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Role of CaMKII in Controlling the Morphology and Motility of Neurite Growth Cone in *Lymnaea stagnalis* Neurons

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ABSTRACT

Calcium ion (Ca^{2+}) and Calmodulin (CaM) are important signaling molecules that have been shown to play a significant role in a wide variety of neuronal functions, including neurite outgrowth of axon and dendrites. The growth cone is located on the tip of the growing neurite. The morphology and motility of the growth cone depend on Ca^{2+} and the stimulation of Ca^{2+} dependent protein kinases (CaMK) in developing neurons. CaM-kinase II (CaMKII) is a member of the CaMK family, and it is highly expressed in the cytosol of developing neurons, including the growth cone. We hypothesized that CaMKII activity could be necessary for growth cones morphology and motility. To test this possibility, the identified Pedal A (PeA) neurons from the central ring ganglia of the freshwater pond snail Lymnaea stagnalis were cultured for 24 hrs -48 hrs in a 2 ml brain conditioned medium. Following neurite outgrowth, we have examined the effect of CaMKII inhibitor on growth cone structure and motility rate by using a CaMKII specific inhibitor KN-93 and its inactive analog KN-92; both drugs were pressure applied directly onto individual growth cones. Here we demonstrate that inhibition of CaMKII dramatically changes the morphology and motility of growth cone in cultured neurons. Growth cones of neurons treated with KN-93 exhibit collapsed morphology. In contrast, growth cone exposure to KN-92 did not affect growth cone morphology and motility rate. Taken together, the data presented here provide the first direct evidence for the involvement of CaMKII activity to maintain growth cone morphology and motility in the freshwater pond snail Lymnaea stagnalis.

Keywords: Calcium, Cytoskeleton, Kinase, Lymnaea stagnalis, Neurons, Neurite growth cone

INTRODUCTION

Axonal degeneration is commonly observed following nervous system injury and other neurodegenerative diseases like Alzheimer's Disease (AD) brain and spinal cord injury. Traumatic nervous system injuries initiate several cellular and molecular changes in and around the injury site. These cellular and molecular changes contribute to axonal damage and neuronal and non-neuronal cell death, leading to functional deficits [1,2]. Degeneration of axons occurs both below and above the level of injury [1]. Many therapies and strategies have been used to enhance and promote axonal regeneration. Regenerative therapies focus on promoting axonal regrowth and neuronal replacement through the application of growth and neurotrophic factors, applying chemicals and neutralizing the inhibitory molecules to

make growth permissive environment, cell transplantation, and application of various paradigms of electrical stimulation [3-12].

Electrical Stimulation (ES) plays a vital role in a variety of common and severe health problems [4]. ES has become one of the most promising therapies for various neurological disorders [3,13,14]. ES such as epidural electrical stimulation, transcranial electrical stimulation, and direct current stimulation has successfully demonstrated the neuronal axon's elongation and regeneration [3,15-18]. ES also induces axonal outgrowth toward the cathode, with axons aligning with the direction of current flow. ES can alter the neuronal cell membrane potential and causes the depolarization of the cell membrane which ultimately assembles the actin filaments in the growth cones cytoskeleton to promote neurite growth [19,20]. In addition, ES changes the electric fields of extracellular matrix proteins which eventually promote elongation of neurite and growth cones [19,21].

Despite the significant progress in neuroscience and regenerative medicine, however, effective nerve repair and regeneration of axons to restore normal function following nerve injury remain a challenge to the research scientist. In the last few decades, tissue engineering introduced the use of natural and synthetic biomaterials or nanomaterials to create a favorable microenvironment to promote the regeneration of nerve and axon [6,22]. Among these biomaterials, conductive materials have been used to mediate the stimulation of electrical currents to regulate cell and tissue growth [20,23]. A variety of conductive materials, including Graphene Oxide (GO), alone or in combinations have been extensively used in nerve regeneration to promote axon and neurite growth [20,23,24].

Currently, promising bio-applications of graphene-based biomaterial in neural network regeneration and nervous system repairing have drawn much attention because the unique electrical properties of graphene can effectively contribute to the electrical stimulations of neurons and the regeneration of nervous systems [20,25]. The previous study has shown that graphene and graphene oxide can alter neuronal cell behaviors, including attachment of growth cone, growth, proliferation, and differentiation of the neuronal cell [25,26]. Graphene can increase the local concentration of extracellular matrices such as collagen, laminin, and fibronectin. Recently, nanocarbon materials such as graphene and carbon nanotubes have been considered new effective electrode materials with high conductivity [13,20]. A recent study has shown that graphene oxide conductive materials combined with electrical stimulation enhance axonal extension and growth [20,25,27].

