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REVIEW

Cerebellar Endocannabinoids: Retrograde Signaling from Purkinje Cells

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Abstract The cerebellar cortex exhibits a strikingly high expression of type 1 cannabinoid receptor (CB1), the cannabinoid binding protein responsible for the psychoactive effects of marijuana. CB1 is primarily found in presynaptic elements in the molecular layer. While the functional importance of cerebellar CB1 is supported by the effect of gene deletion or exogenous cannabinoids on animal behavior, evidence for a role of endocannabinoids in synaptic signaling is provided by in vitro experiments on superfused acute rodent cerebellar slices. These studies have demonstrated that endocannabinoids can be transiently released by Purkinje cells and signal at synapses in a direction opposite to information transfer (retrograde). Here, following a description of the reported expression pattern of the endocannabinoid system in the cerebellum, I review the accumulated in vitro data, which have addressed the mechanism of retrograde endocannabinoid signaling and identified 2-arachidonoylglycerol as the mediator of this signaling. The mechanisms leading to endocannabinoid release, the effects of CB1 activation, and the associated synaptic plasticity mechanisms are discussed and the remaining unknowns are pointed. Notably, it is argued that the spatial specificity of this signaling and the physiological conditions required for its induction need to be determined in order to understand endocannabinoid function in the cerebellar cortex.

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Introduction

Cannabinoids are molecules related to the psychoactive components of marijuana. In vertebrates, cannabinoids and endocannabinoids (their endogenous analogs) mediate most of their effects by binding to two types of G protein-coupled receptors: type 1 and 2 cannabinoid receptors (CB1 and CB2) [1]. CB1 is preponderant in the brain [2] and highly expressed [3, 4]. It has even been proposed to be the most abundant G protein-coupled receptor [5, 6], with a notable high expression in the cerebellum [4]. The cerebellar cortex is made of a highly conserved and repeated microcircuit with inputs converging onto Purkinje cells (PC), the dendrites of which span the molecular layer. CB1 is primarily found on axons in the molecular layer: those of granule cells (GC), basket and stellate cells, and climbing fibers [7, 8]. PC axon collaterals are probably devoid of CB1 since no mRNA expression has been detected in PCs [9, 10].

CB1 is located adjacent to synapses [11]. Although CB1 is highly expressed at interneuron to PC synapses [3, 7, 11, 12], expression at the GC to PC synapse quantitatively dominates due to the abundance of this synapse type. In rat, the PC dendritic tree bears 175,000 granule cell (GC) to PC synapses [13]. The total number of these synapses may be estimated as about 50 10^{12} in human, a number derived from the averaged density of GC to PC synapses in the rat molecular layer (1 per μm^3; [14]) and the approximated volume of the human molecular layer (50 cm^3; [15]). The CB1 expression at the most abundant synapse type in the cerebellar cortex suggests that endocannabinoids are essential to cerebellar function.

Accumulating evidence supports a general role of endocannabinoids in synaptic plasticity [16]. Since the excitatory GC to PC synapse is generally believed to be a site of information storage [17], most research has naturally focused on endocannabinoid signaling at this synapse [18]. Furthermore, in contrast to inhibitory synaptic transmission, GC to PC synaptic transmission can trigger endocannabinoid release, the mechanism of which is key to understand, but remains largely mysterious.

Contrary to the expectation from CB1 high expression, the CB1 knockout phenotype appears rather mild [19, 20], suggesting that endocannabinoid signaling function is subtle. Specific cerebellum-dependent behavioral paradigms have been required to demonstrate the importance of CB1 for cerebellar operation. Eyeblink conditioning, a well-established paradigm demonstrated to involve the cerebellar cortex [21], is impaired in CB1 knockout [22] or in chronic marijuana users [23, 24], supporting the importance of endocannabinoid signaling for normal cerebellar cortex function. However, some of these effects may be caused by long-term compensatory changes, such as increased CB1 expression [25, 26], CB2-modulated microglia activation [27], or changes in GC to PC synaptic strength [28].

A neurodevelopmental role of endocannabinoid signaling is documented from prenatal to adolescent stages [29–31]. CB1 expression by granule cell precursors has been demonstrated [32]. At this early developmental stage, CB1 activation by low concentration of exogenous cannabinoid promotes granule cell proliferation [32] while higher concentration induces apoptosis [32, 33]. Furthermore, pharmacological manipulations enhancing endocannabinoid effects have appeared to prevent trauma-induced granule cell degeneration [34]. In light of these reports, clinical applications for cannabinoids, like counteracting remote cell death in the cerebellum following brain injury [35], may be considered.

Most data on endocannabinoid signaling in the cerebellum have been obtained from rodent acute cerebellar slices superfused with extracellular fluids, a convenient in vitro preparation for electrophysiological and calcium imaging experiments. These data indicate that endocannabinoids can signal in response to neuronal activity, and shape information transfer and storage. In this review, a brief description of the expression of the molecular actors of endocannabinoid signaling in the cerebellar cortex is followed by the assessment of accumulated in vitro data to dissect the mechanism of endocannabinoid signaling and address its role in synaptic plasticity.

