Published in final edited form as:

Neuroscience. 2011 March 31; 178: 1–12. doi:10.1016/j.neuroscience.2011.01.027.

Stimulation-mediated translocation of calmodulin and neurogranin from soma to dendrites of mouse hippocampal CA1 pyramidal neurons

Kuo-Ping Huang, Freesia L. Huang, and Pavan K. Shetty¹

Program of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA

Abstract

Calmodulin (CaM) and neurogranin (Ng) are two abundant neuronal proteins in the forebrain whose interactions are implicated in the enhancement of synaptic plasticity. To gain further insight into the actions of these two proteins we investigated whether they co-localize in principle neurons and whether they respond to high frequency stimulation in a coordinated fashion. Immunohistochemical staining of CaM and Ng in mouse hippocampal slices revealed that CaM was highly concentrated in the nucleus of CA1 pyramidal neurons, whereas Ng was more broadly localized throughout the soma and dendrites. The asymmetrical localization of CaM in the nucleus of pyramidal neurons was in sharp contrast to the distribution observed in pyramidal cells of the neighboring subiculum, where CaM was uniformly localized throughout the soma and dendrites. The somatic concentrations of CaM and Ng in CA1 pyramidal neurons were approximately ten- and two-fold greater than observed in the dendrites, respectively. High frequency stimulation (HFS) of hippocampal slices promoted mobilization of CaM and Ng from soma to dendrites. These responses were spatially restricted to the area close to the site of stimulation and were inhibited by the N-methyl-D-aspartate receptor antagonist 2-amino-5-phosphonopentanoic acid. Furthermore, HFS failed to promote translocation of CaM from soma to dendrites of slices from Ng knockout mice, which also exhibited deficits in HFS-induced long-term potentiation. Translocated CaM and Ng exhibited distinct puncta decorating the apical dendrites of pyramidal neurons and appeared to be concentrated in dendritic spines. These findings suggest that mobilization of CaM and Ng to stimulated dendritic spines may enhance synaptic efficacy by increasing and prolonging the Ca²⁺ transients and activation of Ca²⁺/CaM-dependent enzymes.

Keywords

calmodulin; neurogranin; hippocampus; translocation; dendritic spines; LTP

Calmodulin (CaM) is a highly conserved protein known to participate in a broad range of cellular functions. In contrast, neurogranin (Ng) is expressed predominantly in the forebrain of vertebrates and is believed to be involved primarily in the regulation of neural function.

Correspondence: Kuo-Ping Huang (huangk@mail.nih.gov) or Freesia L. Huang (fhuang@mail.nih.gov), Building 49, Room 6A36, NIH, Bethesda, MD 20892-4510, Phone: 301-496-7827; Fax: 301-496-7434.

¹Present address: Division of Neurosurgery, Department of Surgery, Duke University Medical Center, Durham, North Carolina, 27710

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

CaM contains four EF-hand Ca^{2+} -binding sites and exhibits several different conformational states depending upon the degree of saturation by Ca^{2+} . Each of these states has a specific set of targets (Chin and Means, 2000). Thus, CaM is endowed with multiple functionalities to activate many enzymes and some of them exert opposite effects, such as kinases and phosphatases. Because CaM can interact with a large number of proteins, this intracellular Ca^{2+} sensor often becomes the limiting factor for various competing reactions across different cellular compartments (Xia and Storm, 2005). Thus, a fine-tune mechanism for the spatial and temporal distribution of this protein appears essential for normal cellular function.

In the CNS, Ng is a prominent apo-CaM binding protein and is believed to play a role in adjusting the availability of free CaM and augmenting the Ca^{2+} transients critical for the enhancement of synaptic plasticity and learning and memory (Gerendasy and Sutcliffe, 1997; Pak et al., 2000; Miyakawa et al., 2001; Huang et al., 2004; Zhabotinsky et al., 2006; Kubota et al., 2007; Kubota et al., 2008; Zhong et al., 2009). *In vitro*, the interactions of Ng and CaM are weakened by increasing Ca^{2+} concentration, and by covalent modification of Ng by phosphorylation and oxidation (Sheu et al., 1996; Li et al., 1999; Huang et al., 2000). The phosphorylated form of Ng stimulates G-protein-coupled phosphoinositide second messengers to trigger the mobilization of Ca^{2+} from intracellular stores (Cohen et al., 1993). Furthermore, Ng impedes the direct interaction of Ca^{2+} with CaM and promotes the dissociation of Ca^{2+} from Ca^{2+} /CaM complexes resulting in an enhancement of Ca^{2+} transients (Gaertner et al., 2004; Huang et al., 2004). Deletion of Ng in mice causes severe deficits in learning and memory and high frequency stimulation (HFS)-induced long-term potentiation (LTP) in the hippocampal CA1 region (Pak et al., 2000; Miyakawa et al., 2001; Huang et al., 2004; Huang et al., 2006). Recently, it was reported that deletion of a copy of the Ng gene from human chromosome 11q caused deficits in cognitive function of patients (Coldren et al., 2009). Single nucleotide polymorphism-based genome-wide association studies have also identified variants of Ng gene as conferring a higher risk of schizophrenia (Ruano et al., 2008; Stefansson et al., 2009).

