Long-Term Potentiation at the Mossy Fiber–Granule Cell Relay Invokes Postsynaptic Second-Messenger Regulation of Kv4 Channels

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Mossy fiber afferents to cerebellar granule cells form the primary synaptic relay into cerebellum, providing an ideal site to process signal inputs differentially. Mossy fiber input is known to exhibit a long-term potentiation (LTP) of synaptic efficacy through a combination of presynaptic and postsynaptic mechanisms. However, the specific postsynaptic mechanisms contributing to LTP of mossy fiber input is unknown. The current study tested the hypothesis that LTP induces a change in intrinsic membrane excitability of rat cerebellar granule cells through modification of Kv4 A-type potassium channels. We found that theta-burst stimulation of mossy fiber input in lobule 9 granule cells lowered the current threshold to spike and increases the gain of spike firing by 2- to 3-fold. The change in postsynaptic excitability was traced to hyperpolarizing shifts in both the half-inactivation and half-activation potentials of Kv4 that occurred upon coactivating NMDAR and group I metabotropic glutamatergic receptors. The effects of theta-burst stimulation on Kv4 channel control of the gain of spike firing depended on a signaling cascade leading to extracellular signal-related kinase activation. Under physiological conditions, LTP of synaptically evoked spike output was expressed preferentially for short bursts characteristic of sensory input, helping to shape signal processing at the mossy fiber–granule cell relay.

Key words: cerebellum; granule cell; Kv4; long-term potentiation; mossy fiber

Significance Statement

Cerebellar granule cells receive mossy fiber inputs that convey information on different sensory modalities and feedback from descending cortical projections. Recent work suggests that signal processing across multiple cerebellar lobules is controlled differentially by postsynaptic ionic mechanisms at the level of granule cells. We found that long-term potentiation (LTP) of mossy fiber input invoked a large increase in granule cell excitability by modifying the biophysical properties of Kv4 channels through a specific signaling cascade. LTP of granule cell output became evident in response to bursts of mossy fiber input, revealing that Kv4 control of intrinsic excitability is modified to respond most effectively to patterns of afferent input that are characteristic of physiological sensory patterns.

Introduction

Potassium channels of the Kv4 family are responsible for generating A-type potassium current (I_A) in the majority of central

neurons (Jerng et al., 2004). A-type channels are unique in exhibiting a low voltage for activation and rapid inactivation that can modify channel availability depending on recent fluctuations in membrane potential. As a result, I_A can shape neuronal activity from membrane potentials below rest to the peak of an action potential, creating a dynamic regulation of the latency, threshold, and rate of spike firing. Kv4 channels are expressed at particularly high levels in granule cells of cerebellum (Serôdio and Rudy, 1998; Amarillo et al., 2008; Jerng and Pfaffinger, 2012). This is important because granule cells receive excitatory mossy fibers from both sensory afferents and pontine nuclei to form the pri-

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mary synaptic relay into cerebellum. Any factors that regulate I_A in granule cells are thus in a privileged position to influence signal processing across cerebellar lobules. For instance, it was shown recently that Kv4 channels can form a signaling complex with T-type (Cav3) calcium channels to produce a calcium-dependent increase in Kv4 current availability (Anderson et al., 2010a, 2010b). A differential pattern of Cav3 channel expression in granule cells allows the Cav3–Kv4 complex to shape mossy fiber inputs between cerebellar lobules (Heath et al., 2014).

Mossy fiber input to granule cells can exhibit a long-term potentiation (LTP) that depends on both NMDA and metabotropic glutamatergic (mGlu) receptor activation (Rossi et al., 1996; D'Angelo et al., 1999; Hansel et al., 2001; Gall et al., 2005; Andreescu et al., 2011). LTP at this synapse can further include a postsynaptic increase in granule cell excitability and probability of spike output (Armano et al., 2000), which, on inspection, exhibits characteristics that are consistent with a reduction in Kv4 channel activity. LTP of Schaeffer collateral input to CA1 pyramidal cells invokes a hyperpolarizing shift in I_A voltage for inactivation that reduces I_A availability (Frick et al., 2004). Whereas the molecular mechanism for this finding was not determined, Kv4 channel biophysics can be regulated by the actions of protein kinase A, protein kinase C, and extracellular signal-related kinase (ERK) (Watanabe et al., 2002; Yuan et al., 2002; Schrader et al., 2006; Hu et al., 2007; Rosenkranz et al., 2009). However, the mechanisms by which mossy fiber input triggers a long-term change in intrinsic excitability of cerebellar granule cells has not been determined.

We report that LTP at the mossy fiber–granule cell synapse leads to a 50% reduction in the availability of Kv4 current in lobule 9 granule cells by shifting channel voltage dependence. Postsynaptic modification of granule cell excitability involves an ERK-mediated phosphorylation process activated in a glutamate-receptor-specific manner. LTP of synaptically evoked spike output was most evident for short bursts of mossy fiber input, allowing potentiation to enhance the response to signals characteristic of sensory input. The data thus reveal a key role for a postsynaptic signaling cascade that modifies Kv4 channel properties to regulate granule cell intrinsic excitability and LTP at the mossy fiber–granule cell relay.

Materials and Methods

Animal care and cerebellar slice preparation

Sprague Dawley rats were obtained from Charles River Laboratories and maintained according to the guidelines of the Canadian Council of Animal Care and the Animal Care Committee of the University of Calgary. Established protocols were used for tissue dissection and slice preparation from male pups between postnatal day 19 (P19) and P23 (Anderson et al., 2010b; Heath et al., 2014). All chemicals were obtained from Sigma-Aldrich unless otherwise indicated.

Rats were anesthetized by inhalation of isoflurane until unresponsive to tail pinch. The cerebellum was dissected out and parasagittal (250–300 μ m) slices prepared from the vermis with a vibratome (Leica VT1000 S) in ice-cold artificial CSF (aCSF) composed of the following (in mM): 125 NaCl, 3.25 KCl, 1.5 CaCl_2 , 1.5 MgCl_2 , 25 NaHCO_3 , and 25 D-glucose preoxygenated by carbogen (95% O_2 , 5% CO_2) gas. Slices were then transferred to a carbogen-gassed chamber maintained at 37°C for 10–30 min before being stored at room temperature in aCSF. For recordings, slices were transferred to the stage of an Olympus BX51W1 or a Zeiss Axioskop II microscope equipped with differential interference contrast optics and infrared light transmission.

Electrophysiology

Electrophysiological recordings were performed at 32°C from lobule 9 granule cells (the majority in lobule 9c). Mossy fibers were stimulated

using a bipolar concentric tungsten electrode (Frederick Haer) connected to a stimulus isolation unit (Digitimer). All voltage- and current-clamp recordings were conducted using a Multiclamp 700 B amplifier and Digidata 1440A analog–digital converter. Data were recorded using PClamp version 10.0 software and analyzed using Clampex software or custom Matlab scripts.

