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Clathrin Adaptor AP2 and NSF Interact with Overlapping Sites of GluR2 and Play Distinct Roles in AMPA Receptor Trafficking and Hippocampal LTD

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Summary

Proteins that bind to the cytoplasmic tails of AMPA receptors control receptor trafficking and thus the strength of postsynaptic responses. Here we show that AP2, a clathrin adaptor complex important for endocytosis, associates with a region of GluR2 that overlaps the NSF binding site. Peptides used previously to interfere with NSF binding also antagonize GluR2-AP2 interaction. Using GluR2 mutants and peptide variants that dissociate NSF and AP2 interaction, we find that AP2 is involved specifically in NMDA receptor-induced (but not ligand-dependent) internalization of AMPA receptors, and is essential for hippocampal long-term depression (LTD). NSF function, on the other hand, is needed to maintain synaptic AMPA receptor responses, but is not directly required for NMDA receptor-mediated internalization and LTD.

Introduction

AMPA receptors mediate most of the fast excitatory synaptic transmission in mammalian brain (Dingledine et al., 1999). In recent years, evidence has accumulated that AMPA receptors can undergo dynamic redistribution in and out of the postsynaptic membrane on a rapid time scale. Activation of NMDA receptors stimulates surface/synaptic delivery of GluR1-containing AMPA receptors from intracellular pools (Shi et al., 1999, 2001; Hayashi et al., 2000; Passafaro et al., 2001). On the other hand, NMDA and factors such as AMPA and insulin can promote endocytosis of AMPA receptors in neurons (Carroll et al., 1999; Man et al., 2000; Ehlers, 2000; Lin et al., 2000; Beattie et al., 2000). Internalized AMPA receptors recycle back to the surface in a manner regulated by synaptic activity (Ehlers, 2000; Lin et al., 2000). The controlled delivery and removal of synaptic AMPA receptors likely represent important postsynaptic mechanisms contributing to synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD) (for reviews, see Sheng and Lee, 2001; Lüscher et al., 2000; Carroll et al., 2001; Malinow and Malenka, 2002).

Despite intense research, the molecular mechanisms that control AMPA receptor trafficking remain largely unknown. Specific proteins that bind to the cytoplasmic tails of AMPA receptor subunits are implicated in the exocytosis and endocytosis of AMPA receptors (for reviews, see Sheng and Pak, 2000; Scannevin and Huganir, 2000; Sheng and Lee, 2001). The GluR2 subunit has received particular attention because it binds to a variety of cytoplasmic proteins. The PDZ domain-containing proteins GRIP/ABP and PICK-1 bind to the C terminus of GluR2 and GluR3. Interfering with the GRIP/ABP or PICK-1 interaction attenuates the synaptic clustering or surface expression of AMPA receptors (Dong et al., 1997; Osten et al., 2000). Postsynaptic injection of peptides that block GluR2-PDZ interactions prevents induction of hippocampal LTD (Daw et al., 2000; Xia et al., 2000; Kim et al., 2001). However, the exact mechanisms of these effects are still contentious (Carroll et al., 2001).

Apart from the C-terminal PDZ binding site, a membrane-proximal region of the GluR2 cytoplasmic tail interacts with NSF, a hexameric ATPase involved generally in membrane fusion events (Hay and Scheller, 1997; Osten et al., 1998; Nishimune et al., 1998; Song et al., 1998). In hippocampal neurons, postsynaptic infusion of a peptide corresponding to the NSF binding site of GluR2 (variously termed "pep2m" or "G10") caused run-down of synaptic currents over ~30 min (Nishimune et al., 1998; Lüthi et al., 1999; Song et al., 1998; Noel et al., 1999; Lüscher et al., 1999). This finding suggests that the GluR2-NSF interaction is somehow required for maintaining the normal synaptic level of AMPA receptors. The run-down of AMPA EPSCs mediated by pep2m peptide blocks subsequent LTD, and conversely, prior LTD induction occludes the pep2m effect, suggesting that LTD and pep2m act on the same pool of AMPA receptors. However, the precise function of the GluR2-NSF interaction remains unclear. NSF might be involved in the stabilization of synaptic AMPA receptors, for instance, preventing their internalization from the surface membrane. Alternatively, in the light of recent findings that GluR2 is constitutively delivered to and recycled from synapses (Shi et al., 2001; Passafaro et al., 2001), NSF might play a role in the recycling of GluR2-containing receptors back to the surface, thereby maintaining stable synaptic levels of AMPA receptors.

Internalization of AMPA receptors occurs through dynamin-dependent clathrin-mediated processes (Carroll et al., 1999; Man et al., 2000; Lin et al., 2000; Wang and Linden, 2000). Clathrin adaptor proteins link membrane proteins to clathrin and promote assembly of clathrin coats. Therefore, binding of clathrin adaptors to the cytoplasmic domains of receptors is a key early step in endocytosis (Kirchhausen, 1999). The best-characterized clathrin adaptor involved in endocytosis from the

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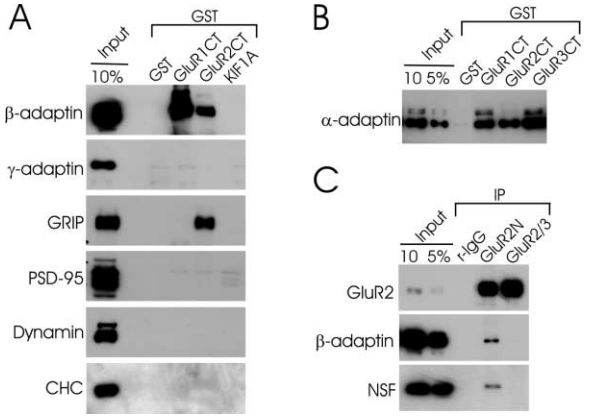


Figure 1. Interaction of AP2 Complex with Cytoplasmic Tails of AMPA Receptor Subunits

(A) AP2 pull-down by GluR1 and GluR2. GST-fusion proteins of the cytoplasmic tails of GluR1 and GluR2 (GluR1CT and GluR2CT) were incubated with brain cytosolic extracts in a GST pull-down assay. Bound proteins were immunoblotted for the indicated proteins. AP2 was examined by immunoblotting for β-adaptin. GST alone and GST-fusion protein of KIF1A served as controls. CHC, clathrin heavy chain.

(B) AP2 pull-down by GluR3. GST fusion proteins of the cytoplasmic tails of GluR1, GluR2, or GluR3 were incubated with brain cytosolic extracts. AP2 binding was examined by immunoblotting for α-adaptin.

(C) Coimmunoprecipitation of AMPA receptors with AP2 complex from brain. Deoxycholate extracts of synaptosomal (P2) fraction of rat brain were immunoprecipitated with nonimmune rabbit immunoglobulins (r-IgG), or antibodies to the C terminus of GluR2/3 or extracellular epitope of GluR2 (GluR2N). Immunoprecipitates were immunoblotted for GluR2, β-adaptin, and NSF.

plasma membrane is the AP2 complex, comprised of four subunits, α-, β-, σ-, and μ-adaptins. Here we show that AP2 associates with a region of GluR2 that closely overlaps with the NSF binding site—indeed, previously used NSF-interfering peptides such as pep2m disrupt AP2 as well as NSF binding to GluR2. By differentially interfering with these protein interactions, we reveal distinct roles for NSF and AP2 in the maintenance and regulated removal of AMPA receptors at the synapse.

Results

Pull-Down of AP2 Complex by Cytoplasmic Tails of AMPA Receptors

We previously showed that a subset of AMPA receptors could be coimmunoprecipitated from brain extracts using antibodies against AP2 (β-adaptin) (Man et al., 2000). To explore the mechanism of this association, we performed GST pull-down assays from rat brain extracts using the cytoplasmic tails (CT) of AMPA receptor subunits. Both GluR1CT and GluR2CT effectively precipitated α-adaptin and β-adaptin (subunits of AP2) (Figures 1A and 1B). On the other hand, γ-adaptin (a subunit of the AP1 clathrin adaptor complex), PSD-95, dynamin, and clathrin heavy chain were not retained by GST-GluR1CT or -GluR2CT. As a further indication of specificity, GRIP was bound by GST-GluR2CT but not by -GluR1CT (Figure 1A). The cytoplasmic tail of GluR3 also bound the AP2 complex in GST pull-down assays (Figure 1B).

Biochemical Association of AMPA Receptors with AP2 Complex in Brain

To confirm the association of AP2 with AMPA receptors in vivo, GluR2 subunits were immunoprecipitated from detergent extracts of P2 (crude synaptosome) fraction of brain and examined for coprecipitation of AP2. GluR2N antibody (raised against N-terminal extracellular domain of GluR2) coprecipitated AP2 subunits β-adaptin (Figure 1C) and α-adaptin (data not shown). Interestingly, a polyclonal antibody raised against the C-terminal 13 amino acids of GluR2 (which also recognizes the highly similar GluR3 subunit, and hence termed "GluR2/3") failed to coprecipitate AP2, even though it immunoprecipitated more GluR2 than did anti-GluR2N antibodies. Similarly, the coprecipitation of NSF with GluR2 from brain extracts was only detected using GluR2N antibodies (Figure 1C). The different abilities of these two GluR2 antibodies to precipitate a complex of GluR2 and AP2 might be explained if the binding of AP2 (a large protein complex of ~300 kDa) sterically prevents binding of antibodies to the C terminus of GluR2, or if GluR2/3 antibody binding to the C-terminal region induces a conformational change that disrupts AP2 interaction with the cytoplasmic tail. Similar steric hindrance has been reported for the interaction of NSF with AMPA receptors (Osten et al., 1998).

