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Synaptic Strength Regulated by Palmitate Cycling on PSD-95

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Summary

Dynamic regulation of AMPA-type glutamate receptors represents a primary mechanism for controlling synaptic strength, though mechanisms for this process are poorly understood. The palmitoylated postsynaptic density protein, PSD-95, regulates synaptic plasticity and associates with the AMPA receptor trafficking protein, stargazin. Here, we identify palmitate cycling on PSD-95 at the synapse and find that palmitate turnover on PSD-95 is regulated by glutamate receptor activity. Acutely blocking palmitoylation disperses synaptic clusters of PSD-95 and causes a selective loss of synaptic AMPA receptors. We also find that rapid glutamate-mediated AMPA receptor internalization requires depalmitoylation of PSD-95. In a nonneuronal model system, clustering of PSD-95, stargazin, and AMPA receptors is also regulated by ongoing palmitoylation of PSD-95 at the plasma membrane. These studies suggest that palmitate cycling on PSD-95 can regulate synaptic strength and regulates aspects of activity-dependent plasticity.

Introduction

Excitatory signaling in the brain occurs primarily at synapses that employ glutamate as a neurotransmitter. The ionotropic glutamate receptors are clustered with associated downstream signaling enzymes in the postsynaptic density (PSD), a thickening of the cytoskeleton beneath the plasma membrane (Kennedy, 1997; Walters and Matus, 1975). This clustered arrangement facilitates rapid and efficient transmission; however, the PSD is also a dynamic structure. Changes in glutamate receptor number at the PSD provide a critical mechanism for rapidly altering synaptic strength (Malenka and Nicoll, 1999; Malinow et al., 2000). Interestingly, the two principle classes of ionotropic glutamate receptors are differentially anchored at the PSD. The N-methyl-D-aspartate (NMDA) receptors are firmly tethered to the PSD (Moon et al., 1994), whereas the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors are more loosely attached (Malenka and Nicoll, 1999; Malinow et al., 2000). Dynamic cycling of AMPA receptors on and off the synaptic membrane occurs in an activity-dependent fashion and appears to underlie aspects of synaptic plasticity (Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000; Lüscher et al., 1999; Man et al., 2000).

Although mechanisms that control glutamate receptor numbers at the synapse are uncertain, proteins containing PDZ domains play a general role in recruiting receptors to synapses and other sites of cell-cell contact (Craven and Bredt, 1998; Garner et al., 2000; Kornau et al., 1997; Lee and Sheng, 2000). PDZ domains are modular protein motifs that bind to the extreme C termini of interacting proteins (Kim et al., 1995; Kornau et al., 1995) or with each other through PDZ-PDZ interactions (Brenman et al., 1996a). The major PDZ protein at excitatory synapses is PSD-95/SAP-90 (Cho et al., 1992; Kistner et al., 1993), a membrane-associated guanylate kinase (MAGUK), which functions as a multivalent synaptic scaffolding protein. The PDZ domains from the postsynaptic density protein (PSD-95) were first shown to bind the C-terminal tails of NMDA receptor type-2 subunits (Kornau et al., 1995) and Shaker-type K^+ channels (Kim et al., 1995). However, the PSD-95 family of MAGUKs can also interact with AMPA receptors. The C terminus of glutamate receptor subunit-1 (GluR1) binds directly to MAGUK protein SAP-97 (Leonard et al., 1998); furthermore, the AMPA receptor trafficking protein, stargazin, associates with PDZ domains from several neuronal MAGUKs (Chen et al., 2000).

By interacting with glutamate receptors, cell adhesion molecules, and cytoskeletal elements (Irie et al., 1997; Kim et al., 1997; Kornau et al., 1997), MAGUK proteins regulate synapse development and plasticity. Disruption of *discs large* (DLG), a *Drosophila* MAGUK, perturbs postsynaptic structure and blocks clustering of Shaker-type K^+ channels that normally associate with DLG (Tejedor et al., 1997). In cultured hippocampal neurons, overexpression of PSD-95 accelerates development of excitatory synapses and selectively enhances clustering of AMPA receptors (El-Husseini et al., 2000b). On the other hand, targeted disruption of PSD-95 causes severe abnormalities of synaptic plasticity, such that long-term potentiation (LTP) is enhanced and long-term depression (LTD) is eliminated (Migaud et al., 1998). These abnormalities in synaptic plasticity presumably explain why PSD-95 mutant mice are impaired in spatial learning (Migaud et al., 1998).

How might PSD-95 dynamically regulate synaptic glutamate receptors and synaptic plasticity? Phosphorylation of DLG within PDZ1 by calcium/calmodulin-dependent protein kinase regulates clustering at the *Drosophila* neuromuscular junction (Koh et al., 1999), but it is uncertain whether this mechanism applies for PSD-95 in the brain. In addition to its PDZ motifs, PSD-95 contains several other domains that could help explain its role. The C-terminal half of PSD-95 comprises an SH3 and a

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guanylate kinase domain, which both function as protein-protein interaction motifs (Craven and Bredt, 1998; Garner et al., 2000; Lee and Sheng, 2000). Interestingly, these domains also can bind each other in both intra- and intermolecular orientations (McGee and Bredt, 1999; Shin et al., 2000). However, it is not yet clear how the SH3 or GK domains might be regulated in an activity-dependent fashion.

PSD-95 also contains two critical cysteine residues at positions 3 and 5, which are sites of protein palmitoylation (Topinka and Bredt, 1998). This posttranslational modification involves addition of palmitate, a 16-carbon fatty acid, via a labile thioester linkage (Dunphy and Linder, 1998; Resh, 1999). Palmitoylation of PSD-95 is essential for clustering at the PSD (Craven et al., 1999). Akin to protein phosphorylation, palmitoylation is a reversible process that is dynamically regulated by specific cellular stimuli (Milligan et al., 1995; Mumby, 1997). We therefore asked whether this lipidation of PSD-95 is activity dependent at the synapse and whether this might regulate glutamate receptor function.

Here, we identify palmitate turnover on PSD-95 at the synapse. This palmitate cycling occurs in an activity-dependent fashion, and acutely disrupting palmitoylation with 2-bromopalmitate disperses synaptic clusters of PSD-95. This activity-dependent dispersion of PSD-95 results in a selective loss of AMPA receptor subunits and AMPA receptor activity at synapses. Preventing depalmitoylation of PSD-95 blocks glutamate-mediated internalization of AMPA receptors. Surface clusters of PSD-95, stargazin, and AMPA receptor subunits that form in transfected heterologous cells also require ongoing palmitoylation of PSD-95 at the plasma membrane. These studies suggest that protein palmitoylation is regulated in an activity-dependent fashion at the synapse and suggest mechanisms for plasticity regulated by palmitate cycling on PSD-95.

Results

Dynamic Turnover of Palmitate on PSD-95 at the Synapse

Previous studies have shown that palmitoylation of PSD-95 is essential for its clustering at synapses (Craven et al., 1999). To determine whether palmitoylation of PSD-95 is a dynamic process at the synapse, we treated cultured hippocampal neurons (DIV 14) with 2-bromopalmitate, a drug that potently blocks palmitoylation by blocking protein acyl-transfer (Webb et al., 2000). We found that incubation of neurons with 10 μM 2-bromopalmitate dispersed synaptic clusters of PSD-95 (Figures 1A and 1B) and blocked palmitoylation of PSD-95 (Figure 1C). This treatment did not affect clustering of synaptophysin, a presynaptic marker used as a control (Figure 1A). This effect was also seen with 2-bromopalmitoyl-CoA (Figure 1B), which also inhibits protein palmitoylation (data not shown). Synaptic clusters of PSD-93 (Brenman et al., 1996b; Kim et al., 1996), a palmitoylated MAGUK (El-Husseini et al., 2000c) related to PSD-95, are also dispersed by 2-Br palmitate (Supplemental Figure S1, http://www.cell.com/cgi/content/full/108/6/849/DC1). As a control, we found that palmitate at a 10 times higher dose significantly enhanced clustering of PSD-95 (Figures 1A and 1B). The effects of 2-Br palmitate on clustering are not simply due to disrupting palmitoylation of newly synthesized PSD-95, as cycloheximide (CHX), which effectively blocks synthesis of PSD-95 (Supplemental Figure S2, http://www.cell.com/cgi/content/full/108/6/849/DC1), does not disperse PSD-95 synaptic clusters (Figures 1A and 1B).

Many palmitoylated proteins, including PSD-95, are insoluble in nonionic detergents (Cho et al., 1992, Kistner et al., 1993). As a surrogate assay for palmitoylation, we monitored the solubility of PSD-95 in neurons treated with 2-bromopalmitate. Neurons were homogenized, lysed in hypotonic buffer, and lysates were centrifuged to separate cytosolic (soluble) and membrane (particulate) fractions. The ratio of PSD-95 in the soluble/particulate fractions (S/P ratio) was quantified by gel densitometry. As previously described, the majority of PSD-95 in cultured hippocampal neurons is insoluble (S/P $= 34 \pm 12$, n $= 6$); however, treatment with 2-bromopalmitate dramatically increased the soluble fraction of PSD-95 (S/P $= 79 \pm 8$, n $= 6$; Figure 1D). Interestingly, treatment with palmitate itself reduced the soluble fraction of PSD-95 (S/P $= 19 \pm 9$, n $= 5$), suggesting it may not be fully palmitoylated in normal culture conditions.

