Mechanisms of Potentiation of Mossy Fiber EPSCs in the Cerebellar Nuclei by Coincident Synaptic Excitation and Inhibition

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Neurons of the cerebellar nuclei receive synaptic excitation from cerebellar mossy fibers. Unlike in many principal neurons, coincident presynaptic activity and postsynaptic depolarization do not generate long-term potentiation at these synapses. Instead, EPSCs are potentiated by high-frequency trains of presynaptic activity applied with postsynaptic hyperpolarization, in patterns resembling mossy-fiber-mediated excitation and Purkinje-cell-mediated inhibition that are predicted to occur during delay eyelid conditioning. Here, we have used electrophysiology and Ca imaging to test how synaptic excitation and inhibition interact to generate long-lasting synaptic plasticity in nuclear cells in cerebellar slices. We find that the extent of plasticity varies with the relative timing of synaptic excitation and hyperpolarization. Potentiation is most effective when synaptic stimuli precede the postinhibitory rebound by ~ 400 ms, whereas with longer intervals, or with a reverse sequence, EPSCs tend to depress. When basal intracellular Ca is raised by spontaneous firing or reduced by voltage clamping at subthreshold potentials, potentiation is induced as long as the synaptic-rebound temporal sequence is maintained, suggesting that plasticity does not require Ca levels to exceed a threshold or attain a specific concentration. Although rebound and spike-dependent Ca influx are global, potentiation is synapse specific, and is disrupted by inhibitors of calcineurin or Ca-calmodulin-dependent protein kinase II, but not PKC. When IPSPs replace the hyperpolarizing step in the induction protocol, potentiation proceeds normally. These results lead us to propose that synaptic and inhibitory/rebound stimuli initiate separate processes, with local NMDA receptor-mediated Ca influx “priming” synapses, and Ca changes from the inhibition and rebound “triggering” potentiation at recently activated synapses.

Key words: deep cerebellar nuclei; interpositus; Purkinje cell; synaptic plasticity; eye blink; long-term potentiation; coincidence detection; non-Hebbian

Introduction

Neurons of the cerebellar nuclei, which receive excitatory synaptic input primarily from mossy fibers and inhibitory synaptic input from Purkinje cells, form the major output of the cerebellum. Consequently, most cerebellar learning must manifest itself as changes in the activity of cerebellar nuclear neurons. For instance, in delay eyelid conditioning, well timed increases in nuclear neuron activity allow trained animals to close their eyelids in response to initially neutral “conditioned” stimuli (carried by mossy fibers) that predict reflex-eliciting “unconditioned” stimuli (carried by climbing fibers), such as an air puff to the eye (McCormick and Thompson, 1984; Mauk et al., 1986; Steinmetz et al., 1989; Hesslow et al., 1999). Behavioral studies demonstrate that this learning process occurs in two stages, with synaptic

and/or excitability changes occurring first in Purkinje cells, and second in nuclear cells (Hesslow, 1994; Garcia and Mauk, 1998; Ohyama and Mauk, 2001; Ohyama et al., 2006). Moreover, modeling studies predict that, during acquisition of conditioned responses, Purkinje neurons generate instructive signals that regulate the strength of mossy fiber synapses onto nuclear cells, ultimately changing nuclear cell output to produce adaptive responses (Miles and Lisberger, 1981; Medina and Mauk, 1999).

This prediction, which requires nuclear cells to integrate inhibitory and excitatory stimuli, is supported by studies of synaptic plasticity in nuclear neurons in cerebellar slices (Pugh and Raman, 2006). This work indicates that EPSCs from mossy fiber afferents are potentiated by high-frequency trains of synaptic excitation only when they are combined with hyperpolarization of the postsynaptic nuclear neuron. Potentiation requires a Ca rise in the postsynaptic cell, activation of NMDA receptors, and concerted activation of Ca channels after the applied hyperpolarization, which produces the “rebound” depolarization (or rebound current) that typifies many nuclear neurons (Llinás and Mühlethaler, 1988; Aizenman and Linden, 1999). Although nuclear neurons generally fire spontaneously (Jahnsen, 1986a), potentiation does not require spiking and can occur under voltage clamp.

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The induction protocols in our previous study mimicked the predicted time course of excitation and inhibition during delay eyelid conditioning. The conditions were not maximally physiological, however, in that spontaneous firing was prevented, current injection replaced synaptic inhibition, and, in some experiments, cells were voltage clamped. Nevertheless, the fact that these protocols induced plasticity at synapses resistant to Hebbian conditioning (Aizenman and Linden 2000) suggests that they indeed converged on a physiological mechanism. This idea raises the question of how the key elements of the initial protocols might occur under physiological conditions to generate potentiation. Here, we have combined electrophysiology, Ca imaging, and pharmacology to assess the interactions between signals produced by synaptic excitation and by the hyperpolarization and subsequent rebound. The results suggest that potentiation of EPSCs can occur against a wide range of baseline Ca levels, providing evidence against an induction rule requiring dendritic Ca to reach a threshold concentration. The data instead lead to the hypothesis that excitatory synaptic stimulation provides a cellular signal that “primes” specific synapses, whereas the inhibition/rebound yields a distinct signal that “triggers” potentiation at primed synapses.

Materials and Methods

Preparation of cerebellar slices. In accordance with institutional guidelines, cerebellar slices containing the lateral and interpositus nuclei were prepared from 13 to 16-d-old C57BL/6 mice (Charles River Laboratories) as previously described (Telgkamp and Raman, 2002). Mice were deeply anesthetized with halothane and perfused with ice-cold (4°C) artificial CSF (ACSF) (in mM: 123.75 NaCl, 3.5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 1.5 CaCl₂, 1 MgCl₂, and 10 glucose, pH 7.4). After decapitation, the cerebellum was removed and placed in ice-cold ACSF. Parasagittal slices were cut using a Vibratome (model VT 100S; Leica) and incubated in warm (35°C) oxygenated (95% O₂/5% CO₂) ACSF for at least 1 h before recording.