AP2 Interaction Site of GluR2 Overlaps but Is Distinct from the NSF Binding Site

To define the sequence determinants required for interaction with AP2, we tested a series of deletion mutants of the GluR2 cytoplasmic tail in the GST pull-down assay (Figure 2). C-terminal deletions of GluR2 abolished GRIP binding, as expected, but truncation of up to the last 30 amino acids of GluR2 (ΔC30) did not affect AP2 pull-down. However, deleting the C-terminal 35 residues (ΔC35) abolished AP2 retention by the GluR2 tail (Figure 2B). The most membrane-proximal 10 amino acids of GluR2CT (ΔN10) were dispensable for AP2 pull-down. These results suggest that a 10 amino acid segment (K844-Q853) is necessary for AP2 interaction. Strikingly, this 10 amino acid stretch corresponds to pep2m, the 10 residue peptide derived from the mouse GluR2 sequence that was used in earlier studies to interfere with NSF binding to GluR2 (Nishimune et al., 1998; Lüthi et al., 1999; Noel et al., 1999; Lüscher et al., 1999). In rat GluR2 (which is used in our binding studies), the sequence of the K844-Q853 peptide (termed pep2r for convenience) differs from pep2m at only one position, where proline (P852, pep2r) is substituted for alanine (A852, pep2m) (see Figure 6A). A GST fusion protein of the 10 residue pep2r (K844-Q853) pulled down AP2 at least as effectively as the GST fusion protein containing the entire C-terminal tail of GluR2 (Figure 2C). An internal deletion of this region (ΔK844-Q853) abolished AP2 precipitation by GluR2. Thus, amino acids K844-Q853 ("pep2r") in the GluR2 tail are necessary and sufficient for association with AP2. The N-terminal half of this peptide sequence is particularly conserved, containing a KRMK motif present in GluR1, GluR2, and GluR3. Inter-

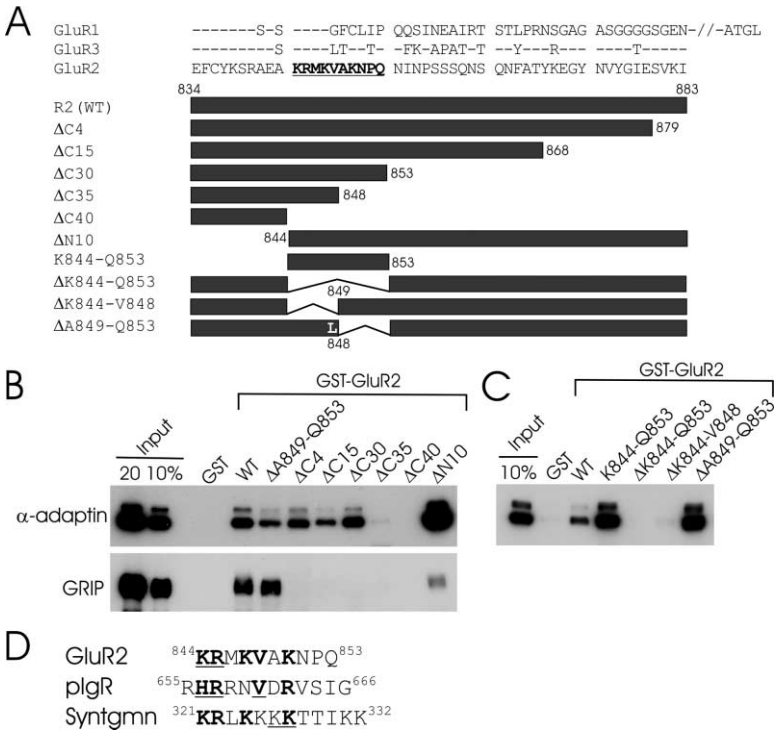


Figure 2. AP2 Interaction Site Overlaps with NSF Binding Site in GluR2

(A) Diagram of the various deletion constructs of GluR2 aligned below a sequence comparison of GluR1, GluR2, and GluR3 cytoplasmic tails.

(B and C) AP2 pull-down by C-terminal and N-terminal deletion mutants (B) or internal deletion mutants (C) of GluR2 cytoplasmic tail. GST pull-downs were performed from brain extracts as in Figure 1, and immunoblotted for AP2 (α-adaptin) and GRIP.

(D) Sequence similarity between the AP2 interaction site of GluR2 and regions of the polymorphic immunoglobulin receptor (pIgR) and synaptotagmin (Syntgmn). Numbers indicate respective amino acid residue positions.

nal deletion of these residues (ΔK844-V848) in GluR2 also abolished AP2 pull-down (Figure 2C).

Since the K844-Q853 region was also identified as the minimal NSF binding domain of GluR2 (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998), we sought to dissociate AP2 and NSF binding by further mutagenesis of this segment. AP2 and NSF interaction of GluR2 mutants were tested by GST pull-down assays; the NSF binding to GluR2 mutants was additionally assayed in the yeast two-hybrid system. Deletion of the last 5 amino acids in the K844-Q853 region (ΔA849-Q853) abolished NSF binding by GluR2 (Figures 3A and 3C), but did not affect AP2 (Figures 2C and 3B). Moreover, GluR1 and GluR3, which do not bind NSF (Osten et al., 1998; Nishimune et al., 1998; Song et al., 1998), interact with AP2 as effectively as GluR2 (Figure 1B). These results indicate that AP2 can associate with AMPA receptor subunits independently of NSF.

More subtle mutations were introduced into the NSF/AP2 binding region. Alanine substitutions in the conserved N-terminal portion (K844A and R845A) had different effects on NSF and AP2 binding. The K844A mutation abolished both NSF and AP2 interaction, while the R845A mutation strongly impaired AP2 pull-down but had little effect on NSF binding (Figures 3A–3D). Mutations in the C-terminal part of the K844-Q853 segment (P852A and double mutant N851S/P852A) did not affect AP2 or NSF interaction. This is perhaps not surprising since the P852A mutation merely mimics the mouse (pep2m) sequence in this region of GluR2. As expected,

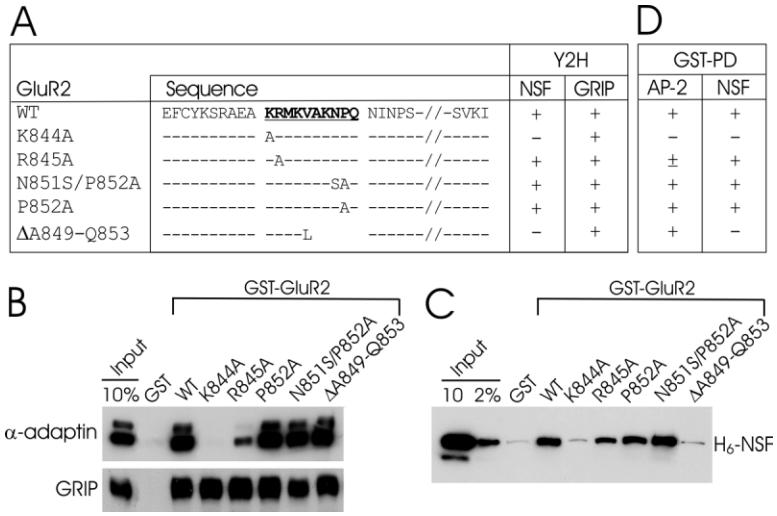


Figure 3. Dissociation of AP2 and NSF Interaction by Mutations in GluR2

(A) Sequence of GluR2 mutants and their binding to NSF and GRIP, as measured by yeast two-hybrid assays. +, <30 min β-gal detection time; −, no detectable β-gal signal after 3 hr.

(B) GST pull-down assay for AP2 and GRIP binding of GluR2 mutants versus wild-type (wt) and GST alone.

(C) Binding of purified recombinant NSF (H_6-NSF) to wt and mutant GluR2 in the GST pull-down assay.

(D) Summary of results from (B) and (C), tabulated for comparison with NSF binding in (A). +, ±, and − indicate strong, weak, and no binding, respectively.

