

N-ethylmaleimide-sensitive factor is required for the synaptic incorporation and removal of AMPA receptors during cerebellar long-term depression

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Cerebellar long-term depression (LTD) is a persistent attenuation of synaptic transmission at the parallel fiber–Purkinje cell synapse mediated by the removal of GluR2 subunit-containing α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. The removal of AMPA receptors requires protein kinase C phosphorylation of the GluR2 subunit within its carboxyl-terminal PSD-95/Discs Large/Zona Occludens-1 (PDZ) ligand and binding of the PDZ domain-containing protein, PICK1. The sequence of the GluR2 subunit is similar to that of the GluR3 and GluR4c subunits, which also contain PDZ ligands and protein kinase C consensus sites. Although GluR3 and GluR4c are also expressed in Purkinje cells, we have previously shown that cerebellar LTD is absent in GluR2^{-/-} mice, suggesting that these subunits are unable to substitute functionally for GluR2. Here, we examine the apparent difference in the regulation of these AMPA receptor subunits by attempting to rescue LTD in GluR2^{-/-} Purkinje cells with WT and mutant GluR2 and GluR3 subunits. Our results show that the selective interaction of the GluR2 subunit with the *N*-ethylmaleimide-sensitive factor protein is required for synaptic, but not extrasynaptic, incorporation of AMPA receptors as well as for their competence to undergo LTD. In addition, perfusion of a synthetic peptide that acutely disrupts the interaction of GluR2 with *N*-ethylmaleimide-sensitive factor selectively depletes GluR2-containing receptors from synapses and occludes LTD. These findings demonstrate that interaction of AMPA receptors with *N*-ethylmaleimide-sensitive factor plays a critical role in incorporation of AMPA receptors into synapses and for their subsequent removal during cerebellar LTD.

cerebellar LTD | GluR2

Cerebellar long-term depression (LTD) at the parallel fiber–Purkinje cell synapse is a persistent form of synaptic plasticity that may be important for certain types of motor learning. Cerebellar LTD occurs when parallel fiber and climbing fiber inputs to a Purkinje cell are repeatedly coactivated and has been shown to involve the removal of postsynaptic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors from the plasma membrane of Purkinje cell dendrites (reviewed in refs. 1 and 2). Induction of cerebellar LTD is thought to require activation of AMPA receptors, voltage-gated calcium channels, and metabotropic mGluR1 receptors. Simultaneous activation of these receptors and channels induces a rise in postsynaptic Ca²⁺ and the liberation of diacylglycerol, which then work together to activate the α isoform of protein kinase C (3). This leads to phosphorylation of the AMPA receptor GluR2 subunit on Ser-880 within its carboxyl-terminal PSD-95/Discs Large/Zona Occludens-1 (PDZ) ligand and the consequent removal of AMPA receptors by clathrin-mediated endocytosis (4–7). The removal of AMPA receptors also appears to require interaction of the GluR2 subunit with the PDZ domain-containing protein, PICK1 (8). A model based on these findings proposes that PICK1 binds GluR2 after protein kinase C-mediated phosphorylation at Ser-880. PICK1 binding then

facilitates endocytosis of GluR2-containing AMPA receptor complexes or, alternatively, their stabilization in internalized pools.

GluR2 belongs to a class of AMPA receptor subunits that contain short intracellular carboxyl-terminal domains. Among the others in this class are the GluR3 and GluR4c subunits. Sequence alignment indicates that both GluR3 and GluR4c contain protein kinase C phosphorylation consensus sites (Ser/Thr-X-Lys/Arg; ref. 9) similar to that surrounding GluR2 Ser-880. Moreover, like GluR2, the GluR3 and GluR4c subunits terminate in PDZ ligands, which serve as binding sites for the PDZ domain-containing proteins, PICK1 and GRIP1 and 2 (ref. 2). Given the similarity of GluR3 and GluR4c to GluR2 with regard to their putative protein kinase C phosphorylation sites and PICK1-binding capabilities, it remains unclear why LTD cannot be induced in cultured Purkinje cells prepared from GluR2^{-/-} mice despite the continued expression of GluR3 and GluR4c (7).

