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Contributions of T-Type Voltage-Gated Calcium Channels to Postsynaptic Calcium Signaling within Purkinje Neurons

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Abstract Low threshold voltage-gated T-type calcium channels have long been implicated in the electrical excitability and calcium signaling of cerebellar Purkinje neurons although the molecular composition, localization, and modulation of T-type channels within Purkinje cells have only recently been addressed. The specific functional roles that T-type channels play in local synaptic integration within Purkinje spines are also currently being unraveled. Overall, Purkinje neurons represent a powerful model system to explore the potential roles of postsynaptic T-type channels throughout the nervous system. In this review, we present an overview of T-type calcium channel biophysical, pharmacological, and physiological characteristics that provides a foundation for understanding T-type channels within Purkinje neurons. We also describe the biophysical properties of T-type channels in context of other voltage-gated calcium channel currents found within Purkinje cells. The data thus far suggest that one specific T-type isoform, $Ca_v3.1$, is highly expressed within Purkinje spines and both physically and functionally couples to mGluR1 and other effectors within putative signaling microdomains. Finally, we discuss how the selective potentiation of $Ca_v3.1$ channels via activation of mGluR1 by parallel fiber inputs affects local synaptic integration and how this interaction may relate to the overall excitability of Purkinje neuron dendrites.

Keywords T-type · Calcium channel · $Ca_v3.1$ · Purkinje · Parallel fiber · mGluR1

Abbreviations

AP	Action potential
CF	Climbing fiber
CNS	Central nervous system
HVA	High-voltage activated
IP_3R	Inositol trisphosphate receptors
KO	Knock-out
LTD	Long-term depression
Ni^{2+}	Nickel
PCs	Purkinje cells
PF	Parallel fiber
RAMP	Random access two photon microscopy
sEPSC	Slow excitatory postsynaptic current
sEPSP	Slow excitatory postsynaptic potential
PSD	Postsynaptic density
WT	Wild-type

Introduction

The discovery of calcium spikes in Purkinje cell (PC) dendrites by Llinas and Sugimori [1] and their subsequent

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seminal studies initiated an era of unraveling the roles of dendritic voltage-dependent conductances in neurons. Since the characterization of high-voltage activated (HVA) P-type calcium channels in PCs [2, 3], and the finding that the selective pharmacological block of P-type channels inhibited calcium spikes in PC dendrites [3, 4], the study of calcium signaling in PC dendrites has largely been focused on the P-type calcium channel. However, the spatial resolution of calcium imaging techniques available in the 1980s could not adequately discriminate between secondary or tertiary dendrites and individual spines, while the temporal resolution did not allow for the fine description of calcium spikes. For almost 20 years now, robust low threshold calcium currents have been recorded from PCs, yet few physiological implications have been demonstrated from these studies. After a brief discussion of relevant T-type calcium channel properties, we will develop new hypotheses linking both T-type and P-type channels to functional dendritic integration of synaptic inputs in PC dendrites.

T-Type Channel Biophysics

T-type channels have many characteristic biophysical properties unique among voltage-gated calcium channels which enable them to serve specialized functions within the nervous system (see "Physiological Roles of T-Type Channels in the CNS: Promoting Bursting Modes"). These include fast activation and inactivation kinetics, a relatively hyperpolarized voltage-dependence of activation and inactivation, and slow deactivation kinetics [5]. The activation and inactivation kinetics of T-type channels are strongly voltage-dependent. After initial cloning, the recombinant T-type isoforms were biophysically well characterized, and these properties have been thoroughly reviewed [5]. In brief, comparison of the three rat Ca_v3 T-type isoforms revealed that $Ca_v3.1$ and $Ca_v3.2$ have properties similar to "typical" native T-type currents, while $Ca_v3.3$ possesses distinct biophysical properties. The $Ca_v3.1$ and $Ca_v3.2$ isoforms have fast activation and inactivation kinetics while $Ca_v3.3$ channel activation and inactivation kinetics are much slower [6]. A recent paper comparing T-type biophysical parameters at room temperature and at a physiological mammalian temperature (37°C) demonstrated that increasing the recording temperature dramatically alters many of these properties in a non-linear, isoform-specific manner [7]. In this regard, caution should be used when extrapolating specific T-type parameters measured at room temperature to models of physiological neuronal excitability. Alternative splicing creates additional functional diversity in T-type channel activity. For example, alternative splicing in the human $Ca_v3.1$ channel leads to multiple variants including those that shift the voltage dependence of inactivation in the hyperpolarizing direction and also increase inactivation kinetics [8]. Also, thalamic splice variants of the $Ca_v3.2$ channel are differentially affected by a missense mutation from the genetic absence epilepsy rats from Strasbourg model of absence epilepsy [9].

T-Type Channel Pharmacology and Modulation

The study of T-type currents in native systems has been historically hindered by two major issues: (1) some HVA calcium channels in fact activate at relatively negative potentials (e.g., $Ca_v1.3$ L-type and $Ca_v2.3$ R-type) and contaminate what were previously thought to be pure low threshold T-type currents and (2) unlike the HVA calcium channel classes, no high affinity channel antagonists are commercially available that clearly distinguish T-type currents from HVA currents or that distinguish between individual T-type channel subtypes. These issues were compounded in early investigations of native T-type currents, where biophysical properties and sensitivities to pharmacological antagonists such as nickel (Ni^{2+}) varied depending on the cell type. The cloning and characterization of the three main T-type isoforms has helped explain these divergent properties and has provided some clarification concerning the limitations and suitable uses of the pharmacological tools presently available.

While one of the earliest T-type current antagonists to be identified was Ni^{2+}, the sensitivity to this agent is highly variable between different native systems. For example, Ni^{2+} inhibits T-type currents in chick skeletal muscle cells with an IC_{50} of 21 μM [10], while it is a much less effective T-type current inhibitor (IC_{50}=110 μM) in cerebellar PCs [11]. Molecular identification of the three Ca_v3 channels revealed that $Ca_v3.2$ is the only T-type isoform highly sensitive to Ni^{2+}, with an IC_{50} of 12 μM compared to IC_{50} values of 250 and 216 μM for $Ca_v3.1$ and $Ca_v3.3$, respectively [12]. Furthermore, Ni^{2+} blocks $Ca_v1.2$ L-type and $Ca_v2.3$ R-type channels with a higher potency than either of the $Ca_v3.1$ or $Ca_v3.3$ T-type isoforms [12, 13]. Therefore, low concentrations (e.g., ~50 μM) of Ni^{2+} can only be used to selectively block $Ca_v3.2$-mediated T-type currents (with minimal blockade of $Ca_v3.1$- and $Ca_v3.3$-mediated T-type currents) but notably cannot reliably distinguish between $Ca_v3.2$- and $Ca_v2.3$-mediated R-type currents at this concentration.

