Mechanism of receptor trafficking regulation by mGluR5 phosphorylation

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Mechanism of receptor trafficking regulation by mGluR5 phosphorylation

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늦게 귀가해서 바로 방으로 쉬러 들어가는 아들에게 섭섭하다고 말씀하시지만 누구보다 큰 힘이 되어주시는 아버지, 어머니 항상 사랑한다고 말씀 드리고 싶습니다. 그리고 툴툴거리지만 형은 잘 할 거라고 응원해 주는 동생, 서른 살이 넘은 외손주 아침을 먹여서 학교에 보내겠다고, 오실 때 마다 아침잠 줄여가며 신경 쓰시는 외할머니, 자주 전화도 못 드리는 제게 항상 우리 손자가 제일이라고, 항상 믿음을 가지고 생활하라고 기도해 주시는 우리 할머니 그리고 큰 조카라고 항상 응원해 주는 여러 가족들에게도 항상 감사하고 사랑한다고 전하며 글을 맺습니다.

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Mechanism of receptor trafficking regulation by mGluR5 phosphorylation

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Glutamate plays an important role in CNS as an excitatory neurotransmitter, and exerts its action through the ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). mGluR5, a subtype in the group I mGluRs, is widely expressed in hippocampus and pre-frontal cortex, and modulates synaptic transmission. It was shown that mGluR5 serine 901 (S901) is a novel phosphorylation site of PKC. The surface expression of mGluR5 was reduced by S901 phosphorylation by inhibiting the binding of CaM to the receptor. The

underlying mechanism of CaM-dependent mGluR5 trafficking, however, was poorly understood. Recent works have shown that seven in absentia homolog-1A (Siah-1A) mediates the ubiquitination and degradation of the group I mGluRs, and that Siah-1A competes with calmodulin (CaM) for binding to the group I mGluRs.

In this study, a novel trafficking mechanism of mGluR5, which is regulated by competitive interaction between CaM and Siah-1A, an E3 protein ubiquitin ligase, is presented. It was found that the protein stability of mGluR5 S901D is lower than that of WT, and that the stability of S901D is affected by the Siah-1A binding. It was also found that S901 phosphorylation induces CaM displacement and Siah-1A binding to mGluR5, and that the competitive interaction between CaM and Siah-1A affects the CaM-dependent regulation of mGluR5 trafficking. Important residues were identified on the mGluR5 C-terminus for Siah-1A binding, and it was shown that Siah-1A binding is a critical factor for the regulation of mGluR5 trafficking. Siah-1A binding decreased the membrane stability of mGluR5 in the hippocampal neurons, and Siah-1A binding to the receptor affects the mGluR5 endosomal trafficking. Taken together, these data indicate that CaM regulates mGluR5 trafficking through the PKC-dependent regulation of the receptor-binding proteins. Furthermore, it expects that these results could enhance to understand the GPCR functions and the pathophysiology of mGluR5-related diseases in the CNS.

Key Words: mGluR5, CaM, Siah-1A, receptor phosphorylation, receptor trafficking

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I. INTRODUCTION

1. Glutamate receptor

Glutamate (Glutamic acid) is an essential excitatory neurotransmitter in mammalian central nervous system and presents in more than 50 % of nervous tissue. Glutamate is also used as a precursor of GABA (γ-aminobutyric acid) which is the important inhibitory neurotransmitter and plays an important role in neuronal excitability regulation[1]. Glutamate is

stored in vesicles of chemical synapse, and nerve impulses trigger glutamate release[2]. Glutamate receptors can be divided into two groups, ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs).

1) Ionotropic glutamate receptor

Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that mediate the major excitatory neurotransmission. The iGluRs are divided into three groups according to their agonist selectivity: N-methyl-D-aspartate (NMDA) receptor, α-amino-3-hydroxy-5-methylisoxazol-4-isoxazolepropionic acid (AMPA) receptor, and kainate (KA) receptor. These receptors depolarize the neuronal membranes by passing ions, but they have distinct functions at the synapse and in the neuronal activity. The AMPA receptors are abundantly expressed in excitatory synapse and mediate fast excitatory synaptic neurotransmission in the CNS. The activation mechanism of the NMDA receptor depends on the coincidence of presynaptic activity (glutamate release) and postsynaptic activity (depolarization of the membrane by the excitatory input from the other receptors). These receptors are activated by glutamate, and their major secondary messenger mechanism is the Ca^{2+} influx in the neurons[3-4].

2) Metabotropic glutamate receptor

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors that modulate neurotransmitter release and ion channel function. The activation

of the glutamate receptors regulates the synaptic transmission and synaptic plasticity, which are responsible for learning and memory[5-7]. Thus, the glutamate receptors might be important therapeutic targets for neurological and psychiatric disorders such as pain, drug addiction, schizophrenia, Alzheimer's disease and Parkinson's disease.

The mGluRs are seven transmembrane domain receptors that are linked via G-proteins to intracellular signaling cascades. Unlike the iGluRs, which are directly coupled with the ion channel, mGluRs are coupled with G-proteins, which regulate second-messenger signaling. The mGluRs have eight different subtypes (mGluR1-8), which are subdivided into three groups. This categorization is based on the agonist/antagonist selectivity, pharmacological properties and associated second-messenger signaling. The group I mGluRs (mGluR1 and mGluR5) activate the phospholipase C (PLC) via $G_q/_{11}$-protein. Stimulating the receptors causes the associated enzyme PLC to hydrolyze the phosphoinositide phospholipids in the plasma membrane. Thus, the group I mGluRs lead to the formation of inositol-1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG)[3]. The group II mGluRs include mGluR2 and mGluR3, and the group III mGluRs include mGluR4, mGluR6, mGluR7 and mGluR8. The activation of both the group II and III mGluRs reduces the cAMP level by activating the inhibitory G-protein, G_i, which inhibits adenylyl cyclase.

The mGluRs have three distinct domains: an extracellular N-terminal domain,

a seven-transmembrane domain, and an intracellular C-terminal domain. The extracellular N-terminal domain contributes to glutamate binding. The N-terminal and intracellular C-terminal domains are connected by the seven-transmembrane domains located in the hydrophobic regions. The second intracellular loop and the amino portion of the C-terminal are possible interaction regions of G-protein binding. The first and third intracellular loops seem to be important regions for G-protein activation[8]. In the C-terminus tail, several phosphorylation sites exist; these regions are the target sites for several kinases that regulate receptor activity. All the mGluR subtypes show sequence similarity, and 19 cysteine residues are conserved in all the mGluRs[9].

Table I. Characteristics of metabotropic glutamate receptors

Family	Receptors	Mechanism	Agonist	Antagonist	Expression
Group I	mGluR1	G_q, $Na^+\uparrow K^+\downarrow$	DHPG, CHPG	MPEP, MTEP	Postsynaptic
	mGluR5				
Group II	mGluR2	G_i/G_o	LY379268, LY314582	EGLU, APICA	Presynaptic
	mGluR3				
Group III	mGluR4	G_i/G_o	L-AP4	MSOP	Presynaptic
	mGluR6				
	mGluR7				
	mGluR8				

3) Metabotropic glutamate receptor 5

mGluR5 is mainly localized in the post-synaptic neurons and has been implicated in excitatory synaptic transmission and activity-dependent short- and long-term synaptic plasticity[10]. Also, mGluR5 has important roles in the

neuronal plasticity processes, which are involved in synapse development and removal[11-13]. In addition, like other glutamate receptors, mGluR5 has been involved in learning and memory as well as in many neurological and psychiatric disorders[14]. These reports indicate that mGluR5 could be a putative drug target for many neurological diseases.

2. Receptor phosphorylation

Receptor phosphorylation is the most powerful post-translational modification that regulates protein expression and function. The phosphorylation of the receptor mediates cellular response from external stimuli in the nervous system. Since 20 years ago, it has been known that the G-protein-coupled receptors (GPCR) are regulated by phosphorylation[15]. GPCR phosphorylation occurs in a stimulus-dependent manner and is mediated by more than one protein kinase family. For example, studies on the β_2-adrenergic receptor determined that both protein kinase A (PKA) and β-adrenergic receptor kinase (GRK2)[16-17] are involved in receptor phosphorylation. These protein kinases were able to phosphorylate different sites of the receptor and to activate different kinds of signaling[18]. Like other GPCRs, mGluR was phosphorylated by several different protein kinases, including PKC. The phosphorylation of mGluR5 affected the receptor desensitization, trafficking, signaling, and other features[19-21]. The phosphorylation-dependent mGluR5 trafficking mechanism, however, is poorly understood.

3. Receptor trafficking

Receptor trafficking is the intracellular movement from the receptor synthesis sites to the surface membrane and to the degradation sites. Using exo- and endocytosis mechanisms, receptors are inserted into and removed from the membrane and diffuse within the plasma membrane[22]. In neurons, receptor trafficking regulates numerous neuronal functions, such as neuronal movement and synaptic transmission. These functions are controlled by setting the capacity of a neuron to respond to an external cue. For example, the postsynaptic density can be regulated by a number of surface receptors, which respond to the neurotransmitter from a presynaptic neuron. As the amount of released neurotransmitters often outweighs the number of available postsynaptic receptors, the process of receptor trafficking can control the efficiency and amplitude of the postsynaptic response[23]. In neurons, the surface expressions of the receptors in both the presynaptic and postsynaptic regions are often in a state of dynamic equilibrium with intracellular pools of receptors, so that the rapid changes in the surface populations can be mediated. The trafficking of receptors in the subcellular organelle is the fundamental function of the neurons, and it gives rise to many of the mechanisms underlying synaptic plasticity. Like other receptors, the surface distribution of the mGluRs can also be rapidly changed. For example, the activation of mGluR1 undergoes rapid internalization through an arrestin- and dynamin-dependent processes[24]. In addition, mGluR5 has high mobility on the neuronal surface, and the agonist-induced activation of mGluR5 increases its mobility[25]. Therefore, the

distribution of mGluRs is dynamically regulated in response to neuronal activity, and thereby regulates synaptic plasticity.

4. Synaptic plasticity

Synaptic plasticity is the strength of the connection between two neurons. Plastic change is regulated by changing the number of receptors on the plasma membrane on a synapse[26-27]. Synaptic plasticity has been found in both the excitatory and inhibitory synapses, and it is dependent on the postsynaptic calcium release[26]. Synaptic plasticity is divided into short- and long-term plasticity.

1) Short-term plasticity

Short-term plasticity acts within milliseconds to a few minutes and either strengthens or weakens the synaptic connection.

