receptor endocytosis during metabotropic glutamate receptor (mGluR)-dependent LTD at hippocampal synapses (11-14). However, whether Y876 phosphorylation is involved in cerebellar LTD, which is dependent on mGluR, is unknown. In addition, its relationship to S880 phosphorylation is unclear.

In this study, we showed that activity-dependent phosphorylation of S880 of GluA2 AMPA receptors was impaired in *GluD2*-null cerebellum. In contrast, basal-state phosphorylation of Y876 of GluA2 was increased in *GluD2*-null cerebellum. Interestingly, Y876 phosphorylation inhibited subsequent S880 phosphorylation. Conversely, Y876 dephosphorylation restored S880 phosphorylation and LTD induction in *GluD2*-null Purkinje cells. Furthermore, interaction with the tyrosine phosphatase PTPMEG (15) at the C terminus of GluD2 was necessary and sufficient for GluD2 to regulate LTD induction. These results indicate that GluD2 serves as a master switch to gate inducibility of LTD by coordinating interaction between two phosphorylation sites of GluA2 via its interaction with PTPMEG. Since PTPMEG and GluD1, a family protein of GluD2, are expressed in regions outside the cerebellum, similar regulatory mechanisms of AMPA receptor endocytosis may also be operational in other brain regions.

Results

Increased Basal-State Phosphorylation of Tyrosine in *GluD2*-Null Purkinje Cells. Phosphorylation of S880 (6-10) or Y876 (11, 12, 14, 16) of GluA2 (Fig. 1*A*) is suggested to play important roles in AMPA receptor endocytosis and LTD. Thus, we first examined basal-state phosphorylation of GluA2 in the synaptosomal fraction of the cerebellum from postnatal day (P) 21–30 wild-type (WT) and *GluD2*-null mice.

Immunoblot analysis using phosphorylation-specific antibodies revealed weak basal-state phosphorylation of S880 and Y876 of GluA2 in WT cerebellum (Fig. 1*A*). Basal phosphorylation levels of S880 of GluA2 were similar in WT and *GluD2*-null cerebellum (0.77 ± 0.19 fold vs. WT, n = 7 each, $P = 0.15$). Interestingly, basal phosphorylation levels of Y876 of GluA2 were significantly increased in *GluD2*-null cerebellum compared to those in WT cerebellum (1.29 ± 0.11 fold vs. WT, n = 11 each, $P = 0.02$; Fig. 1*A*). Thus, loss of GluD2 from parallel fiber (PF)–Purkinje cell synapses increased tyrosine phosphorylation of GluA2 in the cerebellum.

To clarify whether increased phosphorylation levels of Y876 of GluA2 observed in immunoblot analyses (Fig. 1*A*) reflected changes at PF synapses of Purkinje cells, we next examined PF–evoked slow excitatory postsynaptic currents ($_{slow}$EPSCs), which is known to be regulated by tyrosine phosphorylation levels in Purkinje cells (17). We performed patch-clamp recordings in acutely prepared slices from P21–35 mice and adjusted PF stimulus intensity to evoke similar amplitudes of fast EPSCs mediated by AMPA receptors ($_{AMPA}$EPSCs) between WT and *GluD2*-null Purkinje cells. Then, to evoke and isolate $_{slow}$EPSCs, tetanic stimulation (2 to 10 pulses at 100 Hz) was applied to PFs in the presence of an AMPA receptor antagonist NBQX. We found that amplitudes (Fig. 1*B*) and transferred charges of $_{slow}$EPSCs, which were normalized by those of $_{AMPA}$EPSCs, were significantly smaller in *GluD2*-null cells than those in WT Purkinje cells (Fig. 1*B*; $P = 0.001$ in amplitudes and $P = 0.006$ in transferred charges). PF–evoked $_{slow}$EPSCs are regulated by metabotropic glutamate receptor 1 (mGluR1), which is coupled to opening of canonical transient receptor potential channel 1 (TRPC1) or TRPC3 (18, 19). However, immunohistochemical analyses did not show any difference in expression patterns of mGluR1, TRPC1, or TRPC3 between WT and

GluD2-null Purkinje cells (Fig. S1). Indeed, functions of mGluR1 itself were reported as normal in *GluD2*-null Purkinje cells (20). These data suggest that phosphorylation levels of tyrosine are increased in *GluD2*-null Purkinje cells.

To further test this hypothesis, we introduced a PP1 analog (10 μM for 20 min), a specific Src family tyrosine kinase (SFK) inhibitor, into WT and *GluD2*-null Purkinje cells through a patch pipette. As reported previously (17), $_{slow}$EPSCs evoked by a submaximal tetanus stimulus (4 pulses at 100 Hz) to PFs significantly increased by an intracellular loading of a PP1 analog into WT Purkinje cells (Fig. 1*C* and 1*D*). Importantly, reduced $_{slow}$EPSC amplitudes in *GluD2*-null Purkinje cells were rescued by treatment with a PP1 analog (Fig. 1*C* and 1*D*). A PP1 analog exerted stronger enhancing effects on $_{slow}$EPSC amplitudes in *GluD2*-null compared to WT Purkinje cells; this may reflect higher basal-state tyrosine phosphorylation in *GluD2*-null Purkinje cells. Together, these results indicate that phosphorylation levels of tyrosine (including Y876 of GluA2) are generally increased at postsynaptic sites of PF–Purkinje cell synapses in *GluD2*-null cerebellum.

