Cellular/Molecular

Enhanced Inhibitory Synaptic Transmission in the Cerebellar Molecular Layer of the GluRδ2 Knock-Out Mouse

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A novel ionotropic glutamate receptor subunit δ2 (GluRδ2), which is specifically expressed in cerebellar Purkinje neurons (PNs), is implicated in the induction of long-term depression. Mutant mice deficient in GluRδ2 ($\delta2^{-/-}$) have abnormal cerebellar synaptic organization and impaired motor coordination and learning. Previous *in vivo* extracellular recordings in $\delta2^{-/-}$ revealed that PN activity distinct from that in wild-type (WT) mice is attributable to enhanced climbing fiber activity. Here, we report that GABAergic synaptic transmission was enhanced in the molecular layer of the cerebellar cortex in $\delta2^{-/-}$. Optical recordings in cerebellar slice preparations indicated that application of bicuculline, a $GABA_A$ receptor antagonist, increased the amplitude and area of excitation propagation more in $\delta2^{-/-}$ than in WT. Whole-cell patch-clamp recordings from PNs demonstrated that miniature IPSC (mIPSC) amplitude were larger in $\delta2^{-/-}$ than in WT. Also, rebound potentiation (RP), a type of long-lasting inhibitory synaptic potentiation inducible by postsynaptic depolarization of PNs in WT, was not induced in slices prepared from $\delta2^{-/-}$. In contrast, RP was induced in cultured PNs prepared from $\delta2^{-/-}$. Pharmacologic activation of climbing fibers in WT *in vivo* increased mIPSC amplitudes in PNs and prevented RP induction. These results suggest that enhanced climbing fiber activity in $\delta2^{-/-}$ potentiates IPSC amplitudes in PNs through RP *in vivo*, resulting in the prevention of additional RP induction.

Key words: **cerebellum; Purkinje cell; receptor; glutamate; inhibitory synapse; synaptic plasticity**

Introduction

Ionotropic glutamate receptor channels (GluRs) mediate the majority of fast excitatory synaptic transmission in the vertebrate CNS. GluRs are classified into AMPA, kainate, and NMDA subtypes. These receptors are heteromeric tetramers composed of various subunits that have been identified by molecular cloning studies (Hollmann and Heinemann, 1994; Rosenmund et al., 1998). GluRδ2 is classified into a novel subgroup of GluR subunits based on the amino acid sequence (Araki et al., 1993; Lomeli et al., 1993). GluRδ2 protein is selectively localized on the postsynaptic membrane at the excitatory glutamatergic parallel fiber–Purkinje neuron (pf-PN) synapse (Takayama et al., 1996; Zhao et al., 1997) and is required for the induction of long-term depression (LTD) (Hirano et al., 1994, 1995; Kashiwabuchi et al., 1995; Jeromin et al., 1996). How GluRδ2 is involved in LTD, however, is not clear. GluRδ2 might regulate trafficking of AMPA receptors (Hirai, 2001; Hirai et al., 2003) and have a role in synaptic stabilization and elimination (Morando et al., 2001; Cesa et al., 2003).

Mutant mice deficient in GluRδ2 ($\delta2^{-/-}$) have impaired LTD, motor coordination, and motor learning (Funabiki et al., 1995; Kashiwabuchi et al., 1995; Kishimoto et al., 2001; Yoshida et al., 2004). In $\delta2^{-/-}$, the number of pf-PN synapses is reduced and PNs are innervated by multiple climbing fibers (CFs) (Kurihara et al., 1997; Hashimoto et al., 2001; Ichikawa et al., 2002). In adult wild-type (WT) mice, PNs are innervated by single CFs (Ito, 1984). Recently, *in vivo* extracellular recording from cerebellar PNs demonstrated characteristic CF activity with a 10 Hz rhythm in $\delta2^{-/-}$ that is directly related to involuntary spontaneous eye movements (Yoshida et al., 2004). The authors suggested that multiple CF innervation, reduction in the number of pf-PN synapses, and LTD failure-dependent increase in error signals conveyed by CFs might enhance the effects of CF activity on PNs in $\delta2^{-/-}$.

We hypothesized that enhanced CF activity in $\delta2^{-/-}$ might also affect inhibitory synaptic transmission in the molecular layer (ML), because synaptic plasticity whose induction depends on CF inputs occurs at GABAergic synapses on PNs (Kano et al., 1992). Transmission at inhibitory interneuron (basket and stellate cells)–PN synapses is potentiated for longer than 30 min by postsynaptic depolarization of PNs. This plasticity was designated rebound potentiation (RP), and CF activation induces sufficient depolarization for RP induction (Kano et al., 1992). Therefore, the altered CF activity in $\delta2^{-/-}$ might affect RP induction and potentiate the amplitude of inhibitory synaptic inputs on PNs. We examined this issue by applying optical recording and whole-cell patch-clamp recording techniques to slice and culture preparations.

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Materials and Methods

Animals

$\delta 2^{-/-}$ were generated previously by Kashiwabuchi et al. (1995). The experimental procedure was performed in accordance with the guidelines for animal experimentation by the Unites States National Institutes of Health and Kyoto University and was approved by the local committee for handling experimental animals in the Graduate School of Science, Kyoto University.

Optical recording

Horizontal cerebellar slices (300 μm) were prepared from 22- to 24-d-old mice as described previously (Imamura et al., 2000) and were continuously perfused with oxygenated Krebs' solution containing the following (in mM): 124 NaCl, 1.8 KCl, 1.24 KH_2PO_4, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose (saturated with 95% O_2 and 5% CO_2 at 22–24°C). Slices were stained with a voltage-sensitive dye RH482 (0.2 mg/ml, NK3630; Nippon Kankoh-Shikiso Kenkyusho, Okayama, Japan) (Konnerth et al., 1987) for 15 min and placed in a chamber on an inverted microscope (IMT-2; Olympus Optical, Tokyo, Japan) equipped with a camera head containing a 128 × 128 photodiode array (HR Deltaron 1700; Fuji Film, Tokyo, Japan). Bicuculline (50 μM; Sigma, St. Louis, MO), a $GABA_A$ receptor antagonist, was applied to the Krebs' solution and perfused for >30 min. A tungsten bipolar electrode was placed in the white matter to apply electrical stimulation (100 μsec, 500–1200 μA). The optical recording method was similar to that used in previous studies (Tanifuji et al., 1994; Imamura et al., 2000). In each trial, an image was taken ~500 msec before stimulation and stored in one memory frame (reference image). Subsequent frames taken every 0.6 msec were subtracted from the reference image, amplified, and stored in another memory frame (difference image). The difference images were averaged for 16 trials, and each frame was divided by the real-time image to express the optical response as the percentage change in light absorption. The decay of the optical response attributable to photobleaching was calculated by linearly interpolating a baseline and then corrected (Tanifuji et al., 1994; Imamura et al., 2000). The summary data are presented as mean ± SEM, unless otherwise stated.