Palmitate Cycling on PSD-95 Is Regulated by Synaptic Activity

As palmitate turnover on the α subunit of certain G proteins is regulated by activation of their upstream receptors (Wedegaertner and Bourne, 1994), we asked whether palmitate turnover on PSD-95 might be regulated by glutamate receptor activity. Indeed, we found that blocking glutamate receptors with kynurenic acid, an ionotropic glutamate receptor antagonist, blocked the declustering of PSD-95 by 2-bromopalmitate (Figures 2A and 2B). Similarly, we found that blocking glutamate receptors with a combination of CNQX, which blocks AMPA receptors and APV, which blocks NMDA receptors, also inhibited declustering of PSD-95 by 2-bromopalmitate (Figure 2B). To determine if calcium is required for this process, we treated neurons with 2-Br palmitate in the absence of extracellular calcium and found that removing calcium protected clustering and palmitoylation of PSD-95 (Figures 2A and 2B). These results indicate that calcium entry associated with glutamate receptor activity is critical for depalmitoylation. In contrast, treatment with glutamate receptor antagonists or removing extracellular calcium alone did not detectably change PSD-95 clustering (Figure 2B).

The activity-dependent loss of synaptic PSD-95 that occurs in the presence of 2-bromopalmitate has two possible explanations: (1) depalmitoylation of PSD-95 is accelerated by glutamate receptor activity, or (2) diffusion of palmitate-free PSD-95 from the synapse requires glutamate receptor activity. To help to distinguish between these two possibilities, we quantitated the cycling of palmitate on PSD-95. Palmitate turnover on PSD-95 in neurons was monitored by metabolically labeling hippocampal cultures with [^{125}I]palmitate, which is a sensitive radioactive precursor that is incorporated faithfully into palmitoylated proteins (Berthiaume et al., 1995).

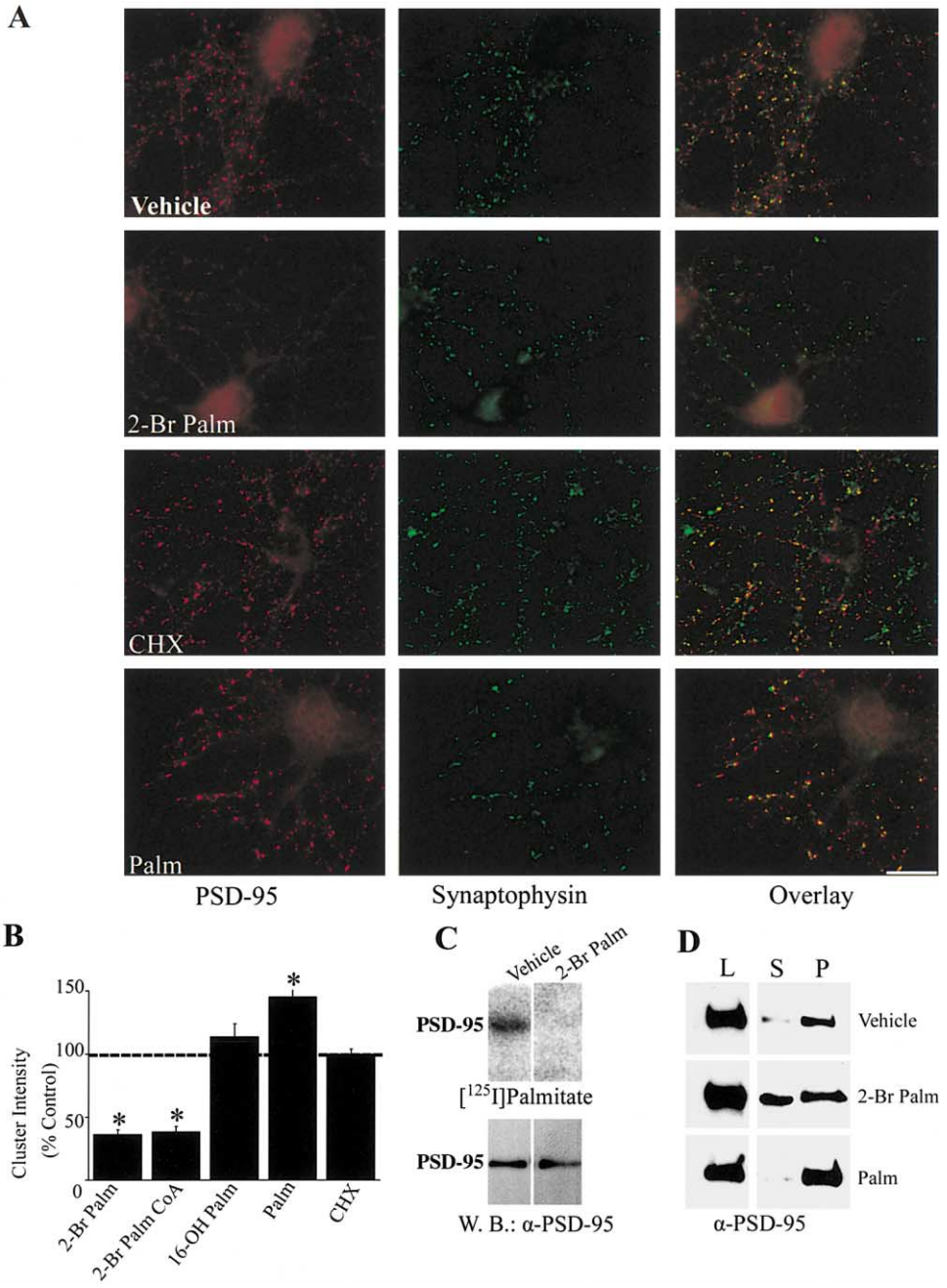

Figure 1. Blocking Palmitoylation Disperses Synaptic Clusters of PSD-95

(A) Cultured hippocampal neurons (DIV 14) were treated for 8 hr with 10 μM 2-bromopalmitate (2-Br Palm), 2-bromo palmitoyl-CoA (2-Br Palm CoA), 10 μg/ml cycloheximide (CHX), 16-hydroxypalmitate (16-OH Palm), or 10 μm palmitate (Palm), or palmitate. Neurons were then fixed and stained for PSD-95 and synaptophysin. Treatment with agents that block protein palmitoylation results in declustering of PSD-95. In contrast, treatment with palmitate slightly enhanced clustering of PSD-95. Cycloheximide did not alter PSD-95 clustering, and none of the treatments changed synaptophysin staining.

(B) Quantitative analysis of the intensity of PSD-95 clusters after these treatments. Treatment with 2-Br Palm or 2-Br Palm-CoA significantly reduced PSD-95 clustering ($p < 0.001$), whereas treatment with palmitate significantly enhanced ($p < 0.01$) PSD-95 clustering when compared to cells treated with vehicle.

(C) 2-Bromopalmitate blocks palmitoylation of PSD-95. Neurons were treated with [^{125}I]palmitate and either vehicle or 10 μM 2-bromopalmitate for three hours. PSD-95 was purified by immunoprecipitation; [^{125}I] incorporation was visualized by autoradiography; and PSD-95 protein was visualized by Western blotting (W.B.).

(D) Blocking palmitoylation increases the solubility of PSD-95. After treatment with 2-bromopalmitate or palmitate, neurons were harvested and the lysate (L) was subjected to protein fractionation (see Experimental Procedures). Treatment with 2-bromopalmitate increased the amount of PSD-95 in the soluble fraction (S). In contrast, PSD-95 in untreated or palmitate-treated cultures was primarily in the pellet (P). Scale bar, 10 μm.

