ESSENTIAL ROLE FOR P62 IN AMPA RECEPTOR TRAFFICKING AND

SYNAPTIC PLASTICITY

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Jianxiong Jiang

Certificate of Approval:

_____	_____
Jacek Wower Professor Animal Sciences	Marie W. Wooten, Chair Professor Biological Sciences
_____	_____
Vishnu Suppiramaniam Associate Professor Pharmacal Sciences	Elaine S. Coleman Associate Professor Anatomy, Physiology and Pharmacology

_____

Joe F. Pittman
Interim Dean
Graduate School

ESSENTIAL ROLE FOR P62 IN AMPA RECEPTOR TRAFFICKING AND SYNAPTIC PLASTICITY

Jianxiong Jiang

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ESSENTIAL ROLE FOR P62 IN AMPA RECEPTOR TRAFFICKING AND SYNAPTIC PLASTICITY

Jianxiong Jiang

_____
Signature of Author

_____
Date of Graduation

VITA

Jianxiong Jiang, was born December 04, 1975, in Tianmen, Hubei, China. He graduated from Huazhong Agricultural University with a Bachelor of Science degree in Plant Genetics in July, 1996. Then he entered East China University of Science and Technology and received Master of Science degree in Biochemistry and Molecular Biology in July, 1999. After working as a project supervisor of R&D in Sino-American Shanghai RAAS Blood Products Corporation for nearly five years, he entered the Graduate School, Auburn University, with Cellular and Molecular Biosciences Fellowship, in August, 2004.

DISSERTATION ABSTRACT

ESSENTIAL ROLE FOR P62 IN AMPA RECEPTOR TRAFFICKING AND SYNAPTIC PLASTICITY

Jianxiong Jiang

Doctor of Philosophy, May 10, 2008
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(B.S., Huazhong Agricultural University, 1996)

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AMPA-type glutamate receptors (AMPARs) mediate major fast excitatory synaptic transmission in the mammalian central nerve system (CNS). Trafficking of AMPA receptors to and away from the postsynaptic membrane is fundamental to many forms of neuronal plasticity, including long-term potentiation (LTP) and long-term depression (LTD), which contribute to be the molecular and cellular basis for learning and memory. During LTP expression, more AMPARs are delivered to the postsynaptic membrane. In contrast, LTD induces receptor internalization. Therefore, alterations in synaptic strength are directly related to the receptor exocytosis and endocytosis. AMPA receptor trafficking is primarily regulated through receptor-associated proteins and post-

translational modifications, principally phosphorylation of GluR1. The mechanism for regulation of AMPAR trafficking has been reviewed in detail in the chapter I.

In the chapter II, the atypical protein kinase C (aPKC) scaffold, p62, was identified as the first non-C-terminal AMPA receptor interactor. AMPA receptor subunit intracellular loop L2-3 and the ZZ-type Zinc finger domain of p62 are essential for the interaction between these two proteins and for surface delivery of the receptor. This intracellular loop L2-3 is completely conserved throughout AMPA receptor subunits GluR1-4, but not other types of Glutamate receptor subunits. Furthermore, LTP was impaired in p62 knock-out mice in an age-dependent manner with normal basal synaptic transmission. Surface delivery of the AMPA receptor subunit GluR1 induced by cLTP (chemical LTP) was impaired and paralleled an absence of phosphorylation at S818, S831, and S845 in brain slices from p62 knock-out mice. These findings reveal that p62 plays a role in AMPA receptor trafficking and LTP expression. In addition, a possible conserved sequence motif (ISExSL) shared by all p62 interacting-aPKC substrates was discovered. Altogether, these findings indicate that p62 may regulate AMPAR trafficking and synaptic plasticity through recruiting AMPAR to aPKC for phosphorylation. A molecular model is presented depicting the mechanism whereby p62 may regulate AMPA receptor surface expression through aPKC-mediated phosphorylation.

Finally, based on these findings, it is reasonable to hypothesize that overexpression of p62 in hippocampus, a brain region important for learning and memory, could facilitate aPKC-mediated GluR1 pS818 phosphorylation as well as trafficking,

resulting in increased GluR1-containing AMPA receptors at the postsynaptic surface initiated by tetanic stimulation. Therefore, enhanced neuronal abilities including learning and memory, and synaptic plasticity are predicted for transgenic mice overexpressing p62. On the other hand, p62 has multiple functions in the cell and its expression is tightly regulated. Therefore, overexpression of p62 even in a mild manner could be detrimental. If this is the case, the p62 transgenic mice might develop unexpected developmental disorders. A p62 mutation P392L has recently been reported to increase osteoclastogenesis and cause a predisposition to the development of Paget disease. P392 is a major ubiquitin binding site in the UBA domain of p62 and P392L mutant p62 lacks of ubiquitin binding. As a result, ubiquitinated proteins cannot bind p62 for proteasome degradation. These ubiquitinated proteins accumulate in the cells and might induce hippocampal oxidative stress. Transgenic mice that overexpress this mutant p62 are likely to display disorders related to ubiquitination.

To examine those possibilities, p62 transgenic mice which overexpress p62 in hippocampus will be generated. In this study, a Thy1 promoter was used to generate a p62 expression cassette. The full length p62 cDNA under the control of the Thy1 promoter will only be expressed in the central nervous system (CNS), specifically hippocampus and cortex, without affecting other tissues. In chapter III, the cDNA constructs of GFP-p62 (WT) and GFP-p62 (P392L) under the control of Thy1 promoter were generated and will be employed for microinjection of embryos. The future studies for generation of p62 transgenic mice also have been introduced and discussed in the chapter III.

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LIST OF ABBREVIATIONS

ABP	AMPAR binding protein
AID	aPKC interacting domain
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-proprionate
AMPAR	AMPA-type glutamate receptor
AP2	Adapter protein 2
aPKC	Atypical protein kinase C
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic AMP
CREB	cAMP response element binding protein
cLTP	Chemically induced LTP
CNS	Central nervous system
D_2 DAR	D_2 Dopamine receptor
ECL	Enhanced chemiluminescence
E-LTP	Early phase LTP
EPSPs	Excitatory postsynaptic potentials
ER	Endoplasmic reticulum

fEPSPs	field EPSPs
GluR1-4	AMPA-type glutamate receptor subunit 1-4
Grb14	Growth factor receptor-bound protein 14
GRIP1	Glutamate receptor interacting protein 1
KA	Kainate
KAR	Kainate-type glutamate receptor
KO	Knock-out
HEK	Human embryonic kidney
HFS	High frequency stimulation
HIP1	Huntingtin interacting protein 1
IP	Immunoprecipitation
L-LTP	Late phase LTP
LTD	Long-term depression
LTP	Long-term potentiation
NMDA	*N*-methyl-D-aspartate
NMDAR	NMDA-type glutamate receptor
NSF	*N*-ethylmaleimide-sensitive fusion protein
PAGE	Polyacrylamide gel electrophoresis
PB1	Phox and Bem1
PBS	Phosphate buffer saline
PICK1	Protein interacting with C kinase 1
PSD	Postsynaptic density

PKA	Protein kinase A
PKC	Protein kinase C
PKCι/λ	Protein kinase C ι/λ
PKCζ	Protein kinase ζ
PKMζ	Protein kinase Mζ
PMSF	Phenylmethylsulfonyl fluoride
RIP	Receptor interacting protein
SAP97	Synapse associated protein 97
SDS	Sodium dodecyl sulfate
SNAP	Soluble NSF attachment protein
SQSTM1	Sequestosome 1
TARP	Transmembrane AMPAR regulatory protein
TBS	Theta burst stimulation
TM	Transmembrane spanning domain
TRAF6	Tumor necrosis factor receptor associated factor 6
TrkA	Tropomyosin receptor kinase A
Ub	Ubiquitin
UBA	Ubiquitin-associated domain
UPS	Ubiquitin proteasome system
WB	Western blot
WT	Wild-type
ZIP	Zeta protein kinase C interacting protein
ZZ	ZZ-type Zinc finger domain

INTRODUCTORY OVERVIEW

The amino acid glutamate is the primary neurotransmitter in excitatory synapses of the mammalian central nervous system (CNS) and AMPA-type glutamate receptors (AMPARs) mediate the majority of fast excitatory synaptic transmission. It is widely believed that trafficking of AMPARs to and away from the postsynaptic membrane is the molecular basis to many forms of neuronal plasticity, including long-term potentiation (LTP) and long-term depression (LTD), which are contributing mechanisms for learning and memory. During LTP expression, more AMPARs are delivered to the postsynaptic membrane; whereas LTD induces receptor internalization. In another words, alterations in synaptic strength are directly related to the receptor exocytosis and endocytosis. However, the detailed mechanism remains are not fully understood (Malinow and Malenka, 2002).

AMPAR trafficking is regulated through receptor-associated proteins and post-translational modifications, principally phosphorylation of GluR1 subunit. Three phosphorylation sites in the GluR1 C-terminus have been discovered: S818, S831 and S845. Phosphorylation at all sites is necessary for AMPAR surface delivery to enhance the synaptic strength. GluR1 S818 and S831 are protein kinase C (PKC) phosphorylation

sites and all isoforms of PKC including atypical PKC (aPKC) can phosphorylate these two residues (Boehm et al., 2006).

Sequestosome 1 (SQSTM1) / p62 is a cytoplasmic and membrane-associated protein. It is also named zeta protein kinase C interacting protein (ZIP) because this protein interacts with the pseudosubstrate region in the regulatory N-terminus of aPKCs. By binding aPKC, p62 serves as a scaffold protein to recruit substrates to the kinase (Geetha and Wooten, 2002; Geetha, et al., 2005). This suggests that p62 might be involved in the regulation of AMPAR trafficking and synaptic plasticity through its interaction with aPKC.

To test this possibility, we first examined the possible interaction formed by p62, aPKC and AMPAR through immunoprecipitation. These proteins formed a ternary complex *in situ*. In addition, all AMPAR subunits can interact with p62. The mapping results revealed that AMPAR subunit intracellular loop L2-3 and the ZZ-type Zinc finger domain of p62 are essential for the interaction between the two proteins. The intracellular loop L2-3 is completely conserved throughout AMPA receptor subunits GluR1-4, but not other types of Glutamate receptor subunits. Furthermore, the p62/AMPAR interaction is necessary for the receptor surface delivery.

To further investigate the physiological function of p62, we examined the synaptic plasticity in p62 knock-out mice. Hippocampal CA1 LTP was impaired in p62 knock-out mice in an age-dependent manner with normal basal synaptic transmission.

These findings suggest a potential postsynaptic malfunction probably caused by a deficit of synaptic targeting of AMPARs. To test this possibility, biotinylation was employed to label the surface proteins of the hippocampal slices from the wild-type and p62 knock-out mice. Consistently, surface delivery of the AMPAR subunit GluR1 induced by cLTP was impaired and paralleled an absence of phosphorylation at S818, S831, and S845 in brain slices from p62 knock-out mice. These findings reveal that p62 regulates AMPA receptor trafficking and LTP expression through phosphorylation of GluR1 subunit. In addition, a possible conserved binding motif (ISExSL) shared by all p62 interacting-aPKC substrates was unveiled.

In conclusion, p62 regulates AMPAR trafficking and synaptic plasticity through recruiting AMPAR to aPKC for phosphorylation. Those phosphorylated AMPARs can stabilize in the synaptic membrane to enhance synaptic strength. Once the AMPARs are dephosphorylated, the synaptic receptors will be internalized and may recycle back to the surface through regained aPKC-mediated phosphorylation.

Loss-of-function and gain-of-function are two common approaches to study the function of a gene during development (Brinster, 1993; Shuldiner, 1996; Wassarman, and DePamphilis, 1993). The loss-of-function approach has been very successful to study gene function during development in recent years. The most common loss-of-function approach is to study the consequence of removing a gene from a model system, most commonly the mouse. This technique, termed gene knock-out, utilizes homologous recombination to replace the endogenous functional gene with a modified nonfunctional

one. Analyzing the phenotype of knock-out mouse can assist in deducing the function of this gene. In this study, reduced hippocampal LTP and a deficit of GluR1 phosphorylation was observed in the p62 knock-out mice, which would indicate that p62 plays important roles in synaptic plasticity through aPKC-mediated phosphorylation.

In contrast, the transgenic mouse technique is the most common approach to study gain-of-function of a gene in mouse. The idea is to overexpress a gene during development and study its consequences (Clarke, 2002). Overexpression of p62 in hippocampus, the brain region important for learning and memory, could facilitate aPKC-mediated GluR1 phosphorylation as well as trafficking, resulting in more GluR1-containing AMPA receptors inserting into the postsynaptic surface. Therefore, enhanced neuronal abilities including learning and memory, and synaptic plasticity are predicted in the p62 transgenic mice. In addition, a p62 mutation P392L has been recently reported to increase osteoclastogenesis and cause a predisposition to the development of Paget disease. Indeed P392 is a major ubiquitin binding site in the UBA domain of p62. Therefore, P392L mutant p62 lacks its ubiquitin binding ability (Seibenhener et al., 2004). As a result, ubiquitinated proteins cannot bind p62 for proteasome degradation. These ubiquitinated proteins accumulate in the cells and might induce hippocampal oxidative stress. Transgenic mice that overexpress this mutant p62 are likely to display disorders related to ubiquitination.

To investigate these possibilities, p62 transgenic mice which overexpress p62 in hippocampus will be generated. The gene under the control of the Thy1 promoter will

only be expressed in the CNS, specifically hippocampus and cortex, without affecting other tissues (Ingraham, et al., 1986). The cDNA constructs of GFP-p62 (WT) and GFP-p62 (P392L) controlled by Thy1 promoter were generated and are ready for microinjection into embryos. The transgenic mice will be characterized employing standard methods and examined to determine specific effects which overexpression of the p62 gene has upon brain neuropathology, alterations in synaptic physiology, biochemical markers, and changes in anxiety, short and long term memory. Collectively, these results will help elucidate the molecular and cellular mechanism that p62 and aPKC regulate AMPAR trafficking and synaptic plasticity.

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CHAPTER I - LITERATURE REVIEW

POST-TRANSLATIONAL MODIFICATIONS AND RECEPTOR-ASSOCIATED PROTEINS IN AMPA RECEPTOR TRAFFICKING AND SYNAPTIC PLASTICITY

SUMMARY

AMPA-type glutamate receptors (AMPARs) mediate most fast excitatory synaptic transmission in the mammalian brain. It is widely believed that the long-lasting, activity-dependent changes in synaptic strength, including long-term potentiation (LTP) and long-term depression (LTD), could be the molecular and cellular basis of experience-dependent plasticities, such as learning and memory. Those changes of synaptic strength are directly related to AMPAR trafficking to and away from the synapse. There are many forms of synaptic plasticity in the mammalian brain. The prototypic form, hippocampal CA1 LTP, has received the most intense investigation. After synthesis, AMPAR subunits undergo post-translational modifications such as glycosylation, palmitoylation, phosphorylation and potential ubiquitination. In addition, AMPAR subunits spatiotemporally associate with specific neuronal proteins in the cell. Those post-translational modifications and receptor-associated proteins play critical roles in AMPAR trafficking and regulation of AMPAR-dependent synaptic plasticity.

INTRODUCTION

Glutamate and Receptors

The amino acid glutamate is the primary neurotransmitter in excitatory synapses of the vertebrate central nervous system (CNS). After release from the presynaptic nerve terminal, glutamate binds to specific receptors on the postsynaptic membrane to conduct excitatory transmission. There are two main types of glutamate receptors (GluRs): ionotropic glutamate receptors (iGluRs), which are ligand-gated ion channels that mediate the fast synaptic transmission, and metabotropic glutamate receptors (mGluRs), which are associated with GTP-binding proteins to exert their physiological effects. Based on their affinity for selective structural analogues of glutamate, iGluRs can be classified into three subtypes: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type receptor (AMPAR), Kainate (KA) type receptor (KAR), and *N*-methyl-D-aspartate (NMDA) type receptor (NMDAR) (Figure 1-1A) (Dingledine et al., 1999; Hollmann and Heinemann et al., 1994; Wisden and Seeburg, 1993).

AMPAR Structure

AMPARs are heterotetrameric cation channels composed of 4 subunits: GluR1-4 (or GluRA-D), each of which comprises about 900 amino acids with a molecular weight of about 105 kDa, and shares 68-74% amino acid sequence identity. There is also about 70% sequence similarity among genes encoding each subunit (Figure 1-1A) (Collingridge et al., 2004). AMPARs are highly conserved among mammals. For example, rat, mouse and human GluR1s share 96-97% identical amino acid sequence. Heteromers of GluR1-GluR2 and GluR2-GluR3 predominantly appear in the mature mammalian hippocampus;

whereas GluR4-containing forms mainly appear in early postnatal development (Fukata et al., 2005; Gardner et al., 2005). Each AMPAR subunit consists of an extracellular N-terminus, three transmembrane spanning domains (TM1, TM3 and TM4), one re-entrant transmembrane domain (TM2) and an intracellular C-terminus. Therefore, the transmembrane domains form two intracellular loops (L1-2 and L2-3) and one extracellular loop (L3-4). The extracellular N-terminus has two domains: the X-domain and the S1 ligand binding domain. The X-domain comprises about 400 amino acids and its function is unknown. The S1 ligand binding domain comprises about 100 amino acids. The other extracellular binding domain S2 is in the L3-4 extracellular loop. S1 and S2 domains form the ligand-binding core. The re-entrant TM2 domain contributes to the cation pore channel. The intracellular C-terminus is the interaction site for a variety of proteins, which are related to the receptor modification, trafficking and signaling (Figure 1-1B) (Hollmann and Heinemann, 1994; Palmer et al., 2005).

Post-Transcriptional Modifications and Receptor Characteristics

In addition to the variation in combinations of subunits, some post-transcriptional modifications also contribute to AMPAR diversity (Figure 1-1C). All four AMPAR subunits are subject to alternative splicing that can produce two forms: flip and flop. These are encoded by exons 14 and 15 in GluR2, respectively. The splicing position immediately precedes the TM4 domain (Monyer et al., 1991). The pharmacological properties of the two spliced forms do not show too much difference, but the flip channels are more efficient because they desensitize at a rate that is four times slower than the flop channels. Expression of the two spliced forms shows different cellular

distribution and developmental regulation in the brain. For example, in hippocampus, only flip forms are found in the CA3 pyramidal cells; whereas there are more flop forms than flip forms in dentate gyrus granule cells. Moreover, flip forms predominantly appear before birth. In contrast, the expression level of flop forms continuously increases after birth up to a level similar to that of the flip forms in the adult (Wisden and Seeburg, 1993). In addition, GluR2 and GluR4 also undergo another alternative splicing in the C-terminal tail to produce long isoforms and short isoforms (Gallo et al., 1992; Köhler et al., 1994). Only a small percentage of GluR2 displays a long C terminus; whereas the long isoform of GluR4 is dominant. The short isoform of GluR4 is mainly found in the cerebellum (Gallo et al., 1992). GluR1 exists only as the long isoforms and the GluR3 exists only as the short isoforms because of the lack of C-terminal splicing sites.

RNA editing also increases the molecular diversity of AMPAR. In the GluR2, a glutamine codon (CAG) within the TM2 domain is edited to an arginine codon (CIG) (Q/R). Therefore, the GluR2-containing AMPAR, the dominant form in the hippocampus, shows low channel conductance and impermeability to Ca^{2+}, and allows ion influx and efflux, depending on the cell membrane potential. By contrast, the AMPAR lacking GluR2 subunit only allows ion flow into the cell (Swanson et al., 1997; Wenthold et al., 1996). In addition, in GluR2, GluR3 and GluR4 mRNA, an arginine codon (AGA) preceding the flip/flop site can be altered to a glycine codon (IGA) (R/G), which changes the receptor kinetics properties of desensitization and resensitization (Lomeli et al., 1994). The process of RNA editing is fulfilled by RNA-dependent adenosine deaminase 2 (ADAR2) (Bass et al., 1997; Higuchi et al., 2000; Ohman et al., 2000).

Composition of the subunits, post-transcriptional modifications, and especially RNA editing, endow AMPARs with substantial diversity and also determine the ion channel characteristics of the receptor. In response to ligand binding, cations Na^+ and Ca^{2+} flow into the cell through AMPARs. Consequently, a fast excitatory postsynaptic response is transmitted. Ca^{2+} is an important second messenger that activates a variety of protein kinases to initiate a series of signaling pathways, which in turn mediate AMPAR modifications and trafficking to promote and maintain synaptic plasticity (Palmer et al., 2005; Mayer et al., 2005).

AMPAR and Synaptic Plasticity

In 1973, Bliss and Lømo showed that repetitive activation of excitatory synapses with high frequency stimulation (HFS) in the hippocampus, a brain area essential for learning and memory, could cause an enhancement in synaptic strength that could last for hours, days, and even weeks. They defined this long-lasting enhancement in synaptic strength as long-term potentiation (LTP) (Bliss and Lømo, 1973). In the past two decades, it has been widely accepted that this long-lasting, activity-dependent change in synaptic strength could be the molecular and cellular basis of experience-dependent plasticities, including learning and memory (Bliss and Collingridge, 1993; Kandel, 1997; Malinow and Malenka, 2002; Lynch, 2004; Pastalkova et al., 2006), and even drug addiction (Malenka, 2003; Malenka and Bear, 2004). Since the discovery of hippocampal CA1 LTP, a variety of other forms of synaptic plasticity have been discovered in the CNS including NMDAR-dependent long-term depression (LTD), NMDAR-independent LTP at moss fiber synapses in the hippocampus, mGluR-dependent LTD in the cerebellum,

and endocannabinoid-mediated LTD (Malenka and Bear, 2004). The prototypic form of plasticity, NMDAR-dependent LTP, generated at excitatory synapses in hippocampal CA1 pyramidal cells, received the wide spread attention (Kandel, 1997, Malenka, 1994).

The process of LTP can be divided into two phases: induction or early LTP (E-LTP), initiating synaptic strength, and maintenance or late LTP (L-LTP), retaining the enhancement of synaptic strength for a long time. Interestingly, there is a parallel between LTP and memory, which also has two components: short-term memory and long-term memory (Lynch, 2004). To induce LTP, glutamate is released from the presynaptic membrane and binds to AMPARs and NMDARs. Release of Mg^{2+} blockade activates NMDARs to allow Na^{+} and Ca^{2+} to flow into the dendritic spine through NMDARs. Increasing Ca^{2+} in the dendritic spine binds to calmodulin to activate the calcium/calmodulin-dependent protein kinase II (CaMKII) (Malenka, 1994, Malenka and Nicoll, 1999). Other kinases involved in LTP induction include cAMP-dependent protein kinase (PKA) (Esteban, 2003; Frey et al., 1993), protein kinase C (PKC) (Boehm et al., 2006; Boehm and Malinow, 2005; Roche et al., 1996), extracellular signal-regulated kinase (ERK) in the mitogen-activated protein kinase (MAPK) cascade (Atkins et al., 1998), phosphatidylinositol 3-kinase (PI3-kinase) (Opazo et al., 2003, Stellwagen et al., 2005), and tyrosine kinase Src (Hayashi and Huganir, 2004). Some phosphatases are also critical for regulation of LTP (Lee et al., 2000). The induction of LTP may be involved in multiple intracellular cascades that are redundant and as yet unidentified (Sheng and Kim, 2002; Thomas and Huganir, 2004). Maintenance of LTP requires the synthesis of new proteins. These proteins include AMPAR subunits, some transcriptional factors, and

structural proteins to enhance existing synapses by enlarging of dendritic spines and forming new connections (Matus, 2000; Fukazawa et al., 2003).

