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Review Article (Invited)

LOV2-based photoactivatable CaMKII and its application to single synapses: Local Optogenetics

Yutaro Nagasawa[1,2], Hiromi H. Ueda[1,2], Haruka Kawabata[1,2], Hideji Murakoshi[1,2]

[1] *Supportive Center for Brain Research, National Institute for Physiological Sciences, Okazaki, Aichi 444-8585, Japan*
[2] *Department of Physiological Sciences, SOKENDAI (The Graduate University for Advanced Studies), Okazaki, Aichi 444-8585, Japan*

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Optogenetic techniques offer a high spatiotemporal resolution to manipulate cellular activity. For instance, Channelrhodopsin-2 with global light illumination is the most widely used to control neuronal activity at the cellular level. However, the cellular scale is much larger than the diffraction limit of light (<1 µm) and does not fully exploit the features of the "high spatial resolution" of optogenetics. For instance, until recently, there were no optogenetic methods to induce synaptic plasticity at the level of single synapses. To address this, we developed an optogenetic tool named photoactivatable CaMKII (paCaMKII) by fusing a light-sensitive domain (LOV2) to CaMKIIα, which is a protein abundantly expressed in neurons of the cerebrum and hippocampus and essential for synaptic plasticity. Combining photoactivatable CaMKII with two-photon excitation, we successfully activated it in single spines, inducing synaptic plasticity (long-term potentiation) in hippocampal neurons. We refer to this method as "Local Optogenetics", which involves the local activation of molecules and measurement of cellular responses. In this review, we will discuss the characteristics of LOV2, the recent development of its derivatives, and the development and application of paCaMKII.

Key words: LOV2, photoactivatable CaMKII (paCaMKII), 2-photon excitation, dendritic spine, excitatory neuron

◀ ***Significance*** ▶

Optogenetic techniques are valuable for the precise manipulation of cells in both space and time. Nevertheless, the absence of adequate optogenetic molecular tools makes it difficult to manipulate intracellular molecules in restricted regions like synapses. The best way for local manipulation is to combine 2-photon excitation and LOV2 since it is efficiently activated by 2-photon excitation. This review presents the optogenetic tools based on the LOV domain developed so far, which we hope will facilitate their comprehension and further advancement. Moreover, we will provide an example of "Local Optogenetics" involving paCaMKII and two-photon excitation.

Introduction

Optogenetic techniques have been extensively utilized in recent decades to manipulate cellular activity [1,2]. In optogenetics, light is combined with genetically-encoded photoactivatable proteins to control and manipulate the activity of specific cells in living organisms. Compared to conventional cell manipulation techniques such as drug treatment and gene down-regulation, optogenetics offers several advantages, including high spatiotemporal resolution, cell-type specificity, and reversibility. Opsins, including Channelrhodopsin-2 (ChR2), in combination with global light

Corresponding author: Hideji Murakoshi, National Institute for Physiological Sciences, 38 Nishigonaka, Myodaiji, Okazaki, Aichi 444-8585, Japan. ORCID iD: https://orcid.org/0000-0002-8938-368X, e-mail: murakosh@nips.ac.jp

illumination, are the most widely used to control neuronal activity at the cellular level (Fig. 1A) [1,3]. However, the application of optogenetics to the subcellular level has been limited despite its importance. For instance, since individual synapses operate independently on a neuron, being able to manipulate them at the single-synapse level optically would be an extremely valuable technique in studying neuronal functions and memory (Fig. 1B). However, while current optogenetic tools primarily enable manipulation at the neuronal level (a scale of several tens of μm), manipulating small areas such as synapses (less than 1 μm) has not progressed due to the lack of appropriate optogenetic tools. For instance, although ChR2 can be locally activated by 2-photon excitation, the induction of synaptic plasticity at the level of single synapses has not been achieved [4-6].

The Light-oxygen-voltage (LOV) domain 2 (LOV2) of Phototropin 1, a plant photoreceptor protein, is a soluble protein in the cytosol (Fig. 2A). It has a higher two-photon absorption efficiency than other photoresponsive molecules such as Cryptochrome 2 (Cry2), making it ideal for two-photon excitation [7-9]. The creation of LOV2 mutants with various properties has been widely explored, and the crystal structures of LOV2 are available, providing an opportunity for developing novel optogenetic tools. Thus far, a wide range of optogenetic tools based on LOV2 have been created, enabling the induction of various biological events such as transcription and cell shape changes through light (Table 1). Recently, we engineered a photoactivatable CaMKII by fusing LOV2 with Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [10]. This new tool can be activated by blue light (400–500 nm) or near-infrared (700–980 nm) two-photon excitation. By activating paCaMKII in excitatory neurons by two-photon excitation, synaptic plasticity can be induced at the single synapse level, allowing to study the functions of CaMKII and synaptic plasticity [10,11]. This review will discuss the features of LOV2 domains, recent advances in their mutants, and the development and applications of paCaMKII as an extended version of our Japanese article [12].