Activity-Dependent Increased Y876 Phosphorylation Inhibits Activity-induced S880 Phosphorylation in *GluD2*-Null Purkinje Cells. To biochemically determine changes in the phosphorylation state of GluA2 during cerebellar LTD, we employed a chemical LTD protocol, which is a combination of 50 mM K^+ and 10 μM L-glutamate (K-glu), to mimic the climbing fiber–evoked depolarization of Purkinje cells and PF–induced activation (21). Cell lysates from WT and *GluD2*-null cerebellar slices treated with K-glu for 5 min were subjected to immunoblot analysis with phosphorylation-specific antibodies. We confirmed that S880

phosphorylation significantly increased on K-glu treatment in WT cerebellum (1.84 ± 0.44 fold vs. no treatment control, n = 8 each, $P = 0.04$; Fig. 2*A*), as reported previously (21). In contrast, K-glu treatment failed to induce S880 phosphorylation in *GluD2*-null cerebellum (1.15 ± 0.16 fold vs. control, n = 13 each, $P = 0.56$; Fig. 2*B*). These data support the concept that phosphorylation of S880 of GluA2 was essential for cerebellar LTD (8, 10) and indicate that this step is impaired in *GluD2*-null cerebellum.

Although rapid dephosphorylation of tyrosine of GluA2 (12, 22), especially at Y876 (14), was reported in mGluR-dependent LTD in the hippocampus, whether Y876 phosphorylation is involved in cerebellar LTD has been unclear. We found that K-glu treatment also significantly decreased phosphorylation levels of Y876 in WT cerebellum (0.58 ± 0.12 fold vs. control, n = 8 each, $P < 0.01$; Fig. 2*A*). Interestingly, K-glu treatment failed to induce changes in phosphorylation levels of Y876 in *GluD2*-null cerebellar slices (0.93 ± 0.11 fold vs. control, n = 13 each, $P = 0.50$; Fig. 2*B*). Thus, basal-state phosphorylation of tyrosine was not only increased but also remained high after stimulation with K-glu in *GluD2*-null cerebellum.

Increased Y876 Phosphorylation Inhibits Activity-Induced S880 Phosphorylation in *GluD2*-Null Purkinje Cells. Why did K-glu treatment fail to phosphorylate S880 and also fail to dephosphorylate Y876 in *GluD2*-null cerebellum? Since these two sites are located close to each other (Fig. 1*A*), we hypothesized that Y876 phosphorylation may have interfered with subsequent S880 phosphorylation. To examine this hypothesis, we first performed an *in vitro* phosphorylation assay using the GluA2 C-terminal region fused to glutathione S-transferase (GST-GluA2-CT). Immunoblot analyses using phosphorylation-specific antibodies confirmed that

GST-GluA2-CT was phosphorylated at Y876 and S880 *in vitro* by purified Src and protein kinase C (PKC), respectively. Interestingly, prior treatment of GST-GluA2-CT with Src, but not with boiled Src, significantly decreased subsequent S880 phosphorylation by PKC (0.66 ± 0.06 fold vs. boiled Src treatment, n = 13, $P < 0.001$), whereas addition of PKC itself did not affect phosphorylation levels of Y876 (Fig. 2*C*). In contrast, when Y876 was replaced with unphosphorable phenylalanine (GST-GluA2^{Y876F}-CT), prior treatment with Src did not affect subsequent S880 phosphorylation by PKC (1.15 ± 0.13 fold vs. boiled Src treatment, n = 6, $P = 0.13$; Fig. 2*D*). These results indicate that phosphorylation of Y876 of GluA2 specifically regulates subsequent phosphorylation of S880 by PKC *in vitro*.

To further examine this hypothesis in a cellular context, we treated *GluD2*-null cerebellar slices with a PP1 analog and examined whether K-glu treatment could induce S880 phosphorylation. Immunoblot analyses confirmed that incubation with PP1 analog significantly reduced basal-state phosphorylation of Y876 without changes in S880 phosphorylation in *GluD2*-null cerebellar slices (Fig. S2). As observed in WT cerebellar slices (Fig. 2*A*), subsequent K-glu treatment induced a decrease in Y876 phosphorylation (0.83 ± 0.09 fold vs. control slices, n = 16, $P = 0.04$) as well as an increase in S880 phosphorylation (1.37 ± 0.19 fold vs. control, n = 17, $P = 0.04$) in *GluD2*-null cerebellar slices, which had been incubated with a PP1 analog (Fig. 2*E*). Together, these results indicate that increased phosphorylation of Y876 of GluA2 was responsible for reduced S880 phosphorylation by K-glu in *GluD2*-null cerebellum.

