Neuron, Vol. 24, 649–658, November, 1999, Copyright ©1999 by Cell Press

Role of AMPA Receptor Cycling in Synaptic Transmission and Plasticity

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Summary

Compounds known to disrupt exocytosis or endocytosis were introduced into CA1 pyramidal cells while monitoring excitatory postsynaptic currents (EPSCs). Disrupting exocytosis or the interaction of GluR2 with NSF caused a gradual reduction in the AMPAR EPSC, while inhibition of endocytosis caused a gradual increase in the AMPAR EPSC. These manipulations had no effect on the NMDAR EPSC but prevented the subsequent induction of LTD. These results suggest that AMPARs, but not NMDARs, cycle into and out of the synaptic membrane at a rapid rate and that certain forms of synaptic plasticity may utilize this dynamic process.

Introduction

Work over the past decade has shown that excitatory synapses in the central nervous system have a remarkable degree of plasticity. The most thoroughly studied forms of plasticity are NMDA receptor (NMDAR)-dependent long-term potentiation (LTP) and long-term depression (LTD). Numerous recent studies strongly suggest that an important mechanism underlying these activity-dependent forms of plasticity involves alterations in the responsiveness of the postsynaptic AMPA receptors (AMPARs) to glutamate. Various mechanisms have been proposed for this altered responsiveness, including the state of phosphorylation of the AMPARs (Barria et al., 1997; Lee et al., 1998) and the redistribution of AMPARs into and out of the synapse (Carroll et al., 1999a; Shi et al., 1999). The actual mechanisms responsible for the redistribution of AMPARs are not clear. We have recently demonstrated that in response to ligand activation, AMPARs undergo a dynamin-dependent endocytosis (Carroll et al., 1999b). In addition, we have found that substances that block membrane fusion can interfere with LTP (Lledo et al., 1998). These results raise the possibility that AMPARs may be inserted into and removed from the postsynaptic membrane on a relatively rapid time scale.

Consistent with this hypothesis are recent reports that N-ethylmaleimide-sensitive fusion protein (NSF), a protein involved in numerous membrane fusion events, interacts with the C terminus of the AMPAR subunit GluR2 (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998). This interaction appears to be essential for the maintenance of stable synaptic currents (Nishimune et al., 1998; Song et al., 1998) as well as the surface expression of AMPARs (Noel et al., 1999). These results raise the intriguing possibility that not only are AMPARs redistributed in response to changes in the patterns of synaptic activity, but they may also constituitively cycle into and out of the synaptic postsynaptic membrane. We have explored this possibility by applying directly into the postsynaptic cell a number of agents known to block various steps in exocytosis and endocytosis. The results provide evidence for a remarkably dynamic system for the control of the surface expression of synaptic AMPARs.

Results

Inhibition of Exocytosis

We first examined the effects of loading cells with the light chains of type B botulinum toxin (Botox), which is known to inactivate v-SNAREs and prevent exocytosis (Huttner, 1993). Soon after establishing the whole cell configuration, the size of the AMPAR EPSCs began to decrease and this rundown stabilized after approximately 20 min at ~40% reduction (Figure 1A1, n = 5). No change in the responses was observed when interleaved recordings were made using only the vehicle (DTT 5 mM) in the pipette (Figure 1A1, n = 7). This action of Botox was selective for the AMPAR EPSC in that when these same experiments were repeated while monitoring the NMDAR EPSC, no effect was detected (Figure 1A2, n = 5).

Inhibition of Endocytosis

Given the results with Botox and the evidence that AMPARs can undergo endocytosis (Carroll et al., 1999b), we wondered whether interfering with endocytosis might have the opposite effect. We used two approaches to disrupt endocytosis. The first approach took advantage of the finding that the endocytosis of AMPARs requires the GTPase activity of dynamin (Schmid et al., 1998; Carroll et al., 1999b). Since it is now established that a common mechanism for the stimulation of dynamin GTPase activity involves the promotion or stabilization of GDP and gamma phosphate-dependent self-assembly of dynamin (Takei et al., 1996), we loaded cells with the inhibitor GDPβS. This manipulation caused a slowly developing enhancement of the AMPAR EPSC that reached a magnitude of greater than 2-fold (Figure 1B, filled squares, n = 4). In striking contrast, the NMDAR EPSC was unaffected by this manipulation (Figure 1B,

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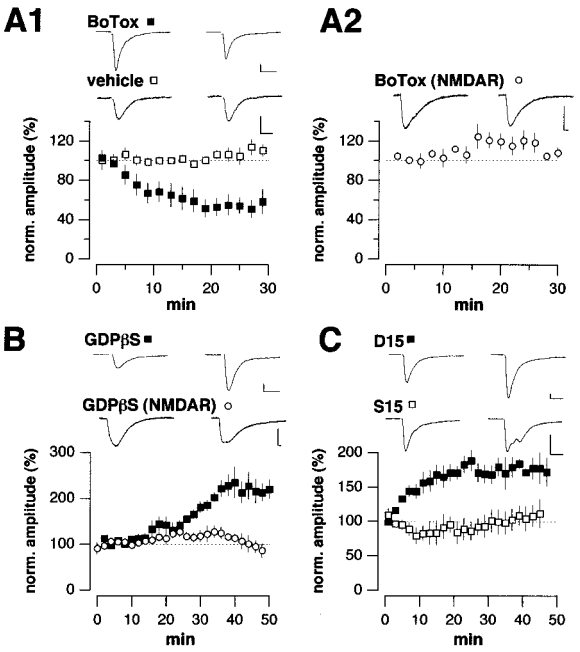

Figure 1. Inhibition of the Exocytotic and Endocytotic Machinery of the Postsynaptic Cell Modulates AMPAR Responses

(A1) An internal solution containing the light chains of botulinum toxin type B (Botox), an enzyme known to cleave v-SNAREs, causes a rundown of the AMPAR responses (filled squares, n = 5). Control experiments using only DTT (reducing agent used as vehicle, open squares, n = 7) showed stable synaptic responses.

(A2) Botox had no effect on pharmacologically isolated NMDAR responses (open circles, n = 5).

(B) GDPβS, a nonhydrolyzable GDP analog, caused a more than 2-fold increase of AMPAR responses (filled squares, n = 4). Isolated NMDR responses recorded using the same internal solution remained unchanged (open circles, n = 4).

