Synaptic transmission and neuronal excitability depend on the concentration of extracellular calcium ([Ca\textsubscript{o}], yet repetitive synaptic input is known to decrease [Ca\textsubscript{o}] in numerous brain regions. In the cerebellar molecular layer, synaptic input reduces [Ca\textsubscript{o}] by up to 0.4 ms in the vicinity of stellate cell interneurons and Purkinje cell dendrites. The mechanisms used to maintain network excitability and Purkinje cell output in the face of this rapid change in calcium gradient have remained an enigma. Here we use single and dual patch recordings in an in vitro slice preparation of Sprague Dawley rats to investigate the effects of physiological decreases in [Ca\textsubscript{o}] on the excitability of cerebellar stellate cells and their inhibitory regulation of Purkinje cells. We find that a Ca\textsubscript{3}–K\textsubscript{4} ion channel complex expressed in stellate cells acts as a calcium sensor that responds to a decrease in [Ca\textsubscript{o}] by dynamically adjusting stellate cell output to maintain inhibitory charge transfer to Purkinje cells. The Ca\textsubscript{3}–K\textsubscript{4} complex thus enables an adaptive regulation of inhibitory input to Purkinje cells during fluctuations in [Ca\textsubscript{o}], providing a homeostatic control mechanism to regulate Purkinje cell excitability during repetitive afferent activity.

**Introduction**

The probability of transmitter release and membrane excitability of neurons is markedly influenced by calcium influx from the extracellular space. Calcium measurements have established that the resting level of [Ca\textsubscript{o}] in the brain ranges from 1.1–2.0 mM (Heinemann et al., 1977; Nicholson et al., 1978; Stöckle and ten Bruggencate, 1980), with repetitive synaptic input rapidly decreasing [Ca\textsubscript{o}] within the surrounding extracellular environment in several brain regions (Heinemann et al., 1977; Stöckle and ten Bruggencate, 1978; Egelman and Montague, 1999; Stanley, 2000; King et al., 2001; Rusakov and Fine, 2003). In the cerebellum, repetitive excitatory input quickly lowers [Ca\textsubscript{o}] by ~0.4 ms in the molecular layer (Nicholson et al., 1978; Stöckle and ten Bruggencate, 1978). Synaptically evoked decreases in [Ca\textsubscript{o}] are expected to reflect calcium influx into presynaptic terminals as well as postsynaptic cells. A prominent postsynaptic contribution to changes in [Ca\textsubscript{o}] is predicted in the molecular layer, where climbing fibers evoke a calcium-dependent complex spike that conducts throughout the Purkinje cell dendritic tree (Llínas and Sugimori, 1980b; Kitamura and Häusser, 2011). However, the effects of a decrease in [Ca\textsubscript{o}] on cell excitability or circuit function in the cerebellum has not been determined.

Stellate cells positioned within the molecular layer provide inhibitory input to Purkinje cell dendrites to suppress calcium transients associated with climbing fiber-evoked complex spikes (Callaway et al., 1995; Kitamura and Häusser, 2011). Any factor that affects stellate cell excitability then has the potential to exert considerable control over the output of the cerebellar cortex. In this regard, stellate cells express K\textsubscript{4} (A-type) potassium channels that associate with potassium channel interacting protein 3 (KChIP3) (Kollo et al., 2006; Burgoyne, 2007; Anderson et al., 2010b). A protein interaction between Ca\textsubscript{3} (T-type) calcium channels and K\textsubscript{4} potassium channels allows Ca\textsubscript{3} calcium influx and the KChIP3 subunit to regulate A-type current (I\textsubscript{A}) in stellate cells. Specifically, Ca\textsubscript{3} calcium influx invokes a depolarizing shift in the voltage dependence of I\textsubscript{A} inactivation to decrease stellate cell excitability (Anderson et al., 2010a,b). This is important, as synaptically evoked decreases in [Ca\textsubscript{o}] in the molecular layer may affect the Ca\textsubscript{3}–K\textsubscript{4} interaction and change stellate cell excitability.

The present study tested the hypothesis that the Ca\textsubscript{3}–K\textsubscript{4} complex can respond to physiologically relevant decreases in [Ca\textsubscript{o}], that occur during synaptic activity to modulate cerebellar stellate cell output. We find that even subtle decreases in [Ca\textsubscript{o}], are detected by the Ca\textsubscript{3}–K\textsubscript{4} complex to increase stellate cell gain.
and maintain inhibitory control of Purkinje cells in the face of reduced synaptic efficacy. Moreover, the calcium-dependent decrease in stellate cell $I_{\text{S}}$ is rapidly evoked by repetitive climbing fiber input, indicating an active response of the Ca$_{3–}$K$_{4}$ complex to physiologically relevant stimuli. The Ca$_{3–}$K$_{4}$ complex thus acts as a novel calcium sensor to adaptively regulate inhibitory input in relation to local changes in the extracellular milieu that accompanies repetitive synaptic input.

**Materials and Methods**

**Animal Care.** Sprague Dawley rat pups were obtained from Charles River Laboratories and maintained according to guidelines of the Canadian Council for Animal Care.

**Slice preparation.** Cerebellar slices were prepared from male rats of postnatal day 15 (P15) to P24. All chemicals were obtained from Sigma unless noted otherwise. Briefly, rats were anesthetized with halothane and the cerebellum removed in artificial CSF (aCSF) composed of (in mM) 125 NaCl, 3.25 KCl, 1.5 CaCl$_2$, 1.5 MgCl$_2$, 25 NaHCO$_3$, and 25 glucose. The internal solution for current- and dynamic-clamp whole-cell recordings was (in mM): 120 NaCl, 3 NaHCO$_3$, 4.2 KCl, 1.2 KH$_2$PO$_4$, 1.5 CaCl$_2$, 1.5 MgCl$_2$, 10 n-glucose, and 10 HEPES, pH adjusted to 7.3 with NaOH. The internal solution for voltage clamp of Kv4.2 expressed in tSA-201 cells was as follows (in mM): 110 K-glucuronate, 30 KCl, 1 EtGTA, 5 HEPES, and 0.5 MgCl$_2$, pH adjusted to 7.3 with KOH.

**Recording solutions.** On-cell recordings for both stellate and Purkinje cells were performed with an internal electrolyte of HEPES-buffered aCSF containing the following (in mM): 150 NaCl, 3.25 KCl, 1.5 CaCl$_2$, 1.5 MgCl$_2$, 10 HEPES, and 20 n-glucose, pH 7.3 with NaOH. The internal solution for current- and dynamic-clamp whole-cell recordings was (in mM) 150 K-glucuronate, 0.1 EtGTA, 10 HEPES, 7 NaCl, and 0.3 MgCl$_2$, pH 7.3 with KOH, and 5 di-Tris-creatine phosphate, 2 Tris-ATP, and 0.5 Na-gluconate. Cells were washed with fresh media 1 h after and moved to 29°C for 2–3 d. For tSA-201 cell recordings, the extracellular solution contained (in mM) 120 NaCl, 3 NaHCO$_3$, 4.2 KCl, 1.2 KH$_2$PO$_4$, 1.5 CaCl$_2$, 1.5 MgCl$_2$, 10 n-glucose, and 10 HEPES, pH adjusted to 7.3 with NaOH. The internal solution for voltage clamp of K$_{4.2}$ expressed in tSA-201 cells was as follows (in mM): 110 K-glucuronate, 30 KCl, 1 EtGTA, 5 HEPES, and 0.5 MgCl$_2$, pH adjusted to 7.3 with KOH. To isolate $I_{\text{S}}$ using internal blockers, the internal solution also contained the following (in mM): 5 tetraethylammonium (TEA), 5 QX-314 bromide, 0.1 phenanthroline, 1 MK-801, and 0.1% BSA. To isolate $I_{\text{S}}$ in the presence of Ca$_{3}$-mediated calcium current ($I_{\text{CA}}$), the bathing medium contained the following (in mM): 0001 TTX, 5 TEA, and 2 CsCl. To isolate $I_{\text{S}}$ in stellate cells during climbing fiber stimulation, the internal solution was as follows (in mM): 100 CsCl, 10 HEPES, 2.5 MgCl$_2$, 10 EtGTA, with the addition of 5 QX-314 bromide, 5 EtGTA, 5 4-A P, 0.1 phenanthroline, 1 MK-801, and 0.1% BSA. To isolate spontaneous or mini IPSCs in Purkinje cells, the internal solution contained the following (in mM): 100 CsCl, 10 EtGTA, 10 HEPES, and 3 MgCl$_2$, pH 7.3 with CsOH. When perfusing a change in bath $[\text{Ca}^2+]_o$ the $[\text{Mg}^2+]_o$ was adjusted to yield a total divalent cation concentration of 3 mM. Except where noted, all recordings were performed in synaptic blockers that were bath applied after obtaining the initial seal: picrotoxin (50 µM), 6-aminopropionic acid (AP5; 25 µM), 6,7-dinitroquinoxaline (DNQX; 10 µM; Torcis Cookson), and (2S)-3-[(15-1-3-(4-dichlorophenyl)ethyl)amino-2-hydroxystyryl]-phenylmethyl phosphonic acid (GP55845; 1 µM; Torcis Bioscience). 2-Methyl-6-phenylethynyl pyridine hydrochloride (MPEP) (1 µM), 3,4-Dihydro-2H-pyran-2,2-biquolinol-7-yl)-(cis-4-methoxy-clohexyl)-methanone (JNJ-16259685) (1.5 µM), and AM-251 (2.5 µM) were used where indicated. Mouse monoclonal antibodies directed at either KChIP1 or KChIP3 isoforms (NeuroMab) were included in the internal electrolyte at 1:100 dilution.

