Postsynaptic $GABA_B$ receptor signalling enhances LTD in mouse cerebellar Purkinje cells

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Long-term depression (LTD) of excitatory transmission at cerebellar parallel fibre–Purkinje cell synapses is a form of synaptic plasticity crucial for cerebellar motor learning. Around the postsynaptic membrane of these synapses, B-type γ-aminobutyric acid receptor ($GABA_BR$), a $G_{i/o}$ protein-coupled receptor for the inhibitory transmitter GABA is concentrated and closely associated with type-1 metabotropic glutamate receptors (mGluR1) whose signalling is a key factor for inducing LTD. We found that in cultured Purkinje cells, $GABA_BR$ activation enhanced LTD of a glutamate-evoked current (LTD_{glu}), increasing the magnitude of depression. It has been reported that parallel fibre–Purkinje cell synapses receive a micromolar level of GABA spilt over from the synaptic terminals of the neighbouring GABAergic interneurons. This level of GABA was able to enhance LTD_{glu}. Our pharmacological analyses revealed that the $\beta\gamma$ subunits but not the α subunit of $G_{i/o}$ protein mediated $GABA_BR$-mediated LTD_{glu} enhancement. $G_{i/o}$ protein activation was sufficient to enhance LTD_{glu}. In this respect, LTD_{glu} enhancement is clearly distinguished from the previously reported $GABA_BR$-mediated augmentation of an mGluR1-coupled slow excitatory postsynaptic potential. Baclofen application for only the induction period of LTD_{glu} was sufficient to enhance LTD_{glu}, suggesting that $GABA_BR$ signalling may modulate mechanisms underlying LTD_{glu} induction. Baclofen augmented mGluR1-coupled Ca^{2+} release from the intracellular stores in a $G_{i/o}$ protein-dependent manner. Therefore, $GABA_BR$-mediated LTD_{glu} enhancement is likely to result from augmentation of mGluR1 signalling. Furthermore, pharmacological inhibition of $GABA_BR$ reduced the magnitude of LTD at parallel fibre–Purkinje cell synapses in cerebellar slices. These findings demonstrate a novel mechanism that would facilitate cerebellar motor learning.

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In the cerebellar cortex, individual Purkinje cells integrate excitatory synaptic inputs from numerous parallel fibres, the axons of granule cells which receive mossy fibre inputs that convey sensory information arising from various parts of the body and motor command signals from the upper centres (Thach *et al.* 1992; Llinas *et al.* 2003). The efficacy of transmission at certain parallel fibre–Purkinje cell synapses undergoes long-term depression (LTD) following correlated transmission at these synapses and climbing fibre–Purkinje cell synapses (Ito, 2002). Cerebellar LTD modifies information flow along the cerebellum and is therefore crucial for cerebellar motor learning (Ito, 2002).

Purkinje cells express a high density of B-type γ-aminobutyric acid receptor ($GABA_BR$), a $G_{i/o}$ protein-coupled receptor for the inhibitory neurotransmitter GABA (Jones *et al.* 1998; Kaupmann *et al.* 1998; Kuner *et al.* 1999). Interestingly, $GABA_BR$ is concentrated on the annuli of parallel fibre-innervated dendritic spines (Ige *et al.* 2000; Kulik *et al.* 2002) where LTD occurs. When the neighbouring interneurons are stimulated at a relatively high frequency, $GABA_BR$ in the dendritic spines may receive 5–10 μM GABA spilt over

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from the synapses of the interneurons (Dittman & Regehr, 1997). $GABA_BR$ has a high enough affinity (EC_{50}, ~1 μM) to sense this level of GABA (Sodickson & Bean, 1996). $GABA_BR$ activated by spilt-over GABA could possibly influence the signalling of type-1 metabotropic glutamate receptor (mGluR1), a $G_{q/11}$ protein-coupled receptor, because these receptors colocalize at the annuli of Purkinje cell dendritic spines (Lujan *et al.* 1997; Kulik *et al.* 2002) and are likely to form complexes (Tabata *et al.* 2004). mGluR1 signalling is an essential factor for inducing LTD (Conquet *et al.* 1994; Shigemoto *et al.* 1994; Ichise *et al.* 2000; Ito, 2002); this signalling leads to the facilitated endocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor at parallel fibre–Purkinje cell synapses (Matsuda *et al.* 2000; Wang & Linden, 2000; Chung *et al.* 2003). Therefore, possible influence of $GABA_BR$ on mGluR1 could modulate the induction of LTD.

In this study, we tested this possibility and explored the underlying mechanism. In cerebellar slice preparations, pharmacological manipulation of $GABA_BR$ should affect glutamate release by the parallel fibres and climbing fibres through modulating the presynaptic mechanisms (Dittman & Regehr, 1996). This masks the postsynaptic action of $GABA_BR$ on LTD. Thus, we employed an *in vitro* experimental system using cultured Purkinje cells, in which parallel and climbing fibre inputs are replaced with local application of glutamate and somatic depolarization, respectively (Linden *et al.* 1991). In this system, a glutamate-evoked current corresponds to parallel fibre-mediated excitatory postsynaptic current and undergoes LTD following conjunctive glutamate/depolarization stimuli (LTD_{glu}).

We show here that postsynaptic $GABA_BR$ activation enhances LTD_{glu}, increasing the magnitude of depression. Our pharmacological analyses indicate that this LTD_{glu} enhancement is likely to result from $G_{i/o}$ protein-mediated augmentation of mGluR1 signalling. Furthermore, we show that $GABA_BR$ indeed influences LTD of parallel fibre–Purkinje cell excitatory postsynaptic currents (EPSCs) in cerebellar slices.

These findings together demonstrate a novel mechanism that would facilitate cerebellar motor learning.

Methods

Cell Culture

Cerebellar cell culture was prepared as described elsewhere (Tabata *et al.* 2000). Briefly, perinatal C57BL/6 embryos were caesarean-sectioned from pregnant mice deeply anaesthetized and killed with diethylether or isoflurane. The embryos were deeply anaesthetized by cooling in chilled phosphate-buffered saline and then killed by decapitation. Cerebellar neurons were obtained from these embryos, dissociated with trypsin, and plated on plastic dishes or low-fluorescence plastic films (Sumilon, MS-92132, Sumitomo, Tokyo, Japan). The cells were cultured in a low-serum medium for 9–22 days. Purkinje cells were identified by their large somata (18 μm or greater) and/or thick primary dendrites. This procedure fully conforms to the guidelines administered by the committee on animal experiments of Osaka University.

Slice preparation

Cerebellar slices were prepared as described elsewhere (Kakizawa *et al.* 2000; Kakizawa *et al.* 2005; Kakizawa *et al.* 2007). Briefly, 3-week-old C57BL/6 mice were deeply anaesthetized with diethylether and then killed by decapitation. Cerebella obtained from these mice were sliced along the parasagittal planes to a thickness of 250 μm. This procedure fully conforms to the guidelines established by the Animal Welfare Committee of the University of Tokyo.

Electrophysiology

Somatic whole-cell voltage-clamp recordings were made from cultured Purkinje cells in dishes, using a perforated-patch technique (holding potential after the correction of a liquid junction potential between the pipette and bath solutions, −70 mV). The recording pipette contained (mM): 95 Cs_2SO_4, 15 CsCl, 0.4 CsOH, 8 $MgCl_2$, 10 Hepes and 500 μg ml^{-1} amphotericin B (pH 7.35). The bath was perfused at a rate of 1–2 ml min^{-1} with a saline consisting of (mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10 Hepes, 0.5 μM tetrodotoxin, and 10 μM (−)-bicuculline methochloride (pH 7.3, 25°C). Signals were sampled at 5 kHz, using an EPC-8 amplifier (HEKA, Lambreht, Germany) driven by PULSE software (versions 8.77, HEKA).

In the measurements using cerebellar slices, somatic whole-cell voltage-clamp recordings (holding potential after the correction of the liquid junction potential, −80 to −90 mV) were made from Purkinje cells, using pipettes (2.5–3.5 MΩ) containing (mM): 60 CsCl, 40 caesium D-gluconate, 20 TEA-Cl, 1 EGTA, 4 $MgCl_2$, 4 ATP, 0.4 GTP, and 30 Hepes (pH 7.3). The bath solution contained (mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 20 glucose (24–25°C), which was bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Bicuculline (10 μM) was always present in the saline to block spontaneous inhibitory postsynaptic currents. Parallel fibre–Purkinje cell EPSCs were monitored delivering electrical pulses (duration, 0.1 ms) to the parallel fibres through a glass pipette filled with the bath solution at 0.1 Hz except for the period of LTD induction. The intensity of the pulses was adjusted as to evoke parallel fibre–Purkinje cell EPSCs with a basal amplitude of 100–150 pA. Signals were sampled at 20 kHz, using an EPC-9 amplifier (HEKA). After stable basal

recording was obtained for at least 10 min, LTD was induced with a conjunctive stimulation protocol stated in the corresponding figure legend.

Ca^{2+} imaging

Cerebellar neurons on a film were incubated with fura-2 acetoxymethyl ester (5 μM) at 37°C for 15 min. Then, the film was placed on a glass-based recording chamber and perfused at a rate of ∼0.8 ml min^{-1} with saline (see above) supplemented with 6-nitro-7-sulfamoylbenzo(*f*)quinoxaline-2,3-dione (NBQX) and (3-[(*R*)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (*R*-CPP). Intracellular free calcium ion concentration ($[Ca^{2+}]_i$)-dependent fluorescence signals were captured at 2–5 Hz, using an imaging system (Polychrome II, TILL, Planegg, Germany) attached to an inverted microscope (IX70 with a ×20 objective lens of NA 0.75, Olympus, Tokyo, Japan). The amplitude of a $[Ca^{2+}]_i$ rise was expressed as a change in the ratio of somatic fluorescence signals excited at 340 and 380 nm (exposure duration, 40 and 20 ms, respectively) (F_{340}/F_{380}).