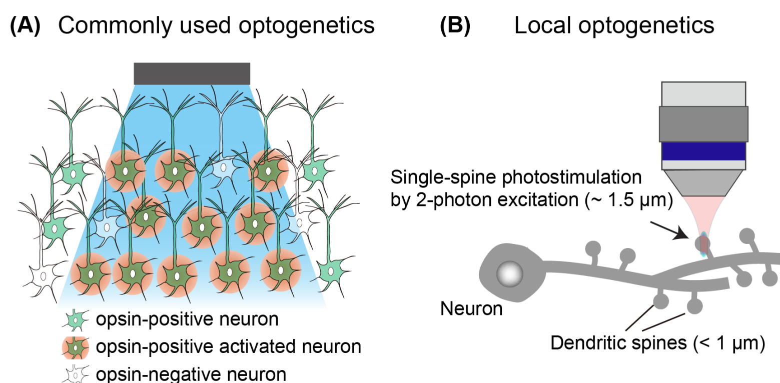

Figure 1 (A) Schematic of multi-neuronal photostimulation. Opsins such as ChR2 are expressed in neurons of a mouse brain and activated by global blue light illumination. (B) Schematic of single-spine photostimulation. Since the diameter of spines are smaller than 1 μm, 2-photon excitation with the size of 1.5 μm can be used to photostimulate single spines.

Characteristics of the LOV2 Domain and its Variants

The LOV domain is a photosensory domain that absorbs blue light [13], and it has been identified in bacterial, algal, fungal, and plant species. The LOV2 domain, which is derived from *Avena sativa* Phototropin 1 (AsLOV2), is a representative LOV domain [14]. It consists of 143 amino acids (404–546 a.a. in Phototropin 1), and its C-terminal Jα helix tightly binds to the Period-ARNT-Singleminded (PAS) core of LOV2 in the dark state (Fig. 2A). When LOV2 is exposed to blue light, flavin mononucleotide (FMN) embedded in LOV2 is excited and forms a covalent bond with Cys450 (Fig. 2A). This covalent bond induces a conformational change in LOV2, causing the release of the Jα helix from the PAS core of LOV2 in a reversible manner, with a deactivation half-time of approximately 40 seconds in the dark state. Since the FMN is also excited by the broad wavelength range of 2-photon excitation, it should induce the structural change of LOV2 (Fig. 2B). The deactivation half-time can be adjusted to range from seconds to hours by introducing mutations in LOV2 [13,15,16]. Typically, when designing optogenetic tools based on LOV2, an active protein such as small GTPase Rac1 is C-terminally fused to Jα helix of LOV2, with linker optimization to inhibit or inactivate the active protein [17]. Upon blue light illumination, the Jα helix is released from LOV2, allowing the sterically hindered active protein to gain its native function. Recently, circularly permuted LOV2 (cpLOV2) has been developed. It allows the fusion of a protein to the N-terminal of LOV2 [18,19]. cpLOV2 is created by fusing the Jα helix (517–546 a.a. of LOV2) to the N-terminal of LOV2 (404–516 a.a.) with a flexible linker.

A variety of optogenetics tools based on LOV2 have been developed to manipulate GTPase signaling [17,20], ion channel [21,22], nuclear transfer signaling, nuclear export signaling [23-25], and many other uses (Table 1). Furthermore, LOV2-based translocation, oligomerization, and dissociation tools have been developed, including tunable light-inducible dimerization tags (TULIPs) [26], improved light-induced dimer (iLID) [27], and LOV2 trap and release of protein

(LOVTRAP) [28]. TULIPs are composed of a modified PDZ domain (ePDZ) and LOVpep. LOVpep is a fusion of LOV2 and an ePDZ-binding peptide (pep). In the dark state, the pep fused to LOVpep cannot bind to ePDZ due to steric hindrance by the LOV2-Jα interaction. Upon blue light illumination, the Jα helix and pep dissociate, and the pep becomes exposed, allowing it to bind to ePDZ. iLID consists of LOV2-Jα-SsrA and SspB. Upon exposure to blue light, they form heterodimers similar to TULIPs. In contrast, LOVTRAP consists of LOV2 and its synthetic binding partner Zdark (Zdk), which recognizes the dark state of LOV2 and forms a heterodimer. Upon blue light, the structural change of LOV2 occurs, leading to the dissociation of Zdk from LOV2. These LOV2-based dimerization and dissociation systems have been widely employed in developing various optogenetic tools (Table 1).

