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Expression of Cerebellar Long-Term Depression Requires Postsynaptic Clathrin-Mediated Endocytosis

Yu Tian Wang* and David J. Linden†‡
*Programme in Brain and Behaviour and
Division of Pathology
Hospital for Sick Children
555 University Avenue
Toronto, Ontario M5G 1X8
Canada
†Department of Neuroscience
Johns Hopkins University School
of Medicine
Baltimore, Maryland 21205

Summary

Cerebellar long-term depression (LTD) is a cellular model system of information storage that may underlie certain forms of motor learning. While cerebellar LTD is expressed as a selective modification of postsynaptic AMPA receptors, this might involve changes in receptor number/distribution, unitary conductance, kinetics, or glutamate affinity. The observation that GluR2-containing synaptic AMPA receptors could be internalized by regulated clathrin-mediated endocytosis suggested that this process could underlie LTD expression. To test this hypothesis, we postsynaptically applied dynamin and amphiphysin peptides that interfere with the clathrin endocytotic complex and found that they blocked LTD expression in cultured Purkinje neurons. In addition, induction of LTD and attenuation of AMPA responses by stimulation of clathrin-mediated endocytosis occluded each other. These findings suggest that the expression of cerebellar LTD requires clathrin-mediated internalization of postsynaptic AMPA receptors.

Introduction

Long-term synaptic depression (LTD) is a use-dependent reduction of synaptic strength that, together with the converse process, long-term potentiation (LTP), has been suggested to underlie information storage in the brain and refinement of neural circuits during development. One particular form of LTD, found in the cerebellum, has been suggested to contribute to certain forms of motor learning, such as associative eyeblink conditioning and adaptation of the vestibulo-ocular reflex (Ito, 1989; Raymond et al., 1996). In cerebellar LTD, a persistent, input-specific attenuation of the parallel fiber–Purkinje neuron synapse is produced when parallel fiber and climbing fiber inputs to a Purkinje neuron are stimulated together at low frequency. Cerebellar LTD is generally understood to have three initial requirements for induction, all of which are postsynaptic: Ca^{2+} influx through voltage-gated channels triggered by the climbing fiber excitatory postsynaptic potential (EPSP), and mGluR1 and AMPA receptor activation produced by activation of parallel fibers. Cerebellar Purkinje neurons do not express functional NMDA receptors after the first 2 weeks of postnatal life, and at no point are they required for cerebellar LTD. The three initial signals are followed by stimulation of postsynaptic protein kinase C (PKC), the brief activation of which is also required. In addition, involvement of a nitric oxide/cGMP pathway in cerebellar LTD induction has been suggested (see Daniel et al., 1998, for review).

In contrast to most other model systems of synaptic plasticity, there seems to be general agreement that cerebellar LTD is expressed postsynaptically as an attenuation of AMPA receptors. In place of test shocks delivered to the parallel fibers, cerebellar LTD may be detected as a decrease in Purkinje neuron responses to exogenous pulses of glutamate (Ito et al., 1982; Crepel and Krupa, 1988). Using cultured mouse Purkinje neurons bathed in tetrodotoxin, simplified postsynaptic preparations have been developed for the study of cerebellar LTD in which iontophoretic glutamate pulses and Purkinje neuron depolarization are substituted for parallel fiber and climbing fiber stimulation, respectively. LTD induced in this manner may be seen as a reduction of the AMPA-evoked current, as measured with a patch electrode attached to the Purkinje neuron soma (Linden et al., 1991; Linden and Connor, 1991). These experiments have been extended with ultrareduced preparations that completely lack functional presynaptic terminals (Linden, 1994), including outside-out dendritic macropatches and acutely dissociated Purkinje neurons (Narasimhan and Linden, 1996; Narasimhan et al., 1998), which provide definitive evidence for a postsynaptic locus of expression.

The attenuation of postsynaptic AMPA receptor function that underlies cerebellar LTD could potentially result from several factors (or a combination thereof). These include changes in receptor number/distribution, unitary conductance, kinetics, or glutamate affinity. At present, there is very little evidence that would allow one to distinguish between these possibilities. Some investigators have suggested that cerebellar LTD is expressed as an increase in the kinetics of AMPA receptor desensitization based on interactions with the drug aniracetam (Hemart et al., 1994), but this seems unlikely, as parallel fiber excitatory postsynaptic currents (EPSCs) do not change their shape after LTD induction (see Khodakhah and Armstrong, 1997, for a nice example).

Internal plasma membrane proteins have their number/distribution regulated at the cell surface through a balance of delivery (via membrane fusion) and internalization (via endocytosis). Many (but not all) of these proteins undergo endocytosis, which is dependent upon the formation of clathrin-coated pits and the subsequent recruitment of a protein complex, including an adapter protein (AP2 in the case of many neuronal proteins, β-arrestin in the case of seven transmembrane domain receptors), the SH3 domain–containing protein amphiphysin, and its binding partner, the multidomain GTPase dynamin (see Schmid et al., 1998; Marsh and McMahon,

‡To whom correspondence should be addressed (e-mail: dlinden@jhmi.edu).

1999, for review). Recent reports have shown that GluR2-containing AMPA receptors at the postsynaptic plasma membrane of hippocampal neurons are rapidly and selectively internalized in a manner that requires this clathrin complex (Carroll et al., 1999b; Lüscher et al., 1999; Man et al., 2000 [this issue of *Neuron*]). To test the hypothesis that the expression of cerebellar LTD involves regulation of AMPA receptor number/distribution through clathrin-mediated endocytosis, we have utilized whole-cell patch-clamp recording in cultured cerebellar Purkinje neurons, together with reagents, to activate or inhibit this form of receptor internalization.

Results

Man et al. (2000) have shown that brief application of insulin can specifically induce clathrin-mediated internalization of AMPA receptors. Importantly, insulin did not induce internalization of either NMDA receptors or AMPA receptors that lacked the GluR2 subunit. These experiments were performed using immunocytochemical measurements in a heterologous expression system (HEK293 cells) and cultured hippocampal neurons, as well as measurements of EPSCs at Schaffer collateral–CA1 pyramidal cell synapses in hippocampal slices. Thus, our first task was to attempt to replicate the basic electrophysiological findings using the cerebellar Purkinje neuron in culture. While mature Purkinje neurons do not express functional NMDA receptors, they are found on both cultured embryonic Purkinje neurons and acutely dissociated Purkinje neurons in early postnatal life (Linden and Connor, 1991; Rosenmund et al., 1992). Purkinje neurons also express AMPA receptors of the GluR2-containing, Ca^{2+}-impermeable variety (Linden et al., 1993; Tempia et al., 1996), as well as receptors for insulin-like growth factor–1 (IGF-1, also known as somatomedin C; Werther et al., 1990; Bondy et al., 1992; Garcia-Segura et al., 1997; Sherrard et al., 1997). The IGF-1 receptor binds both insulin and IGF-1, although the latter is bound with ~100-fold greater affinity. Insulin and IGF-1 receptors are highly homologous and seem to couple to the same signaling cascades through an intracellular tyrosine kinase domain (see Heidenreich, 1993, for review).

Whole-cell voltage-clamp recordings were made from Purkinje neurons in cultures derived from embryonic mouse cerebellum. Alternating iontophoretic test pulses of AMPA and NMDA were delivered to the same site on the proximal dendrite of a Purkinje neuron 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. Following acquisition of baseline responses, either insulin (500 nM) or IGF-1 (50 ng/ml) was applied externally and was maintained for the duration of the recording (Figure 1A). These treatments induced an attenuation of AMPA currents (insulin: 59% ± 8% of baseline at t = 40 min, mean ± SEM, n = 6; IGF-1: 52% ± 8% of baseline, n = 7) but not NMDA currents (insulin: 103% ± 7%; IGF-1: 105% ± 7%), which reached a steady-state level within ~20 min and then persisted for the duration of the recording. Pilot experiments indicated that similar results were produced with IGF-1 at a concentration of 150 ng/ml (data not shown). Figure 1B illustrates that a 10 min long exposure to insulin or IGF-1 produced an attenuation of AMPA current similar to that seen with continuous exposure (insulin: 62% ± 8% of baseline at t = 40 min, n = 5; IGF-1: 54% ± 9%, n = 6). Neither compound produced a significant alteration of R_{series} or R_{input}, as assessed using voltage steps from −80 to −90 mV, monitored during application, at t = 8 min (insulin: 103% ± 4% of baseline, n = 5; IGF-1: 100% ± 5%, n = 6) or after application at t = 33 min (insulin: 99% ± 5%; IGF-1: 103% ± 7%). Current–voltage relations of the AMPA-evoked currents plotted at t = −7 min and t = 36 min showed no significant alteration in either reversal potential (insulin: −2 ± 4 mV [pre], −4 ± 5 mV [post]; IGF-1: −5 ± 3 mV [pre], −5 ± 4 mV [post]) or rectification (data not shown) following insulin or IGF-1 treatment.

Several experiments were performed to determine whether the attenuation of AMPA currents by IGF-1 was mediated by activation of IGF-1 receptors (Figure 2A). Cultures were pretreated with external application of a monoclonal antiserum directed against the IGF-1 receptor (3 μg/ml), which has been shown to block IGF-1 binding (Kull et al., 1983). This treatment, but not preincubation with preimmune mouse immunoglobulin G (IgG) as a control, completely blocked the effect of IGF-1 (anti-IGF-1-R: 105% ± 7% of baseline at t = 40 min, n = 6; control IgG: 53% ± 10%, n = 7). A similar blockade (99% ± 6%, n = 5) was also seen using pretreatment with a peptide (CYAAPLPAKSC, 1 μg/ml) reported to block IGF-1 receptor activation (Pietrzkowski et al., 1992). Finally, inclusion in the internal saline of an inactivating monoclonal antiserum directed against the tyrosine kinase domain of the insulin receptor (Morgan and Roth, 1987) also produced a near complete blockade of the IGF-1 effect on AMPA currents (95% ± 6%, n = 5), as the tyrosine kinase domains of the insulin and IGF-1 receptors are highly conserved (Heidenreich, 1993). Taken together, these experiments indicate that the rapid attenuation of AMPA currents by IGF-1 is mediated by IGF-1 receptor activation.