2) Long-term plasticity

Long-term potentiation (LTP) and long-term depression (LTD) are widespread phenomena expressed at every excitatory synapse in the mammalian brain. It acts within a minutes to hours and occurs at the excitatory synapses. LTP is an increase in synaptic response. It increases the potentiating pulse via electrical stimuli. The pulses are sustained at a level higher than the baseline response. In addition, LTP is related to the interactions between the postsynaptic neurons and the presynaptic inputs.

This interaction is important in synaptic association and is stimulated by the synaptic transmission pathway[28]. LTD is a reduction in the neuronal synapse efficacy lasting hours or longer following a stimulus. In addition, LTD is caused by an increase in the intracellular calcium level at the postsynaptic neuron, and by a minimum level of postsynaptic depolarization[29].

Activity-dependent synaptic plasticity has been implicated as the basic mechanism underlying learning and memory. The mGluRs are also important for synaptic plasticity[30]. Most notably, they can function as triggers for some forms of LTD, particularly the NMDAR-independent forms[31-32], and can also contribute to and modulate the induction of LTP[31]. Therefore, the trafficking of mGluRs is likely to influence synaptic plasticity.

5. Recent works and hypothesis

A recent work showed that calmodulin (CaM) dynamically regulates mGluR5 trafficking through the PKC phosphorylation of the serine 901 (S901) of the mGluR5 C-terminus. The PKC phosphorylation of mGluR5 S901 decreases the surface expression of the receptor by disrupting the CaM binding[33]. The CaM-dependent mGluR5 trafficking mechanism, however, is poorly understood.

CaM is a calcium-binding messenger protein expressed in all the eukaryotic cells, where it participates in the signaling pathways that regulate many processes, such as growth, proliferation, and movement[34-35]. Many researches have reported that CaM binds with numerous neuronal GPCRs, such as the

serotonin, dopamine, opioid, and adenosine receptors[36-39]. The CaM-binding proteins are involved in the functional complex with ion channels such as NMDA, and with voltage-gated calcium channels, and this functional complex mediates synaptic plasticity[40].

Seven in absentia homolog (Siah)-1A is an E3 ubiquitin protein ligase. It belongs to the E3 ubiquitin ligase family, which has a RING-finger protein motif[41]. Deleted in colorectal cancer (DCC), synaptophysin and Numb are the target proteins of Siah, which enhances the degradation of such proteins[42-44]. In the previous study, it was shown that Siah-1A competes with CaM for mGluR5 binding *in vitro*[45], and the group I mGluRs were ubiquitinated and degraded by Siah-1A[46]. As the interaction between mGluR5 and CaM was regulated by S901 phosphorylation[33], and Siah-1A was associated with mGluR5 for degradation and ubiquitination, it was hypothesized that the competitive binding CaM and Siah-1A to mGluR5 might affect the CaM-dependent regulation of mGluR5 trafficking.

6. Present study

It was shown herein that the interaction between mGluR5 and CaM was decreased by the phosphorylation of mGluR5. When phosphorylated, the mGluR5 binding with Siah-1A is increased by the displacement of CaM, and the Siah-1A binding leads to decreased mGluR5 surface expression. In addition, an important Siah-1A-binding motif was found on the mGluR5 C-terminus, which was different from that of CaM. It was also shown through the Siah-1A

binding site mutations on the mGluR5 C-terminus that the direct binding between Siah-1A and mGluR5 is an important factor for mGluR5 trafficking. Then it was confirmed that the role of Siah-1A, which also regulated the mGluR5 surface expression in neurons. From these data, a dynamic model is suggested showing that the trafficking of mGluR5 is regulated by CaM, and that the receptor trafficking is mediated by PKC dependent regulation of the receptor-binding protein as seen as Siah-1A.

II. MATERIALS AND METHODS

1. Cell culture and Transfection.

HeLa cells were incubated with Dulbecco Modified Eagle Medium (DMEM) media containing 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) at 37℃, 5% CO_2. Primary hippocampal neurons were cultured as previously described[47] with some modifications. Primary neuron culture method performed in accordance with the Yonsei University College of Medicine Animal Care (Project license number: #00062) and Use Committee or NIH Guide for the Care and Use of Laboratory Animals. Explain shortly, primary neurons were cultured from embryonic day 18 Sprague-Dawley (SD) rat. The first hippocampus was dissected, and isolated by trypsin and trituration. The neurons were grown in Neurobasal media (Invitrogen, Carlsbad, CA, USA) containing 2% B-27 supplement and 2 mM L-glutamine. According to the manufacturer's protocol, Polyplus reagent (Polyplus transfection, New York, NY, USA) was used for DNA transfection.

2. Constructs and siRNA.

pCBA mammalian expression vector was made with chicken β-actin promoter. mGluR5 DNA was subcloned into pCBA vector. Then, manufactured mutant constructs mGluR5-S901A, S901D by site-directed mutagenesis methods. Then Siah-1A binding disrupting mutant constructs mGluR5-T913D, I916A and S901A/I916A also manufactured. pRK5 mGluR5 construct has a Myc tag which was inserted between amino acids 22 and 23. *Cam2* (Clontech, Mountain View, CA, USA) was inserted into pcDNA3.1 and pPTuner-IRES. pCI-neo-Siah-1A and pPTuner-IRES-Siah-1A were subcloned. For pull-down assay, the first one-third DNA fragment of mGluR5 C-terminus was inserted into pGEX-4T-1 and pBHA vector. Then, Siah-1A cDNA was subcloned into the pRSET bacterial expression vector (Invitrogen, Carlsbad, CA, USA). For immuno-fluorescence, Siah-1A DNA was subcloned into pEGFP-C vector (Clontech, Mountain View, CA, USA). The human Siah-1A siRNA pool (L-012598-00-0050, Pierce, Waltham, CA, USA) and human hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) shRNA (Sigma-Aldrich, St. Louis, MO, USA) were purchased for knock down experiments.

3. Western blot and Immuno-precipitation.

DNA transfected samples were homogenized for 10 min with lysis buffer (1% Triton X-100 in PBS) on ice with protease inhibitors (Complete EDTA-free inhibitor cocktail, Roche Applied Science, Penzberg, Germany). Protein concentration was determined using a BCA Protein Assay Kit (Pierce, Waltham, CA, USA). Equal amounts of protein were loaded on a SDS-polyacrylamide gel for separation and then transferred on a 0.45 μm size pore PVDF membrane. Membranes were blocked with 5% nonfat dry milk and then incubated overnight at 4℃ using one of these antibodies: mGluR5 (Epitomics, Burlingame, CA, USA), mGluR7 (Millipore, Billerica, MA, USA), FLAG M2 (Sigma-Aldrich, St. Louis, MO, USA), Siah-1A (Abcam, Cambridge, UK), CaM (Millipore, Billerica, MA, USA), neomycin phosphotransferase II (NPT-II, Millipore, Billerica, MA, USA), α-tubulin (Hybridoma Bank, Iowa city, IA, USA), and β-actin (Santa Cruz, Dallas, TX, USA). For immuno-precipitation assay, lysates containing equal protein amounts were incubated overnight with primary antibodies at 4℃ with gently agitating. Then, samples were incubated with protein A-Sepharose beads (GE health, Little Chalfont, UK) for 2 h at 4℃ with gently agitating. After then, the supernatants were removed, and beads were washed four times with lysis buffer, treated with 2 X sample buffer containing 4% β-mercaptoethanol, and then incubated for 30 min at 37℃ for elution. Samples were then subjected to SDS-PAGE and Western blotting.

4. Real-time Quantitative PCR.

According to the manufacturer's protocol, total RNA of mGluR5 was isolated using miRNeasy kit (Qiagen, Hilden, Germany). A minimum of 250 ng of total RNA was reverse-transcribed into first-strand cDNA using a mix of random primer and poly-dT. Reverse transcription was performed with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for 1 h at 42℃, inactivated for 15 min at 70℃ and cooled to 4℃. Two μl of cDNA were used in a PCR reaction containing 250 nM primer pairs, and added 10 μl SYBR green mixtures (Applied Biosystems, Carlsbad, CA, USA) in a total reaction volume of 20 μl. The PCR reaction protocols were as follows: 10 min at 95℃, then 40 cycles of 95℃ for 20 s, 60℃ for 40 s, and 70℃ for 30 s. The values were normalized to that of the housekeeping gene β-actin.

5. Pull-down Assay and PKC *in vitro* Phosphorylation.

Using glutathione Sepharose 4B beads (GE Health, Little Chalfont, UK), mGluR5 GST fusion protein was purified. Purified mGluR5 fusion protein was incubated with *in vitro* phosphorylation buffer (20 mM HEPES, 1.67 mM $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$) and 1pmol of [γ-32^P] ATP (3000 Ci/mmol) containing active PKC (25 ng, Promega, Fitchburg, WI, USA) at 30℃ for 30

min. mGluR5 fusion proteins were phosphorylated by PKC, and incubated with CaM (Sigma Aldrich, St. Louis, MO, USA) or Siah-1A His-tag-fusion protein at 4℃ for 2 h. Incubated samples were washed several times, and the bound protein was eluted by 2 X sample buffer containing 4% β-mercaptoethanol at 37℃ for 10 min. Eluted samples were subjected to Western blotting and CaM and Siah-1A were detected with their antibodies, and phosphorylated mGluR5 was detected with S901 phospho-specific antibodies[25].

6. Biotinylation Assay.

HeLa cells were administered mGluR5 WT with Siah-1A or scrambled DNA and incubated 24 h for expression. Cells were washed three times with ice-cold base solution (1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ in PBS) three times and incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-SS-biotin (Pierce, Waltham, CA, USA) in base solution for 5 min at 4℃ with gentle shaking. Excess non-reactive biotinylation reagent was quenched by washing four times with 50 mM glycine in base solution. Samples were lysis buffer (1% Triton X-100 in PBS), and the insoluble pellet was removed by centrifugation at 13,000 rpm for 10 min at 4℃. The supernatant was then incubated with NeutrAvidin agarose resin (Pierce, Waltham, CA, USA) for 2 h at 4℃. The resin was washed four

times with lysis buffer (1% Triton X-100 in PBS), and the bound proteins were eluted by mixing and incubating with 2 X sample buffer at 37℃ for 30 min. The eluted sample was analyzed by Western blotting. For the endocytosis assay, HeLa cells were transfected with mGluR5 WT or mGluR5 S901A mutant and samples were incubated with 1 mg/ml EZ-Link Sulfo-NHS-SS-Biotin in PBS for 20 min at 4℃. The cells were treated with glutamate (100 μM) for 5 min at 37℃ and then the remaining biotinylated proteins on the cell surface were cleaved using 50 mM reduced glutathione (Roche Applied Science, Penzberg, Germany). After cell lysis, NeutrAvidin agarose resin was added to the lysates for 2 h at 4℃ with gentle agitation. After washing the resin three times with lysis buffer, bound proteins were eluted with 2 X sample buffer containing 4% β-mercaptoethanol, and then subjected to Western blotting.

