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FORUM REVIEW ARTICLE

Imaging of Ca^{2+} and Related Signaling Molecules and Investigation of Their Functions in the Brain

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Abstract

Intracellular Ca^{2+} signaling, and related mechanisms involving inositol 1,4,5-trisphosphate (IP_3), nitric oxide, and the excitatory neurotransmitter glutamate, play a major role in the regulation of cellular function in the brain. Due to the complex morphology of central neurons, the correct spatiotemporal distribution of signaling molecules is essential. Thus, imaging studies have been particularly useful in elucidating the functions of these signaling molecules. The advancement of imaging methods, together with the development of a new method for the specific inhibition of intracellular IP_3 signaling, have made it possible to identify pathways that are regulated by Ca^{2+} signals in the brain, including Ca^{2+}-dependent synaptic maintenance and glial cell-dependent neurite growth. Further investigation of Ca^{2+}-related signaling is expected to increase our understanding of brain function in the future. *Antioxid. Redox Signal.* 14, 1303–1314.

Introduction

Intracellular Ca^{2+} concentration regulates a myriad of cell functions, such as contraction, secretion, immune responses, transcription, fertilization, and development. Ca^{2+} signals display complex spatiotemporal patterns, and this is believed to be why this signaling molecule is so versatile (9). In the brain, intracellular Ca^{2+} signals regulate many important functions: neurotransmitter release, synaptic plasticity, and cell death, to name but a few. The spatiotemporal features of Ca^{2+} signaling are of extreme importance in neurons because these cells have a complex morphology and well-defined polarity. Ca^{2+} imaging in neurons has been important in the understanding of neuronal function (18, 21, 55, 63, 66, 73, 92, 93, 96, 101, 103, 105). It follows that the spatiotemporal dynamics of signaling molecules upstream and downstream of the Ca^{2+} signal should also provide important information about neural function. The advent of fluorescent Ca^{2+} indicators based on small molecules (25) or the green fluorescent protein (GFP) (61) has greatly advanced the field. Similarly, generation of new signaling indicators has promoted the study of signaling molecules other than Ca^{2+}. In the first part of this review, we discuss new methods to image signaling molecules upstream and downstream of the Ca^{2+} signal, thus clarifying the spatiotemporal dynamics of Ca^{2+}-related signaling molecules in the brain. We will also discuss the physiological significance of these signaling mechanisms.

Although many Ca^{2+} signaling-related functions in the brain have been described, it is likely that there are many others that remain unrecognized. Intracellular Ca^{2+} concentration is regulated by the influx of Ca^{2+} *via* the plasma membrane and/or release of Ca^{2+} from the intracellular Ca^{2+} store, which is predominantly the endoplasmic reticulum (ER). Voltage-dependent Ca^{2+} channels, Ca^{2+}-permeable glutamate receptors, and transient receptor potential channels are involved in the transport of Ca^{2+} across the plasma membrane in neurons. Two types of Ca^{2+}-release channels, the ryanodine receptor and the inositol 1,4,5-trisphosphate receptor (IP_3R), are expressed in the brain and are involved in intracellular Ca^{2+} mobilization (8). In the second part of this review, we discuss newly recognized neuronal and glial functions that are regulated by the Ca^{2+} release mechanism *via* IP_3R.

Imaging Studies of Ca^{2+}-Related Signaling Molecules in the Brain

IP_3 signaling and synaptic plasticity in the cerebellum

Purkinje cells (PCs) are the sole output neurons from the cerebellar cortex, and they receive two types of excitatory glutamatergic input: numerous inputs from parallel fibers (PFs) originating from the granule cells, and a strong input from usually single climbing fibers (CFs) originating from the inferior olive (37). One of the striking features of PCs is their extremely high expression level of the type 1 IP_3R (IP_3R1) Ca^{2+}-release channels (86). In addition, the type 1 metabotropic glutamate receptor (mGluR1), which is coupled to β isozymes of phospholipase C (PLCβ) via the G-protein to generate IP_3, is also highly expressed at PF–PC synapses

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(7, 88). The high expression levels of proteins involved in IP_3-Ca^{2+} signaling suggest the importance of Ca^{2+} mobilization from the intracellular store in the regulation of PF–PC synaptic function. Indeed, the properties and physiological roles of IP_3-Ca^{2+} signaling in neurons have been most extensively studied in PF–PC synapses.

The primary electrical transmission in PF–PC synapses is mediated by presynaptic glutamate release and the postsynaptic ionotropic glutamate receptor α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) (48). Thus, the PF input generates excitatory postsynaptic currents (EPSCs) to depolarize the PC dendrites. Ca^{2+} imaging studies in PF–PC synapses have shown that physiologically plausible burst PF inputs generate two types of local Ca^{2+} signal in the spines and dendrites of PCs receiving the PF inputs (21, 93). The first is generated by an influx of Ca^{2+} *via* voltage-gated ion channels in response to AMPAR-dependent depolarization. This is followed by a delayed Ca^{2+} signal that is dependent on mGluR1. The delayed Ca^{2+} response is blocked by the intracellular application of heparin, an inhibitor of IP_3R (93). Thus, the mGluR1-dependent Ca^{2+} signal appears to be mediated by IP_3-induced Ca^{2+} release. The transmission at CF–PC synapses is also mediated by presynaptic glutamate release and the postsynaptic AMPAR (48). A CF input generates a large EPSC, which then induces action potentials that are called complex spikes in PCs. Complex spikes activate Ca^{2+} influx *via* voltage-gated Ca^{2+} channels and induce an increase in the intracellular Ca^{2+} concentration throughout the dendritic arborization of PCs (47).

The synaptic strength of PF–PC synapses undergoes long-term depression (LTD) when PF inputs are coupled with CF inputs at ≈ 1 Hz for ≈ 5 min (37). This cerebellar LTD is considered to underlie certain forms of motor learning, such as the vestibulo-ocular reflex (37). Mounting evidence suggests that the IP_3 signaling pathway in PCs plays a critical role in the induction of LTD. First, LTD was blocked by the disruption of the *mGluR1* gene (2, 15, 33) or the *IP_3R1* gene (36). In addition, no LTD was observed in mutant mice in which there is no Ca^{2+} release from the intracellular Ca^{2+} store in PC spines (60). In addition, activation of PFs can be replaced by increasing IP_3 concentration using caged IP_3 to induce LTD (21, 40, 41).