Does IGF-1 receptor activation attenuate AMPA currents through stimulation of clathrin-mediated endocytosis? Unfortunately, while insulin/IGF-1 receptor activation is known to activate this process, the intermediate signals comprising this activation have yet to be clearly defined. There are, however, several tools available for inhibiting clathrin-mediated endocytosis, and we have used them to address this question (Figure 2B). First, a high concentration of Ca^{2+} chelator (bis-[o-aminophenoxy]-N,N,N′,N′-tetraacetic acid [BAPTA], 20 mM) was added to the internal saline. This treatment, which has been shown to block clathrin-mediated endocytosis in rat brain synaptosomes (Marks and McMahon, 1998) resulted in a near complete blockade of the IGF-1-induced attenuation of AMPA currents (90% ± 7% of baseline at t = 40 min, n = 6). Second, the internal saline was supplemented with a glutathione S-transferase fusion peptide derived from the SH3 domain of amphiphysin, the region that binds the proline-rich domain of dynamin I (Grabs et al., 1997). Introduction of this peptide, but not a mutant control peptide, into the presynaptic terminal of the lamprey giant reticulospinal synapse has previously been shown to result in a dramatic impairment of clathrin-mediated synaptic vesicle endocytosis

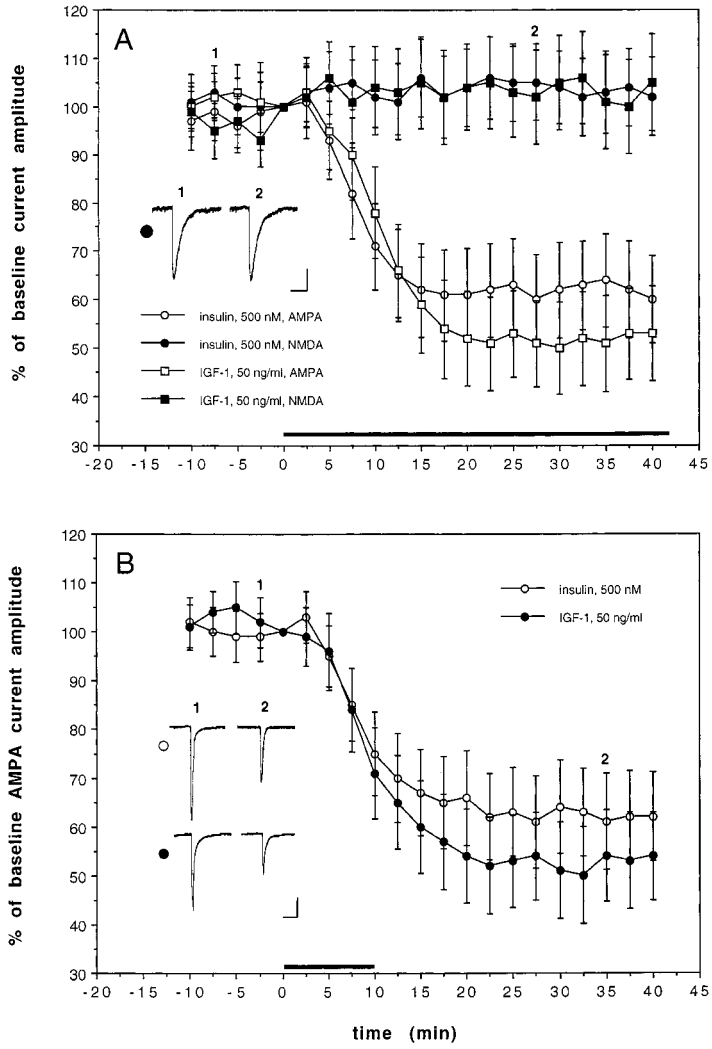

Figure 1. Insulin and IGF-1 Selectively Attenuate AMPA Receptor–Mediated Currents in Cultured Cerebellar Purkinje Neurons

(A) Alternating iontophoretic pulses of AMPA and NMDA were applied to the same dendritic location. $[Mg]_o$ 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. Following acquisition of baseline responses, either insulin (500 nM, n = 6) or IGF-1 (50 ng/ml, n = 7) was applied starting at t = 0 min, as indicated by the horizontal bar. Each point represents the mean ± SEM. Representative current traces were acquired at the times indicated on the graph. Scale bars, 20 pA, 2 s.

(B) Brief application (t = 0–10 min) of either insulin (500 nM, n = 5) or IGF-1 (50 ng/ml, n = 6) produced a sustained attenuation of AMPA current amplitude. $[Mg]_o$ = 0.8 mM and V_{hold} = −80 mV for this and all subsequent experiments, except where noted. Scale bars, 40 pA, 1 s.

(Shupliakov et al., 1997). In the present case, postsynaptic application of amphiphysin SH3 domain peptide (100 μg/ml), but not an inactive form containing two mutations in the dynamin-binding domain (100 μg/ml), strongly impaired the effect of IGF-1 on AMPA currents (AmphSH3: 87% ± 9%, n = 5; AmphSH3mut: 64% ± 10%, n = 5). Third, the internal saline was supplemented with a synthetic peptide derived from the proline-rich domain of dynamin I (Grabs et al., 1997). This peptide, but not a scrambled control peptide, impaired clathrin-mediated synaptic vesicle endocytosis in both the lamprey reticulospinal synapse (Shupliakov et al., 1997) and a rat brain synaptic vesicle preparation (Marks and McMahon, 1998). In the cultured Purkinje neuron, the dynamin proline-rich domain peptide (QVPSRPNRAP, 100 μg/ml) blocked the effect of IGF-1 (93% ± 8% of baseline, n = 7), but the scrambled control peptide (QPPASNPRVR, 100 μg/ml) did not (57% ± 8%, n = 5). These three results indicate that postsynaptic clathrin-mediated endocytosis is required for the rapid attenuation of AMPA currents that is produced by IGF-1 receptor activation. Further experiments using transient transfection with a dominant-negative dynamin construct (as performed in Man et al., 2000) could not be completed, as this treatment rendered the Purkinje neurons insufficiently healthy for either whole-cell or perforated-patch recording.

Recall that the clathrin-mediated internalization of AMPA receptors in a heterologous expression system required the presence of the GluR2 subunit (Man et al., 2000). Cultured cerebellar glial cells have robust responses to AMPA receptor agonists but do not express GluR2 (Burnashev et al., 1992; Linden, 1997). Cerebellar glia do express IGF-1 receptors (Garcia-Segura et al., 1997). Thus, as a further control for possible nonspecific effects of IGF-1, this compound was applied to cerebellar glial cells. Application of IGF-1 in a manner that produced strong attenuation of AMPA responses in Purkinje neurons (50 ng/ml, t = 0–10 min) failed to produce either a transient (101% ± 6% of baseline at t = 10 min, n = 5) or a sustained (98% ± 8% of baseline at t = 40 min) alteration. This failure is likely to reflect the absence of GluR2 in these cells, but it also could result from the absence of a portion of the signaling mechanisms that couple IGF-1 receptor activation to the activation of clathrin-mediated endocytosis.

While experiments that utilize exogenous AMPA test pulses are useful in that they allow for the analysis of

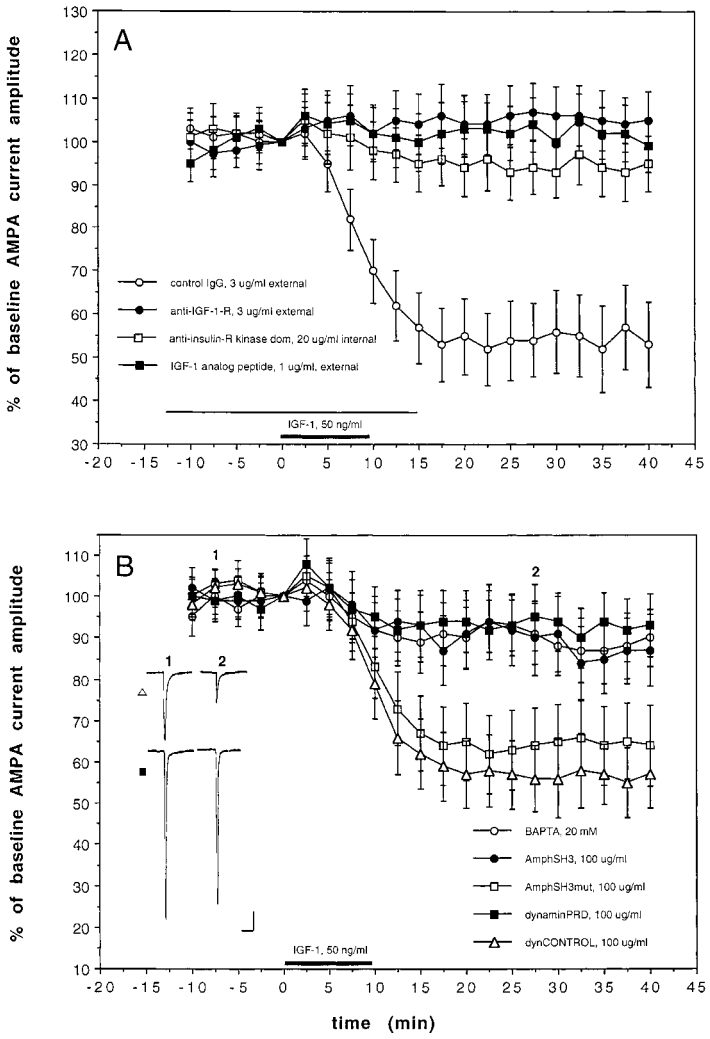

Figure 2. The Attenuation of AMPA Receptor–Mediated Currents by IGF-1 Is Blocked by Inhibitors of IGF-1 Signaling and Clathrin-Dependent Endocytosis

(A) Attenuation of AMPA receptor–mediated currents by brief application IGF-1 (50 ng/ml, t = 0–10 min, heavy horizontal bar) was blocked by external application of an inhibitory IGF-1 analog peptide (1 μg/ml, n = 5) or an antibody directed against the external portion of the IGF-1 receptor (3 μg/ml, n = 6) but not control mouse IgG (3 μg/ml, n = 7). These compounds were applied from t = −15 to 15 min, as indicated by the light horizontal bar. Inclusion in the patch-pipette saline of an antibody directed against the insulin receptor kinase domain (which is highly conserved in the IGF-1 receptor) was also effective in blocking the effect of IGF-1 (20 μg/ml, n = 5).