Electrophysiological recording. Cerebellar slices were placed in the recording chamber and bathed in warmed (33–35°C) oxygenated ACSF containing the GABA_A receptor antagonist SR95531 (10 μM) (Tocris) unless otherwise noted. GABA_B receptor-mediated currents do not contribute significantly to postsynaptic currents in nuclear neurons (Pugh and Raman, 2005), even with high-frequency stimulation of Purkinje afferents (Morishita and Sastry, 1995; Mouginot and Gähwiler, 1995; Telgkamp et al., 2004). Large projection neurons of the cerebellar nuclei (soma 15–25 μm) were identified visually with infrared differential interference contrast microscopy. Patch pipettes were pulled from borosilicate glass to a resistance of 3–4.5 MΩ on a Sutter P97 puller (Sutter). Pipettes were filled with intracellular solution containing the following (in mM): 130 K-gluconate, 2 Na-gluconate, 6 NaCl, 2 MgCl₂, 14 Tris-creatine phosphate, 4 MgATP, 0.3 Tris-GTP, 1 EGTA, 10 HEPES, and 10 sucrose, buffered to pH 7.4 with KOH. Where indicated, calcineurin autoinhibitory fragment (EMD Chemicals), tacrolimus (FK-506; Sigma), Ca-calmodulin-dependent protein kinase II (CaMKII) fragment 290–309 (Sigma), or PKC inhibitor peptide 19–31 (EMD Chemicals) were added to the intracellular solution, and KN62 (Tocris) and FK-506 were added to the bath. Recordings were made with an Axopatch 200B amplifier (Axon Instruments). Data were filtered at 2 kHz and sampled at 50 kHz with pClamp acquisition software (Axon Instruments).

EPSCs were evoked by stimulating the white matter surrounding the cerebellar nuclei with 45 μs voltage pulses delivered through a concentric bipolar electrode (FHC). The stimulation is likely to activate primarily mossy fibers, which form the bulk of the excitatory synapses onto the proximal dendrites of nuclear cells, although inferior olivary collaterals also form synapses onto the more distal dendrites (Chan-Palay, 1977; Zhang and Linden, 2006; Pugh and Raman, 2006). Baseline EPSC amplitudes were measured at –65 mV by stimulating pairs of EPSCs separated by 50 ms once per 15 s for 5–10 min. Cells with unstable baselines were discarded. After recording baseline EPSCs, the amplifier was switched to

current clamp mode (except as noted) to allow the cell to fire normally during the application of the induction protocol. Holding current was applied as indicated. Induction protocols consisted of a 250 ms, 133 Hz train of excitatory synaptic stimuli paired with either a 150 ms hyperpolarizing current injection or, as noted, a 100-Hz train of inhibitory synaptic stimuli. This pattern of stimulation was repeated 30 times at 5-s intervals. After induction, EPSC amplitudes were again measured at –65 mV by stimulating pairs of EPSCs once per 15 s for 20–30 min. For all cells, the percentage change in EPSC amplitude was calculated by comparing the mean baseline amplitude to the mean of all EPSCs recorded after stabilization of the postinduction amplitude.

In experiments in which stimulation of IPSPs replaced hyperpolarizing current injections, SR95531 was omitted from the bathing solution and two stimulating electrodes were inserted into the white matter surrounding the cerebellar nuclei. One electrode was placed near the base of the cerebellar nuclei where mossy fibers entering the cerebellum are abundant and Purkinje axons are relatively sparse. The majority of responses evoked by stimulating this area are exclusively excitatory. This general observation was confirmed at the beginning of each experiment by measuring the evoked current at –35 mV, a voltage at which inhibitory currents, if they are present, are expected to dominate. If outward current was detected at –35 mV, the electrode was repositioned until the outward current was eliminated, or the cell was not analyzed further. EPSCs evoked by this electrode were used to monitor changes in synaptic strength before and after the induction protocol. A second stimulating electrode, placed in or near the corticonuclear tract, was used to elicit IPSPs during the induction protocol. This electrode generally evoked a mix of excitatory and inhibitory responses, but IPSPs dominated such that the net effect was to silence firing in the nuclear neuron.

Ca imaging. EGTA was omitted from the intracellular solution and 200 μM Oregon Green BAPTA 488-1 (Invitrogen) was applied through the patch pipette. Cells were imaged with an Andor EMCCD camera (model DV885KCS-VP, Andor Technology) and TILLvisION software (Till Photonics). Images were captured at 12.5–20 Hz. Basal fluorescence was sufficient to allow visualization of dendrites to ~100 μm from the soma, which is ~60–100% of the length of dendrites of these neurons (Uusisaari et al., 2007). Images were collected only from short stretches (20–50 μm) of dendrite, because most dendrites did not remain in the focal plane for longer distances.

Excitatory synapses were identified in voltage clamp by evoking 100 Hz trains of 40 EPSCs while imaging a stretch of dendrite and then searching (on-line) for regions along the dendrite that displayed a stimulus-dependent increase in the fluorescent signal. These regions could generally be isolated to a 2–3 μm length of the dendrite. After putative synapses were identified, fluorescence signals were recorded from that region during application of induction protocols judged as “effective” and “ineffective” in potentiation experiments. Photobleaching was minimized by limiting the exposure to short (10–20 ms) durations. After subtraction of background fluorescence, the signal was calculated as the change in fluorescence over baseline ($\Delta F/F$). Dye saturation during induction protocols was assessed by switching the amplifier to current-clamp mode and measuring the fluorescence signal evoked when cells held at hyperpolarized potentials were made to produce bursts of action potentials by injection of depolarizing current steps. These fluorescence changes were generally an order of magnitude larger than changes produced by the induction protocols in voltage clamp, suggesting that the signals of interest were not near saturation.

Data were analyzed with IGOR software (Wavemetrics) and are presented as mean ± SE. Statistical significance was assessed with Student's two-tailed paired *t* tests, except as noted. Stimulus artifacts have been digitally removed or reduced. All reported voltages are corrected for the junction potential of 5 mV.

Results

Plasticity depends on the relative timing of excitation and hyperpolarization

Our previous work (Pugh and Raman, 2006) indicated that mossy-fiber-mediated EPSCs in cerebellar nuclear neurons were potentiated by repeated applications of a train of EPSCs followed

Figure 1. Plasticity of EPSCs in the cerebellar nuclei depends on the relative timing of synaptic excitation and the rebound. **A**, Left, Representative responses evoked by three different induction protocols. Cells were held near -65 mV. Protocols consisted of a 250 ms, 133 Hz stimulation of excitatory afferents and a 150 ms current injection to hyperpolarize the membrane to approximately -85 mV. Bars above each trace indicate the time and duration of synaptic excitation. Right, Representative EPSCs before and after the induction protocols at left. **B**, Top, Mean change in EPSC amplitude before and after induction protocols with the onset of synaptic stimulation occurring 400 ms before the rebound (closed black circles), 600 ms before the rebound (open, gray-lined triangles), and 150 ms after the rebound (open, gray-lined squares). Induction protocols in this and similar graphs were applied at time 0. Bottom, Mean paired-pulse ratio (50 ms interval) before and after induction of potentiation with the topmost protocol shown in **A**. **C**, Percentage change in EPSC amplitudes versus the onset of synaptic stimulation relative to the offset of the hyperpolarization (onset of rebound). Circles indicate mean and SE. Bars indicate the time and duration of synaptic stimulation. Solid black and gray bars correspond to data in **A** and **B**. Dotted bars represent data from Pugh and Raman (2006).