Drug application

Test drugs were dissolved into water to concentrations 1000 times higher than the final levels, kept at 4 or −20°C until use, and diluted into saline on the day of recordings unless otherwise stated. Forskolin and thapsigargin were dissolved into DMSO to concentrations of 5000 and 10 000 times higher than the final levels, respectively, and kept at −20°C until use. Glutamate was applied iontophoretically (30 ms, 0.05 Hz) through a glass pipette filled with 10 mM L-glutamate and 10 mM Hepes (pH adjusted to 7.1 with NaOH, kept at −20°C until use) and located about 20 μm from the branching point of a primary dendrite of the examined cell. The level of ejection current was adjusted (200–800 nA, 30 ms) so that the peak amplitude of basal glutamate-evoked currents were in a range of 100–300 pA. Bath application of baclofen, GABA, mastoparan, and forskolin was done by perfusing the recording chamber at rate of 1–2 ml min^{-1} with the drug-containing saline. Local application of (R,S) - 3,5-dihydroxyphenylglycine (DHPG), baclofen, and K^+-rich saline was done by delivering the drug-containing saline through a wide-tipped pipette located near the examined cell under the control of gravity. In some experiments, pertussis toxin (PTX, reconstituted in the culture medium at a concentration 100 times higher than the final level and kept at 4°C until use) was added to the culture medium 16–24 h before recordings.

Data analysis

The peak amplitude of a response was measured as a difference from the prestimulus level to the maximal deflection throughout the record (glutamate-evoked current) or during a 10 s (DHPG-evoked $[Ca^{2+}]_i$ rises) or 1 s (K^+-evoked $[Ca^{2+}]_i$ rises) agonist application.

In measurements using cerebellar slices, the amplitudes of parallel fibre–Purkinje cell EPSCs were normalized to mean over a 10 min basal recording. For each Purkinje cell, the magnitude of LTD was evaluated as the mean of parallel fibre–Purkinje cell EPSCs over 26–30 min after the conjunctive stimuli. Data were discarded in the following cases: (i) either the series resistance or membrane resistance changed by more than 10% during a recording session, (ii) the slope of parallel fibre–Purkinje cell EPSC amplitude changed by more than 2% during the 10 min basal recording, and/or (iii) the amplitude continued to change over 30 min after the formation of whole-cell configuration (Namiki *et al.* 2005; Kakizawa *et al.* 2007).

Groups of numerical data are presented as means ± S.E.M. Statistical differences were examined by two-sided Mann–Whitney's *U* test unless otherwise stated.

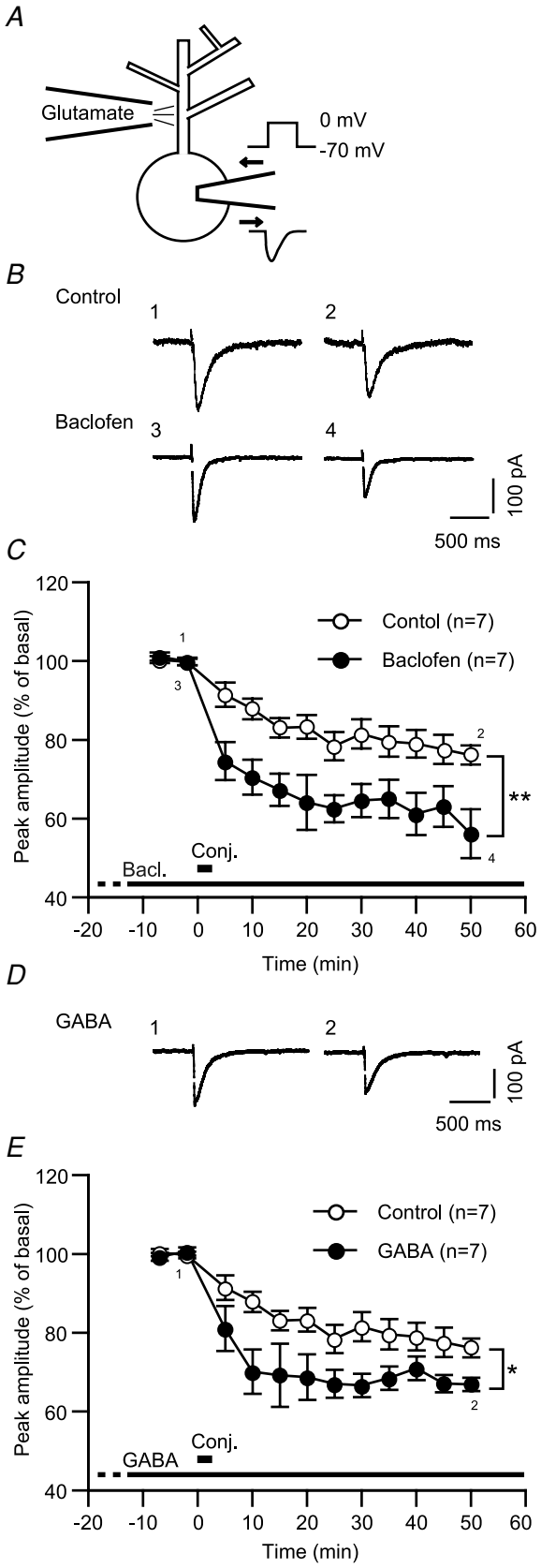
Immunohistochemistry

Cerebellar cells cultured on films were fixed sequentially with 0.1 M sodium phosphate buffer containing 4% paraformaldehyde at room temperature for 30 min and with 100% methanol at room temperature for 10 min. The fixed cells were rinsed with phosphate-buffered saline (PBS) for three times and then treated with PBS containing 0.1% Triton X-100 and 10% normal donkey serum at room temperature for 30 min (Kamikubo *et al.* 2006). The treated cells were incubated with a primary antibody against phospholipase C (PLC) β3 and PLCβ4 (raised in guinea pigs and rabbits, respectively; Nakamura *et al.* 2004; Nomura *et al.* 2007), GABA$_B$R1 subunit (GBR1; raised in rabbits; Kulik *et al.* 2002), mGluR1 (raised in guinea pigs; Tanaka *et al.* 2000), or calbindin (raised in mice; C9848, Sigma, St Louis, MO, USA) (1 μg ml^{-1} in the Triton X-100/serum-containing PBS) at 4°C overnight and then treated with a secondary antibody conjugated with indocorbocyanine (Cy3; Jackson ImmunoResearch, West Grove, PA, USA) or Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) 1 : 200 in the Triton X-100/serum-containing PBS at room temperature for 2.5 h. The film was mounted on a glass slide with Perma Fluor Aquaus mounting medium (434980, Thermo, Pittsburgh, PA, USA), and the cells were examined using a confocal laser microscope (LSM510, Carl Zeiss Göttingen, Germany). The fluorescent signals of Cy3 (red) and Alexa Fluor 488 (green) were excited at 543 and 488 nm, respectively.

Results

GABA$_B$R activation enhances LTD$_{glu}$

We first assessed whether and how GABA$_B$R activation affects LTD of a glutamate-evoked current (LTD$_{glu}$) in



cultured Purkinje cells. We iontophoretically applied glutamate to the dendrite of the examined cell for every 20 s and monitored glutamate-evoked inward currents in a perforated-patch whole-cell mode (holding potential, −70 mV) (Fig. 1*A*). After the basal recording, we gave six sets of depolarizing voltage steps (0 mV, 3 s) through the recording pipette in conjunction with glutamate iontophoresis (Fig. 1*A*). In the normal saline (control), the glutamate-evoked current underwent depression lasting over 50 min following the conjunctive stimuli (Fig. 1*B* and *C*; 'Control'). The peak amplitude of the glutamate-evoked current was reduced to $76.2 \pm 2.4\%$ of the basal level ($n = 7$) at the end of the recording sessions (48–52 min after the conjunctive stimuli) (Fig. 1*C*). We found that $GABA_BR$ activation enhanced LTD_{glu}, increasing the magnitude of depression (Fig. 1*B* and *C*). In the continuous presence of baclofen, a $GABA_BR$-selective agonist (3 μM), the peak amplitude was reduced to $56.2 \pm 6.2\%$ ($n = 7$) at the end of the recording sessions (Fig. 1*C*). This value was significantly smaller than the control (Fig. 1*C*). LTD_{glu} enhancement emerged immediately after the conjunctive stimuli. As early as 5 min after the conjunctive stimuli, the peak amplitude with baclofen ($74.6 \pm 4.8\%$, $n = 7$) was already significantly smaller than that with the normal saline ($91.4 \pm 3.0\%$, $n = 7$) (Fig. 1*C*). The early emergence of enhancement indicates that $GABA_BR$ signalling may modulate the induction process of LTD_{glu}. Moreover, baclofen (3 μM, 15 min) did not reduce the peak amplitude of a glutamate-evoked inward current as its direct effect ($102.7 \pm 1.6\%$, $n = 7$; data not illustrated). Thus,

Figure 1. $GABA_BR$ activation enhances LTD_{glu} in cerebellar Purkinje cells
A, setting of the measurement of long-term depression of glutamate-evoked current (LTD_{glu}). Glutamate was iontophoretically applied to the first branching point of a dendrite of the examined cell (100–300 pA, 30 ms) for every 20 s. Glutamate-evoked inward currents were monitored under voltage clamp at −70 mV. To induce LTD_{glu}, six sets of depolarizing voltage steps (0 mV, 3 s) were given through the recording pipette in conjunction with glutamate iontophoresis. *B* and *C*, baclofen, a $GABA_BR$-selective agonist enhanced LTD_{glu}. *B*, each pair of traces indicates sample glutamate-evoked currents of a cell before and after the conjunctive depolarization/glutamate stimuli in the absence ('Control') or continuous presence of baclofen (3 μM). The time when the traces were recorded are indicated by the corresponding integers in the plots (*C*). *C*, each plot indicates the time course of the mean peak amplitude of the glutamate-evoked current in the absence or continuous presence of baclofen. Thick bar, timing of the conjunctive stimuli. Dot, data binned for each 5 min period. Error bar, ± S.E.M. $^*P < 0.05$; $^{**}P < 0.01$, Mann–Whitney's *U* test. These conventions of data acquisition and representation also apply to the following figures. *D* and *E*, GABA, a native $GABA_BR$ agonist enhanced LTD_{glu}. *D*, sample responses of a cell before and after the conjunctive stimuli in the continuous presence of GABA (3 μM). *E*, time courses of the mean peak amplitudes of glutamate-evoked currents in the absence or continuous presence of GABA.