In this study we employed acute hippocampal slices to investigate the molecular dynamics of Ng and CaM following HFS. Our results reveal that Ng and CaM were co-localized in principle neurons in the hippocampus and under basal conditions both CaM and Ng were concentrated primarily in the soma with a much lower abundance observed in the dendrites. HFS promoted the translocation of CaM and Ng to dendrites, where they both appear to concentrate in the dendritic spines.

1. Experimental procedures

1.1 Animals and reagents

All animal procedures were approved by the National Institute of Child Health and Human Development Animal Care and Use Committee. All wild type and Ng knockout (NgKO) mice used were littermates obtained from mating of Ng^{+/-} mice. These mice were the progeny of the original F1 of 129/sv x C57BL/6 mixed background mutant mice (Pak et al., 2000) and have since been back-crossed into C57BL/6 for more than ten generations. All animals were housed in standard cages on a 12-h light/dark cycle and provided normal chow and water *ad libitum*. An antibody against Ng (Ab#2641) (available through Millipore as anti-neurogranin antibody, #07-425) was raised in rabbit against the C-terminal sequence (GARGGAGGGPSGD) of the protein. The antibody recognized a single protein band in mouse whole brain extracts by Western blot and did not stain any cells in fixed brain sections obtained from NgKO mice (Pak et al., 2000). All other reagents were obtained from commercial sources: mouse anti-CaM (#13-6900) and anti- α CaMKII (#13-7300) from Zymed Laboratories; mouse anti-actin (clone JLA 20) (#CP01) from Calbiochem; Bradford

protein assay reagent, horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG from Bio-Rad; BCA protein assay reagent from Pierce; ImmPRESS peroxidase-conjugated anti-mouse and anti-rabbit IgG, horse serum, and Vectashield from Vector Laboratories; CaM from Calbiochem; Alexa Fluor 594 carboxylic acid succinimidyl ester and 5-(and-6)-carboxyfluorescein (FITC) succinimidyl ester from Invitrogen; DL-2 amino-5-phosphonopentanoic acid (AP5) and anisomycin from Tocris; and tyramine hydrochloride, sodium borate, and hydrogen peroxide-urea adduct tablet from Sigma-Aldrich.

1.2. Quantification of mouse hippocampal Ng and CaM

Mouse hippocampi were removed immediately after decapitation and kept frozen at -80°C until processing. Each hippocampus was homogenized by sonication in 0.25 ml of buffer (50 mM Tris-Cl buffer, pH 7.6, 1 mM EDTA, 1 mM EGTA, and 1% SDS), and the homogenate used for both protein determination (BCA protein assay reagent), SDS-PAGE (10–20% gradient gel), and immunoblot analysis. The hippocampal concentrations of Ng and CaM were estimated by quantitative immunoblot analyses using the purified proteins as standards. We also estimated the concentration of α CaMKII in the same hippocampal extracts to serve as a reference as its content has been determined previously (Erondu and Kennedy, 1985). The hippocampal levels of Ng and α CaMKII were determined in the same blot with increasing concentrations of both purified proteins as standards. The blots were incubated with a mixture of three primary antibodies (rabbit anti-Ng, 1/1,000; mouse anti- α CaMKII, 1/1,000; and mouse anti-actin, 1/10,000) followed by incubation with a mixture of horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG. A linear relationship between the concentration of purified Ng or CaMKII and immunoreactivity (IR) revealed by ECL was established before their use for quantification of the unknown samples. Each hippocampal extract from three wild type mice was analyzed at least three times and the intensities of immunoreactive bands were quantified using Kodak Image Station analysis software. Purified mouse forebrain CaMKII consisted of α and β subunits in a 5 to 1 ratio as determined by Coomassie blue staining and the protein concentration of α CaMKII was estimated accordingly. The combination of three primary antibodies provided the detection of α CaMKII, Ng, and actin in one blot and the IR of actin served as an internal control for protein loading of different samples. CaM, which migrated close to Ng in the SDS-gel, was quantified in a separate blot using mouse anti-CaM antibody (Zymed, 1/200) as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG as secondary antibody. This antibody detected a major protein band (~ 17 kDa), corresponding to the purified CaM, in the hippocampal extracts by Western blot.

1.2. Treatment of hippocampal slices

Following preparation, adult mice (mixed gender, 3–6 months old) hippocampal slices (400 μm) were kept for ~ 2 h in oxygenated (95% O_2 /5% CO_2) artificial cerebrospinal fluid (ACSF) comprised of the following (in mM): 124 NaCl, 4.9 KCl, 1.3 MgSO_4 , 2.5 CaCl_2 , 1.2 KH_2PO_4 , 25.6 NaHCO_3 , 10 D- glucose, pH 7.4. Slices were then submerged in a chamber superfused with oxygenated ACSF at a flow rate of ~ 2 ml/min. Glass electrodes (1–4 M Ω) filled with oxygenated ACSF were used both for the stimulation of Schaffer collateral/commissural fibers and for the recording of field excitatory postsynaptic potential (fEPSP) and population spike (POPS) from the CA1 area. The slope of the fEPSP and the amplitude of POPS were calculated for indexing the synaptic response. After maintaining a control baseline of synaptic transmission (evoked at 0.05 Hz) using stimulus intensity that generated events $\sim 1/3$ of the maximal fEPSP response for at least 20 min, slices were stimulated by HFS (1 $\times$ 100 Hz for 1s). Control slices were pretreated with 100 μM AP5 for 20 min before HFS and maintained in the same solution throughout the experiment. The treated tissue slices were quickly removed from the recording chamber and fixed in 4%

paraformaldehyde in PBS. Synaptic responses were amplified using an AxoClamp 2B or Multiclamp 700B, digitized by CED Power 1401 and analyzed by Signal 4 software (Cambridge Electronic Design).

