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Cerebellar Long-Term Depression Requires PKC-Regulated Interactions between GluR2/3 and PDZ Domain–Containing Proteins

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Summary

Cerebellar LTD requires activation of PKC and is expressed, at least in part, as postsynaptic AMPA receptor internalization. Recently, it was shown that AMPA receptor internalization requires clathrin-mediated endocytosis and depends upon the carboxy-terminal region of GluR2/3. Phosphorylation of Ser-880 in this region by PKC differentially regulates the binding of the PDZ domain–containing proteins GRIP/ABP and PICK1. Peptides, corresponding to the phosphorylated and dephosphorylated GluR2 carboxy-terminal PDZ binding motif, were perfused in cerebellar Purkinje cells grown in culture. Both the dephospho form (which blocks binding of GRIP/ABP and PICK1) and the phospho form (which selectively blocks PICK1) attenuated LTD induction by glutamate/depolarization pairing, as did antibodies directed against the PDZ domain of PICK1. These findings indicate that expression of cerebellar LTD requires PKC-regulated interactions between the carboxy-terminal of GluR2/3 and PDZ domain–containing proteins.

Introduction

Cerebellar long-term depression (LTD) is a cellular model system of memory that has been suggested to comprise a portion of the engram for some forms of motor learning (see Ito, 1989; Mauk, 1997; Bear and Linden, 2000, for review). It is a persistent, input-specific attenuation of the parallel fiber–Purkinje cell synapse that is produced when parallel fiber and climbing fiber inputs to a Purkinje cell are briefly coactivated at low frequency, and, in recent years, considerable progress has been made toward defining the molecular events necessary for its induction. These include three initial postsynaptic events: mGluR1 activation, AMPA receptor activation, and Ca^{2+} influx via voltage-gated Ca^{2+} channels. These three signals appear to converge upon the activation of protein kinase C (PKC) as inhibition of PKC blocks cerebellar LTD induction and application of an exogenous PKC activator produces an LTD-like phenomenon (Crepel and Krupa, 1988; Linden and Connor, 1991; Hartell, 1994; Narasimhan and Linden, 1996; De Zeeuw et al., 1998; Freeman et al., 1998; Matsuda et al., 2000). In addition, inhibition of postsynaptic protein phosphatase activity through a cascade involving NO, cGMP and cGMP-dependent protein kinase (PKG) may also be important (see Daniel et al., 1998; Bear and Linden, 2000, for review).

Progress has also been made toward a molecular understanding of the cerebellar LTD expression mechanism. Cerebellar LTD may be detected using test pulses of AMPA receptor agonists such as glutamate, quisqualate or AMPA in intact (Ito et al., 1982), slice (Crepel and Krupa, 1988), and cell culture preparations (Linden et al., 1991, 1993). Furthermore, cerebellar LTD may be seen with AMPA receptor agonist test pulses in reduced preparations that lack functional presynaptic terminals (Linden, 1994) including dendritic macropatches and acutely dissociated Purkinje cells (Linden, 1996; Narasimhan and Linden, 1996; Narasimhan et al., 1998). Thus, it appears as if cerebellar LTD expression involves some form of postsynaptic AMPA receptor downregulation. This could potentially involve changes in AMPA receptor unitary conductance, kinetics, glutamate affinity, or number/distribution of receptors in the postsynaptic membrane.

Support for this last idea has come from experiments showing that postsynaptic manipulations that interfere with clathrin-mediated endocytosis block the induction of cerebellar LTD in culture, while manipulations that induce postsynaptic internalization of AMPA receptors (such as bath application of insulin or IGF-1) produce an LTD-like effect that mutually occludes LTD induced by synaptic pairing (Wang and Linden, 2000). Interestingly, similar results have been found in studies of hippocampal LTD. Treatments that interfered with postsynaptic clathrin-mediated endocytosis prevented both pairing-induced LTD at the Schaffer collateral/commissural synapse (Luscher et al., 1999; Man et al., 2000) and persistent internalization of AMPA (but not NMDA) receptors induced by brief exposure to AMPA (Carroll et al., 1999b; Luscher et al., 1999) or insulin (Man et al., 2000) in cultured hippocampal neurons. These findings suggest that cerebellar and at least one form of hippocampal LTD may share a common expression mechanism (see Luscher et al., 2000; Turrigiano, 2000, for review).

How might PKC activation be linked to clathrin-mediated internalization of AMPA receptors to result in the expression of cerebellar LTD? Several lines of evidence are suggestive. First, experiments using a heterologous expression system have shown that insulin-induced internalization of AMPA receptors requires the presence of GluR2/3, specifically, the carboxyl terminus (Man et al., 2000). This region contains a conserved PDZ binding motif that serves as a binding site for glutamate receptor interacting protein/AMPA receptor binding protein (GRIP/ABP) and protein interacting with C-kinase 1 (PICK1) as well as a separate site that binds N-ethylmaleimide-sensitive factor (NSF; see Kim and Huganir, 1999; Braithwaite et al., 2000, for review). Second, a specific residue within this PDZ binding motif, GluR2 Ser-880, is phosphorylated by PKC, and this results in a strong decrease in the binding of GRIP/ABP (Matsuda et al., 1999; Chung et al., 2000) but not PICK1 (Chung et al., 2000). Third,

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activation of PKC is also associated with a translocation of Ser-880-phosphorylated GluR2 and PICK1, but not GRIP/ABP immunoreactivity from dendritic shafts to spines in cultured hippocampal neurons (Chung et al., 2000). Fourth, PKC activating phorbol esters result in GluR2 internalization in neocortical neurons (Chung et al., 2000) and mutation of GluR2 Ser-880 to alanine prevents this in a heterologous expression system (H. J. Chung et al., submitted).

While these biochemical experiments are suggestive, their relevance for synaptic plasticity remains undetermined. Thus, we have performed whole-cell patch clamp recordings and microfluorimetric Ca^{2+} imaging of cerebellar Purkinje cells in culture together with manipulations to modify the interactions between GluR2/3, GRIP/ABP, and PICK1. In so doing, we sought to test the hypothesis that LTD expression is achieved by phosphorylation of GluR2 at Ser-880 (or GluR3 at the analogous residue, Ser-885) by PKC, which regulates the interaction of GluR2 with GRIP/ABP and PICK1, thereby priming AMPA receptors for clathrin-mediated endocytosis.

Results

Initially, we wished to determine whether the relevant proteins, GluR2/3, GRIP/ABP, and PICK1, were expressed in cerebellar Purkinje cells (Figure 1). Previous immunocytochemical experiments have shown that both GRIP/ABP (Wyszynski et al., 1998; Burette et al., 1999; Dong et al., 1999) and GluR2/3 (Petralia and Wenthold, 1992; Landsend et al., 1997; Zhao et al., 1998; Burette et al., 1999) are strongly expressed in Purkinje cells and that they colocalize in both somata and dendrites at the level of light microscopy. Using immunohistochemistry in sections of adult rat cerebellum, we confirmed these previous observations and extended them by showing that a purified antibody directed against Ser-880-phosphorylated GluR2 C-terminal peptide (GluR2-pS880; Chung et al., 2000) also binds to Purkinje cell somata and dendrites. PICK1 immunoreactivity was strong in Purkinje cell somata but was weaker in dendrites. Anti-GluR2-pS880 and anti-PICK1 antibody also revealed diffuse immunoreactivity in the cerebellar molecular layer that did not appear to be associated with Purkinje cell dendrites and yet is likely to represent specific binding as it is strongly reduced in control sections in which the antibody was preabsorbed with the peptide used for its generation.

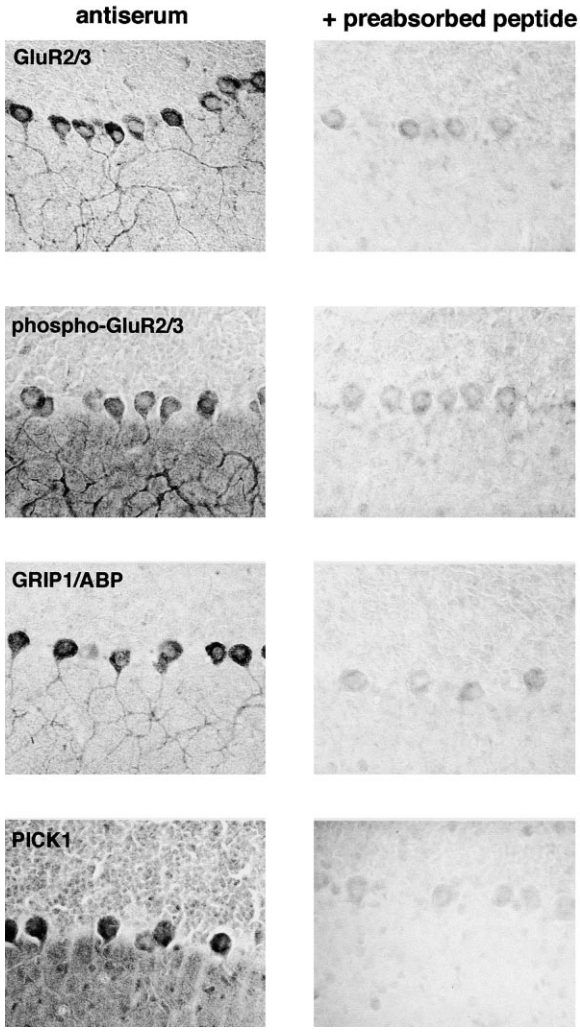

Figure 1. Immunohistochemical Localization of GluR2/3 PDZ Domain–Interacting Proteins in Cerebellar Purkinje Cells

The left hand panels show sagittal sections of adult rat cerebellar cortex stained with various polyclonal antisera generated in rabbits and visualized with a secondary antiserum conjugated to horseradish peroxidase and reacted with diaminobenzidine. The following concentrations of primary antiserum were used: GluR2/3, 1:5,000; GluR2/3 phosphorylated at Ser-880, 1:200; GRIP1/ABP, 1:10,000; and PICK1, 1:1,000. The right hand panels show control sections in which the primary antisera were preabsorbed to an excess of the peptide used for their generation to reveal nonspecific immunoreactivity.