There are a number of clinically used agents that also nonspecifically target T-type channels. Mibefradil (now withdrawn from the market) is a prime example of an agent with good efficacy in blocking T-type currents. For research purposes, mibefradil was found to selectively inhibit T-type currents (IC_{50} ranging from 14 nM to 1 μM) over HVA currents in some native systems, with state-dependent block causing greater inhibition of T-type currents at more depolarized potentials [14]. However, other studies showed

that mibefradil can potently block R-type currents in NG108-15 cells [15] and can also block N-type, L-type, and P-type calcium currents at concentrations of ~1 μM in spinal motor neurons [16]. Due to the nonspecificity of mibefradil action, this compound is now deemed to be a relatively nonspecific T-type antagonist. In recent developments, pharmaceutical companies are beginning to identify small organic molecules that are proving highly efficacious (nanomolar affinity) in blocking T-type channels [17]. One such compound, TTA-P2, potently (IC_{50}=20 nM) blocks T-type currents in thalamocortical and reticular thalamic neurons without altering HVA calcium currents, sodium currents, or glutamatergic and GABAergic synaptic currents [17].

T-type channels were originally thought to be largely resistant to modulation by intracellular signaling pathways. Subsequent studies on T-type modulation in native systems revealed a number of discrepancies, with individual neurotransmitter types being reported to inhibit, stimulate, or have no effect on T-type currents depending upon the tissue and cell type being examined (reviewed in [18]). After a lull following their initial cloning and characterizations, the study of recombinant T-type channel modulation is emerging as an essential tool aimed at shedding further light on low threshold current regulation within native systems. Of the three T-type isoforms, $Ca_v3.2$ is the prototypical modulated T-type isoform as it appears to be specifically targeted by Gβγ [19, 20], CAMKII [21], and redox agents [22]. More recent studies are also now identifying signaling pathways that act on the $Ca_v3.1$ and $Ca_v3.3$ isoforms [23–26]. As discussed in subsequent sections, we have identified a differential modulation of T-type isoforms that results in the potentiation of $Ca_v3.1$-mediated T-type currents within PCs [27].

Physiological Roles of T-Type Channels in the CNS: Promoting Bursting Modes

The low threshold activation of T-type currents enables them to open in response to relatively small membrane depolarizations and to generate "window currents" whereby a fraction of channels are tonically open at resting membrane potentials. Both of these properties are highly dependent on the resting membrane potential, as T-type channels become completely inactivated at more depolarized potentials and are subsequently not available to open without a de-inactivating hyperpolarization [28]. In thalamocortical relay cells, the T-type window current contributes directly to the membrane potential [17] and has been suggested to be essential for the slow sleep oscillations of thalamic neurons [29].

The above properties enable T-type currents to be "first responders" to changes in membrane potential, which potentially impacts both calcium-mediated signaling pathways as well as electrical firing patterns. For example, T-type calcium spikes can have profound effects on global neuronal excitability. Low-threshold calcium spikes were first identified from brain slices of the inferior olive, where removal of T-type inactivation with hyperpolarization initiated a spontaneous "rebound-burst" spike [30]. T-type channels have now been shown to underlie regenerative low-threshold spikes and burst firing in neurons throughout the central nervous system (CNS), including in the thalamus, inferior olive, cerebellum, hippocampus, cortex, and neocortex (reviewed in [31]). In some neurons, low threshold spikes and burst firing can alter neuronal oscillations, causing cells to switch from tonic firing to a phasic mode with regular intervals of high frequency bursts of spikes [32, 33]. Within the thalamus, T-type-mediated changes in rhythmic oscillations directly underlie physiological sleep–wake gating and pathophysiological epileptic absence seizure activity [34, 35].

Recent immunostaining experiments have revealed a differential subcellular localization of the three T-type isoforms in the spines, dendrites, and soma of neurons throughout the CNS [36, 37]. The predominant expression of T-type currents in neuronal dendrites also implicates their potential involvement in signal integration at synaptic inputs. In both pyramidal cortical and hippocampal CA1 neurons, subthreshold excitatory postsynaptic potentials (EPSPs) can activate T-type currents and generate a localized increase in dendritic calcium levels [38, 39]. This T-type activity may act to boost dendritic depolarizations and, therefore, increase excitability, or conversely, may activate calcium-activated potassium currents to cause membrane hyperpolarizations [40]. T-type channel inputs have also been linked to synaptic plasticity [41, 42], although the use of imperfect pharmacological tools make these postulations preliminary. More thorough investigations involving high-resolution two-photon calcium imaging, Ca_v3 knock-out (KO) mice, Ca_v3 RNAi-mediated knock-down, and/or more specific T-type antagonists are required to help elucidate the exact physiological roles of dendritic T-type currents in both plasticity and excitability.

Expression and Localization of Voltage-Gated Calcium Channels in Purkinje Neurons

Voltage-Gated Calcium Channel Distribution and Functional Expression

Of the various calcium channel classes, P-type channels ($Ca_v2.1$) display the highest functional expression within PCs, forming whole-cell currents on the order of several nanoamps [2, 3, 43]. $Ca_v2.1$ channel proteins are expressed throughout the PC dendrites, soma, and even axons, but are

most highly expressed within dendritic spines that are postsynaptic to parallel fiber (PF) inputs [44, 45]. There is also both immunohistochemical [46] and electrophysiological [47] evidence for the expression of functional L-type channels ($Ca_v1.2$, $Ca_v1.3$) in the soma of PCs, but the relative expression of L-type channels appears to decrease during PC maturation [43], potentially due to the increased expression of other calcium channel classes within developing PC dendrites. There is little evidence for the expression of ω-conotoxin-sensitive N-type currents ($Ca_v2.2$) in PCs [48] and while the R-type channel isoform, $Ca_v2.3$, has been shown to be expressed at the protein level in PCs [49, 50] there has yet to be evidence of functional expression.