Rescue of Impaired LTD in *GluD2*-Null Purkinje Cells by Inhibiting Phosphorylation of Y876 of GluA2. To establish the causal relationship between

increased Y876 phosphorylation and impaired LTD, we next applied a PP1 analog to *GluD2*-null Purkinje cells through a patch pipette. Inclusion of a PP1 analog did not affect baseline PF–evoked AMPA receptor-mediated EPSCs (PF–EPSCs; Fig. S3) in WT and *GluD2*-null Purkinje cells. In addition, application of conjunctive stimulation (CJ-stim; consisting of 30 cycles of PF stimulation plus Purkinje cell depolarization at 1 Hz) robustly induced LTD in WT Purkinje cells with (Fig. 3*B* and 3*C*; 72 ± 5% at 25–30 min after CJ-stim, n = 8) or without a PP1 analog (Fig. 3*A* and 3*C*; 74 ± 4% at 25–30 min after CJ-stim, n = 9, $P = 0.56$ vs. with a PP1 analog). In contrast, while CJ-stim failed to induce LTD in *GluD2*-null Purkinje cells (Fig. 3*D* and 3*F*; 99 ± 2% at 25–30 min after CJ-stim, n = 8) as reported previously (2), it induced LTD in Purkinje cells with a PP1 analog (Fig. 3*E* and 3*F*; 80 ± 5% at 25–30 min after CJ-stim, n = 10; $P = 0.006$ vs. without PP1 analog). These results indicate that impaired LTD in *GluD2*-null Purkinje cells was successfully rescued by application of a PP1 analog to Purkinje cells and that increased tyrosine phosphorylation was responsible for impaired LTD in *GluD2*-null Purkinje cells.

A PP1 analog could affect tyrosine phosphorylation of many proteins in Purkinje cells. Thus, to examine the specific role of GluA2 phosphorylation at Y876 in LTD, we expressed GluA2 mutants, in which tyrosine phosphorylation sites were disrupted, in *GluD2*-null Purkinje cells using a Sindbis virus vector. CJ-stim failed to induce LTD in *GluD2*-null Purkinje cells expressing WT GluA2 (Fig. 3*G* and 3*J*; 89 ± 6% at 25–30 min after CJ-stim, n = 6) or a mutant GluA2, in which phenylalanine replaced two tyrosine residues at 869 and 873 ($GluA2^{Y869F,Y873F}$; Fig. 3*I* and 3*J*; 91 ± 6% at 25–30 min after CJ-stim, n = 6). In contrast, CJ-stim induced LTD in *GluD2*-null Purkinje cells expressing a mutant GluA2, in which phenylalanine replaced tyrosine at 876

(GluA2^{Y876F}; Fig. 3*H* and 3*J*; 66 ± 5% at 25–30 min after CJ-stim, n = 5, P = 0.038 vs. GluA2^{WT} and P = 0.048 vs. GluA2^{Y869F,Y873F}). Surface biotinylation assays showed that cell surface expression of GluA2^{Y876F} and GluA2^{Y869F,Y873F} was comparable to that of WT GluA2 in HEK293 cells (Fig. S4). These results indicate that increased Y876 phosphorylation, but not other tyrosine residues, at the C terminus of GluA2 was indeed responsible for impaired LTD and its dephosphorylation was sufficient to restore LTD in *GluD2*-null mice.

Phosphorylation of Y876 Inhibits LTD Independent of BRAG2–Arf6 Pathway in the Cerebellum. Recently, BRAG2, a guanine-nucleotide exchange factor for Arf6, has been shown to bind to the C terminus of GluA2 in a manner dependent on Y876 dephosphorylation, and thereby regulating AMPA receptor endocytosis at hippocampal synapses during LTD (14). Thus, increased Y876 phosphorylation in *GluD2*-null Purkinje cells may inhibit the BRAG2–Arf6 pathway in addition to S880 phosphorylation. To examine this possibility, we used synthetic peptides derived from the sequence in the C terminus of GluA2 between positions 869 and 877 (^{869}**Y**KEG**Y**NV**Y**G^{877}; Fig. 4*A*) that contained three tyrosine residues. Unphosphorylated peptides (pep-3Y), but not the unphosphorable peptides (pep-3A), in which alanine replaced three tyrosine residues, were previously shown to inhibit LTD induction in amygdala (23), nucleus accumbens (24), and hippocampus (11, 14). In these studies, pep-3Y may have inhibited LTD induction by serving as a decoy peptide for BRAG2 (14) or as a pseudo-substrate for SFKs (16). In contrast to these previous reports, application of these peptides to WT Purkinje cells via a patch pipette did not affect CJ-stim–induced LTD in the cerebellum (Fig. 4*B*, 4*D* and 4*E*; 66 ± 4% and 69 ±

7% at 25–30 min after CJ-stim in pep-3Y and pep-3A, respectively, n = 8 each). Instead, application of phosphorylated peptides, in which all tyrosine residues were phosphorylated (pep-3pY), significantly inhibited LTD induction in WT Purkinje cells (Fig. 4*C* and 4*E*; 90 ± 6% at 25–30 min after CJ-stim, n = 8, $P = 0.035$ vs. pep-3Y and $P = 0.047$ vs. pep-3A). We did not detect differences in EPSC amplitudes just after breaking into whole-cell mode and 9–10 min later in any experiments using peptides, (Fig. S5), suggesting that peptides did not affect basal PF–Purkinje cell synaptic transmission. Since BRAG2 does not bind to phosphorylated GluA2 peptides (14), these results suggest that the BRAG2–Arf6 signaling pathway may not play a major role in CJ-stim–induced LTD in Purkinje cells. Instead, pep-3pY most likely served as a pseudo-substrate for tyrosine phosphatases. Thus, increased Y876 phosphorylation in *GluD2*-null Purkinje cells inhibits LTD induction mainly by preventing S880 phosphorylation.