Whole-cell voltage-clamp recordings were made from Purkinje cells in cultures derived from embryonic mouse cerebellum. We wished to determine whether postsynaptic application of peptides based on the GluR2 carboxy-terminal PDZ binding motif would affect basal responses mediated by AMPA and NMDA receptors (Figure 2). As the peptides perfused the postsynaptic cell, alternating iontophoretic test pulses of AMPA and NMDA were delivered to the same site on the proximal dendrite of a Purkinje cell at a frequency of 0.05 Hz. To allow for the recording of NMDA currents, $[Mg]_o$ was reduced to 0.2 mM, 20 μM glycine was added to the external saline, and V_{hold} was set to −50 mV. The following peptides were added to the internal saline at a concentration of 200 μM: KEGYNVYGIESVKI (SVKI, the wild-type sequence from rat GluR2); KEGYNVYGIESGKA (SGKA, a mutant peptide that will not function as a PDZ binding motif); KEGYNVYGIESVAI (SVAI, a mutant peptide that will function as a PDZ binding motif, but not as a substrate for phosphorylation by PKC[Li et al., 1999]); and KEGYNVYGIES-(PO_4)-VKI (phospho-SVKI, the wild-type sequence phosphorylated at Ser-880, which will bind PICK1 but not GRIP/ABP [Matsuda et al., 1999; Chung et al., 2000; H. J. Chung et al., submitted]). SVKI peptide produced a very small decrease in evoked AMPA currents that became stable ~15 min following the beginning of whole-cell recording (90% ±

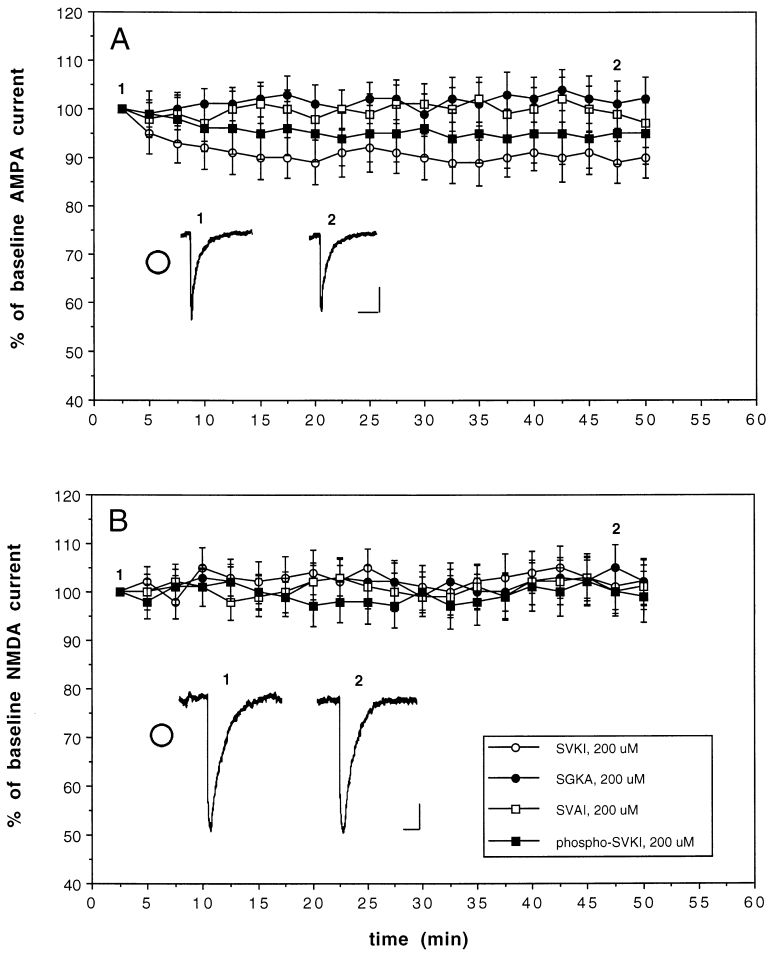

Figure 2. Postsynaptic Application of Peptides Based on the Carboxy-Terminal PDZ Binding Motif of GluR2 Does Not Alter Responses Evoked by AMPA and NMDA Test Pulses

Alternating iontophoretic pulses of AMPA and NMDA were applied to the same dendritic location. $[Mg^{2+}]_o$ was reduced to 0.2 mM, 20 μM glycine was added, and V_{hold} was set to −50 mV to allow for NMDA current recordings. Each point represents the mean ± SEM. SVKI peptide, n = 7; SGKA peptide, n = 6; SVAI peptide, n = 5; phospho-SVKI peptide, n = 5. Representative current traces were acquired at the times indicated on each graph.

(A) Responses to AMPA test pulses. Scale bars: 50 pA, 1 s.

(B) Responses to NMDA test pulses. Scale bars: 20 pA, 1 s.

5% of baseline at t = 15 min, mean ± SEM, n = 7). This decrease was not significant ($p > 0.05$ by Student's t test) when compared with that produced by the control peptide SGKA (102% ± 4% of baseline at t = 15 min, n = 6). SVAI and phospho-SVKI peptides also failed to significantly affect basal AMPA responses (101% ± 4% of baseline at t = 15 min, n = 5, and 95% ± 5% of baseline at t = 15 min, n = 5, respectively). None of the four peptides produced significant alterations in the amplitude of evoked NMDA currents (SVKI: 102% ± 4% of baseline at t = 15 min; SGKA: 100% ± 3% of baseline; SVAI: 101% ± 4% of baseline; phospho-SVKI: 101% ± 4% of baseline).

While the four peptides based on the GluR2 PDZ binding motif do not significantly affect the responses to exogenous test pulses of AMPA (or NMDA), it remains possible that exogenously applied agonists could activate a higher proportion of extrasynaptic receptors than

Table 1. Effects of Experimental Treatments on AMPA-mEPSCs in Cultured Purkinje Neurons

Treatment	Amplitude (pA)	Frequency (s^{-1})	10%–90% Rise (ms)	Half Decay (ms)
Control	27 ± 7	5.9 ± 2.9	2.2 ± 0.3	6.8 ± 0.5
SVKI, 200 μM	23 ± 6	5.6 ± 2.8	2.0 ± 0.4	6.3 ± 0.7
SGKA, 200 μM	28 ± 5	7.0 ± 2.0	2.5 ± 0.3	7.2 ± 0.6
SVAI, 200 μM	27 ± 7	8.4 ± 3.3	2.3 ± 0.4	7.2 ± 0.7
Phospho-SVKI, 200 μM	25 ± 9	6.8 ± 2.9	2.0 ± 0.3	7.0 ± 0.7
Anti-N-PICK1, 20 μg/ml	29 ± 7	7.3 ± 2.8	1.9 ± 0.3	6.7 ± 0.6
+ preabsorbed N peptide	26 ± 6	5.5 ± 2.5	2.3 ± 0.2	6.5 ± 0.6
Anti-rapsyn, 20 μg/ml	26 ± 8	6.0 ± 3.2	2.0 ± 0.4	7.0 ± 0.7
GST-PICK1, 50 μg/ml	30 ± 8	5.9 ± 3.3	2.7 ± 0.5	7.9 ± 0.8
GST backbone, 50 μg/ml	28 ± 5	8.0 ± 2.8	2.2 ± 0.3	7.3 ± 0.6
GST-PICK1ΔCC, 50 μg/ml	26 ± 7	7.2 ± 2.7	2.5 ± 0.4	6.8 ± 0.5
GST-PICK1ΔCC, 150 μg/ml	30 ± 7	6.5 ± 2.5	2.8 ± 0.4	6.1 ± 0.7
GST-PICK1ΔPDZ, 50 μg/ml	27 ± 6	8.6 ± 3.0	2.0 ± 0.3	6.5 ± 0.5

Values are mean ± SEM. n = 10 Purkinje neurons/treatment group. mEPSC measurements were made using a 500 s long sample recorded in external saline supplemented with 100 μM D-AP5. V_{hold} was set to −90 mV to increase driving force.

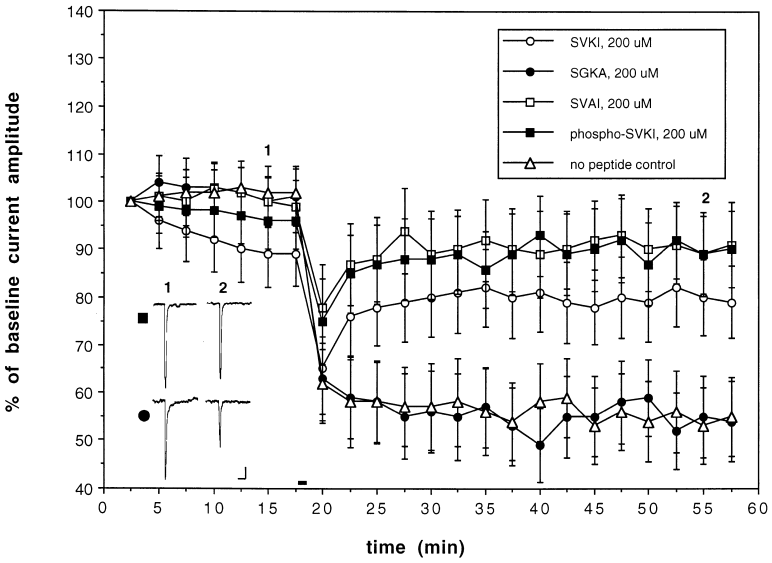

Figure 3. Peptides that Disrupt the Interaction between the GluR2/3 PDZ Binding Motif and PICK1 Block Induction of Cerebellar LTD

Following acquisition of baseline responses to quisqualate (an agonist of both AMPA and mGluRs), LTD was induced by quisqualate/depolarization conjunction at t = 17.5 min as indicated by the horizontal bar. Representative current traces were acquired at the times indicated on the graph. Scale bars = 20 pA, 1 s. SVKI peptide, n = 7; SGKA peptide, n = 6; SVAI peptide, n = 6; phospho-SVKI peptide, n = 7; no peptide control, n = 5.

does glutamate released from synaptic terminals. To address the possibility that the effect of these peptides is weak or absent at synaptic AMPA receptors on Purkinje cells, they were applied together with monitoring of AMPA receptor–mediated mEPSCs (using external saline supplemented with 100 μM D-AP5; Table 1). None of the four peptides produced significant alterations in either the amplitude or frequency of AMPA-mEPSCs. Furthermore, the rise and decay times of these events were also unchanged. Thus, in cultured Purkinje cells, peptides based upon the GluR2 carboxy-terminal PDZ binding motif do not appear to significantly affect the basal electrophysiological properties of synaptic or extrasynaptic AMPA receptors.