Electrophysiology

Whole-cell patch-clamp recording in slice preparations. The methods of thin-slice whole-cell patch-clamp recording were similar to those described previously (Konnerth et al., 1992; Kashiwabuchi., 1995). Horizontal slices (200 μm) prepared from 22- to 24-d-old mice were maintained in Krebs' solution as described previously. PNs were whole-cell voltage clamped with a patch pipette filled with internal solution (pH 7.3) consisting of the following (in mM): 150 CsCl, 15 CsOH, 0.5 EGTA, 10 HEPES, 2 Mg-ATP (Sigma), and 0.2 Na-GTP (Sigma). Mg-ATP and Na-GTP were used to minimize the rundown of $GABA_A$ receptor responsiveness. The electrode resistance of the patch pipette was 2–3 MΩ, and the pipette was coated with silicon to minimize stray capacitance. The holding potential was −70 mV, and junction potentials were offset. At this potential and with the internal solution containing 150 mM Cl^-, both EPSCs and IPSCs were recorded as inward currents. Input resistance (>150 MΩ) and series resistance (15–25 MΩ) were monitored throughout the experiments by applying an 80 msec voltage pulse to −80 mV every 2.5 min. Experiments were terminated when one of these values changed by >20%. The IPSCs were recorded in the presence of 10 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX) (Tocris Cookson, Bristol, UK), an AMPA/kainate receptor antagonist. In PNs, NMDA receptors do not contribute to EPSCs (Hirano and Hagiwara, 1988; Konnerth et al., 1990). To stimulate climbing fibers, a glass pipette filled with Krebs' solution was positioned in the granular layer (GL) ~50 μm away from the recorded cell body. The EPSC evoked by CF stimulation was identified by the all-or-none characteristic, a large amplitude, and paired-pulse depression (Konnerth et al., 1990). Miniature postsynaptic currents, corresponding to responses to single synaptic vesicle release, were measured under action potential suppression by 1 μM tetrodotoxin (TTX) (Wako, Osaka, Japan), a voltage-dependent Na^+ channel blocker. To activate inferior olivary neurons pharmacologically *in vivo*, harmaline (Sigma) was injected intraperitoneally (20–30 mg/kg). Electrophysiological recordings were performed 18–24 hr after the injection.

Culture. Methods for preparing primary cultures of cerebellar neurons were similar to those of previous studies with slight modifications (Kawaguchi and Hirano, 2000, 2002). Briefly, cerebella were dissected out from either WT or $\delta 2^{-/-}$ newborn mice and were incubated in Ca^{2+} and Mg^{2+}-free HBSS containing 0.1% trypsin and 0.05% DNase for 15 min at 37°C. Neurons were dissociated by trituration and seeded in DMEM/F-12-based medium containing 2% fetal bovine serum (Furuya et al., 1998). The next day, 75% of the medium was replaced with the basal medium Eagle-based serum-free medium. Half of the medium was replaced with serum-free medium every 5 d. To inhibit glial proliferation, AraC (4 μM) was added to the medium starting 1 week after dissociation.

Whole-cell voltage-clamp recording from cultured PNs. Whole-cell patch-clamp recordings from cultured PNs were performed in external solution containing the following (in mM): 145 NaCl, 5 KOH, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.3, at room temperature. The external solution also contained 10 μM CNQX (Tocris Cookson) to suppress glutamatergic EPSCs mediated by AMPA/kainate receptors and 1 μM tetrodotoxin. The method of iontophoretic applications of GABA was similar to that used in previous studies (Kawaguchi and Hirano, 2000, 2002). A glass pipette containing 10 mM GABA and 10 mM HEPES was aimed at a proximal or a secondary dendrite of a PN, and 20 msec positive voltage pulses were applied every minute.

Results

Enhanced synaptic inhibition in the molecular layer of $\delta 2^{-/-}$

We obtained optical recordings using a voltage-sensitive dye, RH482, to examine the spatiotemporal excitation propagation in horizontal cerebellar slice preparations. A brief electrical pulse (200 μsec) was applied to the white matter just beneath the GL, which evoked a change in optical signal that spread horizontally within the GL and propagated vertically into the ML (Fig. 1). Our previous study (Imamura et al., 2000) suggested that the major source of change in optical signal in the GL is monosynaptically induced depolarization of granule neurons, and that in the ML is disynaptically induced depolarization of PN dendrites. IPSPs are likely to contribute to the optical signal by decreasing depolarization.

To evaluate the basal contribution of inhibitory synaptic transmission to the excitation propagation in each layer, we applied bicuculline (50 μM), which increased the amplitude of change in the optical signal in the GL and ML (Figs. 1, 2). In $\delta 2^{-/-}$, the peak amplitude of change in the optical signal in the ML increased to 2.44 ± 0.14 times ($n = 6$), which was significantly larger than that in WT (1.78 ± 0.23 times; $n = 8$; $p < 0.05$; Student's t test). The optical signal in the GL increased to 1.44 ± 0.11 times in WT and to 1.45 ± 0.15 times in $\delta 2^{-/-}$ (NS) (Fig. 2*A*,*B*). Thus, GABAergic inhibition in the ML is stronger in $\delta 2^{-/-}$ than in WT. We also examined the effect of bicuculline on the time course of the optical signal (Fig. 2*C*,*D*). There were no significant differences in the half-height width between WT and $\delta 2^{-/-}$ in either the GL or ML before and after bicuculline application.