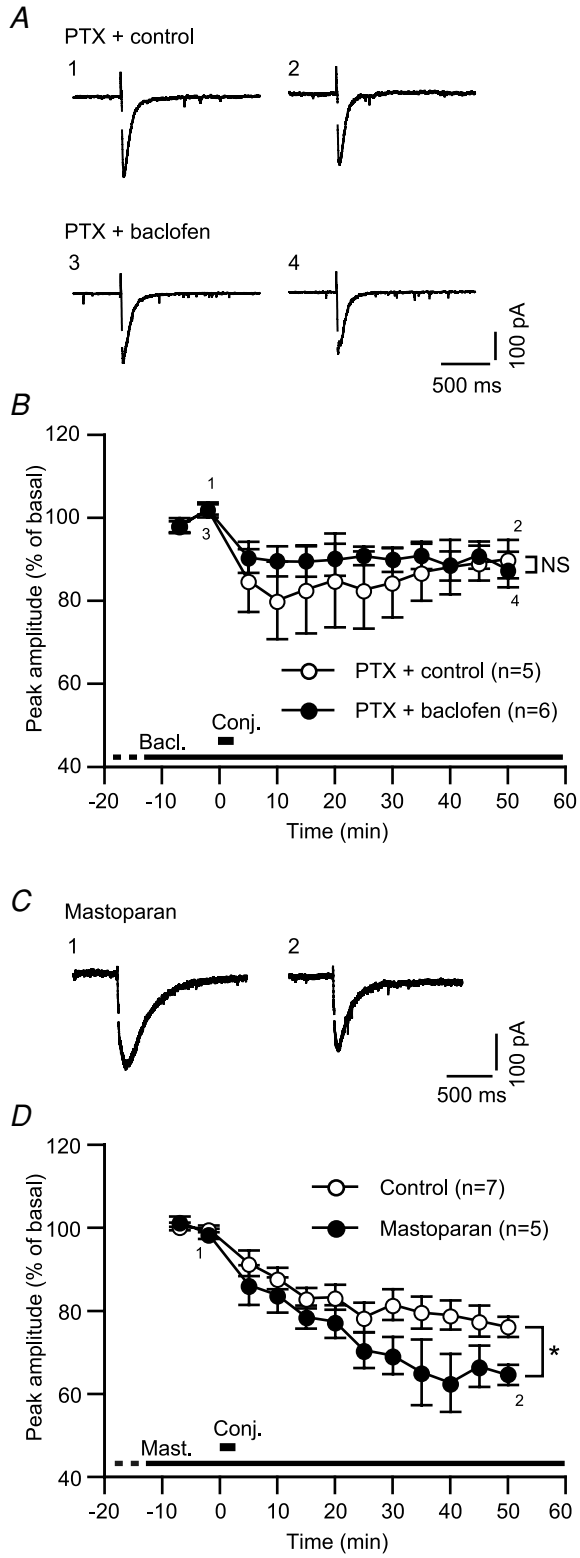


Figure 2. $G_{i/o}$ protein mediates GABA$_B$R-mediated LTD_{glu} enhancement
A and *B*, PTX, a $G_{i/o}$ protein inhibitor abolished baclofen-induced LTD_{glu} enhancement. *A*, each pair of traces indicates sample glutamate-evoked currents of a PTX (500 ng ml^{-1}, > 16 h)-pretreated cell before and after the conjunctive depolarization/glutamate stimuli in the absence ('Control') or continuous presence ('Baclofen') of baclofen (3 μM). *B*, time courses of the mean peak amplitudes of glutamate-evoked currents in the absence and continuous presence of baclofen. *C* and *D*, mastoparan, a $G_{i/o}$ protein agonist, mimicked GABA$_B$R-mediated LTD_{glu} enhancement. *C*, each pair of traces indicates sample glutamate-evoked currents of a cell before and after the conjunctive depolarization/glutamate stimuli in the absence or continuous presence of mastoparan (1 μM). *D*, time courses of the mean peak amplitudes of glutamate-evoked currents in the absence ('Control') and continuous presence of mastoparan.

LTD_{glu} enhancement cannot be ascribed to modulation of AMPA-type glutamate receptor channels by GABA$_B$R.

A previous study (Dittman & Regehr, 1997) suggests that the parallel fibre-innervated dendritic spines of Purkinje cells may receive 5–10 μM GABA spilt over from neighbouring inhibitory neurons' synapses *in situ*. In the continuous presence of a comparable dose (3 μM) of GABA, the peak amplitude of the glutamate-evoked current ($66.9 \pm 1.7\%$, $n = 7$ at 48–52 min after the conjunctive stimuli) was significantly smaller than the control (Fig. 1*D* and *E*). Thus, GABA$_B$R-mediated LTD_{glu} enhancement could occur under physiological conditions.

The $\beta\gamma$ subunits of $G_{i/o}$ protein may mediate GABA$_B$R-mediated LTD_{glu} enhancement

In the following sections, we explored mechanisms underlying GABA$_B$R-mediated LTD_{glu} enhancement. To test the involvement of $G_{i/o}$ protein, the primary messenger coupled to GABA$_B$R, we assessed LTD_{glu} in Purkinje cells pretreated with pertussis toxin (PTX), a $G_{i/o}$ protein inhibitor (500 ng ml^{-1}, > 16 h). This pretreatment is reported to eliminate a $G_{i/o}$ protein-coupled inwardly rectifying K^+ current in Purkinje cells (Tabata *et al.* 2005). In the normal saline, the conjunctive stimuli induced LTD_{glu} in the PTX-pretreated cells while the magnitude of LTD_{glu} (peak amplitude at 48–52 min after the conjunctive stimuli, $90.1 \pm 4.6\%$, $n = 5$) was small compared with that in the untreated cells (Fig. 2*A* and *B*; cf. Fig. 1*C*). PTX pretreatment by itself might impede LTD_{glu} (see Discussion). Addition of baclofen (3 μM) to the saline did not increase the magnitude of LTD_{glu} in the PTX-treated cells ($87.5 \pm 4.3\%$, $n = 6$) (Fig. 2*A* and *B*). This result suggests the involvement of $G_{i/o}$ protein in GABA$_B$R-mediated LTD enhancement.

In supporting the above result, addition of mastoparan, a $G_{i/o}$ protein agonist (1 μM) to the saline significantly increased the magnitude of LTD_{glu} (peak amplitude at 48–52 min after the conjunctive stimuli, $64.6 \pm 2.4\%$, $n = 5$). This drug directly activates $G_{i/o}$ protein by facilitating GTP binding to $G_{i/o}$ protein without the aid of G protein-coupled receptors (Higashijima *et al.* 1988; Shpakov & Pertseva, 2006). Thus, activation of $G_{i/o}$ protein is sufficient to enhance LTD_{glu}.

Upon activation, $G_{i/o}$ protein is cleaved into the α and $\beta\gamma$ subunits ($G_{\alpha i/o}$ and $G_{\beta\gamma i/o}$, respectively). We determined the relative contribution of these subunits to LTD_{glu} enhancement. The main action of active $G_{\alpha i/o}$ is inhibition of adenylyl cyclase, which results in a reduction in cAMP level. We interfered with this action by adding forskolin (20 μM), a potent activator of adenylyl cyclase to the saline. This dose of forskolin is shown to indeed raise cAMP level in cultured Purkinje cells (Tabata *et al.* 2007). It is reported that in a neuron, a similar dose (10 μM) of forskolin activates adenylyl cyclase regardless of the presence or absence of micromolar levels of baclofen (Onali *et al.* 2003). Despite such an effect of forskolin, baclofen enhanced LTD_{glu} (peak amplitude at 48–52 min after the conjunctive stimuli, $55.7 \pm 4.3\%$, $n = 7$) (Fig. 3*A* and *B*). This result suggests that $G_{\beta\gamma i/o}$ may primarily mediate LTD_{glu} enhancement.

$GABA_BR$-mediated LTD_{glu} enhancement is attributable to augmentation of mGluR1 signalling

The immediate emergence of $GABA_BR$-mediated LTD_{glu} enhancement (see above, Fig. 1*A* and *B*) indicates that $GABA_BR$ signalling may modulate mechanisms underlying LTD induction. This possibility is further supported by experiments with pin-point application of baclofen at either the induction phase or expression/maintenance phase of LTD_{glu}. When applied only during the conjunctive stimuli, baclofen enhanced LTD_{glu} (Fig. 4*A* and *B*). By contrast, when applied after the conjunctive stimuli, baclofen failed to enhance LTD_{glu} (Fig. 4*C* and *D*).