(C) Disruption of the protein–protein interaction between dynamin and amphiphysin using D15, which consists of the 15–amino acid portion of the PRD domain of dynamin, also led to an increase of synaptic AMPAR responses (filled squares, n = 5). Open squares are the group data of the control cells filled with the scrambled peptide (S15), n = 5). Insets show averaged representative sweeps during the initial and final 7 min, respectively.

Scale bars, 40 pA/20 ms.

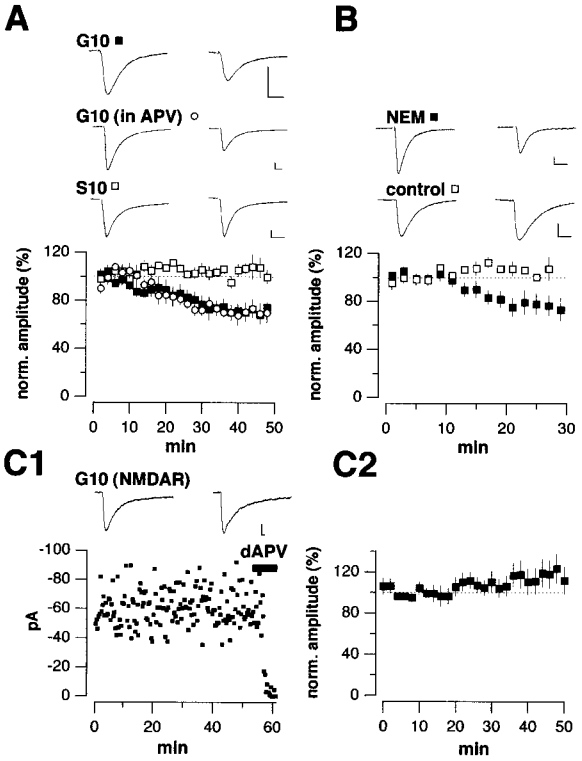

Figure 2. The Stability of AMPAR Responses Depends on the Interaction of NSF and GluR2

(A) Disruption of the NSF–GluR2 protein–protein interaction by introducing G10 into the postsynaptic cell caused a decrease of the synaptic response (filled squares, n = 6). This effect persisted in the presence of the NMDAR blocker D-APV (open circles, n = 5). In contrast, a peptide made with the identical set of amino acids but in a scrambled order, S10), showed stable AMPAR responses (open squares, n = 5) during the time of the recording.

(B) Inhibition of NSF by NEM led to a decrease of the synaptic responses (filled squares, n = 5), while recordings using the normal internal solution remained stable (open squares, n = 7).

(C1) Intracellular G10 did not affect the pharmacologically isolated NMDAR responses.

(C2) Group data showing that the G10 had no effect on the NMDAR responses (n = 8). Insets show averaged representative sweeps during the initial and final 7 min, respectively.

Scale bars, 40 pA/20 ms.

open circles, n = 4). While the effect of GDPβS was dramatic, a limitation of this manipulation is that GTPase activity is involved in a number of cellular processes, including exocytosis (Gasman et al., 1997). In an attempt to block endocytosis more selectively, we used a peptide that is known to interfere with the binding of amphiphysin with dynamin, an interaction that is considered important for endocytosis to occur (Wigge and McMahon, 1998). This 15–amino acid peptide (D15) was found to rapidly enhance the AMPAR EPSC (Figure 1C, filled squares, n = 5). As a control, we performed interleaved experiments in which we loaded cells with a scrambled form of D15 (S15). This had no significant effect on the AMPAR EPSC (Figure 1C, open squares, n = 5).

Disruption of GluR2–NSF Interaction

It has recently been reported that NSF binds to the C-terminal tail of GluR2 (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998). Evidence that this interaction has functional relevance was provided by the finding that introduction into cells of a 10–amino acid peptide (G10) encoding the NSF-interacting region of GluR2 depresses AMPAR EPSCs (Nishimune et al., 1998; Song et al., 1998). We have repeated these experiments and have confirmed that this peptide causes a rapid inhibition of AMPAR EPSCs (Figure 2A, filled squares, n = 6). This effect appeared to be specific in that a scrambled peptide (S10) containing the same amino acids failed to alter the AMPAR EPSC (Figure 2A, open squares, n = 5). Furthermore, the effect of the peptide did not depend on the activation of NMDARs, since an identical inhibition was observed in the presence of the NMDAR antagonist D-APV (50 μM) (Figure 2A, open circles, n = 5). A similar depression of the AMPAR EPSCs was seen when N-ethylmaleimide (NEM, 1 mM), which is known to inhibit NSF function, was loaded into cells (Figure 2B, filled squares, n = 5), while interleaved control cells recorded

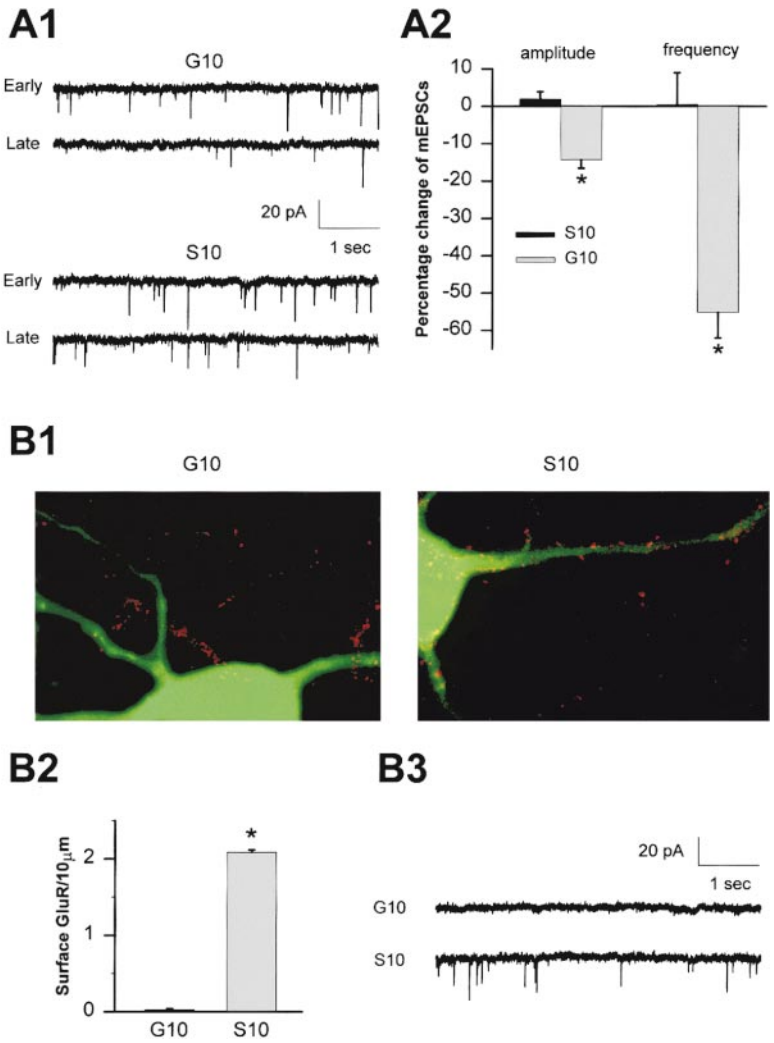

Figure 3. Disruption of the Interaction between NSF and GluR2 by the G10 Peptide Causes a Dramatic Reduction of mEPSCs and Surface Expression of AMPARs