(B) IGF-1-evoked attenuation of AMPA currents was also blocked by manipulations that interfere with clathrin-mediated endocytosis, such as inclusion in the patch-pipette saline of BAPTA (20 mM, n = 6), a peptide based on the sequence of the amphiphysin SH3 domain (AmphSH3, 100 μg/ml, n = 5), or a peptide based on the amphiphysin binding motif from the proline-rich domain of dynamin I (dynaminPRD, 100 μg/ml, n = 7). A mutant form of the amphiphysin SH3 peptide that does not bind dynamin I (AmphSH3mut, 100 μg/ml, n = 5) and a scrambled dynamin control peptide (dynCONTROL, 100 μg/ml, n = 5) failed to block the effect of IGF-1. Representative current traces were acquired at the times indicated on the graph. Scale bars, 30 pA, 1 s.

postsynaptic effects in isolation, they have a disadvantage in that the exogenously applied AMPA could activate a higher proportion of extrasynaptic AMPA receptors than does glutamate released from synaptic terminals. To address the possibility that the effect of IGF-1 is weak or absent at synaptic AMPA receptors on Purkinje neurons, this compound was applied together with monitoring of AMPA receptor–mediated miniature EPSCs (mEPSCs) (using external saline supplemented with 100 μM D-[-]-2-amino-5-phosphonopentanoic acid [D-AP5]; Figure 3). When IGF-1 (50 ng/ml) was applied for 10 min, and mEPSCs were monitored for a 200 s interval immediately before application and again 30 min after application, a large reduction in mEPSC amplitude was seen (63% ± 9% of baseline, n = 7). This was accompanied by a reduction in mEPSC frequency (79% ± 7%). A small portion of this reduction in frequency is probably attributable to the amplitude reduction of small events, which caused them to disappear into the noise. However, experiments using application of cyclothiazide subsequent to IGF-1 showed a significant increase in mEPSC amplitude (175% ± 19% of baseline, n = 5), but only a very small, statistically insignificant ($p > 0.20$) rescue of mEPSC frequency (83% ± 6%), suggesting that almost all of the IGF-1 effect represents a true reduction in mEPSC frequency, possibly due to the complete silencing of a fraction of excitatory synapses (see Man et al., 2000, for a similar effect in hippocampal slice). Cumulative probability histograms for mEPSC amplitude and interval (Figures 3A and 3B) show a leftward shift for amplitude and a rightward shift for interval, consistent with such an effect of IGF-1. No alterations of mEPSC kinetics were observed (Table 1).

Attenuation of AMPA mEPSCs by IGF-1 was blocked by a treatment that interfered with IGF-1 receptors. Cultures pretreated with external application of an inactivating monoclonal antiserum directed against the IGF-1 receptor, but not preimmune mouse IgG, showed no effect of IGF-1 on AMPA mEPSC amplitude (anti-IGF-1-R: 105% ± 5% of baseline, n = 6; control IgG: 66% ± 10%, n = 5). Similarly, when clathrin-mediated endocytosis was blocked by internal application of a peptide derived from the proline-rich domain of dynamin I, this treatment blocked the effect of IGF-1 (dynaminPRD: 100% ± 4% of baseline, n = 8), but the scrambled control peptide did not (dynCONTROL: 62% ± 9%, n = 5). These results are consistent with the experiments that used exogenous AMPA pulses (Figures 1 and 2)

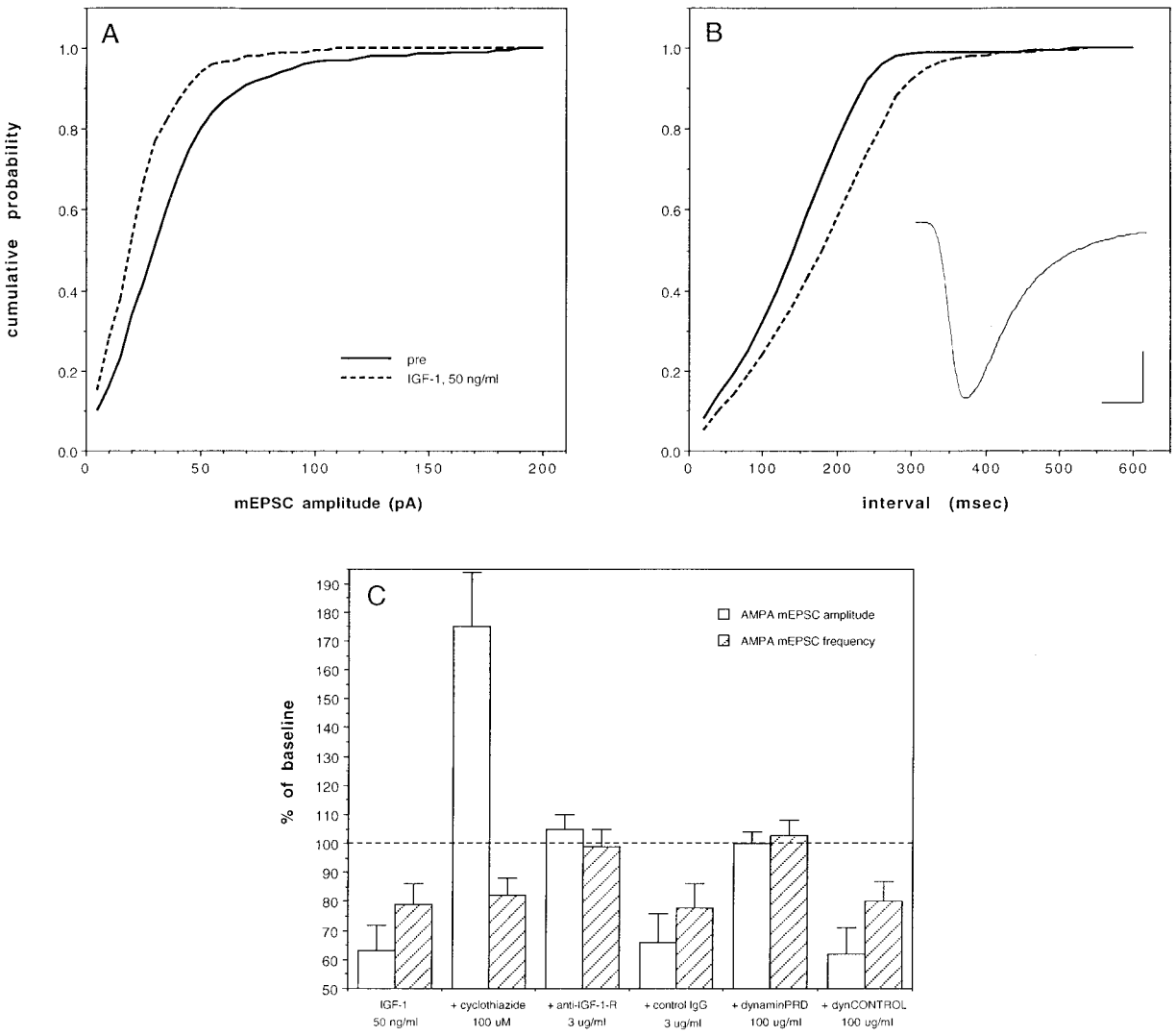

Figure 3. IGF-1 Reduces Both Amplitude and Frequency of AMPA Receptor–Mediated mEPSCs

(A and B) Recordings were made with $V_{hold} = -90$ mV to increase driving force and with the external saline supplemented with 100 μM D-AP5 to block NMDA receptors. Cumulative probability histograms for AMPA mEPSC amplitude (A) and interval (B) are shown for a representative Purkinje neuron recorded for 500 s immediately before the addition to 50 ng/ml IGF-1 for 10 min (pre) and for an additional 500 s starting 20 min after the end of the IGF-1 application. Inset in (B) shows a trace averaged from 20 consecutive mEPSC events prior to IGF-1 addition. Scale bars, 5 ms, 8 pA.

(C) Measures of AMPA mEPSC amplitude and frequency were made from a population of Purkinje neurons treated with IGF-1 (50 ng/ml, n = 7) and from a subset of these cells, which were subsequently exposed to the AMPA receptor desensitization blocker cyclothiazide (100 μM, n = 5). Separate groups of Purkinje neurons were pretreated for ≥12.5 min with the following drugs prior to IGF-1 application: anti-IGF-1-R (3 μg/ml, external, n = 6), control IgG (3 μg/ml, external, n = 5), dynaminPRD (100 μg/ml, internal, n = 8), and dynCONTROL (100 μg/ml, n = 5).

and suggest that the populations of AMPA receptors sampled by these two different methods are similarly regulated.

