Intermediate conductance calcium-activated potassium channels modulate summation of parallel fiber input in cerebellar Purkinje cells

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Encoding sensory input requires the expression of postsynaptic ion channels to transform key features of afferent input to an appropriate pattern of spike output. Although Ca2+-activated K+ channels are known to control spike frequency in central neurons, Ca2+-activated K+ channels of intermediate conductance (KCa3.1) are believed to be restricted to peripheral neurons. We now report that cerebellar Purkinje cells express KCa3.1 channels, as evidenced through single-cell RT-PCR, immunocytochemistry, pharmacology, and single-channel recordings. Furthermore, KCa3.1 channels coinmunoprecipitate and interact with low voltage-activated Cav3.2 Ca2+ channels at the nanodomain level to support a previously undescribed transient voltage- and Ca2+-dependent current. As a result, subthreshold parallel fiber excitatory postsynaptic potentials (EPSPs) activate Cav3 Ca2+ influx to trigger a KCa3.1-mediated regulation of the EPSP and subsequent after-hyperpolarization. The Cav3-KCa3.1 complex provides powerful control over temporal summation of EPSPs, effectively suppressing low frequencies of parallel fiber input. KCa3.1 channels thus contribute to a high-pass filter that allows Purkinje cells to respond preferentially to high-frequency parallel fiber bursts characteristic of sensory input.

Central neurons receive an enormous number of spontaneously active synaptic inputs, but exhibit the capacity to differentiate features of sensory input from background noise. Cerebellar Purkinje cells are contacted by up to ~150,000 parallel fibers from granule cells, of which only a subset will convey sensory information at any given time. The activation of a peripheral receptive field is transmitted to the cerebellar cortex by mossy fibers in the form of high-frequency spike bursts (1). The resulting temporal summation of excitatory postsynaptic potentials (EPSPs) generates a similar high-frequency burst in granule cells (2). Purkinje cells should then also possess the means to respond effectively to bursts of parallel fiber input that convey sensory information compared with background activity.

Postsynaptic membrane excitability can be controlled by activation of K+ channels. There are two established types of Ca2+-activated K+ (KCa) channels in CNS neurons: small conductance (SK, KCa2.1) and big conductance (BK, KCa1.1) (3, 4). A third class of intermediate conductance (KCa3.1, SK4, IK1) KCa channel is thought to be expressed only in microglia and endothelial cells in the CNS (3, 5, 6). KCa3.1 channels are gated by calmodulin in a similar manner to KCa2.x channels but are insensitive to block by aminophylline and tetraethylammonium (TEA) (6–8). Instead, the KCa3.1 α-subunit, encoded by the gene KCNN4, has specific residues that bind charybdotoxin and 1-(2-chlorophenyl)diphenylmethyl)-1H-pyrazole (TRAM-34) (5, 7, 9, 10).

In cerebellar Purkinje cells, KCa1.1 and KCa2.2 channels are activated during a spike by high voltage-activated (HVA) P-type Ca2+ channels (11). In contrast, low voltage-activated (LVA) Cav3 (T-type) Ca2+ channels that are active during the subthreshold interspike interval (12–14) have not been associated with any specific KCa channel. We now report that KCa3.1 channels are expressed in cerebellar Purkinje cells and act to shape the parallel fiber EPSP waveform, providing unique evidence for a functional role for KCa3.1 channels in a CNS neuron. Moreover, KCa3.1 channels colocalize with Cav3.2 Ca2+ channels to allow even subthreshold parallel fiber EPSPs to activate KCa3.1 and suppress temporal summation, contributing to a high-pass filter that allows Purkinje cells to respond to parallel fiber input indicative of sensory input.

Results

Parallel Fiber EPSPs Activate a Ca2+-Dependent After-Hyperpolarization. To examine the potential for parallel fiber EPSPs to activate postsynaptic currents, we stimulated parallel fiber inputs in the presence of picrotoxin and CGP53845 to block GABAergic transmission. The subthreshold parallel fiber-evoked EPSP was followed by a graded after-hyperpolarization (AHP) of up to ~250-ms duration (Fig. S1A). To avoid any effects on presynaptic transmission during pharmacological tests, we simulated EPSPs by injecting excitatory postsynaptic current (EPSC) waveforms at the soma (simEPSCs) in the presence of inhibitory and excitatory synaptic blockers, which produced very similar voltage responses (Fig. S1A). Some portion of this AHP can be attributed to I\textsubscript{AHP} (15). However, given that blocking I\textsubscript{AHP} substantially changes the membrane time constant, all experiments were conducted in the presence of I\textsubscript{AHP} from a membrane potential of −75 mV.

We first tested the ability for blockers against HVA or LVA Ca2+ channels to affect the simEPSP-AHP. We found no significant effect on the simEPSP-AHP by the Cav2.1 (P-type) channel blocker ω-agatoxin IVA (AgTx; n = 4, P = 0.93) (Fig. L4). Similarly, no effects were obtained upon pressure ejection of a mixture of other HVA Ca2+ channel blockers, including ω-conotoxin GVIA, nifedipine, and SNX-482 (n = 6, P = 0.084), or the general HVA blocker Cd\textsuperscript{2+} (n = 5, P = 0.097) (Fig. L4 and Fig. S2). However, both the simEPSP rate of decay and AHP were significantly reduced by low concentrations of the putative T-type channel blockers Ni\textsuperscript{2+} (n = 6, P = 0.00050) and mibefradil (n = 8, P = 0.00018) (Fig. L4) (16, 17). Indeed, Ni\textsuperscript{2+} could affect several aspects of the simEPSP-AHP (Fig. S1B), but the most reliable effect was a reduction in the simEPSP rate of decay (Fig. L4).