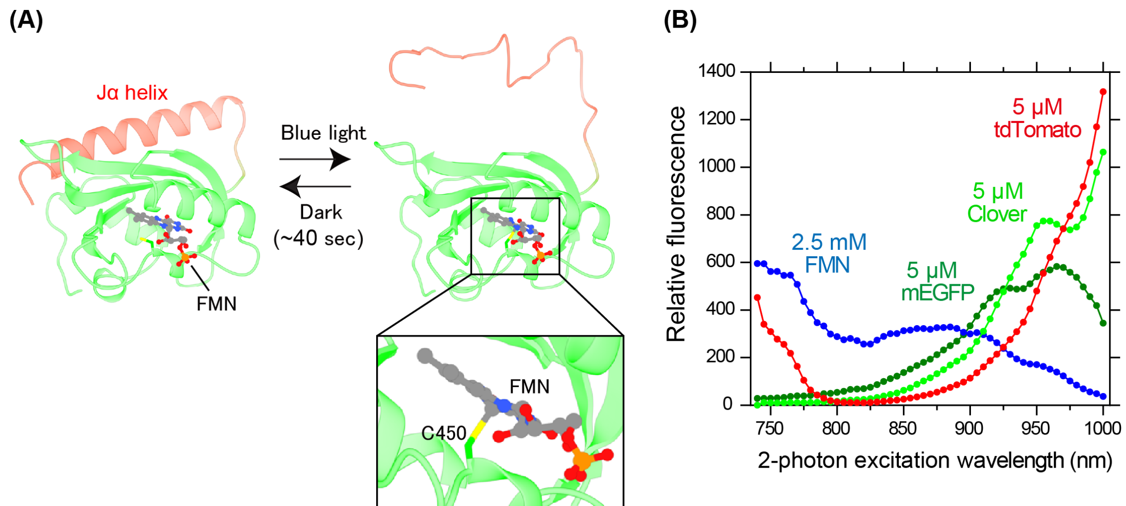

Figure 2 (A) Light-induced conformational change of LOV2 and Jα helix. Blue light induces a temporary covalent bond between the Cys450 LOV2 and the FMN, resulting in a conformational change of LOV2 and the displacement of the Jα helix from LOV2. The structure of the LOV2 in its dark state is modified from PDB ID: 2V0U, and in its light state from PDB ID: 2V0W by using UCSF ChimeraX. Jα helix of LOV2 in a light state is a putative structure. (B) Two-photon excitation spectra of purified proteins (5 μM mEGFP, 5 μM Clover, 5 μM tdTomato, 2.5 mM FMN). FMN absorption spectrum should almost correspond to the absorption spectrum of LOV2 activation. mEGFP, Clover, and tdTomato are examples of fluorescent proteins that can be used with LOV2. These fluorescent proteins can be excited without exciting LOV2 by using excitation light above 1000 nm. A Maitai HP laser (Spectra-Physics) with the power of 3.4–4.5 mW at the respective wavelength under the objective lens was used to excite the purified fluorescent proteins as described elsewhere [29]. Raw fluorescence intensity values were corrected by dividing them by the squared laser power used for each wavelength.

Moreover, *Arabidopsis thaliana*-derived LOV2 (AtLOV2) shares a similar structure and amino acid sequence with LOV2, exhibiting 80% homology with a major difference in the linker region between LOV2 and Jα helix [30]. Using AtLOV2, a light-dependent degradation system was developed by fusing a proteasome recognition sequence, cODC1, with AtLOV2 [31].

The LOV domain derived from *Botrytis cinerea* (BcLOV4) can bind anionic phospholipids. Upon blue light illumination, the membrane-binding region in the LOV domain becomes exposed and subsequently binds to the cell membrane [32,33]. By fusing BcLOV4 with the small G protein RhoA and expressing it in HEK293T cells, local light stimulation successfully induces cell contraction by recruiting RhoA to the cell membrane [34]. One notable advantage of BcLOV4 is that it is possible to construct a light-dependent membrane translocation system using a single component.

The EL222 transcription factor, derived from *Erythrobacter litoralis*, comprises LOV and DNA-binding helix-turn-helix (HTH) domains. In its dark state, it remains monomeric; however, upon exposure to blue light, it forms a homodimer and binds to DNA [35]. The EL222 has been fused with the V16 transcriptional activation domain and co-expressed with the target gene possessing five consecutive EL222 binding sites (C120) upstream of the TATA box. Upon blue light illumination, EL222 binds to C120, activating the target gene's transcription [35].

The LOV domain-containing flavin-binding Kelch repeat F-box1 (FKF1) forms heterodimers with the GIGANTEA protein (GI) upon blue light illumination [36]. One notable characteristic of FKF1 is its irreversibility. By fusing FKF1 with Rac1 and adding a CAAX motif to GI to localize it to the membrane, lamellipodia formation can be induced by recruiting Rac1 to the membrane upon blue light illumination [36].

The LOV domain of the transcription factor Aureochrome 1 derived from *Vaucheria frigida* (VfAU1) exists in a monomeric form in the dark and forms a homodimer upon blue light illumination [37]. One application of VfAU1 is the activation of receptor tyrosine kinase (RTK) signaling. By fusing VfAU1 to the C-terminal of the intracellular domain of a receptor tyrosine kinase FGFR1, which can be activated in a light-dependent manner [37].

The LOV domain of *Rhodobacter sphaeroides* (RsLOV) consists of an N-terminal Aα helix and C-terminal Jα and Kα helices. It forms a homodimer in the dark state and dissociates into monomers upon blue light illumination [38]. A tool that utilizes RsLOV to regulate Cas9 has been developed. The fusion of RsLOV and Cas9 forms a dimer in the dark state, inhibiting Cas9 activity. Upon blue light illumination, the dimer dissociates, restoring Cas9 activity [39].

YtvA, which contains a LOV domain derived from *Bacillus subtilis*, forms a homodimer in the dark state. Upon blue light illumination, each unit of the homodimer undergoes a 5-degree rotation, leading to a structural change [40]. One application of YtvA is for the control of histidine kinases. By replacing the N-terminal PAS domain of the histidine kinase FixL with YtvA, the kinase is activated in the dark state and inactivated upon blue light illumination [41].

The LOV domain of the Vivid protein (VVD) from *Neurospora crassa* forms a stable homodimer upon blue light illumination with a slow dissociation rate of several hours [42,43]. Furthermore, a modified variant called Magnets, which forms heterodimers between pMag (positive Magnet, I52R/M55R mutant) and nMag (negative Magnet, I52D/M55G mutant), has been developed [43]. An application of VVD is the regulation of light-dependent gene expression by combining it with the GAL4/UAS gene expression system [42]. By using Magnets, PI3 kinase can be recruited to the cell membrane to alter membrane morphology [43]. Similarly, the LOV domain of White Collar-1 from *Neurospora crassa* (NcWC1) forms a heterodimer with VVD upon blue light illumination [44] and is applied to the gene expression system [44].