by a post-hyperpolarization rebound depolarization (or current). To investigate the temporal relationship between excitation and hyperpolarization in potentiation of mossy fiber EPSCs, we developed a series of induction protocols in which the onset of synaptic excitation was varied relative to postsynaptic hyperpolarization. Each induction protocol consisted of a 250 ms, 133 Hz stimulation of excitatory afferents (the “synaptic” stimulus) and a 150 ms hyperpolarization to approximately -85 mV, which was followed by spontaneous, postinhibitory action potentials (the “rebound”). The onset time of the synaptic stimuli was varied from 600 ms before the rebound to 150 ms after the rebound. Enough holding current was applied to keep the membrane potential near -65 mV before stimulation, and was maintained throughout induction. When the synaptic stimulus began 400 ms before the rebound, potentiation was both robust, increasing EPSC amplitudes by $39 \pm 7\%$ ($N = 9$, $p < 0.001$), and reliable, potentiating EPSCs by a criterion level of 15% in eight of nine cells (Fig. 1A, B, top). As reported previously (Pugh and Raman, 2006), after application of the induction protocol, EPSCs in most cells were initially depressed. Over the next few minutes, their amplitudes increased, reaching a stable level of potentiation after ~ 10 min. Potentiation did not change either the variance of EPSC amplitudes (2328 ± 871 pA² vs 3327 ± 962 pA², $p = 0.1$) or the paired-pulse ratio of EPSCs (50 ms interval, 0.82 ± 0.05 vs 0.79 ± 0.05 , $p = 0.5$) (Fig. 1B, bottom), consistent with a postsynaptic locus of plasticity.

When the synaptic stimulus began >400 ms before the rebound, however, EPSCs failed to potentiate, instead depressing by $11 \pm 5\%$ ($N = 7$, $p = 0.09$) (Fig. 1A, middle, B). Similarly, EPSCs tended to depress when synaptic stimuli followed the rebound by 150 ms ($-11 \pm 5\%$, $p = 0.06$, $N = 8$) (Fig. 1A, bottom, B), consistent with work showing that pairing synaptic excitation and postsynaptic depolarization (without hyperpolarization) induces depression (Zhang and Linden, 2006). These results demonstrate that potentiation depends not only on the sequence of stimuli but also on the interval between excitation and inhibition, suggesting that the synaptic stimulus produces a transient signal that lasts a few hundred milliseconds (Fig. 1C).

Our previous work indicates that an increase in postsynaptic Ca is required for plasticity, because potentiation is blocked by NMDA receptor antagonists, by 10 mM BAPTA (1,2-bis-(O-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid) in the cerebellar nuclear cell, and by voltage ramp protocols that prevent concerted activation of voltage-gated Ca channels after the period of hyperpolarization (Pugh and Raman, 2006). The simplest interpretation of these data is that the synaptic stimulus activates Ca influx through NMDA receptors and the rebound, through voltage-gated Ca channels, both of which are necessary for plasticity. It is also possible that the hyperpolarization itself plays a specific signaling role, although neither our previous studies nor the data of Figure 1 provide evidence for or against this possibility. The additional requirement for a precise temporal relationship between the synaptic and rebound stimuli raises multiple possibilities for how the signals that they generate might interact to induce plasticity. Potentiation may follow a “threshold rule,” in which two stimuli generate intracellular Ca signals that combine to produce a Ca increase large enough to induce potentiation, possibly incorporating Ca-induced Ca release or a Ca spike (Wang et al., 2000; Brenowitz and Regehr, 2005; Egger et al., 2005). A corollary of this possibility is that potentiation may follow an “inverse-threshold rule,” such that moderate Ca rises induce potentiation, but large Ca rises promote depression, as at parallel fiber synapses onto Purkinje cells (Coessmans et al., 2004;

Belmeguenai and Hansel, 2005). Alternatively, potentiation may follow a “priming rule,” in which synaptic stimuli initiate a signaling cascade that provides a short-lived label of activated synapses, and the rebound initiates a separate cascade that triggers potentiation only at “primed” synapses, loosely analogous to synaptic tagging (Frey and Morris, 1997; Martin et al., 1997).

Regarding any type of threshold rule, a potentially complicating factor is that cerebellar nuclear cells fire spontaneously at 20–30 spikes/s (Thach, 1968; Jahnsen, 1986a; Raman et al., 2000; Telgkamp and Raman, 2002), which may generate a considerable Ca influx through voltage-gated Ca channels. Imaging studies have demonstrated the presence of voltage-gated Ca channels on nuclear cell dendrites (Muri and Knöpfel, 1994; Gauck et al., 2001) and, because most excitatory synapses are on dendritic shafts rather than spines of nuclear cells (Chan-Palay, 1977), synaptic sites may not be fully isolated from Ca influx associated with spiking. In all induction protocols that we have tested, however, spontaneous firing was prevented by tonic current injection or by voltage clamp (Pugh and Raman, 2006), which is likely to minimize basal Ca levels. If potentiation is sensitive to absolute levels of Ca, an increase in basal Ca during spontaneous firing might modify the robustness or reliability of potentiation in response to protocols previously characterized as effective.

Therefore, to test whether ongoing firing influences the extent or sign of potentiation, we applied the most effective induction protocol of Figure 1, i.e., with the synaptic stimulus beginning 400 ms before the rebound, to current-clamped neurons that were allowed to fire spontaneously at 20–30 Hz between each of the 30 repetitions of the protocol. Under these conditions, EPSCs were potentiated in all six cells tested, increasing by $60 \pm 13\%$ ($p = 0.01$) (Fig. 2*A*, top, *B*). In general, inducing potentiation against a background of spontaneous firing appeared more reliable (100%) and more robust ($>30\%$ amplitude increase in all cells) than when cells were held silent between each sweep. When the synaptic stimulation began 150 ms after the rebound, however, EPSCs did not potentiate ($-2 \pm 2\%$ relative to control, $N = 5$, $p = 0.8$), despite the presumed elevated level of baseline Ca (Fig. 2*A*, middle, *B*).

As mentioned, synaptic stimuli before a post-hyperpolarization rebound current can potentiate EPSCs even when action potential firing is prevented by voltage clamp (Pugh and Raman, 2006) (data replotted in Fig. 2*C*). To test the sensitivity of the voltage-clamp induction protocol to the temporal sequence of the synaptic and rebound stimuli, we repeated the experiment with synaptic stimulation applied after the rebound (Fig. 2*A*, bottom, *B*). Consistent with the current-clamp experiments, this pattern of stimulation did not potentiate EPSCs ($-24 \pm 25\%$ relative to control; $N = 4$, $p = 0.63$). Figure 2*C* summarizes the extent of potentiation measured in current clamp with spontaneous firing permitted, in current clamp with spontaneous firing prevented, and in voltage clamp with no firing at all. Although the amount of potentiation appears greater in protocols with more spiking, the primary observation is that potentiation does not depend on action potential firing. Instead, under all conditions, potentiation is predicted by the order of the synaptic and rebound stimuli, suggesting that the temporal pattern of stimulation is of greater importance than absolute Ca levels.