7. Yeast two-hybrid Assay.

To identify interaction between Siah-1A and mGluR5, yeast two hybrid assays was performed using the L40 yeast strain. mGluR5 DNA was inserted into pBHA vector which containing LexA-DNA and pGAD vector which containing GAL4 activation domain subcloned with Siah-1A were co-administered to L40 yeast. The yeasts were grown at 30℃ in complete synthetic medium lacking leucine and tryptophan. This stock was used to

generate a 10-fold dilution series. Using a replica platter, the yeasts in each well were plated as a spot on a synthetic complete medium agar plate lacking tryptophan, leucine and histidine. The plates were then placed in a 30℃ incubator for 2–3 d.

8. Immuno-staining for Surface Expression.

HeLa cells were cultured on poly-D lysine (Sigma-Aldrich, St. Louis, MO, USA) coated glass coverslip and expressing N-Myc-tagged mGluR5 and GFP fused Siah-1A. Cells were washed with ice-cold PBS and surface mGluR5 was labeled by mouse Myc (9E10) antibody (Sigma-Aldrich, St. Louis, MO, USA) for 15 min on 4℃ with gentle shaking. After washing with ice-cold PBS, the cells were fixed by 4% paraformaldehyde/sucrose in PBS for 15 min at room temperature and washed with ice-cold PBS three times. And then the cells were permeabilized by permeabilization solution (0.1% Triton X-100 in PBS) for 10 min at room temperature. The permeabilized samples were blocked with 1% BSA in permeabilization solution and then incubated with rabbit mGluR5 antibody for overnight at 4℃. Following multiple washes with ice-cold PBS, the cells were incubated with Alexa 568 anti-rabbit, and Alexa 647 anti-mouse secondary antibodies (Invitrogen, Carlsbad, CA, USA) at 1: 500 for 1 h at room temperature. The samples were washed several times with ice-cold PBS and

then mount with the ProLong Antifade Kit (Invitrogen, Carlsbad, CA, USA). The cells were visualized using a Zeiss LSM710 confocal microscope (Carl Zeiss, Jena, Germany). EGFP empty vector and EGFP fused Siah-1A were administered to primary cultured hippocampal neurons and surface expression of mGluR5 levels were monitored by mGluR5 N-terminus antibody (Alomone, Jerusalem, Israel). Alexa 568 anti-rabbit antibody was used for detection of mGluR5. The mGluR5 positive signal was captured and fluorescence intensity of mGluR5 was measured by MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

9. Proximity ligation assay.

In situ interactions were detected by the Duolink proximity ligation assay kit (Olink bioscience, St. Louis, MO, USA). Primary cultured hippocampal neurons were grown on glass coverslips and samples were fixed by 4% paraformaldehyde/sucrose and permeabilized by permeabilization solution (0.1% Triton X-100 in PBS). The coverslips were blocked with the Duolink blocking solution for 30 min at 37℃ incubator. After blocking, the samples were incubated with mGluR5 and Siah-1A antibodies in humidity chamber for overnight at 4℃. Slides were washed three times in PBS for 10 min. Duolink PLA probes which detecting rabbit or goat antibodies were diluted in the

blocking agent in a concentration of 1: 5 and applied to the slides followed by incubation for 2 h in a humidity chamber at 37℃. After washing three times with PBS, the coverslips were subjected hybridization, ligation and amplification for 1 h. The cells were incubated with detection solution for 1 h in humidity chamber at 37℃. Afterwards the slides were mounted with DAPI containing Prolong artificial gold kit (Invitrogen, Carlsbad, CA, USA). After experiments, cells were monitored by LSM710 confocal microscope (Carl Zeiss, Jena, Germany). The samples were captured by Z-stack method at 0.22 μm intervals were converted to maximal projection. The experiments data was analyzed by MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

10. Statistical analysis.

All data are reported as means ± SEM. Statistical comparisons were made using two-tailed unpaired *t* test or one-way ANOVA followed by Bonferroni-Dunn test for preplanned multiple comparisons as appropriate. Data were analyzed using Prism software (GraphPad, La Jolla, CA, USA).

III. RESULTS

1. mGluR5 protein stability affected by mGluR5 S901 phospho-mimetic mutation.

According to a previous study, the PKC phosphorylation of mGluR5 inhibits CaM binding to mGluR5[32], and that Siah-1A competes with CaM for binding to the group I mGluRs *in vitro*[44] (Fig. 1A). To check the effect of S901 phosphorylation on the stability of mGluR5, S901 phospho-mimetic form (S901D) and non-phospho from (S901A) were manufactured from mGluR5 expression construct. HeLa cells expressing mGluR5 (WT, S901D, S901A) were subjected to Western blotting. The protein expression level of S901D was significantly lower than that of mGluR5 WT and S901A. Neomycin phosphotransferase II (NPT-II) was also measured. As the NPT-II expression sequence was encoded in all the mGluR5 DNA constructs, NPT-II expression was used to confirm equal amount of the transfected DNA (Fig. 1B). To determine whether the decrease in the S901D expression level was due to the reduction of the mGluR5 mRNA expression, the mGluR5 (WT, S901A, S901D) mRNA level was measured via real-time PCR (Fig. 1C). The mRNA levels of mGluR5 were not different among wild-type and mutants. This indicates that the S901D mutant reduced the mGluR5 protein stability, without changes in the protein synthesis of mGluR5. A chase experiment was performed to verify the protein stability of mGluR5 (WT, S901A, S901D) using the protein

biosynthesis inhibitor cycloheximide. The expression of the mGluR5 protein levels was monitored for 6 h, and samples of each time point were measured via Western blotting. The half-life of the mGluR5 S901D protein level significantly decreased compared with that of WT (Fig. 1D, E). This suggests that the phosphomimetic mutation of mGluR5 S901 accelerated the protein degradation.

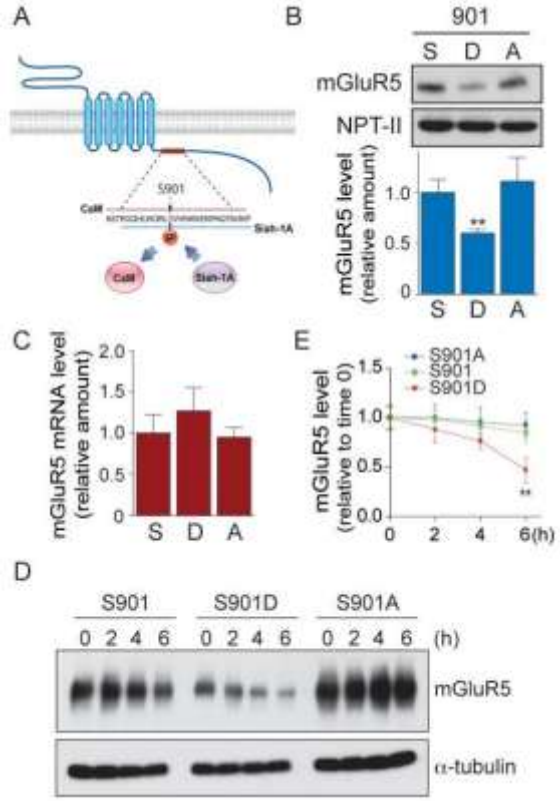

Figure 1. S901 phospho-mimetic mutation affects protein stability of mGluR5.

A) Siah-1A and CaM have similar binding motif on mGluR5 C-terminus. B) mGluR5 S901 site was mutated to phospho-mimetic form (S901D, aspartic acid), and non-phospho form (S901A, alanine). Protein expression level of mGluR5 was measured by Western blotting. The level of mGluR5 S901D was decreased; it assumes that S901 phosphorylation of mGluR5 increased Siah-1A binding by displacing of CaM. NPT-II expression was detected by the NPT-II antibody; it was used for checking an equal amount of DNA transfection. The results represented by histogram are the averages of five independent experiments. Data represent the means ± SEM. One- way ANOVA ($F_{(2, 12)} = 13.41$, $p = 0.0009$), followed by Bonferroni-Dunn test for preplanned multiple comparisons. $^{**}p < 0.01$ compared with mGluR5 WT (S). C) Expression levels of mGluR5 mRNA were measured by quantitative realtime PCR. D) Chase experiment of mGluR5. HeLa cells expressing mGluR5 (WT, S901D, or S901A) were incubated with cycloheximide (200 μg/ml) for 2 h and then collected the samples at the indicated times. Proteins levels were analyzed by Western blotting. E) Quantitative analysis of figure D). The results represented by histogram are the averages of five independent experiments. Data represent the means ± SEM. One-way ANOVA ($F_{(2, 12)} = 121.5$, $p < 0.0001$), followed by Bonferroni-Dunn test for preplanned multiple comparisons. $^{**}p < 0.01$ compared with mGluR5 WT (S).

2. Siah-1A is an important factor for accelerated degradation of mGluR5 S901D.

To determine if the degradation of mGluR5 was regulated by Siah-1A, HeLa cells were co-transfected with mGluR5 WT and Siah-1A, and the protein levels were measured via Western blotting. The protein level of mGluR5 decreased when Siah-1A overexpressed (Fig. 2A). The protein level of mGluR7 was also tested for the specific regulation of Siah-1A for the group I mGluRs, and the result showed that mGluR7 was not changed by Siah-1A overexpression (Fig. 2B). This result was consistent with that of a previous study.[45] To clarify the Siah-1A effect, Siah-1A siRNA was administered into the HeLa cells which were transfected with mGluR5 (WT, S901A, S901D). In the control sample SiCONT, the mGluR5 S901D expression level decreased compared with WT, but when Siah-1A was knocked down by siRNA, the mGluR5 S901D expression level was recovered to the WT level (Fig. 2C). These data indicate that the protein stability of mGluR5 was regulated by Siah-1A. In a previous study, CaM binding to mGluR5 was decreased by S901 phosphorylation[32], and it was speculated that increasing the binding between Siah-1A and mGluR5 S901D would decrease the mGluR5 protein levels. The interaction between Siah-1A and mGluR5 was tested via immuno-precipitation assay to determine if the reduction of the S901D protein levels is caused by the differential binding of CaM and Siah-1A to mGluR5. Siah-1A and mGluR5 (WT, S901D, S901A) were co-transfected into HeLa cells, and receptors were immuno-precipitated with an mGluR5 antibody. The interaction between Siah-1A and mGluR5

S901D increased, but the CaM binding with mGluR5 S901D decreased (Fig. 2D). This suggests that the S901D mutant preferred Siah-1A binding over CaM; thus, the protein stability of the S901D mutant could be lower than that of WT. Next, *in-vitro* pull-down assay was performed to determine if the interaction between Siah-1A and mGluR5 is directly affected by S901 phosphorylation in the absence of CaM. The CaM and mGluR5 binding was decreased by the PKC phosphorylation of mGluR5 S901, but the Siah-1A binding to mGluR5 did not change (Fig. 2E). This indicates that the mGluR5 and Siah-1A binding was directly affected by CaM but not by the S901 phosphorylation.