The induction of LTD therefore requires IP_3 signaling and the coincidence of PF and CF inputs. What mechanism underlies the coincidence detection? Because IP_3Rs require both IP_3 and Ca^{2+} simultaneously for their activation (10, 35), IP_3Rs may function as the molecular coincidence detector. Indeed, the pairing of the PF input (generating a localized dendritic IP_3 signal) with the CF input (generating a Ca^{2+} signal throughout the dendritic arborization in PCs) supralinearly enhances Ca^{2+} release *via* IP_3Rs within the spines receiving PF inputs (101). Thus, conjunctive inputs from PFs and a CF may be integrated at the IP_3R to induce Hebbian synaptic plasticity in PCs. The coincidence-detector property of IP_3Rs and the supralinear increase in local Ca^{2+} concentration has also been reported for pyramidal neurons in the hippocampus and neocortex (49, 66), indicating a ubiquitous involvement of IP_3 signaling in the signal integration in the brain.

Ca^{2+}-induced IP_3 production revealed by IP_3 imaging in PCs

The roles of IP_3-Ca^{2+} signaling in cerebellar LTD have been studied by genetic and pharmacological methods, neither of which can provide spatiotemporal information. Because synaptic plasticity including cerebellar LTD is induced in an input-specific manner, spatiotemporal analysis of intracellular IP_3 dynamics at synapses is required to understand the underlying mechanisms. Hirose *et al*. developed the GFP-tagged pleckstrin homology domain of PLCδ1 (GFP-PHD), a fluorescent IP_3 indicator molecule, to visualize intracellular IP_3 dynamics within intact cells (32). GFP-PHD binds to phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane and translocates to the cytoplasm upon increase in the cytoplasmic concentra-

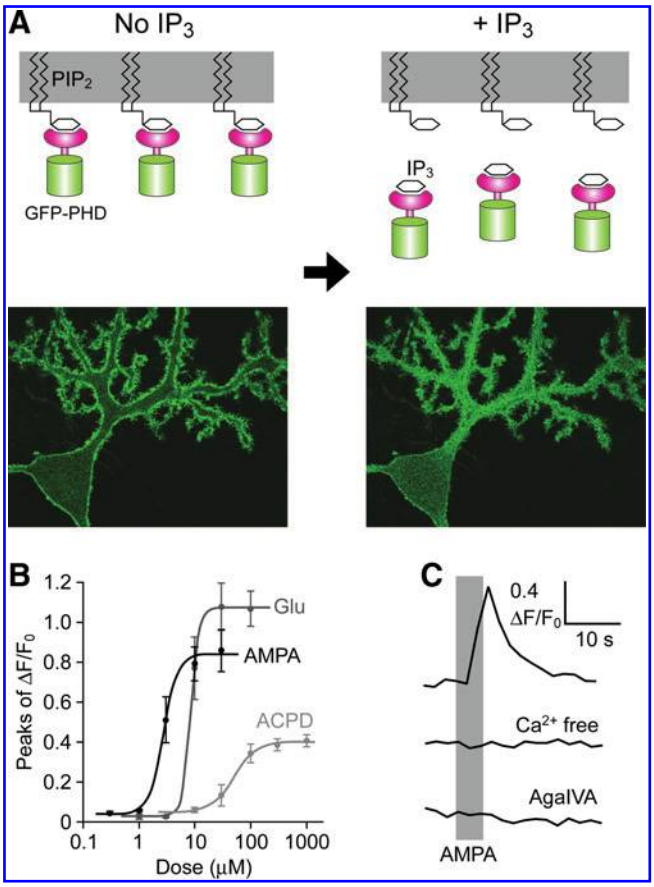

FIG. 1. Imaging of AMPAR-mediated IP_3 production in PCs. (A) *Upper panels*: Schema of IP_3 imaging using GFP-PHD. GFP-PHD translocates from the plasma membrane to the cytoplasm in response to an increase in the IP_3 concentration. *Lower panels*: a cultured PC expressing GFP-PHD imaged by a confocal microscope. GFP-PHD translocation was observed in response to the application of 30 μ*M* glutamate. **(B)** Dose-response relationship of IP_3 production induced by glutamate, AMPA, and (±)-1-Aminocyclopentane-trans-1,3-dicarboxylic acid (ACPD), an mGluR agonist. Note that the ionotropic glutamate receptor agonist AMPA induces considerable IP_3 production. $\Delta F/F_0$ is fractional change in the fluorescence intensity. **(C)** IP_3 production in response to the application of 30 μ*M* AMPA. This AMPAR-mediated IP_3 production was abolished in the absence of Ca^{2+} in the extracellular medium and the presence of 100 n*M* ω-agatoxin IVA (AgaIVA), a blocker of the P-type voltage-gated Ca^{2+} channel. Modified from Ref. (74). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

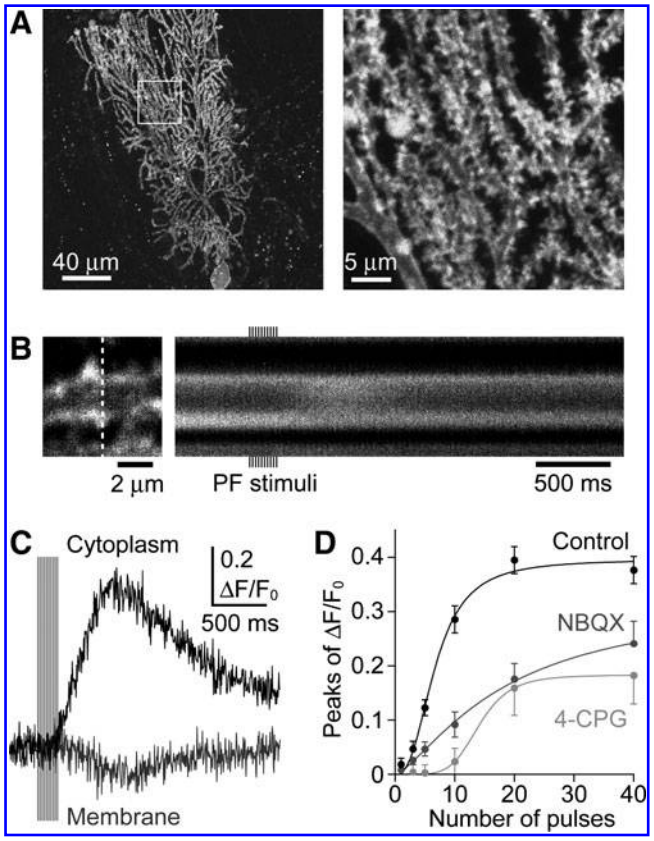

FIG. 2. Imaging of PF-induced IP_3 signaling in PC dendrites using GFP-PHD. (A) Sindbis virus-mediated expression of GFP-PHD in a PC in a cerebellar slice. The *right-hand image* is a magnification of the region indicated by the *white box* in the *left-hand image*. **(B)** Line-scan imaging across a fine dendrite as shown by the *white broken line* shows translocation of GFP-PHD from the plasma membrane to the cytoplasm upon PF stimulation (10 pulses at 50 Hz). **(C)** Time course of the fluorescence intensity changes within the membrane region and cytoplasmic region of the line-scan data shown in **B**. **(D)** Cooperative IP_3 production by AMPAR and mGluR. The number of PF stimulation pulses varied between 1 and 40, at a constant frequency of 50 Hz, in the absence and presence of the glutamate receptor antagonist. 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f] quinoxaline-7-sulfonamide (NBQX), AMPAR antagonist; (*S*)-4-Carboxyphenylglycine (4-CPG), mGluR antagonist. Modified from Ref. (75).