Numerous studies have demonstrated that graphene-based nano-materials play a significant role in promoting neuronal axon regeneration, neurite outgrowth, and differentiation of various cells, including neuronal stem cells into neuronal cells [20,24,28,29]. Another research has shown that graphene-based nano-materials efficiently enhanced the expression of Growth-Associate Protein-43 (GAP-43), promoting neurite sprouting and outgrowth of the growth cone to the maximal extent in growing hippocampal cultures without compromising the morphology and viability of cells [22,28].

Axonal regeneration is the primary failure to regain function following axonal injury and neurodegenerative diseases; therefore, it is critically needed to understand the cellular mechanism of neurite outgrowth and the morphology and motility of the growth cone. The previous study has shown that calcium influx occurred following axonal injury and this influx of extracellular calcium plays a vital role in the growth of axons [30]. The Calcium ions (Ca^{2+}) play a significant role in signal transduction pathways that control various neuronal functions such as neurite outgrowth of the axon, dendrites, and synaptic plasticity [31]. Ca^{2+} signaling is mediated through several Ca^{2+}-binding proteins, including Calmodulin (CaM). CaM is a regulatory protein that modulates the activity of several signaling molecules such as Protein Kinase C (PKC), phosphorylase kinase, myosin light chain kinase, calcineurin, and family of Ca^{2+}/calmodulin-dependent protein kinase (CaMK) [32]. The previous study has demonstrated that overexpression of CaMKII promotes neurite outgrowth of neuroblastoma cells [33].

Multifunctional, Ca^{2+}/calmodulin-dependent protein kinase or (CaM kinase II) is one of the calmodulin-regulated enzymes that mediates its effect through calcium signaling in neurons [34-36]. CaMKII is one of the most highly expressed proteins in neurons, comprising 1%-2% of the total protein concentration [37]. The unique properties of CaMKII, including its abundance, multifunctional nature, key location, and sophisticated regulation, may allow the enzyme to take part in important synaptic functions, including neurotransmitter synthesis and release, modulation of ion channels activity, synaptic plasticity, and gene expression [38-40].

Growth cones are the motile, well-organized dynamic structure located on the motile tips of growing axons and neu-

rites. Growth cones play a critical role in axonal pathfinding to navigates and directs axonal elongation to find out their appropriate synaptic connections during neural development [41,42]. The growth cone is a conical-shaped structure comprising finger-like projection called filopodia containing actin protein and a membranous structure known as lamellipodia (Figure 1). Filopodia and lamellipodia, both structures are necessary for the motility and advancement of the growth cone [34,43]. Growth cones establish the direction of axonal extension by detecting and responding to complex guidance cues and signaling molecules such as cell surface and extracellular matrix molecules including Netrins, Slits, Semaphorins, and Ephrins in the nervous system environment [44,45]. Growth cones are involved in neurite or axonal pathway finding in the developing nervous system [46-49]. Research in the past several decades has identified several families of chemical ligands and their corresponding receptors on growth cone and downstream signaling cascades involved in neuronal outgrowth and guidance during neuronal development [48]. These environmental cues can be chemoattractive or chemorepulsive and can steer extending neuronal growth cones at both long and short ranges, during neural development [50]. Previous research has shown that chemical ligands activate the receptors on growth cones that ultimately initiate the intracellular signals cascade to direct cytoskeletal changes [51].