T-type calcium channel activity was first identified in PCs using sharp electrode intracellular recordings in adult rat cerebellar slices. These recordings on mature PCs revealed a prominent inward-rectifying hyperpolarization-activated current (Ih) as well as a putative low threshold calcium conductance that was de-inactivated by hyperpolarization [51]. One single channel recording study failed to identify low threshold calcium currents in acute cerebellar slices from adult guinea pigs and so the presence of T-type currents in mammalian PCs was initially somewhat controversial [3]. T-type currents were however subsequently identified in PCs of both juvenile and adult rats and mice through recordings on acutely dissociated and primary cultured PCs, PCs from slice cultures and PCs from acute brain slices [11, 52–58]. Purkinje neuron T-type currents possess all of the hallmark T-type current biophysical properties, including low threshold activation ranging between −60 and −40 mV, small single channel conductance between 7 and 9 pS, a relatively hyperpolarized voltage dependence of inactivation, fast activation and inactivation kinetics, and relatively slow deactivation kinetics [11, 52–54, 57]. Cell-attached recordings on PCs from newborn rat slice cultures demonstrated that T-type currents are distributed more densely on dendritic membranes compared to somatic membranes [54]. This finding is consistent with the observation that in a cell culture model of PC development T-type currents are only present in PCs that have developed a dendritic structure [58]. We furthered these investigations by using a combination of two photon calcium imaging and voltage-clamp recordings on PCs from juvenile rat acute cerebellar slices to show that T-type calcium currents are present in both the spines and dendrites of PCs [57]. The T-type currents have a large peak amplitude (approx. −2 nA at postnatal day 10) that increases with developmental age, which indicates that T-type currents are functionally expressed in adult rodent PCs [27, 57] (Fig. 1a). Several studies have now reached the consensus that P-type and T-type currents comprise the vast majority (up to 95%) of calcium channel currents in mature cerebellar PCs [3, 56, 57, 59]. The specific Ca_v3 isoform(s) that compose the native T-type currents within PCs is discussed in "The $Ca_v3.1$ Isoform is the Major Functional T-Type Calcium Channel Expressed in Rodent Purkinje Cells."

There are several technical limitations and caveats to recording calcium currents in PCs that are not always explicitly addressed in studies but that should be highlighted. The high expression of calcium channels in the extensive dendritic arbor of PCs means that space clamp issues arise during whole-cell recordings from intact PCs in cerebellar slices. Thus, whole-cell calcium currents can only be adequately clamped up to approximately postnatal day 14 or even younger (especially for large P-type currents) in PCs from slices ([57] and our unpublished observations), which inversely correlates with dendritic development during PC maturation [60, 61]. In fact, in more mature PCs, the space clamp can become so poor that dendrites are able to fire calcium spikes [56]. Cell-attached recordings can be performed on the soma and dendrites of PCs although this technique is technically challenging, restricts quantitative investigations, and is affected by heterogeneity in channel distribution [43]. Space clamp issues are minimized in recordings on acutely dissociated PCs, but these neurons lack the dendritic tree where many calcium channels predominate and the protease treatments used during dissociation can significantly alter calcium channel expression compared to recordings on equivalent PCs from slices (our unpublished observations and [62]). Similarly, cultures of isolated PCs or cerebellar slices are taken from newborn or juvenile rats and subsequent dendritic growth and channel expression is determined by *in vitro* culture conditions [63]. A combination of pharmacological tools, genetically engineered mice strains, and two-photon calcium imaging has thus far been the only configuration to date that allows adequate study of calcium channel activity in mature animals [27].

The $Ca_v3.1$ Isoform is the Major Functional T-Type Calcium Channel Expressed in Rodent Purkinje Cells

The properties of T-type currents recorded from PCs include low sensitivity to Ni^{2+}, fast inactivation kinetics, and slow deactivation kinetics, which are more characteristic of $Ca_v3.1$ currents than either $Ca_v3.2$ or $Ca_v3.3$ currents [11, 57] (Fig. 1a, b). In support, the overall consensus from *in situ* hybridization and immunohistochemical experiments is that T-type channels are robustly expressed in the soma and dendrites of PCs, with predominant expression of $Ca_v3.1$ and the potential expression of $Ca_v3.3$ in a subset of cells [27, 36, 64–67].

We have further provided several lines of evidence which demonstrate that the $Ca_v3.1$ T-type isoform conducts