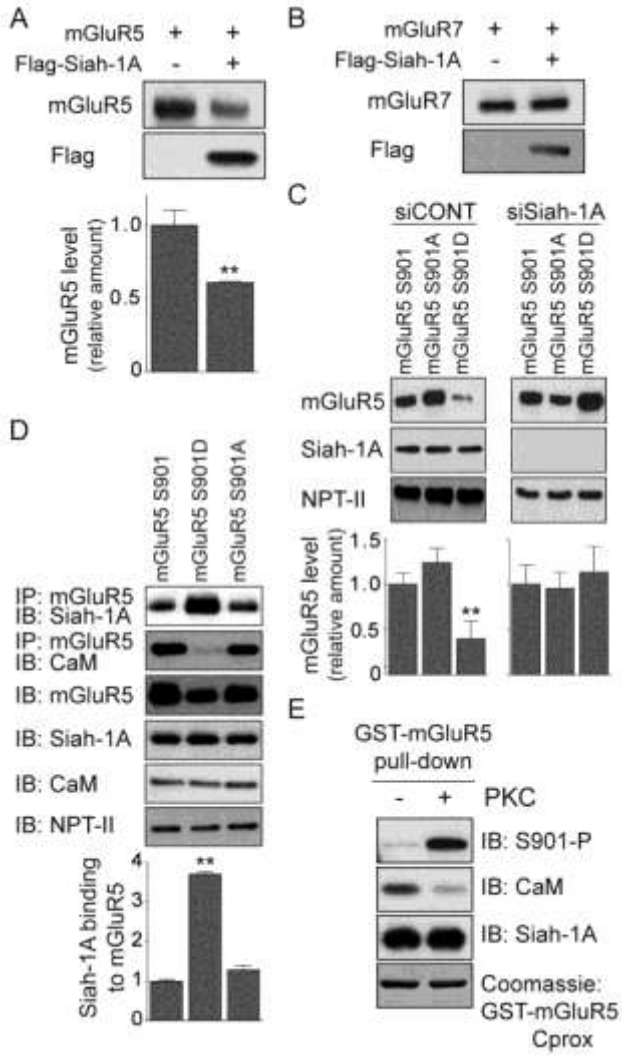

Figure 2. Regulation of mGluR5 protein stability by Siah-1A.

A) HeLa cells were transfected with mGluR5 WT or control vector or Flag-Siah-1A constructs. mGluR5 and Siah-1A protein levels were measured by Western blotting. Unpaired *t* test (**$p < 0.01$) versus mGluR5 WT (S). B) HeLa cells expressing mGluR7 and control vector or the Flag-Siah-1A

constructs were subjected to Western blotting. C) HeLa cells expressing mGluR5 (WT, S901D, or S901A) were transfected with siCONT (nontargeted control siRNA) or Siah-1A siRNA and the protein levels were measured by Western blotting. D) HeLa cells were transfected with mGluR5 (WT, S901D, or S901A). Receptors were immuno-precipitated with mGluR5 antibody, and then immuno-precipitates were subjected to Western-blotting using Siah-1A or CaM antibody. The results represented by histogram are the averages of at least four independent experiments. Data represents the means ± SEM. One-way ANOVA (C: $F_{(2, 12)} = 22.50$, $p < 0.0001$; D: $F_{(2, 9)} = 536.6$, $p < 0.0001$), followed by Bonferroni-Dunn test for preplanned multiple comparisons. **$p < 0.01$ compared with mGluR5 WT (S). E) GST-fusion protein of mGluR5 was phosphorylated by PKC, and a GST pull-down assay was performed. mGluR5 fusion proteins were incubated with either CaM or Siah-1A. In each sample, protein level was measured by Western blotting. Samples were detected by S901 phospho-specific antibody, CaM or Siah-1A antibody.

3. Competitive interaction between Siah-1A and CaM for binding to mGluR5.

HeLa cells were transfected with mGluR5 WT and Siah-1A or CaM. To determine if there is a competitive interaction between Siah-1A and CaM for binding to mGluR5, immuno-precipitation assay was performed with an mGluR5 antibody. It was found that the CaM overexpression increased the

CaM binding to mGluR5, but at that time, the Siah-1A binding decreased. Also, the Siah-1A overexpression increased the Siah-1A binding to mGluR5, but the CaM binding decreased. This indicates that CaM and Siah-1A had a competitive interaction for binding to mGluR5, such as an antagonistic effect on each other, in the test that was conducted (Fig. 3A).

To determine if increasing the CaM binding level on mGluR5 can regulate protein stability, and if it is responsible for the Siah-1A binding to mGluR5, the destabilization domain (DD) fused to CaM or Siah-1A was also used. The DD induces the rapid degradation of the entire fused protein.[48] Shield1, which binds with DD and protects the DD-fused protein from degradation, was also used (Fig. 3B). CaM and Siah-1A were fused with DD and were introduced into HeLa cells with mGluR5 constructs. HeLa cells expressing mGluR5 WT and DD-fused Siah-1A were treated with Shield1. As the Siah-1A protein degradation was protected by Shield1, the protein level of mGluR5 was decreased. This data is consistent with the Siah-1A overexpression data shown in Fig. 2A. Also, DD-CaM and mGluR5 were co-introduced into the HeLa cells, and cells were treated with Shield1. The CaM degradation was protected by Shield1, and the mGluR5 protein level increased (Fig. 3C). This indicates that CaM and Siah-1A have a competitive interaction between them for binding to mGluR5, and this interaction regulates the mGluR5 stability.

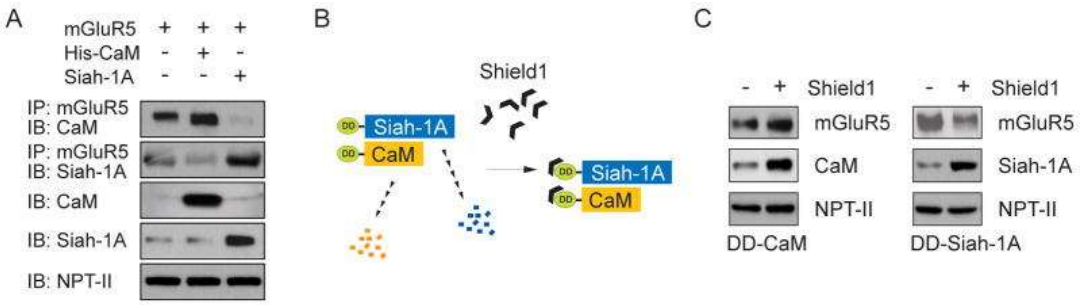

Figure 3. CaM and Siah-1A competition for mGluR5 binding.

A) HeLa cells expressing mGluR5 and CaM or Siah-1A were subjected to immuno-precipitation experiments with mGluR5 antibody, and then the immuno-precipitates were electrophoresed and blotted using CaM or Siah-1A antibodies. B) CaM and Siah-1A were fused with DD. Protein stability of CaM and Siah-1A was increased by Shield1 treatment. C) After Shield1 treatment to medium for 3 h (CaM) or 5 h (Siah-1A), mGluR5, CaM, and Siah-1A levels were measured by Western blotting.

4. Regulation of mGluR5 trafficking by Siah-1A and CaM interaction.

Surface biotinylation assay was performed to determine if the trafficking of mGluR5 is regulated by the competition between Siah-1A and CaM. HeLa cells expressing mGluR5 WT and Siah-1A were used, and surface biotinylation assay was performed. In the Siah-1A over-expressing sample, the surface level

of mGluR5 significantly decreased, but there was no effect on the mGluR7 surface expression level (Fig. 4A). The mGluR5 (WT, S901D, S901A) mutants' surface expression was tested, and like the result of the total lysate in the Western blot, the surface expression level of S901D was lower than the mGluR5 WT and S901A levels (Fig. 4B, upper part). Next, to determine if the decrease in the surface level of mGluR5 is related with the decrease in the total expression, mGluR5-transfected cells were treated with chloroquine, a lysosomal degradation inhibitor. The mGluR5 S901D surface level decreased, but the total level was not changed by the chloroquine treatment. This indicates that Siah-1A regulated mGluR5 trafficking first before it promotes degradation of mGluR5 (Fig. 4B, lower part). Next, the Siah-1A E3-ligase activity that could be required for the effect of Siah-1A on the mGluR5 levels was tested using the Siah-1A ring domain mutant Siah-1A H59Y, which has no E3-ligase activity. In HeLa cells expressing mGluR5 and Siah-1A H59Y mutant, it was confirmed that the Siah-1A H59Y mutant did not affect the mGluR5 protein levels[49] (Fig. 4C).

Next, a test was conducted to identify the effects of Siah-1A, and to determine if the mGluR5 trafficking is regulated by the interaction of Siah-1A with Hrs. Hrs is a key component of the ESCRT-0 complex, which interacts with ubiquitinated proteins, and sorts out and transmits the cargo proteins to the multivesicular system. The ESCRT machinery is an essential step for the lysosomal degradation of the cargo proteins[50]. HeLa cells expressing mGluR5

and Hrs were used, and lysates were immuno-precipitated with an mGluR5 antibody and were detected with an Hrs antibody (Fig. 4D). Compared with WT, the S901D mutant binding to Hrs increased (Fig. 4E). This indicates that S901 phosphorylation is required for Hrs binding to mGluR5. Next, the mGluR5 protein stability was tested when Hrs was knocked down. HeLa cells expressing mGluR5 WT and Hrs shRNA were used, and the protein levels were measured by Western blotting (Fig. 4F). It was found that total protein levels of mGluR5 S901D were recovered to the level of the mGluR5 WT when Hrs shRNA was transfected. This shows that Hrs is an important factor for regulating the protein stability of the mGluR S901D mutant. Together, these data indicate that Siah-1A seems to regulate mGluR5 trafficking through the lysosomal degradation pathway. Receptor endocytosis assay was performed in both cells expressing mGluR5 WT and mGluR5 S901A to know whether S901 phosphorylation is necessary to initiate receptor endocytosis. Cells were treated with glutamate for a short time (5 min), and the receptor endocytosis was observed. Glutamate promoted endocytosis of both mGluR5 and mGluR5 S901A (Fig. 4G). This result showed that S901 phosphorylation and the change in the interaction between CaM and Siah-1A are not absolute factors for mGluR5 endocytosis.