Cerebellar Molecules Involved in Endocannabinoid Signaling

The endocannabinoids and the proteins required for their production, transport, action, and degradation define the endocannabinoid system. Two arachidonic acid derivatives, N-arachidonoylethanolamide (AEA or anandamide) and 2-arachidonoylglycerol (2-AG) are considered the preponderant endocannabinoids in the CNS [36]. Their metabolisms involve different enzymatic pathways (Fig. 1a). Proteins other than CB1 or CB2 may mediate endocannabinoids' actions. The most established such proteins are GPR55, TRPV1 receptors, and PPARs [37]. Although these receptors appear to be expressed in the cerebellar cortex [38–41], their role is currently unknown. CB2 coding mRNAs have been detected in the cerebellum [42] and in cultured GCs [43]. CB2 is generally believed to have an immune function linked to expression by microglia throughout the CNS [44]. The apparent staining of GC axons by anti-CB2 antibodies [45] might suggest that CB2 function in the cerebellum goes beyond the traditionally attributed immune function. However, the reliability of antibodies used to detect specifically CB2 by immuno-staining remains unclear [46]. Endogenous CB1 allosteric modulators have recently started to be identified [47] and may add a new level of complexity to the endocannabinoid system. Their physiological role remains unknown.

In vitro experiments support that 2-AG is the main endocannabinoid mediating phasic signaling in the cerebellar cortex (see below). This may not sound surprising given that the average 2-AG concentration in brain tissue is about a thousand times higher than that of anandamide [48]. Most of this 2-AG is inside cells, and its extracellular concentration is much lower, but it is still considered about five times higher than that of anandamide [48]. Amongst the several metabolic pathways proposed for 2-AG synthesis [49–51], the hydrolysis of arachidonic acid-containing membrane phospholipids into arachidonic acid-containing diacylglycerol (DAG), catalyzed by $G_{q/11}$-activated phospholipase Cβ (PLCβ), followed by conversion into 2-AG catalyzed by *sn*-1-specific DAG lipase (DAGL), is considered as the main pathway (Fig. 1a) [48]. For transient action of 2-AG (see below), the termination of 2-AG action may involve its degradation. Pharmacological and genetic studies indicate that the main pathway of 2-AG degradation is its hydrolysis catalyzed by the monoacylglycerol lipase (MAGL) (Fig. 1a) [52–54].

The subcellular location of these enzymes in the cerebellum is documented. The α type DAGL [55]) exhibits high expression in PCs [56–58], which thus appear to be the main producer of 2-AG. PLCβ4 is also highly expressed in PC dendrite [59, 60] and is the best candidate to convert membrane phospholipid phosphatidylinositol-4,5-diphosphate (PIP_2) into DAG in PCs [61]. In contrast, MAGL has been reported to be localized in presynaptic compartments [62]. Together with the presynaptic localization of CB1, the localization of the enzymes involved in 2-AG metabolism is consistent with the notion that 2-AG signals from the PC dendrites to presynaptic elements (Fig. 1b), as demonstrated by