***PTPMEG*-Null and *GluD2*-Null Cerebellum Show Similar GluA2 Phosphorylation Patterns.** Why do *GluD2*-null mice show increased phosphorylation of Y876 of GluA2? Since the C-terminal domain of GluD2 plays a crucial role in LTD induction (3, 4), we first examined whether the C terminus of GluD2 also regulated tyrosine phosphorylation at PF–Purkinje cell synapses by measuring $_{slow}$EPSCs. We used transgenic mice that express WT GluD2 (*Tg*WT) or mutant GluD2 lacking the seven C-terminal residues (*Tg*ΔCT7) on *GluD2*-null background. In contrast to *GluD2*-null mice, the number of PF–Purkinje cell synapses and climbing fiber innervation patterns in *GluD2*-null/*Tg*ΔCT7 cerebellum have previously been shown to be comparable to those in *GluD2*-null/*Tg*WT cerebellum (4). Nevertheless, amplitudes

and transferred charges of $_{slow}$EPSCs were significantly smaller in *GluD2*-null/*Tg*ΔCT7 than in *GluD2*-null/*Tg*WT Purkinje cells (Fig. 5*A*; $P = 0.001$ in amplitudes and $P = 0.001$ in transferred charges). These results indicate the C terminus of GluD2, to which various intracellular PDZ proteins bind, play a crucial role in regulation of tyrosine phosphorylation levels in Purkinje cells.

Among various PDZ proteins that bind to GluD2, PTPMEG is a good candidate since it has a catalytically active protein tyrosine phosphatase domain (15). Indeed, *PTPMEG*-null mice displayed impaired motor learning and abrogated LTD (25), although its underlying mechansims remained unknown. Thus, we hypothesized that GluD2 may regulate tyrosine phosphorylation levels at PF synapses via its interaction with PTPMEG. Indeed, immunoblot analyses of basal-state phosphorylation of GluA2 in the synaptosomal fraction revealed that Y876 phosphorylation was significantly increased in *PTPMEG*-null compared to WT cerebellum (Fig. 5*B*; 1.84 ± 0.35 fold vs. WT, n = 10 each, $P = 0.04$), while no differences were observed in phosphorylation levels of S880 between WT and *PTPMEG*-null cerebellum (Fig. 5*C*; 1.05 ± 0.42 fold vs. WT, n = 7 each, $P = 0.85$). Furthermore, K-glu treatment failed to induce a decrease in Y876 phosphorylation (0.99 ± 0.15 fold vs. WT, n = 15, $P = 0.97$) and an increase in S880 phosphorylation (0.86 ± 0.14 fold vs. WT, n = 15, $P = 0.36$) in *PTPMEG*-null cerebellar slices (Fig. 5*D*). These results were very similar to those observed in *GluD2*-null cerebellar slices (Fig. 1*A*, 1*B* and 2*B*) and suggest that it is PTPMEG that binds to the C terminus of GluD2 and regulates tyrosine phosphorylation at PF–Purkinje cell synapses.

Interaction with PTPMEG Is Necessary and Sufficient for GluD2 to Induce

LTD. To further clarify the significance of the interaction between GluD2 and PTPMEG in regulating LTD, we next expressed mutant GluD2 lacking the seven C-terminal residues (GluD2ΔCT7), which did not bind PTPMEG (15), in *GluD2*-null Purkinje cells using a Sindbis virus vector. As previously reported, unlike WT GluD2 (3), GluD2ΔCT7 could not rescue impaired LTD in *GluD2*-null Purkinje cells (Fig. 6*A* and 6*C*; 90 ± 8% at 25–30 min after CJ-stim, n = 8). In contrast, when GluD2ΔCT7-PTP, in which the catalytic phosphatase domain of PTPMEG was fused to the C terminus of GluD2ΔCT7, was expressed in *GluD2*-null Purkinje cells, CJ-stim successfully induced LTD (Fig. 6B and 6*C*; 76 ± 6% at 25–30 min after CJ-stim, n = 11, $P = 0.04$ vs. GluD2ΔCT7). These results suggest that the presence of the phosphatase domain of PTPMEG near GluD2 was sufficient to restore impaired LTD in *GluD2*-null Purkinje cells.

