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Regulation of AMPA Receptor–Mediated Synaptic Transmission by Clathrin-Dependent Receptor Internalization

Heng-Ye Man,*† Jerry W. Lin,‡ William H. Ju,*† Gholamreza Ahmadian,*† Lidong Liu,*† Laurence E. Becker,*† Morgan Sheng,‡ and Yu Tian Wang*†§

*Programme in Brain and Behaviour and Division of Pathology
The Hospital for Sick Children
555 University Avenue
Toronto, Ontario M5G 1X8

†Department of Laboratory Medicine and Pathobiology
University of Toronto
Toronto, Ontario M5G 1L5
Canada

‡Howard Hughes Medical Institute and Department of Neurobiology
Massachusetts General Hospital and Harvard Medical School
Boston, Massachusetts 02114

Summary

Redistribution of postsynaptic AMPA- (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid–) subtype glutamate receptors may regulate synaptic strength at glutamatergic synapses, but the mediation of the redistribution is poorly understood. We show that AMPA receptors underwent clathrin-dependent endocytosis, which was accelerated by insulin in a GluR2 subunit–dependent manner. Insulin-stimulated endocytosis rapidly decreased AMPA receptor numbers in the plasma membrane, resulting in long-term depression (LTD) of AMPA receptor–mediated synaptic transmission in hippocampal CA1 neurons. Moreover, insulin-induced LTD and low-frequency stimulation–(LFS-) induced homosynaptic CA1 LTD were found to be mutually occlusive and were both blocked by inhibiting postsynaptic clathrin-mediated endocytosis. Thus, controlling postsynaptic receptor numbers through endocytosis may be an important mechanism underlying synaptic plasticity in the mammalian CNS.

Introduction

The AMPA- (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid–) subtype excitatory amino acid receptor is the principal receptor mediating fast synaptic transmission at most CNS excitatory synapses (Hollmann and Heinemann, 1994). Plastic changes to the strength of AMPA receptor–mediated synaptic transmission are involved with normal physiological brain functions, including learning and memory, as well as with many neuropathological disorders, such as cerebral ischemic associated neurotoxicity. Although the mechanisms by which modifications of synaptic strength are achieved remain intensely debated (Bliss and Collingridge, 1993; Malinow, 1998; Malenka and Nicoll, 1999), it has recently been proposed that rapid changes in functional postsynaptic AMPA receptor numbers may be an important means of controlling synaptic efficacy (Craig, 1998; Malinow, 1998; Malenka and Nicoll, 1999). This has gained support from studies of synaptic plasticity in several experimental preparations (Kullmann, 1994; Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; Wan et al., 1997; O'Brien et al., 1998; Lissin et al., 1999). However, the mechanisms by which receptor numbers in the postsynaptic plasma membrane surface are rapidly regulated are poorly understood. Most integral plasma membrane proteins are trafficked between the plasma membrane and the intracellular compartments via vesicle-mediated membrane fusion (insertion) and endocytosis (internalization). Regulation of these processes is an important means of controlling the cell surface expression and hence, function, of many proteins, such as the opioid receptor (Chu et al., 1997), the β-adrenergic receptor (Karoor et al., 1998), and the GLUT4 glucose transporter (Pessin et al., 1999). It is not unreasonable to speculate that AMPA receptor plasma membrane expression is also subject to similar modes of regulation, and such regulation at the postsynaptic membrane may be a powerful means of controlling synaptic efficacy (Carroll, 1999b; Lüscher et al., 1999; Lüthi et al., 1999).

Insulin is expressed in discrete regions throughout the brain. Neurons synthesize and release insulin in response to membrane depolarization, and peripheral insulin penetrates the blood–brain barrier, entering the brain (Wozniak et al., 1993). Insulin receptors are also highly expressed in CNS neurons and localized to synapses (Wozniak et al., 1993; Abbott et al., 1999). As glucose utilization in neurons is largely insulin independent, CNS insulin may be involved in activities other than the regulation of glucose homeostasis, and recent evidence is consistent with a wide range of neuronal functions for insulin, including neuromodulation, growth and maturation, neuronal protection, and learning and memory (Wozniak et al., 1993; Wickelgren, 1998). However, the detailed mechanisms by which brain insulin modifies neuronal function remain to be determined. Analogous to its function in peripheral tissues, where insulin produces rapid GLUT4 translocation to the plasma membrane to increase glucose uptake in these cells (Pessin et al., 1999), we recently demonstrated that insulin rapidly recruited functional $GABA_A$ (type A γ-aminobutyric acid) receptors to postsynaptic domains in mature CNS neurons, resulting in a long-lasting enhancement of $GABA_A$ receptor–mediated synaptic transmission (Wan et al., 1997). Others have also shown that insulin can regulate the cell surface expression and hence the function of various other ion channels and neurotransmitter receptors (Karoor et al., 1998; Kanzaki et al., 1999). Hence, insulin may function as an important CNS neuromodulator by regulating the intracellular trafficking and plasma membrane expression of ion channels and neurotransmitter receptors.

We set out to determine if the cell surface expression of AMPA receptors could be rapidly regulated by insulin

§To whom correspondence should be addressed (e-mail: y.t.wang@utoronto.ca).

and, if so, whether this could lead to alterations of synaptic strength. Our data indicate that AMPA receptors are subject to constitutive clathrin-mediated endocytosis. By stimulating the rate of this clathrin-mediated endocytic removal of AMPA receptors, insulin produces long-term depression (LTD) of AMPA receptor–mediated synaptic transmission in hippocampal CA1 cells. Insulin-induced LTD and low-frequency stimulation– (LFS-) evoked homosynaptic LTD are mutually occlusive, and blocking postsynaptic endocytosis also prevents LFS-evoked LTD. Thus, our data suggest that regulation of receptor endocytosis may be a common and important mechanism of synaptic plasticity in the mammalian CNS.

Results

Insulin Decreases Cell Surface AMPA Receptors via a GluR2 Subunit–Dependent Mechanism

Native AMPA receptors are predominantly assembled from heteromeric combinations of four known subunits: GluR1–4. However, when expressed in mammalian cells, individual GluR subunits can form functional homomeric AMPA channels (Hollmann and Heinemann, 1994). To study AMPA receptor surface expression, GluR1 and GluR2 cDNAs were tagged with the hemagglutinin (HA) epitope in their amino-terminal extracellular domains. After transient transfection into HEK293 cells, we were able to distinguish between receptors expressed on the cell surface and intracellular receptors by antibody labeling of the HA epitope under nonpermeant and permeant conditions. With confocal microscopy, we found that homomeric HA-GluR1 and HA-GluR2 AMPA receptors were expressed both on the plasma membrane and in intracellular compartments (Figure 1A). Cell surface receptor expression (quantitative colorimetric) assays revealed that the proportion of cell surface receptors was ~50% of the total expressed receptors for both GluR1 and GluR2 (Figure 1D).

Similar to other integral membrane proteins, AMPA receptors are likely constitutively trafficked between the plasma membrane and intracellular compartments via plasma membrane insertion and internalization. Presumably, AMPA receptor numbers on the cell surface reflect a balance between these two processes, and factors affecting insertion or internalization should change the density of surface AMPA receptors. We investigated this by acutely treating HA-GluR1- or HA-GluR2-transfected cells with insulin, as brief insulin stimulation affects membrane insertion (Pessin et al., 1999) and endocytosis (Karoor et al., 1998) of numerous plasma membrane proteins. As a control, we transfected HEK cells with the $GABA_A$ receptor, as we have previously found that insulin produces a rapid increase in cell surface $GABA_A$ receptor numbers in the same expression system (Wan et al., 1997). As shown in Figures 1B and 1C, brief insulin exposure (0.5 μM, 10–15 min) resulted in a rapid and persistent reduction in GluR2 receptors on the plasma membrane. Cell surface GluR2 receptors were depleted to 32.1% ± 2.4% within 10 min, and this lasted for more than 1 hr after insulin washout (Figures 1C and 1D). Prolonged insulin exposure did not further increase GluR2 depletion (Figure 1E). Interestingly, insulin treatment did not alter the cell surface expression of the GluR1 subunit. Conversely, surface $GABA_A$ receptors increased in the same experiments, consistent with our previous findings (Wan et al., 1997) (Figure 1D). Thus, the reduction of cell surface GluR2 by insulin is subunit specific and the opposite of the effect of insulin on $GABA_A$ receptors.

As native AMPA receptors are largely heteromeric complexes, often containing GluR2 (Hollmann and Heinemann, 1994), it was important to determine if GluR2 could confer insulin-regulated surface expression on heteromeric receptors. Insulin stimulation of cells co-transfected with HA-GluR1 and wild-type GluR2 decreased cell surface expression of HA-GluR1 to a level comparable to that seen in cells expressing HA-GluR2 receptors alone (Figure 1D). Furthermore, reductions in cell surface GluR2 were related neither to epitope tagging nor to a cell line–specific phenomenon, as similar reductions in cell surface receptors in response to insulin were found in Chinese hamster ovary (CHO) cells expressing human insulin receptors and wild-type GluR2 or GluR1 + GluR2 cDNAs (using extracellular domain antibodies of these subunits; data not shown).