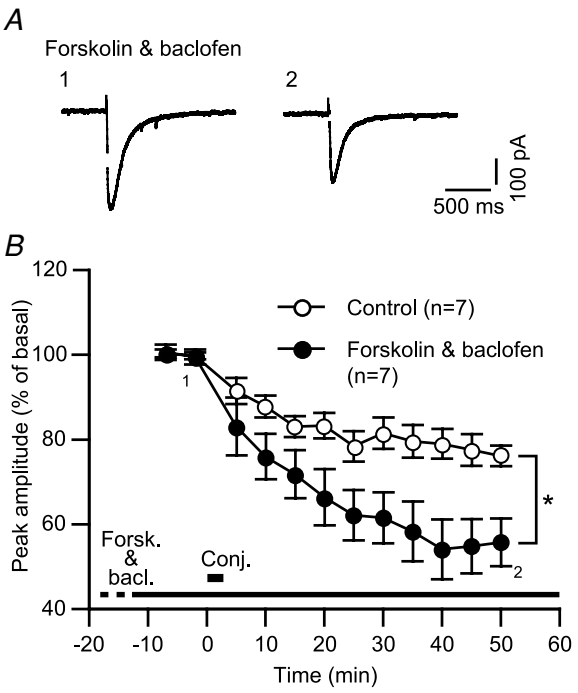


Figure 3. $G_{\alpha i/o}$ does not mediate $GABA_BR$-mediated LTD_{glu} enhancement
A and *B*, forskolin, an adenylyl cyclase activator did not abolish baclofen-induced LTD_{glu} enhancement. *A*, sample glutamate-evoked currents of a cell before and after the conjunctive depolarization/glutamate stimuli in the continuous presence of forskolin (20 μM) and baclofen (3 μM). *B*, time courses of the mean peak amplitudes of glutamate-evoked currents in the absence ('Control') and the continuous presence ('Forskolin & Baclofen') of forskolin (20 μM) and baclofen (3 μM).

In cultured Purkinje cell preparations, the key factors for triggering LTD induction are glutamate-evoked mGluR1 signalling and depolarization-evoked Ca^{2+} influx (Linden *et al.* 1991; Ito, 2002). $GABA_BR$ could enhance LTD_{glu} by augmenting either or both of these factors. We tested this possibility, using Ca^{2+} imaging. First, we examined the effect of baclofen on an mGluR1-mediated $[Ca^{2+}]_i$ rise evoked by DHPG, a group I mGluR agonist (5 μM, 10 s) (Fig. 5). In the recording conditions used here (the excitability of the cells was pharmacologically attenuated, see Methods), this response mainly reflects Ca^{2+} release from the intracellular stores (Sato *et al.* 2004) (see Fig. 7*C*). mGluR1 promotes the opening of Ca^{2+}-permeable inositol trisphosphate receptor channel (IP_3R) on these stores via the $G_{q/11}$ protein-phospholipase C (PLC)-IP_3 cascade (Ito, 2002). Baclofen (3 μM) but not the normal saline augmented the peak amplitude (expressed in a change in F_{340}/F_{380}) of the $[Ca^{2+}]_i$ rise ($154.9 \pm 19.4\%$, $n = 12$ and $98.8 \pm 8.0\%$, $n = 11$, respectively; Fig. 5*A* and *D*). Pretreatment with PTX (500 ng ml^{-1}, > 16 h) abolished this augmentation (Fig. 5*B* and *D*). These results suggest that $GABA_BR$ potentiates mGluR1 signalling via $G_{i/o}$ protein. The resting $[Ca^{2+}]_i$ level (mean F_{340}/F_{380} over 5 s prior to DHPG application) changed little during application of the normal saline or baclofen ($99.2 \pm 1.1\%$, $n = 11$ and $92.0 \pm 3.2\%$, $n = 12$, respectively; data not illustrated). Thus, the augmentation cannot be ascribed to $[Ca^{2+}]_i$-dependent facilitation of Ca^{2+} release (Llano *et al.* 1994). Moreover, baclofen augmented the $[Ca^{2+}]_i$ rise in the presence of SKF96365, an antagonist against receptor-operated Ca^{2+} channels including TRPC1 (30 μM, Fig. 5*C*). The extent of this augmentation with SKF96365 ($184.2 \pm 36.0\%$, $n = 8$; Fig. 5*D*) was comparable with that in the normal saline (Fig. 5*A* and *D*). This result suggests that this augmentation is due largely to an increase of Ca^{2+} release from the intracellular stores; the contribution of an increase of Ca^{2+} influx through receptor-operated channels might be small although we do not exclude that the $[Ca^{2+}]_i$ rise includes such Ca^{2+} influx. These results suggest that $GABA_BR$ signalling potentiates mGluR1 signalling via $G_{i/o}$ protein.

Second, we examined the effect of baclofen on a $[Ca^{2+}]_i$ rise evoked by application of K^+-rich saline ($[K^+] = 75$ mM, 1 s) (Fig. 6). This response was susceptible to ω-agatoxin IVA (100 nM), an antagonist against

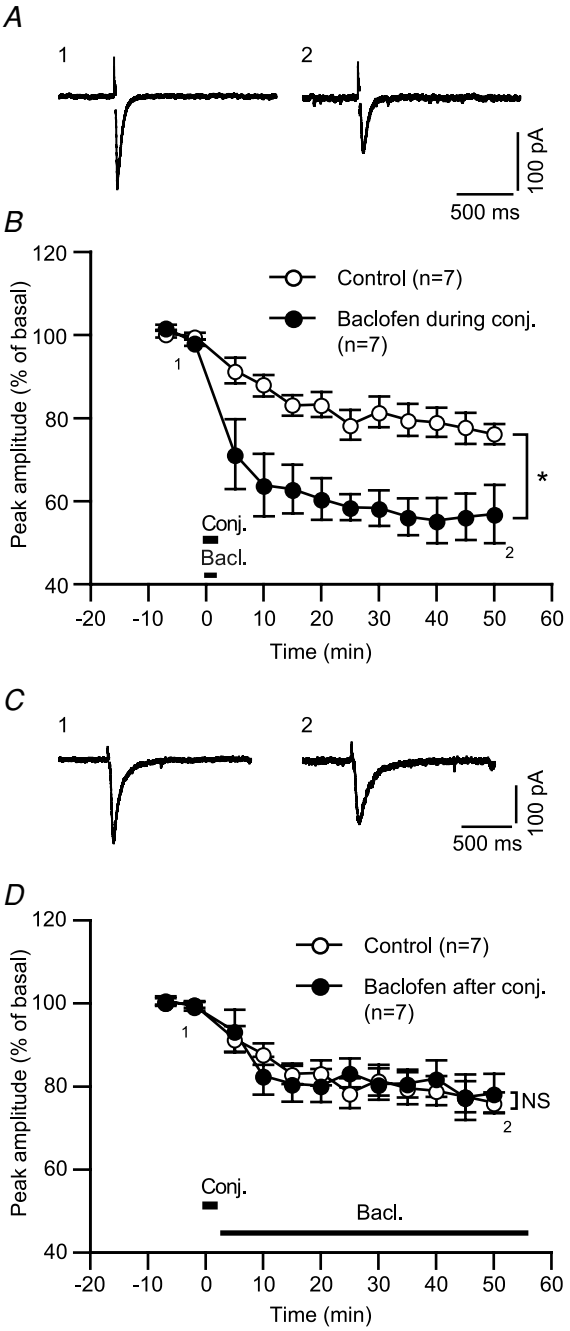


Figure 4. GABA$_B$R activation during the induction phase of LTD$_{glu}$ is sufficient for LTD$_{glu}$ enhancement
A and *B*, baclofen applied during the conjunctive depolarization/glutamate stimuli enhanced LTD$_{glu}$. *A*, sample glutamate-evoked currents of a cell before and after the conjunctive depolarization/glutamate stimuli with baclofen (3 μM) application during the stimuli. *B*, time courses of the mean peak amplitudes of glutamate-evoked currents with or without ('Control') baclofen application during the conjunction stimuli. *C* and *D*, baclofen applied after the conjunctive depolarization/glutamate stimuli did not enhance LTD$_{glu}$. *C*, sample glutamate-evoked currents of a cell before and after the conjunctive depolarization/glutamate stimuli with baclofen (3 μM) application after the stimuli. *D*, time courses of the mean peak amplitudes of glutamate-evoked currents with or without ('Control') baclofen application after the conjunction stimuli.