Next, to examine whether interaction between endogenous GluD2 and PTPMEG was indeed necessary for LTD induction in WT Purkinje cells, we expressed a phosphatase inactive mutant of PTPMEG ($PTPMEG^{DA}$; see next section) in WT Purkinje cells using a Sindbis virus vector. Since CJ-stim no longer induced LTD in WT cells expressing $PTPMEG^{DA}$ (Fig. 6*D* and 6*F*; 89 ± 4% at 25–30 min after CJ-stim, n = 8), $PTPMEG^{DA}$ probably exerted a dominant-negative effect on endogenous PTPMEG. In contrast, expression of another mutant ($PTPMEG^{DA}$-ΔPDZ), which lacked the PDZ domain necessary for binding to GluD2 (15), no longer inhibited LTD induction in WT Purkinje cells (Fig. 6*E* and 6*F*; 66 ± 4% at 25–30 min after CJ-stim, n = 9, $P = 0.004$ vs. $PTPMEG^{DA}$). Taken together, these results indicate that direct interaction between GluD2 and PTPMEG and its phosphatase activity at postsynaptic sites are necessary and sufficient for LTD induction in Purkinje cells.

GluA2 Is a Substrate for PTPMEG. Finally, to examine whether GluA2 serves as a substrate for PTPMEG, we performed a "substrate trapping" assay. Various substrates of tyrosine phosphatases have been identified using substrate trapping mutants, in which mutations in the catalytic center abrogated its enzymatic activity by trapping substrates (26). For example, a substrate trapping mutant of PTPH1, the most closely related phosphatase to PTPMEG in the phylogenetic tree, was produced by replacing aspartate with alanine in the catalytic center and used to identify valosin-containing protein (VCP) as a substrate (27). Thus, we introduced similar mutations to the catalytic center of PTPMEG to produce a possible trapping mutant $PTPMEG^{DA}$. Since many proteins are phosphorylated at tyrosine residues by endogenous tyrosine kinase activities in HEK293 cells, the lysate of HEK293 cells were pulled down by the GST fused with the catalytic domain of WT PTPMEG (GST-PTP^{WT}) or $PTPMEG^{DA}$ (GST-PTP^{DA}). As reported for PTPH1 (27), GST-PTP^{DA}, but not GST-PTP^{WT}, pulled down endogenous VCP in HEK293 cells (Fig. 7A). In contrast, unlike VCP, endogenous N-ethylmaleimide sensitive factor (NSF), a related member of the ATPases associated with a variety of cellular activities (AAA) family, did not interact with GST-PTP^{DA} (Fig. 7*A*), indicating that PTP^{DA} specifically traps its substrates. Importantly, GST-PTP^{DA} also pulled down GluA2 from the lysate of HEK293 expressing GluA2 (Fig. 7*A*), suggesting that GluA2 is a substrate for PTPMEG.

To examine whether PTPMEG directly dephosphorylates tyrosine residues at the C terminus of GluA2, we performed an *in vitro* dephosphorylation assay using pep-3pY as a substrate (Fig. 4*A*). GST-PTP^{DA} and GST-PTP^{CS}, in which the cysteine residue in the catalytic center was replaced with serine, were used as PTPMEG mutants with

reduced phosphatase activities. Isobaric tag-based mass spectrometric quantification of phosphorylation of pep-3pY peptides revealed that all tyrosine residues were dephosphorylated by incubation with GST-PTP^{WT} (0.06 ± 0.02 fold as compared with pep-3pY peptides incubated with GST only, n = 6, Fig. 7*B*). In contrast, relative phosphorylation levels of pep-3pY incubated with GST-PTP^{DA} (1.1 ± 0.3, n = 6) or GST-PTP^{CS} (1.0 ± 0.1, n = 6) were significantly higher than those treated with PTP^{WT} (WT vs. DA or CS, $P < 0.05$; Fig. 7*B*). These results indicate that phosphorylated tyrosine residues in the GluA2 C terminus are directly dephosphorylated by PTPMEG *in vitro*.

To further confirm that PTPMEG dephosphorylates Y876 of GluA2 in a cellular context, we examined whether full-length PTPMEG could dephosphorylate GluA2 in HEK293 cells. Immunoblot analyses of the lysate of HEK293 cells transfected with GluA2 revealed that GluA2 was weakly phosphorylated at Y876 by endogenous tyrosine kinases in HEK293 cells. We found that Y876 phosphorylation was significantly higher in HEK293 cells coexpressing a phosphatase-inactive mutant PTPMEG^{DA} (3.6 ± 0.9 fold, vs. PTPMEG^{WT}, n = 8 each, $P < 0.05$; Fig. 7*C*) than cells coexpressing PTPMEG^{WT} or an empty vector (1.4 ± 0.3 fold vs. PTPMEG^{WT}, n = 8 each, $P < 0.05$; Fig. 7*C*). There was no statistically significant difference in phosphorylation levels of Y876 between cells expressing PTPMEG^{WT} and an empty vector, suggesting that endogenous tyrosine phosphatase may dephosphorylate Y876 in HEK293 cells. Together, these results indicate that Y876 of GluA2 serves as a direct substrate for PTPMEG.