Subunit Rules in Receptor Trafficking

The C-terminal cytoplasmic tail of each AMPAR subunit is unique and could associate with specific regulatory proteins. These proteins impart distinct spatiotemporal patterns of trafficking and localization to each subunit. Combining the subunits to form heterotetramers introduces more complications. Interestingly, in GluR1 knockout mice, the hippocampal CA1 LTP is absent, and a specific spatial working memory is impaired (Chan et al., 2006; Reisel et al., 2002; Zamanillo et al., 1999). No GluR1-containing AMPARs were delivered to the cell membrane, and no LTP was expressed. These results suggest that the AMPAR subunit GluR1 is critical for LTP expression. Consistently, GluR1 exerts dominant effects over GluR2 in the GluR1-GluR2 heteromeric receptors and controls receptor trafficking and distribution (Lee et al., 2004; Passafaro et al., 2001; Shi et al., 2001). Next, we will focus on the roles of post-translational modifications and AMPAR-associated proteins in receptor trafficking during synaptic plasticity.

AMPAR TRAFFICKING REGULATED BY POST-TRANSLATIONAL MODIFICATIONS

Since AMPAR mediates most fast excitatory transmission, its trafficking and cellular distribution must be highly regulated to properly conduct the correct neuronal signals (Esteban, 2003; Kennedy and Ehlers, 2006). After synthesis, AMPAR subunits form heteromers in the endoplasmic reticulum (ER) and the N-terminus of each subunit is

critical for the assembly. GluR1-GluR2 heteromers are transferred from the ER to the *cis* face of Golgi apparatus rapidly through interaction between the GluR1 C-terminus and PDZ protein synapse associated protein 97 (SAP97) (Sans et al., 2001); GluR2-GluR3 heteromers exit the ER much more slowly and require interaction between the GluR2 C-terminus and protein interacting with C kinase 1 (PICK1), another PDZ protein (Figure 1-2A left). A portion of GluR2 is retained in the ER as a complex with some chaperones and the function is unknown (Rubio and Wenthold, 1999), but the unique GluR2 Q/R RNA-editing may be critical (Greger et al., 2002). Most of AMPARs are found in the cell body, so they need to undergo a long journey from the *trans* face of the Glogi apparatus to the dendritic spine, where the receptors can exert their functions (Figure 1-2A right). The process is microtubule and actin filament-based and requires some AMPAR subunit interaction proteins as adapters, such as PDZ protein glutamate receptor interacting protein 1/AMPAR binding protein (GRIP1/ABP) (Goldstein and Yang, 2000; Setou et al., 2002), and protein 4.1 N (Shen et al., 2001). The detailed mechanism will be discussed later. In the last step, AMPARs reach the synapses through two different pathways (Figure 1-2B). GluR2-GluR3 receptors continuously and rapidly cycle in and out of synapses to maintain the amount of AMPARs at synapses; whereas GluR1-GluR2 receptors and GluR4-containing receptors are added into synapses during synaptic plasticity (Malinow et al., 2000; Malinow and Malenka , 2002; Passafaro et al., 2001; Shi et al., 2001). In basal conditions, surface insertion of GluR1-GluR2 receptors is slow. While in response to NMDAR activation, receptors are driven into the dendritic spine and redistributed in the dendrites (Shi et al., 1999). The delivery routes of the two AMPARs are also different: GluR1-GluR2 receptors are inserted into the extrasynaptic membrane,

and later delivered laterally along dendrites to reside stably at the synapse in response to the afferent stimulation; whereas GluR2-GluR3 receptors are inserted more directly into the synaptic membrane (Passafaro et al., 2001; Shi et al., 2001). Real-time receptor trafficking also revealed that intracellular AMPARs are delivered into the synaptic membrane via the extrasynaptic and somatic surface, whereas the direct exchange of intracellular receptors with synaptic receptors is slow (Adesnik et al., 2005).

It is clear that trafficking of AMPARs to and away from synapses is a mechanism to modulate synaptic strength (Barry and Ziff, 2002; Carroll et al., 2001; Ehlers, 2000; Esteban, 2003; Lin et al., 2000; Lynch and Baudry, 1984; Malenka, 2003; Malenka and Nicoll, 1999; Malinow, 2003; Malinow and Malenka, 2002; Rumpel et al., 2005; Sheng and Lee, 2001; Sheng and Lee, 2003; Song and Huganir, 2002). During LTP expression, more AMPARs are delivered to the postsynaptic membrane. In contrast, LTD induces receptor internalization. These findings support the idea that alterations in synaptic strength are directly related to the receptor exocytosis and endocytosis, while AMPAR trafficking is primarily regulated through post-translational modifications and receptor-associated proteins (Barry and Ziff, 2002; Bredt and Nicoll, 2003).

Glycosylation and Receptor Stability

Glycosylation can protect proteins from proteolytic degradation. Each AMPAR subunit can be *N*-glycosylated at 4 to 6 different sites located in the extracellular domains of the protein. Glycosylation increases the receptor subunit molecular weight by about 4 kDa. Ligand binding on crystallized unglycosylated GluR2 S1-S2 domains shows that this

protein modification is not necessary for ligand recognition (Armstrong et al., 1998). However, the desensitization-inhibiting lectin concanavalin A (ConA) can potentiate AMPAR subunit except GluR2 currents by direct binding to these carbohydrate side chains (Everts et al., 1997). Therefore, *N*-glycosylation mainly facilitates the AMPAR maturation, protects the receptor from proteolytic degradation, and may affect receptor current amplitudes as well. However, the lack of *N*-glycosylation does not significantly affect AMPAR subunit synthesis, assembly, or trafficking (Dingledine et al., 1999; Everts et al., 1997).

Palmitoylation and Receptor Localization

Palmitoylation of protein is a reversible fatty acylation and regulates protein trafficking and cellular localization. All AMPAR subunits can be palmitolylated at two cysteine sites: one in the transmembrane domain TM2, the other in the intracellular C-terminal region. TM2 palmitoylation results in accumulation of AMPARs in the Golgi apparatus and consequently, a decrease of receptor expression in the cell surface. C-terminal palmitoylation reduces the interaction between receptor and protein 4.1 N and mediates agonist-induced AMPAR internalization. Therefore, depalmitoylated receptors are stabilized at the cell surface; whereas palmitolylated receptors are more susceptible to internalization triggered by ligand binding. In addition, activation of GluRs by glutamate stimulation decreases receptor palmitoylation and recruits more AMPARs to the cell surface to mediate synaptic plasticity (Hayashi et al., 2005).

Phosphorylation and Receptor Activation

Phosphorylation is the most important molecular mechanism to regulate the ligand-gated ion channels. It can regulate the physiological properties of the channel as well as protein trafficking. With the exception of GluR3, all AMPAR subunits have been reported to be phosphorylated on several amino acid residues by a variety of kinases.

To date, three phosphorylation sites of GluR1 have been reported. All sites are located at the intracellular C-terminus of GluR1. Serine 831 (S831) can be phosphorylated by both PKC (Roche et al., 1996) and CaMKII (Mammen et al., 1997); serine 845 (S845) is a protein kinase A (PKA) phosphorylation site (Roche et al., 1996). Phosphorylations in S831 and S845 are thought to be important for regulating the GluR1-containing AMPAR trafficking and differentiation of two prototypic synaptic plasticities: hippocampal NMDA-dependent LTP and LTD. According to plasticity history, synapses can be classified into three groups: depressed, naive, and potentiated. LTD induction decreases phosphorylation of S845 in naive synapses and phosphorylation of S831 in potentiated synapses; whereas LTP induction increases phosphorylation of S831 in naive synapses and the phosphorylation of S845 in depressed synapses. In the process, dephosphorylation is introduced by phosphatase PP1/2A (Lee et al., 2000). The details that describe how phosphorylation regulates surface expression of AMPAR are not clear, but S845 phosphorylation may facilitate extrasynaptic delivery of GluR1-containing AMPARs and NMDAR activation drives receptors to synapses from extrasynaptic sites by lateral diffusion (Passafaro et al., 2001; Oh et al., 2006). It is important to note that both S831 and S845 are necessary, but not sufficient to deliver AMPARs into synapses.

Therefore, other signaling events may be also involved (Esteban et al., 2003; Hayashi et la., 2004). In addition, transgenic mice with mutations in S831 and S845 still show reduced LTP, although a lack of LTD (Lee et al., 2003). Another PKC phosphorylation site in the C-terminus of GluR1, serine 818 (S818), has been reported recently (Boehm et al., 2006). Phosphorylation of GluR1 S818 is critical in LTP-driven incorporation of AMPARs into the postsynaptic membrane and is suggested to exert its function by facilitating the interaction between GluR1 and a delivery or tethering protein.

PKC phosphorylates GluR2 at serine 880 (S880) in the C-terminal sequence (IESVKI) for PDZ domain binding to differentially regulate the interaction with PDZ proteins: GRIP1/ABP and PICK1 (Chung et al., 2000, Seidenman et al., 2003). Phosphorylation of GluR2 S880 by PKC activation decreases receptor binding to GRIP1, and recruits PICK1 to synapses and facilitates rapid internalization of surface receptors. This indicates GluR2 phosphorylation of S880 is particularly important in regulating the AMPAR internalization during synaptic plasticity. In fact, GluR2 phosphorylation of S880 is a critical event in the induction of cerebellar LTD, not an NMDAR-dependent, but a metabotropic glutamate receptor mGluR1-dependent form of plasticity (Chung et al., 2003). Interestingly, another phosphorylation on tyrosine 876 (Y876) of GluR2 introduced by Src family tyrosine kinases has almost the same effects on GluR2 binding to GRIP1/ABP and PICK1, and in turn facilitates the AMPA or NMDA-induced receptor internalization (Hayashi et al., 2004). In addition, serine 863 of the GluR2 C-terminus is another potential PKC phosphorylation site and its function remains unknown (McDonald et al., 2001).

Like GluR1, GluR4 is a long isoform of AMPAR subunit. The expression of GluR1 and GluR4 in hippocampus is altered during development. GluR4 is critical for the synaptic plasticity during the early postnatal period when GluR1 expression in the hippocampus is low; whereas in the adult hippocampus GluR4 expression level is low and GluR1 is critical for synaptic function (Zhu et al., 2000). These two long isoform subunits share the conserved PKA phosphorylation site: S845 of GluR1 and S842 of GluR4. Interestingly, phosphorylation of GluR1 S845 is required, but not sufficient for LTP induction, which also needs S818 and S831 phosphorylations, and the PDZ binding site. In contrast, phosphorylation of GluR4 S842 is both necessary and sufficient to deliver the receptor to the synapse and induce plasticity in early postnatal development (Esteban et al., 2003). In addition, PKCγ is reported to directly interact with the GluR4 and phosphorylate it at serine 482 (S482) *in vitro*, thereby increasing recombinant GluR4 surface expression to mediate the function of GluR4-containing AMPARs (Correia et al., 2003). Threonine 830 (T830) is another potential PKC phosphorylation site of GluR4 and its function remains unknown (Carvalho et al., 1999).

Taken together, receptor phosphorylation by kinases, specifically CaMKII and PKC, play critical roles in AMPAR trafficking and plasticity expression. Notably, several members of the PKC family directly phosphorylate AMPAR subunits to mediate plasticity (Boehm et al., 2006; Carvalho et al., 1999; Chung et al, 2000; Correia et al., 2003; McDonald et al., 2001; Roche et al., 1996). Recently, a special atypical PKC (aPKC) isoform, PKMζ has received considerable attention and undergoes intense

investigation. Vertebrates express 10 PKC isoforms classified into three groups: conventional PKC (cPKC: α, βI, βII and γ), novel PKC (nPKC: δ, ε, η/L and θ), and atypical PKC (aPKC: ζ and ι/λ) (Figure 1-3A). Each isozyme consists of an N-terminal regulatory region and a conserved C-terminal catalytic domain. The regulatory region contains a pseudosubstrate, an autoinhibitory sequence, which automatically interacts with the catalytic domain and blocks the enzymatic activity. Release of the pseudosubstrate by stimulation of second messengers activates the enzyme (Newton, 2001). PKMζ is an isoform of PKCζ without the regulatory region (Figure 1-3B). This unique characteristic enables the enzyme to be constitutively activated (Hernandez et al., 2003; Sacktor et al., 1993). PKMζ is produced from PKMζ mRNA, transcribed from an internal promoter within the PKCζ gene (Figure 1-3C) (Hernandez et al., 2003). Interestingly, phosphorylation introduced by PKMζ is necessary and sufficient for LTP maintenance (Ling et al., 2002; Serrano et al., 2005). In addition, a PKMζ inhibitor, myristoylated ζ-pseudosubstrate inhibitory peptide (ZIP), both reverses LTP maintenance *in vivo* and leads to loss of spatial information. These findings suggest that the LTP maintenance mediated by PKMζ sustains spatial memory (Pastalkova et al., 2006). A recent study showed that two sequential steps are involved in LTP maintenance: synthesis and activation of PKMζ. PI3-kinase, CaMKII, MAPK, PKA, mTOR (mammalian target of rapamycin) and preexisting PKMζ regulate *de novo* synthesis of PKMζ and phosphoinositides-dependent protein kinase-1 (PDK1) activates PKMζ via phosphorylation of its activation loop (Kelly et al., 2007). In deed, PKMζ causes synaptic potentiation by increasing the number of active AMPARs at the postsynaptic membrane without affecting the unit conductance of receptor channels (Ling et al., 2006). This

indicates that phosphorylation induced by PKMζ mediates AMPAR trafficking, instead of altering channel properties.

The profile of induction and maintenance of LTP is becoming clear. Learning or other afferent stimulation activates the NMDARs and causes Ca^{2+} influx into the dendrite, which in turn activates some Ca^{2+}-dependent kinases. Consequently, the conductance of receptor channels is enhanced to induce LTP expression or early LTP (E-LTP). On the other hand, the influx of Ca^{2+} also triggers an unknown signaling cascade toward the nucleus and initiates the internal promoter of the PKCζ gene to produce PKMζ mRNA. The PKMζ mRNA is actively transported to dendrites and translated to produce PKMζ, which is activated by PDK1 during LTP maintenance or late LTP (L-LTP) (Figure 1-3C). The consistent second messenger-independent phosphorylation by PKMζ increases and maintains the amount of active AMPARs at the synapse (Bliss et al., 2006). Although both GluR1 and GluR2 C-termini are PKMζ substrates *in vitro* (Ling et al., 2006), it would be more reasonable to propose that PKMζ phosphorylates some receptor-associated protein to regulate AMPAR trafficking, instead of directly modifying the receptor itself to change channel characteristics. Continuing to investigate possible candidates for PKMζ substrates is important for uncovering the roles of PKMζ in the maintenance of synaptic plasticity.

Coincidently, both PKCι/λ and PKMζ, but not PKCζ, are expressed in the mammalian brain at a high level, especially in the hippocampus (Oster et al., 2004). PKCι/λ and PKMζ have a similar pattern of distribution in the human brain and both are

activated after tetanization (Crary et al., 2006; Hernandez; 2003; Sacktor et al., 1993). In addition, the catalytic domains of PKCι/λ and PKMζ are almost identical. The PKMζ inhibitors chelerythrine and ZIP also theoretically inhibit other aPKCs (Ling et al., 2002; Pastalkova et al., 2006). Therefore results concerning PKMζ cannot exclude PKCι/λ involvement in synaptic plasticity. Moreover, it is possible that PKCι/λ plays a similar role as PKMζ does before the expression of PKMζ is promoted by afferent stimulation. It is also possible that PKCι/λ and PKMζ possess roles at different sites within the cells, because activated PKCι/λ is bound to the cell membrane, whereas PKMζ is mainly located in the cytosol, due to its lack of a regulatory region. Application of chelerythrine, an aPKC inhibitor, and a dominant negative inhibitory form, PKMζ-K281W, also completely eliminated induction of LTP (Ling et al., 2002). This would suggest that full-length PKCι/λ might be involved in LTP induction. If so, phosphorylation by PKCι/λ may be necessary for LTP induction; whereas later PKMζ is expressed in dendrites and maintains LTP. In addition, a cytoplasmic and membrane-associated protein named p62 interacts with the pseudosubstrate region in the regulatory N-terminus of aPKC but not classic PKCs (Puls et al., 1997; Gong et al., 1999). By binding aPKC, p62 serves as a scaffold protein to recruit substrates to the kinase. This suggests to us that p62 might be involved in the regulation of synaptic plasticity through its interaction with PKCι/λ. The details will be discussed later.

Ubiquitination and Receptor Endocytosis

Ubiquitin has 76 amino acids and belongs to a family of small proteins highly conserved in structure. Ubiquitination, the attachment of a single ubiquitin or a polymeric ubiquitin

chain to a protein, can regulate the proteins in many different ways. The prototypical function of ubiquitination is proteolysis through proteasomal pathway. Later, numerous studies revealed that ubiquitination also plays important roles in many other pathways beyond proteolysis (Hochstrasser et al., 1996; Schnell and Hicke, 2003), such as protein location, activity, etc. The potential functions of the ubiquitin proteasome system (UPS) in synaptic strength have been investigated widely in many species including *C. elegans*, *Aplysia*, *Drosophila*, and mammal (Burbea et al., 2002; DiAntonio et al., 2001; DiAntonio and Hicke, 2004; Hegde and DiAntonio, 2002; Kato et al., 2005; Murphey and Godenschwege, 2002; Speese et al., 2003). Surprisingly, UPS regulates the abundance of both presynaptic and postsynaptic proteins (Ehlers, 2003; Speese et al., 2003) to exert multiple roles in synaptic development, presynaptic function and neurotransmitter release, alternation of postsynaptic density (PSD) and plasticity, spine growth and stability (Yi and Ehlers, 2005). Here, we will focus on the recent progress in understanding ubiquitination as related to AMPAR trafficking and synaptic plasticity.

As mentioned above, trafficking of AMPARs to and away from the postsynaptic membrane is the molecular basis for the change of synaptic strength. The amount of AMPARs at the synapse is regulated through exocytosis and endocytosis mediated by clathrin-coated vesicles (Carroll et al., 1999, Carroll et al., 2001; Wang and Linden, 1999). The disruption of components of clathrin-coated vesicles such as dynamin and huntingtin interacting protein 1 (HIP1) resulted in neurological deficits and a decrease of AMPAR trafficking (Carroll et al., 1999; Metzler et al., 2003). Recent studies reveal the UPS plays important roles for AMPAR endocytosis (Ehlers, 2003; Patrick et al., 2003),

which is critical for LTD (Carroll et al., 2001), induced by treatment of receptor agonists such as NMDA or hormones such as insulin (Wang and Linden, 2000). Ubiquitination also was reported to play important roles in hippocampal LTP, but the detailed mechanism remains unknown (Jiang et al., 1998). Theoretically, the substrates of ubiquitination could be either the glutamate receptors themselves or receptor-associated proteins (Patrick et al., 2003). So far, no evidence of direct ubiquitination of AMPAR in mammals has been reported. However, GLR-1, the ortholog of mammalian GluR1 subunit in *C. elegans*, can be ubiquitinated in the intracellular C-terminus. The ubiquitination site (LxEFxYK/RSRxD/EAK) is conserved in all mammalian AMPAR subunits and shares homology with the ubiquitin/endocytic signal of yeast proteins Ste2P and Ste6P (Burbea et al., 2002; Lin et al., 2000). These findings suggest that ubiquitination of GLR-1 may mediate the synaptic strength and GLR-1-containing synapses through regulating the amount of receptor at the synapse in a clathrin adaptin protein (AP180)-dependent manner (Burbea et al., 2002). Interestingly, ubiquitination of KEL-8, a neuronal protein localized adjacent to GLR-1 on the postsynaptic membrane, is necessary for proteolysis of GLR-1 receptors and may regulate GluRs localization and signaling in postmitotic neurons (Schaefer and Rongo, 2006).

In response to the synaptic activity, the PSD proteins, including some postsynaptic scaffolds, become highly ubiquitinated and those alterations, in turn, change downstream effectors like cyclic AMP (cAMP) response element binding protein (CREB) and ERK-MAPK (Ehlers et al., 2003). In particular, PSD-95, a major PSD scaffolding protein, mediates synaptic plasticity by anchoring NMDARs and AMPARs to the

neuronal cytoskeleton or other receptor-associated proteins (Cho et al., 1992). In response to NMDAR activation, PSD-95 is ubiquitinated and removed from the synaptic sites by UPS (Bingol and Schuman, 2004; Colledge et al., 2003). This results in a decrease of AMPAR expression in the synaptic surface during synaptic plasticity. Interestingly, β-amyloid (Aβ), a peptide that is derived from amyloid precursor protein (APP) in neurons and widely believed to underlie the pathophysiology of Alzheimer's disease, can drive AMPAR endocytosis and cause loss of dendritic spines and inhibit hippocampal LTP (Hsieh et al., 2002; Walsh et al., 2002).

Following endocytosis, AMPARs in the early endosome are sorted via two different pathways. They are either recycled back to the cell surface or degraded through the lysosome, depending on the activations of NMDARs and AMPARs. With NMDAR activity stimulated by glutamate, AMPARs are internalized in a Ca^{2+}-dependent manner, dephosphorylated by protein phosphatases, rephosphorylated by PKA, and at last rapidly reinserted in the synaptic membrane via recycling endosomes. On the other hand, without NMDAR activity, AMPARs are internalized in a Ca^{2+}-independent manner, and then targeted to the lysosomes for degradation via late endosomes (Ehlers, 2000). Consistently, in response to AMPA stimulation, internalized AMPARs enter the recycling system and reappear on the surface quickly; whereas in response to insulin treatment, internalized AMPARs are diverted into a distinct compartment, a non-recycling pathway (Lin et al., 2000). Those reinserted AMPARs are a very important source of receptors for LTP expression (Park et al., 2004). The recycling endosomes and vesicles are also merged into spines to promote and maintain the growth of spines induced by synaptic plasticity

(Kennedy and Ehlers, 2006, Park et al., 2006). Interestingly, chemically induced LTP (cLTP) can drive strong exocytosis of AMPARs, but with a small loss of NMDARs on the spine surface. In addition, spines grow larger before collecting AMPARs on their surface (Kopec et al., 2006). So it is possible that recruitment of AMPARs to the spine surface requires accumulation of certain spine components, specifically, tethering proteins at the spine site.

AMPAR INTERACTING PROTEINS IN RECEPTOR TRAFFICKING

Unquestionably, the localization and trafficking of AMPARs are both an extremely complicated and highly regulated process. Many proteins interact with different AMPAR subunits to regulate receptor trafficking (Braithwaite et al., 2000; Henley, 2003; Palmer et al., 2005). We will discuss some of the more critical proteins.

