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On the Induction of Postsynaptic Granule Cell–Purkinje Neuron LTP and LTD

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Abstract In the last decade, several experimental studies have demonstrated that particular patterns of synaptic activity can induce postsynaptic parallel fiber (PF) long-term potentiation (LTP). This form of plasticity can reverse postsynaptic PF long-term depression (LTD), which has been traditionally considered as the principal form of plasticity underlying cerebellar learning. Postsynaptic PF-LTP requires a transient increase in intracellular Ca^{2+} concentration and, in contrast to PF-LTD, is induced without concomitant climbing fiber (CF) activation. Thus, it has been postulated that the polarity of long-term synaptic plasticity is determined by the amplitude of the Ca^{2+} transient during the induction protocol, with PF-LTP induced by smaller Ca^{2+} signals without concomitant CF activation. However, this hypothesis is contradicted by recent studies. A quantitative analysis of Ca^{2+} signals associated with induction of PF-LTP indicates that the bidirectional induction of long-term plasticity is regulated by more complex mechanisms. Here we review the state-of-the-art of research on postsynaptic PF-LTP and PF-LTD and discuss the principal open questions on this topic.

Keywords Parallel fiber · Ascending fiber · Purkinje neuron · LTP · LTD

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Introduction

According to the classical theory, the activity-dependent weakening of erroneously activated cerebellar granule cell (CGC) to Purkinje neuron (PN) synapses is the most important form of synaptic plasticity in the cerebellum [1, 2]. This view gained importance with the discovery that pairing parallel fiber (PF) activity and climbing fiber (CF) activity could induce long-term depression (LTD) of PF inputs [3, 4]. An important characteristic of the depression of PF excitatory postsynaptic potentials (EPSPs) is the postsynaptic site of induction and expression. PF-LTD requires elevation of free intracellular calcium concentration ($[Ca^{2+}]_i$) [5] and activation of metabotropic glutamate receptor 1 (mGluR1) [6–8], leading to a decrease in α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor efficacy [9] and internalization [10]. For PF silencing to be reversed, the necessity of a "reset" mechanism has been postulated [11–13].

In the last decade, the existence of postsynaptic forms of PF long-term potentiation (LTP) has been demonstrated [11, 12]. This form of plasticity is a potential reset mechanism because it shares the same site of expression as PF-LTD. It was first shown that postsynaptic PF-LTP could be induced by repetitive (1 Hz) PF stimulation without concomitant CF activation. The induction mechanism required elevation of $[Ca^{2+}]_i$, but smaller than that associated with the induction of CF-dependent PF-LTD [12]. This evidence suggested that the polarity of PF plasticity could be given by the size of the $[Ca^{2+}]_i$ transient, which is higher for LTD [12, 13]. The idea of different Ca^{2+} thresholds being responsible for plasticity mechanisms of different polarities was proposed in 1982 [14] and termed the "BCM rule." According to the BCM rule, lower and higher Ca^{2+} transients are associated with the induction of LTD and

LTP, respectively. Here, we will use the term "inverse BCM rule" [14] to refer to a scenario where lower and higher Ca^{2+} transients are associated with the induction of LTP and LTD, respectively.

Recently, it has been shown that local $[Ca^{2+}]_i$ elevation associated with bursts of PF-EPSPs can be much larger than that associated with CF-EPSPs, but repetitive application of this protocol leads to postsynaptic PF-LTP [15]. This finding, together with evidence that LTP could not be induced solely by local Ca^{2+} photorelease [16], suggested that bidirectional induction of plasticity cannot be explained only in terms of $[Ca^{2+}]_i$ signal amplitude.

Here we review the most recent findings on PF-LTP induction mechanisms and discuss our current understanding.

Revisiting Bidirectional Induction of Postsynaptic Plasticity

PF-LTD is classically induced by pairing PF input (either single stimuli or bursts) with CF activation also substituted by postsynaptic depolarization [17, 18]. PF-LTD is also induced by strong PF stimulation without pairing with CF activity [19]. Although some molecular pathways leading to synaptic depression may be similar in different induction protocols, the expected time course and the spatial localization of triggering signals remain essentially diverse. The same argument applies to PF-LTP. Thus far, LTP was induced by three stimulation protocols, namely, "one-pulse-induced PF-LTP," "burst-induced PF-LTP," and "single-train-induced PF-LTP" (see Fig. 1).