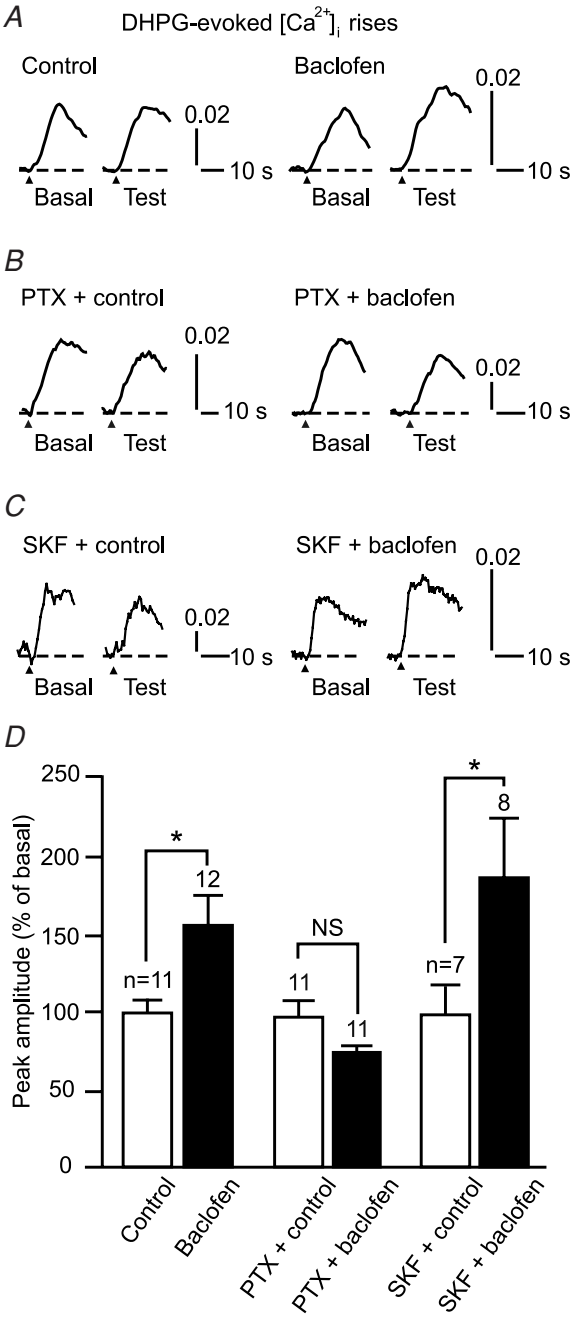


Figure 5. GABA$_B$R activation augments mGluR1 signalling in a G$_{i/o}$ protein-dependent manner
A–D, baclofen induced a G$_{i/o}$ protein-dependent augmentation of a DHPG-evoked $[Ca^{2+}]_i$ rise that reflects mGluR1-coupled intracellular Ca^{2+} store release. *A–C*, sample responses of cells untreated (*A*), pretreated with PTX (500 ng ml^{-1}, > 16 h; *B*), and perfused with SKF96365 (30 μM, *C*). Each pair of traces indicates sample DHPG (5 μM, 10 s)-evoked responses of a cell before ('Basal') and after ('Test') a 12 min application of the normal saline ('Control') or baclofen (3 μM). Arrowhead, DHPG onset. Vertical scale bar, change in F_{340}/F_{380}. *D*, mean peak amplitudes of the DHPG-evoked $[Ca^{2+}]_i$ rise after application of the normal saline or baclofen (expressed as percentage of the basal levels).

the dominant type (P/Q-type) of voltage-gated Ca^{2+} channels in Purkinje cells ($n = 6$, data not illustrated), suggesting that this response reflects Ca^{2+} influx through voltage-gated channels. Baclofen did not augment the K^+-evoked $[Ca^{2+}]_i$ rise but rather reduced it (peak amplitude, $79.0 \pm 2.8\%$, $n = 15$) (Fig. 6*A* and *C*). Pretreatment with PTX (500 ng ml^{-1}, > 16 h) abolished the baclofen-induced reduction of the K^+-evoked $[Ca^{2+}]_i$ rise (Fig. 6*B* and *C*).

The results in Fig. 5 imply that $GABA_BR$-mediated LTD_{glu} enhancement could be due partly to augmentation of mGluR1-coupled Ca^{2+} release from the intracellular stores. This notion would be in a good agreement with previous reports that mGluR1-coupled Ca^{2+} release is essential for inducing LTD in cerebellar slices (e.g. Inoue *et al.* 1998). However, there is a report (Narasimhan *et al.* 1998) that this is not the case in certain cultured Purkinje cell preparations. We checked the dependence of LTD_{glu} upon mGluR1-coupled Ca^{2+} release in our cultured Purkinje cell preparations (Fig. 7). We depleted Ca^{2+} from the intracellular stores by adding thapsigargin, an endoplasmic reticular Ca^{2+}-ATPase inhibitor, to the saline. Under this condition, the conjunctive stimuli failed to induce LTD_{glu} ($105.5 \pm 7.0\%$ at 48–52 min after the conjunctive stimuli, $n = 5$; Fig. 7*A* and *B*). Thapsigargin (1 μM, 12 min) suppressed the DHPG

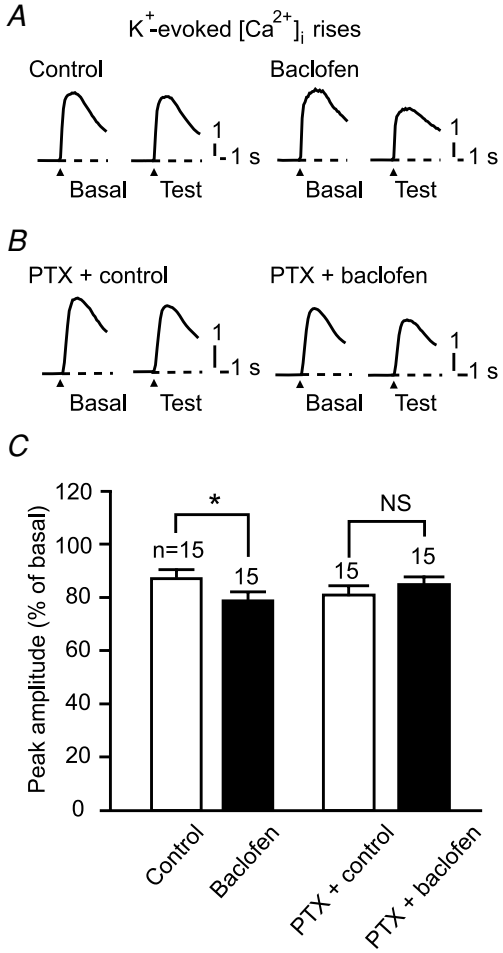


Figure 6. $GABA_BR$ activation does not augment depolarization-evoked Ca^{2+} influx
A–*C*, baclofen did not augment but rather reduced a $[Ca^{2+}]_i$ rise evoked by the K^+-rich saline ($[K^+] = 75$ mM, 1 s). *A* and *B*, sample responses of untreated or PTX (500 ng ml^{-1}, > 16 h)-pretreated cells. Each pair of traces indicates sample K^+-evoked $[Ca^{2+}]_i$ rises of a cell before and after a 12 min application of the normal saline ('Control') or baclofen (3 μM). Arrowhead, K^+-rich saline onset. *C*, mean peak amplitudes of the DHPG-evoked $[Ca^{2+}]_i$ rise after application of the normal saline or baclofen (expressed as percentage of the basal levels).

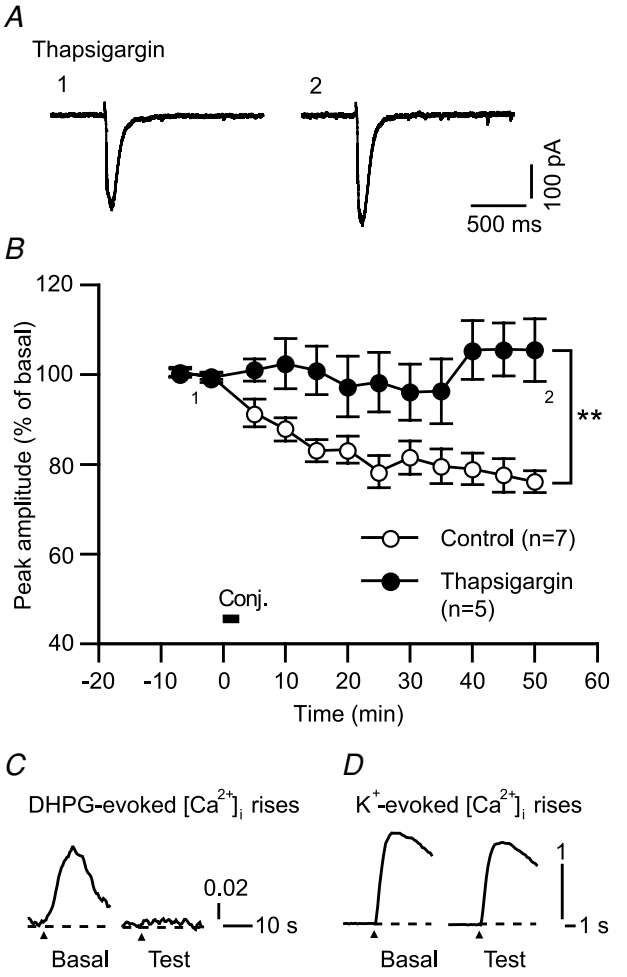


Figure 7. mGluR1-mediated Ca^{2+} release from the intracellular stores is important for inducing LTD_{glu}
A and *B*, thapsigargin (endoplasmic reticular Ca^{2+}-ATPase inhibitor), which was expected to deplete Ca^{2+} in the intracellular stores, abolished LTD_{glu}. *A*, sample glutamate-evoked currents of a cell before and after the conjunctive depolarization/glutamate stimuli in the continuous presence of thapsigargin (1 μM). *B*, time courses of the mean peak amplitudes of glutamate-evoked currents in the absence and continuous presence of thapsigargin. *C*, thapsigargin indeed abolished mGluR1-mediated Ca^{2+} release from the intracellular stores. Traces indicate sample DHPG (5 μM, 10 s)-evoked $[Ca^{2+}]_i$ rises of a cell before and after application of thapsigargin (1 μM, 12 min). Similar results were obtained from 4 cells. *D*, tharpsigargin did not abolish depolarization-evoked Ca^{2+} influx. Traces indicate sample K^+ (75 mM, 1 s)-evoked $[Ca^{2+}]_i$ rises of a cell before and after application of thapsigargin (1 μM, 12 min). Similar results were obtained from 9 cells.

(5 μM, 10 s)-evoked $[Ca^{2+}]_i$ rise as its direct effect (peak amplitude, 13.6 ± 3.6%, $n = 4$; Fig. 7*C*), confirming that thapsigargin indeed abolished mGluR1-mediated Ca^{2+} release from the intracellular stores. On the other hand, thapsigargin (1 μM, 12 min) little affected the K^+ (75 mM, 1 s)-evoked Ca^{2+} influx (98.8 ± 8.0%, $n = 9$, Fig. 7*D*). These results suggest that mGluR1-coupled Ca^{2+} release from the intracellular stores is important for inducing LTD_{glu} at least in our preparations.

