THE REGULATION OF AMPA RECEPTOR TRAFFICKING AND CHANNEL PROPERTIES THROUGH GLUR1 SUBUNIT PHOSPHORYLATION

By

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CERTIFICATE OF APPROVAL

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ABSTRACT

Synaptic plasticity, the ability of neurons to undergo activity-dependent changes in the strength of synaptic transmission, occurs in many different parts of the brain and accounts for important functions of the brain, such as learning and memory formation. Both presynaptic and postsynaptic mechanisms of synaptic plasticity have been identified and characterized, which occur in various parts of the brain and at specific synapses. In the CA3-CA1 synapses of the hippocampus, the synapses of main interest in this thesis, synaptic plasticity occurs mostly through postsynaptic mechanisms. These postsynaptic mechanisms ultimately consist of two main mechanisms that regulate α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)-type glutamate receptors: 1) changes in channel properties and 2) number of receptors in synapses. Evidence that both of these mechanisms are critically regulated by phosphorylation of the GluR1 subunit, the main regulatory subunit of AMPA receptors in the hippocampus CA1 region, is presented in this thesis.

Past studies have shown that AMPA receptors are not static, but are dynamically modulated in properties and numbers in synapses according to the history of pattern and strength of past activity in a given synapse. Strength of synaptic transmission can be modified bi-directionally, resulting in a stably potentiated state called long-term potentiation (LTP) or a depressed state called long-term depression (LTD). These changes in the strength of synaptic transmission are thought to be critical for the ability of the brain to acquire and store new information.

The GluR1 subunit contains two key regulatory phosphorylation sites in the C-terminus. Serine at amino acid position 831 (S831) is phosphorylated by

calcium/calmodulin-dependent protein kinase II (CaM-KII), whereas serine at position 845 (S845) is phosphorylated by cAMP-dependent protein kinase (PKA). The main goal of this thesis work was to determine how AMPA receptor function, in both channel properties and delivery to synapses, is regulated differently by phosphorylation at these two sites.

Previous work in our lab has shown that phosphorylation of S831 by CaM-KII in homomeric GluR1 expressed in HEK293 cell results in increased single-channel conductance. Because the majority of AMPA receptors in the hippocampus consist of GluR1/GluR2 heteromers, studies were performed to determine the functional effect of S831 phosphorylation in heteromeric AMPA receptors. We found that although CaM-KII phosphorylation of the GluR1 subunit at S831 is unchanged, the presence of GluR2 completely blocked the potentiating effects of S831 phosphorylation in the GluR1/GluR2 heteromers. Furthermore, GluR1/GluR2 heteromers were significantly lower in single-channel conductance compared to GluR1 homomers, suggesting that subunit recomposition in synapses can significantly modify the strength of synaptic transmission.

Finally, the hypothesis that S845 phosphorylation plays an important role in regulating the delivery of GluR1-containing AMPA receptors to the surface membrane was tested. Interestingly, S845 phosphorylation resulted in selective delivery of GluR1-containing AMPA receptors to extrasynaptic sites. Synaptic activity was required for synaptic incorporation of extrasynaptic AMPA receptors, and thus, S845 phosphorylation regulates the pool of AMPA receptors available for synaptic incorporation. Thus, this thesis provides evidence and mechanisms for the role of S831 and S845 phosphorylation of the GluR1 subunit during bi-directional synaptic plasticity in the hippocampus.

CHAPTER 1

INTRODUCTION

Synaptic plasticity, the activity-dependent changes in the strength of synaptic transmission, is thought to contribute to the complex neural processes such as learning and memory formation. Numerous studies have elucidated many different molecular mechanisms that regulate bi-directional synaptic plasticity, such as LTP and LTD. These long-term changes in synaptic transmission can be divided into early (~1-2 hours) and late phases (2 hours or longer). The early phase requires kinase activation and protein phosphorylation (1), whereas late phase requires gene expression and new protein synthesis (2). One of the most important mechanisms for the early phase of synaptic plasticity is the phosphorylation of synaptic proteins, which mediate the changes in synaptic strength (1, 3). Activation of key protein kinases during this phase has been shown to be critical for initiating signaling cascades that result in enhanced synaptic transmission. Among many known synaptic substrates, the GluR1 subunit, the main subunit of AMPA-type glutamate receptors in the CA1 region of hippocampus, has been identified as a key substrate during synaptic plasticity (4). AMPA receptors mediate most of the excitatory transmissions in the brain, and thus, modulating this receptor's function through phosphorylation can significantly influence synaptic transmission. So far, two different kinases that phosphorylate GluR1 have been identified that affect AMPA receptor function. First, CaM-KII phosphorylates GluR1 (5, 6), specifically at S831 on the cytoplasmic C-terminus of GluR1 (7-9). This phosphorylation is increased during LTP (10, 11) and enhances the single-channel conductance of AMPA receptors (12-14). Second, PKA phosphorylates S845 (8, 9), also on the C-terminus, and enhances open probability (15, 16). Furthermore, a recent study showed that the phosphorylation

states at these two sites are differentially regulated during LTP, and its counterpart, LTD (11). The question still remains, however, as to what percentage of receptors are phosphorylated at these sites under basal and stimulated conditions, and how activity-regulated phosphorylation affect physiological functions of AMPA receptors. These are the main questions examined in this thesis. Thus, the main components of this thesis are divided into two chapters (**Chapters 2 and 3**), with each chapter focusing on the specific functional role of regulatory phosphorylation at the two sites in GluR1 C-terminus.

1.1 AMPA RECEPTOR STRUCTURE AND FUNCTION

There are four types of glutamate receptors in the central nervous system: AMPA receptors, N-methyl-D-aspartate (NMDA) receptors, kainate receptors, and metabotropic glutamate receptors (mGluRs). The AMPA, NMDA and kainate receptors are fast acting excitatory channels, each with very different properties (17). They are found throughout the central nervous system and are the main mediators of excitatory transmission. Both AMPA and NMDA receptors are highly concentrated in the dendrites of hippocampal CA1 pyramidal neurons. mGluRs are slow acting, G protein-coupled receptors with typical seven transmembrane spanning regions. These receptors are found in the perisynaptic regions and in presynaptic boutons, where they modify synaptic transmission by regulating postsynaptic receptors or presynaptic release of neurotransmitters, respectively (18). The main focus of this thesis is the AMPA receptors, which are the most common excitatory receptors in the brain, responsible for most of the excitatory currents in the CA3-CA1 synapses in the hippocampus. Thus, the following background information will focus primarily on the AMPA receptors.

Four subunits that form AMPA receptors have been cloned, named GluR1-4 or GluRA-D (GluR1-4 nomenclature is used in this thesis) (17, 19-23). In addition, GluR5-7 and Ka1-2 subunits have been cloned for kainate receptors, NR1, NR2A-D, and NR3A-B for NMDA receptors, although NR3A and NR3B form excitatory glycine receptors in combination with NR1 (24), and mGluR1-8 for mGluRs. Each AMPA receptor subunit is able to form functional homomeric receptors when expressed in heterologous system, such as oocytes or HEK293 cells, although GluR2 homomers conduct very small currents (25). The four AMPA receptor subunits are similar in size (~900 amino acids) and share 68-73% amino acid sequence identity (21). However, their channel properties are very different. Each subunit comprises its own channel properties, such as single-channel conductance, desensitization and deactivation kinetics, calcium permeability, rectification (sensitivity to polyamines), and open probability. These differences in subunit channel properties and the ability of four subunits to form different heteromers or homomers greatly expand the molecular diversity of AMPA receptors (17, 21). In hippocampal CA1/CA2 pyramidal neurons, however, two main populations of AMPA receptors exist, consisting of GluR1/GluR2 heteromers and GluR2/GluR3 heteromers (26). A few GluR1 and GluR3 homomers are also present. Among GluR1-containing AMPA receptors, about 92% are in complexes with GluR2 subunits as heteromers, while the remaining 8% are GluR1 homomers. Approximately 70% of GluR2 subunit is co-immunoprecipitated with GluR1, and the remaining 30% is completely immunodepleted with the anti-GluR2/3 antibody. This suggests that 70% of GluR2 subunits form GluR1/GluR2 heteromers, while remaining 30% form GluR2/GluR3 heteromers. GluR4 subunit expression is relatively low compared to other subunits in the 4-6 weeks old rats.

However, GluR4 subunit expression is more predominant during early development and is highly expressed in the thalamus and cerebellum (22, 27). Thus, it is clear that AMPA receptor subunit assembly is not random, but forms heteromeric AMPA receptors with a preferred subunit stoichiometry. In support of this, GluR1 homomers form stochastically, while GluR1/GluR2 heteromers form preferentially with a stoichiometry of two GluR1 subunits with two GluR2 subunits in a symmetric arrangement (28). Thus, subunit composition likely plays a significant role in regulating synaptic AMPA receptor function by conveying different channel properties.

Initially, AMPA receptors were presumed to be members of the ligand-gated ion channel family, exemplified by the nicotinic acetylcholine (nACh) receptors. Because nACh receptors form pentamers with four transmembrane domains, and GluR subunits also contain four transmembrane domains, it was assumed that AMPA receptors also form pentamers. Recent studies, however, suggest that AMPA receptors form tetrameric structures shared with the voltage-gated potassium channels (29). The average conductance of AMPA receptors is determined by the number of subunits bound to agonist. The topology of AMPA receptor was also adapted initially from the nACh receptors, which placed both N- and C-termini in the extracellular matrix. However, N-glycosylation site tagging experiment determined that AMPA receptor subunits contain a membrane re-entrant loop (P loop) in the second transmembrane region, which places the C-terminus in the cytoplasm with the N-terminus in the extracellular matrix (**Figure 1.1**) (30). This study was pivotal for understanding AMPA receptor function, because the cytoplasmic C-terminus, through phosphorylation and protein-protein interactions,

critically affects AMPA receptor function by regulating channel properties and synaptic delivery (3, 31, 32).

Figure 1.1

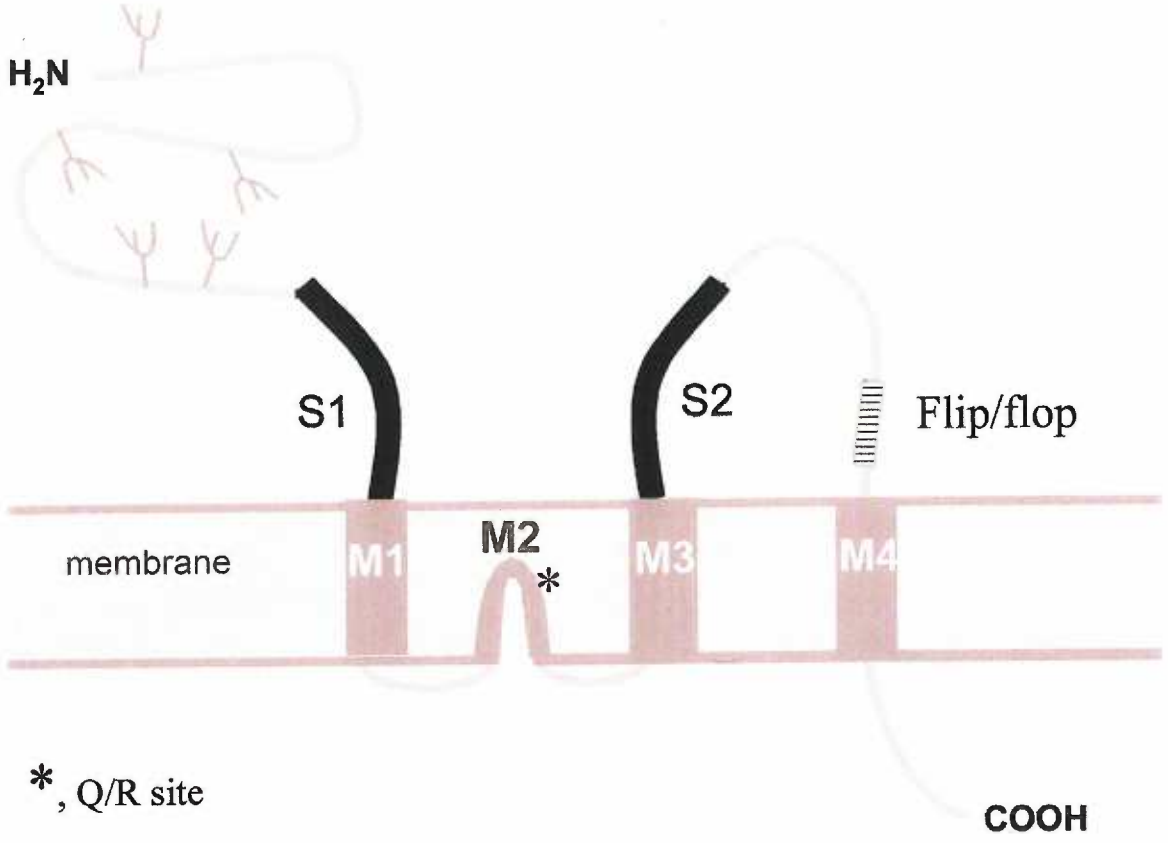

Figure 1.1 Membrane topology of AMPA receptor subunits determined by N-glycosylation site tagging experiment (30). AMPA receptor subunits contain four transmembrane domains (M1-M4), where M2 forms a membrane re-entrant loop (P loop). The C-terminus is cytoplasmic. M2 region forms part of the conducting pore, where * indicates post-transcriptional editing site in the GluR2 subunit (Q/R site). S1 and S2 denote the two ligand-binding domains, and Flip/flop indicates alternatively spliced exons. Glycosylation sites are indicated as trees in the N-terminus. From (17).

1.2 TRANSCRIPT EDITING: ALTERNATIVE SPLICING

AMPA receptor properties are determined by factors other than subunit composition. These factors can be broadly divided into transcript editing (alternative splicing), post-transcriptional editing (RNA editing), and post-translational modifications (phosphorylation and protein-protein interactions). All four subunits are alternatively spliced during transcription, resulting in either flip or flop variants (**Figures 1.1 and 1.2**). Flip variants are expressed prenatally and continue to be expressed in the mature brain, whereas the flop variants start expressing around postnatal day 7-8 and reach similar expression levels as the flip variants in the mature brain. Flip and flop variants convey different channel properties depending on the subunit, but generally, flip variants desensitize more slowly and less profoundly (17). This suggests that flip variants in immature brain may conduct more current compared to the flop variants in mature brain. Thus, developmental regulation of flip and flop variants may fine tune the kinetics of excitatory transmission during development. Alternative splicing also results in either short or long C-terminus forms of GluR2 and GluR4 subunits (**Figure 1.3**). GluR2 is mostly expressed in short form (50 amino acid residues in its C-terminus), but GluR2-long (67 amino acid residues), which is homologous to GluR1 and GluR4-long, is also expressed in young animals (33, 34). GluR4, which is normally expressed as long form (68 amino acid residues), is also expressed in short form, homologous to GluR2-short and GluR3, in cerebellum (35). GluR1 is expressed in long C-terminus form only (82 amino acid residues) and GluR3 in short form only (50 amino acid residues) (for simplicity, all GluR1 and GluR4 refer to long forms and GluR2 and GluR3 to short forms, unless noted otherwise). The different lengths of the C-terminus has implications for synaptic

plasticity, because long forms contain additional or different phosphorylation and protein interaction sites that can significantly affect channel function (**Figure 1.3**) (36). Furthermore, C-terminal domains affect surface delivery, synaptic incorporation, synaptic retention, and recycling to and from the surface membrane with internal stores, all through subunit-specific protein interactions (36-39). For example, GluR1-containing AMPA receptors are delivered in an activity-dependent manner, while GluR2 receptors are constitutively delivered (40). Moreover, GluR2/GluR3 heteromers can replace GluR1/GluR2 heteromers following synaptic potentiation (41), and this difference in functional roles of GluR1 and GluR2 subunits are, in part, due to the difference in PDZ (PSD-95/discs large/zona occludens-1) interacting sequences (ATGL for GluR1, SVKI for GluR2-short) at the very end of C-terminus. For example, the PDZ interacting domain of the GluR1 subunit is important for the activity-dependent delivery of new AMPA receptors during LTP (42). Other long forms of AMPA receptor subunits, GluR4 and GluR2-long, also contain PDZ interacting domains (**Figure 1.3**), although their functional role has not been identified. Recent study also showed that selectively blocking GluR2-long delivery to surface membrane in CA1 pyramidal neurons decreases approximately 35% of basal AMPA receptor transmission in young animals, suggesting that this subunit is responsible for a significant portion of AMPA receptor function during early development (34). Blocking GluR2-long delivery also reduces LTP by 50% in wild-type mice and completely blocks LTP in GluR1 knock-out mice, suggesting that GluR2-long receptor is responsible for about half the synaptic potentiation in young animals. Thus, developmental regulation of selective subunit expression is another mechanism by which AMPA receptor channel properties are modulated.

Figure 1.2

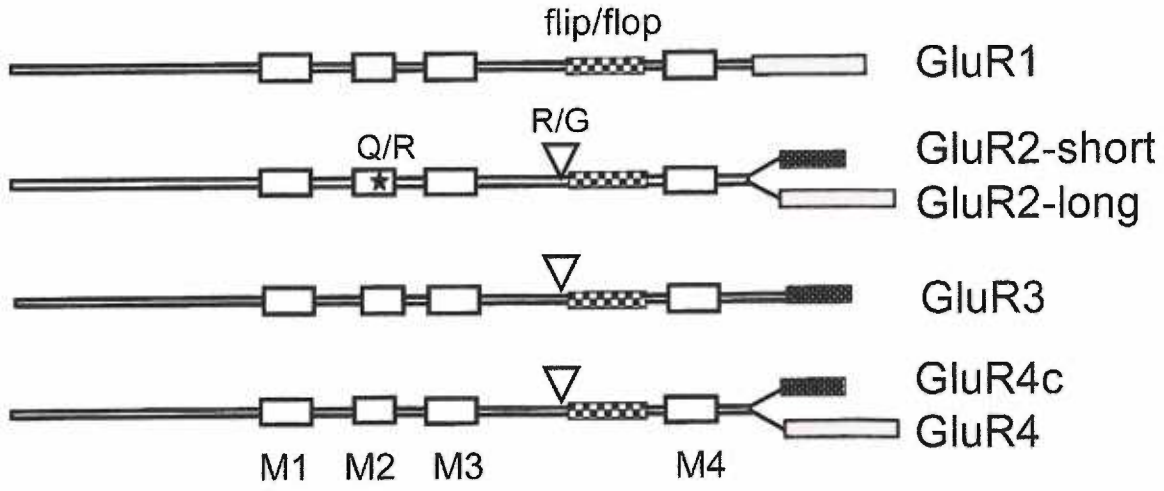
flip/flop
GluR1
R/G
Q/R
GluR2-short
GluR2-long
GluR3
GluR4c
GluR4
M1
M2
M3
M4

Figure 1.2 Transcript and post-transcriptional editing in AMPA receptor subunits. Transcript editing consists of alternative splicing, resulting in either flip or flop variants in all four subunits and long or short C-terminus variants in GluR2 and GluR4 subunits. Post-transcriptional editing, denoted by R/G, is present in GluR2, GluR3, and GluR4 subunits, while Q/R editing is only present in the GluR2 subunit. M1-M4 denotes three transmembrane domains (M1, M3, and M4) and one membrane re-entrant loop (M2). From (17).

Figure 1.3

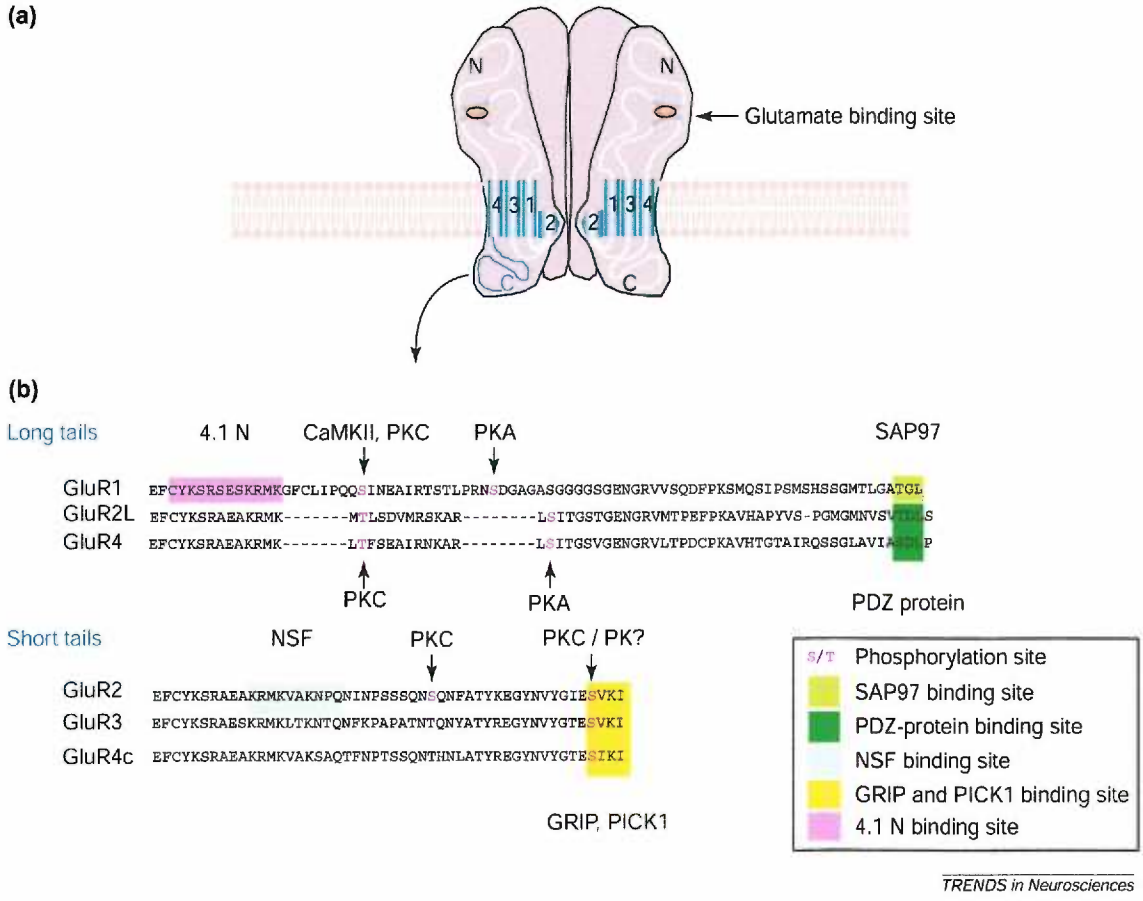
(a)
N
N
Glutamate binding site
4 3 1
2
2
1 3 4
C
C
(b)
Long tails
4.1 N
CaMKII, PKC
PKA
SAP97
GluR1 EFCYKSRSESKRMKGFCLIPQQSINEAIRTSTLPRNSDGAGASGGGGSGENGRVVSQDFPKSMQSIPSMSHSSGMTLGATGL
GluR2L EFCYKSRAEAKRMK-------MTLSDVMRSKAR--------LSITGSTGENGRVMTPEFPKAVHAPYVS-PGMGMNVSVTLLS
GluR4 EFCYKSRAEAKRMK-------LTFSEAIRNKAR--------LSITGSVGENGRVLTPDCPKAVHTGTAIRQSSGLAVIASDLP
PKC
PKA
PDZ protein
Short tails
NSF
PKC
PKC / PK?
GluR2 EFCYKSRAEAKRMKVAKNPQNINPSSSQNSQNFATYKEGYNVYGIESVKI
GluR3 EFCYKSRAESKRMKLTKNTQNFKPAPATNTQNYATYREGYNVYGTESVKI
GluR4c EFCYKSRAEAKRMKVAKSAQTFNPTSSQNTHNLATYREGYNVYGTESIKI
GRIP, PICK1
S/T Phosphorylation site
SAP97 binding site
PDZ-protein binding site
NSF binding site
GRIP and PICK1 binding site
4.1 N binding site
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Figure 1.3 C-terminus of AMPA receptor subunits affect receptor function by phosphorylation and interactions with PDZ proteins. (**a**) Topology of AMPA receptor subunit is shown, with three transmembrane domains (1, 3, and 4) and one membrane re-entrant loop (2). The C-terminus faces the cytoplasmic side. (**b**) Amino acid residues of subunits with long tails and short tails are shown. Arrows indicate known phosphorylation sites, and shaded areas represent known protein-protein interaction domains. Modified from (36).