One known difference between the GluR2 and GluR3/4c subunit carboxyl-terminal domains lies in the membrane-proximal region. In this region, GluR2 contains a stretch of 10 amino acids that confer binding to the *N*-ethylmaleimide-sensitive factor (NSF), a ubiquitous protein involved in vesicular trafficking events (10). Several studies in hippocampal slices or cultured hippocampal neurons have suggested that NSF binding to GluR2 is important for the maintenance of AMPA receptors at synapses. For example, intracellular perfusion of synthetic peptides that interfere with the NSF–GluR2 interaction has been shown to result in a rapid rundown of AMPA receptor-mediated synaptic currents (11–16). Moreover, in one study, viral expression of a fusion protein in hippocampal neurons designed to interfere with binding of NSF to GluR2 was found to reduce the surface expression of AMPA receptors visualized by immunocytochemistry (13).

To investigate the potential role of the GluR2 NSF-binding site in cerebellar LTD, we attempted to rescue LTD in cultured GluR2^{-/-} Purkinje cells with WT and mutant GluR2 and GluR3 subunits. In addition, in GluR2^{+/+} Purkinje cells, we investigated the acute effects of interfering with the NSF–GluR2 interaction on synaptic transmission and LTD. Our results suggest that NSF binding to GluR2 is required for the mobilization of GluR2-containing receptors from the extrasynaptic to synaptic membrane and for the induction of cerebellar LTD.

Materials and Methods

Genotyping and Preparation of Dissociated Purkinje Cell Cultures. To determine the genetic identity of embryonic days 18–19 off-

Abbreviations: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; LTD, long-term depression; EPSC, excitatory postsynaptic current; mEPSC, miniature EPSC; PICK1, protein interacting with C-kinase 1; NSF, *N*-ethylmaleimide-sensitive factor; PDZ, PSD-95/Discs Large/Zona Occludens-1.

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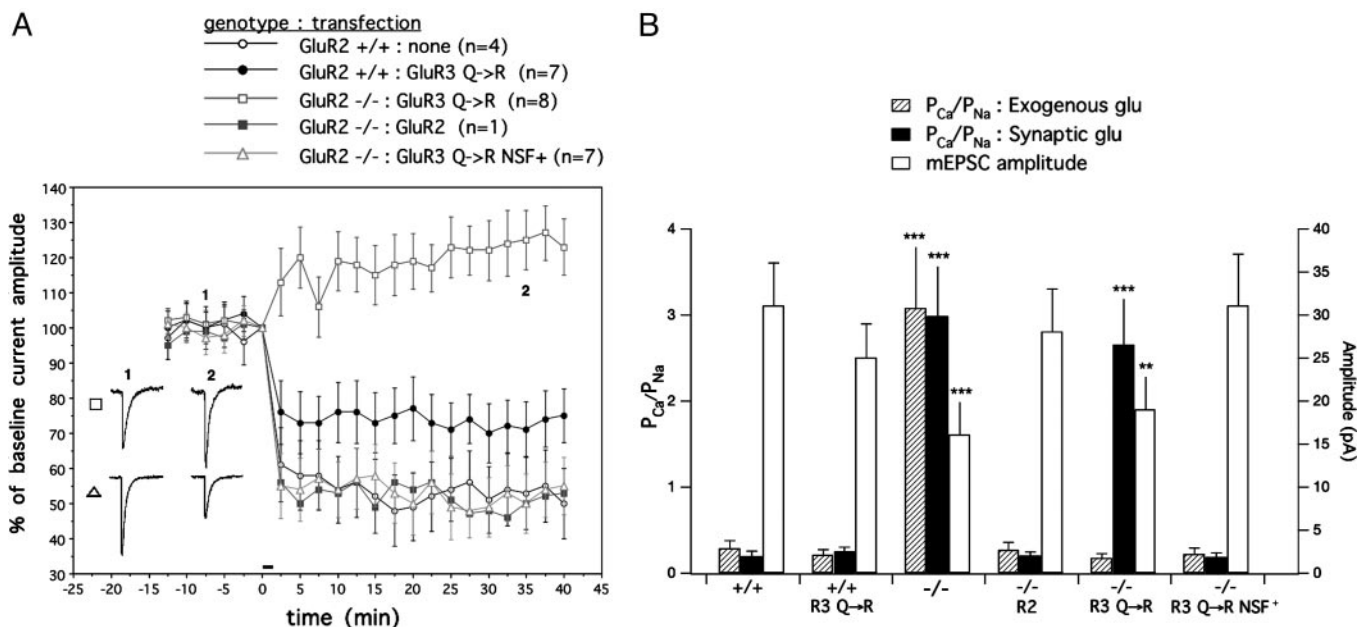