**Electrophysiology.** Neuronal current- and voltage-clamp recordings were performed at 33–35°C and tSA-201 cell recordings at 21°C using a Multiclamp 700B amplifier and pClamp 10.1 software (Molecular Devices). Borosilicate-etched (1.5 mm outer diameter) had a resistance of 6–8 MΩ with access resistance 8–15 MΩ (80–90% compensation in voltage clamp). Cells were rejected if access resistance drifted by >20%. Climbing fibers were stimulated with a monopolar stimulating electrode placed in the granule cell layer. A calculated junction potential of −10.7 mV in current-clamp recordings was subtracted from all data, while a negligible junction potential for voltage-clamp recordings was not subtracted. For on-cell recordings of Purkinje cell firing frequency, the pipette was held at a command potential of −40 mV to promote spontaneous spike discharge.

**Dynamic Clamp.** Dynamic-clamp recordings used a National Instruments BNC-2090 Digital/Analog converter and a computer running 64-bit OpenSuSE 11.1 with a real-time kernel. Real-time Experimental Interface (Dorval et al., 2001) was used for data recording and real-time calculations of injected conductances using voltage samples collected at 40 kHz. A measured junction potential of −10.7 mV was subtracted from all recordings and taken into account for conductance calculations. Custom scripts were used to simulate K$_{4}$ currents. To adjust the biophysical properties of the K$_{4}$ current under control and low $[\text{Ca}^2+]_o$ conditions, two K$_{4}$ currents were jointly simulated using the Hodgkin–Huxley formalism, and current calculations were made based on somatic voltage. The two currents differed only by their $V_{\text{h}}$ of inactivation, which was determined using the following equation:

$$ V_{\text{h}} = \frac{-81.12 + 67.96}{1 + \frac{1}{0.098}} - 67.96 $$

where $[\text{Ca}^2+]_o$ is extracellular calcium concentration in mM.

One of the K$_{4}$ currents ($I_{\text{K,exp}}$) had a $V_{\text{h}}$ of inactivation corresponding to the experimental $[\text{Ca}^2+]_o$ while the other current ($I_{\text{K,mod}}$) had a $V_{\text{h}}$ determined by a hypothetical $[\text{Ca}^2+]_o$. The difference between $I_{\text{K,exp}}$ and $I_{\text{K,mod}}$ represented the change in $I_{\text{S}}$ due to changes in calcium and corresponding physiological shifts in $V_{\text{h}}$. The difference current was injected at the somatic level, adjusting the biophysical parameters of the neuronal current online. To ensure the conductance density was within a physiological range, cell capacitance was first measured and then used to estimate cell surface area. This area was then multiplied by 42.6 pA/pF, the average conductance density of K$_{4}$ channels in stellate cells. Conductance density was then adjusted to within 10% of this estimate.

**Spike threshold.** The spike threshold was detected using a custom Matlab script by conducting a first derivative analysis of the voltage record to identify the absolute voltage at which instantaneous velocity was 3 SDs greater than the velocity of baseline voltage changes.

**Paired recordings.** For paired recordings, simultaneous whole-cell recordings were obtained under current clamp from stellate cells and voltage clamp from the soma of Purkinje cells, respectively. Cell pairs were obtained by first securing a whole-cell recording from a Purkinje cell and then a stellate cell contained within the expected span of the Purkinje cell dendritic arbor in the molecular layer directly above. In some cases stellate cells immediately adjoined the recorded Purkinje cell were verified through direct visualization using differential interference contrast optics. Stellate cells were held just below threshold at rest and spike output evoked using 1 s current pulses, while Purkinje cells were maintained at a holding potential of −65 mV to record IPSCs. In all paired recordings, excitatory synaptic transmission was blocked by bath-applied t-Ap5 (25 µM) and DNQX (10 µM) and inhibitory transmission by bath applied GPP55845 (1 µM).

**Spike-triggered averages (STAs) were calculated off-line in Matlab by time stamping the occurrence of stellate cell spikes and reverse correlating a period of 50 ms following the spike in the Purkinje cell record to extract postsynaptic events, with all events averaged in relation to presynaptic spike times. Failures were identified visually and were defined by...
a lack of a sharp, well-defined inward current of at least 5 pA within 5 ms of the spike peak.

Charge transfer for spontaneous IPSCs was determined by dividing each trace into sections (1 s) and summing the charge transferred for currents greater than one SD (of the control condition) above baseline. To compensate for long-term drift in the recordings, the mean was subtracted from each time section before analysis was performed. To calculate the charge transfer of single stellate cell IPSCs under different conditions, STAs were taken from dual recordings of connected stellate and Purkinje cells. STAs from each condition were fit with the sum of two exponentials of the form $y = Ae^{-t/\tau_{1}} + Be^{-t/\tau_{2}}$. For $1.5\text{mM }\text{Ca}^{2+}$, $A = 95\text{pA}$, $B = 85\text{pA}$, $\tau_{1} = 0.4\text{ms}$, and $\tau_{2} = 11\text{ms}$. For $0.1\text{mM }\text{Ca}^{2+}$, $A = 40\text{pA}$, $B = 37\text{pA}$, $\tau_{1} = 0.4\text{ms}$, and $\tau_{2} = 9\text{ms}$.

**Data and statistical analyses.** Voltage-clamp analysis using OriginLab consisted of fitting steady-state inactivation curves with Boltzmann functions of the following form:

$$Y = \frac{A_{1} - A_{2}}{1 + e^{\frac{x-x_{0}}{k_{B}}}} + A_{2}$$

Dose–response relationships were fit with the following Hill equation:

$$y = \frac{[x]^n}{k^n + [x]^n}$$

Spontaneous IPSC (sIPSC) frequency and amplitudes were measured using a custom Matlab program. Current traces were filtered and local minima and maxima were identified. A local current deviation was defined as a value determined to reliably identify IPSCs based on visual inspection. This method likely underestimates the frequency of sIPSCs and is sensitive to changes in the parameters (amplitude and velocity threshold). However, the ratio of frequencies and amplitudes among all three conditions was consistent for all sets of parameters.

Average values are indicated as mean ± SEM. Unless noted otherwise, all statistical tests were paired t-tests or one-way ANOVA with post hoc Tukey’s HSD test.

