein and distribution in cells. Visual arrestins (β-arrestin1 and β-arrestin2) are specifically expressed in cone otherwise non-visual arrestins are widely expressed all over the brain region. (20)

Nedd4 (Neural precursor cell-expressed developmentally down-regulated), a homologous to E6-associated protein carboxylic terminus (HECT) domain-containing family of E3 Ub ligase which has been well characterized to regulate the turnover and trafficking of ion channels and GPCRs present in the neurons such as sodium channel, AMPA receptor, GluN2D-containing NMDA receptor and β-2-adrenergic receptor. (34)

Based on previous studies, I focused on the mGluR7A ubiquitinylation which of mechanisms and co-relation of β-arrestin proteins for identifying the meaning of the phenomenon. First of all I identified that mGluR7A undergo ubiquitinylation pathway at both C-terminal tail and intracellular loops of mGluR7A in HEK293T cells and neuronal cultured cells. And this ubiquitinylation of mGluR7A is dependent on activation of mGluR7A by agonist stimulation. And then I get a hint of inspiration from the previous study of beta-2-adrenergic receptor which showed that interaction with β-arrestin proteins and E3 ligase Nedd4 (31), I found that β-arrestin1 and Nedd4 are both required for mGluR7A ubiquitinylation.

Moreover these components, β-arrestin1 and Nedd4, bind to mGluR7A and make a multi-complex. Finally I disclosed that this multi-complex of beta-arresitn1-Nedd4-mGluR7A can control mGluR7A cell surface level and trafficking in cultured neuronal cells.

These results can provide that new insight of mGluR7 regulation and novel drug target for neural disorders.

MATERIALS AND METHODS

1. Maintaining cells and Cultures

HEK293T cells were maintained at 37℃ incubator with 10%FBS, 1%L-glutamine in DMEM (WELGENE; Cat.LM001-05).

Primary neuron culture was conducted with E18 SD Rat embryo. Hippocampus and cortex were isolated and dissociated in the clean-bench. Isolated cells were on poly-D-lysine (SIGMA; Cat.P7280-5MG) coated plates and maintained with neuro-basal media (INVITROGEN; Cat.21103-049) added B-27 supplement (INVITROGEN; Cat.17504-044) and L-Glutamine (SIGMA; Cat.G-7513).

2. DNA transfection

DNA constructs and were transfected using lipofectamine2000 (INVITROGEN; Cat.11668-019) followed the manufactural protocols.

Lipofecatamine2000 RNAi-MAX reagent (INVITROGEN; Cat.13778-150) was used in siRNA transfection to HEK293T cells.

3. Cloning and Sequences

Beta-arrestin1 siRNA and scrambled siRNA were purchased at Bioneer Corp. and that of target sequence is specific for human : 5’-AAAGCCUUCUGCGCGGAGAAU-3’

Rat targeting beta-arrestin1 and beta-arrestin2 knock-down virus cloning and generation were starting to order target primer and PCR with cloning vector. Rat beta-arrestin1 KD target sequence is 5'-AGCCTTCTGTGCTGAGAAC-3'. Rat-beta-arrestin2 KD target sequence is 5'-GGACCGGAAAGTGTTTGTG-3'.

4. Western blotting and Immunoprecipitation

HEK293T cells or Neuronal cells were lysised with 0.2%SDS, 1%Triton-X100, 50mM-Tris-HCL, 150mM NaCl buffer added protease inhibitor (ROCHE; Cat.11873580001) and phosphatase inhibitor (genDEPOT; Cat. P3200-001).

For immunoprecipitation, cell lysate was incubated with 0.5μg of antibody and protein G beads at 4℃ for 3hrs. Depending on the species of antibody, protein G beads (GE HEALTHCARE; Cat.17-0618-01) or protein A beads (SIGMA; P3391) was used for incubating lysates. Washed lysates bound beads were loaded at polyacrylamide gels after dissolving step. Proteins were transferred to PVDF membrane and incubated with 5% skim milk at RT for 30min. Washed blots incubate with primary antibody for overnight. Next morning, membrane was incubated with secondary antibody and washed out to ECL. ECL reagent (THERMO SCIENTIFIC; Cat. 34080) was used for detecting protein to film.

5. Surface biotinylation assay in neuronal cells

Cell surface receptor was labeled with the EZ-Link Sulfo-NHS-SS-biotin reagent (THERMO SCIENTIFIC; Cat.21331) and biotinylated receptor was detected with

western blotting.

In neuronal cells L-AP4 0.4mM was treated for 30min for this assay.

6. Hippocampal neuron preparation and internalization assay

DNA constructs were transfected when hippocampal neuron was DIV11 and assay was conducted at DIV14. Anti-Myc (mouse) or anti-Flag (mouse) antibody was used amount of 0.5μg per each cover-slips. Primary antibody was incubated with cells at RT. L-AP4 0.4mM was treated to cells for 15min at 37℃ incubator. And then cells were fixed with 4%SCU/PFA for 15min. After that cells were blocked with 10% normal goat serum for 30min. Surface fractions were staining with Alexa Fluor 568. Neuronal cells were put to 0.25% Triton-X100 solution for 5min to become permeable and blocked 10% normal goat serum for 1hr. The internalized fractions were stained with Alexa Fluor 488. Samples were mounted with Prolong anti-fade kit solution. Slides were took confocal microscopy (Nickon A1) and Z-stack with 40X objective. Images were maximum projected and analyzed with Metamorph software to quantify internalized receptors.

7. Immunostaining to HEK293T cells

HEK293T cells were plated in low density and DNA constructs were transfected cells 2dasy before staining. Anti-Myc (mouse) or anti-Flag (mouse) antibody was used amount of 0.5μg per each cover-slips. Primary antibody was incubated with cells at RT. L-AP4 0.8mM was treated to HEK293T cells at 37℃ incubator. For the next step is same with

the internalization assay staining except that permeable step. In this staining I stained total cells so after the fixation step cells needed to be permeable.

8. GST pull down assay

GST and GST fusion protein with mGluR7WT, mGluR7 mutants were produced in E.coli BL21. The culture was grown at 37℃ shacking incubator. GST and GST fusion protein were induced with using IPTG. These GST fusion proteins were incubated with Glutathione beads at 4℃ for 1hr. Washed beads were used at GST-pull down assay. Cortical neuron lysates were incubated with the GST-fusion proteins beads at 4℃ for 3hrs. These beads were washed out and used for WB.

RESULT

mGluR7A undergoes to ubiquitinylation pathway in HEK293T cells and Neuronal cultured cells

For the first time to identify whether mGluR7A is ubiquitinylated or not Myc-mGluR7A and HA-Ub DNA constructs were co-transfected to HEK293T cells. Immunoprecipitation with anti-Myc antibody and western blot was conducted to detect ubiquitinated mGluR7. Result of (Fig.1A) represents mGluR7 undergoes ubiquitinylation pathway in HEK293T cells.

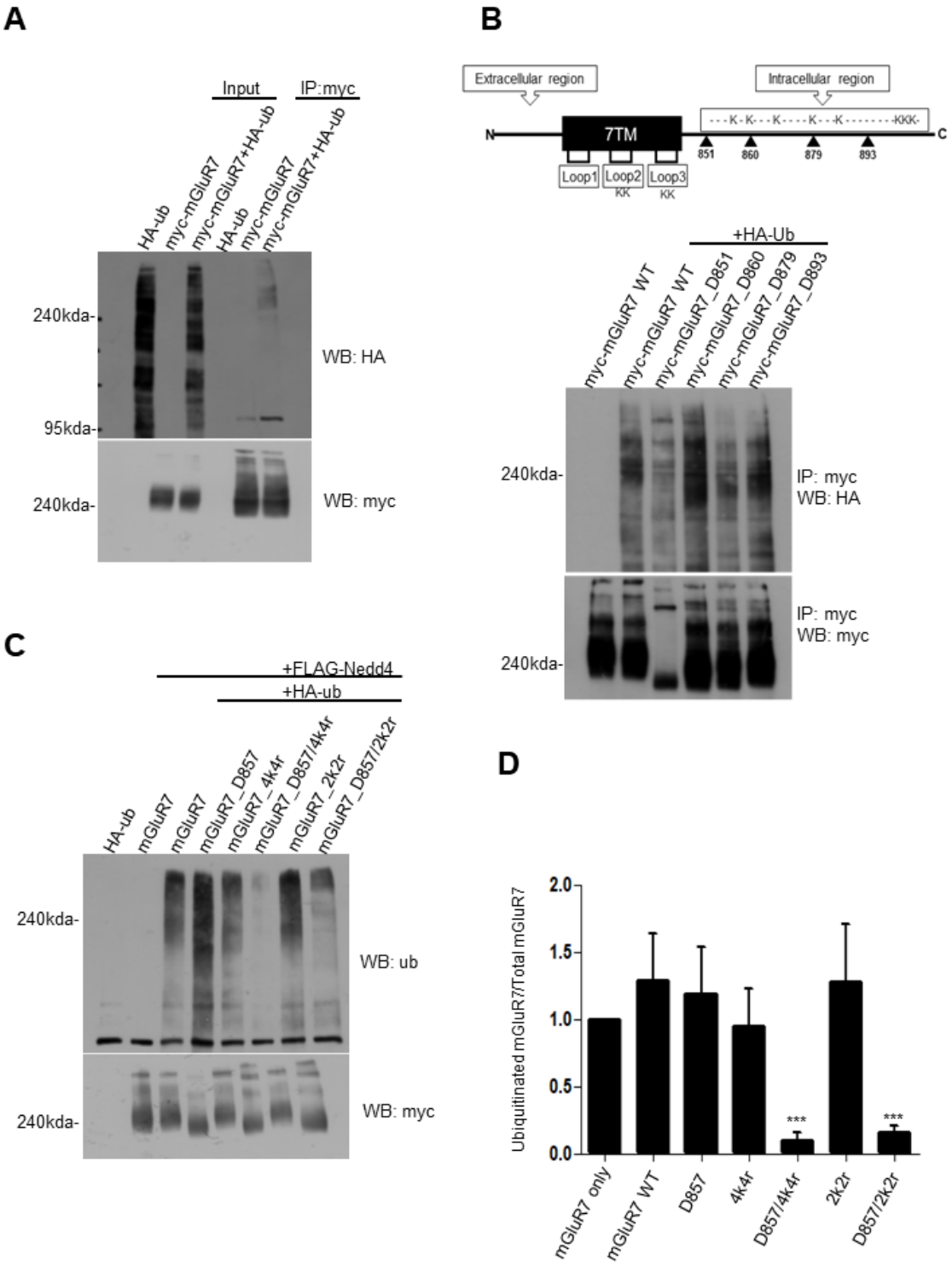
mGluR7A have C-terminus tail and 3 intracellular loops. To dissect the region of ubiquitinylation I used mGluR7A WT DNA and mGluR7A mutants DNAs. Mutants DNAs were produced by site-directed-mutagenesis method.