The relative lack of specificity of T-type channel blockers leaves open the possibility that these effects were not entirely mediated through T-type channels. First, Ni\textsuperscript{2+} and mibefradil


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also block R-type Ca\(^{2+}\) channels (18, 19). However, the lack of effect by either SNX-482 or Cd\(^{2+}\) on the simEPSP rules out R-type channel involvement (Fig. 1D and Fig. S2). The effects of Ni\(^{2+}\) on the simEPSP-AHP were also separate from those of blocking IbTx (Fig. S1C). The effects of Ni\(^{2+}\) were further maintained in the presence of internal heparin (4 mg/mL) and bath-applied cyclopiazonic acid (6 μM) (n = 4, P = 0.0032), indicating no involvement of IP\(_3\)-mediated Ca\(^{2+}\) release or Ca\(^{2+}\) -ATPases (Fig. S1D). Ryanodine also had no significant effect on the simEPSP rate of decay (n = 4, P = 0.12), indicating no role for ryanodine receptor-mediated Ca\(^{2+}\) -induced Ca\(^{2+}\) release (Fig. S1E). Taken together, these data suggest that the simEPSP activates Ca\(^{2+}\) influx through Cav3 Ca\(^{2+}\) channels to control an outward current with an onset early enough to affect both the EPSP rate of decay and AHP.

**EPSP Rate of Decay Is Shaped by a K\(^{+}\) Current Consistent with KCa3.1**

We next examined the identity of K\(^{+}\) channels to control an outward current with an onset early enough to affect both the EPSP rate of decay and AHP. The simEPSP rate of decay was reduced 41.7 ± 6.4% by charrybotoxin (ChTx; n = 6, P = 0.00459) (Fig. 1B). ChTx is known to block Ca\(^{2+}\)-activated KCa1.1 and KCa3.1 channels and specific Kv1.x channels (7, 20). KCa3.1 channels are apamin-insensitive but have specific binding sites for ChTx and the clotrimazole-related compound TRAM-34 (9, 21). TRAM-34 has been established as a selective KCa3.1 blocker (Fig. 1D) (7), despite con-

**Fig. 1. Subthreshold parallel fiber EPSPs generate an AHP consistent with activation of KCa3.1.** All records were evoked using simEPSCs to test post-
synaptic channel contributions, with drug effects on the rate of EPSP decay normalized to the control 5 mV simEPSP. (A and B) Representative record-
ings (Left) and bar plots (Right) showing the effects of Ca\(^{2+}\) and K\(^{+}\) channel blockers. (A) The simEPSP rate of decay is not significantly affected by AgTx (200 nM) or other HVA Ca\(^{2+}\) channel blockers (−conotoxin GVIA, 1 μM; ni-
fedipine, 1 μM; SNX-482, 200 nM) or Cd\(^{2+}\) (30 μM), but is reduced by putative T-type Ca\(^{2+}\) channel blockers Ni\(^{2+}\) (100 μM) and mibefradil (Mib, 1 μM). (B) The simEPSP rate of decay is unaffected by the KCa2.x blocker apamin (100 nM), or KCa1.1 blockers IbTx (200 nM), TEA (5 mM), or paxilline (100 mM), but is significantly reduced by ChTx (100 nM) and TRAM-34 (100 nM). (C) Internal dialysis of Camstatin (5 μM) occludes the effect of TRAM-34 on the simEPSP rate of decay. (D) Pretreatment with Ni\(^{2+}\) occludes the action of TRAM-34 on the simEPSP rate of decay. Sample numbers are shown in brackets at the base of bar graphs. Average values are mean ± SEM; ***P < 0.01, **P < 0.001.

**Purkinje Cells Express KCa3.1 Channels.** Identifying an outward current with KCa3.1-like properties was unexpected. Although KCNN4 mRNA has been detected in the cerebellar Purkinje cell (10, 20), KCa3.1 expression has not been reported in CNS neurons (3). There was also no difference between the effects of Ni\(^{2+}\), mibefradil, ChTx, or TRAM-34 on the simEPSP rate of decay (F = 2.37, one-way ANOVA), suggesting actions on a common pathway. In fact, the effects of TRAM-34 on the simEPSP-AHP were occluded by prior Ni\(^{2+}\) treatment (Fig. 1D) (n = 4, P = 0.67). Taken together, these data reveal a postsynaptic current with a pharmacological profile that is unique to KCa3.1 channels (10, 20) and activated by Cav3-mediated Ca\(^{2+}\) influx.

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electrodes were filled with HEPES-buffered artificial cerebrospinal fluid (aCSF) to provide a physiological level of Ca^{2+} outside, along with tetrodotoxin (TTX), Cd^{2+}, apamin, TEA, 4-AP, and Cs+ in both the electrode and external medium to block sodium, HVA Ca^{2+}, KCa2.2, KCa1.1, Kv, and HCN channels (Materials and Methods and Fig. S2). T-type channel blockers were specifically excluded and the availability of Cav3 channels promoted by applying a +60 mV potential to the pipette at rest to hyperpolarize the patch transmembrane potential. Channel conductance was determined by applying steady-state commands (5 min) up to +30 mV from the resting condition to depolarize the patch to the subthreshold voltage range. Spontaneous channel openings were readily detected in all patches, with either single- or multiple-channel openings per patch that changed linearly in amplitude with applied voltage (Fig. 3 A and B). Mean conductance, as determined from unit amplitude over a range of voltage steps, was 36.3 ± 0.1 pS (n = 5) (Fig. 3B), a value within the range previously reported for KCa3.1 channels (6). Importantly, bath application of the Ca^{2+} ionophore A23187 (n = 6) dramatically increased single channel activity (Fig. 3C), indicating an increase in probability for activation with Ca^{2+} influx. Moreover, channel openings were blocked by perfusion of TRAM-34 (n = 6), a lipophilic drug that acts at the cytoplasmic face (Fig. 3C) (9). These results, combined with the mRNA, immunolabel, and pharmacological profiles established in Figs. 1 and 2, provide strong evidence that KCa3.1 channels are expressed in Purkinje cells.