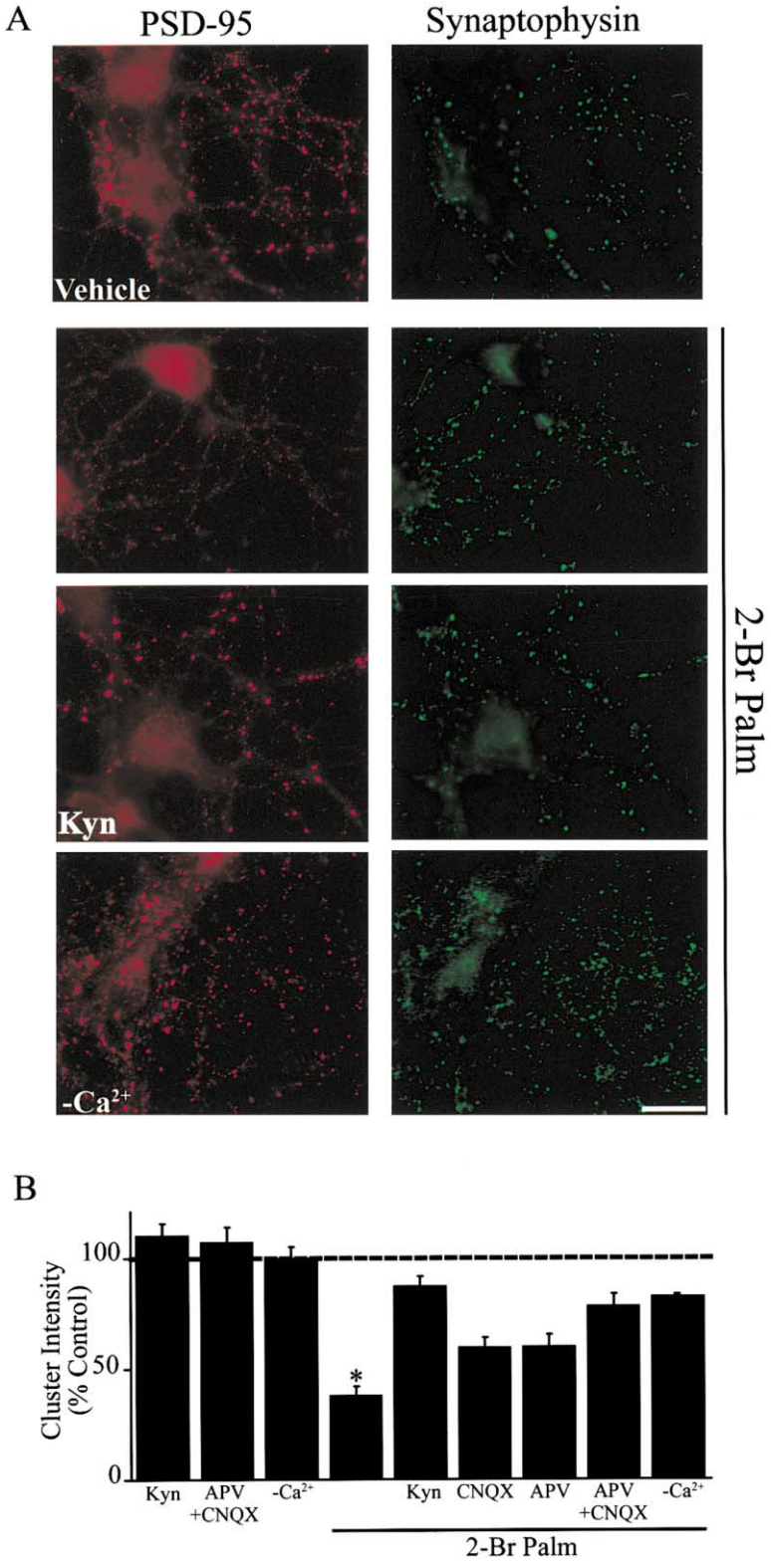

Figure 2. Synaptic Clustering of PSD-95 Is Regulated by Glutamate Receptor Activity

(A) Declustering of PSD-95 by 2-bromopalmitate (2-Br Palm) requires glutamate receptor activity. Cultures were treated with vehicle or with 2-bromopalmitate in the absence or presence of 10 mM kynurenate (Kyn) or low-extracellular calcium ($-Ca^{2+}$). Blocking glutamate receptors or eliminating extracellular calcium blocks the effect of 2-bromopalmitate on declustering of synaptic PSD-95 (red), but had no effect on the presynaptic marker, synaptophysin (green).

(B) A graph summarizes the quantitative changes in the intensity of PSD-95 clusters after these treatments, or 100 μM CNQX and/or 100 μM APV. In all treatments with glutamate receptor antagonists, PSD-95 clustering was significantly higher than in cells treated with 2-Br-palmitate alone ($p > 0.001$). Scale bar, 10 μm.

We found that the half-life of palmitate on PSD-95 is approximately 2 hr, whereas the half-life of PSD-95 protein itself is much longer, approximately 36 hr (Figures 3A and 3B, and Supplemental Figure S2, http://www.cell.com/cgi/content/full/108/6/849/DC1). Importantly, the half-life of palmitate on PSD-95 was substantially increased in the presence of kynurenic acid (Figures 3A and 3B). Conversely, glutamate treatment (10 μM) re-

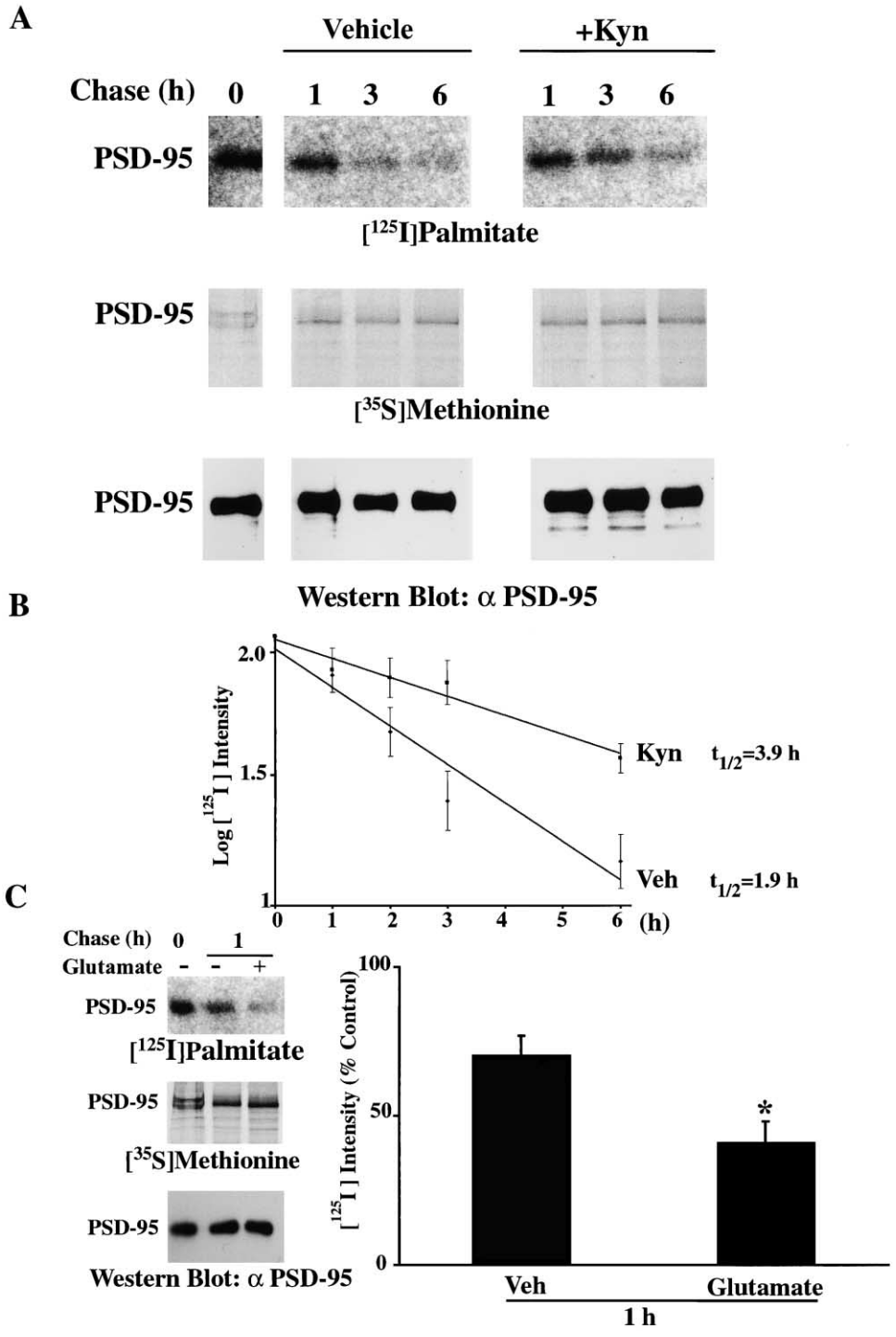

Figure 3. Regulation of Palmitate Turnover on PSD-95 by Glutamate Receptor Activity

(A) Turnover of palmitate on PSD-95 is regulated by glutamate receptor activity. Hippocampal neurons were labeled with [^{125}I]palmitate for 3 hr or with [^{35}S]methionine for 1 hr. Cultures were treated with vehicle (Veh) or with 10 mM kynurenate for 0, 1, 3, and 6 hr; cells were lysed; and PSD-95 was immunoprecipitated from the solubilized material. Immunoprecipitates were loaded onto gels that were analyzed by fluorography for [^{125}I]palmitate (upper gel), [^{35}S]methionine (middle gel), or were immunoblotted for PSD-95 (lower gel).

(B) A graph showing that the half-life ($t_{1/2}$) of palmitoylated PSD-95 is prolonged by blocking glutamate receptors with kynurenate.

(C) Treating neurons with glutamate (10 μM) significantly accelerates ($p < 0.001$) the turnover of [^{125}I]palmitate on PSD-95. Metabolic pulse-chase labeling of cultures and analysis of PSD-95 was done as described in (A).

duced the half-life of palmitate on PSD-95 (Figure 3C). These data indicate that glutamate receptor activity stimulates palmitate turnover on PSD-95.

Palmitate Cycling on PSD-95 Regulates Synaptic AMPA Receptors

NMDA receptors directly bind PSD-95 (Kornau et al., 1995) whereas AMPA receptors, which are indirectly associated (Chen et al., 2000), are recruited to synapses by PSD-95 overexpression (El-Husseini et al., 2000b). We therefore asked whether the 2-bromopalmitate-induced loss of synaptic PSD-95 affects associated glutamate receptors. Immunohistochemical staining of hippocampal cultures treated with 2-bromopalmitate showed synaptic clustering of the AMPA receptor subunit GluR1 is diminished (Figures 4A–4C). The loss of GluR1 at synapses was quantitatively similar to that of PSD-95. On the other hand, synaptic clustering of NMDA receptors as measured by NMDA receptor subunits 1 or 2B (NR1 or NR2B) was unaffected by 2-bromopalmitate (Figures 4B and 4C)

We next asked whether disrupting PSD-95 clustering with 2-bromopalmitate causes specific changes in glutamatergic function. AMPA-mediated miniature excitatory postsynaptic currents (mEPSCs) were monitored with whole-cell recordings from cultured hippocampal neurons by an investigator blinded with regard to 2-bromopalmitate treatment. These experiments revealed a significant reduction in the amplitude and frequency of synaptic events in 2-bromopalmitate-treated neurons (Figures 5A–5C). These data suggest a decrease in the number of functional AMPA receptors at synaptic sites and are consistent with the reduction in punctate GluR1 immunostaining. The selective reduction in AMPA receptor immunostaining relative to NMDA receptor staining in 2-bromopalmitate-treated cells predicts a reduction in the ratio of synaptic AMPA receptors to NMDA receptors at individual synapses. We therefore recorded dual component mEPSCs using conditions that allowed us to assay AMPA and NMDA currents simultaneously. We found that the ratio of AMPA-mediated currents to NMDA-mediated currents was significantly reduced in the 2-bromopalmitate-treated cells (Figures 5D and 5E), demonstrating a selective effect of 2-bromopalmitate on synaptic AMPA receptor function.