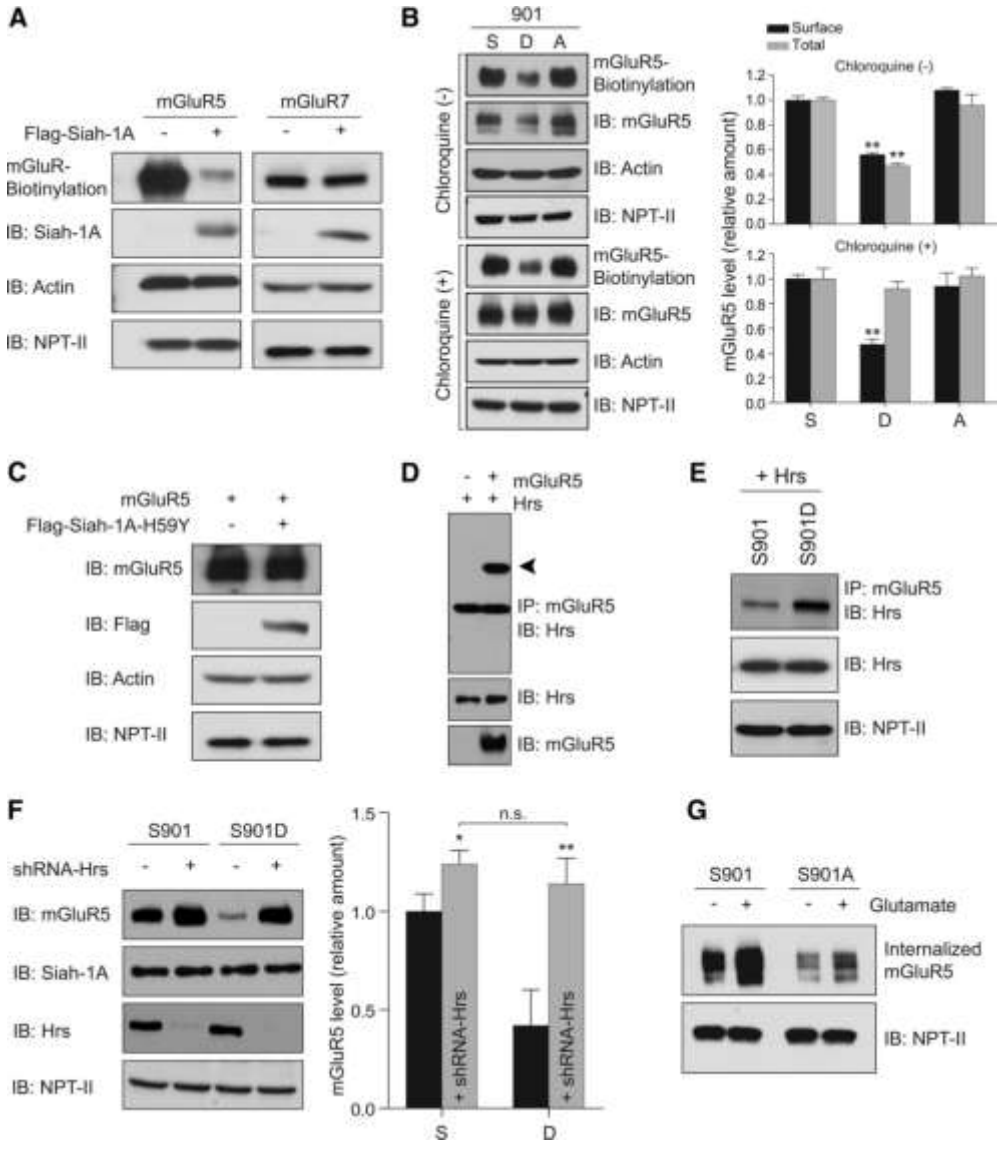

Figure 4. Regulation of mGluR5 trafficking by Siah-1A.

A) Using surface biotinylation assay, mGluR5 surface expression levels were measured. HeLa cells were transfected with mGluR5 with or without Siah-1A. mGluR7 level was also analyzed in the same experiment condition. B) HeLa

cells were transfected with mGluR5 (WT, S901D, S901A) and surface biotinylation assay was performed after chloroquine (25 μM) treatment for 6 h. Surface biotinylated mGluR5 levels were shown by quantitative analysis (right). The results represented by histogram are the averages of four independent experiments. Data represent the means ± SEM. One-way ANOVA [surface/chloroquine (-): $F_{(2, 9)} = 198.1$, $p < 0.0001$; total/chloroquine (-): $F_{(2, 9)} = 52.68$, $p < 0.0001$; surface/chloroquine (+): $F_{(2, 9)} = 49.26$, $p < 0.0001$; total/chloroquine (+): $F_{(2, 9)} = 0.9102$, $p < 0.4365$], followed by Bonferroni-Dunn test for preplanned multiple comparisons. ** $p < 0.01$ compared with mGluR5 WT (S). C) HeLa cells were transfected with mGluR5 and control vector or Siah-1A-H59Y mutant. Protein levels of mGluR5 were measured by Western blotting. D) HeLa cells were transfected with mGluR5 and Hrs. Cell lysates were immuno-precipitated with mGluR5 antibody. Arrow head indicates the band representing Hrs. E) HeLa cells were transfected with Hrs and mGluR5 WT or mGluR5 S901D. Samples were immuno-precipitated with mGluR5 antibody and measured by Western blotting with Hrs antibody. F) HeLa cells were cotransfected with mGluR5 (WT, S901D) with control (non-targeted shRNA) or Hrs shRNA. mGluR5 levels were measured by Western blotting. The results represented by histogram are the averages of four independent experiments. One-way ANOVA ($F_{(3, 12)} = 36.85$, $p < 0.0001$), followed by Bonferroni-Dunn test for preplanned multiple comparisons. *$p < 0.05$ compared with mGluR5 WT (S) with nontargeted shRNA and **$p < 0.01$ compared with mGluR5 S901D with nontargeted shRNA. G) mGluR5 (WT,

S901A) was introduced into HeLa cells. Cells were biotinylated after treatment with glutamate (100 μM) for 5min. To detect internalized biotinylated mGluR5, remaining biotinylated mGluR5 on the surface membrane was cleaved by reduced glutathione.

5. Critical binding residues of Siah-1A and trafficking of mGluR5.

In the experiments performed in this study, the S901D mutant reduced the CaM binding but did not directly affect the Siah-1A binding to mGluR5. The Siah-1A critical binding residue on the mGluR5 C-terminus was then investigated, with focus on the classical Siah-1A binding motif (PXAXVXP, X = any amino acid) (Fig. 5A), which has been reported[51]. Several residues were chosen, putative Siah-1A binding site disrupting mutants were fabricated, and two yeast hybrid assays were performed. In the tests, the mutation of isoleucine 916 to alanine (I916A) or glutamic acid (I916E) and the mutation of threonine 913 to aspartic acid (T913D) were completely disrupted by the Siah-1A binding to mGluR5, but the mutation of proline 918 to alanine (P918A) had no effect on the Siah-1A binding to mGluR5 (Fig. 5B). Using immuno-precipitation assay, results similar to those of the two yeast hybrid assays were obtained. HeLa cells expressing mGluR5 (WT, T913D, I916A) and mGluR5 proteins were immuno-precipitated with an mGluR5 antibody and were measured via Western blotting with a Siah-1A antibody (Fig. 5C). From these data, it was found that the Siah-1A binding site was different from the CaM binding site on

the mGluR5 C-terminus, and that the hydrophobic interaction is important for Siah-1A binding. To determine the specific role of Siah-1A, surface biotinylation assay was performed. HeLa cells expressing mGluR5 (WT, T913D, I916A) and biotinylated protein were purified with neutravidin beads, and the mGluR5 surface expression levels were measured via Western blotting. The surface expression of mGluR5 T913D, I916A mutants that disrupted the Siah-1A binding to mGluR5, significantly increased (Fig. 5D). Similar results were obtained from immuno-staining (Fig. 5E). GFP-tagged Siah-1A was used to find transfected cells and N-Myc tagged mGluR5 was used to detect surface expression of mGluR5. Almost no surface expression of mGluR5 WT was observed when Siah-1A was overexpressed (Fig. 5E, top part). In contrast, two mutants (T913D, I916A) of mGluR5 were well detected on the plasma membrane when Siah-1A was overexpressed in the cells (Fig. 5E, middle and bottom parts). These data show consistent results with the surface biotinylation. Surface biotinylation assay was performed to determine the effect of S901D and I916A double mutation on mGluR5 surface expression. In the case of double mutation, the mGluR5 surface expression did not decrease (Fig. 5F). This suggests that Siah-1A binding is an important factor regulating the mGluR5 surface expression, and that the effects of CaM on mGluR5 trafficking are related with Siah-1A.