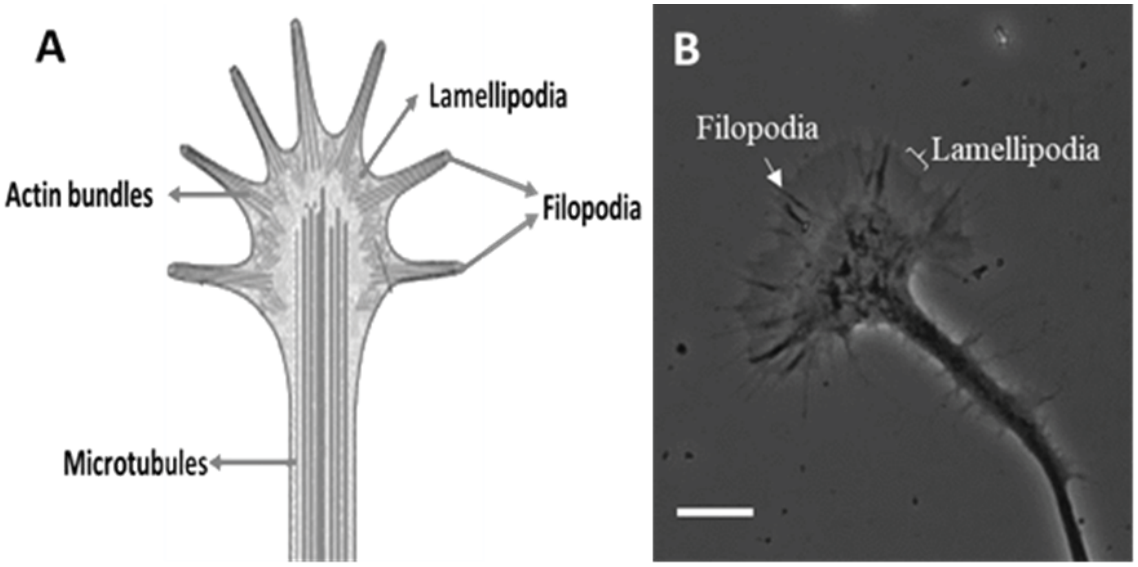

Figure 1 Structure of typical growth cone present at the tip of growing neurite; (A): Illustration of a typical growth cone showing long finger-like cytoplasmic processes filopodia that contain actin protein bundles and flat, sheet-like projections Lamellipodia, Microtubules distributed along the shaft of neurite protrude into the central region of the growth cone; (B): Photomicrograph of growth cone extending from Pedal A, neuron of *Lymnaea stagnalis* in cell culture, filopodia and lamellipodia can be seen, Scale bar 30 µm

The cytoskeleton of the growth cone comprises Actin and tubulin microtubule; this cytoskeleton of the growth cone is highly sensitive to change in the extracellular environment [52]. The changes in the organization of the Actin and tubulin in the cytoskeleton cause the change in morphology and motility of the growth cone [53-55]. The behavior, morphology, and motility of the growth cone are highly sensitive to changes in calcium concentration in the growth cone. The morphology and motility of the growth cone depend on Ca^{2+} and the stimulation of Ca^{2+} dependent protein kinases (CaMK) in developing neurons [34,43,56]. CaMKII is highly expressed in the cytosol of developing neurons, including the growth cone. Therefore, we hypothesize that CaMKII activity could regulate axonal regeneration and maintain the growth cones morphology and motility. In this study, we have investigated the role of multifunctional key synaptic signaling protein CaMKII in maintaining and controlling the morphology and motility of growth cone in Pedal A neurons of freshwater snail *Lymnaea stagnalis*.

Taken together, the present research provided first direct evidence that CaMKII activity is necessary to maintain growth cone morphology and the motility rate of the growth cone of growing neurite of identified neurons of freshwater snail *Lymnaea stagnalis*. Moreover, it enhances our knowledge to understand the mechanisms of neurodegenerative diseases like Alzheimer's Disease (AD) and following axonal injury.

MATERIALS AND METHODS

Animals

The freshwater snail, *Lymnaea stagnalis* were maintained at room temperature in well-aerated, de-chlorinated tap water, and kept at a 12/12 hours light/dark cycle. Animals were fed lettuce and fish food. For cell culture, snails 1 to 2 months old (10 mm-15 mm shell length) were used, whereas the brain Conditioned Medium (CM) was prepared from 2 to 3 months old snails (15 mm-25 mm shell length).

Cell Culture

Animals required for cell culture and CM preparation were deshelled with forceps and anesthetized for 7 minutes-10 minutes in 10% Listerine (21.0% ethanol, 0.042% menthol; Pfizer Canada, Toronto, Ontario, Canada) in normal *Lymnaea* saline (51.3 mM NaCl, 1.7 mM KCl, 4.0 mM $CaCl_2$, 1.5 mM $MgCl_2$), buffered to pH 7.9 with 2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES) [57-59]. All instruments required for dissections were sterilized with 70% ethanol. The animals were pinned down through their body wall to the bottom of the silicone rubber dissection dish (General Electric, RTV 616) containing antibiotic saline. An incision made through the dorsal midline with fine scissors, and the Central Ring Ganglia (CRG) was exposed by pinning down the body wall inside out. The esophagus, which passes through the CRG, was removed using fine scissors.

