Long-Term Depression at Parallel Fiber to Golgi cell Synapses

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Running head

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Abstract

Golgi cells (GoCs) are the primary inhibitory interneurons of the granular layer of the cerebellum. Their inhibition of granule cells is central to operate the relay of excitatory inputs to the cerebellar cortex. Parallel fibers (PFs) establish synapses to the GoCs in the molecular layer; these synapses contain AMPA-, NMDA-, and mostly group II metabotropic glutamate receptors. Long-term changes in the efficacy of synaptic transmission at the PF-GoC synapse have not been described previously. We used whole-cell patch-clamp recordings of GoCs in acute rat cerebellar slices to study synaptic plasticity. We report that high-frequency burst stimulation of PFs, using a current-clamp or voltage-clamp induction protocol, gave rise to long-term depression (LTD) at the PF-GoC synapse. This form of LTD was not associated with persistent changes of paired-pulse ratio, suggesting a postsynaptic origin. Furthermore, LTD induction was not dependent on activation of NMDA receptors. PF-GoC LTD does require activation of specifically group II metabotropic glutamate receptors and of protein kinase A.

Keywords

LTD – Golgi cell – cerebellum - synaptic plasticity – mGluR2
Introduction

The cerebellum plays an important role in several forms of motor learning. For example, there is evidence supporting the requirement of the cerebellar cortex in associative eyelid conditioning (McCormick and Thompson 1984; Koekkoek et al. 2003). Thus activity-dependent alterations in the efficacy of cerebellar synaptic transmission (i.e. potentiation, depression) are thought to underlie learning and possibly the storage of motor patterns. From early theoretical work it was anticipated that long-term depression (LTD) at parallel fiber (PF) to Purkinje cell (PC) synapses underlies motor learning (Marr-Albus-Ito models; Marr 1969; Albus 1971; Ito 1984). Such plasticity was later experimentally demonstrated by applying in vivo conjunctive stimulation of PFs and climbing fibers (Ito and Kano 1982). An elaborate literature exists on the synaptic plasticity of the PF-PC synapse (for reviews see Ito 2001; Jörntell and Hansel 2006) and on plasticity at other cerebellar synapses, such as the climbing fiber to PC synapse (Hansel and Linden 2000; Coesmans et al. 2004). Other synapses in the cerebellum thought to be important for motor learning are the input stage of the cerebellar cortex at the mossy fiber (MF) to granule cell (GC) synapses (D'Angelo et al. 2004) and the MF to deep cerebellar nuclei synapses of which synaptic plasticity was also long theorized but only recently experimentally shown (Medina and Mauk 2000; Zhang and Linden 2006; Pugh and Raman 2009). Furthermore, synaptic inputs to interneurons, like the PF to stellate cell synapses in the molecular layer (ML), have shown to be plastic (Jörntell and Ekerot 2002; Rancillac and Crépel...
We chose to study whether the PF to Golgi cell synapse shows long-term plastic changes.

Golgi cells (GoCs) are the main inhibitory interneurons of the granule cell layer (GCL), gating the activity of as many as 100 billion granule cells. They typically have a large soma emitting a series of basolateral dendrites in the GCL and 3-4 long and thin apical dendrites, going into the ML where they branch. In the GCL, GoCs form a large ramified axonal plexus, allowing each cell to contact several thousands GCs (D'Angelo 2008). The central role of the GoC in the cerebellar cortex is exemplified by the extensive interconnections of the cell (Geurts et al. 2003; Watanabe et al. 1998). GoCs receive excitatory inputs from MFs, which form synapses on their basolateral dendrites, from PFs in the molecular layer (in the order of 5000 inputs per cell) (Pellionisz and Szentágothai 1973; Ito 2006), and possibly also from climbing fibers (Shinoda et al. 2000). GoCs also receive inhibitory inputs from stellate and basket cells in the ML and from Lugaro cells in the GCL (Palay and Chan-Palay 1974; Dieudonné and Dumoulin 2000). The main function of the GoCs is to tonically and phasically inhibit GCs by their GABAergic output (Rossi et al., 2003). Apart from GABA, GoCs co-release glycine at their synaptic terminals (Dugué et al. 2005). In vivo, GoCs have an ongoing irregular, low-frequency firing activity and react to afferent stimulation with a burst of action potentials followed by a pause (Vos et al. 1999; Tahon et al. 2005). In vitro, GoCs behave like regular pacemakers and show phase-reset and resonant activity in the “theta” frequency-band (i.e. 4-7 Hz; Forti et al. 2006; Solinas et al. 2007).
Several studies have shown that PF to GoC synapses have functional glutamate receptors of the AMPA-, NMDA-, and kainate-type (Dieudonné 1998; Misra et al. 2000; Bureau et al. 2000; Kanichay and Silver 2008). Their AMPA receptors (AMPA_R) are composed of GluR2 subunits, which are characterized by low Ca^{2+} permeability and linear I/V relationships (Menuz et al. 2008). The NMDA receptors (NMDA_R) contributing to the EPSCs evoked by stimulating the PFs have a high-conductance, arising from NR2B-containing receptors, and are present both on the synaptic and extrasynaptic membrane. Low-conductance components arising from NR2D-containing receptors are extrasynaptically localized and do not contribute to the EPSC (Misra et al. 2000). Kainate receptors (KA_R) have small amplitudes and slow kinetics, and summate in response to presynaptic tetanic stimulation, thus allowing the GoC to integrate excitatory inputs at different time scales (Bureau et al. 2000). Metabotropic glutamate receptors (mGlu_R) are also expressed postsynaptically on PF-GoC synapses. Both group I (mGlu_R1 and 5) and group II (mGlu_R2 and 3) are expressed, but mGlu_R2 are the most abundant (Watanabe and Nakanishi 1998) and serve as a histological marker of GoCs (Geurts et al. 2001). Activation of mGlu_R2 enhances an inward rectifier potassium current leading to a slow inhibitory postsynaptic current (IPSC) capable of silencing GoC activity following high-intensity PF input (Watanabe and Nakanishi 2003).