First time, I assumed that mGluR7A ubiquitinylation is occurred at C-terminus region because there are many lysine residues. mGluR7A mutant DNAs which have stop-codon on the C-terminus were over-expressed in HEK293T cells and ubiquitinylated mGluR7A was pulled down with IP (Fig.1B). Contrary to my expectations, mGluR7A ubiquitinylation was observed even in the mGluR7A D851 mutant which rarely have C-terminus tail amino acids.

In the second place I mutated Lysine to Arginine in the mGluR7 intracellular loop2, loop3 and made C-term/Loop region double mutants. These DNAs were transfected to HEK293T cells and IP was conducted (Fig.1C). This result showed mGluR7A ubiquitinylation occur in both C-terminus tail and intracellular Loop2 region.

These results were quantified with image J software (Fig.1D). Graph of quantification is shown that the mean SEM of four independent experiments. Paired t test was conducted with Prism program to detect statistical significance: ***p<0.0005(D857/4k4r), ***p<0.0004(D857/2k2r)

Next, I tested activity dependent mGluR7A ubiquitinylation in HEK293T cells (Fig.1E) and neuronal cultured cells (Fig.1F). In HEK293T cells mGluR7A ubiquitinylation was increased in depend on L-glutamate treatment times. L-AP4, agonist of mGluR group III, promoted mGluR7 ubiquitinylation while other drugs did not have effect in cortical neurons. Bicuculline is antagonist of GABA-A receptor and it blocks the inhibitory action of GABA receptor. As a result, the neural activity could be enhanced when bicuculline was treated to neuronal cultured cells. AMN082 is allosteric agonist of mGluR7A but did not have effect on activity-dependent ubiquitinylation of mGluR7A. DHPG is agonist of metabotropic glutamate rceptor group I and was used for negative control of L-AP4 treatment.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HA-ub
myc-mGluR7
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95kda-
WB: HA
240kda-
WB: myc
B
Extracellular region
Intracellular region
7TM
N
C
Loop1
Loop2
KK
Loop3
KK
851
860
879
893
+HA-Ub
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myc-mGluR7 WT
myc-mGluR7_D851
myc-mGluR7_D860
myc-mGluR7_D879
myc-mGluR7_D893
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240kda-
WB: myc
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Ubiquitinated mGluR7/Total mGluR7
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1.5
1.0
0.5
0.0
***
***
mGluR7 only
mGluR7 WT
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4k4r
D857/4k4r
2k2r
D857/2k2r

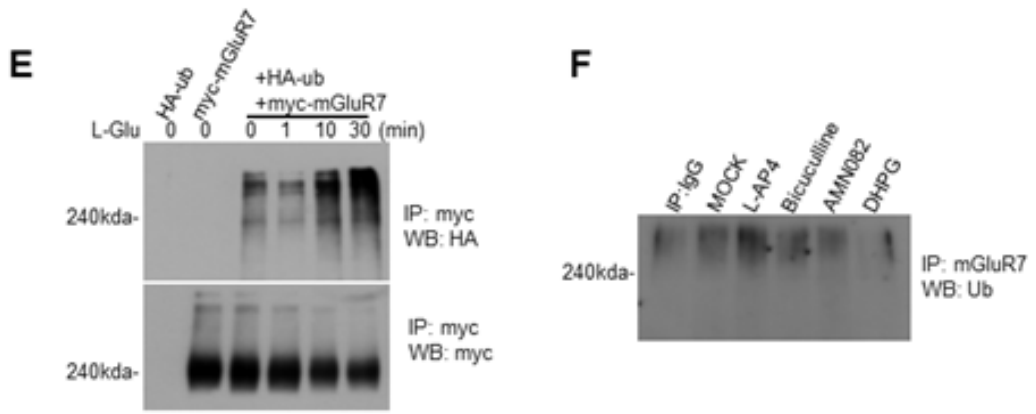


Figure.1 mGluR7A is underwent to ubiquitination in the HEK293T cells and Neuronal cells. (A) HA-Ub and Myc-mGluR7 DNA constructs were overexpressed in HEK293T cells. IP with Myc-specific antibody and did western blot for detecting ubiquitinylated mGluR7A. This result show mGluR7A is constitutively ubiquitinylated in cells. (B) mGluR7A DNA was mutated using site-directed mutagenesis method. mGluR7A WT is full-length and mutants - (Deletion851, Deletion860, Deletion879, Deletion893) have short length on C-terminus of mGluR7A. mGluR7A WT and all that mutants show there is no change of mGluR7A ubiquitination on the mutation.(C) Intracellular loops of mGluR7A were mutated with sited-directed mutagenesis. And Flag-Nedd4 which is E3 ligase was overexpressed to HEK293T cells. At this results I found out mGluR7A ubiquitination was around on the C-terminus and also intracellular loop2. (D) This graph is shown that the mean SEM of four independent experiments. Paired t test was conducted with Prism program to detect statistical significance: ***$p<0.0005$(D857/4k4r), ***$p<0.0004$(D857/2k2r) (E) L-glutamic acid 1mM was treated to HEK293T cells as a agonist of mGluR7A for the indicated times. Ubiquitination

of mGluR7A was increased by activity dependent manner. (F) In cortical neurons Ubiquitination of mGluR7A was increased by L-AP4 treatment.

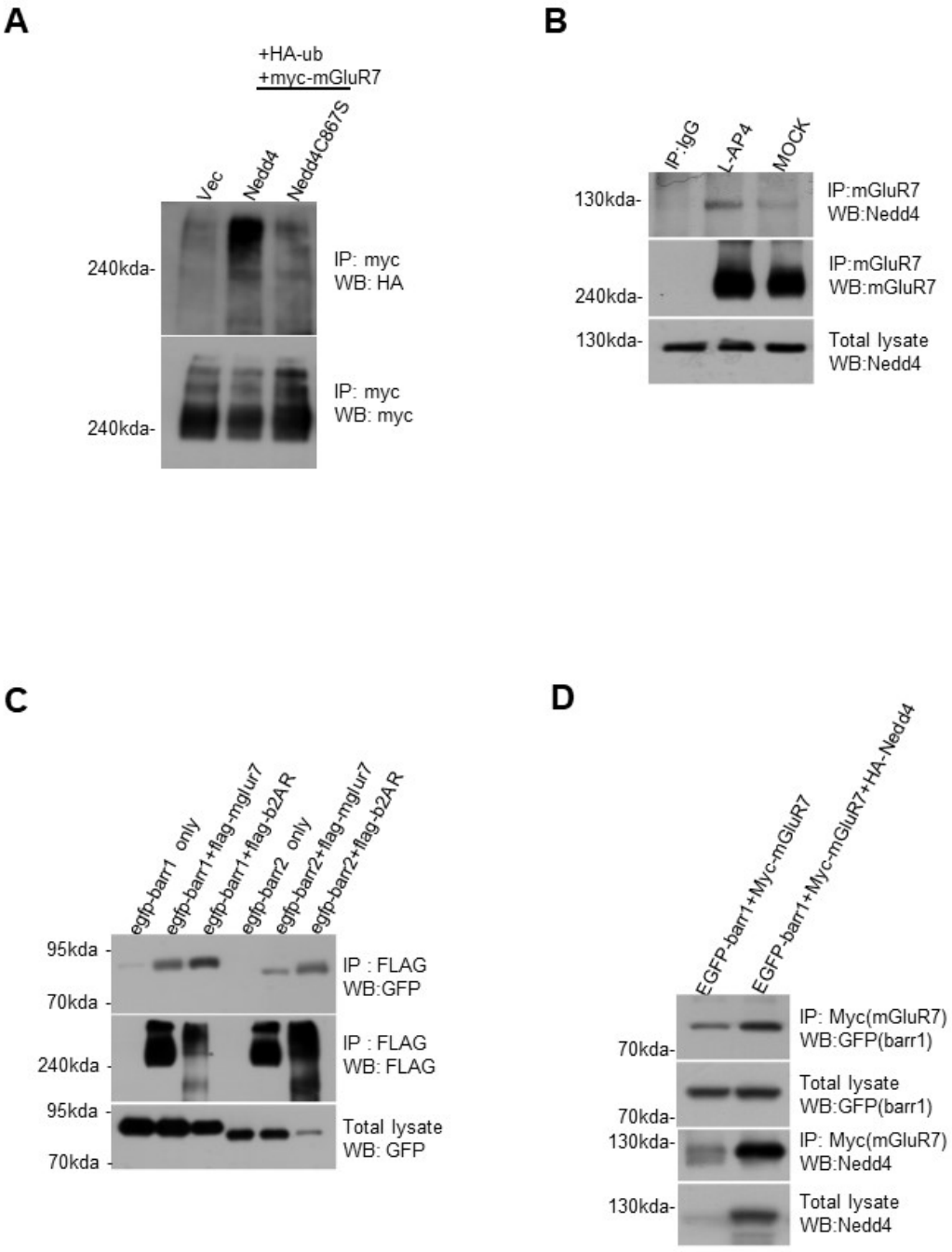
Nedd4 promotes mGluR7A to be ubiquitinylated and Nedd4 binds to mGluR7A in Neuronal cells. Also β-arrestin1 strongly binds to mGluR7A and Nedd4 facilitate the binding

To assess the element which is required for mGluR7A ubiquitinylation I used well-known E3 ligase, Nedd4 for detecting mGluR7A ubiquitinylation in HEK293T cells (Fig.2A). Dominant-Negative mutant, Nedd4C867S was mutated on Nedd4 WT for template DNA and used for the control of Nedd4 WT comparing ubiquitinylation matter of degree. Only in Nedd4 and mGluR7A co-transfected cells mGluR7A ubiquitinylation was increased on agonist stimulation in HEK293T cells. Furthermore, result of (Fig.2B) shows that Nedd4 binds to mGluR7A in cortical neurons. Also when neuronal cells were treated with L-AP4 binding of mGluR7A-Nedd4 was increased.