Cryptochrome 2 (Cry2) is another type of blue light-responsive protein that differs from LOV2 but is used extensively. The N-terminal photolyase homology region (PHR) of Cry2 contains flavin adenine dinucleotide (FAD), and Cry2 forms heterodimers or homomultimers upon blue light illumination [45,46]. Heterodimer formation occurs between wild-type Cry2 and CIB1 [47,48]. Modification has made dimer formation between the PHR of Cry2 and the truncated C-terminal of CIB1 (CIBN) possible [48-50]. Homomultimer formation of Cry2 has been reported not only for the wild-type but also for improved versions such as Cry2olig and Cry2clust [51-53].

Spine Signaling and Structural Long-Term Potentiation

Long-term potentiation (LTP), a type of synaptic plasticity, refers to a long-lasting strengthening of synaptic transmission. The mechanism of LTP has been extensively studied in the excitatory synapses of the hippocampus [54]. First, presynaptic glutamates bind to N-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDARs) on the surface of the dendritic spine, which is the small protrusion (<1 μm) emanating from the dendrite and contains the postsynapse (Fig. 1B). Second, Ca^{2+} influx is triggered through the glutamate-bound NMDARs [55]. This Ca^{2+} increase leads to the activation of various intracellular signaling molecules, including calmodulin [56,57], which binds to CaMKII [56-59]. As a result of the structural change in CaMKII, its kinase activity increases [60,61], which in turn phosphorylates and recruits signaling molecules [62-64]. These events cause spine enlargement and accumulation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptors (AMPARs) in the postsynaptic density, which is known as LTP [65-67]. It is worth noting that previous studies have shown that the activation of CaMKII alone can induce LTP [10,68-70]. As prior research indicates that persistent spine enlargement corresponds with an increase in AMPA currents (i.e., LTP) [71-73], this spine enlargement is referred to as structural LTP (sLTP).

CaMKII

CaMKII is a crucial signaling protein for inducing LTP [56-59] and is abundant in cortical and hippocampal neurons (1–2% of total protein in neurons). CaMKII is a serine/threonine protein kinase consisting of 12–14 α and β subunits (50 and 60 kDa, respectively) at a 3:1 ratio [74,75]. Upon binding of Ca^{2+}/calmodulin to the regulatory region of CaMKII, a conformational change occurs in CaMKII, leading to its activation [57,76]. This activated kinase autophosphorylates the Thr286 in the regulatory domain of neighboring subunits [57,76]. Furthermore, two-photon excitation fluorescence imaging has demonstrated that glutamate stimulation recruits CaMKII into stimulated spines [77,78], induces the activation of small GTPases and actin polymerization, and eventually leads to sLTP and LTP [60,61,79,80]. The importance of CaMKII function in synaptic plasticity has been extensively studied using loss-of-function assays with inhibitory drugs, peptides, or gene silencing [56-59,81]. However, because of the lack of suitable tools, the direct effect of CaMKII activation in single spines has remained elusive. We, therefore, designed and developed paCaMKII with the idea that light could induce synaptic plasticity (i.e., sLTP) in spines.

Table 1 LOV2-based optogenetic tools

	Mechanisms of action	Example applications
LOV2	Conformational change (undocking of Ja helix from PAS core)	GTPase signaling [17,20], ion channel [21,22], intrabody regulation [82,83], CaMKII regulation [10,84], kinase inhibitor [85-87], protein degradation [88,89], nuclear transfer or export [23-25], peroxisome translocation [90], myosin and kinesin motors [91], condition-dependent transcriptional regulation [92-95], Cre recombinase [96], Cas9 regulation [97,98], Endonucleases [99], RNA polymerase [100], apoptosis [101], membrane binding [102]
TULIP	Heterodimerization between LOVpep and ePDZ	organelle transport [103], GTPase signaling [104,105]
iLID	Heterodimerization between iLID (SsrA) and SspB	GTPase signaling [106-108], microtubule dynamics [109], ion channel [110], inter-organelle contacts [111], phase separation [112,113], mitophagy [114], actomyosin contractility [115], organelle transport [116]
LOVTRAP	Dissociation between LOV2 and Zdk	microtubule dynamics [117], transcriptional regulation [89], GTPase signaling [118,119], steric inhibition of protein active sites [120]
AtLOV2	Conformational change (undocking of Ja helix from PAS core)	protein degradation [31]
BcLOV4	The membrane binding region masked by RGS domain is exposed and binds to the membrane	GTPase signaling [34,121,122]
EL222	Homodimerization of DNA binding domain	transcriptional regulation [35,123]
FKF1	Heterodimerization between FKF1 and GIGANTE	GTPase signaling [36], transcriptional regulation [36,124], ion channel [125]
VfAU1	Homodimerization	RTK signaling [37,126,127], Nodal signaling [128], ion channel [129]
RsLOV	Dissociation	transcriptional regulation [130], Cas9 regulation [39]
YtvA	Conformational change in the homodimer (rotation of each unit)	transcriptional regulation [131-133], Histidine kinases [41], Gac/Rsm signaling [134]
VVD	Homodimerization and Heterodimerization between VVD and NcWC1	transcriptional regulation [42,135], RNA regulation [136], Cre recombinase [137,138], organelle transport [116], Tyrosine kinases [139], Zn^{2+} binding [140]
Magnets	Heterodimerization between positive Magnet and negative Magnet	transcriptional regulation [141], RNA polymerases [142], Cre recombinase [143,144], Cas9 regulation [145,146], inter-organelle contacts [147], cell-cell adhesions [148], intrabody regulation [149]
NcWC1	Heterodimerization between VVD and NcWC1	transcriptional regulation [44]