Discussion

LTD at PF–Purkinje cell synapses, which is believed to play important roles in motor learning in the cerebellum, absolutely requires functional GluD2 in Purkinje cells (28). Nevertheless, how and why GluD2 regulates LTD in the cerebellum remains elusive. In this study, we have demonstrated that basal-state phosphorylation of Y876 of GluA2 was increased (Fig. 1); phosphorylation at Y876 prevented subsequent phosphorylation at S880 (Fig. 2), an essential phosphorylation site for AMPA receptor endocytosis during LTD in both hippocampus (6, 7) and cerebellum (8-10). Indeed, dephosphorylation of Y876 restored AMPA receptor endocytosis (Fig. 2) and LTD (Fig. 3) in *GluD2*-null mice. Association of PTPMEG to the C terminus of GluD2 was necessary and sufficient for LTD induction in Purkinje cells (Fig. 3–6). Furthermore, PTPMEG directly dephosphorylated Y876 phosphorylation of GluA2 (Fig. 7). Therefore, we propose that GluD2 serves as a master switch to gate inducibility of LTD by coordinating interaction between two phosphorylation sites of GluA2 via its interaction with PTPMEG (Fig. 8).

Interaction between Y867 and S880 Phosphorylation during LTD. Whether Y876 phosphorylation is involved in cerebellar LTD has been controversial. In early studies, cerebellar LTD was reported to be blocked by broad-spectrum tyrosine kinase inhibitors, such as genistein and lavendustin A, suggesting that LTD depends on tyrosine phosphorylation events (29, 30). In contrast, application of a more specific Src inhibitor PP2 to the bath solution (31) or PP1 to a patch pipette (Fig. 3*A*–3*C*) did not affect LTD induction in WT Purkinje cells. Conversely, inclusion of purified Src in the patch pipette blocked LTD induction in Purkinje cells (31). Furthermore, phosphorylation of Y876 of GluA2 was reduced in the synaptosomal fraction of WT

cerebellar slices after chemical LTD by K-glu treatment (Fig. 2*A*). These results indicate that, like mGluR-dependent LTD in the hippocampus (32, 33), cerebellar LTD, which is also dependent on mGluR, is accompanied by dephosphorylation of Y876 of GluA2.

By studying *GluD2*-null and *PTPMEG*-null cerebellum, we found an unexpected interaction between Y876 and S880 phosphorylation sites of GluA2. When basal-state phosphorylation of Y876 increased in *GluD2*-null (Fig. 1*A*) or *PTPMEG*-null (Fig. 5*B*) cerebellum, K-glu treatment failed to increase S880 phosphorylation (Figs. 2*B* and 5*D*). Application of a PP1 analog not only reduced basal-state phosphorylation of Y876 but also restored K-glu–induced S880 phosphorylation (Fig. 2*E*) and CJ-stim–induced LTD (Fig. 3*D–F*) in *GluD2*-null Purkinje cells. LTD was also restored in *GluD2*-null Purkinje cells expressing GluA2^{Y876F} (Fig. 3*G–J*). Similar interaction between serine and tyrosine phosphorylation sites has been reported in other signaling molecules. For example, serine phosphorylation of insulin receptor substrate has been shown to hinder its tyrosine phosphorylation levels (34). Serine phosphorylation of the C terminus of the NMDA receptor subunit GluN2B also interferes with subsequent phosphorylation at a closely located tyrosine residue (35). In these cases, however, dissociation of interacting proteins by serine phosphorylation affects tyrosine phosphorylation in the locality. In contrast, a phosphomimetic peptide pep-3pY, which should facilitate LTD induction by competing for such interacting proteins, inhibited LTD induction in WT Purkinje cells (Fig. 4*C* and 4*E*). Furthermore, *in vitro* phosphorylation assay revealed that Y876 phosphorylation by Src specifically inhibits S880 phosphorylation by PKC *in vitro* (Fig. 2*C* and 2*D*). These results indicate that S880 phosphorylation is inhibited in *cis* by Y876 phosphorylation on single AMPA receptors by direct mechanisms, such as conformation or electrostatic charges associated with Y876 phosphorylation. Further structural studies

are warranted to clarify this unique interaction between Y876 and S880 phosphorylation sites of GluA2.

Dephosphorylation of Y876 of GluA2 plays an essential role in hippocampal LTD induced by the mGluR agonist 3,4-dihydroxyphenylglycine (DHPG) (32, 33). Specific binding of BRAG2 to dephosphorylated Y876 was shown to regulate AMPA receptor endocytosis in the hippocampal neurons by activating Arf6, which turns on phosphatidylinositol 4-phosphate 5-kinase (PIP5K) to produce phosphatidylinositol (4,5)-bisphosphate and recruit adaptor protein-2 (AP-2) and clathrin (14). In contrast to hippocampal LTD, however, cerebellar LTD was not inhibited by application of pep-3A (Fig. 4*D* and 4*E*), which competes for BRAG2 (14). Thus, the BRAG2–Arf6 pathway may not play a major role in cerebellar LTD. Since accumulation of AP-2 and clathrin is required for AMPA receptor endocytosis during cerebellar LTD (9), we suspect that PIP5K may be activated by other pathways. For example, PIP5K could be activated by calcineurin in low frequency stimulation-induced LTD in the hippocampus (36). Interestingly, PTPMEG is also expressed in the hippocampus (15). In addition, GluD1, a family member of GluD2 that also binds PTPMEG (Fig. S6), is expressed in the hippocampus (37). Considering that phosphorylation of S880 of GluA2 is involved in hippocampal LTD under certain conditions (7, 38), we suggest that Y876 dephosphorylation could also regulate hippocampal LTD induction by direct interaction with the S880 sites of GluA2, as we observed in Purkinje cells.