Previous studies of β-2-adrenergic receptor which is well-known GPCR revealed that both β-arrestin1 and β-arrestin2 bind to β-2-adrenergic receptor. This being so, I tested whether β-arrestins bind to mGluR7A or not (Fig.2C). Because β-arrestin2 have low binding affinity to mGluR7A, I used β-2AR-IP for control of this results. In this result it seems that β-arrestin1 strongly binds to mGluR7A while β-arrestin2 weekly binds to mGluR7A. These consequences aroused my curiosity about relationship of this triple complex – mGluR7A, β-arrestin1 and Nedd4. EGFP-β-arrestins, Myc-mGluR7A and HA-Nedd4 DNA constructs were expressed in HEK293T cells and co-IP experiments were conducted (Fig.2D, 2E). When Nedd4 was over-expressed in HEK293T cells binding of mGluR7A-β-arr1 was increased rather than Nedd4 was not transfected lane. It may be Nedd4 promotes binding of mGluR7A-β-arr1. On the other hand, Nedd4 did not affect to binding of mGluR7A-β-arr2.

In sequence, I dissected the region of mGluR7A where Nedd4 and β-arrestin1 bind (Fig.2F). The short sequence of mGluR7A C-terminus tail, mGluR7A-Loop1, mGluR7A-Loop2 and mGluR7A-Loop3 DNAs were transformed to BL21 competent cells and then I produced proteins induced GST beads. I used these GST beads for GST pull-down assay in cultured neurons. And protein quantities were confirmed with code-gel-blue staining (bottom panel).

This result shows that both Nedd4 and beta-arrestin1 binds to C-terminus and Loop2 of mGluR7A. I could not find out beta-arrestin2 binding regions because of low affinity in endogenous.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+myc-mGluR7
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Nedd4C867S
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IP: myc
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240kda-
IP: myc
WB: myc
B
IP:IgG
L-AP4
MOCK
130kda-
IP:mGluR7
WB:Nedd4
240kda-
IP:mGluR7
WB:mGluR7
130kda-
Total lysate
WB:Nedd4
C
egfp-barr1 only
egfp-barr1+flag-mglur7
egfp-barr1+flag-b2AR
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70kda
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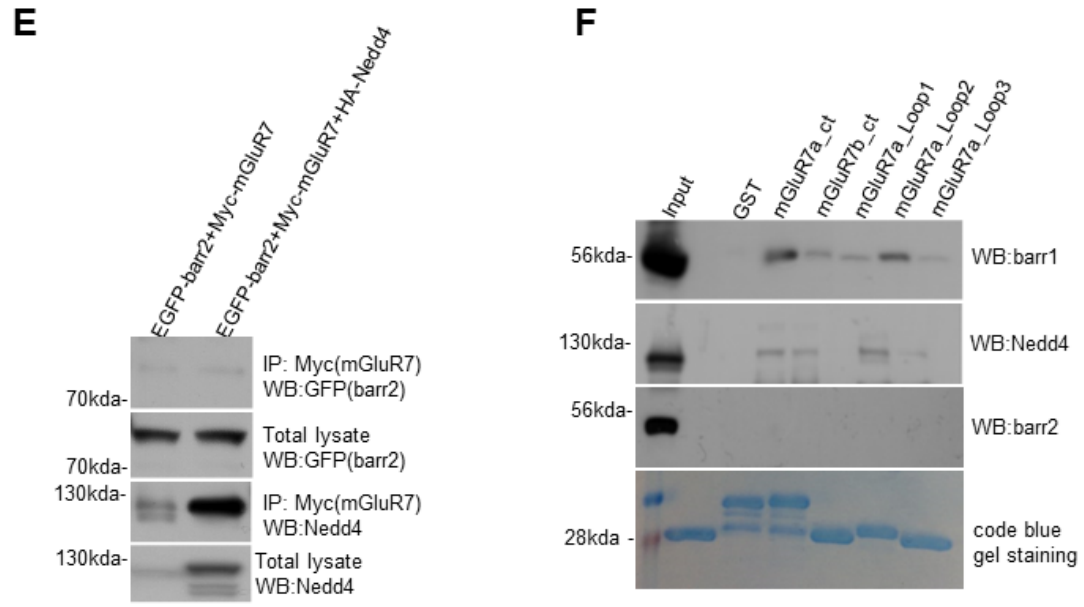


Figure.2 Nedd4 promotes mGluR7 to be ubiquitinated and Nedd4 binds to mGluR7 in Neuronal cells. Beta-arrestin1 strongly binds to mGluR7 and Nedd4 facilitate the binding. (A) HA-Ub, Myc-mGluR7, Nedd4 or Nedd4CS DNA constructs were transfected to HEK293T cells. Ubiquitinylated mGluR7A was IP with Myc-tag specific antibody and western blot by HA specific primary antibody. Ubiquitination of mGluR7A was increased only Nedd4 overexpressed lane. (B) DIV14 cortical neuron was treated with mGluR groupIII agonist, L-AP4 0.4mM for 5min. co-IP was conducted with endogenous mGluR7A specific antibody and then western blot Nedd4 antibody to detect binding of two molecules. This result is analogous to result of (fig.1E) so that binding of mGluR7A and Nedd4 was increased only in stimulation of L-AP4. (C) EGFP-β-arrestin1, Flag-mGluR7 and flag-β-2-AR DNA constructs were overexpressed in HEK293T cells. Co-IP with Flag-β-2-AR was used experimental control which is well-known to interact

with β-arrestin2. This results show that β-arrestin1 binds to mGluR7A while β-arrestin2 weekly binds to mGluR7A. (D) HA-Nedd4, EGFP-β-arrestin1and Flag-mGluR7A were co-transfected to HEK293T cells. In this result, it is reasonable to assume that binding of mGluR7A and β-arrestin1 could be facilitated by Nedd4. (E) In the same way to (fig.2D) there is difficulty to detect binding of mGluR7A and β-arrestin2 because of low affinity of β-arrestin2. And there is no increased binding of mGluR7A and β-arrestin2 by Nedd4. (F) To identify the binding region of mGluR7A mutated mGluR7A-GST protein beads were generated and confirmed protein quantity with code-gel-blue staining. mGluR7A deletion mutant protein induced GST bead and cultured neuronal cell lysates are incubated at 4℃ for binding assay. This result represents β-arrstin1 and Nedd4 bind to mGluR7A C-terminus tail and intracellular loop2 while the region of β-arrestin2 binding couldn’t find out because of low affinity.

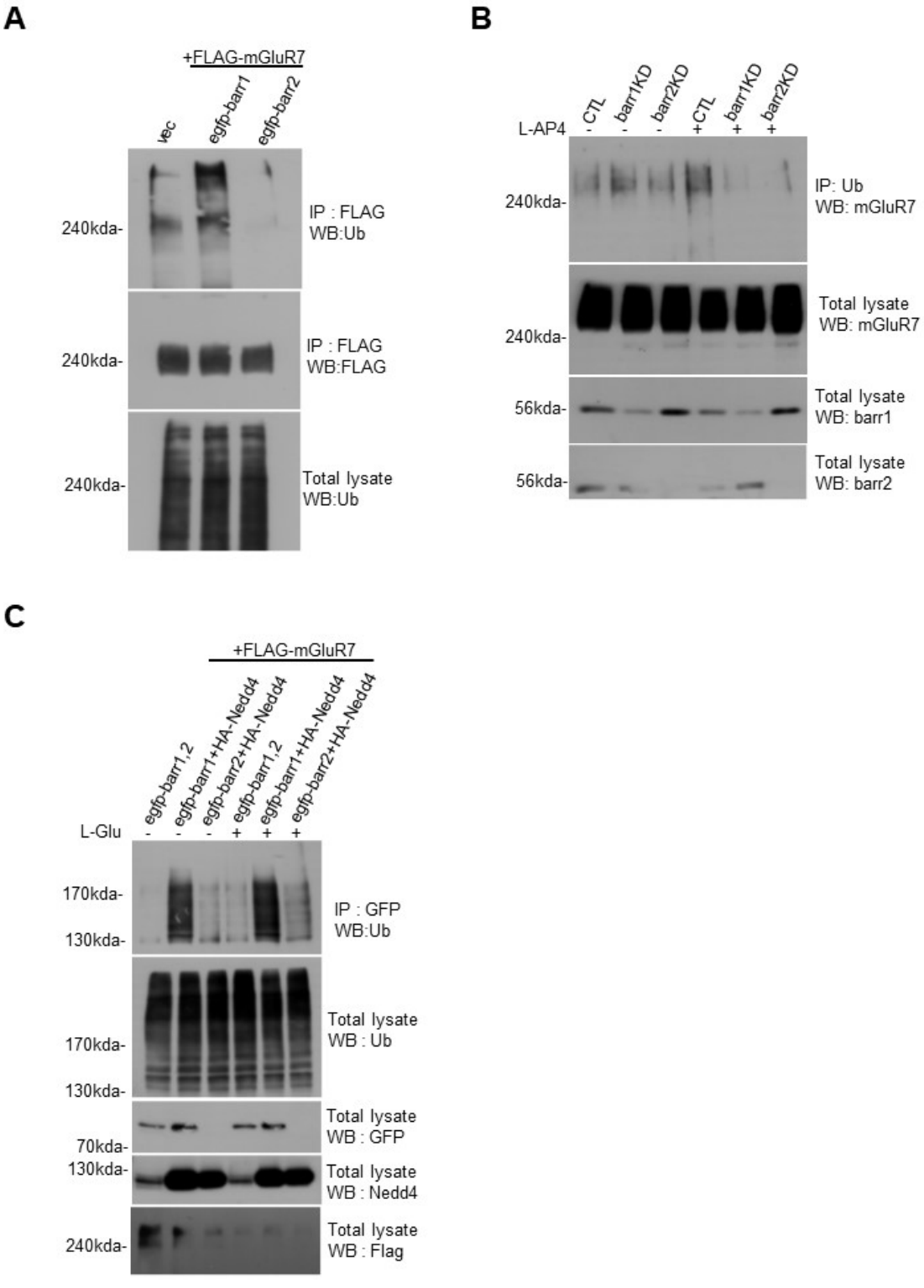
β-arrestin1 is required for ubiquitinylation of mGluR7A and Nedd4 ubiquitinates β-arrestin1

