Long-Term Depression at the Mossy Fiber–Deep Cerebellar Nucleus Synapse

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Several lines of evidence have indicated that the deep cerebellar nuclei (DCN) are a site of memory storage for certain forms of motor learning, most notably associative eyelid conditioning. In particular, these experiments, together with network models, have implicated the excitatory glutamatergic synapse between mossy fibers and DCN neurons in this memory trace. However, to date, evidence for persistent use-dependent change in the strength of this synapse has been almost entirely absent. Here, we report that high-frequency burst stimulation of mossy fibers, either alone or paired with postsynaptic depolarization, gives rise to long-term depression (LTD) of the mossy fiber–DCN synapse. This form of LTD is not associated with changes in the paired-pulse ratio and is blocked by loading with a postsynaptic Ca^{2+} chelator but not by bath application of an NMDA receptor antagonist. Mossy fiber–DCN LTD requires activation of a group I metabotropic glutamate receptor (mGluR) and protein translation. Unlike mGluR/translation-dependent LTD in other brain regions, this form of LTD requires mGluR1 and is mGluR5 independent.

Key words: mGluR1; mGluR5; slow EPSC; synaptic plasticity; protein synthesis; burst detection

Introduction

The cerebellum plays a major role in motor coordination, as well as certain forms of motor learning. The neurons of the deep cerebellar nuclei (DCN) comprise the main output stage of the cerebellum through their projections to premotor centers and the inferior olive. The DCN neurons receive GABAergic inhibitory drive from Purkinje cells in the cerebellar cortex and glutamatergic excitatory drive from mossy fiber collaterals (which also innervate granule cells) and climbing fiber collaterals (which also innervate Purkinje cells).

In the cerebellum, learning and memory can be studied at the level of neural circuits. For example, in associative eyelid conditioning, certain mossy fibers convey the conditioned stimulus (CS) and certain climbing fibers convey the unconditioned stimulus (US) (Kim and Thompson, 1997). From theoretical considerations, the engram must develop at sites in which the information about the US and CS converge. Two cerebellar sites satisfy this criterion, the DCN and the Purkinje cells. Previous lesion and inactivation studies have suggested that the cerebellar cortex learns to modulate the timing and amplitude of conditioned response (CR), whereas the DCN is essential for the performance of the CR (Medina et al., 2000).

Accumulating evidence has implicated the DCN in the memory acquisition and storage of associative eyeblink conditioning (Lavond, 2002). Lesions, reversible inactivation, and local blockade

of protein synthesis in the DCN (particularly the interposed nucleus) can prevent acquisition of conditioned eyelid response when applied before training and can eliminate the memory for training when applied afterward (Clark et al., 1992; Krupa et al., 1993; Bracha et al., 1998). Furthermore, extracellular recordings made from behaving rabbits have shown that neuronal activity within the DCN is highly correlated with the development of the CR. As animals acquire the CR, DCN neurons begin to fire strongly shortly before the onset of the US (McCormick and Thompson, 1984).

The persistent use-dependent changes in both synaptic efficacy [long-term potentiation (LTP) and long-term depression (LTD)] and intrinsic excitability of DCN neurons have been proposed as cellular mechanisms for DCN-dependent learning and memory. However, there have been few physiological studies on long-term plasticity in DCN. Our previous work has shown that the intrinsic excitability of the DCN can be modulated by high-frequency synaptic stimulation, which can be expressed as an increase in firing frequency or a change in firing pattern (Aizenman and Linden, 2000; Zhang et al., 2004). In addition, the GABAergic Purkinje cell–DCN synapse is capable of persistent bidirectional changes in synaptic efficacy (Morishita and Sastry, 1993; Aizenman et al., 1998). Last, an electron microscopy study in rabbits after undergoing associative eyeblink conditioning has shown an increase in the density of excitatory mossy fiber–DCN synapses (Kleim et al., 2002). Although behavioral and computational studies suggest that associative training may induce LTP at the mossy fiber–DCN synapse (Tracy et al., 1998; Medina et al., 2000), electrophysiological studies of plasticity at this synapse are almost nonexistent, the one exception being a brief report of LTP using field recording (Racine et al., 1986). Here, we sought to test the hypothesis that particular, behaviorally salient patterns of synaptic activation, most notably brief bursts, could give rise to plasticity at the mossy fiber–DCN synapse.

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Materials and Methods

Slice preparation. All experiments were performed in accordance with the guidelines approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine. Juvenile (12- to 14-d-old) Sprague Dawley rats were killed by decapitation, and the cerebellum was quickly removed and cooled with ice-cold modified artificial CSF (ACSF) containing the following (in mM): 120 choline-Cl, 2.5 KCl, 1.2 NaH₂PO₄, 25 NaHCO₃, 1.0 CaCl₂, 7.0 MgCl₂, 2.4 Na pyruvate, 1.3 Na ascorbate, and 20 D-glucose (oxygenated with 95% O₂/5% CO₂). Coronal slices of the cerebellum (250 μ m) were prepared using a vibrating tissue slicer (VT1000S; Leica, Nussloch, Germany). After cutting, slices were recovered in a submerged chamber containing oxygenated normal ACSF at 35°C for 30 min and further incubated at room temperature for at least 1 h before they were used for recording. The normal ACSF contained the following (in mM): 124 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 26.2 NaHCO₃, 1 NaH₂PO₄, and 11 D-glucose (equilibrated with 95% O₂/5% CO₂). During recording, slices were placed in a submerged chamber and continuously perfused at 30°C with normal ACSF at 3 ml/min. Inhibitory synaptic transmission was blocked by adding 200 μ M picrotoxin and 20 μ M SR95531 [6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid hydrobromide], GABA_A receptor antagonists, and 1 μ M strychnine, a glycine receptor antagonist in the normal ACSF.