This loss of synaptic GluR1 in 2-bromopalmitate-treated cultures may result from changes in palmitoylation of PSD-95 or from loss of palmitate on other proteins. To help to assess this, we employed a palmitoylation-deficient PSD-95 mutant (PSD-95-prenyl) that is isoprenylated at its C terminus (Figure 6A) and recruited to synapses independent of palmitoylation (El-Husseini et al., 2000a). We transfected neurons with constructs encoding GFP fused either to wild-type PSD-95 or to PSD-95-prenyl and assessed the effects of 2-bromopalmitate on synaptic clustering. As previously published (El-Husseini et al., 2000b), AMPA receptor clusters are enhanced in neurons transfected with PSD-95 (Figures 6B and 6E), and these clusters are diminished following treatment with 2-bromopalmitate (Figures 6C–6E). PSD-95-prenyl also enhanced synaptic clustering of GluR1 in transfected cells (Figures 6C and 6E). However, these synaptic AMPA receptor clusters, as well as those of prenyl-PSD-95, were resistant to treatment with 2-bromopalmitate (Figures 6C–6E).

We used the PSD-95-prenyl mutant to assess a possible role for palmitate cycling on PSD-95 in activity-dependent turnover of AMPA receptors. These experiments employed an "antibody feeding" immunofluorescence internalization assay to quantify AMPA receptor endocytosis. As previously reported (Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000; Zhou et al., 2001), 15 min exposure to glutamate (100 μM) enhances internalization of AMPA receptor subunit GluR1 (Figure 7). The internalized AMPA receptor vesicles accumulate in both the cell body and dendritic processes. This glutamate-mediated AMPA receptor internalization was enhanced in cells transfected with wild-type PSD-95 (Figure 7); this enhancement likely results from the increased synaptic expression of AMPA receptors in PSD-95-transfected neurons (El-Husseini et al., 2000b). Importantly, glutamate-mediated AMPA receptor internalization was abolished in cells transfected with PSD-95-prenyl (Figure 7), indicating that palmitoylation is critical for this regulated AMPA receptor turnover. Neither wild-type PSD-95 nor PSD-95-prenyl affected basal AMPA receptor internalization (Figure 7).

Regulation of AMPA Receptor Clustering by Palmitate Turnover on PSD-95

To determine whether the role of palmitate cycling in clustering of PSD-95 and AMPA receptors can be reproduced in a model system that lacks the molecular complexity of neuronal synapses, we evaluated clustering in heterologous cells. In this model, cotransfection of COS cells with PSD-95, the glutamate receptor targeting protein stargazin, and the AMPA receptor subunit GluR4, yields clusters of the three proteins that appear as patches along the plasma membrane (Chen et al., 2000). Treating cultures with 2-bromopalmitate dispersed these preformed clusters and caused all three proteins to redistribute to perinuclear vesicles that suggest an intracellular accumulation (Figure 8A). This vesicular distribution of PSD-95 and stargazin/GluR4 is also observed in transfections with a palmitoylation-deficient mutant of PSD-95 (Figure 8A). This localization resembles the ER/golgi accumulations of palmitoylation-deficient PSD-95 cotransfected with Kv1.4 (Tiffany et al., 2000). To verify that the effects of 2-bromopalmitate on PSD-95/stargazin/GluR4 in heterologous cells are specific for palmitoylation of PSD-95, we used the prenylated mutant of PSD-95. When this construct was cotransfected with stargazin and GluR4, receptor clustering is also observed (Figure 8B). Although these clusters are smaller than those observed with the palmitoylated version, they appear to be on the plasma membrane and otherwise resemble those found with palmitoylated PSD-95. Importantly, these clusters formed with PSD-95-prenyl are resistant to treatment with 2-bromopalmitate, confirming the specificity of this drug for palmitoylated PSD-95 (Figure 8B).

Discussion

This study identifies palmitate turnover on PSD-95 as a critical regulatory mechanism at the synapse. This cy-

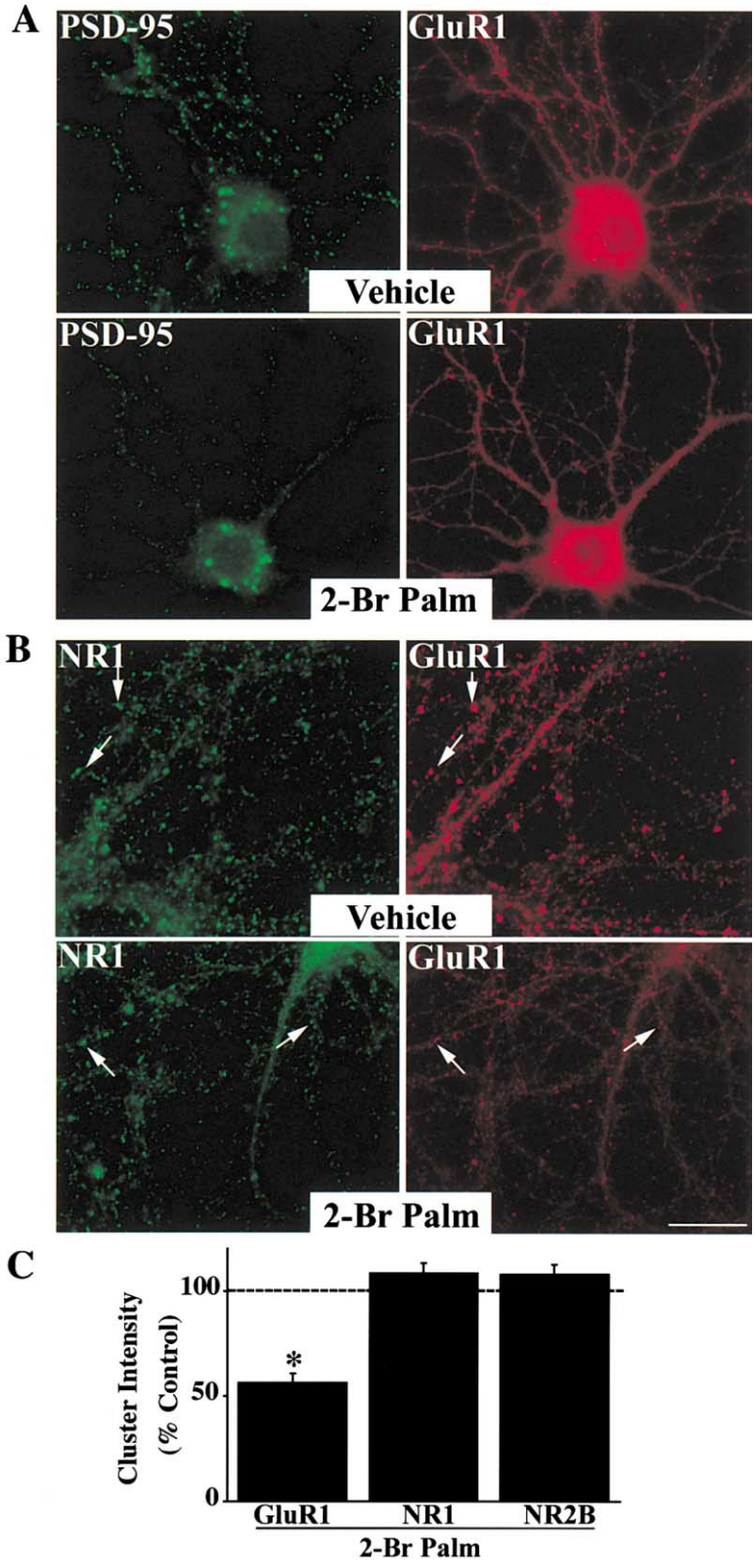

Figure 4. Blocking Protein Palmitoylation Declusters Synaptic AMPA but Not NMDA Receptors

(A) Treatment of neurons with 2-bromopalmitate (2-Br Palm) yields a reduction of synaptic GluR1 clustering that correlates with the depalmitoylation-induced loss of synaptic PSD-95. Hippocampal neurons were treated with 10 μM 2-bromopalmitate for 8 hr, then fixed and stained for PSD-95 (green) and GluR1 (red).

(B) 2-Bromopalmitate disperses synaptic clusters of GluR1 but not NR1. Hippocampal neurons treated with 2-bromopalmitate were fixed and stained for NR1 (green) and GluR1 (red).