1.3 POST-TRANSCRIPTIONAL EDITING: RNA EDITING

Post-transcriptional editing occurs in AMPA and kainate receptor subunits, where adenosines are deaminated to inosines by dsRNA adenosine deaminases (43, 44). Inosines mimic guanosines during translation and changes the amino acid codon. Two different sites are edited in AMPA receptor subunits. Genomically encoded arginine (AGA) is replaced with glycine (IGA) at the R/G site just before the flip/flop region in GluR2, GluR3, and GluR4 subunits, and this shortens the recovery from desensitization (**Figure 1.2**) (45). More extensively studied editing is the glutamine (CAG) to arginine (CGG) editing at the Q/R site, which is only present in GluR2 among AMPA receptor subunits, but is also present in GluR5 and GluR6 subunits of kainate receptors. Q/R editing is located in M2, the membrane re-entrant loop of GluR2 subunit, which forms part of the channel pore. Because of its location, Q/R editing conveys very significant changes to receptor properties. Virtually 100% of all GluR2 subunits expressed are Q/R edited throughout all developmental stages (46), and AMPA receptors containing Q/R edited GluR2 subunits are low in calcium permeability (47, 48), low in single channel conductance (**Figure 2.6**) (25) and have linear rectification (**Figure 2.4**) (49). Although the exact reason for post-transcriptional editing is unknown, it is clear that editing adds to the molecular diversity of AMPA receptors. It has been hypothesized that the number of calcium permeable AMPA receptors is regulated in neurons by controlling the expression level of edited GluR2. GABAergic interneurons in hippocampus (50, 51) and stellate cells in cerebellum (52, 53), for example, express very low amounts of GluR2 subunit, and thus most AMPA receptors expressed in these neurons are highly calcium permeable, sensitive to polyamines and Joro spider toxin (54), and have inward rectification.

Another potential function of Q/R editing involves regulation of receptor tetramerization during the assembly of GluR1/GluR2 heteromers (55). Arginine residue at the Q/R site seems to function as a retention signal for endoplasmic reticulum, such that edited GluR2 remain in the endoplasmic reticulum until dimerizing with unedited GluR1. Two GluR1/GluR2 dimers then dimerize to form tetramers, which efficiently exit the endoplasmic reticulum.

1.4 POST-TRANSLATIONAL MODIFICATIONS: PHOSPHORYLATION AND SUBUNIT-SPECIFIC PROTEIN INTERACTIONS

Numerous phosphorylation and subunit-specific protein interaction sites in AMPA receptor subunits have been identified and characterized (**Figure 1.3 and 1.4**). These post-translational modifications regulate both channel properties and receptor delivery to synapses and are critical components of synaptic plasticity. Interestingly, subunit-specific protein interactions can affect receptor function through regulating phosphorylation. Synapse-associated protein 97 (SAP-97) interacts with the PDZ interacting domain of GluR1 (ATGL) (56), and this is thought to be important for local recruitment of PKA to post-synaptic densities (PSDs) through A kinase anchoring protein (AKAP79/150) interaction with SAP-97 (57). Local targeting of PKA to GluR1-containing AMPA receptors may promote S845 phosphorylation and therefore, may have implications for affecting channel property and synaptic delivery during LTP (42, 58, 59). Interestingly, phosphorylation and synaptic delivery of GluR1-containing AMPA receptors may be coupled. S845 phosphorylation rapidly reinserts recycling AMPA receptors to surface membrane, rather than targeting them for endosomal and lysosomal

degradation (60). Furthermore, over-expression of activated CaM-KII can drive GluR1-containing AMPA receptors into synapses, but this delivery is completely blocked when S845 phosphorylation is inhibited with a PKA inhibitor or when S845 is mutated to alanine (58). Stimulation with forskolin (adenylate cyclase activator) and IBMX (general phosphodiesterase inhibitor), a treatment that increases S845 phosphorylation, is not sufficient alone to drive GluR1-containing AMPA receptors into synapses. Thus, S845 phosphorylation regulates the pool of receptors available for synaptic incorporation (58).

Interestingly, stargazin interacts directly with the GluR1 subunit and PSD-95, and other AMPA receptor subunits, likely playing an important role in synaptic delivery of AMPA receptors (61). More importantly, stargazin over-expression alone delivers AMPA receptors selectively to extrasynaptic sites, while stargazin-PSD-95 interaction is important for synaptic incorporation (62). Thus, stargazin-mediated delivery of AMPA receptors to extrasynaptic sites complements the finding of S845 phosphorylation results described above: both mechanisms may regulate the pool of AMPA receptors available for synaptic incorporation. Data also suggest that stargazin is an integral member of AMPA receptors (63) and affects channel properties by reducing desensitization and slowing deactivation (64).

Figure 1.4

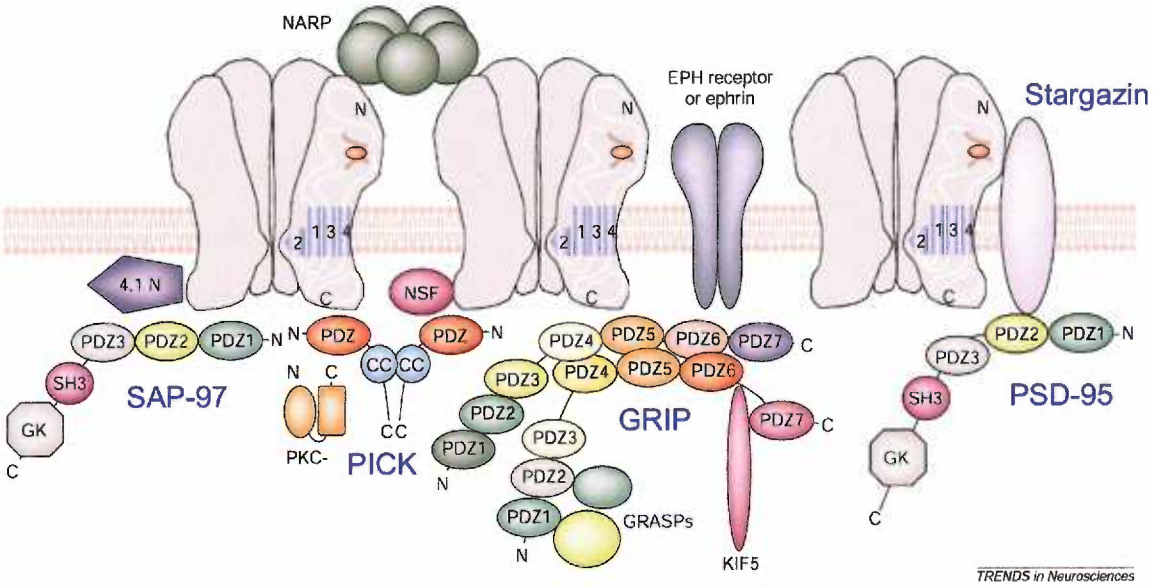
NARP
EPH receptor
or ephrin
Stargazin
4.1 N
NSF
PDZ3
PDZ2
PDZ1
SH3
GK
SAP-97
PDZ
CC
PKC-
PICK
PDZ4
PDZ5
PDZ6
PDZ7
GRIP
GRASPs
KIF5
PSD-95
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Figure 1.4 AMPA receptor-associated protein-interactions. GluR1 interacts with SAP-97 through PDZ interaction, while 4.1N binds to membrane proximal region of C-terminus. GluR1 is localized to synapses by stargazin-PSD-95 interaction. GluR2 C-terminus interacts with GRIP1, GRIP2 (ABP), PICK, and NSF. Only selected proteins of interest are reviewed in this thesis. Modified from (36).

Besides the two major regulatory phosphorylation sites in GluR1 (S831 and S845), numerous other regulatory phosphorylation sites have been identified in both long and short forms of AMPA receptor subunits (**Figure 1.3**). GluR2 contains two PKC phosphorylation sites, S863 and S880 (65), and GluR4 is phosphorylated by PKA, PKC, and CaM-KII at S842, and possibly at S830 by PKC (66). The functional role of GluR4 phosphorylation has not been identified, although GluR4-containing AMPA receptors can be delivered to synapses in activity- and NMDA receptor-dependent, but CaM-KII-independent, manner (67). Thus, it remains to be seen whether phosphorylation plays an important role during synaptic delivery of GluR4-containing AMPA receptors.

There are numerous data supporting the role of GluR2 S880 phosphorylation by PKC during LTD (68-71). S880 is within the PDZ interacting domain of GluR2 (SVKI). This domain interacts with scaffolding proteins, such as glutamate receptor interacting protein (GRIP1) (72), AMPA receptor binding protein (ABP; also called GRIP2) (73, 74), and protein interacting with C kinase (PICK1) (75, 76) (**Figure 1.3 and 1.4**). Furthermore, PKC phosphorylation of S880 decreases GluR2 interaction with GRIP1, but not with PICK1, and promotes AMPA receptor endocytosis (69). This mechanism is likely important for LTD expression in cerebellum (68, 71) and in hippocampus (70, 77). GluR2 also interacts with N-ethylmaleimide-sensitive factor (NSF) (78-80) and clathrin adaptor protein (AP2) (81) through the same site near the membrane proximal region in C-terminus (**Figure 1.3**). NSF is an ATPase involved in regulating membrane fusion events, such as synaptic vesicle exocytosis (82). Disruption of NSF-GluR2 interaction decreases synaptic AMPA receptor responses, suggesting that this interaction is involved in regulating exocytosis of GluR2-containing AMPA receptors to the surface membrane

(78, 80). By contrast, AP2 binds to the cytoplasmic domains of receptors and recruits clathrin, promoting the formation of clathrin-coated pits for endocytosis (83, 84). AP2-GluR2 interaction is involved in NMDA-dependent removal of AMPA receptors, likely through clathrin- (81) and dynamin-mediated (85) endocytosis, and is required for LTD. Thus, both exocytosis and endocytosis of AMPA receptors are critically important for regulating basal synaptic transmission and expressing bi-directional synaptic plasticity (39, 86-89).

1.5 BI-DIRECTIONAL SYNAPTIC PLASTICITY IN THE CA1 REGION OF HIPPOCAMPUS

The CA1 region of hippocampus has been extensively studied as a model system for synaptic plasticity, because excitatory synapses at this site have the ability to undergo bi-directional changes in synaptic strength (90, 91). In other words, the strength of synaptic transmission can be either potentiated (LTP) or depressed (LTD), depending on the pattern and strength of stimulus given and the signaling cascades activated. Acutely prepared hippocampal slices are most commonly used, where electrical stimulation of the Schaffer collateral/commissural afferents from the CA3 region results in postsynaptic potentiation or depression of AMPA receptor responses in the CA1 pyramidal neurons. Because electrical stimulation used in the field recordings is thought to only activate a small population of synapses, analyzing biochemical changes that occur in CA1 during LTP or LTD have been difficult, but possible (10, 11, 92). Thus, chemically induced plasticity may be preferable, in some cases, where whole population of neurons in a given preparation can be stimulated (93-97), although this method has the disadvantage of

losing selective activation of specific synaptic inputs and may not mimic the physiological mechanisms.

The postsynaptic membranes on the CA1 pyramidal neurons contain mainly two excitatory glutamate receptor channels, the NMDA and AMPA receptors. Both of these receptors are fast acting, glutamate-gated ion channels that mediate rapid excitatory synaptic transmission. Under basal conditions, NMDA receptors are not activated due to the voltage-dependent Mg^{2+} block. This Mg^{2+} block is removed when the postsynaptic cell is depolarized due to a strong AMPA receptor activation. Once activated, NMDA receptors are highly permeable to calcium, an important second messenger that can activate a variety of signaling cascades and is critical for synaptic plasticity (98-102). Thus, NMDA receptors act as "coincidence detectors," allowing calcium influx only when a presynaptic release of glutamate is coupled with a strong postsynaptic depolarization. Importantly, calcium can bind to calmodulin to activate CaMK-II (103-105) and CaM-sensitive adenylate cyclase (106) to activate PKA and induce synaptic plasticity. It is generally believed that calcium influx through the NMDA receptors modulates synaptic plasticity by activating specific kinases and phosphatases, as blocking NMDA receptors can prevent both LTP and LTD (107-110). Since the primary focus of this thesis is on the regulatory phosphorylation of GluR1, background will be limited to the two pertinent kinases, CaM-KII and PKA, which are involved in regulating GluR1 function during the early phase of LTP.

1.6 ROLE OF CaM-KII in LTP

CaM-KII is densely concentrated in the postsynaptic spine, where it plays an important role in modulating postsynaptic responses to neurotransmitter release (105, 111, 112). CaM-KII has unique characteristics that make it a viable candidate for decoding calcium signals. CaM-KII forms a multimeric structure composed of twelve monomers arranged in two hexameric rings. Each monomer is composed of catalytic, overlapping autoinhibitory, calmodulin binding, and subunit interaction domains. Under basal conditions, CaM-KII is inactive due to the intramolecular interaction between the autoinhibitory and the catalytic domains. As intracellular calcium concentration rises, calcium/calmodulin complex binds to CaM-KII monomers, freeing the catalytic domain from the autoinhibitory domain. CaM-KII is catalytically activated by this calcium/calmodulin binding and subsequently phosphorylates its substrates, including its autophosphorylation site at T286. This intersubunit autophosphorylation renders the kinase active, even when calcium levels return to basal levels, since autophosphorylated T286 prevents the interaction between autoinhibitory and catalytic domains (**Figure 1.5**) (113). Thus, autophosphorylated CaM-KII is calcium-independent and prolongs the calcium signal. Phosphorylated Thr286 is eventually dephosphorylated by protein phosphatase 1 (PP1) and protein phosphatase 2a (PP2a) (114), which terminates CaM-KII activity. Autophosphorylation of CaM-KII is an important component of hippocampal plasticity, since mice with a mutation at this site to block autophosphorylation (T286) has inhibited LTP and spatial learning in the Morris water maze (115). Interestingly, an *in vitro* study has shown that CaM-KII can also decode the frequency of calcium oscillations (116, 117).

An increasing amount of evidence suggests that CaM-KII is a critical regulator of synaptic plasticity in the hippocampus (3, 112). First, inducing LTP increases CaM-KII activity. Stimulation paradigms resulting in LTP causes a long lasting increase in both calcium-independent and total CaM-KII activity (118, 119). Second, blocking CaM-KII activity prevents the induction of LTP, although adding CaM-KII inhibitor after inducing LTP has no effect (103, 120-122). Third, LTP and CaM-KII-mediated synaptic potentiation share the same mechanisms. Expressing constitutively active form of CaM-KII in hippocampal slices potentiates synaptic transmission and occludes LTP, suggesting that CaM-KII is involved in signaling pathways that induce LTP (123). Furthermore, directly infusing constitutively active CaM-KII into CA1 pyramidal neurons enhances AMPA receptor-mediated currents, while occluding LTP (124). In the same experiment, previously induced LTP also occludes the synaptic potentiating effects of CaM-KII. Finally, CaM-KII mutant mice are deficient in hippocampal LTP (125) and show impaired spatial learning abilities (126).

Some recent studies also suggest that CaM-KII can be translocated to the dendritic spines, specifically to PSDs, in an activity-dependent manner (127, 128). Subsequently, CaM-KII binds to an NMDA receptor subunit, NR2B (129), locking CaM-KII in an active conformation (130). This translocation of CaM-KII to PSD is likely an important step leading to the phosphorylation of postsynaptic proteins, including GluR1, and provides a new mechanism through which CaM-KII can be activated in PSDs (131).

Figure 1.5

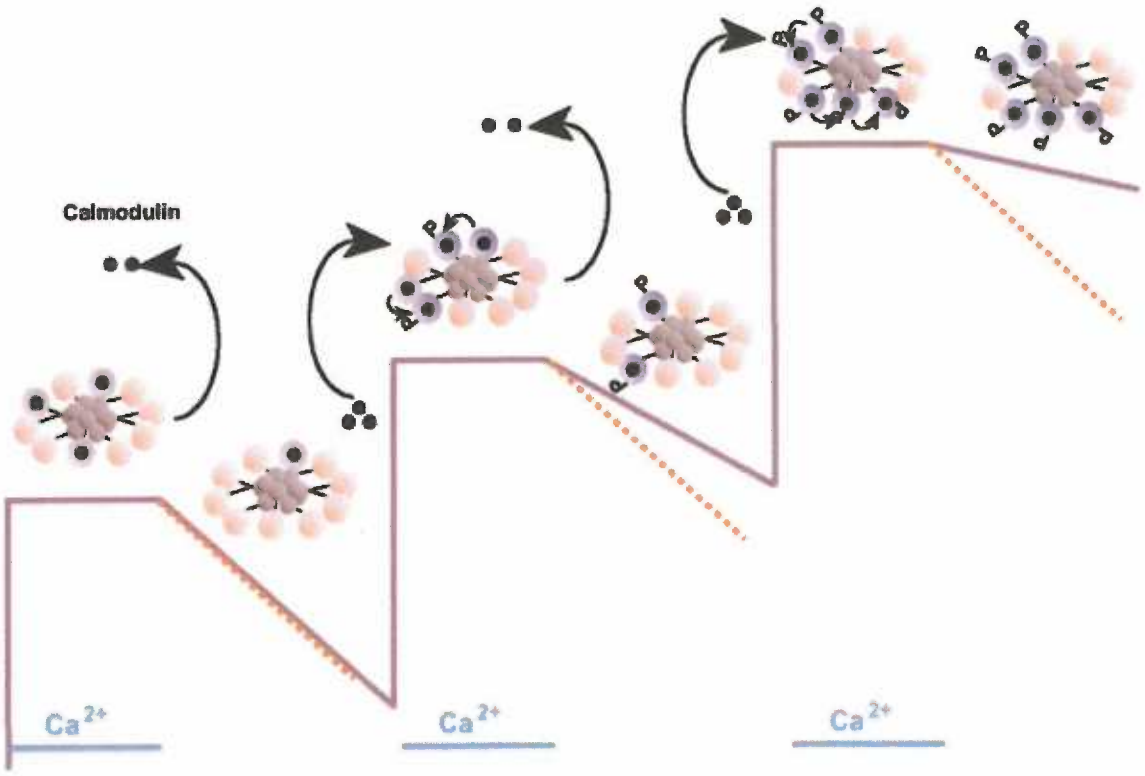


Figure 1.5 Calcium signal-decoding by multi-functional CaM-KII. Increase in intracellular calcium (blue bar) allows binding of calmodulin to CaM-KII (depicted as a decamer for simplicity), which activates CaM-KII. As calcium levels decline to baseline, some calmodulin dissociates from CaM-KII and kinase activity falls (kinase activity is denoted on the vertical scale), while one calmodulin remains bound. During second calcium signal, three more calmodulin binds, resulting in intersubunit autophosphorylation at T286 (denoted by P). This autophosphorylated CaM-KII retains more kinase activity (purple line) compared to non-phosphorylated kinase (dotted orange line). With another calcium transient, more subunits are autophosphorylated, increasing the kinase activity of CaM-KII. From (113) (figure was originally donated by Dr. Howard Schulman of Stanford University).

1.7 ROLE OF PKA IN LTP

Unlike CaM-KII, the direct role of PKA in the early phase of LTP is unclear. A study using an *in vitro* kinase assay to measure PKA activity showed that PKA is only transiently activated for 2 to 10 min after LTP-inducing stimulus (132). Other studies suggest that PKA activity is mainly required for the late phase of LTP, a phase that requires new protein synthesis (133-135). Thus, PKA activity seems to be required during the induction phase of LTP, where PKA may be activating substrates and other signaling pathways that are necessary for the maintenance and the late phase of LTP. Specific roles of PKA may include increasing new AMPA receptor synthesis (136), surface delivery of GluR1-containing AMPA receptors (60), and modulating the "priming" step of AMPA receptors for synaptic incorporation through GluR1 phosphorylation at S845 (58).

Studies also indicate that PKA can "gate" CaM-KII activity during LTP (137, 138). PKA can phosphorylate inhibitor-1 at T35, and phosphorylated inhibitor-1 blocks PP1 activity. Inhibition of PP-1 may allow CaM-KII to be persistently autophosphorylated, and thus, autophosphorylated CaM-KII activity is prolonged with concomitant PKA activation. Because the majority of protein phosphatases in the PSD is PP1 (139), PKA inhibition of PP1 through inhibitor-1 could significantly potentiate CaM-KII activity in dendritic spines. PKA gating CaM-KII activity during LTP was confirmed in a study where postsynaptic injection of thiophosphorylated inhibitor-1 blocked the ability of PKA inhibitors to suppress LTP (137). This reversal of LTP inhibition correlates with increased autophosphorylation and calcium-independent activity of CaM-KII, suggesting that inhibitor-1 blocked PP1. Furthermore, transgenic mice expressing

inducible inhibitor-1 has a lower threshold for learning and memory with higher CaM-KII autophosphorylation when the inhibitor-1 gene is turned on (140). These results suggest that PKA and CaM-KII pathways exert crosstalk during LTP expression.

The ability of PKA to directly potentiate synaptic transmission during the early phase of LTP is controversial, however, since PKA antagonists do not seem to affect this phase of LTP (133-135). However, many studies do support synaptic potentiating effects of PKA in the hippocampus. For example, a study has shown that application of forskolin, an activator of adenylate cyclase, can potentiate AMPA receptor-mediated currents in cultured hippocampal neurons by increasing open probability and mean open time (16). In another study, forskolin treatment in hippocampal slices led to an increase in mEPSC frequency, but not amplitude (141). Although this increase in mEPSC frequency could be due to presynaptic mechanisms, it is unlikely since the majority of evidence suggests that presynaptic potentiating mechanisms do not exist at the CA3/CA1 synapses (142, 143). Other cell-permeable activators of PKA or direct infusion of catalytically active PKA can also increase kainate-induced whole-cell currents in cultured hippocampal neurons (144). This potentiation may be due to direct phosphorylation of GluR1 (15) or GluR6, kainate receptor subunit (145, 146).

1.8 REGULATION OF AMPA RECEPTOR FUNCTION BY GLUR1 PHOSPHORYLATION DURING SYNAPTIC PLASTICITY

GluR1 function is modulated by two different mechanisms: phosphorylation and delivery of new receptors to synaptic membrane (3, 31, 32, 143). Interestingly, CaM-KII can modulate GluR1 function by using both of these mechanisms (**Figure 1.6**). Direct

phosphorylation of GluR1 at S831 by CaM-KII increases the single-channel conductance, thereby increasing the excitatory postsynaptic responses (12-14, 147). Inducing LTP in hippocampal slices is coupled to persistent increase in S831 phosphorylation, becoming significant around 15 min after induction and persisting up to 60 min (10, 11). S831 phosphorylation and LTP is blocked with general CaM-Kinase inhibitors, suggesting that CaM-KII can directly phosphorylate GluR1 during LTP. Depotentiation, a reversal of LTP, is correlated with a decrease in S831 phosphorylation, while having no effect on S845 phosphorylation (11, 148). By contrast, LTD is associated with a decrease in S845 phosphorylation, with no change in S831 phosphorylation (11, 93, 94). Furthermore, de-depression, a reversal of LTD, is associated with a selective increase in S845 phosphorylation, suggesting that the two phosphorylation sites on GluR1 are differentially regulated during bi-directional synaptic plasticity (11). Thus, CaM-KII seems to be involved in potentiating GluR1 responses, while PKA maintains the strength of basal transmission. From these results, a model of GluR1 phosphorylation during bi-directional synaptic plasticity has been proposed (**Figure 1.7**).

Figure 1.6

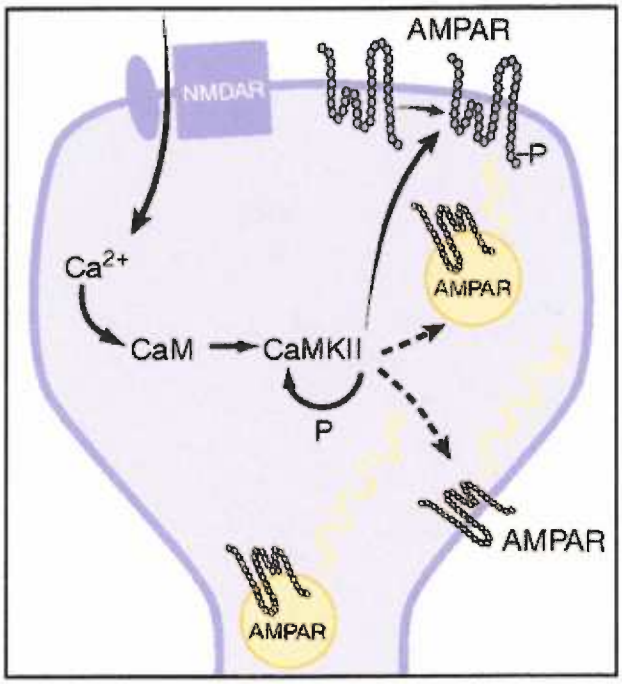


Figure 1.6 CaM-KII potentiates postsynaptic AMPA receptor responses by two different mechanisms. Calcium influx through the NMDA receptors during induction of LTP protocol binds to calmodulin (CaM) and activates CaM-KII. Binding to calcium/calmodulin leads to autophosphorylation of CaM-KII, allowing it to become persistently activated. CaM-KII can then directly phosphorylate GluR1 at S831, thereby enhancing single-channel conductance, or drive AMPA receptors into synapses. The exact substrate and mechanism of the latter step is unknown. Modified from (3).