(A) Acute application of G10 (2 mM), but not S10 (2 mM), causes a rapid decrease in mEPSC amplitude and frequency.

(A1) Representative traces of mEPSCs from the first 5 min of recording (Early) and from 15 to 20 min after break-in (Late) in cells loaded with either G10 (top traces) or S10 (lower traces) peptides.

(A2) Quantitative analysis of the mean change in mEPSC amplitude and frequency 15–20 min after break-in ($n = 5$ for each group; * indicates $p < 0.05$).

(B) Transfection of G10 causes dramatic loss of AMPA receptor surface staining and mEPSCs.

(B1) Representative cell transfected with G10 (left) shows no surface AMPAR staining (green cell), while untransfected cell in the same field shows clear surface AMPAR puncta. Representative cell transfected with S10 (right) also shows clear surface AMPAR puncta.

(B2) Quantitative analysis of the effects of G10 and S10 on surface expression of AMPARs (* indicates $p < 0.01$).

(B3) Representative recordings from cells transfected with G10 or S10 constructs.

with normal internal solution exhibited no significant change in their AMPAR EPSCs (Figure 2B, open squares, $n = 7$). In a final set of experiments, we examined the effects of G10 (Figure 2C, $n = 8$) on NMDAR EPSCs and found that these responses were unaffected by this peptide.

Why does G10 cause a decrease in the AMPAR EPSC? It has been proposed that the NSF–GluR2 interaction may be required either for the delivery of AMPARs to the synaptic plasma membrane or the stabilization of AMPARs after their arrival. In either case, G10 should cause a decrease in the surface expression of AMPARs. To test this prediction, we turned to cultured hippocampal neurons, a preparation in which it is possible to label surface AMPARs on living cells (Noel et al., 1999; Carroll et al., 1999b). To confirm that the G10 peptide also depressed synaptic responses in these cells, we first acutely loaded individual cells with G10 by making whole-cell recordings with pipettes filled with either G10 or S10. Consistent with the results in slices, we observed a rapid decrease in both the frequency and amplitude of miniature EPSCs (mEPSCs) in cells loaded with G10 ($n = 5$), whereas no significant change in mEPSC amplitude or frequency occurred in cells loaded with S10 ($n = 5$) (Figures 3A1 and 3A2).

To examine the effects of G10 on the surface expression of AMPARs, we transfected cultured hippocampal cells with G10 and compared the number of surface AMPAR puncta with cells expressing S10. As shown in Figure 3B1, G10 caused a nearly complete loss of surface AMPAR staining. Note the absence of staining (red puncta) in the transfected cell (green), while neighboring nontransfected processes express clear surface puncta. In contrast, transfection of S10 leaves surface AMPAR puncta intact. This was seen in all experiments, which are summarized in Figure 3B2 (G10, $n = 5$; S10, $n = 4$ from three independent transfections, $p < 0.01$, t test). Consistent with the loss of AMPAR surface puncta in cells transfected with G10, extremely few mEPSCs were observed in recordings from these cells ($n = 4$, only 1 or 2 mEPSCs were observed per minute), whereas frequent mEPSCs (>1 Hz) were observed in recordings from cells transfected with S10 ($n = 3$, Figure 3B3).

If the NSF–GluR2 interaction is required for the stabilization or delivery of AMPARs in the plasma membrane, disruption of this interaction would be expected to enhance the ligand-dependent internalization of AMPARs, which we have recently described (Carroll et al., 1999b; Lissin et al., 1999). To test this prediction, we examined the effects of NEM on the AMPA-induced internalization

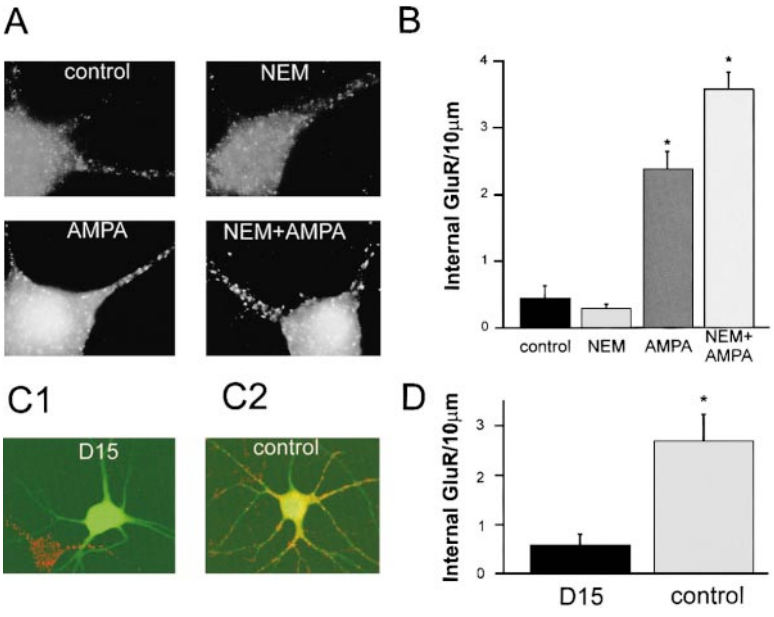

Figure 4. Effects of NEM and D15 on AMPA-Induced Endocytosis of AMPA Receptors

(A) Application of NEM (1 mM) to cultured neurons enhances the AMPAR endocytosis induced by 5 min AMPA (100 μM) treatment. Internalized AMPAR puncta were visualized using the acid stripping procedure described in the Experimental Procedures. Panels are representative immunofluoresence pictures of control neurons or neurons treated with NEM alone, AMPA alone, or NEM and AMPA together.