(C) A graph summarizes the quantitative changes in the intensity of GluR1, NR1, and NR2B after treatment with 2-bromopalmitate. Treatment with 2-bromopalmitate significantly reduced GluR1 clustering ($p < 0.001$), but not NR1 ($p > 0.1$) or NR2B ($p > 0.2$). Scale bar, 10 μm.

cling of palmitate is regulated by glutamate receptor activity, and interrupting this cycle with 2-bromopalmitate disperses PSD-95 and AMPA receptor clusters. Disrupting palmitate cycling on PSD-95 blocks glutamate-mediated AMPA receptor internalization. Regulated palmitoylation of PSD-95 therefore represents a novel physiological mechanism for activity-dependent changes in synaptic strength.

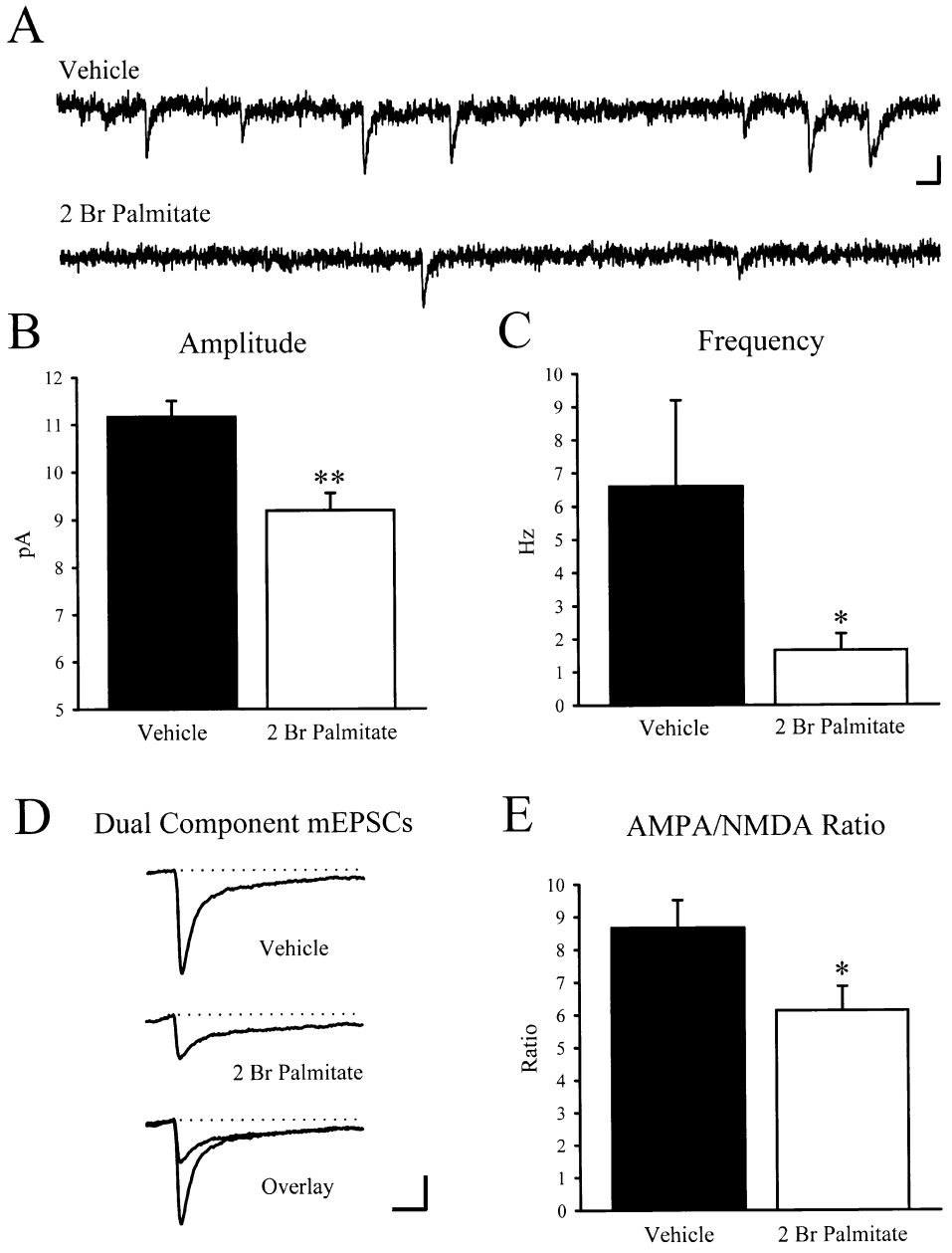

Figure 5. Alteration of AMPA-Receptor Mediated Synaptic Activity by Inhibition of Palmitoylation

(A) 2-Bromopalmitate treatment reduces the amplitude and frequency of AMPA-receptor mediated miniature postsynaptic currents (mEPSCs). Hippocampal neurons were treated with vehicle (control) or with 20 μM 2-Br Palm for 8 hr and whole-cell voltage clamp recordings were made in the presence of picrotoxin and TTX to isolate AMPA-mediated spontaneous synaptic responses. Sample traces from representative neurons are shown. Scale bars 10 pA, 20 msec.

(B) Mean mEPSC amplitude is reduced in 2-Br Palm treated cells (vehicle, $n = 14$; 2 Br Palmitate, $n = 15$; $p < 0.005$).

(C) Mean mEPSC frequency is also reduced by 2-Br Palm treatment ($p < 0.05$).

(D) Cultured hippocampal neurons were recorded in the presence of 20 μM glycine and 0 mM magnesium to evoke dual component (AMPA + NMDA) responses at –70 mV. Representative averaged mEPSCs from control and 2-Br Palm treated neurons are shown, demonstrating a reduction in the AMPA-mediated (fast) component of the response and preservation of the NMDA-mediated (slow) component. Scale bars 10 pA, 10 msec.

(E) The ratio of the AMPA-receptor-mediated component to that of the NMDA-receptor mediated component is significantly reduced in 2-Br palmitate treated cells (vehicle, $n = 12$; 2 Br Palmitate; $n = 11$; $p < 0.05$).

Regulation of the Dynamic Pool of AMPA Receptors by PSD-95

Previous studies have shown that PSD-95 and related MAGUKs interact with glutamate receptors and other ion channels at the synapse (Craven and Bredt, 1998; Garner et al., 2000; Hayashi et al., 2000; Lee and Sheng, 2000; Tejedor et al., 1997). PDZ domains from PSD-95 directly bind the C termini of NMDA receptor subunits

and other proteins terminating with a tSXV motif (Kim et al., 1995; Kornau et al., 1997). This PDZ binding sequence is also present on the AMPA receptor trafficking protein, stargazin (Chen et al., 2000). Stargazin is a tetraspanin transmembrane protein that interacts with AMPA receptors and is essential for their targeting to the plasma membrane (Chen et al., 2000; Tomita et al., 2001). By binding to the PDZ domain of PSD-95, stargazin indirectly links AMPA receptors to PSD-95. Through these PDZ interactions, PSD-95 can mediate clustering of either NMDA receptors (Lee and Sheng, 2000) or stargazin/AMPA receptors (Chen et al., 2000) in heterologous cells.

An essential role for PSD-95 in glutamate receptor clustering at the synapse is less certain. Dislodging PSD-95 from synapses with peptides that block its binding to the cytoskeletal protein CRIPT does not disrupt synaptic NMDA receptor clusters (Passafaro et al., 1999). The converse strategy—overexpressing PSD-95 in neurons—causes a selective enhancement in synaptic AMPA receptors whereas NMDA receptors are unaltered (El-Husseini et al., 2000b). Furthermore, in mutant mice lacking PSD-95, there is no change in the distribution or activity of NMDA receptors though there are clear changes in synaptic plasticity (Migaud et al., 1998). Our studies show that disrupting palmitoylation of PSD-95 with 2-bromopalmitate decreases the number and frequency of miniature EPSCs consistent with the decreased immunohistochemical staining for AMPA receptor subunits. Additionally, 2-bromopalmitate selectively affects AMPA receptors, but not NMDA receptors. Taken together, these studies suggest that PSD-95 is not essential for recruiting the constitutive pool of synaptic NMDA receptors, but that PSD-95 regulates a dynamic pool of AMPA receptors.

Palmitate Turnover Regulated by Synaptic Activity

Palmitoylation of residues of 3 and 5 plays an essential role in synaptic targeting of PSD-95 and in clustering of associated receptors (Craven et al., 1999; Topinka and Bredt, 1998). Palmitoylation of PSD-95 occurs first in the neuronal cell body and targets PSD-95 to a perinuclear endomembrane system (El-Husseini et al., 2000a). The pathway for trafficking PSD-95 from perinuclear endosomes to synaptic clusters is uncertain, but some insights have been gained from studies of receptor clustering with PSD-95 in heterologous cells. In COS cells cotransfected with PSD-95 and an interacting ion channel, clusters of both proteins form on the plasma membrane. Live cell-imaging shows that these surface patches are derived from dynamic vesiculotubular transport intermediates for PSD-95-GFP that fuse with the plasma membrane (El-Husseini et al., 2000a), suggesting that PSD-95 regulates vesicular trafficking of receptors. On the plasma membrane, these PSD-95/ion channel clusters are immobile (Burke et al., 1999); however, studies here indicate that these are not static structures. Acutely blocking palmitoylation disperses these clusters and causes both PSD-95 and the interacting stargazin/AMPA receptors to redistribute to perinuclear endosomal structures, suggesting that depalmitoylation enhances receptor endocytosis. These data are consistent with previous studies showing that the palmitoylation-deficient mutant PSD-95 (C3, 5S) enhances internalization of the associated ion channel Kv 1.4 (Jugloff et al., 2000).