Next, we investigated the extent of excitation propagation in each layer. We counted the number of pixels with an optical signal change greater than an arbitrarily set threshold (>70% of the peak change in each layer) (Fig. 2*E*,*F*). The bicuculline-induced increase in the depolarized area in the ML was significantly larger in $\delta 2^{-/-}$ than in WT (at 6 and 100 msec; $p < 0.05$; Student's t test). Thus, GABAergic inhibition suppressed the spread of excitation in the ML more potently in $\delta 2^{-/-}$ than in WT. These results suggest that $\delta 2^{-/-}$ PNs receive stronger synaptic inhibition than those in WT.

Large miniature IPSCs in δ2 −/−

Enhancement of synaptic inhibition in the ML can be induced by the potentiation of individual synaptic transmission, the increase in number of inhibitory synapses, or the facilitation of presynaptic inhibitory neuronal activity. Individual synaptic transmission can be potentiated through an increase in either postsynaptic responsiveness or presynaptic release probability of synaptic vesicles. Here we examined the possibility that the increase in postsynaptic responsiveness contributed to the enhanced synaptic inhibition in δ2 −/−, because a potentiation of $GABA_A$ receptor responsiveness (i.e., RP) is induced by depolarization of the PN in δ2 −/− (Kano et al., 1992).

To address this issue, we recorded miniature IPSCs (mIPSCs) from PNs in slices using the whole-cell voltage-clamp technique. The mIPSC time course and frequency were not significantly different between δ2 −/− and WT (Fig. 3*A*, Table 1). However, the average mIPSC amplitude was 31.8 ± 3.7 pA ($n = 21$) in WT and 45.4 ± 5.3 pA ($n = 18$) in δ2 −/− (Student's t test; $p < 0.05$) (Fig. 3*B*).

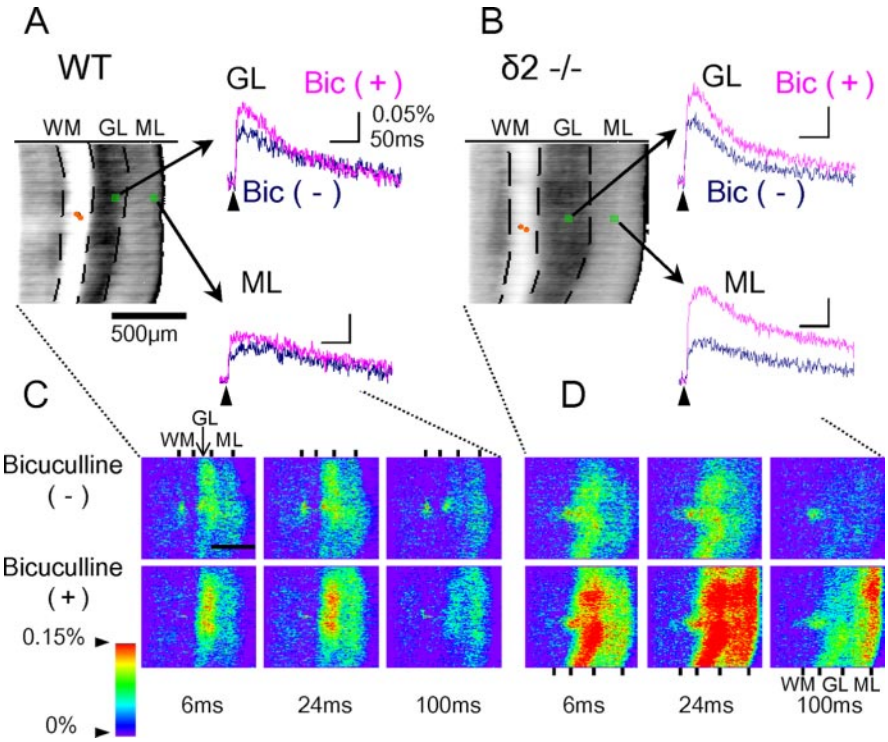

Figure 1. Spatiotemporal pattern of excitation propagation in the cerebellar cortex. *A*, *B*, Slice preparations and time courses of the optical signal change in GL and ML before and after application of bicuculline (Bic) in WT (*A*) and δ2 −/− (*B*). Each trace presents the averaged time course of the optical signal changes ($n = 16$; 5 × 5 pixels, at green squares). Stimulating electrodes were placed in the white matter (WM; orange circles). Stimulation was applied at the arrowhead in each trace. *C*, *D*, Pseudocolor images representing optical signal changes before and after the application of bicuculline. The stimulus was applied at 0 msec.

RP induction failure in δ2 −/− slices

We next addressed the mechanism underlying the larger mIPSC amplitudes in δ2 −/−. In δ2 −/−, the effects of CF activity on PNs, which could induce RP, are enhanced through multiple innervation and increased CF action potential frequency (Yoshida et al., 2004). Therefore, we considered that the enhanced climbing fiber activity in δ2 −/− might potentiate IPSC amplitudes through *in vivo* RP induction.

We recorded spontaneous postsynaptic currents in PNs before and after repetitive conditioning stimulation of CF (five stimulations at 0.5 Hz) in a slice preparation. In this experiment, we used an internal solution containing 150 mM Cl^- for the whole-cell patch-clamp recordings. Thus, both spontaneous EPSCs (sEPSCs) and IPSCs (sIPSCs) were recorded as inward currents [spontaneous compound PSCs (scPSCs)]. Kano et al. (1992) reported that most scPSCs are actually sIPSCs. The conditioning CF stimulation increased the scPSC amplitudes to 137 ± 4% ($n = 7$; $p < 0.01$; Student's t test) 25 min after the conditioning stimulation in WT, as expected. In contrast, the scPSC amplitudes did not change significantly in δ2 −/− (103 ± 4%; $n = 5$; at 25 min) (Fig. 4), suggesting that RP cannot be induced in slices prepared from δ2 −/−. We next estimated the contribution of sEPSCs to scPSCs by applying bicuculline (30 μM), which clearly decreased scPSC amplitude and frequency in both δ2 −/− and WT. The sEPSC amplitude and frequency were 11.8 ± 1.9 pA and 3.7 ± 1.2 Hz ($n = 7$) in WT and 11.6 ± 1.2 pA and 2.5 ± 0.5 Hz ($n = 4$) in δ2 −/−, after bicuculline application. Taking the average amplitude and frequency of mIPSCs (31.8 ± 3.7 pA and 8.5 ± 0.9 Hz in WT; 45.4 ± 5.3 pA and 10.5 ± 1.3 Hz in δ2 −/−) into account, the average scPSC amplitudes mainly reflected those of sIPSCs. Thus, it is suggested that the conditioning CF stimulation induces RP in WT but not in δ2 −/−.