1.3. Immunochemical staining

Each fixed tissue slice (400 μm) was glued directly to the specimen mounting block with a thin layer of cyanoacrylate based adhesive and sectioned into approximately $8 \times 50 \mu\text{m}$ -sections using a Vibratome 1000. Tissue sections were lined up in sequential order in a 96-well plate for free-float staining. They were treated sequentially with PBS containing 0.5% NP-40 and 3% H_2O_2 each for a minimum of 15 min and blocked with 2.5% horse serum. Tissue was then incubated with a combination of rabbit and mouse primary antibodies (rabbit anti-Ng, 1/1,250; mouse anti-CaM, 1/500) in PBS containing 0.025% horse serum and 0.1% thimerosal overnight at room temperature. The IR of CaM was first revealed by incubation of the tissue with ImmPRESS peroxidase-conjugated anti-mouse IgG for 4 h and freshly prepared fluorescence dye/ H_2O_2 solution (stock solution contained one hydrogen peroxide-urea adduct tablet and 1 μl stock Alexa 594-tyramine or 2 μl FITC-tyramine in 10 ml of 50 mM Tris-Cl buffer, pH 8.0) at room temperature for 10 min. The reaction was terminated with 10 mM HCl for 30 min. Afterward, Ng was revealed by incubation with ImmPRESS anti-rabbit IgG for 4 h, second dye/ H_2O_2 solution for 10 min, and terminated with 10 mM HCl. In between incubations, the tissue was washed 10 min each with TTBS (20 mM Tris-Cl, pH 7.5, containing 0.5 M NaCl and 0.05% Tween 20) once and PBS twice. Serial sections from each slice were aligned in their cutting order on a glass slide, spread with Vectashield, and covered with a glass slip. The reaction time of peroxidase-conjugated ImmPress IgG with dye/ H_2O_2 was chosen to minimize background staining and the termination of the reaction with 10 mM HCl for 30 min was confirmed to completely inactivate the enzyme. Tissue sections incubated either without the first or secondary antibody exhibited only background staining. Alexa 594-tyramine was synthesized by incubation of 1 mg carboxylic acid succinimidyl ester of the dye in 12 μl DMSO with 4 μl 0.3 M tyramine hydrochloride and 40 μl 0.1 M sodium borate buffer, pH 8.6, at room temperature in a dark vessel overnight. FITC-tyramine was similarly prepared by incubation of 10 mg carboxylic acid succinimidyl ester of the dye in 70 μl DMSO with 70 μl 0.3 M tyramine and 15 μl 0.2 M sodium borate buffer, pH 8.6.

1.4. Confocal microscopy and quantitative analysis

Stained tissue sections were examined using a Zeiss LSM 510 inverted microscope with a pin hole setting for the red channel (543 nm) at 1 air unit and the pin holes of other channels (488 nm and 405 nm) optimized to achieve a similar optical slice depth with each objective prior to scanning. Images were captured in 8-bit mode (256 scales) at a high resolution ($1,024 \times 1,024$ pixels). For quantification of the fluorescent intensity, the acquired multi-channel images were analyzed off-line using LSM 510 Live analysis software that measured the profiles along a straight line stretching from the distal dendrites to the cell layer or using the histogram program for a selected area. Scoring of IR quantification was blinded to the identity of the samples. The digitized fluorescence intensity was imported into Excel for subsequent analysis. Images of individual 50 μm serial sections derived from a 400 μm slice were taken initially at low magnification (5x or 10x) to identify the location of the stimulating and recording electrodes, which appeared as holes in the stained tissues. While the staining intensity (especially for CaM) was prominent in the dendritic area near the stimulating electrode tip, we found that the same area deeper in the serial sections exhibited diminishing staining intensity. The staining patterns of those sections 100–150 μm beneath the electrode tip were similar to those of either un-stimulated samples or those treated with 100 μM AP5. Thus, they were used as controls for estimation of stimulation-mediated translocation. This approach was taken to illustrate the stimulating specificity of HFS at the

depth dimension (Z axis). To determine the relative changes among serial sections, the fluorescence intensity ratio of the stimulated dendritic area ($\sim 40,000 \mu\text{m}^2$ surrounding the tip of stimulating electrode) over the immediate cell layer ($\sim 15\text{--}18,000 \mu\text{m}^2$) was compared to that of the corresponding area of the deeper section beneath the tip of stimulating electrode. For statistical analysis, results were expressed as mean $\pm$ S.E., and comparisons between groups were made using Sigma Stat one way analysis of variance or *t* test. In all cases, $p < 0.05$ was considered significant and the “N’s” represented the number of slices analyzed. Higher magnification (63x or 100x) was used for capturing the images of CaM/Ng staining patterns in dendrites and in the soma.