(B) Quantification of AMPAR endocytosis. Number of AMPAR puncta per 10 μm of proximal dendrites of neurons under the four conditions. NEM treatment alone shows no significant difference compared to control, but both are significantly different from AMPA treatment. Moreover, NEM and AMPA together cause a significantly higher degree of internalized AMPARs than AMPA alone (control, n = 12 cells; NEM, n = 12 cells, AMPA alone, n = 27 cells; AMPA + NEM, n = 28 cells, $p < 0.03$, t test).

(C and D) Expression of D15 blocks the AMPA-induced endocytosis of AMPARs in cultured neurons.

(C1) Example of a neuron expressing D15 (identified by cotransfection with GFP) that does not show AMPAR endocytosis, while an adjacent untransfected cell does.

(C2) Example of a neuron expressing GFP alone that exhibits AMPAR endocytosis following AMPA treatment.

(D) Quantification of the effects of D15 compared to control cells transfected with GFP alone (n = 10 cells in each group, $p < 0.01$).

of AMPARs in cultured neurons (1 mM for 15 min prior to and during 5 min AMPA treatment). Application of NEM alone had no detectable effect on the magnitude of AMPAR internalization when compared to untreated control cells (Figures 4A and 4B; control, n = 12 cells; NEM, n = 12 cells). However, it significantly enhanced the internalization caused by AMPA (Figures 4A and 4B; AMPA alone, n = 27 cells; AMPA + NEM, n = 28 cells, $p < 0.03$, t test).

We also examined the effects of the D15 peptide on the AMPA-induced internalization of AMPARs. As shown in Figures 4C and 4D, the endocytosis of AMPARs was dramatically inhibited in cells expressing this peptide, whereas untransfected cells on the same cover slip (Figure 4C1) or cells transfected with GFP alone (Figure 4C2) showed normal internalization of AMPARs. A quantitation of the effects of D15 compared to control cells expressing GFP alone is shown in Figure 4D (n = 10 in each group).

Activity Dependence of AMPAR Cycling

The results reported thus far suggest that synaptic AMPARs constitutively cycle into and out of the plasma membrane and that changes in the AMPAR EPSC occurring as a result of interfering with this cycling reflects a change in the number of functional AMPARs in the PSD. An important question is whether this cycling is dependent on synaptic activity. To address this issue experimentally, two independent pathways that form synapses onto the same cell were alternately stimulated. Within the first 5 min following initiation of the whole-cell recording, baseline synaptic responses were recorded for both pathways. Stimulation of pathway 1 was then stopped while continuing to monitor the EPSCs generated by pathway 2. After the effects of the reagent being tested had stabilized, stimulation of pathway 1 was resumed and the size of the synaptic responses generated by the two pathways was compared. Using this protocol, we first tested the activity dependence of Botox (n = 4). As can be seen in Figure 5A, Botox had an identical depressant action on both of the pathways, indicating that synaptic stimulation was not required for the depression of AMPAR EPSCs by Botox.

Using the same two-pathway protocol, we then tested whether the enhancing effects of D15 and GDPβS required synaptic stimulation. As shown in Figure 5B, D15 increased the AMPAR EPSC to the same extent in both pathways (n = 4). Similar results were obtained with GDPβS (n = 2, data not shown). These results suggest that there is a constitutive component of endocytosis of AMPARs.

In the final set of these two-pathway experiments, we examined whether the action of G10 required synaptic stimulation. Surprisingly, when stimulation was resumed on the unstimulated pathway, the responses were identical to those recorded just before the stimulation was paused (Figure 6, n = 5). Upon resumption of the stimulation, the responses decreased at a more rapid rate than the depression observed on the continuously stimulated pathway. Presumably in the continuously stimulated pathway the rate at which the depression occurs is dependent both on the time required for the peptide to diffuse to the synapses and on the activity dependence of the G10 action, while on the second pathway the rate of diffusion is not a factor.

Inhibition of LTD

A number of recent observations suggest that AMPARs are rapidly redistributed during NMDAR-dependent LTD (Carroll et al., 1999a) and LTP (Shi et al., 1999). If this is

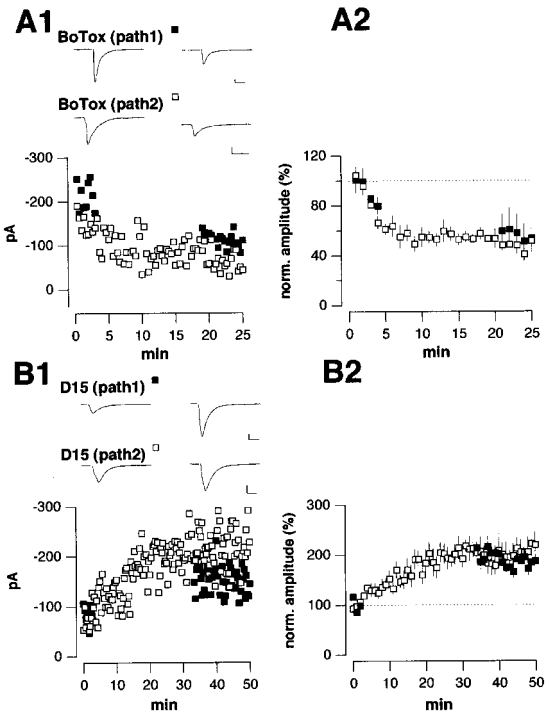

Figure 5. Modulation of AMPAR Responses by Botox and D15 Does Not Require Synaptic Activity

(A1) Two independent pathways converging onto the same postsynaptic cell were stimulated alternately for 5 min. One pathway was then paused for 15 min, while the second was stimulated every 20 s. When stimulation in the first pathway was resumed, responses had decreased by an amount similar to that of the second pathway. Insets show averaged representative sweeps during the initial and final 5 min, respectively.

(A2) Group data, indicating that the rundown was identical in the two pathways (n = 4).

(B1) The increase caused by D15 was also independent of activity, as shown using a protocol similar to that in (A).

(B2) Group data showing identical increase in the constantly stimulated pathway versus the paused input (n = 4).

Scale bars for all insets showing averaged traces of the initial and final 5 min are 40 pA/20 ms.