Dendritic Ca signals evoked by synaptic and rebound stimuli

To begin to investigate this idea directly, we measured relative changes in Ca-dependent fluorescence signals ($\Delta F/F$, referred to as Ca signals) evoked by synaptic and rebound stimuli in cerebel-

Figure 2. Potentiation is independent of the amount of spiking during induction. *A*, Representative responses of nuclear neurons to induction protocols consisting of synaptic stimuli (bars) before (top) or after (middle) the hyperpolarizing current injection and subsequent rebound, applied against a background of spontaneous firing, or synaptic stimuli applied after the rebound in voltage clamp (bottom). The voltage steps, from -65 to -80 to -55 mV, are shown above the current trace. *B*, Percentage change in EPSC amplitudes before and after induction protocols shown in *A*. VC, voltage clamp; CC, current clamp; syn, synaptic stimuli; rbd, rebound stimuli. *C*, Mean change in EPSC amplitudes after induction protocols consisting of synaptic stimuli either before or after the rebound. Induction protocols were given in current clamp with and without spontaneous firing and in voltage clamp holding the cell at -65 mV between each sweep. The data point for VC, syn before rbd, is from Pugh and Raman (2006).

Figure 3. Ca signals evoked in nuclear cells by rebound and synaptic stimuli. **A**, Top, Voltage-clamp protocol and overlaid currents evoked by 300 ms steps from -65 mV to -75 , -95 , and -115 mV, followed by either a step (black) or a 400 ms ramp (gray) to -55 mV. Bottom, Ca signals evoked in a dendrite during the step and ramp voltage-clamp protocols shown above (same cell). Responses to the three step protocols are concatenated, with conditioning at -75 mV shown first. Voltage protocol is shown beneath the trace. **B**, Image of a nuclear neuron (left) and of a single dendrite (right) filled with Oregon Green BAPTA 488-1. Scale bars, 25 μm (left) and 5 μm (right). **C**, Ca signals evoked by synaptic stimulation in a nuclear cell held at -65 mV. Numbered traces indicate Ca signals from sites corresponding to numbered circles in **B**. Inset, EPSCs simultaneously recorded in the nuclear cell. **D**, Mean Ca signals measured along the length of dendrites during synaptic stimulation (syn stim.) of 40 EPSCs at 100 Hz (closed black squares), rebound stimulation (rebound stim.) of steps from -75 to -55 mV (open triangles), or no stimulation (no stim.; closed gray circles). For each cell, a distance of zero was defined as the site producing the maximal synaptically evoked Ca signal, and distances were measured relative (rel.) to that location.

lar nuclear neurons. We first tested whether both synaptic and rebound stimuli-evoked Ca transients could be detected under our recording conditions. The heavy myelination of this region of the cerebellum presents a challenge in the detection of dendritic Ca signals. We therefore used the relatively high-affinity Ca indicator Oregon Green BAPTA 488-1 (200 μM). The advantage of this indicator is that it permitted the visualization of Ca signals evoked by synaptic as well as by rebound stimuli. The disadvantage is that the high affinity of the indicator is expected to prolong and spread the measured Ca signals. Bearing in mind the limitations imposed by the dye, we reasoned that we might nevertheless be able to detect salient phenomena such as changes in baseline Ca with spiking, or the interaction of synaptic and rebound Ca currents to generate Ca spikes.

To elicit rebound Ca signals, cells were voltage-clamped at -65 mV, stepped to voltages from -75 mV to -115 mV for 300 ms, and then depolarized to -55 mV. These conditioning steps were used because they span the voltages over which T-type Ca channels recover from inactivation (Fox et al., 1987; McDonough and Bean, 1998; McRory et al., 2001). The step to -55 mV evoked an inward current, probably dominated by low-voltage-activated T-type and possibly R-type Ca currents (Fig. 3A, top, black). Simultaneous monitoring of Ca signals in the soma and dendrite indicated that, on depolarization to -55 mV from -115 mV, the Ca signal increased modestly at the soma ($4.6 \pm 1.1\%$, $N = 13$) and to a larger extent in the dendrites 20–50 μm from soma ($17.9 \pm 3.4\%$, $N = 13$) (Fig. 3A, bottom, black). The rebound Ca increase was evident throughout the dendrite and tended to become larger at progressively more distal locations (Fig. 3D), consistent with previous reports that low-voltage-activated Ca channels are present at higher densities in the dendrites of these neurons (Muri and Knöpfel, 1994; Gauck et al., 2001). The peak amplitude of current recorded in the soma after the step back to -55 mV was not strongly correlated with the peak amplitude of the Ca signal recorded in the dendrite ($R^2 = 0.14$, $N = 13$) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). This lack of correlation might arise if small currents flowed into small compartments, producing large local Ca changes, or if the voltage-gated Ca influx induced release from Ca stores. Alternatively, in some cases the imaging sites might have been electrically remote, although this possibility seems less likely, given that these sites were relatively close to the soma (<100 μm).

To test whether voltage-clamp protocols that eliminate (somatic) Ca current were effective at reducing the rebound Ca signal, we repeated the experiments but with voltage ramps after the step hyperpolarizations. Indeed, the peak dendritic signal during a 400 ms ramp to -55 mV was reduced by $72 \pm 8\%$ relative to that produced by the step ($N = 4$) (Fig. 3A, gray), suggesting that the dendritic recording sites were reasonably voltage-clamped. Moreover, because our previous work showed that voltage ramps applied after hyperpolarizations block the induction of plasticity (Pugh and Raman, 2006), these data support the idea that a necessary consequence of the hyperpolarization during effective potentiation protocols is a Ca increase resulting from synchronous activation of voltage-gated Ca channels after hyperpolarization is rapidly relieved.

Next, to measure synaptic signals, neurons were voltage-clamped at -65 mV. Previous studies indicate that NMDA receptors of cerebellar nuclear neurons pass current in standard ACSF even at these negative voltages, owing to a weak Mg^{2+} block (Audinat et al., 1992; Anchisi et al., 2001; Pugh and Raman, 2006). Therefore, to identify sites of putative synaptic Ca influx,

we imaged a length of dendrite while applying a 100 Hz train of stimuli to excitatory afferents. Scanning the dendrite revealed relatively small ($\sim 2\text{--}3\ \mu\text{m}$ diameter) dendritic regions in which synaptically evoked Ca transients could be observed, with little or no change in fluorescence in adjacent sites (Fig. 3*C,D*), suggesting that these regions included activated synapses. Synaptically evoked relative Ca changes within these putative synaptic sites ranged from 4 to 23% with a mean change of $12.9 \pm 1.6\%$ ($N = 15$ synapses on 12 cells).