The first induction protocol to be characterized was the one-pulse-induced PF-LTP (Fig. 1a), a repetition of a single pulse of PF stimulation at 1 Hz for 5 min [11, 12]. Pairing a PF-EPSP with a CF-EPSP, with the CF-EPSP following the PF-EPSP, reversed LTP to LTD [12]. Both plasticity forms required transient $[Ca^{2+}]_i$ elevation, and a 20 mM injection of the high-affinity buffer BAPTA could lead to LTP induction with CF pairing [12]. To interpret this result, it has been suggested that PF plasticity follows an inverse BCM rule, compared to hippocampal synapses [13]. According to this view, LTP would be induced by a smaller $[Ca^{2+}]_i$ signal compared to LTD, and the additional Ca^{2+} would be provided by CF depolarization. Further support for this hypothesis came from a previous report showing that CF-EPSP was associated with a supralinear Ca^{2+} signal and that, for minimal PF activation, this signal was mGluR1 dependent and limited to dendritic spines [20]. The inverse BCM rule hypothesis, however, did not take into account two factors that were reported in that study. First, the protocol used for pairing consisted of a burst of PF-EPSPs (typically 5–10 pulses at 100 Hz) and not a single pulse. Second, by increasing the number of stimulated PF, a more

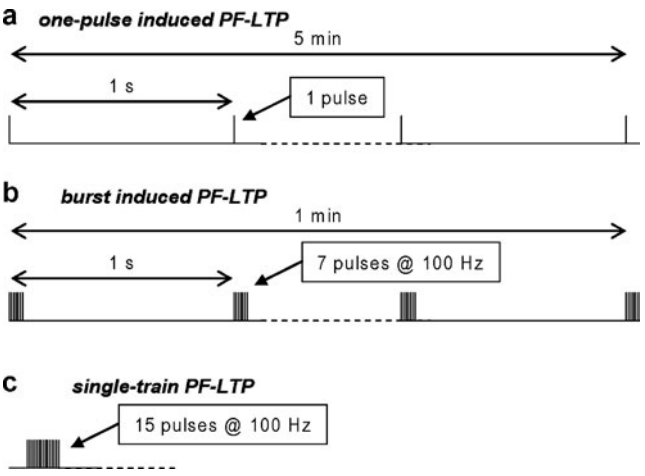

Fig. 1. Protocols that induce postsynaptic PF-LTP. **a** One-pulse-induced parallel PF-LTP: one PF stimulus repeated every second for 5 min; characterized in the brain slices of the rat on postnatal days 17–21 [11] and postnatal days 18–27 [12]. **b** Burst-induced parallel PF-LTP: a train of seven PF stimuli at 100 Hz repeated every second for 1 min; characterized in the brain slices of the mouse on postnatal days 25–25; resistant to blocking of $GABA_A$ receptors, NMDA receptors, and mGluR1 [14]. **c** Single-train-induced parallel PF-LTP: one train of 15 PF stimuli at 100 Hz; characterized in the mouse in vivo on postnatal months 5–8; abolished by blocking mGluR1 [19]

prominent mGluR1-independent supralinear Ca^{2+} signal in a larger portion of the dendrite was observed. It was later reported that the size of this supralinear Ca^{2+} signal depended on the delay between the PF burst and the CF-EPSP, but this delay was different from that associated with mGluR1-dependent short-term synaptic depression [21] and PF-LTD [22]. Indeed, it has been shown that mGluR1-independent supralinear Ca^{2+} summation is due to the local and transient saturation of the endogenous Ca^{2+} buffer and can elevate $[Ca^{2+}]_i$ to micromolar concentrations, much higher compared to $[Ca^{2+}]_i$ elevation associated with a CF-EPSP [15]. Repetition of a burst of seven PF-EPSPs at 1 Hz for 1 min induced postsynaptic PF-LTP (burst-induced PF-LTP; Fig. 1b). The evidence that a $[Ca^{2+}]_i$ signal larger than a CF-mediated $[Ca^{2+}]_i$ signal induces LTP (and not LTD) led to a revision of the simple inverse BCM rule proposed previously [12, 13].