Next, we tried to confirm the above results by monitoring only mIPSCs in the presence of NBQX (10 μM) and TTX. Because NBQX suppressed the CF response (data not shown), the conditioning CF stimulation could not be used. Direct depolarization of a PN (five 500 msec pulses to 0 mV at 0.5 Hz) induces RP in rat slice preparations and also in culture (Kano et al., 1992; Kawaguchi and Hirano, 2000, 2002). In our mouse slice preparations, however, the same conditioning depolarization failed to induce RP in WT (data not shown). We used postnatal day 22–24 mice, whereas Kano et al. (1992) used postnatal day 12–16 rats. It is possible that direct depolarization applied to the PN soma from a more mature mouse might not spread into the dendrites in which RP should occur. Thus, we attempted to induce stronger depolarization in PN dendrites and found that, in the presence of 1 mM 4-aminopyridine (Sigma), a K^+ channel blocker, RP was induced by direct conditioning PN depolarization (five 500 msec pulses to −20 mV at 0.5 Hz) in cerebellar slices prepared from WT. The mIPSC amplitude increased to 157 ± 14% at 30 min after the conditioning ($n = 5$; $p < 0.01$; Student's t test). In δ2 −/−, this conditioning failed to increase mIPSC amplitudes (101 ± 10%; $n = 5$; at 30 min) (Fig. 5). We also tested a stronger conditioning stimulation (20 1 sec pulses to −20 mV at 0.5 Hz), which failed to induce RP in δ2 −/− (109 ± 8%; $n = 6$; at 30 min). Furthermore, we examined whether RP saturation occurs in WT. A second conditioning depolarization (five 500 msec pulses) applied 5 min after the first conditioning depolarization did not increase mIPSC amplitudes further (150 ± 8%, $n = 5$, at 30 min after the first conditioning, not significantly different from those without the second conditioning, 153 ± 9%, $n = 5$). These results, together with the fact that the mIPSC amplitudes in δ2 −/− were larger than those in WT, suggest that RP was induced *in vivo* and that the IPSC amplitudes reached the saturation level, which pre-

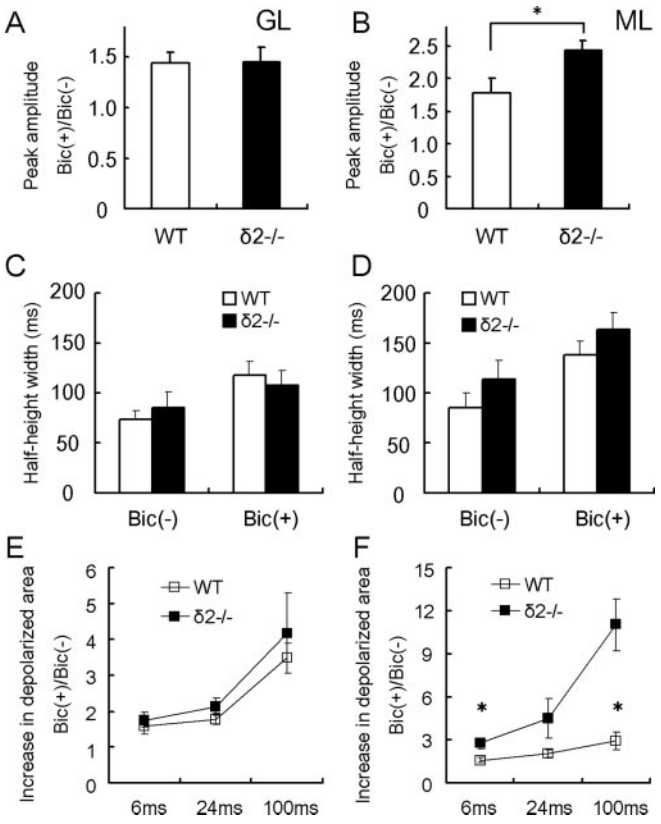

Figure 2. Effects of bicuculline (Bic) on the optical signal. *A*, *B*, The increase in peak amplitude of the optical signal change by bicuculline application. Ratio of the peak amplitudes before and after the application is shown. *$p < 0.05$ indicates that a significant difference was detected between WT and δ2 $^{-/-}$ in the ML (Student's *t* test). *C*, *D*, The half-height width of the optical signal change in the GL (*C*) and ML (*D*) before and after bicuculline application. *E*, *F*, The expansion of depolarized area in the GL (*E*) and ML (*F*) by bicuculline application. The ratio of the depolarized area before and after bicuculline application (6, 24, and 100 msec after the electrical stimulation) is presented. *$p < 0.05$ indicates that significant differences were detected in the ML between WT and δ2 $^{-/-}$.