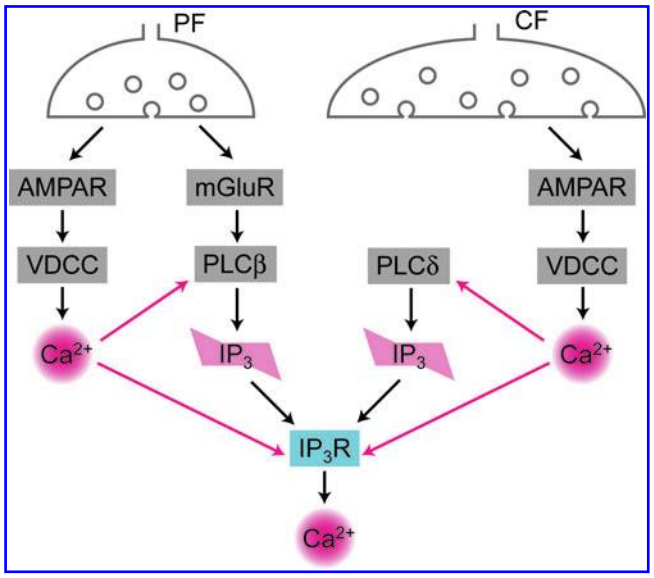

FIG. 3. Schematic diagram of cross talk between Ca^{2+} and IP_3 signaling in PCs induced by PF and CF inputs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

tion of IP_3 because of ≈ 20-fold higher affinity for IP_3 than for PIP_2 (Fig. 1A, upper panels) (32). Changes in the intracellular IP_3 concentration can therefore be measured by imaging the intracellular translocation of GFP-PHD.

Using GFP-PHD as the fluorescent IP_3 probe, intracellular IP_3 dynamics in the fine dendrites of PCs have been monitored in real-time (74) (Fig. 1A, lower panels). Surprisingly, stimulation of the AMPAR, which is not directly coupled to PLC, generated an IP_3 signal (Fig. 1B). This AMPAR-mediated IP_3 production was dependent on Ca^{2+} influx *via* the P-type voltage-gated Ca^{2+} channel (Fig. 1C). Furthermore, CF stimulation induced IP_3 signals within the PCs in acute cerebellar slices (74). Thus, the AMPAR/Ca^{2+} influx-induced IP_3 production appears to be physiologically relevant. These results revealed a new mGluR-independent IP_3 signaling pathway in PCs. Because the PLCδ isozyme is activated primarily by Ca^{2+} concentrations ranging from 0.1 to 10 μM, this may be the enzyme that mediates the Ca^{2+}-induced IP_3 production (3, 5). Indeed, PLCδ2 is expressed at relatively high levels in PCs (59).

What is the physiological role of CF stimulation-dependent IP_3 signaling? Intracellular release of IP_3 by the photolysis of caged IP_3 has suggested that the activation of IP_3Rs expressed in PCs requires a concentration of IP_3 more than 10-times higher than that expressed in glial cells or in peripheral tissue cells (42, 43). To test this, the IP_3 sensitivity of Ca^{2+} release was examined using real-time imaging of the luminal Ca^{2+} concentration in the intracellular Ca^{2+} store of PCs whose plasma membrane was permeabilized (22). By analyzing the rate of Ca^{2+} release upon application of a known concentration of IP_3, it was shown that IP_3R in PCs has a >20-fold lower affinity for IP_3 than that in other types of cell (22). It is, therefore, conceivable that coactivation of both PF- and CF-mediated IP_3 production is required to attain sufficiently high IP_3 concentrations for the activation IP_3R at the synaptic spines of PCs. Thus, the CF stimulation-mediated IP_3 production may provide another mechanism for the coincidence detection during the induction of cerebellar LTD, in addition to the aforementioned IP_3R-dependent coincidence detection.

Cross-talk between AMPAR and mGluR in IP_3 production at PF–PC synapses

In many excitatory glutamatergic synapses including PF-PC synapses, both AMPARs and mGluRs are closely distributed on the postsynaptic membrane (95). At PF–PC synapses, the simultaneous activation of AMPARs and mGluRs is necessary for the induction of LTD (39, 54). However, the mechanism and physiological significance of functional interactions between the two types of glutamate receptor is not fully understood.

To address the interaction between AMPARs and mGluRs at PF–PC synapses in the generation of IP_3, local IP_3 dynamics were visualized using GFP-PHD during a brief train of PF inputs to the fine dendrites of PCs (75) (Figs. 2A–2C). The contribution of mGluRs and AMPARs to PF-induced IP_3 production was studied using specific antagonists for these receptors. Surprisingly, PF-induced IP_3 production was blocked not only by the mGluR antagonist but also by the AMPAR antagonist (*i.e.*, IP_3 production was supralinearly increased upon the simultaneous activation of AMPARs and mGluRs) (Fig. 2D). Cross-talk between AMPARs and mGluRs mediated cooperative production of IP_3 at PF–PC synapses. Simultaneous imaging of IP_3 and Ca^{2+} revealed that PF-induced IP_3 production was highly enhanced upon AMPAR-triggered Ca^{2+} influx *via* voltage-gated Ca^{2+} channels. When *O,O'*-Bis(2-aminophenyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid (BAPTA) was applied through a patch pipette to buffer intracellular Ca^{2+}, negligible IP_3 was produced, indicating the involvement of intracellular Ca^{2+} in PF-induced IP_3 production. The functional interaction between AMPARs and mGluRs may also play an important role in the generation of sufficiently high intracellular IP_3 concentrations to activate the low-sensitivity IP_3R in PCs.

How do AMPARs and mGluRs cooperate with each other in IP_3 generation? The PLCβ isozyme, which is abundantly expressed in PCs, may be involved. Although the activity of PLCβ is primary regulated by G-protein, it is also dependent on the intracellular Ca^{2+} concentration (32, 97). Thus, PLCβ may be cooperatively activated to generate IP_3 by both mGluR-mediated G-protein activation and AMPAR-mediated Ca^{2+} influx (Fig. 3).