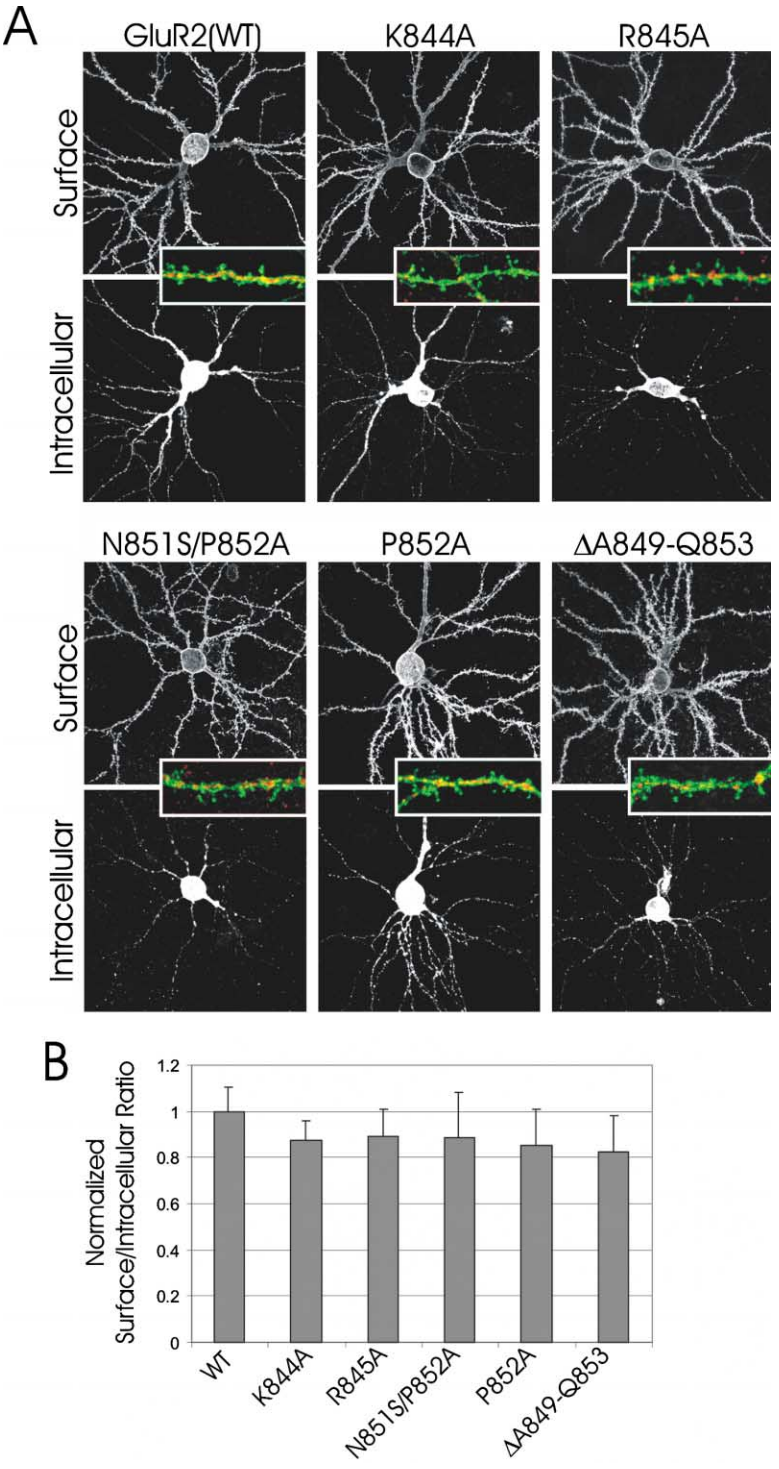


Figure 4. Steady-State Levels of Surface and Intracellular HA-Tagged Wild-Type and Mutant GluR2 in Transfected Hippocampal Neurons

(A) Representative double-label images of surface and intracellular expression of HA-GluR2 constructs (wild-type or mutants as indicated) in transfected neurons. Insets show enlargement of a dendritic segment: surface HA staining in green, intracellular in red (overlap yellow).

(B) Quantitation of surface/intracellular ratio of mutant HA-GluR2 constructs, normalized to wild-type. Histograms show mean and SEM (n = 15 cells for each construct).

none of these internal mutations affected the binding of GluR2 to GRIP in yeast two-hybrid or pull-down assays (Figures 3A and 3B).

AP2 Association Is Required for NMDA-Induced Internalization of AMPA Receptors

To address the potential function of NSF and AP2 in AMPA receptor internalization, we overexpressed in cultured hippocampal neurons various mutants of GluR2 that are defective in AP2 and/or NSF interaction (these constructs were HA tagged in the extracellular N-terminal region to allow surface labeling). At DIV19 (5 days after transfection), wild-type HA-GluR2 and all examined mutants were well expressed on the neuronal surface, including on dendritic spines (Figure 4A). The ratio of steady-state surface versus intracellular levels of HA-GluR2 was similar for wild-type and mutants in transfected neurons (Figure 4B). Thus none of these internal

cytoplasmic tail mutations strongly affected surface expression of GluR2.

We used a fluorescence-based "antibody feeding" assay (with HA antibodies) on live transfected neurons to quantify the degree of internalization of HA-GluR2 occurring in the 10 min following surface labeling (Figure 5A). Wild-type HA-GluR2 showed a constitutive level of internalization in the 10 min that was increased ~2.2-fold upon AMPA or NMDA treatment. GluR2 mutants K844A, R845A, N851S/P852A, and P852A showed constitutive internalization similar to wild-type, and the internalization of all these mutants was significantly enhanced by AMPA. The exception was the NSF binding-defective mutant ΔA849-Q853, whose internalization did not increase in response to AMPA stimulation (Figure 5B). However, the lack of AMPA responsiveness may be explained, at least in part, by the relatively high degree of constitutive internalization of mutant ΔA849-Q853 (~1.4-fold greater than wt) (Figures 5A and 5B).

The most striking result of this analysis was that specific mutations differentially affect the internalization of GluR2 in response to AMPA or NMDA. Internalization of the ΔA849-Q853 (NSF-defective) mutant, which showed little response to AMPA, was still strongly inducible by NMDA (~2.3-fold, cf. 2.1-fold for wild-type HA-GluR2; Figures 5A and 5B). This result implies that NSF binding is not essential for NMDA receptor-dependent internalization of GluR2. Conversely, mutants K844A and R845A (both defective in AP2 pull-down) failed to show increased internalization with NMDA, despite their ability to respond to AMPA (Figure 5). N851S/P852A and P852A (mutants that interact with both NSF and AP2) showed NMDA responses similar to wild-type GluR2. These results demonstrate that distinct sequence determinants are required for NMDA- versus AMPA-stimulated GluR2 internalization. In particular, the data suggest that an interaction with AP2 (but not NSF) is required for the internalization of GluR2 induced by NMDA receptor activation.

AP2 Blocking Peptides Inhibit Hippocampal LTD without Causing Run-Down of EPSC

We also examined the functional significance of AP2-AMPA receptor interaction in a more physiological context by looking at low-frequency stimulation (LFS)-induced hippocampal LTD. To accomplish this, we took a peptide interference strategy similar to that used for studying the GluR2-NSF interaction (Nishimune et al., 1998; Lüthi et al., 1999; Noel et al., 1999; Lüscher et al., 1999). Peptides (10-mers) corresponded to the minimal NSF/AP2 binding segment (K844-Q853) from rat GluR2 (pep2r), mouse GluR2 (pep2m), and from the corresponding region of rat GluR4c (the short splice variant of GluR4; pep4cr) (Gallo et al., 1992). The sequence of pep4cr is equivalent to that of N851S/P852A double mutant of GluR2 (see Figures 3A and 6A). Mutant 10-mer peptides pep-K844A, pep-R845A, and pep-ΔA849-Q853 (see Figure 6A) contain the respective mutations analyzed above in the context of the GluR2 tail.

First, we tested the ability of these 10-mer peptides to bind NSF and AP2 (Figure 6). As expected, pep2r and pep2m were sufficient to bind NSF in the yeast two-hybrid system. Pep4cr showed weaker but significant NSF binding, consistent with a previous report (Song et al., 1998; but see Nishimune et al., 1998). Pep-K844A lost NSF binding, whereas pep-R845A exhibited NSF binding similar to pep2r and pep2m (Figure 6A). Pep-ΔA849-Q853 failed to interact with NSF, as anticipated. AP2 binding of the 10-mer peptides was assessed by their ability to "compete" in GST pull-down assays. Pep2r, pep2m, and pep-ΔA849-Q853 (200 μM) inhibited AP2 pull-down by GST-GluR2CT. Pep4cr was a less effective competitor, implying a weaker affinity for AP2. Neither pep-K844A nor pep-R845A inhibited AP2 pull-down by GST-GluR2CT (Figure 6B). Overall, the NSF and AP2 binding properties of the mutant 10-mer peptides are consistent with the effects of the same mutations when assayed in the context of the full GluR2 cytoplasmic tail (compare Figures 6 and 3).

Based on these results, we conclude that pep2r and pep2m should block GluR2 interaction with both AP2 and NSF. Pep-R845A should specifically inhibit the NSF interaction, while pep-ΔA849-Q853 should selectively block AP2. On the other hand, pep-K844A should have no effect on either interaction.