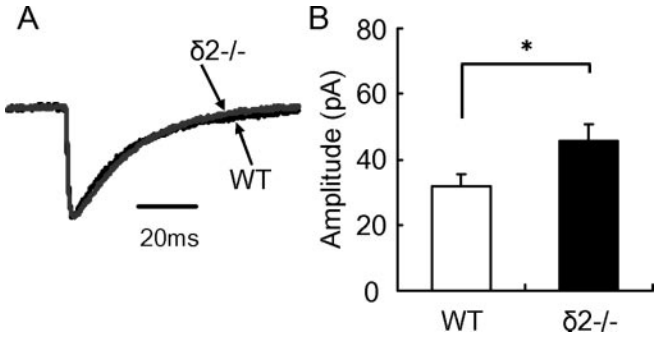

Figure 3. mIPSCs in slice preparations. *A*, Averaged and scaled mIPSC traces in WT and δ2 $^{-/-}$ ($n = 60$ for each). *B*, Averaged mIPSC amplitudes recorded from PNs in WT ($n = 25$) and δ2 $^{-/-}$ ($n = 19$). *$p < 0.05$; Student's *t* test.

vents additional RPs in δ2 $^{-/-}$. This reasoning, however, implies that the RP induced *in vivo* was retained during preparation of cerebellar slices. In slice preparations, no spontaneous CF activity was recorded.

Pharmacologic activation of CFs saturates RP

The above results suggest that the enhancement of basal CF activity *in vivo* induces RP and increases mIPSC amplitude in δ2 $^{-/-}$. We next examined whether enhanced CF activity causes RP saturation in WT. To this end, we used harmaline. Harmaline is a plant-derived indole alkaloid that produces tremors and causes sustained rhythmic activation of inferior olivary neurons *in vivo* and *in vitro* (Llinas and Volkind, 1973; Llinas and Yarom, 1986; Beitz and Saxon, 2004). Harmaline appears to act through serotonin receptors (Grella et al., 1998), which are expressed in the inferior olivary nuclei (Fay and Kubin, 2000). One day after intraperitoneal injection of harmaline, the mIPSC amplitudes in WT were 45.7 ± 5.5 pA ($n = 17$), significantly larger than those in untreated WT (31.8 ± 3.7 pA; $n = 21$) and in the saline-injected control WT (28.1 ± 3.5 pA; $n = 16$) (Fig. 6). Injection of neither harmaline nor saline significantly affected the time course or frequency of mIPSCs (Table 1). In the harmaline-treated WT, conditioning depolarization in the presence of 4-aminopyridine failed to induce RP (101 ± 4%; $n = 5$; at 30 min). In contrast, RP was induced in the saline-injected WT (154 ± 11%; $n = 5$; at 30 min) (Fig. 6*C*). These results support the hypothesis that the *in vivo* increase in CF activity saturates RP.

RP induction in δ2 $^{-/-}$ culture

So far, we have suggested that enhanced CF activity in δ2 $^{-/-}$ *in vivo* might be responsible for the RP induction failure. It is also possible, however, that GluRδ2 is directly linked to the molecular machinery responsible for the RP induction within PNs. To test whether the GluRδ2 deficiency by itself suppresses RP induction, we examined RP induction in GluRδ2-deficient PNs. In the culture, there were no CFs; therefore, their contribution to RP induction could be excluded. We recorded mIPSCs from cultured PNs. The mIPSC amplitudes were 38.0 ± 3.4 pA ($n = 27$) in WT and 33.3 ± 2.0 pA ($n = 23$) in δ2 $^{-/-}$ (NS) (Fig. 7). We also did not detect significant differences in the mIPSC time course or frequency (Table 1) between WT and δ2 $^{-/-}$. The conditioning depolarization (five 500 msec pulses to 0 mV at 0.5 Hz) of cultured PNs increased the amplitudes of mIPSCs in both WT (168 ± 11%; $n = 6$) and δ2 $^{-/-}$ (162 ± 16%; $n = 5$).

Depolarization of PNs enhances the response to GABA application (Llano et al., 1991; Kano et al., 1992). To directly monitor the postsynaptic responsiveness, we iontophoretically applied GABA to dendrites of PNs through a glass pipette (Kawaguchi and Hirano, 2000, 2002). The GABA response was potentiated by the same conditioning depolarization (156 ± 12%, $n = 5$ in WT and 143 ± 11%, $n = 5$ in δ2 $^{-/-}$ at 30 min) (Fig. 8). Together, the above results suggest that GluRδ2 is not directly implicated in the RP induction within PNs. Note that the RP time course was somewhat different for mIPSCs and GABA responses. The latter had a transient peak at ~5 min. This might be attributable to differences in the populations of activated $GABA_A$ receptors. In our experimental condition, the synaptic receptors both on the soma and dendrites were responsible for the mIPSCs, and both synaptic and extrasynaptic receptors on the dendrites were responsible for the GABA responses.

Discussion

Enhanced inhibitory synaptic transmission in δ2 $^{-/-}$

We have suggested that, in δ2 $^{-/-}$, the enhanced CF activity induces RP *in vivo* and potentiates GABAergic synaptic inhibition in PNs. First, using an optical recording technique, we demonstrated that excitation propagation in the cerebellar cortex was suppressed by GABAergic inhibition more strongly in δ2 $^{-/-}$ than in WT. Next, by applying a whole-cell patch-clamp technique to cerebellar slices, we demonstrated that mIPSC amplitudes in PNs were larger in δ2 $^{-/-}$ than in WT and that RP was not induced in δ2 $^{-/-}$. Tonic pharmacologic activation of CFs by

Table 1. Time course and frequency of mIPSCs

	Slice				Culture	
	WT	δ2$^{-/-}$	WT + harmaline	WT + saline	WT	δ2$^{-/-}$
10–90% rise time (msec)	2.7 ± 1.4	2.6 ± 1.6	2.5 ± 1.2	2.2 ± 1.3	3.2 ± 0.9	2.9 ± 0.9
Half-height width (msec)	16.1 ± 4.9	16.9 ± 7.8	16.9 ± 5.5	16.6 ± 7.3	19.0 ± 5.0	18.6 ± 4.1
Frequency (Hz)	8.5 ± 4.2	10.5 ± 5.5	9.3 ± 3.4	7.0 ± 5.3	15.0 ± 5.8	15.0 ± 5.0

The 10–90% rise time, half-height width, and frequency of mIPSCs in slices and in culture are shown. Data are presented as mean ± SD. $n = 16–21$.