NMDA receptors were blocked when necessary through bath application of 25 μ M DL-AP5 and stimulated using NMDA application in the presence of its coagonist, 10 μ M glycine. mGluRs were blocked using 10 μ M CPPCCOEt, 1.5 μ M JNJ 16259685, and 1 μ M MPEP (Tocris Bioscience) and activated by direct application of 50 μ M DHPG (Abcam). The stocks of DNQX, DL-AP5, S-DHPG, NMDA, glycine, and MPEP were dissolved in water and frozen at -20°C before dilution in aCSF to the desired final concentration. The stocks of CGP55845 (Abcam), PD 98059, CPPCCOEt, and JNJ 16259685 were dissolved in DMSO and frozen at -20°C before dilution in aCSF to the desired final concentration. Selumetinib (AZD6244; Selleck Chemicals) was dissolved in DMSO and frozen at -20°C before addition to internal electrolyte (1:100). A mouse monoclonal antibody directed against all KChIP isoforms (Pan-KChIP; NeuroMab, UC Davis) was included in the patch electrode at a 1:100 dilution in specific experiments.

Current-clamp recordings

Whole-cell current-clamp somatic recordings were made using a DC-10 kHz band-pass filter. Current-clamp recordings used an electrolyte modified from that of Gall et al. (2003) containing the following (in mM): 126 K-gluconate, 4 NaCl, 5 HEPES, 1 MgSO_4 , 0.15 BAPTA, and 0.05 CaCl_2 , pH 7.25, via KOH, with 5 di-Tris-creatine phosphate, 2 Tris-ATP, and 0.5 Na-GTP added from fresh frozen stock each day. This internal solution buffered the resting internal concentration of calcium [Ca] to 100 nM (Gall et al., 2003). Electrodes had a resistance of 6–8 M Ω and access resistance of 20–40 M Ω .

Granule cells were distinguished as exhibiting a membrane capacitance of 3–8 pF and a regular firing pattern to current injection with no spike accommodation. Cells were accepted for recording if the input resistance was $>1\text{ G}\Omega$, if they exhibited an resting membrane potential (RMP) between -50 and -60 mV in the absence of bias current injection (RMP, -60.4 ± 5.4 , $n = 8$), if the peak spike amplitude exceeded 0 mV with no spontaneous firing at rest, and if the mossy fiber stimulation evoked an EPSP. The gain of firing was calculated using linear regression to obtain the slope over regions of spike firing on frequency–current ($F-I$) plots.

Voltage-clamp recordings

Isolation of I_A . A combination of external and internal ion channel blockers was used to allow voltage-clamp recording of I_A in granule cells while preserving mossy fiber-evoked EPSCs. Recordings were considered acceptable for an access resistance in the range of 10–50 M Ω that did not drift $>20\%$ over the time course of the recording (5 min baseline recordings and 15 min after LTP). Voltage-clamp recordings used an internal electrolyte solution consisting of the following (in mM): 140 KCl, 10 HEPES, 2.5 MgCl_2 , 0.15 BAPTA, 0.05 CaCl_2 , 5 TEA, and 0.1 QX-314, pH 7.25 via KOH, with 5 di-Tris-creatine phosphate, 2 Tris-ATP, and 0.5 Na-GTP added from fresh frozen stock each day. This internal solution was calculated to buffer the internal [Ca] to 100 nM (MaxChelator) and to establish $E_K = -98\text{ mV}$. The external aCSF during recordings of I_A further contained 50 μ M picrotoxin, 1 μ M CGP55845, 2 mM CsCl, and 5 mM TEA unless otherwise indicated.

Inactivation plots of I_A were constructed using Origin version 8.0 software (OriginLab). Inactivation curves were fitted according to the Boltzmann equation: $I = 1/[1 + \exp(\{V - V_h\}/k)]$, where V_h is the half-inactivation potential and k is the slope factor. Activation curves were also fit according to the Boltzmann equation as follows: $I = 1/[1 + \exp(\{V_a - V\}/k)]$, where V_a is the half activation potential and k is the slope factor. Activation plots were constructed using GraphPad Prism software.

LTP protocol. Mossy fibers were stimulated with a quasiphenological protocol as in Sola et al. (2004) to induce LTP using a “theta-burst stimulus” (TBS) pattern (eight bursts of 10 impulses at 100 Hz, 250 ms

interburst interval) at a stimulus intensity that initially generated a submaximal EPSC or EPSP from a holding potential of -70 mV. For current-clamp recordings, TBS was delivered from a holding potential of ~ -65 mV. For voltage-clamp recordings of I_A , the TBS was also paired with a postsynaptic voltage step from -70 mV to -40 mV for the duration of the TBS synaptic train (3 s total) as in D'Angelo et al. (1999). Current-clamp recordings were maintained at -80 mV resting potential and in 14/17 cases, used only synaptic stimulation without a postsynaptic depolarization given the occurrence of adverse effects on spike amplitude when both were applied. A 5 min period was used to establish baseline synaptic amplitudes before presenting TBS and EPSCs/EPSPs recorded every 7 min for 15–25 min to monitor the induction of LTP.

Immunocytochemistry

Tissue slices prepared for immunocytochemistry were obtained from male rats (P25–P35) deeply anesthetized by isoflurane inhalation until unresponsive to ear pinch followed by intracardial perfusion with 250 ml of 0.1 M phosphate buffer (PB, pH 7.4) and then 100 ml of 4% PFA, pH 7.4, at room temperature (RT). Brains were placed into 4% PFA at RT for 1 h and left overnight in new 4% PFA at 4°C. Sagittal cerebellar sections of 40–50 μ m thickness were cut by vibratome (Leica VT1000 S) in PB.

Primary antibodies were mouse monoclonal anti-Kv4.2 and anti-Kv4.3 (1:500; Neuromab, UC Davis) used in Figure 2, A and B, respectively, or rabbit polyclonal anti-Kv4.3 (1:500, Alomone Labs) in Kv4.2–Kv4.3 dual-labeling experiments (see Fig. 2C–E). Controls consisted of omitting the primary antibodies to compare the relative labeling intensity between sections exposed to primary and secondary antibodies or only secondary antibodies. Tissue sections were reacted in a working solution consisting of 3% normal donkey serum, 0.2% DMSO, and 0.1% Tween 20. Primary antibodies were included in the working solution for 24–72 h with gentle agitation on a rocker at 4°C. After thorough washing in working solution, sections were exposed for 2–3 h at RT to Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000; Invitrogen) or Alexa Fluor 594-conjugated donkey anti-mouse IgG (1:1000). Sections were washed 3×20 min in PB, mounted in Fluoromount antifade medium, and stored at -20°C . Fluorescence labeling was imaged on a Zeiss Axioimager (Zen software) equipped with Colibri LED illumination and optical sections obtained via Apotome grid illumination using a 0.27 μ m section thickness. Postprocessing was restricted to image-wide adjustments to brightness/contrast (Zen software). Low-power montages of cerebellar tissue sections were compiled from multiple frames (80–90) imaged at $10\times$ (Zen software).