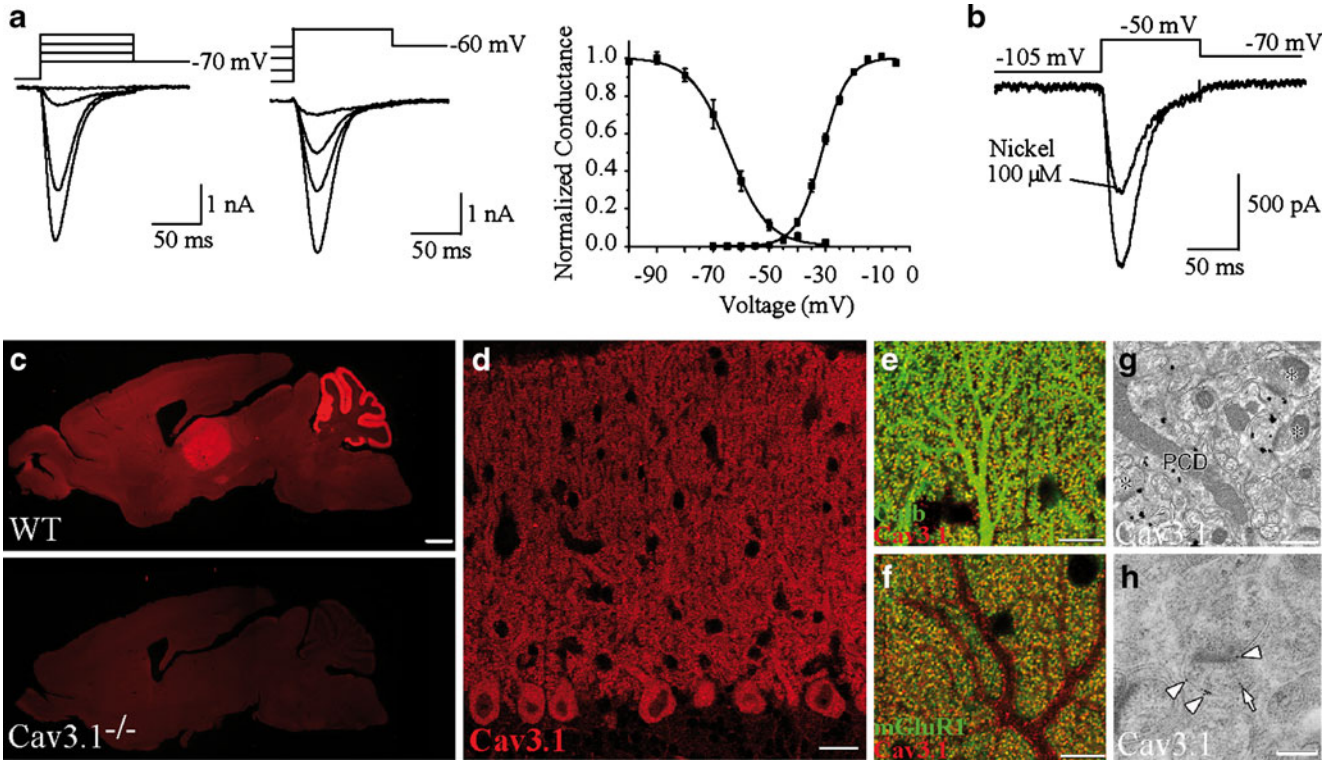


Fig. 1 Biophysical properties of T-type currents in juvenile rodents and $Ca_v3.1$ expression in adult rodents. **a** T-type current activation and inactivation in juvenile rats. *Left panel* depolarizing steps to −65, −55, −45, and −40 mV were applied from −105 mV. *Middle panel* depolarizing steps to −40 mV were preceded by conditioning steps to −80, −75, −70, and −60 mV. *Right panel* normalized conductance curves for activation and steady-state inactivation fitted with Boltzmann equations. **b** Effect of nickel application (100 µM) on T-type current (mean inhibition was 35±15%, n=4). **c–h** Immunofluorescence showing predominant distribution of $Ca_v3.1$ in the dendritic spines of cerebellar Purkinje cells. **c** Specificity of $Ca_v3.1$ antibody in mouse brain. Note intense labeling in the cerebellum (*Cb*) and thalamus (*Th*) of WT but not $Ca_v3.1^{-/-}$. *Scale bar* 1 mm. **d** Immunofluorescence for $Ca_v3.1$ in the cerebellar cortex, *scale bar* 20 µm. **e**, **f** Double immunofluorescence for $Ca_v3.1$ (*red*) and calbindin (*green*) in (**e**) and mGluR1 (*green*) in (**f**), *scale bar* 10 µm. Note the colocalization in spines. **g**, **h** Pre-embedding silver-enhanced immunogold in (**g**) and postembedding immunogold in (**h**) showing $Ca_v3.1$ perisynaptic localization. *Scale bar* 500 nm in (**g**) and 200 nm in (**h**). Panel a from Isope and Murphy [57] and panels c–h from Hildebrand et al. [27] with permission

the majority of functional T-type currents within PCs of both juvenile and adult rodents [27]. Recordings on both juvenile and mature PCs from transgenic $Ca_v3.1$ KO mice show a near complete elimination of T-type currents and calcium transients compared to wild-type (WT) mice. Furthermore, immunofluorescence with a $Ca_v3.1$-specific antibody reveals a robust expression of $Ca_v3.1$ in PCs that rivals the thalamus for the highest overall $Ca_v3.1$ expression in the brain (Fig. 1c). At a subcellular level, confocal and electron microscopy demonstrate that $Ca_v3.1$ is predominantly localized to dendritic spines at PF synapses in both juvenile and adult mice (Fig. 1d–h).

Native $Ca_v3.1$ T-Type Channels are Potentiated by mGluR1

Both the synaptic and pharmacological activation of mGluR1 results in a robust and reversible potentiation of $Ca_v3.1$-mediated T-type transients within PCs. This potentiation involves both an increase in maximal currents and a slight hyperpolarizing shift in the voltage-dependence of T-type channel activation, enabling T-type channels to more potently respond to small depolarizations near the PC resting membrane potential [27]. Interestingly, the potentiation of T-type currents by mGluR1 occurs through the same non-canonical G-protein mediated pathway that couples mGluR1 to the TRPC-mediated slow excitatory postsynaptic current (sEPSC) within PCs [68, 69]. Extensive pharmacological investigations revealed that both the activation of the sEPSC and the potentiation of T-type currents by mGluR1 involve an intracellular pathway that is independent of phospholipase C and other classical downstream mGluR1 effectors but that is dependent upon tyrosine phosphorylation/dephosphorylation [27, 70]. Both sEPSC activation and T-type potentiation are blocked by tyrosine phosphatase antagonists such as orthovanadate and bpV(phen) and are conversely potentiated by PP1, an inhibitor of Src-family tyrosine kinases (Fig. 2a). The augmentation of T-type currents by mGluR1 also involves

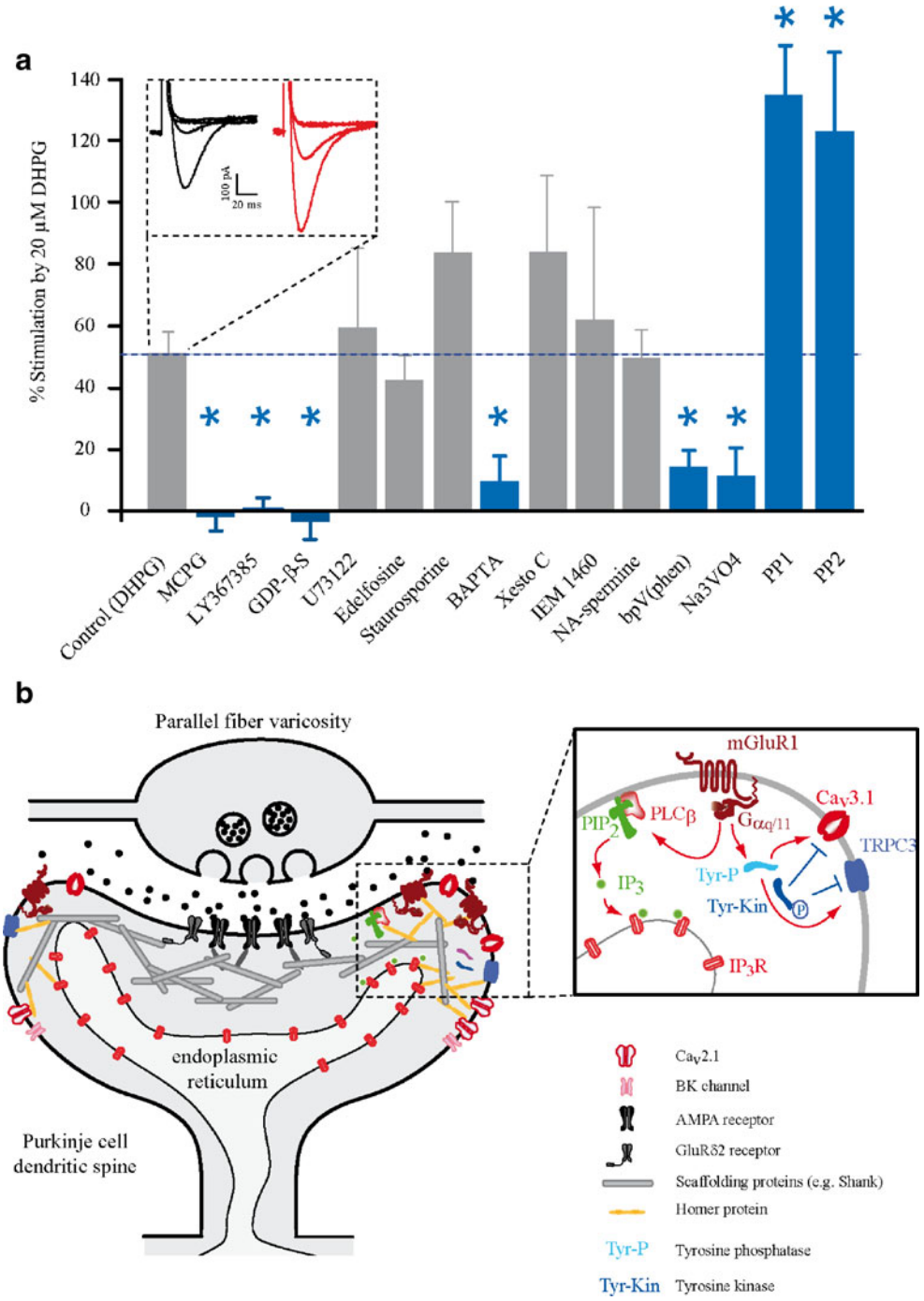
intracellular calcium signaling but does not require the activation of the sEPSC channels themselves [27]. As sEPSCs are activated by PF stimulation within PC dendritic spines [71], the above observations have led us to hypothesize that $Ca_v3.1$ T-type channels are physically coupled with mGluR1 and various other signaling elements within postsynaptic PC spines (Fig. 2b).