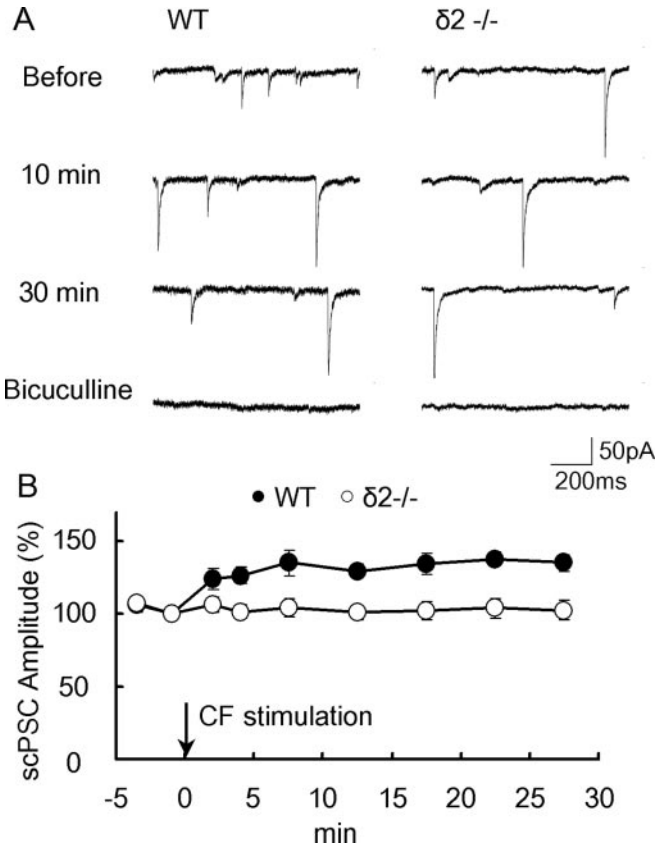

Figure 4. scPSCs and RP induction. *A*, Representative traces of scPSCs in WT and δ2$^{-/-}$ before and 10 and 30 min after the conditioning stimulation of climbing fibers (CF stimulation). Both spontaneous IPSCs and EPSCs were recorded as inward currents, because the internal solution contained a high concentration of Cl^-. Most scPSCs were sIPSCs, however, because bicuculline application abolished most scPSCs (bottom traces). *B*, The time courses of averaged scPSC amplitudes before and after the conditioning CF stimulation. CF stimulation was applied at 0 min (arrow). In each trial, PSC amplitudes were normalized, taking the mean value between −2.5 and 0 min as 100%. Other points in the graph represent data obtained between ±2.5 min of the indicated times, except at 2 and 4 min (±1 min).

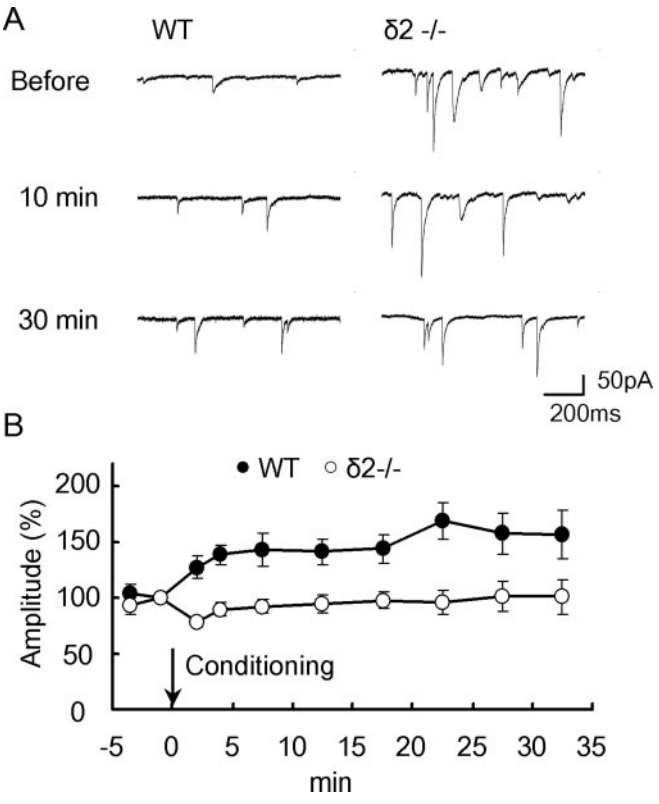

Figure 5. mIPSCs and RP induction. *A*, Representative traces of mIPSCs in WT and δ2$^{-/-}$. Postsynaptic depolarization of PNs was used as a conditioning stimulation. *B*, The time courses of IPSC amplitudes before and after the conditioning (0 min, arrow). $n = 5$ for each.

harmaline in WT also increased mIPSC amplitudes and suppressed RP induction. While in culture, the mIPSC amplitudes recorded from GluRδ2-deficient PNs were not significantly different from those in wild-type PNs, and RP was induced in both WT and δ2$^{-/-}$. Taking all these results into account, we reason that, in δ2$^{-/-}$, RP is induced *in vivo* so that the mIPSC amplitudes reached the saturation level, resulting in enhanced synaptic inhibition in the ML. It should be noted, however, that we have not directly demonstrated RP saturation *in vivo* because of technical difficulties.

The potentiation of postsynaptic responsiveness reflected by the increase in mIPSC amplitudes and GABA responsiveness in PNs should contribute to the enhancement of synaptic inhibition in the ML. Other factors, such as the increase in presynaptic release probability, the increased activity of presynaptic inhibitory neurons, and the increase in the number of inhibitory synapses, can also contribute to the enhanced synaptic inhibition in δ2$^{-/-}$. At present, these possibilities cannot be excluded. A similar scPSC frequency in WT and δ2$^{-/-}$ suggests that presynaptic neuronal activities are not so different between WT and δ2$^{-/-}$, and a similar mIPSC frequency suggests that the presynaptic release mechanism responsible for spontaneous release of synaptic vesicles is not altered in δ2$^{-/-}$. Recently, Duguid and Smart (2004) reported that the GABA release at interneuron–PN synapses is enhanced by depolarization of the postsynaptic PN through retrograde activation of presynaptic NMDA receptors. This potentiation, however, lasts for only ~10 min.