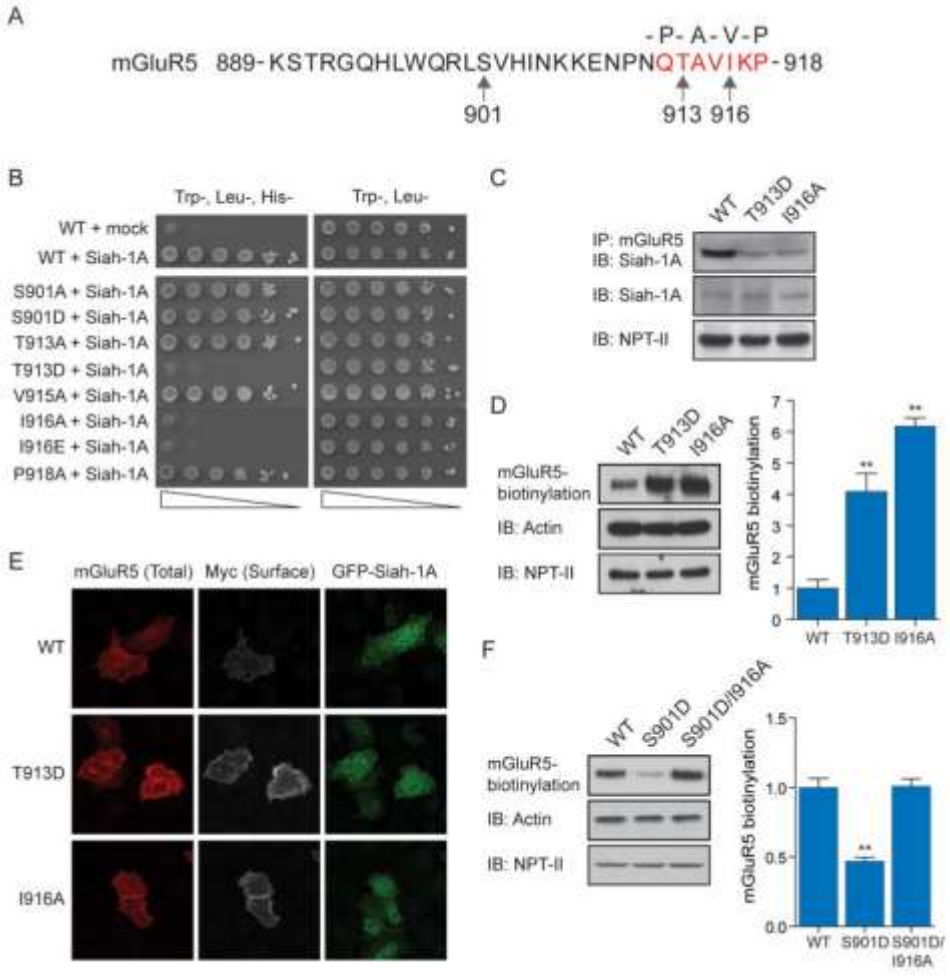

Figure 5. mGluR5-Siah-1A interaction disrupting mutations and there effects on trafficking of mGluR5.

A) Critical binding domains on mGluR5 C-terminus (889 –918; CaM binding domain 889 –917 and Siah-1A-binding domain 892 –918). The Siah-1A-binding consensus sequence (PXAXVXP, X=any amino acid) is indicated at the top. Arrows indicate S901, T913, and I916 on mGluR5. B) mGluR5 T913D, I916A, I916E did not interact with Siah-1A in yeast two-hybrid assays. Yeasts

were co-transformed with LexA-mGluR5 (828 –944) (WT, S901A, S901D, T913A, T913D, V915A, I916A, I916E, or P918A) and Gal4-Siah-1A, and growth was evaluated. Results shown are 10 -fold serial dilutions of yeast cells. All data shown are representative of at least three independent experiments. C) HeLa cells expressing mGluR5 WT, T913D or I916A mutants were subjected to co-immuno-precipitation experiment with mGluR5 antibody. Immuno-precipitated samples were then analyzed by Western blot. D) HeLa cells were transfected with mGluR5 WT, T913D, or I916A mutants. Surface expression levels were measured by a surface biotinylation assay. NPT-II was used to normalize the mGluR5 transfection efficiency. The results represented by histogram are the averages of five independent experiments. Data represents the means $\pm$ SEM. One-way ANOVA ($F_{(2, 12)} = 174.5$, $p < 0.0001$), followed by Bonferroni-Dunn test for preplanned multiple comparisons. $^{**}p < 0.01$ compared with mGluR5 WT (S). E) HeLa cells were transfected with GFP fused Siah-1A and Myc-mGluR5 WT, T913D, or I916A mutants and immuno-staining assay was performed. Surface expression levels of mGluR5 were detected with Myc antibody (surface). F) Lysates from the cells transfected with mGluR5 WT, S901D, or S901D and I916A double mutants. mGluR5 surface expression levels were measured by surface biotinylation assay. Data represent the means $\pm$ SEM. The results represented by histogram are the averages of five independent experiments. One-way ANOVA ($F_{(2, 12)} = 53.60$, $p < 0.0001$), followed by Bonferroni-Dunn test for preplanned multiple comparisons. $^{**}p < 0.01$ compared with mGluR5 WT (S).

6. Agonist-induced change of mGluR5 surface expression by Siah-1A binding.

To examine the surface expression of mGluR5, HeLa cells expressing mGluR5 WT and S901A constructs were used. Cells were treated with glutamate (100 μM) for 10 min, and surface biotinylation assay was performed. The surface level was measured via Western blotting. In a previous study, the PKC phosphorylation of S901 was induced by the glutamate treatment of mGluR5[33]. The surface expression of mGluR5 was decreased by the glutamate treatment, but surface expression of the mGluR5 S901A mutant did not decrease (Fig. 6A). In addition, in the Siah-1A siRNA-treated sample, the agonist-induced surface expression of the mGluR5 levels did not decrease (Fig. 6B). These data indicate that Siah-1A is an important regulating factor for the trafficking of mGluR5 following agonist-induced phosphorylation.

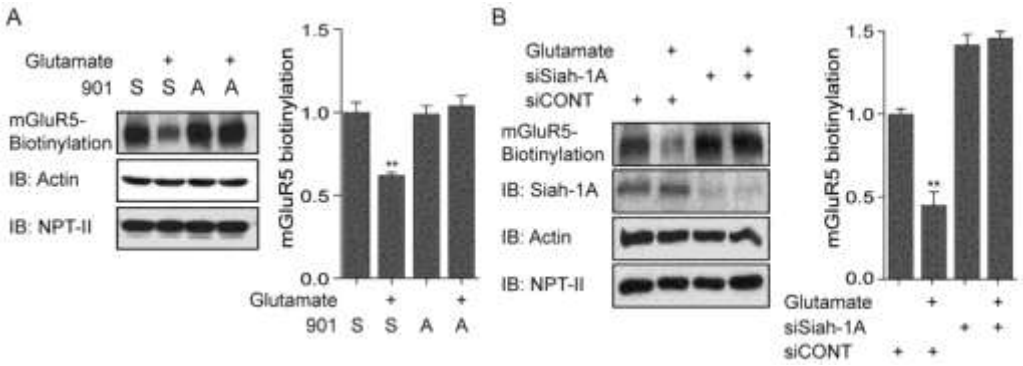

Figure 6. Siah-1A effects on mGluR5 surface expression.

A) HeLa cells were transfected with mGluR5 WT or S901A and then treated with glutamate (100 μM) for 10 min. The results represented by histogram are the averages of four independent experiments. Data in the histogram represents the means ± SEM. One-way ANOVA ($F_{(3, 12)} = 41.64$, $p < 0.0001$), followed by Bonferroni- Dunn test for preplanned multiple comparisons. **$p < 0.01$ compared with mGluR5 WT (S) without glutamate. B) HeLa cells were transfected with mGluR5 WT and siCONT (non-targeted siRNA pool) or siSiah-1A (siRNA pool against Siah-1A) and then stimulated with glutamate (100 μM) for 10 min. Cell lysates were subjected to a surface biotinylation assay. Data in the histogram represent the means ± SEM. The results represented by histogram are the averages of five independent experiments. One-way ANOVA ($F_{(3, 12)} = 72.06$, $p < 0.0001$), followed by Bonferroni-Dunn test for preplanned multiple comparisons. **$p < 0.01$ compared with mGluR5 WT (S) without glutamate.

7. Detection of mGluR5 and Siah-1A interaction *in situ*.

To determine if the competitive interaction between CaM and Siah-1A for binding to mGluR5 also occurs in primary cultured hippocampal neurons, the interaction between Siah-1A and mGluR5 was demonstrated *in situ* using Duolink PLA. This system can be used for detecting protein-protein interaction, modification, or expression *in situ*. The neurons were labeled with Siah-1A and

mGluR5 antibodies and were incubated with species-specific secondary antibodies. The secondary antibody attaches DNA strands, which participate in a series of reactions (ligation, replication, and hybridization with fluorescently labeled detection oligonucleotides) that can occur only when the two proteins are closely interacting with each other (≤ 40 nm). The interaction between Siah-1A and mGluR5 in the brain was tested. Brain lysates were immuno-precipitated with an mGluR5 antibody and were detected with a Siah-1A antibody (Fig. 7A). Siah-1A and mGluR5 were labeled with a PLA probe, and the fluorescent spots were detected with a confocal microscope. Compared with the control, the red fluorescent spots increased in the DHPG treatment sample (Fig. 7B, C). These data indicate that Siah-1A and mGluR5 also interact in the neuronal cells and mGluR5 activation can promote mGluR5-Siah-1A interaction.

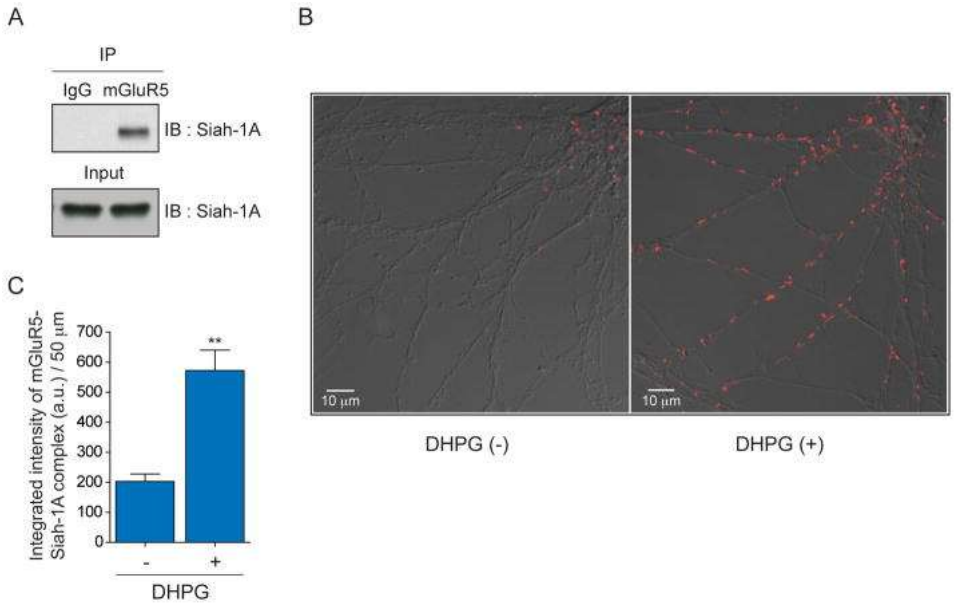

Figure 7. Interaction between mGluR5 and Siah-1A in neurons.

A) Rat hippocampus was immuno-precipitated with mGluR5 antibody and measured by Western blot. B) Using hippocampal neurons at 14 DIV, *in situ* PLA was performed using mGluR5 and Siah-1A antibodies C) Quantification of experiments shown in B). Data were averaged from 150 to 200 dendritic fragments from six independent experiments and expressed as the means ± SEM. Unpaired *t* test (**$p < 0.01$) versus no treatment control.