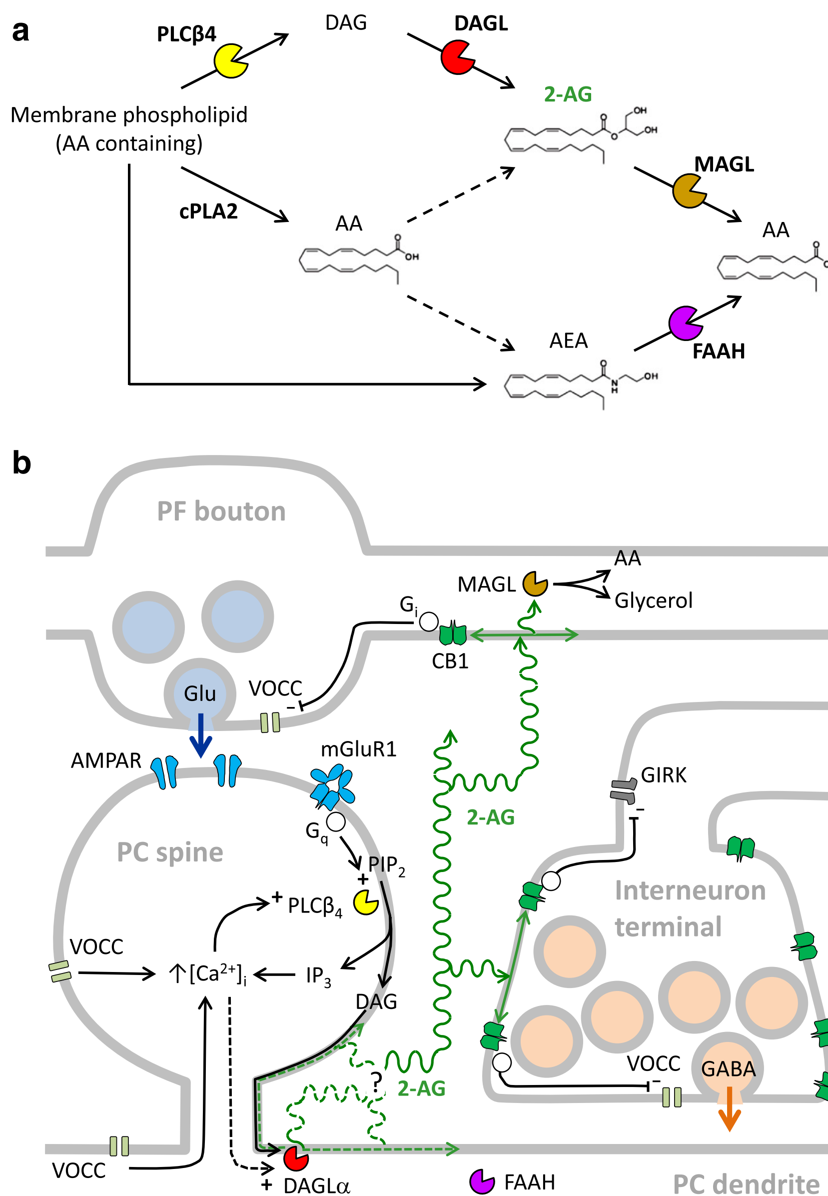

Fig. 1 Known components of the endocannabinoid system in the cerebellum. **a** Biochemical synthesis and degradation pathways for endocannabinoids. Enzymes, the expression of which is reported in the cerebellar cortex, are represented with *colored symbols* also displayed in (**b**). *DAG* diacylglycerol, *AA* arachidonic acid, *2-AG* 2-arachidonoylglycerol, *AEA* N-arachidonoylethanolamine (anandamide). **b** Simplified representation of the two cerebellar synapses where CB1 is the most highly expressed: the GC (PF) to PC and interneuron (basket or stellate cell) to PC synapses. Postsynaptic calcium rise and mGluR1 activation both stimulate 2-AG mobilization from PCs. Although the subcellular site of 2-AG release is unknown, it is likely to coincide with DAGL*α* location, reported near the base of the spine neck [58]. The action of 2-AG on CB1 is hypothesized to follow 2-AG insertion in the cell membrane [115]. Quantitative immunogold labeling has revealed contrasting features for CB1 expression in PF and interneuron terminals [11] which are taken into account in the schematic diagram: CB1 is preferentially found in axonal parts of PFs in contrast to its location in synaptic boutons made by interneurons; CB1 is about six times more concentrated in interneuron terminals [11]

electrophysiological studies (see below). Despite the high expression of CB1 by molecular layer interneuron terminals, MAGL appears mainly compartmented in GC terminals [62], suggesting other enzymes might help to terminate 2-AG action at inhibitory synapses. ABHD6 is currently the main alterative candidate to mediate significant 2-AG degradation [63]. However, the lack of effect of a demonstrated ABHD6 blocker on 2-AG-mediated plasticity in the cerebellum of MAGL knockout mice, or in the presence of the MAGL-specific blocker JZL184, appears to rule out its role in the termination of 2-AG signaling [64].

Although *N*-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) has been shown to mediate anandamide synthesis [65], its role has been questioned by gene

deletion studies that revealed the existence of other metabolic pathways [48, 66]. The fact remains that NAPE-PLD has been reported to be expressed in the cerebellar cortex [67], and its function remains unclear. The pathway of anandamide degradation is more solidly established (Fig. 1a): the role of fatty acid amide hydrolase (FAAH) in the conversion of anandamide into arachidonic acid and ethanolamine [68] has been confirmed by gene deletion and pharmacology studies [48, 69]. High expression of FAAH in PC dendrites [62] suggests that anandamide, in addition to 2-AG, is involved in cerebellar physiology. However, to date, no physiological study has provided evidence for a role of anandamide in the cerebellum.

Endocannabinoid Release from Purkinje Cells

In vitro electrophysiology experiments have demonstrated that 2-AG release can be triggered by depolarization-mediated calcium transients in PC dendrites or by activation of type 1 metabotropic glutamate receptors (mGluR1) borne by PC spines. The two effects synergize when wide beams of densely packed parallel fibers (PFs, granule cell axons) are activated (see below). Since "on demand", increased 2-AG synthesis may cause its release and since the release mechanism itself (i.e., transport across the cell membrane) remains poorly understood [48], "mobilization" is used to designate on demand synthesis and/or release [16].

Postsynaptic Calcium Rise

In acute cerebellar slices, depolarization of PCs through the recording electrode at the soma can evoke short-term depression of GABA inputs onto PC [70]. This synaptic plasticity mechanism, also found in other brain areas, was named depolarization-induced suppression of inhibition (DSI; [71]) and was later extended to excitatory inputs onto PC: depolarization-induced suppression of excitation (DSE; [72]). In both plasticity mechanisms, postsynaptic depolarization mediates a presynaptic reduction in neurotransmitter release [70, 72]. This indicates the existence of signaling in the opposite direction to information transfer: retrograde signaling [16]. Endocannabinoids have been proposed to operate as retrograde messengers [73]. Consistent with this hypothesis, DSI and DSE have been shown to be blocked or occluded by the CB1 antagonist AM251 or agonist WIN 55,212-2, respectively [72, 74] (for a review on the DSI/DSE saga, see [75]). More recently, the endocannabinoid mediating DSI or DSE has been identified as 2-AG: DSI and DSE are abolished in DAGLα knockout mice [56, 76], and strongly prolonged when 2-AG degradation is inhibited by the highly selective MAGL blocker JZL184 [64, 77].

DSI and DSE are both abolished by strong buffering of postsynaptic calcium [70, 72], demonstrating that postsynaptic calcium rise is required to mobilize 2-AG. Calibration of the depolarization-evoked calcium transients in PC dendrites indicates that, while a brief (few seconds) Ca^{2+} transient must reach 5 μM, a longer (15 s) transient only needs to reach 0.4–1 μM to induce 2-AG release [78]. So, both the amplitude and the duration of the calcium transient count. How does postsynaptic calcium induce 2-AG mobilization? Is postsynaptic calcium rise sufficient or is depolarization an additional requirement? Are calcium microdomains required? Indeed, various sources of calcium coexist in PC dendrites [79, 80], and they may exert specific effects depending on their colocalization with calcium-sensing effectors. The effect of caged-calcium photorelease and improved calcium channel pharmacology would be insightful to address these unresolved issues.