The result in Fig. 3 suggests that $G_{\beta\gamma i/o}$ mediates GABA$_B$R-mediated LTD_{glu} enhancement. In various cell types, $G_{\beta\gamma i/o}$ augments $G_{q/11}$ protein-coupled Ca^{2+} release from the intracellular stores by priming some PLC isoforms including PLCβ3 (for review, Park *et al.* 1993;

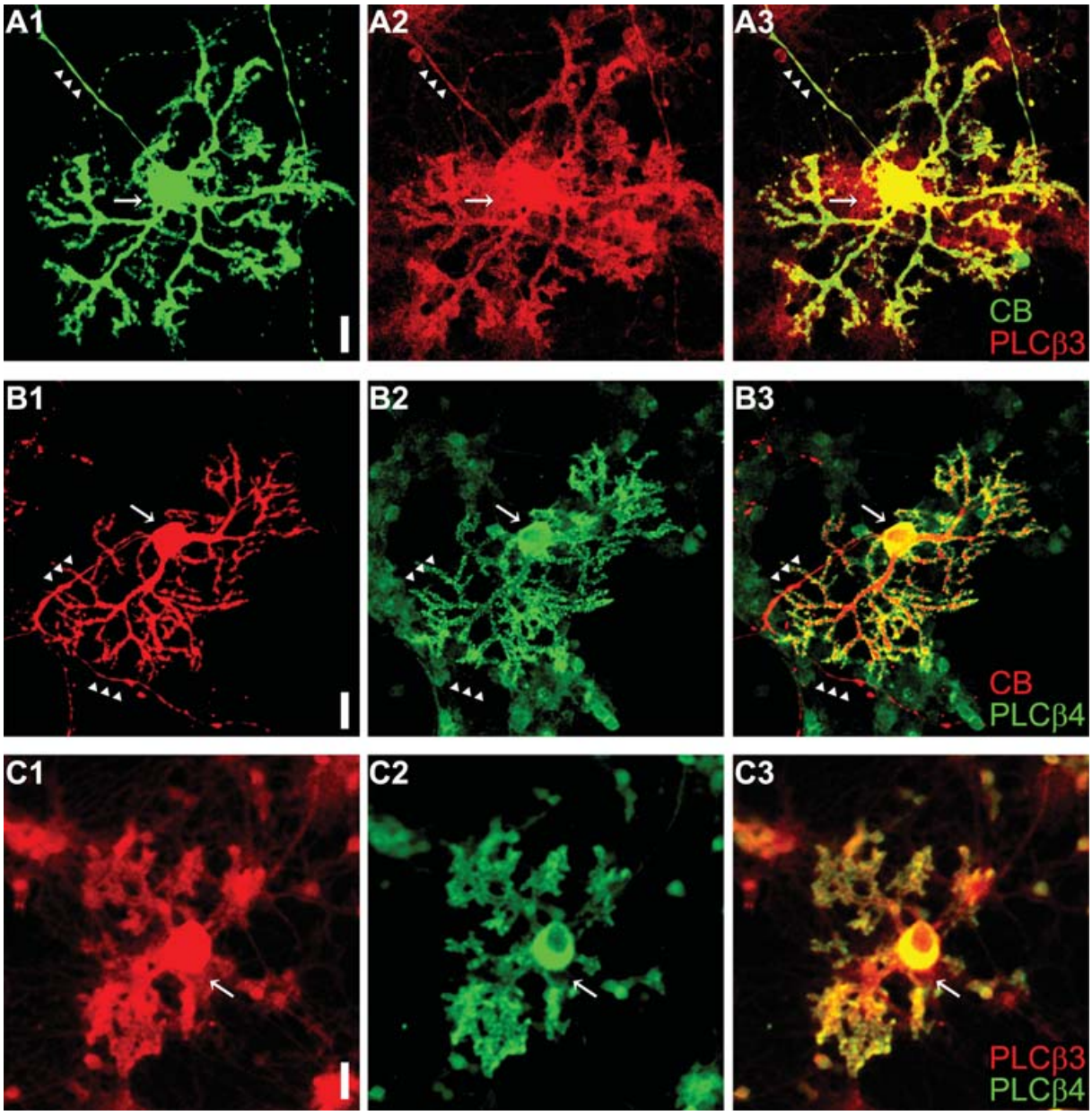
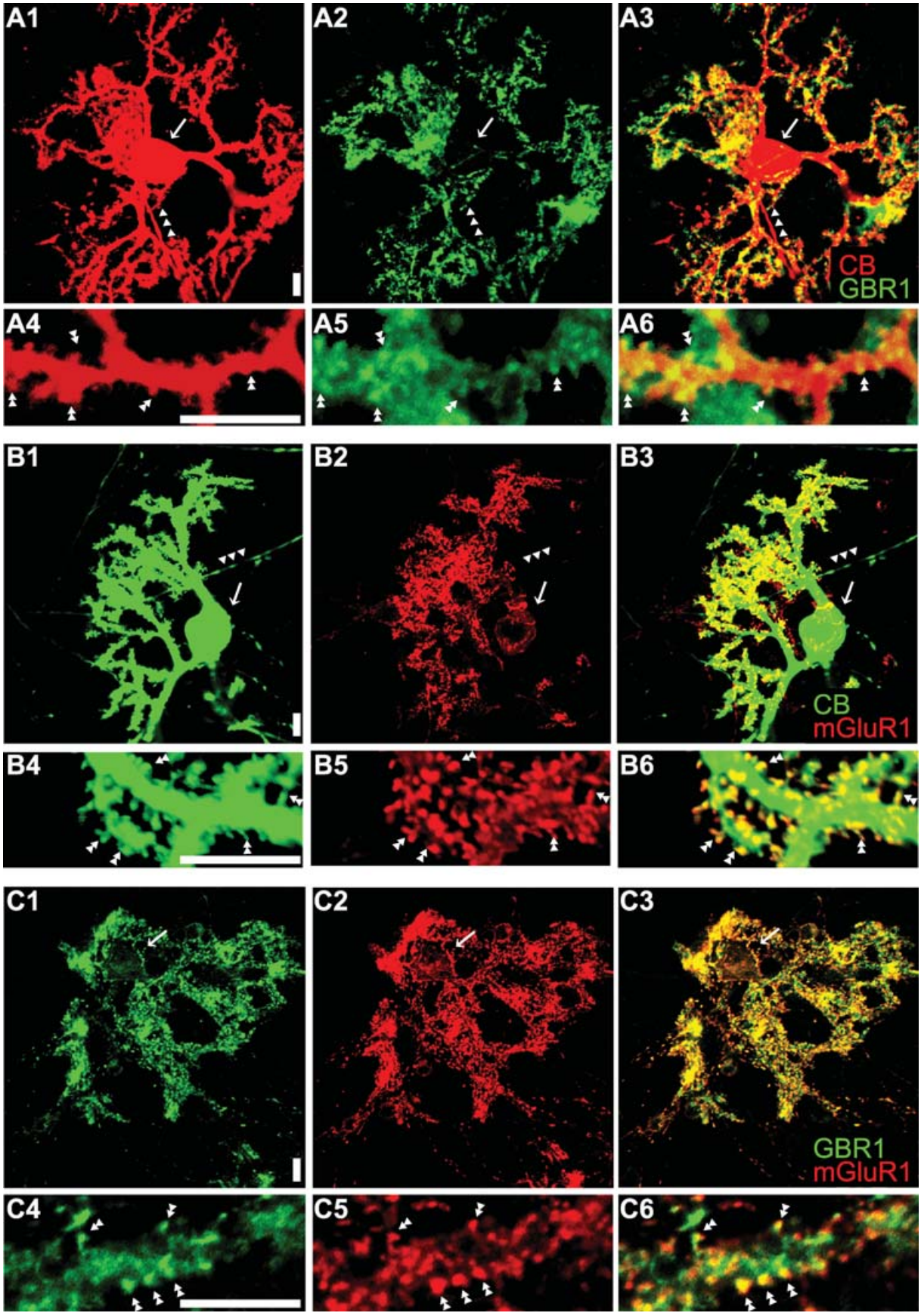


Figure 8. Cultured Purkinje cells express both PLCβ3 and β4
A and *B*, Purkinje cells (14 days old *in vitro*) identified by calbindin ('CB') immunoreactivity (green in *A1* and red in *B1*) are immunostained with anti-PLCβ3 (red in *A2*) and anti-PLCβ4 (green in *B2*) antibodies. Arrows and arrowheads, somata and axons, respectively. Scale bar, 20 μm. The almost complete overlap of PLCβ3 and calbindin signals of the cell (superimposed images in *A3*) suggests that PLCβ3 is expressed throughout the soma, dendrites and axon of the Purkinje cell. By contrast, PLCβ4 immunoreactivity is found in the soma and dendrites but not in the axon (*B3*). *C*, a Purkinje cell (14 days old *in vitro*) displays both PLCβ3 and PLCβ4 immunoreactivities.



Selbie & Hill, 1998). Such priming could underlie $GABA_BR$-mediated augmentation of mGluR1-coupled Ca^{2+} release because Purkinje cells more or less express PLCβ3 *in vivo* (Kano *et al.* 1998; Nomura *et al.* 2007). We checked whether cultured Purkinje cells express PLCβ3 by immunohistochemistry. Cultured Purkinje cells were identified by immunoreactivity against calbindin (green in Fig. 8*A1* and red in Fig. 8*B1*). For all the calbindin-positive cells, PLCβ3 immunoreactivity was found throughout the somata, axons and dendrites ($n = 34$, Fig. 8*A*). A similar subcellular distribution is seen in PLCβ3-positive Purkinje cells *in vivo* (Nomura *et al.* 2007). It is reported that a subpopulation of Purkinje cells *in vivo* express a low level of PLCβ3 while complementarily expressing a high level of PLCβ4 (Sarna *et al.* 2006) that is insensitive to $G_{\beta\gamma i/o}$ (Park *et al.* 1993). Such heterogeneity of PLC isoforms was absent in the culture system. All of the calbindin-positive cells displayed PLCβ4 immunoreactivity ($n = 42$, Fig. 8*B*). PLCβ4 immunoreactivity was confined to the somata and dendrites as shown *in vivo* (Nakamura *et al.* 2004). Furthermore, all of the cells showing PLCβ3 immunoreactivity also displayed PLCβ4 immunoreactivity in the culture system ($n = 28$, Fig. 8*C*). These results suggest that priming of PLCβ3 by $G_{\beta\gamma i/o}$ may occur in most cultured Purkinje cells.