Regulation of LTD by GluD2-PTPMEG Signaling. In addition to PTPMEG, other proteins, such as delphilin, bind to the C terminus of GluD2. Notably, both motor learning and LTD induction are facilitated in mice lacking delphilin (39). The FH1

domain of delphilin binds to the SH3 domain of Src in the yeast two-hybrid system (40). We also found that SFK was coimmunoprecipitated with delphilin in transfected HEK293 cells (Fig. S7). Thus, although the regulation of the binding of PTPMEG and delphilin is unclear, we suggest that delphilin binding may upregulate phosphorylation of Y876 of GluA2 and inhibit LTD, whereas PTPMEG binding downregulates Y876 phosphorylation and enhances LTD. Thus, Y876 phosphorylation levels, regulated by GluD2's C-terminus, may serve as a regulator of metaplasticity at PF–Purkinje cell synapses to determine LTD inducibility.

Although striatal-enriched protein tyrosine phosphatase (STEP) plays a crucial role in DHPG–induced mGluR-dependent LTD in the hippocampus (41), it remains unclear whether Y876 of GluA2 serves as a substrate for STEP. In addition, little STEP mRNA is expressed in the cerebellum (42). In the present study, using a substrate trap mutant of PTPMEG, we identified GluA2 as a substrate for PTPMEG (Fig. 7*A*). An *in vitro* dephosphorylation assay using pep-3pY (Fig. 7*B*) and GluA2-expressing cells (Fig. 7*C*) confirmed Y876 of GluA2 as a substrate for PTPMEG. We also identified VCP as a substrate for PTPMEG using a substrate trap assay. PTPMEG is also shown to interact with and dephosphorylate the T cell receptor ζ (43). Therefore, in addition to GluA2, other proteins could be dephosphorylated by PTPMEG in Purkinje cells. Since many synaptic proteins, such as β-catenin, N-cadherin, and ephrinB, are tyrosine phosphorylated and regulate various aspects of synaptic functions, future studies are warranted to further identify substrates of PTPMEG.

A major remaining question is how increased neuronal activity decreases Y876 dephosphorylation. In the hippocampus, STEP is rapidly translated in response to mGluR activation (41). Since protein translation is suggested to play a role in cerebellar

LTD (44), PTPMEG may also be one of the molecules whose translation is induced by mGluR. PTPMEG is also shown to be activated 4–8-fold upon calpain-induced cleavage (45), which could be triggered by increases in intracellular Ca^{2+} concentrations during LTD induction. Another possibility is that SFKs may be downregulated during LTD (31), thereby decreasing Y876 phosphorylation. Finally, activities of protein phosphatases are reported to be regulated by its oligomerization status (46). Since Cbln1, which is released from granule cells, causes clustering of GluD2 by binding to the N-terminal domain of GluD2 (47), it may activate PTPMEG by bringing PTPMEG close together at PF synapses in Purkinje cells. D-Ser, which is released from Bergmann glia in an activity-dependent manner in immature cerebellum, binds to the ligand-binding domain of GluD2 and facilitates AMPA receptor endocytosis and LTD (48). Thus, conformation changes induced by binding of D-Ser may also regulate PTPMEG activities. Future studies are warranted to clarify whether and how GluD2–PTPMEG signaling is regulated to balance tyrosine phosphorylation and dephosphorylation levels of GluA2 for fine-tuning of AMPA receptor endocytosis.

Materials and Methods

Electrophysiology. Parasagittal cerebellar slices (200-μm thick) were prepared from wild-type, *GluD2*-null or *PTPMEG*-null mice on postnatal day 21–35 (P21–35) as described previously (48). Whole-cell patch-clamp recordings were made from visually identified Purkinje cells using a 60× water-immersion objective attached to an upright microscope (BX51WI, Olympus Optical, Tokyo, Japan) at room temperature. The resistance of patch pipettes was 3–5 MΩ when filled with an intracellular solution of