Do treatments that interfere with insulin/IGF-1 receptor signaling or clathrin-mediated endocytosis produce an alteration in AMPA receptors when applied alone? In Man et al. (2000), it is shown that these reagents do not affect basal AMPA receptor expression measured at the cell surface using immunocytochemical or electrophysiological measures in a heterologous expression system or in hippocampal neurons. However, other studies using cultured hippocampal neurons have found that either transfection with a mutant form of dynamin or a dynamin proline-rich domain peptide can block internalization of AMPA receptors, as measured immunocytochemically (Carroll et al., 1999b; Lüscher et al., 1999). Furthermore, postsynaptic application of a dynamin proline-rich domain peptide to a hippocampal pyramidal neuron in a slice preparation resulted in a gradual increase in the amplitude of AMPA EPSCs (Lüscher et al., 1999). In the present system, we have found that treatments that interfere with insulin/IGF-1

Table 1. Effects of Various Treatments on AMPA-mEPSCs in Cultured Purkinje Cells

Treatment	Amplitude (pA)	Frequency (s^{-1})	10–90% Rise (ms)	Half Decay (ms)
Control	24 ± 6	7.4 ± 2.3	2.4 ± 0.2	7.3 ± 0.6
Insulin, 500 nM	16 ± 5[a]	5.6 ± 2.0	2.4 ± 0.3	7.0 ± 0.5
IGF-1, 50 ng/ml	15 ± 5[a]	5.4 ± 2.1	2.5 ± 0.2	7.1 ± 0.6
Control IgG, 3 μg/ml	26 ± 8	7.5 ± 2.9	2.2 ± 0.2	7.3 ± 0.7
Anti-IGF-1-R, 3 μg/ml	26 ± 7	6.8 ± 3.0	2.3 ± 0.3	7.4 ± 0.6
Anti-insulin-R kinase dom, 20 μg/ml	23 ± 7	7.7 ± 2.6	2.5 ± 0.2	7.2 ± 0.5
IGF-1 analog peptide, 1 μg/ml	24 ± 8	7.8 ± 2.5	2.6 ± 0.2	7.5 ± 0.6
BAPTA, 20 mM	29 ± 8	8.0 ± 2.9	2.4 ± 0.3	7.1 ± 0.5
AmphSH3, 100 μg/ml	23 ± 7	7.1 ± 2.5	2.2 ± 0.4	7.2 ± 0.7
AmphSH3mut, 100 μg/ml	26 ± 6	8.0 ± 2.8	2.4 ± 0.3	7.2 ± 0.6
DynaminPRD, 100 μg/ml	28 ± 8	6.7 ± 2.6	2.3 ± 0.4	7.3 ± 0.7
DynCONTROL, 100 μg/ml	24 ± 7	7.6 ± 2.6	2.5 ± 0.3	7.4 ± 0.5

Values are mean ± SEM; n = 10 Purkinje neurons/treatment group. mEPSC measurements were made using a 500 s sample recorded in external saline supplemented with 100 μM D-AP5.
[a] $p < 0.05$ compared with control, t test.

receptor signaling or clathrin-mediated endocytosis do not produce significant changes in the frequency, amplitude, or kinetics of AMPA mEPSCs (Table 1). One possible explanation for this result is that in cerebellar Purkinje neurons, constitutive insertion and internalization of AMPA receptors are tightly coupled. In this way, blocking internalization could result in a concomitant reduction of insertion to produce a relatively constant level of surface AMPA receptor expression.

Previous reports have indicated that activation of neuronal insulin/IGF-1 receptors can be coupled to Ca^{2+} mobilization (Torres-Aleman et al., 1997; see Heidenreich, 1993, for review) and to transient facilitation of voltage-gated Ca^{2+} currents (Kleppisch et al., 1992; Selinfreund and Blair, 1994; Blair and Marshall, 1997). To determine whether such signals were involved in the present experiments, insulin (500 nM) and IGF-1 (50 ng/ml) were applied from t = 0–10 min, and depolarization-evoked Ca^{2+} influx was measured by ratio imaging in cerebellar cells loaded with fura-2 and subjected to the same depolarization pulse used in LTD induction (3 s pulse to 0 mV). Neither insulin (500 nM, t = 0–10 min) nor IGF-1 (50 ng/ml) produced significant alterations of basal Ca^{2+} or depolarization-evoked Ca^{2+} influx measured at t = 5–7 min (Figure 4). Measurements were made in Purkinje neurons, granule cells, and fusiform glial cells. Similar results were found at t = 1–2 min and t = 10–12 min (data not shown). While these data preclude large, distributed Ca^{2+} signals evoked by insulin or IGF-1, it is possible that Ca^{2+} signals restricted to a small domain (say, within 100 nm of synaptic IGF-1 receptors) would escape detection by the present imaging system.

Does the attenuation of AMPA currents evoked by IGF-1 receptor activation have anything to do with the expression mechanism of cerebellar LTD? If cerebellar LTD, which is expressed as a rapid and specific reduction of AMPA currents, is mediated by the same mechanisms that underlie the attenuation of AMPA currents by IGF-1, then induction of cerebellar LTD should occlude subsequent attenuation of AMPA currents by IGF-1. Figure 5 shows an experiment to test this idea in which quisqualate, an agonist of both mGluRs and AMPA receptors, was used for test pulses, as postsynaptic mGluR1 activation is required for the induction of LTD (Linden et al., 1991; Narasimhan and Linden, 1996; Narasimhan et al., 1998). Previous work has shown that currents evoked by brief (40–110 ms) quisqualate test pulses in cultured Purkinje neurons are completely blocked by an AMPA receptor antagonist, such as

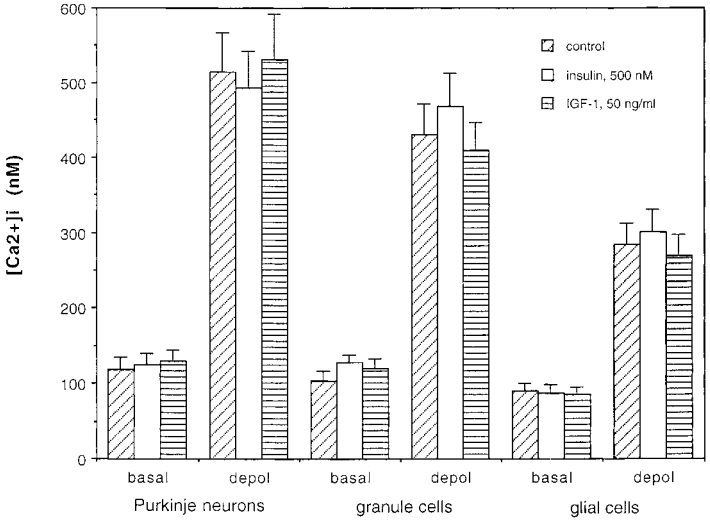

Figure 4. Brief Application of Insulin or IGF-1 Does Not Significantly Alter Basal or Depolarization-Evoked Ca^{2+} Signals in Cerebellar Cultures

Insulin (500 nM) and IGF-1 (50 ng/ml) were applied from t = 0–10 min. Ca^{2+} measurements were made at t = 5–7 min. Values are peak proximal dendritic Ca^{2+} concentration for Purkinje neurons and peak somatic Ca^{2+} for granule neurons and glia. 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. Depolarization-evoked values were measured as the peak during a 30 s measuring period following the onset of depolarization; n = 10 cells/group.

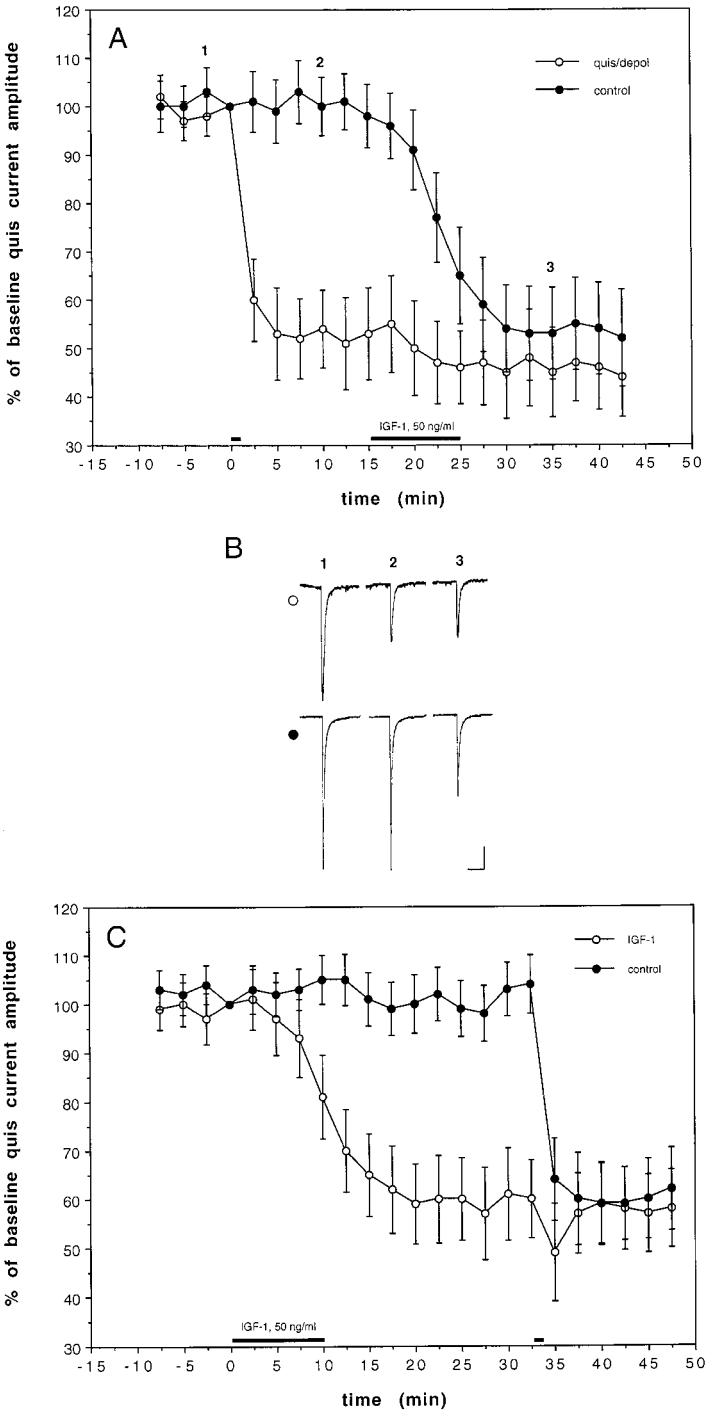

Figure 5. Induction of LTD and Attenuation of AMPA Receptor–Mediated Currents by IGF-1 Mutually Occlude Each Other

(A) Following acquisition of baseline responses to quisqualate (an agonist of both AMPA and mGluRs), LTD was induced by quisqualate/depolarization conjunction (quis/depol) at t = 0 min. Subsequently, IGF-1 was applied (50 ng/ml, t = 15–25 min), and test pulses were continued (n = 7). In a control group, the quisqualate/depolarization conjunction was omitted (n = 6).