GluR1 Interacting Proteins

Synapse associated protein 97 (SAP97) is the first protein reported to directly interact with GluR1 subunit (Leonard et al., 1998). This PDZ-containing protein belongs to a protein family named synapse associated protein (SAP) and other family members include some NMDAR subunit interacting proteins such as SAP90 (PSD95), chapsyn110 (PSD93) and SAP102 (Braithwaite et al., 2000). SAP97 has three PDZ domains and interacts with the very C-terminal of GluR1 with its second PDZ domain (Cai et al., 2002; Leonard et al., 1998). SAP highly accumulates at GluR1-containing synapses, so SAP may serve as an anchoring molecule to help GluR1 reside stably in the synaptic surface (Valtschanoff et al., 2000). The interaction between SAP97 and GluR1 first

occurs in the receptor secretory pathway and is essential for the transport of the receptor from the endoplasmic reticulum to the *cis* face of Golgi apparatus (Figure 1-2A left) (Sans et al., 2001). The interaction of SAP97 with A kinase anchoring protein 79 (AKAP79) can target PKA to GluR1 for S845 phosphorylation and may be involved in the LTD induction (Colledge et al., 2000; Tavalin et al., 2002). Phosphorylation by CaMKII regulates SAP97 targeting and interaction with NMDAR subunit NR2A (Gardoni et al., 2003; Mauceri et al., 2004). Interestingly, PDZ domain-containing proteins including SAP 97 are widely believed to play critical roles in AMPAR trafficking and LTP expression (Shi et al., 2001; Hayashi et al., 2000). However, transgenic mice lacking the C-terminal PDZ binding site of GluR1 show normal GluR1 synaptic localization and unimpaired hippocampal CA1 LTP (Kim et al., 2005).

Proteins 4.1G/N are the other GluR1 interacting proteins (Shen et al., 2000). They belong to a family of multifunctional cytoskeletal components (4.1B/G/N/R) originally isolated from erythrocytes and are essential for assembly and maintenance of the actin cytoskeleton. 4.1N is enriched in synapses; whereas 4.1G is expressed in all cells (Walensky et al., 1999). Both 4.1G and 4.1N bind to the intracellular membrane proximal region of GluR1. Particularly, 4.1N interacts with GluR1 *in vivo* and colocalizes with AMPARs at excitatory synapses. Destruction of actin filaments in cultured cortical neurons significantly decreases GluR1 surface expression. Taken together, 4.1G/N may serve as adapters to link GluR1 to the actin cytoskeleton (Shen et al., 2000). The actin filaments, but not microtubules, are abundant in the dendritic spine (Fischer et al., 1998). These observations suggest that GluR1-containing AMPARs are delivered to synapses

along actin filaments (Figure 1-2A right). Interestingly, GluR4 also has a 4.1 binding site and the 4.1 proteins may affect GluR4 trafficking and localization in a manner similar to that found for GluR1 (Coleman et al., 2003).

GluR2 Interacting Proteins

GRIP/ABP binds to the C-terminus of GluR2/3 via PDZ domains. GRIP contains seven PDZ domains with a molecular weight of 130 kDa. Its fourth and fifth PDZ domains bind the very C-terminal motif of GluR2/3 (ESVKI) (Dong et al., 1997). ABP is a close relative of GRIP and has two splice variants: the short 98 kDa isoform named ABP-S has six PDZ domains and the third, fifth and sixth mediate binding to GluR2/3; the long 130 kDa isoform named ABP-L or GRIP2 has seven PDZ domains (Srivastava et al., 1998). Both GRIP and ABP are ubiquitously expressed in the CNS and enriched in the PSD, but GRIP can be detected before the emergence of AMPARs during embryonic development; whereas the expression pattern of ABP resembles AMPARs (Dong et al., 1999). The function of GRIP has not been fully elucidated, but its multiple PDZ domains may endow the protein to function as an adapter. In fact, several proteins can bind to its PDZ domains other than the fourth and the fifth (Ye et al., 2000). Some other proteins may regulate AMPARs signaling and trafficking via GRIP. For example, GRIP-associated proteins-1 (GRASP-1) is a rasGEF in neurons and associated with GRIP and AMPARs *in vivo*. Overexpression of GRASP-1 in cultured neurons causes a down-regulation of synaptic targeting of AMPARs (Ye et al., 2000). Through GRIP, AMPARs are linked to activity-dependent Ras signaling and trafficking events and the caspase pathway (Ye et al., 2002). In another case, both of the Eph receptors, a family of receptor tyrosine kinases, and their

ephrin ligands, bind to the sixth and seventh PDZ domains of GRIP (Torres et al., 1998) and the interaction that may be involved in neurite extension and axonal guidance (Gale et al., 1997), and recruitment of intracellular GRIPs to membrane lipid rafts (Brückner et al., 1999). The interactions of GRIP with kinesin heavy chain (Setou et al., 2002) and microtubule-associated protein (MAP)-1B light chain (LC) (Seog et al., 2004) may link AMPAR to microtubular motor protein trafficking. Liprin-α interacts with GRIP and is necessary for AMPAR targeting (Wyszynski et al., 2002). Liprin-α can also can associate with KIF1A, another member of the kinesin superfamily of molecular motors (Shin et al., 2003). In addition, interaction between liprin-α and GIT1 is also necessary for AMPAR targeting (Ko et al., 2003). Taken together, AMPAR trafficking along the microtubular cytoskeleton in the dendrite is mediated by interaction between GRIP and several microtubular motor proteins (Figure 1-2A, right).

Protein interacting with C kinase (PICK1) has a BAR (Bin/amphiphysin/Rvs) domain and a PDZ domain, through which the dimer of PICK1 could bind to the C-terminus of GluR2 (Xia et al., 1999) and the catalytic domain of PKCα (Dev et al., 2004). So, PICK1 may target the AMPARs to an active PKCα (Perez et al., 2001). S880 phosphorylation of GluR2 by PKC upon phorbol ester (TPA) induction releases GluR2-containing AMPARs from GRIP1 binding, and increases receptor binding to PICK1. As a result, more PICK1 traffic to synapses and GluR2-containing AMPARs are internalized rapidly to result in cerebellar and hippocampal LTD (Chung et al., 2003; Seidenman et al., 2003). T876 phosphorylation has the similar effects on GluR2 binding to ABP/GRIP and PICK1 (Hayashi et al., 2004). An interesting model may explain the functions of

PICK1 and GRIP/ABP in GluR2-containing AMPAR internalization. PICK1 BAR domain interacts with its PDZ domain and also interacts with GRIP/ABP linker II region. Binding to GluR2 or PKCα interrupts the intramolecular interaction and facilitate the intermolecular interaction of PICK1 BAR domain with GRIP/ABP linker II region. Then PKCα phosphorylates S880 of GluR2 and the phosphorylated GluR2 is released from GRIP/ABP to bind to PICK1. Finally, the PICK1 BAR domain directs receptor internalization (Lu et al., 2005). PICK1 may also be involved in the formation of extrasynaptic membrane pools of GluR2-containing AMPAR which later could be targeted to the synaptic membrane via lateral trafficking (Gardner et al., 2005). The interaction between PICK1 and GluR2 is also necessary for receptor exit from the ER (Figure 1-2A left) (Greger et al., 2002; Lu et al., 2005). Considering PICK1 interacts with more than a dozen proteins besides GluR2, PKCα, and GRIP/ABP (Meyer et al., 2004), the AMPAR trafficking regulated by GRIP is expected to be more complicated. A recent study revealed that the neuronal endosomal protein NEEP21 may be involved in the GluR2-containing AMPAR sorting and reinsertion into the cell surface by binding with GRIP and GluR2 subunit (Steiner et al., 2005).

The interaction between ATPase *N*-ethylmaleimide-sensitive fusion protein (NSF) and AMPAR subunit GluR2 was first detected by using the yeast two-hybrid screen (Henley et al., 1997; Nishimune et al., 1998). A region in the GluR2 C-terminus (KRMKVAKNPQ) is responsible for NSF binding with asparagine 851 playing an essential role (Nishimune et al., 1998). NSF is a well-known multihomomeric ATPase that plays a central role in docking and fusion of synaptic vesicles (Schiavo et al., 1995).

Perfusion of a synthetic peptide pep2m mimicking the NSF binding site on GluR2 to postsynaptic sites partially blocked synaptic transmission (Lee et al., 2002).Therefore, NSF may play a role in the regulated delivery of GluR2-containing AMPARs from the postsynaptic surface to the lysosome during LTD (Lee et al., 2004). Overexpression of NSF in cultured hippocampal neurons caused a down-regulation in surface expression of AMPARs, but the total AMPAR expression was unaffected (Noel et al., 1999). Interestingly, both PICK1 and NSF are necessary for calcium-permeable AMPAR plasticity (CARP), and dynamically exchange AMPARs lacking GluR2 with GluR2-containing AMPARs in the synapse during CARP to mediate the calcium permeability of AMPAR (Gardner et al., 2005).

AP2 belongs to the family of assembly particles and other members include AP1 and AP3. Particularly, AP2 complex, a well-characterized clathrin adapter involved in endocytosis from the cell surface, consists of four subunits: α, β, σ and μ adaptins. Clathrin adapters are essential for endocytosis by linking membrane proteins to clathrin and facilitating assembly of clathrin coats. Therefore, association of clathrin adapters to the cytoplasmic domains of receptors is a key to promote receptor endocytosis (Kirchhausen, 1999). Activity-dependent AMPAR internalization occurs via dynamin-dependent clathrin-coated vesicles (Carroll et al., 1999; Carroll et al., 2001; Wang and Linden, 2000). Interestingly, AP2 associates with GluR2 in almost the same region as the NSF binding site. In fact, application of pep2m blocks both AP2 and NSF binding to GluR2 (Lee et al., 2002). Although sharing the same binding site on GluR2, AP2 and NSF play distinct roles in the maintenance and removal of AMPARs at the synapse.

Other AMPAR Interacting Proteins

Transmembrane AMPAR regulatory protein (TARP) family has four members: γ-2 (or stargazin), γ-3, γ-4 and γ-8. Stargazin, the prototypical TARP, is the first identified transmembrane interactor of AMPARs (Chen et al., 2000; Fukata et al., 2005). Stargazin is mutated in the stargazer mice that show a phenotype of the absence epilepsy and cerebellar ataxia, which results from the lack of functional AMPAR channels in cerebellar granule cells (Chen et al., 2000; Hashimoto et al., 1999; Schnell et al., 2002). These four proteins may function as the auxiliary components of neuronal AMPARs (Fukata et al., 2005, Tomita et al., 2003) and are spatiotemporally expressed during differentiation and development in the CNS. γ-4 is dominant during early development; stargazin is the only expressed TARP in cerebellar granule cells, but lacking from the hippocampal CA1 region; γ-8 is highly expressed allover the hippocampus (Tomita et al., 2004). Different distribution of TARPs would implicate differential and developmental control of activity-dependent trafficking affairs. Stargazin is a four transmembrane protein and targets AMPARs to the granule cell surface (Tomita et al., 2004). The intracellular C-terminus of stargazin binds to some PDZ proteins including PSD-95 to mediate synaptic trafficking and clustering of AMPARs (Chen et al., 2000). Stargazin also can be phosphorylated in an activity-dependent pattern to regulate hippocampal synaptic strength (Tomita et al., 2005a; 2005b). In addition, stargazin controls AMPAR channel gating by slowing glutamate-induced AMPAR deactivation and desensitization, and enhancing the channel conductance (Tomita et al., 163a, Nicoll et al., 2006). Both extracellular and intracellular regions of stargazin interact with AMPARs (Tomita et al.,

2004) and the interaction may be direct because immunoprecipitation of stargazin also pulled down other AMPAR interacting proteins (Fukata et al., 2005), although which domain of GluRs is responsible for stargazin binding remains unknown. A recent study revealed that a point mutation in the glutamate-binding region of GluR1 destroys stargazin's effects on receptor trafficking and channel gating. This finding suggests that the glutamate-binding domain of AMPARs could be the extracellular binding site for TARPs (Tomita et al., 2006).

Soluble NSF attachment protein (SNAP) has three forms: α, β and γ, and is critical in membrane fusion. Particularly, α- and β-SNAPs interact with the C-terminus of the GluR2 subunit (Osten et al., 1998). As the name indicated, SNAPs can bind NSF (Clary et al., 1990), but they associate with AMPARs independently with an increase of the amount of AMPARs binding SNAP in the presence of NSF because AMPARs can recruit more SNAPs indirectly via NSF (Osten et al., 1998). SNAP is another AMPAR interactor involved in vesicle budding and fusion beside NSF and may play a role in regulating synaptic expression of AMPARs through a direct association with receptors (DeBello et al., 1995; Lledo et al., 1998). In addition, SNAPs and NSF can disassemble the GluR2-PICK1 complex to mediate AMPAR surface expression (Hanley et al., 2002).

Lyn is a Src-family non-receptor protein tyrosine kinase and is highly expressed in CNS. About 1-2% of Lyn in the cerebellum associates with GluR2-containing or GluR3-containing AMPARs via its Src homology region consisting of SH2 and SH3 domains (Hayashi et al., 1999). Lyn is activated by AMPAR in a Ca^{2+} and Na^{+}-

independent manner and promotes the MAPK signaling cascade to initiate expression of brain-derived neurotropic factor (BDNF), which is involved in the regulation of synaptic plasticity in addition to that of neuronal survival and differentiation (Caldeira et al., 2007; Poo et al., 2001; Yamada et al., 2002; Zakharenko et al., 2003). Therefore, in addition to synaptic transmission via cation influx, AMPARs may be directly involved in mediating intracellular signal from the cell surface to the nucleus through the Lyn-MAPK cascade and mediate synaptic plasticity by regulating BDNF expression (Hayashi et al., 1999).

LIN-10 is a membrane-associated protein that mediates localization of GLR-1 in *C. elegans* (Rongo et al., 1998). Its human ortholog, mLin-10, directly associates with GluR1 and GluR2 subunits through a PDZ domain-dependent mechanism and mediates the receptor trafficking (Stricker et al., 2003). X11L, a homolog of mLin-10 can bind the p65 subunit of NF-κB to regulate Aβ production in neurons and may indicate a novel means to control the progression of Alzheimer's disease (Tomita et al., 2000). In addition, GluR1 subunit was detected in association with the guanine-nucleotide-binding protein ($G\alpha_{il}$) and stimulation with AMPA can regulate $G\alpha_{il}$ function in a Ca^{2+} and Na^{+}-independent manner (Wang et al., 1997). This suggests that AMPAR could exert a metabotropic function besides the ion channel.

CONCLUSION AND FUTURE DIRECTION

Cumulative studies reveal that AMPAR interacting molecules play critical roles in AMPAR-related synaptic plasticity. Some interacting molecules regulate post-translational modification of the receptors, while others mediate receptor trafficking,

cellular localization and signal transduction cascades. Undoubtedly, AMPAR post-translational modifications and AMPAR-associated proteins are the key to mediate receptor trafficking, which is the underlying molecular mechanism for synaptic plasticity. Despite more than two decades of intense research (Lynch and Baudry, 1984), the complete mechanism of AMPAR trafficking is not clear. For proteins whose binding sites on AMPAR subunits are determined such as 4.1N, SAP97, GRIP/ABP, PICK1, AP2, NSF and TARPs, their functions in AMPAR trafficking are basically understood (Figure 1-4). For those proteins whose binding sites on the AMPAR subunit remain unknown, continued efforts to characterize the binding sites will shed light on their functions in the regulation of synaptic strength (Henley, 2003). Unquestionably, more AMPAR-interacting proteins will be discovered and will allow us to develop a more complete picture of AMPAR interactions. Those AMPAR-associated proteins could be newly discovered or some existing proteins with novel functions in plasticity. As discussed previously, p62, the aPKC adapter, is a possible AMPAR-associated protein. p62 is a cytoplasmic and membrane-associated protein with a molecular weight of about 62 kDa. It is also named zeta protein kinase C interacting protein (ZIP, different from ZIP inhibitor of PKMζ) and was initially identified as a phosphotyrosine-independent ligand of the Src homology 2 (SH2) domain of $p56^{lck}$ (Puls et al., 1997). The p62 protein possesses 6 domains, which endow it with the ability to associate with many other proteins (Figure 1-5): a PB1 domain for aPKC binding, a ZZ-type Zinc finger domain for binding aPKC substrates (Cariou et al., 2002; Croci et al., 2003; Gong et al., 1999; Sanz et al., 1999), a tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) binding domain, two PEST domains for proteolytic recognition, and a ubiquitin-associated (UBA)

domain. p62 is concentrated in hippocampus (Gong et al., 1999). Based on its specific structure, p62 serves as a scaffold to recruit substrates of aPKC through its PB1 domain and ZZ-type finger domain (Moscat et al., 2006). In addition, p62 also functions as an adapter protein in the process of polyubiquitination of many proteins, especially membrane-associated receptors, and specifically binds proteins with K63-polyubiquitin attached through its C-terminal UBA domain, which initiates ubiquitination-mediated receptor endocytosis (Geetha et al., 2005). Furthermore, p62 may function as an intracellular shuttling factor (Seibenhener et al., 2004). Interestingly, the p62 knockout mice exhibit impaired spatial learning and memory, a hippocampal-dependent process (Wooten, unpublished). Defective spatial learning and memory often correlates with impaired hippocampal LTP. These results suggest that p62 may play a yet to be defined role in regulating synaptic transmission.

Theoretically, PKCι/λ may play an important role for early expression of LTP (E-LTP); whereas PKMζ is essential for LTP maintenance (Ling et al., 2002; Pastalkova et al., 2006). If so, p62 could target potential substrates to PKCι/λ to mediate E-LTP through aPKC-dependent phosphorylation. In addition, p62 could also serve as an adapter to facilitate the ubiquitination at the PSD to mediate endocytosis of the receptor itself or of some receptor-associated proteins (Patrick et al., 2003). Investigating the possible roles of p62 in synaptic plasticity may help to uncover additional information on the molecular and cellular mechanisms of synaptic plasticity.

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Figure 1-1. AMPAR Structure and Post-Transcriptional Modifications

(A) Phylogenetic tree of ionotropic glutamate receptor (iGluR) subunits. AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor; KAR, kainate receptor; NMDAR, *N*-methyl-D-aspartate receptor; GluR, glutamate receptor subunit; KA, KAR subunit; NR, NMDAR subunit.

(B) Schematic of AMPAR subunit at the synaptic membrane. Each subunit consists of an extracellular N-terminus, four hydrophobic regions (TM1-4), two intracellular loops, one extracellular loop and an intracellular C-terminus. TM2 domain is a re-entrant transmembrane hairpin structure that forms the cation pore channel. The extreme N-terminus contains an X-domain and its function remains unknown. The ligand binding core is composed of two ligand binding domains: S1 and S2. The intracellular C-terminus is the interaction site for several proteins.

(C) Post-transcriptional modifications of AMPAR subunits. All subunits undergo the flip/flop alternative splicing. GluR2 and GluR4 also contain another alternative splicing site in the C-terminus to produce the long and short isoforms; whereas GluR1 only has the long isoform and GluR3 only has the short isoform. In addition, GluR2 contains the Q/R RNA editing site within the TM2 domain, and GluR2, 3 and 4 have the R/G RNA editing site right preceding the flip/flop site.

Figure 1-1.

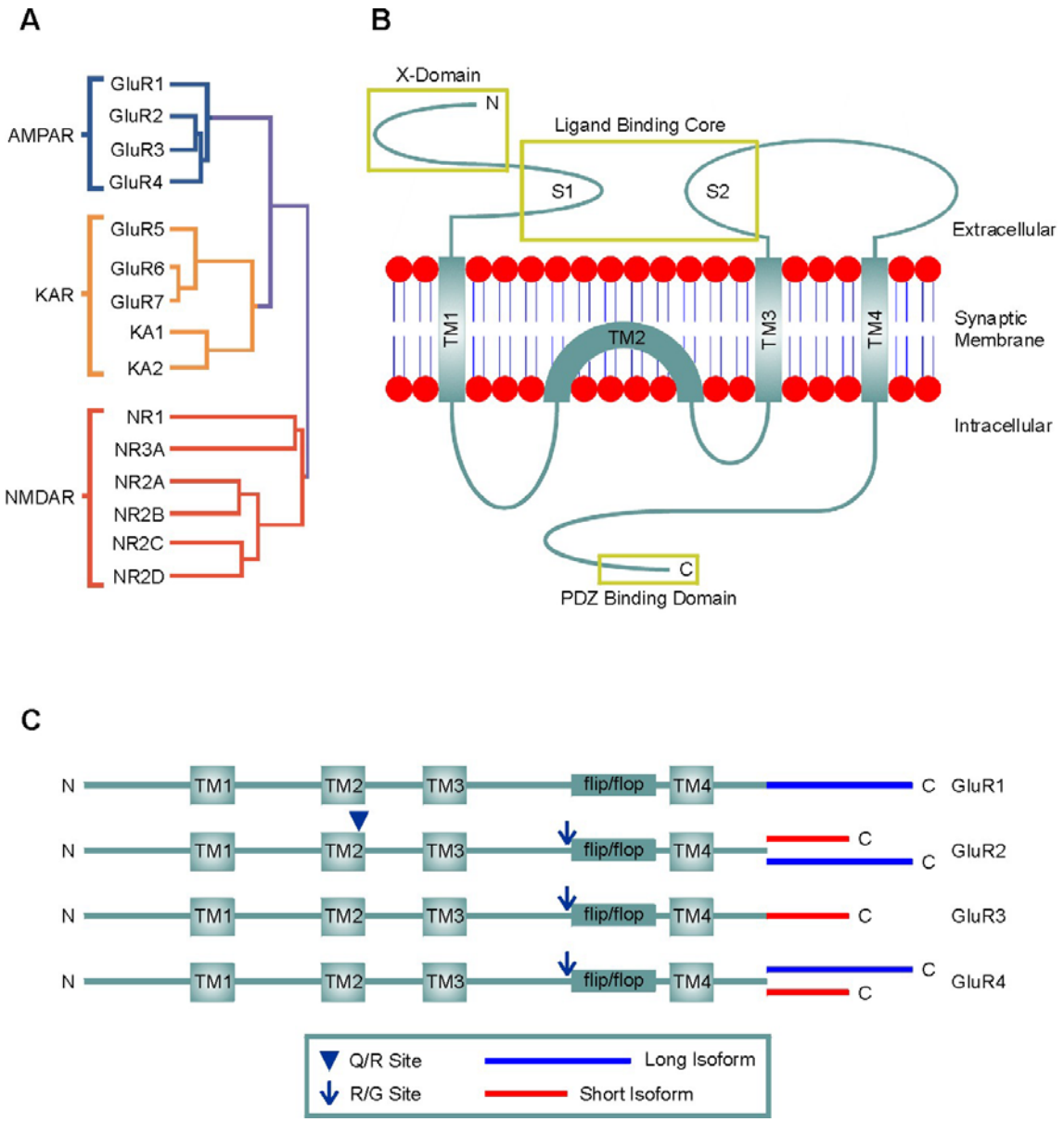

A
AMPAR
GluR1
GluR2
GluR3
GluR4
KAR
GluR5
GluR6
GluR7
KA1
KA2
NMDAR
NR1
NR3A
NR2A
NR2B
NR2C
NR2D
B
X-Domain
N
Ligand Binding Core
S1
S2
Extracellular
TM1
TM2
TM3
TM4
Synaptic Membrane
Intracellular
C
PDZ Binding Domain
C
N
TM1
TM2
TM3
flip/flop
TM4
C
GluR1
GluR2
GluR3
GluR4
Q/R Site
R/G Site
Long Isoform
Short Isoform

Figure 1-2. AMPAR Trafficking from ER to Golgi Apparatus and Synapse

(A) Left, after assembly, GluR1-GluR2 heteromers are rapidly transferred from ER to the *cis* face of Golgi apparatus through interaction between the GluR1 and PDZ protein synapse associated protein 97 (SAP97); whereas GluR2-GluR3 heteromers exit the ER much more slowly and require interaction between GluR2 C-terminus and protein interacting with C kinase 1 (PICK1), another PDZ protein. A fraction of GluR2 is retained in the ER with chaperones for an unknown reason. Right, the interaction between the GluR2 C-terminus and PDZ protein glutamate receptor interacting protein 1/AMPAR binding protein (GRIP1/ABP) is necessary for AMPAR trafficking in dendrites along the microtubular cytoskeleton. Liprin-α serves as an adaptor to link GRIP/ABP with KIF1A, a motor protein. Later, AMPARs are targeted to the dendritic spine along actin filaments through the interaction between the C-terminus of GluR1/GluR4 and protein 4.1N.