The activities of PLCβ4 and DAGLα are modulated by calcium [55, 81]. However, a role of PLCβ4 in DSI and DSE has been ruled out by the lack of effect of acute PLCβ4 block by U73122 [82] or PLCβ4 knockout [61]. DSI and DSE are abolished in a DAGLα knockout [56] and DSI is blocked by DAGL blockers [77, 82]. But the role of DAGLα in DSE remains controversial. The DAGL blocker tetrahydrolipstatin (THL; 2 μM) dialyzed into PCs through the patch pipette (to avoid side effects on presynaptic CB1 receptors) has been reported to be ineffective in blocking DSE [78, 83] while, in the same experimental conditions, it blocked synaptically-induced endocannabinoid release [83] (see below). A similar observation was made with the other DAGL blocker RHC-80627 [83]. To reconcile these data with the abolition of DSE in DAGLα knockout mice, one might propose that depolarization stimulates the release mechanism of "pre-formed 2-AG pools" [16, 84], which would be depleted in DAGLα knockout animals. However, the effect of acute block of DAGLα has been re-evaluated recently with the use of a blocker more specific than THL, OMDM-188 [77]. In contrast to DSI, DSE was only partially blocked following slice incubation with 2 μM OMDM-188, complete block being obtained when using 5 μM [77]. Although the pharmacological targeting of DAGL remains imperfect [85], these recent data support the hypothesis that the effect of postsynaptic depolarization and calcium rise on 2-AG mobilization is mediated by an activation of DAGLα, i.e., the endocannabinoid is synthesized on demand [5, 86].

Is DAGLα directly modulated by postsynaptic calcium? The limited available data supporting such modulation by calcium [55] reports a calcium concentration dependency which does not match the physiological calcium changes occurring in PC dendrites [78]. So, as for other parts of the brain, the mechanism linking postsynaptic calcium to 2-AG mobilization remains unclear [75]. Recently, an alternative pathway for 2-AG synthesis has been suggested [87]. DSE has been reported to be abolished in a cytosolic phospholipase A2

(cPLA2) knockout or by acute cPLA2 block by $AACOCF_3$ [87], suggesting a role for arachidonic acid (AA) metabolism in PCs (Fig. 1a). In this study, the identification of 2-AG as the retrograde messenger was confirmed by showing that dialysis of PC cytoplasm with MAGL (to deplete 2-AG in PCs) prevented DSE induction [87]. Future studies are required to reconcile these data with the data supporting a role for DAGL [56, 77] and establish a link between AA and 2-AG metabolism in PCs. 2-AG transport across the plasma membrane, the mechanism of which is unknown [48, 75, 88], might also mediate the modulation of 2-AG mobilization by calcium.

Depolarization-evoked endocannabinoid release has been qualified as "global" since it affects all the synapses (including the CF synapse) contacting the recorded PC [18]. A plasticity mechanism similar to DSE can also be spatially restricted to a region of the PC dendrites when large and non-propagating calcium transients are elicited locally. Half of PFs are estimated to contact each PC they cross [89]. In typical experimental protocols, beams of hundreds of adjacent PFs are stimulated [90–92]. Such stimulation with tetanic stimuli (typically 10 pulses at 100–200 Hz) can evoke regenerative depolarization in PC dendrites, identified as calcium spikes, which do not propagate and remain localized to a few branchlets [93]. The stimulation threshold to evoke these calcium spikes has been found to be identical to that evoking short-term depression of glutamate release [93], a posttetanic depression (PTD; [91]), with a time course similar to that of DSE and abolished by AM251 [90, 93, 94]. This endocannabinoid-mediated PTD is abolished by knockout or acute block of DAGL [56, 77, 83], and is strongly prolonged in the presence of JZL184 [95], indicating that it is mediated by 2-AG. Similarly to the "global" DSE produced by depolarization of the entire dendrites, this spatially localized PTD is abolished by chelation of postsynaptic calcium [90, 93]. In addition to postsynaptic calcium, tetanic stimulation of PF inputs activates mGluR1 receptors, which also induce 2-AG release from PCs.

Postsynaptic mGluR1 Activation

The activation of mGluR1 by its specific agonist DHPG induces negligible postsynaptic calcium changes compared to those evoked by depolarization. However, DHPG induces a transient depression of PF, CF, and interneuron inputs, shown to be endocannabinoid-mediated [61, 94, 96, 97]. Thus, mGluR1 activation is sufficient to trigger endocannabinoid release. Since burst stimulation of beams of PFs activates mGluR1s [94, 98], which are highly expressed in PC spines [58, 99], their role in PTD is expected.

The discovery of PTD at PF synapses [91] was soon followed by the demonstration that it was mediated by endocannabinoid retrograde signaling [56, 90, 94, 95, 100]. In addition to being abolished by postsynaptic calcium chelation [90, 93], this PTD has been reported to be partially blocked by mGluR1 antagonists [91, 93, 95]. To date, it remains unclear whether the relative dependencies on postsynaptic calcium transients and on mGluR1 activation vary depending on conditions (stimulation strength, animal age, temperature, sub-location within the molecular layer, neuromodulation, PC activation state…). Nevertheless, a synergetic effect of postsynaptic calcium and mGluR1 activation has been demonstrated (see below; [61, 101]). The mechanism downstream of mGluR1 activation involves PLCβ4, the suppression of which abolishes the endocannabinoid-mediated PTD [61]. Thus, in PTD, "on demand" synthesis not only involves the modulation of DAGL (as in DSI or DSE), but also the modulation of the upstream step, DAG formation by PLCβ4.

Burst stimulation of PFs also evokes transient endocannabinoid-mediated depression of CF inputs [96], and inhibitory inputs [97, 102, 103]. The mechanisms of these heterosynaptic PTDs have been less thoroughly investigated. Although the subcellular DAGL localization [58] suggests that 2-AG might spill over micrometers in the extracellular space before reaching presynaptic CB1 (Fig. 1b), diffusion of the postsynaptic effects of PF synapse activation (extracellular glutamate spillover, postsynaptic calcium rise, increased DAG production, 2-AG diffusion in PC membranes) may also cause heterosynaptic 2-AG mobilization. Indeed, the stimulation of beams of PFs is required (see below), a situation activating clusters of PF synapses, enhancing postsynaptic calcium transients [93] and glutamate spillover effect on mGluR1 activation [94].

