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·Review·

Chromophore-assisted laser inactivation in neural development

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Abstract: Chromophore-assisted laser inactivation (CALI) is a technique that uses photochemically-generated reactive oxygen species to acutely inactivate target proteins in living cells. Neural development includes highly dynamic cellular processes such as asymmetric cell division, migration, axon and dendrite outgrowth and synaptogenesis. Although many key molecules of neural development have been identified since the past decades, their spatiotemporal contributions to these cellular events are not well understood. CALI provides an appealing tool for elucidating the precise functions of these molecules during neural development. In this review, we summarize the principles of CALI, a recent microscopic setup to perform CALI experiments, and the application of CALI to the study of growth-cone motility and neuroblast asymmetric division.

Keywords: chromophore-assisted laser inactivation; growth cone; neuroblast; asymmetric cell division

1 Introduction

Loss-of-function analysis of target proteins provides important insights for understanding neural development. Gene knockout and RNA interference have been intensively used to identify molecules essential for the generation of the nervous system. Antibodies and pharmacological chemicals have also been applied to experimental systems where conventional genetic approaches are not available. However, the cellular events underlying neural development are highly dynamic; many key molecules only function in a given subcellular compartment during a certain developmental stage (e.g. growth-cone motility and neuroblast asymmetric division). To understand the molecular and cellular basis of neural development, it is necessary to inactivate the protein of interest in living neurons, non-invasively and with high spatial and temporal precision, which genetic or chemical manipulation by itself cannot accomplish. Chromophore-assisted light inactivation (CALI) is a light-mediated technique that provides an alternative to the above inactivation strategies with the special advantage of high spatiotemporal resolution[1-4].

2 Principles of CALI

CALI experiments consist of two essential steps[1-4]; a chromophore or photo-sensitizer is first tagged to the target protein in the cell, and then laser irradiation of the chromophore is used to generate highly reactive free radicals such as reactive oxygen species (ROS) that inactivate proximate proteins (Fig. 1). The short life of free radicals determines the specificity of CALI because these radicals produce damage within a radius of 1.5–6 nm (Table 1) and

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only destroy proteins immediately adjacent to the chromophore[1-4]. The time between free radical generation and protein inactivation is less than one second, so the real-time manipulation of protein activity can be achieved. Moreover, laser irradiation can be confined within a subcellular area at micrometer accuracy, which offers high spatial resolution of the regulation of protein activity.

Different types of chromophores have been used to generate ROS for CALI (Table 1)[1-4]. The original CALI experiments used the dye malachite green (MG) as the photosensitizer[5,6]. MG-conjugated non-function-blocking antibodies were delivered to cytosolic targets by microinjection or to cell-surface targets by incubation. The action radius of MG is within 1–3 nm (Table 1)[5,6]. The excitation laser wavelength of MG is 620 nm, which does not cause non-specific light damage. Fluorescein-labeled antibodies were later used since fluorescein is a better generator of ROS than MG and its action radius is 3–4 nm. The major limitations of MG and fluorescein in CALI are the generation of antibodies and microinjection (Table 1).

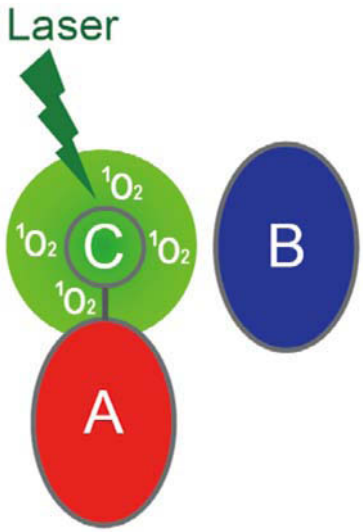

Fig. 1. Principles of CALI. Laser irradiation of a chromophore (C) generates $^1O_2$ that inactivates an adjacent protein (A) without affecting other proteins (B).