(B) Left, GluR2-GluR3 receptors continuously and rapidly cycle in and out of synapses to maintain the total number of AMPARs at synapses; whereas GluR1-GluR2 receptors and GluR4-containing receptors are added into synapses via the extrasynaptic surface during expression of LTP. Right, with induction of LTD, receptors are internalized rapidly.

Figure 1-2.

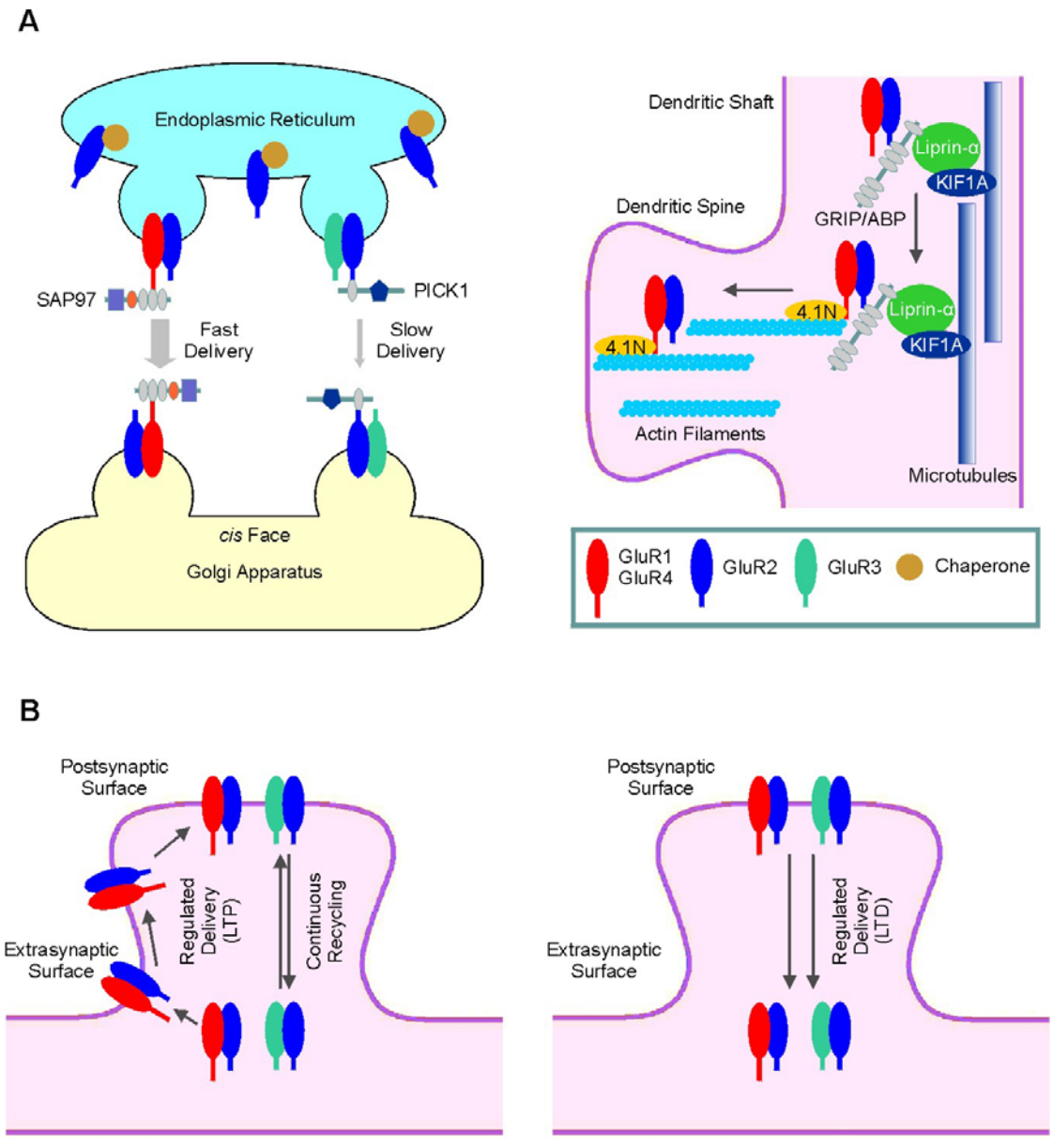

A
Endoplasmic Reticulum
SAP97
PICK1
Fast Delivery
Slow Delivery
cis Face
Golgi Apparatus
Dendritic Shaft
Dendritic Spine
Liprin-α
KIF1A
GRIP/ABP
4.1N
4.1N
Liprin-α
KIF1A
Actin Filaments
Microtubules
GluR1 GluR4
GluR2
GluR3
Chaperone
B
Postsynaptic Surface
Extrasynaptic Surface
Regulated Delivery (LTP)
Continuous Recycling
Postsynaptic Surface
Extrasynaptic Surface
Regulated Delivery (LTD)

Figure 1-3. The Specific Structure Enables PKMζ to Maintain LTP

(A) PKC family has 10 isoforms classified into three groups: conventional PKC (cPKC: α, βI, βII and γ), novel PKC (nPKC: δ, ε, η/L and θ), and atypical PKC (aPKC: ζ and ι/λ). Each isozyme consists of an N-terminal regulatory region, which contains cysteine-rich domains (C-1 and C-2) and a pseudosubstrate, and a conserved C-terminal catalytic domain with of serine/threonine kinase activity.

(B) The pseudosubstrate in the regulatory region automatically interacts with the catalytic domain and blocks the activity of the enzyme. Release of the pseudosubstrate by stimulation of the second messengers can activate the enzyme. PKMζ is an isoform of PKCζ without the regulatory region, so this enzyme is constitutively activated.

(C) Afferent stimulation activates the NMDARs and causes Ca^{2+} influx into the dendrite, which in turn activates various Ca^{2+}-dependent kinases to enhance the conductance of receptor channels, to induce LTP expression or early LTP (E-LTP). The influx of Ca^{2+} also triggers an unknown signaling cascade toward the nucleus. As a result, a transcriptional factor binds the internal promoter (P2) of the PKCζ gene to initiate the PKMζ mRNA transcription. The PKMζ mRNA is actively transported to dendrites and produces PKMζ, which is activated by PDK1 during LTP maintenance or late LTP (L-LTP). Consistent phosphorylation by PKMζ even without second messengers increases and maintains the amount of active AMPARs at the synapse to sustain LTP.

Figure 1-3.

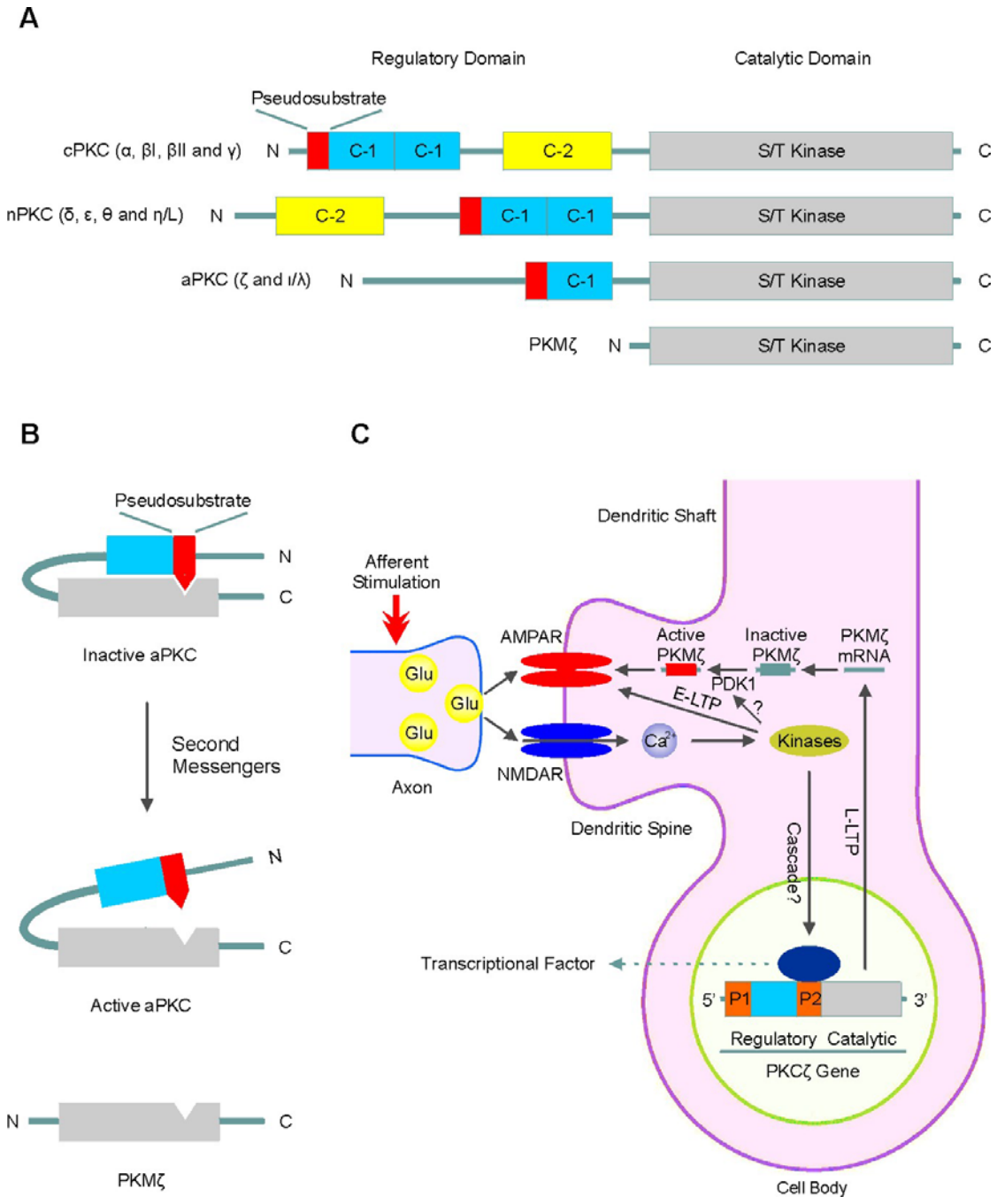

A
Regulatory Domain
Catalytic Domain
Pseudosubstrate
cPKC (α, βI, βII and γ)
N
C-1
C-1
C-2
S/T Kinase
C
nPKC (δ, ε, θ and η/L)
N
C-2
C-1
C-1
S/T Kinase
C
aPKC (ζ and ι/λ)
N
C-1
S/T Kinase
C
PKMζ
N
S/T Kinase
C
B
Pseudosubstrate
N
C
Inactive aPKC
Second Messengers
N
C
Active aPKC
N
C
PKMζ
C
Afferent Stimulation
Glu
Glu
Glu
Axon
AMPAR
NMDAR
Dendritic Shaft
Active PKMζ
Inactive PKMζ
PKMζ mRNA
PDK1
E-LTP
?
Ca2+
Kinases
Dendritic Spine
Cascade?
L-LTP
Transcriptional Factor
5'
P1
P2
3'
Regulatory
Catalytic
PKCζ Gene
Cell Body

Figure 1-4. Interactions between AMPARs and Associated Proteins

4.1G/N and SAP97 interact with the GluR1 subunit; whereas AP2, NSF, GRIP/ABP and PICK1 interact with the GluR2 subunit. Both extracellular and intracellular regions of TARPs interact with AMPARs, but the intracellular binding site is unknown. Other AMPAR-associated proteins include Lyn, LIN-10 and Gα but the binding sites are unclear.

Figure 1-4.

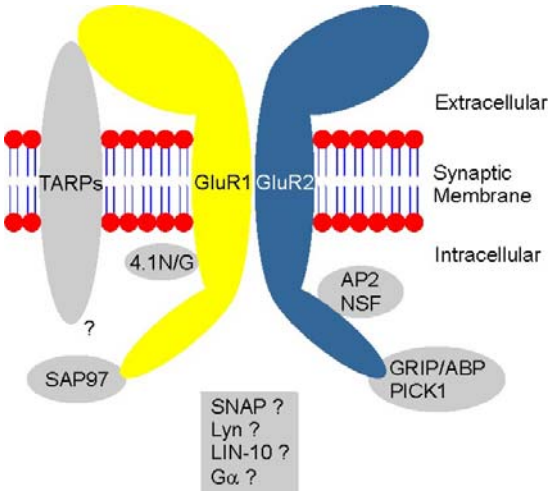
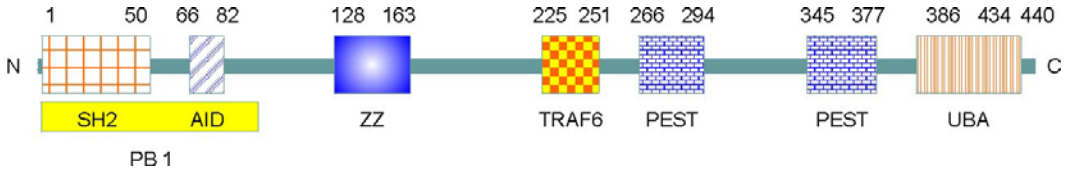

Extracellular
Synaptic Membrane
Intracellular
TARPs
GluR1
GluR2
4.1N/G
AP2
NSF
?
SAP97
GRIP/ABP
PICK1
SNAP ?
Lyn ?
LIN-10 ?
Gα ?

Figure 1-5. p62 Primary Structure

p62 possesses 6 domains: a PB1 domain consisting of a SH2 binding site and an aPKC interaction domain (AID), a ZZ-type finger domain binds aPKC substrates, a tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) binding domain, two PEST domains for proteolytic recognition, and a ubiquitin-associated (UBA) domain.

Figure 1-5.


1
50
66
82
128
163
225
251
266
294
345
377
386
434
440
N
C
SH2
AID
PB 1
ZZ
TRAF6
PEST
PEST
UBA

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CHAPTER II - P62 REGULATES AMPA RECEPTOR TRAFFICKING AND SYNAPTIC PLASTICITY

SUMMARY

Trafficking of AMPA receptors to and away from the postsynaptic membrane is fundamental to many forms of plasticity, including long-term potentiation (LTP). In this chapter, we identified the atypical protein kinase C (aPKC) scaffold, p62, as the first non-C-terminal AMPA receptor interactor. AMPA receptor intracellular loop L2-3 and the ZZ-type Zinc finger domain of p62 are essential for interactions between the two proteins and for surface delivery of the receptor. Furthermore, LTP was impaired in p62 knock-out mice with normal basal synaptic transmission. Surface delivery of the AMPA receptor subunit GluR1 induced by cLTP (chemical LTP) was impaired and paralleled an absence of phosphorylation at S818, S831, and S845 in brain slices from p62 knock-out mice. These findings reveal that p62 regulates AMPA receptor trafficking and expression of LTP. Lastly, we identify a possible conserved sequence (ISExSL) shared by all p62 interacting-aPKC substrates.

INTRODUCTION

AMPA-type glutamate receptors (AMPARs) mediate major fast excitatory synaptic transmission in the mammalian brain. Structurally, AMPARs are heterotetrameric cation

channels composed of 4 subunits: GluR1-4, each of which comprises about 900 amino acids with a molecular weight of about 105 kDa, and shares 68-74% amino acid sequence identity. Each AMPAR subunit consists of an extracellular N-terminus, three transmembrane spanning domains (TM1, TM3 and TM4), a re-entrant transmembrane domain (TM2) and an intracellular C-terminus. Therefore, the transmembrane domains form two intracellular loops (L1-2 and L2-3) and one extracellular loop (L3-4). The re-entrant TM2 domain contributes to the cation pore channel. The C-terminal cytoplasmic tail of each AMPAR subunit is unique and could be associated with specific regulatory proteins, which are related to receptor modification, trafficking and signaling (Dingledine et al., 1999; Hollmann and Heinemann, 1994).

It is widely believed that long-term potentiation (LTP) and long-term depression (LTD) form the cellular and molecular basis for neuronal plasticity, including learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Malinow and Malenka, 2002). Trafficking of AMPARs to and away from synapses is a mechanism to modulate synaptic strength (Lynch and Baudry, 1984; Malenka and Nicoll, 1999). During LTP expression, additional AMPARs are delivered to the postsynaptic membrane (Shi et al., 1999). In contrast, LTD induces receptor internalization (Sheng and Lee, 2001). Therefore, alterations in synaptic strength are directly related to the receptor exocytosis and endocytosis (Malinow and Malenka, 2002). AMPAR trafficking to the surface is primarily regulated by two mechanisms, receptor associated proteins that aid in delivery of the receptor to the surface membrane (Hayashi et al., 2000; Henley, 2003; Fukata, 2005; Palmer et al., 2005), and phosphorylation (Lee et al., 2000, 2003; Oh et al., 2006;

Roche et al., 1996; Song and Huganir, 2002). For example, two phosphorylation sites in the C-terminus of GluR1 subunit exist, S845 is a protein kinase A (PKA) site, S831 is a site phosphorylated by calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC). However, the detailed cellular and molecular mechanism for AMPAR trafficking is not yet fully understood.

Several members of the PKC family directly phosphorylate AMPAR subunits to mediate plasticity (Boehm et al., 2006; Carvalho et al., 1999; Chung et al., 2000; Correia et al., 2003; Lee et al., 2007; McDonald et al., 2001). The atypical PKC (aPKC) PKCι/λ is the major PKC isoform in the brain (Oster et al., 2004). In addition, the aPKC scaffold, p62/Sequestosome 1 (SQSTM1), is primarily expressed in hippocampus (Gong et al., 1999), the primary brain region responsible for learning and memory. The possibility that the aPKC adaptor functions as a scaffold for aPKC-mediated phosphorylation of the AMPAR has not been investigated. p62 is a cytoplasmic and membrane-associated protein that possesses 6 domains: a PB1 domain consisting of SH2 binding motif and aPKC interacting domain (AID), a ZZ-type Zinc finger domain for binding aPKC substrates, a tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) binding domain, two PEST domains for proteolytic recognition, and a ubiquitin-associated (UBA) domain (Moscat et al., 2007). These domains endow p62 with the ability to associate with many other proteins and enables p62 serve as a scaffold to recruit substrates of aPKC through its PB1 domain and ZZ-type finger domain (Cariou et al., 2002; Croci et al., 2003; Gong et al., 1999; Kim, 2006; Puls et al., 1997; Sanz et al., 1999). In addition, p62

also functions as a shuttling protein for endocytosis of polyubiquitinated proteins by interaction with its UBA domain (Geetha et al., 2005).

Here we demonstrate that p62 is an AMPAR interacting protein. The interaction between p62 and AMPAR is mediated through the AMPAR subunit intracellular loop L2-3 and the ZZ-type Zinc finger domain of p62. Furthermore, long-term potentiation (LTP) was significantly reduced in p62 knock-out mice with normal basal synaptic transmission and short-term plasticity. The delivery of GluR1 to the surface and phosphorylation triggered by chemical LTP (cLTP) decreased in hippocampal slices obtain from p62 knock-out mice. Altogether, our results suggest that p62 plays an important role in synaptic plasticity via regulating AMPAR trafficking.

RESULTS

p62 Interacts with AMPAR Subunits

All PKC isoforms, including aPKC, phosphorylate the AMPAR subunit GluR1 both *in vitro* and *in vivo* (Boehm et al., 2006; Lee et al., 2007; Roche et al., 1996). Therefore, we hypothesized that an interaction between the AMPAR and the aPKC adaptor, p62, might occur. To examine this possibility, brains from 6 month-old mice were homogenized in ice-cold Triton lysis buffer, followed by addition of SDS at various concentrations and incubation at 4ºC for 1 hour to release the membrane-bound proteins. Co-immunoprecipitation revealed that the AMPAR subunit GluR1 interacts with p62 and PKCι/λ *in situ* (Figure 2-1A). The concentration 0.05% of SDS was optimal for the release of GluR1 from the cell membrane without affecting the interaction among these

proteins. The interaction between GluR1 and p62 was recapitulated in HEK cells cotransfected with GFP-tagged GluR1 and Myc-tagged p62 cDNA constructs (Figure 2-1B and C). AMPAR subunits share 68-74% amino acid identity, which provides the subunits with similar binding characteristics (Palmer et al., 2005). In particular, the long isoform subunits (GluR1 and GluR4) share interacting proteins; while the short isoform subunits (GluR2 and GluR3) also interact with the same proteins (5). We next investigated whether p62 interacts with other AMPAR subunits expressed in mouse brain. To our surprise, AMPAR subunits GluR2 and GluR3 also interacted with p62 in transfected HEK cells (Figure 2-2). As an alternative means to examine interaction, colocalization of GluR1-3 and p62 were tested in cotransfected HEK cells, followed by immunofluorescent microscopy. Consistently, GluR1, GluR2 and GluR3 colocalized with p62 (Figure 2-3).

Next, the interaction between p62 and AMPAR subunits in brain lysates prepared from wild-type (WT) and p62 knock-out (KO) mice was analyzed via co-immunoprecipitation. Compared to wild-type mice, the interaction between p62 and AMPAR subunits was abolished in the p62 knock-out mice (Figure 2-4). These results validate that p62 interacts with all AMPAR subunits both *in situ* and *in vivo* and suggest a physiologically significant role for this protein.

p62 Interacts with GluR1 Subunits Through its ZZ-type Zinc Finger Domain

p62 possesses six domains, which enable the protein to recruit many different interactors (Moscat et al., 2007). To investigate which region of p62 is responsible for AMPAR

interaction, C-terminal and N-terminal truncated p62 constructs were used to examine protein-protein interaction by co-immunoprecipitation (Figure 2-5A). Interestingly, the C-terminal truncated p62 (ΔUBA) still bound GluR1; whereas the N-terminal truncated p62 (ΔPB1, ΔZZ) lost the capacity to interact with GluR1 (Figure 2-5B). N-terminal truncation does not affect the p62 protein folding and functions exerted via C-terminal domains (Seibenhener et al., 2004). These findings indicate that the N-terminus of p62 is essential for AMPAR interaction. There are three functional regions located on the p62 N-terminus: SH2 binding site, AID motif and ZZ-type Zinc finger domain. To further investigate which domain in the N-terminus of p62 interacts with the AMPAR, a series of p62 deletion constructs were employed to test the capacity for GluR1 binding by transfection in HEK cells and coimmunoprecipitation (Table 2-1). Among these p62 deletion constructs, ΔZZ totally abolished interaction with GluR1. We conclude that the ZZ-type Zinc finger domain mediates interaction with the AMPAR.