Development of a Photoactivatable CaMKII

We aimed to develop photoactivatable CaMKII (paCaMKII) as a new optogenetic tool, which can be used to induce LTP/sLTP in a single spine by two-photon excitation. To achieve this, we opted to fuse LOV2-Jα and CaMKII genetically (Fig. 3). The light-absorbing cofactor of LOV2, FMN, has a relatively large 2-photon absorption cross-section (0.5–0.9 GM in 800–900 nm) [8], which is larger than that of other light-absorbing proteins such as Cry2 (0.02–0.04 GM in 800–900 nm, FAD) [7]. Given the availability of crystal structures of human CaMKII (PDB ID: 3SOA) [150] and LOV2-Jα (PDB ID: 2V1B) [151], we utilized them as references for our design. We first determined the insertion site for LOV2 by constructing and testing dozens of DNAs. We found that the CaMKII hinge region (residues 275–278) was suitable (Fig. 3A), i.e., in the dark state, CaMKII activity was inhibited by fusing LOV2 at this position. Next, we introduced various mutations in the regulatory domain, ensuring that the kinase and regulatory domain bind in the dark and release in the light (Figs. 3B,3C). As a result, we successfully developed a paCaMKII. Biochemical assays confirmed that the Thr286 site of paCaMKII is autophosphorylated by light irradiation (an indication of CaMKII activity), and is dephosphorylated within a few minutes upon returning to the dark state. We also confirmed that paCaMKII is incorporated into the endogenous CaMKII dodecamer in neurons (Figs. 3D,3E). This is not surprising since our paCaMKII has an association domain similar to that of endogenous CaMKII.

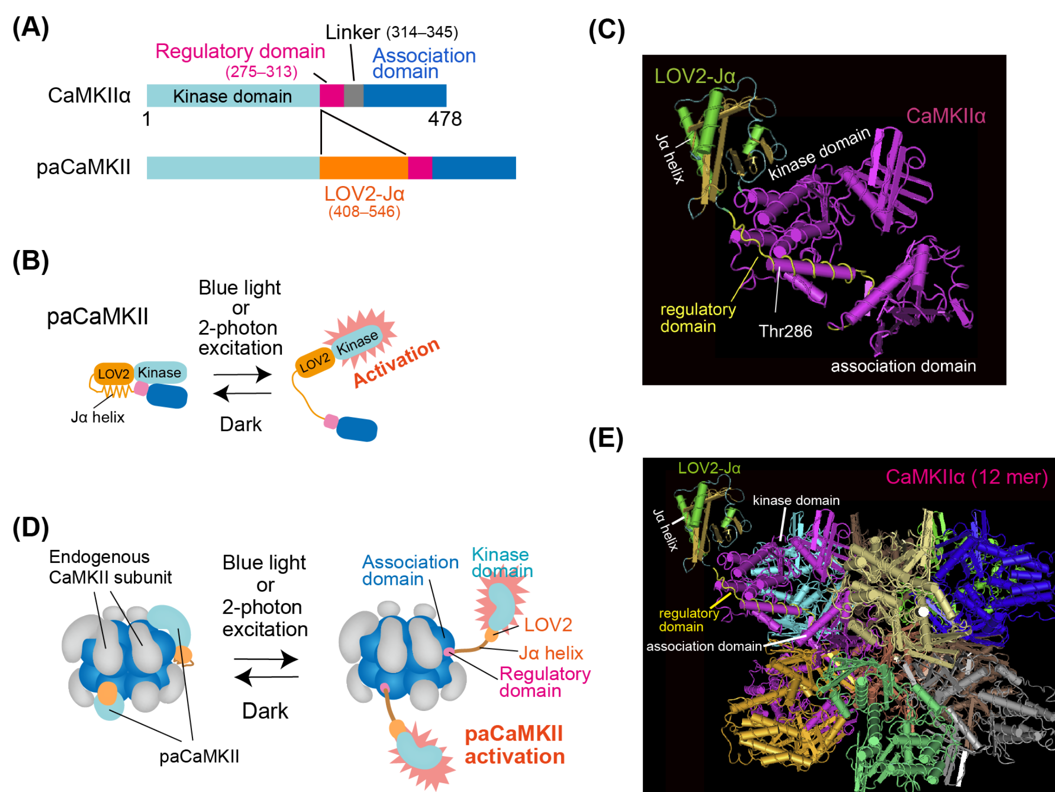

Figure 3 (A) CaMKIIα and paCaMKII domain structures. Figures adapted, with permission from the previous paper [10], also for (B) and (D). (B) Schematic drawing of paCaMKII activation. (C) Putative structure of monomeric paCaMKII created by available structural data (PDB ID: 3SOA and 2V1B) [150,151]. (D) Schematic drawing of paCaMKII activation in the oligomeric state. Note that most CaMKII and paCaMKII likely exist in the oligomeric form in neurons. (E) Putative structure of dodecameric paCaMKII created by available structural data (PDB ID: 3SOA and 2V1B) [150,151]. Monomeric paCaMKII shown in (C) is incorporated in CaMKII dodecamer.