Fig. 1. Rescue of cerebellar LTD and synaptic Ca^{2+} permeability in $GluR2^{-/-}$ Purkinje cells requires NSF binding. (A) LTD was induced by iontophoretic glutamate in conjunction with depolarization at $t = 0$ min (horizontal bar). Error bars represent the SEM in this and all other graphs. Representative raw current traces were acquired at the indicated times. (Scale bars: 1 sec, 50 pA). (B) Basal synaptic properties of cultured Purkinje cells in each of the five conditions depicted in A. P_{Ca}/P_{Na} values are plotted on the left axis, whereas mEPSC amplitudes are plotted on the right axis. **, $P < 0.02$ compared with $GluR2^{+/+}$ and ***, $P < 0.001$ compared with $GluR2^{+/+}$.

transfected cells vs. 2.98 ± 0.60 in untransfected $GluR2^{-/-}$ and 0.19 ± 0.07 in $GluR2^{+/+}$). Interestingly, $GluR3$ Q→R transfection completely restored a low Ca^{2+}/Na^{+} permeability ratio of AMPA receptors in response to exogenously applied glutamate (0.17 ± 0.06 in $GluR3$ Q→R-transfected cells vs. 3.08 ± 0.73 in untransfected $GluR2^{-/-}$ and 0.28 ± 0.09 in $GluR2^{+/+}$). Our interpretation of these findings is that the overexpressed $GluR3$ Q→R subunit reaches the surface plasma membrane in $GluR2^{-/-}$ Purkinje cells, but fails to become incorporated into synapses.

Transfer of the NSF-Binding Sequence into $GluR3$ Allows Rescue of Basal Synaptic Properties and Cerebellar LTD in $GluR2^{-/-}$ Purkinje Cells. To test whether NSF binding could explain the functional differences between the $GluR2$ and $GluR3$ subunits, we created an NSF-binding site in $GluR3$ by mutating the membrane-proximal region of the $GluR3$ Q→R construct to resemble that of $GluR2$ ($GluR3$ Q→R NSF⁺; Fig. 2). When transfected into $GluR2^{-/-}$ Purkinje cells, this construct provided a complete rescue of LTD ($55 \pm 8.2\%$ of baseline, $n = 7$; Fig. 1A) as well as the basal mean mEPSC amplitude (31 ± 6 pA in $GluR3$ Q→R NSF⁺ transfected cells vs. 16 ± 4 pA in untransfected $GluR2^{-/-}$ and 31 ± 5 pA in $GluR2^{+/+}$; Fig. 1B). $GluR3$ Q→R NSF⁺ also completely rescued the Ca^{2+}/Na^{+} permeability ratio to WT levels in response to both exogenous (0.22 ± 0.08 in $GluR3$ Q→R NSF⁺ transfected cells vs. 3.08 ± 0.73 in untransfected $GluR2^{-/-}$ and 0.28 ± 0.09 in $GluR2^{+/+}$) and synaptic glutamate (0.18 ± 0.06 $GluR3$ Q→R NSF⁺ transfected cells vs. 2.98 ± 0.60 in untransfected $GluR2^{-/-}$ and 0.19 ± 0.07 in $GluR2^{+/+}$; Fig. 1B). These data suggest a critical role for NSF binding in the movement of $GluR2$ -containing receptors from the extrasynaptic to synaptic membrane and their subsequent removal upon induction of cerebellar LTD.

Acute Peptide-Mediated Disruption of the $GluR2$ -NSF Interaction in Purkinje Cells Reduces Synaptic Currents and Occludes Cerebellar LTD. To further probe the role of NSF binding, we acutely perfused peptides that interfere with the $GluR2$ -NSF interaction into

$GluR2^{+/+}$ Purkinje cells in culture. When a previously characterized peptide specific for the NSF- $GluR2$ interaction (KAMKVAKNPQ; ref. 16) was introduced into Purkinje cells, a gradual rundown in EPSCs evoked by stimulation of granule cell-Purkinje cell pairs was observed, which became stable at ≈ 45 min ($51 \pm 6.7\%$ of baseline at $t = 50$ min, $n = 6$; Fig. 3A). No rundown was observed with a control peptide (ARMKVAKNPQ; ref. 16) over this same time period ($102 \pm 7.5\%$ of baseline, $n = 6$). Interestingly, the rundown in current observed with the NSF-interfering peptide after 45 min of recording was