To examine if these interactions possessed a physiological role, we next examined whether p62 regulated GluR1 localization by confocal microscopy (Figure 2-6). Wild-type p62 and p62-ΔUBA colocalized with GluR1 in the cell membrane; whereas p62 (Δ N-term) and p62 (ΔZZ) failed to colocalize with GluR1. Interestingly, expression of GluR1 with the ΔZZ construct resulted in accumulation of GluR1. These results indicate that interaction of GluR1 with the ZZ-type Zinc finger domain of p62 may be necessary for AMPAR surface expression.

Intracellular Loop L2-3 of GluR1 is Critical for p62 Interaction

So far, most AMPAR associated proteins that have been discovered interact with the intracellular C-terminus of the receptor (Malinow and Malenka, 2002). Therefore, we hypothesized that p62 might also interact with the AMPAR C-terminus. To test this possibility, a series of C-terminal truncated GluR1 constructs were used to map the interaction between the GluR1 and p62 (Shen et al., 2000). Surprisingly, all C-terminal truncated GluR1 constructs were observed to interact with p62 by co-immunoprecipitation. Even if the C-terminus was totally truncated, GluR1 was still able to interact with p62 (Table 2-2). Therefore, the intracellular C-terminus of GluR1 is not involved in mediating interaction with p62.

The three transmembrane domains of AMPAR subunit form three intracellular regions: C-terminus, loop TM1-TM2 (L1-2) and loop TM2-TM3 (L2-3) (Hollmann and Heinemann, 1994). Since GluR1 C-terminus is not responsible for the interaction with p62, these two intracellular loops could be a p62 interaction site. To test this possibility, two GluR1 deletion constructs were generated: GluR1 (DL1-2) and GluR1 (DL2-3), which lacks the intracellular loop L1-2 and loop L2-3, respectively (Figure 2-7A). Compared to the wild-type GluR1, deletion of L1-2 had no effect on GluR1/p62 interaction; whereas deletion of L2-3 significantly reduced the GluR1/p62 interaction to 22.92% of control (N = 4, $P < 0.005$) (Figure. 2-7B). These results suggested that the intracellular loop L2-3 of GluR1 is critical for GluR1/p62 interaction. Alignment of GluR family members reveals that the intercellular loop L2-3 is completely conserved among all AMPAR subunits family (Figure 2-7C). This finding is consistent with the

observation that p62 interacts with all AMPAR subunits (Figure 2-2 and 2-3). The functional role for this interaction was tested by colocalization by confocal microscopy. Interestingly, in the absence of the L1-2 region, GluR1 still colocalizes with p62 at the cell surface; whereas the deletion of L2-3, the p62 interaction site, from GluR1 impaired colocalization with p62 at the cell membrane (Figure 2-7D). These results further suggest that interaction between p62 and the AMPAR may be necessary for surface delivery of the receptor.

p62 Facilitates Synaptic Plasticity Through Surface Expression of Phosphorylated GluR1

The p62 knock-out mice exhibit defective spatial learning (Wooten, unpublished) which often correlates with impaired LTP (Chan et al., 2006). Basal synaptic transmission in the Shaffer collateral-CA1 synapses of the hippocampus was examined using extracellular recordings in transverse of hippocampal slices. Slices from p62 knock-out mice revealed no significant reduction in the excitatory postsynaptic potentials across a range of stimulus intensities (Figure 2-8A). To further evaluate the properties of these synapses, LTP induced by theta burst stimulation (TBS) was measured at 1, 2, 6 and 12 months-of-age in hippocampal slices (Figure 2-8A). A decline in LTP was noted by 2 months-of-age in the knock-out mice, and completely absent by 6 months-of-age. Since there were no significant changes in stimulus-response curves and paired-pulse facilitation, the failure to induce LTP is not likely due to impaired basal synaptic transmission.

Phosphorylation is one of the mechanisms by which AMPAR trafficking is regulated (Lee et al., 2000, 2003; Oh et al., 2006; Roche et al., 1996; Song and Huganir, 2002). A newly identified PKC phosphorylation site, S818, in GluR1 is necessary for AMPAR surface delivery and maintenance of GluR1 at the postsynaptic membrane triggered by chemical LTP (cLTP) (Boehm et al., 2006). Interestingly, GluR1 S818 is phosphorylated by all PKC isoforms, including aPKC. Given that hippocampal LTP in the p62 knock-out mice was impaired, and interaction with p62 was necessary for cell surface delivery of AMPAR *in situ*, we hypothesized that an absence of p62 may also have an effect on amounts of total GluR1 and phosphorylated GluR1 at the postsynaptic membrane *in vivo*. To investigate this possibility, we performed surface biotinylation and immunoblot analysis of acute hippocampal slices from 6 month-old mice after induction of chemically induced form of LTP (cLTP). A combination of Rolipram, Forskolin and Picrotoxin can activate PKA and NMDA receptors, and then drive GluR1 into synapses (Boehm et al., 2006; Oh et al., 2006). In response to cLTP induction, increased GluR1 and pS818 GluR1 were observed at the surface of slices from wild-type mice. In contrast, cLTP failed to induce surface delivery of GluR1 or phosphorylation of S818 in the p62 knock-out mice (Figure 2-8B). The defect was more obvious when the phosphorylation of GluR1 at S831 and S845 were examined.

CONCLUSION AND DISCUSSION

Several proteins have been reported to regulate synaptic plasticity through interacting with AMPARs either directly or indirectly (reviewed in Jiang et al., 2007). Proteins such as Stargazin, 4.1N, AP2, PI3-Kinase have been reported to regulate receptor trafficking.

In this study, we identified a novel AMPAR interacting protein, p62/SQSTM1. The ZZ-type Zinc finger domain of p62 and the intracellular loop L2-3 of AMPAR subunit are critical for these protein-protein interactions. Interestingly, p62 is the first protein that has been discovered to interact with the AMPAR subunit outside of the C-terminus with an impact upon surface delivery and expression of LTP.

Sequestosome 1/p62 possesses six functional domains, which endow the protein with an ability to interact with many different molecules to exert multiple-functions. To date, most p62-interacting proteins have been observed to interact with its N-terminal ZZ-type Zinc finger domain or the C-terminal UBA domain (Cariou et al., 2002; Croci et al., 2003; Geetha et al., 2005; Gong et al., 1999; Kim, 2006; Puls et al., 1997; Sanz et al., 1999). The UBA domain of p62 interacts with K63-polyubiquitinated membrane-bound proteins to initiate ubiquitin-dependent receptor endocytosis (Geetha et al., 2005), whereas the ZZ-type Zinc finger domain interacts with substrates of aPKC (Cariou et al., 2002; Croci et al., 2003; Gong et al., 1999; Kim, 2006; Puls et al., 1997; Sanz et al., 1999). Therefore, p62, aPKC and the substrate are able to form a ternary complex. So far, several receptors and non-receptor proteins have been discovered to bind to the ZZ-domain of p62. Those proteins include: D_2 dopamine receptor (DAR), $GABA_C$ receptor subunit ρ1-3, growth factor receptor-bound protein 14 (Grb14), receptor interacting protein (RIP), and potassium channel subunit Kvβ2 (Table 2-3) (Cariou et al., 2002; Croci et al., 2003; Gong et al., 1999; Kim, 2006; Puls et al., 1997; Sanz et al., 1999). p62 interacts with the intracellular loop (TM3-TM4) of $GABA_C$ receptor, ID (intermediate domain) of RIP and PIR (phosphorylated insulin receptor) domain of Grb14; while in our

study, the intracellular loop L2-3 of AMPAR subunits was revealed to be critical for p62 interaction. Alignment of all p62-interacting sites in each protein reveals a potential conserved consensus sequence, ISExSL (where x is any amino acid) (Figure 2-9A). We hypothesize this site might serve as a putative protein binding motif to recruit the substrate for phosphorylation by aPKCs (Table 2-3). In fact, interactions of p62 with Kvβ2, $GABA_C$ receptor, RIP and Grb14 regulate phosphorylation mediated by aPKC (Table 2-3).

Receptor phosphorylation by kinases, specifically CaMKII and PKC, play critical roles in AMPAR trafficking (Jiang et al., 2007). To date, four phosphorylation sites have been discovered in the GluR1 C-terminus: S818 and T840 are PKC sites, S831 is both a PKC and CaMII site, and S845 is phosphorylated by both PKA and cGKII (cGMP-dependent protein kinase II) (Boehm et al., 2006; Esteban et al., 2003, Lee et al., 2003; Lee et al., 2007; Roche et al., 1996; Serulle et al., 2007). Phosphorylation stabilizes the AMPAR in the synaptic membrane to mediate plasticity (Lee et al., 2000). As a scaffold p62 may thereby recruit GluR1 for phosphorylation by aPKC (Figure 2-9B). More importantly, our results reveal that p62 plays a critical role in mediating the delivery of GluR1 to the cell surface *in vivo*. In this regard, p62 has been shown to interact with MAP1B a component of the cytoskeleton (Pankiv et al., 2007), and MAP1B knock-out mice are likewise deficient in LTP (Zervas et al., 2005). Thus, p62 may be directly involved in trafficking of the phosphorylated receptor to the cell surface by interaction with the cytoskeleton as well as regulating aPKC phosphorylation of GluR1. Studies are underway to define p62-aPKC-GluR1 interaction with MAP1B.

The p62 knock-out mice exhibit age-dependent loss in synaptic plasticity with normal basal neurotransmission (Figure 2-8A). Potentiation was normal immediately after theta burst stimulation (TBS) in p62 knock-out adult mice, suggesting that LTP induction by TBS which is dependent on NMDA receptors and L-type Ca^{2+} channels (Huber et al., 1995; Morgan and Teyler, 2001) is not impaired. Analysis of the NMDA receptor reveals that the putative p62-interacting consensus sequence is absent and would suggest that NMDA receptor function might be regulated independent of p62. After the initial induction phase, the EPSPs rapidly declined to basal level indicating a possible impairment of AMPAR delivery to the postsynaptic membrane. In fact, cLTP resulted in decreased GluR1 at the cell surface along with impaired phosphorylation at S818, S831 and S845. Thus, cLTP of slices from p62 knock-out mice leads to internalization of existing membrane receptors without recruitment of new GluR's to the surface.

Both GluR1 and GluR4 are long isoforms of the AMPAR subunit. The expression of GluR1 and GluR4 in hippocampus is regulated during development (Jiang et al., 2007; Malinow and Malenka, 2002). GluR4 is critical for the synaptic plasticity during the early postnatal period when GluR1 expression in the hippocampus is low; whereas in the adult hippocampus GluR4 expression level is low and GluR1 is critical for synaptic function (Zhu et al., 2000). GluR1 and GluR4 share the conserved PKA and cGKII phosphorylation site: S845 of GluR1 and S842 of GluR4. In fact, phosphorylation of GluR4 S842 is sufficient to deliver the receptor to the synapse and induce plasticity in early postnatal development (Jiang et al., 2007). However, GluR4 lacks the aPKC S818

phosphorylation site. Therefore, an absence of p62 is more likely to affect the function of GluR1. Our findings are consistent with this expression pattern of GluR1 and GluR4. As a consequence of p62 deficiency in brain, the effect on LTP would be less in the 1 month-old juveniles, compared to complete loss in 6 month-old adult mice. In addition, coincident with the loss of LTP the p62 knock-out mice display impaired working memory (Wooten, unpublished). Collectively the electrophysiology and behavioral phenotype of p62 knock-out reveal a notable similarity with those reported for the GluR1 knock-out mice (Reisel et al., 2002; Zamanillo et al., 1999). Additionally, the p62 knock-out mice display mature-onset obesity (Rodriguez et al., 2006), which could amplify the deficits in AMPAR surface delivery and synaptic plasticity. Altogether, our study reveals a novel and critical role for p62 in AMPAR trafficking and synaptic plasticity.

EXPERIMENTAL PROCEDURES

Generation of p62 $^{-/-}$ Mice

Knock out mice (p62 $^{-/-}$) were generated as described previously (Duran et al, 2004). For the duration of the study all mice were housed in a pathogen-free barrier environment. The animals were handled according to the NIH and Auburn University IACUC guidelines.

Antibodies

The mouse GluR1 and GFP monoclonal, rabbit HA and Myc polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GluR1 polyclonal, GluR2 and GluR3 monoclonal antibodies, GluR1 phosphorylation antibodies: pS831 and pS845

were from Chemicon (Temecula, CA). GluR1 phosphorylation antibody pS818 was a kind gift from R.L. Huganir from Johns Hopkins University (Baltimore, MD). p62 monoclonal antibody and PKCι/λ monoclonal antibody were purchased from BD Biosciences (San Jose, CA). Rabbit p62 polyclonal antibodies were raised against the full-length p62 (Geetha et al., 2005).

Generation of Deletion Constructs

The cDNA deletions were performed with a QuickChange® II XL Site-Directed Mutagenesis Kit according to the manufacturer's standard protocol (Stratagene, La Jolla, CA). The primers used for loop L1-2 deletion: Forward 5'-GGAGTGAGCGTCGTCCTCTTCCTGGTCAGCTTTGGCATATTCAACAGCCTGTGGTTCTCC-3'; Backward 5'-GGAGAACCACAGGCTGTTGAATATGCCAAAGCTGACCAGGAAGAGGACGACGCTCACTCC-3'. The primers used for loop L2-3 deletion: Forward 5'-CCCTGGGGGCCTTCATGCAGCAAGGATGTATCGTCGGCGGCGTCTGGTGGTTCTTCAC-3'; Backward 5'-GTGAAGAACCACCAGACGCCGCCGACGATACATCCTTGCTGCATGAAGGCCCCCAGGG-3'. The deletions were confirmed by DNA sequencing and the absence of any nonspecific mutation was confirmed for the GluR1 cDNA.

Preparation of Mouse Brain Lysate

Adult mouse brains were homogenized in ice-cold Triton lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin and 2 μg/ml aprotinin) plus

SDS at different concentration range. The homogenate was sonicated on ice for 10 s three times, followed by rotating the lysates for 30 min and centrifuged for 10 min at 4ºC. The protein concentration was estimated by Bio-Rad DC assay (Hercules, CA), with 1 mg/ml BSA (Sigma-Aldrich, St. Louis, MO) as a standard.

HEK Cell Culture and Transfection

Human embryonic kidney (HEK) 293 cells were maintained as previously described (31). The HEK cells were transfected with the calcium phosphate method by using a Mammalian Cell Transfection Kit (Millipore, Billerica, MA) or with cationic lipid method by using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA). The cells were harvested and lysed with Triton lysis buffer (31). Protein concentration was estimated by Bradford assay.

Co-immunoprecipitation and Western Blotting

HEK Cell lysates (750 μg) or brain lysates (1 mg) were diluted in l ml Triton lysis buffer and incubated with 4 μg of primary antibodies at 4ºC for 3 hr. The immunoprecipitates were collected with agarose-coupled secondary antibodies (Sigma) for at least 2 hr, and then were washed three times with lysis buffer. Proteins bound to the antibodies were released using sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (2% SDS, 1% β-mercaptoethanol, 0.005% Bromophenol blue, 2% glycerol, and 0.05 M Tris-HCl, pH6.8). The brain lysates, cell lysates, brain-slice lysate, or immunoprecipitated proteins were resolved by (10% w/v) SDS-PAGE under reducing conditions (1% β-mercaptoethanol) and transferred onto nitrocellulose membranes

(Amersham Biosciences). The blots were incubated with appropriate antibodies at 4ºC overnight. After incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), the blots were analyzed using by enhanced chemiluminescence (ECL) and X-film (Amersham Biosciences). Captured images were scanned and quantified using the UN-SCAN-IT gel software (Silk Scientific, Orem, UT). Data in figures are representative of at least three independent experiments.

Immunocytochemistry

For localization of AMPAR subunits and p62, HEK cells were cotransfected with Myc- or HA-tagged p62 and GluR1, GluR2, or GluR3 with or without GFP tag as indicated in the figures. After 48 hr, the cells were fixed, permeabilized and were incubated with rabbit anti-Myc or HA IgG and mouse anti-GluR1, GluR2 or GluR3 IgG for the AMPAR subunit construct without GFP tag. Labeled the cells with Texas Red-conjugated anti-rabbit antibodies (red) and Oregon Green-conjugated anti-mouse antibodies (green). The colocalization of GluR1 and p62 was determined by confocal immunofluorescence microscopy and analyzed on a Bio-Rad MRC 1024 Laser Scanning Confocal Microscope.

Hippocampal Slice Preparation

The mice were anaesthetized with carbon dioxide, decapitated and brains were rapidly isolated in ice cold dissection buffer (250 mM sucrose, 25 mM $NaHCO_3$, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, and 1.5 mM $MgCl_2$, pH 7.3). Hippocampal slices (transverse, 300 μm thickness) were cut in ice cold dissection buffer continuously bubbled with a mix of 95% O_2 and 5% CO_2 using a Tissue Sectioning

System (Vibratome, St. Louis, MO). The slices were then transferred to an incubating chamber filled with artificial cerebrospinal fluid (ACSF) (124 mM NaCl, 25 mM $NaHCO_3$, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, and 1.5 mM $MgCl_2$, pH 7.3) and equilibrated with a mix of 95% O_2 and 5% CO_2 and incubated for at least 1.5 hr prior to use.

Electrophysiology

Field excitatory postsynaptic potentials (fEPSPs) from the Shaffer collateral-CA1 synapses in hippocampal slices were recorded (Bukalo and Dityatev, 2006). Current intensity of test stimuli was set to produce 50% of subthreshold maximum and the test stimuli was applied every 15 sec. Stimulus response and paired pulse ratio experiments were performed by varying the stimulus intensity and interpulse interval. LTP was induced by 5 trains of theta burst stimulation (TBS) (Each train consisted of 10 bursts at 5 Hz; each burst consisted of four pulses delivered at 100 Hz with 15 sec interval). Values of LTP were calculated as the increase in the mean slopes of fEPSPs measured 50-60 min after TBS.

Induction of cLTP

Chemical LTP (cLTP) was induced as described (17) with minor modifications. In brief, the hippocampal slices were subjected to cLTP solution consisting of the ACSF lacking $MgCl_2$ and containing 100 nM Rolipram (Calbiochem, San Diego, CA), 100 μM Forskolin (Sigma-Aldrich), and 100 μM Picrotoxin (Sigma-Aldrich) gassed with a mix of 95% O_2 and 5% CO_2 for 16 min at 30ºC.

Surface Protein Biotinylation

After induction of cLTP, hippocampal slices were transferred to ice-cold ACSF for 2 min, followed by biotinylation in 1 mg/ml of EZ-Link Sulfo-NHS-SS-Biotin (Pierce) for 45 min with slow agitation at room temperature. The slices were rinsed in cold Tris-based ACSF (HEPES replaced by Tris) for three times to quench free biotin. The slices then lysed in cold homogenized buffer (50 mM NaCl, 10 mM EDTA, 10 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, 25 mM NaPPi, 1 mM –glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 μM microcystine, 2 μg/ml aproptinin, 2 μg/ml leupeptin, 1% Triton X-100, and 50 mM HEPES, pH 7.5). The homogenates were sonicated the on ice for 5 s three times and rotated at 4ºC for 1 hr. The lysates were centrifuged for 10 min to pellet the insoluble fraction. The protein concentration was estimated with Bradford assay. For each sample, 400 μg protein in 500 μl homogenization buffer was immunoprecipitated with 50 μl of 50% avidin-agarose beads (Pierce, Rockford, IL) for 3 hr at 4ºC. The beads were pelleted, rinsed three times with homogenization buffer, and heated in 60 μl 1×SDS sample buffer (surface fraction). The samples were resolved by SDS-PAGE and visualized by Western blot.

Statistics

Data were expressed as the mean ± S.E.M. Statistical analyses were performed using Excel (Microsoft, Redmond, WA). P values < 0.05 were judged statistically significant.

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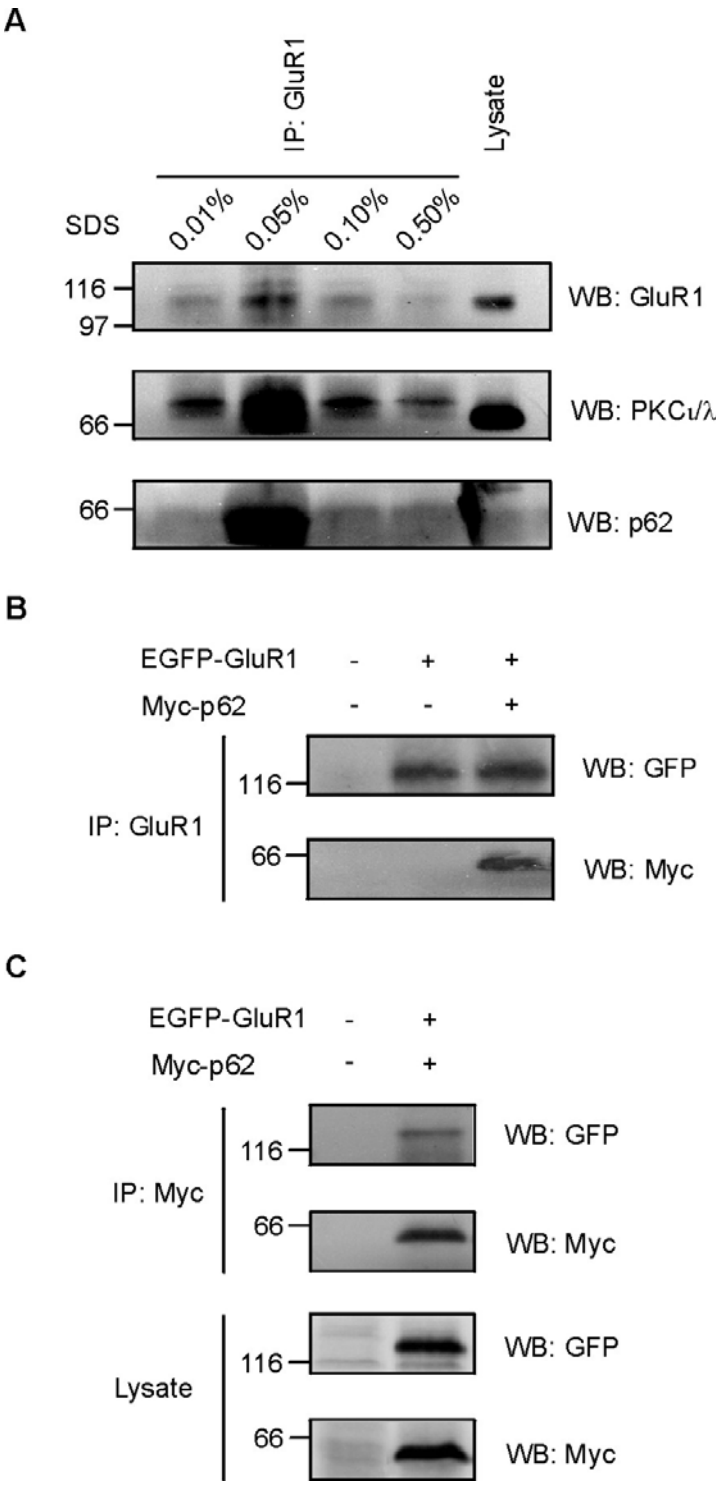
Figure 2-1. GluR1 interacts with p62 both *in situ* and *in vivo*

(A) Mouse brain was homogenized in Triton lysis buffer, SDS was added into the lysate to various concentrations as shown, followed by incubation in 4°C for 30 min to release the membrane-bound proteins. The lysates were immunoprecipitated with anti-GluR1, and then Western blotted with anti-GluR1, anti-PKCι and anti-p62. The lysate (50 μg) was also Western blotted. GluR1 interacts with PKCι and p62 to form a ternary complex *in situ*.