β-arrestin proteins are widely known for function of adaptor proteins so that they can recruit E3 ligase to receptors and affect to receptor ubiquitinylation. Because β-arrestin1 binds to mGluR7A it may be influence to mGluR7A ubiquitinylation. So I tested that β-arrestin1 over-expressed condition could affect to mGluR7A ubiquitinylation in HEK293T cells (Fig.3A) like likewise when Nedd4 was over-expressed (Fig.2A). In lane transfected β-arrestin1, mGluR7A ubiquitinylation was increased. However β-arrestin2 did not affect to mGluR7A ubiquitinylation. For the precise comparison I used rat hippocampal cultured neurons and β-arrestin1,2 knock-down lenti-viruses were infected for this experiments (Fig.3B). The basal level of mGluR7A ubiquitinylation had little variations. Comparing with non-stimulation condition and L-AP4 treatment condition, mGluR7A ubiquitinylation was increased in control lane when cells were treated L-AP4. Otherwise, when β-arrestin1 or β-arrestin2 was knock-downed the increased mGluR7A ubiquitinylation could not be observed.

These results mean that β-arrestin proteins can be adaptor protein for E3 ligase of mGluR7A ubiquitinylation. And also β-arrestin proteins are generally known to be self-ubiquitinylation for its own. Because Nedd4 let binding of mGluR7A-β-arr1 to promote, I assumed that Nedd4 ubiquitinates β-arrestin proteins (Fig.3C). I transfected triple DNAs to HEK293T cells and IP with β-arrestin proteins. The very β-arrestin proteins over-expression made mGluR7A ubiquitinylation to be increased. Moreover, on the agonist treatment mGluR7A ubiquitinylation was increased in both β-arrestin1 over-expression

lane and β-arrestin2 over-expression lane.

This data suggests that mGluR7A agonist activation regulates β-arrestin proteins ubiquitinylation.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vec
egfp-barr1
egfp-barr2
240kda-
IP : FLAG
WB:Ub
240kda-
IP : FLAG
WB:FLAG
240kda-
Total lysate
WB:Ub
B
L-AP4
- CTL
- barr1KD
- barr2KD
+ CTL
+ barr1KD
+ barr2KD
240kda-
IP: Ub
WB: mGluR7
240kda-
Total lysate
WB: mGluR7
56kda-
Total lysate
WB: barr1
56kda-
Total lysate
WB: barr2
C
+FLAG-mGluR7
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egfp-barr1+HA-Nedd4
egfp-barr2+HA-Nedd4
egfp-barr1,2
egfp-barr1+HA-Nedd4
egfp-barr2+HA-Nedd4
L-Glu
- - - + + +
170kda-
130kda-
IP : GFP
WB:Ub
170kda-
130kda-
Total lysate
WB : Ub
70kda-
Total lysate
WB : GFP
130kda-
Total lysate
WB : Nedd4
240kda-
Total lysate
WB : Flag

Figure.3 β-arrestin1 is required for ubiquitination of mGluR7A and Nedd4 ubiquitinates β-arrestin1 (A) Flag-mGluR7A, EGFP-β-arrestin1 and EGFP-β-arrestin2 were co-transfected to HEK293T cells. Flag-mGluR7A was immune-precipitated and ubiquitinylated mGluR7A was immunoblotted. In the middle lane which EGFP-β-arrestin1 is overexpressed, ubiquitination of mGluR7A was increased. Meanwhile β-arrestin2 overexpression was not influent to ubiquitination of mGluR7A. (B) For this experiment, rat-β-arrestin1 and rat-β-arrestin2 knock-down lentivirus cloning and production were preceded. Cultured neuronal cells were infected β-arrestin1, 2 KD lentivirus at DIV7 and matured until DIV14. Endogenous Ub was immune-precipitated and ubiquitinylated mGluR7A was immunoblotted by mGluR7A antibody. When β-arrestin1 was knock-down ubiquitination of mGluR7A was abolished. (C) HEK293T cell was transfected Flag-mGluR7A, HA-Nedd4 and EGFP-β-arrestin1 or EGFP-β-arrestin2. In the lane co-transfected with Nedd4 and β-arrestin1, β-arrestin1 ubiquitination was dramatically increased while β-arrestin2 ubiquitination was slightly increased. And this phenomena undergo activity dependent manner.

β-arrestin1 binds to mGluRA7 and Nedd4 by activity-dependent manner and makes multi-complex

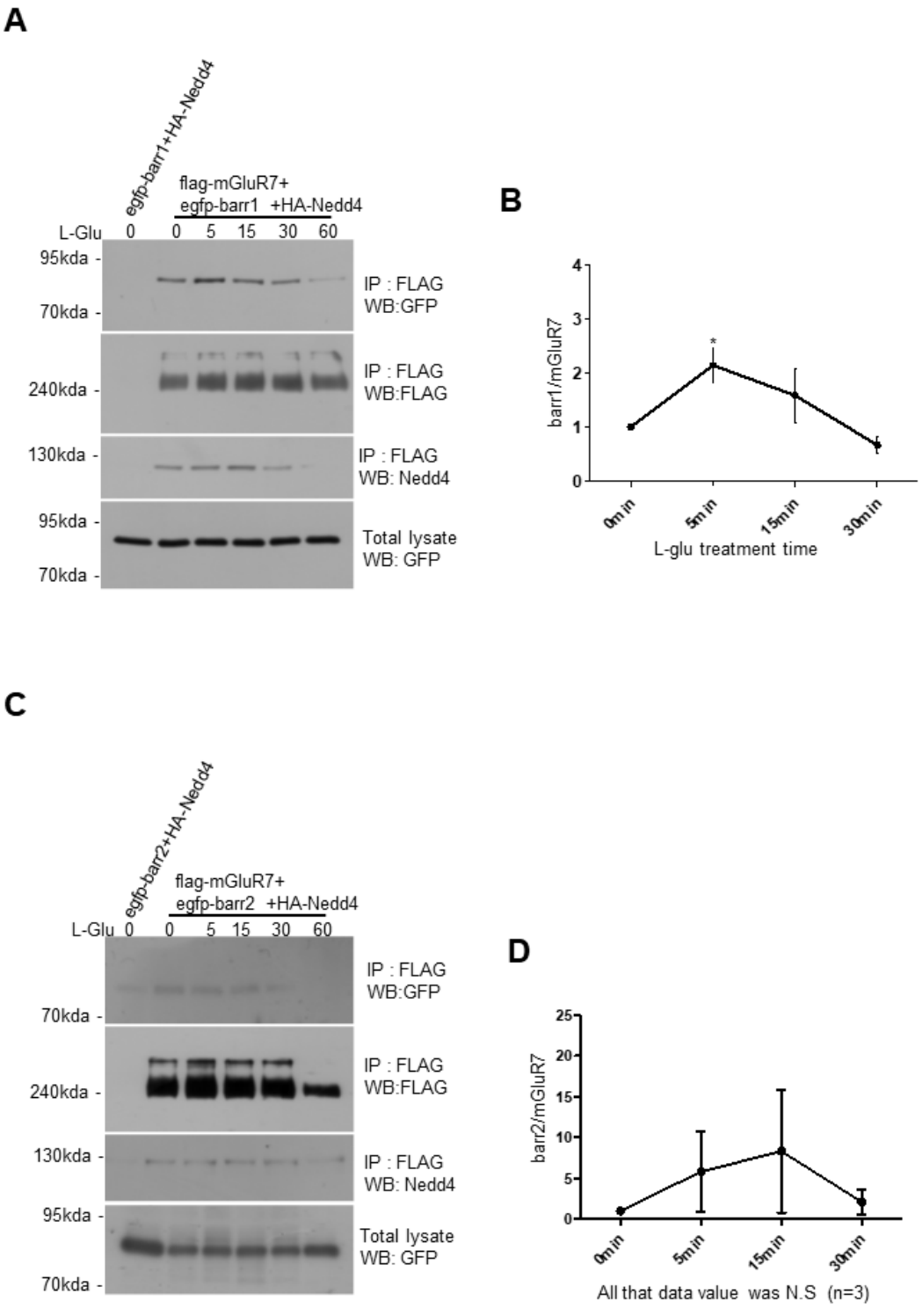
To find out whether β-arrestin1-mGluR7A-Nedd4 indeed interact each other in activity-dependently, I analyzed what if activation of mGluR7A leads to increasing β-arrestin1-mGluR7A binding and Nedd4-mGluR7A binding. In that of bindings, β-arrestin1 could be bridge of mGuR7A-Nedd complex. When I tested activity-dependent binding of mGluR7A-β-arrestin1 I observed increased binding of mGluR7A-β-arrestin1 upon agonist stimulation (Fig.4A). However β-arrestin2 was not regulated by mGluR7A activation (Fig4C). And also binding of mGluR7A-Nedd4 was dynamic when β-arrestin1 was overexpressed in HEK293T cells meanwhile whereas β-arrestin2 did not work (Fig4. and Fig.4B). mGluR7A-β-arrestin binding portion was quantified and analyzed with image J software and Prison software (Fig.4B and Fig.4D).