PaCaMKII Activation by Two-photon Excitation Induces sLTP and Functional LTP

To assess whether paCaMKII activation can induce sLTP within individual dendritic spines, we quantified the change in spine volume following 2-photon paCaMKII activation in single spines (Fig. 4). Biolistic gene transfer was employed to introduce a DNA plasmid encoding paCaMKII and the tdTomato (tdTomato-P2A-paCaMKII) to CA1 pyramidal neurons in cultured hippocampal slices. We assessed spine volume change by monitoring tdTomato red fluorescence by

2-photon excitation at 1000 nm. To elicit paCaMKII activation within an individual dendritic spine, we administered a low-frequency train of 2-photon excitation pulses to the spine (900 nm, 30 pulses, 1 Hz, 80 ms duration per pulse, 4 mW). Since the region where two-photon excitation occurs (~1.5 μm) and the size of the spine (< 1 μm) are nearly identical, only paCaMKII in a single spine can be activated.

Activation of paCaMKII led to a rapid increase in spine volume (Figs. 4A,4B), which subsequently relaxed to an elevated level of 150% after 20–30 minutes. In contrast, adjacent spines that were not stimulated by light did not exhibit an increase in volume, indicating that changes in spine volume are specific to a single spine (Fig. 4B). Long-term observation of dendritic spines after paCaMKII activation revealed that paCaMKII-induced sLTP persists for over 4 hrs (Fig. 4C). Activation of the kinase-dead mutant, K42M, did not change spine volume (Fig. 4B). Activation of the T286A autophosphorylation-deficient mutant produced a smaller and transient increase in spine volume compared to paCaMKII activation (Fig. 4B). These findings suggest that the increase in spine volume is attributable to the light-induced rise in kinase activity and autophosphorylation of paCaMKII. In addition, it is confirmed that paCaMKII activation induces spine-specific functional LTP, possibly through the recruitment of AMPAR to the stimulated spines [10].

Given that glutamate-induced sLTP may rely on protein synthesis in certain conditions [73,152], we investigated whether paCaMKII-dependent sLTP also relies on protein synthesis. Our results indicate that two different protein synthesis inhibitors, anisomycin or cycloheximide, reduced the transient volume change and inhibited persistent sLTP (Fig. 4C), suggesting that de novo protein synthesis is required for long-lasting sLTP.

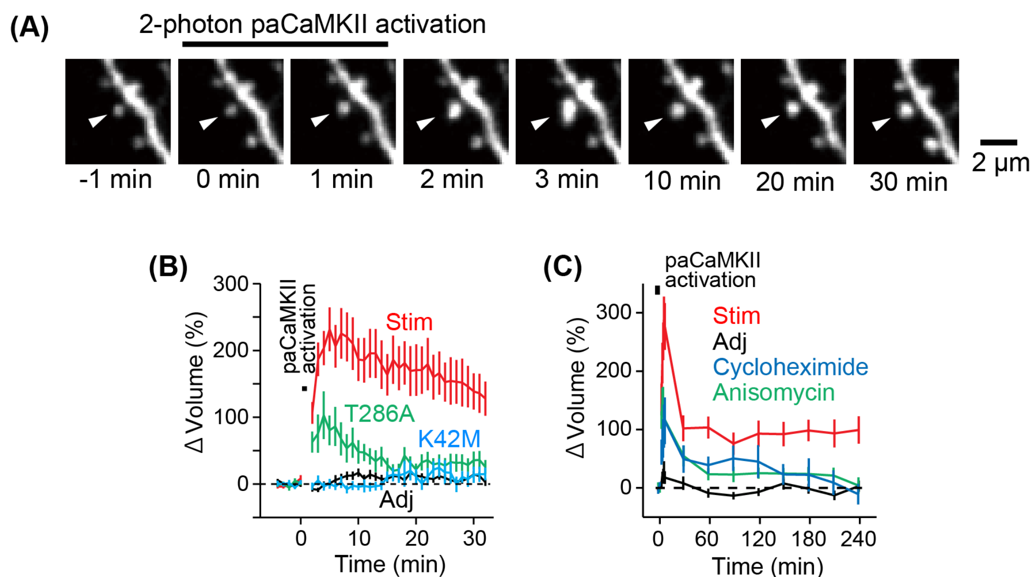

Figure 4 (A) Two-photon fluorescence images of dendritic spines after 2-photon paCaMKII activation. tdTomato-P2A-paCaMKII -expressing neuron was observed by 2-photon excitation at 1000 nm, and paCaMKII was activated at 900 nm in a spine indicated by white arrows. Figures adapted, with permission from the previous paper [10], also for (B) and (C). (B) Averaged time-courses of spine volume change in the stimulated spine (Stim) and adjacent spines (2–10 μm, Adj). Data using paCaMKII mutants (T286A and K42M) are also shown. (C) Averaged time-courses of long-term spine observation of the stimulated spine (Stim) and adjacent spines (Adj). In addition, the groups of neurons treated with protein synthesis inhibitors (50 μM cycloheximide and 50 μM anisomycin) are also shown.