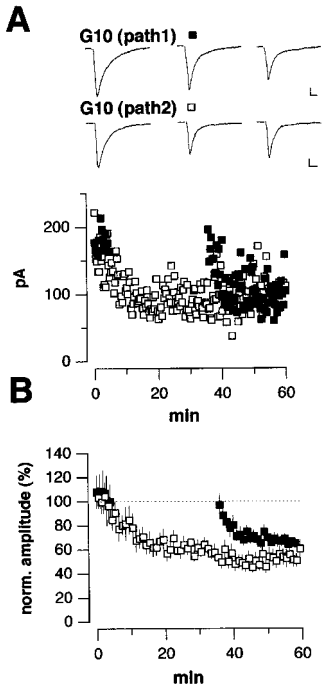

Figure 6. The G10-Mediated Decrease of Synaptic Responses Is Activity Dependent

(A) Two independent pathways converging onto the same postsynaptic cell were stimulated alternately for 5 min. One pathway was then paused for 30 min, while the second was stimulated every 20 s and ran down. When stimulation in the first pathway was resumed, responses remained unchanged. Insets show an average of representative sweeps during the initial 5 min, the 5 min after the first pathway was paused, and the final 5 min. Scale bars 40 pA/20 ms.

(B) Summary of group data (n = 5).

correct, AMPAR endocytosis or exocytosis may play important roles in mediating these phenomena, and therefore inhibition of these processes would be expected to impair LTD and LTP. Because the preceding experiments involved whole-cell recording, which causes washout of the ability to generate LTP (Malinow and Tsien, 1990), and we have previously examined the role of postsynaptic membrane fusion in LTP (Lledo et al., 1998), we focused our efforts on the examination of LTD. Figure 7A shows that following the depression of AMPAR EPSCs caused by Botox, an LTD-inducing stimulus (see Experimental Procedures) did not elicit any significant change in synaptic strength (n = 5), while interleaved cells recorded for a similar period of time with the vehicle for Botox exhibited stable LTD (n = 6). Similarly, after the growth of the AMPAR EPSC in response to D15 (n = 6) or GDPβS (n = 4) had stabilized, LTD could not be elicited (Figure 7B, open circles and filled squares, respectively). Again stable LTD was induced in a set of interleaved control cells (n = 6) that were recorded for a similar time before the LTD-inducing stimulus was applied (Figure 7B, open squares). Finally, loading cells with G10 was also found to block the ability to generate LTD (Figure 7C, filled squares, n = 4), while in control cells recorded for a similar period of time with pipettes containing S10, LTD could be generated (Figure 7C, open squares, n = 3). These results suggest that in addition to the constitutive endocytosis of AMPARs, there is also a regulated component that may be utilized by LTD.

Discussion

Postsynaptic Blockade of Exocytosis Depresses the AMPAR EPSC

We have carried out a number of experiments to determine whether AMPARs constitutively cycle into and out of the postsynaptic density (PSD) at excitatory synapses on hippocampal CA1 pyramidal cells. To address the possible role of exocytosis in the delivery of AMPARs to synapses, we loaded CA1 cells with botulinum toxin type B (Botox), which disrupts membrane fusion by cleaving the v-SNARE synaptobrevin (Huttner, 1993). This caused a significant rundown of the AMPAR EPSC but had little effect on the NMDAR EPSC. The depressant action of Botox did not require synaptic stimulation, since the magnitude of the depression observed on an

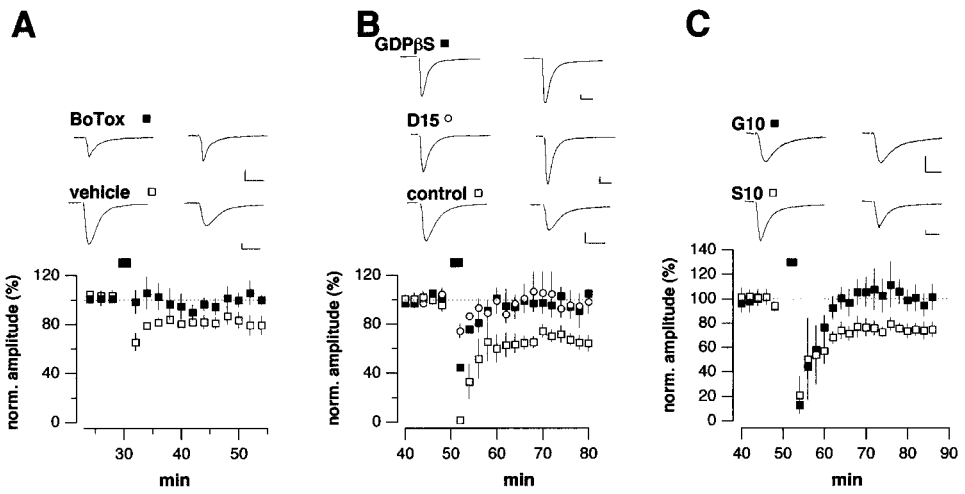

Figure 7. BoTox, G10, GDPβS, and D15 Prevent LTD

(A) Following the Botox-induced rundown, the application of an LTD-inducing protocol failed to induce LTD (filled squares, n = 5). Control cells perfused only with the vehicle (DTT, 5 mM) show LTD (open squares, n = 6).

(B) After the effects of D15 (open circles, n = 6) or GDPβS (filled squares, n = 4) had stabilized, an LTD induction protocol was applied but failed to elicit LTD, whereas LTD was induced in control cells (open squares, n = 6).

(C) LTD was not induced in cells after the G10-mediated rundown had leveled off (filled squares, n = 4), but substantial LTD was observed in cells loaded with the scrambled peptide (S10) (open squares, n = 3). Scale bars for all insets showing averaged traces of the initial and final 5 min are 40 pA/20 ms. Time on the x axis reflects the time following break-in.

unstimulated pathway was the same as that on a stimulated pathway. This result suggests that the level of functional AMPARs, but not NMDARs, at the synapse depends on a relatively rapid constitutive insertion of AMPARs that is independent of activity. In a previous study using sharp microelectrodes, we found that intracellular application of Botox inhibited the ability to generate LTP but had no obvious effect on baseline synaptic responses (Lledo et al., 1998). Presumably, the higher concentrations and more rapid application afforded by the whole-cell recording used in the present study accounts for the difference in the present results. Furthermore, sharp electrode recordings are known to invariably cause an initial increase of the leak of the cell, which usually seals over in about 5–10 min, the time period during which we have observed most of our effects under whole-cell conditions. The depression of the AMPAR EPSC by inhibition of exocytosis cannot explain the impairment in LTP (Lledo et al., 1998), since the NMDAR EPSC was unaffected and the tetanus-induced envelope of depolarization was unaltered.