The general validity of the inverse BCM rule was also explored in a study where plasticity induction was tested by local Ca^{2+} photorelease. It was found that at high concentrations, Ca^{2+} released in spiny peridendritic regions could induce LTD, but no LTP was observed for smaller Ca^{2+} signals [16].

Both one-pulse-induced PF-LTP and burst-induced PF-LTP require repetitive application of the stimulating protocol. More recently, it was found that postsynaptic LTP can be also induced in vivo by a single train of 15 stimuli at 100 Hz (single-train-induced PF-LTP; Fig. 1c) [23].

The interpretation of these experimental observations points to more complicated scenarios underlying the

induction of postsynaptic PF-LTP, but also of PF-LTD. In the case of postsynaptic PF-LTD, the induction can occur with moderate PF stimulation and pairing with a CF-EPSP, or using a stronger PF stimulation intensity [15, 19] without concomitant CF stimulation. The burst of PF-EPSPs that induces PF-LTD is associated with a $[Ca^{2+}]_i$ signal >2 μM [15], and the evidence that Ca^{2+} photorelease can induce this form of plasticity [16] suggests that a large Ca^{2+} signal is sufficient to induce PF-LTD. In contrast, both postsynaptic PF-LTP and postsynaptic PF-LTD induced by weaker PF stimulation seem to require additional signalling associated with PF synaptic transmission. This signalling, which may involve well-defined spatiotemporal patterns of Ca^{2+} signals and Ca^{2+}-independent biochemical pathways, may also be different for different protocols of plasticity induction. For instance, activation of mGluR1 was shown to be necessary for single-train-induced PF-LTP [20], but not for burst-induced PF-LTP [15].

The electrophysiological induction protocol is the first step of one or more sequences of events leading to a change in the efficacy or in the number of functional synaptic receptors. In this dynamic process, a measurable fundamental molecule is Ca^{2+}. Thus, the first aspect that will be carefully analyzed is the spatial distribution and time course of Ca^{2+} signals associated with PF-EPSPs and CF-EPSPs during different induction protocols. Ca^{2+} signals are fundamental variables in biochemical cascades activated by induction mechanisms where other proteins are involved. For many different molecules, although a role in synaptic plasticity has been demonstrated, the direct association with a particular pathway was limited by the available pharmacological or genetic tools. Some controversial issues arising from molecular analysis are discussed later as the second aspect. Finally, upstream of the molecular pathways, induction protocols are produced by artificial electrical stimulation at a given site. Several studies have shown that induction protocols in brain slices can produce different results according to the position of the stimulating electrode and the orientation of the slice. These discrepancies may be due to the architecture of the presynaptic fibers and the localization of synaptic contacts, and the use of a spatially well-defined stimulation may help to isolate specific signalling pathways. In addition, this information can shed light on the functional organization of the cerebellar circuitry. Thus, this is the third aspect addressed in this review.

Potential Ca^{2+} Signals Involved in Postsynaptic PF-LTP Induction

Although the size of $[Ca^{2+}]_i$ transients cannot be directly correlated with the polarity of long-term synaptic plasticity, the spatial distribution and the time course of Ca^{2+} signals can be determinants of the bidirectional induction of plasticity. Indeed, the contribution of Ca^{2+} to a particular pathway may, in general, depend on its colocalization with a particular Ca^{2+}-binding protein (localization of Ca^{2+} signal), as well as on the kinetics of the interaction with the various Ca^{2+}-binding proteins expressed in PNs (see, for example, Section IV in Ito [17]).