LTP Induction by paCaMKII Activation *in Vivo*

Optogenetic tools offer significant advantages as they can be used on living animals. To verify if paCaMKII activation can trigger sLTP *in vivo*, we injected adeno-associated viral vectors (AAVs) encoding paCaMKII and Clover to express them in layer 2/3 neurons of the mouse cortex (Fig. 5A). For the sparse labeling of Clover, a double-floxed inverted open reading frame (DIO) system combined with low Cre expression system was used. The sparse labeling of the neurons enables us to visualize the individual spines. Using a 2-photon microscope, we imaged the neurons expressing Clover and induced sLTP at multiple spines by raster scanning in a view field with 2-photon excitation. paCaMKII activation resulted in sLTP over 30 min at multiple spines in anesthetized mice (Fig. 5B), demonstrating that paCaMKII can be used to induce sLTP *in vivo*.

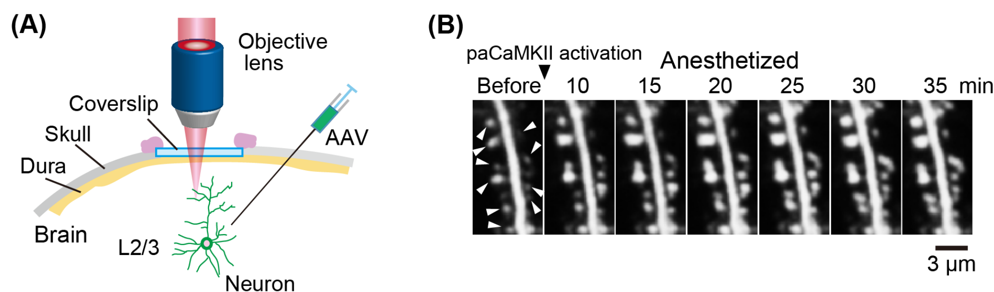

Figure 5 (A) Schematic drawing of *in vivo* imaging. A yellow-green fluorescent protein, Clover, and paCaMKII were expressed in neurons. Figures adapted, with permission from the previous paper [10], also for (B). (B) Two-photon fluorescence images of dendritic spines of layer 2/3 pyramidal neurons of the cortex. The paCaMKII was activated by global scanning with 2-photon excitation at 920 nm. The images were taken in anesthetized states. White arrows indicate the enlarged spines by paCaMKII activation.

PaCaMKII-dependent sLTP in Chronically Excited Neurons

The activity state of a neuron is well known to alter its sensitivity to synaptic inputs. For example, neurons in an aberrant neuronal excitation state increase the threshold for LTP induction [153-155]. However, it is unclear whether sLTP is also depressed in excited neurons, and molecular mechanisms of the regulation of synaptic plasticity are poorly understood.

To test if sLTP occurs in chronically excited neurons, we used two-photon paCaMKII activation [10] in cultured hippocampal slices [11]. We co-infected AAVs encoding tdTomato-P2A-paCaMKII and ESARE-d2Achilles (Fig. 6A). To label the chronically excited neurons, we utilized a synthetic activity-dependent promoter, ESARE [156], in combination with a fast-maturation mutant of yellow fluorescent proteins called Achilles [157] with a destabilization signal [158], which we referred to as d2Achilles. After 5–8 days, we incubated the slices in culture media containing bicuculline (10 µM), a GABAA receptor antagonist, for 24 hrs and successfully observed the fluorescence of d2Achilles, indicating chronically excited neurons (Figs. 6B,6C).

To induce paCaMKII activation in single spines, we applied a low-frequency train of two-photon excitation pulses to single spines (820 nm, 30 pulses, 0.5 Hz, 80 ms duration/pulse, 4 mW). Interestingly, paCaMKII activation in chronically excited neurons failed to induce sLTP (Figs. 6D,6E), whereas a control experiment shows spine enlargement. Since the chronic activation of neurons leads to protein synthesis [159-161], we examined whether the suppression of paCaMKII-induced sLTP requires the newly synthesized proteins during chronic neuronal activation. Notably, the inhibition of sLTP was reversed by the inhibition of protein synthesis (Figs. 6D,6E). These results suggest that sLTP inhibition may be caused by newly synthesized proteins, inhibiting the downstream signaling of CaMKII.

Summary and Future Perspective

In this review, we have introduced recent optogenetic tools based on LOV2. These tools are highly valuable as they allow the direct activation of molecules in a complex molecular network within living cells using light. This enables us to understand their molecular functions. Many researchers have successfully developed various optogenetic tools that enable us to manipulate intracellular signaling molecules. However, most of these tools only change the localization of the active molecule by light, potentially disturbing the intracellular signalings.

Conversely, photo-activatable proteins that change their activity through light are useful since their expression rarely alters the cellular state, but not many have been developed to date. Developing such molecules requires inactivating the active site of the target molecule with LOV2 and then releasing it with light, which is challenging. Although designing molecules by referring to crystal structures is an effective method, it is almost impossible to design a molecule without one. Furthermore, even with a crystal structure, designing a molecule that suppresses molecular activity in the dark is difficult. Thus, future advances in the development of light-manipulated molecules will require breakthroughs. One strategy could be to develop and utilize protein design tools such as AlphaFold2 and other computational methods.