Extraction of Identified Neurons

Neurons were isolated individually and cultured, as described previously [58,59]. The central ring ganglia were isolated and then sterilized with three, fifteen-minute antibiotic washes (50 µg/ml gentamycin in normal saline). Ganglia were subsequently treated with trypsin (2 mg/ml, Sigma type III; Sigma Chemical Company, St. Louis, MO) for 23 minutes and then soybean trypsin inhibitor (2 mg/ml, Sigma type 1-S; Sigma Chemical Company) for 15 minutes, each dissolved in 50% L-15 (Gibco special order) Defined Medium (DM). DM was prepared with added inorganic salts (similar concentration as saline) and 20 µg/ml gentamycin, with the pH adjusted to 7.9 with 1N NaOH. Following enzymatic treatment, the ganglia were pinned to the bottom of a dissection dish containing high osmolarity DM (DM with added 20 mM D-glucose) and unsheathed with a pair of fine forceps. Suction pressure was applied through fire-polished, Sigmacoated pipettes (50-100 µm tip diameter) to remove desired neurons individually [60]. Isolated neurons were cultured on poly-L-lysine treated dishes either in the presence or absence of CM. The dishes were left undisturbed overnight.

Pressure Injection of Drugs

Chemicals. KN-93 (Calbiochem), KN-92 (Sigma) was first dissolved in dimethylsulfoxide (DMSO; Sigma). The stock solution (2 mM) was subsequently diluted in *Lymnaea* saline to make a final concentration of 100 nM immediately before the application. Final DMSO concentration in the diluted working solution was less than 0.01 % (DMSO<0.01%), and the drugs were pressure ejected (model 5242 (Eppendorf Scientific, Westbury, NY); 10-30-sec pulses, 6-10 psi, pipette tip diameter of 2-5 mm) directly onto the individual growth cones.

Images of growth cones were captured using a Contax (Toronto, Canada) camera mounted on a Zeiss (Oberkochen, Germany) Axiovert 135 inverted microscope. Growth cones were visually scored for a collapsed morphology.

Statistics

Data expressed as Mean ± Standard Errors of the Mean (SEM). Statistical analysis of the data conducted by using SPSS (IBM SPSS statistics 25) and graphs was constructed with Microsoft Office Excel 2019. The significant difference between various groups was determined by a t-test. Statistical significance was assumed if the p-value was less than 0.05 (*$p<0.05$).

RESULT

CaMKII is involved in Maintaining the Morphology of Neurite Growth Cone

To determine whether Ca^{2+}/calmodulin-dependent protein kinase activity is necessary to maintain the structure and function of the neurite growth cone of isolated molluscan neurons. Individually identified Pedal A (PeA) neurons were isolated from the *Lymnaea* CNS and plated on poly-L-lysine coated dishes containing CM and left undisturbed

overnight. Neurite outgrowth was monitored and visualized under the microscope 24 hours-48 hours after initial plating. All neurons following neurite outgrowth have examined the effect of CaMKII inhibitor on growth cone structure and motility by using a CaMKII specific inhibitor KN-93. KN-93 (2 µl; 100 nM) was pressure applied directly onto individual growth cones. Figure 2 shows the structure of neuronal growth cones from intact Pedal A, neurons cultured in 2 ml of CM, before and after the application of CaMKII inhibitor KN-93. Within a few minutes of KN-93 application, the growth cone not only halts the motility but also exhibited collapse morphology and growth cone retraction in cultured neurons.

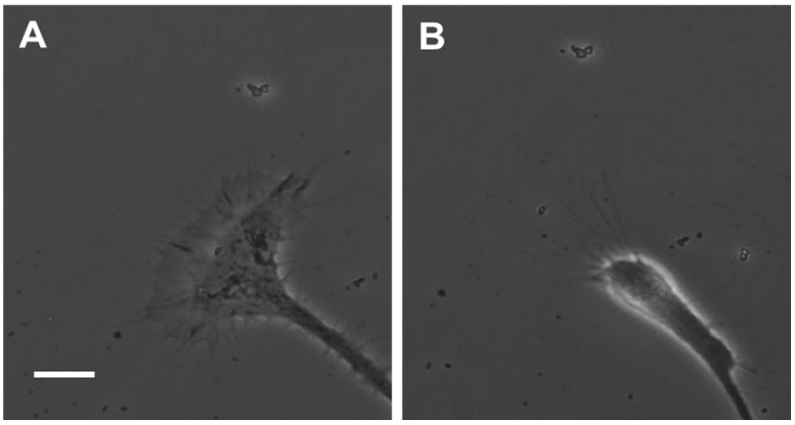

Figure 2 Effect of CaMKII inhibitor KN 93, on growth cone; (A): Photomicrograph showing growth cone of growing neurite of *Lymnaea stagnalis* before adding CaMKII specific inhibitor KN-93; CaMKII inhibitor KN-93, when added to the growth cone of growing neurites, induces a dramatic response with the collapse of the growth cone and retraction of the neurite; (B): Scale bar 30 µm