**Results**

The Cav3–Kv4 complex is modulated by a range of $[\text{Ca}^{2+}]_{o}$

Measurements of $[\text{Ca}^{2+}]_{o}$ in the cerebellar molecular layer in vivo have revealed a decrease in $[\text{Ca}^{2+}]_{o}$ from 1.2 mm to as low as 0.8 mm during 5 Hz climbing fiber stimulation (Stöckle and ten Bruggencate, 1978). We showed previously that blocking Cav3.3 calcium current causes a hyperpolarizing shift in the half-inactivation voltage ($V_{1/2}$) of stellate cell $I_{A}$, effectively reducing the availability of $I_{A}$ (Anderson et al., 2010b). However, the ability for the Cav3–Kv4 complex in stellate cells to shift in $[\text{Ca}^{2+}]_{o}$ is not known.

We measured the effects of bath perfusing various concentrations of $[\text{Ca}^{2+}]_{o}$ (0.1–2.2 mm) on the inactivation voltage of $I_{A}$ in stellate cells. $I_{A}$ was isolated pharmacologically by blocking sodium, hyperpolarization-activated cyclic nucleotide-gated cation (HCN), and noninactivating potassium currents through bath application of 100 mM TTX, 2 mM Cs+, and 5 mM TEA, respectively. Calcium channel blockers were specifically excluded to allow low voltage-activated Cav3-mediated calcium influx ($I_{CA}$) during voltage commands. Under these conditions, the primary current recorded is a LVA, fast inactivating $I_{A}$ (Fig. 1A). The $V_{0}$ of $I_{A}$ was determined using 1 s conditioning steps from $-110$ to 0 mV in 10 mV steps followed by a test pulse to $-30$ mV. A test pulse of $-30$ mV was used, as it corresponds to the peak of $I_{A}$ activation and is within the range of $I_{A}$ activation, but below that of high voltage-activated (HVA) calcium currents. In this regard, previous work has shown that stellate cells exhibit little HVA calcium current (Anderson et al., 2010b), and that none of the HVA calcium channels modulate the Kv4–KChIP3 interaction even when overexpressed in a heterologous system (Anderson et al., 2010b).

Under control conditions of 1.5 mM $[\text{Ca}^{2+}]_{o}$, stellate cells exhibit an $I_{A}$ with a $V_{0}$ of $-69.5 ± 1.9$ mV ($n = 7$). Decreasing bath $[\text{Ca}^{2+}]_{o}$ to 0.1 mM resulted in an approximately $-10$ mV hyperpolarizing shift in $V_{0}$ for $I_{A}$ to $-80.8 ± 2.0$ mV ($n = 7$) (Fig. 1A). In contrast, increasing $[\text{Ca}^{2+}]_{o}$ to 2.2 mm did not shift the $V_{0}$ of $I_{A}$ (Fig. 1B; $n = 5$, $p > 0.05$, unpaired t-test), indicating that the $V_{0}$ recorded under control conditions could not be further shifted in the depolarizing direction by increasing calcium conductance. As $[\text{Ca}^{2+}]_{o}$ was decreased to levels between 1.5 and 0.1 mM $[\text{Ca}^{2+}]_{o}$, the $V_{0}$ exhibited a graded leftward hyperpolarizing shift, reaching a stable saturation point of $-80.1 ± 1.2$ mV at $0.8$ mM $[\text{Ca}^{2+}]_{o}$ ($n = 5$, $p < 0.001$; Fig. 1B). The relationship between $V_{0}$ and $[\text{Ca}^{2+}]_{o}$ was readily fit with the Hill equation ($k = 1.1 ± 0.1$ mm; Hill coefficient, $N = 8.3 ± 2.4$) with a midpoint of $-75.3 ± 1.3$ mV at 1 mM $[\text{Ca}^{2+}]_{o}$ ($n = 6, p < 0.05$; Fig. 1B). The effects of low $[\text{Ca}^{2+}]_{o}$ on $I_{A}$ availability were not due to a change in divalent charge screening,
as changes in [Ca]o were always offset by a corresponding change in [Mg]o, to yield a total divalent cation concentration of 3 mM. We further confirmed that the mean V0 of Ia in stellate cells was not significantly different in 1.1 mM [Ca]o if [Mg]o was not elevated (n = 5, p > 0.05, unpaired t-test; data not shown), and that elevating [Mg]o by 0.4 mM did not affect Ca3.3 calcium current measured in tsA-201 cells (n = 7; data not shown). The effects of decreasing [Ca]o on K4 Vh are thus interpreted as a decrease of calcium influx through Ca3.3 T-type calcium channels.

To further examine the relationship between [Ca]o and the Ca3–K4 interaction, we recorded K4.2 current in tsA-201 cells coexpressing cDNA for Ca3.3, K4.2, KChIP3, and DPP10c as a representative complement of the Ca3–K4 complex in stellate cells (Anderson et al., 2010b). tsA-201 cells were bathed in an external medium containing 1.5 mM [Ca]o at rest. Under these conditions, the K4.2 Vh was −57.4 ± 0.6 mV (n = 30), a baseline value lower than that encountered in stellate cells, but not unexpected for differences in absolute voltage dependence when comparing cDNA expressed in a heterologous system. Importantly, varying [Ca]o between 0.1 and 2.2 mM again shifted the voltage dependence of K4 inactivation over a range of ~10 mV, similar to that found in stellate cells (Fig. 1C). K4.2 Vh reached a maximum hyperpolarized value of −66.5 ± 1.3 mV (n = 6) for a [Ca]o of 0.8 mM, with a Vh of −62.9 ± 0.9 mV at 1.1 mM [Ca]o (n = 5; k = 1.2 ± 0.1 mV; N = 11.2 ± 5.4). Importantly, the Ca3–K4 interaction has been shown to depend on KChIP3, an accessory calcium-sensing protein of the K4 complex (Anderson et al., 2010b). When cDNA encoding for KChIP3 proteins was omitted from the transfection medium, the dose–response relationship between [Ca]o and K4.2 Vh was abolished, with K4.2 Vh remaining stable at approximately −70 mV for all values of [Ca]o (Fig. 1C). These data indicate that the effects of a decrease in [Ca]o on Ia availability derive at least in part from an interaction between Ia and KChIP3. Our understanding of the interaction between Ca3 and K4 channels suggests that the dose–dependent response of K4 Vh to [Ca]o reflects a shift in Vh due to a calcium-dependent KChIP3 interaction. The prediction is that K4 current density should plateau once all KChIP3 proteins are maximally bound as calcium influx increases with [Ca]o. To test this we measured the peak density of Ia under different [Ca]o (Fig. 1D). Ia density was assessed with Ia left intact, and then Ia was isolated in the same cell by bath application of 4-AP (20 mM) and evoked using the same step command (Fig. 1D). Control tests established that 20 mM 4-AP has no effect on the current–voltage relationship of either Ca3.2 or Ca3.3 channels, the two Ca3 channel isoforms expressed by stellate cells (n = 6; data not shown) (Molineux et al., 2006). While Ia could be recorded over a range of 0.8–2.2 mM [Ca]o, Ia could not be reliably resolved below 1.1 mM [Ca]o, restricting Ia density measurements to 1.1–2.2 mM [Ca]o. These tests showed that Ia peak density exhibited a linear increase over the full range of 1.1 to 2.2 mM [Ca]o, as predicted by an increased driving force and the number of permeant ions as [Ca]o was raised. In contrast, Is peak density followed the shift in Vh established for different levels of [Ca]o, in Figure 1B, reaching a plateau of maximal density for [Ca]o levels of 1.5 mM or greater (Fig. 1D).

These data establish that K4 availability can be modulated within the range of shifts in [Ca]o that have been reported to occur under physiological conditions in vivo. The results also indicate that reductions in [Ca]o will decrease Ca3.3-mediated calcium influx and reduce the availability of K4 current in a manner consistent with an action through the Ca3–K4 complex.