the following composition (in mM): 110 K-gluconate, 50 HEPES, 10 KCl, 4 $MgCl_2$, 4 Na_2ATP, 1 Na_2GTP and 5 sucrose (pH 7.23, 298 mOsm/kg) for $_{slow}$EPSC recordings and 65 Cs-methanesulfonate, 65 K-gluconate, 20 HEPES, 10 KCl, 1 $MgCl_2$, 4 Na_2ATP, 1 Na_2GTP, 5 sucrose and 0.4 EGTA (pH 7.25, 295 mOsm/kg) for LTD recordings. Pep-3Y (YKEGYNVYG), pep-3pY (pYKEGpYNVpYG) or pep-3A (AKEGANVAG) was added to the patch pipette solution at the concentration of 500 μM for the decoy-peptide experiments. The peptides were provided by Operon Biotechnologies (Tokyo, Japan). PP1 analogue (10 μM, Calbiochem/Merck Biosciences, Darmstadt, Germany) was also applied through the pipette. The extracellular solution (artificial cerebrospinal fluid; ACSF) used for slice storage and recording consisted of 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 D-glucose (in mM), bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Picrotoxin (100 μM; Sigma, St. Louis, MO) was always added to ACSF to block inhibitory synaptic transmission. PF–EPSCs were elicited by application of square pulses to a stimulating electrode placed on the molecular layer, and the selective PF stimulation was confirmed by paired-pulse facilitation of EPSCs with a 50-ms inter-stimulus interval.

$_{slow}$EPSCs were elicited from Purkinje cells voltage-clamped at −80 mV by the application of tetanic stimulation (2–10 times at 100Hz) to PFs in the presence of NBQX (100 μM). Before the $_{slow}$EPSC recordins, AMPA receptor-mediated PF–EPSCs were recorded in the absence of NBQX in order to normalize $_{slow}$EPSCs amplitudes and transferred charges. For the experiments of the LTD session, PF–EPSCs were recorded successively at a frequency of 0.1 Hz from Purkinje cells voltage-clamped at −80 mV. After stable PF–EPSCs were obtained at least for 10 min, a conjunctive (CJ) stimulation

(30 × [PF stimulus together with 500-ms depolarizing pulses from −60 to +20 mV] at 1 Hz) was applied. Access resistance was monitored every 10 sec by measuring the peak currents evoked by 2-mV, 50-ms hyperpolarizing steps throughout the experiments. The measurements were discarded if access resistance changed by more than 20% of its original value. Current responses were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), and the pCLAMP system (version 9.2; Molecular Devices) was used for data acquisition and analysis. The signals were filtered at 1 kHz and digitized at 4 kHz.

Virus Vector Constructs and *in vivo* Microinjection. For transduction of mutant transgenes into cerebellar Purkinje cells, we utilized a modified Sindbis virus vector (Invitrogen, Carlsbad, CA), which contained an additional subgenomic promoter and a green fluorescent protein (3, 4). When we transduced GluA2, we used GluA2 which carried glutamine (Q) in its Q/R RNA editing site to increase cell surface expression.

Surgery for microinjection into the mouse cerebellum were described previously (3, 4). Briefly, mice were anesthetized by an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (20 mg/kg; Sigma). A glass pipette was inserted into the cerebellum 500–800 μm from the surface, and 0.5–2 μl of the virus solution was injected using a Nanoliter (World Precision Instruments, Sarasota, FL). The mice were subjected to further experimental manipulations 15–36 h after the virus injection. All procedures relating to the care and treatment of animals were performed in accordance with NIH guidelines and permitted by Keio University Experimental Animal Committee.

GluA2 Phosphorylation Levels in the Cerebellum. To study baseline phosphorylation of GluA2-Y876 and -S880, the synaptosomal fraction was extracted from wild-type, *GluD2*-null or *PTPMEG*-null cerebella. The whole cerebellum from a P21–30 mouse was quickly dissected out and homogenized in 0.32 M sucrose in buffer A, composed of 5 mM HEPES (pH 7.3) and 0.1 mM EDTA, supplemented with protease inhibitors (Calbiochem), serine/threonine phosphatase inhibitors (Sigma) and 1 mM Na vanadate. After 1,000 × *g* centrifugation for 5 min at 4°C, the supernatant was collected and centrifuged at 12,000 × *g* for 20 min. The resulting pellet was suspended with 0.32 M sucrose/buffer A, overlaid on a gradient of 0.8 and 1.2 M sucrose/buffer A and ultra-centrifuged at 105,000 × *g* for 2 h at 4°C. The synaptosomal fraction was suspended in the 5 × volume of 0.32 M sucrose/buffer A and centrifuged at 12,000 × *g* for 10 min. We added 200 μl of 2 × SDS-PAGE buffer to the pellet and boiled it for 5 min. Protein concentration was estimated by BCA protein assay kit (Thermo Scientific, Rockford, IL). Ten to fifteen μg of synaptosomal fraction protein was electrophoresed on the 5–20% gradient SDS polyacrylamide gel and transferred to the PVDF membrane (Immobilon; MilliPore, Billerica, MA). The antibody against Y876-phosphorylated GluA2 was kindly donated by Dr. R. Huganir or purchased from Cell Signaling Technology (Danvers, MA). To detect S880 phosphorylation, we generated the polyclonal antibody against S880-phosphorylated GluA2 using a phosphorylated synthetic peptide of the most C-terminal 11 amino acids of GluA2 (YNVYGIEpSVKI) as an antigen. The specificity of the antibody was confirmed by *in vitro* phosphorylation assay using the C-terminal of GluA2 (Fig. S8). Immunoreactive materials were detected with Immobilon Western (MilliPore) and its chemiluminescence signals were obtained