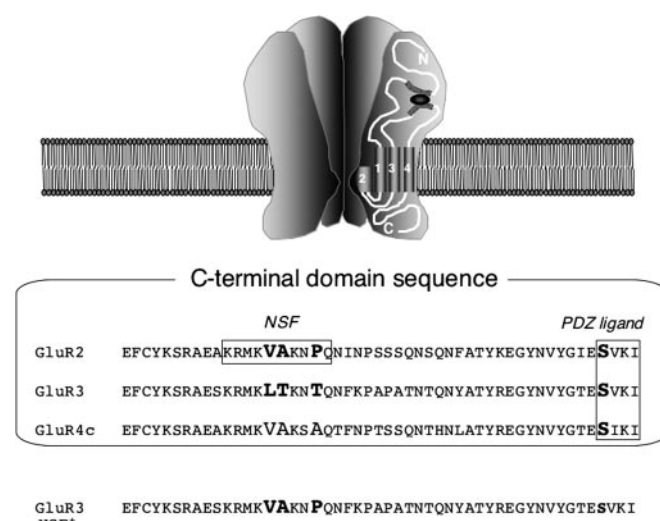


Fig. 2. Sequence alignment of $GluR2$, $GluR3$, and $GluR4c$, shown for rat ($\geq 98\%$ identity to mouse homologs). Bold residues denote differences between $GluR2$ and $GluR3$ in the NSF-binding region. $GluR3$ NSF⁺ mutation = $GluR3$ L853V/T854A/T857P. Ser-880, located within the $GluR2$ PDZ ligand, as well as corresponding serines in other subunits, are also shown in bold.

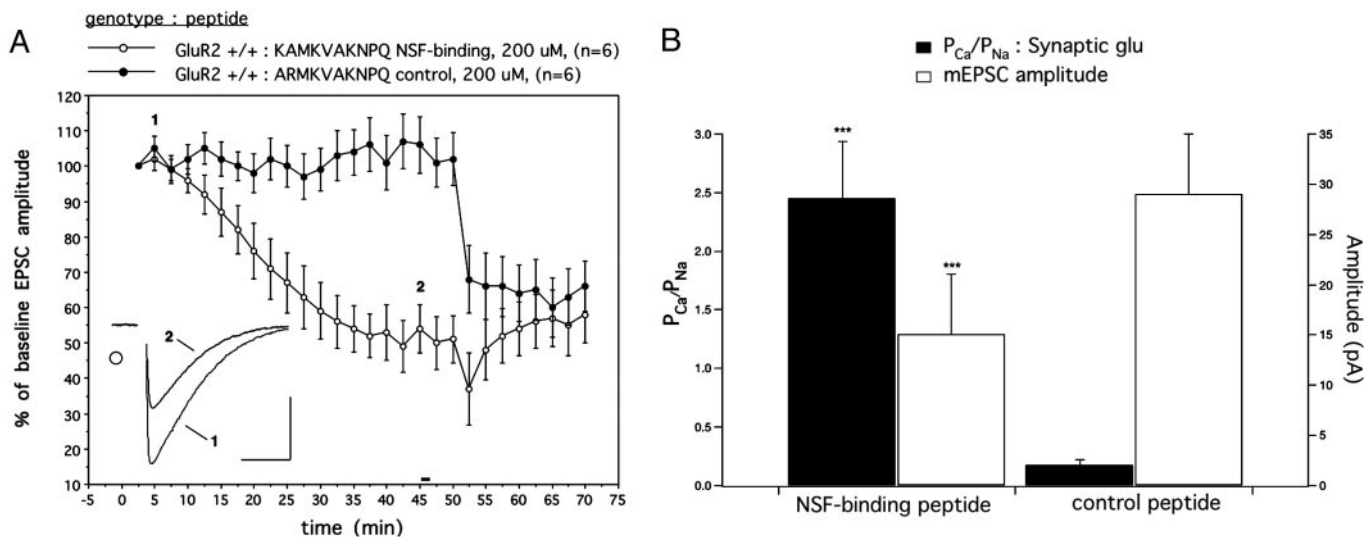


Fig. 3. Acute peptide-mediated disruption of the NSF-GluR2 interaction causes gradual rundown of the synaptic EPSC amplitude during the baseline period and occludes subsequent LTD induction. (A) LTD was induced by pairing granule cell-evoked EPSCs with Purkinje cell depolarization after a stable baseline was achieved (horizontal bar at $t = 50$ min). Representative current traces are the averages of five consecutive responses. (Scale bars: 5 ms, 50 pA). (B) Basal synaptic properties of GluR2^{+/+} Purkinje cells perfused with NSF-binding or control peptides for ≥ 60 min (a separate population from that shown in A). $P_{Ca^{2+}/P_{Na^{+}}}$ values are plotted on the left axis, whereas mEPSC amplitudes are plotted on the right axis. NSF-binding peptide, $n = 10$; control peptide, $n = 9$. ***, $P < 0.001$ compared with control.