Whole-cell patch-clamp recordings and data analysis. Whole-cell patch-clamp recordings were made from DCN neurons located in the interpositus nuclei. The position of the interpositus nuclei was identified in the slices using a 5 $\times$ objective mounted on an upright microscope (Axioskop 2 FS; Zeiss, Oberkochen, Germany), and DCN neuron somata were then visualized through a 40 $\times$ water immersion objective using infrared differential interference contrast optics. In the present study, recordings were made from large neurons with somatic diameter larger than 20 μ m, most of which are thought to correspond to the glutamatergic projection neurons of the cerebellar nuclei (Batini et al., 1992; De Zeeuw and Berrebi, 1995; Aizenman et al., 2003). It is possible, however, that a small number of our recordings were from the largest of the DCN GABAergic inhibitory projection neurons.

The recording electrodes (resistance, 3–5 M Ω) were typically filled with a solution containing the following (in mM): 122 CsOH, 122 D-gluconic acid, 10 HEPES, 0.3 EGTA, 4 Na₂ATP, 0.4 Na₃GTP, 5 QX314-Cl [2(triethylamino)-*N*-(2,6-dimethylphenyl) acetamine chloride], 2 MgCl₂, and 10 phosphocreatine, pH 7.3. For experiments in which bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetate (BAPTA) was added to reduce rises in [Ca²⁺]_i, the solution contained the following (in mM): 44 CsOH, 44 gluconic acid, 10 HEPES, 5 QX314-Cl, 2 MgCl₂, 4 Na₂ATP, 0.4 Na₃GTP, 10 phosphocreatine, 4 CaCl₂, 40 Cs₄-BAPTA or 29 CsOH, 29 gluconic acid, 10 HEPES, 5 QX314-Cl, 2 MgCl₂, 4 Na₂ATP, 0.4 Na₃GTP, 10 phosphocreatine, 14 CaCl₂, and 40 Cs₄-BAPTA, pH 7.3. Whole-cell voltage-clamp recording was performed with an Axopatch 200B amplifier (Molecular Devices, Palo Alto, CA). Holding potential was -70 mV unless otherwise indicated. Currents were filtered at 2 kHz, digitized at 5 kHz, and acquired using pClamp9 software (Molecular Devices). Series resistance and input resistance were monitored using a 2.5 mV hyperpolarizing voltage step throughout the experiments, and recordings were discontinued if changes in series resistance or input resistance were larger than 20%. Data were analyzed offline using pClamp9 software (Molecular Devices) and Igor Pro software (WaveMetrics, Portland, OR). For extracellular stimulation, a monopolar glass electrode filled with ACSF was placed in the white matter adjacent to the nuclei. To monitor changes in synaptic efficacy, EPSCs were evoked by using paired pulses (8–16 μ A, 100 μ s) with a 100 ms interval at a frequency of 0.05 Hz, and EPSC amplitudes were measured at the peak. The stimulation intensity was adjusted so that the EPSC amplitudes were in the range of 50–250 pA. Only recordings in which the EPSCs showed paired-pulse depression (PPD) were selected for the present study.

To induce LTD, DCN neurons were voltage clamped at -70 mV, and high-frequency synaptic stimulation (10 pulses at 100 Hz) was applied 20 times at 0.5 Hz. In most cases, each burst was paired with a 200-ms-long depolarization of the DCN neuron to -5 mV. During the LTD induction, the stimulation intensity was increased by increasing the pulse

width to 300 μ s. In the experiments to characterize the mechanisms of LTD induction (see Figs. 4–7), all drugs were added at the beginning of the experiment and remained in the perfusate thereafter.

Slow EPSCs (sEPSCs) were evoked with excitatory synaptic bursts consisting of a train of 10 pulses (8–16 μ A, 300 μ s) applied at 100 Hz. The bursts were given 2 min apart. The sEPSC amplitudes were measured as a mean value over a manually set 500 ms [in the presence of DL-threo- β -benzyloxyaspartic acid (DL-TBOA)] or 200 ms (in the absence of DL-TBOA) time window around the peak to minimize the noise. The fast EPSC (fEPSC) charge transfer was determined by measuring the area of the 10 fEPSCs starting from the onset of the stimuli to 10 ms after the end of the stimuli. The sEPSC charge transfer was determined by measuring the sEPSC area starting from 10 ms after the end of the stimuli to the point at which the current returned to baseline.

All group data are reported as mean $\pm$ SEM and compared statistically by using 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.

Drugs. D-2-Amino-5-phosphonopentanoic acid (D-APV), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro-benzo(*f*)quinoxaline-7-sulfonamide disodium salt (NBQX-Na₂), DL-TBOA, 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP hydrochloride), 7-(hydroxyimino)cyclopropyl(b)chromen-1a-carboxylate ethyl ester (CPCCOEt), SR95531 hydrobromide, actinomycin D [2-amino-(*N,N*)-1-bis(hexadecahydro-6,13-diisopropyl-2,5,9-trimethyl-1,4,7,11,14-penta-oxo-1*H*-pyrrolo[2,1]-[1,4,7,10,13]oxatetraazacyclohexadec-10-yl)-4,6-dimethyl-3-oxo-3*H*-phenoxazine-1,9-dicarboxamide], and cycloheximide [4-(2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl)-2,6-piperidinedione] were purchased from Tocris Cookson (Ellisville, MO). Cs₄-BAPTA was purchased from Invitrogen (Carlsbad, CA). All other drugs were purchased from Sigma (St. Louis, MO).