In neurons, blocking palmitoylation also disperses preformed synaptic clusters of PSD-95. This suggests that maintenance of PSD-95 synaptic clusters requires ongoing palmitoylation. Importantly, palmitate turnover on PSD-95 is regulated by glutamate receptor activity, as glutamate receptor antagonists prolong the half-life of palmitate on PSD-95 and prevent declustering of PSD-95 by 2-bromopalmitate. This stimulation of palmitate turnover by activation of an upstream glutamate receptor is reminiscent of receptor-activated palmitate cycling on signaling proteins in nonneuronal cells. An elegant series of studies has shown that activation of β adrenergic receptors stimulates palmitate turnover on $G\alpha s$ (Wedegaertner and Bourne, 1994). This depalmitoylation downregulates G protein signaling by both (A) removing $G\alpha$ from the plasma membrane and (B) enhancing deactivation of $G\alpha$ by RGS proteins (Resh, 1996; Tu et al., 1997). Agonist-stimulated depalmitoylation of endothelial nitric oxide synthase also disrupts its membrane localization and activity (Robinson et al., 1995). The cytosolic tails of some receptors are also palmitoylated, and this can regulate receptor-mediated endocytosis (Alvarez et al., 1990). By analogy, our studies show that activity-dependent depalmitoylation causes PSD-95 to diffuse from the PSD and downregulates synaptic AMPA receptors (Figure 8C). Taken together, these data suggest that agonist-stimulated palmitate turnover may represent a common mechanism for dampening receptor activated signaling.

Palmitate Turnover Regulating Synaptic Function

The enzymes that regulate palmitoylation of PSD-95 at the synapse remain unidentified. Our previous studies showed that the N terminus of PSD-95 contains a five amino acid consensus sequence that determines palmitoylation (El-Husseini et al., 2000a). However, the protein acyl-transferase that recognizes this sequence and mediates palmitoylation and the protein thioesterase that removes the palmitate remain uncertain. For this reason, it is unclear how glutamate receptor activity stimulates palmitate turnover. One possibility is that the protein thioesterase is a second messenger-regulated enzyme whose activity is stimulated by calcium influx or protein phosphorylation at the postsynaptic density. Alternatively, synaptic activity may make the N terminus of PSD-95 more accessible to the thioesterase in a mechanism akin to that proposed for receptor-stimulated palmitate turnover on G protein subunits. Isolation of the proteins that mediate addition and removal of palmitate will help address these issues.

PSD-93, a close homolog of PSD-95 is palmitoylated, expressed in hippocampal neurons, and can interact with stargazin. Furthermore, synaptic clusters of PSD-93 are dispersed by 2-Br palmitate. This isoform redundancy may explain why basal synaptic transmission remains intact in PSD-95 mutant mice (Migaud et al., 1998). In addition to PSD-95 and PSD-93 (El-Husseini et al., 2000c), a variety of proteins associated with gluta-

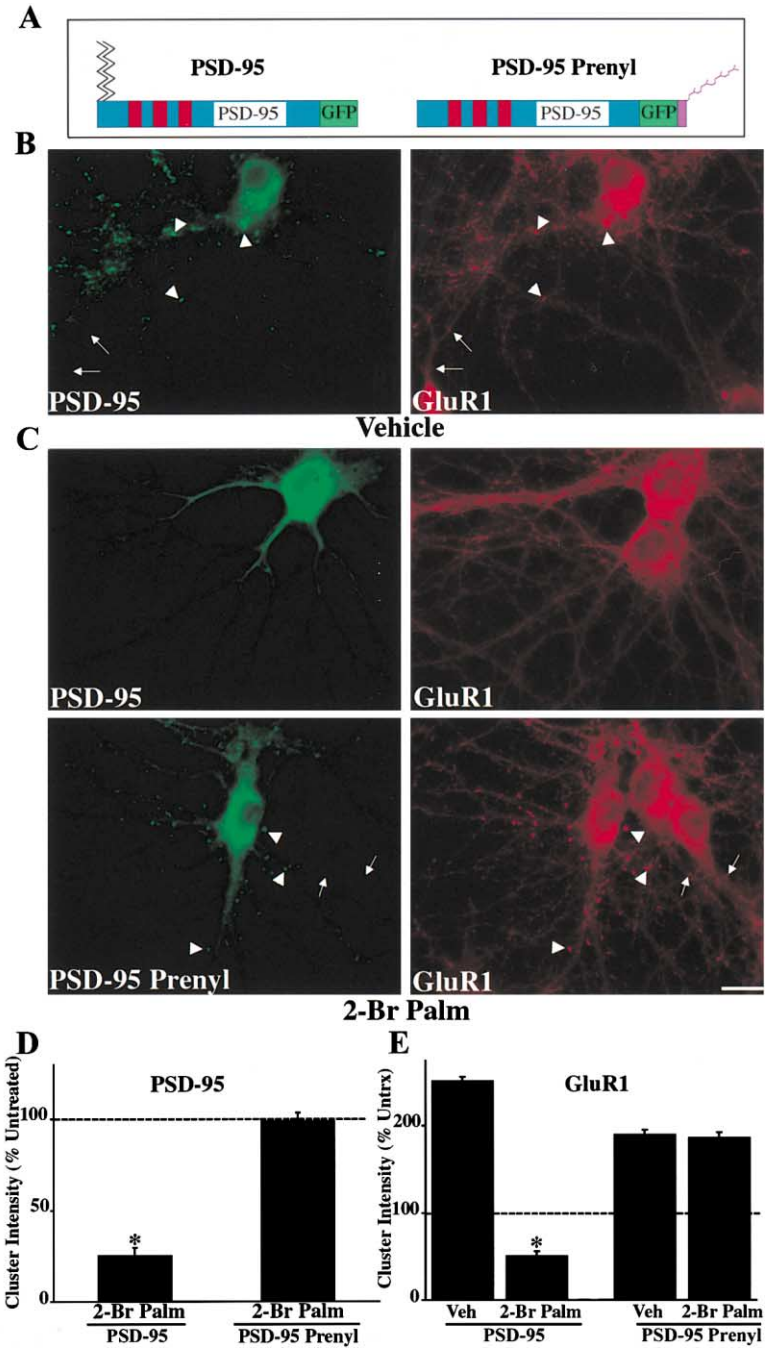

Figure 6. Palmitoylation-Dependent Clustering of AMPA Receptors Specifically Involves PSD-95

(A) Schematic diagrams of palmitoylated PSD-95 GFP and palmitoylation-deficient PSD-95 GFP containing a C-terminal prenylation motif (PSD-95-Prenyl). (B and C) Hippocampal neurons were transfected with either PSD-95 GFP (PSD-95) or with PSD-95-GFP-Prenyl (PSD-95 Prenyl) and were treated with vehicle or with 10 μM 2-bromopalmitate (2-Br Palm) for 8 hr. Neurons were then fixed and stained for GluR1 (red) and the PSD-95 was visualized on the green channel.

(B) GluR1 clusters in neurons transfected with PSD-95 GFP (arrowheads) were enhanced relative to untransfected neurons (arrows).

(C) Treatment with 2-bromopalmitate diminished clustering of PSD-95 GFP and GluR1. However, the prenylated PSD-95 GFP and associated GluR1 clusters were resistant to 2-bromopalmitate treatment. Note in (C) that the neuron transfected with PSD-95-GFP-prenyl shows strong GluR1 clusters (arrowheads) that are resistant to 2-bromopalmitate, whereas the untransfected neighbors show minimal GluR1 clusters (arrows). Scale bar, 10 μm.

(D) Quantitative analysis shows that 2-bromopalmitate significantly reduces synaptic clusters of transfected PSD-95-GFP ($p < 0.001$), but has no significant effect on synaptic clustering of PSD-95-GFP-prenyl.

(E) Quantitative analysis shows that synaptic clusters of GluR1 in cells transfected with PSD-95-GFP are reduced by 2-bromopalmitate ($p < 0.001$). By contrast, GluR1 clusters in cells transfected with PSD-95-GFP-prenyl are resistant to 2-bromopalmitate.

matergic transmission are regulated by palmitoylation, and inhibiting their palmitoylation could influence synaptic function. These include the metabotropic glutamate receptor mGluR4 (Alaluf et al., 1995), the kainate receptor subunit GluR6 (Pickering et al., 1995), and the glutamate receptor interacting protein GRIP (Yamazaki et al., 2001). Changes in palmitoylation of these proteins are unlikely to explain the effects observed here, as we found that expression of a palmitate-resistant mutant of PSD-95 (PSD-95-prenyl) blocked the effects of 2-bromopalmitate on AMPA receptor clustering and prevented glutamate-mediated AMPA receptor internalization. Nevertheless, palmitoylation of numerous proteins at the PSD suggests that this protein modification may play multiple important roles at the synapse. Our studies of activity-regulated cycling of palmitate on PSD-95 should serve as a prototype for understanding how this mechanism can regulate synaptic structure and function.