Table 1. Photo-sensitizers for chromophore-assisted laser inactivation (CALI)

Chromophore	Tagging / Delivery	Action radius	Limitation	References
Malachite green	Antibody-conjugation; microinjection/incubation	1–3 nm	Specific antibodies and microinjection are required.	[16]
Fluorescein isothiocyanate	Antibody-conjugation; microinjection/incubation	3–4 nm	Specific antibodies and microinjection are required.	[3]
Eosin	Incubation & HaloTag fusion	N.D.	Dyes may not easily enter live animals.	[37]
ReAsH/FlAsH	Incubation & genetic modification	N.D.	Dyes may not easily enter live animals.	[7]
eGFP	Genetic modification	6 nm	CALI efficiency of GFP is not the highest.	[8, 11]
KillerRed	Genetic modification	N.D.	KillerRed forms dimers.	[9, 10]

GFP, green fluorescent protein.

More advanced versions of CALI use genetically-tagged photo-sensitizers (Table 1)[1,2]. Tsien and colleagues fused calcium channels with one or two small tetracysteine (TC) motifs, which specifically bind to the membrane-permeant biarsenical dyes ReAsH (resorufin-based arsenical hairpin binder) and FlAsH (fluorescein-based arsenical hairpin binder). The TC-labeled calcium channels lost 90% of their activity after <30 s of wide-field illumination[7]. Using green fluorescent protein (GFP)[8] or the photo-toxic red fluorescent protein KillerRed[9,10] in CALI does not require the introduction of fluorescent dyes, which is particularly useful for CALI in live animals (Table 1). GFP is an effective CALI chromophore with an irradiation radius of ~6 nm (Table 1). GFP-tagged cytoskeleton proteins such as α-actin[8] and cap protein[11], or the gap junction protein connexin 43[12] or Aurora B kinase[12] dur-

ing mitosis were inactivated by CALI (reviewed by[2]). The photo-toxicity of KillerRed was estimated to be at least 1 000-fold higher than GFP[9,10]. Recent crystallographic evidence showed that KillerRed has a long water-filled channel reaching the chromophore area from the end cap of the β-barrel, and this unique structure facilitates the transit of ROS and might be responsible for the high photo-toxicity[13,14]. When using KillerRed, one has to be aware of the tendency of this protein to dimerize, which may alter the activity of the target protein (Table 1).

CALI has been shown to inactivate a wide array of proteins which localize to the plasma membrane, cytosol or nucleus (reviewed by[1,3,15]). The target proteins tested so far include membrane receptors, phosphatases and kinases in signaling pathways, the actin cytoskeleton and microtubules as well as their associated proteins, molecular motors, transcription factors, and metabolic enzymes. In principle, any protein can be inactivated by CALI as long as a non-function-blocking antibody or GFP/KillerRed tagging is available; however, some proteins, such as hexokinase, were not inactivated by CALI[16].

CALI has been used to study neural development in cultured neurons or neural progenitor cells in live animals (reviewed by[1,3,15]). CALI was first used to study the axon adhesion and outgrowth of grasshopper pioneer neurons, and has been extensively used to study the function of membrane proteins, the cytoskeleton, and motor proteins in growth-cone motility in chicken dorsal root ganglia neurons. In model organisms, GFP or KillerRed can be used as the photo-sensitizer by fusion to the target protein and expression in particular neurons using tissue-specific promoters. In *C. elegans*, CALI was successfully used to study myosin polarization during Q neuroblast asymmetric cell division[17]. In *Drosophila* embryos, CALI of the segment-polarity gene, patched, changed the number of neurons in the Bolwig organ[18], and CALI of the myosin light chain inhibited cell mixing at compartmental boundaries and cytokinesis[19].

3 Microscopy apparatus for CALI

The experimental setup for CALI experiments is similar to that for photo-bleaching and photo-activation, i.e., the equipment has facilities to deliver high-intensity light to a region of interest in the sample. In principle, it is possible to perform CALI experiments with a standard epifluorescence microscope. By closing down the field diaphragm in the epi-illumination pathway one can restrict illumination to a small area where chromophore-assisted inactivation will take place. In a more efficient incarnation, light from a laser is focused on the object plane. This results in very high power densities and fast and efficient bleaching and inactivation of the chromophore. Ideally, the experimenter can manipulate the location and size of the illuminating spot or even pre-program spatial illumination patterns. Such control is achieved by steering the beam with galvanometrically-controlled mirrors.