$Ca_v3.1$ Channels are Embedded in a Scaffolding and Calcium Signaling Complex within Dendritic Spines

Unraveling the fine microstructure and protein scaffolding organization within PC dendritic spines is yielding exciting new results towards understanding local synaptic integration. Biochemical assays have demonstrated physical interactions between several calcium signaling pathways and the structural organization of the spine [72, 73] (Fig. 2b). The scaffolding of postsynaptic densities (PSDs) in PC spines includes tight interactions between PDZ domain proteins such as PSD-93 and Shank [72, 74, 75]. AMPA receptors (containing GluR2) that mediate fast synaptic transmission are linked to this structure via GRIP and PICK1 proteins [76, 77]. Purkinje cell spines that receive PF inputs contain a specific PSD that lacks NMDA receptors but includes the GluRδ2 receptor, a GluR subunit with thus far no assigned ionotropic activity [78]. After 3 weeks of age, GluRδ2 is specifically expressed at the PF-PC synapse and is essential for both stabilization and plasticity (long-term depression; LTD) of this synapse via an interaction between its C terminus and PSD scaffolding proteins such as Shank [79, 80] [72]. Homer 3 is another highly expressed and critical scaffolding protein that forms a lattice around the PSD of PF-PC synapses [81–83]. Homer also binds calcium signaling proteins; indeed, homer co-immunoprecipitates with GluRδ2, Shank, mGluR1a, PLCβ4 and IP_3 receptors (IP_3Rs) [72, 84]. This architecture surrounds the PSD like a belt and links it to the endoplasmic reticulum via IP_3Rs, suggesting that proteins of the mGluR1/PLC/IP_3 pathway, involved in the induction of LTD (for review, see [85]), are tethered to each other by homer proteins. Electron microscopy studies have shown that mGluR1 and PLCβ4 receptors are highly expressed at the perisynaptic site of the PF synapse [73, 86]. Upon glutamate binding to mGluR1, the coupled $G\alpha_{q/11}$ activates two independent intracellular pathways: one leads to calcium release from internal stores through IP_3Rs [87–89], while a second pathway activates a slow EPSP [90] mediated by a nonselective cation channel that is calcium permeable and recently identified as the TRPC3 channel [68] (Fig. 2b). Like IP_3-mediated calcium release, this sEPSC has a delayed onset in the range of hundreds of milliseconds [90]. PTPMEG tyrosine phosphatase is highly expressed in PC spines and binds to GluRδ2 receptors in the PSD structure and thus, could be directly involved in the tyrosine-phosphatase dependent activation of sEPSCs (see "Native $Ca_v3.1$ T-Type Channels are Potentiated by mGluR1"). Interestingly, PTPMEG KO mice and TRPC3 KO mice exhibit severe impairment in rotarod tests and walking coordination, respectively [68, 91].

Although membrane depolarization is thought to spread throughout the spines and dendrites of PCs, suggesting that voltage-dependent channels do not need to be part of a microdomain to be activated, recent studies have demonstrated that calcium channels are also part of this scaffolding belt surrounding the PSD in PF spines and that they can be modulated by physical interactions. Functional and physical interactions between T-type and P-type calcium channels and scaffolding architecture might impact calcium signaling within PC spines and dendrites. Electron microscopy and biochemical assays have demonstrated a physical link between mGluR1s and $Ca_v2.1$ (P-type) channels in spines via their carboxyl terminal intracellular domains, leading to a decrease in calcium conductance [44, 92]. Conversely, a coactivation of mGluR1 and $Ca_v2.1$ channels can also induce large calcium increases within PCs [92]. Furthermore, functional and anatomical studies have demonstrated that a combination of P-type calcium channel activity and mGluR1 activity can activate BK potassium