The Effect of CB1 Activation

The overall effect of presynaptic CB1 activation is a decrease in neurotransmitter release probability. Bath-applied CB1 agonists have been shown to increase paired pulse facilitation and coefficient of variation (which are generally considered to correlate negatively with release probability) of GC to PC transmission [104] and to reduce basal spontaneous inhibitory postsynaptic current (IPSC) frequency [105]. Furthermore, the effect of CB1 agonists on PF stimulation-evoked presynaptic calcium transients and excitatory postsynaptic current (EPSC) size is consistent with the predicted reduction in EPSC size arising solely from alterations in action potential-driven presynaptic Ca influx [106]. In DSI, similarly evoked at synapses made by stellate cells or basket cells [7], the CB1-mediated endocannabinoid action reduces GABA release by at least two mechanisms. The reduction of miniature IPSCs (recorded in the presence of TTX) demonstrates an effect on vesicular release [7, 107]. Additionally, in absence of TTX, an effect on the interneuron spontaneous firing rate has been reported [107, 108]. This effect may be caused by CB1-mediated

activation of a G protein-activated inward rectifier potassium channel [108] (Fig. 1b). In DSE and endocannabinoid-mediated PTD, CB1-mediated increase in paired pulse facilitation and reduction in action potential-driven presynaptic calcium transient are consistent with depression being entirely mediated by inhibition of presynaptic calcium signaling [72, 90, 95].

The mechanism linking CB1 activation and neurotransmitter release has been investigated in most detail for PF synapses, which can be readily bulk loaded with calcium dyes due to their geometrical organization in transverse slices. The mechanism has been proposed to involve inhibition of presynaptic potassium channels [109, 110]. However, this would be expected to affect the presynaptic action potential waveform. The lack of detectable change in the PF volley (an indirect measurement of the presynaptic action potential waveform) or in presynaptic calcium influx kinetics argues against this hypothesis [104, 106, 109]. Instead, current data are consistent with pertussis toxin-sensitive G protein-mediated inhibition of presynaptic voltage-operated calcium channel, irrespective of their type (P/Q, N, or R) [106, 110].

Amplitude and Time Course of Endocannabinoid Effects

DSI, DSE, and endocannabinoid-mediated PTDs amplitudes reach 85 % [7], 85–95 % [83, 103], and 80–85 %, respectively [93, 94], while external application of saturating WIN 55-212 concentration reduces interneuron-evoked IPSCs and PF-evoked EPSCs and by up to 90 % [7, 105, 106]. These reductions in neurotransmitter release are comparable in amplitude, suggesting that mobilized 2-AG reaches saturating concentrations in the vicinity of presynaptic CB1 receptors. DSE, DSI, and endocannabinoid-mediated PTD are short-term and their time courses are similar, consistent with being mediated by similar mechanisms. The depression peaks 5–20 s following the beginning of the stimulus and synaptic transmission recovers in 20–90 s [7, 64, 72, 74, 77, 82, 83, 87, 90, 93, 94, 103, 111, 112]. The time course of recovery is complicated by the occurrence of overlapping longer-term depressions [95, 111] and its dependence on temperature [108]. The use of the specific MAGL blocker JZL184 significantly prolongs the endocannabinoid-mediated PTD [95] but also delays the peak of the effect, suggesting that 2-AG mobilization is a slow process and that the time course of the 2-AG-mediated depression results from a balance between 2-AG mobilization and degradation.

Spatial Specificity of Endocannabinoid Effects

DSI spreads to synapses contacting non-depolarized PCs up to 75 μm away from the depolarized PC [113]. This spatial spread of DSI has been shown to be mediated by a decrease of spontaneous interneuron firing [108, 113]. It is absent in TTX. Thus, it does not reflect the spread of extracellular endocannabinoid diffusion. Simultaneous whole-cell recording from nearby PCs has enabled to detect some spread of the DSE produced in one PC to synapses on the neighboring PCs, at room temperature. However, at near physiological temperature, in contrast to DSI, no spread of DSE could be detected [108]. The limited spread of extracellular endocannabinoid diffusion is best demonstrated for PTD. When the PC is dialyzed with the calcium buffer BAPTA and the irreversible G protein inhibitor GDP-βs, the endocannabinoid-mediated PTD is prevented [90]. This lack of PTD, despite the fact that the induction protocol (stimulation of wide PF beams) presumably induces 2-AG release from neighboring (not dialyzed) PCs in the slice, has been interpreted as the evidence that 2-AG signaling is highly spatially restricted [90]. Assuming that postsynaptic internal BAPTA and GDP-βs do not interfere with presynaptic CB1-mediated signaling, and that a large fraction of PCs are preserved in the slice, these data suggest that diffusion of 2-AG does not spill over to synapses on adjacent PCs. This is not unexpected given the propensity of 2-AG to bind nonspecifically to cell membranes due to its hydrophobicity [114, 115]. However, a quantification of the spread of endocannabinoid effects over a scale corresponding to the actual distance between synapses is lacking [75]. The average distance between neighboring PF synapses is about 1 μm, with on average 17 spines per linear micrometer of spiny branchlets [14]. Furthermore, DAGL is preferentially localized at the base of dendritic spine necks [58], a location equidistant to several nearest presynaptic boutons (Fig. 1b). Unless newly synthetized 2-AG is transported to the spine head before being released, the DAGL location suggests that 2-AG signaling is not spatially restricted enough to achieve synapse specificity (i.e., 2-AG mobilized by the stimulation of one bouton cannot act solely on CB1 borne by this same bouton).