Experimental Procedures

cDNA Cloning and Mutagenesis

Constructs encoding PSD-95 and PSD-95 (C3, 5S) fused to GFP in GW1 vectors were described previously (Craven et al., 1999). The PSD-95-GFP-prenyl chimera was generated by appending oligos encoding the prenylation motif of paralemmin (amino acids:

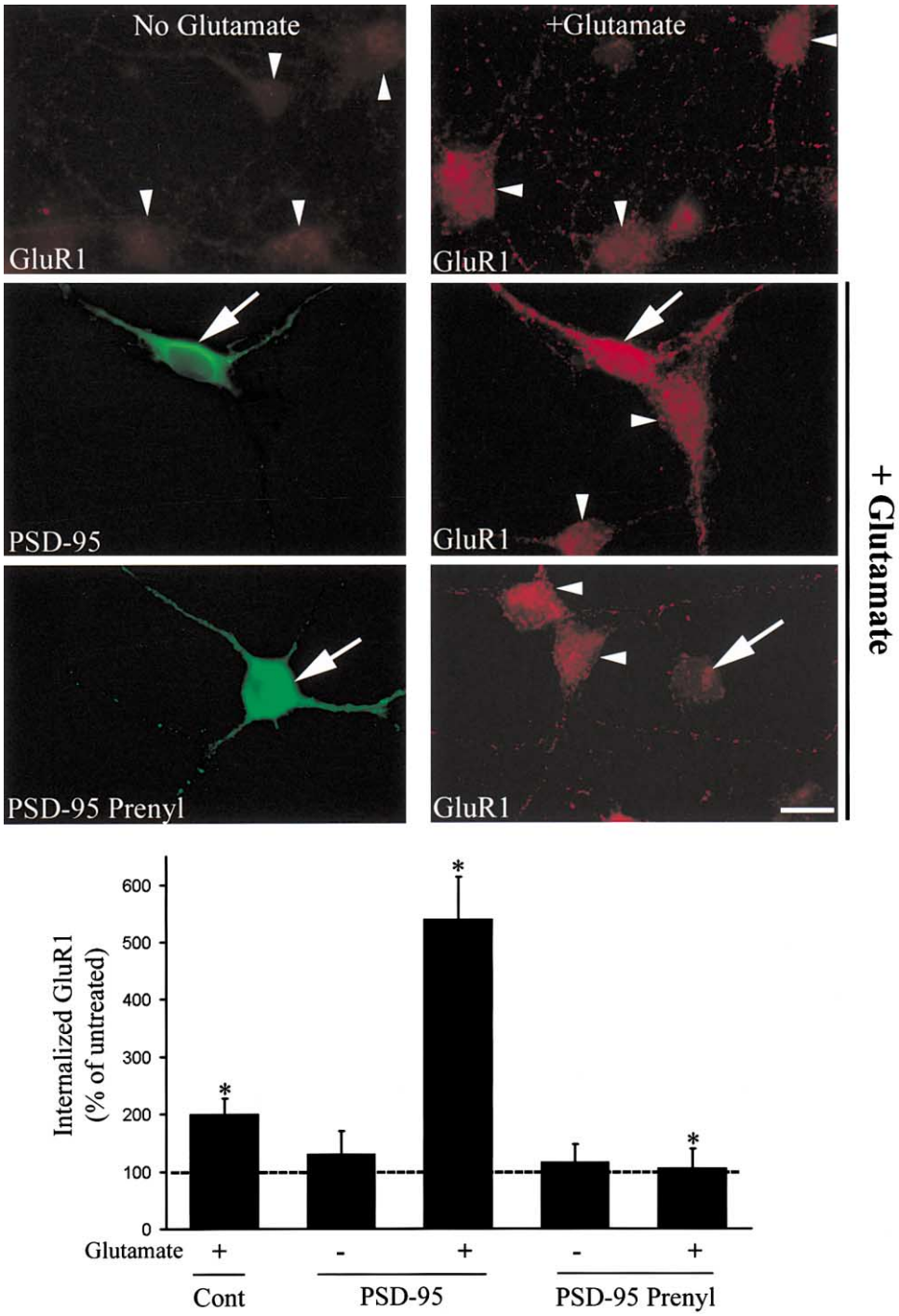

Figure 7. Palmitoylation-Regulated Internalization of AMPA Receptors Mediated by PSD-95

Endocytosis of AMPA receptors (GluR1) was analyzed by an immunofluorescence internalization assay. Hippocampal neurons were transfected with either PSD-95 GFP (PSD-95) or with PSD-95-GFP-prenyl (PSD-95 Prenyl), and following glutamate treatment (15 min, 100 μM), neurons were fixed and stained for internalized AMPA receptors (GluR1; red). Glutamate enhances internalization of GluR1 in untransfected cells (top panels). PSD-95-GFP overexpression (arrows) enhances the effect of glutamate on internalization of GluR1 as compared to untransfected cells (arrowheads; middle panels). In contrast, the prenylated PSD-95-GFP (arrows) prevents glutamate-induced GluR1 internalization as compared to neighboring untransfected cells (arrowheads; lower panels). Quantitative analysis shows that glutamate increases GluR1 internalization relative to untreated cells ($p < 0.01$), that overexpression of PSD-95-GFP significantly enhances this internalization ($p < 0.01$), and that PSD-95GFP-prenyl abolishes the glutamate-stimulated internalization ($p < 0.01$). Scale bar, 10 μm.

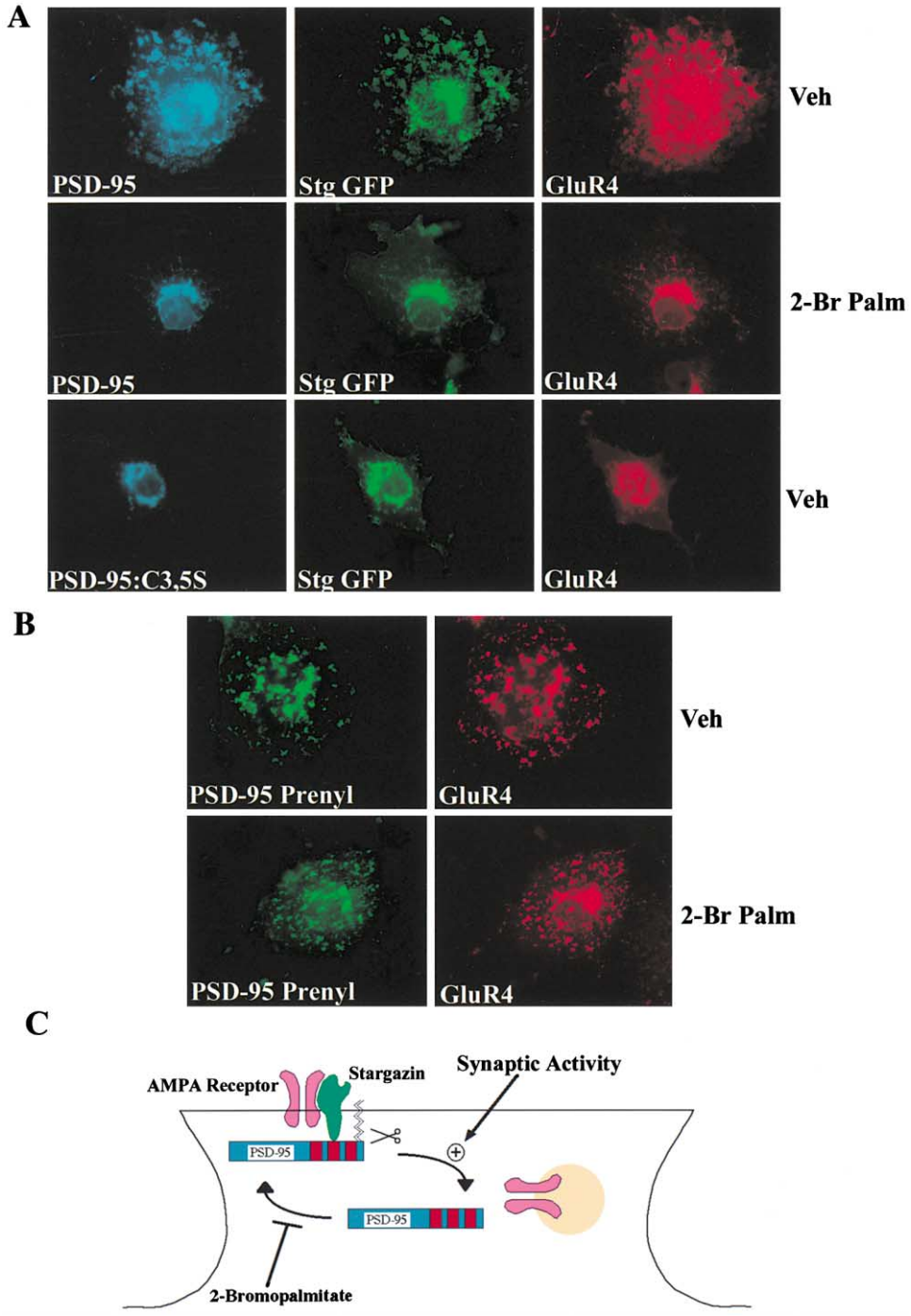

Figure 8. Assembly of the Stargazin-Glutamate Receptor Complex by PSD-95 Is Modulated by Palmitoylation

(A) Depalmitoylation of PSD-95 inhibits clustering of stargazin and GluR4. COS cells were cotransfected with GluR4, stargazin-GFP (Stg GFP), and wild-type PSD-95. Twelve hours posttransfection, cells were treated with vehicle or 10 μM 2-bromopalmitate (2-Br Palm) for 8 hr then were stained with antibodies to PSD-95 (blue) and GluR4 (red); stargazin was visualized on the green channel. Treatment with 2-bromopalmitate blocks clustering of PSD-95, stargazin, and GluR4, and causes all three proteins to accumulate in a perinuclear pattern that resembles the distribution of the proteins in cells cotransfected with the palmitoylation-deficient mutant of PSD-95 (PSD-95:C3,5S).