The GluR2 Carboxyl Tail Determines the Subunit Specificity of Insulin Effects

GluR subunits display sequence divergence within the carboxy-terminal (CT) cytoplasmic tail, and this region has been shown to mediate subunit-specific interactions with various cytoplasmic proteins (Dong et al., 1997; Lin and Sheng, 1998; Srivastava et al., 1998; Xia et al., 1999). To test whether the signals that confer subunit specificity of the insulin effect reside within the GluR2 CT region, we exchanged the CT domains of GluR1 and GluR2, creating chimeric HA-GluR1/$_{2CT}$ and HA-GluR2/$_{1CT}$ receptors. Replacing the GluR2 CT with that of GluR1 (GluR2/$_{1CT}$) completely blocked the insulin-induced decrease in cell surface receptors (Figure 1F). In contrast, insulin treatment decreased the cell surface expression of GluR1/$_{2CT}$, making it comparable to that of wild-type GluR2 (Figure 1F). These results demonstrate that the GluR2 CT region contains a signal that is necessary and sufficient for the insulin-induced decrease in the cell surface expression of the GluR2-containing AMPA receptors.

Insulin Selectively Facilitates the Rate of GluR2 Receptor Endocytosis

Insulin treatment reduced the number of AMPA receptors expressed on the plasma membrane surface without altering the total number of receptors expressed in these transfected cells (Figure 1C), indicating that relative rates of protein synthesis and degradation were not affected. Changes in AMPA receptor surface expression therefore result from inhibition of membrane insertion and/or facilitation of endocytosis. To discriminate between these possibilities, we employed an internalization assay previously used in measuring the endocytosis of other plasma membrane receptors (Chu et al., 1997). Surface HA-GluR1 and HA-GluR2 receptors in live cells were prelabeled with anti-HA antibody at 4°C (a temperature that blocks endocytosis; Figure 2A). Cells were returned to 37°C to allow endocytosis to resume, and

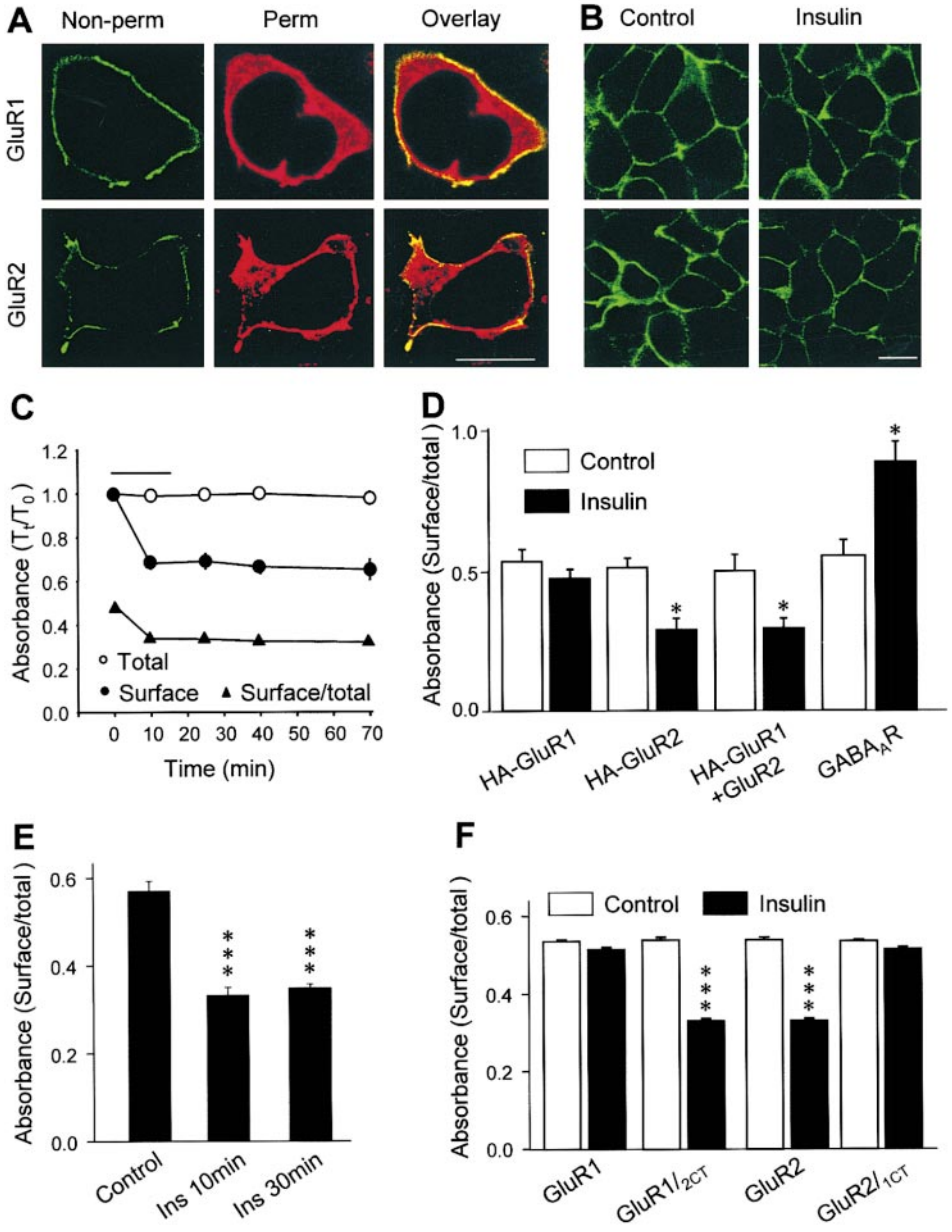

Figure 1. Insulin-Induced Depletion of Cell Surface AMPA Receptors in HEK293 Cells

(A) Confocal sections show subcellular homomeric HA-GluR1 or HA-GluR2 AMPA receptors. Receptors on the cell surface were labeled with antibody under nonpermeant conditions (green), and the total receptor population was probed under permeant conditions (red).

(B) Representative optical sections of cells stained under nonpermeant conditions show that insulin reduces cell surface GluR2, but not GluR1, AMPA receptor expression. Scale bars, 10 μm (A and B).

(C) Time course of insulin-induced reduction in cell surface GluR2 receptors using quantitive colorimetric assays (Wang et al., 1998). Insulin (closed bar) induces a rapid, long-lasting decrease in cell surface GluR2 receptors ("Surface," closed circles) without affecting the total number of GluR2 receptors ("Total," open circles), resulting in a lower proportion of surface receptors ("Surface/total," closed triangles). Data are expressed as the ratio between absorbance readings at the indicated time point ("T_t") and the initial absorbance ("T_0") (37°C, n = 6 for each time point).

(D) Insulin-induced reduction in cell surface AMPA receptors is GluR2 dependent. Cell surface expression of HA-GluR receptors in HEK cells was determined using the ratio of absorbance readings obtained with HA antibody labeling under nonpermeant (surface) versus permeant (total) conditions. In cells transfected with $GABA_A$ receptor $\alpha_1^{FLAG}\beta_2\gamma_2$ cDNAs, $GABA_A$ receptors translocated to the plasma membrane in response to insulin.

(E) Reduction in HA-GluR2 receptor cell surface expression by insulin ("Ins 10 min") plateaus within 10 min and prolonged stimulation ("Ins 30 min") fail to further reduce cell surface receptors.

(F) Exchanging carboxyl tails between HA-GluR1 and HA-GluR2 subunits demonstrates that insulin-induced cell surface AMPA receptor reduction is dependent on the presence of the GluR2 CT. Colorimetric assays were performed using anti-HA antibodies. In (D) through (F), paired t tests were used to analyze differences between control and insulin-treated groups; asterisk and triple asterisk denote $p < 0.05$ and $p < 0.001$, respectively (mean ± SE, n = 6 in each group).