This spatial specificity issue is important to address if we are to understand the function of 2-AG signaling. It is generally accepted that the GC to PC synapse is a site of information storage [17]. Independent operation of neighboring synapses is predicted to maximize the information storage capacity [116], and the GC to PC synapse appears ideally designed to operate independently from its neighbors as it is tightly wrapped by Bergmann glia processes expressing high density of glutamate transporters which help to prevent the glutamate spillover to neighboring synapses [117, 118]. On these theoretical grounds, synaptic plasticity mechanisms mediating information storage might be expected to be synapse-specific. The lack of synapse specificity of 2-AG signaling would argue for a role which might be more homeostatic. Alternatively, it

would suggest that the information storage unit is larger than the single spine.

Physiological Relevance of 2-AG-Mediated Short-Term Plasticity

DSI and DSE are robust synaptic plasticity mechanisms, which revealed the operation of the endocannabinoid system as a retrograde signaling mechanism at central synapses, and are expected to be useful to continue to explore how 2-AG is mobilized. DSI and DSE are achieved with cesium-based intracellular media in order to improve voltage-clamp and enhance the spread of the depolarization from the soma to distal parts of the PC dendrites. What is the physiological relevance of these plasticity mechanisms? Their dependence on endocannabinoids may depend on postnatal development [111]. Besides, they are evoked by sustained postsynaptic depolarization, the physiological correlate of which is unclear. CF activation evokes depolarization spreading to distal parts of the dendritic tree [80, 119]. However, endocannabinoid release has not been reported to be induced by CF stimulation, unless postsynaptic calcium changes are enhanced by experimental manipulations, like the block of calcium-activated potassium channels [93]. Only sustained CF stimulations at more than 5 Hz, the physiological relevance of which remains to be shown, has been reported to induce endocannabinoid-mediated inhibition of interneuron inputs [120]. The observation that 2-AG release occurs in depolarized states of PCs (bursting mode) [78] only shifts the question: does the burst mode occur in vivo [121–123]? Thus, the physiological relevance of this depolarization-evoked endocannabinoid release producing a "global" reduction of neurotransmitter release at all synapses on PCs remains unclear.

In vivo, GCs have been reported to fire in short high frequency bursts [124, 125] similar to those required to induce the endocannabinoid-mediated PTD. However, experimental induction critically depends on the spatial pattern of the input (Fig. 2a–d) [94, 100]. Traditionally, GC to PC synaptic transmission is studied in acute cerebellar slices by stimulating PFs in the molecular layer (Fig. 2a). This configuration leads to the stimulation of hundreds of adjacent parallel fibers [92]. The geometric architecture of the cerebellar microcircuit enables comparison of this dense pattern of synapse activation with sparse patterns obtained by stimulation in the granular layer [92] (Fig. 2b). These sparse patterns do not include synapses made by GC ascending axons when the stimulating pipette is sufficiently far from the PC sagittal plane [100]. To date, endocannabinoid-mediated PTD has never been observed for spatially sparse patterns of synapse activation [92, 94, 95, 100, 102, 103], even when PF stimulation is paired with CF stimulation [94] (Fig. 1c, d). Our recent data based on the mapping of PF input using an optogenetic approach indicate that, in young mice, a density of activated PF larger than 3 μm^{-2} in the sagittal plane is required to reliably induce endocannabinoid-mediated PTD [92]. Dense input leads to enhanced postsynaptic calcium transients [93] and glutamate spillover effects (mGluR1 activation) due to local saturation of glutamate transporters [94, 117]. Both effects are expected to enhance 2-AG mobilization.

The in vitro observation of the PTD dependency on the spatial pattern of inputs raises questions about its physiological occurrence. A PC receives more than 100,000 PF inputs. Considering that about 90 % of them may be silent and that no more than 200 non-silent synapses need be activated simultaneously to evoke a postsynaptic action potential [126], a physiological input is unlikely to involve more than 2000 simultaneously firing PFs, i.e., less than 2 % of the PFs contacting the PC. If homogeneously distributed, 2 % of inputs would correspond to an average input density of 0.12 μm^{-2}, which is too low to enable the induction of endocannabinoid-mediated PTD [92]. However, random distributions of these inputs are expected to include clusters of PFs which are, by chance, in close proximity [92]. Furthermore, data based on in vivo mapping of PF firing following their bulk-loading with a calcium dye suggest that physiological stimuli produce spatially clustered inputs [127]. Thus, dense PF input may occur in physiological conditions. The 2-AG-mediated PTD resulting from such occurrence would be an efficient homeostatic mechanism which, by reducing glutamate release, would downregulate glutamate spillover effects and promote independent operation of neighboring synapses, thereby maximizing the storage capacity of the cerebellum [94, 116]. 2-AG-mediated PTD has been shown to reduce glutamate spillover effects following the activation of synapses in close proximity (Fig. 2e) [94]. This homeostatic function is further supported by the lack of synapse specificity of 2-AG signaling: its synthesis site is equidistant to several presynaptic terminals and 2-AG release requires a large number of neighboring synapses to be simultaneously activated [94, 100].