When infused through the recording pipette into CA1 pyramidal cells of acute hippocampal slices, pep2r and pep2m caused run-down of EPSCs over a time course of ~20 min (−26% ± 6% and −30% ± 8%, respectively; $p < 0.001$) (Figures 7A and 7B). Pep2r and pep2m also largely prevented subsequent LFS-induced LTD (only −6% ± 4% and −5% ± 9% depression from the run-down baseline, $p = 0.59$ and 0.78, respectively). Control recordings (no peptide) showed no run-down (0% ± 2%) and robust LTD following LFS (−39% ± 4%, $p < 0.0001$). Pep4cr, which binds weakly to NSF and AP2, had little effect on EPSC (−7% ± 5%, $p = 0.22$) or LTD induction (−34% ± 5% after LFS, $p < 0.0005$) (Figure 7G). These results are generally consistent with previous reports (Nishimune et al., 1998; Song et al., 1998; Lüthi et al., 1999; Noel et al., 1999; Lüscher et al., 1999).

In contrast, pep-R845A (which binds NSF but not AP2) evoked a run-down of EPSC amplitude (−22% ± 5%, $p < 0.001$) but did not block subsequent LTD (−23% ± 5% from the run-down baseline, $p < 0.01$) (Figure 7D). Most strikingly, the AP2-specific blocking peptide (pep-ΔA849-Q853) did not affect basal synaptic transmission (3% ± 6%, $p = 0.7$), but completely abolished LFS-induced LTD (−2% ± 7%, $p = 0.89$) (Figure 7E). As predicted, pep-K844A (which binds neither NSF nor AP2) did not inhibit basal synaptic transmission (−3% ± 4%, $p = 0.48$) or LTD (−38% ± 5%, $p < 0.0001$) (Figure 7C). In summary, blocking the GluR2-AP2 interaction results in loss of LTD but no run-down, whereas blocking the NSF interaction leads to EPSC run-down but does not abolish subsequent LTD.

To corroborate the latter conclusion, we also tested an NSF-interfering peptide (pep-NSF3), which specifically inhibits NSF function by blocking the ATPase activity of NSF stimulated by the soluble NSF attachment protein (Schweizer et al., 1998). Since pep-NSF3 is completely unrelated to GluR2 in sequence (see Experimental Procedures) and does not inhibit AP2 pull-down by GluR2CT (Figure 6B), it should serve as an independent inhibitor of NSF function. Similar to pep-R845A, pep-NSF3 produced run-down of EPSC (−20% ± 5%, $p < 0.005$) in neurons, but did not prevent subsequent induc-

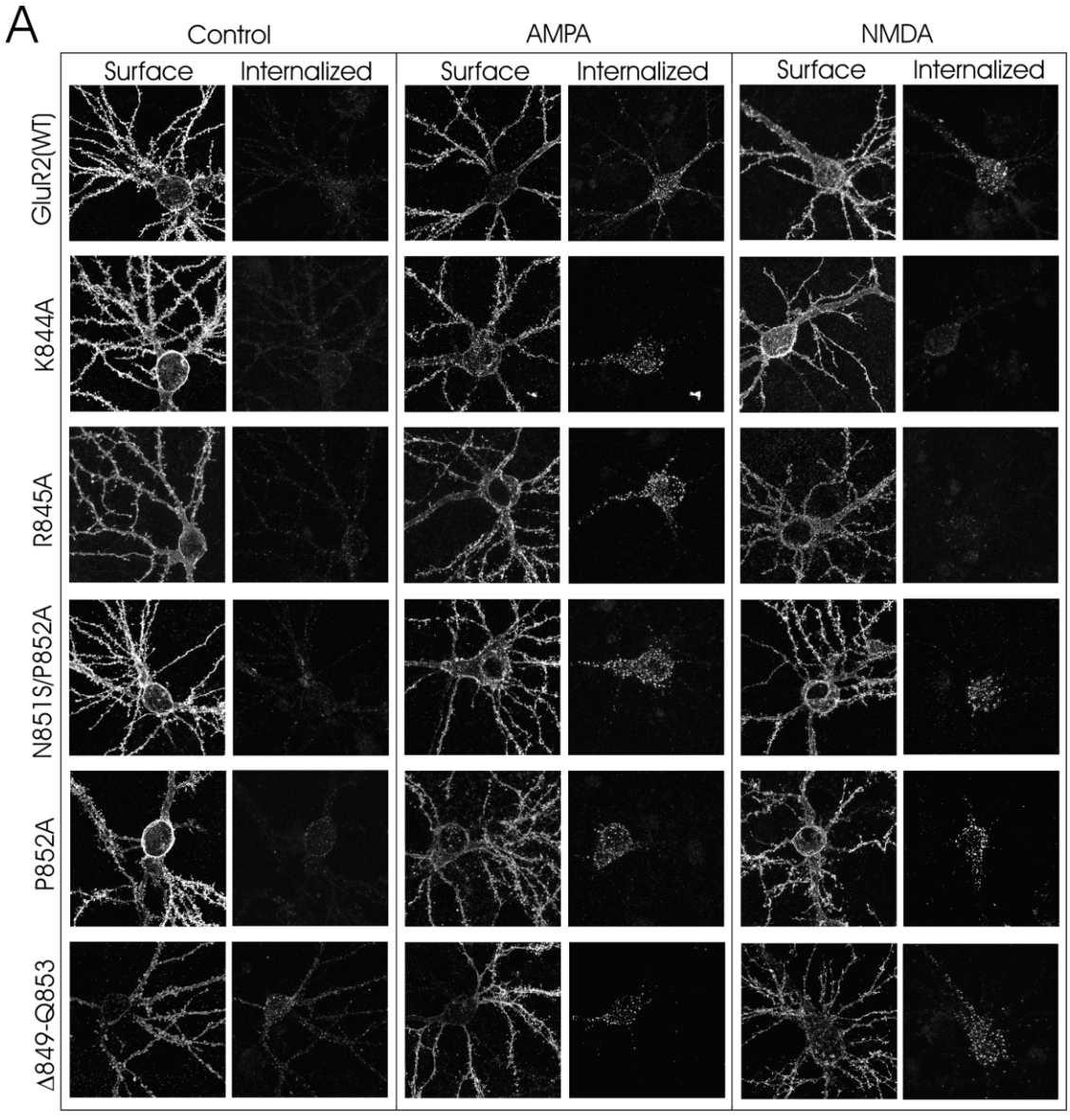


Figure 5. Differential Sequence Requirements for NMDA- and AMPA-Induced Internalization of GluR2

HA-tagged wild-type and mutant GluR2 were transfected into hippocampal neurons and their internalization measured by fluorescence-based antibody-feeding assay. Surface HA-GluR2 was labeled with HA antibody in live neurons. Following a 10 min period to allow for internalization, HA antibody remaining on the surface was stained with Alexa488-secondary antibody in nonpermeabilized conditions ("Surface"), and internalized HA antibody was labeled with Cy3-secondary antibody in permeabilized conditions ("Internalized").

A

	Sequence	Y2H NSF	GST-PD AP-2
pep2r	KRMKVAKNPQ	+	+
pep2m	--------A-	+	+
pep4cr	-------SA-	±	±
pep-K844A	A---------	−	−
pep-R845A	-A--------	+	−
pep-ΔA849–Q853	----LNI--S	−	+

B

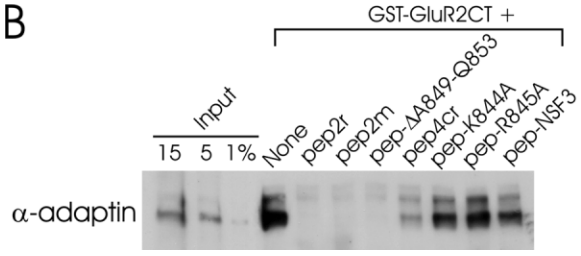


Figure 6. AP2 and NSF Binding Properties of pep2m, pep2r, and Mutant Peptides

(A) Sequence of pep2r, pep2m, and mutant peptides and their ability to bind to NSF in yeast two-hybrid assays. +, <30 min β-gal detection time; ±, 30–60 min β-gal detection time; −, no detectable β-gal signal after 3 hr.

(B) AP2 interaction of individual peptides analyzed by competitive inhibition of GST pull-down of AP2 (α-adaptin) by GST-GluR2CT. GST pull-down assays were performed with brain extracts preincubated with individual peptides (200 μM).

(C) Tabulated summary of peptide interaction with AP2 based on (B). +, ±, and − indicate strong, weak, and no binding, respectively.

tion of substantial LTD (−30% ± 4%, $p < 0.001$) (Figure 7F).