To test for the specificity of CaMKII inhibitor KN-93 effects on growth cone morphology, we used the inactive analog KN-92 (2 µl; 100nM) to see its effects on growth cone morphology. Despite multiple applications of KN-92 on the growth cone, the growth cone morphology and motility remain unchanged, suggesting that the KN-92 did not affect the structure of the cytoskeleton in the growth cone (Figure 3). These results indicate that: a) CaMKII specific inhibitor KN-93 indeed perturb the morphology of the growth cone in the *Lymnaea* model, and b) that CaMKII is necessary to maintain the structure of the cytoskeleton of the growth cone.

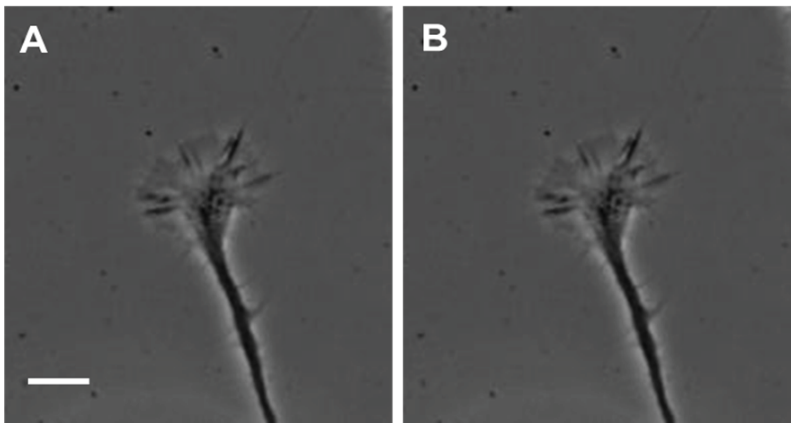

Figure 3 The inactive analog of CaMKII inhibitor KN-92 did not affect the neurite growth cone; (A): Photomicrograph of the growth cone of *Lymnaea stagnalis* before adding the inactive analog of CaMKII inhibitor KN-92 and (B): growth cone after adding the inactive analog of CaMKII inhibitor KN-92, Scale bar 30 µm

Effect of CaMKII Inhibitor on Neurite Growth Cone Motility Rate

To determine the effect of CaMKII on the growth cone motility rate of the neurite growth cone, the identified Pedal A (PeA) neurons from the central ring ganglia of the freshwater pond snail *Lymnaea stagnalis* were cultured for

24 hrs-48 hrs in a 2 ml brain conditioned medium. To investigate the involvement of CaMKII in motility rate and growth cone advancement, we pressure applied CaMKII inhibitor (final concentration of DMSO<0.01%), KN-93 (2 µl; 100 nM in DMSO) directly onto individual growth cones. The growth cone motility rate of the growth cone was monitored before and after the applications of drugs. Within 20 minutes of its application, the growth cone motility rate was significantly reduced ($p<0.05$) (Figure 4). Specifically, the control growth cone motility rate was 27 ± 0.57 µm/h (n=10), which was reduced significantly in the presence of pressure applied KN-93 to -91 ± 0.93 µm/h (n=10; Figure 4). To test the specificity of KN-93 effects and control for the carrier solution (DMSO), we used the inactive analog KN-92 (also 100 nM). KN-92 or DMSO alone did not affect the growth cone motility rate, (control motility rate 27 ± 0.57 nm/h (n=10); KN-92 26± 0.61 nm/h (n=10; Figure 4)). These results indicate that CaMKII specific drugs indeed perturb its function in the *Lymnaea* model and that CaMKII is involved in controlling the growth cone motility rate.