Long-term changes at GoC synapses have not been studied previously. A good candidate is the PF-GoC synapse since PF inputs are in general associated with activity-dependent plastic phenomena. Because of its central
position in the control of information transfer to Purkinje cells, a change in the synaptic input to the GoC, even if subtle, could have a significant effect on network activity in the GCL. Here we demonstrated that brief trains of high-frequency synaptic activation can induce LTD of the PF excitatory inputs to GoCs. This PF-GoC LTD is demonstrated using induction protocols (IPs) in voltage-clamp (V-clamp) and current-clamp (C-clamp). The PF-GoC LTD induced by a C-clamp IP is further pharmacologically analyzed.
Materials and methods

Slice preparation and electrophysiology

Parasagittal and coronal cerebellar slices were acutely prepared from Wistar rats (P15-22) as previously reported (Dugué et al. 2005) and in agreement with institutional, federal and European ethical guidelines and laws for animal experimentation. Briefly, the brain was quickly removed after decapitation and 250μm tissue slices were cut by a vibratome (VT 1000 S; Leica, Germany) in a ice-cold solution containing (in mM): 130 K-gluconate, 15 KCl, 0.5 EGTA, 20 HEPES, and 25 glucose, with pH adjusted to 7.4 by KOH (oxygenated with 100% O₂) (Dugué et al. 2005). Slices were then transferred to an incubation chamber, heated at 32ºC for at least 45 minutes, and later stored at room temperature. The artificial cerebrospinal fluid (ACSF) employed for slice incubation, storage, and for continuous perfusion during the electrophysiological recordings, contained (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 25 glucose, 2 CaCl₂, and 1 MgCl₂ (compensated with 95% O₂, 5% CO₂).

Recordings were carried out at room temperature from the soma of Golgi cells (GoCs), visually identified by infrared differential interference contrast (DIC) videomicroscopy. The identity of GoCs was routinely assessed using previously established criteria (Dieudonné 1995) and upon observation of excitatory synaptic currents (EPSCs) evoked by extracellular stimuli delivered in the molecular layer (ML) (Bureau et al. 2000; Beierlein et al. 2007). Additional morphological confirmation was obtained in a subset of cells (n =
155) filled with a low concentration (0.05%) of Lucifer Yellow and imaged by epifluorescence. Giga-seal patch-clamp recordings were obtained, under the whole-cell configuration, using 2-3 MΩ borosilicate-glass pipettes. Intracellular solution contained (in mM): 135 potassium gluconate, 10 KCl, 10 Hepes, 0.2 EGTA, 4 Mg-ATP, 0.4 Na₃GTP and either 10 Na₂-Phosphocreatine and 10 sucrose or 14 Na₂-Phosphocreatine, with pH 7.25-7.3 titrated with KOH. For experiments in which BAPTA was added to strongly buffer free-intracellular calcium, at increasing concentrations (i.e. 10, 20, and 40mM) the composition of the intracellular solution was adjusted by decreasing the concentration of potassium gluconate (i.e. 135, 90, and 35 mM, respectively). Voltage- and current-clamp recordings were performed with an EPC 10 amplifier (HEKA, Lambrecht, Germany). See Supplementary Materials and Figure S1 for description of the voltage-clamp methods and results on parasagittal slices. All results discussed here are from recordings in coronal slices. In coronal slices Cₘ was 149 ± 12 pF (range: 34-392 pF; n=36) and Rₘ was 290 ± 24 MΩ (range: 79-708 MΩ; n=36). These results are in close agreement with Forti et al. (2006). A theta-glass pipette (1401016, Hilgenberg, Malsfeld, Germany) filled with ACSF, and accommodating two platinum-wires, was employed to deliver bipolar extracellular electrical stimuli. This electrode was placed in the lower half of the ML, at lateral distances of 150-300μm. The stimulation artifacts were small. To monitor changes in synaptic efficacy, EPSCs were systematically evoked by paired-pulses stimuli with a 50 ms inter-pulse
interval and repeated at a frequency of 0.1 Hz. Current-controlled stimuli had amplitude and duration in the ranges 8-100μA and 100-200μs, respectively. The stimulation intensity was adjusted so that the equivalent EPSP had a peak value of 5-10 mV. GoCs are in general parasagittally organized (Sillitoe et al. 2008; Barmack and Yakhnitsa 2008). But it was much easier to evoke and obtain EPSCs in the coronal plane where responses up to 2 nA were not unusual. No more than one or two position adjustments of the stimulating electrode in the ML were necessary.

The LTD-induction protocol (IP) consisted of a high-frequency extracellular electrical stimulation (20 pulses at 100Hz), repeated 30 times with an interval of 2 seconds. For the C-clamp IP recordings the cells were held in current clamp during the IP, injecting negative current to hold the cell around -70 mV but allowing the cell to spike freely during the tetanic stimulation. For the V-clamp IP recordings the cells were held under voltage clamp at -70 mV during the IP.