The contribution of Ca^{2+} entry via AMPA receptors is negligible in mature PNs [24], and there is no evidence of dendritic Ca^{2+} entry via NMDA receptors, although there is recent evidence that these receptors are still expressed in PNs in mature mice [25]. Thus, following excitatory postsynaptic activity, $[Ca^{2+}]_i$ elevation is due to Ca^{2+} entry via voltage-gated Ca^{2+} channels (VGCCs) activated by dendritic depolarization, or by secondary pathways triggered by mGluR1 activation in the case of PF activity (Fig. 2a). Activation of VGCCs following dendritic depolarization triggers local dendritic Ca^{2+} spikes [26, 27]. A single dendritic Ca^{2+} spike can be elicited either by a CF-EPSP, in the bulk of the dendritic arbor, or by one or more PF-EPSPs locally [15]. The fast $[Ca^{2+}]_i$ elevation associated with a calcium spike has a peak of ~100–200 nM and a duration of a few milliseconds, as measured using low-affinity dyes [15, 28] and as depicted in Fig. 2b. When several calcium spikes occur during a high-frequency burst, the transient saturation of the endogenous calcium buffer can elevate $[Ca^{2+}]_i$ to higher concentrations (see Fig. 2c), depending on the number and the architecture of the PFs activated. This phenomenon can be observed only in association with PF-EPSP bursts, or with a CF-EPSP following a PF-EPSP burst [15]. The time course of the $[Ca^{2+}]_i$ elevation following a PF-EPSP burst depends on the timing of calcium spikes. The different spatial distribution of calcium spikes elicited either by CF stimulation or by PF stimulation can play an important role in the bidirectional induction of plasticity, and this hypothesis should be further investigated. Activation of mGluR1 is associated with PF-EPSPs. Currently, there is evidence for two Ca^{2+} signals mediated by mGluR1 activation [29]. The faster signal is Ca^{2+} release from stores via $InsP_3$ receptors. Evidence from several laboratories indicates that Ca^{2+} release from stores can be localized in dendritic spines [29–31]. In rats, this signal has well-defined kinetics, as depicted in Fig. 2d, and it peaks □100 ms after mGluR1 activation [29]. It is primed by Ca^{2+} influx [29] and facilitated by a concomitant CF-EPSP [32]. A slower signal is Ca^{2+} influx via nonselective cation conductance [33]. A recent study suggested that this conductance is the TRPC3 channel widely expressed in PNs [34]. This signal is slower and smaller compared to Ca^{2+} release from stores [29], as depicted in Fig. 2e.

Figure 2f shows the four $[Ca^{2+}]_i$ transients reported in Fig. 2b–e normalized and superimposed. The time course of different $[Ca^{2+}]_i$ transients can play a role in synaptic