Fig. 2 The spine machinery: intracellular pathway involved in the functional coupling between $Ca_v3.1$ and mGluR1. **a** Bar graph showing T-type channel potentiation values during various pharmacological treatments compared with the control (DHPG) potentiation value (indicated by *dashed line*). Blocking mGluR1 receptors with 500 μM MCPG ($n=6$) or 100 μM LY367385 ($n=8$), inhibiting G-protein signaling with 2 mM GDP-β-S ($n=5$), buffering intracellular Ca^{2+} through inclusion of 20 mM BAPTA ($n=6$) in the pipette, and blocking tyrosine phosphatases with 100 μM bpV(phen) ($n=6$) or 1 mM Na_3VO_4 ($n=5$) all significantly (*blue bars*; $p<0.02$) reduced the DHPG-mediated increase. Conversely, blocking Src-family tyrosine kinases with inclusion of 10 μM PP1 ($n=5$) or 10 μM PP2 ($n=6$) in the pipette significantly ($p<0.02$) augmented the DHPG-induced increase. Blocking phospholipase C with 1 μM U73122 ($n=6$) or 10 μM edelfosine ($n=7$), serine/threonine kinases (such as protein kinase C) with 1 to 2.5 μM staurosporine ($n=8$), IP_3Rs with 1 μM xestospongin C ($n=6$), and sEPSC currents with 250 μM IEM 1460 ($n=6$) or 100 μM NA-spermine ($n=5$) all caused no significant ($p>0.05$) change in the level of DHPG-mediated increase in T-type currents. *Inset* Representative voltage-clamped current traces from rat PCs during depolarizations to potentials ranging from –60 to –30 mV before (*left*; *black*) and after (*right*; *red*) mGluR1 was activated with 30 μM DHPG. From Hildebrand et al. [27] with permission. **b** Synaptic scaffolding of the parallel fiber to Purkinje cell synapse. Glutamate AMPA and GluRδ2 receptors are embedded in a subsynaptic ultrastructure composed notably by Shank and Homer proteins. Homer proteins also tether various calcium signaling receptors and channels to scaffolding proteins, creating a perisynaptic molecular microdomain ideally suited for calcium signal integration within Purkinje cell spines. *Inset* mGluR1 receptor activation leads either to a release of calcium from internal stores via the PLCβ/IP_3 pathway or to activation of $Ca_v3.1$ and TRPC3 via tyrosine phosphatases



currents in PC spines, indicating that this channel may also be closely associated with mGluR1 at the PF-PC synapse [93–95]. In a recent study [27], we suggest that $Ca_v3.1$ and mGluR1 are tightly linked together within PC spines. Although, $Ca_v3.1$ channels and mGluR1s are also found in PC dendrites, they are both highly expressed at the perisynaptic site around the PSD (Fig. 1f–h). Furthermore, when internal stores are blocked, the mGluR1-mediated augmentation of T-type calcium transients is observed in PC spines, but not their parent dendrites, indicating that the modulation occurs in a microdomain within spines. This evidence suggests either a physical link between $Ca_v3.1$ and mGluR1 or that key elements of the modulation pathway are restricted to PC spines. We have also demonstrated that $Ca_v3.1$ channels are potentiated by the same unique mGluR1 signaling pathway as for sEPSCs (see "Native $Ca_v3.1$ T-Type Channels are Potentiated by mGluR1"). Thus, we propose that activation of mGluR1-mediated pathways can increase intracellular calcium levels within PF-PC spine microdomains through the activation of

both voltage-dependent ($Ca_v3.1$ and $Ca_v2.1$) and voltage-independent (sEPSC) membrane calcium channels (Fig. 2b). The specific physiological roles of both $Ca_v3.1$ and $Ca_v2.1$ channels are discussed in "PF Inputs Induce Spine-Specific Fast Calcium Signaling Mediated by $Ca_v3.1$ T-Type Channels."

Although direct evidence has not yet linked all of these signaling elements together within a single PC distal dendritic spine, this proposed organization of microdomains below the PSD of the PF-PC synapse is likely to play a major role in calcium signaling and thus in the regulation of synaptic transmission. Indeed, in mice with mutations in myosin Va, the endoplasmic reticulum and associated IP_3Rs do not enter into the spine head of the PC [96]. In these mice, basic excitatory transmission is normal and all the synaptic proteins are functional; however, IP_3-mediated calcium signaling in spines is altered and LTD induction is impaired [96]. This is the first demonstration in PCs of the requirement for the structural integrity of the subsynaptic scaffolding microdomain.

T-type Channels Promote Bursting Behavior in Reduced Preparations

Recent studies have implicated T-type calcium channels as having a significant role in dendritic calcium spikes and the resultant burst firing within the soma and proximal dendrites of PCs (reviewed in [97]). For over 25 years, it has been accepted that sodium-driven action potentials (APs) are produced at the PC soma while calcium-driven APs are produced in the PC dendrites and that both are generally restricted to their respective compartments. The dendritic calcium spikes originally studied in guinea pig PCs were shown to include both calcium-dependent plateau potentials as well as calcium spikes [1, 98], and P-type calcium channels were thought to generate these calcium spikes [3, 56]. Studying PCs from rat organotypic cerebellar slice cultures revealed that T-type calcium channels underlie the dendritic calcium spikes while P-type channels underlie a plateau potential that is unmasked when potassium channels are blocked [99]. In fact, pharmacological blockade of the P-type current promoted propagation of the low threshold calcium spikes to the PC soma [100]. The robust P-type dendritic currents in PCs have been shown to activate calcium-dependent BK and SK potassium channels, which induces afterhyperpolarizing potentials and alters the frequency of PC firing [94, 95]. It is proposed that dendrosomatic propagation of T-type-dependent calcium spikes is inhibited by this activation of calcium-dependent potassium channels and that these low threshold spikes may underlie the CF-induced complex spike [100, 101]. In separate experiments on acutely dissociated PCs and PCs from acute cerebellar slices, it has been shown that P-type currents are required to sustain the spontaneous firing of PCs, while T-type currents have a substantial contribution to calcium currents generated during interspike intervals of spontaneous bursting PCs [59, 102].

Although this collection of studies implicates T-type calcium channels in generating calcium-dependent bursting in PC dendrites, with potentially large physiological implications, several limitations of these studies should be noted. Firstly, the overall structure and native composition of PCs is not well maintained in most of the *in vitro* systems used. Acutely dissociated PCs lack the dendritic tree where T-type calcium channels predominate [59] while cerebellar slice cultures are taken from newborn rats and subsequent dendritic growth and channel expression is determined by *in vitro* culture conditions [63, 99–101]. Indeed, in acute slices from young and mature rodents, T-type calcium currents are not directly involved in the generation of PC bursting behavior [59, 102–104] and except during the CF response, potassium channels likely prevent the generation of calcium spikes in Purkinje cell dendrites [95, 103, 105–107].