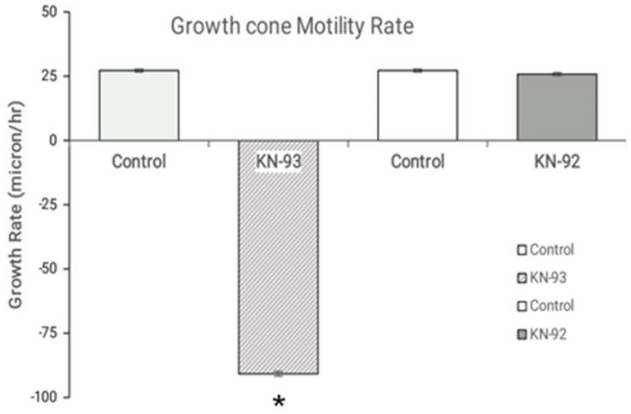

Figure 4 The growth cone motility rate significantly decreases when CaMKII activity is blocked with KN-93; Bar graph showing that the growth cone motility rate significantly reduced (*$p<0.05$) when CaMKII activity was blocked with KN-93 compared with control; there was no significant difference ($p>0.05$) in the growth cone motility rate treated with inactive analog KN-92 and control

DISCUSSION

In the present study, we have demonstrated that inhibition of CaMKII activity in the growth cone, with its specific inhibitor KN-93, dramatically changes the morphology and motility of the growth cone in identified cultured neurons. In addition, growth cones of neurons treated with KN-93 exhibit collapsed morphology. Blocking of CaMKII activity alters the growth cone morphology through the changes in cytoskeleton protein. Moreover, the assembly of cytoskeleton elements is responsible for the advancement and elongation of the neurite growth cone. This finding indicates that CaMKII serves as a pivotal molecule to maintain the structure and function of the neurite growth cone present at the tip of the growing neurite.

Calcium ion (Ca^{2+}) and calmodulin (CaM) are important signaling molecules that have been shown to play a significant role in a wide variety of neuronal functions, including synaptic plasticity, neurite outgrowth of axon and dendrites [35,36]. Ca^{2+} signaling is mediated through several Ca^{2+}-binding proteins, including CaM [35,61]. CaM is a regulatory protein that modulates the activity of several signaling molecules such as Protein Kinase C (PKC), phosphorylase kinase, myosin light chain kinase, calcineurin, and family of Ca^{2+}/calmodulin-dependent protein kinase (CaMK) [32].

Growth cones present at the tips of growing neurites are responsible for axonal pathfinding, target cell selection, and specific synapse formation [62,63]. The morphology and motility of the growth cone depend on Ca^{2+} and the stimulation of Ca^{2+} dependent protein kinases (CaMK) in developing neurons. Cytosolic free Ca^{2+} and Ca^{2+} influx into axoplasm appear to regulate growth cone behavior by activating different signaling cascades, including cAMP and CaMKII [43,56,64-66]. CaMKII is a member of the CaMK family, and it is highly expressed in the cytosol of developing neurons, including the growth cone. CaMKII is critically involved in synaptic plasticity in the brain. Neuronal activity and the Ca^{2+} influx activate the CaMKII [34,35].

The movement of filopodia-like extensions is a necessary step for neuronal growth cones to find new connection sites that can then advance into nascent synapses and mature into functional synaptic connections [67-70]. Intracellular Ca^{2+} plays an essential role in promoting growth cone [34,71,72]. Ca^{2+} controls cytoskeletal dynamics of the growth cone through the modulation of polymerization and depolymerization of cytoskeletal proteins, including microtubules and F-actin [33,73,74]. Previous studies have shown that motile growth cones have higher free Ca^{2+} levels than growth that stops growing [75-77]. A moderate level of intracellular Ca^{2+} is necessary to neurite outgrowth, and extension of growth cone, very high or very low intracellular Ca^{2+} level impairs the neurite growth and halts the growth cone extension at the tip of the neurite [34,64]. The moderate Ca^{2+} level of the active growth cone of Snail Helisoma is an estimated range of 200 nm-1000 nm, whereas the non-motile growth cone has a 30 nM-70 nM intracellular Ca^{2+} level [34]. Previous studies indicate that the overexpression of CaMKII in neuron culture increases filopodia motility, dendritic arborization, and spine density. CaMKII protein expresses in the neurite growth cone and interacts with F-actin proteins to regulate the stability of cytoskeletal elements in the growth cone [56,78-80].

CONCLUSION

In summary, our findings suggest that CaMKII activity is critically needed to maintain the growth cone morphology and for the advancement and elongation of the growth cone to regulate the motility rate. Together with previous studies, our finding underscores the importance of CaMKII activity to maintain the structure and motility of growth cone in neuronal cultured.

DECLARATIONS

Conflicts of Interest

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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