In sequence, I co-IP β-arrestin1 and Nedd4 in HEK293T cells under mGluR7A activation with agonist. As I expected β-arrestin1 binds to Nedd4 activity-dependently (Fig.4E). Especially, at L-glutamate treatment time 1min and 10min Nedd4-β-arrestin1 binding was dramatically increased in HEK293T cell. But drug treatment could not prolonged the increasing. These data were analyzed and quantified and show (Fig.4F) graph.

Through above data, I assumed that β-arrestin1 have function as adaptor of mGluR7A-Nedd4 binding. So I tested what if β-arrestin1 were abolished, activity-dependent increasing of mGluR7A-Nedd4 binding also disappeared in HEK293T cells (Fig.4G). Using β-arrestin1-siRNA, endogenous β-arrestin1 was knock-downed in HEK293T cells.

In Control (CTL) which was transfected scrambled siRNA, binding of mGluR7A-Nedd4 was augmented upon L-glutamic acid. In the contrast, the dynamic of mGluR7A-Nedd4 binding did not work after β-arrestin1 KD.

This data verify that β-arrestin1 is bridge of mGluR7A-Nedd4 and regulate activity-dependent dynamic of mGluR7A.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flag-mGluR7+
egfp-barr1 +HA-Nedd4
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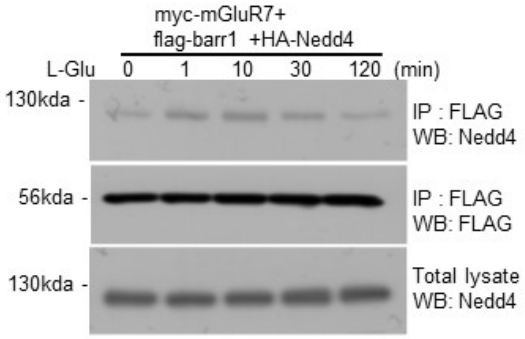
70kda -
IP : FLAG
WB:GFP
240kda -
IP : FLAG
WB:FLAG
130kda -
IP : FLAG
WB: Nedd4
95kda -
70kda -
Total lysate
WB: GFP
B
barr1/mGluR7
0
1
2
3
4
*
0min
5min
15min
30min
L-glu treatment time
C
egfp-barr2+HA-Nedd4
flag-mGluR7+
egfp-barr2 +HA-Nedd4
L-Glu 0 0 5 15 30 60
70kda -
IP : FLAG
WB:GFP
240kda -
IP : FLAG
WB:FLAG
130kda -
IP : FLAG
WB: Nedd4
95kda -
70kda -
Total lysate
WB: GFP
D
barr2/mGluR7
0
5
10
15
20
25
0min
5min
15min
30min
All that data value was N.S (n=3)

E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flag-barr1 +HA-Nedd4
L-Glu 0 1 10 30 120 (min)
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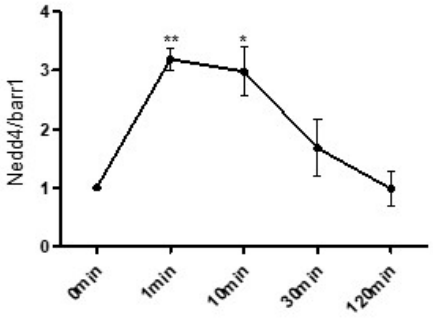
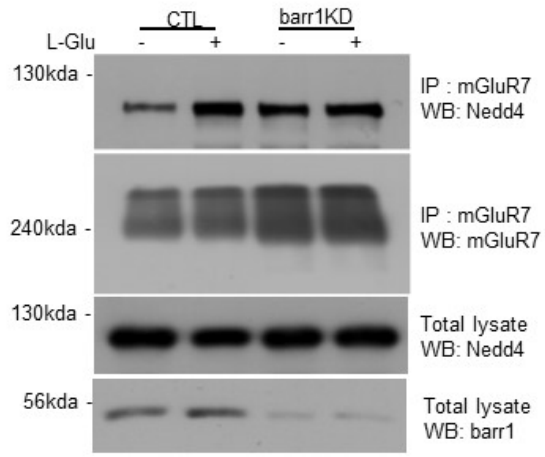
IP : FLAG
WB: Nedd4
56kda -
IP : FLAG
WB: FLAG
130kda -
Total lysate
WB: Nedd4

F


Nedd4/barr1
0
1
2
3
4
**
*
0min
1min
10min
30min
120min

G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IP : mGluR7
WB: mGluR7
130kda -
Total lysate
WB: Nedd4
56kda -
Total lysate
WB: barr1

Figure.4 β-arrestin1 binds to mGluR7A and Nedd4 by activity-dependent manner and makes multi-complex. (A) HEK293T cells were transfected EGFP-β-arrestin1, FLAG-mGluR7A and HA-Nedd4. And L-glutamic acid 1mM was treated to HEK293T cells for the indicated times. β-arrestin1 was immune-blotted with GFP specific antibody after mGluR7A was immune-precipitated with FLAG antibody. β-arrestin1 binds to mGluR7A activity dependently and time dependently. (B) This result is shown that the mean SEM of three experiments. Paired t test was conducted with prism program to detect statistical significance: *P<0.0349 Binding of mGluR7A and β-arrestin1 is significantly increased at L-glutamic acid treatment for 5min. (C) HEK293T cells were transfected EGFP-β-arrestin2, FLAG-mGluR7A and HA-Nedd4.And L-glutamic acid 1mM was treated to HEK293T cells for the indicated times. β-arrestin2 constitutively binds mGluR7A and there is no change over the agonist stimulation. (D) This result is quantification graph of (fig.4C). (E) HEK293T cells were transfected Myc-mGluR7A, Flag-β-arrestin1 and HA-Nedd4. L-Glutamic acid 1mM was treated to cells for the indicated time scales. β-arrestin1 was co-immuno-precipitated with FLAG antibody and Nedd4 was detected with Nedd4 antibody with immune-blotting. Binding of β-arrestin1 and Nedd4 was increased at time point of 1min and 10min. (F) This graph represent the value of mean SEM of three experiments. Paired t test was conducted with prism program to detect statistical significance: *P<0.0396, **P<0.0069 β-arrestin1 binds to E3 ligase Nedd4 on the activity-dependent manner and the binding was dramatically increased at 1min treatment. (G) Myc-mGluR7A was overexpressed and β-arrestin1 siRNA was transfected to HEK293T cells which is endogenously expressed β-arrestin1.

Scrambled siRNA (CTL) was also transfected to HEK293T cells. And L-glutamic acid was treated for 10min. Activity dependent increasing of mGluR7A and Nedd4 was detected in the CTL while the increasing was abolished when endogenous β-arrestin1 was knock-down.

Beta-arrestin1 and mGluR7 change the distribution upon GluR7 agonist treatment

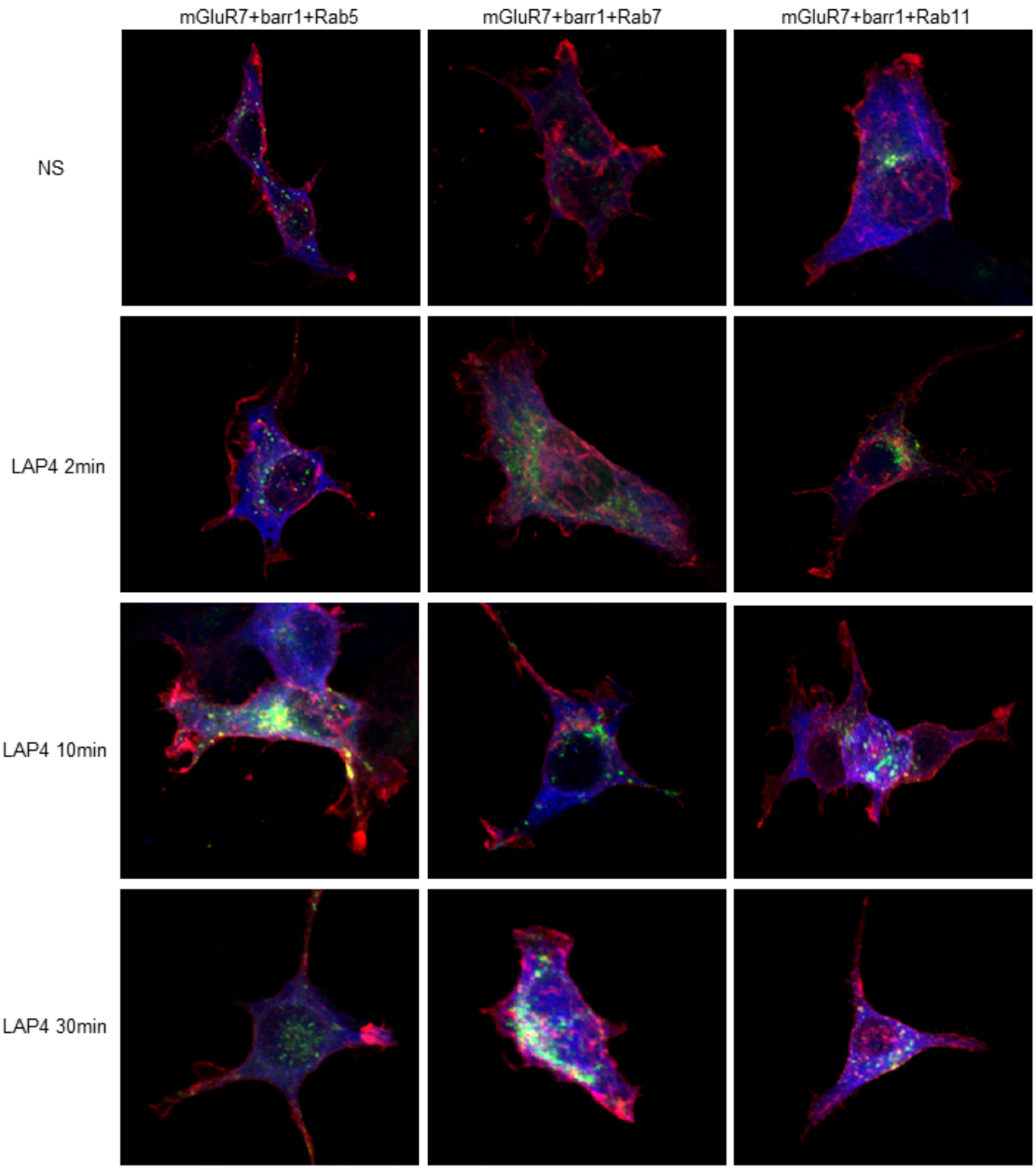
Now I know that β-arrestin1 can move with mGluR7A on agonist stimulation and mGluR7A goes ubiquitinylation pathways, I tested to find out where mGluR7A-β-arrestin1 (Fig.5A). I co-transfected triplet DNAs, mGluR7A-β-arrestin1-Rab family, to HEK293T cells. (Rab5 is early endosomal marker, Rab7 is late endosomal marker and Rab11 is recycling endosomal marker) β-arrestin1 diffused in entire cells and co-localized with mGluR7A even if before agonist treatment. When L-AP4 treatment in 10min, mGluR7A was co-localized with early endosomal marker and after that time they came off each other. When treatment time at 30min, late endosomal marker and recycling marker were co-localized with mGluR7A.