PLC activity produces not only IP_3 but also diacylglycerol, which is a precursor of endocannabinoids (90). Cooperative activation of PLCβ by mGluR activation and Ca^{2+} influx may also lead to the generation of endocannabinoids, which function as retrograde messengers to modulate the transmission efficacy at various brain synapses including PF-PC synapses. Concordantly, the simultaneous activation of mGluRs and Ca^{2+} influx supralinearly increases the production of endocannabinoids in PCs and hippocampal neurons (27, 56). Furthermore, studies using mice deficient for PLCβ isozymes have shown that PLCβ4 and PLCβ1 are involved in the cooperative generation of endocannabinoids in PCs (56) and hippocampal neurons (27), respectively.

Nitric oxide signaling and synaptic plasticity in the cerebellum

Bidirectional changes in synaptic strength have been reported for many excitatory glutamatergic synapses in the brain. Long-term potentiation (LTP) is also induced in PF–PC synapses and allows reversible modulation of the synaptic gain in combination with LTD. This cerebellar LTP is induced by PF inputs at 1 Hz for 5 min without coupling with CF inputs (52). It is possible that the LTP extinguishes LTD in PF–PC synapses to remodel neural circuit connections that regulate motor behaviors (14, 51).

Cerebellar LTP is dependent on nitric oxide (NO) production during PF input (52). Furthermore, the application of an NO donor induces cerebellar LTP (52). NO is generated from arginine by NO synthase (NOS), and is an important intercellular messenger (34). In the brain, neuronal NOS (nNOS) is expressed in particular neurons and glial cells, and its activity is regulated by the intracellular Ca^{2+} concentration (104). In the cerebellum, nNOS is expressed in granule cells but not in PCs. It is likely that PFs generate NO during activation, and NO then spreads to PCs to induce LTP. Indeed, PF activity-dependent increases in NO have been observed in cerebellar slices using NO-sensitive electrodes (87).

Because cerebellar LTP is induced in an input-specific manner, measurement of NO dynamics at synapses is critical for understanding synaptic plasticity. However, NO-sensitive electrodes have a limited spatial resolution; thus, it is difficult to measure the spatial distribution of NO signals. To overcome this, small molecule NO indicators including 4,5-diaminofluorescein (DAF-2) have been developed (46). To mediate a change in the fluorescence intensity of DAF-2 and its sister indicators, multi-step reactions of NO with ambient O_2, and subsequent covalent binding of the oxidized product with the indicator molecule, are required. These complex reactions, and their irreversible nature, would limit measurement of the spatiotemporal distribution of NO signals in the cerebellum. Thus, another type of NO indicator is required.

NO signaling mechanisms revealed by NO imaging in cerebellar slices

A protein-based, genetically encoded NO indicator with PC-specific expression would allow higher spatial resolution than does the nonspecific labeling with small molecule indicators. A new NO indicator was generated in the following way. Soluble guanylyl cyclase (sGC) is a physiological target molecule of NO. sGC is a heme protein and generates cyclic GMP from GTP upon binding to NO. Unlike other heme proteins that bind both NO and O_2, sGC has the unique property of binding to NO but not O_2. Therefore, sGC may be used for the recognition of NO. The heme binding capacity of a series of deletion mutants of sGC was assessed (67). The N-terminal region of the sGC β1 subunit was found to be essential for heme binding and NO recognition, and was termed the heme-binding region (HBR). A panel of HBR–GFP fusion proteins was assessed for NO-dependent fluorescence intensity change. One of them showed a 14% increase in the fluorescence intensity upon NO binding, and could therefore be used as a new NO indicator (68).

Imaging the fluorescence intensity changes of HBR–GFP expressed in PCs clearly showed PF stimulation-induced NO signals within PCs (68) (Fig. 4A). The amplitude of the PF-induced NO signals depended uniquely on the PF stimulation frequency. Burst PF stimulation (BS) of five pulses at 50 Hz (mimicking the physiological firing pattern) was repeated at different frequencies. There was a frequency-dependent increase in the NO signal intensity upon BS at 0.25–1 Hz (Fig. 4B). Unexpectedly, BS at higher frequencies (2–4 Hz) resulted in a decrease in the NO signal intensity (Fig. 4B). The inhibitory effect of high-frequency BS is due to retrograde endocannabinoid signaling (see above), which causes inhibition of presynaptic Ca^{2+} influx through voltage-gated Ca^{2+} channels (11). The resulting decrease in the presynaptic Ca^{2+} concentration is responsible for the decrease in the NOS activity. Taken together, NO signaling at PF–PC synapses depends on the frequency of PF inputs and thus functions as a frequency discriminator, converting specific patterns of PF activity to an NO signal.