Cav3 and KCa3.1 Channels Exhibit Nanodomain Coupling to Evoke a Transient Voltage-Dependent K+ Current. The selective reduction of the simEPSP rate of decay by T-type Ca^{2+} channel blockers (Fig. 1) raised the possibility that Cav3 and KCa3.1 channels are part of a physical signaling complex. We tested for an association between Cav3.2 and KCa3.1 channels, finding that Cav3.2 channels coimmunoprecipitated with KCa3.1 channels from homogenates of rat cerebellum (Fig. 4A). In comparison, we found no coimmunoprecipitation between KCa3.1 and Cav2.1 channels that are expressed by Purkinje cells (Fig. S5A).
fast inactivating \((t_{1/2} = 22.6 \pm 1.4 \text{ ms})\) (Fig. 4B and Table S1). Importantly, this outward current cannot reflect activation of KCa1.1, KCa2.2, Kv1, Kv3, or Kv4 channels, as these are substantially blocked by apamin or 5 mM TEA and 4-AP \((20)\). HVA Ca\(^{2+}\) currents (including R-type) are also highly effectively blocked by the 30 \(\mu\text{M} \text{Cd}^{2+}\) included here (Fig. S2), leaving Cav3 channels as the predicted primary source for Ca\(^{2+}\) influx in these recordings. We further isolated the Ni\(^{2+}\) and TRAM-sensitive currents evoked by steps from \(-110 \text{ mV}\) using the same conditions of HVA Ca\(^{2+}\) and K\(^+\) channel block. These tests showed that TRAM-34 \((n = 4)\) or Ni\(^{2+}\) \((n = 4)\) blocked a fast inactivating current of up to 30 pA, with mean values of 11.4 \(\pm\) 2.3 pA and \(t_{1/2}\) inactivation of 23.6 \(\pm\) 6.0 ms at \(-30 \text{ mV}\) \((n = 8)\) (Fig. 4B and Table S1). TRAM-34 and Ni\(^{2+}\)-sensitive currents also activated in the low voltage range in a manner similar to the inactivating current isolated by membrane voltage (Fig. 4C). In support of this, current-clamp recordings indicated a clear threshold for activation of a Ni\(^{2+}\)-sensitive AHP for membrane potentials positive to \(-80 \text{ mV}\), while voltage-ramp commands revealed Cav3 and KCa3.1 activation from membrane voltages between \(-80\) and \(-90 \text{ mV}\) (Fig. S6). Finally, to test the proximity of Cav3 to KCa3.1 channels, we used outside-out recordings to measure TRAM-34–sensitive current in the presence of either EGTA or BAPTA \((10 \text{ mM})\) in the internal electrolyte. TRAM-34–sensitive current was only recorded in the presence of internal EGTA \((n = 5)\) but not BAPTA \((n = 6)\) (Fig. 4D), indicating an interaction at the nanodomain level \((30)\).

Given that KCa3.1 channel open probability is only a function of internal Ca\(^{2+}\) concentration \((31)\), the voltage dependence of KCa3.1 activation must indirectly reflect the voltage dependence of Ca\(^{2+}\) influx through Cav3 channels. Taken together, these data indicate that an association between Cav3 and KCa3.1 channels at the nanodomain level allows T-type Ca\(^{2+}\) influx to activate KCa3.1 as a Ca\(^{2+}\)-dependent and transient outward current in the low voltage range.

**Cav3–KCa3.1 Interaction Controls Temporal Summation of Parallel Fiber EPSP Trains.** To test the hypothesis that the Cav3–KCa3.1 complex could affect summation of EPSPs, we stimulated parallel fiber inputs at 25 Hz for 2 s. Initial EPSP amplitude was 2 mV, with presynaptic facilitation causing the EPSP amplitude to reach \(-5 \text{ mV}\). Under control conditions, an initial summation of the EPSP was reduced within 250 ms to a lower, stable amplitude that remained below spike threshold for the duration of the stimulus train (Fig. 5A). However, we found a rapid temporal summation of EPSPs within the first five stimuli after applying either Ni\(^{2+}\) \((n = 8, P = 0.00012)\) or TRAM-34 \((n = 4, P = 0.019)\) to block the Cav3–KCa3.1 interaction (Fig. 5A and B). Measuring the baseline membrane voltage immediately preceding each stimulus indicated a rapid increase over the first \(\sim 20\) stimuli that reached a sustained level 8–10 mV beyond initial baseline (Fig. 5A and B). As a result, parallel fiber-evoked EPSPs surpassed spike threshold within 5–10 stimuli to reliably generate spike output. We found no role for mGluR1 receptor activation \((14)\) or presynaptic endocannabinoid receptors \((32)\), as neither JNJ16259685 nor the CBI receptor blocker AM-251 occluded the effect of Ni\(^{2+}\) \((n = 4)\) during these stimuli. In addition, the parallel fiber-evoked EPSP paired-pulse ratio was unaffected by either Ni\(^{2+}\) \((n = 7, P = 0.95)\) or TRAM-34 \((n = 7, P = 0.582)\) (Fig. S7A and B), indicating that the increase in temporal summation did not involve presynaptic effects.

Temporal summation of parallel fiber EPSPs has also been shown to be controlled through feed-forward inhibition \((33)\). However, when we repeated these tests in coronal slices in the absence of picrotoxin to preserve feed-forward inhibition, TRAM-34 rapidly increased temporal summation of parallel fiber-evoked EPSPs (Fig. 5C) \((n = 5, P = 0.04,\text{ one-way ANOVA with post hoc Tukey test})\). Subsequent addition of picrotoxin to block GABAergic inputs led to a further increase in temporal summation during the stimulus train (Fig. 5C) \((n = 5, P = 0.08,\text{ one-way ANOVA with post hoc Tukey test})\). These results indicate that postsynaptic control of temporal summation by the Cav3–KCa3.1 complex is effective in the presence of feed-forward inhibition.