2. Results

2.1. Localization of CaM and Ng in hippocampal CA1 pyramidal neurons

In rodent, Ng and CaM are thought to interact with each other based on yeast two-hybrid screening (Prichard et al., 1999) and *in vitro* affinity chromatography (Huang et al., 1993). However, no experimental data exists to indicate that these two proteins co-localize in hippocampal neurons. First, we employed acute hippocampal slices routinely used for electrophysiological experiments to investigate the localization of both Ng and CaM by immunohistochemical staining (Fig. 1). These tissue slices (400 μm) were incubated in oxygenated ACSF for $\sim 2\text{h}$, followed by fixation with 4% paraformaldehyde. Tissue was then further sub-sectioned into 50 μm thickness. The IR of Ng (panel B), as shown previously (Represa et al., 1990; Alvarez-Bolado et al., 1996; Singec et al., 2004), was prominent in the soma and dendrites of CA1 pyramidal neurons, whereas CaM (panel A) was predominantly observed in the soma. In double-stained sections, CaM and Ng exhibited a strong co-localization in the soma but less significant co-localization in the dendrites. The somatic CaM IR was concentrated in the nucleus as seen in the merged images (panel C), in which the nucleus was surrounded by a ring of cytosolic Ng IR. The unusually high concentration of CaM in the nucleus of the CA1 pyramidal neurons was in sharp contrast to the neighboring neurons of the subiculum (panels D, E, and F), where CaM and Ng IRs were evenly distributed throughout the soma and dendrites. It should be noted that neither CaM nor Ng sequences possess a nuclear translocation signal, suggesting that they are probably sequestered there by their nuclear binding partner(s) (Agell et al., 1998).

2.2. Estimation of Ng and CaM concentration in CA 1 pyramidal neurons

The hippocampal levels of Ng and CaM were determined by quantitative immunoblot analysis using each of the purified proteins as standards. In addition, we determined the level of α CaMKII for comparison with the published data (Erondy and Kennedy, 1985). The concentrations of Ng and α CaMKII were determined in the same blot (Fig. 2A); whereas CaM, which migrated closely to Ng in SDS-PAGE, was determined in a separate blot (Fig. 2B). The estimated concentration of CaM was $2.75 \pm 0.26 \mu\text{g}/\text{mg}$ ($n = 3$ mice) and those of Ng and α CaMKII were $2.16 \pm 0.12 \mu\text{g}/\text{mg}$ ($n = 3$ mice) and $21.7 \pm 0.29 \mu\text{g}/\text{mg}$ ($n = 3$ mice), respectively. The concentrations of Ng and α CaMKII determined here were similar to previously reported values (Erondy and Kennedy, 1985; Huang et al., 2004). Assuming the hippocampal protein content is 0.25 g/ml cell volume, the concentrations of CaM (16.8 kDa) and Ng (7.5 kDa) would be approximately 40 and 60 μM , respectively. The estimated concentration of CaM in the hippocampus as a whole was less than that observed in cultured neurons ($\sim 60 \mu\text{M}$) (Grant et al., 2008). This perhaps is expected because glial cells and interneurons express much less CaM at basal conditions (Ikeshima et al., 1993; Sola et al., 1997). The mean fluorescence intensity of CaM and Ng within the CA1 region (including stratum radiatum (Sr), stratum oriens (So), and pyramidal cell layer) was $86.1 \pm 8.4\%$ (SD, $n = 27$ slices) and $101.3 \pm 5.9\%$ (SD, $n = 27$ slices) of their respective values observed in the entire hippocampus. Thus, the concentrations of CaM and Ng in the CA1 region were

estimated to be 34 and 60 μM , respectively. For mouse CA1 pyramidal neurons, the soma diameter is $\sim 12\text{--}14\ \mu\text{m}$ and that of the nucleus is $\sim 10\ \mu\text{m}$ and the diameter of the apical dendritic trunk is $1\text{--}2\ \mu\text{m}$ with a length of $\sim 300\ \mu\text{m}$ (Fig. 3). Consequently the estimated volume of the CA1 pyramidal cell body is $\sim 1,150\ \mu\text{m}^3$ (diameter of $13\ \mu\text{m}$ sphere) and that of the nucleus $\sim 520\ \mu\text{m}^3$ (assuming a $10\ \mu\text{m}$ sphere). The somatic volume of the mouse hippocampal CA1 pyramidal neurons estimated here was approximately 50% that of the rat reported previously (Ishizuka et al., 1995). The estimated volumes of rat hippocampal CA1 dendrites (apical plus basal) vary greatly among several reports (see Ambros-Ingerson and Holmes, 2005). Assuming the somatic volume is equal to that of the dendrites according to Ishizuka et al. (Ishizuka et al., 1995), the dendritic volume of CA1 pyramidal neurons will be $\sim 1,150\ \mu\text{m}^3$. The relative fluorescent intensities of Ng and CaM in the dendrites were estimated at $41 \pm 1.9\%$ (SE, $n = 18$) and $10.8 \pm 0.7\%$ (SE, $n = 16$) of those seen in the soma, respectively. Based on their relative staining intensity, the estimated concentrations of CaM in the nucleus and dendrites are 110 and 12 μM , respectively. Similar estimation of the Ng concentrations in the soma and dendrites were 85 and 35 μM , respectively.