Figure 1.7

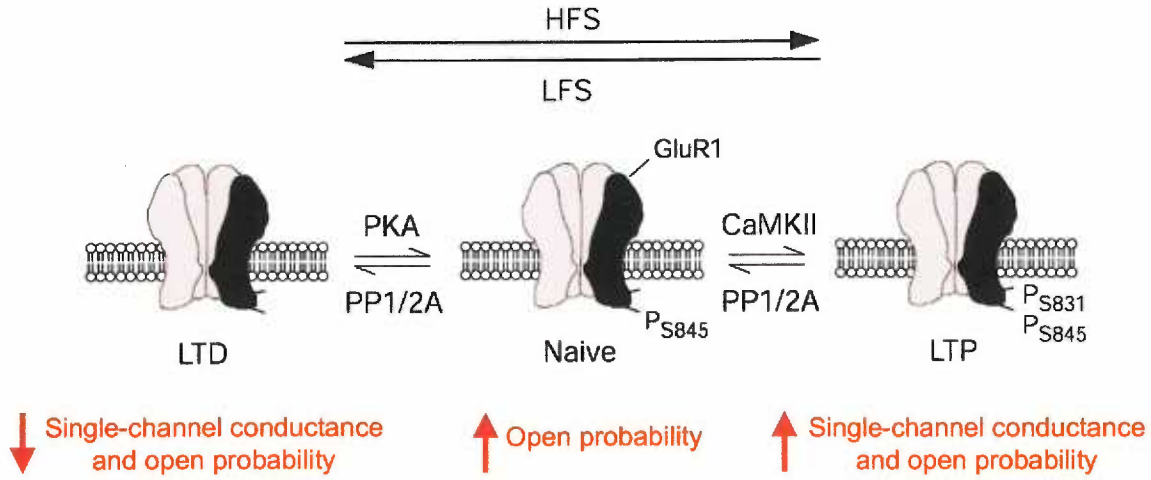

HFS
LFS
GluR1
PKA
PP1/2A
CaMKII
PP1/2A
P_{S845}
P_{S831}
P_{S845}
LTD
Naive
LTP
Single-channel conductance and open probability
Open probability
Single-channel conductance and open probability

Figure 1.7 The current model of GluR1 phosphorylation during bi-directional synaptic plasticity. In basal conditions (Naive), GluR1 is proposed to have high S845 phosphorylation. Induction of LTP with high-frequency stimulation (HFS) leads to the activation of CaM-KII, resulting in phosphorylation of S831 and enhanced single-channel conductance. During LTD induction with low-frequency stimulation (LFS), PP1 and PP2A are activated resulting in dephosphorylation of S845, which decreases open probability of the channel. Depotentiation and de-depression are the reverse of these processes. Modified from (11).

New GluR1 can be delivered to synapses in an activity-dependent manner (40-42, 149), likely though exocytosis of GluR1-containing vesicles (95, 150). This activity-dependent delivery requires NMDA receptor activation, and may be the mechanism by which silent synapses are activated (97). However, NMDA receptor mediated synaptic potentiation seems to occur only when synaptic NMDA receptors are activated, since activation of extrasynaptic NMDA receptors by bath application of NMDA induces S845 dephosphorylation (93, 151), GluR1 endocytosis (152), and LTD (94). The differences in signaling cascades activated from these two different populations of NMDA receptors and why they modulate synaptic strength differently remain unanswered.

CaM-KII activity is required for delivering new GluR1 receptors to the postsynaptic membrane during LTP, although this does not require a direct phosphorylation of GluR1 at S831 (**Figure 1.6**) (42). Instead, the PDZ-interacting domain of GluR1 (ATGL) is required, since mutating this domain (AAGL) prevents CaM-KII-mediated synaptic potentiation and LTP (42). Furthermore, only GluR1-containing GluR1/GluR2 heteromers are delivered in an activity-dependent manner, while GluR2/GluR3 heteromers continuously replace the existing receptors (41). Results also indicate that GluR1 is inserted at extrasynaptic sites under basal conditions, while GluR2 is rapidly inserted more directly near synapses (40). The substrates of CaM-KII involved in GluR1 delivery are still unknown.

The role of PKA during GluR1 delivery to synapses is yet to be defined. However, recent studies indicate that S845 phosphorylation is involved in regulating the surface delivery of GluR1 (58, 60). Furthermore, PKA can be localized to the PSDs through its interaction with AKAP79/150, and this localization of PKA to PSDs is

important for modulating AMPA receptor function (153). PKA/AKAP interaction may even promote GluR1 phosphorylation, since SAP-97, a member of the PDZ-containing scaffolding protein, can bind to both GluR1 and AKAP79/150, and thus, co-localize PKA with GluR1 (57). However, there is some controversy regarding the ability of PKA to phosphorylate GluR1 directly, since PKA is not able to directly phosphorylate GluR1 in certain conditions (5, 6, 154). In addition, PKC may phosphorylate GluR1 at S831 (8, 9), but the extent and functional consequence of this phosphorylation in neurons is unknown.

The importance of GluR1 subunit during hippocampal plasticity is clearly exemplified by the GluR1 knockout mice. LTP is completely absent in the GluR1 knockout mice (155, 156), even though the spatial learning in the Morris water maze is normal (156). However, knockout mice have specific hippocampal spatial working memory impairments, such as in spatial discrimination tasks (157, 158), spatiotemporal context learning (159), and spatial reference learning (160, 161). Interestingly, restoration of GluR1 in the knockout mice restored hippocampal LTP, suggesting that mature hippocampal neurons have the capacity to express GluR1-dependent LTP even in the absence of GluR1 during development (155) and emphasizes the importance of GluR1 in hippocampal synaptic plasticity..

1.9 AMPA RECEPTOR SUBUNIT RECOMPOSITION

A recently identified mechanism for the activity-dependent changes in synaptic strength is AMPA receptor subunit recomposition. Under basal conditions, parallel fiber synaptic inputs to stellate cells in cerebellum are primarily mediated by highly calcium-permeable AMPA receptors, suggesting that these receptors lack edited GluR2 subunits.

Upon 50 Hz repetitive synaptic activation, calcium-permeable AMPA receptors are rapidly and persistently replaced by calcium-impermeable AMPA receptors in cerebellar stellate cells (53). New receptors are GluR2-containing AMPA receptors since they are low in calcium permeability with linear rectification. Thus, calcium influx through N-type calcium channel can drive GluR2-containing AMPA receptors into these synapses, resulting in a switch of subunit composition. Subunit switch from GluR2-lacking receptors to GluR2-containing receptors can also occur by increased spontaneous synaptic activity (52). Thus, activity-dependent AMPA receptor subunit recomposition may represent a potential mechanism of changes in synaptic strength during synaptic plasticity.

Several other studies support this hypothesis. Over-expression of PICK1, GluR2-interacting PDZ protein, in CA1 hippocampal neurons results in increased AMPA receptor rectification and sensitivity to polyamines, suggesting that synaptic GluR2-containing AMPA receptors are removed (162). AMPA receptor responses are increased with PICK1 over-expression, and immunocytochemistry experiments suggest that GluR2 surface receptors are decreased, with no change in GluR1 surface expression. Thus, PICK1 is able to cause selective removal of the GluR2 subunits from the surface membrane. This increases the strength of synaptic transmission, likely due to the fact that GluR2-lacking AMPA receptors have higher single-channel conductance compared to GluR2-containing receptors (**Figure 2.6**) (25). This new form of synaptic plasticity, termed calcium-permeable AMPA receptor plasticity (CARP), seems to involve both PICK1 and NSF (163). An important question during CARP, however, is how does phosphorylation of the GluR1 subunit regulate channel properties during subunit

recomposition. The effect of GluR1 phosphorylation on channel properties were only studied for homomeric GluR1 receptors (13-15), and thus, these studies need to be extended for the heteromeric AMPA receptors.

1.10 AMPA RECEPTOR TRAFFICKING BY LATERAL DIFFUSION

Ever since AMPA receptor trafficking to and from the synaptic locations was first hypothesized as a possible mechanism to modify the strength of synaptic transmission (164), direct exocytosis and endocytosis have been considered as key mechanisms regulating trafficking (32, 149, 165-167). After exocytosis, AMPA receptors are thought to be stabilized in PSDs by interacting with cytoskeletal elements through scaffolding proteins (37, 168). However, more recent data suggest that synaptic AMPA receptors are not stable, but rapidly diffuse in and out of synapses and even diffuse within synapses (169, 170). Thus, lateral diffusion of extrasynaptic AMPA receptors into synapses has been proposed as another mechanism for synaptic incorporation of AMPA receptors (171-173).

Although there is no direct evidence so far showing that AMPA receptors are synaptically incorporated by lateral diffusion during LTP, recent data suggest lateral diffusion, rather than direct exocytosis at synapses, as a more likely mechanism to increase AMPA receptor numbers at synapses. Several lines of evidence support this hypothesis. First, PSDs are detergent-insoluble regions, consisting of dense concentrations of scaffolding and cytoskeletal proteins. Exocytosis of new receptors directly at membrane regions lined with such a dense population of proteins seems highly unlikely, and direct evidence of a vesicle undergoing exocytosis in the PSD is lacking. In

support of this hypothesis, GluR1-containing AMPA receptors, which are delivered to the surface membrane in an activity-dependent manner, are first delivered to extrasynaptic locations, followed by slower synaptic incorporation (40).

Second, synaptic receptors reside in two different populations: synaptic and extrasynaptic (149, 174-177). Both AMPA and NMDA receptors are found evenly distributed along the dendritic membrane before synaptogenesis, followed by clustering of receptors at synaptic sites during synaptogenesis (178). Although the delivery mechanism of AMPA and NMDA receptors to synapses during synaptogenesis is not well understood, the persistence of extrasynaptic receptors following synaptogenesis implies that these receptors serve an important function, possibly as a reserve pool that can be rapidly mobilized to synapses by lateral diffusion. Since synapses occupy only 1-2% of a hippocampal neuron's total surface area (179), extrasynaptic receptors likely make up a significant portion of the total receptor pool.

Third, synaptic receptors are not permanently anchored within a synapse. Most studies in the past have relied on immunocytochemistry to track localization of receptors on the surface membrane. This method gives information in a single point in time and cannot reflect the dynamics of receptors on the surface membrane, including synaptic receptors. Recent advancements in molecule imaging techniques have allowed real time tracking of single receptors. Nicotinic acetylcholine receptors at neuromuscular junctions were the first receptors proposed to cluster at junctional zones by lateral diffusion from nonjunctional zones (180, 181). Since then, studies have shown that synaptic receptors in the central nervous system diffuse within a synapse and can also diffuse between multiple synaptic sites by lateral diffusion (169, 170, 182, 183). Fluorescent tracking of single

AMPA receptors, labeled with latex beads coated with GluR2 antibody, shows rapid diffusion on the surface membrane of cultured hippocampal neurons. Diffusion of surface AMPA receptors decrease with maturation, in parallel with synaptogenesis, suggesting that the formation of synapses stabilizes laterally diffusing AMPA receptors (182). One limitation of this study is that the latex beads are too large (500 nm) to enter synaptic clefts. However, more recent imaging using the single-molecule fluorescence imaging approach (184), where a small proportion of surface AMPA receptors are labeled with Cy5- or Alexa-647-tagged GluR2 antibody, have confirmed rapid dynamics of extrasynaptic and synaptic AMPA receptors (169, 170). In fact, extrasynaptic AMPA receptors diffuse quite freely, reaching Brownian movements, and about half of synaptic AMPA receptors are mobile, and not permanently anchored (170). Furthermore, bath application of glutamate, a treatment known to mimic LTD by decreasing surface AMPA receptors (85, 165, 166, 185, 186), causes an increase in diffusion of synaptic AMPA receptors, decrease in proportion of immobile synaptic receptors, and an increase in the proportion of receptors in the surrounding region of synapses. These results all suggest that synaptic receptors become more mobile and diffused out of synapses into perisynaptic regions by lateral diffusion during glutamate-induced LTD (170). Thus, the traditional views of AMPA receptor "stabilization" and "concentration" in synapses and the concept that receptors are "stably anchored" at synapses clearly need to be reconsidered.

Besides AMPA receptors, other receptors, such as the glycine receptors (187), mGluRs (183), and NMDA receptors (188), diffuse freely in and out of synapses from extrasynaptic sites on the surface membrane. Glycine receptors, a major inhibitory

receptor in the spinal cord, are composed of three α and two β subunits that are highly localized in microdomains of receptor clusters. These microdomains of receptors are stabilized by β subunit interaction with a cytoplasmic scaffolding protein, gephyrin. The glycine receptor subunit α1 diffuse freely on the surface membrane when expressed alone or with gephyrin in COS7 cells or cultured spinal cord neurons (187). However, chimeric α1 subunit, containing the gephyrin binding domain of the β subunit, forms clusters that colocalize with gephyrin clusters. More importantly, studies that photographically tracked a single glycine receptor show that gephyrin clusters are able to confine freely diffusing glycine receptors on the surface membrane. Homer, the scaffolding protein for mGluRs, has a similar function as gephyrin in stabilizing mGluR5 on the surface membrane of cultured hippocampal neurons (183). Moreover, Homer-mGluR5 stabilization is reversible, because mGluR5 diffuse in and out of Homer clusters at fast rates. These results suggest that synaptic receptors are not permanently anchored, but are dynamic in their movements and in their interactions with cytoplasmic scaffolding proteins. The exchange of receptors from a freely diffusing extrasynaptic state to a confined synaptic state likely represents a physiological mechanism of receptor clustering at synapses.

Fourth, activating signaling cascades that are strongly correlated with synaptic potentiation has been shown to stabilize rapidly diffusing AMPA receptors on the surface membrane. Raising local intracellular calcium by photo-release of caged calcium results in local accumulation of AMPA receptors on the surface membrane, and this is correlated with an abrupt decrease in the diffusion coefficient of labeled, single AMPA receptors (182). Uncaging glutamate extracellularly has smaller, but similar effect of immobilizing

surface AMPA receptors. The results from this study strongly support the hypothesis that synaptic activity, resulting in increase in intracellular calcium, is necessary to immobilize laterally diffusing receptors at synaptic sites.

And finally, removal of surface receptors by endocytosis occurs in areas away from PSDs. Postsynaptic membrane, lined by PSDs, only make up 10-15% of the dendritic spine membrane (189). In recent studies, endocytosis has been localized to the other 85-90% of membrane making up the dendritic spine. Electron microscopy of the distribution of clathrin, AP2, and dynamin, the key components of endocytosis, shows that these molecules are localized to the lateral regions of dendritic spines, away from PSDs (190, 191). Clathrin assembly and disassembly occurs rapidly in "hot spots" in dendrites and at the tips of dendritic filopodia in young neurons (192). In mature neurons, clathrin localizes to lateral portions of dendritic spines, where it is completely exclusive with PSD-95 localization. In agreement with these data, endocytosis of AMPA receptors occurs first in the extrasynaptic regions followed by a decrease in synaptic AMPA receptors, suggesting that receptors are removed from synapses by lateral diffusion (193). Thus, these results together imply that removal of synaptic AMPA receptors during LTD may be a reverse of synaptic AMPA receptor delivery during LTP.

1.11 HYPOTHESES

What are the roles of GluR1 S831 and S845 phosphorylation in regulating AMPA receptor channel properties and number in synapses during synaptic plasticity? This was the main question examined in this thesis. Specifically, what functional role does S831 phosphorylation by CaM-KII play in context of GluR1/GluR2 heteromers, and what

functional role does S845 phosphorylation play in the context of delivery of AMPA receptors to synapses during LTP? These two questions are separately addressed in the following two chapters (**Chapter 2 and 3**).

Functional changes in channel properties through direct phosphorylation have been studied for GluR1 homomers (13-15). However, recent studies suggest that subunit recomposition may be responsible for changing synaptic strength. Thus, further examination of heteromeric receptor properties in response to direct phosphorylation is needed. Moreover, GluR1/GluR2 heteromers make up the majority of GluR1-containing AMPA receptors in the hippocampus (26) and thus, understanding how these receptors are modulated by phosphorylation will be critical for deciphering the molecular mechanisms of synaptic plasticity.

Many phosphorylation sites were initially characterized by 2-D phospho-peptide mapping, non-quantitative *in vitro* ^{32}P-incorporation kinase assays, or immunoblotting with phospho-specific antibodies. These are all very sensitive but non-quantitative ways to determine protein phosphorylation. Thus, it is not clear whether all identified phosphorylation sites in the AMPA receptor subunits (**Figure 1.3**) are significantly phosphorylated in neurons. Without this knowledge, phosphorylation-functional effect relationship cannot be fully comprehended. For example, a poor phosphorylation site with basal phosphorylation of 1-2% may be phosphorylated two fold, which can be easily detected by a very sensitive phospho-specific antibody. Although this two fold increase in phosphorylation may be statistically significant, the functional effects of this phosphorylation (i.e. 2-4% net phosphorylation) is likely insignificant. Therefore, it is critical to quantify the net phosphorylation changes to ensure that the physiological

mechanisms being studied are actually due to the changes in phosphorylation state. One of the main goals in this thesis was to develop an assay to quantify the net phosphorylation at S845 in GluR1 subunit during bi-directional synaptic plasticity, and use this information to correlate how S845 phosphorylation affects surface delivery of GluR1-containing AMPA receptors. This new approach to studying phosphorylation-functional effect relationship will likely create new ideas and views about examining the molecular mechanisms involved in synaptic plasticity.

CHAPTER 2

DOMINANT ROLE OF THE GLUR2 SUBUNIT IN REGULATION OF AMPA RECEPTORS BY CAM-KII

Oh and Derkach. ***Nat Neurosci*** **(2005), in press.**

2.1 ABSTRACT

GluR1 and GluR2 subunits compose AMPA receptors in the mature hippocampus, and both GluR1 subunit and CaM-KII are required for synaptic plasticity, memory and learning. In this study, we investigated how the presence of GluR2 subunit in the receptor modulates its functional properties and the regulation by CaM-KII. Strikingly, while GluR1 phosphorylation by CaM-KII was preserved, the functional regulation of AMPA receptors by phosphorylation was lost in the presence of GluR2 subunit. Therefore, our findings define a previously unknown, dominant role of GluR2 subunit for the signaling mediated by CaM-KII at AMPA receptors.

2.2 INTRODUCTION

In the mature brain, LTP of synaptic efficacy in the CA1 region of hippocampus and several forms of learning and memory require the GluR1 subunit of the AMPA receptor and CaM-KII (115, 159, 161, 194). Activated CaM-KII increases the trafficking of AMPA receptors at excitatory glutamatergic synapses (31, 32, 42, 58) and phosphorylates S831 in the C-terminus of GluR1 subunit (7-11). S831 phosphorylation enhances the single-channel conductance of GluR1 homomers (13, 14) and this tightly correlates with the increased channel conductance of synaptic AMPA receptors during LTP (12, 31). Thus, S831 phosphorylation is considered as one of several molecular mechanisms contributing to LTP (3, 112). AMPA receptor single-channel conductance is also altered by edited GluR2 subunit (25), and mature hippocampal AMPA receptors are heteromers composed of mainly GluR1 and GluR2 subunits (26). Therefore, we investigated the role of GluR2 subunit in the regulation of GluR1/GluR2 heteromeric AMPA receptors (GluR1/GluR2 heteromers), and thus synaptic strength, by CaM-KII.

2.3 MATERIALS AND METHODS

Cultured Hippocampal Neurons

Hippocampal neurons were cultured from postnatal day 1-2 Sprague-Dawley rats as previously described (195, 196). Animal use and procedures were reviewed and approved by the Institutional Animal Care and Use Committee and the Department of Comparative Medicine at Oregon Health & Sciences University. Hippocampi were dissected in ice cold dissecting media (1x Hank's buffer supplemented with 50 mM HEPES, pH 7.4; Gibco) and cut into 1-2 mm pieces. Hippocampal pieces were digested with 2 mg/mL papain (Worthington) in the dissecting media for 20 min at 37°C and triturated using a sterile polished glass pipette (0.8-1 mm pore diameter). Dissociated hippocampal cells were plated on 6-well plates (35 mm well diameter), precoated with 1 mg/mL poly-L-lysine (Sigma), in plating media (Neurobasal A media supplemented with 2% B-27 and 2 mM GlutaMAX^{TM}-I supplement; Gibco) at a density of 4 x 10^5 cells per well. 3-4 hours after the initial plating, the media was replaced with fresh pre-warmed plating media. After 4 days *in vitro* (DIV), 2.5 μM cytosine-β-D-arabino furanoside was added to the plating media. Cultures were fed twice a week by replacing half of the media with fresh plating media and used for experiments at 13-15 DIV.

Glycine-Induced Synaptic Potentiation (GISP)

We used GISP paradigm (197, 198) to induce synaptic plasticity in cultured hippocampal neurons and to assay GluR1 S831 phosphorylation in endogenous GluR1 homomers and GluR1/GluR2 heteromers. Prior to GISP, the cultures were incubated for 30 min in the extracellular control solution (in mM): 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 2

$CaCl_2$, 33 D-glucose, 0.02 D-APV (NMDA-R blocker), 0.003 strychnine (inhibitory glycine receptor blocker), 0.02 bicuculline ($GABA_A$ receptor blocker), 0.0005 TTX (to block action potentials), and 25 HEPES, pH 7.3. GISP was induced by treating cultures for 10 min with the same control solution as above, but with glycine (100-200 μM) and without TTX, Mg^{2+} and D-APV. After glycine treatment, cultures were returned to the control solution for electrophysiology, or immediately scraped in homogenization buffer for Western blotting (see below).

Immunoprecipitation and Western Blotting

For biochemical analysis of endogenous AMPA receptors, cultured hippocampal neurons were stimulated by GISP, followed by immunoblotting with the S831 phospho-specific antibody (UBI). Cells were scraped immediately after GISP in cold 1% Triton X-100 homogenization buffer (in mM): 50 NaCl, 10 EDTA, 10 EGTA, 1 Na_3VO_4, 50 NaF, 25 NaPPi, 1 β-glycerophosphate, 1 PMSF, 0.001 microcystine, 1 protease inhibitor cocktail tablet per 50 mL (Roche), and 50 HEPES, pH 7.5. 300 μL of the homogenization buffer was used per well, and samples from 2 wells were combined for each condition. Scraped cells were sonicated for 20 min in ice-cold water bath and spun at 10,000g for 20 min. GluR1/GluR2 heteromers in the supernatant were immunoprecipitated by incubating with 4 μg of anti-GluR2 antibody (Santa Cruz) for 1 hour on ice, followed by 3 hour incubation with 40 μl of 50% Protein G Sepharose 4 Fast Flow (Amersham) on a rocker at 4°C. After the initial spin, the supernatant was saved and the precipitated pellet was rinsed four times with the homogenization buffer. This resulted in complete immunodepletion of GluR2 from the supernatant (**Figure 2.3a**), suggesting that AMPA

receptors remaining in the supernatant were GluR2-lacking. Preliminary blots were run to normalize for differences in GluR1 amount between precipitate and supernatant (data not shown). Normalized volumes of samples were resolved in 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted using infra-red dye-coupled secondary antibodies (anti-rabbit IgG Alexa Fluor 680, Molecular Probes; anti-mouse IgG IRdye800, Rockland). Image acquisition and data quantitation were performed on the Odyssey Infrared Imaging System (Li-Cor). Relative phosphorylation for each receptor composition and condition was calculated by normalizing the phospho-S831 readings to the total GluR1 protein. Primary antibodies used were: anti-phospho-S831-GluR1 (UBI), anti-GluR1 (UBI), anti-GluR2 (Santa Cruz), anti-phospho-Thr286-CaM-KII (ABR), and anti-CaM-KII (ABR).

To analyze S831 phosphorylation of recombinant GluR1/GluR2 heteromers expressed in HEK293 cells, we followed the same immunoprecipitation protocol described above using the anti-GluR2 antibody. For GluR1 homomers, supernatant from cells transfected with GluR1 subunit alone was used. Phosphorylation of S831 was analyzed for each receptor type with and without co-expressing constitutively active CaM-KII (H282R mutant).