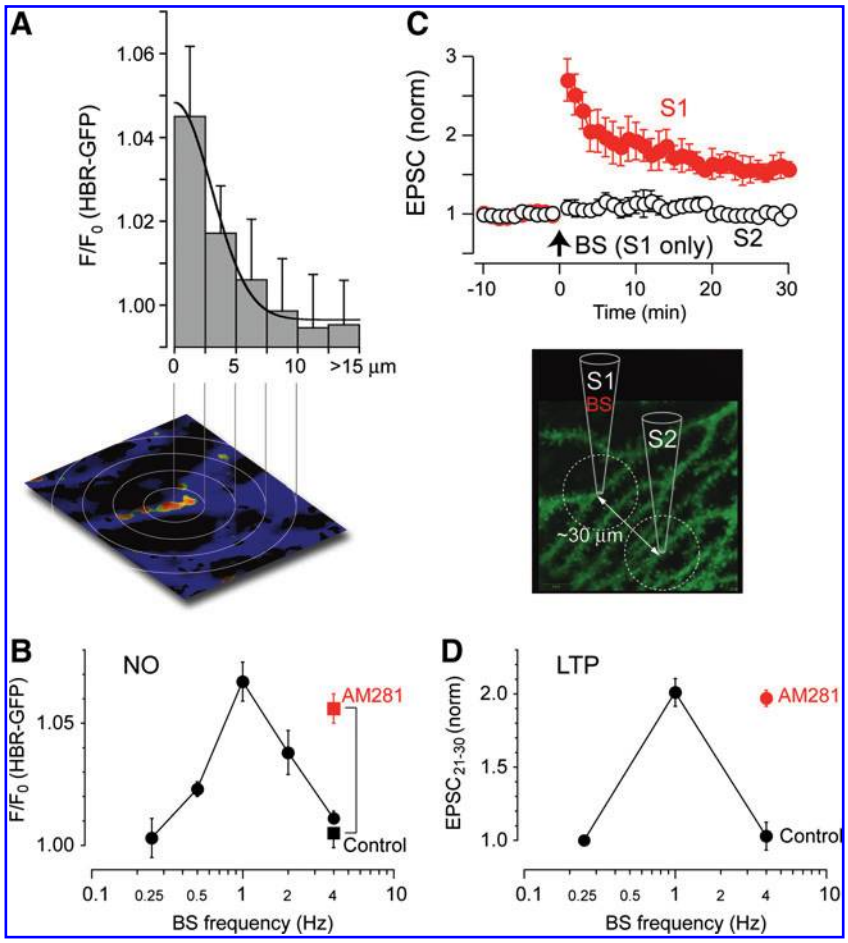

FIG. 4. PF-mediated NO signaling and cerebellar LTP. (A) Spatial distribution of NO signal around the stimulated population of PFs imaged using HBR-GFP. **(B)** Dependence of NO signal amplitude on the frequency of BS (*closed circles*). Another set of experiments in the presence and absence of the CB1 receptor antagonist AM281 at 4 Hz BS (*squares*), showed the endocannabinoid-mediated inhibition of NO production. **(C)** Spatial distribution of 1 Hz BS-induced LTP examined using two stimulating electrodes, S1 and S2. Nonoverlapping PFs were stimulated by S1 and S2, and 1 Hz BS was applied only to S1. LTP was observed only in S1 pathway but not in S2 pathway, indicating the input specificity in cerebellar LTP. **(D)** Dependence of LTP induction on the frequency of BS (*black circles*). AM281 rescued LTP at 4 Hz BS (*red circle*). Modified from Ref. (68).

The frequency-dependent NO signaling at PF–PC synapses correlated with the induction of cerebellar LTP. LTP was efficiently induced by 1 Hz BS, which generated the NO signal. However, LTP was not induced by 0.25 or 4 Hz BS, which generated very little NO signal (Fig. 4D). The LTP induced by 1 Hz BS was inhibited in the presence of a NOS inhibitor, confirming that NO is essential in the induction of cerebellar LTP. Thus, NO signaling appears to filter PF inputs with specific frequencies at the PF–PC synapse to induce cerebellar LTP in a frequency-dependent manner.

NO has often been regarded as a far-reaching messenger. Therefore, the spatial distribution of 1 Hz BS-induced NO signal was imaged using HBR–GFP. The HBR–GFP signal was observed around activated PFs but decreased sharply with the distance from the center of the activation (Fig. 4A). This indicates that NO is an input-specific messenger, which is contrary to the conventional concept but consistent with input-specific induction of cerebellar LTP. Confirming this, LTP was generated in PF–PC synapses that received BS, but not in adjacent nonoverlapping synapses that lay outside the BS-induced NO signal (Fig. 4C).

New Roles for Ca^{2+} Signaling in Neurons and Glia

Ca^{2+}-dependent maintenance of synaptic strength in the cerebellum

Activity-dependent changes in synaptic function are essential in both developing and adult brains. Other synapses must be sustained at a basal level for the maintenance of neural circuits. Because synaptic activity is required for the stabilization of synapses at adult neuromuscular junctions (4), it is possible that ongoing synaptic activity is required for the maintenance of synaptic function in the brain. To examine this, the effect of chronic inhibition of the activity-dependent signaling cascade on synaptic function was examined in PF–PC synapses (23). Because mGluR1 is located perisynaptically at PF–PC synapses (7), repetitive stimulation of PFs at high frequencies is required for mGluR1 activation, which then leads to intracellular IP_3 signaling in PCs (21, 75, 93). The spontaneous activity of PFs (simple spikes), with their low firing rate (0.5 Hz) (12), is insufficient for the activation of mGluR1-mediated IP_3 signaling (Fig. 2D). However, sensory inputs to the granule cells *via* the mossy fibers generate high-frequency bursts (several pulses in the order of 100 Hz) of PF action potentials (12), and are expected to activate mGluR1-IP_3-Ca^{2+} signaling in PCs (Fig. 2D). In other words, IP_3-Ca^{2+} signaling in PCs may function *in vivo* as an activity sensor for the sensory inputs to the cerebellum.

For the specific inhibition of IP_3 signaling in PCs *in vivo*, IP_3 5-phosphatase (5ppase), which specifically hydrolyzes IP_3 to generate inactive inositol 1,4-bisphosphate, was used (50, 58). 5ppase was expressed in PCs by injecting Sindbis virus-encoded 5ppase into the mouse cerebellum. The magnitude of EPSCs (output) recorded in PCs in response to varying PF stimulus (input) intensities was measured to assess the strength of the PF–PC synapse. There was a

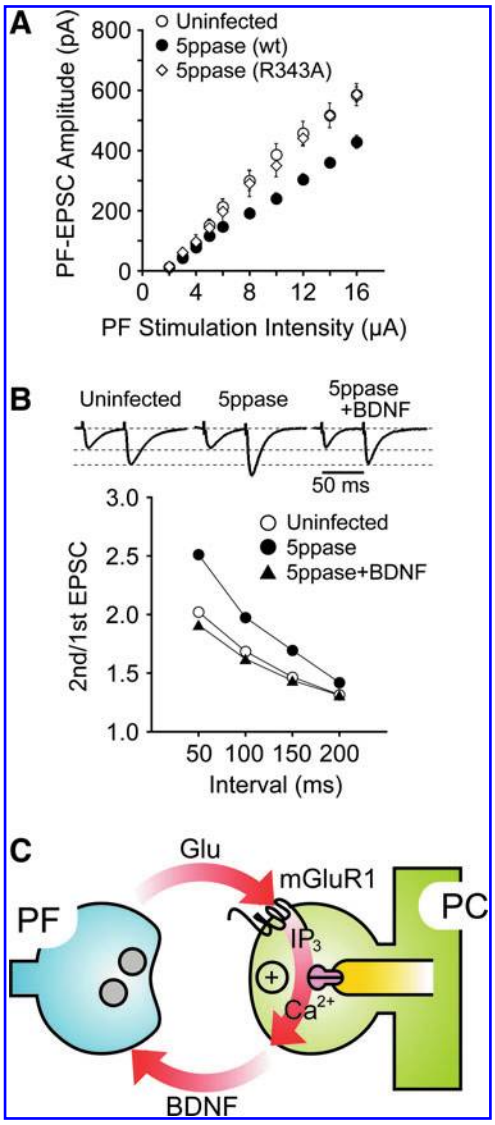

FIG. 5. IP_3-Ca^{2+}-dependent maintenance of PF-PC synapses. (A) The input-output relationship at the PF-PC synapse in uninfected, 5ppase (wild type)-expressing and 5ppase (R343A)-expressing PCs. The magnitude of PF-EPSC was plotted against the stimulus intensity of PFs. Ongoing IP_3 signaling *in vivo* is indispensable for the maintenance of PF synapses. **(B)** Paired-pulse ratios (PPRs) in uninfected and 5ppase-expressing PCs plotted against the stimulus interval. The increased PPR in 5ppase-expressing PCs was reversed by chronic application of BDNF. **(C)** Schematic drawing showing a possible mechanism for the maintenance of presynaptic function in PF–PC synapses. Modified from Ref (23). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