Confocal scanning laser microscopes containing galvo mirrors are often the most accessible equipment for CALI experiments, although such systems are not necessarily optimal for live samples. It is also possible to project patterns of light onto the sample using digital mirror devices or spatial light modulators. However, in most cases this results in the energy of the light source being distributed evenly over the object (the devices block light from reaching certain areas of the sample) and the resulting low power densities may necessitate very long illumination times to achieve full inactivation. Using spatial light modulators that manipulate both phase and amplitude of the illuminating light makes it possible to concentrate the available energy on freely definable areas in the sample plane, and it appears likely that this will eventually be the method of choice for CALI experiments.

Often, it is possible to convert a through-the-objective type of total internal reflection microscope (TIRFM) into a CALI microscope[17]. In the TIRFM, laser light is focused on the back-focal plane of the objective. By placing an appropriate lens in the light path, light can be focused on the sample plane instead. A convenient place to mount this lens is the dichroic filter cube holder. Some microscope companies (e.g. Nikon) have such a lens as a catalog item, whereas for other microscopes the lens can be procured from third parties. We converted a Zeiss Axiovert 200M microscope with a TIRF illuminator into a system capable

of photo-bleaching and CALI by inserting a biconcave lens (Optosigma 017-0275, D = 25.40, focal length 89.7 mm) into the reflector cube at the position of the emission filter along with either a 488-nm reflecting long-pass mirror or a 100% reflecting mirror in the cube. Two milliwatts of laser power and a 1-μm spot size resulted in a power density of 250 kW/cm^2. It is important to know in advance where the bleaching spot is located so that the area of interest can be moved to the bleaching position. To determine the location of the bleaching spot we use coverslips coated with the lipophilic carbocyanine dye DiI as well as coverslips coated with a fluorescent marker[17]. Such samples can also be used to focus the laser beam (focusing gives coarse control of the size of the illumination spot).

4 CALI in neuritogenesis

CALI has mostly been used to study neuritogenesis, a process driven by the motility of the neuronal growth-cone. The growth-cone is a highly dynamic structure and can be divided into three continuous regions: the peripheral (P) domain contains actin cytoskeleton-based lamellipodia and filopodia, the central (C) domain between the P domain and the axon shaft is composed of microtubule cytoskeleton and many organelles and vesicles, and the transition domain between the P and C domains contains actomyosin contractile structures and regulates actin and microtubules in the growth-cone. To accomplish directed motility, the signaling molecules on the growth-cone membrane detect environmental cues and transduce them to cytoskeleton-associated proteins, which regulate cytoskeletal dynamics and molecular motors to generate force for motility[20]. A number of proteins have been identified in the growth-cone; however, the direct dissection of their activities in motility requires a technique that allows the inhibition of their function specifically in the growth-cone during its migration. Such a spatiotemporal perturbation cannot be achieved by traditional loss-of-function assays such as genetic deletion, RNA interference, antibody microinjection or pharmacological treatment.

CALI was shown to be an efficient strategy to inhibit the function of growth-cone proteins during motility (Table 2). The pioneering CALI experiments in neuritogenesis were performed by Jay *et al*. By using MG-labeled antibodies against fasciclin family proteins and microinjection, CALI of fasciclin I specifically disrupted axon adhesion while CALI of fasciclin II inhibited axon outgrowth in grasshopper pioneer neurons[5,6]. More recently, Jay and others have used CALI to systematically dissect the functions of cytoskeletal proteins and signaling molecules in growth-cone motility and guidance[4]. Most experiments were carried out using fluorescent dyes as the photosensitizers and dorsal root ganglia neurons as the model system[4].