To assess the potential role of the GluR2/3 carboxy-terminal PDZ binding motif in the expression of LTD, peptides were perfused into Purkinje cells prior to LTD induction (Figure 3). During the baseline recording period, quisqualate test pulses were applied at 0.05 Hz. At t = 17.5 min, LTD was produced by six conjunctive stimuli, each consisting of a quisqualate pulse together with a 3 s long depolarizing step to 0 mV (Linden et al., 1993). Perfusion of SVKI peptide produced a small attenuation of basal test pulses (89% ± 7% of baseline at t = 15 min, n = 7, similar to that seen in Figure 2A) and a large attenuation of LTD (80% ± 8% of baseline at t = 55 min, as compared with 54% ± 8% of baseline, n = 6 for SGKA control peptide and 53% ± 8% of baseline, n = 5, for a no peptide control). In addition to blocking the interactions of PICK1 and GRIP/ABP, SVKI peptide serves as a PKC substrate and thereby could function as a PKC inhibitor by competing with native substrates for the catalytic site. Since PKC inhibitors are known to block induction of cerebellar LTD (Linden and Connor, 1991; Hartell, 1994; Narasimhan and Linden, 1996; De Zeeuw et al., 1998; Freeman et al., 1998), it became necessary to distinguish this potential side effect from direct effects on the GluR2/3 carboxy-termi-

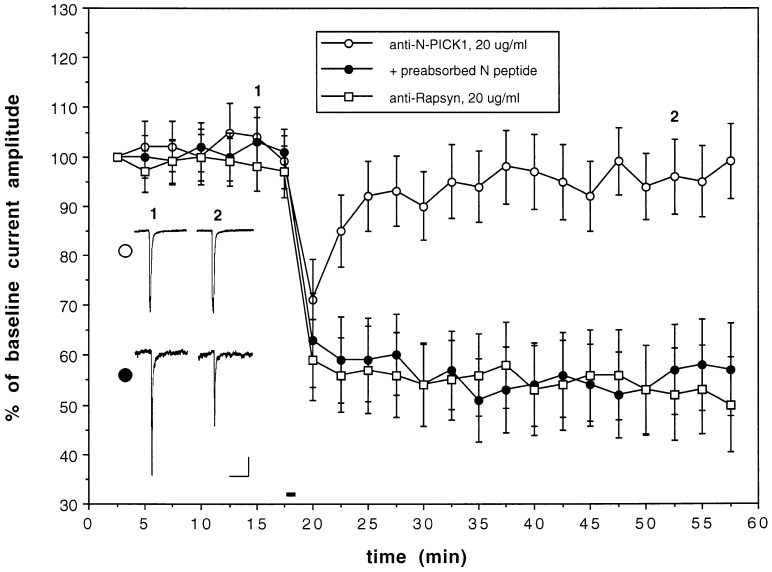

Figure 4. An Antiserum Directed against the Amino-Terminal PDZ Domain of PICK1 Blocks Induction of Cerebellar LTD

Following acquisition of baseline responses to quisqualate LTD was induced by quisqualate/depolarization conjunction at t = 17.5 min. Scale bars: 30 pA, 2 s. Treatments included intracellular application of an affinity-purified rabbit antiserum directed against the amino-terminal PDZ domain of PICK1 (anti-N-PICK1, n = 7), this antiserum preabsorbed with the peptide used for its generation (n = 6) and, as a control, an affinity-purified rabbit antiserum directed against the nonneuronal protein rapsyn (n = 5).

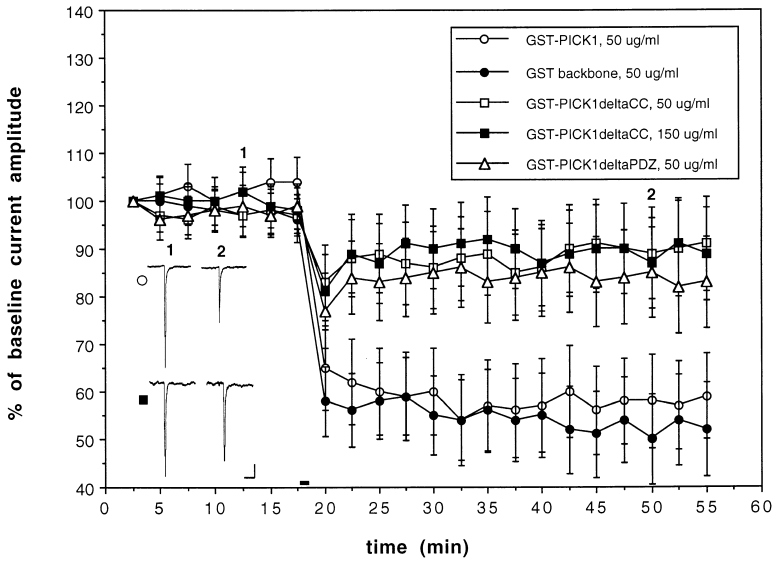

Figure 5. Mutant GST-PICK1 Fusion Proteins Block Induction of Cerebellar LTD

Following acquisition of baseline responses to quisqualate, LTD was induced by quisqualate/depolarization conjunction at t = 17.5 min. Scale bars: 35 pA, 2 s. Treatments included intracellular application of a wild-type GST-PICK1 fusion protein (GST-PICK1, 50 μg/ml, n = 5), the GST backbone protein (GST backbone, 50 μg/ml, n = 4), a mutant GST-PICK1 fusion protein with a deletion of the coiled-coil multimerization domain (GST-PICK1ΔCC; 50 and 150 μg/ml, n = 7 and 5, respectively), and a mutant GST-PICK1 fusion protein in which the PDZ domain had been rendered nonfunctional (GST-PICK1ΔPDZ, 50 μg/ml, n = 6).

nal PDZ binding motif. To accomplish this, we applied SVAI peptide, which interferes with PICK1-GRIP/ABP-GluR2/3 interactions but is not a substrate for PKC. This treatment also produced a strong blockade of LTD (89% ± 9% of baseline at t = 50 min, n = 6). Finally, to distinguish between PICK1-GluR2/3 interactions and GRIP/ABP-GluR2/3 interactions, we used phospho-SVKI peptide, which binds endogenous PICK1 but not GRIP/ABP (Matsuda et al., 1999; Chung et al., 2000). Infusion of phospho-SVKI peptide also produced a strong blockade of LTD (90% ± 8% of baseline at t = 50 min, n = 7).

The finding that infusion of phospho-SVKI peptide blocked cerebellar LTD induction suggests that the binding of GluR2/3 to PICK1, but not GRIP/ABP, is required. To provide a further test of this idea, we internally perfused an affinity-purified antiserum directed against the amino-terminal PDZ domain of rat PICK1 (anti-N-PICK1, 20 μg/ml; see Figure 4). This antiserum had no significant effect on the amplitude of basal quisqualate pulses (104% ± 6% of baseline at t = 15 min, n = 7), nor did it effect the amplitude, frequency, or kinetics of AMPA-mEPSCs (Table 1). However, it did produce a strong blockade of LTD induction (95% ± 7% of baseline at t = 55 min). Two control experiments indicate that the blockade of LTD induction by this antiserum results from a specific action at the PICK1 PDZ domain. First, preabsorption of the antiserum with the PICK1 PDZ domain peptide used for its generation resulted in a complete reversal of the LTD-blocking effect (59% ± 9% of baseline at t = 55 min, n = 6). Second, perfusion of an affinity-purified antiserum directed against the nonneuronal protein rapsyn, had no effect on LTD induction (53% ± 9% of baseline at t = 55 min, n = 5).

How might binding of PICK1 to GluR2/3 trigger LTD? In addition to its amino-terminal PDZ domain, which can bind GluR2/3 and PKCα, PICK1 has a coiled-coil domain that could potentially allow for multimerization (see Kim and Huganir, 1999; Xia et al., 1999; Braithwaite et al., 2000, for review). Thus, it is possible that multimerization of PICK1-GluR2/3 and/or PICK1-PKCα complexes may be required to induce LTD. As an initial test of this hypothesis, mutant GST-PICK1 fusion proteins were added to the internal saline and allowed to perfuse Purkinje cells prior to LTD induction (Figure 5). Perfusion of a mutant GST-PICK1 fusion protein with a deletion of the coiled-coil domain (GST-PICK1ΔCC; 50 and 150 μg/ml) produced no significant alteration of baseline responses to quisqualate (97% ± 4% and 102% ± 4% of baseline at t = 17.5 min, n = 7 and 5, respectively) when compared with two controls, wild-type GST-PICK1 (GST-PICK1, 50 μg/ml, 104% ± 5% of baseline, n = 5) and the GST backbone (GST backbone, 50 mg/ml, 96% ± 5% of baseline, n = 4). However, perfusion of GST-PICK1ΔCC did result in an attenuation of subsequently induced LTD (GST-PICK1ΔCC; 50 and 150 μg/ml, 91% ± 10% and 89% ± 10% of baseline at t = 55 min, respectively) as compared with GST-PICK1 (59% ± 9% of baseline) and GST backbone (52% ± 10% of baseline). If multimerization of PICK1-GluR2/3 or PICK1-PKCα complexes is required for LTD induction, then one would predict that application of an excess mutant GST-PICK1 fusion protein in which the PDZ domain had been rendered nonfunctional might function as a dominant–negative for this process as it could multimerize with native PICK1 but not bind GluR2/3 or PKCα. When this protein (GST-PICK1ΔPDZ, 50 μg/ml, n = 6) was infused into Purkinje cells, no significant alteration in baseline responses to test pulses of quisqualate was seen (99% ± 5% of baseline at t = 17.5 min), but the amplitude of subsequent LTD induction was significantly reduced (83% ± 10% of baseline at t = 55 min).