Electrophysiology

AMPA receptor miniature excitatory postsynaptic currents (mEPSCs) were recorded in the extracellular control solution indicated above. Cs^{2+}-based intracellular solution was used to block majority of K^+ conductance (in mM): 100 Cs-methanosulfonate, 25 $CsCl_2$, 2 $MgCl_2$, 0.4 EGTA, 4 ATP, 0.4 GTP, 10 phospho-creatine,

and 10 HEPES, pH 7.3. Input and serial resistances (<10-15 MΩ, 70-80% compensated) were monitored throughout the experiments, and cells with deviations >15% were discarded. Individual mEPSCs were detected using the template algorithm (AxoGraph 4.0, Axon Instruments), and their representative amplitudes and kinetic properties were determined based on averaged mEPSC (200-300 currents averaged) and from corresponding cumulative distributions. These measurements were made every 5 min of spontaneous activity to monitor changes in activated synapses.

HEK293 cells were transfected 24-36 hours after plating. Currents from all types of receptors were recorded either in whole-cell (cells lifted from the bottom) or outside-out patch-clamp configurations. Glutamate (10 mM, 100 ms pulses, 5 s interval) was delivered to receptors either by a piezo-driven application system or puffing, as described previously (13). Homomeric GluR1 and GluR2 receptors (flip isoforms) were recorded by expressing these subunits alone. Because edited GluR2 homomers produced very small currents (**Figure 2.4d**) (199), we increased the concentration of GluR2 cDNA during transfection by four fold. To obtain GluR1/GluR2 heteromers, GluR1 and GluR2 cDNAs were co-transfected at a ratio of 1:1 (0.8 μg of total cDNA per 35 mm well) using Lipofectamine 2000 transfection kit (GibcoBRL). Extracellular solution for HEK293 cell recordings contained (in mM): 155 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 D-glucose, and 5 HEPES, pH 7.3. Intracellular solution was same as above. Currents were recorded at 2 kHz bandwidth and digitized at 20 kHz.

Non-Stationary Fluctuation Analysis (NSFA)

Because single-channel conductance of GluR1/GluR2 heteromers and GluR2 homomers was very low (**Figure 2.6**) (25), a reliable single-channel or silence analyses were not feasible (14), and therefore, we used NSFA as a principle approach to measure channel properties throughout all experimental conditions in HEK293 cells and for all types of receptors tested. NSFA was performed essentially as described (13, 14). The quality of individual registrations and fitting were tested as described (15), and 31-78 currents were selected for each measurement. All statistics were evaluated by Student's two-tailed *t*-test, and all data are presented as mean ± s.e.m. ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

2.4 RESULTS

We first determined whether CaM-KII can regulate GluR1/GluR2 heteromers. Surprisingly, infusion of activated CaM-KII to GluR1/GluR2 heteromers expressed in HEK293 cells failed to enhance their currents (**Figure 2.1a**). This was in striking contrast to the potentiation of GluR1 homomers by CaM-KII (13) (**Figure 2.1b**). One possibility for the lack of GluR1/GluR2 heteromer regulation by CaM-KII is that GluR1-GluR2 interactions prevent S831 phosphorylation by CaM-KII. First, we tested this hypothesis for endogenous AMPA receptors in cultured rat hippocampal neurons by using glycine-induced synaptic potentiation (GISP), which induces NMDA receptor and CaM-KII-dependent long-term increase in synaptic strength (**Figures 2.2a,b**). GISP was associated with long-lasting changes (1-3 hours) in frequency and amplitude of mEPSCs (**Figure 2.2c**) without affecting the kinetics (**Figure 2.2b**) (95). GISP shared many key properties with hippocampal CA1 LTP (**Table 2.1**). We selectively precipitated GluR1/GluR2 heteromers using GluR2 specific antibody (**Figure 2.3a**). Because of complete immunodepletion of GluR2 from the supernatant (**Figure 2.3a**), non-precipitated GluR1 were homomeric or in complexes with GluR3 or GluR4 subunits (26). S831 phosphorylation was increased in both receptor populations to the same extent (**Figures 2.3a-b**) and tightly correlated with CaM-KII activation (**Figure 2.3c**).

Figure 2.1

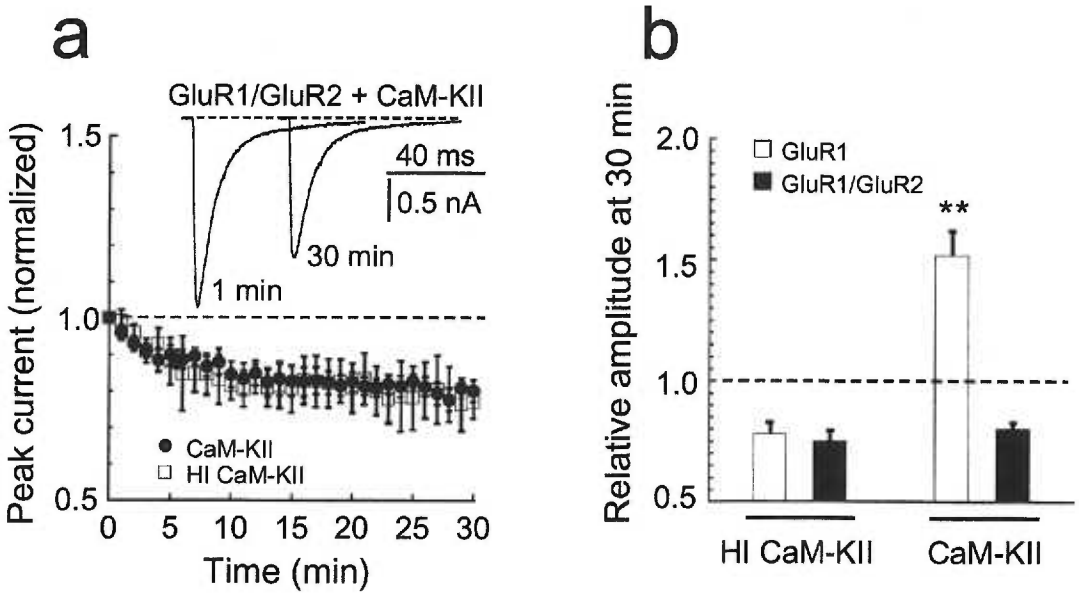

a
GluR1/GluR2 + CaM-KII
40 ms
0.5 nA
30 min
1 min
Peak current (normalized)
1.5
1.0
0.5
CaM-KII
HI CaM-KII
0
5
10
15
20
25
30
Time (min)
b
Relative amplitude at 30 min
2.0
1.5
1.0
0.5
GluR1
GluR1/GluR2
**
HI CaM-KII
CaM-KII

Figure 2.1 GluR2 subunit eliminated the regulation of GluR1/GluR2 heteromers by CaM-KII. (**a**) Intracellular infusion of activated CaM-KII did not potentiate glutamate-induced currents of GluR1/GluR2 heteromers expressed in HEK293 cells (n = 11) compared to the heat-inactivated CaM-KII (HI, control). Insert represents currents of GluR1/GluR2 heteromers evoked by 10 mM glutamate at 1 and 30 min after infusion of activated CaM-KII. (**b**) In contrast, activated CaM-KII did potentiate GluR1 homomers (13) (** $P < 0.01$, $n = 16$, Student's two-tailed t-test.).

Figure 2.2

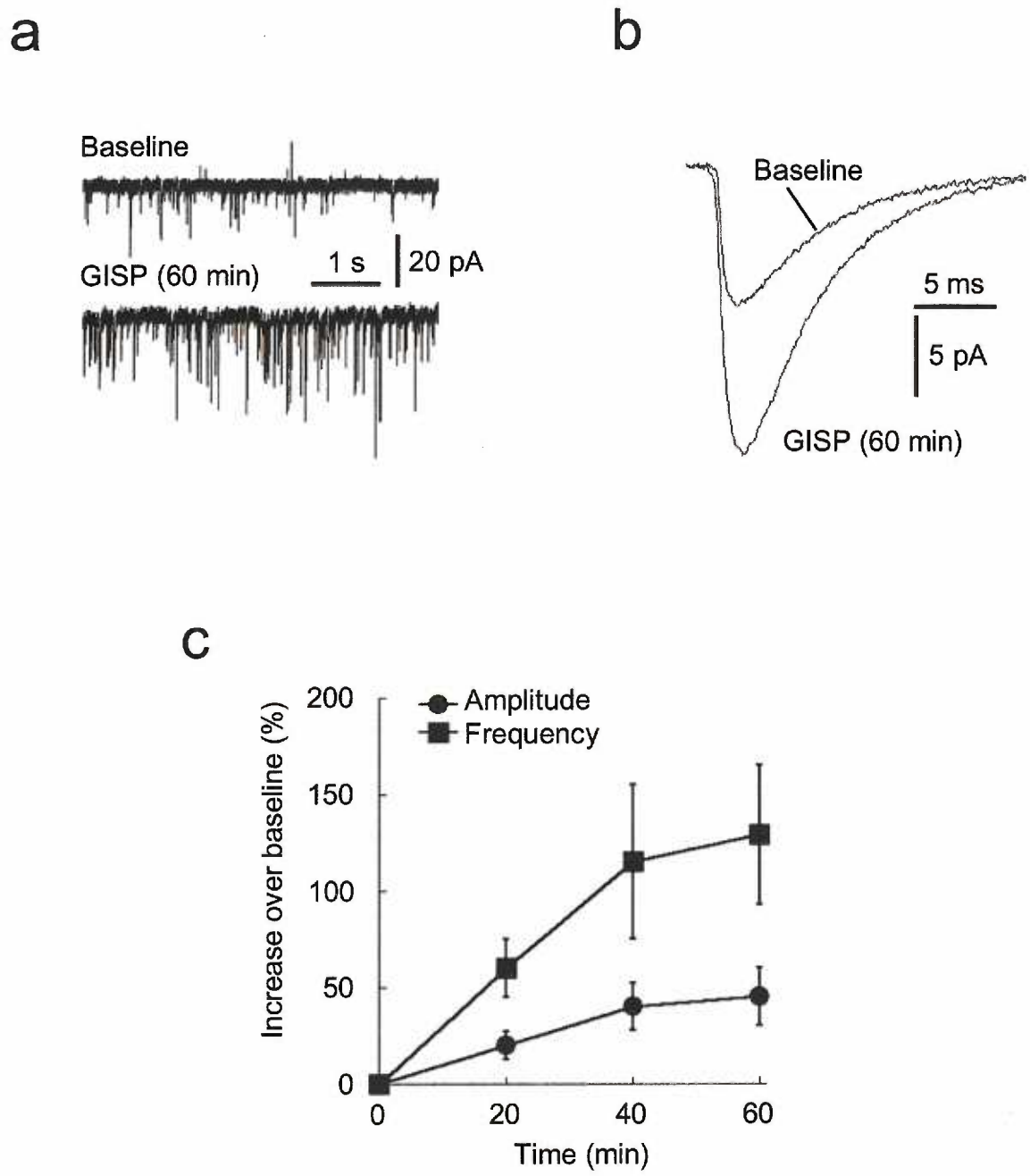

a
Baseline
GISP (60 min)
1 s
20 pA
b
Baseline
5 ms
5 pA
GISP (60 min)
c
Amplitude
Frequency
Increase over baseline (%)
200
150
100
50
0
0
20
40
60
Time (min)

Figure 2.2 GISP evoked a long-lasting potentiation of AMPA receptor mEPSCs in cultured hippocampal neurons. (**a**) Comparison of synaptic activity before (Baseline) and 60 min after GISP. (**b**) Superimposition of averaged mEPSCs from (**a**) before and 60 min after GISP. (**c**) GISP evoked long-lasting changes in frequency and amplitude of mEPSCs ($n = 15$).

Table 2.1 GISP shares many characteristics of LTP in the CA1 region of hippocampus.

Common properties between GISP and CA1 LTP	**References for GISP**
1. Induction requires NMDA receptors (blocked by APV)	(95)
2. Induction is Ca^{2+} dependent	(97)
3. Expression is largely due to AMPA receptors	(95)
4. Results in increased frequency and amplitude of mEPSCs	**Figure 2.2**, (95, 97)
5. Results in CaM-KII-dependent phosphorylation of GluR1 at S831	**Figures 2.3a-b**
6. Results in AMPA receptor, but not NMDA receptor, membrane insertion	(95)
7. "Silent synapses" are converted to functional synapses	(97)
8. Induction requires CaM-K activation.	Blocked by 3 μM KN-93 (data not shown)
9. Results in CaM-KII autophosphorylation at Thr286	**Figures 2.3a,c**

Figure 2.3

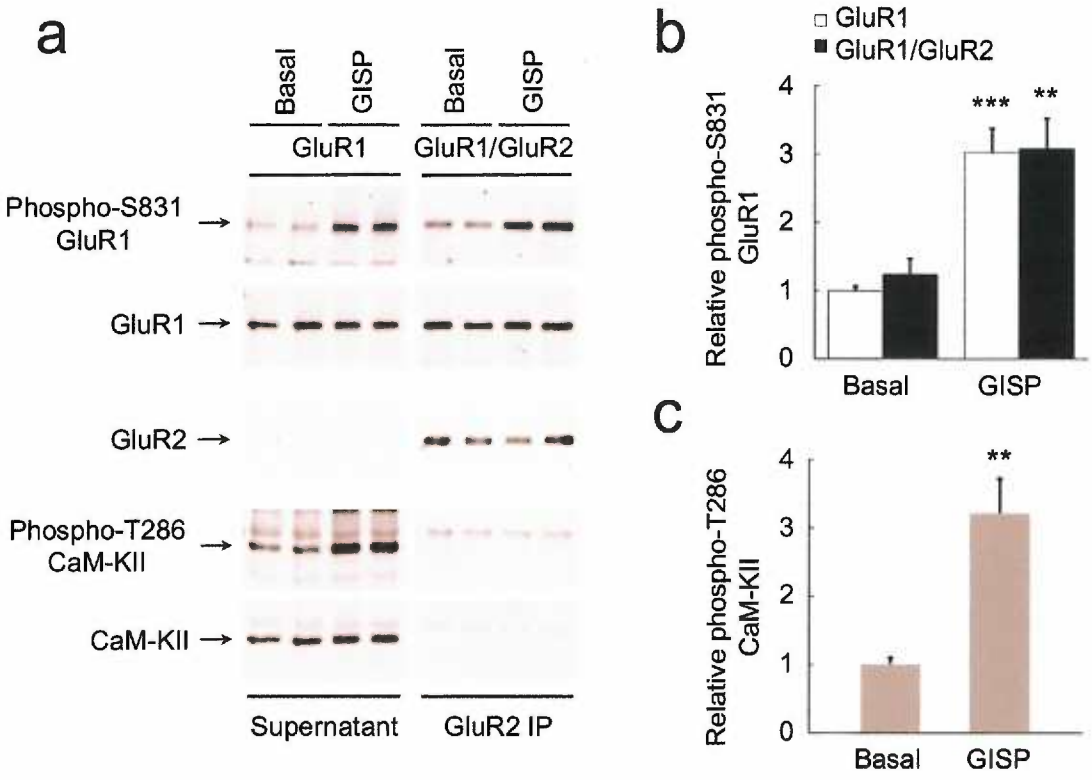

a
Basal
GISP
Basal
GISP
GluR1
GluR1/GluR2
Phospho-S831 GluR1
GluR1
GluR2
Phospho-T286 CaM-KII
CaM-KII
Supernatant
GluR2 IP
b
GluR1
GluR1/GluR2
Relative phospho-S831 GluR1
***
**
Basal
GISP
c
Relative phospho-T286 CaM-KII
**
Basal
GISP

Figure 2.3 GluR2 subunit did not affect CaM-KII phosphorylation of S831 in hippocampal AMPA receptors during synaptic potentiation. (**a**) Endogenous GluR1/GluR2 complexes were immunoprecipitated before and after the induction of NMDA receptor-dependent plasticity, GISP, in cultured hippocampal neurons. The immunoprecipitation was complete, since no GluR2 was detected in the remaining supernatant. (**b**) Phosphorylation of S831 was similarly increased by GISP in both heteromeric GluR1/GluR2 (** $P < 0.01$; $n = 5$) and GluR2-lacking GluR1 complexes (*** $P < 0.001$; $n = 5$), compared to basal S831 phosphorylation. (**c**) Increase in S831 phosphorylation tightly correlated with CaM-KII activation (** $P < 0.01$; $n = 5$).

Next, we tested the phosphorylation of AMPA receptors of defined GluR1 and GluR1/GluR2 subunit composition by co-expressing these receptors with or without constitutively active CaM-KII in HEK293 cells. We used the following criteria to ensure that tested receptors in HEK293 cells represent GluR1/GluR2 heteromers, with no or negligible contribution from GluR1 or GluR2 homomers: 1) absence of rectification (no contribution from GluR1 homomers) (**Figures 2.4a-b**); 2) no sensitivity to joro-toxin, the inhibitor of GluR1 homomers (**Figure 2.4c**); 3) difference in kinetics of GluR1 and GluR2 homomers from the GluR1/GluR2 heteromers (**Figure 2.4d**); and 4) small amplitude of current from edited GluR2 homomers (2-3 order of magnitude smaller at 0.4 μg of cDNA per well) compared to the GluR1/GluR2 heteromers (**Figure 2.4d**) (199). Similar to endogenous AMPA receptors, S831 phosphorylation was also indistinguishable between these receptor populations (**Figure 2.5**). These findings indicate that GluR2 subunit does not modulate S831 phosphorylation by CaM-KII.

Homomeric GluR1 receptors can adopt multiple conductance states (13). S831 phosphorylation significantly increases the contribution of high conductance states to the channel activity, enhancing the macroscopic currents of these receptors (10, 13). Therefore, another possibility for the lack of GluR1/GluR2 potentiation by CaM-KII is that GluR2 subunit can disrupt the coupling of S831 phosphorylation to channel conductance, thus keeping the receptor in a low conductance state, regardless of whether or not S831 is phosphorylated. We tested this hypothesis by measuring the single-channel conductance of GluR1/GluR2 heteromers and GluR1 and GluR2 homomers upon infusion of either heat-inactivated (HI, control) or activated CaM-KII in HEK293 cells (**Figure 2.6**). Indeed, single-channel conductance and open probability of GluR1/GluR2

heteromers were similar to those of GluR2 homomers (**Figures 2.6a-b**), but significantly lower than those of GluR1 homomers (**Figures 2.6c-d**). These results suggest that the GluR2 subunit defines the properties of heteromeric AMPA receptors (25). Moreover, the conductance of GluR1/GluR2 heteromers remained unchanged by CaM-KII, in contrast to the enhanced conductance of phosphorylated GluR1 homomers (**Figure 2.6d**) (13). Furthermore, mutating S831 to either alanine (Ala831) or aspartate (Asp831) to prevent or mimic phosphorylation, respectively, had no effect on single-channel conductance of GluR1/GluR2 heteromers, whereas GluR1 homomers with Asp831 mutation showed increased conductance. (**Figure 2.6d, Table 2.2**).

Figure 2.4

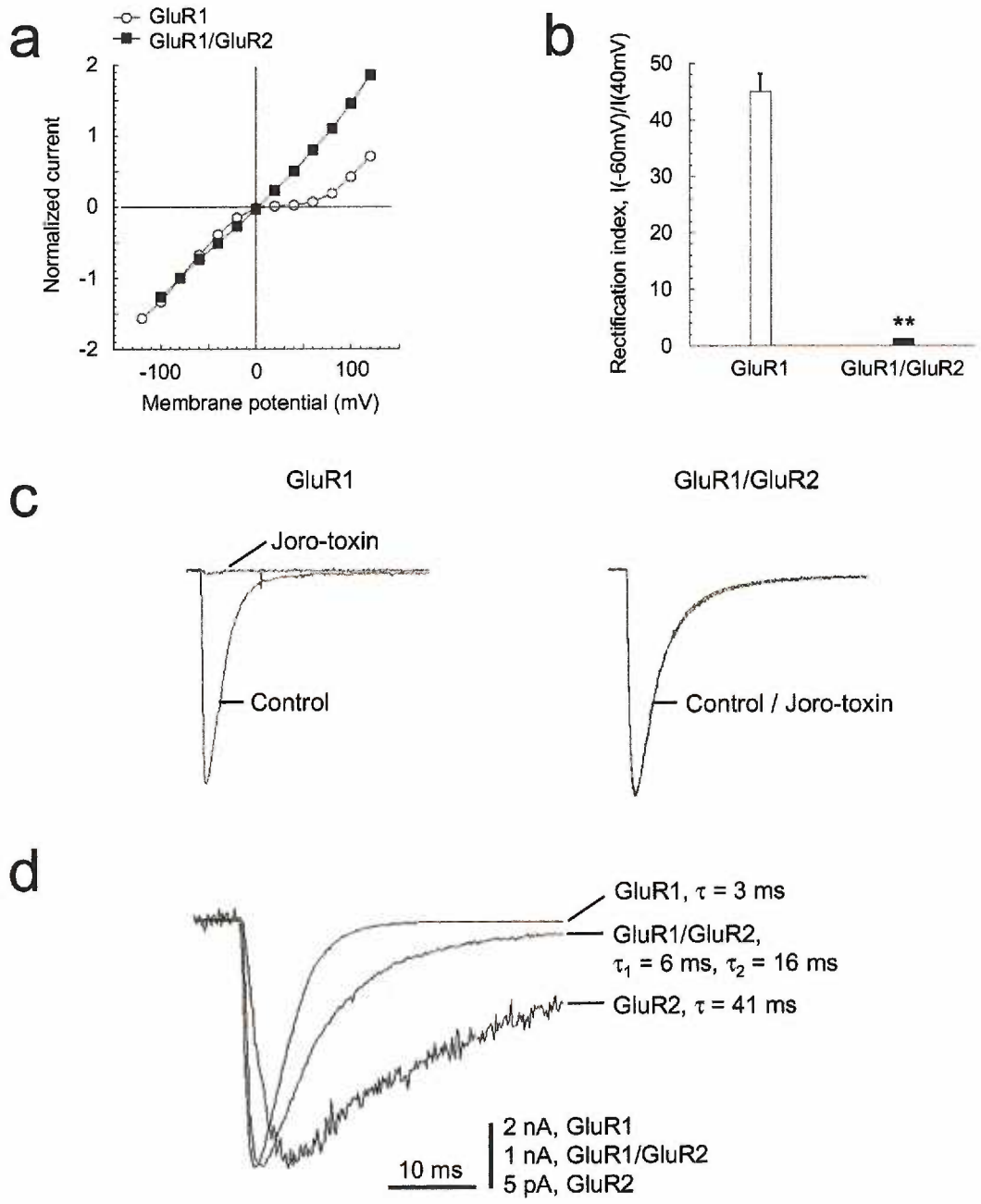
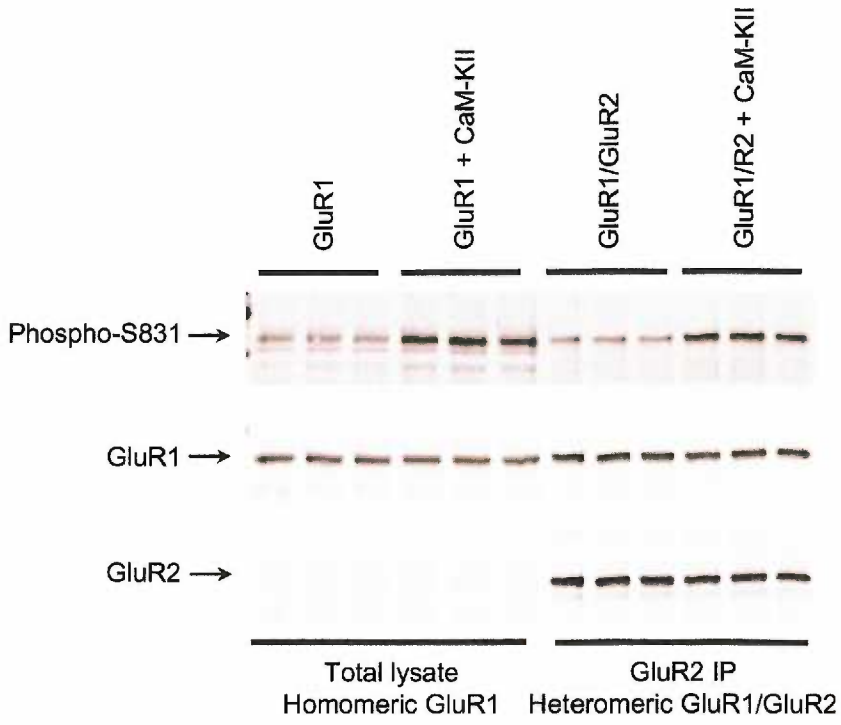

a
GluR1
GluR1/GluR2
Normalized current
2
1
0
-1
-2
-100
0
100
Membrane potential (mV)
b
Rectification index, I(-60mV)/I(40mV)
50
40
30
20
10
0
**
GluR1
GluR1/GluR2
c
GluR1
GluR1/GluR2
Joro-toxin
Control
Control / Joro-toxin
d
GluR1, τ = 3 ms
GluR1/GluR2,
τ1 = 6 ms, τ2 = 16 ms
GluR2, τ = 41 ms
10 ms
2 nA, GluR1
1 nA, GluR1/GluR2
5 pA, GluR2

Figure 2.4 Properties of GluR1/GluR2 heteromers were very different from either GluR1 or GluR2 homomers. (**a**) GluR1/GluR2 heteromers showed non-rectifying I-V curves, in contrast to strong rectification of GluR1 homomers (n = 9-17). (**b**) Comparison of rectification index (amplitude of the current at -60mV divided by the current at 40mV) for GluR1/GluR2 heteromers and GluR1 homomers from (**a**) (** $P <$ 0.01; n = 9-17). (**c**) Joro-toxin (1 μM) selectively inhibited GluR1 homomers but had no effect on GluR1/GluR2 heteromers. (**d**) GluR1 and GluR2 homomers were kinetically very different from GluR1/GluR2 heteromers. Moreover, the currents from GluR2 homomers were two to three fold of magnitude smaller than the currents from GluR1/GluR2 heteromers, indicating a negligible contribution from GluR2 homomers.