(B) Stargazin and GluR4 are also clustered by a prenylated PSD-95 mutant (GFP-PSD-95-prenyl), though the clusters are somewhat smaller and more punctate than with wild-type PSD-95. Importantly, the stargazin/AMPA receptor clusters with GFP-PSD-95-prenyl are resistant to 2-bromopalmitate.

(C) Schematic diagram of activity-dependent palmitate cycling on PSD-95 at the synapse. 2-bromopalmitate causes accumulation of depalmitoylated PSD-95 and decreases synaptic AMPA receptors and synaptic activity enhances depalmitoylation of PSD-95.

DMKKHRSKSCSIM) to the C terminus of the PSD-95 (C3, 5S) GFP construct.

Primary Neuronal Culture, Transfection, and Immunofluorescence

Neuronal cultures were prepared from hippocampi of E18/E19 rats. Hippocampi were dissociated by enzyme digestion with papain followed by brief mechanical trituration. Cells were plated on poly-D-lysine (Sigma) treated glass coverslips (12 mm in diameter), and maintained in neurobasal media (Gibco) supplemented with B27, penicillin, streptomycin, and L-glutamine as described in Brewer et al. (1993). For transfection of PSD-95 GFP and mutant forms, hippocampal cultures were transfected by lipid-mediated gene transfer kit (DOTAP) as described (Craven et al., 1999).

Coverslips were removed from culture wells and fixed in methanol for 10 min. The cells were washed with Tris-buffered saline containing 0.1% Triton X-100 (TBST) and blocked in TBST with 3% normal goat serum for 1 hr. Mouse monoclonal antibodies to PSD-95 (#46; Affinity Bioreagents), NR1 (PharMingen), and synaptophysin (Sigma), and rabbit polyclonal antibodies to GluR1 (Upstate), NR2B (Chemicon), synaptophysin (Sigma), and PSD-93 were used. Primary antibodies were added to blocking solution for 1 hr at RT followed by donkey anti-mouse conjugated to Cy3 fluorophores (diluted 1:200 in blocking solution) for 1 hr. Coverslips were then mounted on slides (Frost Plus; Fisher) with Fluoromount-G (Southern Biotechnology Associates), and images were taken under fluorescence microscopy with a 60× objective affixed to a Zeiss inverted microscope.

Where indicated, protein synthesis blockers and glutamate receptor agonists and antagonists were used at the following concentrations: cycloheximide (10 μg/ml), APV (100 μM), AMPA (100 μM), CNQX (100 μM), and kynurenate (10 mM). For calcium depletion, 4.75 mM EGTA was added to the neurobasal media, and $MgCl_2$ was adjusted to a final concentration of 5 mM.

Quantitative Measurement of Protein Clustering and Receptor Internalization

Images of neurons were acquired with a CCD camera with a 60× oil-immersion objective (NA = 1.4) affixed to a Zeiss inverted microscope and quantitated using Metamorph imaging software (Universal Imaging). Protein clustering was quantified on 10 fields chosen randomly from at least 3 independent neuronal cultures as previously described (El-Husseini et al., 2000b). For each field, protein accumulation in synaptic puncta was determined by calculating the average pixel intensity of puncta for PSD-95, PSD-93, NR2B, NR1, or GluR1, and comparing them to the average pixel intensity of synaptophysin puncta present in the same field. Synaptic puncta were defined as sites containing synaptophysin and PSD-95, NR1, or GluR1 at intensities at least twice the dendritic background. Data were analyzed by t test using two-tailed distribution and two-sample equal variance.

Internalization assays were done as described (Lin et al., 2000). Briefly, live hippocampal neurons were labeled with an antibody (5 μg/ml) to an extracellular epitope of GluR1 (Oncogene Research). After washing with PBS, neurons were incubated with 100 μM glutamate for 15 min. Cells were then washed and fixed with 4% paraformaldehyde + 4% sucrose in PBS for 20 min. Cells were then incubated with unlabeled anti-rabbit secondary antibodies for 1 hr to block staining of surface receptors. Cells were then permeabilized with 0.25% Triton for 5 min, followed by incubation with anti-rabbit Cy3 to visualize internalized AMPA receptors. To avoid observer bias, slides were then coded and image analysis done without knowledge of the cDNA transfected. For image analysis, a cell was centered in the visual field and its body and dendrites within one visual field were analyzed. Automated analysis was performed using custom software routines in Northern Eclipse (EMPIX Imaging). For each cell analyzed, AMPA receptor internalization was quantitated as the fluorescence density of internalized AMPA receptor puncta. This value was normalized to the average of nontransfected, nontreated cells. Statistical analysis was performed using the nonparametric Mann-Whitney test.

Cell Labeling and Immunoprecipitation

Synthesis of [^{125}I]palmitic acid was done as described (Berthiaume et al., 1995). For labeling with [^{125}I]palmitate, cortical neurons (4×10^6 cells/well) were labeled for 3 hr in neurobasal media containing 25 μCi/ml [^{125}I]palmitic acid. For metabolic labeling with [^{35}S]methionine, cells were incubated for 15 min with methionine-free neurobasal media and then for 1 hr in this media containing 300 μCi/ml EXPRE^{35}S^{35}S methionine (1175 Ci/mmol; NEN Life Science Products, Inc.). For cycloheximide treatment, 10 μg/ml was added to neurobasal media. For pulse-chase experiments using [^{125}I]palmitate and [^{35}S]methionine, cells were washed once with neurobasal media and then were incubated for variable times in conditioned neurobasal media containing 100 μM palmitate or 2 mM methionine, respectively. Labeled cells were washed with ice-cold PBS and resuspended in 0.1 ml lysis buffer containing TEE (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM EGTA), 150 mM NaCl, and 1% SDS. After extracting for 5 min at 4°C, Triton X-100 was added to 1% to neutralize the SDS in a final volume of 0.5 ml. Insoluble material was removed by centrifugation at 10,000 × g for 10 min. For immunoprecipitation, the samples were then incubated with PSD-95 antibodies (anti-rabbit, 1:100 dilution) for 1 hr at 4°C. After addition of 20 μl of protein A Sepharose beads (Pharmacia), samples were incubated for 1 hr at 4°C. Immunoprecipitates were washed three times with buffer containing TEE, 150 mM NaCl, and 1% Triton X-100, boiled in SDS-PAGE sample buffer with 1 mM DTT for 2 min, and analyzed by SDS-PAGE. For fluorography, protein samples were separated by SDS-PAGE and dried under vacuum. For ^{35}S-labeled samples, gels were exposed to (Kodak Biomax MS) at −80°C for 3 to 5 days. For ^{125}I-labeled samples, gels were exposed to Kodak X-Omat MR with intensifying screens at −80°C for 15 to 20 days.

Subcellular Cell Fractionation

Cultured cortical neurons (DIV 14–17; 12×10^6 cells) were treated for 8 hr in neurobasal media with or without 100 μM palmitate or 2-Br palmitate. Cells were washed 1× with PBS, harvested, and then suspended in 200 μl sonication buffer (50 mM Tris [pH 7.4], 0.1 mM EGTA) supplemented with a protease inhibitor cocktail (2.5 μg/ml leupeptin, 2.5 μg/ml aprotinin, and 1 μM PMSF). Cells were sonicated briefly and nuclei were pelleted at 14,000 × g at 4°C for 10 min. Lysates were centrifuged at 49,000 × g for 1 hr at 4°C. The supernatants were collected and pellets were resuspended in 150 μl resuspension buffer (RB; 50 mM Tris [pH 7.4], 0.1 mM EGTA, 1 M KCl, 10% glycerol, 1.5 μl/10 ml BME and protease inhibitors). Fractions (10 μl each) were analyzed by SDS-PAGE and immunoblotting for PSD-95.

Electrophysiology

Whole-cell recordings were made from 21-to 22-day-old cultured neurons, with 3–4 MΩ patch pipettes. The pipette solution contained (in mM): 115 Cs-$MeSO_3$, 20 CsCl, 2.5 Mg_2Cl, 10 HEPES, 0.6 EGTA, 4 Na_2ATP, 10 Na-phosphocreatinine, and 0.4 Na_3GTP. Cultures were continuously superfused with buffer containing (in mM) 119 NaCl, 2.5 KCl, 10 glucose, 26 $NaHCO_3$, 1 NaH_2PO_4, 2 $CaCl_2$, 1.3 $MgSO_4$, 0.1 picrotoxin, and 0.001 TTX (saturated with 95%O_2/5%CO_2) at 1 ml/min. Cells were patched under visual guidance using a water immersion microscope. Current records were low-pass filtered at 2 kHz, stored on tape, and digitized off-line at 5 kHz.

Cells were held at −70 mV, and recording stability was monitored in real time using −4 mV steps every 10 s. Series resistances ranged between 10 and 20 MΩ, and did not differ between treatment groups. Recordings were made for up to 5 min from each cell, depending on mEPSC frequency (cells with higher frequencies were recorded for less time). mEPSCs were analyzed with customized software (E. S.), using an amplitude threshold of 5 pA. Prior to recording, cultures were treated for 8 hr with 20 μM 2-Br palmitate (or vehicle control) by a separate experimenter. An experimenter blinded with regard to 2-palmitate treatment performed all recordings and mEPSC analyses. For dual component mEPSC recordings, the external buffer contained 20 μM glycine, and magnesium was omitted.

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