Results

Basal properties of mossy fiber–DCN synapses

Previous studies have indicated that DCN neurons have functional glutamate receptors (GluRs) of both the AMPA and NMDA types (Audinat et al., 1992; Anchisi et al., 2001). The NMDA receptors (NMDA-Rs) mediating EPSCs on the DCN neurons have two components: a conventional Mg²⁺-sensitive voltage-dependent component and a component with low Mg²⁺ sensitivity, weak voltage dependence and relatively fast kinetics (Anchisi et al., 2001). The component with low Mg²⁺ sensitivity is thought to be mediated by NR2D subunit-containing NMDA receptors (Akazawa et al., 1994; Anchisi et al., 2001). Unlike most other glutamatergic excitatory synapses in the brain, in DCN neurons, both AMPA receptors (AMPA-Rs) and NMDA receptors contribute to the excitatory synaptic signals at resting membrane potential in the presence of physiological concentrations of Mg²⁺ (Audinat et al., 1992; Aizenman and Linden, 2000; Anchisi et al., 2001).

Visualized whole-cell patch-clamp recordings were made from large DCN neurons (somatic diameter, >20 μ m) located in the interpositus nuclei in cerebellar slices prepared from juvenile (12- to 14-d-old) rats. The stimulating electrode was placed in the white matter adjacent to the nuclei. To isolate glutamate-evoked currents, slices were bathed in normal ACSF supplemented with 200 μ M picrotoxin and 20 μ M SR95531 to block GABA_A receptors and 1 μ M strychnine to block glycine receptors. Climbing fiber–DCN synapses are thought to be sparse at postnatal day 12 (P12) to P14 (Chan-Palay, 1977; Nicholson and Freeman, 2004). Hence, we believe that the evoked EPSCs examined in the present study were mainly mediated by mossy fibers, although we cannot rule out the possibility that a small number of climbing fiber collaterals were also activated. EPSCs were evoked by a paired test pulse with a 100 ms interpulse interval. At the mossy fiber–DCN synapse, the EPSCs showed PPD, suggesting that the mossy fibers have a high release probability (Fig. 1*A*). At -70 mV holding potential, the EPSC was partially blocked in the presence of 20 μ M

Figure 1. EPSCs evoked by paired and burst stimulation at the mossy fiber–DCN synapse. **A**, In these recordings, $200\ \mu\text{M}$ picrotoxin, $20\ \mu\text{M}$ SR95531, and $1\ \mu\text{M}$ strychnine were applied to block synaptic inhibition, and a command potential of $-70\ \text{mV}$ was applied. EPSCs were evoked by a paired-pulse stimulation with a $100\ \text{ms}$ interpulse interval in control conditions (black). EPSCs were partially blocked in the presence of $20\ \mu\text{M}$ NBQX (red) and completely blocked in the presence of $50\ \mu\text{M}$ D-APV and $20\ \mu\text{M}$ NBQX (blue). Traces shown are the average of six consecutive responses. **B**, EPSCs evoked by a stimulus consisting of 10 pulses at $100\ \text{Hz}$ have two components: an sEPSC (top black trace) and a series of fEPSCs (bottom black trace). In the presence of $50\ \mu\text{M}$ D-APV, both the sEPSC (top red trace) and the fEPSCs (bottom red trace) were partially blocked. In the presence of $50\ \mu\text{M}$ D-APV and $20\ \mu\text{M}$ NBQX, the fEPSCs were completely blocked (bottom blue trace) but not the sEPSC (top blue trace). DL-TBOA at $50\ \mu\text{M}$, a glutamate transporter blocker, potentiated the sEPSC (top gray trace). Traces shown are the average of three consecutive responses.

NBQX, an AMPA-R antagonist, and additional application of $50\ \mu\text{M}$ D-APV, a specific NMDA receptor antagonist, completely blocked the residual EPSC (Fig. 1A). The charge transfers of AMPA-R EPSC and NMDA-R EPSC were -0.77 ± 0.05 and $-0.28 \pm 0.07\ \text{pC}$, respectively ($n = 4$). Consistent with a previous report, we found at this synapse that the NMDA-R EPSC had a relatively fast onset kinetics as by the time-to-peak (AMPA-R EPSC, $1.4 \pm 0.1\ \text{ms}$; NMDA-R EPSC, $5.0 \pm 0.5\ \text{ms}$; $n = 4$) (Anchisi et al., 2001). These results indicate that both AMPA and NMDA receptors contribute to the EPSC at resting membrane potential in the presence of physiological concentrations of Mg^{2+} at the mossy fiber–DCN synapse (Audinat et al., 1992; Aizenman and Linden, 2000; Anchisi et al., 2001).

When high-frequency burst stimulation, consisting of 10 pulses at $100\ \text{Hz}$, was applied, the resultant EPSCs had two components: a decrementing series of fEPSCs, followed by an sEPSC (Fig. 1B). In the presence of $50\ \mu\text{M}$ D-APV, both the fEPSCs and the sEPSC were partially blocked, as indicated by a decrease in charge transfer to $63 \pm 2\%$ of control for the 10 fEPSCs and to $68 \pm 8\%$ of baseline for the sEPSC ($n = 5$). Additional application of $20\ \mu\text{M}$ NBQX completely blocked the fEPSCs (the charge transfer was reduced to $-1 \pm 0\%$ of control). NBQX treatment had little effect on the sEPSC because the charge transfer showed only a slight additional reduction, to $59 \pm 12\%$ of control ($n = 5$). The AMPA-R- and NMDA-R-independent sEPSCs had a small amplitude ($-11.6 \pm 1.6\ \text{pA}$; $n = 5$), even when strong synaptic stimuli that can evoke large first fEPSCs ($-708.0 \pm 69.9\ \text{pA}$; $n = 5$) were applied. The sEPSC had a time-to-peak of $0.6 \pm 0.0\ \text{s}$ and a 50% decay time of $1.9 \pm 0.3\ \text{s}$ ($n = 5$). These characteristics of the sEPSC are approximately similar to that of metabotropic GluR1 (mGluR1)-mediated slow EPSCs that have been found in other brain regions (Tempia et al., 1998; Dzubay and Otis, 2002; Huang et al., 2004). Activation of perisynaptically localized mGluRs requires glutamate “spillover” from the synaptic cleft, which is controlled by glutamate transporters. Previously, it has been shown that inhibiting glutamate uptake using glutamate transporter antagonists can enhance mGluR1-mediated sEPSCs (Brasnjic and Otis, 2001; Huang et al., 2004). The sEPSCs in DCN neurons were strongly enhanced by bath-applied $50\ \mu\text{M}$ DL-TBOA, a glutamate transporter antagonist, as indicated by the dramatic increase in the charge transfer (control, $-55.5 \pm 15.8\ \text{pC}$; DL-TBOA, $-319.8 \pm 17.3\ \text{pC}$; $n = 4$) (Fig. 1B). These results indicate that the ionotropic glutamate receptor-independent sEPSC in DCN neurons is mediated by glutamate and is probably attributable to activation of perisynaptic group I mGluRs.