At 15 min after break-in (formation of whole-cell patch), when the effect of NSF-interfering peptides on EPSC was near maximal, there was no difference in paired pulse ratio between control cells (no peptide) and cells injected with pep-K844A, pep-R845A, or pep-ΔA849-Q853 (see Supplemental Figure S1A at http://www.neuron.org/cgi/content/full/36/4/661/DC1). These results indicate that presynaptic function was not affected by the peptides. In addition, we examined whether the peptides impair NMDA receptor function, which might also influence the induction of LTD. We found no difference in the ratio of EPSC at −60mV/EPSC at +40mV (the −60/+40 ratio), or in summation of the responses, in control versus pep-K844A- or pep-ΔA849-Q853-injected cells (see Supplemental Figure S1B at http://www.neuron.org/cgi/content/full/36/4/661/DC1), implying no loss of NMDA receptor activity. Moreover, neither pep-K844A nor pep-ΔA849-Q853 affected basal synaptic NMDA receptor currents directly measured after blocking AMPA receptors with DNQX (see Supplemental Figure S1C at http://www.neuron.org/cgi/content/full/36/4/661/DC1). These data support our conclusion that the AP2-blocking peptide prevents the *expression* of LTD by blocking NMDA receptor-dependent internalization of AMPA receptors, rather than prevents the *induction* of LTD by inhibiting NMDA receptor function.

Cells infused with NSF-blocking pep-R845A showed a small reduction in the −60/+40 ratio (~10%, not statistically significant) and in the sum of responses (~25%) (see Supplemental Figure S1B at http://www.neuron.org/cgi/content/full/36/4/661/DC1), reflecting a decrease in the +40 as well as the −60 response. Since the +40 response contains both AMPA and NMDA components, the small reduction in the +40 measurement may have been due to the effect of the peptide on the AMPA component, the NMDA component, or other factors affecting postsynaptic excitability. However, direct measurements showed that pep-R845A did not impair pharmacologically isolated NMDA receptor currents (see Supplemental Figure S1C3 at http://www.neuron.org/cgi/content/full/36/4/661/DC1). This is consistent with a previous report that pep2m (which binds both NSF and AP2) had no effect on pharmacologically isolated NMDA receptor responses (Lüscher et al., 1999). Thus we conclude that none of the tested peptides had an adverse effect on NMDA receptor function.

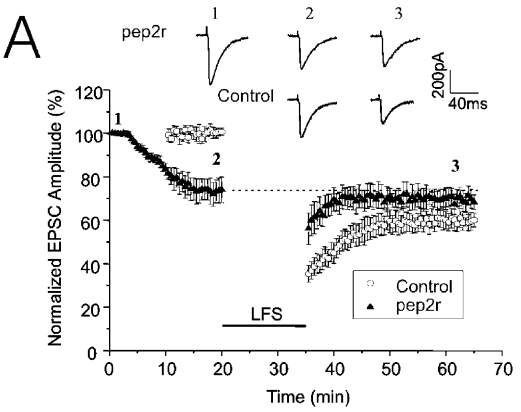
Role of NSF in Synaptic Expression of AMPA Receptors

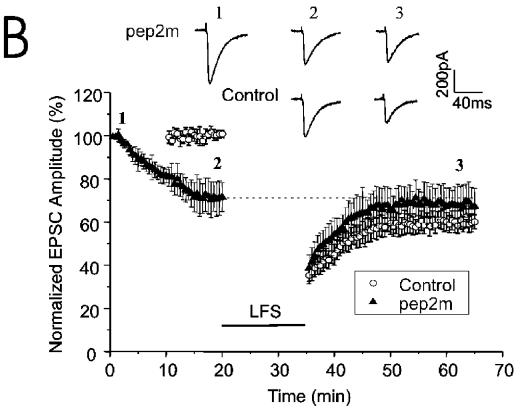
The NSF-blocking peptides caused run-down of EPSCs (e.g., pep-R845A, Figure 7D), implying loss of synaptic AMPA receptors. Does NSF interference also affect extrasynaptic AMPA receptors? To test this, we measured the response to exogenously applied AMPA in CA1 neurons of hippocampal slice in which pep-R845A was acutely introduced through the patch pipette. Responses to exogenous AMPA were recorded by puffing 200 μM AMPA for 50 ms on the soma while concomitantly monitoring synaptic EPSCs. Interestingly, the NSF-interfering peptide pep-R845A did not affect the "extrasynaptic" response to exogenously applied AMPA, even while synaptic EPSCs showed run-down (Figure 7H). These electrophysiological results imply that NSF is important for the synaptic expression, but not for total surface expression, of AMPA receptors. This interpretation is supported by the GluR2 mutant ΔA849-Q853, which showed normal steady-state surface levels in neurons despite being unable to bind NSF (see Figure 4).

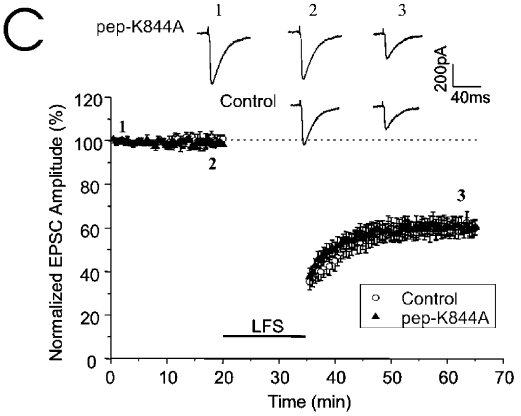
To explore this issue further, we infected hippocampal neurons with attenuated Sindbis virus vectors overexpressing NSF- or AP2-interfering peptides (pep2m, pep-R845A, and pep-ΔA849-Q853, fused to EGFP for easy identification of infected cells). Sixteen to twenty-four

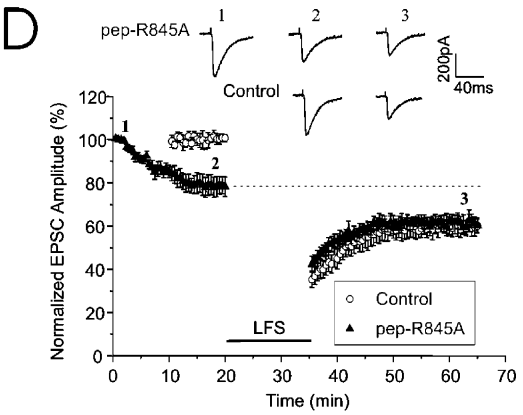
(A) Representative images of neurons (transfected as indicated) stained for surface and internalized HA-GluR2, following 10 min incubation in conditioned medium (Control); 10 min in conditioned medium containing 100 μM AMPA (AMPA); or 2 min incubation in conditioned medium containing 50 μM NMDA plus 50 μM CNQX followed by additional 8 min in conditioned medium (NMDA).

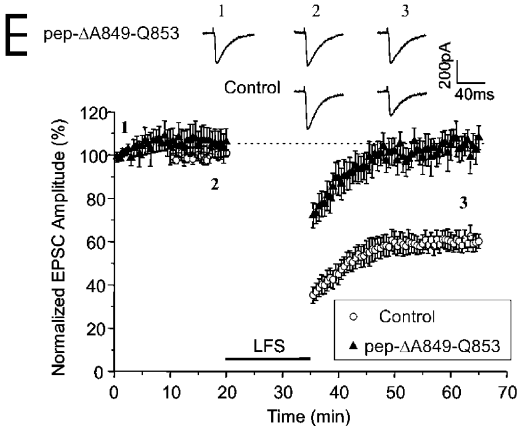
(B) Quantitation of internalization assays, measured as the ratio of internalized/surface fluorescence (Internalization Index), normalized to wild-type 10 min control. Histograms show mean ± SEM (n = 15–25 for each condition). ***$p < 0.001$ compared with control; **$p < 0.01$ compared with control; #$p < 0.05$ compared with control for wild-type GluR2.

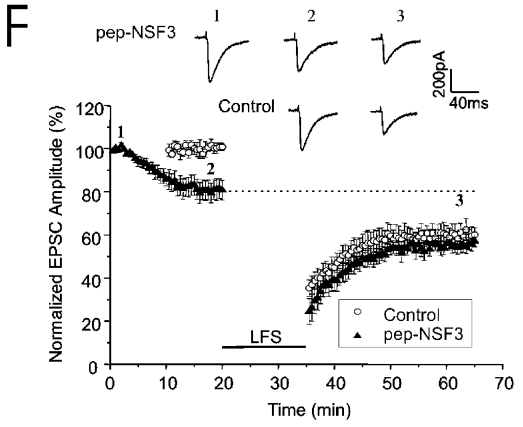












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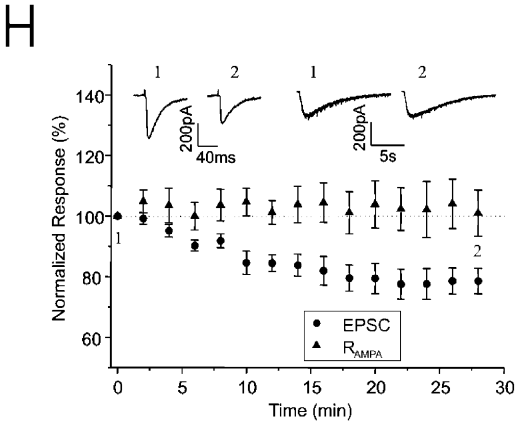
Peptide	Rundown (%)	Depression (%)
Control	–0 ± 2	–39 ± 4
pep2r	–26 ± 6	–6 ± 4
pep2m	–30 ± 8	–5 ± 9
pep4cr	–7 ± 5	–34 ± 5
pep-K844A	–2 ± 4	–38 ± 5
pep-R845A	–22 ± 5	–23 ± 5
pep-ΔA849-Q853	+3 ± 6	–2 ± 7
pep-NSF3	–20 ± 5	–30± 4



hours after infection, we directly measured the total surface expression of endogenous AMPA receptors by surface immunostaining for GluR1 and GluR2. None of these peptides (including the NSF-blocking peptides pep2m and pep-R845) had a significant effect on AMPA receptor surface levels (Figures 8A and 8B). The lack of effect is unlikely due to inadequate expression of the EGFP-peptide fusions, since cells infected with EGFP-pep2m or -ΔA849-Q853 showed grossly dysregulated AMPA receptor internalization (see below). Thus, these immunocytochemical data corroborate that NSF is not important for the total surface expression of AMPA receptors.