The most common way to photo-stimulate optogenetic tools has been through widefield illumination in combination with channelrhodopsin. However, the complexity of brain architecture has made it necessary to switch to two-photon excitation, which provides better spatial specificity and deeper penetration in scattering tissue [4,162]. For instance, single-cell resolution optogenetics based on 2-photon holographic light-targeting approaches allows the manipulation of individual cells selected by users and the generation of precise spatiotemporal neuronal activity patterns. Nevertheless, optogenetic manipulation in smaller compartments such as synapses, referred to as "Local Optogenetics", remains challenging.

Recently, several genetically-encoded photoactivatable signaling proteins have been developed and used to study the functions of synapses [10,50,84,87,163-167]. These optogenetic tools are revolutionizing neuroscience and, more broadly, molecular cell signaling studies. In particular, optical induction of shrinkage of subsets of activated spines *in vivo* has been achieved recently [163,167]. Here, we introduced a photoactivatable CaMKII, which can be used to induce sLTP/LTP. As paCaMKII allows LTP induction at the level of single synapses, it should be possible to combine paCaMKII with activated-synapse tagging technology to boost the activity of specific subsets of spines *in vivo*. Such technology will allow the identification of the direct link between synaptic plasticity and animal behavior.

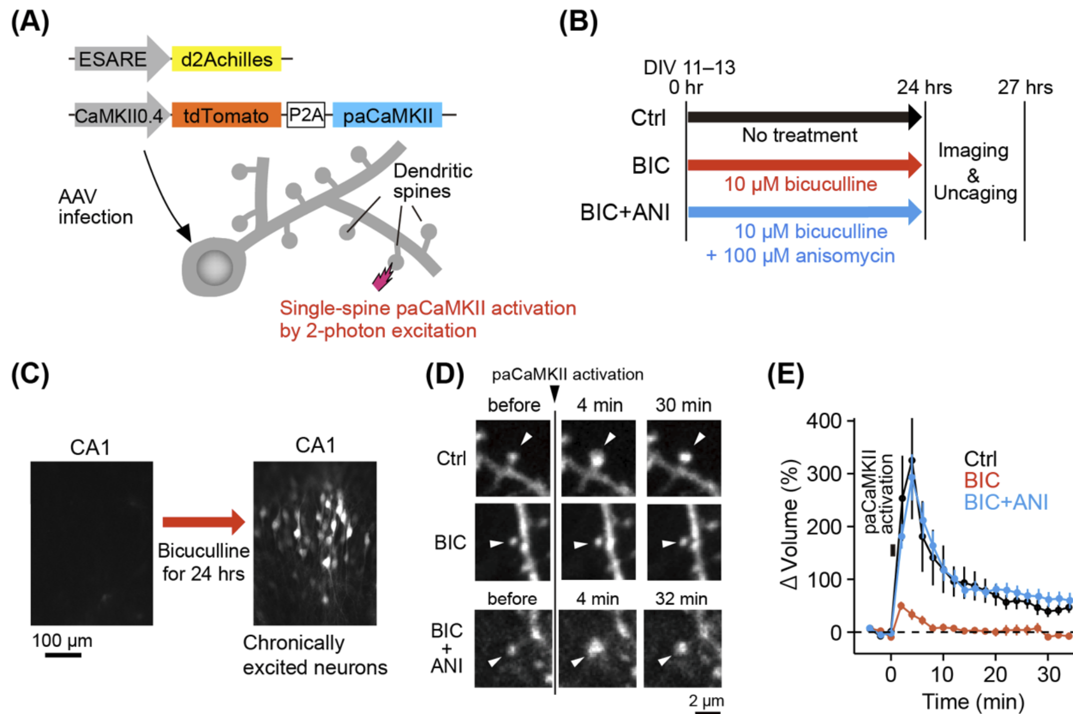

Figure 6 (A) Schematic of sLTP induction by paCaMKII activation. AAVs encoding CaMKII0.4-tdTomato-P2A-paCaMKII and ESARE-d2Achilles were co-transfected into the neurons. Figures adapted, with permission from the previous paper [11], also for (B–E). (B) The timelines of control (Ctrl), bicuculline treatment (BIC), and anisomycin with bicuculline (BIC+ANI) treatment. Hippocampal slices were incubated with the drags for 24 hrs to excite the neurons chronically. Subsequently, the slices were imaged for up to 3 hrs. (C) Epifluorescence images of hippocampal slices transfected with ESARE-d2Achilles. After the chronic neuronal excitation, a number of neurons expressed d2Achilles. (D) Two-photon fluorescence images of dendritic spines during the induction of sLTP by two-photon paCaMKII activation. Hippocampal CA1 neurons expressing Achilles or d2Achilles and tdTomato-P2A-paCaMKII were observed by two-photon excitation at 1010 nm, and paCaMKII was activated at 820 nm (30 trains, 0.5 Hz, 80 ms duration/pulse, 4 mW) in the spine indicated by white arrowheads. (E) Averaged time course of the change in spine volume upon paCaMKII activation after the bicuculline (BIC) or anisomycin with bicuculline (BIC+ANI) treatment.

Conflict of Interest

The author declares no conflict of interest.

Author Contributions

Y.N., H.U., H.K., and H.M. wrote the manuscript.

Data Availability

The data shown in the manuscript are available from the corresponding author on reasonable request.

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