### Climbing fiber input decreases stellate cell Ia and IA

To be physiologically relevant, the low calcium-induced change in K4 availability should be reproduced by direct activation of synaptic inputs in the molecular layer. In this regard, climbing fibers present an especially interesting case for potential regulation of the Ca3–K4 complex. Climbing fiber boutons have been shown to overlap with the soma and dendritic processes of stellate cells (Nishiyama et al., 2007; Brown et al., 2012), while ultrastructural analyses reveal boutons that are tightly apposed to stellate cell membranes, but without evidence for vesicular release machinery (Hámori and Szentágothai, 1980; Kollo et al., 2006). In fact, the locations at which climbing fiber boutons align with stellate cells closely matches the distribution pattern of K4 channel immunolabel in stellate cell membranes, a highly unusual relationship for which there was no known function (Kollo et al., 2006). Although the distribution of Ca3 channels has not been determined at the ultrastructural level, the known association between Ca3 and K4 channels within a functional nanodomain (<50 nm) (Anderson et al., 2010b) suggests that Ca3 channels will be found in close proximity to some number of the K4 channels at these junctions. Climbing fiber activation is further interesting in reportedly generating a reduction in [Ca]o (Stöckle and ten Bruggencate, 1980) in the course of generating a calcium-dependent complex spike in Purkinje cells that conducts throughout the dendritic tree (Llinás and Sugimori, 1980b; Kitamura and Häusser, 2011), where stellate cells are embedded.

### Sensing changes in calcium driving force

We wished to test whether repetitive climbing fiber stimulation could evoke a decrease in [Ca]o, that in turn alters the availability of Ia through the Ca3–K4 interaction. To date, the only measure of a change in [Ca]o during climbing fiber stimulation was obtained using calcium-selective microelectrodes in vivo (Heinemann et al., 1977; Nicholson et al., 1978; Stöckle and ten Bruggencate, 1980; McCrery and Agnew, 1983). The ability to evoke a decrease in [Ca]o in vitro during repetitive synaptic input must also contend with the technical necessity of perfusing a fixed level of calcium in the bathing medium. We note, however, that the recordings of Ia in stellate cells in Figure 1D indicate that a change in [Ca]o in the bathing medium can be detected as a change in driving force, as reflected by a linear reduction in Ia density as [Ca]o is lowered, with an ~30% decrease in Ia when [Ca]o was lowered from 1.5 to 1.1 mM. We thus tested whether repetitive climbing fiber stimulation could decrease Ia in a manner consistent with a change in the driving force for calcium. In this way we could effectively use the stellate cell itself as a delicate sensor for any changes in [Ca]o in the surrounding microenvironment during in vitro recording conditions.

To examine the effect of climbing fiber stimulation on Ia in stellate cells, we simultaneously recorded from the soma of a Purkinje cell and that of a stellate cell visualized directly above in the molecular layer, and thus presumably within or near the dendritic arbor of the recorded Purkinje cell. In some cases this could be confirmed by tracking the course of a Purkinje cell dendritic tree through DIC-IR optics to the position of the targeted stellate cell. We then stimulated climbing fiber input to simultaneously monitor complex spike discharge in the Purkinje cell and Ia in the stellate cell. Ia was isolated by including in the electrode 5 mM QX-314 to block sodium channels and 5 mM 4-AP and 5 mM TEA to block Ia and any delayed rectifying potassium channels. Although internal QX-314 can affect some calcium currents (Talbot and Sayer, 1996), we confirmed that we could record Ia with a stable baseline amplitude of 8.4 ± 1.9 pA (n = 6; maxi-
mum 20 pA) that reliably recovered to control levels following trains of synaptic stimulation. Blockade of any remaining potassium channels was ensured by using a CsCl-based internal electrolyte. Synaptic activation of stellate cells (Jörntell and Ekerot, 2003; Szapiro and Barbour, 2007; Barmack and Yakhnitsa, 2008) was blocked by internal perfusion of 100 μM phosphatidylcholine to block AMPA receptors (Nilsen and England, 2007) and 1 mM MK-801 to block NMDA receptors, and bath application of 1 mM MPEP, 1.5 μM INJ-16295685, and 2.5 μM AM-251 to block potential activation of group I metabotropic glutamate or CB1 receptors (Gatley et al., 1996; Gasparini et al., 1999; Crepel and Daniel, 2007; Knöpfel, 2007). Under these conditions, the majority of synaptic currents were blocked with only occasional fast spontaneous synaptic currents in four of six cells (mean of −57.8 ± 20.0 pA amplitude; n = 4). Spontaneous synaptic events were not associated with climbing fiber stimulation and were readily distinguishable from I_{T}, but any records with overlapping spontaneous synaptic currents were discarded. In this way, I_{T} could be recorded in stellate cells while preserving climbing fiber-evoked complex spike discharge in nearby Purkinje cells.

Voltage pulses were delivered to stellate cells from a holding potential of −110 to −45 mV (50 ms) at a 5 Hz base rate (Fig. 2A) to measure I_{T} near its peak voltage on I-V plots. After 2.5 s of control recording, a climbing fiber stimulus was interleaved with step commands by stimulating at 10 Hz (10 pulses), and recordings of I_{T} continued for at least 5 s after stimulation was terminated. Data from recordings were accepted when I_{T} density was sufficient to evoke a clearly resolved transient inward current, a complex spike was reliably evoked in the Purkinje cell, and recovery of I_{T} peak amplitude was apparent after synaptic stimulation. I_{T} peak amplitude was stable during control pulses, but declined rapidly during climbing fiber stimuli (Fig. 2A), reducing from an original peak value of 8.4 ± 1.9 pA to 5.5 ± 1.8 pA (n = 6) by the end of the 10 pulse stimulus train, reflecting a 42.2 ± 11.8% decrease in amplitude (n = 6, p < 0.05, one-way ANOVA, Tukey’s pairwise comparison). Within 1 s following a stimulus train, I_{T} peak amplitudes had recovered to 8.2 ± 1.5 pA (n = 6, p = 0.98), a level that was not significantly different from control. Full recovery was apparent by 5 s (8.45 ± 1.92 pA, n = 6).

A reduction in I_{T} density could come about through a change in Ca_{3,3} channel insertion in the membrane, a reduction in conductance through the action of a ligand or a shift in voltage-dependent properties, or a reduction in calcium driving force. It seems highly unlikely that the density of Ca_{3,3} channels in the plasma membrane could be reduced and restored to control levels within ~1 s time in relation to climbing fiber stimulation. We note that we cannot fully exclude the possibility that an unknown ligand (such as that producing occasional spontaneous synaptic events observed in stellate cells under our conditions) could have affected Ca_{3,3} channel conductance, although we did not detect any synaptic responses evoked by climbing fiber stimuli per se. We also have no evidence to support a shift in the voltage dependence of Ca_{3,3} channels, a parameter that would be difficult to test in the short time frame associated with changes in I_{T} density recorded here. Rather, the results presented in Figure 1D indicate that even subtle decreases in [Ca\textsubscript{o}] are sufficient to substantially alter I_{T} density, and in a linear fashion as predicted for a shift in calcium driving force. In fact, comparing the 40% mean reduction in I_{T} density recorded during climbing fiber stimulation (Fig. 2D) to that recorded during bath perfusion of different levels of [Ca\textsubscript{o}] predicts a potential climbing fiber-induced change in [Ca\textsubscript{o}] of 0.63 mM (from 1.5 mM down to 0.87 mM). These data are important in revealing that climbing fiber stimulation reduces I_{T} in a manner consistent with a decrease in [Ca\textsubscript{o}] in the microenvironment surrounding stellate cells even under in vitro recording conditions.