Co-localization of $GABA_BR$ and mGluR1 in cultured Purkinje cells

To explore the possible site for the functional interaction of $GABA_BR$ and mGluR1 signalling cascades, we immunostained cultured Purkinje cells with antibodies against these receptors. Calbindin-positive cells had clear immunoreactivities for GBR1 (Fig. 9*A*) and mGluR1 (Fig. 9*B*). Higher levels of immunoreactivities were found at the dendritic spines of these cells (Fig. 9*A4–6* and *B4–6*). Co-immunostaining with the anti-GBR1 and anti-mGluR1 antibodies shows that the subcellular distributions of $GABA_BR$ and mGluR1 overlap each other (Fig. 9*C*). These results suggest that co-localization of these receptors at the dendritic spines seen *in situ* (Lujan *et al.* 1997; Kulik *et al.* 2002) is preserved in our cultured cell preparations.

$GABA_BR$ enhances LTD *in situ*

Lastly, we tested whether $GABA_BR$ indeed enhances LTD of parallel fibre–Purkinje cell synaptic transmission, using cerebellar slices. The extracellular fluid in the cerebellar slices might contain GABA because the cerebrospinal fluid contains GABA and cerebellar interneurons synaptically release GABA (see Discussion). We examined the effect of CGP55845, a $GABA_BR$-selective antagonist that is expected to interfere with GABA action on $GABA_BR$. We induced LTD with conjunctive stimuli (30 sets at 0.5 Hz) each of which consisted of a train of electrical pulses to the parallel fibres and a depolarizing voltage step to the Purkinje cell (Fig. 10*A*). The electrical pulses were given in relatively high-frequency bursts (100 Hz) to facilitate GABA release from interneurons activated by the parallel fibre stimuli (Dittman & Regehr, 1997; Hirono *et al.* 2001). Under the control condition, parallel fibre–Purkinje cell EPSC was depressed over 30 min after the conjunctive stimuli (Fig. 10*B*). The continuous presence of CGP55845 (2 μM) abrogated initial depression after the conjunctive stimuli and slowed down the development of LTD (Fig. 10*B*). For both conditions, LTD appeared to develop to the maximal extents at 17–21 min after the conjunctive stimuli (mean amplitudes of parallel fibre–Purkinje cell EPSC at this period were not significantly different those at 27–31 min after the conjunctive stimuli, $P > 0.1$, paired Student's t test). Over 30–31 min after the conjunctive stimuli, the mean relative amplitude of parallel fibre–Purkinje cell EPSC with CGP55845 ($81.1 \pm 5.9\%$ of the basal level, $n = 5$) was significantly larger than that of the control ($61.1 \pm 4.4\%$, $n = 5$) (Fig. 10*B*). This result indicates that $GABA_BR$-mediated enhancement may occur for LTD *in situ*.

Discussion

We found that $GABA_BR$ activation enhanced LTD_{glu}, increasing the magnitude of depression (Fig. 1*B* and *C*). $GABA_BR$-mediated LTD_{glu} enhancement emerged immediately after the induction phase of LTD_{glu} (Fig. 1*C*). $GABA_BR$ agonist application within the period of the induction stimuli was sufficient to enhance LTD_{glu} (Fig. 4).

Figure 9. Co-localization of $GABA_BR$ and mGluR1 in cultured Purkinje cells
A and *B*, Purkinje cells (20 days old *in vitro*) identified by calbindin ('CB') immunoreactivity (*A1* and *B1*) are immunostained with anti-GBR1 (*A2*) and anti-mGluR1 (*B2*) antibodies (*A3* and *B3*, superimposed images). Arrows and arrowheads, somata and axons, respectively. GBR1 and mGluR1 immunoreactivities are detected at most dendritic spines (double arrow heads in the close-up images, *A5* and *6* and *B5* and *6*). *C*, co-immunostaining of Purkinje cells (20 days old *in vitro*) with the anti-GBR1 (*C1*) and anti-mGluR1 (*C2*) antibodies shows the overlapping subcellular distribution of these antigens (*C3*, superimposed image). Co-localization of these antigens is found at dendritic spines (double arrow heads in the close-up images, *C4–C6*). Scale bars, 10 μm.

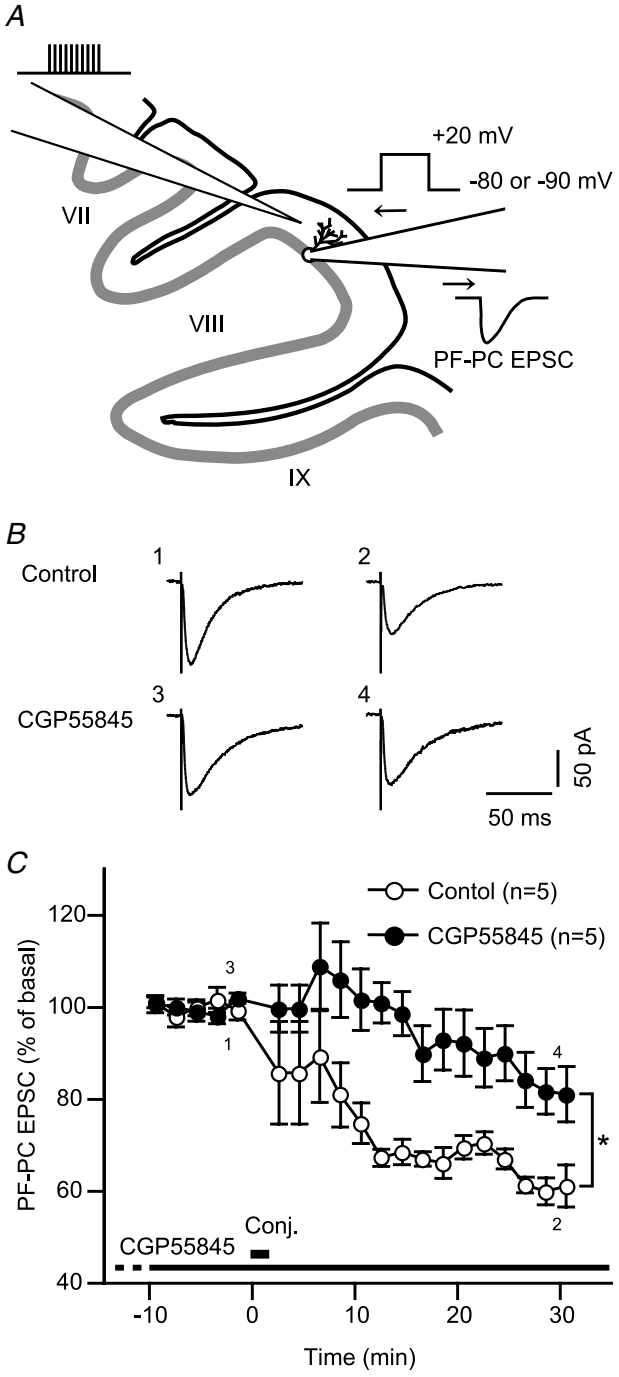


Figure 10. $GABA_BR$-mediated LTD enhancement may occur *in situ*

A, as schematically shown, we measured LTD of parallel fibre–Purkinje cell (PF-PC) EPSC in cerebellar slices. Whole-cell voltage-clamp recording was made from a Purkinje cell in the lamina VII or VIII. Parallel fibres were stimulated by electrical pulses delivered through a glass pipette positioned within a 1/3-part amid the molecular layer. LTD was induced with 30 sets of conjunctive stimuli (repeated at 0.5 Hz) each of which consisted of 10 pulses to the parallel fibres (100 Hz) and a depolarizing voltage step (+20 mV, 100 ms) to the Purkinje cell through the recording pipette. *B*, relative amplitude of parallel fibre–Purkinje cell (PF-PC) EPSC in the absence (control, $n = 5$) or continuous presence ($n = 5$) of CGP55845, a $GABA_BR$-selective antagonist (2 μM) plotted against time after the conjunctive stimuli ('Conj.'). CGP55845 was included in the bath solution from at least 30 min before the conjunctive stimuli. Dot and error bar, the mean and S.E.M. of the data for each 2 min period. Mean value over 29–30 min after the conjunctive stimuli significantly differs between the two conditions (*$P < 0.05$, Mann–Whitney's *U* test).

These results suggest that $GABA_BR$ signalling modulates mechanisms underlying LTD_{glu} induction (Fig. 11). In cultured Purkinje cell preparations, glutamate-evoked mGluR1 signalling and depolarization-evoked Ca^{2+} influx are known as the key factors for triggering LTD induction (Fig. 11*A*) (Linden *et al.* 1991; Ito, 2002). $GABA_BR$ activation augmented DHPG-evoked mGluR1 signalling (Fig. 5*A* and *D*; for details, see below) while reduced K^+-evoked Ca^{2+} influx (Fig. 6*A* and *C*). A previous study (Mintz & Bean, 1993) shows that $GABA_BR$ negatively regulates the P/Q-type channels in Purkinje cells. These results suggest that $GABA_BR$ signalling augments mGluR1 signalling in Purkinje cells and thereby facilitate LTD_{glu} induction (Fig. 11*B*).