These data shows mGluR7A activation with β-arrestin1 and agonist regulates mGluR7A trafficking in HEK293T cells.

I used vector DNA instead of FLAG-β-arrestin1 plasmid DNA to compare the moving of mGluR7A and β-arrestin1 (Fig.5B). In this case, there is no dramatic change of mGluR7A distribution.

These results show that β-arrestin1 let mGluR7A internalize in HEK293T cells. Although there is still endogenous β-arrestin1 in HEK293T cells, over-expressed β-arrestin1 clearly affects on distribution of mGluR7A.

A


mGluR7+barr1+Rab5
mGluR7+barr1+Rab7
mGluR7+barr1+Rab11
NS
LAP4 2min
LAP4 10min
LAP4 30min

B

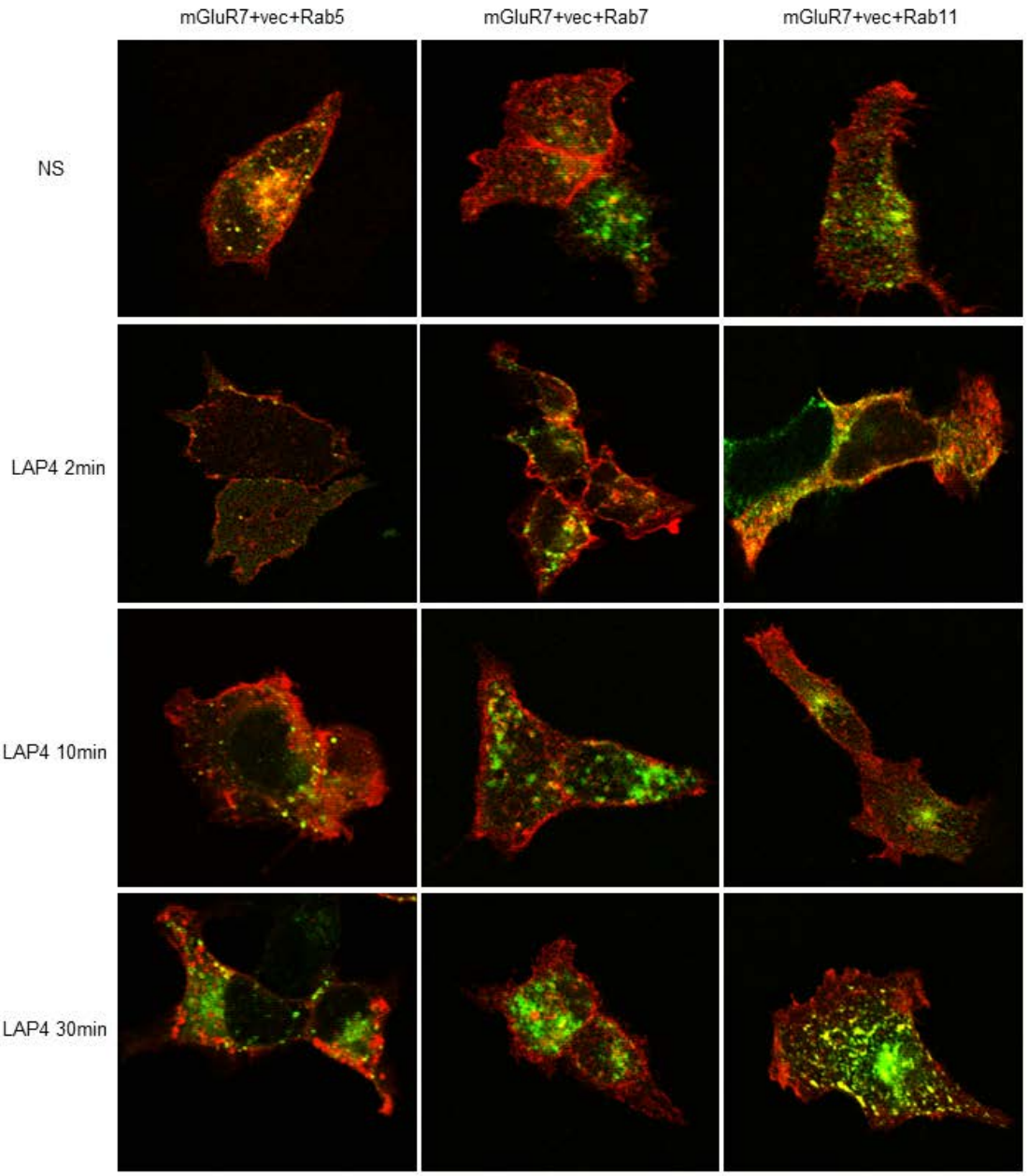

mGluR7+vec+Rab5
mGluR7+vec+Rab7
mGluR7+vec+Rab11
NS
LAP4 2min
LAP4 10min
LAP4 30min

Figure.5 β-arrestin1 and mGluR7A change the distribution on the mGluR7A agonist treatment. (A) Flag-β-arrestin1, myc-mGluR7A and GFP-Rab5 or Rab7 or Rab11 were co-transfected to HEK293T cells and L-AP4 0.4mM was treated for 15min. And the process was conducted on the confocal microscopy (Nickon A1), Z-stack with 60X objective. Flag-β-arrestin1 was immuno-stained with anti-FLAG (M2) antibody is shown in the blue channel, and GFP-Rab5 or Rab7 or Rab11 is shown in green, Myc-mGluR7A was immune-stained with 9E10 antibody is shown in the red channel. Co-localization of mGluR7A and Rab family constructs could be detectable on the yellow color in the merged images. Co-localization of mGluR7A and β-arrestin1 can be detectable on the purple color in the merged images. This data shows that β-arrestin1 exists in diffused around in HEK293T cells. At the L-AP4 treatment for 10min, mGluR7A and early endosome marker were co-localized. In the 30min late endosomal marker, recycling endosome marker and mGluR7A were co-localized. Furthermore, mGluR7A, recycling marker and β-arrestin1 makes triplet complex on 30min. (B) Instead of Flag-β-arrestin1, pcDNA3-vector plasmid DNA was transfected to HEK293T cells. Other transfected DNAs and immuno-staining conditions are in same with (fig.5A).

β-arrestin proteins and Nedd4 allow mGluR7A to be internalized in Neuronal cells

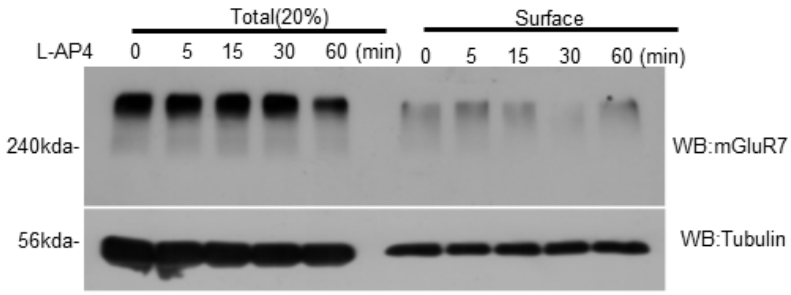
To make sure activity-dependent mGluR7A trafficking, mGluR7A surface biotinylation assay was conducted in cortical neuron cells (Fig.6A). Most of mGluR7A was internalized on L-AP4 treatment at 30min and recycled at 60min.

Accordingly, I tested that if β-arrestin1 is knock-down or β-arrestin2 is knock-down or Nedd4 is knock-down in cultured neuronal cells (Fig.6B). When β-arrestin1 or Nedd4 was knock-downed, activity-dependent internalization of mGluR7A could not observed. Otherwise β-arrestin2 KD did not work at mGluR7A internalization.

To confirm this I conducted mGluR7A internalization assay and immune-staining in hippocampal neurons (Fig.6C and Fig.6E). I transfected mGluR7A and β-arrestin proteins DNAs to hippocampal neurons using tag-specific antibody, mGluR7A surface was stained in red color and internalized mGluR7A was visualized with green color. Even only when β-arrestin1 or β-arrestin2 was over-expressed, mGluR7A internalization was increased (Fig.6C and Fig.6D).

Also Nedd4 over-expression affects to mGluR7A internalization while Nedd4 DN mutant did not (Fig.6E and Fig.6F). These consequences indicate that β-arrestin proteins–Nedd4-mGluR7A multi-complex regulates mGluR7A trafficking in neurons.