2.3. HFS enhanced translocation of CaM and Ng from soma to dendrites

The relatively low levels of CaM in the dendrites compared to that observed in the soma prompted us to investigate whether stimulation of the CA1 Schaffer-collateral/commissural fibers might result in a redistribution of CaM from soma to distal dendrites. Immunohistochemical staining of CaM of serially sectioned tissue ($50\ \mu\text{m}$) derived from hippocampal slices ($400\ \mu\text{m}$) 60 min after stimulation by HFS revealed a significant increase in dendritic CaM IR in area close to the stimulating electrode (Fig. 4). Tissue sections deeper and beneath the tip of the stimulating electrode exhibited diminishing CaM staining intensity (Fig. 4A). Experiments using two stimulating electrodes (S1 and S2) (Fig. 5A), with one (S1) delivering HFS and the other (S2) only test pulse (0.05Hz), showed that LTP was induced by S1 but not by S2 (Fig. 5D). The stained tissue section at a higher magnification revealed more CaM-positive “spots” near the stimulating electrode S1 (Fig. 5B) than observed at the control S2 electrode (Fig. 5C). The intensity of CaM IR in the stratum radiatum (Sr) progressively reduced at greater distance from the stimulating electrode. These findings indicate that the increase in dendritic CaM following HFS is specific to the stimulated pathway. Another control experiment was carried out using slices obtained from Ng knockout (NgKO) mice, which exhibit deficits in HFS-induced LTP (Fig. 6B). In these slices there was no significant increase in the dendritic CaM IR surrounding either the stimulating electrode or in the two consecutive sections beneath (Fig. 6A).

To quantify the extent of translocation of CaM and Ng from soma to dendrites, we scanned the CaM and Ng IRs of a dendritic area ($\sim 40,000\ \mu\text{m}^2$) nearby the tip of the stimulating electrode and its immediate cell layer ($\sim 15\text{--}18,000\ \mu\text{m}^2$) (Fig. 7A, boxed areas) for comparison with those of the corresponding areas from a deeper section as control (Fig. 7B). The ratios of the fluorescence intensities of the dendritic areas over those of the cell layers were compiled for statistical analysis. The increase in dendritic CaM IR of stimulated wild type (WT) mice slices was significantly greater than those of both slices from NgKO or wild type slices treated with $100\ \mu\text{M}$ AP5 (WT+AP5) (WT, 1.21 ± 0.03 , $n = 19$ slices vs. NgKO, 1.03 ± 0.02 , $n = 19$ slices, $p < 0.001$; WT vs. WT+AP5, 1.04 ± 0.02 , $n = 6$ slices, $p < 0.05$). No significant difference was found between NgKO and WT+AP5 slices. The increase of Ng IR of stimulated slices was slightly greater than in those treated with AP5 (WT, 1.06 ± 0.02 , $n = 19$ slices vs. WT+AP5, 0.96 ± 0.02 , $n = 5$ slices, $p < 0.05$) (Fig. 7C). The enhancement in dendritic CaM IR was detected as early as 10 min post HFS. Finally the increase in both CaM and Ng IR observed 60 min after HFS were not inhibited by $20\ \mu\text{M}$ of anisomycin (data not shown), suggesting that those changes are not due to the accumulation of newly synthesized protein.

2.4. Co-localization of CaM and Ng within dendritic spines

CaM IR in the stimulated CA1 region exhibited a punctate staining profile and, at a higher magnification appeared to decorate the surface of apical dendrites in Sr (Fig. 8). These prominent CaM- and Ng-positive puncta were not evenly distributed in all dendritic branches, suggesting that certain branches may have received a stronger stimulus input than others (panel A). Further magnification of a selected area (dotted rectangle) revealed that CaM (panel B) was co-localized with Ng (panel C) in a number of dendritic spines (panel D). The diameters of these spines were slightly less than 1 μm , which is comparable to the reported spine head diameters of apical dendrites of mouse CA1 pyramidal neurons (Konur et al., 2003). The immunochemical identification of Ng in the dendritic spine by confocal microscopy confirmed previous studies using electron microscopy (Watson et al., 1992).

Stimulation of the Schaffer-collateral/commissural fibers in the CA1 region caused potentiation of fEPSP in the dendritic field and the amplitude of POPS observed at the cell body layer. The subsequent accumulation of CaM and Ng in the stimulated dendritic branches and spines may contribute to the potentiation of the fEPSP by increasing the Ca^{2+} -transients that promote the Ca^{2+} - and Ca^{2+} /CaM-mediated signaling pathways and rearrangement of cytoskeleton and neurotransmitter receptors. At the pyramidal cell layer of stimulated slices, certain neurons exhibited an increase in Ng IR in the cytoplasm relative to nuclei (Fig. 9B) when compared to cells from control slices that exhibited a similar intensity between cytoplasm and nuclei (see Fig. 1B). Among them, some cells exhibited a greater accumulation of Ng at the neck between cell body and proximal dendrite. For CaM, such an enhanced cytoplasmic staining comparable to that of Ng was not apparent; however, faint CaM IRs at the proximal dendrites, likely projecting from the nucleus, was detectable (Fig. 9A). These findings suggest that an increase in the cytoplasmic Ng and CaM could contribute to the potentiation of the amplitude of POPS seen near the cell layer.