Testing a threshold rule for potentiation

Having verified that we could detect synaptic and rebound Ca signals, we next evaluated the threshold rule by testing whether induction protocols that are effective, i.e., that reliably and robustly induce potentiation, evoke a Ca rise that is larger than that produced by ineffective protocols at the synapse. In these experiments, we identified putative synaptic sites as in Figure 3, and then recorded Ca signals at these sites against a background of spontaneous firing. We first elicited synaptic and rebound Ca signals in isolation (Fig. 4*A*, top two traces, *B*), and then the two stimuli were applied together to generate either an effective potentiation protocol, with the synaptic stimulus beginning before the rebound, or an ineffective potentiation protocol, with the synaptic stimulus coinciding with the rebound (Fig. 4*A*, bottom two traces, *B*). In all protocols, the effect of synaptic stimulation was either to increase the firing rate or to drive cells into depolarization block, both of which raised Ca above the spontaneously firing baseline level. Conversely, hyperpolarization interrupted firing, leading to a decrease in dendritic Ca. On resumption of firing, Ca rose again, to a level that sometimes but not always exceeded the baseline level.

To quantify these data, the maximal Ca change from the level maintained during spontaneous firing was measured during three time windows: the 250 ms preceding the hyperpolarization, the 150 ms hyperpolarization, and the 800 ms after the end of the hyperpolarization. The mean data are plotted in Figure 4*C*. Notably, the Ca levels attained by the effective (synaptic preceding rebound) induction protocol are intermediate to those achieved with the other protocols, which are ineffective at potentiating EPSCs. With the imaging techniques used, however, the Ca signals in both current clamp and voltage clamp are likely to be highly filtered, so that local transients were either prevented or not detected, and the mobile dye is expected to extend the spatial range of the signals. Nevertheless, it is evident that the combination of synaptic and rebound stimulation does not produce an overtly supralinear Ca increase, suggesting that regenerative Ca spikes are not triggered by the combined stimuli, unlike at other excitatory cerebellar synapses (Brenowitz and Regehr, 2005). Moreover, because synaptic stimulation alone generates the largest, longest Ca increase, these data provide evidence against a threshold rule. They also do not support the inverse-threshold rule, because the ineffective (rebound preceding synaptic) induction protocol produces a smaller Ca rise.

This interpretation is subject to two concerns, however. First, potentiation was not measured in these experiments, because the time required to locate putative synaptic sites and then to induce and measure plasticity exceeded the possible recording durations. Therefore, it is possible that binding of Ca by the indicator might have prevented release from Ca stores or interfered with activation of Ca-dependent enzymes, so that the Ca signals recorded corresponded to levels that would not have been able to induce plasticity. We therefore tested whether potentiation could be induced in the presence of the indicator (without concurrent

Figure 4. *A–C*, Ca signals elicited in nuclear cell dendrites by effective and ineffective current-clamp induction protocols. Representative voltage traces (*A*) and Ca signals (*B*) evoked at putative synaptic sites by synaptic stimulation applied alone (syn only), hyperpolarization and rebound response alone (rbd only), the synaptic train 400 ms preceding the rebound response (syn-rbd), and the synaptic train 150 ms after the rebound response (rbd-syn). Changes are measured relative to the mean Ca level maintained during spontaneous firing. Vertical lines indicate the onset of synaptic and rebound responses in the syn-rbd graph and define windows for measurements in *C*. *C*, Mean maximal Ca changes (increases or decreases) from the four conditions in *A*, measured during the period preceding the hyperpolarization (pre), during the hyperpolarization (hyper), and during rebound (rbd).

imaging). Indeed, with $200\ \mu\text{M}$ Oregon Green BAPTA 488-1 in the pipette, the effective protocol of Figure 4 still potentiated EPSCs by $20.3 \pm 7.5\%$ ($N = 5$) (data contributed by Dr. A. L. Person, laboratory of I.M.R.). These results rule out the possibility that the Ca signals measured in the presence of indicator correspond to protocols that could not have induced potentiation.

The second concern results from the fact that highly localized Ca transients were beyond the resolution of our recording techniques. It therefore remains possible that the synaptic and rebound stimuli in the effective protocol may have either produced

Figure 5. Ca signals elicited in nuclear cell dendrites by effective and ineffective voltage-clamp induction protocols. **A**, **B**, Representative currents (**A**) and Ca signals (**B**) evoked at putative synaptic sites by synaptic stimulation applied alone (syn only), hyperpolarization and rebound current alone (rbd only), the onset of the synaptic train 350 ms preceding the rebound current (syn-rbd), and the onset of the EPSC train coinciding with the rebound current (rbd-syn). Vertical lines indicate the onset of synaptic and rebound currents in the syn-rbd graph. **C**, Mean Ca signals averaged >350 ms beginning at the time of the peak rebound current. Striped bar indicates the sum of the signals evoked by synaptic and rebound stimuli applied alone. Asterisk indicates statistically significant difference; NS, nonsignificant difference.

a particularly high level of Ca or generated the precise local Ca concentrations necessary to induce potentiation. To explore these possibilities, we altered the background Ca level by repeating recordings in voltage-clamped neurons. The suppression of spikes allowed Ca changes from the synaptic and rebound stimuli to be recorded in isolation. Under voltage clamp, the baseline levels of Ca at putative synaptic regions were reduced by $18.4 \pm 4.8\%$ ($N = 7$), relative to when cells were spontaneously firing. We again recorded Ca signals resulting from either synaptic or rebound stimulation, as well as the Ca signals obtained when the stimuli were combined in the temporal sequence that either favors or fails to induce potentiation (Fig. 5A,B). For each condition, the Ca signal was measured for 350 ms after the peak of the

rebound current. Within this time window, synaptic or rebound stimulation applied alone elicited relative Ca changes of $4.4 \pm 0.9\%$ or $6.8 \pm 1\%$, respectively (Fig. 5C). When the stimuli were applied concurrently in the effective protocol, however, the total Ca signal was not significantly different from the linear sum of its components ($10.4 \pm 1.6\%$ vs $11.2 \pm 1.6\%$, $p = 0.52$, $N = 8$ synapses from 6 cells) (Fig. 5C), again suggesting that no additional Ca sources were recruited by the combined stimuli. Moreover, in the ineffective protocol, the combined stimuli consistently produced a larger peak Ca signal than in the effective protocol ($14.9 \pm 2.9\%$ vs $10.4 \pm 1.6\%$, $N = 5$, $p = 0.02$) (Fig. 5C). Thus, although the absolute levels of Ca were greatly reduced in voltage clamp because action potentials were suppressed, the Ca changes produced by the effective induction protocol were once again bracketed by those generated by ineffective protocols. Even considering the fact that the high-affinity Ca indicator would have extended the apparent duration of the synaptic and rebound signals, it is unlikely that the effective current-clamp and voltage-clamp protocols produced an identical profile of Ca concentrations, providing further evidence against either a threshold or inverse-threshold rule.