Inhibition of $G_{i/o}$ protein by PTX abolished $GABA_BR$-mediated LTD_{glu} enhancement (Fig. 2*A* and *B*). PTX did not appear to exert an inhibitory effect directly on glutamate-evoked currents in Purkinje cells (see online supplemental material, Supplemental Fig. 1). Thus, the effect of PTX on LTD_{glu} enhancement cannot be ascribed to a PTX-induced reduction of glutamate-evoked currents which would occlude LTD_{glu}. Activation of $G_{i/o}$ protein by mastoparan mimicked LTD_{glu} enhancement (Fig. 2*C* and *D*). Activation of adenylyl cyclase by forskolin did not abolish $GABA_BR$-mediated LTD_{glu} enhancement (Fig. 3). Forskolin at the dose used is thought to increase cAMP level regardless of the presence or absence of micromolar levels of baclofen (Onali *et al.* 2003). Thus, this manipulation should cancel the inhibitory action of $G_{\alpha i/o}$ on adenylyl cyclase. These results raise the possibility that $G_{\beta\gamma i/o}$ primarily mediates $GABA_BR$-mediated LTD_{glu} enhancement (Fig. 11*B*). There are several possible targets for $G_{\beta\gamma i/o}$. In various cell types, $G_{\beta\gamma i/o}$ are known to exert a synergistic action with $G_{q/11}$ protein on some PLC isoforms including PLCβ3 (Park *et al.* 1993). All of the cultured Purkinje cells expressed PLCβ3 (Fig. 8). Thus, in most of the cases in this study, such a synergistic action on PLC should occur, and this might augment downstream mGluR1 signalling mediated by PLC (Fig. 11*B*). Moreover, a study (Zeng *et al.* 2003) has reported a possibility that $G_{\beta\gamma i}$ binds to and exerts a direct agonistic action on type-1 IP_3R, which is important for cerebellar LTD induction (Inoue *et al.* 1998).

Depletion of Ca^{2+} in the intracellular stores by thapsigargin abolished mGluR1-mediated $[Ca^{2+}]_i$ rises (Fig. 7*C*). In the presence of this agent, the conjunctive depolarization/glutamate stimuli did not

induce LTD$_{glu}$ (Fig. 7*A* and *B*). These results suggest that mGluR1-coupled Ca^{2+} release from the intracellular stores is essential for inducing LTD$_{glu}$ in our cultured cell preparations (Fig. 11*A*) as reported in cerebellar slice preparations (Inoue *et al.* 1998). Thus, the GABA$_B$R-mediated augmentation of mGluR1-coupled Ca^{2+} release may contribute to LTD$_{glu}$ enhancement (Fig. 11*B*). A resultant elevation of the cytoplasmic [Ca^{2+}] may promote activation of PKC (Ito, 2002), which in turn facilitates endocytosis of AMPA-type glutamate receptors (Matsuda *et al.* 2000; Chung *et al.* 2003). A recent study (Hansel *et al.* 2006) shows that genetic or pharmacological suppression of α-calcium/calmodulin-dependent kinase II, which is activated at a high [Ca^{2+}]$_i$, switches the polarity of plasticity from depression to potentiation at parallel fibre–Purkinje cell synapses. mGluR1-coupled Ca^{2+} release might ensure induction of LTD$_{glu}$ by boosting the activity of this kinase.

PTX pretreatment by itself attenuated the magnitude of LTD$_{glu}$ (Fig. 2*A* and *B*) as previously reported (Ito & Karachot, 1990). A small amount of G proteins may be active in a constitutive manner due to the ligand-independent basal activity of G protein-coupled receptors (Smit *et al.* 2007). Thus, G$_{i/o}$ protein could always enhance LTD$_{glu}$ slightly unless inhibited by PTX.

Previous studies (Hirono *et al.* 2001; Tabata *et al.* 2004) show that in Purkinje cells, GABA$_B$R activation augments an mGluR1-mediated inward cation current (slow EPSC at parallel fibre–Purkinje cell synapses) by raising the ligand sensitivity of mGluR1 in a G$_{i/o}$ protein-independent manner and by potentiating the mGluR1 signalling cascade in a G$_{i/o}$ protein-dependent manner. GABA$_B$R-mediated LTD$_{glu}$ enhancement resembles the latter action of GABA$_B$R in terms of G$_{i/o}$ protein dependence. However, activation of G$_{i/o}$-coupled receptors other than GABA$_B$R (e.g. A1 adenosine receptor; Tabata *et al.* 2007) fails to augment the slow EPSC (Hirono *et al.* 2001), suggesting that G$_{i/o}$ protein activation is not sufficient to enhance this response. This contrasts with G$_{i/o}$ protein activation being sufficient to enhance LTD$_{glu}$ (Fig. 2*C* and *D*). LTD$_{glu}$ enhancement and cation current augmentation may involve different mechanisms.

Co-localization of GABA$_B$R and mGluR1 at the dendritic spines of Purkinje cells is seen commonly in culture (Fig. 9) and *in situ* (Lujan *et al.* 1997; Kulik *et al.* 2002). Therefore, GABA$_B$R-mediated enhancement described above could possibly occur for mGluR1-mediated LTD at real parallel fibre–Purkinje cell synapses. We confirmed this possibility, using cerebellar slices. A GABA$_B$R-selective antagonist reduced the magnitude of LTD induced by conjunctive parallel fibre stimulation and postsynaptic depolarization (Fig. 10). In mammals, the cerebrospinal fluid contains a few tens of

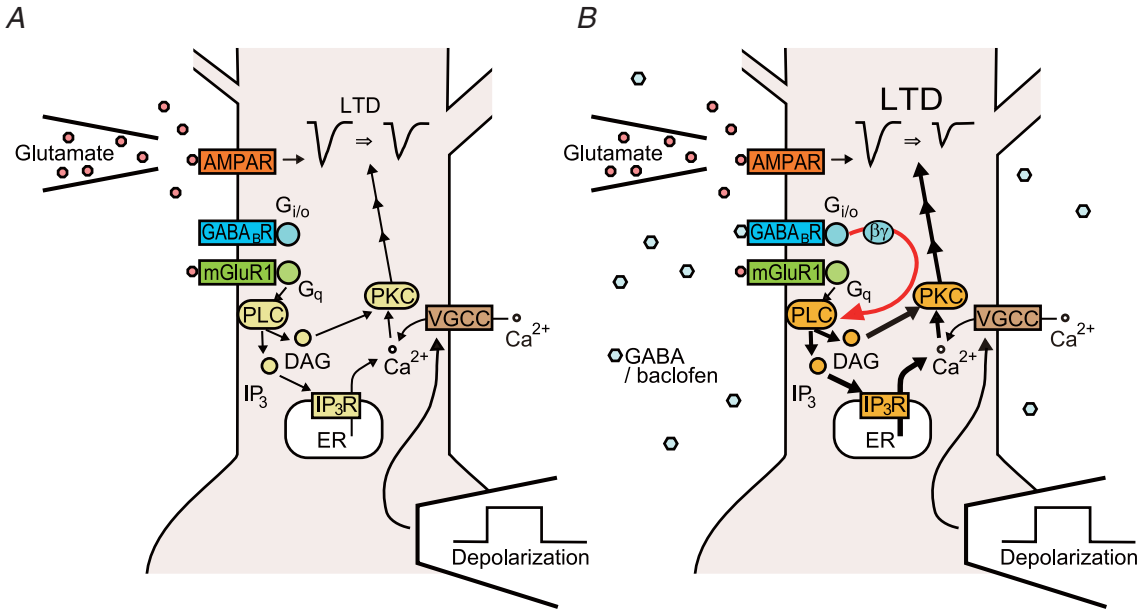


Figure 11. Possible mechanisms underlying GABA$_B$R-mediated LTD$_{glu}$ enhancement
A, in the cultured Purkinje cells, glutamate-evoked mGluR1 signalling including Ca^{2+} release from the intracellular stores and depolarization-evoked Ca^{2+} influx are key factors for inducing LTD$_{glu}$. *B*, GABA and baclofen promotes activation of G$_{i/o}$ protein by GABA$_B$R. Activated G$_{i/o}$ protein is cleaved into the α and $\beta\gamma$ subunits; the latter may be more important for LTD$_{glu}$ enhancement than the former. The $\beta\gamma$ subunits may augment mGluR1-mediated signalling presumably by acting on PLC, and this may result in LTD$_{glu}$ enhancement. For further explanation, see Discussion. AMPAR, AMPA-type ionotoropic glutamate receptor; DAG, diacylglycerol; ER, endoplasmic reticulum; IP$_3$, inositol trisphosphate; IP$_3$R, IP$_3$ receptor; PKC, protein kinase C; PLC, phospholipase C; VGCC, voltage-gated calcium channel.

nanomolar of GABA *in vivo* (Bohlen *et al.* 1979). Some fraction of this GABA could remain in the cerebellar slices. Parallel fibre stimulation may further increase ambient GABA concentration in the cerebellar slices by facilitating GABA release from interneurons activated by the parallel fibre inputs. Released GABA may spill over from the synaptic clefts and act on neighbouring parallel fibre–Purkinje cell synapses (Dittman & Regehr, 1997; Hirono *et al.* 2001). CGP55845 might interfere with GABA action on $GABA_BR$ in the Purkinje cells (Fig. 11). The effect of CGP55845 on LTD is not attributable to its action on presynaptic $GABA_BR$. $GABA_BR$ mediates presynaptic inhibition of parallel fibre–Purkinje cell synapses (Dittman & Regehr, 1996). A $GABA_BR$ antagonist is shown to relieve this inhibition (Hirono *et al.* 2001), and this would rather enhance LTD.

The concentration of GABA contained in the cerebrospinal fluid of healthy animals (see above) is high enough to activate $GABA_BR$ considerably ($GABA_BR$'s affinity for GABA, $\sim$1 μM; Sodickson & Bean, 1996). GABA spilt over from the interneurons' synapses might further facilitate $GABA_BR$ activation in Purkinje cells. Therefore, $GABA_BR$-mediated enhancement of cerebellar LTD could occur under physiological conditions *in vivo*. Our findings demonstrate a novel mechanism that would facilitate cerebellar motor learning.

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Supplemental material

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