(B) HEK cells were cotransfected with GFP-tagged GluR1 and Myc-tagged p62. After 48 hr, the cells were harvested and lysed with Triton lysis buffer. The cell lysates (750 μg) were immunoprecipitated with anti-GluR1, and then Western blotted with anti-GFP (Upper panel) and anti-Myc (Lower panel).

(C) The lysates (750 μg) from transfected HEK cells were immunoprecipitated with anti-Myc, and then Western blotted with anti-GFP and anti-Myc (Upper 2 panels). The cell lysates (50 μg) were also Western blotted with anti-GFP and anti-Myc to verify expression of recombinant proteins (Lower 2 panels). These blots are representative of three different experiments with similar results.

Figure 2-1.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97
WB: GluR1
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IP: Myc
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WB: GFP
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WB: Myc
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WB: GFP
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WB: Myc

Figure 2-2. p62 Interacts with GluR1-3 Subunits *in vivo*

(A-C) HEK cells were cotransfected with GluR1, GluR2 or GluR3 and Myc-tagged p62. After 48 hr, the cells were harvested and lysed with Triton lysis buffer. The cell lysates (750 μg) were immunoprecipitated with anti-GluR1, GluR2 or GluR3, and then Western blotted with anti-GluR1, GluR2 or GluR3 and anti-p62 (Upper panels). The lysates (50 μg) were also Western blotted with GluR1, GluR2 or GluR3 and p62 antibodies to verify expression of recombinant proteins (Lower panels).

Figure 2-2.

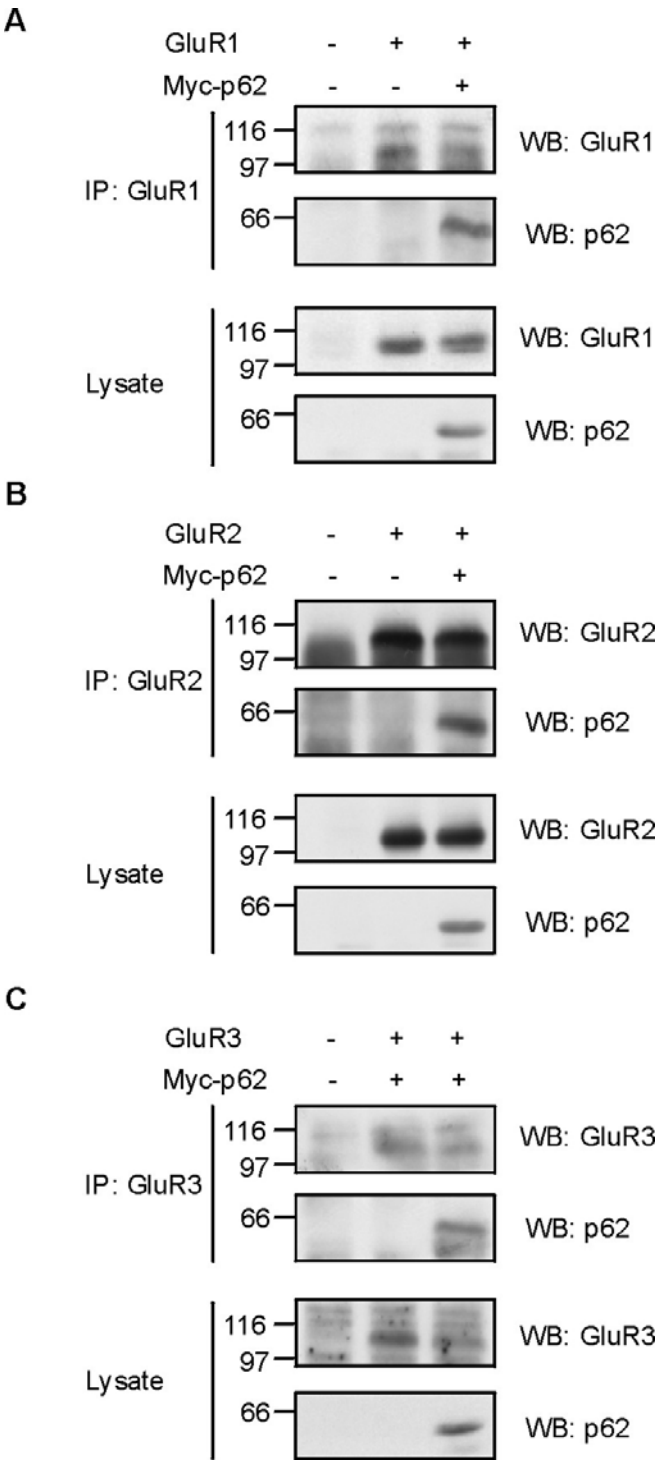
A
GluR1 - + +
Myc-p62 - - +
116
97
66
IP: GluR1
WB: GluR1
WB: p62
Lysate
116
97
66
WB: GluR1
WB: p62
B
GluR2 - + +
Myc-p62 - - +
116
97
66
IP: GluR2
WB: GluR2
WB: p62
Lysate
116
97
66
WB: GluR2
WB: p62
C
GluR3 - + +
Myc-p62 - + +
116
97
66
IP: GluR3
WB: GluR3
WB: p62
Lysate
116
97
66
WB: GluR3
WB: p62

Figure 2-3. p62 Colocalizes with GluR1-3 Subunits

HEK cells were cotransfected with GluR1 (Upper panel), GluR2 (Middle panel) or GluR3 (Lower panel) and HA-tagged p62 cDNA constructs. After 48 hr, the cells were fixed, and incubated with rabbit anti-HA IgG followed by labeling with Texas Red conjugated anti-rabbit antibodies (red) and mouse anti-GluR1, GluR2 or GluR3 and labeled with Oregon Green conjugated anti-mouse antibodies (Green). The colocalization of GluR2 or GluR3 and p62 was determined by confocal microscopy. GluR1, GluR2 and GluR3 colocalize with p62 in the cell membrane (yellow).

Figure 2-3.

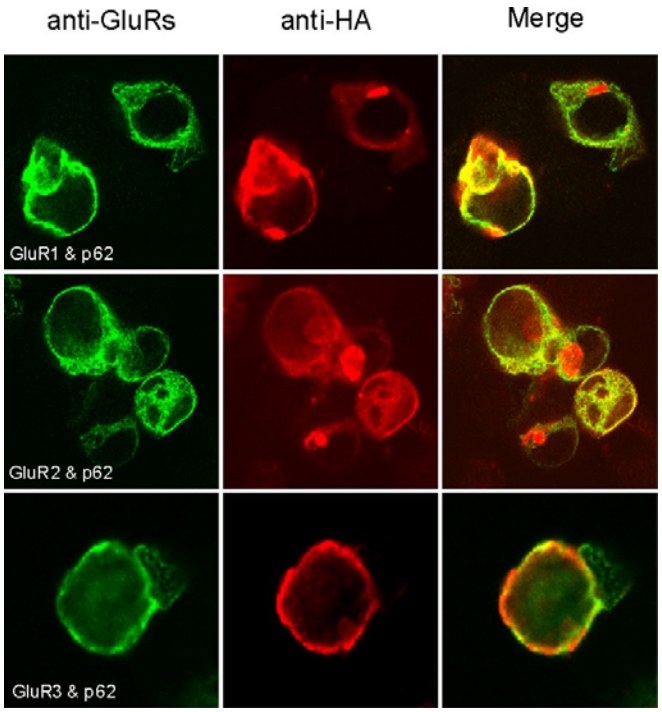

Figure 2-4. AMPAR Subunits GluR1, GluR2 and GluR3 Interact with p62 *in situ*

Brain lysates (1 mg) of wild-type and p62 knock-out were immunoprecipitated with anti-GluR1, anti-GluR2 and anti-GluR3, respectively, and then Western blotted with relevant AMPAR subunit antibodies, and anti-p62 as well. The lysates (40 μg) were also Western blotted with both AMPA receptor subunit and p62 antibodies. The findings are representative of three different experiments with similar results.

Figure 2-4.

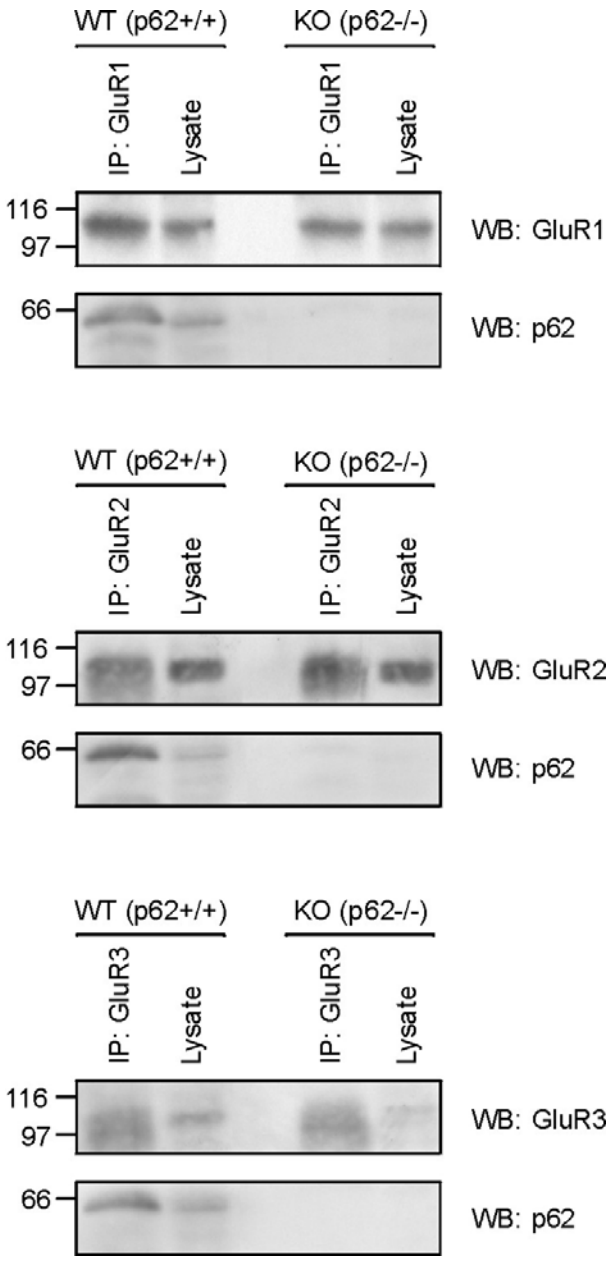
WT (p62+/+)
KO (p62-/-)
IP: GluR1
Lysate
IP: GluR1
Lysate
116
97
66
WB: GluR1
WB: p62
WT (p62+/+)
KO (p62-/-)
IP: GluR2
Lysate
IP: GluR2
Lysate
116
97
66
WB: GluR2
WB: p62
WT (p62+/+)
KO (p62-/-)
IP: GluR3
Lysate
IP: GluR3
Lysate
116
97
66
WB: GluR3
WB: p62

Figure 2-5. N-terminus of p62 is Responsible for AMPAR Subunit Binding

(A) Schematic diagram of Myc-tagged p62 constructs used for mapping of interaction between GluR1 and p62: p62 (WT), p62 (ΔUBA) and p62 (ΔN-term). (B) HEK cells were cotransfected with GFP-GluR1 and Myc-tagged p62 (WT), p62 (ΔUBA) or p62 (ΔN-term). After 48 hr, the cells were harvested and lysed with Triton lysis buffer. The cell lysates (750 μg) were immunoprecipitated with anti-GluR1, and then Western blotted with anti-GFP and anti-Myc (Upper 2 panels). The lysates (50 μg) were also Western blotted with anti-GFP and anti-Myc (Lower 2 panels) to verify expression of the constructs.

Figure 2-5.

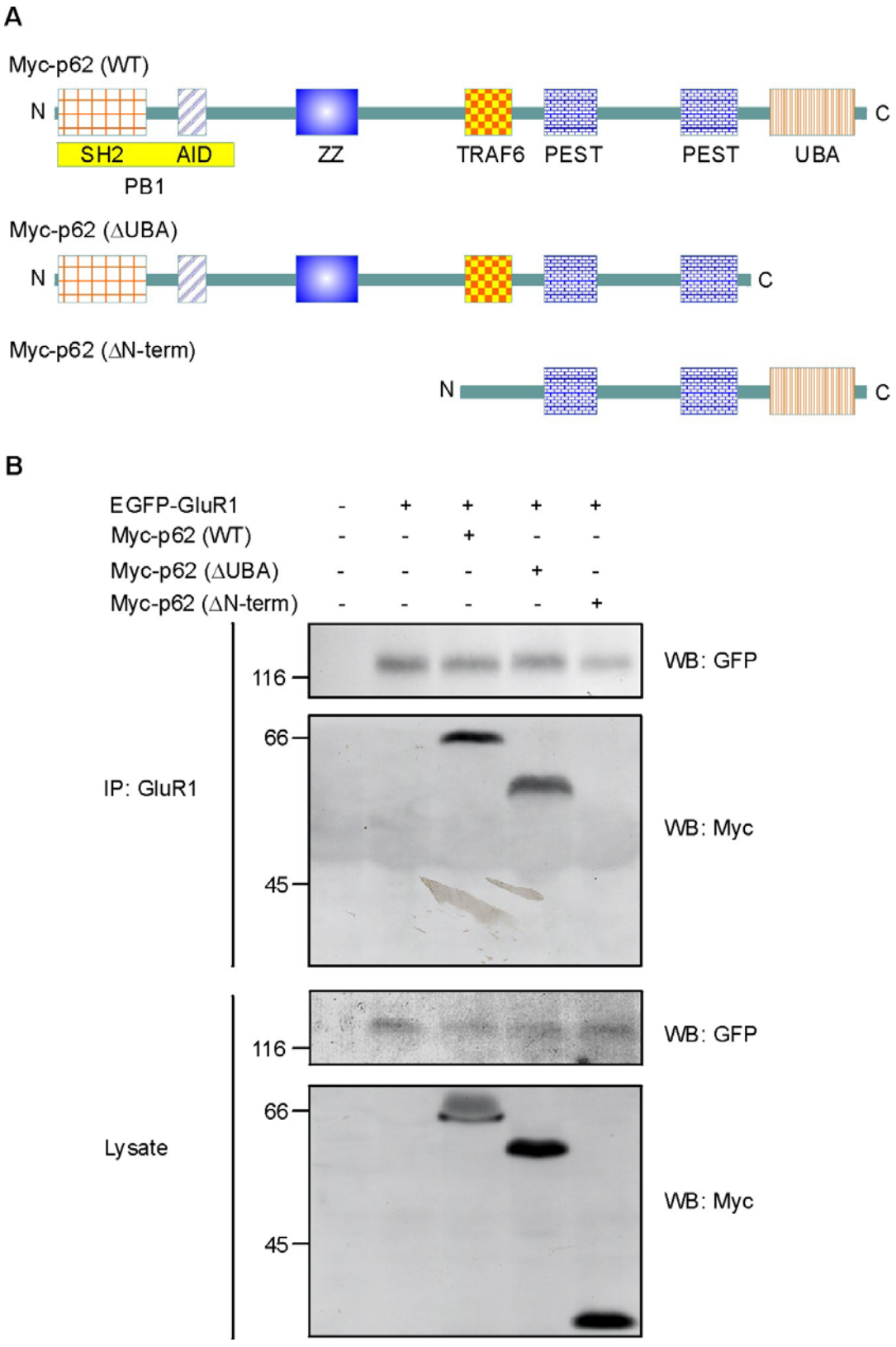
A
Myc-p62 (WT)
N
C
SH2
AID
PB1
ZZ
TRAF6
PEST
PEST
UBA
Myc-p62 (ΔUBA)
N
C
Myc-p62 (ΔN-term)
N
C
B
EGFP-GluR1 - + + + +
Myc-p62 (WT) - - + - -
Myc-p62 (ΔUBA) - - - + -
Myc-p62 (ΔN-term) - - - - +
IP: GluR1
116
66
45
WB: GFP
WB: Myc
Lysate
116
66
45
WB: GFP
WB: Myc

Figure 2-6. ZZ-Type Zinc Finger Domain of p62 is Necessary for GluR1 Recruitment to the Cell Surface

HEK cells were cotransfected with GFP-tagged GluR1 and Myc-tagged p62 (WT), p62 (ΔUBA), p62 (ΔN-term) or p62 (ΔZZ). After 48 hr, the cells were fixed, followed by addition of rabbit anti-Myc IgG and labeled with Texas Red-conjugated anti-rabbit antibodies (red). The colocalization of GluR1 and p62 (yellow) was examined by confocal microscopy.

Figure 2-6.

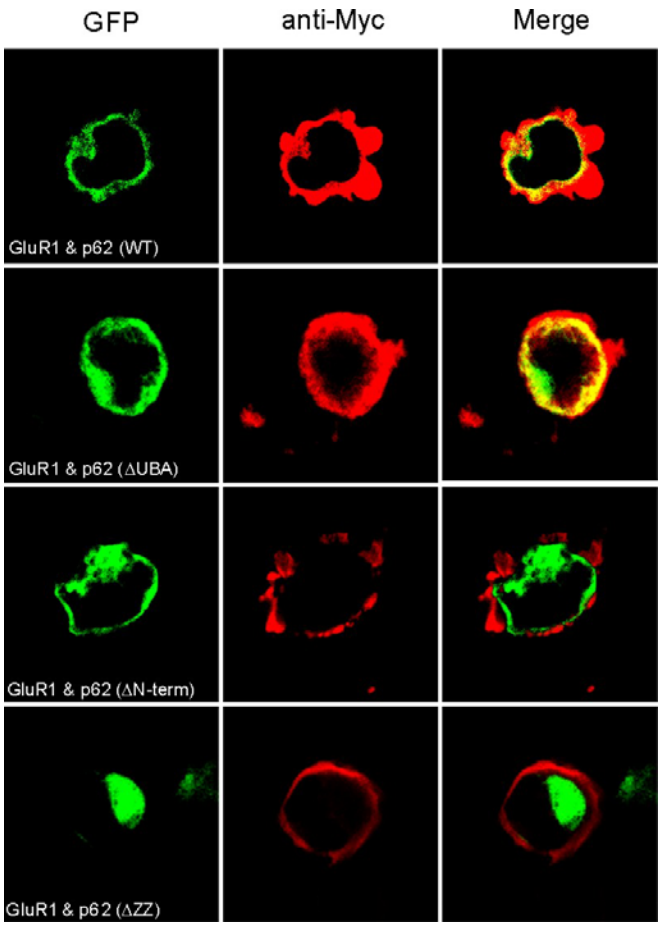
GFP
anti-Myc
Merge
GluR1 & p62 (WT)
GluR1 & p62 (ΔUBA)
GluR1 & p62 (ΔN-term)
GluR1 & p62 (ΔZZ)

Figure 2-7. AMPAR Subunits Bind p62 though the Intracellular Loop L2-3

(A) Schematic diagram of GFP-tagged GluR1 (WT), GluR1 (DL1-2) and GluR1 (DL2-3) mutant constructs.

(B) HEK cells were cotransfected with GFP-tagged GluR1 (WT), GluR1 (DL1-2) or GluR1 (DL2-3) and HA-tagged p62 cDNA constructs. After 48 hr, the cells were harvested and lysed with Triton Lysis buffer. The cell lysates (750 μg) were immunoprecipitated with anti-GluR1, then Western blotted with anti-GluR1 and anti-HA (Left upper 2 panels). The lysates (50 μg) also were Western blotted with anti-GluR1 and anti-HA to verify expression of recombinant constructs. (Right) The relative interaction of p62 with GluR1 mutants was examined by coimmunoprecipitation and the resulting blots scanned. The data represent the mean ± S.E.M. (*, N = 4, $P < 0.005$).

(C) Intracellular loop sequence alignment of GluR1-4.

(D) HEK cells were cotransfected with either GFP-tagged GluR1 (DL1-2) or GluR1 (DL2-3) and HA-tagged p62. After 48 hr, the cells were fixed, followed by incubation with rabbit anti-HA IgG and labeled with Texas Red conjugated anti-rabbit antibodies (red). The colocalization (yellow) was examined by confocal microscopy. GluR1 (DL1-2) colocalized with p62 in the cell membrane (yellow); whereas the deletion of intracellular loop L2-3 significantly reduced the colocalization of GluR1 and p62 at the cell membrane.

Figure 2-7.