Postsynaptic Blockade of Endocytosis Enhances the AMPAR EPSC

We have recently demonstrated that AMPARs undergo dynamin-dependent endocytosis (Carroll et al., 1999b). If endocytosis also occurs constitutively and affects functional AMPARs at the synapse, blockade of endocytosis would be expected to result in an accumulation of surface AMPARs and thereby an increase in the AMPAR EPSC. Consistent with this prediction, blockade of GTP-dependent processes, which are required for endocytosis (Sweitzer and Hinshaw, 1998), with the application of GDPβS resulted in a significant but delayed increase in the AMPAR EPSC. The NMDAR EPSC was unaffected by this manipulation. While this result is in accord with a blockade of constitutive endocytosis, many cellular processes are dependent on GTP hydrolysis. We therefore loaded cells with a 15–amino acid peptide (D15) that prevents the interaction between amphiphysin and dynamin, an interaction that is required for endocytosis (Wigge et al., 1997). Injection of D15, but not a scrambled version of the peptide, led to a rapid increase in the AMPAR EPSC, but no change in the NMDAR EPSC. These results suggest that synaptic AMPARs do in fact undergo a constitutive endocytosis, which functions to limit the number of AMPARs in the PSD. Furthermore, the AMPA-induced translocation of surface AMPARs to internal sites is blocked by expressing D15 in cultured neurons.

Anatomical Receptor Localization and AMPAR Cycling

The results discussed thus far are consistent with a model in which AMPARs, but not NMDARs, are continuously undergoing cycling into and out of the synaptic plasma membrane. Since the changes in synaptic strength caused by the various reagents stabilized within 20–30 min, the rate at which the AMPARs recycle at the synapses must be fairly fast. This implies that there should be a population of AMPARs present in a membranous mobile pool just beneath the PSD. Although membranous structures within the spine have been observed (Spacek and Harris, 1997), none of the recent immunogold studies that have examined the localization of AMPARs (Baude et al., 1995; Nusser et al., 1998; Takumi et al., 1999) has reported such a pool of receptors. Although AMPARs can be found within the dendritic shaft and on occasion in the spine apparatus (Nusser et al., 1998), one might still expect to observe receptors in the spine in transit to the PSD if this pool of receptors was involved in the cycling. Thus, based

on the current anatomical data, it is necessary to postulate a very brief dwell time for AMPARs within the spine, compared to that for the surface synaptic AMPARs in the PSD. Indeed, this conclusion is consistent with our recent observations (Carroll et al., 1999b) in which we detected small amounts of internalized AMPARs under resting conditions. Another surprising implication of our results is that although the PSD appears to be a highly organized and dense structure (Kennedy, 1997; Kornau et al., 1997; Ziff, 1997), AMPARs appear to be able to move freely into and out of it. Of course, we cannot rule out the possibility that our manipulations have interfered with the function or localization of proteins other than AMPARs and as a consequence have affected synaptic transmission.

In marked contrast, NMDARs, over the time scale of our experiments, appear to be remarkably fixed in the PSD, since they were unaltered by any of our manipulations. This extreme difference in the mobility of AMPARs compared to NMDARs is consistent with results involving a number of manipulations, including glutamate receptor blockade (Craig, 1998; Liao et al., 1999), ligand-induced internalization (Carroll et al., 1999b; Lissin et al., 1999), and LTD (Carroll et al., 1999a). Further evidence in support of the idea that AMPARs are much less firmly attached to the PSD than NMDARs comes from the demonstration that brief detergent treatment or disruption of the actin cytoskeleton results in the selective loss of AMPARs from the synapse (Allison et al., 1998). Presumably, this differential stabilization of the two classes of ionotropic glutamate receptors is due to the different set of proteins with which each of the receptors interact (O'Brien et al., 1998; Sheng, 1997; Ziff, 1997). Ultrastructural localization of AMPARs and NMDARs at the synapse has shown that these two types of receptor are intermingled in the PSD (Takumi et al., 1999). Since the present results indicate that AMPARs can be rapidly and selectively removed from the PSD, a mechanism must exist for the selective removal of AMPARs that are located among NMDARs.

Possible Roles for the NSF–GluR2 Interaction

It has recently been reported that the universal membrane fusion protein, NSF, binds the C terminus of the GluR2 subunit of the AMPAR (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998) and that disruption of this interaction by injecting G10, which contains the same sequence as the GluR2-binding site for NSF, results in an inhibition of the AMPAR EPSC. We have confirmed these results and find that the inhibition occurs in the absence of NMDAR activation. The inhibition is selective in that the active peptide has no effect on the NMDAR EPSC and specific because a scrambled version of the peptide did not affect the AMPAR EPSC. Inhibition of NSF with NEM mimicked the action of the active peptide, causing a similar rundown of the AMPAR EPSC, but not the NMDAR EPSC. Consistent with a recent report (Noel et al., 1999), we found that prolonged expression of G10 in cultured hippocampal neurons caused a profound loss of surface AMPARs. Furthermore, disruption of NSF function by treatment of cells with NEM enhanced the AMPA-induced endocytosis of surface AMPARs. These results support the notion that the depression of the AMPAR EPSC caused by NEM and G10 is a consequence of the loss of surface synaptic AMPARs.

A critical question that remains is what exact mechanistic role does the NSF–GluR2 interaction play in the regulation of AMPARs in the PSD? The answer to this question is complicated by the finding that the depressant action of G10 required synaptic stimulation, while the actions of Botox, GDPβS, and D15 did not. Possible models that are consistent with many of the present results are illustrated in Figure 8. Part A shows the insertion of AMPARs into the synaptic membrane via exocytosis and its blockade by Botox, as well as the removal of the receptors via endocytosis and its blockade by GDPβS and D15. In addition, it is postulated that the association of GluR2 with NSF (gray squares) is required for maintaining the AMPARs at the synapse. In this scenario, the G10 binds to free NSF, but because the binding of NSF to GluR2 is strong, there is little rundown in the AMPAR EPSC. When glutamate binds to the receptor, the NSF–GluR2 association is weakened, and no free NSF is now available to bind to the AMPARs. In the absence of NSF binding to the AMPARs, the receptors are no longer retained at the synapse. As a consequence, there is an activity-dependent rundown of the AMPAR EPSC in the presence of G10. A variation on this theme is shown in Figure 8B in which the presence of G10 along with the binding of glutamate to the receptor facilitates endocytosis (green arrow pathway). In this scenario, as well as that shown in Figure 8A, the separation of NSF from GluR2 is nonphysiological and only occurs when G10 is present. However, the data would also be consistent with a model in which the binding of glutamate normally causes the dissociation of NSF, resulting in endocytosis (green arrow pathway) that occurs in parallel with the constitutive pathway (red arrow pathway). In this case, one would have to postulate that in the presence of G10, there is no more free NSF to rebind to the internalized receptor, and the unbound receptor is no longer recycled.