CALI has been used to study the function of membrane receptors and signal transduction molecules in the growth-cone. The type 1 inositol 1,4,5-trisphosphate receptor (IP3R1) localizes to the growth-cone and regulates calcium levels, and local inactivation of IP3R1 by CALI resulted in growth arrest and neurite retraction[21]. CALI of L1 and NCAM-180, two adhesion molecules, on the membrane of the growth-cone generated a slow neurite retraction and filopodial/lamellipodial retraction respectively, suggesting that L1 may be required for neurite extension and NCAM-180 may be involved in the protrusion of the growth-cone[22].

CALI of calcineurin, a calcium/calmodulin-dependent serine threonine phosphatase, caused the retraction of filopodia and lamellipodia, suggesting that it regulates growth-cone outgrowth[23]. CALI of pp60-c-src, a protein tyrosine kinase, reduced protein tyrosine phosphorylation and resulted in accelerated neurite extension, indicating that pp60-c-src negatively controls growth-cone outgrowth[24]. Radixin is an ERM (ezrin-radixin-moesin) protein that acts as a linker between plasma membrane and actin cytoskeleton on the growth-cone, and CALI of radixin reduced lamellipodial area[25].

The spatiotemporal inactivation of actin cytoskeleton-associated proteins by CALI uncovered the *in situ* function of these molecules during growth-cone motility. CALI of vinculin increased the frequency of filopodial bending and buckling, suggesting that it is involved in filopodial integrity[26]. CALI of talin in the growth-cone showed that it may func-

Table 2. Examples of chromophore-assisted laser inactivation in neural development

Topic	Neuron	Target Protein	Observation	References
Neuronal growth-cone	Chick DRG	IP3R	Inositol 1,4,5-trisphosphate receptor regulates neurite growth.	[21]
	neurons	L1 and NCAM-180	L1 regulates neurite extension. NCAM-180 regulates growth-cone protrusion.	[22]
		Calcineurin	Calcineurin regulates motility and outgrowth of the growth-cone.	[23]
		pp60(c-src)	pp60(c-src) negatively regulates laminin-1-mediated neurite outgrowth.	[24]
		Radixin	Radixin controls lamellipodial stability in the growth-cone.	[25]
		Talin and vinculin	Talin couples growth-cone extension and retraction to actin dynamics. Vinculin regulates the structural integrity of filopodia.	[26]
		Myosin 1c and IIB	Myosin 1c and IIB regulate lamellipodial dynamics.	[27]
		Myosin-V	Myosin-V-mediated vesicle transport regulates filopodial extension.	[28]
		Tau	Tau is required for neurite outgrowth.	[29]
		MAP1B	MAP1B regulates growth-cone stabilization and steering.	[30]
		CRMP1 and CRMP2	CRMP1 and CRMP2 function distinctly in neurite outgrowth.	[31]
	Mouse DRG	Dynactin	Dynactin controls growth-cone advance.	[33]
	Rat sympathetic neurons	Kinesin-5	Kinesin-5 regulates microtubule redistribution in the growth-cone.	[32]
Axon adhesion	Grasshopper pioneer neurons	Fasciclin I	Fasciclin I is required for axon fasciculation.	[6]
Axon outgrowth	Grasshopper pioneer neurons	Fasciclin II	Fasciclin II is required for axon outgrowth.	[5]
Axon guidance	Retinal ganglion cells	Repulsive guidance molecule	Repulsive guidance molecule is involved in the guidance of retinal axons.	[38]
Axon arborization	Chick retinotectal projection	Ephrin-A5	Ephrin-A5 restricts arborization.	[39]
Nerve regeneration	Chick retina-optic nerve explants	MAG	MAG inhibits nerve regeneration.	[40]
Neuronal fate	*Drosophila* visual system	Patched	Patched regulates the number of neurons in the Bolwig organ.	[18]
Asymmetric division	*C. elegans* Q neuroblast	Myosin II	Polarized myosin II regulates daughter cell size and fate.	[17]

DRG, dorsal root ganglion; MAG, myelin-associated glycoprotein.

tion in the actin clutch module[26]. CALI also uncovered the function of the myosin family of motor proteins in the growth-cone: myosin 1c is required for retrograde actin flow and regulates lamellipodial retention; myosin II acts in lamellipodial expansion; and myosin V delivers membrane or other cargos to extending filopodia[27,28]. Actin cytoskeleton-associated proteins and myosin motors are present in other parts of the cell and CALI provides a unique tool to dissect their functions in the growth-cone.