**Cav3–KCa3.1 Complex Is Active During Membrane Depolarizations and Tonic Firing.** Given that Cav3 channels inactivate upon depolarization, it is important to determine the availability of Cav3 current at physiological potentials. We thus tested if the Cav3–KCa3.1 interaction is active at more depolarized levels of membrane potential that promote spontaneous spike firing. To measure Cav3 activation and inactivation over a full voltage range, we used whole-cell recordings from P10–P12 Purkinje cells when the dendritic tree has not fully developed to avoid space clamp errors \((n = 7)\) (Fig. 6A and B). The Boltzmann fits for activation and inactivation data (Fig. 6B) indicated that a Cav3 window current exists for membrane voltages up to at least \(-20 \text{ mV}\), and thus well into the suprathreshold range \((12, 34)\) (Fig. 6A and B).

We further tested the degree to which parallel fiber EPSP summation could be modified by the Cav3–KCa3.1 interaction during tonic firing. Bias current was applied to sustain a tonic firing frequency of \(\sim 50 \text{ Hz (49.7 \pm 1.8 Hz, } n = 9)\) before and after drug application. Parallel fiber inputs were stimulated at 100 Hz (five pulses) to mimic frequencies of input relevant to sensory stimulation \((1, 35)\). After application of TRAM-34, there was an increase in both peak baseline voltage \((59 \pm 25.6\%, n = 5, \text{ Fig. 5})\).
A functional coupling between T-type Ca\(^{2+}\) and KCa2.x channels in select neuronal subtypes (39–41) is also reported to operate at the microdomain level compared with the nanodomain demonstrated here. A Cav3–Kv4.3 \(K^+\) channel interacting protein 3 to mediate Ca\(^{2+}\) sensing for voltage-gated Kv4 channels (42, 43). The Cav3-KCa3.1 complex instead depends on calmodulin that has been shown to directly gate the KCa3.1 channel (3).

All members of the KCa2.x and KCa3 families are known to be Ca\(^{2+}\)- but not voltage-dependent (3). However, the close association between Cav3 and KCa3.1 channels allows KCa3.1 to acquire the properties of a low voltage-activated current, as well as the fast inactivating kinetics of Cav3 current. KCa3.1 channels also exhibit a two-to-three times greater sensitivity to internal Ca\(^{2+}\) than Cav3.x channels (36). These properties are consistent with the ability for a transient T-type Ca\(^{2+}\) influx to activate KCa3.1 with little delay at the onset of an EPSP and yet generate an AHP of up to 250-ms duration. KCa3.x channels can thus be activated by single, low-amplitude EPSPs but also exhibit a cumulative activation during repetitive activity, an advantage over other \(K^+\) channels for modulating temporal summation of synaptic depolarizations.

Control over temporal summation of parallel fiber EPSPs in Purkinje cells has also been reported through other mechanisms. One is a role for \(I_{\text{H}}\) to reduce EPSP width and temporal summation (15). Because all tests conducted here were performed with \(I_{\text{H}}\) intact, KCa3.1 clearly has a role distinct from \(I_{\text{H}}\) in producing the AHP and modifying temporal summation. Molecular layer interneurons provide a feed-forward inhibitory influence that reduces parallel fiber EPSP summation (33). This function is again different, in that the Cav3–KCa3.1 complex exerts a significant effect on temporal summation even in the presence of feed-forward inhibition. The influence of the Cav3–KCa3.1 interaction is also fully functional during tonic firing, a result attributable to the wide extent of Cav3 window current in the suprathreshold voltage range. The present results then suggest that the Cav3–KCa3.1 complex functions synergistically with \(I_{\text{H}}\) and feed-forward inhibition to reduce Purkinje cell responsiveness to background granule cell activity yet permit activation by high-frequency trains of parallel fiber input. Taken together, these factors underlie a high-pass filter function that allows spike bursts produced by granule cells to preferentially evoke Purkinje cell spike output (2).

**Materials and Methods**

**Molecular Biology and Cytochemical Methods.** Materials and methods related to coimmunoprecipitation, RT-PCR, immunocytochemistry, and use of TSA-201 cells are found in SI Materials and Methods.

**Animals.** Timed-pregnant Sprague-Dawley rats (Charles River) were maintained according to the Canadian Council on Animal Care; male pups ≥ postnatal day (P) 18 were used, unless otherwise indicated. Rats were anesthetized by inhalation of isoflurane until unresponsive to tail pinch. Tissue dissection and preparation of parasagittal (300 \(\mu\)m) slices from the cerebellar vermis were previously described (24).

**Solutions.** Chemicals were obtained from Sigma unless otherwise indicated. aCSF was composed of 125 mM NaCl, 3.25 mM KCl, 1.5 mM CaCl\(_2\), 1.5 mM MgCl\(_2\), 25 mM NaHCO\(_3\), and 25 mM \(\beta\)-glycose. Picrotoxin (50 \(\mu\)M) and 10 \(\mu\)M 6,7-dinitroquinoxalinedione (DNOX; Tocris), 25 \(\mu\)M DL-AP5 (Ascent Scientific), and 1 \(\mu\)M CGP55485 (Tocris) were added to the aCSF for all recordings that included a change in the amplitude of the evoked EPSP (Fig. S7 C and D). These results demonstrate that both Cav3 and KCa3.1 channels are available at depolarized potentials supporting tonic spike firing, and modulate temporal summation of parallel fiber inputs at physiologically relevant input frequencies.