Statistical analysis

Average values are expressed as mean $\pm$ SEM, where sample values represent the number of individual cell recordings. Statistical significance was set at $p < 0.05$ in all cases. The Shapiro–Wilk normality test was used to ensure that data were drawn from a normally distributed population and statistical significance was determined using the paired Student's *t* test unless otherwise specified (all within-group comparisons for data obtained at baseline recording and 15 min after TBS). The regression slopes of *F–I* plots were compared using a two-tailed test in GraphPad Prism software. One-way repeated-measures ANOVA was used for within-group comparisons of V_a and V_h over time and two-way repeated-measures ANOVA was used for between-group comparisons of V_a and V_h over time, with Tukey's *post hoc* analysis.

Results

LTP increases the postsynaptic excitability of lobule 9 granule cells

Subgroups of cerebellar granule cells can receive mossy fiber input from multiple sensory sources that converge to allow integration of sensory modalities (Azizi and Woodward, 1990; Arenz et al., 2009; Huang et al., 2013; Ishikawa et al., 2015). Many of these inputs arrive as high-frequency, short-duration bursts of mossy fiber discharge (Chadderton et al., 2004; Rancz et al., 2007; Powell et al., 2015). The vermal region of lobule 9 corresponds to the uvula that receives vestibular, somatosensory, and corticopon-

tine mossy fiber inputs involved in mediating aspects of optokinetic and postural responses (Voogd et al., 2012). In lobule 9, bursts of mossy fiber input can range in frequency up to 105 Hz (Arenz et al., 2008). This is important because a TBS pattern using a 100 Hz intraburst frequency is known to induce LTP at the mossy fiber–granule cell relay (D'Angelo et al., 2005). We thus used a TBS protocol consisting of eight bursts of 10 impulses at 100 Hz (250 ms interburst interval) to study LTP in granule cells in lobule 9, with recordings centered primarily near the border of the granule cell layer with the white matter in lobule 9c.

Mossy fiber LTP induced by TBS is expressed as an increase in EPSP amplitude and an increase in spike output from granule cells (Armano et al., 2000; Sola et al., 2004; Nieuw et al., 2006). Postsynaptic increases in intrinsic excitability can be evoked at even low levels of excitation during the TBS (Armano et al., 2000), but is often considered to have a secondary role compared with presynaptic mechanisms in mossy fiber LTP (Maffei et al., 2002; Sola et al., 2004; D'Angelo et al., 2005; Nieuw et al., 2006). Previously, we reported differences in granule cell excitability according to the expression pattern of a Cav3–Kv4 channel complex in the vermis of lobules 2 and 9 (Heath et al., 2014). Here, we specifically examined the effects of delivering TBS to mossy fiber inputs on the contributions of Kv4 A-type current to postsynaptic excitability. Spike firing preceding TBS was assessed by constructing an *F–I* plot (1 s pulse) to establish the threshold for firing and instantaneous firing frequency. The TBS protocol was then delivered and *F–I* measurements repeated every 7 min thereafter until spike amplitude characteristics dropped below acceptable levels (up to 25 min after TBS). Unless otherwise indicated, all recordings were conducted in the presence of 50 μ M picrotoxin and 1 μ M CGP55845 to block GABA-A and GABA-B responses, respectively.

We found that TBS to mossy fibers induced an increase in the intrinsic excitability of granule cells within 10–15 min, apparent as a substantial increase (up to 3-fold) in the instantaneous firing frequency for a given level of current injection (Fig. 1A,B). The *F–I* plots for granule cells after TBS revealed a marked reduction ($\sim 75\%$) in the current threshold to evoke firing and a 2- to 3-fold increase in the gain of firing (baseline gain = 2.3 ± 0.3 Hz/pA; post-TBS gain = 5.7 ± 0.2 Hz/pA, $n = 8$, $p = 0.001$; Fig. 1B). In many cases, the increase in excitability was sufficiently pronounced to cause progressive spike inactivation at current injection levels that were originally capable of evoking stable firing ($n = 7/12$). In four cells, spike accommodation was prominent enough to restrict output to only one or two spikes during current injection. Data from these cells were not included in *F–I* calculations, but they were deemed healthy in retaining the ability to discharge a normal spike in response to a synaptic stimulus. These TBS-induced changes in spike frequency were also accompanied by a substantial reduction in the latency to discharge the first spike from the onset of current injection (Fig. 1C). The increase in excitability was not associated with any significant change in input resistance (baseline $R_{in} = 1.8 \pm 0.3$ G Ω ; post-TBS $R_{in} = 2.4 \pm 0.4$ G Ω , $n = 8$, $p = 0.15$). However, the absolute voltage threshold for spike discharge was substantially reduced by ~ 10 –15 mV for all levels of current injection 15 min after TBS (Fig. 1D), helping to account for the leftward shift in current threshold to discharge a spike on the *F–I* plot (Fig. 1B).

TBS reduces the availability of Kv4 channels

We have shown previously that Cav3 calcium channels allow calcium influx to interact with an associated potassium-channel-interacting protein 3 (KChIP3) subunit to produce a depola-

Figure 1. LTP at the mossy fiber–granule cell synapse increases postsynaptic excitability and properties of I_A . **A**, **B**, Results of delivering TBS of mossy fiber input on spike firing evoked by two levels of current injection (**A**) and mean F – I plots of instantaneous firing rate (**B**). Arrows in **B** indicate the current level to reach spike firing threshold. F – I plots were collected before TBS (baseline) and 15–20 min after TBS. **C**, **D**, Plots of the first spike latency (**C**) and spike voltage threshold (**D**) for different levels of current used

rising shift in the V_h of Kv4 (Anderson et al., 2010a, 2010b; Anderson et al., 2013; Heath et al., 2014). Reducing calcium influx or interfering with the Cav3–Kv4 interaction produces a select leftward shift in V_h that reduces I_A availability. We thus recorded I_A in lobule 9 granule cells under conditions that left postsynaptic calcium influx intact to identify any role for the Cav3–Kv4 complex. I_A was isolated by including 2 mM CsCl (HCN channels) and 5 mM TEA (KCa1.1, Kv1.x, Kv3.x) in the bathing medium (Coetzee et al., 1999; Gutman et al., 2005), with 100 μ M QX-314 (Nav1.x) and 5 mM TEA in the internal solution. Together, these conditions isolated I_A postsynaptically, but produced no significant change in the amplitude of the synaptically evoked EPSC ($n = 5$, $p = 0.54$).