The focal and acute inactivation of microtubule associated proteins (MAPs) and kinesin and dynein motors by CALI defined the functions of these proteins in the growth-

cone. Tau and MAP1B are MAPs that bundle and stabilize microtubules. CALI of Tau and MAP1B reduced the size of lamellipodia and loss of function of Tau also slowed down neurite extension, suggesting that both are required for growth-cone motility[29,30]. Collapsin response mediator protein 1 (CRMP1) and CRMP2 are novel MAPs involved in the semaphorin 3A signaling pathway. Although focal inactivation of both CRMP1 and CRMP2 arrested neurite outgrowth, CALI of CRMP1 and CRMP2 separately generated opposite phenotypes; CALI of CRMP1 caused the growth-cone to turn away from the irradiated area, but CALI of CRMP2 attracted the growth cone to migrate into the irradiated region[31].

Kinesin-5 was previously shown to regulate microtubule invasion of the peripheral domain of the growth-cone, which is critical for turning. However, it was unclear where kinesin-5 functions in the growth-cone. Using CALI, Nadar and colleagues focally inactivated GFP-tagged kinesin-5 in different areas of the growth-cone and found that it suppresses microtubule invasion from the transition zone[32]. The motility of cytoplasmic dynein is regulated by a dynactin protein complex, and dynamitin interacts with p150 Glued in this complex. CALI of dynamitin disrupted this interaction and decreased the rate of growth-cone extension, suggesting that this component in the dynein complex regulates growth-cone motility[33].

5 CALI in neuroblast asymmetric division

CALI has recently been applied to the study of asymmetric division in *C. elegans* Q neuroblasts[17]. The Q neuroblast undergoes three rounds of asymmetric division to generate a ciliated sensory neuron, a mechanosensory neuron and an interneuron (Fig. 2A). The second round is of particular interest because it produces one small apoptotic cell and one large cell that differentiates into one neuron or divides and differentiates into multiple neurons. Understanding of the cellular mechanisms by which two differently-sized daughters arise from an asymmetric cell division has mostly come from the study of early *C. elegans* embryos. In this system, the mitotic spindle is pulled towards one side of the cell by a dynein-dependent force in the posterior cortex. Once the cleavage furrow bisects the middle of the off-center anaphase spindle, the cell is divided into two unequally-sized daughters. The posterior Q daughter cell (Q.p) uses this mechanism. However, the anterior Q daughter cell (Q.a) division starts with a centrally-localized spindle and it also creates two daughter cells of distinct sizes[17]. In the Q.a division, we found that myosin II accumulates at higher levels on the side that will become the smaller daughter cell. However, myosin II is an essential gene, and the uniform knockdown of myosin function by mutation, RNAi or a drug does not provide information