A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0 5 15 30 60 (min)
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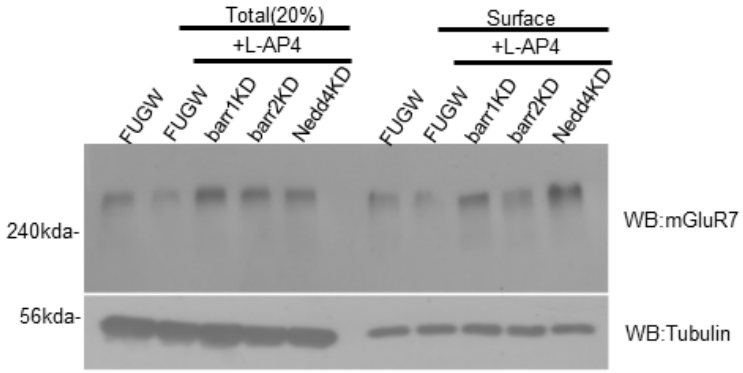
56kda-
WB:Tubulin

B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Surface
+L-AP4
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FUGW
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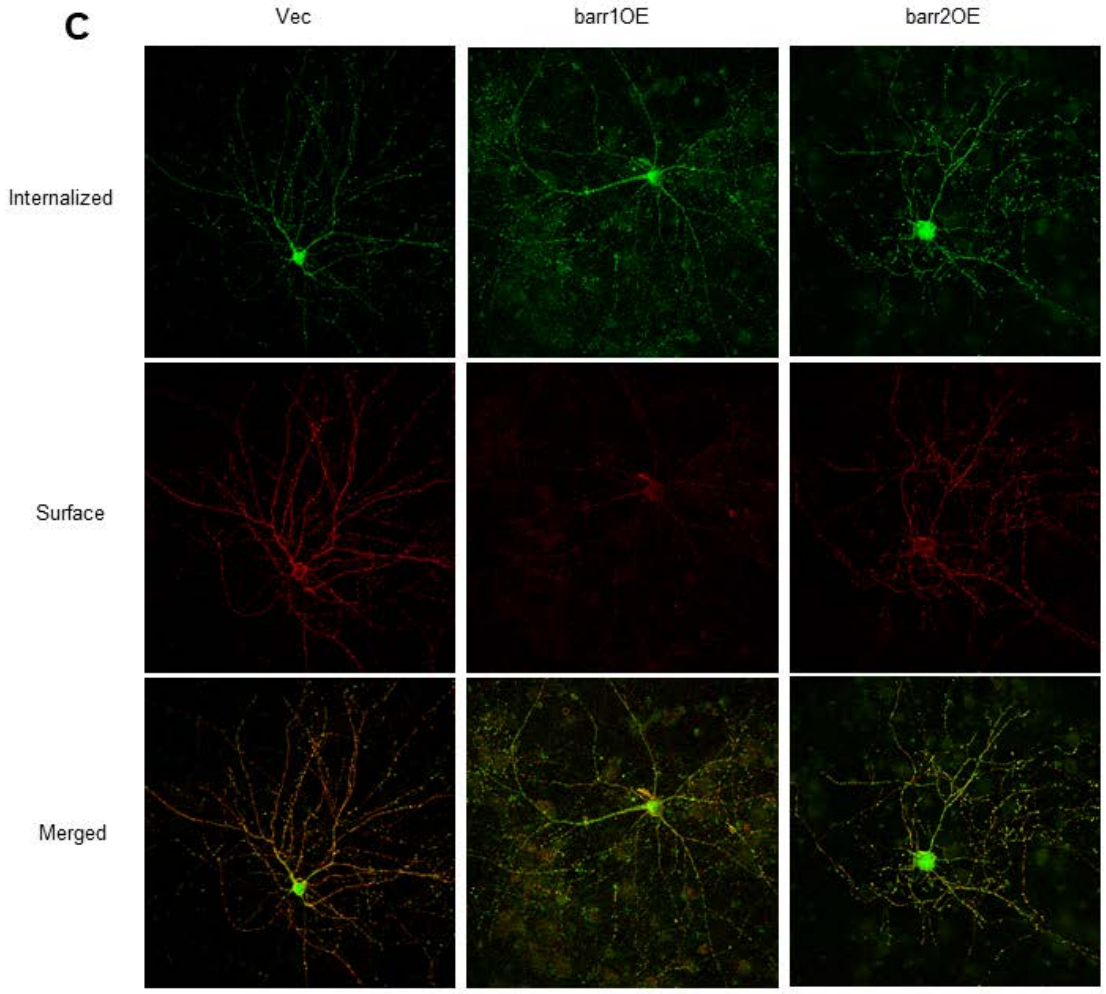
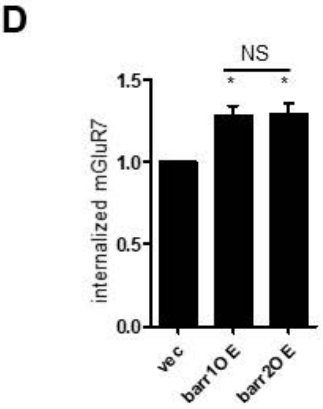

C
Vec
barr1OE
barr2OE
Internalized
Surface
Merged


D
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*
*
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0.5
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internalized mGluR7
vec
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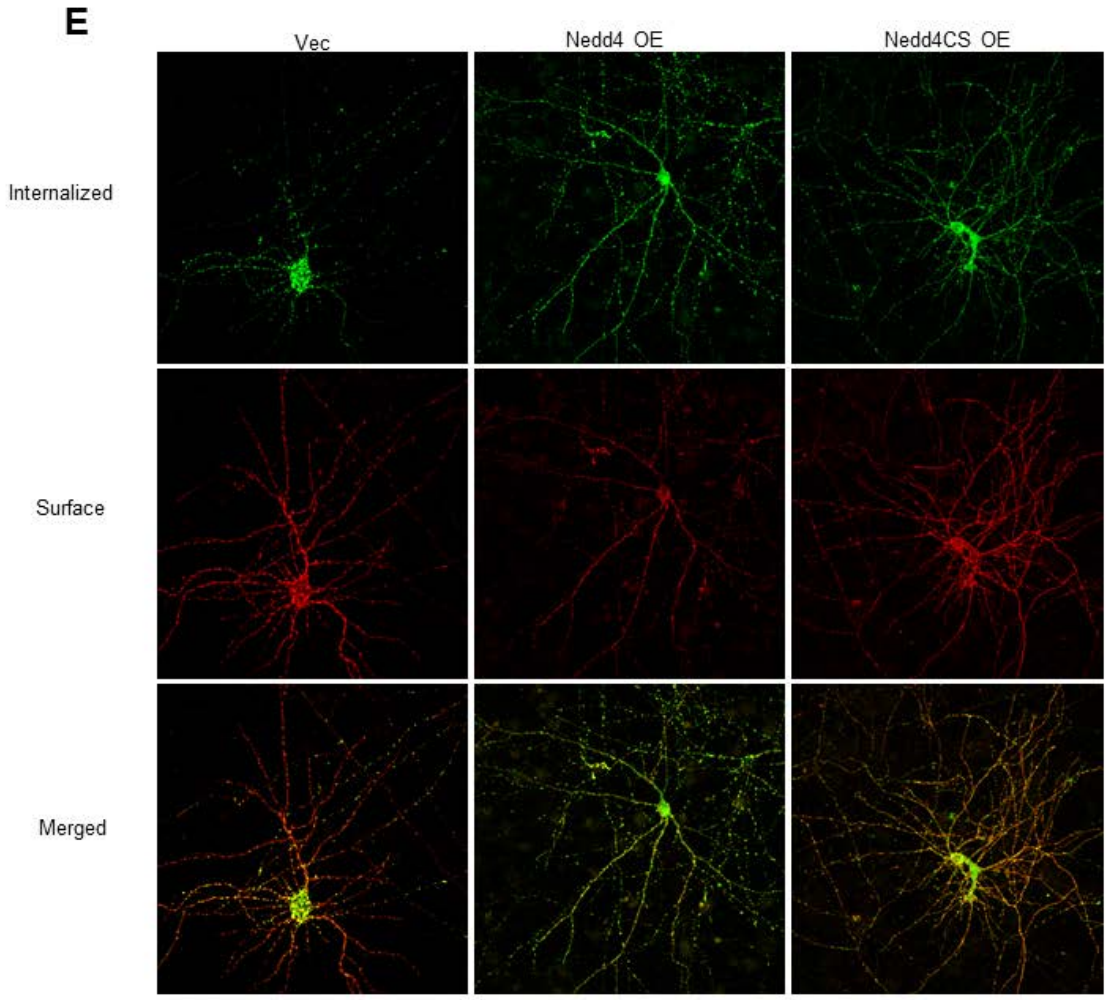
barr2OE


E
Vec
Nedd4 OE
Nedd4CS OE
Internalized
Surface
Merged

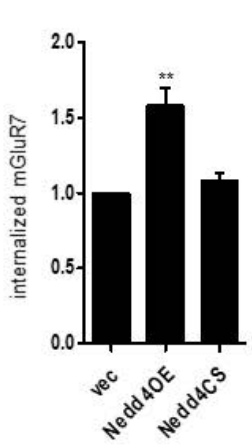

F
internalized mGluR7
2.0
1.5
1.0
0.5
0.0
**
vec
Nedd4OE
Nedd4CS

Figure.6 β-arrestins and Nedd4 allow mGluR7A to be internalized in Neuronal cells. (A) In primary cultured cells mGluR7A surface biotinylation assay was conducted. L-AP4 was treated DIV14 cortical neurons during indicated time scale. I could observe that mGluR7A is internalized when L-AP4 treatment time was at 30min. based on this data, I fixed the L-AP4 treatment time to 30min. (B) DIV14 cortical neuron was infected with β-arresstin1 KD virus, β-arrestin2KD virus and Nedd4 KD virus each and these cells were used to mGluR7A surface biotinylation assay. When β-arrestin1 and Nedd4 were knock-down, internalization of mGluR7A was blocked. On the other hand, β-arrestin2 KD was not affect to stability of mGluR7A. (C) Flag-mGluR7A and HA-β-arrestin1 or HA-β-arrestin2 DNA constructs were co-transfected to DIV11 hippocampal neurons. mGluR7A internalization assay was experimented in DIV14. Internalized fraction of mGluR7A could be shown in green color and surface fraction of mGluR7A is in red channel. And this process was conducted on the confocal microscopy (Nickon A1), Z-stack with 40X objective. mGluR7A internalization was increased when β-arrestin1 and β-arrestin2 were overexpressed in hippocampal cells. (D) This graph is the quantification result of (fig.6c) and represent the value of mean SEM of three experiments. Paired t test was conducted with prism program to detect statistical significance: *P<0.0405(βarr1OE), *P<0.0429(βarr2 OE) (E) Myc-mGluR7A and HA-Nedd4 or HA-Nedd4CS DNA constructs were co-transfected to DIV11 hippocampal neurons. mGluR7A internalization was increased when Nedd4 was overexpressed in hippocampal cells rather Nedd4CS didn't affect to internalization of mGluR7A. (F) This graph is the quantification result of (fig.6E) and represent the value of mean SEM of five independent experiments. Paired t test was conducted with prism program to detect statistical significance: **P<0.00065