Diverse phenotypes of δ2$^{-/-}$ and their interrelationship

Most previous studies on δ2$^{-/-}$ were focused on the properties of excitatory synapses. They reported the impairment of LTD at pf-PN synapses, a decrease in the number of pf-PN synapses, and the multiple innervation of CFs on PNs (Hirano et al., 1994, 1995; Kashiwabuchi et al., 1995; Kurihara et al., 1997). The reduction in the number of pf-PN synapses was suggested to contribute to the multiple CF innervation on PNs (Hashimoto et al., 2001; Ichikawa et al., 2002). Also, δ2$^{-/-}$ have impaired motor coordi-

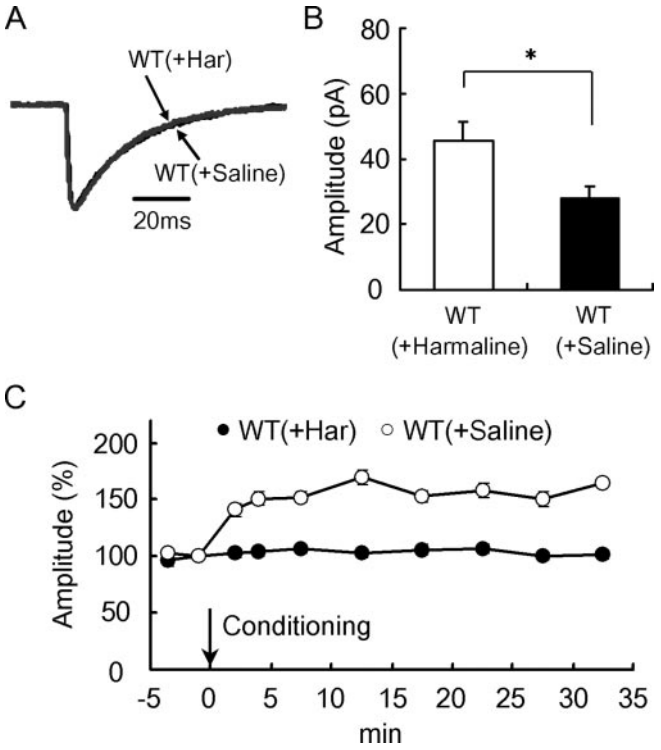

Figure 6. RP induction failure in harmaline (Har)-treated WT. *A*, Averaged and scaled mIPSC traces in harmaline- and saline-injected WT ($n = 60$ for each). *B*, Averaged mIPSC amplitudes ($n = 17$ and 16). *$p < 0.05$; Student's *t* test. *C*, The time courses of averaged mIPSC amplitudes before and after the conditioning depolarization (0 min, arrow). Conditioning depolarization potentiated the mIPSC amplitude in saline-injected WT but not in harmaline-injected WT ($n =$ 5 for each).

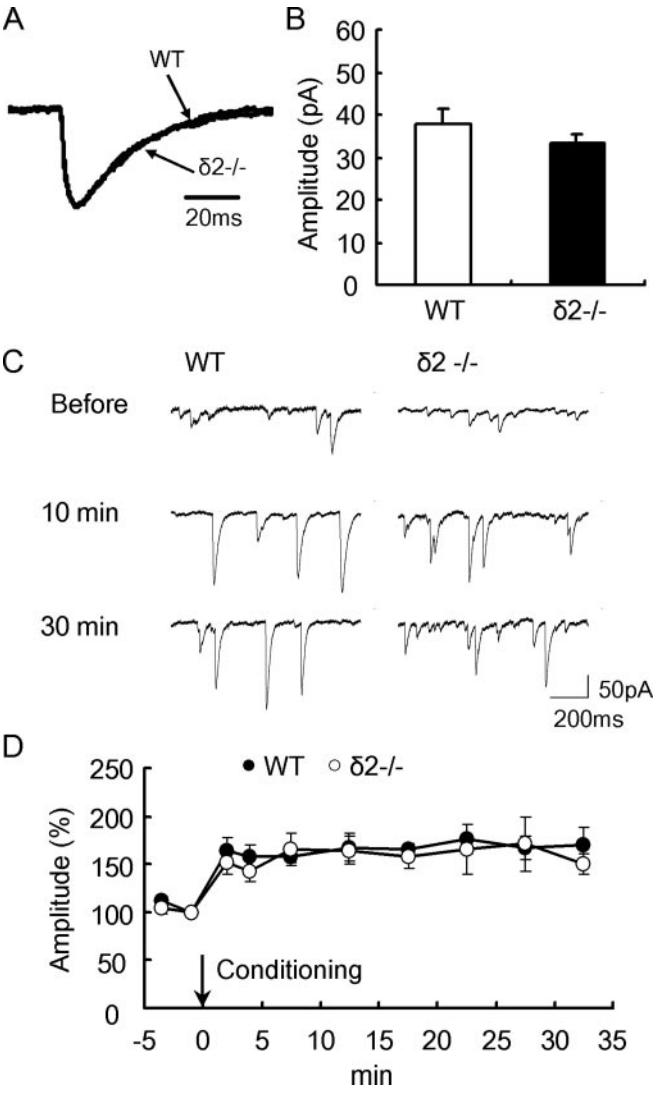

Figure 7. mIPSCs and RP induction in culture. *A*, Averaged and scaled mIPSC traces in WT and δ2$^{-/-}$ ($n = 50$ for each). *B*, Averaged mIPSC amplitude in WT ($n = 27$) and δ2$^{-/-}$ ($n = 23$). *C*, Representative traces of mIPSCs in cultured PNs prepared from WT and δ2$^{-/-}$. Depolarization of a PN was applied as the conditioning stimulation at 0 min. *D*, The time courses of averaged mIPSC amplitudes before and after the conditioning (0 min, arrow). RP was induced in both WT ($n = 6$) and δ2$^{-/-}$ ($n = 5$).

nation and motor learning (Funabiki et al., 1995; Kashiwabuchi et al., 1995; Kishimoto et al., 2001). Recently, Yoshida et al. (2004) demonstrated spontaneous oscillatory eye movement in δ2$^{-/-}$ and suggested that such an involuntary movement disturbs motor coordination. They also suggested that LTD failure, multiple CF innervation, and the reduction in the number of pf-PN synapses together enhance the effects of rhythmic inferior olivary neuronal activity on PNs.