3. Discussion

The current study was initiated to determine whether CaM and Ng closely interact to support normal neuronal function. Our results demonstrate that these two proteins co-localize in principle neurons of the mouse CA1 hippocampus. It is generally believed that small molecular weight proteins, such as CaM and Ng, are freely diffusible throughout the various cellular compartments. Surprisingly, we found that these two proteins were asymmetrically localized in the CA1 pyramidal neurons, where both were more concentrated in the soma. This was especially true for CaM, whose concentration in the nucleus was estimated to be nearly 10-time greater than that observed in the dendrites. For Ng, the concentration in the soma (including nucleus and cytoplasm) was ~ 2.5 -time greater than that in the dendrites. The asymmetrical distributions of CaM and Ng in the CA1 pyramidal neurons were distinct from those of neighboring subiculum neurons, where CaM and Ng appeared to be more uniformly distributed throughout soma and dendrites. Nuclei from different cell types and tissues are known to contain CaM ($\sim 1\text{--}10\ \mu\text{M}$) (Bachs et al., 1992, 1994; Pruschy et al., 1994); yet, the concentration of nuclear CaM observed in hippocampal CA1 neurons ($\sim 110\ \mu\text{M}$) was one- to two-order of magnitude greater than the reported values. Several reports suggested that CaM can enter the nucleus by Ca^{2+} -dependent and – independent mechanisms, likely, by binding to a carrier(s) through facilitated transport (Pruschy et al., 1994; Thorogate and Torok, 2004, 2007). Inside the nucleus, there are numerous binding proteins, including those involved in the actin-myosin contractile system and RNA-processing, nucleolar ribosomal proteins, protein kinases and phosphatases, transcription factors, and chaperones (Agell et al., 1998) that can all sequester CaM inside the nucleus. In spite of the fact that there are also a large number of CaM-binding proteins in the cytoplasm and dendrites (Xia and Storm, 2005), these fail to compete with those in the nucleus for the retention of CaM in resting neurons. A high concentration of CaM in the nucleus may also

retain Ng into this compartment. The presence of high concentrations of CaM and Ng in the nucleus might act to amplify nuclear Ca^{2+} -transients as previously demonstrated in the dendrites (Huang et al., 2004). Recently, nuclear localization of Ng and its activity-dependent shuffling between cytoplasm and nucleus have been reported (Garrido-Garcia et al., 2009).

HFS-induced early LTP was effective in causing a persistent increase in CaM IR in the apical dendrites surrounding the site of the stimulating electrode. Under basal conditions at low $[\text{Ca}^{2+}]_i$ (~ 100 nM), a large fraction of CaM will bind Ng such that free CaM becomes a limiting factor for the activation of CaM-dependent enzymes. Under these conditions enzymes such as calcineurins (PP2B) and cyclic nucleotide phosphodiesterases, those exhibit a high affinity for CaM, will be preferentially activated. Translocation of CaM from the soma to the dendrites will enhance the stimulation of Ca^{2+} /CaM-dependent enzymes, including α CaMK II, adenylate cyclases, and nitric oxide synthases, which require a higher concentration of CaM for optimal activity. Concurrent translocation of Ng with CaM from soma to dendrites and their accumulation within dendritic spines may sensitize these compartments to maintain a higher level of Ca^{2+} -transients for a given Ca^{2+} influx. This is based on our previously proposed “mass-action mechanism” for Ng (Huang et al., 2004), which predicts that the binding of Ng and CaM to form Ng/CaM complexes will impede the direct interaction of Ca^{2+} with CaM and also facilitate the release of Ca^{2+} from CaM/ Ca^{2+} complexes. The coordinated response of CaM and Ng to a stimulus, which promotes their translocation to the spines, is crucial to strengthening the synaptic efficacy. It is interesting to note that Ng knockout mice exhibit deficits in LTP and HFS do not trigger a significant translocation of CaM from nucleus to dendrites. It appears that Ng plays a critical role in promoting the HFS-mediated exit of CaM from soma to the dendrites. In addition, this process is dependent on the activation of NMDA receptors as AP5 inhibited the translocation of CaM from soma to dendrites.

Translocation of signaling proteins from soma and dendritic trunk into spines may serve as a type of molecular mechanism of memory. An increase in CaM/Ng will facilitate the activation and autophosphorylation of α CaMKII, a memory molecule, and potentiate the neurotransmitter receptors and channel functions through both Ca^{2+} - and Ca^{2+} /CaM-dependent signaling pathways. In addition, it has been reported that PKC γ also undergoes stimulation-induced translocation from soma to spines (Craske et al., 2005). The efficacy of the CaM/Ng-regulated signaling could be improved further by a concurrent translocation of PKC γ , which regulates the interaction of CaM and Ng by phosphorylating the latter at a site (Ser-36) within the CaM-binding domain. Phosphorylated Ng no longer binds CaM and, thus, prolongs the availability of free CaM for the activation of CaM-dependent enzymes even after the decline of Ca^{2+} transients. It should be noted that the PKC γ -mediated phosphorylation of Ng occurs only after a rise in $[\text{Ca}^{2+}]_i$ that promotes the dissociation of CaM/Ng complexes. CaM and Ng in its associated form is a poor substrate for the kinase (Huang et al., 1993). One may envisage that a high level of CaM/Ng in the spine will potentiate neurotransmitter-mediated Ca^{2+} -transients (Huang et al., 2004) and the rise in $[\text{Ca}^{2+}]_i$ in turn activates PKC to phosphorylate Ng. The phosphorylated Ng also would trigger the release of Ca^{2+} from internal stores to prolong the Ca^{2+} -transients (Cohen et al., 1993) and the availability of free CaM. These sequences of events are critical for a sustained synaptic activation following the induction of LTP.