(B) Representative current traces taken at the times indicated in (A) Scale bars, 30 pA, 1 s.

(C) Occlusion experiment the reverse of the one illustrated in (A) utilized perforated-patch recording. AMPA currents were attenuated by IGF (50 ng/ml at t = 0–10 min). Subsequently, quisqualate/depolarization conjunction was applied (t = 32.5 min), and test pulses were continued (n = 5). In a control group, the IGF-1 application was omitted (n = 5).

6-cyano-7-dinitroquinoxaline-2,3-dione (Linden et al., 1993). Following acquisition of baseline responses to quisqualate test pulses, six conjunctive stimuli, each consisting of a quisqualate pulse together with a 3 s long depolarizing step to 0 mV, were applied at t = 0 min. This resulted in a rapid depression of subsequent test pulses (54% ± 8% of baseline at t = 10 min, n = 7), as has been previously described (Linden et al., 1991, 1993). When IGF-1 was then applied at t = 15–25 min, further attenuation of quisqualate-evoked currents was minimal (45% ± 10% at t = 35 min). As a control, another group of Purkinje cells received quisqualate test pulses from t = −10 to 15 min without quisqualate/depolarization conjunctive stimulation. When IGF-1 was then applied at t = 15–25 min, quisqualate-evoked currents were strongly attenuated (53% ± 9% at t = 35 min, n = 6). This argues that the failure of IGF-1 to induce further attenuation in the former case results from occlusion by prior LTD induction rather than a nonspecific effect related to the duration of the whole-cell recording. To further address this point, a reverse occlusion experiment was also performed in which prior exposure to

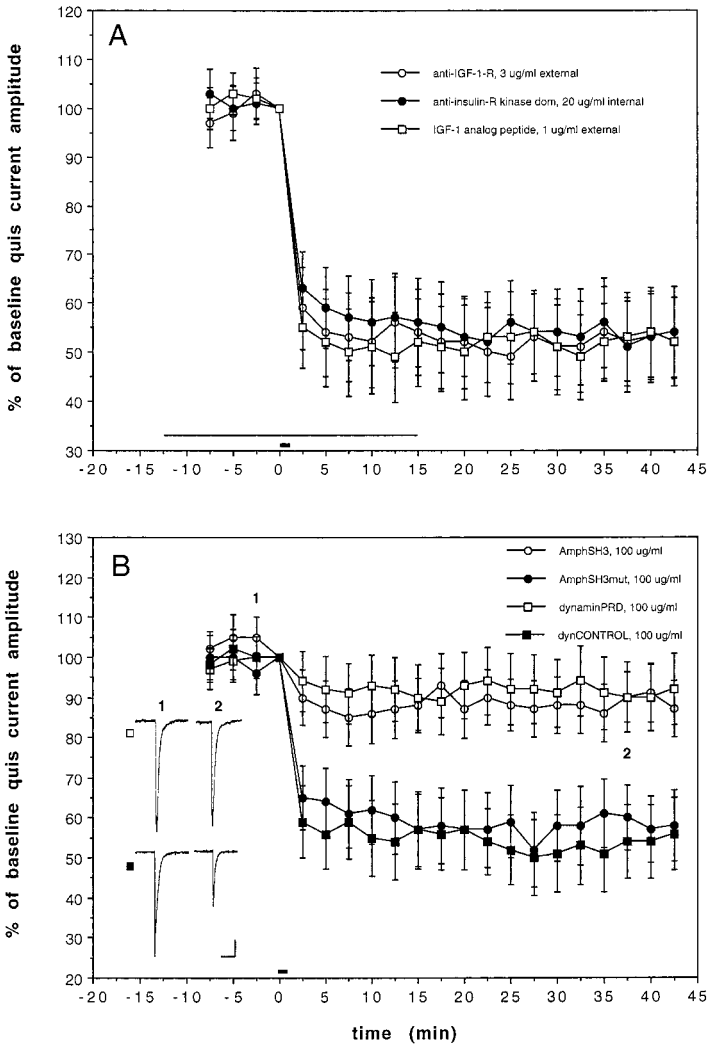

Figure 6. Induction of Cerebellar LTD by Quisqualate/Depolarization Conjunction Is Blocked by Inhibitors of Clathrin-Dependent Endocytosis but Not by Inhibitors of Insulin/IGF-1 Signaling

(A) Induction of LTD by quisqualate/depolarization conjunction (heavy horizontal bar at t = 0 min) was unaltered by externally applied inhibitors of IGF receptors (light horizontal bar at t = −15 to 5 min), such as an inhibitory IGF-1 analog pepide (1 μg/ml, n = 6) or an antibody directed against the external portion of the IGF-1 receptor (3 μg/ml, n = 6). Inclusion in the patch-pipette saline of an antibody directed against the insulin receptor kinase domain also failed to alter LTD induction (20 μg/ml, n = 7).

(B) In contrast, LTD was strongly attenuated by inhibitors of clathrin-dependent exocytosis applied in the patch-pipette saline. These included a peptide based on the sequence of the amphiphysin SH3 domain (AmphSH3, 100 μg/ml, n = 5) or a peptide based on the amphiphysin binding motif from the proline-rich domain of dynamin I (dynaminPRD, 100 μg/ml, n = 7). A mutant form of the amphiphysin SH3 peptide that does not bind dynamin I (AmphSH3mut, 100 μg/ml, n = 5) or a scrambled dynamin control peptide (dynCONTROL, 100 μg/ml, n = 5) had no significant effect on LTD. Representative current traces were acquired at the times indicated on the graph. Scale bars, 30 pA, 1 s.

IGF-1 occluded subsequent induction of LTD by quisqualate/depolarization conjunction (Figure 5C). This experiment required perforated-patch as opposed to whole-cell recording, as whole-cell recording for >30 min begins to nonspecifically reduce the amplitude of cerebellar LTD induction (the so-called "washout effect," which is also seen in hippocampal LTP).

The experimental evidence to this point suggests three possible models to explain the interaction of insulin/IGF-1 effects and LTD expression. First, quisqualate/depolarization conjunction results in the activation of IGF-1/insulin receptors to trigger clathrin-mediated internalization of AMPA receptors and thereby express LTD. Second, quisqualate/depolarization conjunction results in neither insulin/IGF-1 receptor activation nor clathrin-mediated internalization, and LTD is expressed through a different set of mechanisms. Third, quisqualate/depolarization conjunction does not activate insulin/IGF-1 receptors, but, through a converging intermediary step, does activate clathrin-mediated internalization of AMPA receptors to express LTD. To begin to distinguish among these models, LTD was induced by quisqualate/depolarization conjunction in the presence of compounds designed to block IGF-1/insulin receptor signaling (Figure 6A). All three treatments, external application of an inhibitory IGF-1 analog peptide (1 μg/ml), application of an antibody directed against the external portion of the IGF-1 receptor (3 μg/ml), and internal application of an antibody directed against the insulin receptor kinase domain (20 μg/ml), were previously shown to block the attenuation of AMPA currents produced by exogenous IGF-1 (Figure 2A). However, robust and persistent LTD was evoked using all of these treatments (anti-IGF-1-R: 54% ± 9% of baseline at t = 42.5 min, n = 6; anti-insulin-R kinase domain: 53% ± 9%, n = 7; IGF-1 analog peptide: 52% ± 8%, n = 6), indicating that LTD induction does not require activation of insulin/IGF-1 receptors, thereby eliminating the first model described above.