**Data analysis and statistics**

Currents were filtered at 2 kHz, digitized at 5 kHz, and acquired using Pulse software (Heka, Lambrecht, Germany). Data were analyzed offline using custom-made macros written in Igor Pro (Wavemetrics, Lake Oswego, OR, USA). All group data are reported as mean ± SEM and compared statistically by using the Student’s t test. A significance of $p < 0.05$ was indicated by an asterisk, and a significance of $p < 0.01$ was indicated by two asterisks.
The paired-pulse ratios were calculated from the ratio of the second EPSCs over the first EPSCs of the baseline EPSCs, during the 10 minutes pre-IP and a stable recording period 25-35 minutes post-IP (60 data-points each). No corrective measure was applied to the PPR, as for example taking the mean of second EPSCs over the mean of the first EPSCs of a series of consecutive paired EPSCs to compensate for the increase in variability of the PPR post-IP in case of depression of the EPSCs (Kim and Alger 2001; Sims and Hartell 2005).

The main parameter measured to characterize the synaptic responses evoked by extracellular stimulation was the amplitude of the individual EPSCs, although other parameters like the time-to-peak, the slope of the rising-phase, and the decay-phase time constant were computed. An estimate of the charge transfer, corresponding to each EPSC, was also routinely computed by subtracting the time-integral of the steady-state current from the time-integral of total current during the EPSC.

**Pharmacology**

Inhibitory synaptic transmission was blocked by bath-applying a cocktail of 10μM of SR95531 (gabazine) and 1μM strychnine, a GABAA-receptor and glycine-receptor antagonist, respectively. D-(-)-2-amino-5-phosphono-pentanoic acid (D-APV), (S)-α-Methyl-4-carboxyphenylglycine ((S)-MCPG) and LY367385 (S)-(+) -Amino-4-carboxy-2-methylbenzeneacetic acid were also bath-applied to block NMDA-receptors, mGluR and mGluR1 respectively. LY341495 bis-(O-aminophenoxy)-N,N,N',N'-tetraacetic acid and KT5720...
(9R,10S,12S)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-
epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-
carboxylic acid, hexyl ester were prepared in DMSO as stock solutions and
used to specifically block mGluR2 receptors and protein kinase A (PKA)
respectively. Drug-application always followed the establishment of the whole-
cell configuration, preceded the LTD induction protocol and persisted
thereafter. All chemicals were obtained from Sigma-Aldrich, Ascent Scientific
or Tocris Cookson.
Results

Characterization of parallel fiber to Golgi cell synapses

Patch-clamp recordings were established at room temperature, under the whole-cell configuration, from the soma of visually identified putative GoCs in the GCL of cerebellar slices, prepared from 15-22 days old rats. In general, eliciting excitatory post-synaptic currents (EPSCs) by extracellular stimulation of the PFs further confirmed the identity of the cell recorded, complementing standard electrophysiological identification criteria (Dieudonné 1995; 1998). In addition, to isolate glutamatergic PSCs, gabazine (10µM) and strychnine (1 µM) were bath applied for the entire duration of the experiment. A schematic representation of the position of the recording and stimulating electrodes in the coronal slice is shown in Figure 1C.

Characteristic kinetic parameters of the evoked EPSCs are summarized in Table 1. A sample EPSC, average superposed on series of 20 consecutive EPSCs, is shown in Figure 1A. Paired-pulse stimuli with 50 ms inter-pulse interval were systematically used to evoke EPSCs, representative traces and their averages are shown in Figure 1B. Paired stimulation of PF-GoCs with relative short time intervals led to paired-pulse facilitation (PPF), implying that the release probability of the second EPSC is increased. When systematically characterized, the paired-pulse ratio (PPRs, i.e. the ratio of the second EPSC amplitude over the first EPSC of each pair) decreased exponentially (decay time constant 135 ± 16 ms) for increasing inter-pulse intervals, while leveling off at an average value of 2.45 for intervals smaller than 50 ms (Figure 1D, n
The variability of the responses also increased for shorter time intervals. From the traces in Figure 1D, we have an estimated PPR of 2.25 for a time interval of 50 ms between the paired EPSCs.

---FIGURE 1 ABOUT HERE---

Responses to trains of stimulations have previously been studied (Bureau et al. 2000; Kanichay and Silver 2008). A detailed study of short-term plasticity of the PF-GoC synapse was done by Beierlein et al. (2007), showing that short stimulus bursts had little overall effect on the strength of the PF-GoC synapse, GoCs do not release endocannabinoids to regulate their PF synapses, and that short-term post-tetanic potentiation (PTP) is absent beyond the 3rd pulse. When trains of stimulation pulses were delivered, both short-term facilitation and depression of PF-GoC EPSCs were apparent. Figures 1E and F report sample average responses to tetani of 10 pulses at 50Hz and of 20 pulses at 100Hz, respectively. From Figure 1F, the progressive depression of the EPSCs amplitude was particularly prominent at 100 Hz, where separable responses practically vanished by the end of the train.

---TABLE1 ABOUT HERE---
LTD of the parallel fiber to Golgi cell synapse

We performed extensive preliminary work to investigate the parameters required to induce synaptic plasticity at the PF-GoC synapse. In general we employed high-frequency stimulation burst pulses as the IP. This was based on having more consistent results using a high frequency IP as compared to using low-frequency IPs. More specifically we used high-frequency, quasi-physiological IPs similar to some IPs used for LTD of the PF-PC synapse where ‘bursts only’ IPs are used not necessarily combined with postsynaptic depolarization (Hartell 1996; Eilers et al. 1997). To further increase possible physiological relevancy, we performed the high-frequency IP in coronal slices under C-clamp of the postsynaptic cell allowing the cell to spike during the IP. Control experiments were done under V-clamp conditions in coronal slices (see further) and in parasagittal slices (Supplementary Material and Figure S1).