Figure 2.5

a


GluR1
GluR1 + CaM-KII
GluR1/GluR2
GluR1/R2 + CaM-KII
Phospho-S831
GluR1
GluR2
Total lysate
Homomeric GluR1
GluR2 IP
Heteromeric GluR1/GluR2

b

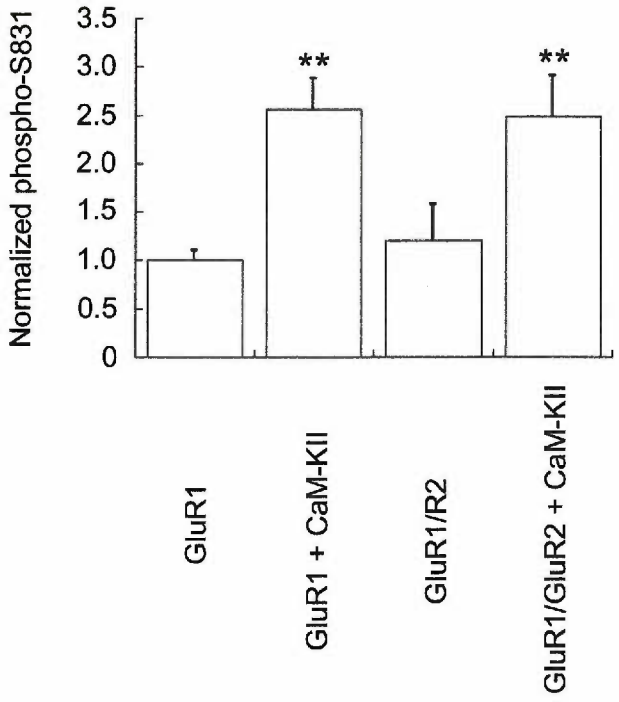

Normalized phospho-S831
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
**
**
GluR1
GluR1 + CaM-KII
GluR1/R2
GluR1/GluR2 + CaM-KII

Figure 2.5 Constitutively active CaM-KII phosphorylated GluR1 S831 similarly in GluR1 homomers and GluR1/GluR2 heteromers expressed in HEK293 cells. (**a**) Representative immunoblots showing that S831 phosphorylation is indistinguishable between GluR1 homomers and GluR1/GluR2 heteromers. Quantitation is shown in (**b**) (** $P < 0.01$ compared to GluR1; $n = 5$).

Figure 2.6

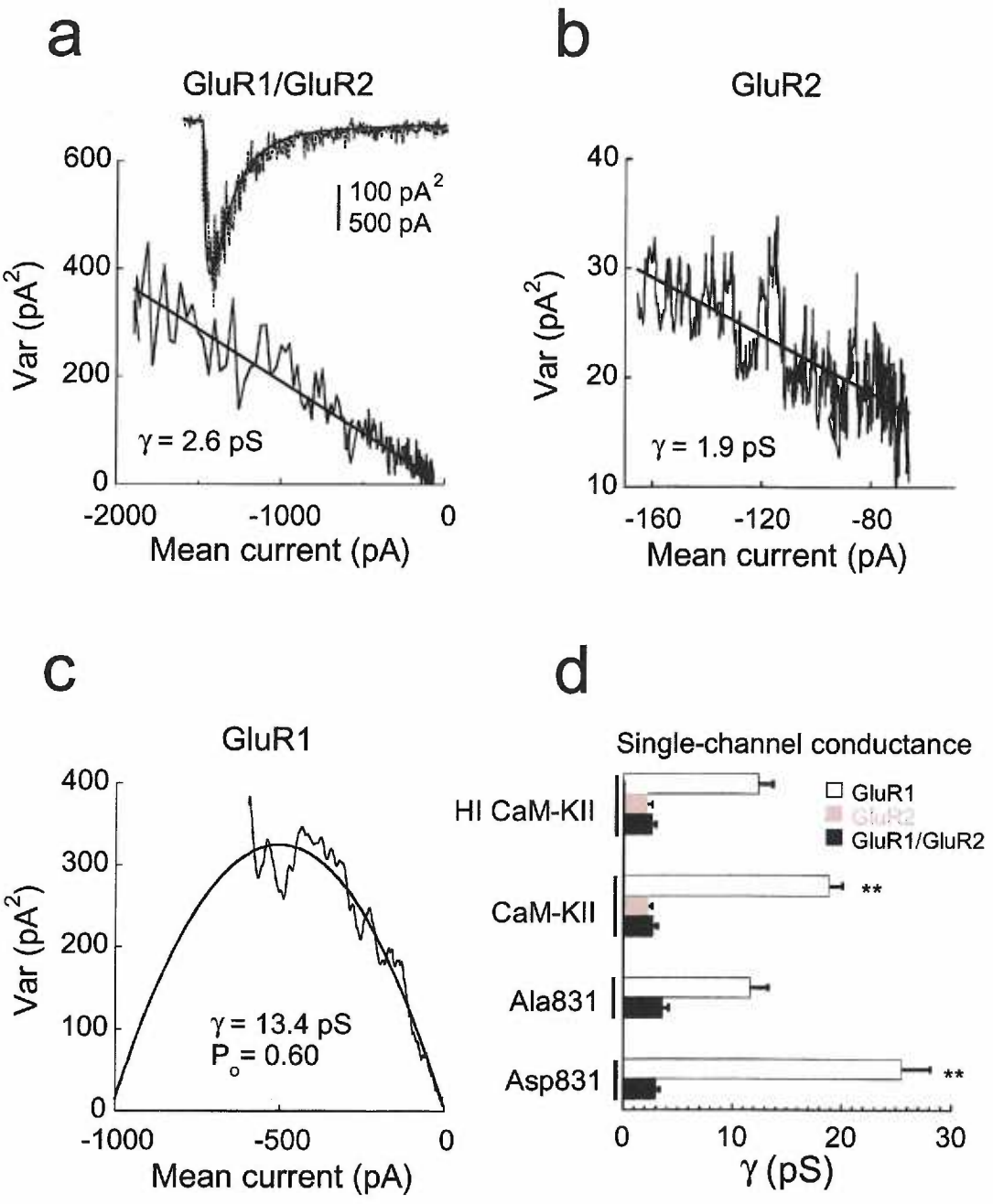

a
GluR1/GluR2
600
400
200
0
Var (pA²)
100 pA²
500 pA
γ = 2.6 pS
-2000
-1000
0
Mean current (pA)
b
GluR2
40
30
20
10
Var (pA²)
γ = 1.9 pS
-160
-120
-80
Mean current (pA)
c
GluR1
400
300
200
100
0
Var (pA²)
γ = 13.4 pS
P_o = 0.60
-1000
-500
0
Mean current (pA)
d
Single-channel conductance
HI CaM-KII
CaM-KII
Ala831
Asp831
GluR1
GluR2
GluR1/GluR2
**
**
0
10
20
30
γ (pS)

Figure 2.6 GluR2 subunit preserves GluR1/GluR2 heteromers in a low conductance state, regardless of S831 phosphorylation. Channel properties of GluR1/GluR2 heteromers (**a**) and GluR2 (**b**) homomers were similar, but very different from GluR1 homomers (**c**). (**d**) Neither activated CaM-KII nor mutation of S831 to aspartate (Asp831) increased the single-channel conductance of GluR1/GluR2 heteromers, but both conditions increased the single-channel conductance for GluR1 homomers (** $P <$ 0.01, n = 6-17, **Table 2.2**). Channel open probabilities were very low for GluR1/GluR2 and GluR2 receptors (**a-b**) and therefore were not determined. Insert in (**a**) is a superimposition of the mean current (solid line) and its variance.

Table 2.2 GluR2 subunit blocks the regulation of single-channel conductance (γ) of GluR1/GluR2 heteromers by CaM-KII or S831 to aspartate mutation (Asp831). ** indicates significance by *t*-test compared to same receptors infused with heat-inactivated (HI) CaM-KII.

Receptor	HI CaM-KII γ, pS	*n*	CaM-KII γ, pS	*n*	Ala831 γ, pS	*n*	Asp831 γ, pS	*n*
GluR1	12.4 ± 1.3	16	18.9 ± 1.2 **	15	11.7 ± 1.6	12	25.5 ± 2.6 **	17
GluR2	2.2 ± 0.5	7	2.3 ± 0.3	6				
GluR1/ GluR2	2.5 ± 0.3	8	2.7 ± 0.4	11	3.6 ± 0.6	9	3.0 ± 0.3	11

2.5 DISCUSSION

We have demonstrated a novel, dominant role of the GluR2 subunit in the regulation of AMPA receptors by CaM-KII and the mechanism for this dominance. The GluR2 subunit disrupts the coupling of S831 phosphorylation to channel conductance in GluR1/GluR2 heteromers, and this keeps receptors in a low conductance state regardless of S831 phosphorylation. However, we cannot exclude the possibility that other unknown protein-protein interactions mediated by CaM-KII may regulate the conductance of GluR1/GluR2 heteromers in neurons. Previous studies indicate that activity-dependent delivery of AMPA receptors to synapses is mediated by GluR1 subunit, while constitutive delivery is due to GluR2 subunit (31, 32, 40). In fact, synaptic activity and phosphorylation can change the balance in subunit composition of synaptic AMPA receptors toward GluR2-lacking receptors (31, 32, 53, 200). Our findings suggest that such subunit recomposition alone can increase synaptic strength by increasing the channel conductance of AMPA receptors and more importantly, allows the regulation of GluR2-lacking AMPA receptors by CaM-KII through S831 phosphorylation (12, 13). Thus, subunit recomposition accompanied by S831 phosphorylation or the delivery of different subtypes of AMPA receptors with a larger single-channel conductance may provide additional mechanisms for the enhanced AMPA channel conductance observed upon LTP induction (12-14).

CHAPTER 3

EXTRASYNAPTIC MEMBRANE TRAFFICKING REGULATED BY GLUR1 SERINE 845 PHOSPHORYLATION PRIMES AMPA RECEPTORS FOR LTP

Oh, Derkach, and Soderling. (2005), Submitted.

3.1 ABSTRACT

Activity-dependent delivery of AMPA receptors (AMPARs) to synaptic sites is a crucial component of long-term potentiation (LTP) in central glutamatergic synapses. Using a quantitative assay to measure net levels of serine 845 (S845) phosphorylation in the GluR1 subunit of AMPARs, we investigated the relationship between phospho-S845, GluR1 surface expression and synaptic strength in hippocampal neurons. Interestingly, S845 phosphorylation was associated with selective delivery of GluR1 to extrasynaptic sites, and their synaptic localization required coincident synaptic activity. Increasing the extrasynaptic pool of AMPA receptors resulted in stronger theta burst LTP. Our results support a two-step model for delivery of GluR1-containing AMPARs to synapses during activity-dependent LTP, where S845 phosphorylation serves as a "priming" step.

3.2 INTRODUCTION

In central glutamatergic synapses, AMPARs are a major target for the regulation of synaptic strength during synaptic plasticity. AMPAR function is dynamically regulated by two distinct mechanisms that can result in bi-directional synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD). First, phosphorylation of GluR1, the main AMPAR subunit, can directly enhance channel properties (13-15). Second, GluR1-containing AMPARs can be delivered to the surface membrane in an activity-dependent manner (40-42, 149), effectively regulating the number of functional receptors at postsynaptic sites. Thus, these two postsynaptic mechanisms tune the amplitude of synaptic transmission at the CA3-CA1 synapses in the hippocampus (3). Recent studies indicate that GluR1 phosphorylation and trafficking are coupled (58, 60), although exactly how GluR1 phosphorylation affects trafficking is unknown.

The GluR1 subunit contains two major phosphorylation sites in the carboxyl-terminus that are important for synaptic plasticity (4). Serine 831 (S831) is phosphorylated by calcium/calmodulin-dependent protein kinase II (CaM-KII) (7, 8), which potentiates single-channel conductance (13, 14). S831 is persistently phosphorylated during LTP (10, 11), suggesting this is one likely mechanism of synaptic potentiation during LTP (12). S845 is phosphorylated by cAMP-dependent protein kinase (PKA) (9), which increases open-probability of the channel (15). Recent studies indicate that S845 phosphorylation can also regulate AMPAR delivery to the surface membrane. Following endocytosis, phosphorylation of S845 in cultured hippocampal neurons increases the pool of GluR1 recycling back to the surface membrane, rather than

being degraded (60). Furthermore, CaM-KII-mediated synaptic incorporation of virally-expressed GluR1 in cultured hippocampal slices requires a serine at position 845 in GluR1, suggesting that its phosphorylation may be critical (58). These results strongly support a role of S845 phosphorylation during activity-dependent delivery of GluR1-containing AMPARs in the hippocampus.

Current understanding of the functional role of S845 phosphorylation in GluR1 during synaptic plasticity is limited because previous studies have analyzed only relative changes in phospho-S845. In fact, the functional significance of these changes is determined by the net level of phospho-S845. One particular model proposes a high basal phosphorylation at S845 (11, 93, 94). However, direct evidence of high basal S845 phosphorylation in hippocampal neurons is lacking. Thus, quantitation of the net phospho- S845 is critical in determining the role of S845 phosphorylation during activity-dependent synaptic plasticity. In this report, we quantified the net phospho-S845 and surface expression of GluR1 in hippocampal neurons during bi-directional synaptic plasticity. We conclude that S845 phosphorylation directly correlates with extrasynaptic surface expression of GluR1-containing AMPARs, which can then be incorporated into synapses by synaptic activity. Thus, S845 phosphorylation serves as a key "priming" step in enhancing synaptic strength during LTP.

3.3 Materials and Methods

In Vitro Kinase Assay.

The carboxyl-terminal 75 amino acids of GluR1 flip were fused to GST in pGEX-4T-3 vector (Amersham) and expressed in *E. coli* BL21(DE3). Proteins were purified on glutathione-sepharose column (Amersham Biosciences). Mutations were made with QuikChange Site-Directed Mutagenesis Kit (Stratagene). GST-GluR1-CTs (10 μM) were phosphorylated with purified CaM-KII (10 nM) or PKA (10 nM) for indicated time points at 30°C in phosphorylation buffer (in mM): 10 Mg^{2+}acetate, 50 HEPES (pH 7.5), 0.4 $AT^{32}P$ (2000 cpm/pmol). CaM-KII assay also contained 1 mM $CaCl_2$ and 2 μM calmodulin. Phosphorylation reactions were spotted in P81 filter papers, rinsed three times in 75 mM phosphoric acid, once in 95% ethanol, dried, and counted in a scintillation counter. For non-radioactive phosphorylation assays, substrates (10 μM) were phosphorylated with purified CaM-KII or PKA (10 nM) with unlabeled 0.4 mM ATP in the phosphorylation buffer for 30 min at 30°C. Reactions were terminated by adding and heating in 2x SDS sample buffer, and 10 ng of each substrate was subjected to 10% SDS-PAGE and probed with either S831 or S845 phospho-specific antibodies. Primary antibodies were: anti-phospho-Ser831-GluR1 (UBI), anti-phospho-Ser845-GluR1 (UBI and Chemicon), anti-GluR1 (UBI), and anti-tubulin (Developmental Studies Hybridoma Bank).

Cultured Hippocampal Neurons and Biochemistry.

Hippocampal neurons were cultured from postnatal day 1-2 Sprague-Dawley rats as previously described (195, 196). All procedures were reviewed and approved by the

Institutional Animal Care and Use Committee and the Department of Comparative Medicine at Oregon Health & Sciences University. Cultures were used for experiments at 13-15 DIV. For chemically-induced synaptic plasticity experiments, cultures were first incubated in ACSF for 30 min at room temperature (in mM): 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 33 D-glucose, and 25 HEPES (pH 7.3; osmolarity adjusted to 320), followed by stimulation in 50 μM forskolin (Sigma) and 0.1 μM rolipram (Calbiochem) in ACSF (no $MgCl_2$) or 50 μM NMDA. After 10 min stimulation, neurons were replaced in regular ACSF and then subjected to surface biotinylation after indicated time points.

Surface Biotinylation.

After F/R or NMDA stimulations, cultured neurons or slices were transferred to ice-cold ASCF for 2 min, followed by biotinylation in 1 mg/mL biotin (EZ-Link Sulfo-NHS-SS-Biotin; Pierce) in ACSF on ice with slow agitation for 20 and 45 min for cultured neurons and slices, respectively. 45 min biotinylation has been shown to completely biotinylate throughout 400 μm hippocampal slices (201). Following biotinylation, cultures and slices were rinsed three times in cold Tris-based ACSF (HEPES replaced with Tris) to quench free biotin. Slices were snap frozen in liquid nitrogen and stored in -80°C. Cultured neurons were immediately scraped in cold 1% Triton X-100 homogenization buffer (150 μL per 35 mm well; in mM): 50 NaCl, 10 EDTA, 10 EGTA, 1 Na_3VO_4, 50 NaF, 25 NaPPi, 1 β-glycerophosphate, 1 PMSF, 0.001 microcystine, 1x protease inhibitor cocktail tablet (Roche), 1x phosphatase inhibitor cocktail set I (Calbiochem), and 50 HEPES (pH 7.5). Homogenates from 2 wells were pooled for each condition and centrifuged at 10,000g for 20 min to pellet the insoluble

fraction. 75 µL of the supernatant was mixed and heated with 25 µL of 6x SDS sample buffer for total fraction (surface plus internal) of GluR1. Biotinylated surface proteins in the remaining supernatant (~225 µL) were immunoprecipitated with 40 µL of 50% avidin-agarose (ImmunoPure Immobilized Avidin; Pierce) for >2 hrs at 4°C. Avidin-agarose beads were then pelleted, and 75 µL of the supernatant was mixed and heated with 25 µL of 6x SDS sample buffer for internal fraction of GluR1. Avidin-agarose beads were rinsed three times with 1% Triton X-100 homogenization buffer and heated in 100 µL of 2x SDS sample buffer to obtain surface GluR1. Equal volumes of the total and internal fractions were subjected to 10% SDS-PAGE, on the same gel, and probed with total GluR1 and tubulin antibodies. Signals from total and internal GluR1 were normalized to tubulin. The surface fraction was calculated by subtracting the fraction of the tubulin-normalized internal over total GluR1 from 1 [surface = 1 – (internal/total)]. Surface GluR1 from avidin-agarose beads were also subjected to 10% SDS-PAGE with calibration samples for quantitative phospho-S845 Western blotting. Two frozen slices were pooled for each condition and were homogenized in 150 µL of cold 1% Triton X-100 homogenization buffer. After homogenization, additional 150 µL of homogenization buffer was added to obtain 300 µL of total homogenate. This homogenate was centrifuged and subjected to immunoprecipitation of surface GluR1 as described above for cultured neurons. Surface fraction and net phospho-S845 of surface GluR1 in slices were also determined as described for cultured neurons.

Quantitative Western Blotting.

All surface GluR1 obtained from cultured neurons and slices were subjected to 10% SDS-PAGE with a set of calibration standards on the same blot for net quantitation of phospho-S845. Calibration samples were prepared by phosphorylating GST-fused CPS mutant for PKA, P842R mutant (10 μM), with PKA (100 nM) *in vitro* for 30 min at 30°C in phosphorylation buffer. These fully phosphorylated proteins (**Figure 3.1d**) were mixed with specific amounts of non-phosphorylated proteins (10 μM) to obtain 4, 8, 12, 16, 20, 40, 60, 80, and 100% phosphorylated calibration standards (**Figure 3.1g**). All biotinylated surface GluR1 were run on 10% SDS-PAGE with at least 5 different calibration standards on the same blot to determine the net phospho-S845. Gels were transferred to nitrocellulose membrane, immunoblotted using infra-red dye-coupled secondary antibodies (anti-rabbit IgG Alexa Fluor 680, anti-mouse IgG IRdye800; Rockland). Image acquisition and data quantitation were performed on the Odyssey Infrared Imaging System (Li-Cor). Preliminary blots were run to normalize for the differences in GluR1 amount between biotinylated GluR1 and calibration standards. Calibration curves were calculated by determining the fraction of phospho-S845 signals over total GluR1 signals, then plotting this fraction against % phospho-S845 of calibration standards. Only linear curves with $R^2 > 0.90$ were used for the analysis (**Figure 3.1g**).

Electrophysiology and Biochemistry of Hippocampal Slices.

Acute hippocampal slices were prepared and recorded from 4-6 wk old male Sprague-Dawley rats as previously described (92, 202). Slice recording buffer was

saturated with 95% O_2/5% CO_2 (in mM): 125 NaCl, 2.5 KCl, 22.6 $NaHCO_3$, 1.25 NaH_3PO_4, 2 $CaCl_2$, 1 $MgCl_2$, and 11.1 D-glucose (pH 7.4). Recordings were digitized at 100 kHz, and initial slope of fEPSPs were analyzed. Basal stimulation was induced every min, and recordings were averaged to 3 min bins for analysis. Control and test conditions were interleaved within each animal to control for differences between animals. After 20 min of stable baseline, 25 μM forskolin and 0.1 μM rolipram (no $MgCl_2$) were added in slice recording buffer for 10 min with or without basal stimulation (**Figures 3.5-3.7**). Theta burst stimulation protocols were used as described (92). After recordings, slices were snap-frozen, micro-dissected for CA1 region, homogenized in 150 μL of ice-cold 1% Triton X-100 homogenization buffer, and subjected to 10% SDS-PAGE with calibration samples for quantitative phospho-S845 Western blotting. For Chem-LTD experiments (**Figures 3.4c,d**), F/R stimulations were immediately followed by 3 min of 25 μM NMDA. Slices were replaced in regular slice recording buffer after stimulation and then subjected to surface biotinylation after indicated time point.

Statistical Analysis.

All statistics were performed by Student's two-tailed, unpaired *t*-test compared to controls or basal (0 min time point) within each group. * indicates $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. All data are presented as mean ± s.e.m.

3.4 RESULTS

GluR1 was phosphorylated *in vitro* for calibration standards

Although relative changes in phospho-S845 of GluR1 in hippocampal neurons have been previously reported using a phospho-specific antibody (11, 58), interpreting the physiological significance of these changes is difficult without knowing the molar levels of phosphorylation. For example, a 3-fold relative change could represent an increase from 1% to 3% or from 15% to 45%. The latter would likely have physiological importance, whereas the former probably would not. We decided to use GST-tagged GluR1 carboxyl-terminus (GST-GluR1-CT) phosphorylated *in vitro* with PKA at S845 as calibration standards for quantitative Western blotting. GST-GluR1-CT with a molar phosphorylation stoichiometry of 1 at S845 can be mixed with specific amounts of unphosphorylated proteins to make different percentages of phosphorylated GluR1 calibration standards.

In vitro phosphorylation assays were performed with γ-^{32}P-labeled ATP using purified kinases to phosphorylate wild-type (wt) and mutant GST-GluR1-CTs to verify that the phosphorylation sites of interest were fully phosphorylated. CaM-KII rapidly phosphorylated wt GST-GluR1-CT with a stoichiometry close to one phosphate per GluR1 molecule, indicating there is only one CaM-KII site in GluR1 carboxyl-terminus (**Figure 3.1a**). Mutating S831 to alanine, but not S845, completely blocked CaM-KII phosphorylation, confirming that S831 is the CaM-KII specific site (7, 8).