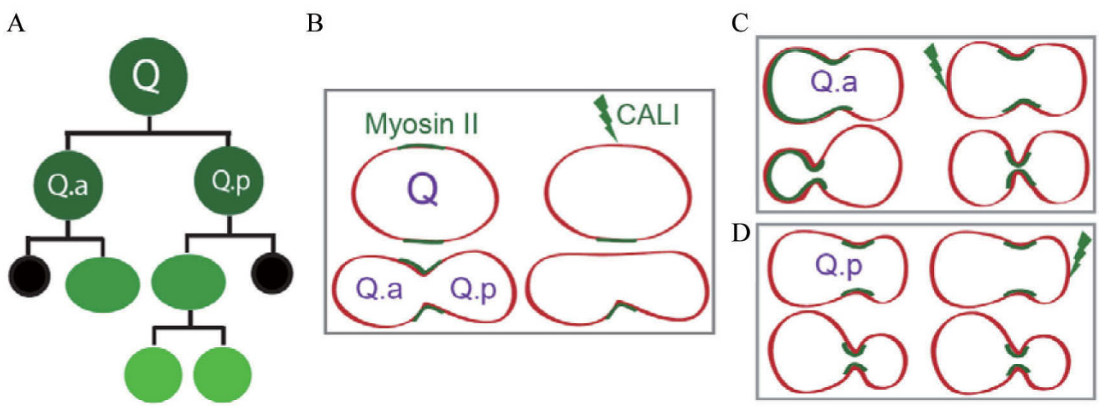

Fig. 2. Application of CALI to asymmetric division in *C. elegans* Q neuroblasts. A: Q neuroblast lineage; the Q neuroblast undergoes three rounds of asymmetric division and generates three different neurons (green) and two apoptotic cells (black)[17]. B: CALI of GFP-tagged myosin on one side of the Q neuroblast during cytokinesis inhibits furrow ingression on this side. C: CALI of GFP-tagged myosin on the anterior of Q.a changes asymmetric to symmetric division. D: As a negative control for (C), laser irradiation of the posterior of Q.p does not change the asymmetric division.

on issues related to myosin II asymmetry. Thus, it was hard to obtain direct evidence for a role of myosin II polarization in daughter cell-size asymmetry by traditional loss-of-function approaches[17].

Using CALI, we inactivated myosin function only in the anterior of the Q.a cell at the onset of cytokinesis inside developing *C. elegans* L1 larvae[17]. Using GFP as a photosensitizer, CALI inhibits GFP-tagged myosin function; CALI of GFP-myosin II applied to one side of an ingressing furrow of a Q neuroblast pauses furrow ingression, while the non-illuminated side continues to ingress (Fig. 2B).

During normal Q.a division, the side of the cell containing more myosin becomes the smaller Q.aa cell and ultimately dies by apoptosis[17]. After CALI of GFP-myosin in the anterior of the dividing Q.a cell, the daughter Q.aa cell becomes larger and in many cases exceeds the size of the Q.ap cell (Fig. 2C). Controls with another GFP-tagged protein did not produce this effect. CALI of the posterior Q.p cell (which does not have myosin asymmetry) did not alter Q.p cell division either (Fig. 2D)[17].

CALI was thought to be generally toxic and could conceivably produce these effects through cell damage. However, the Q cell CALI experiments suggest that quite the opposite is true[17]. Instead of dying, as the Q.aa always does during normal development, the Q.aa cell subjected to CALI often escapes cell death and differentiates into a neuron-like cell that extends a process. This result suggests a link between cell size and apoptosis during *C. elegans* development and shows that manipulating cell size results in a change in cell fate[17]. Such a connection could not have been made without the direct and non-genetic inactivation of protein function offered by CALI.

6 Future perspectives

CALI is a unique approach that can assign protein function to specific cellular areas at particular stages of neural development. The selection of proper photo-sensitizer is one of the keys for success with CALI. Molecular and cellular mechanisms of neural development have been extensively studied in cultured systems, where photo-sensitizers for CALI can be easily introduced by incubation or microinjection. However, the study of neural development in live animals is more demanding, and the improvement of genetically encoded photo-sensitizers for CALI is an important future direction. The efficiency of GFP in CALI is lower than KillerRed, but the dimerization of KillerRed prevents its application to many biological processes in which the protein monomer is the major active form[9,10]. Thus, it is necessary to monomerize KillerRed without affecting its photo-toxicity in the near future. miniSOG (mini singlet oxygen generator), a fluorescent protein of 106 amino-acids, has recently been shown to generate singlet oxygen and ablate *C. elegans* neurons upon blue-light illumination, making it another good photosensitizer to inactivate protein function by CALI in living organisms[34].

CALI provides a loss-of-function analysis for proteins in neural development at precise spatial and temporal resolution. Several approaches offering the spatiotemporal activation of target proteins by illumination have recently emerged[35,36]. The combination of both loss- and gain-of-function assays will advance our understanding of neural development.

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