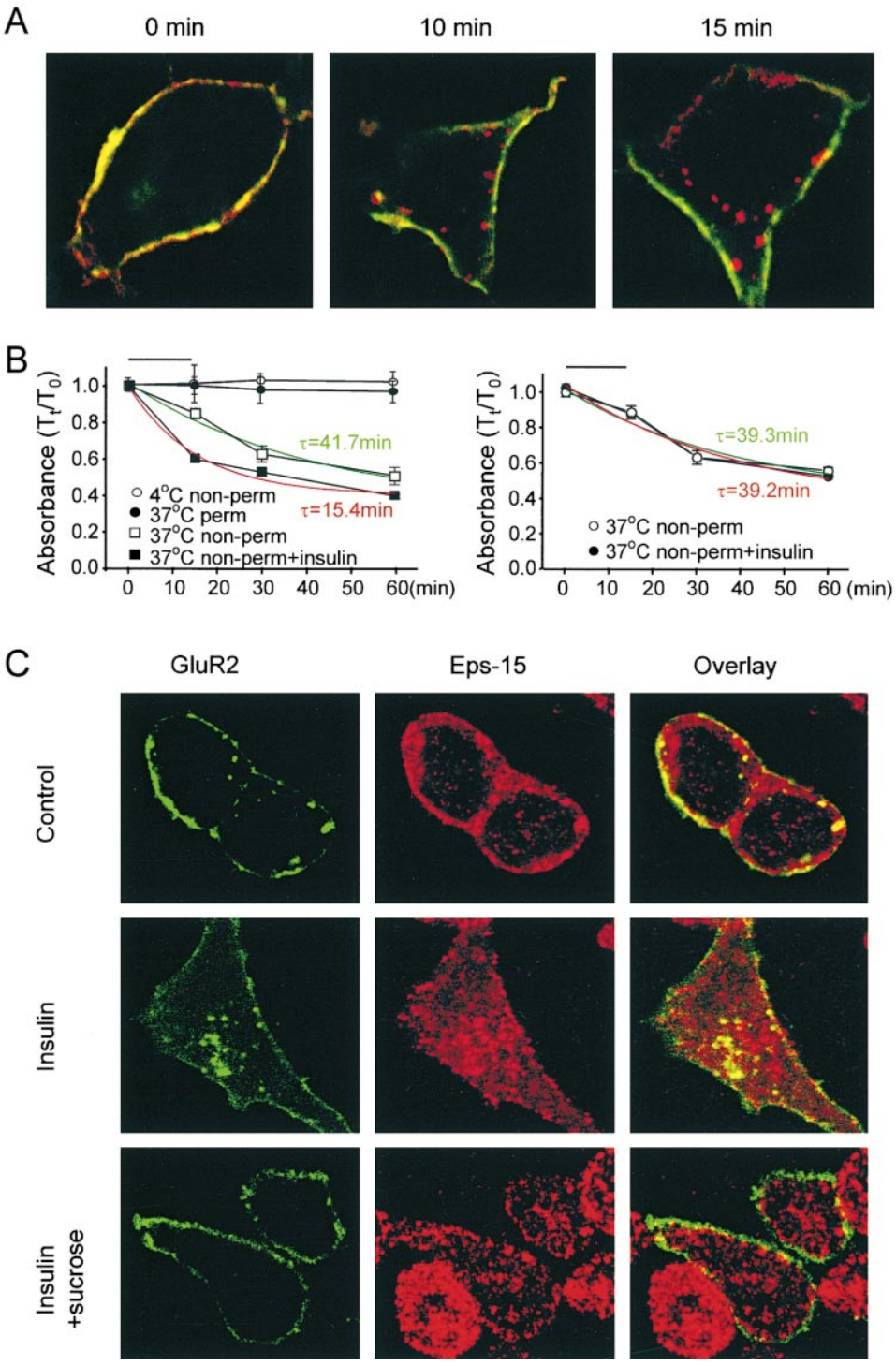

Figure 2. Insulin Stimulates Clathrin-Mediated Endocytosis of HA-GluR2 Receptors in HEK Cells

(A) Superimposed confocal sections of cell surface HA-GluR2 receptors (green) and internalized receptors (red) showing constitutive endocytosis of HA-GluR2. Live cells were prelabeled with anti-HA antibody at 4°C, returned to 37°C to allow constitutive internalization, and fixed at the indicated time points. Cell surface and internalized receptors were sequentially stained with secondary antibodies under nonpermeant (green) and permeant (red) conditions.

(B) Time course of constitutive endocytosis of GluR2 (left) and GluR1 (right) determined using colorimetric assays. Time-dependent loss of surface-bound antibody was observed at 37°C nonperm. This reflects a constitutive endocytosis of the prelabeled cell surface receptors that is due to neither a dissociation of antibody from the receptors, as there is no detectable antibody loss following incubation for various periods of time at 4°C ("4°C nonperm"), nor to antibody instability, as no detectable change in antibody absorbance readings was observed following a 1 hr incubation at 37°C under permeant conditions ("37°C perm"). Brief insulin treatment (closed bar) enhanced both the rate and magnitude of GluR2 receptor internalization but had no effect on those of GluR1 ("37°C nonperm+insulin"). Green and red lines represent the best single exponential fit to the mean data of GluR receptor endocytosis without and with insulin treatment, respectively ($n = 6$ in each group, for each time point).

internalization of the labeled surface receptors at different time points was then visualized using confocal microscopy and quantified by colorimetric assays. GluR2 receptors showed a constitutive time-dependent endocytosis from the cell surface in untreated cells (Figures 2A and 2B, left). After 10 min, most internalized receptors were localized to small punctae closely associated with the plasma membrane that may represent early endosomes. At later times, internalized receptors accumulated in larger punctae farther away from the plasma membrane, which may indicate vesicle fusion or localization in late endosomes. The time course for constitutive internalization (determined by quantitative colorimetric assays) may be described by a single exponential and a time constant of 42 min (Figure 2B, left). Fifteen minutes after the start of endocytosis, ~15% ± 2.1% of the labeled cell surface GluR2 receptors were internalized, and this increased to 48% ± 4.7% by 1 hr (Figure 2B, left; n = 6). In the presence of insulin (0.5 μM, 15 min), the labeled GluR2 receptors were found to be internalized at an accelerated rate following an exponential time course with a time constant of 15 min, nearly three times faster than observed in non insulin-stimulated cells (Figure 2B, left). Insulin stimulation also increased the degree of GluR2 internalization. At 15 min and at 1 hr, 39% ± 1.2% and 58.9% ± 1.0% of receptors were internalized, respectively (Figure 2B, left). Prelabeled GluR1 receptors were also subject to constitutive endocytosis at a rate and degree comparable to that of GluR2 (Figure 2B, right). However, in contrast to its actions on GluR2, insulin failed to alter either the rate or level of GluR1 receptor endocytosis, supporting the dependence of insulin's selective actions on the presence of GluR2 (Figures 1D and 1F). Thus, while constitutive receptor endocytosis may be a common feature of different AMPA receptor subunits, the insulin-stimulated endocytosis is GluR2 specific.

GluR2 Endocytosis Is Mediated by Clathrin-Coated Pits

Most plasma membrane proteins are internalized by clathrin-mediated endocytosis. Consistent with GluR2 endocytosis by clathrin-dependent mechanisms, we found that with both constitutive and insulin-stimulated conditions, internalized GluR2 receptors extensively colocalized with EPS15, an integral component of clathrin-coated pits (van Delft et al., 1997) (Figure 2C). Moreover, pretreatment of cells with hypertonic sucrose (0.45 M, 10–30 min), a blocker of clathrin-mediated endocytosis (Hansen et al., 1993), prevented both constitutive and insulin-enhanced internalization of GluR2 receptors (Figure 2C). These results indicate that AMPA receptors undergo clathrin-mediated constitutive endocytosis and that insulin accelerates this process.

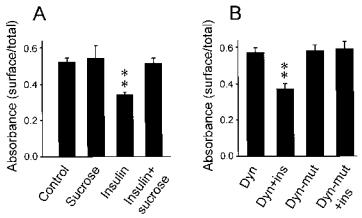

Figure 3. Inhibiting Clathrin-Dependent Receptor Endocytosis Blocks Insulin-Induced Reduction in Cell Surface AMPA Receptors

Colorimetric assays show insulin-induced depletion of cell surface GluR2 receptors is blocked by treatment of cells with hypertonic sucrose solution (A) or coexpression of mutant dynamin K44E ("Dyn-mut") (B). In control experiments, coexpression of wild-type dynamin ("Dyn") does not affect either the basal cell surface GluR2 levels or insulin-induced reduction of cell surface GluR2 (B). Neither sucrose treatment nor Dyn-mut expressions alter basal levels of cell surface AMPA receptors. Data are expressed as cell surface labeling versus total cell labeling ("surface/total"). Paired t tests; double asterisk denotes $p < 0.01$ (mean ± SE; n = 6 in each group).

Facilitated Endocytosis Is Fully Responsible for Insulin-Induced Reduction in Cell Surface GluR2 Receptors

As mentioned previously, reduction of cell surface AMPA receptors by insulin (Figure 1) could result from increased endocytosis, decreased insertion, or a combination of the two processes. As shown in Figure 3A, when AMPA receptor cell surface levels were quantified by colorimetric assays, we found that the insulin-induced reduction was completely abolished by the blockade of clathrin-mediated endocytosis with hypertonic sucrose. Moreover, internalization via the clathrin pathway is known to be dependent on the GTPase dynamin, and dominant-negative mutants of dynamin (K44A or K44E) block clathrin-mediated endocytosis without affecting vesicle exocytosis (Damke et al., 1994) or insulin signaling (Kao et al., 1998). In HA-GluR2 and dynamin-K44E cotransfected HEK293 cells, the basal level of surface receptors was maintained, but insulin-induced decreases in cell surface GluR2 were blocked (Figure 3B). Overexpression of wild-type dynamin did not affect either the basal cell surface GluR2 levels or insulin-induced reductions of cell surface GluR2. Together, these results indicate that insulin can reduce the number of GluR2-containing AMPA plasma membrane receptors over a time course of minutes, primarily by stimulating clathrin-mediated endocytosis.

Clathrin-Mediated Endocytosis of Native AMPA Receptors in Cultured Neurons

To determine if the results observed with recombinant HA-tagged AMPA receptor subunits were consistent for native neuronal AMPA receptors, we investigated AMPA receptor endocytosis in cultured hippocampal neurons

(C) Clathrin-coated pits mediate constitutive and insulin-stimulated GluR2 endocytosis. Individual (GluR2, green; EPS15, red) and superimposed ("Overlay") confocal images show colocalization of endocytosed GluR2 receptors with EPS15 in GluR2-transfected HEK cells. GluR2 receptors were prelabeled with primary antibody at 4°C, and following 10 min incubation at 37°C cells were sequentially stained for GluR2 and EPS15 under permeant conditions. Most internalized GluR2 in the presence ("Insulin") and absence ("Control") of 0.5 μM insulin are colocalized with EPS15. GluR2 endocytosis was blocked by incubation of the cells with hypertonic sucrose solution (0.45 M, 10 min) prior to insulin application ("Insulin+sucrose").