Stimulus-evoked reductions in I_{A} 
We next repeated these tests to examine the effect of repetitive climbing fiber stimulation on the amplitude of I_{A} in stellate cells. We thus obtained dual recordings from the soma of a Purkinje cell and a stellate cell positioned directly above in the molecular layer. I_{A} was isolated in stellate cells by including 5 mM QX-314 and 5 mM TEA in the electrode using a KCl-based internal electrolyte, and complex spike discharge was recorded in the Purkinje cell in current-clamp
mode (Fig. 3A). The same set of internal and external synaptic blockers were applied as for recordings of \( I_{A} \). In a separate set of experiments, we verified that QX-314 had no significant effect on the magnitude of the shift in \( K_{A} \) \( V_{o} \) induced by perfusing 1.1 mm [Ca]o \( (n = 6, p > 0.05) \). The channel was also stable during the control pulses, but showed a progressive decrease during climbing fiber evoked complex spike discharge in a nearby Purkinje cell, reaching an average 15.8 ± 3.2% reduction after 10 stimuli (maximum 30%; \( n = 10, p < 0.001 \); Fig. 3A). At the end of the climbing fiber stimulus train, \( I_{A} \) amplitude gradually recovered, returning to 92.3 ± 1.6% of its original value by 2.4 s \( (n = 10, p < 0.01) \), and was fully recovered at 5 s to 1.0 ± 0.02 of control \( (p = 8, p > 0.05) \); Figs 3A–C). Moreover, the reduction in \( I_{A} \) could be evoked in a reversible manner on repeated occasions at 5 min intervals, ruling out any significant washout over time.

Based on the \( V_{o} \) [Ca]o dose–response curve (Fig. 1B), we predicted that conducting these tests in 2.2 mm [Ca]o should prevent significant \( V_{o} \) shifts by offsetting any reduction in [Ca]o during climbing fiber stimulation. Therefore, in a subset of cells, we subsequently bath applied 2.2 mm [Ca]o \( (n = 4 \) of 10) for 5 min and retested the effects of climbing fiber stimulation on \( I_{A} \) amplitude. No significant decrease in \( I_{A} \) was detected in the presence of 2.2 mm [Ca]o \( (0.8 ± 2.2\% \) decrease, \( n = 4, p = 0.95 \); Fig. 3B–D), even though the preceding trains in this group of cells decreased \( I_{A} \) by 10.6 ± 2.9% when delivered in 1.5 mm [Ca]o \( (n = 4, p < 0.05) \).

Collectively, these results are consistent with the interpretation that repetitive stimulation of complex spike discharge in Purkinje cells promotes a decrease in the driving force of Cav3–Kv4 complex, which is accompanied by a reduction in the availability of \( I_{A} \) in stellate cells, as predicted for actions of the Cav3–Kv4 complex.

**Physiological changes in [Ca]o alter stellate cell excitability**

Outward current generated by \( I_{A} \) is capable of controlling several aspects of spike output, including spike latency, threshold, and frequency (Connor and Stevens, 1971a, b; Hoffman et al., 1997; Molinex et al., 2005; Khalilq and Bean, 2008; Anderson et al., 2010b; Norris and Nerbonne, 2010; Norris et al., 2010; Carrasquillo et al., 2012). We next examined the effect of decreasing [Ca]o, from a resting level of 1.5 to 1.1 mm on firing frequency using on-cell recordings to avoid cell dialysis inherent to whole-cell recordings. Stellate cells exhibited a resting level of spontaneous discharge between 5 and 10 Hz \( (6.6 ± 0.5 \) Hz, \( n = 35) \) in 1.5 mm [Ca]o. Decreasing [Ca]o, to 1.1 mm resulted in a nearly two-fold increase in the rate of spontaneous discharge \( (n = 6) \) that was reversible when [Ca]o was returned to 1.5 mm (Fig. 4A). It is possible that the observed elevation in firing frequency in 1.1 mm [Ca]o could involve a decreased activation of calcium-dependent potassium channels. However, neither the SK channel blocker apamin \( (100 \) nm, \( n = 6, p > 0.05) \) nor the BK channel blocker paxilline \( (100 \) nm, \( n = 5, p > 0.05) \) affected the rate of spontaneous on-cell firing frequency.

A second issue to consider is that repetitive activation of synaptic inputs also elevates the extracellular concentration of potassium ([K]o). In the molecular layer, 5 Hz climbing fiber fiber discharge evoked by peripheral nerve stimulation in vivo produces an increase in [K]o of ~0.7 mm from resting conditions that could affect \( E_{K} \) and the driving force for \( I_{A} \) (Bruggencate et al., 1976; Stöckle and ten Bruggencate, 1980). However, we found that a 0.75 mm elevation of [K]o did not significantly alter either the rate of on-cell spike discharge \( (n = 6, p > 0.05) \) or the amplitude of \( I_{A} \) \( (n = 10, p > 0.05) \). A combined change in [Ca]o and [K]o reduced \( I_{A} \) amplitude \( (n = 6, p < 0.05) \), but it was not significantly different from that of 1.1 mm [Ca]o alone \( (n = 6, p > 0.05) \). These tests reveal that the increases in [K]o reported during physiological levels of climbing fiber stimulation in vivo are not sufficient to account for the effects detected here for associated decreases in

![Figure 3](image-url)
crease in gain was not related to any secondary regulation of internal heparin. 

Internal calcium stores, as the gain of stellate cell firing increased in a similar manner in the presence of internal heparin.

We showed previously that pharmacological blockade of Cav3 calcium channels and its interaction with the K,4 channel complex increased the gain of firing in stellate cells (Anderson et al., 2010b). We next determined the effect of different levels of [Ca]o on stellate cell gain used whole-cell recordings. Applying a decrease in [Ca]o from 1.5 to 1.1 mM to reduce I_A availability revealed an ~65% increase in the gain of stellate cell firing on current–frequency plots (n = 5, p < 0.001; Fig. 4B, C). The increase in gain was not related to any secondary regulation of internal calcium stores, as the gain of stellate cell firing increased in a similar manner in the presence of internal heparin (4 mg/ml) and bath-applied 6 µM cyclopiazonic acid to block any IP3-mediated calcium release and calcium ATPases (n = 8, p < 0.05). Pretreating slices with the Ca,3 channel blocker mibebradil (500 nM) further occluded the effects of 1.1 mM [Ca]o on the gain of firing frequency (n = 8), supporting the interpretation that the increase in gain is mediated by Ca,3 calcium influx (Fig. 4C).

One means of interrupting the Ca,3–KChIP3 interaction is by internally infusing a monoclonal KChIP3 antibody (Anderson et al., 2010b). We thus infused KChIP antibodies (1:100 dilution) through the electrode to test the ability for low [Ca]o to induce a change in the gain of firing. Frequency–current plots were constructed at 10 min intervals for up to 30 min following entry in the whole-cell configuration to allow full dialysis of KChIP antibodies. These tests revealed that infusion of a KChIP3 antibody occluded the ability of 1.1 mM [Ca]o to increase the gain of firing (n = 7; Fig. 4C). By comparison, infusion of a monoclonal antibody against KChIP1 (1:100 dilution), a protein not expressed in stellate cells, did not prevent an increase in gain of firing by 1.1 mM [Ca]o (n = 7, p < 0.001; Fig. 4C).

A closer examination of the process of spike firing revealed that the increase in gain of firing in 1.1 mM [Ca]o was associated with a significant decrease in spike threshold voltage, with the point of membrane potential transition to spike output shifting in a negative direction by 4–10 mV (n = 5, p < 0.05; Fig. 4D). The increase in spike frequency in low [Ca]o could also be accompanied by a decrease in the amplitude of the AHP, although the magnitude of these changes were variable and not statistically significant (data not shown). The loss of I_A and outward current during the interspike interval in low [Ca]o can thus reduce spike threshold, identifying one factor contributing to the increase in firing frequency.