During the C-clamp IP current was applied to hold the membrane potential around -70 mV, although the cell was allowed to spike during the IP stimuli. Quite a regular response developed during the IP, with evoked spiking activity occurring during the first couple of bursts, followed by progressively decreasing postsynaptic and action potentials. By the end of the IP protocol, during the last 5 to 10 bursts, the extracellular pulses elicited practically no effect on the postsynaptic membrane. This is a result of a complete but transient presynaptic exhaustion of the PFs and/or of postsynaptic receptor desensitization. An example of the response of a cell to the repetitive tetanus is shown in Figure S2 (Supplementary Material).
Control experiments, where no IP was applied but EPSCs were continuously monitored at 0.1 Hz, showed a moderate decrease of the EPSC amplitude over the 50 min recording period (-9.9 ± 3.9% change at $t = 25-35$ min; $n=10$) (Figure 2C). No significant changes were seen for the PPR controls (Figure 2D). The C-clamp IP resulted generally in a progressive and sustained LTD of the amplitude of the evoked EPSCs (-26.5 ± 5.1% change at $t = 25-35$ min post-IP; $n = 19$: $p<0.01$ compared to the control group) (Figure 2C and F).

This depression was also reflected by changes in average slope and charge transfer, without any significant changes of kinetic parameters (Table 1). 15 out of 19 experiments showed a progressive and sustained depression following the IP (-34 ± 4% change at $t = 25-35$ min; $n=15$, Figure 2B), 3 showed no change and one resulted in clear potentiation of EPSCs amplitude. Out of the 15 experiments showing LTD, 8 showed a small and transient post-tetanic potentiation (PTP), 10-20 min post-IP, of the order of 10 to 30% compared to baseline. Average EPSCs waveforms, pre- and post-IP, are shown in Figure 2A.

Under the same experimental conditions, changes in the PPR were investigated as a potential landmark of alterations in presynaptic release probability. To check whether our recording setup can detect small changes in PPR at distal dendrites and thus be a reliable test for confirming the post-versus presynaptic locus of the plasticity, control experiments were carried out in which the extracellular Ca$^{2+}$ concentration was systematically lowered (see Supplementary Material and Figure S3). These indicate that because of the small extent of LTD any changes in PPR at electrotionically distant sites may
be difficult to detect. The C-clamp IP recordings were not associated with a
significant increase of the PPR (2.06 ± 0.12 at 10 min pre-IP versus 2.14 ±
0.15 at \( t = 25-35 \) min post-IP; \( n = 19; p=0.57 \)), suggesting a postsynaptic
origin of the LTD (Figure 2E).

To see whether spiking behavior during the C-clamp IP was necessary to
induce LTD we applied the same high-frequency IP while voltage clamping
the cell at -70 mV all through the IP. Passive cell parameters and EPSC
related parameters were comparable for the 2 experimental groups (Table 1).
The V-clamp IP results are shown in Figure 2C and F (n=7). They showed a
similar amount of depression as for the C-clamp IP series (-29.6 ± 4.7% 
change at \( t = 25-35 \) min post-IP; \( n = 7; p<0.01 \) compared to the control
group). Similar changes were seen for the slope of the rising phase and the
charge transfer, while kinetic parameters did not show any significant changes
(Table 1). A more pronounced PTP was observed in 4 of the 7 recordings and
is reflected in the summary plot and in the depression of the PPR right after
the IP. No significant changes of the PPR were observed (2.18 ± 0.17 at 10
min pre-IP versus 2.34 ± 0.22 at \( t = 25-35 \) min post-IP; \( n = 7; p=0.17 \); Figure
2E), suggesting for this IP also a postsynaptic origin of the LTD.

---FIGURE 2 ABOUT HERE---

Using similar IPs as in coronal slices we performed a number of experiments
in parasagittal slices and these data confirm that PF-GoC LTD can be evoked
in parasagittal slices (see Supplementary Materials and Figure S1).
Pharmacology of the LTD at the parallel fiber to Golgi cell synapse