marked reduction in the slope of the input-output relationship in PCs expressing 5ppase (Fig. 5A). On the other hand, the expression of mutant low-activity 5ppase, in which a critical arginine residue was replaced with alanine (R343A), had no significant effect on the synaptic strength (Fig. 5A). Synaptic strength decreases when IP_3 signaling is inhibited in PCs.

The mechanism underlying the reduced synaptic strength at the PF–PC synapse could be either reduced transmitter release from the presynaptic terminal or reduced postsynaptic glutamate sensitivity. The amplitude of quantal EPSCs was not altered, suggesting that postsynaptic glutamate sensitivity remained constant. The paired-pulse ratio (PPR), which is often used to assess the presynaptic transmitter release probability of PF–PC synapses (53), increased, suggesting that the presynaptic function was reduced (Fig. 5B). It is likely that the transmitter release probability of PFs decreases when IP_3 signaling is blocked in PCs.

The above results indicate that postsynaptic IP_3 signaling in PCs is necessary for the maintenance of presynaptic PF terminal function. Therefore, it is likely that a retrograde messenger is released from the postsynaptic PCs to maintain the presynaptic function. What then is the retrograde signal? Brain-derived neurotrophic factor (BDNF) is a neurotrophin that is strongly expressed in PCs (57). Furthermore, TRKB, the BDNF receptor, is expressed in the presynaptic granule cells (24, 85). Thus, BDNF is potentially a retrograde messenger at the PF–PC synapse. Indeed, after chronic application of an anti-BDNF antibody to the cerebellar cortex *in vivo*, the PPR increased, suggesting a decrease in the transmitter release probability. The IP_3 signaling in PCs and the retrograde BDNF signal may share the same mechanism for maintaining presynaptic function, because chronic application of the anti-BDNF antibody had no additional inhibitory effect when the IP_3 signaling was inhibited by 5ppase. Furthermore, the application of extrinsic BDNF reversed the effect of 5ppase. These results suggest that there is a sequence of events from the PF input for the maintenance of synaptic function: mGluR1 activation, IP_3 generation, Ca^{2+} release, BDNF production, and presynaptic maintenance (Fig. 5C). Considering that mGluR1 is preferentially activated by the burst of PF activity induced by sensory input, this mechanism may underlie experience-dependent synaptic maintenance mechanisms. This also suggests that the connection between synapses that are rarely used would become weaker.

Regulation of neurite growth by spontaneous Ca^{2+} oscillations in astrocytes

Glial cells are non-neuronal cells that outnumber neurons in the human brain. Among glial cells, oligodendrocytes/Schwann cells and microglial cells have been recognized by their ability to form myelin sheaths and to govern host immune responses, respectively. In contrast, the function of astrocytes, the predominant glial cells, has not been fully characterized. They were traditionally considered to be mere supporting elements for neurons, because their spatial distribution seems to form a scaffold of neurons ["nerve-glue" or "nerve-cement" (89)] and they are unable to generate membrane action potentials. However, recent results have shown that astrocytes play active roles in the regulation of brain function. Astrocytes display dynamic Ca^{2+} oscillations that are evoked by various stimuli including neurotransmitters, cytokines, cellular metabolites, and mechanical stress. Accumulating evidence supports that the Ca^{2+} oscillations in astrocytes modulate neighboring synaptic transmissions and local blood flow *via* the Ca^{2+}-dependent release of ATP, glutamate, and D-serine (6, 20, 28, 29, 44, 64, 77, 80, 100). Notably, astrocytic Ca^{2+}

oscillations are generated spontaneously even when the neighboring neurons are not firing (30, 70, 78, 79). This is a remarkable feature for nonexcitable cells. However, the physiological significance of the spontaneous Ca^{2+} oscillations remains to be elucidated.

Astrocytes also regulate neuronal growth. They enhance synaptogenesis by providing thrombospondins or the ligands for integrin receptors (13, 26), regulate spine maturation through ephrin/Eph signaling (65, 72), and modulate the homeostasis of synaptic strength by releasing cytokines (91). Furthermore, astrocytes promote neurite growth by providing growth factors and transmembrane/extracellular matrix proteins, such as nerve growth factors, cadherins, laminins, and fibronectins (62, 99). Conversely, reactive astrocytes, which have undergone characteristic differentiation in response to inflammation, inhibit the growth of regenerating axons by providing growth-inhibitory molecules, including proteoglycans, tenascins, and cytokines (81, 83). The relationship between these functions of astrocytes and their Ca^{2+} oscillations remains elusive. Interestingly, spontaneous Ca^{2+} oscillations are lost in reactive astrocytes (1) and they are frequently observed in the early postnatal period, when neural circuits are formed (78, 79). These findings suggest the involvement of Ca^{2+} signals in astrocyte-dependent promotion of neurite growth. It is, therefore, important to examine the physiological role of spontaneous Ca^{2+} oscillations in astrocytes in conjunction with neurite growth.

Monocultures of purified astrocytes retain spontaneous Ca^{2+} oscillations (19, 45). When IP_3 signaling in cultured astrocytes was inhibited by the stable expression of retrovirally-expressed 5ppase, astrocytic Ca^{2+} oscillations were virtually abolished, while the R343A/R350A-mutant inactive 5ppase (in which two critical arginine residues were replaced by alanine residues) had no effect on the Ca^{2+} oscillations (Fig. 6C) (38). These 'Ca^{2+} signal-deficient' astrocytes allowed long-term analysis of the effect of astrocytic Ca^{2+} oscillations on neuronal development *in vitro*. When dissociated hippocampal neurons were cultured on a monolayer of Ca^{2+} signal-deficient astrocytes for over 10 days, the neurons had much shorter dendrites than those cultured on control astrocytes (Figs. 6A and 6B). Thus, astrocytic Ca^{2+} oscillations are necessary for supporting neuronal dendritic growth.