In situ hybridization and immunohistochemistry have shown that both mGluR1 and mGluR5 are present in the DCN (Shigemoto et al., 1992; Romano et al., 1995). To date, throughout the brain, glutamatergic sEPSCs evoked by synaptic stimulation have been blocked by mGluR1 antagonists but not mGluR5 antagonists. However, mGluR5 antagonists have been reported to attenuate responses to exogenous application of combined mGluR1/5 agonists such as (RS)-3,5-dihydroxyphenylglycine (DHPG) (van Hooft et al., 2000; Gee et al., 2003; Rae and Irving, 2004). To test whether the sEPSC at the mossy fiber–DCN synapse is mediated by activation of group I mGluRs and to distinguish which subtype contributes to the sEPSCs, we evoked sEPSCs in the presence of ionotropic glutamate receptors antagonists ($50\ \mu\text{M}$ D-APV and $5\ \mu\text{M}$ NBQX) to minimize the contamination from the tail of AMPA-R or NMDA-R EPSCs (Fig. 1B). To obtain larger currents and evaluate the effects of the mGluR antagonists accurately, the sEPSCs were first enhanced by blocking glutamate transporters with $50\ \mu\text{M}$ DL-TBOA (control, $-13.4 \pm 2.7\ \text{pA}$; DL-TBOA,

-88.9 ± 17.8 pA; $n = 5$) (Fig. 2A). A similar strategy has been used previously to characterize mGluR1-mediated sEPSCs at the climbing fiber–Purkinje cell synapse (Dzubay and Otis, 2002). The enhanced sEPSC was not blocked by $2 \mu\text{M}$ MPEP, an mGluR5-specific antagonist, as indicated by the small decrease in the sEPSC peak to $92 \pm 2\%$ of control and a small decrease in the sEPSC charge transfer to $94 \pm 3\%$ ($n = 5$) of control. Additional application of $125 \mu\text{M}$ CPCCOEt, an mGluR1-specific antagonist, strongly blocked the sEPSC, as indicated by the substantial decrease in sEPSC peak to $16 \pm 1\%$ and sEPSC charge transfer to $10 \pm 2\%$ of control (Fig. 2). The sEPSC partially recovered after a 15–20 min washout of CPCCOEt, with the sEPSC peak and charge transfer recovering to 32 ± 2 and $34 \pm 5\%$ of control, respectively. These results indicate that the sEPSC in DCN neurons is dependent on the activation of mGluR1 but not mGluR5.

LTD at mossy fiber–DCN synapses

Synaptic plasticity at the mossy fiber–DCN synapse has been proposed as a cellular mechanism for DCN-dependent learning and memory, but electrophysiological evidence in support of this hypothesis remains scant. In investigating the parameters required to induce plasticity at this synapse, we tested several quasi-physiological patterns of stimulation and found that LTD can be reliably induced by mossy fiber synaptic burst stimulation. Test pulse pairs were delivered at a frequency of 0.05 Hz, and inhibitory synaptic transmission was blocked. The test pulses alone did not cause a change in EPSC response over the 35 min recording period ($4 \pm 3\%$ change at $t = 30$ – 35 min; $n = 6$) (Fig. 3). In a separate population of neurons, after a 5 min baseline period, a high-frequency synaptic stimulation consisting of 20 bursts of 10 pulses at 100 Hz paired with a postsynaptic depolarization consisting of a 200 ms voltage step to -5 mV was applied with an interburst frequency of 0.5 Hz. The onset of the synaptic stimuli and the postsynaptic depolarizing step were matched. This resulted in a fast, transient decrease followed by a slow, sustained decrease in the evoked EPSCs ($-27 \pm 4\%$ change at $t = 30$ – 35 min; $n = 13$; $p < 0.01$ compared with the control group) (Fig. 3). There was no correlation between the amplitude of the initial depression (measured at $t = 6$ min) and the amplitude of the long-term depression ($r^2 = 0.08$; $n = 13$; $p = 0.34$) measured at $t = 30$ – 35 min. Additional experiments indicated that synaptic bursts alone were sufficient to induce LTD ($-23 \pm 3\%$ change at $t = 30$ – 35 min; $n = 6$; $p < 0.01$) (Fig. 3). However, when the 20 postsynaptic depolarizing steps were applied alone, no LTD was observed ($0 \pm 3\%$ change at $t =$

Figure 2. The burst-evoked sEPSC requires activation of mGluR1 but not mGluR5. **A**, An sEPSC was evoked by a high-frequency stimulus consisting of 10 pulses at 100 Hz (black), with inhibitory synaptic transmission blocked by bath-applied $200 \mu\text{M}$ picrotoxin, $20 \mu\text{M}$ SR95531, and $1 \mu\text{M}$ strychnine and fast excitatory synaptic transmission blocked by bath-applied $50 \mu\text{M}$ D-APV and $5 \mu\text{M}$ NBQX. The sEPSC was enhanced by $50 \mu\text{M}$ DL-TBOA (red). In the presence of $50 \mu\text{M}$ DL-TBOA, the sEPSC was not blocked by $2 \mu\text{M}$ MPEP (blue) but was nearly completely blocked by additional application of $125 \mu\text{M}$ CPCCOEt (gray). The sEPSC partially recovered after a 15–20 min washout of CPCCOEt (green). Traces shown are the average of three consecutive responses. **B**, Summary graph showing the different sensitivity of sEPSC peak and sEPSC charge transfer to MPEP and CPCCOEt ($n = 5$). All of the data were normalized to the value in the presence of $50 \mu\text{M}$ DL-TBOA.