Extensive pharmacological studies were done in coronal slices using the C-clamp IP to elucidate the mechanisms involved in LTD induction. As NMDA$_R$ are involved in many forms of synaptic plasticity and are expressed at PF-GoC synapses we tested whether these receptors are required for PF-GoC LTD. Bath applying 50 µM D-APV from the beginning of the experiment, had no significant effect on the PF-GoC LTD (Figure 3A and 4), compared to the results summarized in Figure 2C (-23.0 ± 7.6% EPSCs amplitude change at $t = 25-35$ min; $n = 10$; $p = 0.73$). Again no significant change was present in the PPR time course (1.93 ± 0.12 at 10 min pre-IP versus 2.13 ± 0.16 at $t = 25-35$ min post-IP; $n = 10$; $p=0.17$) (Figure 3A). Under these conditions, 6 out of 10 experiments showed LTD, while 3 showed no change and one experiment showed LTP. A transient PTP was observed in 7 out of 10 experiments. This was reflected in a depression of the PPRs, during the same 10 min post-IP period, possibly indicating a presynaptic origin of the PTP. Sample average EPSCs waveforms pre- and post-induction are shown in Figure 3A. No significant difference was observed, comparing these EPSCs with those evoked without NMDA$_R$ blockade (Table 1). When comparing the population plot of the V-clamp IP experiments (Figure 2C) with Figure 3A a similar time course is observed: a transient PTP over the first 10 minutes post-IP followed by a progression towards sustained depression at about 20 minutes post-IP. These results suggest that AMPA$_R$-mediated EPSCs at PF-GoC synapses undergo NMDA$_R$-independent LTD but that the extra depolarization caused by NMDA$_R$ activation may cause a faster induction of LTD.
Next, we considered the necessity of an increased intracellular free Ca\textsuperscript{2+} concentration for PF-GoC LTD. Indeed, spiking during C-clamp IP might result in postsynaptic Ca\textsuperscript{2+} transients by Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels, as well as Ca\textsuperscript{2+} release from internal stores. Entry through AMPA\textsubscript{R} and NMDA\textsubscript{R} could also be possible contributing factors. To test the Ca\textsuperscript{2+} requirement, we first employed an intracellular solution containing 10 mM BAPTA (tetraacetic acid). Since this had no effect on the LTD (-25 ± 11% change at \( t = 25-35 \) min; \( n = 4; p = 0.94 \) as compared to results C-clamp IP) (Figure 4), we tested a 20 mM BAPTA concentration for the intracellular solution (BAPTA tetrapotassium salt, with adjustment of the concentration of the potassium gluconate). Under these conditions, no effect on the LTD was observed (-39 ± 5% change at \( t = 25-35 \) min; \( n = 5; p = 0.18 \) as compared to results C-clamp IP) (Figure 4). Since GoC apical dendrites in the ML are very thin, one might expect difficult diffusion of BAPTA to the distant spine-like protrusions that form the postsynaptic part of the PF-GoC synapse and maybe higher concentrations of the chelating agent are necessary to see an effect on PF-GoC LTD. For this reason we added 40 mM BAPTA to the intracellular solution and waited longer than usual after establishing the whole-cell configuration, before applying the C-clamp IP (40 ± 4 min, \( n = 9 \), compared to a standard interval 24 ± 2 min, \( n = 19 \)). As in the previous experiments, no significant effect of this Ca\textsuperscript{2+} chelator on the LTD was observed (-19.5 ± 2.5% change at \( t = 25-35 \) min; \( n = 9; p = 0.23 \) as compared to results C-clamp IP and \( p = 0.01 \) as compared to controls) (Figure 3B and 4). The parameters of the EPSCs and passive cell parameters, recorded
under these conditions are summarized in Table 1. These results do not support an essential role for postsynaptic Ca\textsuperscript{2+} transients in PF-GoC LTD induction, although a partial effect is not excluded since LTD induction was slower and less pronounced with 40 mM BAPTA.

---FIGURE 3 ABOUT HERE---

Finally, mGluRs are known to be expressed postsynaptically at the PF-GoC synapses. Specifically, activation of mGluR\textsubscript{2} by glutamate gives rise to slow inhibitory postsynaptic potentials through the activation of GIRKs (Knoflach and Kemp 1998; Watanabe and Nakanishi 2003). The mGluR\textsubscript{2}-coupling GIRK is predominantly located at GoC dendrites. Since mGluR are in general important mediators for synaptic plasticity phenomena we investigated the effect of blocking these receptors on the PF-GoC LTD.

Because mGluR\textsubscript{1} is also expressed in GoCs, though to a lesser degree than mGluR\textsubscript{2}, we first did a series of experiments whereby an aspecific mGluR blocker (i.e. (S)-MCPG, 500 µM) was bath applied. This resulted in complete blockade of PF-GoC LTD (-4.8 ± 4.4% change at \( t = 25\text{-}35 \text{ min} \); \( n = 4 \); \( p<0.01 \) as compared to results C-clamp IP) (Figure 3C and 4). No significant change was observed in the PPR, although a rather pronounced but transient decrease in the ratio was recorded after the IP (2.30 ± 0.40 at 10 min pre-IP versus 1.77 ± 0.26 at \( t = 25\text{-}35 \text{ min post-IP; } n = 4; p=0.16 \)) (Figure 3C).

Next we studied the effect of treatment with a specific mGluR\textsubscript{2} blocker (LY341495, 1 µM) in DMSO as vehicle. In this series of experiments, PF-GoC
LTD was also completely abolished, confirming the results with the aspecific mGluR blocker and emphasizing the role of mGluR2 in PF-GoC LTD (+0.3 ± 9.5% change at t = 25-35 min; n = 7; p<0.05 as compared to results C-clamp IP) (Figure 3D and 4). In 6 out of 7 experiments, a PTP was observed and it was reflected in a depression of the PPRs during the same 10 min post-IP period. Average EPSCs waveforms pre- and post-induction are shown in Figure 3D. This lack of change was also reflected in the other EPSC parameters (Table 1). No significant change was observed in the PPR values (1.68 ± 0.06 at 10 min pre-IP versus 1.76 ± 0.14 at t = 25-35 min post-IP; n = 7; p=0.46) (Figure 3D). Application of the vehicle alone (0.25% DMSO) had no effect on PF-GoC LTD (-23 ± 7.1% change at t = 25-35 min; n = 7; p = 0.48 as compared to results C-clamp IP; Figure 4).

These experiments do not exclude a role of mGluR1 in the induction and/or expression of LTD. We next used the selective mGluR1 antagonist LY367385 at a concentration of 100 μM in the ACSF. No effect on LTD was observed (-36.0 ± 2.6% change at t = 25-35 min; n = 3; p=0.46 as compared to results C-clamp IP) (Figure 3E and 4). No significant change was observed in the PPR (1.75 ± 0.06 at 10 min pre-IP versus 1.93 ± 0.13 at t = 25-35 min post-IP; n = 3; p=0.26) (Figure 3E).