Input specificity of potentiation

The results are instead more consistent with the “priming rule” mentioned above, in which the synaptic and hyperpolarization/rebound Ca signals act independently to activate distinct intracellular signaling pathways, both (or all) of which are required to induce potentiation. Because this hypothesis requires that the hyperpolarization/rebound triggers potentiation exclusively at previously primed synapses, however, it makes the specific prediction that potentiation must be limited to synapses activated during the induction protocol.

We therefore tested the input specificity of potentiation by recording EPSCs elicited by two independent sets of excitatory synapses onto a nuclear neuron and delivering the induction protocol to only one set of synapses (the “test pathway”). To maximize the probability of activating independent populations of synapses, two stimulating electrodes were inserted far apart in the white matter surrounding the cerebellar nuclei, with one at the ventral surface of the cerebellum and one in the corticonuclear tract. The stimulus intensity was adjusted to produce EPSCs of similar amplitudes in the two pathways. Because they could not always be made identical, sometimes the larger and sometimes the smaller EPSC was selected as the test pathway. Across cells, however, the mean amplitudes of the initial EPSCs in the control and test pathways were nearly the same (256 ± 42 pA and 255 ± 34 pA, $N = 6$). Because these EPSCs depressed only mildly in response to paired-pulse stimulation, an insensitivity of one pathway to previous stimulation of the other pathway did not provide a rigorous test of pathway independence. Instead, independence was tested by evoking EPSCs in the two pathways, first separately and then simultaneously, and then comparing the EPSC evoked by simultaneous stimulation with the sum of the separately evoked EPSCs. Only cells in which the amplitude and kinetics of the summed EPSC overlapped with the EPSC produced by simultaneous stimulation were tested further for potentiation (Fig. 6A).

After establishing a baseline amplitude of EPSCs for each set of synapses, the effective induction protocol of Figure 1A was applied in current clamp to the test pathway, whereas the other “control pathway” was not activated. After induction, EPSCs in the test pathway increased by $57 \pm 20\%$ ($p = 0.06$, $N = 6$), whereas those in the control pathway remained unchanged

Figure 6. Synapse specificity of potentiation. *A*, EPSCs evoked by stimulation of the control pathway (thin gray trace), the test pathway (thick black trace), or both simultaneously (thick gray trace). Thin black trace, the sum of control and test EPSCs, confirming independence by overlap with the thick gray trace. *B*, Mean EPSCs from the control and test pathways before (thick traces) and after (thin traces) induction protocols in which synaptic stimuli were applied only to the test pathway. EPSCs are normalized to the preinduction amplitude and superimposed for comparison. *C*, Mean change in EPSC amplitudes in response to induction in the two pathways.

($-4 \pm 4\%$, $p = 0.6$) (Fig. 6*B,C*). Thus, potentiation is indeed limited to synapses activated by the induction protocol, satisfying the basic requirement of a priming rule, as well as suggesting that the synaptic signal is localized. The results also verify that the rebound stimulus alone is inadequate to potentiate synapses (Pugh and Raman, 2006), instead supporting the idea that the widespread postinhibitory Ca signal is only an effective trigger of potentiation at recently activated synapses.

Inducing plasticity with synaptic inhibition instead of current injections

In all induction protocols tested, we have mimicked postsynaptic inhibition with current injections that hyperpolarize neurons to voltages between -80 and -90 mV. To a first approximation, this stimulus provides a reasonable simulation of inhibition, because it precisely dictates the times when firing ceases and when it resumes. Nevertheless, current injections and Purkinje-mediated IPSPs differ in the extent of hyperpolarization produced. In cerebellar nuclear neurons, the chloride equilibrium potential is

Figure 7. Potentiation in response to synaptic excitation and synaptic inhibition. *A*, Top, Representative response of a spontaneously firing nuclear neuron to the effective induction protocol of Figure 2*A*, but with hyperpolarization replaced by 100 Hz stimulation of mixed IPSPs and EPSPs, with IPSPs predominating. Bottom, EPSC amplitudes before and after stimulation for the cell shown above. Open circles are individual EPSCs; solid circles are the mean of the 10 surrounding EPSCs. Inset, Mean of 10 EPSCs in this cell before and after induction. *B*, Mean change in EPSC amplitude before and after stimulation.

near -75 mV (Jahnsen, 1986b; Aizenman and Linden, 1999), and the membrane potential during trains of IPSPs falls just short of this value (Telgkamp and Raman, 2002). Therefore, physiological inhibition is likely to recruit smaller rebound responses than do steps to more negative voltages, raising the question of the efficacy of “real” IPSPs at providing a triggering signal.

We therefore tested whether potentiation of EPSCs could be induced under more physiological conditions, i.e., with Purkinje-mediated inhibition evoking postinhibitory firing. In these experiments, SR95531 was omitted from the bath to permit activation of GABA_A receptors. Two stimulating electrodes were inserted into the white matter surrounding the cerebellar nuclei (see Materials and Methods). One electrode was positioned to evoke exclusively excitatory responses (in the “test” pathway). The other electrode was placed deep in the corticonuclear tract, where it elicited IPSPs as well as EPSPs (the “mixed PSP pathway”), with IPSPs predominating, resulting in a net inhibition of firing in the nuclear neuron (Fig. 7*A*, top). We substituted stimulation of this pathway for the hyperpolarizing current injection in the effective induction protocol of Figure 1*A* and measured the

effect on EPSCs in the test pathway. To mimic physiological conditions as closely as possible, we again allowed the cell to fire spontaneously in the intervals between applications of the induction protocol. With this induction protocol, EPSCs in the test pathway were potentiated by $30 \pm 10\%$ ($N = 6$, $p = 0.06$; 66% reliability) (Fig. 7A, bottom, B). Thus, under nearly physiological conditions that include background spontaneous firing and Purkinje-mediated inhibition, sequential synaptic excitation and inhibition does indeed potentiate EPSCs. Interestingly, the mixed PSPs hyperpolarized the membrane potential to only -67 ± 1 mV (Fig. 7A). Because this voltage is not negative enough for extensive recovery of most low-voltage-activated Ca channels (Fox et al., 1987; McDonough and Bean, 1998; McRory et al., 2001), it suggests that the majority of postinhibitory rebound Ca flows through high-voltage-activated Ca channels activated by the resumption of firing. Moreover, because the interruption of spontaneous firing reduces basal Ca levels (Fig. 4), these data raise the possibility that the triggering signal may involve a decrease in intracellular Ca while the cell is silenced, as well as an increase in Ca after the inhibition.