Associative Plasticity

Similarly to sparse spatial inputs produced by granular layer stimulation, weak molecular layer stimulation fails to evoke endocannabinoid-mediated PTD [95, 101]. With weak molecular layer stimulation, however, the PTD is rescued when the PF tetanic stimulus is paired with CF stimulation [101]. This associative property of the endocannabinoid-mediated PTD can be explained by the synergic action of PF-mediated mGluR1 activation and the CF-mediated calcium transient propagating through the entire dendritic tree [61]. This synergistic effect does not appear to be sufficient to induce endocannabinoid-mediated PTD for the very sparse spatial pattern of PF firing produced by granular layer stimulation

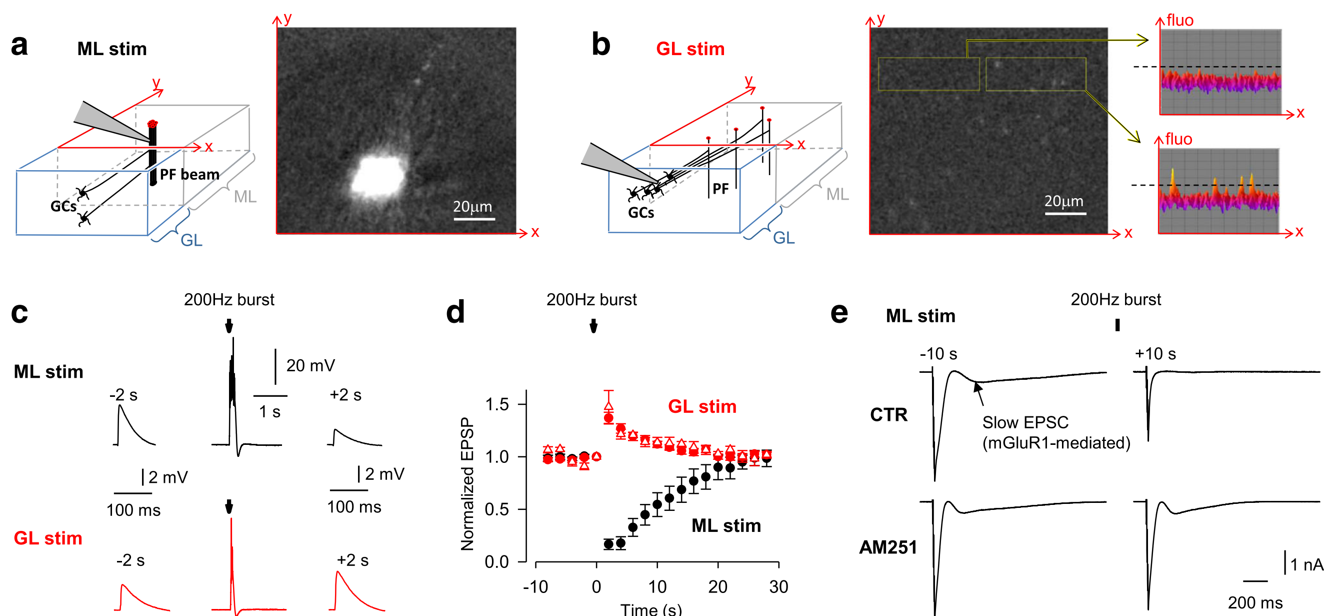

Fig. 2 Cerebellar endocannabinoid signaling dependence on spatial pattern of PF synapse activation. **a** Spatial pattern of PF activation by molecular layer stimulation (*ML stim*) in a sagittal slice. Schematic diagram showing the position of the stimulation electrode, expected to stimulate a beam of PFs perpendicular to the sagittal (x,y) plane. Image of fluorescence change evoked by ML stimulation in a sagittal slice obtained from a mouse expressing the genetically encoded calcium indicator GCaMP2 selectively in granule cells [92]. This fluorescence change is produced by stimulation-evoked presynaptic calcium rise. It maps, in the sagittal (x,y) plane, the section of the beam of adjacent stimulated PFs. **b** Spatial pattern of PF activation by granular layer stimulation (*GL stim*) in a sagittal slice. The stimulation electrode is positioned within the GL. The stimulation-evoked change of fluorescence detected in the ML, in the sagittal (x,y) plane, consists in isolated dots detected over the noise threshold (*insets*). This mapping of PF activation demonstrates the spatially scattered distribution resulting from GL stimulation, as predicted previously [94]. **c** Endocannabinoid-mediated PTD. Electrophysiological traces illustrating the PTD observed at GC to PC synapses 2 s following stimulation of the same synapses with a burst of 10 stimuli at 200 Hz. This PTD is readily observed for ML stimulation, but not for GL stimulation. **d** Time course of endocannabinoid-mediated PTD obtained for ML stimulation (*black symbols*). GL stimulation produces instead a posttetanic potentiation (*red symbols*). The pairing of GL stimulation with CF stimulation does not rescue the PTD (*open triangles*) [94]. **e** Downregulation of glutamate spillover effects following endocannabinoid-mediated PTD. In voltage-clamped PCs, ML stimulation with short burst (4 pulses at 200 Hz) evokes a fast AMPA receptor-mediated EPSC followed by a slow mGluR1-mediated EPSC which little overlap. The slow EPSC detection strongly correlates with the amount of glutamate spillover effect [94]. Following endocannabinoid-mediated PTD induction, the slow EPSC is undetectable, indicating strong reduction of glutamate spillover effects [94]

[94] (Fig. 2d). However, the CF-evoked calcium transient is a highly modulated process, which is compromised in standard in vitro conditions [80]. Future work taking into account this modulation may reveal the occurrence of associative 2-AG-mediated PTD for sparse spatial patterns of PF firing, like the ones obtained with granular layer stimulation.