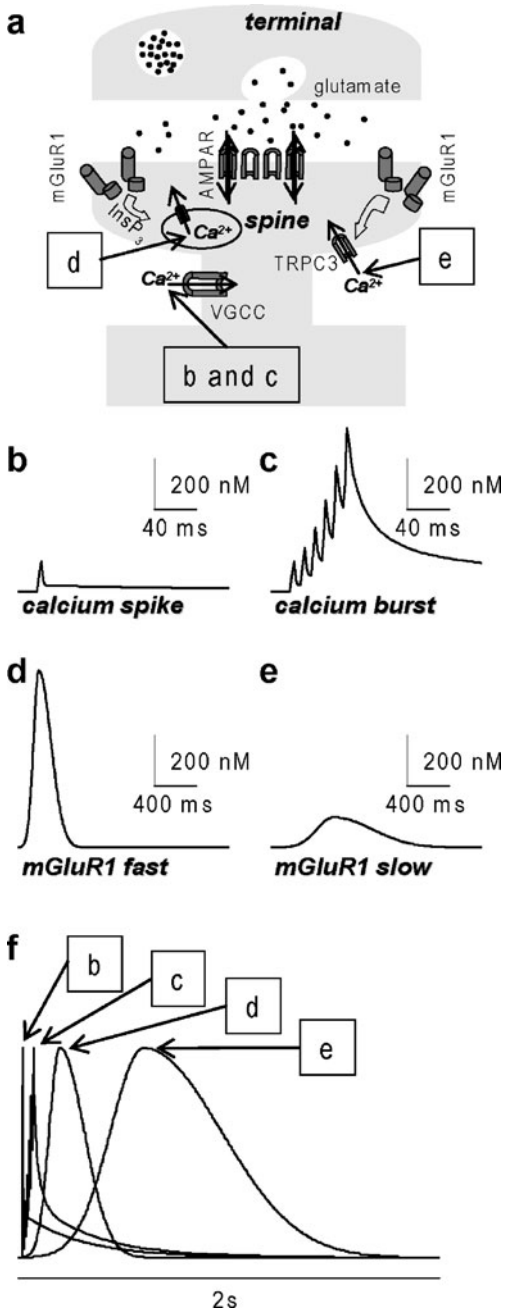

Fig. 2. Schematic of four $[Ca^{2+}]_i$ signals associated with excitatory synaptic transmission. **a** Associated with glutamate release from presynaptic terminals and depolarization due to AMPA receptor (*AMPAR*) activation, $[Ca^{2+}]_i$ can elevate via Ca^{2+} entry through VGCCs (signals b and c), via Ca^{2+} release from stores triggered by mGluR1 activation and $InsP_3$ (signal d), and via Ca^{2+} entry through TRPC3 triggered by mGluR1 activation (signal e). **b** $[Ca^{2+}]_i$ signal associated with one calcium spike: peak, ~100–200 nM; duration, ~10 ms (estimated from Canepari and Vogt [15]). **c** $[Ca^{2+}]_i$ signal associated with a burst of six calcium spikes at 100 Hz (calcium bursts): peak, 0.5–2 μM; duration, ~80 ms (estimated from Canepari and Vogt [15]). **d** $[Ca^{2+}]_i$ signal mediated by mGluR1 and Ca^{2+} release from stores (fast mGluR1): peak, ~1 μM; delay from mGluR1 activation, ~50 ms; duration, ~100 ms (estimated from Canepari and Ogden [29]). **e** $[Ca^{2+}]_i$ signal mediated by mGluR1 and slow Ca^{2+} influx (slow mGluR1): peak, ~100–200 nM; time to peak from mGluR1 activation, ~0.5–1 s; duration, ~1 s (estimated from Canepari and Ogden [29] and Canepari et al. [33]). **f** The four $[Ca^{2+}]_i$ signals normalized in amplitude and superimposed

plasticity by selective activation of proteins with different kinetics and affinities. Both Ca^{2+} influx associated with Ca^{2+} spikes and Ca^{2+} release from stores can, in principle, occur in dendritic spines, in the bulk of the dendrite, or in both, and a different spatial distribution can result in the activation of different molecular pathways. Finally, the interplay of $[Ca^{2+}]_i$ transients with well-defined kinetics can play a crucial role in the timing of signals associated with different patterns of stimulation that induce long-term synaptic plasticity.

In conclusion, it must be pointed out that in induction mechanisms produced by repetitive stimulation, not only the $[Ca^{2+}]_i$ transient associated with one individual stimulation but also its evolution during the repetition and the change in basal $[Ca^{2+}]_i$ must be taken into account.

Molecular Pathways Associated with Repetitive Stimulations

It is well established that changes in the phosphorylation state of AMPA receptor subunits regulate the efficacy of AMPA receptors in glutamate binding and their insertion/internalization balance (for a review, see Roche et al. [35]). Thus, research on molecular cascades leading to different forms of postsynaptic cerebellar plasticity focused on pathways that could produce changes in kinase/phosphatase activation. Several types of kinase inhibitors affect LTD (for a review, see Ito [17]). However, the same kinase/phosphatase may act at different stages of a molecular cascade or within different concomitant pathways. This multiple action of a single enzyme complicates analysis and generates discrepancies in the interpretation of results. A typical example is the old controversy on the role of protein kinase C (PKC). At postsynaptic PNs, PKC can phosphorylate the Ser880 site of GluR2, leading to its internalization [36], but it also interacts with the mitogen-activated protein kinase involved in LTD [37]. Presynaptically, PKC regulates the granule cell synthesis of nitric oxide (NO) [38], which is involved in both LTP [11] and LTD induction [39]. Thus, unspecific blocking of PKC prevents CF-dependent LTD in cultures [40], but this form of plasticity is surprisingly still intact in mice that are deficient in PKC-γ, which is exclusively expressed in PNs [41].

In a recent investigation, it was reported that inhibition of several types of phosphatases prevents postsynaptic LTP, suggesting that bidirectional plasticity may be eventually controlled by the balance of kinase/phosphatase activation [42]. Interestingly, in another report, LTP was prevented by

the blocking of *N*-ethylmaleimide-sensitive factor, suggesting that the binding of this molecule to GluR2—and not GluR2 dephosphorylation—may be responsible for LTP [43].

In summary, it is important to remark that mechanisms underlying different induction protocols are likely to be associated with different molecular pathways, but the interpretation of results from pharmacological and genetic explorations is often limited by the fact that the same molecule may be involved in more steps of a putative molecular pathway underlying synaptic plasticity. In addition, the situation is further complicated by the involvement of the same molecule in pathways occurring in parallel during the application of a particular induction protocol.