**Discussion**

The present study provides unique evidence for the activation of KCa3.1 \(K^+\) channels in a CNS neuron and its role in creating a postsynaptic frequency filter for synaptic input. Although KCa3.1 channel activation has been documented in cells of the enteric and myenteric nervous systems (36, 37), several lines of evidence now support the expression of KCa3.1 channels in Purkinje cells, including single cell RT-PCR, KCa3.1 immunolabel, single channels with intermediate conductance, and macroscopic recordings of KCa currents with a pharmacological profile that is unique to KCa3.1 channels (3, 7). Moreover, Cav3 \(Ca^{2+}\) channels colocalize and associate with KCa3.1 channels to provide \(Ca^{2+}\)-dependent regulation at the nanodomain level to control temporal summation of parallel fiber EPSPs. This unique Cav3–KCa3.1 complex differs significantly from other ion channel complexes involving \(T\)-type channels reported in previous studies. The ability to evoke the Cav3–KCa3.1 interaction using postsynaptic simEPSCs indicates that the complex does not require \(Ca^{2+}\) influx through ligand-gated channels, a known interaction for KCa2.x channels (38). A functional coupling between T-type \(Ca^{2+}\) and KCa2.x channels in select neuronal subtypes (39–41) is also reported to operate at the microdomain level compared with the nanodomain demonstrated here. A Cav3–Kv4.3 \(K^+\) channel complex employs \(K^+\) channel interacting protein 3 to mediate \(Ca^{2+}\) sensing for voltage-gated Kv4 channels (42, 43). The Cav3-KCa3.1 complex instead depends on calmodulin that has been shown to directly gate the KCa3.1 channel (3).
Electrophysiology. Whole-cell current-clamp somatic recordings were made using Axoclamp amplifiers and Digidata 1322 with a DC-10 kHz band-pass filter and pClamp software. Negative bias current of less than 1.0 nA was applied during current-clamp recordings to maintain Purkinje cell resting potential at —75 mV and below the level of tonic firing. See SI Materials and Methods for further details.

Data Analysis and Statistics. Statistical analysis was carried out in OriginPro 8. Unless otherwise indicated, paired-sample Student t tests were used to determine significance. The Tukey HSD post hoc comparison was used to test significance between means following one-way or repeated-measures ANOVA. Average values are expressed as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

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**Supporting Information**

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**SI Materials and Methods**

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**Solutions.** Chemicals were obtained from Sigma unless otherwise indicated. Artificial cerebrospinal fluid (aCSF) was continuously bubbled with carbogen gas. Food coloring (1:100) was included in pressure electrodes to allow visualization of the area of drug ejection. BSA (0.1%) was included to prevent nonspecific adhesion of the drugs. Outside-out recordings of high voltage-activated (HVA) Ca$^{2+}$ currents (Fig. S2) used an electrolyte consisting of 140 mM CsCl, 1 mM MgCl$_2$, 5 mM EGTA, and 10 mM Hepes, pH 7.3 with KOH, with bath perfusion of tetrodotoxin (TTX), apamin, tetraethylammonium (TEA), 4-AP, NiCl$_2$, CsCl, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34), and synaptic blockers.

**Electrophysiology.** Whole-cell current-clamp somatic recordings were made using either an Axoclamp 2A amplifier/Digidata 1322 or a Multiclamp 700B amplifier/Digidata1440A (Molecular Devices). Data were collected with pClamp 8.1 or 10 software with a DC 10-kHz band-pass filter. Single channel recordings in on-cell mode were collected at 40-kHz sample frequency using a 4-kHz Bessel filter and analyzed using Clampfit (1-kHz Bessel filter). Pipettes were constructed from 1.5-mm OD fiber-filled glass (A-M Systems) with resistance of 4–8 MΩ. Series resistance was compensated with the bridge balance circuitry for current-clamp recordings and with up to 80% compensation during voltage-clamp recordings. Single channel recordings were analyzed using Clampfit (1-kHz Bessel filter) to identify channel openings and to calculate amplitude distribution histograms (0.25-pA bin width) and open probability. Commands were delivered from +60 mV to +30 mV for 5 min and then in −10 mV steps to 0 mV to calculate single channel conductance according to the slope of current amplitudes against step potential. Parallel fiber-evoked excitatory postsynaptic potentials (EPSPs) were evoked by a patch electrode containing Heps-buffered aCSF in the distal third of the molecular layer using a stimulus isolation unit (Digitimer; 0.1- to 0.2-ms pulse width). Parallel fiber EPSPs recorded in the presence of feed-forward inhibition were performed in coronal slices and evoked by stimulation in the molecular layer >200 μm lateral to the recording site. Alternatively, postsynaptic ion channel contributions to synaptic depolarizations were measured by injecting simulated parallel fiber excitatory postsynaptic current (si-EPSC) at the soma, as previously described (2, 3). EPSP rate of decay was defined as the slope of the EPSP between 90% and 10% of the EPSP height. Stimulus artifacts were removed before analysis. Analysis was performed in Clampfit 10 or using custom Matlab R2007B scripts.

**tSA-201 Cells.** tSA-201 Cells were maintained as previously described (4). Cav3.2 cDNA was kindly provided by T. Snutch (University of British Columbia, Vancouver, BC, Canada). The tSA-201 cells were transfected with cDNA for Cav3.1, -3.2, and -3.3 calcium channels (5 μg/μL), or cDNA for the Cav2.3 calcium channel (5 μg/μL) coexpressed with α-δ (5 μg/μL) and β-1b (5 μg/μL) subunits. All transfections included cDNA for GFP to identify cells successfully transfected. Currents were recorded at room temperature in aCSF consisting of 120 mM NaCl, 3 mM NaHCO$_3$, 4.2 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.5 mM MgCl$_2$, 10 mM d-glucose, 10 mM Hepes and 1.5 mM CaCl$_2$ (pH adjusted to 7.3 with NaOH). Electrodes were filled with 110 mM potassium gluconate, 30 mM KCl, 1 mM EGTA, 5 mM Hepes, and 0.5 mM MgCl$_2$ (pH adjusted to 7.3 with KOH).