To distinguish between the remaining two models, inhibitors of the clathrin endocytotic complex were applied prior to LTD induction (Figure 6B). Two peptides designed to block the amphiphysin/dynamin I interaction, one based on the dynamin I proline-rich domain and another based on the amphiphysin SH3 domain, both produced a near complete blockade of LTD induced by quisqualate/depolarization conjunction (AmphSH3: 87% ± 7% of baseline at t = 42.5 min, n = 5; dynaminPRD: 92% ± 9%, n = 7). Control peptides that do not block the dynamin I/amphiphysin interaction

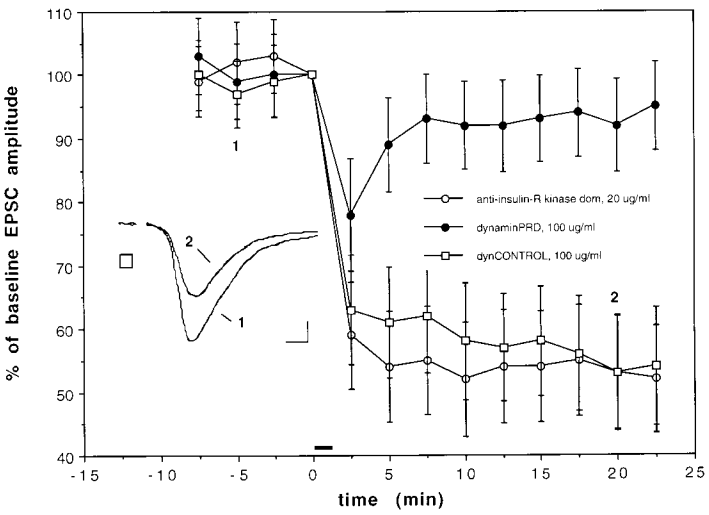

Figure 7. Induction of Cerebellar LTD by EPSC/Depolarization Conjunction in Granule Cell–Purkinje Neuron Pairs Is Blocked by an Inhibitor of Clathrin-Dependent Endocytosis but Not by an Inhibitor of Insulin/IGF-1 Signaling

Cerebellar LTD induction is associated with a decrease in the amplitude of mean evoked EPSCs calculated using all responses, including synaptic failures. LTD was induced by pairing 60 granule cell stimuli delivered at 0.5 Hz, each with a 50 ms long Purkinje cell depolarization to 0 mV (horizontal bar). Anti-insulin-R kinase domain (20 μg/ml, n = 5), dynaminPRD (100 μg/ml, n = 6), and dynCONTROL (100 μg/ml, n = 5) were applied internally. Inset illustrates current traces representing the average of ten consecutive responses (including failures) recorded from a granule cell–Purkinje neuron pair loaded with dynCONTROL peptide. Traces are from the time points indicated on the graph. Scale bars, 5 ms, 5 pA.

were without effect (AmphSH3mut: 58% ± 9% of baseline at t = 42.5 min, n = 5; dynCONTROL: 56% ± 9%, n = 5). High concentrations of a Ca^{2+} chelator have also been shown to suppress clathrin-mediated endocytosis (Marks and McMahon, 1998) and AMPA receptor internalization (Man et al., 2000; Figure 2B). Previous work has shown that cerebellar LTD is blocked by a postsynaptic Ca^{2+} chelator in both slice (Sakurai, 1990; Konnerth et al., 1992) and culture (Linden and Connor, 1991) preparations. However, it is not clear if this represents a direct effect on clathrin-mediated endocytosis or inhibition of some intermediate, Ca^{2+}-sensitive postsynaptic signal, such as activation of PKC (Crepel and Krupa, 1988; Linden and Connor, 1991) or soluble guanylyl cyclase (Hartell, 1994; Lev-Ram et al., 1997). In either case, these results argue for the third model described above, in which expression of cerebellar LTD does not require activation of insulin/IGF-1 receptors, but, similar to IGF-1-induced attenuation of AMPA currents, does require clathrin-mediated internalization of AMPA receptors.

Finally, to address the possibility that the blockade of LTD by reagents that interfere with clathrin-mediated endocytosis is an artifact of an experimental system using exogenous quisqualate test pulses, experiments were conducted using evoked EPSCs in granule cell–Purkinje neuron pairs (Figure 7). Activation of a synaptically connected granule cell–Purkinje neuron pair at 0.1 Hz resulted in a mixture of evoked currents and failures (Linden, 1997; Linden and Ahn, 1999). LTD was induced by applying 60 pulses at 0.5 Hz at the test pulse stimulation strength to the granule cell, with each pulse paired with a 50 ms long depolarization of the Purkinje neuron to 0 mV. This produced a significant decrease in the mean amplitude of evoked EPSCs in Purkinje neurons loaded with the scrambled dynamin control peptide (100 μg/ml, 99% ± 6% of baseline at t = −2.5 min prior to LTP induction, compared with 53% ± 9% at t = 20 min after LTP induction, n = 5) or a reagent to interfere with IGF-1/insulin receptor signaling (anti-insulin-R kinase domain, 20 μg/ml, 53% ± 8% of baseline at t = 20 min, n = 5). In contrast, Purkinje cells loaded with a dynamin proline-rich domain peptide (100 μg/ml) showed a near complete block of LTD monitored and induced with synaptic stimulation (92% ± 7% of baseline at t = 20 min, n = 6).

Discussion

The main finding of this study is that expression of cerebellar LTD requires postsynaptic clathrin-mediated endocytosis. This conclusion is supported by the observation that induction of LTD and clathrin-mediated AMPA current attenuation induced by activation of IGF-1 receptors occluded each other. Furthermore, postsynaptic application of both an amphiphysin SH3 domain peptide and a dynamin I proline-rich domain peptide, but not their inactive controls, blocked expression of cerebellar LTD. The most straightforward explanation for these findings is that AMPA receptors are internalized in a clathrin-dependent manner during the induction of cerebellar LTD. Indeed, both Man et al. (2000) and Lüscher et al. (1999) have shown AMPA receptor internalization directly using immunocytochemical techniques applied to hippocampal neurons and heterologous expression systems. However, in the absence of this form of evidence for cerebellar Purkinje neurons, the formal possibility remains that here it is not AMPA receptors that are internalized but rather, some associated modulatory protein.

While treatments that interfered with clathrin-mediated endocytosis produced a significant attenuation of LTD expression, it was not total (Figures 6 and 7). This could indicate that other expression mechanisms, such as changes in AMPA receptor kinetics, unitary conductance, or glutamate affinity, underlie the small remaining depression. Alternatively, the residual depression could result from a submaximal effect of the amphiphysin and dynamin peptides on clathrin-mediated endocytosis.

A secondary finding of this study (and of Man et al., 2000) is that activation of insulin/IGF-1 receptors can induce rapid clathrin-mediated internalization of neuronal postsynaptic AMPA receptors. It is unlikely that

the attenuation of Purkinje neuron AMPA currents by IGF-1 is a nonspecific effect as it is (1) seen with AMPA but not NMDA test pulses, (2) not seen in cerebellar glial cells that lack GluR2, (3) not associated with significant changes in R_{input}, (4) blocked by three different treatments that interfere with IGF-1 receptor signaling, and (5) blocked by three different treatments that interfere with the clathrin endocytotic complex. The attenuation of AMPA currents by IGF-1 is a phenomenon that affects AMPA receptors localized at the synapse, as it may be detected as a reduction in the amplitude (and frequency) of AMPA receptor–mediated mEPSCs (see also Man et al., 2000). Interestingly, in contrast to the present findings, *Xenopus* oocytes expressing several forms of cloned mouse NMDA receptor subunits have been reported to have NMDA-evoked currents that were potentiated ~2-fold by brief insulin treatment (Liao and Leonard, 1999).

This attenuation of AMPA currents by insulin/IGF-1 receptor activation is consistent with certain recent findings. It has been shown that, like AMPA receptors, the insulin receptor and its substrate, p58/53, are enriched in synapses and, more specifically, in a postsynaptic density fraction (Abbott et al., 1999). More indirectly, conjoint administration of glutamate and IGF-1 through a microdialysis probe implanted in the intact cerebellar cortex has been reported to produce a persistent depression of glutamate-evoked GABA release in fractions collected in the cerebellar deep nuclei, where the axons of the Purkinje neurons terminate (Castro-Alamancos and Torres-Aleman, 1993; Castro-Alamancos et al., 1996). While this measure reflects multiple processes (including AMPA and mGluR1 activation, intrinsic excitability of the Purkinje neuron, network effects on inhibitory tone, and GABA release at the Purkinje neuron terminal), it is possible that some or all of the effect of IGF-1 on glutamate-evoked GABA release could result from internalization of AMPA receptors on Purkinje neuron dendrites.

While activation of insulin/IGF-1 receptors is not required for expression of cerebellar LTD in cultured Purkinje neurons, it is possible that this phenomenon could be required for LTD in other synapses. Alternatively, activation of insulin/IGF-1 receptors could have a modulatory role in use-dependent synaptic plasticity. Either of these models would be consistent with behavioral experiments that have shown a link between insulin/IGF-1 receptor activation and memory. For example, intraventricular administration of streptozocin destroyed insulin receptors in adult rats and produced a long-term and progressive deficit of both working and reference memory in holeboard and passive avoidance tasks (Lannert and Hoyer, 1998). Likewise, infusion in the cerebellum of an antisense oligonucleotide directed against IGF-1 produced a complete blockade for the acquisition (but not retention) of associative eyeblink conditioning in the freely moving rat (Castro-Alamancos and Torres-Aleman, 1994). Conversely, continuous ventricular infusion of IGF-1 ameliorated deficits in novel object recognition and place discrimination tasks in senescent rats (Markowska et al., 1998). In addition, Alzheimer's disease patients have been reported to have a significant reduction in cerebrospinal fluid insulin concentration, as compared with age-matched controls, with the largest reductions seen in those individuals with the most advanced cognitive impairment (Craft et al., 1998). While most workers have interpreted the behavioral effects of insulin/IGF-1 manipulations in terms of possible alterations in cerebral glucose metabolism or slow neurotrophic effects, the present findings suggest that rapid AMPA receptor internalization might also contribute.

What intermediate signals might couple the initial events in cerebellar LTD induction to clathrin-mediated endocytosis? Cerebellar LTD induction requires at least three initial events: activation of AMPA receptors and consequent Na^{2+} influx; activation of mGluR1 and, thereby, phospholipase C; and Ca^{2+} influx through voltage-gated channels. Phospholipase C cleaves phosphatidylinositol-4,5-bisphosphate to yield inositol-1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol. The former binds to specific intracellular IP3 receptors, resulting in the liberation of Ca^{2+} from internal stores, while the latter results in activation of PKC, a process that is also necessary for LTD induction. A requirement for IP3 receptor–mediated Ca^{2+} mobilization in LTD is somewhat controversial, with evidence both for (Khodakhah and Armstrong, 1997) and against (Narasimhan et al., 1998) this process (see Daniel et al., 1998, for review). It may be that these different findings reflect differences in the "basal state" of Purkinje neuron Ca^{2+} homeostasis in different cerebellar preparations. Another issue involves a requirement for signaling cascade in which Ca^{2+} entry into the granule cell terminal activates nitric oxide synthase, resulting in production of NO, orthograde diffusion and activation of soluble guanylyl cyclase in the Purkinje neuron, production of cyclic guanosine 3′,5′ monophosphate (cGMP), and the activation of cGMP-dependent protein kinase (see Daniel et al., 1998, for review). While this signaling cascade may contribute to cerebellar LTD induction, it is clear that it is not absolutely required, as cerebellar LTD may be induced in preparations that completely lack presynaptic terminals (Narasimhan and Linden, 1996; Narasimhan et al., 1998).