Geometric Constraints of Postsynaptic LTP and LTD Induction

In brain slices, EPSPs (and related induction protocols) are evoked by a stimulating electrode positioned at a particular site of the slice cut with a particular orientation. Whereas initial studies on cerebellar plasticity did not take into account this factor, more recently, it was found that an important aspect of postsynaptic PF-LTP (or PF-LTD) induction is its critical dependence on the spatial organization of the synapses with respect to dendritic geometry, which reflects the architecture of afferents. PF synapses, formed along the parallel axons of the molecular layer, comprise ~90% of the contacts between granule cells and PNs, whereas the remaining 10%, called ascending fibers (AFs), are formed in the ascending tracts of granule cell axons [44]. In experiments performed on coronal slices in the rat, where activation of PF and AF synapses can be separated (Fig. 3a), both one-pulse-induced parallel PF-LTP and one-pulse-induced parallel PF-LTD have been shown to be inducible only by PF stimulation and not by AF stimulation [45, 46].

The postsynaptic PF-LTD induced by pairing the one-pulse protocol with CF stimulation is mGluR1 dependent, and a critical correlation was found between glutamate spillover and PF-LTD induction [47]. Thus, in another study, it was suggested that the difference between PF and AF susceptibility reported previously [46] was actually due to the spatial arrangement of afferents [48]. According to this view, PF stimulation results in the activation of afferents contacting postsynaptic PNs at adjacent dendritic spines and in the local glutamate accumulation necessary for spillover and chemical cross-talk among adjacent spines.

Because the one-pulse-induced LTD was shown to be different for the PF pathway and the AF pathway, the same type of analysis in coronal slices was performed for the

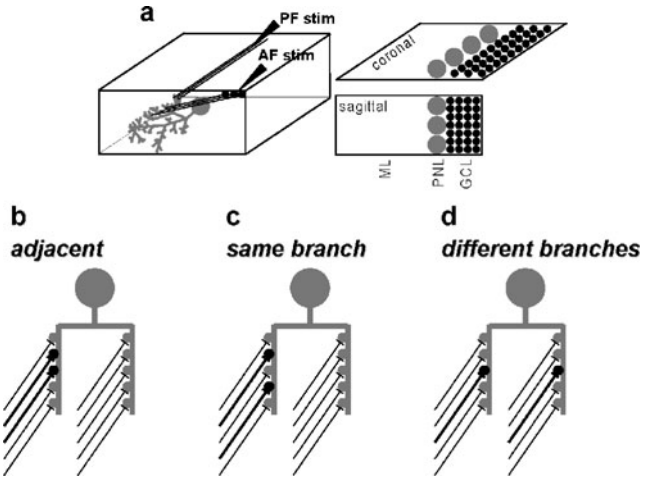

Fig. 3. Possible spatial arrangement of two simultaneously active CGC–PN synapses. **a** Schematic of a sagittal/coronal section of the cerebellum with molecular layer (*ML*), PN layer (*PNL*), and granule cell layer (*GCL*). PF stimulation: stimulation in the ML; AF stimulation: stimulation in the GCL behind the PN. **b** Adjacent: afferents contacting two adjacent spines in the same dendritic branch, allowing for chemical cross-talk between the two synapses. **c** Same branch: afferents contacting nonadjacent spines but in the same dendritic branch, allowing for local depolarization of the dendrite. **d** Different branches: afferents contacting two different dendritic branches

burst-induced PF-LTP [15]. In contrast to that reported for one-pulse-induced plasticity, LTP was found both at the PF pathway and at the AF pathway. This induction protocol did not require mGluR1-mediated Ca^{2+} signals [15] associated with glutamate spillover [47], but required Ca^{2+} influx associated with Ca^{2+} spikes that could be elicited by the activation of both the PF pathway and the AF pathway [15].