Potentiation requires both kinase and phosphatase activity

Together, these observations support the idea that the synaptic and inhibition/rebound stimuli activate distinct intracellular signaling pathways. Because both stimuli affect postsynaptic Ca levels, it is likely that these signaling pathways are sensitive to changes in local Ca concentration. We therefore tested whether Ca-dependent enzymes that are involved in several forms of potentiation and/or depression might also be required for plasticity of EPSCs in the cerebellar nuclei. These included CaMKII (Malinow et al., 1988; Silva et al., 1992a,b), calcineurin (Mulkey et al., 1993, 1994; Belmeguenai and Hansel, 2005), and PKC (Chung et al., 2000; Seidenman et al., 2003). We first blocked CaMKII activity by including a CaMKII-inhibitory peptide (fragment 290–309, 25 μ M) in the intracellular solution and repeating the most effective induction protocol of Figure 1 (with the synaptic stimulus 400 ms before the rebound). After the protocol, EPSCs initially increased in amplitude with a time course similar to that of potentiation under control conditions. Nevertheless, EPSCs consistently returned to the baseline amplitude within ~ 20 min (Fig. 8A) ($N = 7$). To corroborate this result, we repeated the experiment with the membrane-permeant CaMKII inhibitor KN62 included in the bath solution. Under these conditions, EPSCs returned to baseline after induction ($N = 3$). Pooling the data from both drugs indicates that when CaMKII was blocked, induction protocols produced no significant long-lasting change in EPSC amplitudes ($8.6 \pm 4.1\%$, $N = 10$). The mean pooled data are shown in Figure 8B.

When the activation of calcineurin (protein phosphatase 2B) was blocked by either 50 μ M intracellular calcineurin-autoinhibitory peptide ($N = 6$) or 1 μ M extracellular FK-506 ($N = 5$), EPSCs did not potentiate at all and instead depressed by $12 \pm 7\%$ ($p < 0.01$ Fig. 8A,B). Thus, not only is an increase in calcineurin activity required for the induction of potentiation, but the same induction protocols can also elicit a long-lasting depression when this enzyme is suppressed. These results are consistent with the idea that multiple processes can be triggered by the induction protocols and raise the possibility that the slow onset of potentiation may reflect the time course of calcineurin action. Moreover, these data illustrate that activity by both kinase and phosphatase is required for stable potentiation, suggesting that multiple substrates must be modulated for this form of plasticity to occur. In contrast, when PKC activity was reduced by

Figure 8. Requirement for active CaMKII and calcineurin, but not PKC, in potentiation. **A**, Top, Representative response to the effective induction protocol of Figure 1A in a cell containing calcineurin autoinhibitory peptide. Bottom, EPSC amplitudes in three cells before and after induction in the presence of inhibitory peptides (IP) blocking activity of CaMKII, calcineurin, or PKC (as labeled). Insets, Mean of 10 EPSCs before and after induction for each condition. For inhibition of CaMKII, three EPSCs are shown. The first is preinduction, the second 10 min postinduction, and the third 24 min postinduction. **B**, Mean changes in EPSC amplitudes during inhibition of CaMKII (triangles), calcineurin (squares), and PKC (circles).

including the inhibitory peptide PKC_{19–31} (1–2 μ M) in the intracellular solution, potentiation was unchanged relative to control ($p = 0.32$, unpaired t test, $N = 6$) (Fig. 8A, B, circles). The effects of these drugs on plasticity did not appear to result from direct modification of either synaptic currents or intrinsic excitability. Specifically, none of the drugs changed EPSC kinetics relative to control, with the exception of KN62, which slightly slowed the fast decay constant of the EPSC. Additionally, the number of action potentials produced in the first 300 ms of the rebound depolarization was not significantly changed in any of the inhibitors (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Together, these data suggest that neither priming nor triggering depends strongly on the activation of PKC. In contrast, both CaMKII and calcineurin are required for long-term plasticity.

Discussion

These experiments provide evidence that excitation and inhibition interact to potentiate mossy fiber EPSCs in cerebellar nuclear neurons. This interaction occurs when an ~ 250 ms train of synaptic excitation precedes the offset of a 150 ms period of inhibition by ~ 400 ms. Consequently, potentiation requires an approximate coincidence of synaptic excitation and inhibition, a pattern of activity that likely occurs during cerebellar learning. The excitatory synaptic and postinhibitory rebound stimuli activate NMDA receptors and voltage-gated channels, respectively, each of which elevates Ca in the dendritic shaft. The sign of plasticity is unaffected by raising or lowering basal dendritic Ca, providing evidence against the idea that potentiation proceeds when Ca levels reach a threshold. Instead, excitatory and inhibitory stimuli apparently produce functionally distinct local (priming) and global (triggering) signals that induce plasticity through a multistep process, involving the Ca-dependent enzymes calcineurin and CaMKII.

Plasticity rules in the cerebellar nuclei

These rules for inducing potentiation are distinct from classical long-term potentiation (LTP) in the hippocampal CA1 region, in which EPSCs are potentiated by large increases in postsynaptic Ca resulting from a temporal coincidence, on a scale of a few milliseconds, of synaptic excitation and postsynaptic spiking (Markram et al., 1997; Bi and Poo, 1998). The converse occurs at parallel fiber synapses onto Purkinje neurons, where small elevations of Ca promote potentiation (Coesmans et al., 2004; Belmeguenai and Hansel, 2005; Jörntell and Hansel, 2006). In cerebellar nuclear neurons, at least two factors may make such threshold or inverse-threshold rules inappropriate. First, spontaneous firing raises Ca levels in the relatively short dendrites of nuclear cells, near excitatory synapses. Thus, Ca-dependent induction of plasticity of EPSCs must occur against a high, probably fluctuating, background Ca level, which is expected to add noise to a threshold-based induction signal. Second, behaviorally relevant information from the external world likely increases inhibition from Purkinje cells as well as excitation from mossy fibers. Because inhibition decreases Ca, and excitation increases Ca, a simple threshold rule may be inadequate to encode meaningful temporal information that is required for adaptive plasticity.

The alternative hypothesis, supported by our data, is that excitatory inputs generate Ca signals that prime synapses for potentiation, whereas inhibitory inputs trigger potentiation by first decreasing and then increasing free Ca. Because these events must occur serially, the timing of synaptic input relative to the rebound determines whether potentiation occurs. Several studies provide precedent for the idea that the temporal sequence of activation of

different Ca sources sets the sign of plasticity. For instance, in pyramidal neurons of rat somatosensory cortex, synaptic activity preceding spiking induces LTP via NMDA receptor activation, whereas the reverse sequence induces LTD, because voltage-gated Ca influx facilitates metabotropic glutamate receptor-mediated endocannabinoid release (Bender et al., 2006; Nevian and Sakmann, 2006). In the dorsal cochlear nucleus, synaptic and spike-mediated Ca influx activates CaMKII, promoting LTP, and endocannabinoid release, promoting LTD. The former dominates when spiking precedes synaptic stimuli, however; the latter occurs when the order is reversed (Tzounopoulos et al., 2007).