concomitant with a reduction in the mEPSC amplitude and an increase in the synaptic Ca^{2+}/Na^{+} permeability ratio (measured in a separate population of cells), resulting in levels comparable to those observed in GluR2^{-/-} Purkinje cells (mEPSC amplitude: 15 ± 6 pA for NSF-interfering peptide, $n = 10$ vs. 29 ± 6 pA for control peptide, $n = 9$; $P_{Ca^{2+}/P_{Na^{+}}}$: 2.45 ± 0.49 for NSF-interfering peptide vs. 0.17 ± 0.05 for control peptide; Fig. 3B). When LTD-inducing stimuli were applied to cell pairs after a stable baseline was achieved, no further depression was observed in Purkinje cells perfused with the NSF-interfering peptide ($58 \pm 8.0\%$ of baseline vs. $66 \pm 7.0\%$ of baseline for control, measured at $t = 70$ min). Taken together, these results suggest that sustained perfusion of the NSF-interfering peptide eliminates GluR2-containing and LTD-competent AMPA receptors from the synapse.

Discussion

In this study, we investigated the differential ability of the GluR2 and GluR3 AMPA receptor subunits to support LTD in cerebellar Purkinje cells. WT and mutant GluR2 and GluR3 subunits were used to rescue LTD in GluR2^{-/-} Purkinje cells. Altogether, these experiments show that the NSF-binding site on GluR2 is required for synaptic incorporation of AMPA receptors and induction of LTD. The GluR3 subunit, which is unable to bind NSF, is restricted to extrasynaptic sites when transfected into GluR2^{-/-} Purkinje cells as demonstrated by the restoration of a low Ca^{2+}/Na^{+} permeability ratio (by the Q \rightarrow R pore mutant) in response to exogenous, but not synaptic, glutamate. However, when the NSF-binding sequence is inserted into GluR3, the transfected receptor can enter synapses and completely rescue the Ca^{2+}/Na^{+} permeability ratio as well as LTD. These results are consistent with the peptide interference experiments in GluR2^{+/+} Purkinje cells in which sustained disruption of the GluR2-NSF interaction was found to deplete GluR2-containing receptors from the synaptic compartment. After a 45-min rundown period, synapses have electrophysiological properties similar to those of GluR2^{-/-} Purkinje cells: The synaptic AMPA receptor Ca^{2+}/Na^{+} permeability ratio is elevated and LTD cannot be induced.

Our results are consistent with previous studies showing that GluR3 fails to incorporate into synapses upon overexpression in virally infected hippocampal slice cultures (22). In this system, GFP-tagged GluR3 was visualized in spines but failed to change the rectification properties of synaptic AMPA receptors. Such a change would be expected with incorporation of GFP-GluR3-containing receptors into the synaptic plasma membrane be-