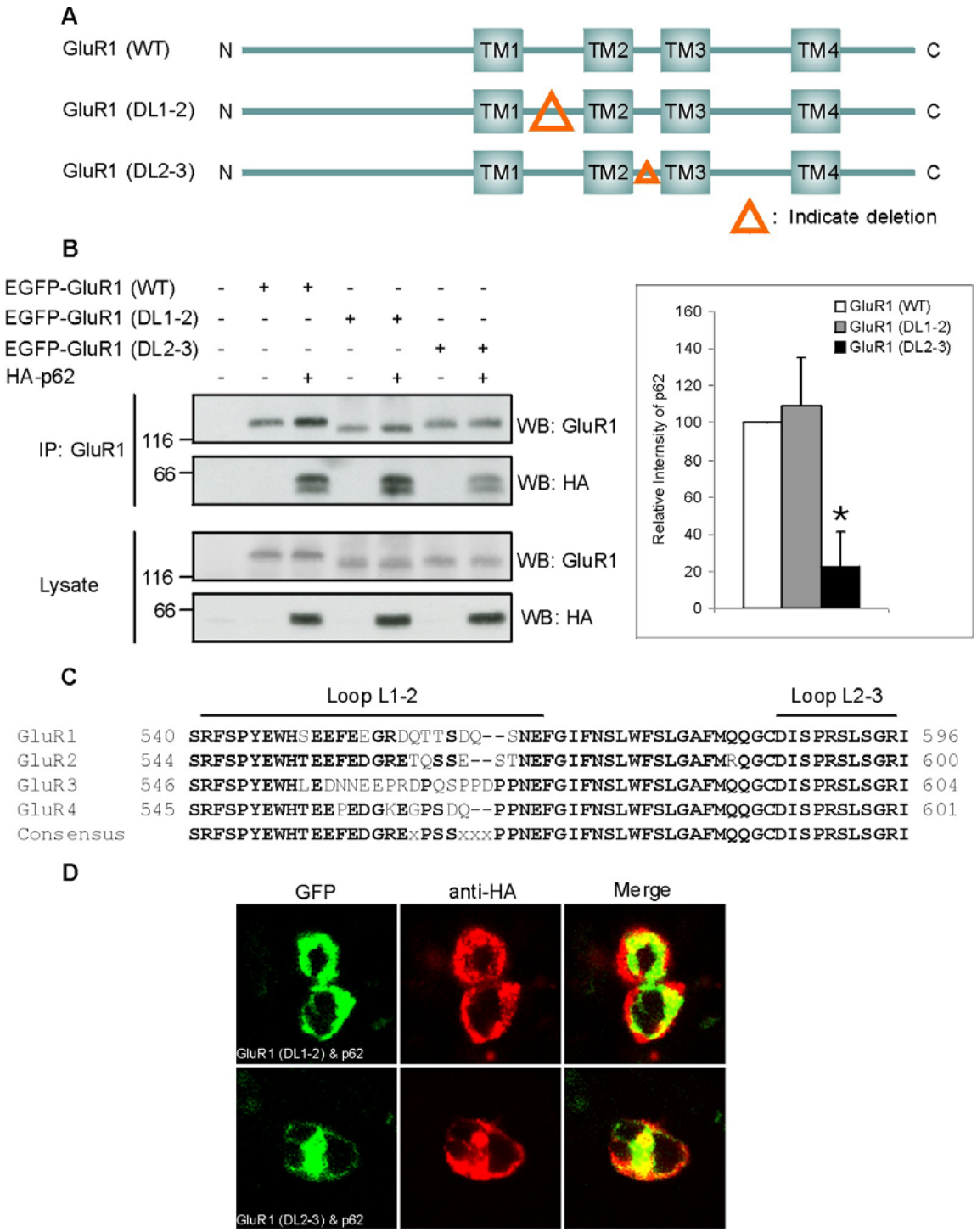
A
GluR1 (WT)
GluR1 (DL1-2)
GluR1 (DL2-3)
N
C
TM1
TM2
TM3
TM4
: Indicate deletion
B
EGFP-GluR1 (WT)
EGFP-GluR1 (DL1-2)
EGFP-GluR1 (DL2-3)
HA-p62
IP: GluR1
Lysate
116
66
WB: GluR1
WB: HA
GluR1 (WT)
GluR1 (DL1-2)
GluR1 (DL2-3)
Relative Internsity of p62
C
Loop L1-2
Loop L2-3
GluR1 540 SRFSPYEWHSEEFEEGRDQTTSDQ--SNEFGIFNSLWFSLGAFMQQGCDISPRSLSGRI 596
GluR2 544 SRFSPYEWHTEEFEDGRETQSSE--STNEFGIFNSLWFSLGAFMRQGCDISPRSLSGRI 600
GluR3 546 SRFSPYEWHLEDNNEEPRDPQSPPDPPNEFGIFNSLWFSLGAFMQQGCDISPRSLSGRI 604
GluR4 545 SRFSPYEWHTEEPEDGKEGPSDQ--PPNEFGIFNSLWFSLGAFMQQGCDISPRSLSGRI 601
Consensus SRFSPYEWHTEEFEDGREXPSSXXXPPNEFGIFNSLWFSLGAFMQQGCDISPRSLSGRI
D
GFP
anti-HA
Merge
GluR1 (DL1-2) & p62
GluR1 (DL2-3) & p62

Figure 2-8. Synaptic Plasticity and Delivery of GluR1 to the Surface is Impaired in p62 Knock-out Mice

(A) Synaptic transmission and plasticity in hippocampal CA1-CA3 synapses of wild-type (WT) and p62 knock-out (-/-) mice at 1, 2, 6, and 12 months, as shown. Data are represented as Mean +/- S.E.M. Each experimental group contains recordings from 7 animals, 2 slices per animal. Statistical significance is accepted at $P < 0.05$. The arrow indicates when theta burst stimulation (TBS) was applied.

(B) Chemical LTP (cLTP) was induced in acute hippocampal slices from wild-type and p62 knock-out mice. The surface receptor was examined by biotinylation and Western blotting for GluR1, p818, p831, and p845. The results are representative of two other experiments with similar results.

Figure 2-8.

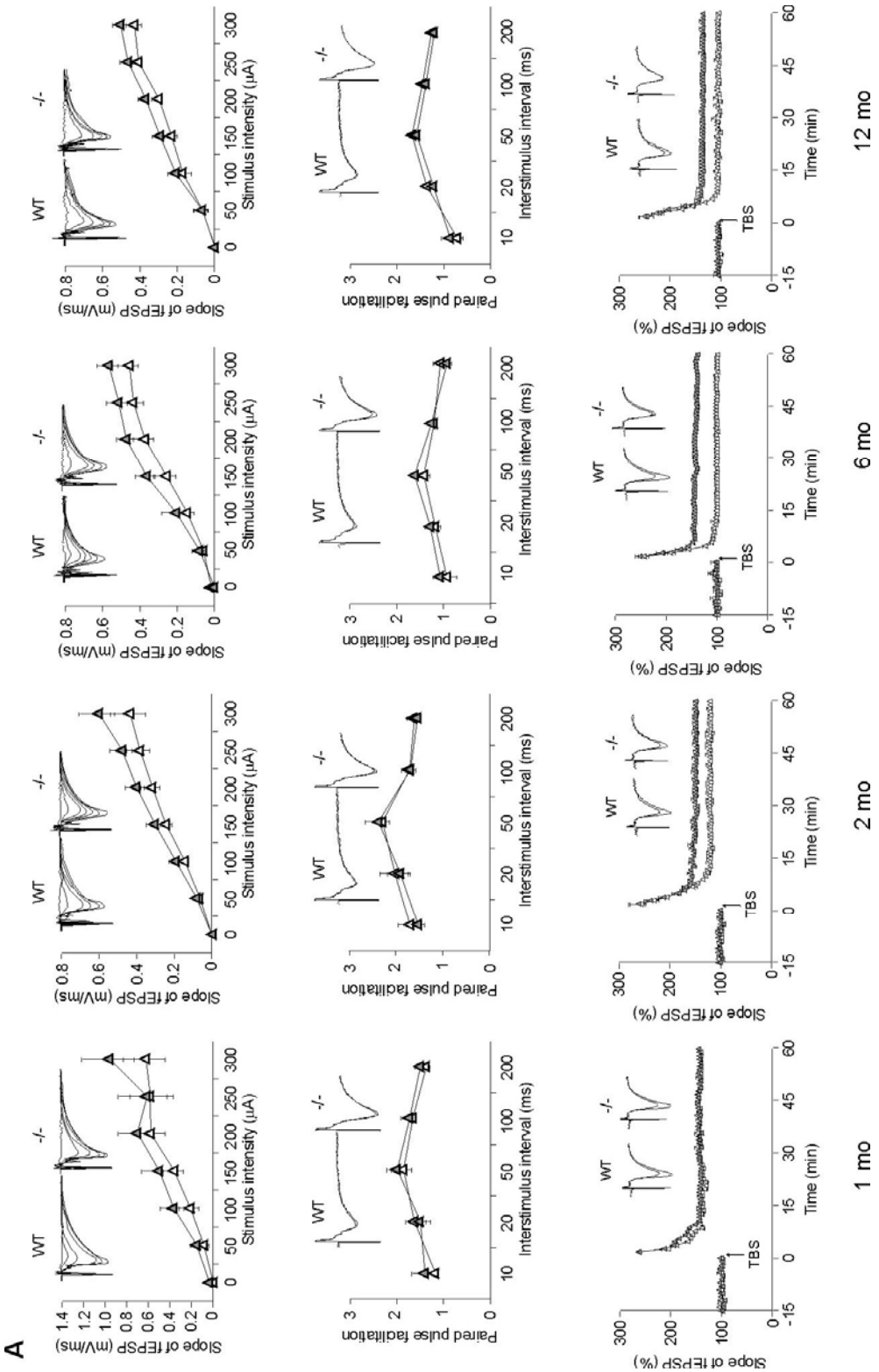
A
WT
-/-
Slope of fEPSP (mV/ms)
Stimulus intensity (μA)
Paired pulse facilitation
Interstimulus interval (ms)
Slope of fEPSP (%)
Time (min)
TBS
1 mo
2 mo
6 mo
12 mo

Figure 2-8.

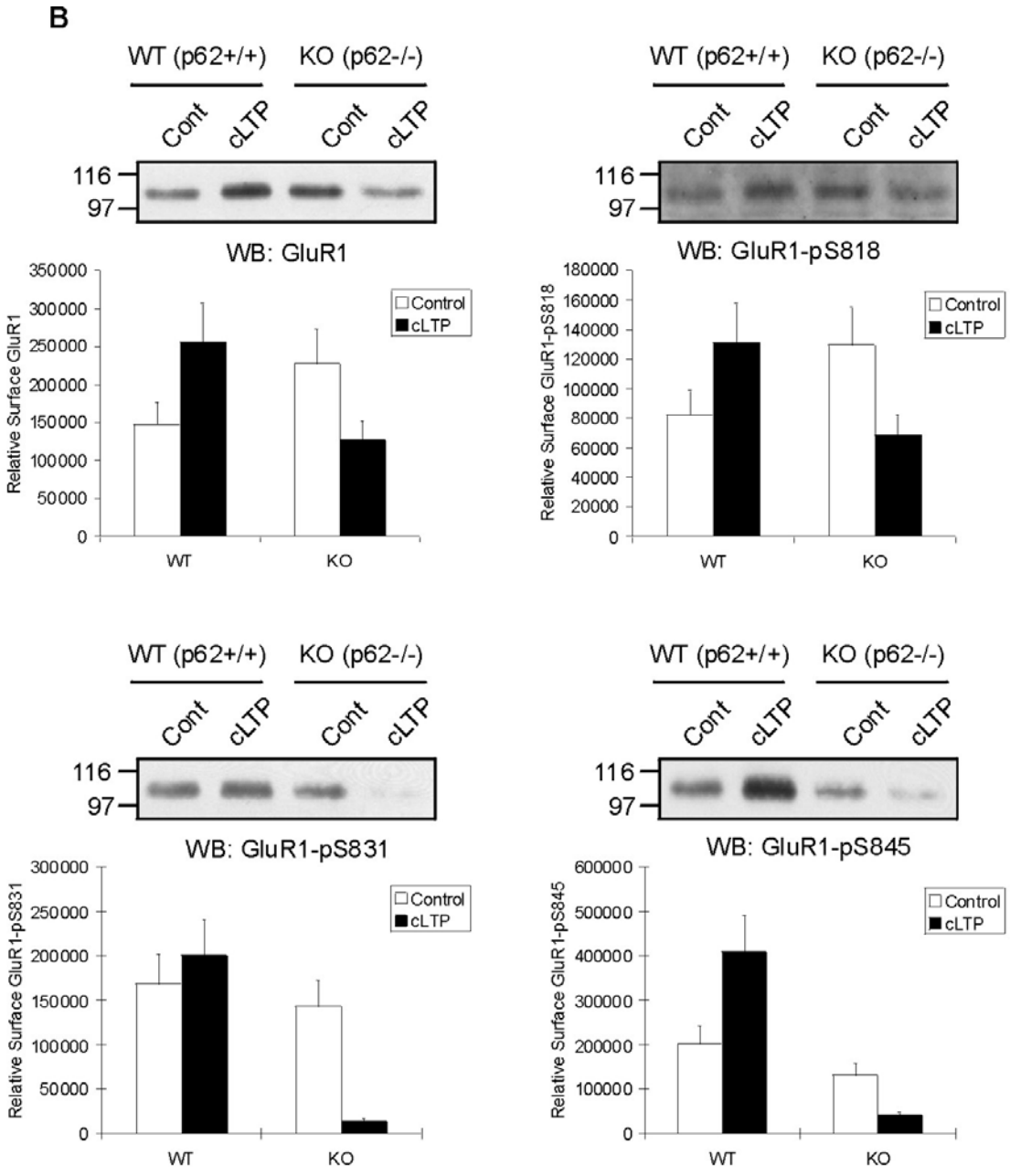
B
WT (p62+/+)
KO (p62-/-)
Cont
cLTP
116
97
WB: GluR1
Relative Surface GluR1
Control
cLTP
WT
KO
WB: GluR1-pS818
Relative Surface GluR1-pS818
WB: GluR1-pS831
Relative Surface GluR1-pS831
WB: GluR1-pS845
Relative Surface GluR1-pS845

Figure 2-9. p62 Serves as a Scaffold

(A) Alignment of the p62 binding sites in AMPAR subunits GluR1-4, $GABA_C$ receptor subunit ρ3, Grb14 and RIP indicates a conserved motif, ISExSL, shared by p62-interacting proteins.

(B) This model illustrates the role of p62 in synaptic targeting of AMPAR. p62 recruits AMPAR through its ZZ domain (a) for phosphorylation mediated by aPKC (b), then phosphorylated AMPAR is delivered in the synaptic surface to mediate LTP (c). Once the receptor is dephosphorylated (d), the AMPAR is internalized (e), and could recycle back to the surface by phosphorylation (a).

Figure 2-9.

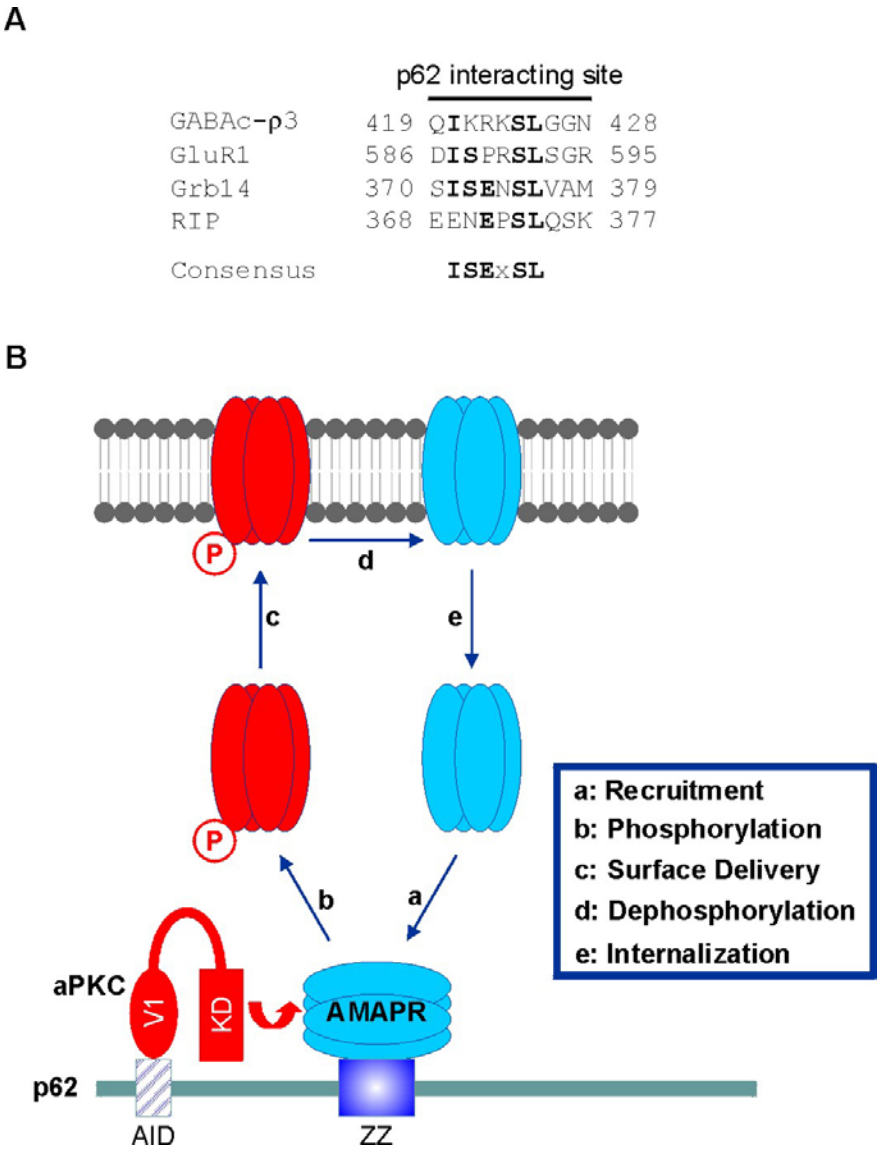
A
p62 interacting site
GABAc-ρ3 419 QIKRKSLGGN 428
GluR1 586 DISPRSLSGR 595
Grb14 370 SISENSLVAM 379
RIP 368 EENEPSLQSK 377
Consensus ISExSL
B
P
d
c
e
P
b
a
a: Recruitment
b: Phosphorylation
c: Surface Delivery
d: Dephosphorylation
e: Internalization
aPKC
V1
KD
AMAPR
p62
AID
ZZ

Table 2-1. Mapping the Interaction of GluR1 with p62

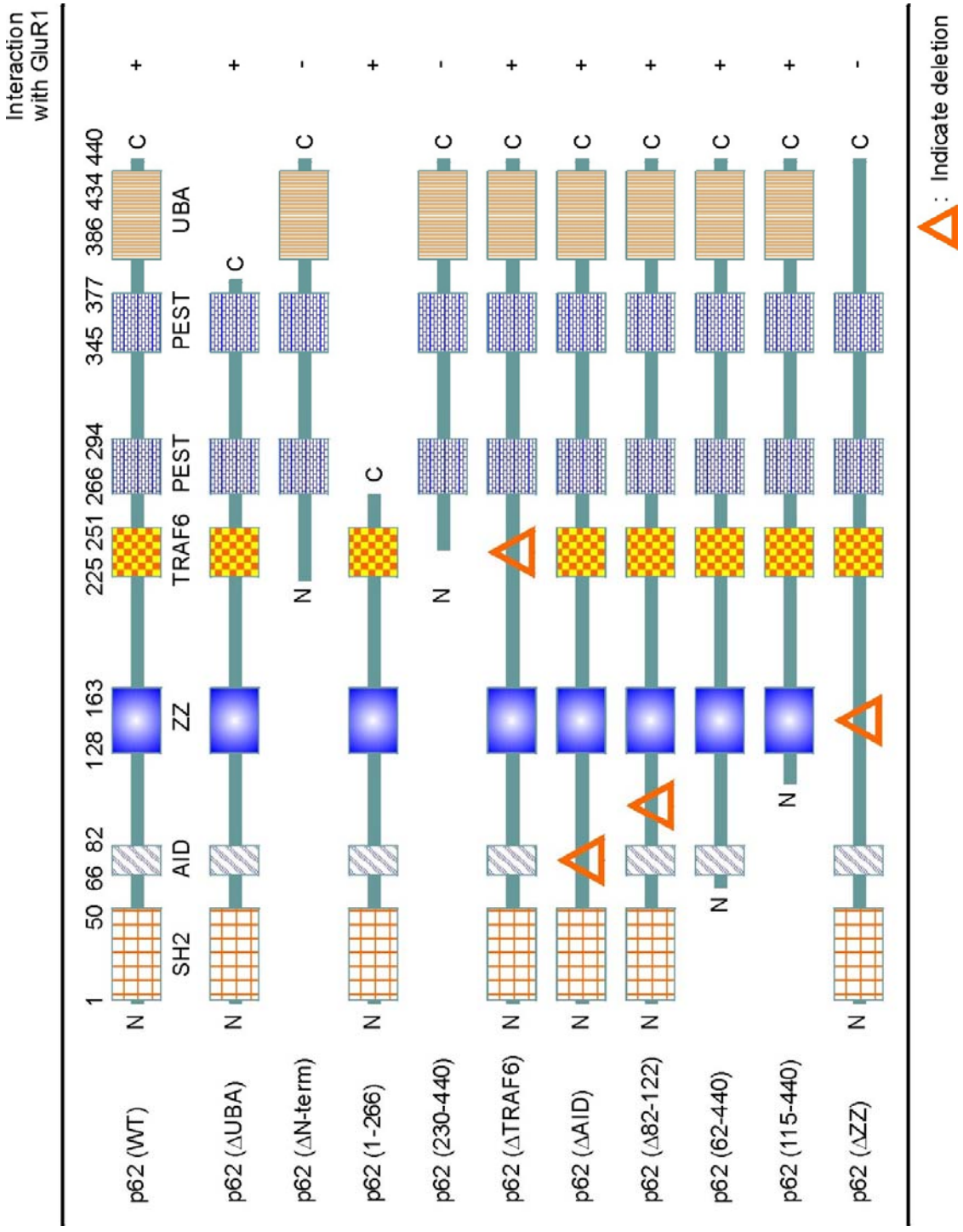

Table 2-2. Mapping the Interaction of p62 with GluR1

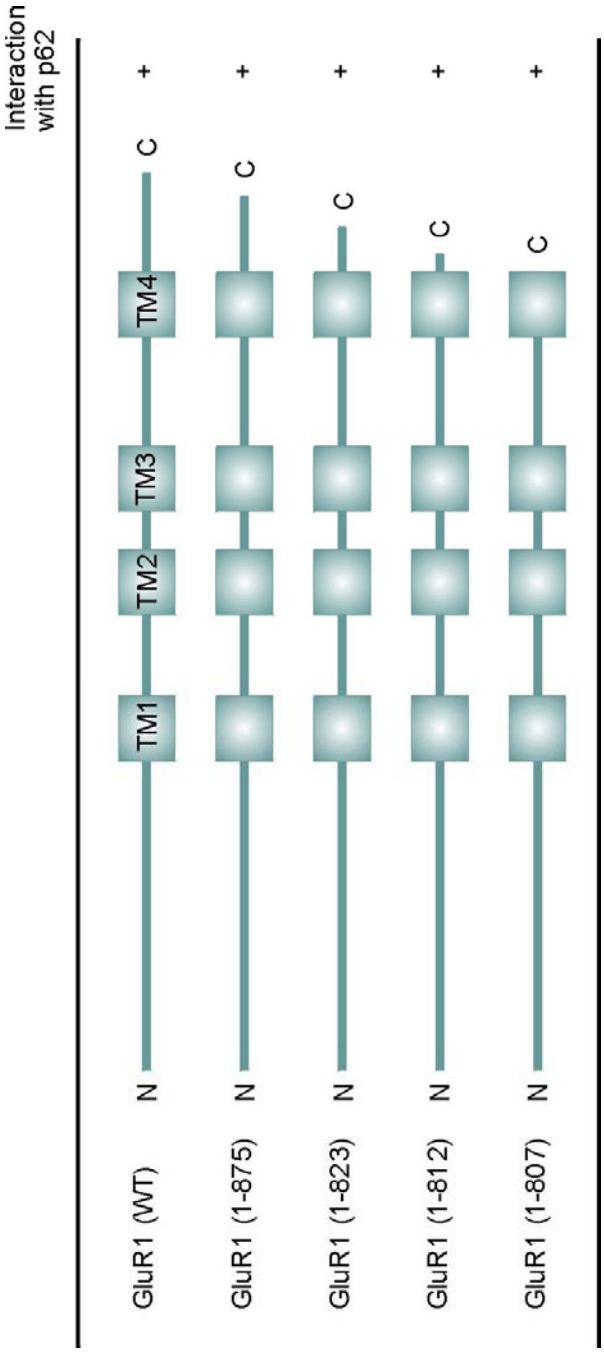

Table 2-3. Proteins Interacting with p62 through ZZ-Type Zinc Finger Domain

	Binding Site on Protein	Role of p62	Reference
Receptor protein			
Kvβ2	Not mapped	Recruiting aPKC for phosphorylation	Gong et al., 1999
D_2 DAR	Not mapped	Ubiquitination related	Kim, 2006
$GABA_C$-ρ3	Intracellular loop (TM3-TM4)	Recruiting aPKC for phosphorylation	Croci et al., 2003
AMPA receptor	Intracellular loop (L2-3)	Trafficking	
Non-receptor protein			
RIP	ID (intermediate domain)	Linking aPKC to NF-κB activation	Sanz et al., 1999
Grb14	PIR (phosphorylated insulin receptor) domain	Recruiting aPKC for phosphorylation	Cariou et al., 2002

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CHAPTER III - CONSTRUCTION OF THY1-GFP-P62 EXPRESSION CASSETTE FOR GENERATION OF P62 TRANSGENIC MICE

SUMMARY

p62 is highly expressed in the mammalian hippocampus and regulate AMPAR trafficking, however, the detailed mechanism of how p62 exerts its functions in synaptic plasticity needs to be further clarified. Transgenic mice which overexpress wild-type p62 in the hippocampal area are expected to display enhanced neuronal plasticities and can help to determine the effects of increased p62 on brain development. In this chapter, the constructs of GFP-p62 (WT) and GFP-p62 (P392L) fusion genes under the control of Thy1 promoter were generated and are ready for microinjection into embryos.