These models suggest that the role of NSF interacting with GluR2 is quite different from the function NSF plays in membrane fusion. It also should be noted that the model does not explain all of our data. For instance, the acute administration of G10 and Botox caused a 40% depression of the EPSC, suggesting a pool of surface AMPARs that is not involved in the rapid cycling. Chronic expression of G10, however, resulted in a profound decrease in the expression of surface AMPARs, suggesting that this pool of more stable AMPARs does interact on a slower time scale with the rapidly cycling pool.

The NSF–GluR2 interaction is presumably not the only mechanism by which AMPARs are localized to synapses because synaptic AMPARs in interneurons can lack the GluR2 subunit and still be localized to the synapse (He et al., 1998). Moreover, in the GluR2 knockout mouse AMPARs are localized at the synapse, albeit at a reduced density (Jia et al., 1996). Perhaps related to these findings is the observation that the depressant action of both Botox and G10 were incomplete, stabilizing at an approximately 40% depression. While this could be due to the incomplete access of the blockers to a pool of AMPARs, the fact that the inhibition by the two markedly different sized molecules was very similar suggests that

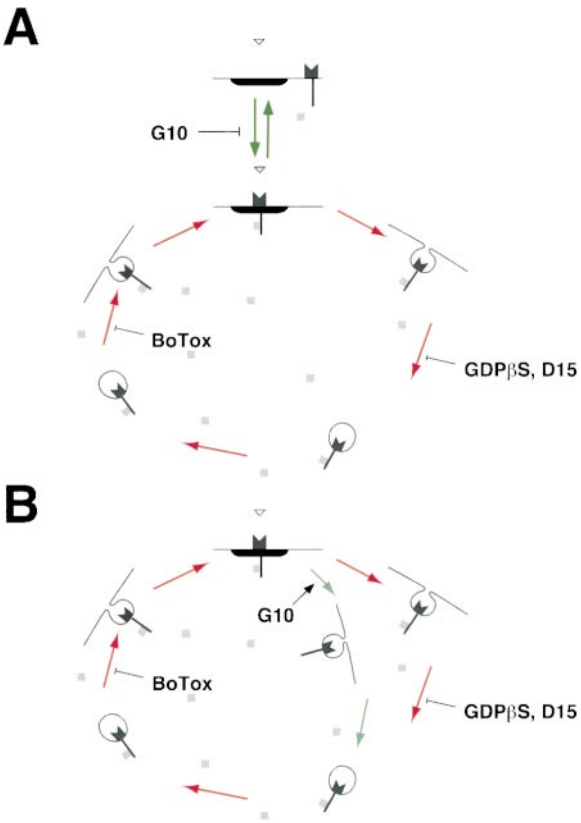

Figure 8. Proposed Model

A constitutive recycling pathway (red arrows) can be disrupted by introducing D15 or GDPβS. Conversely, the exocytotic limb is blocked by Botox. Version A proposes a role of the NSF–GluR2 interaction in preventing lateral redistribution. In version B, two endocytotic pathways are proposed to exist for AMPARs (filled M), one of which is activity dependant (green arrow) and involves the dissociation of the NSF (gray squares)–GluR2 interaction upon binding (open triangle) in the presence of G10.

there is a pool of AMPARs that is resistant to the acute effects of these manipulations. Our results, in combination with recent results of others (Noel et al., 1999), showing a dramatic loss of surface AMPARs after expressing G10 for approximately 3 days, suggest that with time this resistant pool of AMPARs moves into the sensitive pool.

AMPAR Recycling and Synaptic Plasticity

Perhaps the most intriguing aspect of these results is their implication for understanding various aspects of synaptic plasticity. Indeed, it was shown in a previous study that manipulations that altered membrane fusion events were able to disrupt LTP (Lledo et al., 1998). The recent demonstration that LTP-inducing stimuli can cause the movement of GFP-tagged glutamate receptors into the dendritic spine (Shi et al., 1999) provides support for a role of AMPAR trafficking in LTP. In the present study, we found that LTD could not be elicited after the effects of Botox, D15, GDPβS, or G10 had stabilized. The inability to elicit LTD after the depression of AMPAR EPSCs by Botox and G10 could be due to an occlusion because the depression engages the same mechanisms as LTD, or because the depression is independent of LTD and the substances interfere with some step involved in LTD. Similarly, that LTD cannot be induced following manipulations that interfere with endocytosis would be expected if LTD involved an endocytosis of AMPARs, but we cannot exclude the possibility that by interfering with endocytosis, D15 and GDPβS interfere with LTD independent of their inhibition of AMPAR cycling. Thus, these results are consistent with an intact cycling of AMPARs being required for the generation of LTD and that LTD may involve an enhanced endocytosis of surface AMPARs. Interestingly, the magnitude of the depression caused by Botox and G10 was similar to that observed when LTD is saturated. These results are entirely consistent with the finding that in culture, LTD is associated with a decrease in the number of synapses containing AMPARs (Carroll et al., 1999a).

The exact mechanisms by which the induction of LTP or LTD would cause a long-lasting change in AMPAR cycling will require considerably more study. One intriguing possibility is that the state of phosphorylation of the AMPAR may impact on the kinetics of cycling. A great deal of work has been done indicating that CaMKII plays an essential role in LTP, perhaps by the direct phosphorylation of the GluR1 subunit (Derkach et al., 1999). Similarly, LTD is associated with a decrease in the phosphorylation of GluR1 (Lee et al., 1998). Might this phosphorylation impact on AMPAR cycling? Although the reported increase in single channel conductance seen both with CaMKII (Barria et al., 1997; Derkach et al., 1999) and with LTP (Benke et al., 1998) is likely to be independent of AMPAR recycling, the level of phosphorylation of GluR1 or other subunits might in some undefined manner serve to facilitate or depress the expression of AMPARs in the PSD.