To this point, we have shown that treatments that interfere with the interaction between PICK1 and GluR2/3 or PICK1 multimerization produce a strong blockade of cerebellar LTD. One concern in ascribing causality to these observations is that these treatments might have side effects on other processes known to be required for LTD induction. To address this possibility, we examined the effects of GluR2 peptides and PICK1 antiserum on assays of voltage-gated Ca^{2+} channel and mGluR1 function. Purkinje cells were perfused

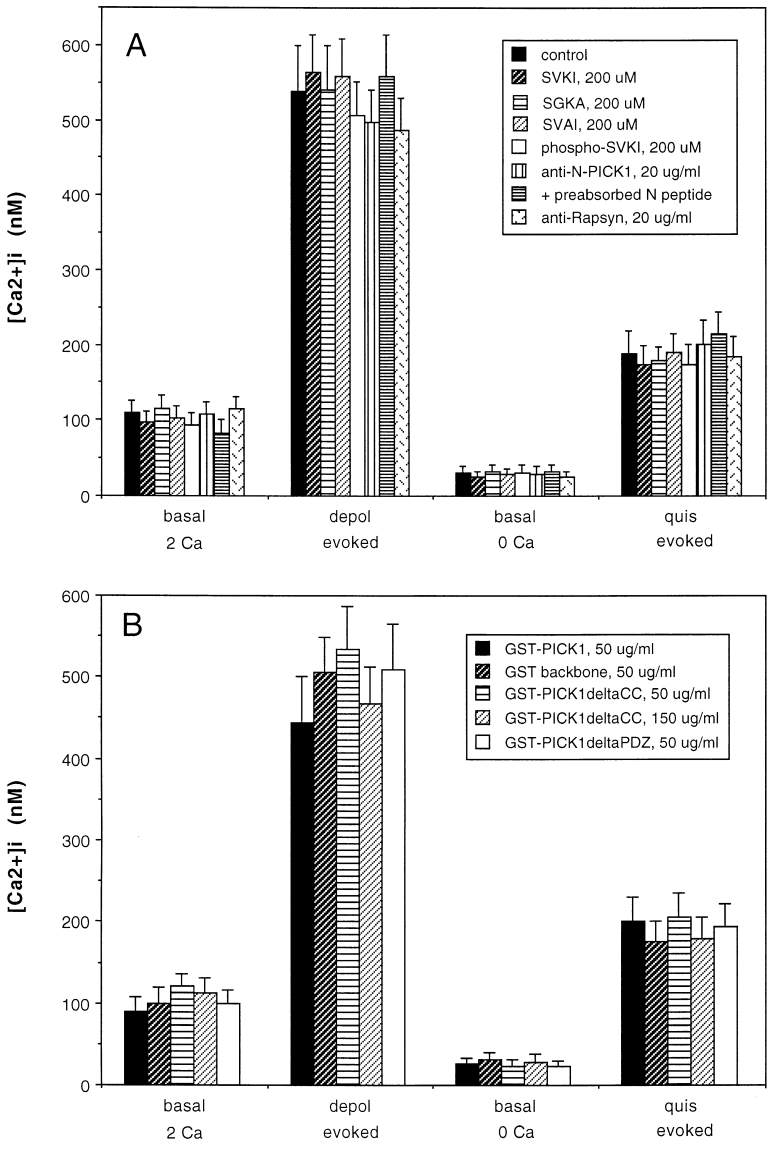

Figure 6. Treatments that Block PICK1-GluR2/3 Interactions and LTD Do Not Alter Ca^{2+} Influx Evoked by Depolarization or Ca^{2+} Mobilization Evoked by Activation of mGluR1

Various compounds were applied in the internal saline. Ca_i measurements were made at t = 17.5–20 min. Values are peak proximal dendritic Ca^{2+} concentration. Depolarization-evoked Ca_i was measured in cells that were incubated in normal external saline and stimulated with a 3 s depolarizing pulse from −80 to 0 mV. Resting values were measured immediately before stimulation. mGluR1 function was measured as the Ca^{2+} mobilization evoked by a 3 s long quisqualate pulse delivered to a Purkinje cell in 0 Ca^{2+}/0.2 EGTA external saline. Quisqualate-evoked Ca_i was measured as the peak during a 30 s measuring period following the onset of depolarization. n = 10 cells/group.

with the various compounds used herein, together with 100 μM bis-fura-2. Following 17.5 min of perfusion in whole-cell mode (to match the interval in LTD experiments), the Ca^{2+} transients evoked by six 3 s long depolarizing pulses were measured and averaged (Figure 6). In a separate set of experiments, Ca^{2+} transients evoked by a 3 s long pulse of quisqualate were applied to Purkinje cells bathed in Ca^{2+}-free medium to provide a measure of mGluR1-evoked Ca^{2+} mobilization. Neither the resting Ca^{2+} levels nor the peak values of the evoked Ca^{2+} transients were significantly altered by any of the treatments that acted upon GluR2/3-PDZ domain protein interactions or PICK1 multimerization (or their corresponding controls).

While cerebellar LTD is typically induced by conjunctive stimulation, an LTD-like phenomenon may also be induced by exogenous application of PKC-activating phorbol ester (Crepel and Krupa, 1988; Linden and Connor, 1991; Matsuda et al., 2000). Phorbol ester–induced depression appears to be similar to LTD induced by conjunctive stimulation as they mutually occlude each other, both result in depression of AMPA but not NMDA currents, and they are both blocked by PKC inhibitors (Linden and Connor, 1991). Therefore, treatments that interfere with PICK1-GluR2/3 interactions, and thereby block LTD induced by conjunctive stimulation, would be predicted to produce a similar blockade of depression induced by exogenous phorbol ester. This prediction was tested by applying phorbol-12,12-diacetate (PDA, 200 nM) to the external saline for the period t = 15–30 min (Figure 7A). In these experiments, test pulses of AMPA, rather than quisqualate were used. When the internal saline was supplemented with the control peptide SGKA, application of PDA resulted in a slowly developing but ultimately stable depression (51% ± 9% of baseline at t = 45 min, n = 5), similar to that reported previously (Linden and Connor, 1991). However, when phospho-SVKI and SVAI peptides were applied (which share blockade of PICK1-GluR2/3 interactions but which differ in terms of their function as PKC substrates and

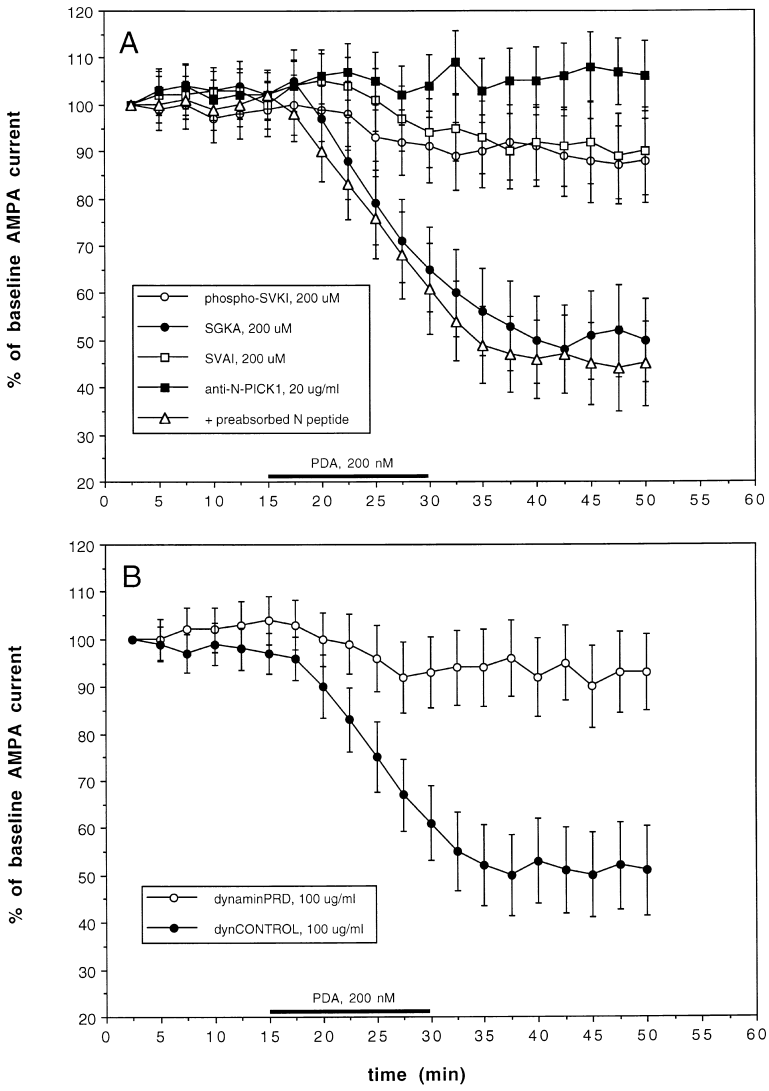

Figure 7. Treatments that Block PICK1-GluR2/3 Interactions Also Attenuate the Depression of AMPA Responses Evoked by Phorbol Ester

Following acquisition of baseline responses to AMPA pulses, phorbol-12,13-diacetate (PDA) was applied in the bath from t = 15–30 min, as indicated by the horizontal bar.