Under baseline conditions, I_A V_h was -71.3 ± 0.8 mV ($n = 5$) and the V_a was -18.9 ± 3.2 mV ($n = 5$; Fig. 1E,F). Delivering the TBS protocol produced a significant hyperpolarizing shift in I_A V_h of ~ -11 mV (15 min post-TBS $V_h = -82.1 \pm 1.4$ mV, $n = 5$, $p = 0.008$). The shift in V_h was apparent within 5 min of delivering the TBS protocol and continued to increase up to 15 min after TBS (two-way repeated-measures ANOVA, V_h : $F_{(1,4)} = 11.7$, $n = 5$, $p = 0.03$; Fig. 1G). TBS also induced an ~ -12 mV hyperpolarizing shift in V_a (15 min post-TBS $V_a = -30.7 \pm 1.5$ mV, $n = 5$, $p = 0.003$) that reached statistical significance at 15 min after baseline recording (two-way repeated-measures ANOVA, V_a : $F_{(1,4)} = 10.18$, $n = 5$, $p = 0.03$; Fig. 1G). Time-matched controls revealed no significant change in either V_h or V_a over time without TBS (Fig. 1G; one-way repeated mea-

to construct F – I plots in **B** before and after TBS stimulation. **E**, **F**, Whole-cell steady-state voltage inactivation and conductance plots for I_A isolated in lobule 9c granule cells before and after TBS. Tests included 2 mM CsCl and 5 mM TEA in the medium and 0.1 mM QX-314 and 5 mM TEA in the electrode to isolate I_A while retaining synaptic responses to deliver TBS. **G**, Bar plots of I_A V_h and V_a measured over time with or without TBS. Data are plotted as the difference from values obtained at the end of 5 min baseline recordings and statistical significance measured with respect to time-matched control values (two-way ANOVA with Tukey *post hoc* tests). The x -axis represents time after the baseline recording. **H**, Step command from a holding potential of -70 mV (nominal resting potential) to -30 mV reveals a net decrease in I_A availability after TBS. Asterisks in **E** and **F** indicate significant shifts in V_h and V_a (Table 1). All recordings were conducted in 50 μ M picrotoxin and 1 μ M CGP55485 to block GABAergic inhibition. Sample values represent number of individual cell recordings. Average values are mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 2. Kv4.2 and Kv4.3 isoforms are coexpressed in cerebellar granule cells. **A, B**, Montage of sagittal cerebellar sections immunolabeled for Kv4.2 or Kv4.3 isoforms indicate that both isoforms are expressed in the granule cell layer, but differentially across lobules. Labeling for both isoforms is detected in the granule cell layer of lobule 9c. **C–E**, Kv4.2 and Kv4.3 channel isoforms are coexpressed in individual lobule 9c granule cells, as indicated by the merged image in **E**. Labeling in **B** was converted digitally to green to retain a consistent presentation. GCL, Granule cell layer; mol, molecular layer. Scale bars: **A, B**, 1000 μm ; **C–E**, 10 μm .

tures ANOVA: V_h , $F_{(2,3)} = 1.88$, $p = 0.29$; V_a , $F_{(2,3)} = 2.55$, $n = 5$, $p = 0.22$). Delivering only the postsynaptic voltage step to -40 mV also did not cause a shift in I_A V_h (baseline $V_h = -74.4 \pm 1.2$ mV; 15 min postvoltage step $V_h = -76.5 \pm 1.1$ mV, $n = 3$, $p = 0.39$) or V_a (baseline $V_a = -18.6 \pm 2.9$ mV; 15 min postvoltage step $V_a = -20.2 \pm 2.1$ mV, $n = 3$, $p = 0.46$).

NMDAR activation has been shown to be capable of reducing Kv4 channel membrane density in hippocampal cells (Kim et al., 2007; Hammond et al., 2008). We tested the potential effects of TBS on I_A density (pA/pF) in granule cells, but found no reliable shift over time compared with a set of equivalent time controls (two-way repeated-measures ANOVA, V_a : $F_{(1,4)} = 0.47$, $n = 4$, $p = 0.53$). Collectively, the changes in Kv4 V_h and V_a induced $>50\%$ reduction in the availability of Kv4 current at -70 mV (the nominal resting membrane potential of granule cells) after TBS ($n = 5$, $p = 0.0001$; Fig. 1H). These results are consistent with the role for Kv4 channels in modulating spike voltage threshold and gain of firing in granule cells (Heath et al., 2014).

Because the Cav3–Kv4 interaction invokes a selective shift in Kv4 V_h (Anderson et al., 2010b; Heath et al., 2014), the TBS-induced hyperpolarizing shift of both V_h and V_a indicates an action on Kv4 function that could be separate or in addition to that mediated by Cav3 channels. We have established that internal perfusion of a PanKChIP antibody blocks the Cav3-mediated effect on Kv4 V_h (Anderson et al., 2010b; Heath et al., 2014). However, including the PanKChIP antibody in the electrode (1:100 dilution) did not impede the synaptically induced hyperpolarizing shift in V_h or V_a (baseline $V_h = -72.7 \pm 3.2$ mV; post-TBS $V_h = -79.9 \pm 3.8$ mV, $n = 6$, $p = 0.046$; baseline $V_a = -21.9 \pm 3.9$ mV; post-TBS $V_a = -32.5 \pm 4.7$ mV, $n = 4$, $p = 0.004$). Therefore, the ability of TBS to induce hyperpolarizing shifts in V_h and V_a under conditions when the Cav3–Kv4 interaction is blocked would suggest that the effects of TBS on I_A occurs through mechanisms beyond a disruption of the Cav3–Kv4 interaction.