**The Ca,3–K,4 complex adjusts firing rate in response to fluctuations in [Ca]o.**

To determine the extent to which a selective shift in the V_h of stellate cell I_A can alter the gain or threshold of stellate cell firing, we applied dynamic clamp. For this we used the established biophysical properties of I_A and I_h in stellate cells (Molineux et al., 2005), the dose–response relationship between [Ca]o and I_h V_h values (Fig. 1B), and the inactivation curves in 1.5 versus 1.1 mM [Ca]o to estimate the h_∞ values reflecting I_A availability. These data were then used to iteratively simulate either condition of [Ca]o and dynamically modulate the I_A available at each voltage sampled during a spike response (25 µs sampling interval). In this way, a cell could be maintained in either 1.5 or 1.1 mM [Ca]o and dynamic clamp used to simulate a shift in I_A availability typical of a change in [Ca]o without altering the driving force for calcium entry. Practically, this equated to calculating the I_A predicted to be available for either [Ca]o and then injecting or subtracting the difference current (Fig. 5). Simulating a shift from an actual level of 1.5 mM [Ca]o in the bath to a “virtual” 1.1 mM [Ca]o thus predicted a net inward current that acted in a depolarizing
manner during the time course of an evoked spike (Fig. 5B). Conversely, if a cell was held in 1.1 mM [Ca]o, we simulated the condition relevant to Iia availability for a virtual increase of [Ca]o, to 1.5 mM by injecting an outward difference current during the course of a spike (Fig. 5C). Thus, the availability of Iia that would be characteristic of a given [Ca]o could be applied on-line without directly manipulating [Ca]o, or Iia.

The effects of dynamically modulating Iia availability are shown in Figure 6. Recordings were first performed in 1.5 mM [Ca]o and the gain of firing frequency measured using current pulses under conventional current clamp to construct a frequency–current plot. Subtraction of Iia using dynamic clamp to simulate a left shift in Vc relevant to 1.1 mM [Ca]o caused a substantial increase in gain of firing (n = 6, p < 0.05; Fig. 6A,C). We note that an additional increase in the initial rate of firing on the frequency–current plot was apparent for the dynamic clamp subtraction of Iia (Fig. 6A), likely reflecting the depolarizing action of Iia that remains intact under these conditions. Recordings from the same neurons were then performed in the presence of 1.1 mM [Ca]o in the bath to reduce the Ca3–K4 interaction and increase gain in the expected manner, as evident by an increased rate of firing and slope of the baseline frequency–current plot (Fig. 6B). Dynamic injection of Iia that would be expected in the presence of 1.5 mM [Ca]o, then restored excitability by decreasing the gain of firing to within control levels (n = 6, p < 0.05; Fig. 6B,C). Consistent with results in bath-applied low [Ca]o, a reduction of Iia via dynamic clamp for cells bathed in 1.5 mM [Ca]o also resulted in a significant decrease in spike threshold (n = 6, p < 0.01; Fig. 6C). Conversely, dynamic addition of Iia in cells perfused initially with 1.1 mM [Ca]o significantly increased spike threshold (n = 6, p < 0.05; Fig. 6C). Collectively, these results demonstrate that a selective shift in the Vc for Iia (without affecting calcium conductance directly) is sufficient to account for the changes in gain of stellate cell firing during fluctuations of [Ca]o again implicating a key role for the Ca3–K4 complex.

The Ca3–K4 complex maintains inhibitory synaptic efficacy during reductions of [Ca]o

Synaptic transmission depends critically on calcium influx into presynaptic terminals to cause vesicle fusion and transmitter release. Any change in [Ca]o would then be expected to reduce release probability within the local environment. This is important, as a decrease in release probability could reduce the net inhibitory charge transferred to Purkinje cells when excitatory synaptic input lowers [Ca]o. We thus tested the effects of low [Ca]o on synaptic transmission between stellate and Purkinje.
had variable amplitudes, attributed to synaptic events occurring at high frequency and originating from varying sites within the dendritic tree that lead to overlap and summation (Fig. 7C). Counter to our predictions, bath application of 1.1 mM [Ca]o substantially increased the proportion of large-amplitude sIPSCs (n = 7, p < 0.001, Kolmogorov–Smirnov test; Fig. 7D). Furthermore, the measured value of sIPSC frequency was not significantly different between 1.5 mM [Ca]o (26.6 ± 2.6 Hz, n = 7) and 1.1 mM [Ca]o (25 ± 4.7 Hz, n = 7, p > 0.05; Fig. 7E). Subsequent application of picrotoxin (50 µM) blocked nearly all synaptic currents (Fig. 7D,E), indicating that the majority of sIPSC activity was mediated by GABA_A receptors.

In considering these results, we noted that large-amplitude events that may constitute simultaneous input from multiple sources would be difficult to distinguish from single events, potentially decreasing sIPSC frequency and increasing the measured proportion of large-amplitude events. We also reasoned that because probability of release decreased in 1.1 mM [Ca]o (Fig. 7C), mIPSCs recorded from a Purkinje cell in the presence of 200 nM TTX to block spike-associated transmitter release from presynaptic axons were considered to be derived from other stellate or basket cells synapsing on the recorded Purkinje cell. Cell pairs that exhibited correlations between presynaptic spikes and eIPSCs were encountered with a success rate of 5/42 cells, as judged in previous studies (King et al., 2001).

Based on these results, we predicted that sIPSCs would show a decrease in amplitude and frequency due to the decreased probability of release. We recorded sIPSCs in Purkinje cells in the presence of DNQX and D-AP5 to block excitatory synaptic events and CGP55845 to block GABA_A receptors. The use of a high CsCl internal solution to block potassium currents reversed the chloride gradient, revealing sIPSCs as inward current deflections. sIPSCs recorded in the slice preparation at 1.5 mM [Ca]o, cells. To understand the effects of low [Ca]o on the probability of transmitter release, we recorded miniature IPSC (mIPSC) frequency in either 1.5 or 1.1 mM [Ca]o, in the presence of TTX (200 nM) to block all spike-associated transmitter release from presynaptic cells. Shifting from 1.5 to 1.1 mM [Ca]o lowered mIPSC frequency from 15 ± 2.9 Hz to 9.7 ± 1.5 Hz (n = 10, p < 0.001), reflecting an ∼35% decrease in release probability at inhibitory synapses onto Purkinje cells (Fig. 7A,B), which is consistent with previous studies (King et al., 2001).

To more closely examine the relationship between presynaptic spike discharge and postsynaptic inhibition in different levels of [Ca]o, we performed dual recordings of synchronously connected stellate and Purkinje cells (Fig. 8). Presynaptic spikes in the recorded stellate cell that were correlated with postsynaptic IPSCs were identified by detecting an IPSC of at least 5 pA within 5 ms of a presynaptic spike peak (Fig. 8A). Spike-associated IPSCs were referred to as evoked IPSCs (eIPSCs) and used to create a spike-triggered average. If a presynaptic spike was not followed by an IPSC, it was taken to reflect a failure of presynaptic transmitter release. Finally, IPSCs with no clear association with spike discharge in the recorded presynaptic stellate cell were considered to be derived from other stellate or basket cells synapsing on the recorded Purkinje cell. Cell pairs that exhibited correlated presynaptic spikes and eIPSCs were encountered with a success rate of ∼10–15% (n = 5/42 cells), as judged in post hoc analysis of spike-triggered responses in Purkinje cells. To improve calculations of the net influence of presynaptic spike discharge on postsynaptic inhibition in low [Ca]o, stellate cells were held below threshold to prevent spontaneous discharge, and 20 current pulses were delivered to evoke repetitive firing (5–8 pA, 1 s duration, 5 s intervals; Fig. 8A).
Spike-triggered averages were calculated both with and without transmission failures to determine the effect on lowering \([Ca]_o\) on eIPSCs. When failures were not included, eIPSC amplitudes decreased from \(-92.8 \pm 4.1\) pA in 1.5 mM \([Ca]_o\) to \(-62.8 \pm 10.6\) pA in 1.1 mM \([Ca]_o\) \((n = 5, p < 0.05; \text{Fig. 8B, top traces})\). When all postsynaptic responses (regardless of failure) were included in the average as a measure of overall inhibitory influence, it was revealed that lowering \([Ca]_o\) decreased the net amplitude of spike-related postsynaptic inhibitory events from \(-62.8 \pm 10.6\) pA in 1.5 mM \([Ca]_o\) to \(-15.6 \pm 4.6\) pA 1.1 mM \([Ca]_o\) \((n = 5976\) spikes over 5 cells, \(p < 0.01; \text{Fig. 8B, right, bottom traces})\). This large decrease in eIPSC amplitude was due to an increase in failure rate at the stellate cell to Purkinje cell synapse from 34.6 \pm 7.7\% in 1.5 mM \([Ca]_o\) to 62.8 \pm 8.0\% in 1.1 mM \([Ca]_o\) \((n = 5, p < 0.01; \text{Fig. 8C})\).