4. Conclusion

CaM and Ng were found to be co-localized in CA1 pyramidal neurons of mouse hippocampus. Both proteins were more concentrated in the soma compared to the dendrites. In particular, CaM level in the nucleus was nearly ten times that observed in the dendrites.

The asymmetrical localization of CaM was in sharp contrast to that observed in neighboring subicular neurons, where CaM was more uniformly distributed throughout the soma and dendrites. HFS of the CA1 Schaffer-collateral/commissural fibers triggered a redistribution of CaM and Ng from soma to the dendritic branches. This process was inhibited by the NMDA receptor antagonist, AP5, and required the presence of Ng as HFS failed to promote a similar translocation of CaM in the Ng knockout mice. Based on the punctate staining patterns of these proteins along the dendritic shaft, we inferred that they were associated with spines. The stimulation-mediated translocation of CaM and Ng to the dendritic spines may act to enhance synaptic responses by increasing Ca^{2+} transients and activation of Ca^{2+} /CaM-dependent enzymes.

Acknowledgments

The research was supported by the Intramural Research Program of the NICHD, NIH. The authors would like to thank Dr. Vincent Schram for his help in confocal microscopy and Dr. Chris McBain for critical reading of the manuscript.

Abbreviations

CaM	calmodulin
Ng	neurogranin
ACSF	artificial cerebrospinal fluid
HFS	high frequency stimulation
LTP	long-term potentiation
fEPSP	field excitatory postsynaptic potential
POPS	population spike
IR	immunoreactivity
NMDA	N-methyl-D-aspartate
AP 5	2-amino-5-phosphonopentanoic acid

References

- Agell N, Aligue R, Alemany V, Castro A, Jaime M, Pujol MJ, Rius E, Serratosa J, Taules M, Bachs O. New nuclear functions for calmodulin. *Cell Calcium* 1998;23:115–121. [PubMed: 9601606]
- Alvarez-Bolado G, Rodriguez-Sanchez P, Tejero-Diez P, Fairen A, Diez-Guerra FJ. Neurogranin in the development of the rat telencephalon. *Neuroscience* 1996;73:565–580. [PubMed: 8783271]
- Ambros-Ingerson J, Holmes WR. Analysis and comparison of morphological reconstructions of hippocampal field CA1 pyramidal cells. *Hippocampus* 2005;15:302–315. [PubMed: 15490464]
- Bachs O, Agell N, Carafoli E. Calcium and calmodulin function in the cell nucleus. *Biochim Biophys Acta* 1992;1113:259–270. [PubMed: 1510999]
- Bachs O, Agell N, Carafoli E. Calmodulin and calmodulin-binding proteins in the nucleus. *Cell Calcium* 1994;16:289–296. [PubMed: 7820848]
- Chin D, Means AR. Calmodulin: a prototypical calcium sensor. *Trends Cell Biol* 2000;10:322–328. [PubMed: 10884684]
- Cohen RW, Margulies JE, Coulter PM 2nd, Watson JB. Functional consequences of expression of the neuron-specific, protein kinase C substrate RC3 (neurogranin) in *Xenopus* oocytes. *Brain Res* 1993;627:147–152. [PubMed: 8293295]
- Coldren CD, Lai Z, Shragg P, Rossi E, Glidewell SC, Zuffardi O, Mattina T, Ivy DD, Curfs LM, Mattson SN, Riley EP, Treier M, Grossfeld PD. Chromosomal microarray mapping suggests a role