Kv4.2 and Kv4.3 are coexpressed in granule cells

The change in spike-firing properties after TBS are consistent with a reduction in the known actions of Kv4 A-type current. Previous work has established a high level of Kv4 expression in granule cells, with opposing gradients in the level of Kv4.2 and Kv4.3 mRNA transcripts and protein across cerebellar lobules (Serôdio and Rudy, 1998; Amarillo et al., 2008). To define the expression pattern for these Kv4 channel isoforms in rats, we conducted immunocytochemical labeling for Kv4.2 and Kv4.3 in sagittal cerebellar tissue sections. We confirmed that Kv4.2 and Kv4.3 immunolabel was detected in the granule cell layer throughout the cerebellum (Fig. 2A,B). Kv4.2 immunolabel also exhibited a gradient of intensity that was strongest in the anterior lobules and weakest in the posterior lobules (Fig. 2A), consistent with previous reports (Serôdio and Rudy, 1998). By comparison, a gradient for Kv4.3 immunolabel in the granule cell layer across lobules was less apparent. Instead, Kv4.3 immunolabel was strongest in the granule cell layer primarily in lobules 9c and 10, with relatively weak immunolabel in lobules 5–8 (Fig. 2B). Interestingly, Kv4.3 labeling was more apparent in the Purkinje cell layer in anterior lobules 1–5, revealing a differential expression for Kv4.3 in this key output cell. When Kv4.2 and Kv4.3 immunolabels were compared at higher magnification, we found expression for both isoforms in a plasma-membrane-like pattern in lobule 9c granule cells (Fig. 2C,D). Dual labeling further verified that Kv4.2 and Kv4.3 isoforms can be coexpressed in almost every granule cell observed in lobule 9c (Fig. 2E). Collectively, these data indicate that both Kv4.2 and Kv4.3 isoforms and heteromeric combinations of these subunits can be expected to contribute to A-type currents in lobule 9 granule cells.

Effects of LTP on Kv4 are mediated by select activation of NMDAR and mGluRs

It was shown that LTP at the mossy fiber–granule cell synapse requires coactivation of NMDAR and mGluRs (Rossi et al., 1996). We thus tested the contribution of these classes of glutamate receptors to LTP.

Figure 3. TBS-evoked effects on I_A depend on activation of specific glutamate receptors. **A–C**, Tests on the effects of applying TBS of mossy fiber input in the presence of mGluR or NMDAR blockers, with schematics of the stimulation protocol. TBS fails to induce a leftward shift in I_A V_h or V_a when delivered in the presence of mGluR1,5 blockers (10 μ M CPCCOEt, 1.5 μ M JNJ 16259685, 1 μ M MPEP) or an NMDAR blocker (25 μ M DL-AP5) applied either alone (**A**, **B**) or together (**C**). **D–F**, Effects of applying agonists to mGluRs (50 μ M S-DHPG) or NMDARs (100 μ M NMDA) on I_A V_h and V_a . All recordings were conducted in 50 μ M picrotoxin and 1 μ M CGP 55845 to block GABAergic inhibition. Sample values represent number of individual cell recordings. Average values are mean $\pm$ SEM. * p < 0.05; ** p < 0.01, Student's paired t test.

mate receptors to the long-term changes in Kv4 properties. For this, we applied the NMDAR blocker DL-AP5 (25 μ M) or a combination of CPCCOEt (10 μ M) and JNJ 16259685 (1.5 μ M), which target the mGluR1 isoform, and MPEP (1 μ M), which targets the mGluR5 isoform. Application of either NMDAR or mGluR blockers did not eliminate mossy fiber-evoked EPSCs entirely, with the additional postsynaptic step command to -40 mV during TBS ensuring a minimal level of postsynaptic depolarization. We found that applying NMDAR or mGluR blockers alone or in combination prevented entirely the TBS-induced shift in I_A V_h and V_a (Fig. 3A–C, Table 1).

We next tested whether direct agonist stimulation of these glutamate receptor subtypes was sufficient to induce a shift in I_A properties. For these tests, TBS was substituted with a 5 min perfusion of an agonist followed by a 15–20 min washout. Voltage for inactivation and activation were tested before and 15–20 min after the end of agonist perfusion. Application of 50 μ M S-DHPG as a group I mGluR agonist slightly left shifted V_h (~ -5 mV), but not V_a (Fig. 3D, Table 1). Conversely, perfusion of 100 μ M NMDA had no effect on Kv4 V_h but did left shift V_a (Fig. 3E, Table 1). Finally, simultaneous application of NMDA and S-DHPG produced large, hyperpolarizing shifts in both V_h

(~ -18 mV) and V_a (~ -30 mV) (Fig. 3F, Table 1). In summary, NMDAR and mGluR agonists were able to induce select but relatively minor shifts in I_A voltage-dependent properties, whereas large effects on both V_h and V_a were encountered upon coactivating NMDAR and group I mGluRs.

To test the calcium dependence of ligand-gated shifts in Kv4 properties, we recorded I_A V_h and V_a in the presence of 10 μ M BAPTA-AM in the bath. These tests revealed that, in the presence of BAPTA-AM, the shift in I_A V_h induced by direct coapplication of NMDA and S-DHPG was blocked (V_h , $n = 4$, $p = 0.83$; Fig. 4A, Table 1). In contrast, NMDA and S-DHPG coapplication in the presence of BAPTA-AM caused a hyperpolarizing shift in V_a ($n = 4$; $p = 0.01$; Fig. 4A, Table 1).

Blocking ERK phosphorylation prevents the postsynaptic effects of TBS stimulation

Coactivation of NMDAR and mGluRs has been shown to have synergistic effects on the activation of ERK (Yang et al., 2004). ERK is part of a signaling cascade activated by an increase in internal calcium that elevates the level of Ras-GTP. Ras-GTP then activates RAF, a protein kinase that activates mitogen-activated protein kinase kinase (MEK). MEK in turn phosphorylates ERK

Table 1. NMDA and mGluR1,5 receptors modulate Kv4 voltage dependence collectively

	Baseline V_h (mV)	Post-TBS or post-agonist V_h (mV)	$p(V_h)$	Baseline V_a (mV)	Post-TBS or post-agonist V_a (mV)	$p(V_a)$
Blocked						
mGluR1,5	-73.0 ± 3.0 (4)	-68.3 ± 1.4 (4)	0.26	-13.6 ± 1.5 (4)	-13.3 ± 2.4 (4)	0.51
NMDAR	-72.7 ± 1.7 (4)	-74.4 ± 2.2 (4)	0.41	-23.0 ± 2.4 (4)	-27.1 ± 1.8 (4)	0.30
mGluR + NMDAR	-71.1 ± 2.0 (4)	-71.5 ± 2.5 (4)	0.19	-17.6 ± 5.7 (4)	-23.6 ± 5.2 (4)	0.10
[Ca] ²⁺ (BAPTA-AM)	-71.6 ± 3.7 (4)	-72.3 ± 3.0 (4)	0.83	-21.4 ± 3.9 (4)	-25.8 ± 3.3 (4)*	0.01
ERK (PD98059)	-73.0 ± 2.7 (5)	-72.0 ± 2.1 (5)	0.83	-25.5 ± 3.7 (5)	-36.5 ± 2.2 (5)*	0.01
Agonists						
DHPG	-71.2 ± 0.3 (6)	-76.7 ± 2.2 (6)*	0.04	-17.6 ± 4.0 (6)	-21.6 ± 3.4 (6)	0.16
NMDA	-69.4 ± 2.0 (6)	-72.5 ± 3.2 (6)	0.40	-19.6 ± 2.6 (6)	-31.5 ± 2.1 (6)*	0.01
DHPG + NMDA	-71.7 ± 2.0 (5)	-89.5 ± 4.6 (5)*	0.01	-18.1 ± 5.3 (5)	-48.2 ± 7.9 (5)*	0.03