Long-Term Plasticity

The best-characterized associative plasticity mechanism at PF synapses is long-term depression (LTD) [17, 128]. Although the protocol traditionally used to induce LTD has been 1 Hz repetitive pairing of CF stimulation with single or double PF stimulation [129, 130], the pairing with short PF bursts has also proved effective [83, 131]. Since PF bursts induce 2-AG mobilization, a role of 2-AG signaling might be expected. Strikingly, LTD induced by such protocols has been shown to be prevented in CB1 knockout mice, or in the presence of AM251 or THL [83]. Unexpectedly, LTD induced by the standard protocol using single PF stimulation (which does not induce 2-AG mobilization) is similarly prevented in CB1 knockout or in AM251 [83]. These data suggest that LTD induction requires CB1 activation but not phasic 2-AG mobilization. Tonic CB1 activation may be required. Selective CB1 gene deletion in GCs has been reported to similarly prevent LTD induction [132], confirming that CB1s required for LTD induction are on PF boutons, despite the well-established postsynaptic expression of LTD [128]. However, these findings are inconsistent with previous report showing a lack of effect of SR141716A (a CB1 antagonist, more potent than AM251) on LTD induction [104]. Thus, the involvement of 2-AG signaling in LTD may depend on experimental conditions. Furthermore, the role of 2-AG remains to be tested for sparse GC inputs which may require different protocols for LTD induction [100].

Recently, 2-AG signaling has also been reported to be required for postsynaptic long-term potentiation (LTP) at GC to PC synapses [133]. Stimulation of PFs at 1 Hz for 5 min induces an LTP expressed postsynaptically [129, 134]. This LTP is blocked by AM251, suggesting that it requires presynaptic CB1 activation [133]. The LTP was prevented when the recorded PC was dialyzed with 0.7 μg/ml MAGL, supporting the hypothesis that LTP induction requires 2-AG originating in PCs [133]. In the absence of data indicating that PF firing at 1 Hz promotes 2-AG mobilization (PF bursting is normally required), the results by [133] suggest that LTP induction requires tonic 2-AG release from PCs. As for LTD [132], the mechanism translating presynaptic CB1 activation into changes in postsynaptic strength remains to be investigated. Endocannabinoids having also been shown to be required for presynaptic LTD induction [135, 136], their role in modulating long-term plasticity at the GC to PC synapse appears central and complex.

Endocannabinoid Tone

If there was an endocannabinoid tone, CB1 antagonists by themselves would be expected to affect synaptic transmission at synapses expressing CB1. The application of AM251 alone on acute rat cerebellar slice has been reported to increase the firing frequency of interneurons [108]. This effect was not observed in another study using the antagonist SR141716A [137]. AM251 has also been reported to produce a small but significant increase in the amplitude of evoked IPSCs [97]. Furthermore, SR141716A and AM251 have been reported to produce 20 and 50 % increases in mIPSC frequency in P9-14 and P21-35 mice, respectively [138, 139]. Overall, these data suggest that there is tonic activity of CB1 on interneurons, which could be due to constitutive activity of CB1 or to an endocannabinoid tone. The lack of reported effect of CB1 antagonists on excitatory transmission onto PCs suggests either that CB1 borne by excitatory terminals are less constitutively active or that excitatory terminals express less CB1 proteins. In this latter hypothesis, supported by quantitative immunocytochemistry [11], tonic CB1 activation would be mediated by a low endocannabinoid tone, the effect of which would be more readily detected at terminals expressing high quantities of CB1.

The hypothesis of a 2-AG tone is supported by the observation that SR141716 induces an increase in baseline EPSC amplitude in MAGL knockout mice [64]. In wild-type animals, this 2-AG tone may be tuned by modulation of 2-AG synthesis, release, uptake, and degradation. This tone might explain how CB1 activation is involved in long-term plasticity induction while the stimulation protocols used do not produce any detectable phasic 2-AG-mediated effects. A contribution of anandamide to this tone remains to be investigated in the light of the report of a daily temporal change in FAAH activity in the mouse cerebellum [140]. Furthermore, the endocannabinoid tone may vary with postnatal development, as suggested by the effect of MAGL gene deletion on DSE, which was not detected at P10-14, but was at P20-25 [64].

Concluding Remarks

In 1990, binding studies using radiolabeled cannabinoid revealed strikingly high CB1 expression in the cerebellum [4]. In 2001, retrograde endocannabinoid signaling at synapses onto Purkinje cells was demonstrated [72]. Thirteen years later, 2-arachidonoylglycerol has been identified as the endocannabinoid mediating this retrograde signaling, the roles of postsynaptic DAGL and presynaptic MAGL have been established [56, 64, 77]. Despite a large amount of work exploring the mechanism of this retrograde signaling, key issues remain unsolved. As in other parts of the brain, it is unclear how postsynaptic calcium triggers endocannabinoid mobilization. In the cerebellar cortex specifically, it remains unclear whether physiological patterns of activity (PC depolarized states, CF or GC synaptic transmission) can actually induce endocannabinoid mobilization. Data on long-term plasticity even suggest that what matters is CB1 tonic activation.

The strong dependency of endocannabinoid signaling on the spatial pattern of GC to PC synaptic activation has led to the proposal that endocannabinoid-mediated depression of transmitter release could be homeostatic, downregulating excessive glutamate release arising from dense spatial pattern of synapse activation, thereby reducing glutamate spillover effects and promoting independent operation of neighboring synapses [92, 94, 95, 100]. This hypothesis would be challenged if experiments were to demonstrate endocannabinoid release induced at individual, spatially isolated, GC to PC synapses. In vivo approaches would help to meet physiological conditions. Alternatively, in acute slice models, one needs to control the spatial pattern of synapse activation and take into account modulation of dendritic electrogenesis [80] and the role of neuromodulators like monoamines [95].

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