Surprisingly, PKA was not able to phosphorylate wt GST-GluR1-CT *in vitro* (**Figure 3.1b**), even at extremely high PKA concentrations of 200 nM (data not shown). An examination of the amino acid sequences around the two phosphorylation sites

indicated that neither S831 nor S845 were consensus phosphorylation sequences for CaM-KII or PKA, respectively (**Figure 3.1e**). Both sites contain a proline residue at the p-3 position, whereas the consensus phosphorylation sequences for both CaM-KII (RXXS/T) and PKA (RRXS/T) have an arginine residue at the p-3 position (203). Therefore, the proline at p-3 position of S845 was mutated to arginine (P842R) to convert S845 into a consensus phosphorylation sequence (CPS) mutant for PKA. Indeed, PKA rapidly phosphorylated P842R mutant with a phosphorylation stoichiometry approaching 1 (**Figure 3.1d**), while similar mutation near the CaM-KII site (P828R) had little effect (**Figure 3.1c**). To confirm these finding, phosphorylation assays were performed with unlabeled ATP, followed by immunoblotting with the phospho-specific antibodies for S831 and S845 (**Figure 3.1f**). Again, wt GST-GluR1-CT was poorly phosphorylated by PKA, while converting S845 into a CPS for PKA allowed very strong phosphorylation at S845. Although PKA phosphorylation of S845 was detectable in wt, S831A, and P828R proteins, further analysis with lower dilutions of phosphorylated P842R samples indicated that wt GST-GluR1-CT phosphorylation at S845 was around 1% of S845 phosphorylation in the CPS P842R mutant (data not shown). These results confirmed that wt GluR1 is not a good substrate for PKA *in vitro*, in contrast to CaM-KII.

Because wt GST-GluR1-CT was not phosphorylated by PKA *in vitro*, we used the P842R mutant as our calibration standards to quantify the net phospho-S845 of endogenous GluR1 in hippocampal neurons. Different percentage phosphorylation ratios of S845 were obtained by mixing specific amounts of non-phosphorylated GluR1 with fully phosphorylated GluR1. Plotting the ratios of phospho-to-total GluR1 signals from

two different immunoblots consistently yielded a very linear relationship with high R^2 values (**Figure 3.1g**; $R^2 > 0.90$ for analysis).

S845 phosphorylation increased surface GluR1 in cultured hippocampal neurons

The *in vitro* phospho-S845 calibration standards were used to quantify the net phospho-S845 of endogenous GluR1 in cultured hippocampal neurons. To induce S845 phosphorylation, we used a recently described chemical stimulation protocol of forskolin plus rolipram (F/R) (96). Stimulation of hippocampal slices with forskolin, an adenylate cyclase activator, and rolipram, phosphodiesterase type IV inhibitor, results in prolonged NMDA receptor (NMDAR)-dependent LTP (cLTP) (96) and recruits CaM-KII to dendritic spines (128). Because S845 is described as a PKA phosphorylation site (9) and forskolin strongly activates PKA, we decided to use this chemical potentiation to evaluate the changes in net phospho-S845 in hippocampal neurons.

Stimulation of mature cultured hippocampal neurons with F/R (50 μM/0.1 μM) for 10 min resulted in persistent phosphorylation of S845 (**Figures 3.2a,b**). Endogenous surface GluR1 had consistently low basal phosphorylation (10-20%), and total GluR1 fraction also had low basal phosphorylation similar to the surface GluR1 (data not shown). F/R treatment resulted in a robust S845 phosphorylation that continued to increase to approximately 60% even after the replacement of F/R at 10 min with regular ACSF. The increase in phospho-S845 was associated with a significant increase in the surface fraction of GluR1 following F/R stimulation (**Figure 3.2c**), suggesting that S845 phosphorylation may play an important role in GluR1 trafficking (58, 60). Blocking NMDARs with D-APV (50 μM) had no affect on phospho-S845 or surface GluR1

delivery by F/R stimulation, suggesting that both do not require NMDAR activation (**Figures 3.2a-c**). In accordance with this finding, tetrodotoxin also did not block F/R-mediated increase in phospho-S845 or surface GluR1 (data not shown). When changes in net phospho-S845 were plotted against normalized surface GluR1 in response to F/R stimulation, a linear relationship was obtained with an R^2 value of 0.997 and slope of 0.75 (**Figure 3.2d**). This is strong evidence that GluR1 S845 phosphorylation regulates surface expression of AMPARs.

Dephosphorylation of S845 was associated with a decrease in surface GluR1

Bath application of NMDA induces chemical LTD (Chem-LTD) and S845 dephosphorylation in hippocampal slices (93, 94). Thus, we hypothesized that blocking S845 phosphorylation during F/R stimulation may also block the surface delivery of new AMPARs. Bath application of NMDA (50 μM) resulted in significant dephosphorylation of S845 of surface AMPARs in cultured hippocampal neurons (**Figures 3.3a,b**). NMDA treatment also prevented subsequent F/R-mediated S845 phosphorylation. Importantly, the decrease in phospho-S845 was associated with a decrease in the surface pool of GluR1 (**Figure 3.3c**). These results further confirmed that the fraction of GluR1 available in the surface membrane is strongly correlated to the net phospho-S845 (60).

S845 phosphorylation was accompanied by an increase in surface GluR1 in hippocampal slices

The functional role of S845 phosphorylation was further investigated by testing whether F/R also increases phospho-S845 and surface GluR1 in acute hippocampal

slices. Surface GluR1 was measured by biotinylation using conditions previously shown to penetrate the entire slice (201). Similar percentages of AMPARs (about 30-35%) were found on the surface in both slices and cultured neurons, indicating that biotinylation was equally effective in these preparations (data not shown). Using the *in vitro* calibration standards as described above (**Figure 3.1g**), we quantified the net phospho-S845 of GluR1 under basal and stimulated conditions. Again, as in cultured hippocampal neurons, the basal phospho-S845 in hippocampal slices was relatively low with net values less than 20%. Moreover, 10 min F/R (25 μM/0.1 μM) stimulation resulted in a robust S845 phosphorylation to approximately 50% that persisted for at least 1 hour after the washout of drugs (**Figures 3.4a,b**). The increase in net phospho-S845 was associated with an increase in surface GluR1 fraction (**Figure 3.4d**), similar to the results found in cultured hippocampal neurons (**Figure 3.2c**). Bath treatment with 25 μM NMDA for 3 min induced Chem-LTD (65 min after NMDA treatment, fEPSP slope: Control = 0.94 ± 0.4, $n = 6$; NMDA = 0.75 ± 0.5, $n = 12$; $P < 0.05$) (202) and completely blocked F/R-mediated S845 phosphorylation (**Figure 3.4c**) as well as surface delivery of GluR1 (**Figure 3.4d**). Thus, pharmacological stimulations that either increase (F/R) or decrease (NMDA) net phospho-S845 significantly increased or decreased the surface pool of GluR1, respectively, supporting the hypothesis that S845 phosphorylation is involved in regulating the magnitude of the surface pool of GluR1.

Forskolin/Rolipram stimulation required synaptic activity for LTP

We evaluated the functional effect of GluR1 S845 phosphorylation on synaptic transmission by recording field excitatory postsynaptic potentials (fEPSPs) from the CA1

region of hippocampal slices. After 20 min of stable baseline, F/R was applied to slices for 10 min. This resulted in a significant postsynaptic potentiation (PTP), followed by chemical LTP (cLTP) (96) that persisted for over an hour (**Figure 3.5a**).

As shown in a previous study (96), the absence of basal stimulation during and for 45 min after F/R treatment prevented cLTP, suggesting that concomitant synaptic NMDAR activation is absolutely required for F/R-induced cLTP (**Figure 3.5a**). We found this result surprising since our biochemical analyses confirmed an increase in the surface pool of GluR1 associated with the F/R stimulation alone (**Figures 3.2-3.4**). Although we did not confirm an increase in the surface pool of GluR1 in slices that we recorded from, extracts prepared from micro-dissected CA1 regions of slices that were stimulated with F/R during recordings showed a 2- to 3-fold increase in net phospho-S845 (**Figures 3.5c,d**), suggesting that the surface pool of GluR1 was elevated in these slices. Thus, we hypothesized that F/R treatment resulted in selective delivery of GluR1-containing AMPARs to extrasynaptic sites in the absence of basal stimulation.

S845 phosphorylation primed GluR1 for synaptic incorporation

To test this hypothesis of extrasynaptic delivery, we designed a "priming" experiment where slices were treated with F/R in the absence of basal stimulation, followed by theta burst stimulation to induce electrical LTP. According to this hypothesis, one would predict that the magnitude of LTP would be greater in slices pretreated with F/R compared to untreated, control slices, because more extrasynaptic AMPARs would be available for synaptic incorporation. This hypothesis strongly favors the model (170, 171, 182) in which the newly delivered synaptic AMPARs originate

from extrasynaptic pools by lateral movement on the surface membrane, rather than direct exocytosis into the post-synaptic densities (PSDs) in synapses.

First, we tested whether priming by F/R pretreatment can enhance the LTP induced by a single train of theta bursts. Hippocampal slices were treated with either F/R or DMSO for 10 min in the absence of basal stimulation. F/R was then washed out for 45 min, followed by 15 min of baseline recording. A single train of theta bursts was then applied, resulting in decremental LTP, with 16% potentiation 50 min after the theta train (1.16-fold over baseline). F/R pretreated slices did not exhibit any enhancement of LTP induced by a single theta stimulation compared to control slices (1.26-fold over baseline, $P > 0.05$) (**Figure 3.6a**). There were no significant changes in PPF ratios during baseline, after F/R treatment, and 50 min after the theta train (**Figure 3.6c**), suggesting that presynaptic components were not affected by F/R treatment in the absence of basal stimulation. However, biochemical analysis of the slices that were micro-dissected for CA1 regions after recordings showed that S845 phosphorylation was significantly increased in slices treated with F/R (**Figure 3.6e**).

We next investigated whether a stronger stimulation could reveal a priming effect on LTP. This seemed possible if LTP occurred via creating more anchors for AMPARs within synapses (204), and weak stimulation only creates few new anchors compared to a stronger stimulation. This model of LTP is supported by recent data indicating that PSD-95 can act as an anchoring site for AMPARs in synapses through stargazin/PSD-95 interaction (61, 62, 205). Indeed, three theta trains resulted in significantly larger LTP in slices pretreated with F/R (1.63-fold over baseline, $P < 0.05$) compared to control slices (1.34-fold over baseline) (**Figure 3.6b**). PPF ratios were not significantly different

between F/R and control groups (**Figure 3.6d**), suggesting that presynaptic components were minimal or absent under these conditions. CA1 regions micro-dissected after recordings had significantly elevated phospho-S845 in slices pretreated with F/R compared to controls (**Figure 3.6f**). However, the increase in net phospho-S845 was similar in slices with either single or triple theta bursts. These results support the hypothesis that GluR1 S845 phosphorylation increased the pool of receptors available for synaptic incorporation (58), most likely through increasing the extrasynaptic pool of AMPARs. Apparently, a strong induction protocol is required for the extrasynaptic AMPARs to either translocate to and/or anchor at synaptic sites and enhance LTP.

Priming GluR1 for Synaptic Incorporation was blocked by Chem-LTD

The results so far indicated that S845 phosphorylation is important for regulating the surface pool of extrasynaptic AMPARs. We further tested this hypothesis by using Chem-LTD, which resulted in dephosphorylation of S845 (**Figures 3.3a,b,3.4c**) (93, 94) and decreased the surface pool of GluR1 (**Figures 3.3c,3.4d**) (152). We predicted that NMDA would block F/R-induced cLTP as well as the priming effect. Indeed, stimulating hippocampal slices with F/R followed by 3 min bath application of 25 μM NMDA completely blocked F/R-induced cLTP in the presence of basal stimulation (data not shown). More importantly, NMDA treatment completely blocked the priming effect of F/R stimulation by three theta bursts (**Figure 3.7a**). As before, these treatment protocols had no significant effect on PPF ratios (**Figure 3.7b**). Biochemical analysis showed that net phospho-S845 was significantly suppressed from 46% (**Figure 3.6f**) in slices treated with F/R alone to 19% (**Figure 3.7d**, $P < 0.001$) in slices treated with

NMDA following F/R. These results suggested that GluR1 S845 phosphorylation play an important role in modulating the dynamic range of LTP amplitude by regulating the amount of extrasynaptic AMPARs available for synaptic incorporation.

Figure 3.1

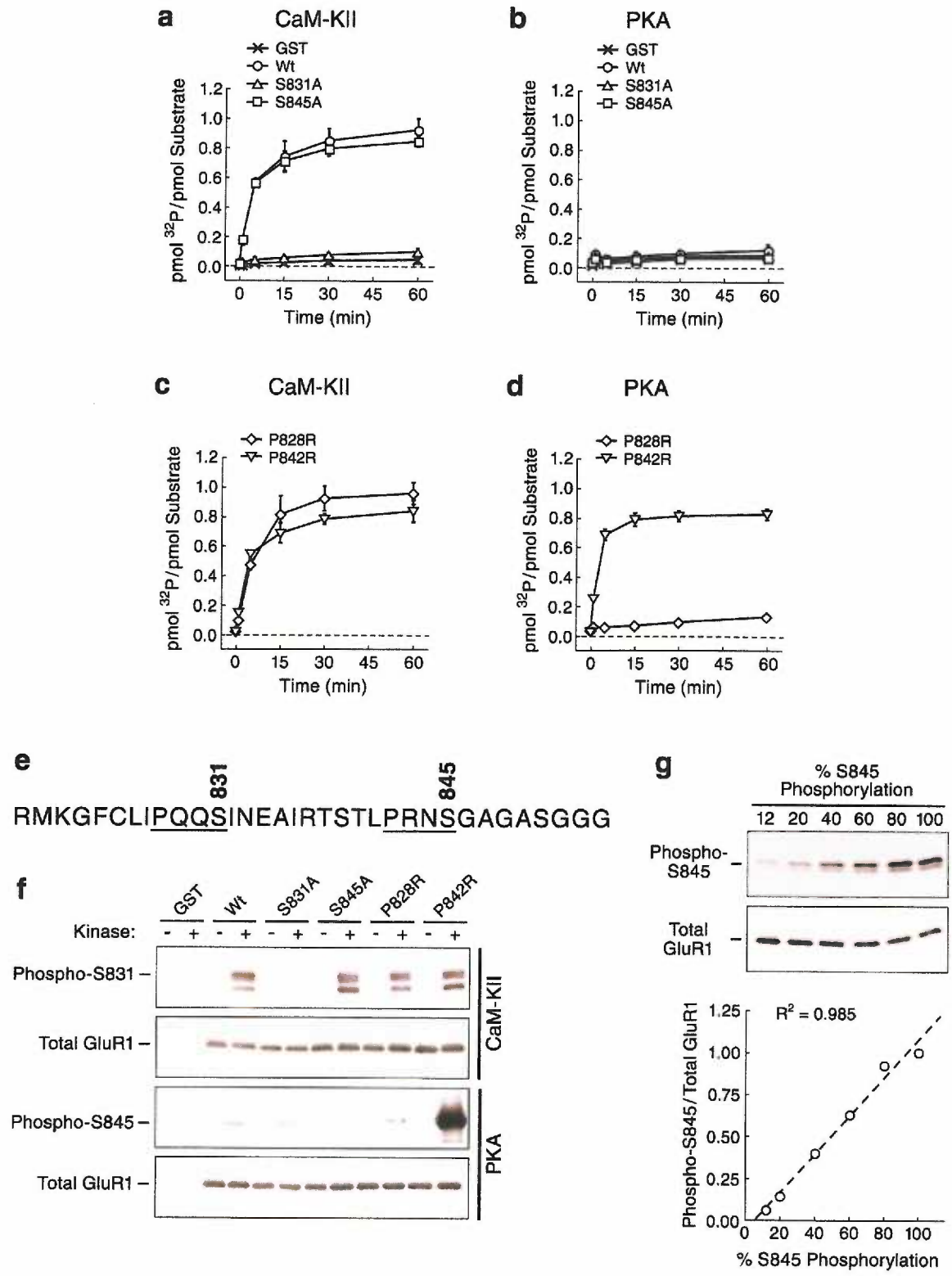

a
CaM-KII
GST
Wt
S831A
S845A
pmol 32P/pmol Substrate
Time (min)
b
PKA
c
CaM-KII
P828R
P842R
d
PKA
e
831
845
RMKGFCLIPQQSINEAIRTSTLPRNSGAGASGGG
f
GST
Wt
S831A
S845A
P828R
P842R
Kinase:
Phospho-S831
Total GluR1
Phospho-S845
CaM-KII
PKA
g
% S845 Phosphorylation
12 20 40 60 80 100
Phospho-S845
Total GluR1
R2 = 0.985
Phospho-S845/Total GluR1
% S845 Phosphorylation

Figure 3.1 *In vitro* CaM-KII and PKA phosphorylation of GST-GluR1 C-terminus. (**a**) CaM-KII (10 nM) phosphorylated wt GluR1 (10 μM) to a molar stoichiometry of approximately 1, and this was completely blocked by S831A mutation. (**b**) PKA (10 nM) did not phosphorylate wt GluR1, but mutating S845 into CPS for PKA (P842R) allowed robust phosphorylation (**d**), while a similar mutation at the CaM-KII site (P828R) had no effect (**c**) (panels **a-d**; $n = 4$). (**e**) Amino acid sequence around the two phosphorylation sites revealed that neither S831 nor S845 sites are consensus phosphorylation sequences for CaM-KII or PKA, respectively, with a proline rather than an arginine at the p-3 position. (**f**) Western blot analysis using phospho-specific and total GluR1 antibodies of *in vitro* CaM-KII and PKA phosphorylated GST-GluR1 C-termini gave similar results as ^{32}P-incorporation assays (**a-d**). (**g**) PKA CPS mutant (P842R) was phosphorylated to a molar stoichiometry with PKA and mixed with specific amounts of non-phosphorylated protein to make calibration standards. Phospho-S845 signal was divided by total GluR1 signal, plotted against % S845 phosphorylation, and fitted with a linear curve (dotted line), resulting in very high R^2 values.

Figure 3.2

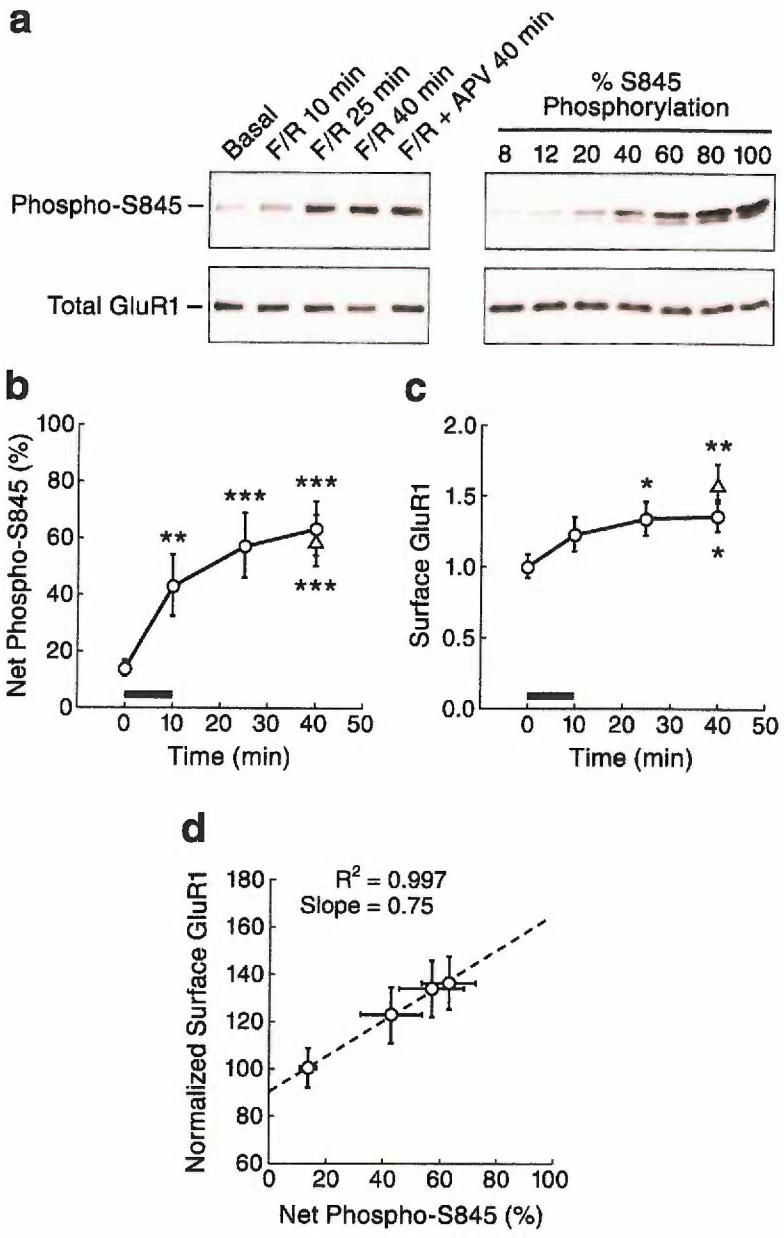

a
Basal
F/R 10 min
F/R 25 min
F/R 40 min
F/R + APV 40 min
% S845 Phosphorylation
8 12 20 40 60 80 100
Phospho-S845
Total GluR1
b
Net Phospho-S845 (%)
Time (min)
c
Surface GluR1
Time (min)
d
R^2 = 0.997
Slope = 0.75
Normalized Surface GluR1
Net Phospho-S845 (%)

Figure 3.2 Surface S845 phosphorylation and surface delivery of GluR1 are tightly correlated in cultured hippocampal neurons. (**a**) 10 min F/R (50/0.1 μM) stimulation significantly increased phospho-S845 of biotinylated surface GluR1 in cultured neurons. S845 phosphorylation continued to increase even after the removal of F/R and persisted up to 40 min after stimulation. D-APV (50 μM) treatment had no effect. Calibration standards used to calculate the net phospho-S845 is shown on right. (**b**) Increase in net phospho-S845 of biotinylated surface GluR1 in response to 10 min (black bar) F/R treatment without (circles, n = 6-8) or with D-APV (triangles, n = 5). (**c**) Normalized increase in surface fraction of GluR1 (fold over baseline, baseline surface GluR1 was 34.2 ± 3.1% of total GluR1) is shown (circles, n = 6-8). D-APV (triangles, n = 5) did not block the increase in surface GluR1. (**d**) Net phospho-S845 of surface GluR1 (**b**) is plotted against normalized surface fraction of GluR1 (**c**, basal surface GluR1 fraction is normalized to 100%). This linear relationship (dotted line, R^2 = 0.997) with a slope of 0.75 indicates a strong correlation between phospho-S845 and surface delivery. * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 3.3

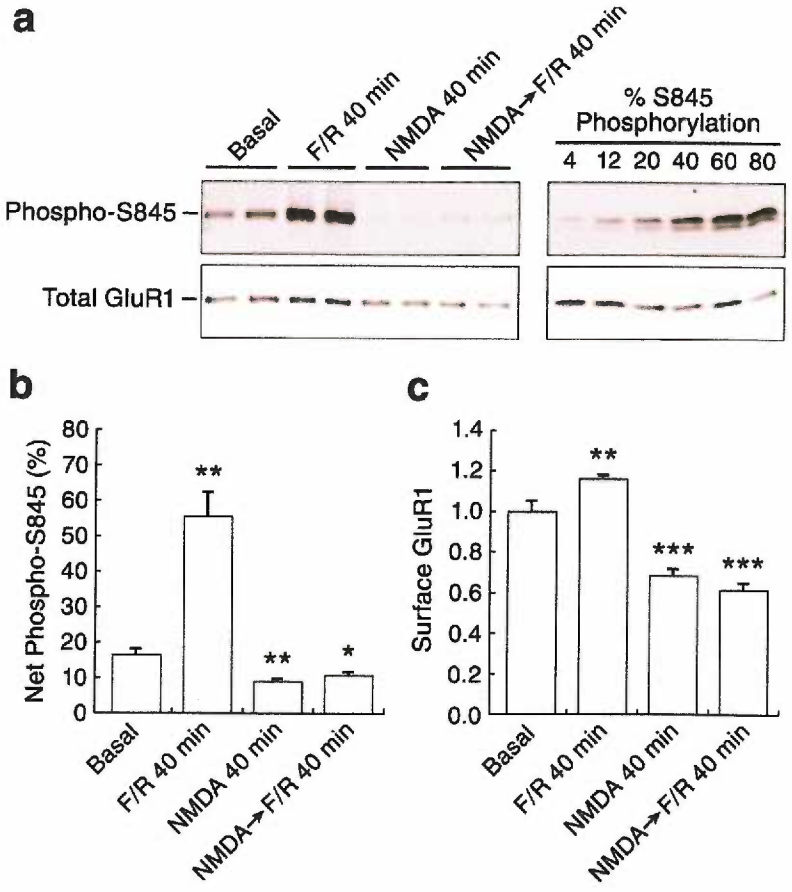

a
Basal
F/R 40 min
NMDA 40 min
NMDA→F/R 40 min
% S845 Phosphorylation
4 12 20 40 60 80
Phospho-S845
Total GluR1
b
Net Phospho-S845 (%)
80
70
60
50
40
30
20
10
0
**
**
*
Basal
F/R 40 min
NMDA 40 min
NMDA→F/R 40 min
c
Surface GluR1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
***
Basal
F/R 40 min
NMDA 40 min
NMDA→F/R 40 min