In this study, we have demonstrated that the enhanced CF activity also potentiated the inhibitory synaptic transmission on PNs in δ2$^{-/-}$. This enhanced GABAergic synaptic transmission would also contribute to the abnormalities in GluRδ2-deficient PN activity. Previous cell-attached recordings in slice preparations indicated that interspike intervals of PNs are longer and more variable in δ2$^{-/-}$ than in WT (Yoshida et al., 2004). Yoshida et al. (2004) also recorded the spontaneous PN activity *in vivo* and reported that spikes induced by CF activity occur more frequently in δ2$^{-/-}$. The overall spike frequencies, however, are not significantly different between WT and δ2$^{-/-}$. Enhanced synaptic inhibition might work to suppress overexcitation in δ2$^{-/-}$. *In vivo* PN activities are more irregular in δ2$^{-/-}$ than in WT. Generation of the irregular PN firing pattern by tonic inhibition was demonstrated in rat cerebellar slice preparations (Hausser and Clark, 1997). A model simulation study also demonstrated that inhibitory synaptic inputs are more influential than excitatory ones in controlling spike timing in PNs (Jaeger et al., 1997). Thus, enhanced synaptic inhibition in δ2$^{-/-}$ might significantly influence PN activity.

Regulation and roles of RP

Long-term synaptic plasticity occurs not only at excitatory synapses (Jeromin et al., 1996; D'Angelo et al., 1999) (see also Ito, 1989; Bliss and Collingridge, 1993; Malinow and Malenka, 2002) but also at inhibitory synapses (Kano et al., 1992; Komatsu and Iwakiri, 1993; Kano, 1995; Aizenman et al., 1998; Nusser et al., 1998) in the mammalian CNS. Inhibitory synaptic plasticity is also induced or regulated in an activity-dependent manner (Aizenman et al., 1998; Kawaguchi and Hirano, 2000, 2002; Komatsu and Yoshimura, 2000). At the inhibitory synapses between PNs and neurons in the deep cerebellar nuclei, the polarity of long-lasting synaptic changes is determined by the activity of the postsynaptic neurons (Aizenman et al., 1998). In visual cortex, maintenance of long-term potentiation at inhibitory synapses requires low-frequency neuronal activity (Komatsu and Yoshimura, 2000). In the cerebellar cortex, CF activation induces RP. Involvement of Ca^{2+}, Ca^{2+}/calmodulin-dependent protein kinase II, and cAMP-dependent protein kinase in RP induction,

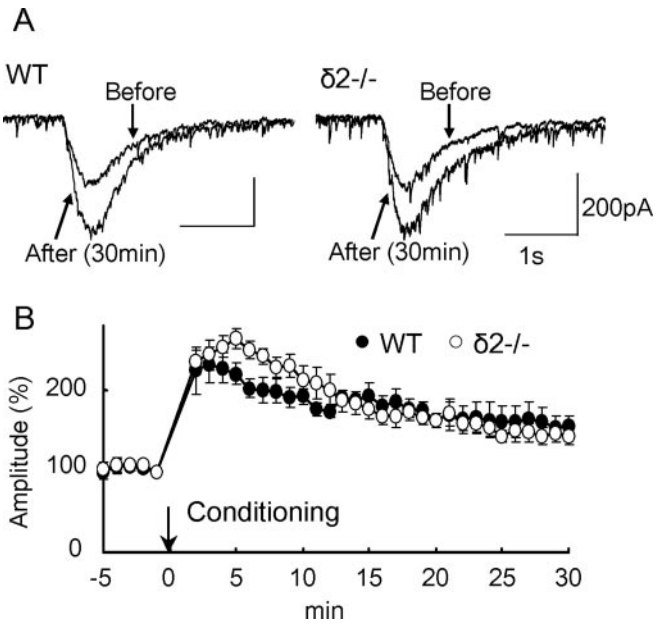

Figure 8. RP of GABA response in cultured PNs. *A*, Representative current responses to GABA application before and after the conditioning depolarization in WT and δ2$^{-/-}$. *B*, The time courses of GABA response amplitudes before and after the conditioning (0 min, arrow). $n = 5$ for each.

and its negative regulation by the presynaptic activity through $GABA_B$ receptors, dopamine and cAMP-regulated phosphoprotein 32, and phosphatases have been reported previously (Kano et al., 1992, 1996; Kawaguchi and Hirano, 2000, 2002).

One possible role of RP is to suppress overexcitation of PNs through enhancement of inhibitory synaptic inputs (Kawaguchi and Hirano, 2000). The present results that RP is saturated in δ2$^{-/-}$, in which excitatory climbing fiber activities are enhanced, and that pharmacologic activation of inferior olivary neurons by harmaline-administration also saturated RP in WT, are consistent with the notion that RP works to suppress overexcitation of PNs. The average firing frequency of a PN might be maintained in a certain range by balancing the strength of excitatory and inhibitory synaptic inputs. Our data also suggest that RP actually occurs *in vivo* under certain conditions.

Homeostasis in neuronal activity

There are a number of homeostatic synaptic plasticity mechanisms contributing to the stabilization of neuronal activity (Turrigiano and Nelson, 2004). Homeostatic changes in intrinsic excitability of neurons have also been reported previously (Zhang and Linden, 2003). It has been suggested that these mechanisms work to prevent neuronal circuits from becoming hyperactive or hypoactive. For example, mEPSC or mIPSC amplitudes change so as to cancel the altered neuronal activity (O'Brien et al., 1998; Turrigiano et al., 1998; Kilman et al., 2002). Our present results provide another example of homeostatic regulation of neural circuit activity. Here, hyperactive excitatory CF inputs potentiated the inhibitory synaptic inputs on the same postsynaptic PNs through RP induction, which counteracts the aberrant excitatory effects of CF inputs on the PN. Although CF activities are clearly enhanced in δ2$^{-/-}$, overall spike frequencies are not significantly different between WT and δ2$^{-/-}$ (Yoshida et al., 2004).

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