30 – 35 min; $n = 5$; $p = 0.41$) (Fig. 3), suggesting that postsynaptic Ca^{2+} influx through voltage-gated Ca^{2+} channels is not sufficient to induce mossy fiber–DCN LTD.

PPD is a form of short-term plasticity, and changes in the degree of PPD have been suggested to reflect presynaptic alterations in release probability. In this vein, it is worth noting that mossy fiber–DCN LTD was not associated with a sustained alteration of PPD in either induction condition (bursts, 0.74 ± 0.05 at $t = 0$ – 5 min, 0.75 ± 0.06 at $t = 30$ – 35 min; bursts paired with

Figure 3. LTD of the mossy fiber–DCN synapse is induced by synaptic burst stimulation. **A**, DCN neurons were voltage clamped at -70 mV, and EPSCs were evoked by paired test pulses with a 100 ms interpulse interval. After a 5 min baseline recording period, a high-frequency synaptic burst stimulation (10 pulses at 100 Hz) was applied 20 times at 0.5 Hz, with or without paired postsynaptic depolarization (from -70 to -5 mV for 200 ms). Representative traces of the EPSC evoked by the first test pulse from time points indicated by **a** (black) and **b** (gray), respectively, on the time course graph (**B**) are shown. Traces shown are the average of 15 consecutive responses. **B**, Time course of the normalized evoked EPSC amplitude under control conditions (open circles; $n = 6$), postsynaptic depolarization alone (filled triangles; $n = 5$), high-frequency synaptic burst stimulation alone (open triangles; $n = 6$), and high-frequency synaptic burst stimulation paired with postsynaptic depolarization (filled circles; $n = 13$). The data from each neuron were normalized to the mean value of the first 5 min of recording. The arrow indicates the time point at which the stimulation was given. **C**, Time course of the paired-pulse ratio (second EPSC/first EPSC) under different conditions. Paired-pulse depression ratio did not change under all conditions over 30 min of recording. **D**, Quantification of the percentage change in EPSC amplitude averaged over the last 5 min. ** $p < 0.01$ compared with control.

depolarization, 0.68 ± 0.05 at $t = 0-5$ min, 0.65 ± 0.03 at $t = 30-35$ min) (Fig. 3C). Although this suggests that the mossy fiber–DCN LTD is not presynaptically expressed, it does not comprise definitive evidence on this point.

In vivo, DCN neurons fire action potentials spontaneously even with intact tonic inhibition from Purkinje cells, and mossy fiber bursts are likely to evoke spiking in DCN neurons. Hence, the pairing protocol consisting of synaptic bursts paired with a depolarizing step serves to mimic the *in vivo* situation when mossy fiber stimuli causes a DCN neuron to fire action potentials, and this relatively more physiological pairing protocol was used for subsequent LTD mechanism studies.

Receptor activation requirements for mossy fiber–DCN LTD

It has been shown that activation of NMDA receptors is required for the induction of heterosynaptic LTP at the Purkinje cell–DCN synapse (Ouardouz and Sastry, 2000) and for a persistent increase in the intrinsic excitability of DCN neurons evoked by mossy fiber bursts (Aizenman and Linden, 2000). To test whether induction of mossy fiber–DCN LTD is also NMDA-R dependent, we added $50 \mu\text{M}$ D-APV to the perfusate starting from the beginning of the experiment. As shown in Figure 4, this treatment had no effect on the mossy fiber–DCN LTD induced by the pairing protocol ($-23 \pm 3\%$ change at $t = 30-35$ min; $n = 9$; $p = 0.42$ compared with control LTD). As described above, both AMPA receptors and NMDA receptors contribute to synaptic signals at resting membrane potential in DCN neurons; hence, this experiment differs from Figure 3 in that the evoked EPSCs were only mediated by AMPA receptors. The mossy fiber–DCN LTD of AMPA-R EPSCs was not associated with a significant change in PPD (0.66 ± 0.04 at $t = 0-5$ min; 0.73 ± 0.05 at $t = 30-35$ min) (Fig. 4C). These results show that AMPA-R EPSCs at mossy fiber–DCN synapse can undergo NMDA-R-independent LTD.