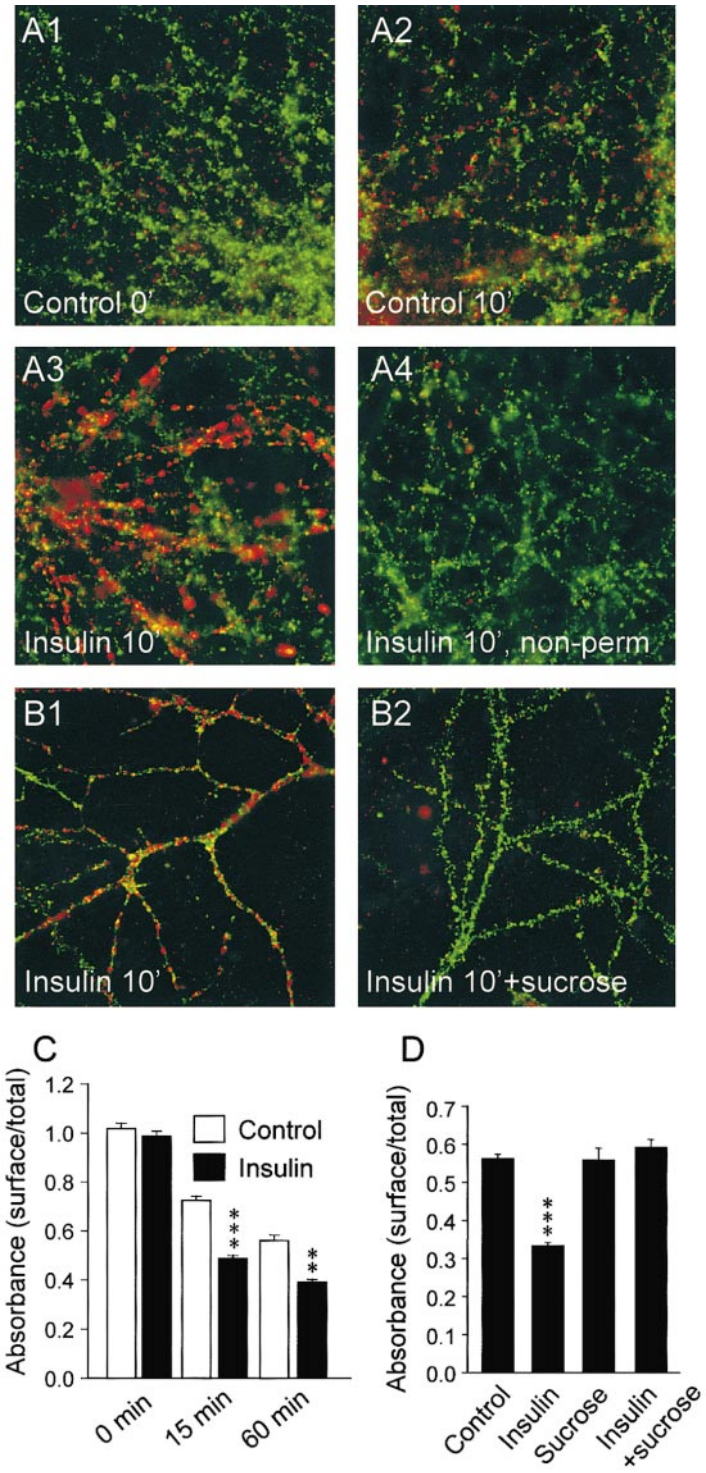

Figure 4. Clathrin-Mediated Endocytosis of AMPA Receptors in Cultured Hippocampal Neurons

(A) Confocal images of internalization of cell surface AMPA receptors at time 0 (A1) and 10 min in the absence (A2) or presence (A3) of insulin treatment. The receptors were prelabeled with an antibody raised against the amino-terminal extracellular epitope of GluR1 in live neurons and allowed to endocytose for varying lengths of time at 37°C. AMPA receptors remaining on the cell surface and those internalized into the interior of the cells were then sequentially stained in green and red under nonpermeant and permeant conditions. The red labeling of endocytosed receptors was specific and not artifactual as, following identical treatment with insulin, no red staining could be visualized without permeabilization (A4).

(B) Inhibition of clathrin-mediated endocytosis by hypertonic sucrose abolishes both constitutive and insulin-stimulated AMPA receptor internalization. Following 10 min hypertonic sucrose (0.45 M) treatment, labeling of internalized AMPA receptors was nearly abolished, even in the presence of insulin stimulation (B2), compared with nontreated control (B1).

(C) Colorimetric quantitation of constitutive and insulin-stimulated endocytosis of AMPA receptors. Following prelabeling of cell surface AMPA receptors, there was a time-dependent decline of cell surface AMPA receptors at 15 and 60 min (open bars). Insulin increased the loss of the surface receptors when compared with control values at all times points (closed bars).

(D) Insulin produces a rapid reduction of cell surface AMPA receptors by facilitating clathrin-mediated endocytosis. After fixation of neurons, AMPA receptors on the surface and in the cell interior were labeled with amino-terminal extracellular antibodies under nonpermeant and permeant conditions, respectively, and changes in cell surface AMPA receptors were quantified using colorimetric assays. Note that hypertonic sucrose blocked insulin-induced reductions of cell surface AMPA receptor expression without altering basal numbers of the cell surface AMPA receptors.

following the prelabeling of cell surface AMPA receptors in live cells and internalization at various lengths of time. In Figure 4A, there was a modest but clear AMPA receptor internalization 10 min after prelabeling under untreated control conditions (Figures 4A1 and 4A2), suggesting constitutive endocytosis of native neuronal AMPA receptors. Insulin treatment greatly enhanced AMPA receptor internalization when compared with the control (Figures 4A1 and 4A3). Internalized receptors under control and insulin-stimulated conditions were characterized by small red punctae in dendritic shafts, typically lying within the boundaries of the plasma membrane delineated by surface-localized (green) receptors. Internalized red staining was virtually abolished when

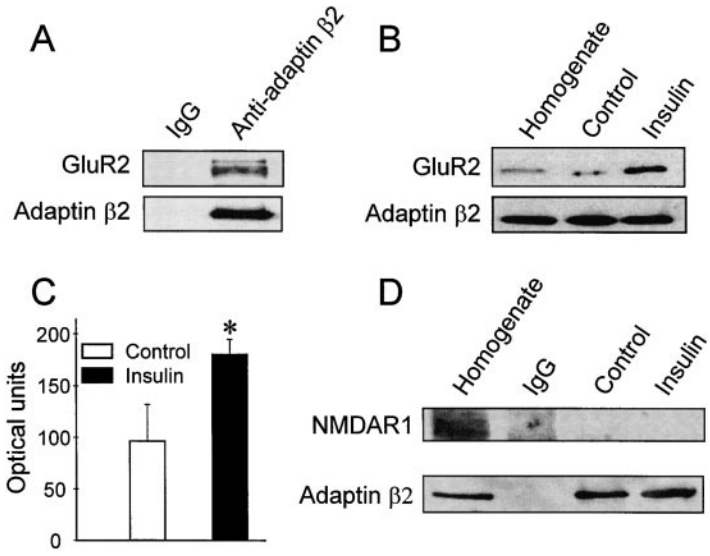

Figure 5. Insulin Increases the Association of Native AMPA Receptors and the AP2 Adaptor Protein Complex In Situ

(A) Antibody to adaptin β2 coimmunoprecipitates GluR2 (and GluR1; data not shown) with adaptin β2 from hippocampal slice homogenate, but control immunoprecipitation with nonspecific mouse IgG precipitates neither. Immunoprecipitates were first immunoblotted for GluR2 ("GluR2") and then stripped and reprobed for adaptin β2 ("Adaptin β2").

(B) Insulin treatment increases the amount of native AMPA receptor associated with the AP2 complex. Coimmunoprecipitates using anti–adaptin β2 antibody from control and insulin-treated (0.5 μM, 10 min) hippocampal slices were sequentially immunoblotted for GluR2 and adaptin β2. Densitometric quantitation from five separate experiments is summarized in the histogram in (C); asterisk, $p < 0.05$.

(D) NMDA receptor does not associate with the AP2 adaptor complex. Protein samples from control and insulin-treated slices were coimmunoprecipitated with anti–adaptin β2 antibody or control IgG and then sequentially probed with anti-NMDAR1 and anti–adaptin β2 antibody.

the permeabilization between the FITC- and Cy3-conjugated secondary antibody detection was eliminated (Figure 4A4), indicating that red punctate labeling truly represents the internalized AMPA receptors. Using colorimetric assays, we further quantified both constitutive and insulin-sensitive receptor endocytosis, and, as shown in Figure 4C, following prelabeling there was a decrease in the number of prelabeled cell surface AMPA receptors to 76% of control values at 15 min that declined to 58% by 60 min, and insulin stimulation reduced these values to 43% and 37% respectively. Thus, native AMPA receptors in cultured hippocampal neurons also undergo both constitutive and regulated endocytosis at rates similar to those of recombinant GluR2-containing AMPA receptors in HEK cells. Constitutive and insulin-stimulated AMPA receptor internalization in neurons were both found to be dependent on clathrin-mediated endocytosis, as they were completely inhibited by hypertonic sucrose treatment (Figure 4B).

We next sought to determine if insulin stimulation was capable of reducing the overall number of AMPA receptors expressed on the cell surface by facilitating AMPA receptor endocytosis. Using quantitative colorimetric assays, we found surface levels of AMPA receptors in cultured hippocampal neurons to be ~56% of the total number of receptors (Figure 4D). As expected, brief insulin stimulation produced a significant reduction in neuronal cell surface expression of AMPA receptors that could be blocked by pretreatment with hypertonic sucrose (Figure 4D). Interestingly, we found that, similar to the results in HEK cells (Figures 2C and 3A), hypertonic sucrose treatment of hippocampal neurons, although blocking constitutive and insulin-stimulated AMPA receptor endocytosis (Figure 4B2), did not alter the basal levels of the receptors expressed on the cell surface (Figure 4D). Taken together, these data provide strong evidence that the native AMPA receptor, like its counterpart in transfected HEK cells, undergoes clathrin-dependent constitutive and regulated endocytosis and that stimulating regulated endocytosis can rapidly reduce the overall number of receptors expressed on the cell surface.