In addition to the effect of 5ppase on dendritic growth, axonal growth cone advancement was also markedly reduced on Ca^{2+} signal-deficient astrocytes (38). The inhibitory effect of Ca^{2+} signal-deficient astrocytes on growth cone advancement may be mediated either by a diffusible factor released from the astrocytes or by a nondiffusible factor expressed on the astrocyte surface. To distinguish between these two possibilities, half of the cells were transduced with 5ppase in a mosaic pattern. If a diffusible factor were responsible, growth cone advancement would be evenly reduced on the culture; if a nondiffusible factor were involved, growth cones would advance faster on nontransduced astrocytes than on transduced (Ca^{2+} signal-deficient) astrocytes. The results clearly supported the latter, indicating the involvement of a nondiffusible factor. Thus, spontaneous Ca^{2+} oscillations in astrocytes enhance neurite growth through the regulation of certain factors expressed on the astrocytic membrane (38).

What is the factor that regulates neurite growth? Astrocytes express on their surface various proteins that either promote or inhibit neuronal growth, such as N-cadherin, laminins, collagens, and proteoglycans (62, 71, 83, 84, 99). None of these proteins displayed altered expression levels in Ca^{2+} signal-deficient astrocytes, except N-cadherin, whose expression was downregulated (Fig. 7). N-cadherin is a transmembrane cell–cell adhesion glycoprotein with important roles in neuronal growth, activity, and the maintenance of morphology (71, 82, 94, 99). The artificial expression of N-cadherin in Ca^{2+} signal-deficient astrocytes partially rescued the neurite

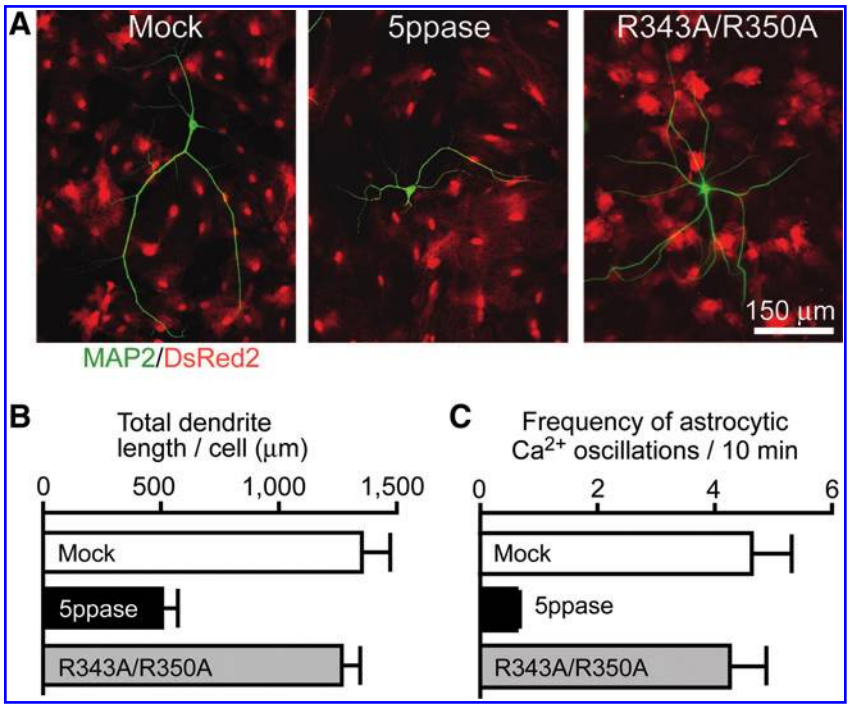

FIG. 6. Reduced neurite growth on Ca^{2+} signal-deficient astrocytes. (A) Representative images of neuron–glia cocultures. Astrocytes expressing wild-type 5ppase (5ppase) showed reduced dendritic growth compared with astrocytes expressing only the infection marker RFP (Mock) or the phosphatase-inactive mutant 5ppase (R343A/R350A). *Red*, astrocytes; *green*, dendrites visualized by immunostaining using anti-MAP2 antibody. **(B, C)** Compiled data of dendritic growth **(B)** and the frequency of astrocytic spontaneous Ca^{2+} oscillations **(C)**. Modified from Ref. (38).

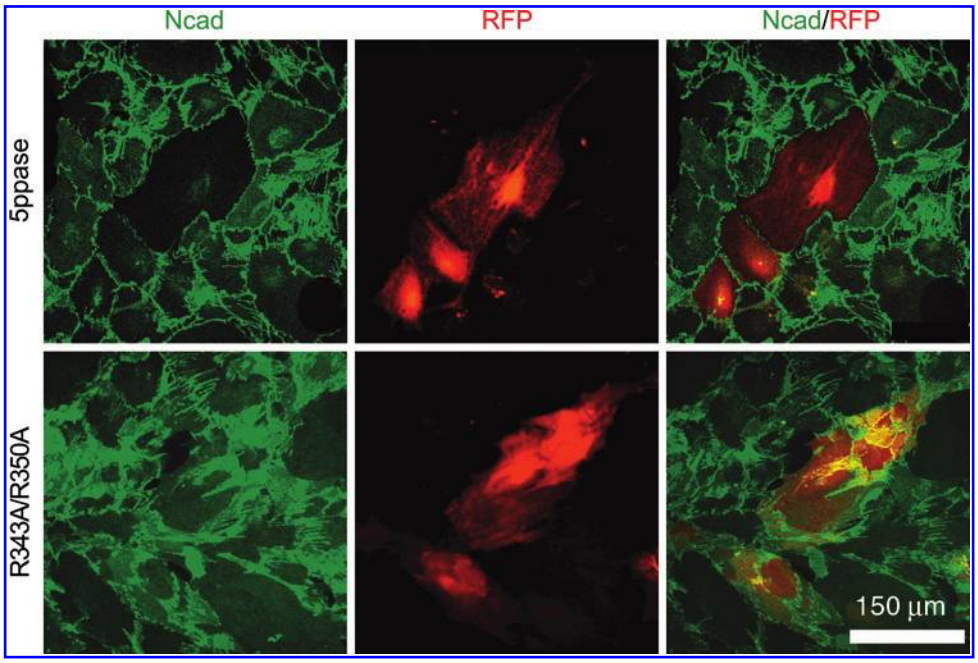

FIG. 7. Reduced expression of N-cadherin in Ca^{2+} signal-deficient astrocytes. Immunocytochemical analysis of astrocytes transduced with 5ppase. *Upper panels*: 5ppase-expressing astrocytes (distinguished by the infection marker RFP) showed reduced expression level of the cell adhesion protein N-cadherin (Ncad) as compared with the surrounding uninfected cells. *Lower panels:* R343A/R350A-mutant inactive 5ppase had no effect on the N-cadherin expression level. Modified from Ref. (38).

growth. Therefore, N-cadherin is at least partly responsible for the regulation of neurite growth downstream of astrocytic spontaneous Ca^{2+} oscillations (38). This indicates a new neuron–glia interaction that depends on Ca^{2+} signaling in astrocytes (Fig. 8). Moreover, Ca^{2+} signals can regulate the level of N-cadherin expression. Further studies are required to uncover the cellular mechanisms of the regulation.