In addition to the NMDA receptors, group I mGluRs located perisynaptically can also serve as “burst detectors” and have been involved in LTP and LTD induction in other brain regions (Anwyl, 1999; Grassi and Pettorossi, 2001). One possibility is that induction of mossy fiber–DCN LTD requires activation of group I mGluRs. To test this hypothesis, we bath applied $125 \mu\text{M}$ CPCCOEt to block mGluR1 and $10 \mu\text{M}$ MPEP to block mGluR5. This manipulation resulted in a

Figure 4. LTD of the mossy fiber–DCN synapse does not require activation of NMDA-Rs. **A**, LTD induced by the pairing protocol was not blocked in the presence of bath-applied 50 μ M D-APV ($n = 9$). Representative traces of evoked EPSCs from the time points indicated by **a** (black) and **b** (gray), respectively, on **B**. **B**, Time course of the normalized evoked EPSC. Arrows indicate the time points at which the pairing protocol was applied. **C**, Time course of the paired-pulse ratio. **D**, Quantification of the percentage change in EPSC amplitude averaged over the last 5 min. The LTD induced by the pairing protocol with NMDA receptor blocked by 50 μ M D-APV was not statistically significant from control LTD.

complete blockade of mossy fiber–DCN LTD ($1 \pm 5\%$ change at $t = 30$ – 35 min; $n = 5$; $p < 0.01$ compared with control LTD) (Fig. 5). Application of the vehicle (0.145% DMSO) had no effect ($-37 \pm 6\%$ change at $t = 30$ – 35 min; $n = 5$; $p = 0.20$ compared with control LTD) (Fig. 5). No change of PPD was observed in either group (DMSO, 0.71 ± 0.05 at $t = 0$ – 5 min, 0.69 ± 0.03 at $t = 30$ – 35 min; CPCCOEt and MPEP, 0.83 ± 0.03 at $t = 0$ – 5 min, 0.79 ± 0.02 at $t = 30$ – 35 min) (Fig. 5C). These results indicate that group I mGluRs are involved in mossy fiber–DCN LTD. In subsequent experiments, pairing protocols were applied in the presence of either CPCCOEt alone or MPEP alone. We found that MPEP affected LTD in a concentration-dependent manner. When 10 μ M MPEP was applied, LTD was partially blocked ($-16 \pm 3\%$ change at $t = 30$ – 35 min; $n = 5$; $p = 0.03$ compared with control LTD). Considering that the IC_{50} of MPEP for mGluR5 is 36 nM (Gasparini et al., 1999), we reduced the MPEP concentration to 2 μ M to minimize its side effects without losing its capability to completely block mGluR5. In the presence of 2 μ M MPEP, the pairing protocol induced a slower but fully developed LTD ($-27 \pm 3\%$ change at $t = 30$ – 35 min; $n = 6$; $p = 0.96$ compared with control LTD) (Fig. 5), without significant

change in PPD (0.66 ± 0.05 at $t = 0$ – 5 min; 0.71 ± 0.05 at $t = 30$ – 35 min) (Fig. 5C), suggesting that the mossy fiber–DCN LTD is mGluR5 independent. However, when 125 μ M CPCCOEt was applied alone, LTD was almost completely abolished ($-6 \pm 5\%$ change at $t = 30$ – 35 min; $n = 5$; $p < 0.01$ compared with control LTD) (Fig. 5), again without significant change in PPD (0.76 ± 0.02 at $t = 0$ – 5 min; 0.74 ± 0.04 at $t = 30$ – 35 min) (Fig. 5C), indicating that induction of mossy fiber–DCN LTD is mGluR1 dependent.

Intracellular signaling requirements for mossy fiber–DCN LTD

Previous studies have shown that both synaptic plasticity (LTP and LTD) at the Purkinje cell–DCN synapse (Morishita and Sastry, 1996; Aizenman et al., 1998; Ouadouz and Sastry, 2000) and modification of intrinsic excitability of DCN neurons (Aizenman and Linden, 2000; Zhang et al., 2004) require postsynaptic Ca^{2+} influx, either through NMDA receptors or voltage-gated Ca^{2+} channels. In the present experiments, application of the pairing protocol may result in a postsynaptic Ca^{2+} transient by Ca^{2+} influx through voltage-gated Ca^{2+} channels, NMDA receptors and potentially mGluR1-coupled transient receptor potential (TRP) channels (Kim et al., 2003), as well as Ca^{2+} mobilization from internal stores by activation of mGluR1. To test the hypothesis that a postsynaptic Ca^{2+} transient is required for LTD induction, DCN neurons were loaded with 40 mM BAPTA, a Ca^{2+} chelator, applied in the pipette solution together with 14 mM Ca^{2+} to yield a predicted basal free Ca^{2+} concentration of ~ 125 nM. This treatment produced a strong blockade of LTD induction ($-5 \pm 4\%$ change at $t = 30$ – 35 min; $n = 6$; $p < 0.01$ compared with control LTD) (Fig. 6A,B,D) without a significant change in PPD (0.74 ± 0.04 at $t = 0$ – 5 min; 0.76 ± 0.05 at $t = 30$ – 35 min) (Fig. 6C). This result was confirmed using 40 mM BAPTA and 4 mM Ca^{2+} , predicted to yield a basal free Ca^{2+} concentration of ~ 25 nM ($-11 \pm 4\%$ change at $t = 30$ – 35 min; $n = 7$; $p < 0.01$ compared with control LTD) without a significant change in PPD (0.71 ± 0.03 at $t = 0$ – 5 min; 0.75 ± 0.05 at $t = 30$ – 35 min). These results suggest that a postsynaptic Ca^{2+} transient is necessary for mossy fiber–DCN LTD.

It has been reported that induction of mGluR5-dependent LTD in the hippocampal CA1 region required rapid dendritic protein synthesis (Huber et al., 2000). Because mGluR5 and mGluR1 have similar downstream signaling pathways, it is possible that activation of mGluR1 also triggers mRNA translation, which may be required for LTD expression. To test whether the mGluR1-dependent mossy fiber–DCN LTD is protein synthesis dependent, we preincubated the slices with 20 μ M anisomycin, an mRNA translation inhibitor, for at least 25 min before the pairing protocol was given. This treatment resulted in a nearly complete blockade of LTD ($-6 \pm 2\%$ change at $t = 30$ – 35 min; $n = 12$; $p < 0.01$ compared with control LTD) (Fig. 7). This result was confirmed using another mRNA translation inhibitor, 60 μ M cycloheximide ($4 \pm 5\%$ change at $t = 30$ – 35 min; $n = 6$; $p < 0.01$ compared with control LTD) (Fig. 7). However, in a separate population of neurons, when 25 μ M actinomycin D, a DNA transcription inhibitor, was applied, the induction of LTD was unaffected ($-25 \pm 3\%$ change at $t = 30$ – 35 min; $n = 5$; $p = 0.58$ compared with control LTD) (Fig. 7). No significant change in PPD was found in any case (anisomycin, 0.69 ± 0.03 at $t = 0$ – 5 min, 0.72 ± 0.03 at $t = 30$ – 35 min; cycloheximide, 0.74 ± 0.02 at $t = 0$ – 5 min, 0.73 ± 0.03 at $t = 30$ – 35 min; actinomycin D, 0.71 ± 0.03 at $t = 0$ – 5 min, 0.76 ± 0.02 at $t = 30$ – 35 min) (Fig. 7C). Additional experiments showed that the mGluR1-