The increase in failure rate of stellate cell synaptic transmission in 1.1 mM \([Ca]_o\) would predict that the level of stellate cell-mediated inhibition will be diminished during repetitive synaptic input that decreases \([Ca]_o\) \textit{in vivo}. On the other hand, the gain of stellate firing is increased in 1.1 mM \([Ca]_o\) through the action of the Ca\(_{3–4}\) complex, suggesting a mechanism to counteract decreased synaptic efficacy at stellate to Purkinje cell synapses. Indeed, the average number of spikes evoked by a 1 s current step in stellate cells increased from 10.9 \pm 1.3 spikes in control to 24.6 \pm 3.3 spikes in 1.1 mM \([Ca]_o\) \((\text{Fig. 8D}; n = 5, p < 0.01)\). We thus examined the combined effects of an increased failure rate of synaptic events, decreased IPSC amplitude, and an increase in spike count on the overall charge transfer. Charge transfer was compared under three conditions: normal gain of stellate cell synaptic events, decreased IPSC amplitude, and an increase in spike count on the overall charge transfer. Charge transfer was calculated by multiplying the area under the simulated IPSCs for 1.5 and 1.1 mM \([Ca]_o\) \((n = 461)\), charge transfer in 1.1 mM \([Ca]_o\) was calculated as the product of spike count in D and fits (inset) to the mean eIPSCs shown in lower records of B. Charge transfer in 1.1 mM \([Ca]_o\) was calculated for both the actual spike count and for a hypothetical situation (1.1 hypoth) where spike count did not change from that recorded in 1.5 mM \([Ca]_o\). Bar plots of spike and eIPSC properties from identified stellate–Purkinje cell pairs in 1.5 and 1.1 mM \([Ca]_o\).

With an average of 11 spikes for a 1 s current step in 1.5 mM \([Ca]_o\) \((n = 5)\), charge transfer was predicted to be 9.7 \pm 1.2 pC \((\text{Fig. 8E})\). Repeating these calculations for 1.1 mM \([Ca]_o\) revealed that spike train charge transfer was maintained in the lower calcium condition when the number of spikes increased to 25 spikes/s \((7.8 \pm 1.0\ pC, n = 5, p > 0.05; \text{Fig. 8E})\). However, in a hypothetical situation where the number of spikes per unit time was unchanged in 1.1 mM \([Ca]_o\) from that in 1.5 mM \([Ca]_o\) (11
spikes/s), the predicted spike train charge transfer was greatly reduced to 3.4 ± 0.4 pC (n = 5; Fig. 8D), a value that is significantly different from both the 1.5 and 1.1 mm [Ca]o conditions (p < 0.01 for both comparisons). Collectively, these data indicate that decreased synaptic efficacy in low [Ca]o is overcome by way of a Ca3–K,4 complex-mediated increase in stellate cell gain of firing.

**An adaptive response of the inhibitory network restrains Purkinje cell firing**

These results would predict that at the network level, the rate of Purkinje cell firing should be maintained in homeostatic manner despite fluctuations in extracellular ionic concentrations during synaptic input. To directly test this, we used on-cell recordings to monitor the rate of spontaneous Purkinje cell firing in response to modulations of [Ca]o, K, characteristic of physiologically relevant inputs. In all of these recordings, DL-AP5 (25 μM) and DNQX (10 μM) were included in the bath to block excitatory synaptic transmission. Cells were recorded from animals at P18–P20 to reduce the probability of inducing trimodal activity (McKay and Turner, 2005), and were excluded if trimodal activity was detected. Cells were recorded in an initial control solution of 1.5 mm [Ca]o, and then perfused with 1.1 mm [Ca]o, and finally 50 μM picrotoxin to block all GABAAergic inputs to assess the relative influence of the inhibitory network on Purkinje cell firing rate under each condition. In 1.5 mm [Ca]o, Purkinje cells discharged at a rate of 43.6 ± 7.8 Hz (n = 8), with no significant increase in firing rate in the presence of lowered [Ca]o (Fig. 8F).

Perfusing picrotoxin on cells maintained in 1.5 mm [Ca]o revealed a modest but significant mean increase in firing rate of ∼5 Hz. However, in the presence of 1.1 mm [Ca]o, picrotoxin unmasked a significant increase in Purkinje cell firing of ∼20–25 Hz over the baseline rate of firing (p < 0.05; Fig. 8F). To fully test the influence of ionic factors that can change during repetitive synaptic inputs, these tests were repeated for exposures to both 1.1 mm [Ca]o and 4.0 mm [K]o, with the same results (data not shown).

These data indicate that the increase in activity of inhibitory interneurons in 1.1 mm [Ca]o acts to lower the excitability and firing rate of Purkinje cells in the order of ∼40% compared to the level that would be encountered without the inhibitory network. The reason for the increase in firing rate of Purkinje cells in the absence of GABAAergic inhibition was not fully determined here. However, it does not relate to a Ca3–K,4 interaction in Purkinje cells, as rat Purkinje cells have not been found to express K,4 current (our unpublished observations). It could instead be due to an increase in excitability caused by decreased activation of calcium-dependent AHPs in Purkinje cells (Hosy et al., 2011; Engbers et al., 2012). These data are then consistent with a compensatory increase in the firing rate of stellate cells via the Ca3–K,4 complex to suppress an increase in Purkinje cell excitability that would occur in low [Ca]o. This adaptive form of inhibitory control then acts in a homeostatic fashion to limit nonspecific increases in Purkinje cell excitability during repetitive afferent activity.

**Discussion**

Early measurements of [Ca]o with ion-selective electrodes reported that peripheral stimulation of afferent inputs to cerebellum rapidly reduce [Ca]o in the order of 0.4 mm or more in the molecular layer (Stöckle and ten Bruggencate, 1980). Subsequent studies established that even minimal rates of spike invasion and transmitter release reliably deplete [Ca]o in a synaptic cleft. Thus, experiments employing ion-selective electrodes (Heinemann et al., 1977; Nicholson et al., 1978; Stöckle and ten Bruggencate, 1978; Torres et al., 2012), dual patch recordings (Borst and Sakmann, 1999; Stanley, 2000), two-photon imaging (Rusakov and Fine, 2003), and modeling (Egelman and Montague, 1999; Wiest et al., 2000; King et al., 2001) all report measureable decreases in [Ca]o, or its predicted consequence on presynaptic and postsynaptic responses during physiological rates of activation. The direct recordings of ITP in stellate cells provided here now confirm that repetitive climbing fiber stimulation reduces the driving force for calcium in a manner consistent with a decrease in [Ca]o.

Stimulus-induced reductions in [Ca]o seem counterintuitive in that the efficacy of transmitter release will be reduced and circuit function altered in the act of processing incoming signals. The mechanism by which cerebellar circuit function is maintained during stimulus-induced changes in membrane currents had not been identified. Our data now provide evidence that the Ca3–K,4 complex alters stellate cell excitability to maintain inhibitory charge transfer to Purkinje cells during a reduction in [Ca]o.