(A) Treatments that interfere with PICK1-GluR2/3 interactions and their controls. Phospho-SVKI peptide, n = 6; SGKA peptide, n = 5; SVAI peptide, n = 5; anti-N-PICK1, n = 6; anti-N-PICK1 preabsorbed with N peptide, n = 5.

(B) A dynamin proline rich domain peptide (dynaminPRD, n = 4) and a scrambled control peptide (dynCONTROL, n = 4) were used to assess the requirement for clathrin-mediated endocytosis in PDA-induced depression of AMPA responses.

their ability to bind GRIP/ABP), both produced a strong blockade of PDA-induced depression (phospho-SVKI: 88% ± 9% of baseline at t = 45 min, n = 6; SVAI: 92% ± 9% of baseline, n = 5). Furthermore, anti-N-PICK1 antiserum produced a similar blockade 108% ± 7% of baseline, n = 6), which was prevented when the antiserum was preabsorbed with PICK1 N-terminal PDZ domain peptide (45% ± 8% of baseline, n = 5).

Previous work has shown that cerebellar LTD induced by conjunctive stimulation is blocked by treatments that interfere with clathrin-mediated endocytosis such as perfusion of a peptide based on the proline-rich domain of dynamin I (Wang and Linden, 2000). As GluR2 carboxy-terminal PDZ domain interactions are regulated by PKC, we sought to determine if depression induced by the PKC activator PDA also requires clathrin-mediated endocytosis. Therefore, the aforementioned dynamin peptide (dynaminPRD, 100 μg/ml) was perfused into Purkinje cells prior to bath application of PDA. The dynamin PRD peptide, but not a scrambled control (dynCONTROL, 100 μg/ml) produced a strong blockade of PDA-induced depression (dynaminPRD: 90% ± 9% of baseline at t = 45 min, n = 4, dynCONTROL: 50% ± 9% of baseline, n = 4). Thus, LTD induced by quisqualate/depolarization conjunction and depression of AMPA responses by an exogenous PKC activator both require PICK1-GluR2/3 interactions and clathrin-mediated endocytosis.

Discussion

The main finding of this study is that treatments that block interactions between the carboxy-terminal PDZ binding motif of GluR2/3 and PICK1 strongly attenuate the expression of cerebellar LTD in cultured Purkinje cells. These manipulations include three different peptides based upon the GluR2 PDZ binding motif (but not an inactive control) and an antiserum directed against the amino-terminal PDZ domain of PICK1 (but not a preabsorbed control; see Figures 3 and 4). In addition, treatments that potentially interfere with PICK1-GluR2/3 multimerization blocked LTD induction (Figure 5). These treatments are likely to be exerting their effects through disruption of PICK1-GluR2/3 interactions rather than

side effects because they did not affect the basal function of AMPA receptors (Figure 2 and Table 1), voltage-gated Ca^{2+} channels or mGluR1 (Figure 6). Thus, blockade of PICK1-GluR2/3 interactions attenuates cerebellar LTD expression in a manner that is independent of the initial signals known to be required for its induction.

While the GluR2/3 carboxy-terminal PDZ binding motif can interact with both PICK1 and GRIP/ABP, the finding that two different treatments (phospho-SVKI peptide and anti-N-PICK1 antiserum) that block PICK1 but not GRIP/ABP interactions attenuate LTD (Figures 3 and 4) suggests that it is the binding of the former that is required. Blockade of the PICK1-GluR2/3 interaction attenuates LTD induced by quisqualate/depolarization conjunction (which has previously been shown to require PKC activation; Linden and Connor, 1991) and an LTD-like phenomenon produced by exogenous PKC-activating phorbol ester (Figure 7A). Both of these forms of depression require clathrin-mediated internalization of AMPA receptors (Wang and Linden, 2000; Figure 7B).

While the present data indicate a role for interactions between GluR2/3 and PICK1 in LTD induction, it should be cautioned that this does not mean that GRIP/ABP is not involved. A recent report has shown that when cultured hippocampal neurons are transfected with mutant GluR2 subunits that can bind PICK1 but not GRIP/ABP, these mutants subunits do not accumulate at synapses to the same degree as wild-type GluR2 (Osten et al., 2000). Furthermore, the observation that PKC-mediated phosphorylation of GluR2 Ser-880 results in a strong decrease in the binding of GRIP/ABP (Matsuda et al., 1999; Chung et al., 2000) suggests a scheme in which the release of GluR2/3 from GRIP/ABP is required for LTD induction. Unfortunately, it is difficult to test this idea directly without a method of stabilizing the GRIP/ABP-GluR2/3 interaction in living Purkinje cells.

A model of cerebellar LTD induction and expression based upon these observations is shown in Figure 8A. Conjoint activation of mGluR1, AMPA receptors and voltage-gated Ca^{2+} channels results in stimulation of PKC. PKC phosphorylates Ser-880 of GluR2 (and possibly other sites as well) resulting in both unbinding of GRIP/ABP and, through an unknown mechanism, the recruitment of PICK1 to synaptic AMPA receptors. PICK1 recruitment together with GRIP/ABP unbinding then primes AMPA receptors for clathrin-mediated internalization. Alternatively, GRIP/ABP unbinding is sufficient to prime synaptic AMPA receptors for internalization but synaptic strength is not depressed unless PICK1 binding to GluR2/3 also occurs, thereby stabilizing the internal pool and attenuating compensatory insertion of AMPA receptors (see Figure 8B). Importantly, in both of these models, application of reagents that block either PICK1-GluR2/3 or GRIP/ABP-GluR2/3 interactions (or both) would not be expected to alter the basal response of Purkinje cells to AMPA test pulses.

These models are consistent with certain recent observations including: (1) activation of PKC resulted in an increase in Ser-880-phosphorylated GluR2 in spines and the translocation of PICK1 immunoreactivity from dendritic shafts to spines in cultured hippocampal neurons (Chung et al., 2000), (2) PKC activation evoked GluR2 internalization in cerebellar (Matsuda et al., 2000), neocortical (Chung et al., 2000), and hippocampal neurons (H. J. Chung et al., submitted), and (3), in a heterologous expression system, mutation of GluR2 Ser-880 to alanine prevented phorbol ester–induced GluR2 internalization (H. J. Chung et al., submitted). However, there are several caveats that also should be mentioned. First, the present experiments were done using immature Purkinje cells in culture and so one must always be concerned that the cellular mechanisms underlying cerebellar LTD might be different in intact, adult tissue. Second, the prior observation that phorbol ester treatment of cerebellar cultures results in GluR2 internalization (Matsuda et al., 2000) is potentially complicated by that fact that these measures were made using immunoblots from cultures in which only about 2.5% of the neurons are Purkinje cells (Furuya et al., 1998). Third, while there is now reason to believe that AMPA receptor internalization plays a role in LTD expression, it would be premature to conclude that it can wholly account for it. Injection of a catalytically active fragment of PKC into cultured hippocampal neurons modulated the decay rate of AMPA-mEPSCs in hippocampal neurons (Wang et al., 1994) and it is not impossible to imagine that a similar mechanism or another utilizing alterations in AMPA receptor unitary conductance (Benke et al., 1998) might be operative in Purkinje cells.

Prior work using other model systems has implicated GluR2/3 interactions in use-dependent synaptic plasticity. In spinal dorsal horn neurons, serotonin can produce a form of LTP that appears to involve the recruiting of AMPA receptors to previously silent synapses (Li and Zhuo, 1998). Like cerebellar LTD, this potentiation can be blocked by postsynaptic PKC inhibitors or mimicked by PKC activators (Li et al., 1999). In addition, peptides based upon the GluR2 carboxy-terminal PDZ binding motif were used (they used 10 amino acid peptides as opposed to 15 in the present study). Postsynaptic application of SVKI peptide did not produce basal alterations in glutamatergic EPSCs but blocked LTP induced by serotonin or phorbol ester. However, in contrast to the present results, Li et al. (1999) found that EVKI peptide (which should be functionally equivalent to our phospho-SVKI) failed to block serotonin-induced LTP. Thus, serotonin-induced LTP in spinal dorsal horn appears to require GRIP/ABP-GluR2/3 rather than PICK1-GluR2/3 interactions supporting the idea that GRIP/ABP-GluR2/3 interactions may stabilize AMPA receptors in the postsynaptic plasma membrane.

GluR2/3 carboxy-terminal PDZ domain interactions have also been implicated in hippocampal LTD. H. J. Chung et al. (submitted) show that internal application of SVKI and phospho-SVKI GluR2 peptides, but not an SVKA control, attenuates LTD at the Schaffer collateral-CA1 synapse in a slice preparation. Furthermore, in separate experiments using field potential recording, LTD induction was associated with an increase in Ser-880-phosphorylated GluR2 as measured by Western blot. This increase was blocked by pretreatments that prevent this form of LTD such as an NMDA receptor antagonist or a protein phosphatase inhibitor. Taken together with previous results showing that both hippocampal and cerebellar LTD require clathrin-mediated endocytosis (Luscher et al., 1999; Man et al., 2000, Wang and Linden, 2000) and are associated with GluR2 internalization (Carroll et al., 1999a; Luscher et al., 1999; Man et