Figure 5. LTD of the mossy fiber–DCN synapse requires activation of mGluR1 but not mGluR5. **A**, LTD induced by the pairing protocol was abolished in the presence of bath-applied 125 μ M CPCCOEt (an mGluR1 antagonist) or in the presence of 125 μ M CPCCOEt and 10 μ M MPEP (an mGluR5 antagonist) but not in the presence of 2 μ M MPEP alone or in the presence of vehicle alone (0.145% DMSO). Representative traces of evoked EPSCs under different conditions from time points indicated by **a** (black) and **b** (gray), respectively, on **B** are shown. **B**, Time course of the normalized evoked EPSC amplitude in the presence of 125 μ M CPCCOEt (filled triangles; $n = 5$), 125 μ M CPCCOEt and 10 μ M MPEP (open circles; $n = 5$), 2 μ M MPEP (open triangles; $n = 6$), and vehicle (filled circles; $n = 5$). The arrow indicates the time point at which the pairing protocol was applied. **C**, Time course of the paired-pulse ratio under conditions shown in **B**. **D**, Quantification of the percentage change in EPSC amplitude averaged over the last 5 min. $^{**}p < 0.01$ compared with control.

mediated sEPSC was not impaired in the presence of 20 μ M anisomycin (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), suggesting that anisomycin does not block mGluR1 activation itself. These results indicate that induction of mossy fiber–DCN LTD requires protein synthesis but not mRNA synthesis.

Discussion

The central finding of our study is that the mossy fiber–DCN synapse can undergo LTD after brief high-frequency synaptic burst stimulation. The mossy fiber–DCN LTD requires a postsynaptic Ca^{2+} transient, activation of group I mGluRs, and mRNA translation but not DNA transcription or NMDA receptor activation. Unlike mGluR/protein synthesis-dependent LTD in other brain regions, this form of LTD is dependent on mGluR1 but not mGluR5. Mossy fiber–DCN LTD is likely to be postsynaptically expressed because it is not associated with changes in PPD. Thus, synaptic plasticity at the mossy fiber–DCN synapses, long predicted by behavioral studies and cerebellar learning models (Tracy et al., 1998; Medina et al., 2000), has now been demonstrated and (at least initially) characterized.

There are several caveats that should be sounded in interpreting the present findings. First, for technical reasons, the present studies were performed using tissue derived from juvenile rats (P12–P14). Eyelid conditioning in rats has been reported to emerge at P17–P24, coincident with maturation of climbing fiber–DCN synapses (Nicholson and Freeman, 2004) and many other developmental events in cerebellum. As such, definitive statements about the relevance of mossy fiber–DCN LTD to eyelid conditioning must await confirmation in older tissue. It is possible that the polarity of synaptic plasticity is developmentally regulated. In the vestibular nuclei, a cognate structure of the DCN for the vestibulocerebellum, field potential recording showed that high-frequency stimulation induced an mGluR5-dependent LTD in juvenile rat but an NMDA-R- and mGluR1-dependent LTP in young adult rat (Puyal et al., 2003).

Second, to evoke EPSCs at the mossy fiber–DCN synapse, the stimulating pipette was placed in the white matter adjacent to the DCN. This region contains a mixture of fibers, including mossy fibers, climbing fibers, and inhibitory fibers from Purkinje cells, that latter of which were pharmacologically inhibited. The DCN also receive extrinsic modulatory fibers,

Figure 6. A postsynaptic Ca^{2+} transient is necessary for induction of LTD at the mossy fiber–DCN synapse. **A**, LTD induced by the pairing protocol was abolished in DCN neurons loaded with 40 mM BAPTA. Representative traces of the evoked EPSC from time points indicated by **a** (black) and **b** (gray), respectively, on **B** are shown. **B**, Time course of the normalized evoked EPSC amplitude ($n = 6$). The arrow indicates the time point at which the pairing protocol was applied. **C**, Time course of the paired-pulse ratio. **D**, Quantification of the percentage change in EPSC amplitude averaged over the last 5 min. $^{**}p < 0.01$ compared with control.

including those using the neurotransmitters acetylcholine (Woolf and Butcher, 1989), serotonin (Takeuchi et al., 1982), noradrenaline (norepinephrine) (Olson and Fuxe, 1971), and histamine (Panula et al., 1989). Therefore, it is also possible that release of these neuromodulators may contribute to the plasticity at this synapse.

Third, we did not perform an exhaustive search of the stimulation parameter space, and it is likely that this synapse can undergo bidirectional modification using other stimulation parameters, particularly those in which the DCN neuron was not voltage clamped. Indeed, another group has recently reported that LTP at the mossy fiber–DCN synapse may be evoked by high-frequency stimulation paired with delayed postsynaptic spiking (Pugh and Raman, 2006).