The results herein suggest that activation of insulin/IGF-1 receptors and LTD-inducing stimulation converge on a common signaling system to stimulate AMPA receptor internalization. In this vein, it is worth noting that while there are several signals that result from insulin/IGF-1 receptor activation, one that is shared with LTD induction is a transient activation of PKC (see Heidenreich, 1993, for review). Thus, it will be informative to test PKC inhibitors to determine if these drugs block the attenuation of AMPA currents by IGF-1.

While models of clathrin-mediated endocytosis are still evolving, several aspects of this process are now well understood (see Schmid et al., 1998; Marsh and McMahon, 1999, for review). Clathrin-mediated endocytosis appears to occur in several steps. Initially, a scaffold of clathrin and AP2 is formed to constitute a coated pit. Amphiphysin dimers, which are associated by their coiled-coil domains, bind clathrin both directly and indirectly via AP2 through a unique domain in the middle of the protein. Amphiphysin also contains a C-terminal SH3 domain, which binds the C-terminal proline-rich domain of dynamin, thereby recruiting it to the invaginating endocytotic structure. GTP binding triggers dynamin self-assembly into "spiral collars," thereby generating force to constrict the neck of the vesicle. Finally, hydrolysis of GTP is required for vesicle budding.

Unfortunately, the regulation of clathrin-mediated endocytosis is only beginning to be understood. One mechanism that appears to be important is the dephosphorylation of dynamin and amphiphysin, a process that appears to be required for the formation of the clathrin protein complex (Slepnev et al., 1998). More specifically, the Ca^{2+}/calmodulin-dependent phosphatase calcineurin appears to be involved, as calcineurin inhibitors have been reported to block clathrin-mediated endocytosis in mammalian nerve terminals (Marks and McMahon, 1998) and adrenal chromaffin cells (Engisch and Nowycky, 1998). In addition, it has recently been reported that in the presence of 100–400 nM free Ca^{2+}, a complex of calcineurin, dynamin I, and amphiphysin is formed and that transfection of cells with a catalytically inactive calcineurin resulted in a ~35% decrease in clathrin-mediated endocytosis (Lai et al., 1999). Interestingly, while calcineurin inhibitors appear to block hippocampal homosynaptic LTD (Mulkey et al., 1994) and depotentiation (O'Dell and Kandel, 1994), they have been reported to induce a form of synaptic depression at parallel fiber–Purkinje neuron synapses in the cerebellum (Ajima and Ito, 1995) and to persistently attenuate the responses to cultured Purkinje neurons to exogenous glutamate (Kasahara and Sugiyama, 1998).

It is likely that clathrin-mediated endocytosis is subject to many forms of regulation. Proteins that bind the proline-rich domain of dynamin include phospholipase Cγ (which is not the form coupled to mGluR1 in Purkinje neurons), Grb2, Src, cortactin, synaptojanin, and the p85 subunit of phosphatidylinositol-3-kinase. One protein of particular interest is endophilin I, which binds both dynamin and the phosphatidylinositol 5-phosphatase synaptojanin and which functions to transfer an acyl group to the membrane lipid lysophosphatidic acid, thereby altering membrane curvature (Ringstad et al., 1999). Dynamin also contains a pleckstrin homology domain that binds phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-3,4,5-triphosphate (Barylko et al., 1998; Klein et al., 1998). One possibility is that the recruitment of these phosphoinositides to the vesicle neck might bind the dynamin I pleckstrin homology domain and thereby promote GTPase activity and the subsequent scission event. Unfortunately, this mechanism does not link clearly to our current understanding of either LTD induction or insulin/IGF-1 signaling. Activation of neuronal insulin/IGF-1 receptors results in the activation of phosphatidylinositol-3-kinase (Heidenreich, 1993; Blair and Marshall, 1997) and the consequent conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-triphosphate, both of which activate the dynamin I GTPase. LTD induction requires activation of phospholipase C, which converts phosphatidylinositol-4,5-bisphosphate into products that do not have a substantial interaction with dynamin. In summary, the protein interactions of the clathrin endocytic complex are indicative of a complex set of regulatory events, none of which is presently an obvious link to signals known to be required for cerebellar LTD induction.

Man et al. (2000) have shown that at least one form of hippocampal homosynaptic LTD is blocked by the amphiphysin SH3 domain peptide used herein. This is consistent with a previous report using cultured hippocampal neurons in which the induction of LTD by field electrode stimulation was associated with a reduction of surface membrane AMPA receptor clusters (Carroll et al., 1999a) and with another report in which postsynaptic application of a dynamin proline-rich domain peptide blocked induction of homosynaptic LTD in area CA1 of a hippocampal slice (Lüscher et al., 1999). Taken together, these results suggest that forms of LTD that are expressed in different brain locations (hippocampus versus cerebellum) and that have different initial signaling requirements (NMDA receptor activation versus Ca^{2+} channel/mGluR1/AMPA receptor activation, respectively) may ultimately utilize similar expression mechanisms involving clathrin-mediated AMPA receptor internalization.

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. The growth medium used for the cell cultures contained 1.7 μM insulin and was completely replaced with insulin-free growth medium 24 hr prior to the onset of electrophysiological experiments. Patch electrodes attached to Purkinje neuron somata were typically filled with a solution containing CsCl (135 mM), HEPES (10 mM), EGTA (1 mM), Na_2-ATP (4 mM), and Na GTP (0.4 mM, adjusted to pH 7.35 with CsOH). In experiments in which 20 mM Cs_4-BAPTA was applied (Figure 2B; Table 1), CsCl was reduced to maintain osmolality. Similarly, for glial cell recordings, the internal saline was supplemented with 10 mM TEA-Cl, and the concentration of CsCl was appropriately reduced. In one experiment (Figure 5C), perforated-patch recording was used. Patch electrodes were filled with a solution containing Cs_2SO_4 (95 mM), CsCl (15 mM), $MgCl_2$ (8 mM), and HEPES (10 mM, pH 7.35 with CsOH). Electrode tips were filled with a small amount of this solution, and the shanks backfilled with this solution, supplemented with amphotericin B at a concentration of 300 μg/ml. Stable access resistance of <12 MΩ could be obtained within 10 min of gigaseal formation.

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 ~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. $V_{hold} = -70$ mV was imposed for experiments in which exogenous pulses of AMPA or quisqualate were applied. To allow for the recording of NMDA currents, as in Figure 1A, $[Mg]_o$ was reduced to 0.2 mM, 20 μM glycine was added to the external saline, and V_{hold} was set to −50 mV. For recording AMPA mEPSCs (Figure 3; Table 1), V_{hold} was set to −90 mV (to increase driving force), and 100 μM D-AP5 was added to the external saline.

One series of experiments (Figure 7) utilized synaptic activation of granule cell–Purkinje neuron pairs, as has been previously described (Linden, 1997; Linden and Ahn, 1999). For these experiments, tetrodotoxin was omitted from the external saline, and granule cells were activated using constant voltage pulses through a loose-patch electrode (5–6 MΩ) filled with external saline. Granule cells were identified as small (<7 μm), round clustering cells with short dendrites that intermittently evoked an EPSC in neighboring Purkinje neurons and that showed paired pulse facilitation when stimulated at an interval of 50 ms. EPSC amplitude was calculated as the average of ten consecutive evoked responses. LTD was induced by pairing each of 60 granule cell stimuli, delivered at 0.5 Hz, with a 50 ms long Purkinje cell depolarization to 0 mV.

Membrane currents were recorded with an Axopatch 200A amplifier (Axon Instruments), low-pass filtered at 2 kHz and digitized at 5

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 3.5 mini analysis software was used, which detected events based on the closeness of the fit of the mEPSC to a template. Events smaller than −5 pA were discarded. A separate template was created for each recording by averaging >50 of its most unambiguous mEPSCs, as selected by human inspection. 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 (Linden et al., 1995). Experiments were conducted at room temperature.

Human recombinant IGF-1 expressed in *Escherichia coli* was obtained from Sigma (I-3769). A monoclonal antibody directed against the IGF-1 receptor was purchased from Calbiochem (GR11). A peptide inhibitor of the IGF-1 receptor (CYAAPLPAKSC) was obtained from Bachem (H-1356). Zn^{2+}-free insulin, amphiphysin SH3 domain peptide, its inactive mutant control peptide, and the antibody directed against the insulin receptor kinase domain were prepared as described in Man et al. (2000). 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. Fura-2 and Cs_4-BAPTA were from Molecular Probes, tetrodotoxin was from Alexis Biochemicals, D-AP5 was from Tocris, and all other reagents were from Sigma.

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References

Abbott, M.-A., Wells, D.G., and Fallon, J.R. (1999). The insulin receptor tyrosine kinase substrate p58/53 and the insulin receptor are components of CNS synapses. J. Neurosci. *19*, 7300–7308.

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

Barylko, B., Binns, D., Lin, K.-M., Atkinson, M.A.L., Jameson, D.M., Yin, H.L., and Albanesi, J.P. (1998). Synergistic activation of dynamin GTPase by Grb2 and phosphoinositides. J. Biol. Chem. *273*, 3791–3797.