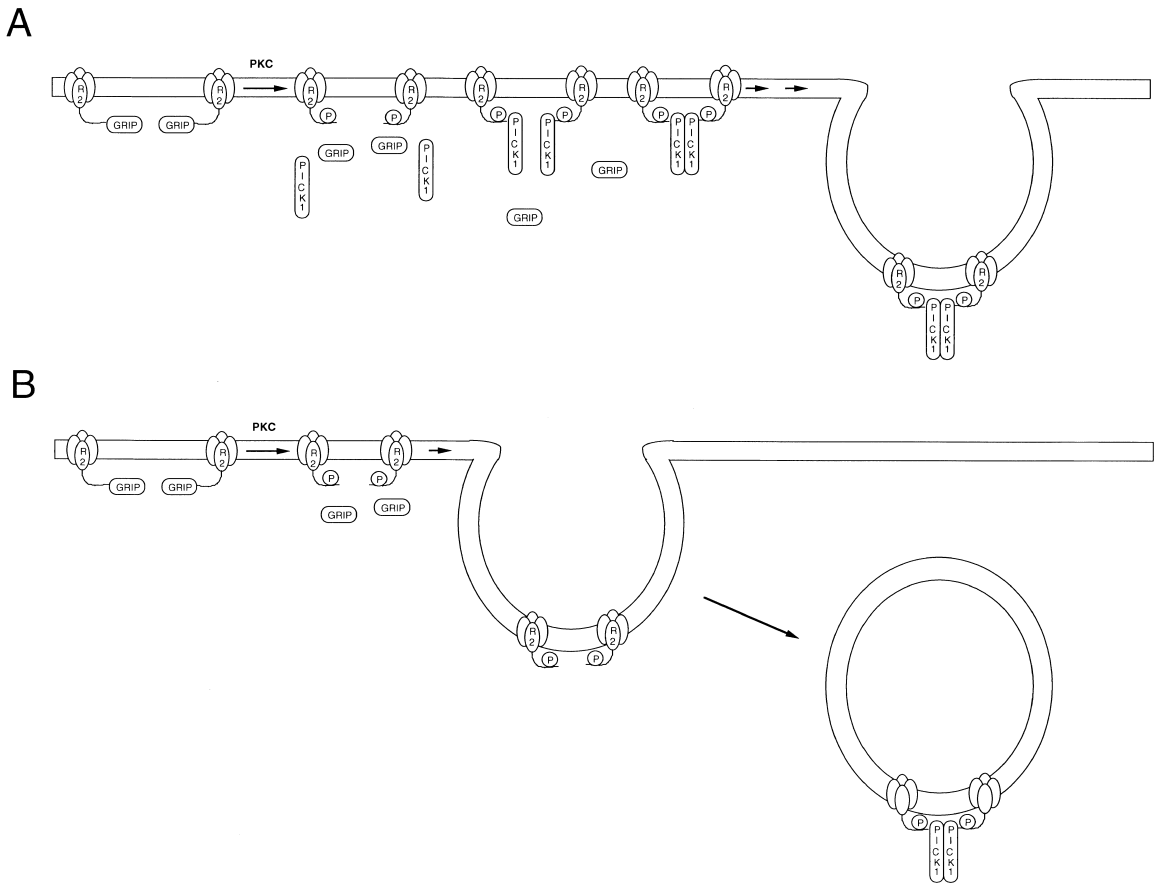

Figure 8. Two Models for Expression of LTD in Cultured Purkinje Cells

(A) Synaptic AMPA receptors containing at least one GluR2/3 subunit are bound to GRIP/ABP in the basal state. Activation of PKC during LTD induction results in the phosphorylation of GluR2 Ser-880, This causes the unbinding of GRIP/ABP and the recruitment of PICK1 to the synapse. PICK1 can multimerize through its coiled-coil domain. Synaptic GluR2/3 containing AMPA receptors in the GRIP/ABP-unbound, PICK1-bound state are then primed for internalization by clathrin-mediated endocytosis.

(B) A variant of the model above. In this scheme, GRIP/ABP unbinding is sufficient to prime synaptic AMPA receptors for internalization. However, PICK1 binds AMPA receptors in the internal pool, thereby stabilizing them and attenuating compensatory AMPA receptor insertion.

al., 2000; Matsuda et al., 2000), the present findings suggest that cerebellar and at least one form of hippocampal LTD may share aspects of a common expression mechanism. However, it should be cautioned that there are important differences as well. PKC-activating phorbol esters do not typically induce either LTD or a decrease in mEPSC amplitude at hippocampal synapses (Carroll et al., 1998). Likewise, while there are varieties of hippocampal LTD that require activation of an mGluR cascade (Stanton et al., 1991; Bolshakov and Siegelbaum, 1994; Yang et al., 1994) and consequent activation of PKC (Oliet et al., 1997; Otani and Connor, 1998; Wang et al., 1998), the more commonly studied form of hippocampal LTD requires activation of NMDA receptors and protein phosphatases and is independent of mGluR or PKC activation (see Bear and Linden, 2000, for review).

In addition to the PDZ binding motif at the extreme carboxyl terminus of GluR2, there is a binding site for the protein NSF between residues 844 and 853 (see Kim and Huganir, 1999; Braithwaite et al., 2000, for review). In recent years, several laboratories have used various manipulations to disrupt this interaction and measure the consequences for synaptic transmission in the hippocampus (see Kullmann, 1999; Luscher et al., 2000; Turrigiano, 2000, for review). While there have been some differences between experiments, in general, peptides designed to block the NSF-GluR2 interaction produce an attenuation of evoked AMPA receptor-mediated EPSCs as well as a reduction in the both the amplitude and frequency of AMPA-mEPSCs (Nishimune et al., 1998; Song et al., 1998; Luthi et al., 1999; Luscher et al., 1999; Noel et al., 1999). The latter presumably represents the complete "silencing" of a fraction of synapses and in consistent with the finding that this treatment causes a reduction in the surface expression of AMPA receptors as determined immunocytochemically (Luscher et al., 1999; Noel et al., 1999). Furthermore, the attenuation of synaptic transmission produced by these peptides occludes subsequent LTD induction at the hippocampal Schaffer collateral-CA1 synapse (Luscher et al., 1999; Luthi et al., 1999), and neither LTD induction nor NSF peptide application appeared to alter EPSC kinetics or AMPA receptor unitary conductance as esti-

mated using nonstationary fluctuation analysis (Luthi et al., 1999). These findings, together with experiments showing that blockers of clathrin-mediated endocytosis can attenuate hippocampal LTD (Luscher et al., 1999; Man et al., 2000) have suggested models in which NSF-mediated constitutive insertion of GluR2-containing AMPA receptors is balanced by both constitutive and regulated endocytosis of GluR2-containing AMPA receptors to control AMPA receptor number and thereby synaptic strength. At present, direct interactions between NSF-GluR2 binding and GRIP/ABP or PICK1-GluR2 binding have been hypothesized (Osten et al., 1998; Song et al., 1998) but have yet to be observed. The potential role of NSF-GluR2 binding in cerebellar LTD remains to be investigated.

There are several additional aspects of the present cerebellar LTD expression model that require further explication. First, the mechanism by which PICK1 is translocated to spine synapses following PKC activation (Chung et al., 2000) remains unclear. While this event is coincident with an increase in Ser-800-phosphorylated GluR2 immunoreactivity in spine synapses, there is no proof that they are causally related. Indeed, in vitro binding and coimmunoprecipitation experiments indicated that GluR2/PICK1 binding was not significantly affected by the phosphorylation state of Ser-880 (Chung et al., 2000). It is possible that PKC-mediated phosphorylation of other sites or other proteins is involved.

Second, how does protein phosphatase activity affect the phosphorylation of GluR2 Ser-880 and other PKC sites? Ajima and Ito (1995) showed that the phosphatase inhibitors microcystin-LR or calyculin A induced an LTD-like effect in cerebellar slices, an effect that was later confirmed in a cell culture preparation (Kasahara and Sugiyama, 1998). Furthermore, a signaling cascade involving NO/cGMP/PKG has been seen to be required for cerebellar LTD induction in many, but not all, preparations (Linden et al., 1995; see Daniel et al., 1998, Bear and Linden, 2000, for review). PKG has been suggested to exert its effect through the phosphorylation of G substrate, which, in its phosphorylated form, functions more effectively as a phosphatase inhibitor (Ito, 1990).

Third, does PICK1 binding to GluR2/3 prime AMPA receptors for clathrin mediated endocytosis (Figure 8A), or does it act subsequent to clathrin-mediated endocytosis to stabilize internal AMPA receptor pools (Figure 8B)? Little is known about the signaling functions of PICK1 that might shed light on either of these possible mechanisms. When PICK1 and GluR2 are coexpressed in a heterologous system, clustering of GluR2 is seen (Dev et al., 1999; Xia et al., 1999). However, it is unclear how clustering in a heterologous expression system relates to the functional status of synaptic AMPA receptors in neurons.

Recently, it has been shown that, in addition to the well-characterized LTD of the parallel fiber–Purkinje cell synapse, LTD of the climbing fiber–Purkinje cell synapse may also be seen (Hansel and Linden, 2000). The climbing fiber–Purkinje cell neuron also uses the transmitter glutamate and expresses postsynaptic GluR2/3 (Zhao et al., 1998). While these synapses are similar, at a molecular level they are not identical. In contrast to parallel fiber synapses, climbing fiber synapses lack both nNOS and GluRδ2. However, at present, no differences have emerged at the molecular level in the induction requirements for these two forms of LTD—both require mGluR1 activation, postsynaptic Ca^{2+} transients and PKC activation. As such, the present cell culture model system may be a model for one or both processes. It will be useful to determine whether the expression mechanism of climbing fiber LTD requires PICK1-GluR2/3 interaction and clathrin-mediated AMPA receptor internalization.

Finally, it should be noted that, like several other forms of synaptic plasticity, cerebellar LTD has a protein synthesis–dependent late phase that begins at about 1 hr (Linden, 1996; Ahn et al., 1999; Murashima and Hirano, 1999). While the late phase of LTD also appears to involve postsynaptic AMPA receptor downregulation (Linden, 1996; Murashima and Hirano, 1999), it is unclear if AMPA receptor number/distribution specifically plays a role, and if so, if it engages molecular mechanisms involving interactions between GluR2/3 and PDZ-domain containing proteins.