T-Type Channels in Local Calcium Signaling in Purkinje Cell Dendrites

PF Inputs Induce Spine-Specific Fast Calcium Signaling Mediated by $Ca_v3.1$ T-Type Channels

Miyakawa et al. [108] first demonstrated that bursts of PF stimulation induce two types of calcium transients in PC spines. While low-intensity PF stimulation induces a barely detectable graded response in the distal part of the dendrites (see also [109]), strong PF stimulation induces clear regenerative calcium spikes in PC dendrites. Using confocal and two photon laser microscopy, Eilers et al. [110] and Denk et al. [111] showed that subthreshold PF stimulation induces localized calcium transients in PC spines. Furthermore, both CNQX, an AMPA receptor antagonist, and hyperpolarization decrease the size of the calcium transients, suggesting that they are mediated by voltage-dependent calcium channels. More recently, we found that T-type channels are expressed in PC spines [57] and used ultrafast random access two photon microscopy (RAMP) [112] to demonstrate that bursts of low-intensity PF stimulation induce fast calcium transients that are mainly mediated by $Ca_v3.1$ T-type channels in individual spines [27]. At low-stimulation intensity, focal PF stimulation (trains of extracellular stimulation in the molecular layer) induces local calcium influx that is reduced by more than 75% in $Ca_v3.1$ KO mice compared to WT mice when internal stores are blocked by heparin. Since P-type

channels are also present in PC spines, why are they not activated by low intensity PF stimulation? As shown by Isope and Barbour [113], the individual synaptic efficacy of granule cell to PC connections is widely distributed. Based on the distribution of individual connections, the conversion factor between EPSCs and EPSPs (8.3 μV/pA, see figure 3 of [113]) and a model of dendritic filtering by Roth and Hausser [114], we can estimate that individual synaptic EPSPs range from tens of microvolts to a few millivolts. Although we certainly underestimate the filtering capacity of the spine neck and the drop of potential between the spine and parent dendrite [115, 116], such small EPSPs are below the threshold for activation of $Ca_v2.1$ P-type channels (around −45 mV). In fact, for moderate PF stimulations, the first EPSP in the train does not induce any detectable calcium influx, suggesting that temporal summation may be required to even reach the activation threshold for low threshold T-type channels (between −60 and −55 mV; Fig. 3). However, when stimulation intensity is raised and individual PF inputs now summate to locally depolarize PC dendrites, regenerative P-type calcium transients can be observed in several spiny branchlets

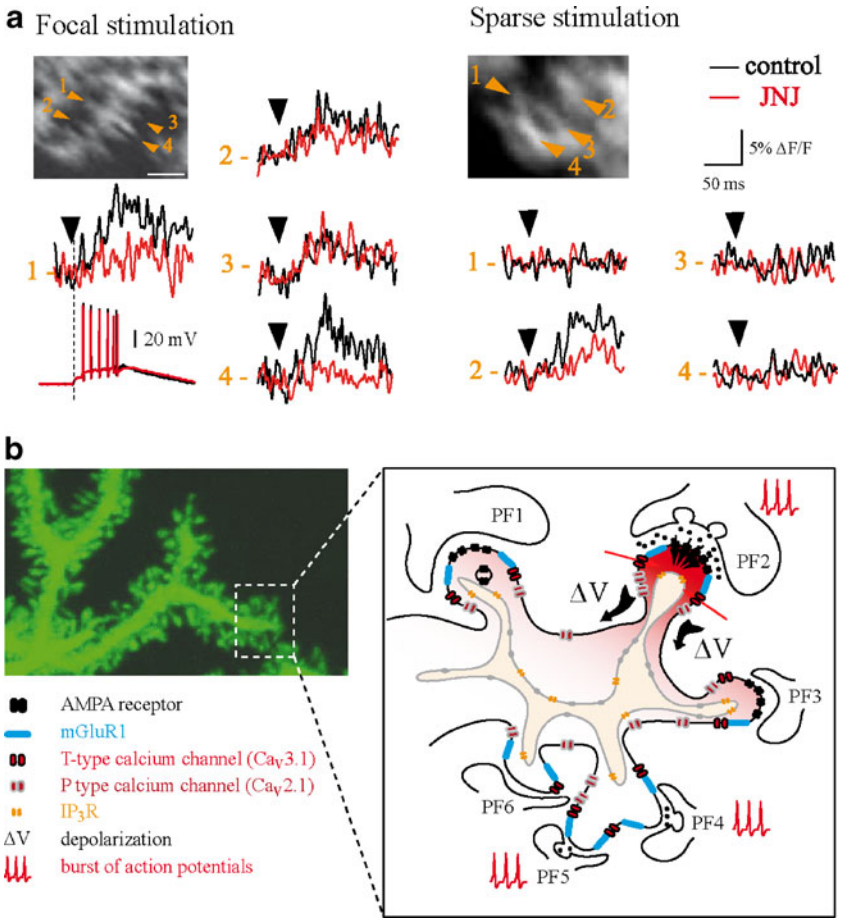


Fig. 3 Spine-restricted fast calcium signaling is mediated by T-type channels. **a** Variation of calcium fluorescence in individual spines following parallel fiber stimulation in control conditions (*black*) and during application of an antagonist of the mGluR1 receptor (*red*, 1.5 μM JNJ16259685; for detailed description, see [27]). In all experiments, calcium release by internal stores was pharmacologically blocked using heparin and cyclopiazonic acid. *Left panel* an example of the EPSPs recorded at the soma. Note that when multiple spines are activated in one spiny branchlet ("focal stimulation"), two types of voltage-dependent calcium transients are observed: in one population of spines (spines 2 and 3), the calcium transients are not blocked by the mGluR1 antagonist, suggesting that depolarization is the sole mediator of T-type channel opening. In a second population (spines 1 and 4), the calcium transients are highly affected by the mGluR1 antagonist, suggesting that they directly receive synaptic input. *Right panel* However, when one spine is active in the spiny branchlet ("sparse stimulation"), the calcium transient is almost completely controlled by mGluR1 activation (spine 2). We postulate that this situation resembles physiological conditions. From Hildebrand et al. [27] with permission. **b** Model of fast calcium signaling in Purkinje cell dendritic spines. When a burst of parallel fiber action potentials release enough glutamate to activate both AMPA and mGluR1 receptors, the combination of local depolarization and potentiation of T-type channel activation mediated by mGluR1 leads to an enhanced calcium transient in the activated spine. Spine specificity is promoted by: (1) the fact that in non-synaptically activated spines, the depolarization is smaller and does not reach the threshold for T-type channel activation (e.g., PF1 and PF3) and (2) the high number of silent synapses (e.g., PF4 and PF5; Isope and Barbour [113]). Spiny branchlet picture courtesy of Boris Barbour

(Supplementary figure 6 in [27] and unpublished data). Interestingly, these calcium spikes do not propagate to the soma and Rancz and Hausser have also demonstrated that calcium-activated BK channels can restrict the spread of calcium spikes in PC dendrites [105].