Figure 3.3 Correlation of surface S845 phosphorylation and surface delivery of GluR1 during bi-directional synaptic plasticity in cultured hippocampal neurons. (**a**) 10 min F/R stimulation strongly stimulated S845 phosphorylation of surface GluR1, 40 min after treatment, while 10 min NMDA (50 μM) stimulation significantly decreased phospho-S845 of surface GluR1. (**b,c**) Quantified changes in net phospho-S845 and surface fraction (fold over basal) of GluR1 in response to cLTP (F/R) or Chem-LTD (NMDA) 40 min after stimulation (n = 5-7). Treatment with NMDA completely blocked subsequent F/R-mediated increase in phospho-S845 and surface GluR1. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 3.4

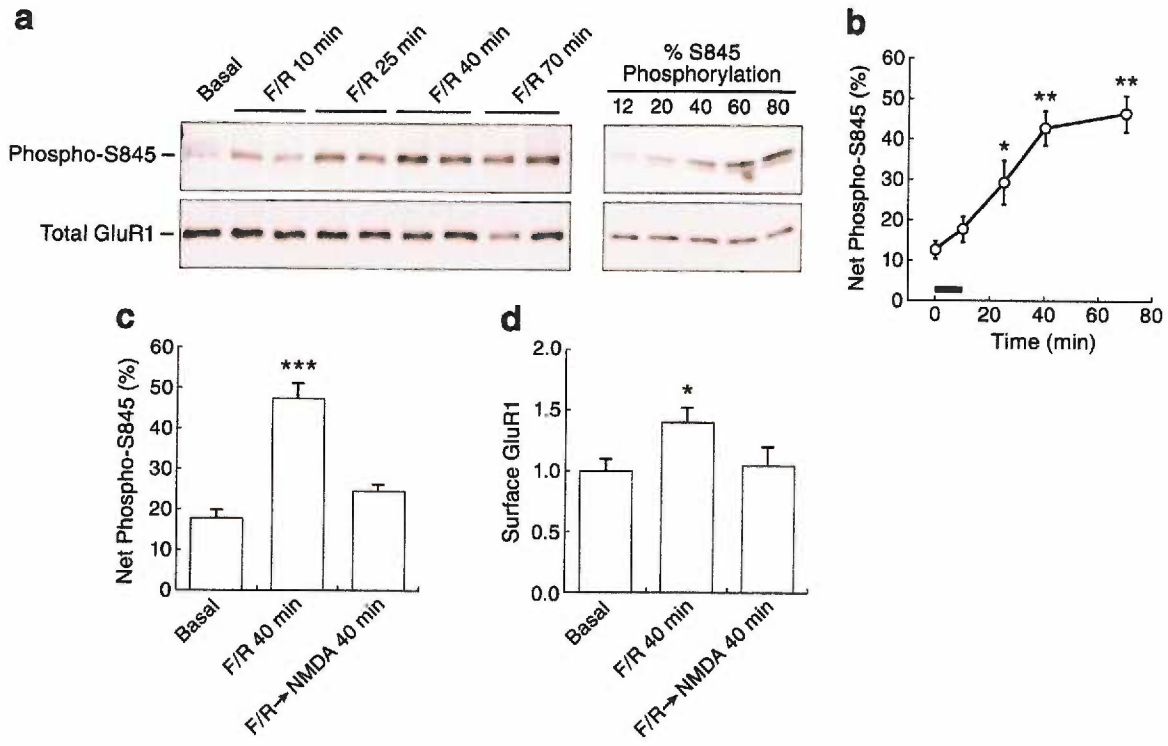

a
Basal
F/R 10 min
F/R 25 min
F/R 40 min
F/R 70 min
% S845 Phosphorylation
12 20 40 60 80
Phospho-S845
Total GluR1
b
Net Phospho-S845 (%)
Time (min)
c
Net Phospho-S845 (%)
Basal
F/R 40 min
F/R→NMDA 40 min
d
Surface GluR1
Basal
F/R 40 min
F/R→NMDA 40 min

Figure 3.4 Parallel changes in GluR1 S845 phosphorylation and surface delivery in acute hippocampal slices. (**a**) 10 min F/R (25/0.1 μM) stimulation increased net phospho-S845 of biotinylated surface GluR1 in hippocampal slices. (**b**) Increase in phospho-S845 was persistent, up to 70 min after stimulation (n = 3-4). (**c,d**) There was a parallel increase in net phospho-S845 (n = 6) and surface GluR1 (n = 6-9) at 40 min after cLTP. Basal surface GluR1 (normalized to 1) represented ~30 % of total GluR1. These F/R changes were completely blocked by Chem-LTD (10 min of F/R followed by 3 min of 25 μM NMDA and then 27 min of basal ACSF, n = 6). * $P < 0.05$; ** $P < 0.05$; *** $P < 0.001$.

Figure 3.5

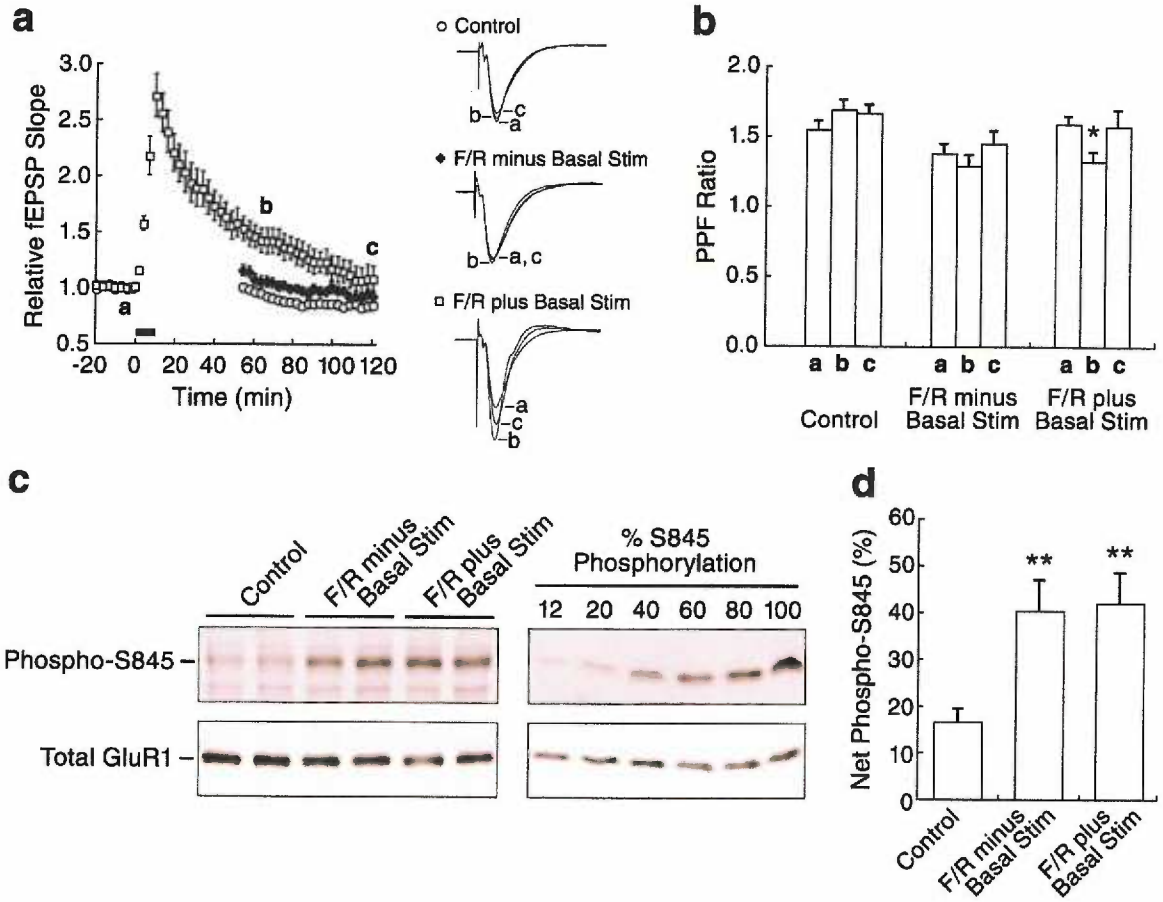

a
Relative fEPSP Slope
3.0
2.5
2.0
1.5
1.0
0.5
-20 0 20 40 60 80 100 120
Time (min)
a
b
c
Control
F/R minus Basal Stim
F/R plus Basal Stim
b
a
c
a, c
b
a
c
b
PPF Ratio
2.0
1.5
1.0
0.5
0.0
a b c
a b c
a b c
Control
F/R minus Basal Stim
F/R plus Basal Stim
c
Control
F/R minus Basal Stim
F/R plus Basal Stim
% S845 Phosphorylation
12 20 40 60 80 100
Phospho-S845
Total GluR1
d
Net Phospho-S845 (%)
60
50
40
30
20
10
0
**
**
Control
F/R minus Basal Stim
F/R plus Basal Stim

Figure 3.5 Increased S845 phosphorylation and surface GluR1 are not sufficient for synaptic potentiation in the absence of synaptic activity. (**a**) fEPSPs from hippocampal slices stimulated with F/R (black bar) with (open squares) and without (solid diamonds) basal stimulations (every min) are shown. Representative traces (a, b, and c time points indicated on graph) are shown on the right. Potentiation occurred only when F/R treatment was combined with basal stimulation (at 60 min after F/R: Control, 0.96 ± 0.02; F/R minus Basal Stim, 1.07 ± 0.05; F/R plus Basal Stim, 1.46 ± 0.10). For Control vs F/R plus Basal Stim, $P < 0.0001$ at 60 min and $P < 0.05$ at 120 min ($n = 9$-12). (**b**) There were no changes in PPF (a, b, and c time points indicated in **a**, $n = 9$-12) in Control and F/R minus Basal Stim groups. F/R plus Basal Stim group had a modest decrease in PPF at 60 min after F/R ($P < 0.05$), suggesting some presynaptic component at this time point. (**c,d**) After recordings, CA1 regions were micro-dissected from slices and subjected to quantitative Western blotting for net phospho-S845. F/R stimulation resulted in significant increase in phospho-S845, suggesting there was an increase in surface GluR1 with F/R stimulation, regardless of basal stimulation ($n = 5$-7). ** $P < 0.05$.

Figure 3.6

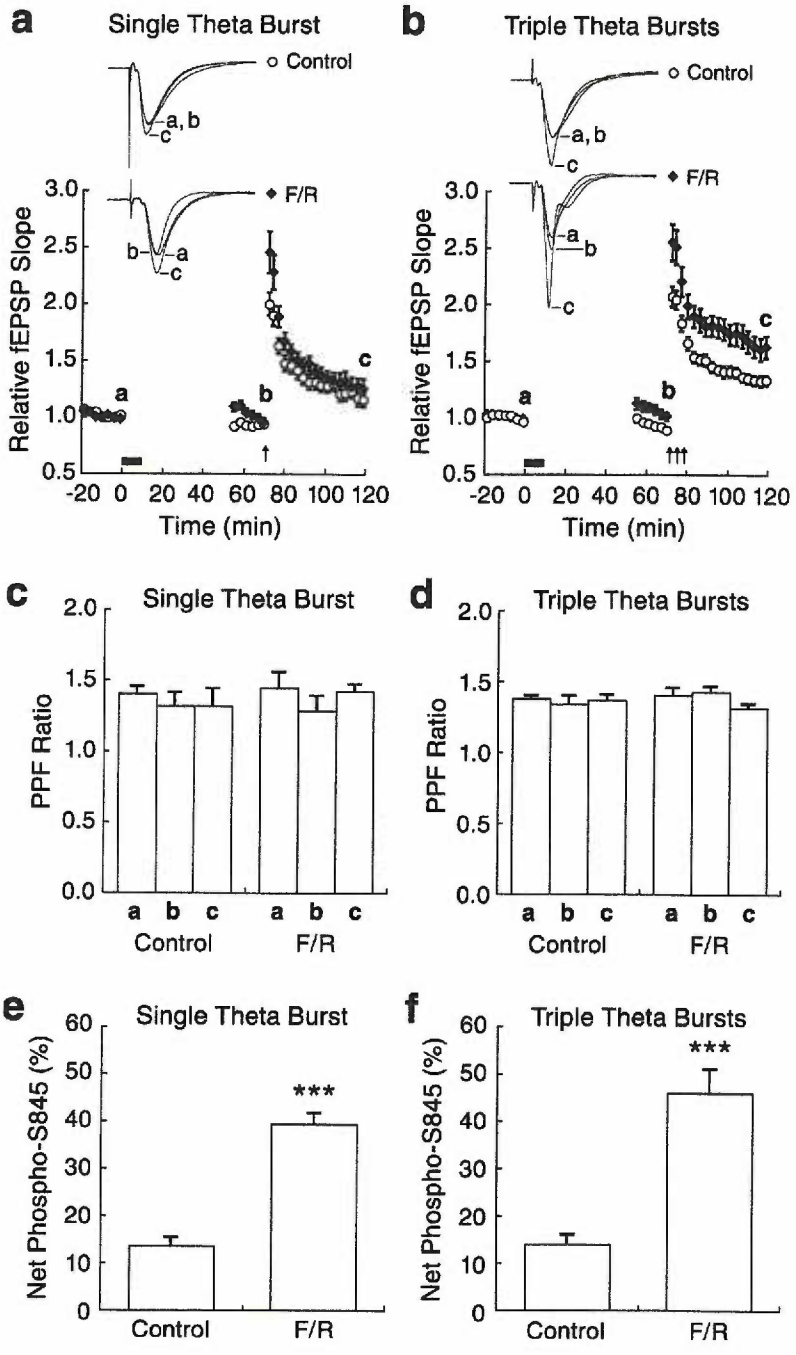

a
Single Theta Burst
Control
F/R
a, b
c
b
a
Relative fEPSP Slope
3.0
2.5
2.0
1.5
1.0
0.5
-20 0 20 40 60 80 100 120
Time (min)
b
Triple Theta Bursts
Control
F/R
a, b
c
a
b
c
Relative fEPSP Slope
3.0
2.5
2.0
1.5
1.0
0.5
-20 0 20 40 60 80 100 120
Time (min)
c
Single Theta Burst
PPF Ratio
2.0
1.5
1.0
0.5
0.0
a b c a b c
Control
F/R
d
Triple Theta Bursts
PPF Ratio
2.0
1.5
1.0
0.5
0.0
a b c a b c
Control
F/R
e
Single Theta Burst
Net Phospho-S845 (%)
60
50
40
30
20
10
0
***
Control
F/R
f
Triple Theta Bursts
Net Phospho-S845 (%)
60
50
40
30
20
10
0
***
Control
F/R

Figure 3.6 GluR1 S845 phosphorylation "primes" AMPARs for synaptic incorporation. (**a,b**) F/R pretreatment (black bar, solid diamonds) without basal stimulation did not result in larger potentiation in response to a single theta burst (single arrow in panel **a**) (at 120 min after F/R: Control, 1.16 ± 0.06, n = 12; F/R, 1.26 ± 0.08, n = 11; P = 0.35). However, a stronger LTP stimulation protocol using triple theta bursts (triple arrows in panel **b**), resulted in significantly larger potentiation (at 120 min after F/R: Control, 1.34 ± 0.04, n = 7; F/R, 1.63 ± 0.10, n = 9; $P < 0.05$). Representative fEPSP traces are shown on top. (**c,d**) There were no differences in PPF changes for all groups (a, b, and c time points indicated in **a** and **b**, n = 5-10). (**e-f**) Net phospho-S845 was significantly elevated in the CA1 region from slices recorded in **a-d** (n = 9-13). *** $P < 0.001$.

Figure 3.7

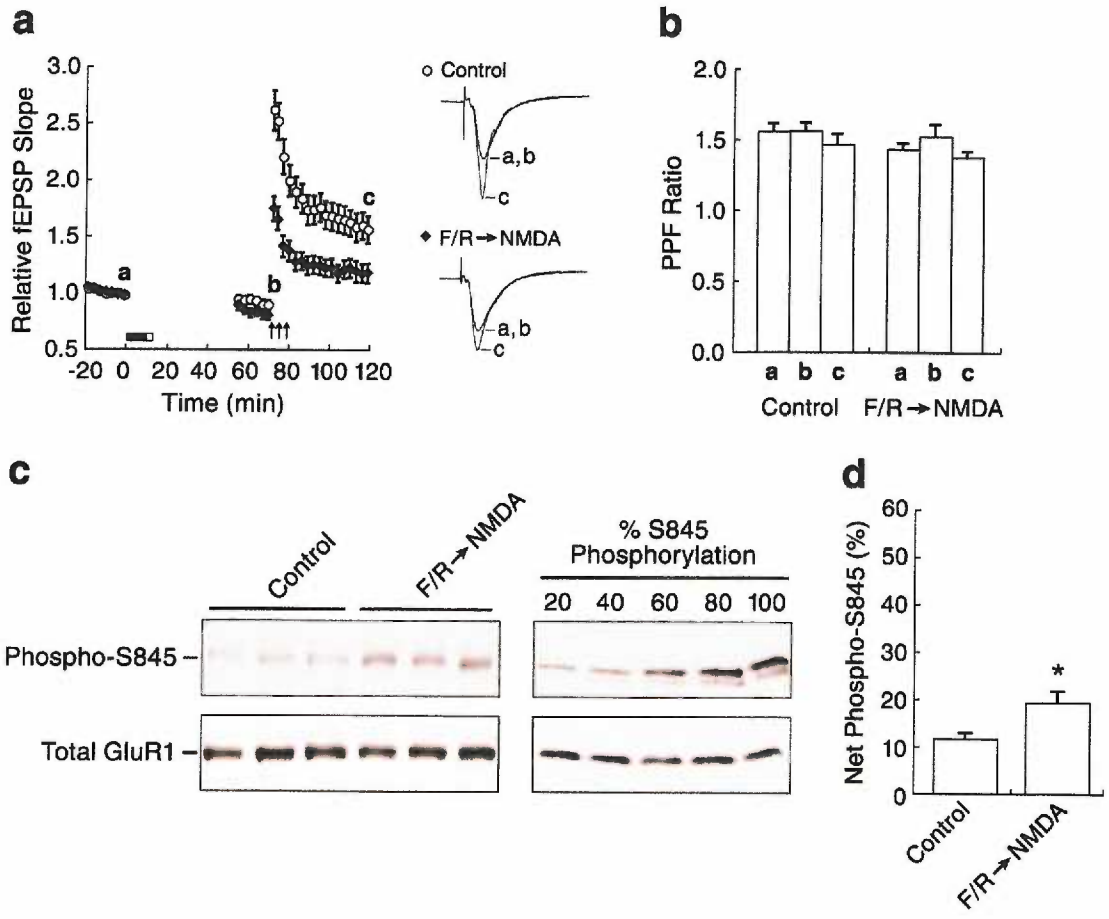

a
Relative fEPSP Slope
3.0
2.5
2.0
1.5
1.0
0.5
-20 0 20 40 60 80 100 120
Time (min)
a
b
c
Control
a,b
c
F/R→NMDA
a,b
c
b
PPF Ratio
2.0
1.5
1.0
0.5
0.0
a b c a b c
Control F/R→NMDA
c
Control
F/R→NMDA
% S845 Phosphorylation
20 40 60 80 100
Phospho-S845
Total GluR1
d
Net Phospho-S845 (%)
60
50
40
30
20
10
0
*
Control
F/R→NMDA

Figure 3.7 Chem-LTD blocks S845 phosphorylation-dependent "priming". (**a**) 10 min F/R (black bar) followed by 3 min of NMDA (open bar, solid diamonds) not only completely blocked the priming effect of S845 phosphorylation on LTP induced with triple theta bursts (compare with **Figure 3.6b**) but largely suppressed LTP itself. Representative fEPSP traces are shown on the right. (**b**) There were no differences in PPF changes (a, b, and c time points indicated in **a**, $n = 13$). (**c**) F/R-mediated phospho-S845 was significantly attenuated by subsequent NMDA treatment. (**d**) Quantitation shows that NMDA significantly attenuated F/R-stimulated phospho-S845 from 46.2 ± 5.4% (see **Figure 3.6h**) to 19.4 ± 2.4% ($P < 0.001$), although phospho-S845 was still slightly increased over the control group ($P < 0.05$, $n = 6$-8). * $P < 0.05$.

Figure 3.8

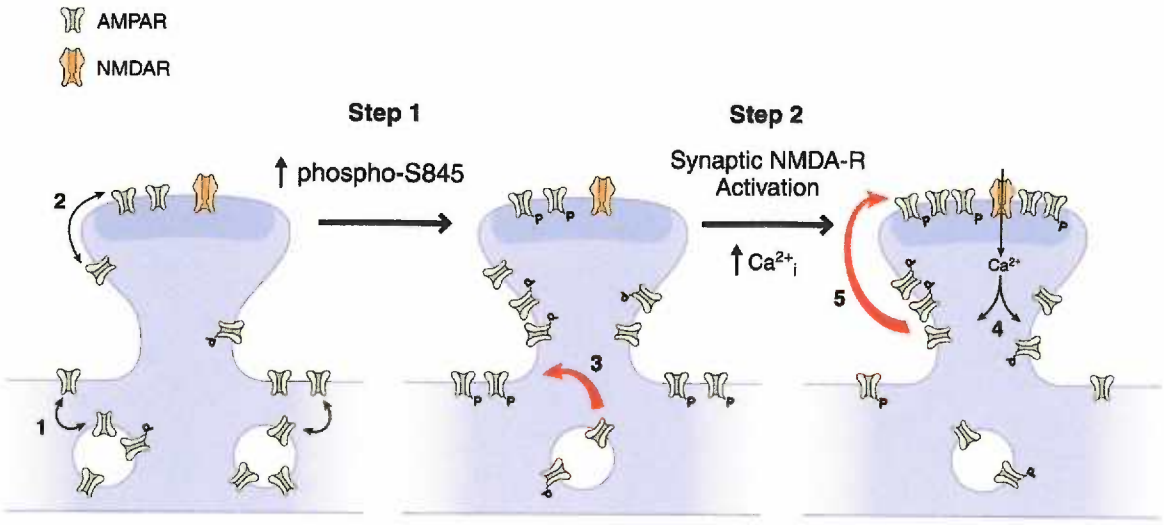

AMPAR
NMDAR
Step 1
↑ phospho-S845
Step 2
Synaptic NMDA-R Activation
↑ Ca^{2+}_i
1
2
3
4
5
Ca^{2+}

Figure 3.8 Two-step model for synaptic delivery of AMPARs during LTP. Under basal conditions (left panel), GluR1 has a low phosphorylation at S845. Constitutive recycling occurs between the surface and internal pools (**1**) and the synaptic and extrasynaptic pools (**2**) of AMPARs. Increasing S845 phosphorylation (**Step 1**) stimulates trafficking of internal GluR1-containing AMPARs to extrasynaptic sites on the surface membrane, which primes AMPARs for synaptic incorporation (**3,** middle panel). During strong synaptic activation (**Step 2**), synaptic NMDARs are activated, resulting in increased intracellular calcium (**4**). Calcium triggers the activation of signaling cascades, likely involving CaM-KII (42, 58), that drives GluR1-containing AMPARs to synapses from extrasynaptic sites by lateral diffusion (**5,** right panel). Thus, the two-step model for synaptic delivery of AMPARs consists of delivery of GluR1-containing AMPARs to extrasynaptic sites in a phospho-S845-dependent manner (**Step 1**; the priming step), followed by synaptic incorporation of AMPARs, which requires synaptic NMDAR activation and calcium (**Step 2**).