These data confirm an essential role for mGluR2 in the induction and/or expression of PF-GoC LTD. Puzzled by the fact that no clear Ca²⁺ dependency was demonstrated, although an effect on the induction of LTD is not excluded, we tested whether PKA might be a downstream mediator of the induction mechanism for LTD. PKA was chosen because this kinase can be
activated without a rise in intracellular Ca\textsuperscript{2+} concentration. As blocker we used the very selective PKA inhibitor, KT5720 dissolved in DMSO, at a concentration of 0.1 \(\mu\)M in the ACSF. In this series of experiments, PF-GoC LTD was also completely abolished (-0.3 ± 4.3\% change at \(t = 25-35\) min; \(n = 6; p<0.01\) as compared to results C-clamp IP) (Figure 3F and 4), suggesting a highly probable role for PKA in the biochemical cascade leading to LTD. In 5 out of 6 experiments, a PTP was observed and it was reflected in a depression of the PPRs during the same 10 min post-IP period. Average EPSCs waveforms pre- and post-induction are shown in Figure 3F. This lack of change was also reflected in the other EPSC parameters (Table 1). No significant change was observed in the PPR values (1.71 ± 0.2 at 10 min pre-IP versus 1.70 ± 0.11 at \(t = 25-35\) min post-IP; \(n = 6; p=0.91\)) (Figure 3F).

A summary of all pharmacology experiments is shown in Figure 4.

---FIGURE 4 ABOUT HERE---
Discussion

We have reported here for the first time that the PF-GoC synapse can undergo sustained LTD after a quasi-physiological, high-frequency synaptic burst stimulation. PF-GoC LTD was shown to be NMDAR-independent. A clear Ca\(^{2+}\) dependence could not be demonstrated. On the other hand, PF-GoC LTD showed a strong dependence on activation of mGlu\(_R\) and more specifically on mGlu\(_R2\), which are characteristic markers for GoCs. It is also shown that PKA acts as a mediator of PF-GoC LTD. The LTD is likely to be postsynaptically expressed because it was not associated with significant changes in the PPR and because mGlu\(_R2\) is only postsynaptically expressed.

The central finding of this work is that the PF-GoC synapse can undergo LTD using quasi-physiological IPs similar to those used in PF-PC LTD experiments. This was inspired by the work of Chadderton et al. (2004) showing that sensory stimulations produce short high frequency bursts of granule cell spikes and the current tendency to use quasi-physiological patterns of stimulation to induce synaptic plasticity in slices (Shin and Linden 2005; Pugh and Raman 2006; Zhang and Linden 2006). No important differences were observed between V-clamp IP and C-clamp IP based experiments.

We regularly observed a transient PTP after the IP, sometimes of quite large amplitude and lasting up to 20 min. This PTP was more pronounced for the V-clamp IP data than for the C-clamp IP data. It is not excluded that more Ca\(^{2+}\) enters the cell during C-clamp IPs since cells were allowed to spike. This could possibly lead to an earlier induction of LTD by itself or through the
mediation of other factors necessary for the induction. We cannot exclude, however, that there could be a washout of factors necessary for LTP of the PF-GoC synapse, as quite often described for plasticity in hippocampal interneurons (Kullmann and Lamsa 2007).

It is remarkable that protocols similar to IPs used for inducing PF-PC LTD (Sims and Hartell 2006; Coesmans et al. 2004), result in LTD of the PF-GoC synapse as well. In the same vein, recent in vivo work reported that conjunctive stimulation of peripheral afferents and climbing fibers causes depression of GoC firing activity (Xu and Edgley, 2008), but the same peripheral stimulation alone does not cause plasticity of activity. The locus of these plastic changes is unclear but is supposed to lie in the inhibitory input from ML interneurons to GoCs. This represents the first experimental indication of long-term activity changes of GoCs, and it adds to recent literature that the cerebellum is highly prone to plastic changes in vivo (Jörntell, 2008).

The control experiments done for the V-clamp and C-clamp IP experiments, showed a rundown of their activity (about -10% of baseline activity pre-IP) over the course of the 50 min duration of the experiments. This depression in activity was rather small and the differences with pre-IP values were not significant, both for the V-clamp and C-clamp controls. It is not excluded that the stimulation of PFs at a frequency of 0.1 Hz could lead to mild mGluR2 activation and thus be the cause of the moderate depression of activities. This hypothesis is supported by the fact that there is no rundown of the EPSCs in the mGluR blocking experiments.
Concerning the induction of PF-GoC LTD we showed that it is NMDA$_R$ independent. This is also reflected by the lack of effect of APV on the kinetic parameters of the evoked EPSCs. This independence of the PF-GoC LTD is in a way remarkable, since it is generally expected that if glutamergic synapses express GluR2-containing AMPA$_R$ they are not permeable to Ca$_{2+}$ and when depolarized they show strong activation of NMDA$_R$ (Kullmann and Lamsa 2007). This did not seem to be the case for Golgi cells because no clear differences were observed when comparing responses to tetanus stimulation with or without NMDA$_R$-blockers. It can of course not be excluded that the NMDA$_R$ conductance undergoes LTD with the same high-frequency IP. Cortical and hippocampal synapses, having a postsynaptic expression of mGlu$_R$2 and showing LTD, also show no dependence on NMDA$_R$ for LTD (Otani et al. 2002; Pöschel and Manahan-Vaughan 2005).