Internalization of endogenous AMPA receptors (GluR2 subunit) was also assayed in EGFP-peptide-infected neurons. None of the peptides (pep2m, pep-R845A, or pep-ΔA849-Q853) affected constitutive or AMPA-induced internalization of AMPA receptors (measured at 10 min; Figure 8D). However, in cells expressing AP2-blocking peptides (pep2m or pep-ΔA849-Q853), NMDA-induced AMPA receptor internalization was drastically reduced (Figures 8C and 8D). In contrast, cells expressing the NSF-specific interfering peptide (pep-R845A) showed robust NMDA-inducible internalization of AMPA receptors, similar to uninfected cells or cells expressing EGFP only (Figures 8C and 8D). These immunocytochemical data are in accord with the electrophysiological experiments (see Figure 7), confirming that AP2, but not NSF, is critical for NMDA receptor-dependent internalization of AMPA receptors.

Discussion

Distinct Roles of AP2 and NSF in LTD

In this study, we defined an interaction of AMPA receptors with the AP2 adaptor complex that is critical for NMDA-induced internalization of AMPA receptors and induction of hippocampal LTD. Interestingly, the site required for AP2 interaction overlaps closely with that of NSF, a hexameric ATPase involved in general membrane fusion. In earlier studies, pep2m peptide was used to block the NSF-GluR2 interaction, causing a run-down of basal AMPA EPSCs (Nishimune et al., 1998; Song et al., 1998; Noel et al., 1999) and leading to reduced surface expression of AMPA receptors (Noel et al., 1999). These findings suggested a role for NSF in the maintenance of surface and synaptic AMPA receptors, via either delivery or stabilization. Pep2m-mediated run-down and LTD mutually occluded each other, giving rise to the proposal that LTD involves a pool of AMPA receptors regulated by NSF-GluR2 interaction (Lüthi et al., 1999; Lüscher et al., 1999). A major finding of our study, however, is that pep2m not only interacts with NSF but also with the AP2 complex, thereby complicating interpretation of previous data. By using a systematic set of GluR2 mutants, and assaying their effects on both internalization and LTD, we now reveal different roles of AP2 and NSF in AMPA receptor trafficking and plasticity.

Peptides that specifically block the GluR2-AP2 interaction (pep-ΔA849-Q853) eliminated LTD without affecting basal synaptic transmission. In contrast, peptides that specifically interfere with the GluR2-NSF interaction (pep-R845A) or NSF activity (pep-NSF3) caused run-down but did not prevent subsequent LTD. Finally, peptides that antagonize both NSF and AP2 (pep2m, pep2r) caused run-down *and* prevented LTD. Thus a simple explanation for the disruption of LTD by pep2m and pep2r is that these peptides interfere with the GluR2 interaction with AP2 (rather than NSF).

It is possible, however, that interference with NSF contributes indirectly to loss of LTD (i.e., that NSF-dependent run-down leads to "occlusion" of LTD). Some observations are consistent with this possibility. For example, the degree of run-down induced by pep2r and pep2m (~26% and 30%, respectively, after 20 min) was greater than for the NSF-interfering peptides that did not abolish LTD (pep-R845A, ~22%; and pep-NSF3, ~20%). This difference may reflect differential efficacy of these peptides in blocking NSF function/binding; i.e., the incomplete occlusion of LTD by pep-R845A or pep-NSF3 may be due to incomplete inhibition of NSF. Our results are therefore not incompatible with the idea that LTD induction involves a pool of AMPA receptors that depends on NSF activity (Lüthi et al., 1999). However, since the AP2 interaction is critical for LTD induction but has no effect on baseline AMPA EPSCs, we propose that AP2 recruitment is the more directly regulated step in NMDA receptor-dependent synaptic depression, presumably leading to clathrin coat assembly and endocytosis of AMPA receptors. In contrast, NSF is important for the maintenance of synaptic AMPA receptors, but is not essential for NMDA-induced internalization of GluR2. Thus, NSF indirectly affects LTD by influencing the availability of synaptic AMPA receptors for internalization.

NSF and Surface Expression of AMPA Receptors

Despite the clear requirement of NSF for synaptic expression of AMPA receptors, we found no evidence that NSF is important for total surface expression of AMPA

Figure 7. Differential Effects of NSF- and AP2-Interfering Peptides on Basal Synaptic Transmission and LTD in Hippocampal CA1 Neurons

(A–F) Normalized EPSC amplitudes of CA1 neurons recorded with pipettes containing standard intracellular solution (control, open circles, n = 7) or solution supplemented with 100 μM of peptide (n = 6). Top of each panel shows representative traces taken at indicated time points. 1, 2, and 3 define time points used to calculate depression level in (G). Infusion of pep2r or pep2m caused run-down of EPSC and prevented low-frequency-stimulation (LFS)-induced LTD (A and B). Pep-K844A did not affect either basal synaptic transmission or LTD (C). Pep-R845A induced a run-down of EPSC amplitude but did not block subsequent LTD induced by LFS (D). Pep-ΔA849-Q853 did not affect basal synaptic transmission but completely blocked LFS- LTD (E). Infusion of NSF interfering peptide, pep-NSF3, produced run-down of EPSC but did not prevent subsequent LFS-LTD (F). (G) Table showing the effects of each peptide (mean ± SEM) on basal synaptic transmission ("Rundown", defined by [EPSC amplitude at time point 2/EPSC amplitude at time point 1] × 100 − 100%), and on LFS-induced synaptic depression ("Depression", defined by [EPSC amplitude at time point 3/EPSC amplitude at time point 2] × 100 − 100%). (H) Acutely applied NSF-blocking peptide (pep-R845A) caused a run-down of AMPA receptor-mediated EPSCs, but did not affect extrasynaptic AMPA receptor responses evoked by exogenous AMPA (200 μM) applied to soma of same cell.