8. Regulation of mGluR5 trafficking by Siah-1A in hippocampal neurons.

To determine the role of Siah-1A on mGluR5 trafficking, a control vector (pEGFP-empty vector) and pEGFP-Siah-1A constructs were transfected into primary cultured hippocampal neurons, and the mGluR5 level was measured through an immuno-fluorescence experiment. Using an antibody that can detect the mGluR5 extracellular domain, the surface expression of mGluR5 was captured. In the Siah-1A transfected neuron, the surface expression of mGluR5 decreased (Fig. 8A, B). This result shows that Siah-1A also affects mGluR5 trafficking in primary hippocampal neurons.

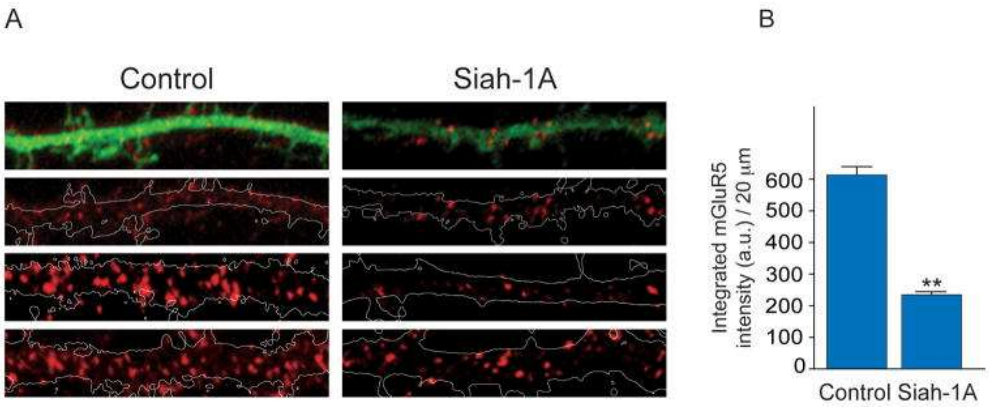

Figure 8. mGluR5 surface levels were changed by Siah-1A in hippocampal neurons.

A) All neurons were labeled by green fluorescence protein and surface expression of mGluR5 was detected with mGluR5 N-terminus antibodies (red spot). B) Acquired images were thresholded, and the integrated mGluR5 immuno-staining intensities in neurons that were measured in 20 μm dendritic segments close to the first branch point. The intensities of mGluR5 were quantified using MetaMorph software (Molecular Devices). Unpaired *t* test (**$p < 0.01$) versus EGFP control. Images were taken from > 50 neurons from four independent experiments.

IV. DISCUSSION

This study suggests that the competitive interaction between Siah-1A and CaM has an important role in the regulation of mGluR5 trafficking by controlling the number of receptors on the plasma membrane. In many researches, protein binding with mGluR5, including Siah-1A and CaM, have been discovered. It was found in a previous study, through *in-vitro* pull-down assay, that Siah-1A and CaM bind with the mGluR5 C-terminus[45]. This interaction has not been confirmed, however, in the molecular levels and in neurons. In addition, receptor binding protein Siah-1A mediates ubiquitination and degradation of group I mGluRs. However, the underlying mechanism that Siah-1A mediates group I mGluRs trafficking, has not been confirmed. The previously obtained data showed that agonist-induced mGluR5 activation leads to PKC phosphorylation of mGluR S901 site, which profoundly inhibited CaM binding to mGluR5 and the phosphorylation of mGluR5 regulates the receptor trafficking[33]. In addition, Siah-1A and CaM have similar binding motifs on mGluR5. Therefore, it can be speculated that the interaction between Siah-1A and mGluR5 would be affected by S901 phosphorylation.

In the present study, it was found that phospho-mimetic mutation of mGluR5 S901 protein level was lower than that of mGluR5 WT, and half-life of the mGluR5 S901D protein level significantly decreased compared with that of mGluR5 WT. These indicate that phospho-mimetic mutation of mGluR5 S901 reduced protein stability of mGluR5 and accelerated the protein degradation. Then, it was found that

the Siah-1A binding decreased mGluR5 protein level. In addition, using immuno-precipitation assay, it was found that binding of mGluR5 S901D with Siah-1A was increased but CaM binding with mGluR5 S901D was decreased. These data indicate that phospho-mimetic mutation of mGluR5 S901 preferred binding with Siah-1A over CaM. Thus mGluR5 S901D protein stability was significantly lower than that of mGluR5 WT. Next, using *in vitro* pull-down assay, it was demonstrated that PKC-dependent mGluR5 S901 phosphorylation decreased CaM binding to the receptor. Siah-1A binding, however, was not directly affected by receptor phosphorylation in the absence of CaM. This indicates that the interaction between mGluR5 and CaM is an important factor for the regulation of Siah-1A binding to the receptor. Using immuno-precipitation assay, it was shown that CaM overexpression increased interaction between CaM and mGluR5, but the binding of mGluR5 with Siah-1A decreased. Also, Siah-1A overexpression increased Siah-1A binding to mGluR5, but the binding with CaM decreased. This indicates that CaM and Siah-1A had competitive interaction for binding to mGluR5. To further validate this hypothesis, several experiments were performed. Using surface biotinylation assay, it was shown that Siah-1A decreased surface expression of mGluR5 protein, and the PKC phosphorylation of mGluR5 increased the binding with Siah-1A. In addition, it was found that Siah-1A interacted with Hrs, which recruited ubiquitinated protein cargo to the multivesicular body, and Hrs overexpression decreased mGluR5 protein expression. Then it was shown that upon knock-down of Hrs with shRNA, phospho-mimetic mutation of mGluR5 S901 protein level recovered to that of WT. These data indicate that phosphorylation of mGluR5

increased the binding with Siah-1A and that it is a critical factor for receptor trafficking to the lysosome for degradation. Also, the critical binding site of Siah-1A to mGluR5 was identified, and it was shown that phosphorylation of the receptor is an important event for Siah-1A binding to the receptor. Next, using proximity ligation assay, it was found that Siah-1A also bound with mGluR5 and affected the mGluR5 trafficking in primary hippocampal neurons. Taken together, the present study strongly indicate that CaM regulates mGluR5 trafficking through the PKC-dependent regulation of the receptor-binding proteins.

A recent study has shown that norbin increases the receptor membrane stabilization, and regulates signaling of mGluR5. Also norbin protein competes with CaM for binding to mGluR5 C-terminus, which was implicated in the development of schizophrenic phenotypes in mice. In this study, binding residues of norbin is highly similar to the CaM binding residue on the mGluR5 C-terminus[36]. Then, the interaction between mGluR5 and norbin is likely to be affected by S901 phosphorylation, which modulates the CaM interaction to the receptor. These results showed that norbin is the other mGluR5 binding protein, which was regulated by CaM.

CaM is a calcium sensor protein and ubiquitously expressed in most eukaryotic cells, which relays Ca^{2+} mediated intracellular signaling[34-35]. Especially, it is highly expressed in the brain and enriched in the postsynaptic density and synaptic vesicles of neurons, thereby it regulates ion channel function, GPCR signaling, and synaptic

plasticity[52-56]. Intriguingly, there have been accumulating evidences suggesting that CaM directly interacts with various GPCRs expressed in neurons such as dopamine[55], opioid[57], serotonin[38], and metabotropic glutamate receptors (mGluRs) [58]. In some cases, CaM is involved in the GPCR trafficking. For examples, CaM binds with mGluR7, which belongs to the group III mGluRs. Interestingly, the CaM displacement from the receptor enhances the mGluR7 protein stabilization on the plasma membrane[47]. In contrast to mGluR5, mGluR7 is not regulated by Siah-1A[46], so it was suggested that other receptor-binding proteins might be competing with CaM to regulate the surface expression of mGluR7[47]. The protein interacting with C-kinase 1 (PICK1), which is adaptor protein that binds to and organizes the subcellular localization of membrane proteins, competes with CaM for binding to mGluR7, and the PICK1-receptor binding increases the mGluR7 surface expression. Interestingly, the PICK1 and CaM binding site are different, and these residues are located very far from each other. This indicates that the binding motif overlapping between CaM and the receptor-binding protein is not a necessary factor for CaM competition. In addition, there are some evidences that CaM regulates the function of G protein-coupled receptor kinases, which are implicated in endocytosis of GPCRs, indicating multiple roles of CaM in regulating GPCR trafficking[59-60]. From these findings, it could be suggested that the function of GPCR can be regulated by CaM binding, which can enhance or reduce the interaction among the GPCR-binding proteins.

As mentioned already, CaM is abundantly expressed in brain. While mGluR5 and mGluR7 are expressed together in one neuron, the trafficking of these two receptors is regulated by CaM in an opposite way. In the case of mGluR5, CaM increased protein stability on the plasma membrane of mGluR5, however, CaM displacement increased protein stability of surface expression of mGluR7. Activation of G_q-coupled metabotropic and tyrosine kinase receptors by stimuli can induce the activation of protein kinase Cs (PKCs) that is crucial mediator for synaptic plasticity. The activation of PKC is especially implicated in the phosphorylation of various CaM binding GPCRs including to 5-HT_{1A} and 5-HT_{2A} receptors[37], mGluR5[58] and mGluR7[61]. Phosphorylation of the CaM binding sites by activated PKC can induce the dissociation of CaM binding from its target GPCRs. For example, phosphorylation of GPCRs such as 5-HT_{1A} receptor[37], mGluR5[58], mGluR7[61] by PKC and CaM binding to the receptors are antagonistic. Moreover, CaM binding can affect the interaction of GPCRs with other binding protein. In the present study, agonist-induced phosphorylation of mGluR5 and consequential CaM dissociation from the receptors can regulate the binding of other receptor-binding proteins. Taken together, this study also suggests that CaM indirectly regulates the receptor functions through modulation the affinity of mGluR5 binding to Siah-1A.

At this point, a question arises how the trafficking of mGluR5 is regulated by Siah-1A. Siah-1A is an enzyme and acts as an E3 ligase for the group I mGluRs (mGluR1 and mGluR5), and induces the ubiquitination and degradation of its substrate[46]. Ubiquitination is the process where 76-amino acid protein ubiquitin

attaches to the lysine residues of substrate protein covalently by the sequential processes of three distinct enzymes[62]. The ubiquitination process starts with an E1-activating enzyme, forming a thioester bond that is required for transfer to E2-conjugating enzymes, and E3 ubiquitin ligase attaches the ubiquitin to substrate proteins[63]. Ubiquitin is responsible for both internalization signal[64] and lysosomal sorting signal[65].