We found that the mossy fiber–DCN LTD is dependent on mGluR1 activation and protein synthesis but not mRNA synthesis. A similar mGluR/translation-dependent LTD has been found in hippocampus. At the hippocampal CA3–CA1 synapse, LTD induced by both chemical (bath application of the mGluR1/5 agonist DHPG) and synaptic (paired-pulse, low-frequency) stimulation was abolished when postsynaptic dendritic protein

synthesis was blocked by adding mRNA translation inhibitors in the external solution or loading the postsynaptic neuron with an mRNA cap analog (m7GpppG), a competitive mRNA translation inhibitor (Huber et al., 2000). Unlike the mGluR1/translation-dependent mossy fiber–DCN LTD, the LTD in hippocampal CA1 region is thought to be mediated by mGluR5. This is supported by the observations that the LTD is absent in the mGluR5 knockout mice (Huber et al., 2001) and can be blocked by selective mGluR5 antagonists but not mGluR1 antagonists (Faas et al., 2002; Hou and Klann, 2004) (but see Volk et al., 2006).

Mossy fiber–DCN LTD induction was blocked by postsynaptic application of a Ca^{2+} chelator, indicating a requirement for a postsynaptic Ca^{2+} transient. The observations that LTD may be induced under postsynaptic voltage clamp and in the presence of an NMDA-R antagonist suggests that Ca^{2+} influx from voltage-sensitive Ca^{2+} channels and NMDA-Rs is not required. Other potential Ca^{2+} sources are influx via mGluR1-coupled TRP channels (Kim et al., 2003) and Ca^{2+} release from internal stores.

The observation herein, that mossy fiber LTD is not associated with changes in PPD, argues against a presynaptic locus of expression. Our finding that mossy fiber–DCN LTD can be induced in the continuous presence of D-APV indicates that the AMPA receptor-mediated component of the EPSC can express LTD. However, additional experiments are required to clarify whether the NMDA receptor-mediated component of the mossy fiber EPSC is also affected by LTD induction. This has been suggested in other brain regions (O'Connor et al., 1995; Snyder et al., 2001).

The molecular mechanisms underlying mGluR/protein synthesis-dependent LTD are only partially understood, both at the mossy fiber–DCN synapse and in other brain regions. One hypothesis, which has come from experiments in hippocampal synapses, is that activating group I mGluRs and its downstream signaling pathways, particularly the extracellular signal-regulated kinase pathway and/or the phosphoinositide 3-kinase–Akt–mammalian target of rapamycin, can facilitate mRNA translation initiation, which produces new proteins required for the internalization of surface AMPA receptors (Snyder et al., 2001; Hou and Klann, 2004). It will be instructive to test this hypothesis at mossy fiber–DCN synapses in which it is possible to measure synaptically evoked mGluR1-mediated sEPSCs as a control measure for presumed downstream manipulations.

Previous anatomical studies have shown that DCN neurons can be divided into at least two and possibly three types. Large glutamatergic neurons project to the premotor nuclei, medium-to-large GABAergic neurons project to the inferior olive, and small GABAergic and/or glycinergic interneurons project their axons locally, although some have claimed that the local projections are collaterals of GABAergic axons that innervate the olive (Batini et al., 1992; De Zeeuw and Berrebi, 1995). In the present study, we performed whole-cell patch recordings from neurons with somatic diameter larger than 20 μm . Hence, the majority of neurons in the present study are presumably glutamatergic projection neurons (Batini et al., 1992; De Zeeuw and Berrebi, 1995; Aizenman et al., 2003). In addition to the glutamatergic projection neurons, it will also be interesting to investigate whether the mossy fiber synapses onto inhibitory neurons in the DCN can also express LTD or LTP.

It has now been >20 years since the DCN was proposed as a site of motor learning for associative eyelid conditioning (McCormick and Thompson, 1984). In this task, repeated pairings of the CS (usually a tone) and US (usually an air puff or mild periorbital shock) lead to gradual learning of the CR (a carefully timed eyelid closure). This process can be reversed by extinction training, in which a trained animal that has already learned the CR is retrained with the CS alone,

Figure 7. LTD of the mossy fiber–DCN synapse requires rapid protein synthesis. **A**, LTD induced by the pairing protocol was abolished in the presence of mRNA translation blockers 20 μ M anisomycin or 60 μ M cycloheximide but not 25 μ M actinomycin D (DNA transcription blocker). Representative traces of the evoked EPSC under different conditions from time points indicated by **a** (black) and **b** (gray), respectively, on **A** are shown. **B**, Time course of the normalized evoked EPSC amplitude in the presence of 20 μ M anisomycin (filled circles; $n = 12$), 60 μ M cycloheximide (open triangles; $n = 6$), or 25 μ M actinomycin D (open circles; $n = 5$). The arrow indicates the time point at which the pairing protocol was applied. **C**, Time course of the paired-pulse ratio under conditions shown in **B**. **D**, Quantification of the percentage change in EPSC amplitude averaged over the last 5 min. ** $p < 0.01$ compared with control.

leading to the active suppression of the CR. *In vivo* recording experiments indicate that the activity of DCN neurons is strongly correlated with the memory acquisition and extinction. The firing rate of the DCN neurons measured during the CS–US interval increased during CR acquisition and decreased to baseline during CR extinction (McCormick and Thompson, 1984). Computational studies suggest that an LTP at the mossy fiber–DCN synapse may contribute

to the increased CS-evoked firing rates in DCN neurons during acquisition (Medina et al., 2000). Similarly, an LTD at the same synapse may play a role in the decrease in DCN firing during extinction. It will be useful to examine whether natural patterns of activity during extinction training can produce mossy fiber LTD in brain slices from mature animals. Furthermore, as our understanding of the molecular mechanisms of this phenomenon grows, it will be useful to determine whether manipulations to selectively interfere with mossy fiber–DCN LTD will affect acquisition or extinction of eyelid conditioning and related forms of cerebellar motor learning.

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