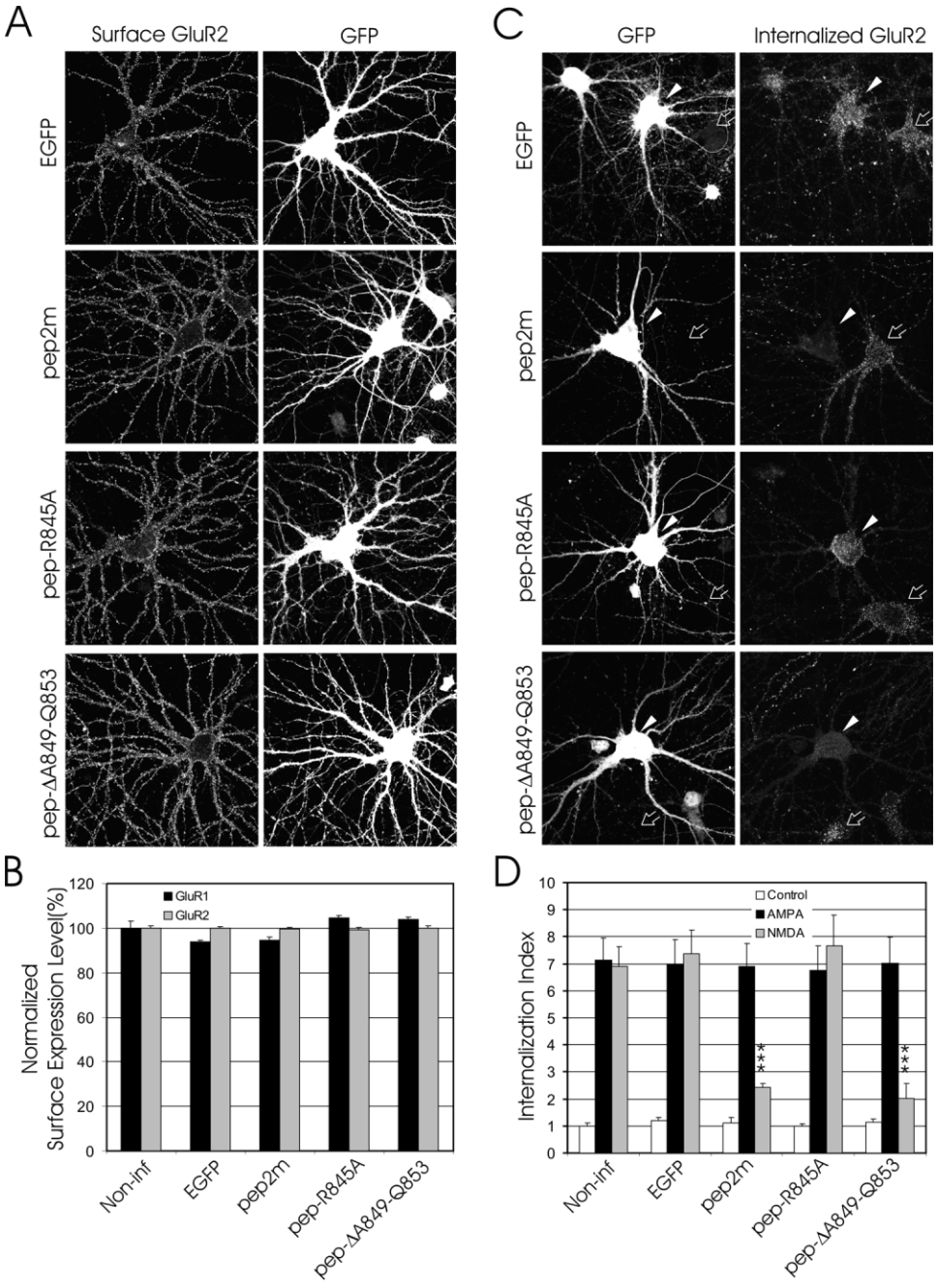


Figure 8. Effect of NSF- or AP2-Interfering Peptides on Surface Expression and NMDA-Induced Internalization of Endogenous AMPA Receptors

Neurons were infected with Sindbis viral vector expressing the indicated peptide fused to the C terminus of EGFP.

(A) Representative double-label images of surface staining for endogenous AMPA receptors (GluR2, left panels) and GFP fluorescence (right panels) in cells infected with the indicated peptide.

(B) Quantification of surface immunofluorescence staining of endogenous GluR1 (image data not shown) and GluR2, normalized to control (noninfected) cells.

(C) Images of infected neurons showing GFP fluorescence (left panels) and internalized GluR2 (antibody feeding assay; right panels) after NMDA stimulation as in Figure 5. Solid arrowheads indicate the cell bodies of infected neurons (expressing EGFP control or EGFP fusions of the indicated peptides), and open arrows indicate the cell bodies of uninfected cells.

(D) Quantification of GluR2 internalization, measured by the amount of internalized receptors per neuron (Internalization Index), normalized to uninfected cells (Non-inf) after 10 min incubation in conditioned medium. Histograms show mean ± SEM (n = 10 for each condition). ***$p < 0.001$ compared with uninfected cells or EGFP control.

receptors. A GluR2 mutant defective in NSF binding (ΔA849-Q853) showed wild-type steady-state surface levels, and NSF-blocking peptides did not affect total surface expression of endogenous AMPA receptors, as assayed immunocytochemically (Figure 8A) or electrophysiologically (Figure 7H). Thus NSF appears to be required specifically for the maintenance of synaptic AMPA receptors. NSF could act either by stabilizing

AMPA receptors in the postsynaptic membrane or by enhancing their recycling to the postsynaptic membrane.

Our results differ from a previous report (Noel et al., 1999) in which viral introduction of pep2m caused a reduction in surface expression of AMPA receptors. Several methodological differences could account for this apparent discrepancy. Noel et al. (1999) used an inducible adenoviral (AdTet-on) vector to overexpress the pep2m peptide fused to a FLAG epitope, thus producing a short peptide (18 residues) that might accumulate to higher molar levels than our EGFP-peptide fusions. The cells were infected for a total of 40 hr and induced with doxycycline for 16 hr. In our case, we used an attenuated Sindbis virus (less cytotoxic than the original viral vector) (Dryga et al., 1997), and the neurons were infected for a shorter duration (16–24 hr). Thus, the loss of surface AMPA receptor expression in the study by Noel et al. (1999) might be attributable to a higher level of interfering peptide or to a longer duration of peptide overexpression.

AP2 and Regulated Endocytosis of AMPA Receptors

Another major finding of this study is that GluR2 possesses different sequence determinants for AMPA- versus NMDA-stimulated internalization. In particular, the ability to associate with AP2 correlates with NMDA-inducible internalization of GluR2, but is not required for AMPA-stimulated internalization. Since NMDA receptor activation is a crucial step in hippocampal LFS-LTD, AP2 recruitment to AMPA receptors is likely to be a key event linking NMDA receptor activation to AMPA receptor endocytosis and synaptic depression. This idea is consistent with previous studies showing common signaling pathways shared between NMDA-induced internalization of AMPA receptors and LTD (Beattie et al., 2000; Ehlers, 2000). It is interesting that AP2 can interact with all three AMPA receptor subunits we tested (GluR1, GluR2, and GluR3), suggesting that regulated endocytosis via this pathway can apply to most if not all AMPA receptor subtypes. This could explain the persistence of LTD in GluR2-deficient mice (Jia et al., 1996).

Is AP2 then dispensable for AMPA-stimulated receptor internalization? Although our mutagenesis data indicate that the AP2 pull-down determinants of GluR2 are not essential for ligand-induced internalization, we cannot exclude the possibility that AP2 interacts with AMPA receptors through other means. For instance, during AMPA-induced endocytosis, AP2 could be recruited to AMPA receptors via intermediary proteins that bind elsewhere on GluR2, analogous to the way that AP2 can interact with β-adrenergic receptors indirectly via β-arrestins (Laporte et al., 1999). However, we were unable to detect β-arrestin in GST pull-down assays with GluR1CT or GluR2CT, or by coimmunoprecipitation with AMPA receptors. Determinants involved in ligand-induced internalization of GluR2 in heterologous cells have been mapped to the most membrane-proximal region of the cytoplasmic tail (Lin et al., 2000), upstream of the AP2/NSF binding region studied here. Further research is required to understand the mechanism of AMPA-induced internalization of AMPA receptors in neurons.

An important remaining question is whether AP2 binds directly to GluR2, and if so, which subunit of the AP2 complex mediates the interaction. The "classical" endocytic signals (the YxxØ motif recognized by μ-adaptin, and the dileucine motif recognized by α-adaptin) (Kirchhausen, 1999) are absent from the cytoplasmic tails of AMPA receptor subunits. Instead, the AP2-interacting site in GluR2 contains a cluster of basic amino acids. Similar sequences have been shown to be important for endocytosis and recycling of the polymeric immunoglobulin receptor (Aroeti and Mostov, 1994), and for the interaction between synaptotagmin and μ-adaptin (Chapman et al., 1998; Haucke et al., 2000) (see Figure 2D).

NMDA Receptor-Dependent Recruitment of AP2 to AMPA Receptors

How might NMDA receptor activation lead to recruitment of the AP2 complex to AMPA receptors? Calcineurin (PP2B) and PP1 activity are required for the NMDA-dependent internalization of AMPA receptors (Beattie et al., 2000; Ehlers, 2000) and for hippocampal LTD (Mulkey et al., 1993, 1994; Morishita et al., 2001). These protein phosphatases have been shown to dephosphorylate proteins involved in endocytosis including AP2, thereby promoting assembly of the endocytic protein complex containing clathrin coat, dynamin, synaptojanin, and amphiphysin. (Slepnev et al., 1998; Chen et al., 1997; Wilde and Brodsky, 1996). Thus, one possibility is that opening of NMDA receptors triggers a calcium signaling cascade that activates calcineurin and PP1, which in turn leads to dephosphorylation of AP2 and its recruitment to AMPA receptors.

Dephosphorylation of the PKA site (serine 845) in the cytoplasmic tail of GluR1 is also correlated with AMPA receptor endocytosis (Ehlers, 2000) and with LTD (Lee et al., 1998, 2000). For GluR2 on the other hand, phosphorylation of S880 correlates with internalization (Chung et al., 2000; Matsuda et al., 2000) and differentially affects GluR2 interaction with PDZ proteins GRIP and PICK1 (reviewed in Carroll et al., 2001; Chung et al., 2000; Matsuda et al., 2000; Kim et al., 2001). Such changes in the phosphorylation state, or the binding interactions, of the cytoplasmic tails of AMPA receptor subunits might also play a role in the regulated recruitment of AP2 to AMPA receptors.

Finally, an intriguing idea is that AP2 and NSF compete for binding to their overlapping sites on GluR2. In such a case, NSF might act to restrain receptor internalization by sterically blocking the association of AP2. NMDA receptor activity would somehow stimulate the dissociation of NSF from GluR2, freeing up access for AP2 and leading to AMPA receptor internalization. However, NSF binding to GluR2 is clearly not necessary for NMDA-induced internalization, as evidenced by the ΔA849-Q853 mutant, therefore arguing against this simple competition model.