Receptor blockers were bath applied throughout recordings and before TBS of mossy fiber afferents. Receptor agonists were bath applied after baseline recordings in normal medium for 5 min in place of TBS. Receptor blockers were as follows: NMDA, DL-AP5 25 μ M; mGluR1,5, CPCCOEt 10 mM, JNJ 16259685 1.5 μ M, and MPEP 1 μ M. Agonists were as follows: NMDA, 100 μ M; mGluR, S-DHPG 50 μ M; BAPTA-AM, 10 μ M; and PD98059, 20 μ M. Sample values represent the number of individual cell recordings. Average values are mean $\pm$ SEM. * $p < 0.05$, Student's paired t test.

to target Ser/Thr phosphorylation sites, three of which have been reported on Kv4.2 channels (Thomas and Huganir, 2004; Schrader et al., 2006). We therefore examined the effects of 20 μ M PD 98059, a cell-permeable inhibitor of MEK (Hu et al., 2006). Bath application of PD 98059 before mossy fiber stimulation had no effect on $I_A V_h$ or V_a (V_h , $n = 5$, $p = 0.96$; V_a , $n = 5$, $p = 0.33$). In the presence of PD 98059, TBS delivered to mossy fibers failed to induce a hyperpolarizing shift in $I_A V_h$, but did produce a hyperpolarizing shift in $I_A V_a$ (Fig. 4B, Table 1). We further note that TBS induced no shift in V_h in the presence of the alternate ERK blocker selumetinib (10 μ M) in the electrode ($n = 3$, $p = 0.93$). Next, we recorded in current-clamp mode and delivered TBS in the presence of PD 98059 to determine the role of ERK in modulating firing frequency. Inhibition of MEK by PD 98059 prevented the TBS-induced increase in granule cell firing evoked by current injection (Fig. 4C). Similarly, direct application of 100 μ M NMDA and 50 μ M S-DHPG in the presence of 10 μ M BAPTA-AM to reduce calcium increases blocked the agonist-induced increase in postsynaptic firing (Fig. 4D).

Altogether, the data suggest that the increase in intrinsic excitability after TBS reflects primarily a hyperpolarizing shift in $I_A V_h$ that involves a calcium-dependent process and activation of an ERK signaling cascade. An additional hyperpolarizing shift in $I_A V_a$ appears different in reflecting a process that develops with a slower time course (Fig. 1G) and can be at least partially evoked by NMDA receptors (Fig. 3E), but is calcium independent (Fig. 4A) and relatively insensitive to an ERK blocker (Fig. 4B). Although a hyperpolarizing shift in V_a should increase I_A availability, the overall reduction in I_A suggests that the effects of TBS on $I_A V_h$ has a greater net influence to increase postsynaptic excitability.

TBS-induced LTP is preserved in the presence of GABAergic circuitry

The excitability of granule cells is closely regulated by Golgi cells, a class of GABAergic interneurons located within the granule cell layer (Mapelli and D'Angelo, 2007; Duguid et al., 2012). Previous reports indicated that GABAergic inhibition is robust enough to prevent induction of LTP of the mossy fiber EPSP and reduce the extent of spatial region expressing LTP (Armano et al., 2000; Mapelli and D'Angelo, 2007). To determine the ability to evoke LTP of postsynaptic excitability when GABAergic systems are intact, we repeated TBS in the absence of picrotoxin and CGP55845. We found that TBS still evoked a long-term increase in the rate of current-evoked firing, along with a reduction in the spike voltage threshold (baseline $V_{\text{threshold}} = -38.7 \pm 2.5$ mV; post-TBS $V_{\text{threshold}} = -52.6 \pm 3.5$ mV, $n = 7$, $p = 0.001$) and increase in

gain of firing on $F-I$ plots (baseline gain = 2.3 ± 0.2 Hz/pA; post-TBS gain = 4.1 ± 0.6 Hz/pA, $n = 7$, $p = 0.01$; Fig. 5A,B).

To again consider the physiological relevance of these changes to modulation of I_A , we recorded the effects of inducing LTP on I_A in medium lacking picrotoxin or CGP55845. The application of TBS to mossy fibers again induced an ~ -10 mV hyperpolarizing shift in $I_A V_h$ (baseline $V_h = -70.5 \pm 2.1$ mV; post-TBS $V_h = -79.9 \pm 3.2$ mV, $n = 7$, $p = 0.0002$) and V_a (baseline $V_a = -16.9 \pm 4.9$ mV; post-TBS $V_a = -29.1 \pm 4.6$ mV, $n = 7$, $p = 0.002$; Fig. 5C). These tests are important in demonstrating that a TBS-evoked increase in postsynaptic excitability of lobule 9 granule cells occurs in the presence or absence of GABAergic inhibition.

Bursts of mossy fiber EPSPs uncover LTP of synaptic efficacy in the intact circuit

It was important to determine the effective contribution of an increase in postsynaptic excitability in spike firing to LTP of the synaptically evoked response. As noted, previous work testing single evoked EPSPs concluded that LTP of the mossy fiber EPSP was only reliably detected when GABAergic circuits were blocked (Armano et al., 2000), raising questions as to the functional role of LTP in the intact system. However, recent work *in vivo* indicates that the physiological pattern of input for mossy fibers that evokes a response in granule cells is in the form of short bursts (Chadderton et al., 2004; Rancz et al., 2007; Duguid et al., 2012; Powell et al., 2015). We thus retested the ability to record LTP of the synaptic response and the effects of a TBS-induced increase in postsynaptic firing in the presence or absence of GABAergic inhibition.