**Immunohistochemistry.** Adult males (~250 g) were deeply anesthetized with an overdose of sodium pentobarbital and perfused intracardially with 250 mL or 0.1 M phosphate buffer (PB, pH 7.4) followed by 100 mL of 4% paraformaldehyde (pH 7.4) at room temperature for 1 h and overnight at 4 °C. Free-floating 40- to 50-μm sections were cut by vibratome and transferred to a working solution of 3% normal donkey or horse serum (Jackson ImmunoResearch), 0.2% Tween, and 2% dimethylsulfoxide in PB. Primary antibodies were reacted for 48 h at 4 °C and washed in working solution 3× 15 min and secondary antibodies for 4 h at room temperature. After washing in PB, sections were mounted on gel-coated slides and coverslipped with antifade medium and stored at ~20 °C. Primary antibodies were mouse monoclonal anti-IK-1(D-5) (1:100; Santa Cruz), rabbit polyclonal anti-Cav3.2 (H-300) (1:200; Santa Cruz), and mouse monoclonal anticalbindin (1:1,000; Swant). Secondary antibodies (1:1,000) were the appropriate AlexaFluor-488 or -594 conjugated donkey IgGs (Molecular Probes). Controls consisted of omitting the primary antibodies. Images were obtained using a Zeiss AxioImager, Apotome grid and Colibri LED illumination and processed in Adobe Photoshop and Illustrator.

**Coimmunoprecipitation Assay.** Fresh rat cerebellum was homogenized in lysis buffer (10% wt/vol, 150 mM NaCl, 50 mM Tris pH 7.5 and 1% Nonidet P-40 including protease inhibitors). The homogenate was centrifuged at ~16,000 × g for 10 min at 4 °C in a microcentrifuge and supernatant collected. Protein concentration was estimated using the Bradford assay (BioRad). The supernatants (containing 200 μg total proteins) were incubated with mouse monoclonal anti-KCa3.1 antibody (sc-365265; Santa Cruz) at a 5 μg/mL final concentration overnight at 4 °C. The mixtures were then incubated with protein G beads (Amersham) for 2 h to bring down the antibody-antigen complexes. The beads were washed three times with lysis buffer by centrifugation (700 × g) per resuspension. The immune complexes were eluted using 40 μL of 2x SDS/PAGE sample buffer and heating for 5 min at 95 °C. Twenty microfilters eluents along with 40 μg of total protein from lysate were separated on SDS/PAGE gels. The primary antibodies used for Western blotting were rabbit polyclonal anti-Cav3.2 (1,2,500) (5) and rabbit polyclonal anti-Cav2.1 (4.75 μg/mL). The bands were detected by HRP-linked secondary antibody (1:5,000) and ECL+ (GE Healthcare).

**RT-PCR. Single-cell samples.** The cytoplasmic contents of Purkinje cells were extracted through whole-cell recordings in the slice preparation for RT-PCR analysis following procedures of Toledo-Rodriguez and Markram (6). Before recording, both the recording bath and lines were cleaned with HCl, and electrodes were cleaned and rechlorided. The internal electrolyte for current-clamp recordings used to identify Purkinje cell firing patterns was the standard potassium gluconate-based solution (detailed in Materials and Methods). To prevent RNA degradation by RNA nucleases, RNase inhibitor (200 U/mL) was in-
cluded in the internal solution. Purkinje cells were patch-
dered visual inspection by infrared-differential interference contrast and the identity further confirmed by injecting depolarizing current steps to identify the pattern of action potential discharge (1). Negative pressure was then applied for 5–10 min and holding current was monitored in voltage clamp to ensure the integrity of the seal. After the internal contents of the cell were extracted, an outside–out patch configuration was attained upon withdrawing the electrode to prevent contamination of the single-cell sample. Samples were transferred to a sterile centrifuge tube and immediately frozen on dry ice and stored at −80 °C.

**Whole cerebellar samples.** Whole cerebella were taken from rats at P18 and put in trizol solution (Invitrogen). The cerebellar RNA lysate was prepared by the acid-guanidinium thiocyanate-phenol-chloroform extraction method (7) with commercially available trireagent/trizol (Invitrogen). This method consists of several steps: cell lysis, phase separation, RNA precipitation, RNA wash, and redissolving RNA. The extract RNA was dissolved and stored as precipitate in RNase-free water at −80 °C.

**RT-PCR reaction.** The mRNA from single cell samples and cerebellar RNA lysate were reverse-transcribed using an Omniscript reverse transcriptase (Qiagen) and oligo(dT) primers in final volume of 20 μL. After 60-min incubation at 37 °C, the cDNA was frozen and stored at −20 °C before further processing. The cDNA samples were then amplified by PCR using Taq DNA polymerase (Invitrogen) and primers for KCa3.1 channels (IKF1: 5′-ATG GCC GGG GAG GAG GCTG CTGG AGT GGG TTC TGG CGG CTG C-3′; IKF2: 5′-GGC CAT GCT GCT ACG TCT CTA CCT GGT GCC TCG-3′; IK3R: 5′-GCT GAT GCC TGC GAG CCG CTC GGG AGT CC-3′; and IK4R: 5′-CTA TGT GCC CTC CTG GAT GGG TCG CTT GCG CTC C-3′), and primers for microglial response factor-1 (MRF-1) (8) (MRF-1: 5′-TCTGAGGAGCTATGAGCC-3′; and MRF2: 5′-TCCACCTCATTAGGGAAC-3′), and primers for KCa1.1 channels (kindly provided by A. Braun, University of Calgary, Calgary, AB, Canada) (hSKCa F: 5′-GGAATTCCAGTATCACAA-CAAGGGCCACTG-3′; and hBKCa R: 5′-GGAATTCAAG-GACAGACCCAGAAGGC-3′), and primers for KCa2.2 channels (kindly provided by A. Braun, University of Calgary, Calgary, AB, Canada) (hBKCaF: 5′-GGAATTCCAGTATCACAA-CAAGGGCCACTG-3′; and hBKCaR: 5′-GGAATTCAAG-GACAGACCCAGAAGGC-3′), and primers for KCa1.1 channels (kindly provided by A. Braun, University of Calgary, Calgary, AB, Canada) (hSKCaF: 5′-GCTAGCATTCCATGCGG-3′; and hBKCaR: 5′-CTAAGACCCAGAAGGC-3′). Primers of KCa3.1 and MRF-1 were based on rat nucleotide sequences, and primers for KCa1.1 and KCa2.2 channels were based on human nucleotide sequences (accession numbers: NM_023021.1 for KCa3.1 channel/KNCN4, AB000818 for MRF-1, NM_181361.1 for KCa1.1 channels, and NM_021614.2 for KCa2.2 channels).