**The Ca3–K,4 complex as a calcium sensor**

Our tests in both stellate cells and tsA-201 cells reveal that decreases in [Ca]o of 0.4 mm are sufficient to shift the V50 of A-type current toward negative potentials, reducing the availability of K,4 current. Indeed, the steepest component of a dose–response curve for [Ca]o, IA, was situated directly within the range of [Ca]o changes reported in vivo (Nicholson et al., 1978; Stöckle and ten Bruggencate, 1978, 1980). Both the shifts in V50 and increases in gain of stellate cell firing were KChIP3-dependent, as expected for the Ca3–K,4 interaction. These data indicate that the Ca3–K,4 complex can effectively act as a novel sensor for [Ca]o.

Calcium sensors that can influence neuronal excitability have been reported previously. Nevertheless, each of these differ from the calcium sensing capabilities of the Ca3–K,4 complex. A calcium-sensing receptor (CaSR) is widely expressed (Ruat et al., 1995; Washburn et al., 1999; Bandyopadhyay et al., 2010), but CaSR immuno label is not detected in stellate cells (Ruat et al., 1995), and activation of CaSR requires an increase in [Ca]o (Washburn et al., 1999; Lu et al., 2010). A sodium-conducting leak channel (NALCN) can increase neuronal excitability when [Ca]o is lowered (Lu et al., 2010), but the low calcium-induced increase in stellate cell firing is occluded by mibebradil (500 nm), which does not affect NALCN channels (Lu et al., 2007). TRPM7 channels activate in low [Ca]o but with an IC50 of 4.1 μM [Ca]o (Li et al., 2007; Wei et al., 2007), well below the range of [Ca]o tested here. Although low calcium-induced increases in excitability can involve charge-screening effects (Formenti et al., 2001), we minimized this by balancing the concentration of extracellular divalent ions with elevated [Mg2+]o. Furthermore, any charge-screening effects are predicted to occur at the extracellular face, while the effects here were occluded by internal infusion of a KChIP3 antibody. Therefore, the changes in stellate cell excitability when [Ca]o is decreased can be attributed to the Ca3–K,4 complex acting as a sensor for changes in [Ca]o.

**Inhibitory control of cerebellar output during changes in [Ca]o**

Stellate cells form an axonal network in the molecular layer that is highly effective in controlling Purkinje cell excitability and spike output (Callaway et al., 1995; Häsuer and Clark, 1997; Mann-
The effects of a decrease in $[\text{Ca}^{2+}]_o$ in the molecular layer on cell excitability and synaptic transmission could then disrupt cerebellar output. This could particularly be the case for climbing fiber input that activates a large-amplitude calcium-dependent complex spike that conducts throughout the Purkinje cell dendritic tree (Llinás and Sugimori, 1980a, b; Kitamura and Häusser, 2011), providing a large postsynaptic sink for calcium from the extracellular space. In addition, glutamate spillover during high-frequency climbing fiber input can reduce GABA release from presynaptic terminals of molecular layer interneurons (Satake et al., 2000; Rusakov et al., 2005), while endocannabinoid release from Purkinje cells can reduce stellate cell firing (Kreitzer et al., 2002). It thus seems difficult to predict how inhibitory tone is maintained on Purkinje cells during repetitive climbing fiber input. Despite this, careful analyses of the role of cerebellar interneurons suggest that stellate cells are the primary elements mediating a climbing fiber-induced reduction of Purkinje cell simple spike discharge (Barmak and Yakhnitsa, 2008).

The actions of a Ca$_3$-K$_4$ complex now reveal a mechanism by which inhibitory charge transfer can be maintained between stellate and Purkinje cells during repetitive climbing fiber input. Our data indicate that the Ca$_3$-K$_4$ complex allows stellate cells to respond to a decrease in $[\text{Ca}^{2+}]_o$, that will occur when climbing fiber input activates a complex spike in nearby Purkinje cell dendrites. Ultramicrographs of K$_4$ channel distribution (Kollo et al., 2006) indicate that K$_4$ channels are concentrated between stellate and climbing fiber boutons and within 300 nm of climbing fiber synapses on Purkinje cell dendrites, increasing the likelihood of being positioned within the zone of calcium depletion. Indeed, the change in $[\text{Ca}^{2+}]_o$ can be expected to be even greater within the narrow space available in the junction that exists between climbing fiber terminals and stellate cells (Kollo et al., 2006; Syková and Nicholson, 2008). Our recordings of $I_T$ in stellate cells established that a change in driving force for calcium is readily detected even in vitro for bath perfusions of different $[\text{Ca}^{2+}]_o$ (Fig. 1D), with a concomitant decrease in $I_T$ and $I_A$ density during climbing fiber stimulation (Fig. 2). In fact, repetitive climbing fiber activation was closely correlated with a reduction in both $I_T$ and $I_A$ in stellate cells, with the effect on $I_A$ blocked when $[\text{Ca}^{2+}]_o$ was raised to 2.2 mM. All these results are consistent with the Ca$_3$-K$_4$ complex responding to changes in $[\text{Ca}^{2+}]_o$ that in turn reduce $I_A$ availability through its action on the K$_4$ voltage-dependence of inactivation.

A reduction in $I_A$ availability is important to functional output because it is associated with a decrease in spike threshold and increase in spike frequency in stellate cells. An increase in stellate firing rate would be predicted to elevate the level of GABAergic inhibition onto Purkinje cells, reducing dendritic calcium spikes and modifying Purkinje cell output. Recordings in Purkinje cells confirmed that the net activity of sIPSCs was substantially increased when $[\text{Ca}^{2+}]_o$ was lowered from 1.5 to 1.1 mM. However, paired recordings also revealed a decrease in the amplitude and increase in the probability of failure of eIPSCs in lowered $[\text{Ca}^{2+}]_o$. A decrease of only 0.4 mM $[\text{Ca}^{2+}]_o$ is then sufficient to reduce the probability of transmitter release and efficacy of transmission between stellate and Purkinje cells (Borst and Sakmann, 1999; Rusakov and Fine, 2003). Despite this, the elevated rate of stellate cell firing in reduced $[\text{Ca}^{2+}]_o$, almost fully offset the associated decrease in IPSC influence. The ability for an enhanced level of inhibition to act at the circuit level to restrain Purkinje cell firing during reductions in $[\text{Ca}^{2+}]_o$ was apparent when the relative effects of picrotoxin on Purkinje cell output were compared under control and test conditions (Fig. 8F).

Adaptive control of inhibition

Together the data indicate that cerebellar circuit function can be maintained in the face of synaptically evoked reductions in $[\text{Ca}^{2+}]_o$ by the actions of a Ca$_3$-K$_4$ complex that adaptively adjusts the level of inhibition to maintain inhibitory charge transfer to Purkinje cells. A schematic representation of the events we propose accompany repetitive climbing fiber input and its effects on $[\text{Ca}^{2+}]_o$, $I_T$, and $I_A$ density, and adaptive control of inhibitory charge transfer to Purkinje cells is summarized in Figure 9. Moreover, by positioning numerous stellate cells in different regions of the Purkinje cell dendritic tree, the Ca$_3$-K$_4$ complex could provide local (single stellate cells) or more global (multiple stellate cells) control of dendritic activity depending on the extent of evoked activity in the dendritic arbor. This will be important in providing a homeostatic balance of inhibition to maintain Purkinje cell output at a level that will allow an appropriate response in the course of processing afferent inputs. Direct recordings of $I_A$ suggest that a synaptically evoked decrease in $[\text{Ca}^{2+}]_o$ can recover within ~1 s in vitro, while ion-selective electrodes suggest that full recovery of $[\text{Ca}^{2+}]_o$...
in the extracellular space \textit{in vivo} may be even longer (Nicholson et al., 1978; Stöckle and ten Bruggencate, 1980). Note that a timeline of only seconds differs substantially from other forms of homeostatic plasticity that require hours or days of intervention to invoke long-term shifts in inhibitory function (Wenner, 2011). Given the widespread expression of Cav3 and Kv4 channels, we predict that the Cav3–Kv4 complex will be an integral part of inhibitory control in many other central neural circuits.

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