To explore in detail the differences in synaptic plasticity regardless of whether synaptic contacts originate from the PF pathway or the AF pathway, we summarize which hypothetical geometric scenarios of CGC–PN synapses can occur under different constraints (Fig. 3a). In the first case, depicted in Fig. 3b, adjacent CGC axons are stimulated. This configuration can occur by stimulating in the vicinity of the PN dendrite in sagittal slices or in the molecular layer in coronal slices. Thus, CGC–PN synapses can target adjacent spines, allowing for glutamate accumulation, transient uptake saturation, and glutamate spillover onto adjacent spines [47, 48]. In the second case, depicted in Fig. 3c, axons that activate spines belonging to the same dendritic branch (but not adjacent) are excited. This configuration may be what happens when AFs are stimulated in coronal slices by positioning the stimulating electrode in the granule cell layer below the PN. Under this condition, synaptic activation may be insufficient for chemical cross-talk, but may still allow localized depolarization and excitation. Finally, sparse spines belonging to different dendritic branches may be

activated (see Fig. 3d). This configuration can hypothetically occur by using more stimulating electrodes.

The evidence that different forms of LTP (and LTD) are remarkably dependent on the physical arrangement of the afferents implies an important functional role of the spatial organization of the CGC–PN synapses. The possibility of synaptic cross-talk, either by activation of adjacent synapses via glutamate spillover or by local summation of depolarization leading to dendritic calcium spikes, may underlie the translation of the spatial organization into a functional organization of PF afferents.

Summary and Future Directions

It is proven that both postsynaptic PF-LTP and postsynaptic PF-LTD require $[Ca^{2+}]_i$ elevation, but the hypothesis that the polarity of postsynaptic PF long-term synaptic plasticity is solely determined by Ca^{2+} signals of different amplitudes appears unlikely. More realistically, $[Ca^{2+}]_i$ elevation following PF and/or CF activation may occur at different spatial and temporal scales, triggering local biochemical pathways and activating sensors with different kinetics. In addition, Ca^{2+}-dependent pathways may interact with Ca^{2+}-independent pathways, as suggested by the evidence that Ca^{2+} photorelease does not mimic the induction of long-term synaptic plasticity observed by synaptic stimulation [16]. In these experiments, PF-LTP was not observed even when $[Ca^{2+}]_i$ was within the range normally associated with LTP. This leaves two options: either the Ca^{2+} signal did not properly mimic the physiological signal or the synaptically evoked Ca^{2+} increase was accompanied by an as-of-yet undetermined signal. The first option might be explored by optimizing the spatiotemporal characteristics of the photoreleased Ca^{2+}. For the second option, some of the candidates of necessary signals that are not present by Ca^{2+} photorelease are presynaptic release of NO or postsynaptic binding of glutamate to the AMPA receptors and subsequent membrane depolarization.

The mechanisms and conditions necessary for the induction of postsynaptic PF-LTP are a cornerstone in the relationship between cerebellar activity and memory formation. Our current understanding on this topic is a mosaic of information with several apparent discrepancies. Part of these discrepancies arises from the fact that postsynaptic PF-LTP has been characterized under different experimental conditions (i.e., different induction stimulation protocols) (see Fig. 1), as well as with different species and at different stages of development. For instance, whereas in vitro studies in rats [11, 12] and mice [15] have been carried out in animals 3–5 weeks old, in vivo LTP has been characterized in adult mice [23]. Interestingly, one-pulse-induced PF-LTP, as well as PF-LTD induced by CF pairing, has been observed also in a nonmammalian nervous system [49]. Other discrepancies originate from the incomplete interpretation of nonquantitative data on Ca^{2+} signalling, missing information on the spatiotemporal distribution of $[Ca^{2+}]_i$ transients associated with the different induction protocols. Advanced optical techniques are available, allowing Ca^{2+} measurements at the level of single spines [50, 51]. In particular, with two-photon microscopy, it is possible to measure $[Ca^{2+}]_i$ transients from individual spines using low-affinity dyes [20] that produce minimal perturbation of physiological $[Ca^{2+}]_i$ dynamics. The use of two-photon voltage measurements, recently available [52], may provide additional information on the spatial distribution of local excitation that appears essential for long-term plasticity induction. A final aspect that may produce controversial results is the spatial arrangement of CGC–PN synapses, which mirrors the highly ordered functional architecture of the cerebellar circuitry. The use of stimulating electrodes can bias the conditions for postsynaptic PF-LTP induction by artificially selecting pathways of stimulation, and these pathways may be very different under different experimental conditions. This aspect must be taken into account especially when performing experiments on sagittal cerebellar slices, which are ideal for imaging studies but preclude the possibility of controlling the arrangements of the stimulated presynaptic afferents.

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Conflict of interest We declare that there are no conflicts of interest with this submission.

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