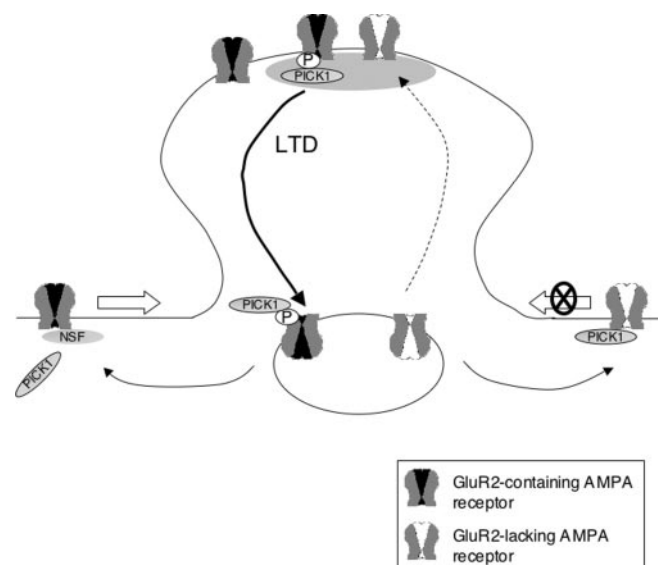


Fig. 4. A model of NSF and GluR2 action in cerebellar LTD based on rescue transfection and peptide perfusion experiments. GluR2-containing AMPA receptor complexes (drawn with a black pore) are Ca^{2+} -impermeable, enter the synaptic compartment from extrasynaptic regions in a manner dependent on NSF, and are internalized upon induction of cerebellar LTD via phosphorylation and binding to PICK1. GluR2-lacking AMPA receptor complexes (drawn with a white pore) are Ca^{2+} -permeable. They cannot bind NSF and remain largely extrasynaptic. A subset of GluR2-lacking AMPA receptors become synaptically localized in an NSF-independent manner (dashed arrow) but are incompetent for LTD.

cause of the inward rectification properties of the GluR3 (Q) subunit (reviewed in ref. 20). Interestingly, although rectification was unchanged in this experiment, a decrease in AMPA receptor-mediated EPSC amplitude was observed. Our results in Purkinje cells suggest that NSF may be required for lateral migration of AMPA receptors from extrasynaptic to synaptic sites in hippocampal neurons as well. Although no direct role for NSF in lateral membrane diffusion has been identified, recent work indicates that NSF can dissociate GluR2–PICK1 complexes through its ATPase activity (23). In an analogous manner, it is conceivable that NSF permits AMPA receptor entry into synapses by disrupting GluR2 interactions with proteins, possibly PICK1, in regions outside the synapse.

Although GluR3 Q→R was delivered to the extrasynaptic plasma membrane in our experiments, it failed to rescue LTD that was both induced and measured by iontophoretic glutamate application. In contrast, GluR3 Q→R NSF⁺ fully rescued LTD by using this same approach. Because iontophoretic glutamate activates synaptic as well as extrasynaptic receptors, our results suggest that entry of AMPA receptors into synapses from extrasynaptic membranes may be a prerequisite for their removal during LTD. Alternatively, NSF may play some specific additional role in regulating the removal of AMPA receptors from the plasma membrane during LTD.

Previous studies performed in hippocampal slices have indicated that the NSF–GluR2 interaction regulates the same pool of receptors that become internalized during hippocampal LTD (14, 15). However, these studies relied on the use of peptides that block the interaction of GluR2 with NSF as well as the interaction of the AP2 clathrin adaptor. AP2 binds to GluR2 in a region that overlaps the NSF-binding site (16). Lee *et al.* (16) distinguished between the effects of NSF and AP2 by designing peptides that selectively interfere with the binding of either protein. When a peptide specific for the NSF–GluR2 interaction (KAMKVAKNPQ) was perfused into CA1 pyramidal cells, a rundown in EPSC amplitude was observed over a period of ≈20 min. In contrast to our results in Purkinje cell cultures by using

the same peptide, Lee *et al.* (16) found that hippocampal LTD could still be induced following this rundown. With an AP2-interfering peptide, Lee *et al.* (16) observed the opposite effect: no rundown of basal EPSCs but complete blockade of LTD induction. Although these results might initially suggest the existence of separate receptor pools regulated by NSF and AP2, Lee *et al.* (16) acknowledged the possibility that interference with NSF might indirectly lead to loss of LTD by means of occlusion. The LTD observed in their experiments by using the NSF-interfering peptide may represent incomplete occlusion, perhaps because of the different combinations of AMPA receptor subunits expressed in hippocampal cells as compared to Purkinje cells. This is consistent with the observation that hippocampal LTD is still present in GluR2^{-/-} mice (21).

Although NSF appears to regulate entry of AMPA receptors into the synaptic compartment, our results indicate that at least some AMPA receptors become synaptically localized in an NSF-independent manner. This population of receptors corresponds to those contributing to the residual mEPSC response in GluR2^{-/-} Purkinje cells and in GluR2^{+/+} Purkinje cells perfused with the NSF-interfering peptide after 45 min of rundown. These residual Ca²⁺-permeable AMPA receptors, however, cannot support LTD because no synaptic depression was observed in the absence of GluR2. This raises the possibility of an additional locus of action of NSF in receptor internalization during LTD, as mentioned above. Alternatively, these Ca²⁺-permeable AMPA receptors at synapses may contain subunits with long intracellular domains such as GluR4 that endow the receptors with other regulatory mechanisms that render them incompetent for LTD (Fig. 4).

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