The above results concur with previous reports that suggest the potential significance of astrocytic spontaneous Ca^{2+} oscillations in normal brain development and pathological conditions. Spontaneous Ca^{2+} oscillations in rat ventrobasal thalamus astrocytes are frequently observed in the early postnatal period, when the axons and dendrites actively grow to form the thalamocortical circuits. These Ca^{2+} signals become less frequent with time (78, 79), suggesting the requirement of astrocytic Ca^{2+} signals for the formation of neural circuits. Ca^{2+} signals may also play an important role in the function of radial glia that are astrocyte precursor cells in the embryonic brain (102). Radial glial cells in developing rat neocortical slices show spontaneous intra/intercellular Ca^{2+} waves. These Ca^{2+} signals require the metabotropic ATP receptor, the $P2Y_1$ receptor and IP_3-Ca^{2+} signaling. Notably, disruption of this signaling by the application of an ATP receptor antagonist reduced the proliferation of neurons, suggesting that the radial glial Ca^{2+} signals promote neuronal proliferation during neocortical development (102). This suggests that spontaneous Ca^{2+} signaling may be a common feature of astrocytes and radial glial cells. Although the effectors of the Ca^{2+} signaling in radial glial cells are unknown, it is an interesting possibility that a similar cellular mechanism is involved in the enhancement of neuronal growth induced by both radial glia and astrocytes.

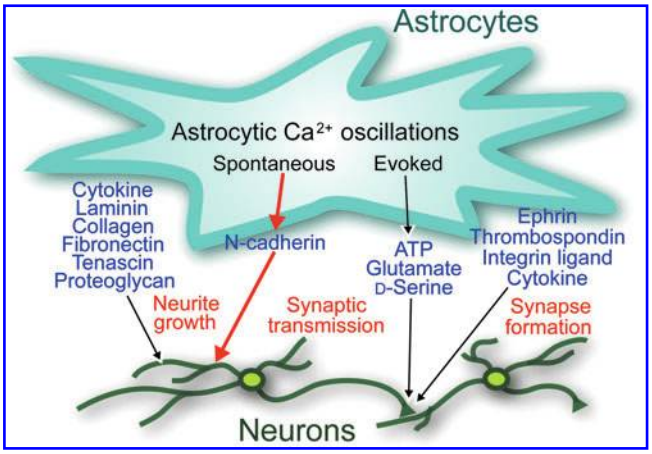

FIG. 8. Schematic diagram of neuron–glia interaction mediated by astrocytes. Astrocytes produce various molecules to modulate neuronal functions such as neurite growth, synaptic transmission, and synapse formation. Whereas ATP, glutamate, and D-serine are released from astrocytes by 'neuron-evoked' astrocytic Ca^{2+} signals to modulate synaptic transmission, 'spontaneous' astrocytic Ca^{2+} oscillations regulate neurite growth by maintaining the N-cadherin expression level on astrocytes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Astrocytic Ca^{2+} signals are also involved in pathological conditions. Astrocytes close to damaged regions of the brain become reactive astrocytes. This differentiation is accompanied by a broad spectrum of changes in the gene expression profile, and the astrocytes become nonpermissive for regenerating growth cones by expressing various growth-inhibitory proteins (81, 83). It was shown that reactive astrocytes do not generate spontaneous Ca^{2+} oscillations in acute brain slice preparations, which were prepared ~2 days after the application of a brain lesion (1). However, using *in vivo* Ca^{2+} imaging, controversial results were recently reported. Damaged mouse neocortical astrocytes showed Ca^{2+} oscillations with large amplitudes and high frequencies 30–150 min after experimentally-induced ischemia (17). Similarly, en-

hanced Ca^{2+} signals in astrocytes have been observed in animal models of epilepsy. Epileptic stimulation to the mouse cortex enhanced astrocytic Ca^{2+} signaling immediately (98) or after a few days (16). Although further observations are required to conclude how astrocytic Ca^{2+} signals are affected by pathological conditions, it is interesting to examine whether the change in astrocytic Ca^{2+} signals are involved in the property of reactive astrocytes, such as proliferation, hypertrophy, and inhibition of axonal regeneration. The 5ppase method applied to astrocytes *in vivo* may provide further insights into glial function.

Perspectives

New indicators for IP_3 and NO have revealed novel important functions for these signaling molecules and will continue to be useful in the investigation of brain function. Recently, new indicators for glutamate, the major excitatory neurotransmitter in the mammalian brain, have been developed (31, 69, 76). Glutamate imaging in intact brain tissues should advance our understanding of this neurotransmitter. In addition, the importance of Ca^{2+} signaling is becoming evident. The results discussed here have revealed new roles for Ca^{2+} signaling in brain neurons and astrocytes and have shed new light on the mechanisms of cellular regulation in the brain.

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Abbreviations Used

AMPAR = α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
BAPTA = *O,O′*-Bis(2-aminophenyl)ethyleneglycol-*N,N,N′,N′*-tetraacetic acid
BDNF = brain-derived neurotrophic factor
BS = burst stimulation
CF = climbing fiber
EPSC = excitatory postsynaptic current
ER = endoplasmic reticulum
GFP = green fluorescent protein
GFP-PHD = GFP-tagged pleckstrin homology domain of PLCδ1
HBR = heme-binding region
HBR-GFP = fusion protein between HBR and GFP
IP_3 = inositol 1,4,5-trisphosphate
IP_3R1 = type 1 IP_3 receptor
LTD = long-term depression
LTP = long-term potentiation
mGluR1 = type 1 metabotropic glutamate receptor
nNOS = neuronal NOS
NO = nitric oxide
NOS = NO synthase
PC = Purkinje cell
PF = parallel fiber
PIP_2 = phosphatidylinositol 4,5-bisphosphate
PLCβ = β isozyme of phospholipase C
PPR = paired-pulse ratio
R343A = arginine residue at 343 replaced with alanine
R343A/R350A = arginine residues at 343 and 350 replaced with alanine
sGC = soluble guanylyl cyclase

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