The concept that synaptic AMPARs may be constitutively cycling over the course of minutes using exocytotic/endocytotic pathways is, in our view, quite remarkable. However, it should be noted that while the evidence for the endocytosis of AMPARs is reasonably strong (see Carroll et al., 1999b), there is as yet little direct evidence for the exocytosis of AMPARs. Nevertheless, the present results combined with other recent results (Carroll et al., 1999a; Engert and Bonhoeffer, 1999; Okabe et al., 1999; Shen and Meyer, 1999; Shi et al., 1999) force a major shift in our concept of the synapse. Instead of a structure in which a dynamic presynaptic terminal acts on a relatively static postsynaptic structure, the postsynaptic membrane appears to be a far more dynamic subcellular compartment than previously imagined and one that can modify its molecular composition rapidly.

Experimental Procedures

Slice Preparation

Hippocampal slices were prepared from P10-P17 Sprague-Dawley rats. The animals were decapitated under deep halothane anesthesia; their brains were removed, rapidly cooled in ACSF, and bubbled continuously with 95% O_2 and 5% CO_2. Coronal sections of the hippocampus (350 μm thick) were obtained using a VT1000 vibratome (Leica, USA). After 1–8 hr, the slices were transferred to a recording chamber and the CA3 region surgically separated from the CA1 region. The slices were superfused (2 ml/min) with an external

solution containing (in mM) NaCl 119, KCl 2.5, $MgSO_4$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26.2, picrotoxin 0.05, and glucose 11. All experiments were carried out at room temperature, except for the experiments involving the light chain of botulium toxin, which were done at 30°C–32°C.

Electrophysiology

Whole cell recordings were made from CA1 pyramidal cells, which were identified under a DIC microscope. Patch pipettes (3–5 MΩ) filled with the following internal solution were used (in mM): $CsCH_3SO_3$ 130, HEPES 10, EGTA 0.5, NaCl 8, MgATP 4, and Na_3GTP 0.3 (pH 7.4). Cells were held at −70 mV, and recordings were amplified with an Axopatch 1D; filtered at 2 kHz and digitized at 5–10 kHz (National Instruments Board MIO-16, NI-DAQ/Igor 3.1 Software, Wave Metrics, Lake Oswego, Oregon); and stored on a hard disk. NMDAR EPSCs were recorded by adding CNQX (10 μM) to the solution and reducing the Mg^{2+} concentration to 0.5 mM. Representative traces are averages of 12–21 sweeps. Data are expressed as means ± SEM.

To ensure stability of the recordings, electrical stimulation was initiated well before the cell was patched. A minimum of 5 min was allowed in the "cell-attached" configuration before break-in in order to wash off any residual internal solution spilled from the approaching pipette. After break-in, recordings were started within 0–5 min. During this time period, stimulation strength was adjusted. The passive membrane properties were monitored throughout the recording, and none of the substances introduced into the neurons had any effect on membrane properties. It might be argued that while the substances were applied into the postsynaptic cell, they may have escaped and entered across the presynaptic membrane to change the release of glutamate. This scenario seems most unlikely for large peptides, and in any event the lack of change in the NMDAR EPSC for all of the manipulations argues against most accepted alterations in transmitter release. To evoke synaptic responses, stimuli (0.1 ms duration) were delivered at 0.05–0.1 Hz (unless stated otherwise) through bipolar (200 μm tip spacing) stainless steel electrodes (FHC, Bowdoinham, Maine). LTD was induced by a 5 Hz–3 min train or a pairing protocol using 100 pulses at 1 Hz at −40mV.

Culture of Hippocampal Neurons

Hippocampi of newborn (postnatal day 0) Sprague-Dawley rat pups were removed, and the dentate gyri were grossly dissected away. Cells derived from the remaining tissue were plated as described previously (Brewer et al., 1993). One-half of the growth medium was exchanged 1 day after plating and weekly thereafter.

AMPAR Endocytosis Assay, Transfection, and Immunohistochemistry

AMPAR endocytosis was assayed using the acid strip method described previously (Carroll et al., 1999b). Oligonucleotides encoding G10 and S10 peptides with HA tags or the untagged D15 oligonucleotide were inserted between HindIII and BamHI sites of a pcDNA3 vector. These constructs were cotransfected with a GFP containing vector using Effectine transfection reagent (Qiagen) (1 μg pcDNA3/0.2 μg GFP per cover slip). Three days after transfection, the cells were subjected to surface labeling of AMPARs: live neurons were incubated with a polyclonal antibody recognizing the N terminal of GluR1 (5 μg/ml; Oncogene) for 15 min and then washed three times with TBS. The neurons were then fixed with 4% paraformaldehyde, 4% sucrose in PBS for 15 min. Cells were then blocked in 2% BSA in TBS for 1 hr before exposure to Cy3 conjugated donkey anti-rabbit secondary antibody for 1 hr. Cells were then washed and mounted for immunofluorescence microscopy using a Nikon 60×, NA 1.4 objective and commercial FITC and Cy3 filter sets (Omega). Images were captured and analyzed as described previously (Carroll et al., 1999b). Transfected cells were identified by the expression of GFP.

Peptides and Drugs

Peptides were dissolved (1–2 mM) in the internal solution described above. The amino acid sequence for G10, which is a segment of the C-terminal region of GluR2, was KRMKVAKNAQ (Nishimune et al., 1998). As a control, a peptide containing the same amino acids in a scrambled order, (S10): VRKKNMAKQA, was used. A 15–amino acid peptide constituting a stretch of the PRD domain of dynamin (Dynamin 828-42, PPPQVPSRPNRAPPG) referred to as D15 was used to disrupt dynamin's interaction with amphiphysin (Schmierer et al., 1998). The corresponding scrambled peptide (S15) was ANVRRGPPPPPQPSP. All custom-made peptides were prepared by the Biomedical Resource Center, UCSF, San Francisco, California, and were purified by reverse phase high-pressure liquid chromatography.

The following drugs were either bath-applied or included in the internal solution and bought from the following sources: picrotoxin (50 μM, Sigma), NBQX (5 μM, Tocris), D-AP5 (100 μM, Tocris), NEM (1 mM, Sigma), light chains of botulinum toxin type B (0.5 μM, List Biological), and GDPβS (0.6 mM, RBI).

Acknowledgments

We thank the members of the Nicoll and Malenka labs for many helpful discussions, and Sue Giller for the preparation of the cultures. C. L. is supported by the Swiss National Science Foundation. R. A. N. is a member of the Keck Center for Integrative Neuroscience and the Silvio Conte Center for Neuroscience Research. He is supported by grants from the National Institutes of Health and the Bristol-Myers Squibb Co. R. C. M. is supported by grants from the National Institutes of Health, Human Frontier Science Project, and an Investigator Award from the McKnight Endowment Fund for Neuroscience.

Received July 26, 1999; revised October 28, 1999.

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