Experimental Procedures

Mouse embryonic cerebellar cultures were prepared as previously described (Linden et al., 1991). Cultures were maintained for 9–16 days in vitro prior to their use in whole-cell patch-clamp or Ca^{2+} imaging experiments. Patch electrodes attached to Purkinje cell somata were filled with a solution containing CsCl (135 mM), HEPES (10 mM), EGTA (0.5 mM), Na_2-ATP (4 mM), and Na-GTP (0.4 mM), adjusted to pH 7.35 with CsOH. Iontophoresis electrodes (1 μm tip diameter) were filled with 10 mM AMPA (in 10 mM HEPES [pH 7.1] with NaOH), 30 mM NMDA (in 10 mM HEPES [pH 7.3]), or 10 mM quisqualate (in 10 mM HEPES [pH 7.1]) and were positioned $\sim$20 μm away from large-caliber dendrites. Test pulses were delivered using negative current pulses (600–900 nA, 30–110 ms duration, 0.05 Hz). Cells were bathed in NaCl (140 mM), KCl (5 mM), $CaCl_2$ (2 mM), $MgCl_2$ (0.8 mM), HEPES (10 mM), glucose (10 mM), tetrodotoxin (0.0005 mM), and picrotoxin (0.2 mM), adjusted to pH 7.35 with NaOH, which flowed at a rate of 0.5 ml/min. Externally applied compounds were dissolved in a separate reservoir of external saline and were applied using a switching valve located upstream from the recording chamber. Patch electrodes were pulled from N51A glass and polished to yield a resistance of 3–5 MΩ. $V_{hold} = -80$ mV was imposed for experiments in which exogenous pulses of AMPA or glutamate were applied. To allow for the recording of NMDA currents, as in Figure 2, $[Mg^{2+}]_o$ was reduced to 0.2 mM, 20 μM glycine was added to the external saline, and V_{hold} was set to -50 mV. For recording AMPA-mEPSCs, V_{hold} was set to -90 mV (to increase driving force) and 100 μM D-AP5 was added to the external saline.

Membrane currents were recorded with an Axopatch 200A amplifier (Axon Instruments), lowpass filtered at 2 or 5 kHz, and digitized at 10 kHz using an ITC-16 interface (Instrutech) and a Macintosh computer running Axodata 1.2.2 software. Recordings in which R_{input} or R_{series} varied by more than 15% were excluded from the analysis. For analysis of AMPA-mEPSCs Axograph 4.2 mini analysis software was used, which detected events based on closeness of fit of the mEPSC to a template. Events smaller than -5 pA were discarded. A separate template was created for each recording by averaging $>$30 of its most unambiguous mEPSCs as selected by eye.

Bis-fura-2 ratio imaging of intracellular free Ca^{2+} was accomplished by measuring the background corrected fluorescence ratio at 340 and 380 nm excitation using a cooled CCD camera system as previously described (Narasimhan et al., 1998). In these experiments, EGTA was removed from the internal saline and replaced with 100 μM bis-fura-2. In a subset of experiments examining quisqualate-evoked Ca^{2+} mobilization, Ca^{2+} was removed from the external saline and was replaced with 0.2 mM EGTA. All electrophysiological experiments were conducted at room temperature.

For GST fusion protein expression and purification, wild-type and mutant rat PICK1 were subcloned into glutathione S-transferase fusion protein expression vector pGEX4T2. PCR primers containing

the desired PICK1 mutations were synthesized using full-length rat PICK1 cDNA as template for mutants generation. PCR mutagenesis were performed using a Quikchange site-directed mutagenesis kit (Stratagene). All mutations were subsequently confirmed by sequencing. To create the ΔPDZ mutation, lysine and aspartic acid at amino acids 27 and 28 of PICK1 were replaced with alanine. The ΔCC mutation was made by deleting amino acids 139–166 of PICK1. All constructs were then transformed to *E. coli* BL21 cells to produce GST fusion proteins. After IPTG induction, GST fusion proteins were liberated by sonication and solubilized by 1% Triton X-100. Glutathione Sepharose 4B (Pharmacia Biotech) was added to the cleared cell lysates. After washing, GST fusion proteins were eluted by reduced Glutathione. Purified GST fusion proteins were then dialyzed against internal solution. Fusion protein concentration was determined by reading of O.D. 280 nm against the standard curve.

The dynamin I proline-rich domain peptide (QVPSRPNRAP) and the dynamin scrambled control peptide (QPPASNPRVR) were synthesized and purified at the Biosynthesis and Sequencing Facility, Department of Biological Chemistry, Johns Hopkins University School of Medicine. The GluR2 peptides (KEGYNVYGIESVKI, KEG YNVYGIESGKA, KEGYNVYGIESVAI, and KEGYNVYGIES-[PO_4]-VKI) were synthesized and purified at the Howard Hughes Medical Institute Biopolymer Facility at Johns Hopkins University School of Medicine. The PICK1 antiserum was generated in rabbits against an N-terminal peptide as previously described (Xia et al., 1999). Bis-fura-2 was from Molecular Probes, tetrodotoxin from Alexis Biochemicals, AMPA, NMDA, and D-AP5 from Tocris, and phorbol-12,13-diacetate was from LC Laboratories. All other reagents were from Sigma.

Immunohistochemistry was performed as described in Dong et al. (1999) with small variations. Adult male Sprague-Dawley rats were anesthetized with pentobarbital and transcardially perfused with phosphate buffered saline (PBS), followed by cold 4% paraformaldehyde in PBS. Following removal, brains were postfixed for 2 days and cryoprotected overnight at 4°C in 30% sucrose/PBS. Sagittal sections (40 μm thick) were washed four times and then blocked for 1 hr at room temperature in a PBS solution containing 0.2%–0.3% Triton and 2%–8% normal goat serum. Blocking solution for sections to be stained for GluR2/3, phospho-GluR2/3, or GRIP1 contained 2%–3% bovine serum albumin, while that for PICK1 contained 5% nonfat dry milk. Sections were incubated with primary polyclonal antisera from rabbits for 2–3 days at 4°C. The generation of antisera directed against GluR2/3 (Dong et al., 1997), phospho-GluR2/3 (Chung et al., 2000), GRIP1/ABP (Dong et al., 1999), and PICK1 (Xia et al., 1999) have previously been described. Control sections were incubated with antisera preabsorbed with an excess of the peptide originally used for their generation. Sections were rinsed three times in PBS and then incubated in biotinylated goat anti-rabbit IgG 1:200 in PBS with 1.5% normal goat serum and processed with a Vectastain IgG Elite Kit for rabbit (Vector Labs). Horseradish peroxidase immunostaining was visualized using diaminobenzidine as a substrate. Sections were viewed using a Zeiss Axioskop and images were acquired with a digital CCD camera and Metamorph software (Universal Imaging).

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References

Ahn, S., Ginty, D.D., and Linden, D.J. (1999). A late phase of cerebellar long-term depression requires activation of CaMKIV and CREB. Neuron *23*, 559–568.

Ajima, A., and Ito, M. (1995). A unique role of protein phosphatases in cerebellar long-term depression. Neuroreport *6*, 297–300.

Bear, M.F., and Linden, D.J. (2000). The mechanisms and meaning of long-term synaptic depression. In The Synapse, W.M. Cowan and K. Davies, eds. (Baltimore, MD: Johns Hopkins University Press), pp. 455–517.

Benke, T.A., Luthi, A., Isaac, J.T., and Collingridge, G.L. (1998). Modulation of AMPA receptor unitary conductance by synaptic activity. Nature *393*, 793–797.

Bolshakov, V.Y., and Siegelbaum, S.A. (1994). Postsynaptic induction and presynaptic expression of hippocampal long-term depression. Science *264*, 1148–1152.

Braithwaite, S.P., Meyer, G., and Henley, J.M. (2000). Interactions between AMPA receptors and intracellular proteins. Neuropharmacology *39*, 919–930.

Burette, A., Wyszynski, M., Valtschanoff, J.G., Sheng, M., and Weinberg, R.J. (1999). Characterization of glutamate receptor interacting protein-immunopositive neurons in cerebellum and cerebral cortex of the albino rat. J. Comp. Neurol. *411*, 601–612.

Carroll, R.C., Nicoll, R.A., and Malenka, R.C. (1998). Effects of PKA and PKC on miniature excitatory postsynaptic currents in CA1 pyramidal cells. J. Neurophysiol. *80*, 2797–2800.

Carroll, R.C., Lissin, D.V., von Zastrow, M., Nicoll, R.A., and Malenka, R.C. (1999a). Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures. Nat. Neurosci. *2*, 454–460.

Carroll, R.C., Beattie, E.C., Xia, H., Luscher, C., Altschuler, Y., Nicoll, R.A., Malenka, R.C., and von Zastrow, M. (1999b). Dynamin-dependent endocytosis of ionotropic glutamate receptors. Proc. Natl. Acad. Sci. USA *96*, 14112–14117.

Chung, H.J., Xia, J., Scannevin, R.H., Zhang, X., and Huganir R.L. (2000). Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain–containing proteins. J. Neurosci. *20*, 7258–7267.

Crepel, F., and Krupa, M. (1988). Activation of protein kinase C induces a long-term depression of glutamate sensitivity of cerebellar Purkinje cells. An *in vitro* study. Brain Res. *458*, 397–401.

Daniel, H., Levenes, C., and Crepel, F. (1998). Cellular mechanisms of cerebellar LTD. Trends Neurosci. *21*, 401–407.

Dev, K.K., Nishimune, A., Henley, J.M., and Nakanishi, S. (1999). The protein kinase Cα binding protein PICK1 interacts with short but not long form alternative splice variants of AMPA receptor subunits. Neuropharmacology *38*, 635–644.

De Zeeuw, C.I., Hansel, C., Bian, F., Koekkoek, S.K.E., van Alphen, A.M., Linden, D.J., and Oberdick, J. (1998). Expression of a protein kinase C inhibitor in Purkinje cells blocks cerebellar long-term depression and adaptation of the vestibulo-ocular reflex. Neuron *20*, 495–508.

Dong, H., O'Brien, R.J., Fung, E.T., Lanahan, A.A., Worley, P.F., and Huganir, R.L. (1997). GRIP: a synaptic PDZ domain–containing protein that interacts with AMPA receptors. Nature *386*, 279–284.

Dong, H., Zhang, P., Song, I., Petralia, R.S., Liao, D., and Huganir, R.L. (1999). Characterization of the glutamate receptor-interacting proteins GRIP1 and GRIP2. J. Neurosci. *19*, 6930–6941.