Insulin Stimulates Association of Native AMPA Receptors with the AP2 Complex

To examine AMPA receptor endocytosis in a more physiological context, we investigated whether clathrin-mediated constitutive and insulin-induced internalization occurs in mature neurons in situ using hippocampal slices prepared from adult rats. Plasma membrane receptor endocytosis is initiated by the recruitment and concentration of receptors in clathrin-coated pits, presumably accomplished by binding of the receptors to a clathrin adaptor protein complex such as AP2 (Schmid, 1997). We examined the association of native AMPA receptors with the AP2 complex in control or insulin-treated (0.5 μM, 10 min) slices. In control conditions (Figure 5A), a small number of AMPA receptor subunits were coimmunoprecipitated with adaptin β2, a key component of the AP2 complex, but not with control immunoglobulin G (IgG) antibody, perhaps reflecting constitutive AMPA receptor internalization. Insulin treatment of the slices increased the amount of AMPA receptor coimmunoprecipitated with adaptin β2 (Figures 5B and 5C). In contrast, under the same conditions, anti–adaptin β2 did not immunoprecipitate the N-methyl-D-aspartate- (NMDA-) subtype glutamate receptors from either control or insulin-treated slice homogenates (Figure 5D). This suggests that in mature neurons, AMPA receptors are constitutively internalized via clathrin-mediated mechanisms and that insulin enhances this process by recruitment of receptor subunits to the AP2 complex.

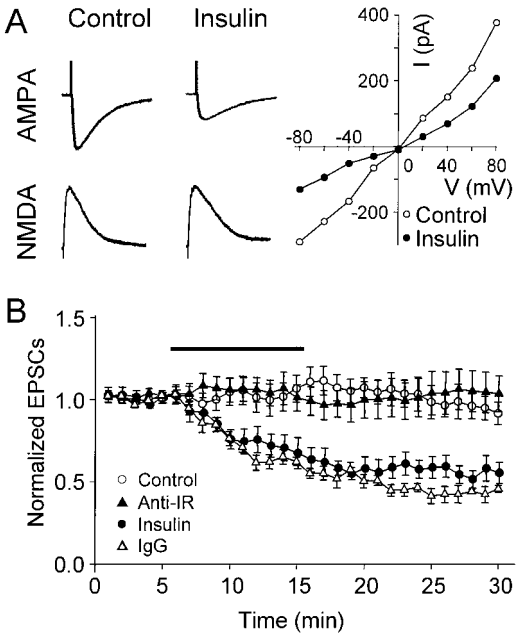

Figure 6. Insulin Induces Long-Lasting Depression of AMPA Receptor–Mediated EPSCs in Hippocampal CA1 Neurons

(A) Bath application of insulin selectively inhibits the AMPA, but not the NMDA, component of EPSCs. Representative EPSCs averaged from four individual recordings before ("Control") or during insulin application (0.5 μM). The NMDA component was recorded at a holding membrane potential of +40 mV. Current–voltage curves were constructed by plotting the AMPA component of EPSCs recorded before ("Control") and after 10 min application of insulin ("Insulin") at a series of membrane holding potentials.

(B) Insulin-induced depression of AMPA EPSCs is dependent on the activation of postsynaptic insulin receptor tyrosine kinase. Normalized EPSCs ($EPSC_t/EPSC_0$) were plotted from neurons recorded with regular intracellular solution without ("Control," n = 10) or with bath application of insulin ("Insulin," n = 10), or from neurons recorded with intracellular solution supplemented with 60 μg/ml insulin receptor–neutralizing antibody ("Anti-IR," n = 6) or 100 μg/ml control mouse IgG ("IgG," n = 5) with insulin in the bath. Time 0, in this as well in all following figures, is defined as the time at which EPSC amplitudes stabilized (typically 10 min after the start of whole-cell recording); at t = 5 min, insulin was added (black bar for "Insulin," "Anti-IR," and "IgG" groups).

Insulin Produces Long-Lasting Depression of the AMPA Component of Hippocampal CA1 EPSCs

To determine the functional consequences of insulin-induced AMPA receptor internalization, we examined insulin's effects on AMPA receptor–mediated synaptic transmission in hippocampal slices. Electrical stimulation of Schaffer collateral–commissural fibers evoked excitatory postsynaptic currents (EPSCs) in CA1 cells, which consist of two pharmacologically distinct components mediated by AMPA and NMDA receptors (Figure 6A). Bath application of insulin (0.5 μM, 10 min) reduced the AMPA component amplitude of EPSCs but had little effect on the NMDA component (Figure 6A). The reduction in AMPA EPSC amplitude was not associated with changes in either the voltage–current relationship or the reversal potential (Figure 6A). Moreover, the time course of EPSC inhibition by insulin (Figure 6B) was similar to that of receptor internalization observed in HEK cells (Figure 1C).

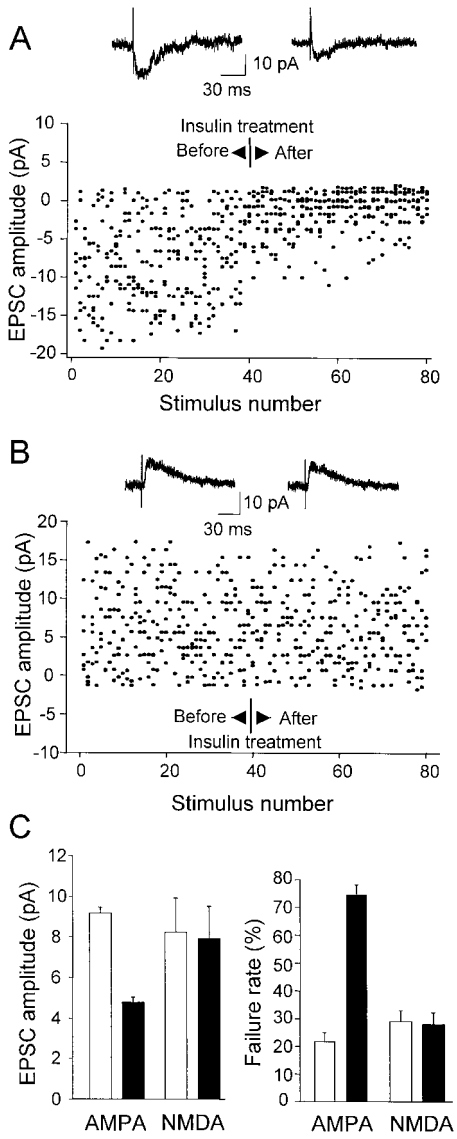

Figure 7. Insulin Selectively Inhibits Minimum Stimulus–Evoked AMPA EPSCs

(A) Plot of the minimum stimulus–evoked AMPA EPSC amplitudes from hippocampal CA1 neurons (n = 8). Individual traces before (left) and after (right) insulin bath application (0.5 μM) are shown above the EPSC plot.

(B) Insulin has little effect on the NMDA component of minimum stimulus–induced EPSCs (n = 6). NMDA EPSCs were recorded in the presence of CNQX (10 μM) at a holding membrane potential of +40 mV.

(C) A bar graph showing insulin's selective effects on both the amplitude and failure rate of the AMPA (n = 8), but not the NMDA (n = 6), component of the minimum stimulus–evoked EPSCs. Open and closed bars represent data obtained before and after insulin treatment, respectively.

The Induction of Insulin-Induced Depression of AMPA EPSCs Is Postsynaptic

Insulin's effects on AMPA receptor EPSCs may be exerted either pre- or postsynaptically, as insulin receptor tyrosine kinases are expressed at both presynaptic and postsynaptic membranes (Jonas et al., 1997; Abbott et al., 1999). The differential effects on AMPA and NMDA

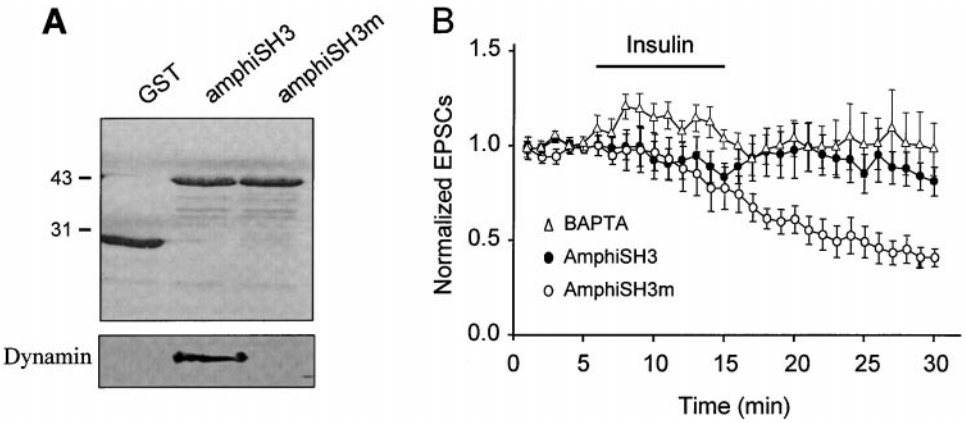

Figure 8. Inhibiting Clathrin-Mediated Endocytosis in Postsynaptic Neurons Blocks Insulin-Induced Depression of AMPA EPSCs

(A) Wild-type but not mutant amphiphysin-SH3 domain GST fusion proteins specifically interact with dynamin. Purified GST alone ("GST"), GST-amphiphysinSH3 ("amphiSH3"), and mutant GST-amphiphysinSH3 ("AmphiSH3m") fusion proteins were quantified using a Coomassie blue–stained gel (top) and added to hippocampal homogenates in a pull-down assay. Precipitated proteins were then immunoblotted with anti-dynamin I antibody (bottom).