DISCUSSION

mGluR7A is pre-synaptically expressed throughout the CNS. Mainly mGluR7A is thought to be a role of inhibitory effects on transmission at the synapses. Thereby if there is a small error of mGluR7A post-translational modification, it could be leaded to severe mental disorder.

Recent studies for mGluR7A using gene knockout mice revealed that mGluR7A regulates emotionality, stress, and fear responses (5-10). Furthermore mGluR7A is implicated in neurological and psychiatric disorder development. For these diseases, mGluR7A could be a new pharmacological target. In the modern society, mental disorder is commonplace. So the demand of treatment method and that of fundamental study must be needed.

Ubiquitinylation is one of the post-translational modification processes of proteins which are conjugated to small ubiquitin protein and typically targeted to the proteosomal pathway. In many previous reports, ubiquitinylation plays a role in protein turn-over and receptor trafficking (13). Although mGluR7A can be an attractive drug target due to the GPCR properties, whether mGluR7A is a target of ubiquitin conjugation has not been identified yet. Ubiquitin conjugation of proteins is carried out by an enzymatic cascade involving the systematical activity of three enzymes (E1, E2, E3 ligase). And these enzymes regulate ubiquitinylation reactions. Unlikely to E1 and E2 ligase, E3 ligases interact directly with their target substrate proteins. Thus, they generally appear the specificity to an ubiquitinylation reaction.

For this reason, which E3 ligase is the main E3 ligase of mGluR7A ubiquitinylation is most important question for this study. Interestingly I identified Nedd4 as a main E3 ubiquitinylation ligase for mGluR7A (Fig.2A).

My hypothesis is that agonist-dependent mGluR7A ubiquitinylation is mediated Nedd4. And this Nedd4 mediated mGluR7A ubiquitinylation would be essential to mGluR7A internalization and trafficking. And first in importance in this study is to find out the mechanism of Nedd4 binding manner to mGluR7A. And the novel finding of this research is this mGluR7A-Nedd4 interaction is mediated with small adaptor protein, β-arrestin1 but not β-arrestin2.

In fact, regulation of mGluR7A has been studied in diverse aspects which are included in phosphorylation and SUMOylation of mGluR7 (14, 4). These previous studies revealed that phosphorylation Ser-862 residue of mGluR7A C-terminus and SUMOylation of mGluR7A are key point of controlling mGluR7A surface stability. Following these studies I tried to test the phosphorylation effect of mGluR7A Ser-862 residue in ubiquitinylation. But I could not find any connection whatsoever.

In this study, I used mGluR7A agonist as L-glutamate or L-AP4 to activate mGluR7A. Because mGluR7A is not sensitive to low-concentration of L-glutamate or L-AP4 I had to treat high concentration drugs to cells (15). Furthermore, many papers report that AMN082, allosteric agonist of mGluR7A, leads to mGluR7A internalization but it was not effect on my experiments (16). When cells were treated high-concentration agonist, mGluR7A was ubiquitinated (Fig.1E, F). This result shows that mGluR7A

ubiquitinylation mechanism is on activity-dependent manner.

To identify the precise mechanic in intra-cellular region of cells, I introduced well-known ubiquitinylation mechanism of GPCR, β2AR receptor model. Arrestin protiens are small adaptor protein which bind to various receptors and regulate receptor cell surface level and signaling (17, 18). In the preceding studies, correlations of GPCR and arrestin proteins have been well established (19). There are two classes of GPCR, Class A GPCR transiently interacts with β-arrestin proteins and preferentially binds to β-arrestin2. But Class B GPCR robust interacts with β-arrestin proteins and binding with both arrestin protein iso-forms, β-arrestin1 and β-arrestin2 (20).

Despite these findings, mGluRs including mGluR7A have not been classified yet. However my results can suggest that mGluR7A is similar to class B. Because (figure.4) and (figure.5) show constitutively binding of mGluR7A-β-arrestin1 and this binding is robustly increase on agonist stimulation although β-arrestin2 very weekly binds to mGluR7A. And mGluR7A-β-arrestin2-Nedd4 activity-dependent dynamics did not observed because there is possibility of other E3 ligase which can interact with β-arrestin2 (Fig.4C, D).

Dissection result of β-arrestin proteins and Nedd4 binding sites of mGluR7A elucidate that both β-arrestin1 and Nedd4 bind to mGluR7A C-terminus and intra-cellular loop2. This data concurrence with dissection of the ubiquitinylation site experiments (Fig.1B, C and Fig.2F). Interestingly, second intra-cellular loop of mGluRs have been known to have important function at regulating receptors (21) and C-terminus of mGluRs are important

site as controlling G-protein coupling. These findings reasonably support my results. (Figure.6) data delineate that β-arrestin proteins and Nedd4 let mGluR7A internalization in cultured neuronal cells. In tern activity dependent mGluR7A ubiquitinylation leads mGluR7A to be internalized. It is not surprising that β-arrestin proteins mediate trafficking of mGluR7A. Because previous studies which in receptor endocytosis provide clues whose co-relation of clathrin-mediated receptor endocytosis and β-arrestin proteins (22-26).

For following study should explore the relevance of signaling with mGluR7A ubiquitinylation. Recently, there was a signaling study on mGluR7A associated with β-arrestin proteins in HEK293T cells not cultured neurons (27). In that paper, it is revealed opposite function of β-arrestin1 and β-arrestin2. β-arrestin1 promotes ERK1/2 signaling and reduce JNK signaling and β-arrestin2 affect to signaling on the other way. But the signaling could be dependent on cell types and conditions-over-expression or gene knock-down (28, 29). So it is valuable to dig the precise signaling pathway related with mGluR7A and β-arrestin proteins in cultured neuronal cells.

In conclusion my results of this study provide the insights of mGluR7A regulation mechanism which occur combine of multi-complex mGluR7A-β-arrestin1-Nedd4 and this complex have dynamics on mGluR7A activation which leads to mGluR7A ubiquitinylation.

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국문 초록

중추신경계에서 널리 발현되는 G단백질공역수용체인 글루타메이트 수용체7은 시냅스에서 신경전달 물질의 방출을 조절하는 것으로 알려져 있다. 그러나 글루타메이트 수용체7의 세포막 에서의 역동적인 움직임에 관한 자세한 조절작용에 대한 기작은 아직 자세하게 연구되어지지 않았다. 이 연구에서 나는 글루타메이트 수용체7이 유비퀴티네이션 현상을 겪게 되며, 이러한 현상이 글루타메이트 수용체7의 세포막에서의 움직임에 관여한다는 사실을 알아냈다. 이러한 과정에는 보조 단백질로 불리는 베타-어레스틴1이 관여하며, E3 유비퀴틴화 효소인 네드4가 주요한 E3 유비퀴틴화 효소로 작용 한다는 것을 역시 밝혀냈다. 글루타메이트 수용체7의 카복실-말단를 변성시킨 DNA와 세포막 내의 고리 부분의 아미노산을 변형시킨 DNA를 이용하여 글루타메이트 수용체7의 카복실-말단과 세포막 내의 고리 부분 둘 다에서 유비퀴티네이션 현상이 일어난다는 것을 밝혔다. 네드4와 베타-어레스틴1을 HEK293T 세포에서 과다발현 시키면 글루타메이트 수용체7의 유비퀴티네이션이 증가되었다. 그러나 베타-어레스틴2의 과다발현은 이에 별다른 영향이 없었다. 또한 베타-어레스틴1과 글루타메이트 수용체7, 그리고 네드4가 서로 글루타메이트 수용체7의 길항제에 반응하여 종합적인 구조물을 이룬다는

사실을 면역침강기법의 실험을 통하여 밝힐 수 있었다. 또한 글루타메이트 수용체7-네드4의 결합은 베타-어레스틴1을 siRNA를 이용해 HEK 293T 세포에서 발현을 저해시키자, 글루타메이트 수용체7의 길항제의 자극에 의한 결합의 증가가 관찰되지 않았다. 또한 베타-어레스틴1이 신경세포 에서 발현이 저해되면 길항제에 의한 글루타메이트 수용체7의 세포막 표면에서의 분포감소가 보이지 않으며, 베타-어레스틴1의 과다발현 되면 글루타메이트 수용체7의 세포 안쪽으로의 이동이 눈에 띄게 증가한다. 이러한 결과 들은 베타-어레스틴1과 네드4가 글루타메이트 수용체7의 길항제의 의한 유비퀴티네이션에 중요하며, 그에 따른 수용체의 이동에 따른 신경전달물질 발현에 중요한 요소라는 사실을 뒷받침 한다고 주장할 수 있다.

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주요어 : G단백질공여수용체, 글루타메이트 수용체7, 네드4, 베타-어레스틴, 길항제, 면역침강법, 유비퀴티네이션

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