Blair, L.A.C., and Marshall, J. (1997). IGF-1 modulates Na and L calcium channels in a PI 3-kinase dependent manner. Neuron *19*, 421–429.

Bondy, C., Werner, H., Roberts, C.T., and LeRoith, D. (1992). Cellular pattern of type-I insulin-like growth factor receptor gene expression during maturation of the rat brain: comparison with insulin-like growth factors I and II. Neuroscience *46*, 909–923.

Burnashev, N., Khodorova, A., Jonas, P., Helm, P.J., Wisden, W., Monyer, H., Seeburg, P.H., and Sakmann, B. (1992). Calcium-permeable AMPA-kainate receptors in fusiform cerebellar glial cells. Science *256*, 1566–1570.

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., Lüscher, 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.

Castro-Alamancos, M.A., and Torres-Aleman, I. (1993). Long-term depression of glutamate-induced γ-aminobutyric acid release in cerebellum by insulin-like growth factor I. Proc. Natl. Acad. Sci. USA *90*, 7386–7390.

Castro-Alamancos, M.A., and Torres-Aleman, I. (1994). Learning of the conditioned eyeblink response is impaired by an antisense insulin-like growth factor I oligonucleotide. Proc. Natl. Acad. Sci. USA *91*, 10203–10207.

Castro-Alamancos, M.A., Arevalo, M.A., and Torres-Aleman, I. (1996). Involvement of protein kinase C and nitric oxide in the modulation by insulin-like growth factor-1 of glutamate-induced GABA release in the cerebellum. Neuroscience *70*, 843–847.

Craft, S., Peskind, E,, Schwartz, M.W,, Schellenberg, G.D., Raskind, M., and Porte, D., Jr. (1998). Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease: relationship to severity of dementia and apolipoprotein E genotype. Neurology *50*, 164–168.

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.

Engisch, K.L., and Nowycky, M.C. (1998). Compensatory and excess retrieval: two types of endocytosis following single step depolarizations in bovine adrenal chromaffin cells. J. Physiol. *506*, 591–608.

Garcia-Segura, L.M., Rodriguez, J.R., and Torres-Aleman, I. (1997). Localization of the insulin-like growth factor I receptor in the cerebellum and hypothalamus of adult rats: an electron microscopic study. J. Neurocytol. *26*, 479–490.

Grabs, D., Slepnev, V.I., Songyang, Z., David, C., Lynch, M., Cantley, L.C., and De Camilli, P. (1997). The SH3 domain of amphiphysin binds the proline-rich domain of dynamin at a single site that defines a new SH3 binding consensus sequence. J. Biol. Chem. *272*, 13419–13425.

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.

Heidenreich, K.A. (1993). Insulin and IGF-1 receptor signaling in cultured neurons. Ann. NY Acad. Sci. *692*, 72–88.

Hemart, N., Daniel, H., Jaillard, D., and Crepel, F. (1994). Properties of glutamate receptors are modified during long-term depression in rat cerebellar Purkinje cells. Neurosci. Res. *19*, 213–221.

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

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. (Lond) *324*, 113–134.

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

Khodakhah, K., and Armstrong, C.M. (1997). Induction of long-term depression and rebound potentiation by inositol trisphosphate in cerebellar Purkinje neurons. Proc. Natl. Acad. Sci. USA *94*, 14009–14014.

Klein, D.E., Lee, A., Frank, D.W., Marks, M.S., and Lemmon, M.A. (1998). The pleckstrin homology domains of dynamin isoforms require oligomerization for high affinity phosphoinositide binding. J. Biol. Chem. *273*, 27725–27733.

Kleppisch, T., Klinz, F.-J., and Hescheler, J. (1992). Insulin-like growth factor I modulates voltage-dependent Ca^{2+} channels in neuronal cells. Brain Res. *591*, 283–288.

Konnerth, A., Dreessen, J., and Augustine, G.J. (1992). Brief dendritic calcium signals initiate long-lasting synaptic depression in cerebellar Purkinje cells. Proc. Natl. Acad. Sci. USA *89*, 7051–7055.

Kull, F.C., Jr., Jacobs, S., Su, Y.F., Svoboda, M.E., Van Wyk, J.J., and Cuatrecasas, P. (1983). Monoclonal antibodies to receptors for insulin and somatomedin-C. J. Biol. Chem. *258*, 6561–6566.

Lai, M.M., Hong, J.J., Ruggiero, A.M., Burnett, P.E., Slepnev, V.I., De Camilli, P., and Snyder, S.H. (1999). The calcineurin–dynamin I complex as a calcium sensor for synaptic vesicle endocytosis. J. Biol. Chem. *274*, 25963–25966.

Lannert, H., and Hoyer, S. (1998). Intracerebroventricular administration of streptozocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. Behav. Neurosci. *112*, 1199–1208.

Lev-Ram, V., Jiang, T., Wood, J., Lawrence, D.S., and Tsien, R.Y. (1997). Synergies and coincidence requirements between NO, cGMP, and Ca^{2+} in the induction of cerebellar long-term depression. Neuron *18*, 1025–1038.

Liao, G.-Y., and Leonard, J.P. (1999). Insulin modulation of cloned mouse NMDA receptor currents in Xenopus oocytes. J. Neurochem. *73*, 1510–1519.

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. (1997). Long-term potentiation of glial synaptic currents in cerebellar culture. Neuron *18*, 983–994.

Linden, D.J., and Ahn, S. (1999). Activation of presynaptic cAMP-dependent protein kinase is required for induction of cerebellar long-term potentiation. J. Neurosci. *19*, 10221–10227.

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.

Lüscher, 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.

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

Markowska, A.L., Mooney, M., and Sonntag, W.E. (1998). Insulin-like growth factor-1 ameliorates age-related behavioral deficits. Neuroscience *87*, 559–569.

Marks, B., and McMahon, H.T. (1998). Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals. Curr. Biol. *8*, 740–749.

Marsh, M., and McMahon, H.T. (1999). The structural era of endocytosis. Science *285*, 215–219.

Morgan, D.O., and Roth, R.A. (1987). Acute insulin action requires insulin receptor kinase activity: introduction of an inhibitory monoclonal antibody into mammalian cells blocks the rapid effects of insulin. Proc. Natl. Acad. Sci. USA *84*, 41–45.

Mulkey, R.M., Endo, S., Shenolikar, S., and Malenka, R.C. (1994). Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. Nature *369*, 486–488.

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^{2+} mobilization is not required for cerebellar long-term depression in reduced preparations. J. Neurophysiol. *80*, 2963–2974.

O'Dell, T.J., and Kandel, E.R. (1994). Low-frequency stimulation erases LTP through an NMDA receptor–mediated activation of protein phosphatases. Learn. Mem. *1*, 129–139.

Pietrzkowski, Z., Wernicke, D., Porcu, P., Jameson, B.A., and Baserga, R. (1992). Inhibition of cellular proliferation by peptide analogues of insulin-like growth factor I. Cancer Res. *52*, 6447–6451.

Raymond, J.L., Lisberger, S.G., and Mauk, M.D. (1996). The cerebellum: a neuronal learning machine? Science *272*, 1126–1131.

Ringstad, N., Gad, H., Low, P., Di Paolo, G., Brodin, L., Shupliakov, O., and De Camilli, P. (1999). Endophilin/SH3p4 is required for the transition from early to late stages in clathrin-mediated synaptic vesicle endocytosis. Neuron *24*, 143–154.

Rosenmund, C., Legendre, P., and Westbrook, G.L. (1992). Expression of NMDA channels on cerebellar Purkinje cells acutely dissociated from newborn rats. J. Neurophysiol. *68*, 1901–1905.

Sakurai, M. (1990). Calcium is an intracellular mediator of the climbing fiber in induction of cerebellar long-term depression. Proc. Natl. Acad. Sci. USA *87*, 3383–3385.

Schmid, S.L., McNiven, M.A., and De Camilli, P. (1998). Dynamin and its partners: a progress report. Curr. Opin. Cell Biol. *10*, 504–512.

Selinfreund, R.H., and Blair, L.A.C. (1994). Insulin-like growth factor-I induces a rapid increase in Ca^{2+} currents and spontaneous membrane activity in clonal pituitary cells. Mol. Pharmacol. *45*, 1215–1220.

Sherrard, R.M., Richardson, N.A., and Sara, V.R. (1997). Localization of insulin-like growth factor-I (IGF-I) immunoreactivity in the olivocerebellar system of developing and adult rats. Dev. Brain Res. *98*, 102–113.

Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., De Camilli, P., and Brodin, L. (1997). Synaptic vesicle endocytosis impaired by disruption of dynamin–SH3 domain interactions. Science *276*, 259–263.

Slepnev, V.I., Ochoa, G.-C., Butler, M.H., Grabs, D., and De Camilli, P. (1998). Role of phosphorylation in regulation of the assembly of endocytic coat complexes. Science *281*, 821–824.

Tempia, F., Kano, M., Schneggenburger, R., Schirra, C., Garaschuk, O., Plant, T., and Konnerth, A. (1996). Fractional calcium current through neuronal AMPA-receptor channels with a low calcium permeability. J. Neurosci. *16*, 456–466.

Torres-Aleman, I., Villalba, M., and Nieto-Bona, M.P. (1997). Insulin-like growth factor-1 modulation of cerebellar cell populations is developmentally stage-dependent and mediated by specific intracellular pathways. Neuroscience *83*, 321–334.

Werther, G.A., Abate, M., Hogg, A., Cheesman, H., Oldfield, B., Hards, D., Hudson, P., Power, B., Freed, K., and Herrington, A.C. (1990). Localization of insulin-like growth factor-I mRNA in rat brain by in situ hybridization—relationship to IGF receptors. Mol. Endocrinol. *4*, 773–778.