We have also shown that mGluR1 gates the T-type calcium transients by shifting the activation curve towards hyperpolarized potentials and increasing maximal conductance. Blockade of mGluR1 leads to a significant decrease in synaptic calcium transients produced by low intensity burst stimulation of PFs. Interestingly, when focusing on individual spiny branchlets, we observed that focal stimulation of PF inputs can induce T-type calcium transients in most spines of a given branchlet, leading to depolarization of the entire branchlet (Fig. 3). In this particular case, "unlocking" by mGluR1 is not necessary for the activation of T-type channels (although mGluR1-mediated potentiation is still observed in most spines). However, at the periphery of the excited PF beam, it is possible to identify spiny branchlets wherein T-type calcium transients are elicited in single spines and are almost fully blocked by mGluR1 antagonist (Fig. 3). We postulate that T-type channel signaling is designed to mediate local calcium signaling selectively in spines that receive a PF input. *In vivo* experiments have revealed a large dynamic range for granule cell activity: although the activity of granule cells is notably sparse, they are also capable of bursting at frequencies exceeding 1 kHz [117–119]. We hypothesize that mGluR1 activation will both amplify the discrepancy between activated spines and their neighbors as well as detect incoming bursts of PF inputs.

Climbing Fiber Signaling: Decoupling Somatic and Dendritic Excitability

During the 1990s, the laboratory of William Ross first demonstrated that distal CF-evoked calcium transients are highly variable compared to proximal ones ([108]; see also figure 1 of [109]), suggesting that regenerative calcium spikes do not always propagate to spiny branchlets. Furthermore, they showed that evoked inhibition by activation of molecular layer interneurons is able to block the CF-activated calcium transient in identified dendritic branches or even the full dendritic tree [120]. Ross and colleagues also showed that the shape of the complex spike recorded at the soma of PCs is not modified by the extent of the dendritic calcium transient, suggesting that sodium channel input is the major component of the plateau depolarization typical of the complex spike waveform. This finding was confirmed in a recent article using simultaneous dendritic and somatic patch-clamp recordings [121]. Since a direct projection of the CF onto molecular interneurons has now been demonstrated both *in vitro* and *in vivo* [122, 123], it appears that synaptic connections between stellate and basket cells might dampen the extent of the calcium transient mediated by the CF input. Finally, the group of Stephane Dieudonné showed that T-type calcium channels are also involved in CF calcium signaling that is dependent on PC dendritic excitability ([124]; see also [125]).

An emerging hypothesis of CF signaling in PCs is that the stereotyped somatic complex spike mediated by sodium channels is decoupled from the dendritic calcium transient that is highly variable and strongly modulated both by intrinsic dendritic conductances and molecular layer interneuron inhibition. How could such a large depolarization by climbing input in the main trunk of PC dendrites decline so rapidly and lead to only small depolarizations in spines? Since both low threshold (K_v1 and $K_v4.3$) and high threshold (K_v3) voltage-dependent potassium channels have been identified in PC dendrites [106, 126–128], one hypothesis suggests that these channels can dampen CF EPSPs. Indirect experiments demonstrate that K_v1 or K_v4 blockade favors the initiation of calcium spikes in the dendrites, suggesting that low threshold potassium channels control dendritic excitability [106, 129]. We hypothesize that high levels of low-threshold potassium channels can dramatically decrease the large CF-EPSP as it travels up to more distal parts of the PC dendrites (as far as 150 μm in adult mice). Furthermore, after calcium spike occurrence, high levels of calcium-activated potassium channels (BK and SK) will help repolarize dendrites and limit spike propagation [95, 105]. These findings help explain the absence of propagation of calcium transients from the dendrites toward the somatic compartment. We further postulate that CF dendritic calcium spike gating regulates the source (T-type vs P-type) and amplitude of CF-mediated calcium signaling and provides for new mechanistic possibilities concerning activity-dependent learning in PCs.

Physiological Consequences and Future Directions

Based on the above discussions, we propose a new picture of voltage-dependent calcium signaling within PC dendrites and make hypotheses that may stimulate new experiments on information processing in the cerebellar cortex.

1. $Ca_v3.1$ T-type channels are highly expressed in PC dendrites and spines. T-type channels are not inactivated at rest and regular spiking at the soma does not interfere with channel availability in spines [27, 54]. Low threshold potassium channels such as K_v1 and $K_v4.3$ channels probably clamp the cell to hyperpolarized potentials [106, 126–128]. As recently described [130], the activity of inhibitory oscillatory networks could also enhance the deinactivation of

T-type channels in distal dendrites in some physiological conditions.

2. Parallel fiber synaptic input induces fast T-type calcium transients in spines following either conjunctive activation of a group of neighboring synapses or by mGluR1 activation, which selectively unlocks T-type channels in individual spines. This effect is specific to PF bursts and occurs during the burst itself with no time lag. The T-type channel conductances contribute to synaptic charge and boost individual synaptic inputs. LTP at PF synapses is mediated by an unknown mGluR1-mediated calcium-dependent pathway [131, 132] and we postulate that T-type channels could be the source of calcium in this plasticity.
3. Although 85% of PF-PC synapses are electrically silent in the adult rat when recorded at the soma, it is likely that sparse glutamate receptor activation at a synaptic site would not induce sufficient charge to be detected at the soma due to dendritic filtering. Indeed, it was demonstrated that all spines possess at least a small number of AMPA receptors [133]. The functional coupling between mGluR1 and T-type channels could then provide a mechanism for inducing calcium inputs in spines where the efficacy of transmission is low because of a small number of receptors. This pathway could potentially lead to the awakening of synaptic transmission to the soma via LTP.
4. We postulate that P-type channels are activated only when a large number of PF inputs impinge simultaneously on the PC dendritic tree, or during simultaneous CF activation. Conjunction of CF inputs and PF inputs could lead to the inactivation of low threshold potassium channels (K_v1, $K_v4.3$) and promote a supralinear calcium increase in spines as shown by Wang and Hausser [109] and LTD induction. T-type channel unlocking in the early phase followed by subsequent IP_3-mediated calcium release from internal stores might combine to reach a high level of calcium both in local microdomains and in the whole spiny branchlets. This second level of voltage-dependent calcium signaling is projected to propagate to groups of spiny branchlets and could trigger release of retrograde signaling via endocannabinoids [105] or glutamate [134]. The two levels of calcium signaling might be tightly regulated in PC dendrites by potassium channels and/or the depolarization state of the PC.

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