A two-round PCR was performed on cDNA of KCa3.1 channels, using the product obtained after the first round as template for the second round; for others single-round PCR was performed. The first PCR of KCa3.1 was performed after adding PCR buffer, MgCl2 (2.5 mM), 2.5 U Taq Polymerase and corresponding primers (IK1F and IK4R) to the room temperature product (final volume 25 μL). Forty-five cycles were performed (denaturation at 95 °C, 1 min; annealing at 65 °C, 2 min for the first five cycles, and at 52 °C, 1 min, for the remaining cycles; extension at 72 °C, 2 min; final elongation at 72 °C, 10 min), and an aliquot (5 μL) of the first-round PCR product was used as template for the second PCR using the nested primers (IK2F and IK3R) with the same cycles and condition as described above.

The single-round PCR tests were performed using 35 cycles (denaturation at 94 °C, 1 min; annealing at 60 °C, 1 min; extension at 72 °C, 1 min; and final elongation at 72 °C, 15 min). Negative controls for contaminations from genomic DNA were run as described above, but without adding reverse transcriptase to the room temperature reaction to ensure that genomic DNA did not contribute to the PCR products. A contamination from extraneous sources was ruled out by replacing the mRNA cellular template with water.

All products were identified by agarose gel electrophoresis (1%, stained with ethidium bromide) and documented with Alpha Innotech Gel Doc System. All KCa3.1 PCR products were confirmed by sequencing analyses.

**Endothelial cell cultures.** Endothelial cells were derived from a cultured human umbilical cell line, EA.hy926, stored as a pellet at −80 °C (kindly provided by A. Braun, University of Calgary, Calgary, AB, Canada). The pellet was lysed in internal solution (the same solution for the Purkinje cell sample solution) and prepared for PCR using primers for KCa3.1 channels and a two-round PCR performed as for KCa3.1 channels. The negative controls were run as for Purkinje cells and whole cerebellar RT-PCR, and all products were identified the same as described above.

**Data analysis and statistics.** Rate of decay was defined as the velocity during the voltage decay as measured by the slope of the response between 90–10% of the EPSP height. Stimulus artifacts were removed before analysis. Analysis was performed either in Clampfit 10 or using custom Matlab R2007B scripts. OriginPro 8 was used for statistical analysis. Unless otherwise indicated, paired-sample Student's t tests were used to determine significance. The Tukey HSD post hoc comparison was used to determine significant difference between means following one-way or repeated-measures ANOVA. Average values are expressed as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. S1. The effects of Ni²⁺ on the simEPSP. (A) Evoked parallel fiber EPSPs generate an after-hyperpolarization (AHP), which lasts up to ~250 ms. Somatic injection of simEPSCs generate similar voltage waveforms to evoked EPSPs (1, 2). (B) Ni²⁺ (100 μM) causes a significant increase in the simEPSP rate of rise and decreases both the simEPSP time to peak and AHP depth. (C) The effect of Ni²⁺ on the simEPSP rate of decay and AHP are distinct from Cs⁺-sensitive i₇. Ni²⁺ application slows the rate of decay of a simEPSP and decreases AHP amplitude whether applied before or after Cs⁺ to block i₇. Shown are superimposed recordings of simEPSPs in control medium (black) and after perfusion of Ni²⁺ (green) or Cs⁺ (blue). ZD-7228 was not used to test i₇ given nonspecific actions on T-type calcium channels (3). (D) Bar plots and representative recordings indicating that the effect of Ni²⁺ on the simEPSP rate of decay is maintained in the presence of cyclopiazonic acid (CPA, 6 μM) and internal heparin (4 mg/mL). (E) Recordings showing the lack of effect by ryanodine (10 μM) on simEPSP rate of decay. Drug effects were normalized to the control 5 mV simEPSP. Sample values are shown in brackets in bar plots and average values are mean ± SEM; **P < 0.05, ***P < 0.01.

Fig. S2. The effects of Cd\(^{2+}\) on HVA calcium currents. (A) Example of Cd\(^{2+}\) (30 μM)-sensitive HVA calcium current (presumed P/Q-type) recorded in outside-out patches from the soma of a P 18–25 Purkinje cell. Patches were stepped from −85 mV to 30 mV to evoke HVA calcium current. Record represents the difference current between control and Cd\(^{2+}\)-treated tissue in the presence of 100 μM Ni\(^{2+}\) to block Cav3 T-type calcium currents. Mean bar plots indicate a near complete block of HVA calcium current by 30 μM Cd\(^{2+}\). (B) Representative current-voltage plot and recording (Inset) of R-type current recorded at room temperature in tSA-201 cells following cotransfection of Cav2.3 cDNA with α2-δ and β1-b subunits. Substantial block is obtained upon perfusion of 30 μM Cd\(^{2+}\). (Right) Bar plot illustrates the average block of R-type calcium current by 30 μM Cd\(^{2+}\) in tSA-201 cells. Average values are mean ± SEM; **P < 0.01; ***P < 0.001.