We first compared the ability to detect LTP of synaptic transmission using single evoked EPSPs in the manner traditionally used in studies of long-term plasticity. The intensity of synaptic stimulation was adjusted to the minimal level required to evoke a subthreshold EPSP, with a stable baseline EPSC or EPSP amplitude confirmed by stimulating 1/min for 5 min before delivering TBS. Testing the effects of TBS on a single evoked EPSP in the presence of 50 μ M picrotoxin and 1 μ M CGP55845 to block GABAergic inhibition consistently revealed a potentiation of the EPSP and an increase in the probability of discharging a spike. Therefore, a single EPSP originally subthreshold for spike firing was increased after TBS to the point of reliably triggering a single spike per stimulus, a potentiated state that persisted for at least 15 min ($n = 6/6$). We also found a potentiation of EPSP amplitude in five of five cells after TBS in the presence of the ERK blocker PD 98059, with three of five cells exhibiting an increase in synaptically evoked spike discharge. However, when the same tests were

Figure 4. TBS-evoked effects on $I_A V_h$ and intrinsic excitability are calcium and ERK dependent. **A**, Effects of applying agonists of mGluR (50 μM S-DHPG) and NMDAR (100 μM NMDA) on $I_A V_h$ (**A**) are blocked in the presence of 10 μM BAPTA-AM, whereas $I_A V_h$ still exhibits a modest hyperpolarizing shift in BAPTA-AM (**A**). **B**, In the presence of the ERK blocker PD98509 (20 μM), the effects of TBS on $I_A V_h$ are blocked, whereas a leftward shift in V_a remains. **C**, TBS-evoked increase in postsynaptic spike firing is blocked by the ERK blocker PD98509 (20 μM). **D**, Increase in spike firing induced by direct application of 100 μM NMDA and 50 μM S-DHPG is blocked in the presence of 10 μM BAPTA-AM. Sample values represent number of individual cell recordings. Average values are mean $\pm$ SEM.

conducted without GABA receptor blockers present, TBS failed to evoke a long-term increase in the amplitude of a single evoked EPSP or spike firing in seven of seven cells ($p = 0.20$), confirming previous reports on the inability to evoke LTP of single evoked EPSPs when GABA circuits are intact (Armano et al., 2000).

We next examined the effects of evoking a short, four-pulse train of mossy fiber EPSPs (10, 20, 50, and 100 Hz) in the absence of GABA receptor blockers to retain GABAergic circuitry. Here, we could record stepwise (quantal) changes in EPSP amplitude and intermittent failures from pulse to pulse, as expected for activation of individual mossy fiber axons (Sola et al., 2004; Nieuwenhuis et al., 2006), along with a temporal summation of the EPSP (Fig. 6A). Applying TBS in the absence of GABA receptor blockers now revealed LTP of synaptic efficacy and increased spike output in response to the mossy fiber burst input in five of five cases (Fig. 6B). Therefore, a short train of EPSPs exhibiting temporal summation but below threshold for spike discharge was converted by TBS to an enhanced EPSP amplitude and summation process that increased spike firing up to eight spikes during a four-pulse train (Fig. 6B). A TBS-induced increase in postsynaptic firing was further verified in a subset of cells using direct current injection ($n = 2$), confirming that the potentiated synaptic response was accompanied by an increase in postsynaptic excitability. In comparison, single evoked EPSPs tested in this group still failed to potentiate compared with the train of EPSPs ($n = 3$; $p = 0.61$). Interestingly, the degree of potentiation of spike output in response to burst input showed evidence for a frequency-dependent influence, such that the greatest degree of synaptically evoked potentiation occurred in the 20–50 Hz range (Fig. 6B). These data are important in establishing that the effects of TBS-induced LTP of synaptic transmission and postsynaptic excitability in granule cells are apparent in preparations with intact GABAergic circuits.

Discussion

The current study reveals that LTP at the mossy fiber–granule cell synaptic relay includes a modulation of postsynaptic Kv4 channels that dramatically increases the excitability of lobule 9 granule cells. A TBS-evoked shift in I_A voltage dependence involves NMDA and mGlu receptors that invoke the signaling cascade leading to ERK activation. The final result is a long-term reduction in postsynaptic I_A that contributes to LTP of mossy fiber

Figure 5. Effects of applying TBS on postsynaptic firing and I_A with GABA circuitry intact. **A**, **B**, Results of TBS of mossy fiber input on postsynaptic firing in the absence of GABA receptor blockers. Representative spike firing to current injection (**A**) with mean $F-I$ plots of instantaneous frequency (**B**) before and 15 min after TBS are shown. Arrows in **B** indicate the current level to reach spike firing threshold. **C**, Voltage inactivation and conductance plots for I_A before and after TBS. I_A was isolated while retaining synaptic responses for TBS using 2 mM CsCl and 5 mM TEA in the medium and 0.1 mM QX-314 and 5 mM TEA in the electrode. I_A exhibits a significant hyperpolarizing shift in V_h and V_a (**C**) after TBS. Data in **C** are normalized to the peak current in baseline conditions. Sample values represent the number of individual cell recordings. Average values are mean $\pm$ SEM.

Figure 6. LTP is expressed for burst-like mossy fiber input patterns in lobule 9 granule cells. All recordings were conducted in the absence of GABA receptor blockers to assess the effects of TBS on synaptically evoked granule cell firing in a circuit in which GABAergic inputs are intact. **A**, Recordings from granule cells indicating the results of TBS on a four-pulse burst of mossy fiber EPSPs at the indicated frequencies. EPSPs initially set subthreshold for spike discharge reliably evoke single or multiple spike discharge in granule cells when tested using a burst of mossy fiber inputs. **B**, Plots of the number of spikes evoked during mossy fiber burst input delivered at the indicated frequencies for five different cells before and after TBS.

inputs that arrive in a burst-like pattern characteristic of physiologically relevant inputs.

LTP at the mossy fiber–granule cell relay

It is known that the mossy fiber–granule cell synapse is capable of exhibiting LTP after theta burst patterns of input both *in vitro* (D'Angelo et al., 1999; Mapelli and D'Angelo, 2007) and *in vivo* (Roggeri et al., 2008). LTP depends on mossy fiber activation elevating postsynaptic calcium concentration after coactivation of NMDA and mGlu receptors (Rossi et al., 1996; D'Angelo et al., 1999; Gall et al., 2005). The source of LTP has been largely attributed to presynaptic mechanisms (Maffei et al., 2002; Maffei et al., 2003; Sola et al., 2004). A second contributing factor is a postsynaptic increase in granule cell excitability, as reflected in a lower spike threshold and increased probability for spike firing (Armano et al., 2000; Nieuws et al., 2006).

In lobule 9, TBS evoked a decrease in the threshold for granule cell firing that supported a 2- to 3-fold increase in spike frequency and a higher probability of firing in response to synaptic input. Although a decrease in the threshold to trigger a spike has been reported previously (Armano et al., 2000), the TBS-evoked increase in excitability detected for lobule 9 granule cells was much more pronounced. Voltage-clamp analysis revealed a rapid and enduring hyperpolarizing shift in $I_A V_h$ after TBS and a hyperpolarizing shift of V_a with a slightly later onset. The balance of these factors decreased I_A availability, a result consistent with the change in spike threshold and rate of firing after TBS. Moreover, the change in $I_A V_h$ was maximal upon coactivation of NMDARs/mGluRs, with an increase in postsynaptic excitability apparent in the presence or absence of GABAergic inhibition. The current results are thus important in identifying modulation of Kv4 channels as a contributing factor to a marked TBS-induced increase of intrinsic excitability in lobule 9 granule cells in relation to physiologically relevant patterns of mossy fiber input.