A number of GPCRs are degraded via lysosomal sorting, and the ubiquitin-attached receptor can initiate degradation[66]. Also, the ubiquitination of the receptor is related to receptor endocytosis. For example, an epidermal growth factor receptor was ubiquitinated by the Cbl family of ubiquitin ligases, which plays an important role in both the endocytosis and translocation of the receptor to lysosomal degradation[67-68]. In addition, the chemokine receptor CXCR4 was ubiquitinated by the Nedd4 family of E3 ubiquitin ligase AIP4, which is responsible for lysosomal degradation of the receptor[69]. In the present study, phospho-mimetic mutation of mGluR5 S901 accelerates protein degradation by enhancing the binding of Siah-1A to mGluR5. When treated with the lysosomal inhibitor chloroquine, however, the total mGluR5 S901D protein levels did not decrease, but the surface levels did. This indicates that Siah-1A actively regulates particular steps in endocytosis or in the lysosomal sorting pathways.

Siah-1A is involved in both mono-ubiquitination and poly-ubiquitination[70-71]. For example, Siah-1A triggers the mono-ubiquitination of α-synuclein[73], which

functions as a molecular chaperone in the formation of SNARE complexes, and the poly-ubiquitination of ELL2 which is an elongation factor; RNA polymerase II[74]. In the ubiquitination system, single ubiquitin addition to a substrate protein is known as mono-ubiquitination. Then, ubiquitin contains seven lysine residues, which means ubiquitin binds together with other ubiquitin by an iterative process, known as poly-ubiquitination, that leads to the formation of the ubiquitin chain attached to a single lysine of substrate protein. Generally, poly-ubiquitinated proteins are sorted to proteasome for degradation, and mono-ubiquitinated proteins are sorted to late endosomes and subsequent to lysosomes for degradation[72].

A GPCR like mGluR5 undergo endocytosis, and the receptor enters into an early endosome, after which the receptor will either recycle back to the plasma membrane or sort into the late endosome and lysosome for protein degradation. For example, membrane receptors such as receptor tyrosine kinases (RTKs) and alpha-factor receptor are undergone mono-ubiquitination, and this modification play a role in the receptor trafficking by enhancing the internalization and protein lysosomal degradation[65, 72]. In this study, mGluR5 and Siah-1A interaction might bring about receptor mono-ubiquitination and sorts the receptor into the late endosome and lysosome for degradation. This model was confirmed by immuno-precipitation assay between Siah-1A and Hrs. Then, it was validated by showing the increased stability of the mGluR5 protein levels after Hrs deletion. Hrs belongs to ESCRT-0 complex, and interacts with ubiquitinated proteins and sorts out the cargo proteins to the multivesicular system for protein degradation. The ESCRT machinery is

multi-subunit machinery that performs a topologically unique membrane bending and scission reaction away from the cytoplasm. This machinery plays an important role in the lysosomal degradation of the cargo proteins[50].

mGluR5 plays important roles in both synaptic plasticity and neuronal development. Glutamate receptors, such as ionotropic glutamate receptors (NMDA, AMPA, kainate) and metabotropic glutamate receptors, play important roles in the regulation of synaptic plasticity. There are two necessary factors for regulating synaptic strength: receptor phosphorylation and receptor induction by increased intracellular calcium levels[16, 76]. In the case of mGluR5, these two processes also exist. Activation of mGluR5 induces calcium release, and the mGluR5 S901 residue is phosphorylated by PKC[25, 77]. This receptor phosphorylation occurs after the receptor activation by agonists. Interestingly, the activation of mGluR5 can initiate these conflicting events. The activation of mGluR5 increases the intracellular calcium levels and enhances the CaM-mGluR5 binding. The receptor-activation-induced PKC phosphorylation of the mGluR5 S901 residue, however, displaces CaM. In the experiment model, the phosphorylation of mGluR5 S901 appeared to avoid enhanced CaM affinity, and the activation of mGluR5 decreased the mGluR5 protein stability on the membrane and increased the Siah-1A binding to mGluR5. In the brain, however, numerous channels and receptors could increase the intracellular calcium levels and/or the activation of protein kinases/phosphatases, and could also be activated under discrete conditions for synaptic plasticity and synaptic transmission. Thus, different synaptic transmission stages may provide

various receptor binding capacities for CaM, which would affect mGluR5 trafficking. These regulatory mechanisms for the CaM-mGluR5 interactions can be seen as contradictory. When these mechanisms are reconciled, CaM can be used as the central mediator for the integration of various synaptic signals.

The activity of mGluR5 is strictly regulated and is important for preventing neurological and psychological diseases. Dysfunction of mGluR5 has been implicated in numerous central nervous system disorders, including anxiety, depression, epilepsy, neuropathic pain, autism, drug addiction, Parkinson's disease, and fragile X mental retardation syndrome[78-84]. For example, the reduction or knock-out of mGluR5 has been shown to inhibit anxiety and depression. In the fragile X mental retardation disease and autism, the absence of the fragile X mental retardation protein induces uncontrolled mGluR5 signaling, and this signaling can be considered as the primary pathological mechanism[84]. Recently, norbin gene deletion, which has positive effects on mGluR5 expression on surfaces like CaM, was reported to have caused schizophrenic symptoms in animal models[36]. This data indicates that strict control of the surface membrane expressed receptor is critical for the normal brain function. In this study, the interaction between CaM and Siah-1A were shown to have important roles in the trafficking of mGluR5 regulation by controlling the number of receptors on the membrane. It remains to be seen, however, why neurons indirectly regulate the receptor binding protein efficacy through CaM rather than directly regulating proteins through receptor modifications, such as phosphorylation.

Present study was shown that Siah-1A and CaM competed each other for binding to mGluR5, and Siah-1A regulated mGluR5 trafficking toward lysosomal degradation. Then, Siah-1A binding capacity was increased by mGluR5 S901 phosphorylation. Taken together, this study suggests a dynamic model that CaM regulates mGluR5 trafficking through the PKC-dependent regulation of the receptor-binding protein Siah-1A. From these finding, I expect that these results could enhance to understand the GPCR functions and the pathophysiology of mGluR5-related diseases in the CNS.

V. CONCLUSION

Present study demonstrated that competitive interaction between Siah-1A and CaM was an important regulatory mechanism of mGluR5 trafficking. The regulation mechanism of the mGluR5 trafficking was concluded as follows:

1. Siah-1A and CaM binding amino acid sequence to mGluR5 C-terminus overlaps considerably.

2. Protein stability of mGluR5 was decreased by Siah-1A. The overexpression of Siah-1A decreased mGluR5 level and knock-down of Siah-1A recovered S901D protein level to that of WT.

3. Siah-1A and mGluR5 binding was affected by interaction with CaM but not by PKC phosphorylation itself of mGluR5 S901.

4. The mGluR5 and Siah-1A interaction induced the acceleration of mGluR5 trafficking into intracellular sites. It reduced total amount of the functional mGluR5 protein on the plasma membrane.

5. Binding of Siah-1A on mGluR5 C-terminus is important for regulation of mGluR5 trafficking.

6. In hippocampal neurons, Siah-1A regulates mGluR5 protein stability and surface expression.

These results indicate that CaM binding which is regulated by S901 phosphorylation plays a central role in regulating interaction between mGluR5 and Siah-1A and subsequent mGluR5 trafficking, and expect that these results could enhance to understand the GPCR functions and mGluR5-related diseases in the CNS.

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ABSTRACT (IN KOREAN)

mGluR5 인산화에 따른 receptor trafficking 조절 기전

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고 석 진

Glutamate는 중추신경계의 흥분성 신호 전달물질로서, ionotropic 과 metabotropic glutamate receptor (mGluRs) 에 모두 작용하여 뇌에서 흥분성 신경 전달을 매개한다. 그 중에서 mGluRs 는 기능과 역할의 차이로 3 가지 그룹으로 구분되며, 신경 전달물질의 유리와 이온채널의 기능을 포함한 다양한 신경기능을 조절한다. Group I 에 속하여져 있는 mGluR5 는 synaptic transmission 을 조절하고 통증, 불안, 약물중독, 그리고 알츠하이머병을 비롯한 많은 신경학적 질병에 연관되어 있다. 기존 연구에서 mGluR5 C-terminus 의 S901 부위는 PKC 에 의해 인산화 되는 부위임이 확인되었고, S901 부위의 인산화는 mGluR5 와 calmodulin (CaM) 사이의 결합을 저해시킴으로써 mGluR5 의 세포막 발현을 감소시킴이 발표되었다. 그러나 CaM 에 의존적인 mGluR5 의 trafficking 조절기전은 잘 알려져 있지 않았다. 최근 연구에 따르면 CaM 과 ubiquitin E3 ligase 인 seven in absentia homology 1A (Siah-1A) 가 서로 경쟁을 통하여

group I mGluRs 에 결합한다고 밝혀졌으며 또한 group I mGluRs 의 ubiquitination 과 분해를 Siah-1A 가 매개한다는 것이 밝혀졌다.

본 연구에서는 CaM 과 Siah-1A 간의 경쟁적 결합에 의해 조절되는 mGluR5 trafficking 기전을 밝혔다. Siah-1A 의 결합에 의해 mGluR5 S901D 변이가 mGluR5 wild-type 에 비하여 단백질의 안전성이 저하되는 것을 확인하였고, mGluR5 S901 부위의 인산화가 mGluR5 와 CaM 사이의 결합을 저해하고, Siah-1A 와의 결합을 증가 시키는 것을 확인하였다. 또한 Siah-1A 와 CaM 의 경쟁적 결합이 CaM 에 의존적인 mGluR5 의 trafficking 을 조절하는 것을 확인하였다. 또한 mGluR5 C-terminus 에 존재하는 Siah-1A 의 중요한 결합부위를 찾아내었고, 그 결합이 mGluR5 의 trafficking 을 조절하는데 중요한 인자라는 것을 발견하였다. 또한, hippocampal neuron 에서 mGluR5 와 Siah-1A 의 결합은 mGluR5 의 세포막 발현을 감소시키고, 이는 이 결합이 mGluR5 의 endosomal trafficking 에 영향을 주어서 발생하는 현상임을 밝혔다. 본 연구는, mGluR5 의 trafficking 이 PKC 인산화에 의존적인 수용체 결합단백에 의해 조절되는 CaM 을 통하여 조절된다는 것을 밝혔고, 이를 통하여, 이 연구결과는 GPCR 의 기능과 mGluR5 와 연관된 중추신경계질병에 대한 이해를 증진시키는데 기여할 것으로 생각한다.

핵심 되는 말: mGluR5, CaM, Siah-1A, 수용체 인산화, 수용체 trafficking