Freeman, J.H., Shi, T., and Schreurs, B.G. (1998). Pairing-specific long-term depression prevented by blockade of PKC or intracellular Ca. Neuroreport *9*, 2237–2241.

Furuya, S., Makino, A., and Hirabayashi, Y. (1998). An improved method for culturing cerebellar Purkinje cells with differentiated dendrites under a mixed monolayer setting. Brain Res. Protocols *3*, 192–198.

Hansel, C., and Linden, D.J. (2000). Long-term depression of the cerebellar climbing fiber–Purkinje neuron synapse. Neuron *26*, 473–482.

Hartell, N.A. (1994). cGMP acts within cerebellar Purkinje cells to produce long-term depression via mechanisms involving PKC and PKG. Neuroreport *5*, 833–836.

Ito, M. (1989). Long-term depression. Ann. Rev. Neurosci. *12*, 85–102.

Ito, M. (1990). Long-term depression in the cerebellum. Seminars Neurosci. *2*, 381–390.

Ito, M., Sakurai, M., and Tongroach, P. (1982). Climbing fibre induced

depression of both mossy fiber responsiveness and glutamate sensitivity of cerebellar Purkinje cells. J. Physiol. *324*, 113–134.

Kasahara, J., and Sugiyama, H. (1998). Modulation of glutamate sensitivities by inhibitors of protein kinase and a protein phosphatase in cultured rat Purkinje cells. Neurosci. Lett. *247*, 139–142.

Kim, J.H., and Huganir, R.L. (1999). Organization and regulation of proteins at synapses. Curr. Opin. Cell Biol. *11*, 248–254.

Kullmann, D.M. (1999). AMPA receptor attrition in long-term depression. Neuron *24*, 288–290.

Landsend, A.S., Amiry-Moghaddam, M., Matsubara, A., Bergersen, L., Usami, S.-I., Wenthold, R.J., and Ottersen, O.P. (1997). Differential localization of δ glutamate receptors in the rat cerebellum: coexpression with AMPA receptors in parallel fiber-spine synapses and absence from climbing fiber-spine synapses. J. Neurosci. *17*, 834–842.

Li, P., and Zhou, M. (1998). Silent glutamatergic synapses and nociception in mammalian spinal cord. Nature *393*, 695–698.

Li, P., Kerchner, G.A., Sala, C., Wei, F., Huettner, J.E., Sheng, M., and Zhou, M. (1999). AMPA receptor-PDZ interactions in facilitation of spinal sensory synapses. Nat. Neurosci. *2*, 972–977.

Linden, D.J. (1994). Input-specific induction of cerebellar long-term depression does not require presynaptic alteration. Learn. Mem. *1*, 121–128.

Linden, D.J. (1996). A protein synthesis-dependent late phase of cerebellar long-term depression. Neuron *17*, 483–490.

Linden, D.J., and Connor, J.A. (1991). Participation of postsynaptic PKC in cerebellar long-term depression in culture. Science *254*, 1656–1659.

Linden, D.J., Dickinson, M.H., Smeyne, M., and Connor, J.A. (1991). A long-term depression of AMPA currents in cultured cerebellar Purkinje neurons. Neuron *7*, 81–89.

Linden, D.J., Smeyne, M., and Connor, J.A. (1993). Induction of cerebellar long-term depression in culture requires postsynaptic action of sodium ions. Neuron *11*, 1093–1100.

Linden, D.J., Dawson, T.M., and Dawson, V.L. (1995). An evaluation of the nitric oxide/cGMP/ cGMP-dependent protein kinase cascade in the induction of cerebellar long-term depression in culture. J. Neurosci. *15*, 5098–5105.

Luscher, C., Xia, H., Beattie, E.C., Carroll, R.C., von Zastrow, M., Malenka, R.C., and Nicoll, R.A. (1999). Role of AMPA receptor cycling in synaptic transmission and plasticity. Neuron *24*, 649–658.

Luscher, C., Nicoll, R.A., Malenka, R.C., and Muller, D. (2000). Synaptic plasticity and dynamic modulation of the postsynaptic membrane. Nat. Neurosci. *3*, 545–550.

Luthi, A., Chittajallu, R., Duprat, F., Palmer, M.J., Benke, T.A., Kidd, F.L., Henley, J.M., Isaac, J.T., and Collingridge, G.L. (1999). Hippocampal LTD expression involves a pool of AMPARs regulated by the NSF-GluR2 interaction. Neuron. *24*, 389–399.

Man, H.Y., Lin, J.W., Ju, W.H., Ahmadian, G., Liu, L., Becker, L.E., Sheng, M., and Wang, Y.T. (2000). Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. Neuron *25*, 649–662.

Matsuda, S., Mikawa, S., and Hirai, H. (1999). Phosphorylation of serine-880 in GluR2 by protein kinase C prevents its C terminus from binding with glutamate receptor-interacting protein. J. Neurochem. *73*, 1765–1768.

Matsuda, S., Launey, T., Mikawa, S., and Hirai, H. (2000). Disruption of AMPA receptor GluR2 clusters following long-term depression induction in cerebellar neurons. EMBO J. *19*, 2765–2774.

Mauk, M.D. (1997). Roles of cerebellar cortex and nuclei in motor learning: contradictions or clues? Neuron *18*, 343–346.

Murashima, M., and Hirano, T. (1999). Entire course and distinct phases of day-lasting depression of miniature EPSC amplitudes in cultured Purkinje neurons. J. Neurosci. *19*, 7326–7333.

Narasimhan, K., and Linden, D.J. (1996). Defining a minimal computational unit for cerebellar long-term depression. Neuron *17*, 333–341.

Narasimhan, K., Pessah, I.N., and Linden, D.J. (1998). Inositol-1,4,5-trisphosphate receptor-mediated Ca mobilization is not required for cerebellar long-term depression in reduced preparations. J. Neurophysiol. *80*, 2963–2974.

Nishimune, A., Isaac, J.T., Molnar, E., Noel, J., Nash, S.R., Tagaya, M., Collingridge, G.L., Nakanishi, S., and Henley, J.M. (1998). NSF binding to GluR2 regulates synaptic transmission. Neuron *21*, 87–97.

Noel, J., Ralph, G.S., Pickard, L., Williams, J., Molnar, E., Uney, J.B., Collingridge, G.L., and Henley, J.M. (1999). Surface expression of AMPA receptors in hippocampal neurons is regulated by an NSF-dependent mechanism. Neuron *23*, 365–376.

Oliet, S.H., Malenka, R.C., and Nicoll, R.A. (1997). Two distinct forms of long-term depression coexist in CA1 hippocampal pyramidal cells. Neuron *18*, 969–982.

Osten, P., Srivastava, S., Inman, G.J., Vilim, F.S., Khatri, L., Lee, L.M., States, B.A., Einheber, S., Milner, T.A., Hanson, P.I., and Ziff, E.B. (1998). The AMPA receptor GluR2 C terminus can mediate a reversible, ATP-dependent interaction with NSF and α- and β-SNAPs. Neuron. *21*, 99–110.

Osten, P., Khatri, L., Perez, J.L., Kohr, G., Giese, G., Daly, C., Schulz, T.W., Wensky, A., Lee, L.M., and Ziff, E.B. (2000). Mutagenesis reveals a role for ABP/GRIP binding to GluR2 in synaptic surface accumulation of the AMPA receptor. Neuron *27*, 313–325.

Otani, S., and Connor, J.A. (1998). Requirement of rapid Ca^{2+} entry and synaptic activation of metabotropic glutamate receptors for the induction of long-term depression in adult rat hippocampus. J. Physiol. *511*, 761–770.

Petralia, R.S., and Wenthold, R.J. (1992). Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. J. Comp. Neurol. *318*, 329–354.

Song, I., Kamboj, S., Xia, J., Dong, H., Liao, D., and Huganir, R.L. (1998). Interaction of the N-ethylmaleimide-sensitive factor with AMPA receptors. Neuron. *21*, 393–400.

Stanton, P.K., Chattarji, S., and Sejnowski, T.J. (1991). 2-Amino-3-phosphonopropionic acid, an inhibitor of glutamate-stimulated phosphoinositide turnover, blocks induction of homosynaptic long-term depression, but not potentiation, in rat hippocampus. Neurosci. Lett. *127*, 61–66.

Turrigiano, G.G. (2000). AMPA receptors unbound: membrane cycling and synaptic plasticity. Neuron *26*, 5–8.

Wang, Y.-T., and Linden, D.J. (2000). Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. Neuron *25*, 635–664.

Wang, L.-Y., Dudek, E.M., Browning, M.D., and MacDonald, J.F. (1994). Modulation of AMPA/kainate receptors in cultured murine hippocampal neurons by protein kinase C. J. Physiol. *475*, 431–437.

Wang, Y., Wu, J., Rowan, M.J., and Anwyl, R. (1998). Role of protein kinase C in the induction of homosynaptic long-term depression by brief low frequency stimulation in the dentate gyrus of the rat hippocampus in vitro. J. Physiol. *513*, 467–475.

Wyszynski, M., Kim, E., Yang, F.-C., and Sheng, M. (1998). Biochemical and immunocytochemical characterization of GRIP, a putative AMPA receptor anchoring protein, in the rat brain. Neuropharmacology *37*, 1335–1344.

Xia, J., Zhang, X., Staudinger, J., and Huganir, R.L. (1999). Clustering of AMPA receptors by the synaptic PDZ domain–containing protein PICK1. Neuron *22*, 179–187.

Yang, X.D., Connor, J.A., and Faber, D.S. (1994). Weak excitation and simultaneous inhibition induce long-term depression in hippocampal CA1 neurons. J. Neurophysiol. *71*, 1586–1590.

Zhao, H.M., Wenthold, W., and Petralia, R.S. (1998). Glutamate receptor targeting to synaptic populations on Purkinje cells is developmentally regulated. J. Neurosci. *18*, 5517–5528.