(B) Normalized EPSCs are plotted in CA1 hippocampal neurons recorded with intracellular solution supplemented with 10 mM BAPTA ($n = 7$), or with 100 μg/ml amphiSH3 ($n = 7$) or 100 μg/ml amphiSH3m ($n = 6$) GST fusion proteins. AmphiSH3, which interacts with and competes for endogenous dynamin (A), thereby being capable of blocking clathrin-mediated endocytosis, reduced insulin's ability to depress AMPA EPSCs. In contrast, the amphiSH3m, which cannot interact with endogenous dynamin (A) and is therefore incapable of inhibiting clathrin-dependent endocytosis, has little effect on insulin-induced depression of AMPA EPSCs.

component EPSCs are more consistent with a postsynaptic action of insulin. To examine insulin's site of action, we postsynaptically applied a membrane-impermeant monoclonal antibody against the tyrosine kinase domain of the insulin receptor, which specifically inhibits the activity of insulin receptors in in vitro kinase assays and in situ when injected directly into cells (Morgan and Roth, 1987). The postsynaptic application of this anti-insulin receptor antibody (but not of the control mouse IgG) eliminated insulin's ability to inhibit AMPA EPSCs (Figure 6B). These results demonstrate that insulin acts postsynaptically via its receptor tyrosine kinase, supporting a postsynaptic locus for insulin-induced depression of AMPA EPSCs.

The Expression of Insulin-Induced Depression of AMPA EPSCs Is Also Postsynaptic

To determine the site(s) of expression of the insulin-induced depression of AMPA EPSCs, we evoked unitary EPSCs by lowering the stimulus intensity to a level that produced a failure rate of $>20\%$. If the expression of depression is presynaptic, insulin should affect only the failure rate and not the amplitude of the weak, stimulus-induced EPSCs. If, on the other hand, expression is postsynaptic, resulting from the removal of postsynaptic AMPA receptors, the altering of channel conductance, or open probability, insulin should alter both the amplitude and failure rate of EPSCs, with the latter due to postsynaptic failure in released transmitter detection (Liao et al., 1995). Consistent with a postsynaptic site of expression, insulin reduced the amplitude of AMPA EPSCs in 8 of 11 cells tested, and this reduction was associated with a dramatic increase in the failure rate (Figures 7A and 7C). In the 3 remaining cells, EPSCs were no longer detected following insulin application (data not shown). This could be a result of the reduction of EPSC amplitude below the detection threshold, but it could also be due to the conversion of active synapses into silent ones. In marked contrast, insulin failed to alter either the amplitude or the failure rate of the minimum stimulus–evoked NMDA component EPSCs (Figures 7B and 7C). These results strongly suggest that the expression of insulin-induced depression of AMPA responses is also postsynaptic.

Insulin-Induced Depression of AMPA EPSCs Is Dependent on Clathrin-Mediated Endocytosis in Postsynaptic Neurons

Insulin's postsynaptic loci of induction and expression are both consistent with its effect being mediated by the clathrin-dependent endocytosis of AMPA receptors. To address this more specifically, we chelated intracellular Ca^{2+} with the postsynaptic application of the membrane-impermeant Ca^{2+} chelator bis-(o-aminophenoxy)-N,N,N′,N′-tetraacetic acid (BAPTA) (10 mM), since clathrin-mediated endocytosis is dependent on intracellular Ca^{2+} (Marks and McMahon, 1998). Following BAPTA application, insulin treatment failed to depress the AMPA EPSC amplitude (Figure 8B). More direct evidence for the involvement of postsynaptic clathrin-mediated endocytosis was obtained by the blockade of the insulin effect with a glutathione S-transferase (GST) fusion protein of the amphiphysin SH3 domain (GST-amphiSH3). It is believed that amphiphysin SH3 domain binding to the proline-rich region of dynamin recruits dynamin to clathrin-coated pits, initiating clathrin/dynamin-dependent endocytosis (Shupliakov et al., 1997). When incubated with hippocampal slice homogenates, GST-amphiSH3 was able to pull down a protein of $\sim$100 kDa, which was recognized by dynamin antibodies on Western blots (Figure 8A). GST alone, and

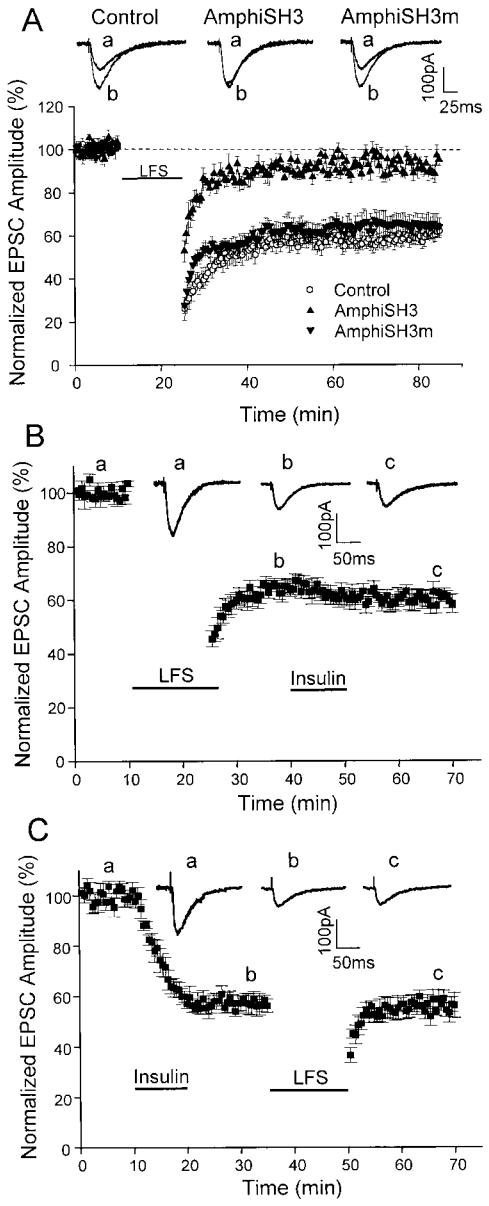

Figure 9. Expression of Homosynaptic Hippocampal CA1 LTD Requires Clathrin-Dependent Endocytosis in Postsynaptic Neurons

(A) Inhibition of clathrin-dependent endocytosis in postsynaptic neurons prevents the expression of LFS-induced LTD in CA1 neurons in a hippocampal slice preparation. Plot of averaged AMPA EPSC amplitudes in recordings with pipettes containing standard recording solution ("Control," n = 6), or solution supplemented with 100 μg/ml GST-amphiSH3 ("AmphiSH3," n = 6) or 100 μg/ml GST-amphiSH3m ("AmphiSH3m," n = 6). Top panels are superimposed individual recordings before ("b") and 60 min after ("a") the LFS–LTD induction.

(B) and (C) Plots of normalized EPSC amplitudes show that LFS- and insulin-induced LTDs mutually occlude each other. Representative traces shown in the plots were taken at time points, as indicated. Following induction of LFS LTD ("LFS"), the ability of insulin ("Insulin," 0.5 μM) to depress AMPA EPSCs was dramatically reduced ([B], n = 6), and, conversely, after the establishment of insulin-induced depression of AMPA EPSCs, LFS protocol failed to induce LTD ([C], n = 6).

GST-amphiSH3 with two mutations in its dynamin binding domain (GST-amphiSH3m) that render it incapable of binding to dynamin (Shupliakov et al., 1997), failed to precipitate endogenous dynamin (Figure 8A). Thus, GST-amphiSH3 specifically interacts with endogenous rat brain dynamin and was therefore used as a competitive inhibitor to disrupt binding between endogenous amphiphysin and dynamin in rat hippocampal neurons. GST-amphiSH3 reduced the ability of insulin to depress AMPA EPSCs (Figure 8B). In contrast, GST-amphiSH3m had little effect on insulin-induced depression of AMPA EPSCs.

Collectively, our biochemical and electrophysiological results from hippocampal slices and cultured hippocampal cells suggest that native AMPA receptors, similar to their recombinant counterparts in HEK cells, are subject to clathrin-mediated endocytosis. Insulin stimulates this process and causes the rapid removal of postsynaptic AMPA receptors, leading to LTD or even the silencing of AMPA receptor–mediated synaptic transmission.