Fig. S3. TRAM-34 has no effect on Cav3 calcium currents. Current-voltage plots are shown for each of the Cav3 calcium channel isoforms expressed in tSA-201 cells. Currents were recorded at room temperature and evoked by steps from −110 mV to 10 mV before and after perfusion of TRAM-34 (100 nM). Average values are mean ± SEM.
Fig. S4.  (A and B) Low-power montage of the cerebellar Purkinje cell layer and proximal molecular layer in a coronal tissue section dual-labeled for calbindin (A) and KCa3.1 (B) showing KCa3.1 expression in Purkinje cell somata and restricted regions of dendrites. (C and D) Higher power image in sections cut in the sagittal plane showing KCa3.1 immunolabel (C) and Cav3.2 immunolabel (D) on Purkinje cell somata and proximal dendrites, with associated controls consisting of omission of primary antibody. Control and test images were matched for light intensity during imaging and processing. (Scale bars: A, 50 μm; C and D, 20 μm.)

Fig. S5.  (A) Western blot showing a lack of coimmunoprecipitation between Cav2.1 (P/Q-type) calcium channels and KCa3.1 from cerebellar homogenates. (B) Outside-out recordings from a Purkinje cell soma in response to steps from −110 mV to 0 mV (Top). (Middle) Voltage-dependent inactivation of a transient outward current at a holding potential of −40 mV. Subtracting currents evoked from −40 mV (Bottom) reveals a fast activating and inactivating outward current (a-b). (C) Recordings of TRAM-34 (100 nM)- and Ni²⁺ (100 μM)-sensitive currents in separate outside-out recordings evoked from a holding potential of −110 mV in steps to 0 mV. Subtraction of test from control records reveals fast inactivating TRAM-34-sensitive and Ni²⁺-sensitive currents. Records represent the full set of data used to calculate currents shown in Fig. 4B.
Fig. S6. (A) The effect of Ni\(^{2+}\) on the rate of decay of simEPSPs was measured over a range of resting membrane potentials. A reduction in the simEPSP rate of decay is apparent for membrane potentials positive to \(-80\) mV (right of dashed line). (B) Whole-cell voltage-clamp recordings from mature (P 18–30) Purkinje cells using K-gluconate recording solutions similar to those used in A. Ni\(^{2+}\)-sensitive subthreshold currents were recorded in separate Purkinje cells evoked by a voltage ramp protocol (−100 to −40 mV over 500 ms; Lower) in the absence (Upper) or presence (Lower) of TRAM-34 (100 nM) in the aCSF. The aCSF also contained 200 nM TTX, 2 mM Cs\(^{+}\), 5 mM TEA, 100 nM apamin, and 30 μM Cd\(^{2+}\), with [K\(^{+}\)]\(_o\) lowered to 1.5 mM to set E\(_K\) at \(-120\) mV to increase the ability to detect K\(^{+}\) current at hyperpolarized voltages. Current traces represent the difference between recordings before and after application of 100 μM Ni\(^{2+}\). In the absence of TRAM-34, an outward current can be seen with an onset voltage between −80 and −70 mV. When slices are preincubated with TRAM-34, an inward current is recorded with an onset between −90 and −80 mV. (C) Averages of Ni\(^{2+}\)-sensitive currents in the absence of TRAM-34 (Upper) show an outward current for potentials positive to −80 mV. When KCa3.1 is blocked with perfusion of TRAM-34 (Lower), an inward current is revealed for potentials positive to −90 mV. Black trace indicates the mean value and gray traces indicate the SEM. A junction potential of −10.7 mV was subtracted from all recordings. Average values are mean ± SEM.

Fig. S7. Actions of TRAM-34 or Ni\(^{2+}\) are not the result of presynaptic effects. Purkinje cells were held at subthreshold potentials and the amplitude of parallel fiber-evoked EPSPs examined before and after application of TRAM-34 or Ni\(^{2+}\). (A and B) Paired-pulse ratio (40-ms interstimulus interval) of the evoked EPSP is not significantly changed following application of either TRAM-34 (100 nM) or Ni\(^{2+}\) (100 μM). (C and D) The primary effect of both TRAM-34 and Ni\(^{2+}\) is on the EPSP rate of decay, with no significant effect on the mean value of EPSP amplitude. Sample values are shown in brackets at the base of bar plots and average values are mean ± SEM.
Table S1. Properties of outward current isolated from Purkinje cell somatic outside-out macropatch recordings under steady-state conditions from a holding potential of −110 mV

<table>
<thead>
<tr>
<th>Means for isolation</th>
<th>n</th>
<th>Amplitude at −30 mV</th>
<th>$t_{1/2}$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage (−40 mV holding potential)</td>
<td>6</td>
<td>24.8 ± 6.2 pA</td>
<td>22.6 ± 1.4</td>
</tr>
<tr>
<td>TRAM-34 (100 nM)</td>
<td>4</td>
<td>8.1 ± 3.3 pA</td>
<td>26.5 ± 9.3</td>
</tr>
<tr>
<td>Ni$^{2+}$ (100 μM)</td>
<td>4</td>
<td>14.8 ± 2.2 pA</td>
<td>20.8 ± 8.7</td>
</tr>
</tbody>
</table>

Currents were isolated by subtraction of test responses recorded in TRAM-34, Ni$^{2+}$, or voltage isolation by holding at −40 mV. The contents of external medium and internal electrolytes are provided in Materials and Methods. Average values are mean ± SEM.