Model for Role of AP2 and NSF in AMPA Receptor Trafficking

Based on the findings of this study and other published work, we propose the following model for NSF and AP2 action. During basal synaptic transmission, NSF maintains synaptic strength by promoting the delivery/recy-

cling of GluR2-containing AMPA receptors to the postsynaptic membrane. Since GluR2 receptors undergo rapid cycling even in basal conditions (Passafaro et al., 2001), disruption of NSF function or NSF interaction with GluR2 leads to run-down of AMPA EPSCs over a time scale of minutes. NSF dissociates from GluR2 once AMPA receptors are recycled to the postsynaptic membrane. During induction of NMDA receptor-dependent LTD, AP2 is recruited to AMPA receptors, thereby promoting assembly of the clathrin coat and endocytosis of AMPA receptors.

The model can explain the activity dependence of pep2m-mediated synaptic run-down (Lüscher et al., 1999), which we envisage arises from an activity-dependent internalization of AMPA receptors occurring in basal conditions (e.g., via AMPA receptor activation). Normally, such internalized receptors are recycled to the synapse (Lüscher et al., 1999; Ehlers, 2000; Passafaro et al., 2001), but this is prevented by pep2m interference of the NSF-GluR2 interaction. Since our data suggest that NSF is not required for delivery of AMPA receptors to the neuronal surface per se, we propose that NSF is involved specifically in receptor incorporation into the synapse. Localization of GluR2 mutants at the ultrastructural level will be helpful to test this hypothesis. Since AMPA receptor migrate in a regulated manner on the neuronal surface (Borgdorff and Choquet, 2002), it seems not unreasonable to invoke a step between the cell surface and the synapse that is dependent on NSF. A similar two-stage delivery of AMPA receptors to the synapse has been inferred from the study of Stargazin-deficient neurons (Chen et al., 2000).

Experimental Procedures

DNA Constructs

GluR1 and GluR2 tagged with HA-epitope in the N-terminal extracellular region were previously described (Man et al., 2000). Mutations in GluR2 were introduced by in vitro mutagenesis using the QuickChange system (Stratagene) and verified by sequencing. Constructs for yeast two-hybrid assays and GST fusion proteins were prepared by PCR-based subcloning of GluR2 fragments into pBHA (Niethammer and Sheng, 1999) or pGEX-4T (Pharmacia), respectively. For viral expression of EGFP-fused peptides, oligonucleotides encoding the specific peptides were first subcloned in frame in pEGFP-C1, and then transferred to a low-cytotoxicity Sindbis virus vector, pSinRep(nsp2S) (Dryga et al., 1997).

GST Pull-Down Assays and Immunoprecipitation

Cytosolic extracts (S3 fraction) were prepared from rat cerebral cortex as described in Lee et al. (2001). S3 fraction was adjusted to 150 mM NaCl and 0.5% Triton X-100, and incubated with glutathione-sepharose beads containing 100 μg of GST fusion proteins for 3 hr at 4°C. After washing three times in Wash buffer (50 mM Tris·Cl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM DTT) for 10 min each, bound proteins were eluted in 2× SDS sample buffer by boiling for 5 min. For peptide competition assays, peptides (Research Genetics) were preincubated with cytosolic extracts at 200 μM concentration for 1 hr at 4°C before GST pull-down with GST-GluR2 beads. Recombinant NSF was purified from Rosetta (DE3) strain bacteria (Novagen) harboring NSF/pQE-9 plasmid that encodes for hexahistidine (H6)-tagged NSF using Ni-NTA agarose (Qiagen) affinity chromatography. In binding assays, 500 ng of H_6-NSF (10 nM) was incubated with 5 μg of GST-GluR2 mutants in Buffer B (25 mM HEPES·KOH, pH 7.3, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 1% Triton X-100, 10% glycerol) containing 1 mM ATP-γ-S for 2 hr at 4°C. After washing four times in Buffer B containing 1 mM ATP, bound NSF was eluted in SDS sample buffer and detected by Western blotting. GST-KIF1A contains a region (Y717-E1030) in the C-terminal tail of kinesin-like motor KIF1A (Okada et al., 1995). The amino acid sequence of NSF3 peptide is TGKTLIARKIGTMLNAREPK (Schweizer et al., 1998). Immunoprecipitations were performed as described using deoxycholate-extracted synaptosome fraction of rat forebrain (Naisbitt et al., 1999).

Hippocampal Neuron Cultures, Transfection, and Sindbis Virus Infection

High-density hippocampal neurons were prepared from E18/19 rat embryos and maintained in MEM containing 10% fetal bovine serum, 25 μg/ml insulin, 100 μg/ml transferrin, 1 mM pyruvate, and 0.6% glucose as described (Sala et al., 2000). Neurons were transfected at DIV14 using calcium phosphate method (Passafaro et al., 2001) and used for internalization experiments at 5–8 days posttransfection (DIV19–22). Neurons were infected with Sindbis virus at DIV19 and examined for endogenous AMPA receptor internalization after 16–24 hr. Recombinant Sindbis virus was produced following a standard procedure described in the Sindbis expression system (Invitrogen) with helper DH-BB(tRNA/TE12) (Dryga et al., 1997).

Internalization Assays

Fluorescence-based internalization assay was performed as described (Lin et al., 2000), with minor modifications. HA-tagged surface AMPA receptors were "live"-labeled with mouse anti-HA monoclonal antibody by incubating neurons in conditioned medium containing the antibody (10 μg/ml) for 10 min at 37°C. For endogenous AMPA receptors, antibodies recognizing extracellular epitopes of GluR2 (Chemicon) or GluR1 (Oncogene) were used. After brief washing in DMEM, neurons were either returned to conditioned medium (control) or medium containing 100 μM AMPA and incubated for 10 min at 37°C. For NMDA treatment, the pre- live-labeled neurons were incubated in 50 μM NMDA and 50 μM CNQX for 2 min and further incubated in normal conditioned medium for 8 min. Subsequently, neurons were fixed in 4% formaldehyde/4% sucrose in PBS for 8 min at room temperature, and surface receptors were visualized with Alexa488-secondary antibody. Internalized receptors were detected with Cy-3-secondary antibody after permeabilizing cells in methanol (−20°C) for 1 min.

Image Acquisition and Quantification

Images were acquired using z-serial section scanning mode on MRC 1024 confocal microscope (BioRad). The same confocal acquisition setting was applied to all samples from the same experiment. Collected z-section images were first converted to projection images and analyzed using Metamorph image analysis program (Universal Imaging Corporation). After setting threshold levels for green and red channels (the same threshold applied for each experimental set of images), integrated fluorescence value (which includes both area and intensity) from each channel was quantified. The "internalization index" for transfected GluR2 was determined by dividing the computed red fluorescence by the green fluorescence. For endogenous receptor internalization, the internalization index was computed by measuring the amount of internalized receptors per neuron.

Electrophysiological Recording

Hippocampal slices (400 μm) were prepared as described (Wan et al., 1997) from Sprague-Dawley rats (16–26 days old) and placed in a holding chamber for at least 1.5 hr prior to recording. Slices were perfused at room temperature with artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose and bubbled with 95%O_2/5%CO_2. Whole-cell recordings of CA1 neurons were performed using the "blind" method with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA) at holding potential −60mV. The recording pipettes (4–5 MΣΩ) were filled with intracellular solution containing 135 mM CsCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM EGTA, 10 mM HEPES, 4 mM Mg-ATP, 0.2 mM GTP, and 5 mM QX-314, pH 7.4, 310 mOsm. Series and input resistance were monitored throughout each experiment; cells were excluded from analysis if they showed >20% change in series or input resistance during the experiment. EPSCs were evoked every 30 s by stimulation (0.05 ms duration) of the Schaffer collateral-commissural pathway

with a bipolar tungsten electrode in the presence of bicuculline methiodide (10 μM). Peptides were dissolved (100 μM) in the intracellular solution. After break-in, baseline EPSCs were recorded for 20 min. During the induction of LTD, the recording was switched to current-clamp mode, and 15 min train stimulation at 1 Hz (900 pulses in total) was delivered from the same stimulating electrode. After stimulation, the recording was switched back to voltage-clamp mode, and EPSC recordings at the baseline stimulus rate were then recorded for 1 hr thereafter.

The NMDA component of EPSC was isolated by perfusing slices with ASCF containing 20 μM DNQX and no $MgCl_2$. Paired-pulse ratios (#2/#1) were measured using paired-pulse stimulation (40 ms interpulse interval) of Schaffer collateral afferents recorded in the CA1 of hippocampal slices 15 min after break-in. AMPA-EPSCs and "AMPA+NMDA" EPSCs were recorded at holding potential −60 and +40mV, respectively, with 2 mM extracellular Mg^{2+}. The amplitude for each cell patched was averaged from five consecutive recordings evoked at 20 s intervals. 135 mM CsCl was substituted with 117.5 mM Cs-gluconate and 17.5 mM CsCl in the intracellular solution for both ratio recordings.

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