An important result of our work is the demonstration that PF-GoC LTD is mGlu$_R$ dependent, and more specifically mGlu$_R$2 dependent and mGlu$_R$1 independent. Postsynaptic mGlu$_R$2 endow the GoC with a capability to sense the strength of presynaptic GC inputs. Weak stimuli hardly activate mGlu$_R$2, while strong stimuli activate these receptors through spillover activation, causing a temporal inhibition of the GoCs. This permits the GoC to discriminate between weak and strong stimuli (Watanabe and Nakanishi 2003). This mechanism has consequences on the network level activity in the GCL since inhibition will relieve GoC-mediated feedback inhibition in a stimulus-dependent manner (Nakanishi 2009). The fact that blocking the mGlu$_R$2 in GoCs leads to relief of LTD of the PF-input adds to the important
role played by the mGluR2 with regard to the spatiotemporal regulation of the cerebellar circuitry.

The possible role of presynaptically expressed mGluR4 autoreceptors, which are the main group III mGluR on PFs, was not investigated because of the lack of a specific blocker (Abitbol et al. 2008; Niswender and Conn 2010). LY341495 at 1 μM has no blocking effect on mGluR4 (Kingston et al. 1998; Johnson et al. 1999; Wright et al. 2000). The specific LY341495 block of PF-GoC LTD makes the contribution of a presynaptic mechanism very unlikely because mGluR2 is only postsynaptically expressed and mGluR4 only presynaptically.

We convincingly show that PKA is involved with the biochemical pathways leading to PF-GoC LTD induction. Group II metabotropic glutamate receptors (mGluR2 and mGluR3) are known to couple to Gi/o proteins, which inhibit cAMP formation and Ca²⁺ channels at pre- and postsynaptic sites, and they are known to activate GIRK channels at postsynaptic sites (Bellone et al. 2008; Knoflach et al. 2001). Activation of mGluR2 can trigger a form of postsynaptically expressed LTD in different areas of the cortex and hippocampus. At Schaffer collaterals to CA1 synapses, LTD has been shown to be dependent on the simultaneous activation of mGluR2 and A1 adenosine receptors by inhibiting adenylate cyclase, leading to less cAMP and reducing PKA activity (Santschi et al. 2006). Less PKA activity promotes the dephosphorylation and endocytosis of AMPAR. This mechanism is thus a plausible biochemical mechanism for PF-GoC LTD since a postsynaptic increase in Ca²⁺ does not seem to be necessary.
While in Marr’s seminal theory (1969) it was suggested that only PF-PC synapses should be plastic, plasticity has now been demonstrated in a number of cerebellar synapses (Hansel et al. 2001). Recently there is a growing interest in the role of inhibitory interneurons and their relation to synaptic plasticity and circuit control. This is specifically true concerning the role of the GoC in cerebellar motor learning (Dugué et al. 2009; Prsa et al. 2009; Xu et al. 2008).

LTD of the PF input on GoCs can have significant effects on the cortical network activity of the cerebellum. GoCs play a central role in the information processing of the cerebellar cortex (Watanabe et al. 1998) by carefully regulating the silencing and the timing of feedback inhibition of GC activity (Vos et al. 1999; De Schutter and Bjaalie 2001; Tahon et al. 2005) and by probably generating regular synchronous oscillations over large GCL fields (Maex and De Schutter 1998; D'Angelo 2008). It is assumed that this oscillatory behavior plays a central role in precise timing of sensorimotor information (Isope et al. 2002) and cognition and memory consolidation by interacting with for example the long-term plasticity at the input stage of the cerebellar cortex (D'Angelo et al. 2009). Recent studies show that GoCs are extensively coupled through electrical synapses and display low-frequency oscillatory synchronization, imposing rhythmic inhibition onto GCs (Dugué et al. 2009). PF-GoC LTD can be envisaged to have a pronounced effect on these oscillations and the timing of feedback inhibition.

PF synapses have a low initial probability of release of glutamate (leading to PPF) and can thus function as high-pass filters which allows the GoC easily to
detect bursts in presynaptic activity (Abbott and Regehr 2004; Beierlein et al. 2007). PF-GoC LTD induced after intense presynaptic stimulation could lead to a diminishment of inhibition at the input stage and could thus allow more activation of PCs in the corresponding cortical field on a longer timescale. We used a synchronous but dispersed stimulation IP, which is a physiologically probable input pattern. Therefore, PF-GoC LTD can serve as an example of use-dependent changes evoked in a relative small number of synapses with possible consequences for the activity of an entire network of interconnected neurons.

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Figure legends

**Figure 1.** Properties of parallel fibers to Golgi cell EPSCs. A: representative (gray) and averaged traces (red) from a series of 20 consecutive EPSCs evoked by extracellular stimulation of PFs in coronal slices. B: in all experiments, EPSCs were evoked with a paired-pulse protocol (10-100 μA, 100-200 μs) with a 50 ms inter-pulse interval. Representative (gray) and averaged (red) traces from 20 consecutive stimulations show paired-pulse facilitation. C: schematic diagram of representative positions of the stimulating and recording electrode in the coronal slice plane. D: on the average, paired pulse ratios (PPRs) were strongly dependent on the inter-pulse intervals (n = 9; 20 PPRs per data-point, with error-bars indicating SEM). E-F: response to tetanic stimulation, averaged over 20 responses, for stimulation at 50 Hz (E) and at 100 Hz (F), the latter was employed to induce long-term depression of EPSCs.