Postsynaptic Clathrin-Mediated Endocytosis Is Required for the Expression of CA1 LTD

Homosynaptic LTD of AMPA receptor–mediated synaptic transmission in hippocampal CA1 neurons is a well-characterized in vitro model of synaptic plasticity (Bear and Malenka, 1994). While it is agreed that induction of the most common form of LTD at this synapse is postsynaptic, mechanisms underlying its expression remain hotly debated (Bear and Malenka, 1994; Bolshakov and Siegelbaum, 1994). Our preceding experiments suggested that enhanced AMPA receptor endocytosis could depress AMPA receptor–mediated transmission, so we investigated if postsynaptic AMPA receptor endocytosis could contribute to homosynaptic LTD and whether LTD and insulin-induced reductions in AMPA EPSCs shared common pathways. Delivering 900 pulses at 1 Hz to the Schaffer collaterals consistently produced LTD of AMPA EPSCs in CA1 neurons, recorded with standard intracellular solution (EPSC amplitude: 62% ± 3% of baseline 60 min poststimulation, n = 6; Figure 9A). However, when 100 μM of GST-amphiSH3 was included in the recording pipette, LTD failed to be induced in every neuron tested (EPSC: 95% ± 3% of control after 60 min post LTD induction, n = 6; Figure 9A). In contrast, inclusion of GST-amphiSH3m altered neither the time course nor the extent of LTD (64% ± 4%, n = 6; Figure 9A). These results indicate that a dynamin-dependent, clathrin-mediated endocytic process is required for the production of this particular form of LTD and further suggest that LFS-induced LTD may use a similar mechanism underlying insulin-induced LTD that involves clathrin-mediated postsynaptic AMPA receptor internalization. Indeed, as shown in Figure 9B, after LFS-induced LTD was fully established, application of insulin failed to further reduce EPSC amplitudes. Conversely, after insulin induced rapid and persistent decreases in AMPA EPSCs (Figure 9C), LFS no longer produced LTD. Thus, LFS and insulin-induced LTD mutually occlude each other, suggesting that the two share a common final pathway, namely the clathrin-mediated endocytosis of postsynaptic AMPA receptors.

Discussion

Constitutive versus Regulated AMPA Receptor Endocytosis

It is becoming clear that clathrin-mediated internalization of plasma membrane proteins is a tightly regulated process and that such regulation is an important means of controlling the cell surface expression, and hence function, of these proteins (Schmid, 1997; Karoor et al., 1998). In the present study, we examined the role of clathrin-mediated endocytosis in the activity-dependent redistribution of AMPA receptors. Using antibodies against extracellular epitopes on AMPA receptors, we selectively visualized the trafficking of AMPA receptors expressed on the surface of live cells and found that both GluR1 and GluR2 receptors undergo constitutive clathrin-dependent endocytosis. One would expect that perturbation of constitutive endocytosis should lead to altered AMPA receptor expression on the plasma membrane. Surprisingly, we found that while hypertonic sucrose treatment and overexpression of the dominant-negative dynamin mutant effectively blocked constitutive AMPA receptor endocytosis, both manipulations failed to alter AMPA receptor numbers on the cell surface. Among many possible explanations, the simplest one is that there is an unknown mechanism tightly coupling the constitutive endocytosis and insertion of AMPA receptors. Thus, factors affecting one pathway would also affect the other through a feedback mechanism to ensure a balance between receptor insertion and removal. The result would produce a constant number of receptors on the cell membrane, and hence stable baseline synaptic transmission, even under conditions in which either constitutive exocytosis (Lledo et al., 1998) or endocytosis (the present work; Wang and Linden, 2000 [this issue of *Neuron*]) has been selectively altered. It should be noted that Lüscher et al. (1999) have recently reported rundown and runup of AMPA receptor–mediated EPSCs in hippocampal slices following postsynaptic injection of putative inhibitors of exocytosis and endocytosis, respectively. Thus, some discrepancies between this and our results remain to be explained.

In addition to constitutive endocytosis, we observed insulin-stimulated, clathrin-dependent endocytosis of AMPA receptors in both transfected HEK cells and cultured hippocampal neurons. Several features associated with this insulin-regulated pathway distinguish it from constitutive endocytosis, the most prominent being its ability to rapidly reduce AMPA receptors on the cell surface, thereby suppressing AMPA receptor–mediated responses. This indicates that insulin may selectively facilitate receptor endocytosis without affecting receptor insertion, hence resetting the equilibrium between receptor removal and insertion. This feature makes regulated endocytosis an attractive mechanism for regulating synaptic plasticity in models such as LTD. Indeed, a similar mechanism appears to be critical in various forms of LTD, as suggested by this study (see below) and the companion paper (Wang and Linden, 2000).

Another feature of regulated endocytosis is its subunit specificity. Unlike the constitutive pathway, which seems to be common across at least the GluR1 and GluR2 receptor subunits, insulin-stimulated endocytosis appears to be GluR2 specific, contingent on unique sequences in its intracellular carboxyl tail. Many intracellular proteins have recently been revealed to be AMPA receptor–interacting proteins (e.g., NSF, PICK1, and GRIPs). It is noteworthy that the majority of these proteins bind specifically to the GluR2 cytoplasmic tail (Dong et al., 1997; Lin and Sheng, 1998; Srivastava et al., 1998; Xia et al., 1999). Although the functions of these GluR2-interacting proteins remain elusive, they may regulate endocytosis or other aspects of AMPA receptor vesicle trafficking via their binding to the GluR2 carboxyl tail, thereby playing an important role in controlling AMPA receptor density in the postsynaptic membrane. Indeed, evidence is accumulating suggesting the important role that some of these proteins may play in regulating the level of cell surface AMPA receptors, and hence the efficacy of the AMPA receptor–mediated synaptic transmission (Li et al., 1999; Lüscher et al., 1999; Lüthi et al., 1999; Noel et al., 1999). For instance, interrupting NSF-GluR2 interaction by postsynaptic injection of the pep2m peptide, like insulin stimulation, produces an LTD of AMPA EPSCs that is also mutually occlusive with the LFS-induced CA1 LTD (Lüscher et al., 1999; Lüthi et al., 1999). As demonstrated in this work, the regulated clathrin-dependent endocytosis of AMPA receptors is contingent upon the GluR2 carboxyl tail, and enhanced endocytotic removal of AMPA receptors clearly contributes to the LTD expression. It would therefore be very interesting to know if NSF actually acts to maintain the levels of AMPA receptor cell surface expression, primarily by preventing the GluR2-dependent regulated AMPA receptor endocytosis. In this case, pep2m, by interrupting NSF-GluR2 interaction, produces its rapid reduction of cell surface expression of AMPA receptors by stimulating the endocytotic arm of the AMPA receptor cycling but not by impairing the exocytotic arm, as originally envisaged.

Thus, from the evidence presented here, we speculate that there are two distinct clathrin-mediated endocytic pathways involved in the removal of cell surface AMPA receptors. Constitutive endocytosis seems to be shared by all GluRs, and its major function would be to counteract constitutive receptor insertion, ensuring a constant number of cell surface AMPA receptors under most conditions. In contrast, the regulated pathway is GluR2 specific and rapidly regulates AMPA receptor cell surface expression. Therefore, this pathway may be important in certain forms of synaptic plasticity involving GluR2-containing AMPA receptors. It remains to be determined how distinct constitutive and regulated endocytotic pathways are in terms of their molecular mechanisms and how they converge on clathrin-mediated internalization as a common final step.

Clathrin-Mediated Endocytosis in Synaptic Plasticity

The "silent synapse" is a recent hypothesis that has been proposed to explain the postsynaptic locus of expression of LTP and LTD (Kullmann, 1994; Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; Malinow, 1998; Malenka and Nicoll, 1999). A silent synapse contains functional NMDA receptors but lacks functional AMPA receptors and becomes activated during the induction of LTP by recruitment of AMPA receptors. By

extrapolation, an active synapse may be silenced during LTD by the loss of functional AMPA receptors. One key question that remains is how synapses are switched between active and silent states. Since plasma membrane insertion and internalization of proteins are highly controlled, regulated exocytosis and endocytosis of AMPA receptors offer attractive mechanisms for activating and silencing synapses, and hence for the expression of LTP and LTD. Indeed, evidence is emerging to support a critical role for postsynaptic membrane fusion (Lledo et al., 1998) and AMPA receptor translocation (Shi et al., 1999) in LTP induction.

We report here several observations that suggest the involvement of clathrin-mediated endocytotic removal of postsynaptic AMPA receptors in LTD. First, rapid endocytosis of AMPA receptors occurs in mature hippocampal neurons, shown by the increased association of AMPA receptors with the AP2 complex following insulin stimulation. Second, facilitation of clathin-mediated endocytosis of postsynaptic AMPA receptors by insulin produces LTD and, in some cases, silencing of AMPA receptor–mediated synaptic transmission. Third, insulin- and LFS-induced CA1 LTDs are mutually occlusive, suggesting a common mechanism mediating the two forms of LTD. Finally, LFS-induced LTD is blocked by postsynaptic microinjection of the amphiphysin SH3 domain. Thus, together with work from Lüscher et al. (1999), our results suggest that rapid, clathrin-mediated endocytotic removal of postsynaptic AMPA receptors may play an important role in LFS-induced hippocampal homosynaptic CA1 LTD production.

Endocytosis of postsynaptic AMPA receptors may not be limited to homosynaptic CA1 LTD, as in a related study (Wang and Linden, 2000) we found that insulin/IGF-I also produces a rapid and long-term depression of AMPA responses mediated by postsynaptic clathrin-dependent endocytosis in cultured cerebellar neurons. The insulin/IGF-I-induced depression of AMPA currents occludes cerebellar LTD, which in turn can be blocked by the inhibition of postsynaptic clathrin-dependent endocytosis (Wang and Linden, 2000). Additionally, Carroll et al. (1999a) have reported a rapid, activity-dependent reduction of postsynaptic AMPA receptors in a culture model of LTD induced by field stimulation. Taken together, these data suggest that rapid, clathrin-dependent removal of postsynaptic AMPA receptors may be a common final step in the expression of certain forms of LTD.