In the case of the cerebellar nuclei, because potentiation requires NMDA receptor activation (Pugh and Raman, 2006), priming of synapses is probably initiated by Ca influx through these receptors, although other Ca-permeable glutamate receptors may contribute as well. Because of a weak Mg^{2+} block of NMDA receptors in nuclear cells (Anchisi et al., 2001; Pugh and Raman, 2006), the synaptic Ca signal is probably minimally affected by postsynaptic spiking. Although most excitatory synapses are located on dendritic shafts, this signal must be highly localized, possibly by endogenous buffering, as in aspiny cerebellar stellate neurons (Soler-Llavina and Sabatini, 2006). Moreover, at other excitatory synapses, calcineurin is localized to the postsynaptic density via A-kinase anchoring proteins (Coghlan et al., 1995; Gomez et al., 2002) and is stimulated by activation of NMDA receptors (Mulkey et al., 1993, 1994; Tong et al., 1995). The biochemical association of NMDA receptors and calcineurin, along with the result that inhibition of calcineurin consistently prevented potentiation, makes it reasonable to hypothesize that activation of the phosphatase by NMDA receptor-mediated Ca influx is a key step in the priming of mossy fiber synapses onto nuclear cells.

The triggering signal, in contrast, appears to be a global signal initiated by inhibition. Triggering may depend on the Ca increase after a period of hyperpolarization via the activation of Ca currents (Llinás and Mühlethaler, 1988; Aizenman and Linden, 1999; Molineux et al., 2006), and/or by the Ca decrease from the silencing of spontaneous firing by inhibition. Our previous work indicated that low-voltage-activated rebound currents were necessary to potentiate EPSCs in nuclear cells that were prevented from firing by voltage clamp (Pugh and Raman, 2006), suggesting that triggering requires a postinhibitory increase in Ca. This result does not, however, rule out the possibility that the interruption of firing itself, and the concomitant decrease in Ca, also contributes to the triggering signal in normally firing cells. Studies of medial vestibular nuclear neurons, which are also Purkinje cell targets, provide precedent for this idea. In these cells, inhibiting spontaneous firing decreases intracellular Ca, reducing the activity of tonically active CaMKII and producing long-term changes in intrinsic excitability (Nelson et al., 2003, 2005). Similarly, in cerebellar nuclear neurons, the basal level of Ca shifts significantly depending on whether or not the cell is firing, raising the possibility that triggering comprises the inhibition of Ca-dependent processes followed by the activation (or resumption) of other Ca-dependent processes.

This scenario is appealing in part because trains of IPSPs actually hyperpolarize cells quite modestly, only to -65 to -70 mV (Telgkamp and Raman, 2002; McKay et al., 2005). This voltage range is unlikely to produce significant recovery of low-voltage-activated Ca currents (McDonough and Bean, 1998; McRory et al., 2001), but potentiation proceeds nonetheless. Therefore, although low-voltage-activated Ca currents have the capacity to carry the triggering signal in voltage-clamped cells, it seems prob-

able that the postinhibitory Ca signal under physiological conditions instead arises from high-voltage-activated Ca currents activated during spiking, which either transiently raise Ca or simply restore it to baseline levels.

Molecular bases for priming and triggering

Although the stability of the paired-pulse ratio as well as the EPSC variance indicates a postsynaptic site of plasticity, the molecular events underlying priming and triggering are unknown. Interestingly, potentiation was more reliable and robust when induction protocols were applied against a background of spontaneous firing, suggesting that synaptic excitation is most effective when Ca levels start high. Mechanistically, these observations suggest that triggering may involve a shift in the balance of two antagonistic processes such as receptor internalization and receptor insertion. If these processes depend on separate signaling pathways with distinct Ca sensitivities (Chung et al., 2000; Hayashi et al., 2000; Seidenman et al., 2003), a drop followed by an increase in Ca might favor receptor insertion at previously primed synapses. Such a situation could arise if phosphatases or kinases activated during priming can regulate PSD-95 (postsynaptic density protein 95) or TARPs (transmembrane AMPA-receptor regulatory proteins) to make a transiently permissive environment for AMPA receptor insertion (El-Husseini et al., 2002; Schnell et al., 2002; Tomita et al., 2005; Kim et al., 2007).

Alternatively, priming might recruit receptor pools to specific synapses. For instance, Esteban et al. (2003) proposed that PKA-mediated phosphorylation readies GluR1 receptors for insertion, whereas CaMKII-mediated phosphorylation triggers fusion of GluR1-containing vesicles into synapses. A parallel mechanism may exist in nuclear neurons, although the receptors involved may differ, because *in situ* hybridizations reveal substantial GluR2, but little GluR1, in the cerebellar nuclei (Lein et al., 2007).

Relating potentiation of EPSCs to cerebellar learning

The form of plasticity described here has several characteristics appropriate for a cellular process relevant to delay eyelid conditioning. In this cerebellar task, conditioned stimuli are carried by mossy fibers, which likely provide nuclear cells with concurrent direct excitation and indirect inhibition, via the granule–Purkinje–nuclear cell circuit (Mauk et al., 1986; Steinmetz et al., 1989; Hesslow et al., 1999). Similarly, at the cellular level, cerebellar nuclear neurons detect the approximate coincidence of synaptic excitation and inhibition, in which a train of excitation precedes a postinhibitory rebound by ~300 ms. Acquisition of conditioned responses is optimal when conditioned stimuli last for 100–500 ms (Ohya et al., 2003b), consistent with the durations of synaptic excitation that effectively potentiate EPSCs. A critical component of cellular plasticity is the relief of inhibition; early in delay eyelid conditioning training, such disinhibition of nuclear cells might occur after the unconditioned stimulus, which activates complex spikes in Purkinje cells. Complex spikes can be followed by pauses in Purkinje cell firing (Sato et al., 1992), which could permit postinhibitory firing in the nuclei. Later in training, Purkinje cells generally slow their firing toward the end of the conditioned stimulus, which would also disinhibit nuclear cells (Green and Steinmetz, 2005; Jirenhed et al., 2007). The latter scenario is consistent with the prediction that synaptic plasticity occurs first in the cerebellar cortex, and second in the nuclei (Medina and Mauk 1999). Finally, the synapse specificity of the potentiation of EPSCs in the cerebellar nuclei, coupled with a relief of Purkinje-mediated inhibition late in the conditioned stimulus, appears well suited to underlie the conditioned-

stimulus specificity of delay eyelid conditioning (Ohya et al., 2003a).

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