**Figure 2.** Long-term depression of the parallel fibers to Golgi cells synaptic responses. A: representative averaged traces of evoked EPSCs waveforms 5 min pre-IP and 30 min post-IP, time points indicated by open bars in (B). B: sample experiment showing LTD of EPSCs amplitude evoked by the high-frequency burst IP under C-clamp. C: population summary showing the time-course of the normalized EPSCs amplitude, comparing control conditions (n=10) with the LTD induced by the high-frequency burst IP under C-clamp (n=19) and under V-clamp mode (n=7). The arrow at time point 0 indicates the occurrence of the 1 min long IPs. D: population summary of the time course of
the PPR for the control condition (n=10). E: population summary of the time
course of the PPR for C-clamp IP and V-clamp IP. F: box plot shows that for
both C-clamp IP and V-clamp IP protocols the percentage changes of EPSC
amplitude averaged over 25 to 35 min post-IP are significantly different from
controls (**p<0.01).

Figure 3. Pharmacology of long-term depression at the parallel fibers to Golgi
cells synapses. A-F: Top: representative traces of paired EPSCs. Traces
shown are the averages of 30 consecutive responses taken from time points
indicated by an open bar before and after IP and centered respectively around
-5 min pre-IP and 30 min post-IP. Center: time course of the normalized
evoked EPSC amplitude under test conditions versus controls (n = 10) and C-
clamp IP LTD (n = 19). The data for each cell were normalized to the mean
value of the EPSC amplitudes 10 min prior to the IP. The arrow at time point 0
indicates the start of the IP lasting one minute. Bottom: time course of the
PPR under test conditions. A: LTD induced by the C-clamp IP was not
blocked in the presence of 50 μM D-APV (n = 10; p = 0.73). No significant
changes in PPR occurred. B: LTD induced by the C-clamp IP was not blocked
in GoCs loaded with 40 mM BAPTA (n = 9; p = 0.21), although LTD is clearly
slower to develop and the amplitude is smaller than usual. No significant
changes in PPR occurred. C: LTD induced by the C-clamp IP was completely
blocked in the presence of 500 μM (S)-MCPG (n = 4; p<0.01). No significant
changes in PPR occurred. D: LTD induced by the C-clamp IP was completely
blocked in the presence of 1 μM LY341495 with DMSO as vehicle (n = 7; p
<0.05). No significant changes in PPR occurred. E: LTD induced by the C-clamp IP was not blocked in the presence of 100 μM LY367385 (n = 3; p=0.46). No significant changes in PPR occurred. F: LTD induced by the C-clamp IP was completely blocked in the presence of 0.1 μM KT5720 with DMSO used as vehicle (n = 7; p <0.05). No significant changes in PPR occurred.

Figure 4. Box plot summary of all recordings showing the percentage change in EPSC amplitude. Summary showing the percentage change in EPSC amplitude averaged over time 25 to 35 min post-IP, centered around 30 min post-IP for the conditions indicated. *p<0.05 and **p<0.01 as compared to C-clamp IP LTD.
Table 1. Passive cell parameters and several EPSC parameters, shown per experimental group and pre- versus post- induction protocol. Data are expressed as mean ± sem. Pre-IP means the baseline activity starting 10 min before start IP. Post-IP means the activity 25-35 min post-IP. p-values for statistical significance have been obtained by applying paired t-tests between values of the same parameter pre- versus post-IP.
Controls
C-clamp IP
V-clamp IP
NMDAR block
Aspecific mGluR block
mGluRI block
mGluRII block
PKA inhibition
DMSO controls
10 mM BAPTA
20 mM BAPTA
40 mM BAPTA

EPSC amplitude (% change)
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<th>NMDAR block</th>
<th>40 mM BAPTA</th>
<th>Aspecific mGluR block</th>
<th>mGluRII block</th>
<th>mGluRI block</th>
<th>PKA block</th>
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<td>(n = 19)</td>
<td>(n = 7)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
<td>(n = 4)</td>
<td>(n = 7)</td>
<td>(n = 7)</td>
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<td>Rinput (Mohm)</td>
<td>271 ± 37</td>
<td>322 ± 52</td>
<td>286 ± 38</td>
<td>255 ± 32</td>
<td>301 ± 61</td>
<td>320 ± 50</td>
<td>573 ± 210</td>
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<td>Cm (pF)</td>
<td>131 ± 13</td>
<td>142 ± 46</td>
<td>152 ± 17</td>
<td>96 ± 30</td>
<td>123 ± 25</td>
<td>141 ± 22</td>
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<td>126 ± 22</td>
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<td>pre-IP EPSCs (pA)</td>
<td>243 ± 24</td>
<td>362 ± 51</td>
<td>277 ± 35</td>
<td>263 ± 27</td>
<td>300 ± 104</td>
<td>308 ± 23</td>
<td>279 ± 16</td>
<td>372 ± 40</td>
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<tr>
<td>post-IP</td>
<td>185 ± 22</td>
<td>253 ± 41</td>
<td>210 ± 36</td>
<td>210 ± 23</td>
<td>284 ± 82</td>
<td>308 ± 39</td>
<td>178 ± 6</td>
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<td>&lt; 0.01</td>
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<tr>
<td>pre-IP</td>
<td>2.06 ± 0.12</td>
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<td>1.75 ± 0.06</td>
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<td>2.12 ± 0.16</td>
<td>1.88 ± 0.07</td>
<td>1.81 ± 0.26</td>
<td>1.75 ± 0.14</td>
<td>1.93 ± 0.13</td>
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<td>pre-IP</td>
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