INTRODUCTION

SQSTM1/p62 plays an essential role in AMPAR trafficking and synaptic plasticity (Jiang et al., 2008), although the detailed mechanism needs further investigation. Interestingly, LTP in the p62 knock-out mice was significantly impaired in an age-dependent manner (Jiang et al., 2008). We propose that p62 might serve as a scaffold to regulate the GluR1 subunit trafficking. It is reasonable to hypothesize that overexpression of p62 in hippocampus, a brain region important for learning and memory, could facilitate aPKC-mediated GluR1 S818 phosphorylation as well as trafficking. Consequently, more

GluR1-containing AMPARs will be delivered to the postsynaptic surface initiated by tetanic stimulation. Therefore, enhanced neuronal abilities including learning and memory, and synaptic plasticity are expected in the p62 transgenic mice (Collins et al., 2004; Tang et al., 1999; Thorsell, et al., 2000). On the other hand, p62 has multiple functions in the cell and its expression is tightly regulated. Therefore, overexpression of p62 even in a mild manner could be detrimental. If this is the case, the p62 transgenic mice might develop unexpected developmental disorders (Collins et al., 2004). The generation of p62 transgenic mice overexpressing p62 in the hippocampus would help to determine the effects of increased p62 on brain development.

In this study, a Thy1 promoter was used to generate a p62 expression cassette. The gene under the control of the Thy1 promoter is exclusively expressed in the central nervous system (CNS), specifically hippocampus and cortex, without expression in other tissues (Lim et al., 2001; Lüthi et al., 1997; Tang et al., 1999; Zhou et al., 2008). In addition, a p62 mutant P392L construct will also be employed to generate transgenic mice which produce this p62 mutant form in the hippocampal region.

RESULTS

Thy1-GFP-p62 Construct

Thy1 expression cassette vector pEX12 contains an 8.2 kb genomic DNA fragment encompassing the mouse *Thy1.2* gene, which is primarily expressed in the hippocampal neurons (Ingraham et al., 1986). This cassette was generated originally by deleting a DNA fragment (from the *Ban*I site in exon 2, upstream of the translation start codon, to a

*Xho*I site in exon 4) and inserting an *Xho*I linker (Figure 3-1 and 3-2) as previously described (Lüthi et al., 1997). This *Xho*I site is the only cloning site for insertion of foreign cDNA fragments (Lim et al., 2001). A DNA fragment containing GFP-p62 was obtained from pGFP-p62 by PCR amplification and the *Sal*I sites on each side were introduced (Figure 3-1). The GFP-p62 cDNA was inserted into the *Xho*I site of the Thy1 cassette in the forward orientation with respect to the *Thy1* promoter. The GFP-p62 encompasses the GFP translation initiation (ATG) codon, signal peptide, and p62 translation initiation (ATG) codon, signal peptide, termination codon (TGA) (Figure 3-1). The entire cDNA sequence and orientation of the fusion genes in the Thy1 expression cassette were confirmed by restriction analysis and DNA sequencing (Figure 3-3).

Th1-GFP-p62 (P329L) Construct

Interestingly, a p62 missense mutation P392L (from C<u>C</u>G to C<u>T</u>G in a GC rich area) was identified by DNA sequencing. This mutation was randomly generated during the PCR process. Unexpectedly, this p62 missense mutation gene has been recently reported to increase osteoclastogenesis and cause a predisposition to the development of Paget disease by increasing the sensitivity of osteoclast precursors to osteoclastogenic cytokines (Kurihara et al., 2006). P392 is a major ubiquitin binding site in the UBA domain of p62 (Seibenhener et al., 2004). Therefore, P392L mutant p62 under the control of Thy1 promoter will only be expressed in CNS and will lack ubiquitin binding ability. As a result, ubiquitinated proteins cannot bind p62 for proteasome degradation (Geetha et al., 2005). These ubiquitinated proteins accumulate in the cells and induce hippocampal oxidation stress. Hence, transgenic mice that overexpress this mutant p62 in CNS are

likely to display disorders related to ubiquitination. To test this possibility, the mutant p62 gene will also be used to generate p62 P392L transgenic mice.

DISCUSSION

p62 conducts multiple functions in hippocampal neurons, but mainly serves as aPKC phosphorylation adaptor and ubiquitin binding scaffold (Jiang et al., 2008; Geetha et al., 2005). Although p62 has been discovered to be highly expressed in the mammalian hippocampus and regulate AMPAR trafficking, the detailed mechanism of how p62 exerts its functions in synaptic plasticity needs to be further clarified. Transgenic mice which overexpress wild-type p62 are expected to display enhanced neuronal plasticities including learning and memory; whereas P392L mutant p62 transgenic mice are likely to develop ubiquitination-related disorders due to accumulation of ubiquitinated proteins. Further investigation on these p62 transgenic mice will help elucidate the roles of p62 in regulation of AMPAR trafficking and neuronal plasticity.

EXPERIMENTAL PROCEDURES

Preparation of Thy1-GFP-p62 Construct cDNA

The fusion genes were excised with *Afl*III and *Pvu*I, and freed from plasmid sequences by agarose gel electrophoresis. Once the location of the fusion genes were located using UV light, insert band were cut from the corresponding areas in another lanes of the gel that had not been exposed directly to UV light. The DNA fragments were further purified with a Qiagen II gel purification kit (Valencia, CA) and were dissolved in microinjection buffer (10 mM Tris, pH7.4, 0.25 mM EDTA) at final concentration of 50-100 ng/μl. The

microinjection buffer was filtered (0.2 μM) and autoclaved before use. The DNA fragments were introduced by pronuclear microinjection into single-cell CBA × C57BL/6 embryos (Botteri et al., 1987; Bürki and Ullrich, 1982).

Generation of p62 Overexpressing Transgenic Mice

For each transgenic mouse to express either the full length or mutant p62 cDNA, 8 donor females will be mated to males, and 150-200 embryos will be obtained. Typically 150 ova will be injected and those that survive will be implanted into a pseudopregnant female. Eight to twelve proestrus females will be mated to 8-12 vasectomized males. Of these, a number mate- then: 5 pseudopregnant females will be used for embryo transfer (20-30 embryos/female). We predict that 20-25 pups will be obtained for further testing (~20% survival of transferred embryos). Of those, 3-6 founders will be determined to be transgenic based on PCR analysis to be mated to wild type mice. Lineages will be evaluated and 1-2 independent lineages propagated to form the nucleus of a colony. The founder mice will be inter-crossed to generate a strain of mice which overexpresses p62 in the hippocampus.

The mice will be characterized employing standard methods. Mice will be examined to determine specific effects which overexpression of the p62 gene has upon brain neuropathology, alterations in synaptic physiology, biochemical markers, and changes in anxiety, short and long term memory (Wooten, unpublished).

The animals expressing high levels of p62 in brain will be used to establish a breeding colony. Any possible adverse effects on animal health that are known or expected to occur in these mice as a result of the gene knockin will be recorded. The mice will be housed in the Biological Research Facility and routinely bred to establish a colony of animals needed for the study. The breeding stock will be continuously maintained as breeding pairs in microisolator cages under barrier conditions. All offspring are weaned at 3 weeks of age. Most of the offspring will be used for the research protocol while some will be used to replace the breeding stock. The animals will be euthanized by administering a lethal dose of CO_2. The brains will be rapidly dissected and transported back to the laboratory for further dissection of hippocampus, or preparation of hippocampal neuronal cultures. The study we are proposing has been modeled using established replicas and animal numbers present in the literature for these types of studies.

Detection of the Recombinant GFP-p62 Fusion Gene in Founder Mice

Founder mice will be identified by PCR and crossed with wild-type C57BL/6 mice. PCR screening will be performed on tail DNA by using the primers Thy1-F5: 5'-CTCCGTGGA TCTCAAGCCCT-3' (in intron A of the mouse *thy1.2* gene); p62-R4: 5'-CAGCAGCCGCTCGCAGGGTC-3' (in the 5' end of p62 cDNA). The PCR with these two primers should produce a 1.1 kb DNA fragment, which contains the entire GFP cDNA and 5' end of p62 cDNA (Figure 3-2). To optimize the cycling parameters and sensitivity issues, the DNA fragments were added into normal mouse DNA with 0-, 1-, 10-, and 100- copy. The PCR was optimized to detect single copy integrants.

Calculation Single Gene Copy/Genome Equivalent

The mouse diploid genome has a mass of 6.42×10^{-12} g and the PCR was conducted with 100 ng mouse DNA per sample. The length of Thy1-GFP-p62 expression cassette is 9.1 kb. Therefore, for 100 ng mouse DNA sample, the single gene copy equivalent is (9100 bp) × (1.07×10^{-21} g/bp) × (1×10^{-7} g) / (6.42×10^{-12} g/diploid genome) = 1.52×10^{-13} g.

Figure 3-1. Construct of Thy1-GFP-p62 Expression Cassette

GFP-p62 cDNA fragment was obtained from pGFP-p62 by PCR and the *Sal*I sites on each side were introduced. The GFP-p62 fragment was inserted into the *Xho*I site of the Thy1 expression cassette vector pEX12 in the forward orientation with respect to the *Thy1* promoter. The fusion genes were excised with *Afl*III and *Pvu*I, and freed from plasmid sequences by agarose gel electrophoresis and further purified with a Qiagen II gel purification kit.

Figure 3-1.

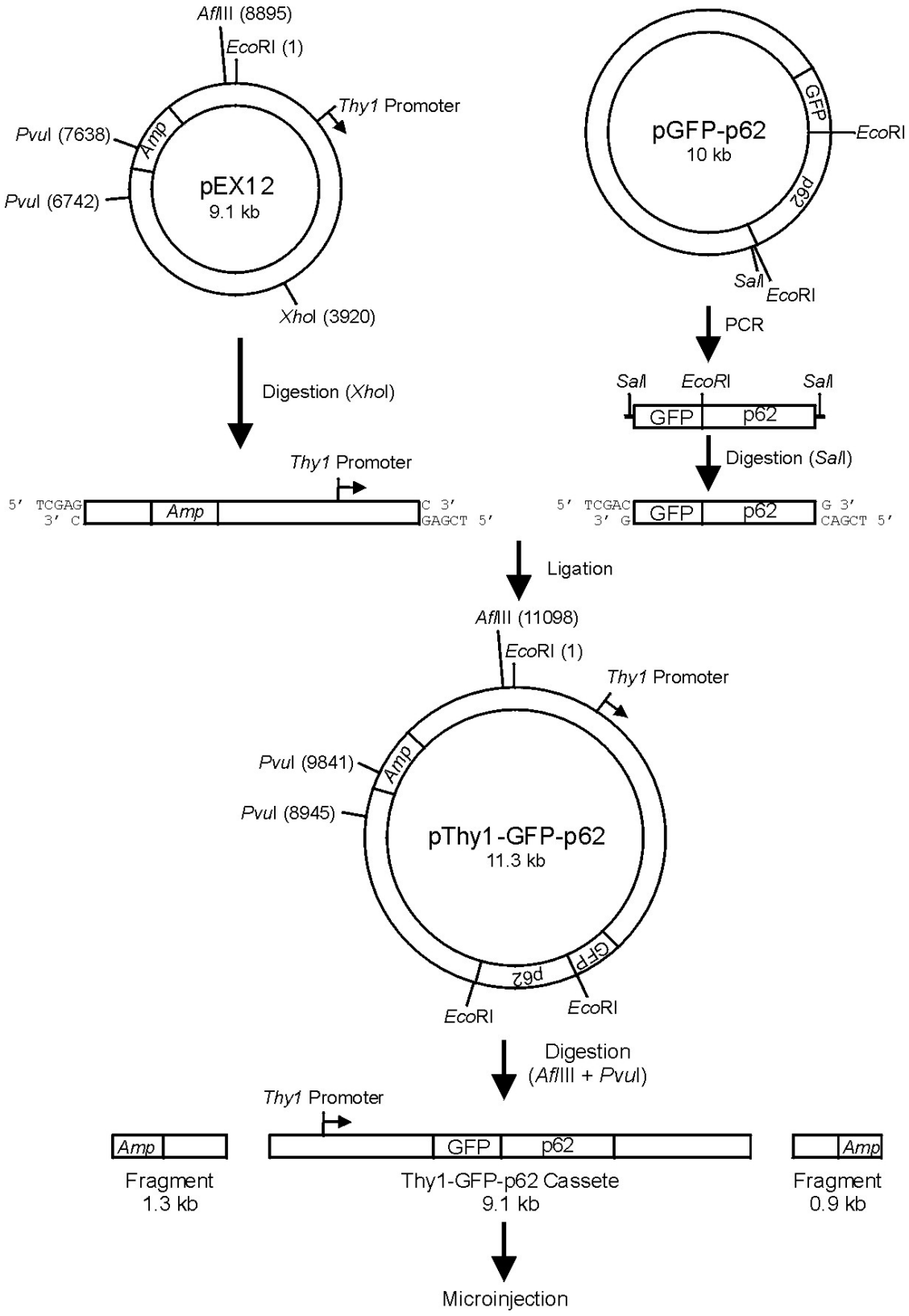

AflIII (8895)
EcoRI (1)
Thy1 Promoter
PvuI (7638)
Amp
pEX12
9.1 kb
PvuI (6742)
XhoI (3920)
Digestion (XhoI)
Thy1 Promoter
5′ TCGAG
3′ C
Amp
C 3′
GAGCT 5′
pGFP-p62
10 kb
GFP
EcoRI
p62
SalI
EcoRI
PCR
SalI
EcoRI
SalI
GFP
p62
Digestion (SalI)
5′ TCGAC
3′ G
GFP
p62
G 3′
CAGCT 5′
Ligation
AflIII (11098)
EcoRI (1)
Thy1 Promoter
PvuI (9841)
Amp
PvuI (8945)
pThy1-GFP-p62
11.3 kb
p62
GFP
EcoRI
EcoRI
Digestion
(AflIII + PvuI)
Thy1 Promoter
Amp
GFP
p62
Amp
Fragment
1.3 kb
Thy1-GFP-p62 Cassete
9.1 kb
Fragment
0.9 kb
Microinjection

Figure 3-2. Thy1.2 Gene and Thy1-GFP-p62 Expression Cassette Structure

Top line, Genomic structure of the mouse Thy1.2 gene, consisting of four exons depicted as solid boxes. Bottom line, Schematic diagram of the 9.1 kb DNA fragment containing the Thy1-GFP-p62 fusion gene that was introduced into the mouse germ line.

Figure 3-2.

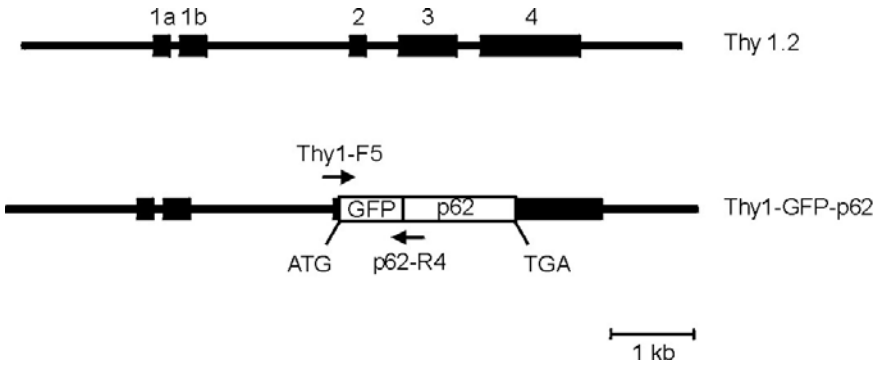
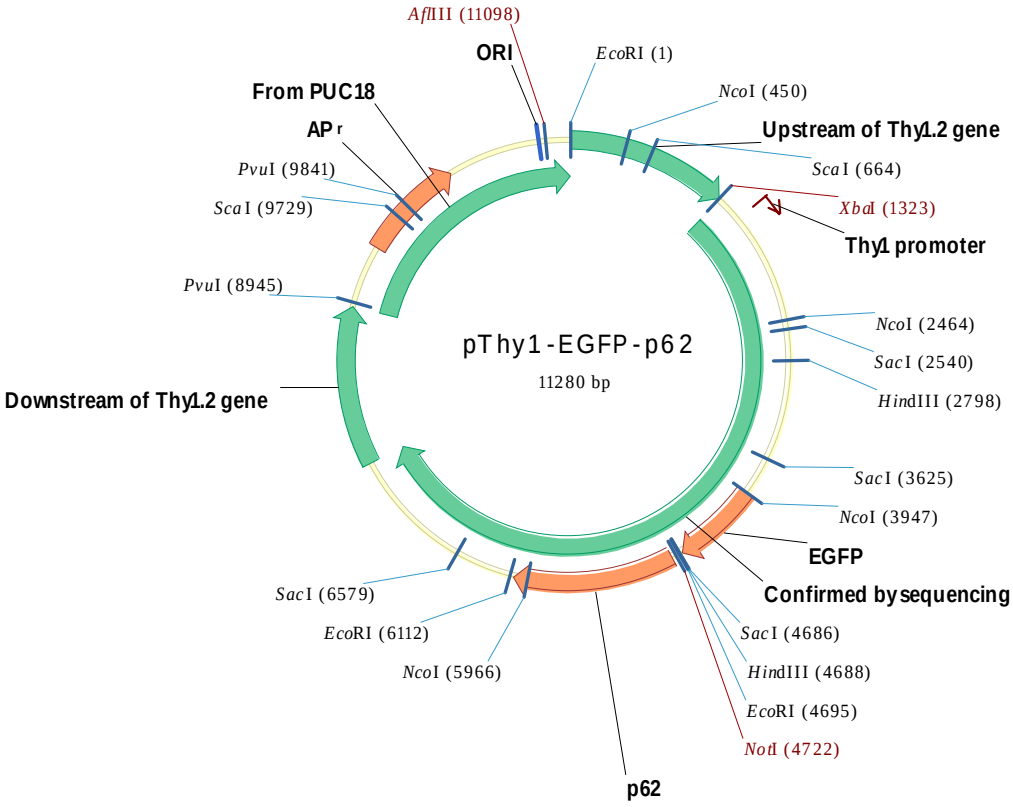


Figure 3-3. Map of Thy1-GFP-p62 Expression Cassette Plasmid

GFP-p62 fusion gene is under the control of Thy1 promoter which is only initiated in CNS. The orientation of the expression construct components: upstream of Thy1.2 gene, Thy1 promoter, exon 1a and 1b, 5' end of exon 2, GFP-p62 fusion gene, 3' end of exon 4, downstream of Thy1.2 gene.

Figure 3-3.


AflIII (11098)
ORI
EcoRI (1)
From PUC18
NcoI (450)
APr
Upstream of Thy1.2 gene
PvuI (9841)
ScaI (664)
ScaI (9729)
XbaI (1323)
Thy1 promoter
PvuI (8945)
pThy1-EGFP-p62
11280 bp
NcoI (2464)
SacI (2540)
Downstream of Thy1.2 gene
HindIII (2798)
SacI (3625)
NcoI (3947)
EGFP
Confirmed by sequencing
SacI (6579)
EcoRI (6112)
SacI (4686)
NcoI (5966)
HindIII (4688)
EcoRI (4695)
NotI (4722)
p62

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SUMMARY AND CONCLUSIONS

AMPARs mediate most of the fast excitatory synaptic transmission in the mammalian CNS. It is widely believed that trafficking of AMPARs to and away from the postsynaptic membrane is the molecular mechanism for many forms of neuronal plasticity, including long-term potentiation (LTP) and long-term depression (LTD). During LTP expression, more AMPAR are inserted into the postsynaptic membrane to enhance the synaptic transmission; whereas LTD induces AMPAR internalization. AMPAR trafficking is highly regulated by receptor-associated proteins and post-translational modifications.

Herein, the aPKC scaffold, p62, was identified as a novel AMPAR-associated protein. AMPAR subunit intracellular loop L2-3 and the ZZ-type Zinc finger domain of p62 are essential for the interaction. The AMPAR/p62 interaction is necessary for surface delivery of the receptor, which is the critical molecular event for LTP introduction. This intracellular loop L2-3, not the loop L1-2, is completely conserved throughout all AMPAR subunits GluR1-4. In addition, the sequence of this loop is unique for AMPAR subunits and is not shared by NMDAR or KAR subunits.

Furthermore, LTP was impaired in p62 knock-out mice in an age-dependent manner with normal basal synaptic transmission. The reduced LTP indicated a postsynaptic malfunction, which might be caused by the instability of AMPAR in the synaptic membrane. Surface delivery of the AMPAR subunit GluR1 induced by cLTP was impaired and paralleled an absence of phosphorylation at S818, S831, and S845 in brain slices from p62 knock-out mice. These findings reveal that p62 is involved in regulation of AMPAR trafficking and LTP expression via phosphorylation of GluR1 subunit. In addition, a possible conserved binding motif (ISExSL) shared by all p62 interacting-aPKC substrates has been discovered. Based on these findings, it is possible that p62 may serve as an adapter to recruit AMPAR to aPKC for phosphorylation through its N-terminal ZZ-type Zinc finger domain. Those phosphorylated AMPAR can stabilize in the postsynaptic membrane to enhance the synaptic transmission and mediate LTP expression. In the absence of phosphorylation, the unstable synaptic AMPAR could be internalized followed by LTP decay.

Like the p62 knock-out mice, the transgenic mice which overexpress wild-type p62 and mutant p62 (P392L) in the CNS, specifically hippocampus, may also help elucidate the function of p62 in neuronal plasticity. An absence of p62 impairs AMPAR surface delivery and results in a deficit of LTP expression in p62 knock-out mice; whereas overexpression of p62 in hippocampus may facilitate AMPAR surface delivery, and then enhance neuronal abilities including learning and memory. In addition, P392 is a major ubiquitin binding site in the UBA domain of p62. Therefore, P392L mutant p62 lacks ubiquitin binding ability. As a result, ubiquitinated proteins cannot bind p62 for

proteasome degradation. These ubiquitinated proteins accumulate in the neurons and might induce hippocampal oxidative stress. Transgenic mice that overexpress this mutant p62 are likely to display disorders related to ubiquitination.

In this study, a Thy1 promoter was used to generate the p62 expression cassette. The p62 gene controlled by the Thy1 promoter will only be expressed in CNS, specifically hippocampus and cortex, without expression in other tissues. Herein, the expression cassettes of GFP-p62 (WT) and GFP-p62 (P392L) under the control of Thy1 promoter have been generated. The linearized cDNA fragments have been further purified and are ready for microinjection into embryos.