Role for kinase activation

The molecular mechanisms underlying a change in postsynaptic excitability in granule cells after LTP had not been identified previously. Our ability to block the TBS-induced hyperpolarizing shift in $I_A V_h$ and the increase in spike output with a MEK blocker strongly implicates an ERK-mediated phosphorylation process. There are three known sites for ERK phosphorylation on Kv4.2 (Schrader et al., 2006). However, we cannot predict the site(s) on Kv4 subunits potentially targeted by ERK because we cannot establish whether the channels reflect homomeric or heteromeric combinations of Kv4.2 and Kv4.3 subunits. We also cannot rule out the possibility that ERK may target other subunits of the Cav3–Kv4 complex, including KChIP and dipeptidyl-peptidase-like subunits. Cav3 calcium channels can also link to a Kv4 complex containing KChIP3 to produce a depolarizing shift in Kv4 V_h to augment I_A (Anderson et al., 2010a, 2010b; Heath et al., 2014). The finding that infusion of an anti-PanKChIP antibody did not prevent the TBS-induced hyperpolarizing shift in V_h suggests that the effects of TBS may be independent of the Cav3–Kv4 complex. Conversely, a phosphorylation site on Kv4.2 at which ERK can induce a rightward shift in V_a in an expression system depends on KChIP3 coexpression (Schrader et al., 2006). The exact role for a Cav3–Kv4 complex in these results thus remains to be identified, as do the site(s) at which ERK acts to modulate I_A in granule cells.

Some of our findings resemble Kv4 modulation after LTP induction in CA1 hippocampal pyramidal cells. A TBS to Schaffer collaterals to induce LTP invoked a select hyperpolarizing shift in

Kv4 V_h that reduced I_A availability and increased the amplitude of dendritic spikes (Frick et al., 2004; Rosenkranz et al., 2009). The increase of dendritic spike amplitude was traced to a PKA–MAPK cascade (Rosenkranz et al., 2009), but the relationship of this kinase activity to the shift in $I_A V_h$ was not established. Instead, phosphorylation by PKA, PKC, or ERK results in a depolarizing shift in Kv4 V_a , with no effects on V_h in hippocampal pyramidal cells (Johnston et al., 1999; Watanabe et al., 2002; Yuan et al., 2002). By comparison, LTP in cerebellar granule cells was traced to an ERK-mediated hyperpolarizing shift in $I_A V_h$, with a second process to shift $I_A V_a$ through mechanisms that remain to be identified. The second messengers triggered by LTP to modify I_A in hippocampus thus differ from cerebellar granule cells.

Functional role of Kv4 modulation of granule cell excitability

The ability to induce LTP and modulate Kv4 channels with stimuli centered on 100 Hz input is important given that a TBS-like pattern of tactile stimuli can induce LTP in granule cells *in vivo* (Roggeri et al., 2008). We view the postsynaptic contributions by Kv4 channels to mossy fiber LTP as an important complement to the established role of presynaptic factors underlying LTP at this synapse. It is known that mossy fiber LTP requires an increase in postsynaptic calcium (Gall et al., 2005). Our results now identify NMDA and mGlu receptor activation as a source for calcium to modulate Kv4 voltage dependence and I_A availability in lobule 9 cells. A key result of I_A modulation is a decrease in spike threshold that will support a shorter first spike latency of granule cell firing, as detected here in response to current injection (Fig. 1C,D). We also document an increase in the probability and number of spikes generated by granule cells after TBS, with a short burst of subthreshold EPSPs transformed into responses capable of triggering up to eight spikes per EPSP.

These data are important to the potential functional significance of LTP of mossy fiber input. Direct recordings *in vivo* establish that granule cells exhibit little response to spontaneous mossy fiber discharge and require burst input to drive spike firing reliably (Chadderton et al., 2004; Rancz et al., 2007; Powell et al., 2015). It is thus important that we found an effect of TBS specifically on the granule cell response to bursts of input compared with single EPSPs. This would indicate that postsynaptic Kv4 channels can contribute to blocking a response to background spontaneous input (Jörntell and Ekerot, 2006; Powell et al., 2015) by lowering granule cell firing probability until a TBS-induced decrease in I_A increases postsynaptic excitability. The number of spikes generated also showed a relation to the frequency of repetitive mossy fiber bursts, suggesting a frequency-filter effect presumably mediated by the combination of EPSP summation, Golgi cell inhibition, and Kv4 regulation of postsynaptic excitability. Interestingly, a frequency filter for input has been reported in Purkinje cells to restrict a response to high-frequency bursts of parallel fibers. In that case, the filter is established by a Cav3–KCa3.1 channel complex that suppresses temporal summation of EPSPs at low frequencies (Engbers et al., 2012). As a result, Purkinje cells respond most effectively to short bursts of parallel fiber input from granule cells.

It is known that cerebellar circuits can shape motor responses with a resolution of <10 ms (D'Angelo and De Zeeuw, 2009). It has been proposed that the combined actions of a burst of mossy fiber excitation and feedforward inhibition by Golgi cells establishes a time window of opportunity for a granule cell to respond with a short-latency first spike and/or burst (D'Angelo and De Zeeuw, 2009; Chadderton et al., 2014). The occurrence of LTP or LTD at this synapse was thus proposed to modulate the timing of

evoked spikes in relation to a ~ 5 ms window for granule cells to respond to sensory inputs. Interestingly, using current injections, we found that first spike latency was reduced by $\sim 2/3$ after TBS (Fig. 1C). Control over the timing of granule cell output through LTP could therefore be another means to influence temporal coding strategies by establishing time delays between populations of granule cells activated by different sensory inputs (adding to that proposed for presynaptic mechanisms of plasticity; Chabrol et al., 2015). Alternatively, LTP-induced shifts in intrinsic excitability could reduce the number of mossy fiber inputs of a specific modality needed to reach threshold for firing (Jörntell and Ekerot, 2006).

The LTP-induced modulation of Kv4 control over granule cell excitability would thus appear to dovetail with other mechanisms in cerebellum that effectively preset the system to respond to bursts of sensory input conveyed by mossy fibers (Roggeri et al., 2008; D'Angelo and De Zeeuw, 2009; Chabrol et al., 2015; Ramakrishnan et al., 2016).

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