3.5 Discussion

The major finding of this study was that GluR1 S845 phosphorylation primes AMPARs for synaptic incorporation by trafficking AMPARs to extrasynaptic sites. Previous studies (11, 15, 58) examined S845 phosphorylation in GluR1 upon various stimulation paradigms, but only relative changes were analyzed, making it difficult to interpret the likely physiological significance of these changes. This report is the first investigation to quantify the net phosphorylation state of S845 in endogenous hippocampal GluR1 during chemically-induced bi-directional synaptic plasticity and show a strong correlation between phospho-S845 and surface GluR1. Under basal conditions approximately 30-35% of GluR1 was on the surface membrane and only 15% was phosphorylated at S845. Induction of cLTP increased surface expression and net phospho-S845, whereas Chem-LTD decreased surface expression and net phospho-S845. Combined with a functional analysis of synaptic responses, we demonstrated that S845 phosphorylation-regulated surface GluR1 delivery was to extrasynaptic sites. Results from this study indicate that S845 phosphorylation can modulate the dynamic range of synaptic plasticity by regulating the pool of AMPARs available for synaptic incorporation. Thus, S845 phosphorylation might be one mechanism that neurons may use to regulate the amplitude of synaptic potentiation.

It is interesting that neither S831 nor S845 in GluR1 are a CPS for either CaMKII or PKA, respectively. They both contain a proline rather than an arginine in the p-3 position. PKA did not phosphorylate S845 in wt GluR1 *in vitro*, but, intriguingly, CaMKII robustly phosphorylated S831 (**Figures 3.1a,b**). When both sites were mutated to a CPS by replacement of the p-3 proline by arginine, S845 became an excellent PKA

site whereas the corresponding mutation had no apparent affect on the rate of CaMKII-mediated S831 phosphorylation (**Figures 3.1c,d**). It is possible that PKA activates some downstream kinase that phosphorylates S845 in neurons. Alternatively, since GluR1 is part of a multi-protein complex in the PSD (36, 37, 86, 206), these interactions may alter the conformation around S845 allowing direct PKA phosphorylation. Using the CPS mutant for PKA (P842R), we obtained S845 phosphorylation with a molar stoichiometry of approximately 1 (**Figure 3.1d**). Mixtures of this fully phosphorylated mutant with non-phosphorylated mutant were used as calibration standards to quantify net phospho-S845 of endogenous AMPARs in cultured hippocampal neurons and slices.

This study presented the first quantified measures of net phospho-S845 in hippocampal neurons. This allowed analyses of functional effects in changes of net phospho-S845 and surface GluR1 delivery during bi-directional synaptic plasticity by combining quantitative immunoblotting, surface biotinylation, and slice field recordings. We found that under basal conditions, about 15% of surface GluR1 was phosphorylated at S845 with approximately 30-35% of receptors on the surface membrane. This suggests that phospho-S845-independent mechanisms may be also involved in maintaining surface GluR1 under basal conditions. However, chemical potentiation with F/R increased phospho-S845 to 40-65%. For every 1% increase in net phospho-S845, there was 0.75% increase in the surface fraction of GluR1 (see the slope of 0.75 in **Figure 3.2d**). This is an important finding indicating that the surface delivery of GluR1 was tightly correlated with an increase in phospho-S845 and strongly implicates the regulation of surface delivery of AMPARs by S845 phosphorylation. In support of this finding, Chem-LTD with NMDA treatment significantly decreased net phospho-S845 to below 10% and the

surface GluR1 fraction in cultured neurons (**Figure 3.3**). Chem-LTD also significantly attenuated F/R-mediated S845 phosphorylation and surface GluR1 delivery in slices (**Figure 3.4**), and more importantly, blocked F/R-mediated cLTP (data not shown). Interestingly, neither S845 phosphorylation nor surface delivery during F/R stimulation was NMDAR dependent (**Figures 3.2a-c**).

Another essential finding was that the surface delivery of AMPARs was not sufficient for synaptic targeting, but required concomitant synaptic activity (**Figure 3.5a**), suggesting that synaptic NMDAR activation is required (96). S845 phosphorylation has previously been proposed to be important for regulating the amount of GluR1-containing AMPARs available for synaptic incorporation, whereas CaM-KII activity appears to be critical for synaptic incorporation (58). Our results provide a specific mechanism for this previous finding, mainly that S845 phosphorylation-dependent delivery is selectively to extrasynaptic sites. The increase in extrasynaptic pool of GluR1 resulted in larger LTP following triple theta burst stimulation (**Figure 3.6b**), suggesting that strong synaptic NMDAR activity can drive GluR1-containing AMPARs into synapses from the extrasynaptic pools. This "priming" effect was suppressed by Chem-LTD, which blocked the F/R-mediated increase in phospho-S845 and surface GluR1 (**Figures 3.3,3.4,3.7**) (152). Therefore, our results support a two-step model for synaptic delivery of AMPARs (**Figure 3.8**), where GluR1-containing AMPARs are first delivered to the extrasynaptic sites in a S845 phosphorylation-regulated manner. Activated synaptic NMDARs then cause an influx of calcium that likely triggers the signal transduction cascades necessary for anchoring AMPARs in synapses. Thus, S845 phosphorylation "primes" the GluR1-containing AMPARs for synaptic incorporation. However, we

cannot exclude that other factors are also important for the initial surface delivery of GluR1. For example, phospho-S845 did not change in response to LTP induced by high-frequency stimulation (100 Hz, 1 sec) (11). However, the role of phospho-S845 during LTP may be more pronounced in younger animals (P7-8) where PKA exerts a major role in LTP expression (194) or under conditions where LTP is induced by different stimulation paradigms, such as theta burst stimulation.

The two-step model for synaptic delivery of AMPARs (**Figure 3.8**) is consistent with reports from other labs. For example, extrasynaptic AMPARS in hippocampal dendrites are increased nearly two fold during LTP (207) and extrasynaptic AMPARs are significantly depleted in GluR1 knockout mice (208). Furthermore, removal of GluR2-containing AMPARs occurs first at extrasynaptic sites, immediately followed by a decrease in synaptic AMPARs (193). AMPARs have been shown to rapidly move in and out of synapses via lateral movement along the surface membrane, which may account for the increase in postsynaptic AMPAR responses during LTP (169-171, 182). Elevating local intracellular calcium causes accumulation of AMPARs on the surface membrane, suggesting that calcium triggers the mechanisms for synaptic anchoring of laterally diffusing AMPARs (170, 182). Whether calcium first increases the number of anchors at synapses or blocks the lateral diffusion of AMPARs, which then recruits the anchors, remains to be determined. Because AMPARs diffuse freely in extrasynaptic sites (170), it seems more likely that synaptic calcium recruits more anchors to PSDs, which then sequester laterally diffusing AMPARs. One potential component of this anchor might be PSD-95, which has been shown to be important for regulating the number of synaptic AMPARs (209-211). Moreover, our data suggest that a certain

threshold or duration of calcium is required to recruit the anchors, since triple theta burst stimulation was required to drive more extrasynaptic AMPARs into synapses.

An important regulator of AMPAR trafficking is stargazin, which has been shown to be important for the delivery of AMPARs to the surface membrane (61). Importantly, stargazin-mediated surface delivery of AMPARs is selective to extrasynaptic sites (62) and stargazin knockout mice lack extrasynaptic AMPARs and have decreased synaptic AMPARs (61). By contrast, PSD-95 over-expression results in synaptic potentiation of AMPARs (62, 209). What might be the Ca^{2+}-sensitive transducer that promotes anchoring of AMPARs at synapses? CaM-KII has been implicated in inserting AMPARs into synapses (42, 58), and F/R stimulation is known to recruit CaM-KII to dendritic spines (128). Thus, calcium influx through synaptic NMDARs can activate CaM-KII (112), which may then drive extrasynaptic AMPARs into synapses. The exact target of CaM-KII important for this delivery is unknown, as it does not appear to be S831 in GluR1 (42). Intriguingly, stargazin has multiple phosphorylation sites, including CaM-KII and PKC sites that appear to be involved in regulating AMPAR trafficking (212). Clearly, additional studies are needed to elucidate the mechanisms of synaptic capture of AMPARs.

CHAPTER 4

SUMMARY AND CONCLUSIONS

The studies presented in the previous two chapters of this thesis provide new insight into the molecular mechanisms of synaptic plasticity through phosphorylation of the GluR1 subunit of AMPA receptors. Because the GluR1 subunit is the predominant subunit of the most common excitatory receptor used in the hippocampus, studies clarifying the molecular regulation of this subunit have wide implications for understanding synaptic function, both during synaptic plasticity and basal neurotransmission. Two known regulatory phosphorylation sites in the GluR1 C-terminus were studied, each chapter focusing on the different role of particular phosphorylation site in modulating AMPA receptor function. These studies not only extend our current understanding of AMPA receptor function, but they provoke new exciting questions in neuroscience as discussed below.

4.1 SUMMARY AND SIGNIFICANCE OF FINDINGS FROM CHAPTER 2

Phosphorylation of the GluR1 subunit at S831 plays an important role during synaptic potentiation (4, 10). NSFA (variance analysis) (13) and silence analysis (14) have determined that S831 phosphorylation by CaM-KII in homomeric GluR1 enhances single-channel conductance, without affecting other channel properties. GluR1 homomers have multiple subconductance states, which are determined by the number of glutamate bound to each subunit in a tetrameric receptor, and S831 phosphorylation increases the probability of higher conductance states when glutamate is bound (13). Thus, coupling of ligand binding and channel opening to higher conductance states is enhanced by S831 phosphorylation. The main question examined in **Chapter 2** was,

"how does S831 phosphorylation by CaM-KII affect channel properties in the context of GluR1/GluR2 heteromers?" This question is an important one, because first, the majority of AMPA receptors in the CA3/CA1 synapses are composed of GluR1/GluR2 heteromers, and second, AMPA receptors may undergo subunit recomposition during synaptic plasticity (52, 53, 213). The latter bolsters the importance of defining the channel properties of different subunit compositions, because without this knowledge, the consequence of subunit recomposition during synaptic plasticity cannot be fully comprehended. For example, replacing calcium-permeable AMPA receptors with calcium-impermeable AMPA receptors, which contain the edited GluR2 subunit, during synaptic plasticity in cerebellar stellate cell does not give any information about whether synaptic transmission is potentiated or depressed, if the differences in channel properties of AMPA receptors with and without GluR2 are unknown. This chapter specifically addressed these questions, such that molecular consequences of subunit recomposition can be better understood.

To achieve this aim, recombinant AMPA receptors of defined subunit compositions were expressed and recorded in HEK293 cells. Studies of AMPA receptor function are suitable in HEK293 cells, because these cells lack glutamate-gated ion channels and have the machinery to express and deliver functional receptors to the surface membrane. Receptor properties characterized in HEK293 cells also seems to mimic that of endogenous receptors found in neurons. Surprisingly, activated CaM-KII did not potentiate glutamate-induced currents in cells expressing GluR1/GluR2 heteromers, whereas GluR1 homomers were significantly potentiated. CaM-KII was able to phosphorylate S831 to the same extent, regardless of the presence of GluR2 subunit,

both in transiently transfected HEK293 cells co-expressed with active CaM-KII and endogenous AMPA receptors in cultured hippocampal neurons stimulated with GISP. These findings suggest that the lack of CaM-KII-mediated potentiation in GluR1/GluR2 heteromers may be due to the loss in coupling S831 phosphorylation with high conductance state by GluR2. NSFA verified this hypothesis, showing that neither S831 phosphorylation nor aspartate mutation enhanced single-channel conductance in the presence of GluR2. This is an important finding that will help tremendously in clarifying the mechanisms of potentiation or depression that may occur during subunit recomposition in glutamatergic synapses.

Because single-channel conductance of GluR1/GluR2 heteromers (2.5 ± 0.3pS) is significantly lower than GluR1 homomers (12.4 ± 1.3pS), subunit recomposition alone can significantly modulate synaptic transmission. This means replacing GluR1/GluR2 heteromers with GluR1 homomers can results in five-fold increase in the average postsynaptic conductance. More importantly, replacing heteromers with GluR1 homomers allows further potentiation by CaM-KII phosphorylation at S831, enhancing the single-channel conductance to even higher levels (18.9 ± 1.2 to 25.5 ± 2.6pS as measured with CaM-KII infusion or with Asp831 mutation, respectively). Phosphorylated GluR1 homomers are thus eight to ten-fold greater in single-channel conductance compared to GluR1/GluR2 heteromers. Furthermore, open probability of GluR1/GluR2 heteromers are so low that accurate estimates could not be obtained by NSFA. Thus, synaptic delivery of phosphorylated GluR1 homomers, which have open probability in the range of 40 to 90% depending on S845 phosphorylation (15), would further contribute to synaptic potentiation.

Subunit recomposition during synaptic plasticity is likely involved not only in LTP, but in LTD as well. Cerebellar stellate cells, for example, contain mostly GluR2-lacking AMPA receptors at synapses innervated by parallel fibers under basal conditions, and activity delivers GluR2-containing AMPA receptors to synapses (52, 53, 213). Likewise, dopaminergic neurons in the ventral tegmental area are innervated by excitatory glutamatergic inputs and contain mostly GluR2-lacking AMPA receptors, as they show strong inward rectification and are blocked by Joro spider toxin. After mGluR-dependent LTD, induced by five stimuli at 66 Hz or mGluR type I (mGluR1 and mGluR5) agonist DHPG, AMPA receptors show linear rectification and become insensitive to Joro spider toxin, suggesting that AMPA receptors are replaced with GluR2-containing ones (214). Because GluR2-containg AMPA receptors are not modulated by CaM-KII phosphorylation and have much lower single channel conductance and open probability compared to GluR1 homomers, synaptic delivery of GluR2-containing AMPA receptors is likely an important mechanism of LTD in cerebellar stellate and dopaminergic neurons in the ventral tegmental area.

Selective delivery of different subunits of AMPA receptors may reflect differences in synaptic plasticity found throughout the brain. CA3 pyramidal cells are innervated by two different afferents, mossy fibers (MF) and associational fibers (AF). Synaptic plasticity at these two synapses is likely due to very different mechanisms, although both synapses are found in the same neuron. Interestingly, two synapses show very different preferences for activity-dependent AMPA receptor delivery (215). Although GluR2 subunits are constitutively delivered to both MF and AF postsynaptic sites under basal conditions, GluR1 homomers are delivered in an activity-dependent

manner only to AF synapses, but not to MF synapses. This activity-dependent delivery of GluR1 homomers in AF synapses is mimicked by co-expression of activated CaM-KII, suggesting that CaM-KII can drive GluR1 into AF synapses as in CA1 pyramidal neurons (42). Furthermore, newly delivered GluR1 homomers to AF synapses allows new NMDA receptor-independent form of LTP, suggesting that calcium influx through these new AMPA receptors are sufficient to activate postsynaptic mechanisms that result in LTP. In contrast, MF synapses do not deliver new GluR1 homomers in response to activity (215) and delivery of calcium-permeable GluR2Q (not edited) does not result in NMDA receptor-independent postsynaptic LTP (216), suggesting that presynaptic mechanisms dominate in these synapses. Thus, two different inputs into CA3 pyramidal neurons show very different preferences for AMPA receptor delivery during synaptic plasticity, and the functional consequences of receptor delivery are distinct in these two synapses. These results suggest that different synapses throughout the brain utilize different mechanisms of subunit-specific delivery and/or subunit recomposition to achieve LTP and LTD.

One criticism of the subunit recomposition hypothesis is that previous studies have not detected changes in the rectification of AMPA receptors during LTP expression in CA3/CA1 synapses (207). If GluR1/GluR2 heteromers are replaced by GluR1 homomers, one would expect to see more inward rectification after LTP expression. However, there are some caveats to this criticism. First, studies that have examined changed in rectification with LTP have not included polyamines, such as spermine, in the intracellular solution. GluR1 homomers have linear rectification in the absence of polyamines, mimicking the properties of GluR1/GluR2 heteromers (49). Thus, lack of

changes in rectification after LTP expression may be due to a "washout" of intracellular polyamines. Second, subunit recomposition may occur with little change in rectification, while having a significant effect on average channel conductance at a synapse. Using the information determined in the **Chapter 2** of this thesis and from the current literature, a specific example can be proposed. The excitatory synapse in the CA1 region of hippocampus is estimated to have around 90 AMPA receptors (although sometimes as few as 10) activated during glutamate release induced by a single presynaptic release event (217-219). About 70% of these receptors is GluR1-containing AMPAR receptors of which 92% is GluR1/GluR2 heteromers and 8% is GluR1 (26). This translates to about 63 GluR1-containg AMPA receptors activated, consisting of 58 GluR1/GluR2 heteromers and 5 GluR1 homomers (for simplicity, only GluR1-containing AMPA receptors are considered). Thus, the estimated conductance from GluR1-containing AMPA receptors is 207pS per synapse during one presynaptic release event [(58 GluR1/GluR2 x 2.5pS) + (5 GluR1 x 12.4pS) = 207pS] (see **Table 2.2**). During LTP, GluR1 is phosphorylated at S831 (7, 8) and also delivered to synapses (41, 149), both being mediated by CaM-KII (10, 11, 42, 58). If only five new phosphorylated GluR1 homomers are delivered to this synapse, and one receptor adds 18.9 to 25.5pS to a synapse (18.9 ± 1.2 to 25.5 ± 2.6pS as measured with CaM-KII infusion or with Asp831 mutation, respectively), this would be equivalent to 46-62% potentiation in total conductance for this particular synapse (18.9pS or 25.5pS x 5 receptors / 207pS = 46-62%). Adding five receptors to a synapse containing 63 receptors (8% increase in number of receptors) may not affect average rectification significantly (less than 10%), while total synaptic conductance would be significantly enhanced (46-62%) as calculated

above. Thus, it is very possible that methods used in the previous studies were not sensitive enough to detect the changes in rectification during LTP. Furthermore, newly delivered GluR1 homomers would have higher open probability compared to the GluR1/GluR2 heteromers, adding to the overall increase in total synaptic current. Thus, more studies directly evaluating the contribution of subunit recomposition during synaptic plasticity are needed.

4.2 SUMMARY AND SIGNIFICANCE OF FINDINGS FROM CHAPTER 3

S845 phosphorylation of GluR1 subunit has been implicated in trafficking of AMPA receptors in response to activity (58, 60) and LTP (4). PKA phosphorylates internalized AMPA receptors at S845, and this promotes recycling of the receptors back to surface membrane rather than targeting them for degradation (60). Furthermore, CaM-KII-mediated synaptic incorporation of GluR1 is completely blocked in conditions that inhibit S845 phosphorylation or with S845A mutation (58). S845 phosphorylation is proposed to regulate the pool of receptors available for synaptic incorporation. Importantly, the results from **Chapter 3** provide a novel mechanism for this process. Mainly, S845 phosphorylation increases GluR1 delivery to extrasynaptic sites, effectively "priming" these receptors for synaptic incorporation.

For the first time, a quantitative assay was developed to measure the net changes in S845 phosphorylation of GluR1 during bi-directional synaptic plasticity. This technique revealed several unique facets of AMPA receptor plasticity in the hippocampus. S845 phosphorylation was consistently low in hippocampal neurons under basal conditions. This was confirmed in both cultured neurons and in slices. Under basal

conditions, about 15% of surface GluR1 was phosphorylated at S845 with approximately 30-35% of receptors on the surface membrane. This suggests that S845 phosphorylation-independent mechanisms may be responsible for maintaining surface GluR1 during basal conditions. Stimulation with forskolin and rolipram significantly elevated net S845 phosphorylation (40-65% 40 to 70 min after stimulation), and more importantly, this was highly correlated with an increase in the surface fraction of GluR1. Specifically, for every 1% increase in surface GluR1 S845 phosphorylation, there was 0.75% increase in the surface fraction of GluR1. This tight correlation strongly supports the role of S845 phosphorylation in activity-dependent delivery of GluR1 to the surface membrane. In support of this, Chem-LTD with NMDA treatment significantly decreased net S845 phosphorylation and surface GluR1 fraction and significantly attenuated the "priming" effects of forskolin and rolipram. Interestingly, F/R stimulation promoted additional GluR1-containing AMPA receptors to extrasynaptic sites, and synaptic potentiation was dependent on concomitant synaptic activity. Increasing the extrasynaptic pool of AMPA receptors resulted in a greater potentiation in response to triple theta burst stimulation, and thus, S845 phosphorylation "primes" the AMPA receptors for synaptic incorporation. This finding supports the "slot" model, where AMPA receptors are synaptically anchored by "slots" (204). Only strong stimulation seems to be able to form new "slots" for AMPA receptors, since the weaker single theta burst stimulation was not sufficient to reveal the priming effect.

The results from this study indicate that S845 phosphorylation can modulate the dynamic range of synaptic plasticity. The degree of synaptic potentiation can be modulated by regulating the pool of AMPA receptors available for synaptic

incorporation. This hypothesis is supported by the finding that increasing the extrasynaptic pool of AMPA receptors resulted in greater LTP, while blocking delivery resulted in smaller LTP. Thus, S845 phosphorylation might be one mechanism that neurons may use to regulate the amplitude of synaptic potentiation.

The findings in this study also emphasize the importance of quantifying phosphorylation. Without determining the net changes in phosphorylation states, correlating functional changes with phosphorylation can be misleading. With quantitative calibration standards on every Western blot, a strong correlation between S845 phosphorylation and surface delivery of GluR1 was validated.

4.3 FUTURE STUDIES AND CONCLUDING REMARKS

The results from the studies presented in this thesis provoke new and exciting questions about AMPA receptor plasticity. One important question is whether subunit recomposition occurs during LTP in CA3/CA1 synapses in the hippocampus. More careful examination of the rectification changes with polyamines included in the intracellular solution would be critical. Imaging studies could also bring new information about subunit recomposition during LTP, although this would likely require experiments to be performed in cultured slices with high resolution imaging capacity. By tagging GluR1 and GluR2 subunits with different fluorescent tags, it would be possible to track surface localizations of different subunits during LTP expression in the CA1 region. This imaging technique could be combined with electrophysiology to track synaptic delivery of specific receptor subunits during LTP. Synaptic incorporation of both GluR1 and unedited GluR2Q subunits can be verified by changes in rectification.

How does S845 phosphorylation promote surface delivery of GluR1 to extrasynaptic sites? Does S845 phosphorylation cause release of GluR1 from the internal anchors as it has been proposed for the GluR4 subunit (58), or are phosphorylated receptors stabilized at the surface membrane through protein-protein interactions, and if so, what proteins stabilize GluR1 at extrasynaptic sites? How are AMPA receptors stabilized at synapses? These are some of the most interesting questions that would need to be addressed to fully map the molecular pathway of AMPA receptor trafficking. Recent studies have demonstrated that experience-driven synaptic potentiation in barrel cortex and fear conditioning in lateral amygdala drive AMPA receptors into synapses (220, 221). As more and more proteins that interact with AMPA receptors are identified, specific proteins important for the learning-dependent delivery of AMPA receptors to synapses can be characterized. Current studies can focus on already identified interacting proteins, such as stargazin, 4.1N, and SAP-97. All of these proteins have been implicated in trafficking of AMPA receptors (61, 62, 205, 222-224). Thus, it would be important to study how these protein interactions affect phosphorylation-regulated channel property and surface delivery of GluR1 during synaptic plasticity.

Interestingly, synaptic activity, likely involving synaptic NMDA receptor activation, was required for driving AMPA receptors into synapses, while activation of extrasynaptic NMDA receptors by bath application of NMDA resulted in a decrease in S845 phosphorylation and surface GluR1. This result clearly suggests that signaling cascades activated by these two populations of NMDA receptors are different. It would be important to dissect out the differences in molecular mechanisms activated by these receptors, as extrasynaptic NMDA receptors could be activated during spillover since

spillover of glutamate is known to activate AMPA and NMDA receptors at neighboring synapses (225-228).

Finally, under what conditions is S845 phosphorylated during LTP and dephosphorylated during LTD? Previous study inducing LTP with high-frequency stimulation (1 sec 100 Hz) could not detect an increase in S845 phosphorylation (11). What signaling cascades lead to S845 phosphorylation? Because S845 was not phosphorylated *in vitro* by PKA, it is possible that PKA does not directly phosphorylate GluR1 but activates a downstream kinase. More detailed biochemical analyses are required to map this signaling pathway.

The studies presented in this thesis greatly extend our current understanding of AMPA receptor functions during synaptic plasticity, specifically through phosphorylation of the GluR1 subunit. It is interesting to note that two phosphorylation sites separated by only 14 amino acids have such distinct regulatory roles and that addition of a small negatively charged phosphate group to a large tetrameric protein complex, where GluR1 alone is made up of 907 amino acids, can induce such profound changes to channel properties and trafficking patterns. Clearly, more studies are needed to define the regulatory roles of other phosphorylation sites in AMPA receptors and how these phosphorylation sites interact and complement each other during synaptic plasticity. Nonetheless, the results presented here clearly strengthen the evidence supporting the role of protein phosphorylation during synaptic plasticity (1).

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