How is the clathrin-dependent endocytosis of AMPA receptors stimulated by LTD-inducing protocols? As neurons contain and are able to release insulin in an activity-dependent manner, and as insulin receptors are concentrated in the postsynaptic density (Wozniak et al., 1993; Abbott et al., 1999), one mechanism may involve the release of insulin presynaptically in response to LFS during LTD induction. Insulin may in turn activate its postsynaptic neuronal receptors to facilitate clathrin-dependent endocytosis of AMPA receptors. However, postsynaptic injection of the insulin receptor–neutralizing antibody, while blocking insulin-induced depression of AMPA EPSCs, had little effect on either hippocampal homosynaptic LTD (L. D. L. and Y. T. W., unpublished data) or cerebellar LTD (Wang and Linden, 2000). These results suggest that insulin is not itself directly involved in mediating the expression of these forms of LTD but rather that insulin and LTD-inducing stimuli may converge to cause AMPA receptor endocytosis. It is likely that multiple signal transduction pathways exist for the regulation of AMPA receptor trafficking, and the elucidation of these pathways will provide further insight into the molecular mechanisms of synaptic plasticity and may ultimately provide mechanistic clues for the role of insulin in learning and memory.

Experimental Procedures

cDNA Plasmids, Cell Cultures, and Plasmid Transfection

Rat GluR1 and GluR2 cDNAs were amplified by PCR and cloned into the XbaI/EcoRI and HindIII/SalI sites, respectively, of the mammalian expression vector GW1 (British Biotechnology). For extracellular HA tagging of GluR1 and GluR2 subunits, site-directed mutagenesis was performed to insert an AscI restriction site after A374 in GluR1 and after G384 in GluR2. An oligonucleotide cassette encoding the HA epitope was inserted into these AscI restriction sites. For exchanges of carboxyl tails between GluR1 and GluR2 subunits, EcoRI sites were generated by silent mutation in the carboxyl termini of HA-GluR1 and HA-GluR2 immediately after the fourth transmembrane domain. After the removal of their cytoplasmic tails using EcoRI digestion, the resulting backbones of HA-GluR1 and HA-GluR2 were ligated, respectively, to the PCR products of the GluR2 and GluR1 carboxy-terminal tails. Both chimeras were confirmed by sequencing. Rat $GABA_A$ receptor subunit cDNAs have been described previously (Wan et al., 1997). Wild-type GluR1 and GluR2 cDNAs were expressed in pCIS2 plasmid vectors. pcDNA3 expression vectors containing wild-type and K44E mutant dynamin I cDNAs were gifts of Dr. R. B. Vallee (Worcester Institute, Shrewsbury, MA). HEK293 (ATCC) and CHO cells overexpressing human insulin receptors (provided by Dr. C. C. Yip, Banting and Best Institute, Toronto) were transfected using the Ca^{2+}-phosphate precipitation method. Thirty-six to forty-eight hours after transfection, cells were washed with extracellular recording solution (ECS, in mM: NaCl, 140; $CaCl_2$, 1.3; KCl, 5.4; HEPES, 25; and glucose, 33 [pH 7.4], 320 mOsm) and incubated in ECS (serum starvation) for at least 1 hr. For insulin treatment, cells were incubated with ECS supplemented with 0.5 μM human recombinant insulin (Sigma) for 10–15 min. Cultured hippocampal neurons were prepared from embryonic Wistar rats (embryonic day 18–19; Charles River) as described previously (Wan et al., 1997). Cells were used following 2–3 weeks growth in culture.

GST Fusion Protein Production

pGEX2T plasmids encoding either wild-type or mutant SH3 domain of human amphiphysin were obtained from Dr. P. De Camilli (Yale University, New Haven). GST fusion proteins were expressed in DH5α *Escherichia coli* and purified from bacterial lysates according to the manufacturer's protocol (Pharmacia). Products were dialyzed in phosphate-buffered saline (PBS) and concentrated using Microcon-10 (Amicon) tubes for intracellular application during whole-cell recordings.

Immunofluorescent Confocal Microscopy

HEK293 cells were transfected with each plasmid described above. For cell surface receptor expression assays, cells at 48 hr post-transfection were fixed with 4% paraformaldehyde in PBS for 10 min. Surface AMPA receptors were first labeled with monoclonal anti-HA antibody (1:5000, Babco, Berkeley, CA) and a FITC-conjugated anti-mouse antibody (1:500, Sigma). Total cellular AMPA receptors were then stained with polyclonal anti-GluR1 (Cedarlane Laboratories) or GluR2 (Chemicon) antibody and Cy3-labeled anti-rabbit antibodies following permeation of the cells with 0.25% Triton X-100 in PBS for 10 min. For the AMPA receptor internalization assay, HEK293 cells transfected with HA-GluR constructs were incubated live at 4°C with 10 μg/ml monoclonal anti-HA antibody for 1 hr to label surface receptors and then incubated at 37°C for various time periods to allow for constitutive internalization of labeled receptors. Following fixation without permeation, receptors remaining on the plasma membrane surface were stained with FITC-conjugated

anti-mouse antibodies. Cells were subsequently treated for 1 min with 100% methanol and stained with Cy3-conjugated anti-mouse antibodies. Colocalization of HA-tagged GluR2 receptors with EPS15 was studied by sequentially staining both monoclonal anti-HA and FITC-conjugated anti-mouse antibody and polyclonal anti-EPS15 and Cy3-conjugated anti-rabbit antibody under permeant conditions. Subcellular localization of fluorescently labeled receptors was examined with a Leica TCS-4D confocal microscope (Wan et al., 1997).

Colorimetric Assays

Colorimetric assays were performed using a protocol modified from a published method (Wang et al., 1998). Briefly, HEK293 cells were transfected by calcium phosphate in 15 cm dishes. Cells were replated 24 hr after transfection into 12-well plates. Treatment and control studies, as well as time course studies, were performed on the same sets of 12-well plates derived from the same population of transfected cells so as to minimize transfection efficiency–related variation. For the assay, cells were fixed in paraformaldehyde as described above. Cells were, under either nonpermeant (PBS alone) or permeant (PBS containing 0.2% Triton X-100) conditions, blocked for 1 hr at room temperature with 3% bovine serum albumin and then incubated overnight at 4°C in anti-HA (1:2000) to detect HA-tagged receptors, polyclonal antibodies raised against the amino-terminal region of GluR1 (1:1000, Oncogene Science) to detect wild-type GluR1, or monoclonal antibody raised against an amino-terminal sequence of GluR2/4 for wild-type GluR2 (1:1000; Pharmingen). After extensive wash, cells were sequentially incubated for 1 hr at room temperature with the appropriate horseradish peroxidase–conjugated secondary antibody (1:800, Amersham) and for 2 min with 1 volume of OPD substrate (Sigma). Reactions were stopped with 0.2 volume of 3N HCl, and the optical density of 1 ml of supernatant was read on a spectrophotometer at 492 nm.

Coimmunoprecipitation and Electrophysiology in Hippocampal Slices

Hippocampal slices (300 μm thickness) were prepared from adult male Sprague-Dawley rats (150–200 g, Charles River) as described (Wan et al., 1997). For immunoprecipitation and immunoblotting, homogenate (~500 μg protein) from control or slices treated with 0.5 μM insulin for 10 min were incubated with anti–adaptin β2 monoclonal antibody (Sigma) in 500 μl of 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Triton X-100 for 4 hr at 4°C. The antibody-protein complexes were then pelleted with protein A–Sepharose beads. Proteins eluted from the beads were subjected to SDS–PAGE and immunoblotting for anti–adaptin β2 (1:5000), GluR2/4 (Pharmingen, 1 μg/ml), GluR1 (1 μg/ml, Oncogene Science), or monoclonal anti-NMDAR1 (1:800, Pharmingen), respectively. For sequential probing of the same membrane, the membranes were stripped of antibody and reprobed. The blot was developed using enhanced chemiluminescence detection methods (Amersham). Band intensities were quantified using Scion Image PC software.

Whole-Cell Recordings

For electrophysiological recordings, slices were perfused at room temperature (22–24°C) with artificial cerebrospinal fluid containing 126 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose and bubbled with 95% O_2/5% CO_2. An incision was made between CA3 and CA1 to reduce epileptiform activity. Whole-cell recordings of CA1 neurons were performed using the "blind" method with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA). Recording pipettes (4–5 MΩ) were filled with intracellular solution containing (in mM): CsCl, 135; EGTA, 0.05; HEPES, 10; Mg-ATP, 4; GTP, 0.2; and QX-314, 5 (pH 7.4), 310 mOsm, or the same solution supplemented with one of the following: 10 mM BAPTA, anti-insulin receptor kinase domain antibody (60 μg/ml) (17A3; Morgan and Roth, 1987), mouse IgG (100 μg/ml), amphiphysin-SH3 domain (100 μg/ml), or mutant amphiphysin-SH3 domain (100 μg/ml). EPSCs were evoked by stimulation of the Schaffer collateral–commissural pathway with a bipolar tungsten electrode (0.05 ms duration at a rate of 0.07 Hz) in the presence of bicuculline (20 μM). Minimum stimulus–evoked EPSCs were recorded following a modified protocol (Liao et al., 1995).

Homosynaptic CA1 LTD was recorded in slices prepared from rats aged 16–26 postnatal days old. Following 20 min recording of baseline EPSCs evoked every 30 s, recording was switched to current-clamp mode, and 15 min of train stimulation at 1 Hz (900 pulses in total) was delivered from the same stimulating electrode. The recording was then switched back to voltage-clamp mode, and EPSC recordings at the baseline stimulus rate were then recorded for more than 1 hr thereafter.

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