- for BSX and Neurogranin in neurocognitive and behavioral defects in the 11q terminal deletion disorder (Jacobsen syndrome). *Neurogenetics* 2009;10:89–95. [PubMed: 18855024]
- Craske ML, Fivaz M, Batada NN, Meyer T. Spines and neurite branches function as geometric attractors that enhance protein kinase C action. *J Cell Biol* 2005;170:1147–1158. [PubMed: 16186260]
- Erondy NE, Kennedy MB. Regional distribution of type II Ca²⁺/calmodulin-dependent protein kinase in rat brain. *J Neurosci* 1985;5:3270–3277. [PubMed: 4078628]
- Gaertner TR, Putkey JA, Waxham MN. RC3/Neurogranin and Ca²⁺/calmodulin-dependent protein kinase II produce opposing effects on the affinity of calmodulin for calcium. *J Biol Chem* 2004;279:39374–39382. [PubMed: 15262982]
- Garrido-Garcia A, Andres-Pans B, Duran-Trio L, Diez-Guerra FJ. Activity-dependent translocation of neurogranin to neuronal nuclei. *Biochem J* 2009;424:419–429. [PubMed: 19751214]
- Gerendasy DD, Sutcliffe JG. RC3/neurogranin, a postsynaptic calpacitin for setting the response threshold to calcium influxes. *Mol Neurobiol* 1997;15:131–163. [PubMed: 9396008]
- Grant PA, Best SL, Sanmugalingam N, Alessio R, Jama AM, Torok K. A two-state model for Ca²⁺/CaM-dependent protein kinase II (alphaCaMKII) in response to persistent Ca²⁺ stimulation in hippocampal neurons. *Cell Calcium* 2008;44:465–478. [PubMed: 18436302]
- Huang FL, Huang KP, Wu J, Boucheron C. Environmental enrichment enhances neurogranin expression and hippocampal learning and memory but fails to rescue the impairments of neurogranin null mutant mice. *J Neurosci* 2006;26:6230–6237. [PubMed: 16763030]
- Huang KP, Huang FL, Chen HC. Characterization of a 7.5-kDa protein kinase C substrate (RC3 protein, neurogranin) from rat brain. *Arch Biochem Biophys* 1993;305:570–580. [PubMed: 8080473]
- Huang KP, Huang FL, Li J, Schuck P, McPhie P. Calcium-sensitive interaction between calmodulin and modified forms of rat brain neurogranin/RC3. *Biochemistry* 2000;39:7291–7299. [PubMed: 10852729]
- Huang KP, Huang FL, Jager T, Li J, Reymann KG, Balschun D. Neurogranin/RC3 enhances long-term potentiation and learning by promoting calcium-mediated signaling. *J Neurosci* 2004;24:10660–10669. [PubMed: 15564582]
- Ikeshima H, Yuasa S, Matsuo K, Kawamura K, Hata J, Takano T. Expression of three nonallelic genes coding calmodulin exhibits similar localization on the central nervous system of adult rats. *J Neurosci Res* 1993;36:111–119. [PubMed: 8230317]
- Ishizuka N, Cowan WM, Amaral DG. A quantitative analysis of the dendritic organization of pyramidal cells in the rat hippocampus. *J Comp Neurol* 1995;362:17–45. [PubMed: 8576427]
- Konur S, Rabinowitz D, Fenstermaker VL, Yuste R. Systematic regulation of spine sizes and densities in pyramidal neurons. *J Neurobiol* 2003;56:95–112. [PubMed: 12838576]
- Kubota Y, Putkey JA, Waxham MN. Neurogranin controls the spatiotemporal pattern of postsynaptic Ca²⁺/CaM signaling. *Biophys J* 2007;93:3848–3859. [PubMed: 17704141]
- Kubota Y, Putkey JA, Shouval HZ, Waxham MN. IQ-motif proteins influence intracellular free Ca²⁺ in hippocampal neurons through their interactions with calmodulin. *J Neurophysiol* 2008;99:264–276. [PubMed: 17959737]
- Li J, Pak JH, Huang FL, Huang KP. N-methyl-D-aspartate induces neurogranin/RC3 oxidation in rat brain slices. *J Biol Chem* 1999;274:1294–1300. [PubMed: 9880498]
- Miyakawa T, Yared E, Pak JH, Huang FL, Huang KP, Crawley JN. Neurogranin null mutant mice display performance deficits on spatial learning tasks with anxiety related components. *Hippocampus* 2001;11:763–775. [PubMed: 11811671]
- Pak JH, Huang FL, Li J, Balschun D, Reymann KG, Chiang C, Westphal H, Huang KP. Involvement of neurogranin in the modulation of calcium/calmodulin-dependent protein kinase II, synaptic plasticity, and spatial learning: a study with knockout mice. *Proc Natl Acad Sci U S A* 2000;97:11232–11237. [PubMed: 11016969]
- Prichard L, Deloulme JC, Storm DR. Interactions between neurogranin and calmodulin in vivo. *J Biol Chem* 1999;274:7689–7694. [PubMed: 10075657]
- Pruschy M, Ju Y, Spitz L, Carafoli E, Goldfarb DS. Facilitated nuclear transport of calmodulin in tissue culture cells. *J Cell Biol* 1994;127:1527–1536. [PubMed: 7798309]

- Represa A, Deloulme JC, Sensenbrenner M, Ben-Ari Y, Baudier J. Neurogranin: immunocytochemical localization of a brain-specific protein kinase C substrate. *J Neurosci* 1990;10:3782–3792. [PubMed: 2269883]
- Ruano D, Aulchenko YS, Macedo A, Soares MJ, Valente J, Azevedo MH, Hutz MH, Gama CS, Lobato MI, Belmonte-de-Abreu P, Goodman AB, Pato C, Heutink P, Palha JA. Association of the gene encoding neurogranin with schizophrenia in males. *J Psychiatr Res* 2008;42:125–133. [PubMed: 17140601]
- Sheu FS, Mahoney CW, Seki K, Huang KP. Nitric oxide modification of rat brain neurogranin affects its phosphorylation by protein kinase C and affinity for calmodulin. *J Biol Chem* 1996;271:22407–22413. [PubMed: 8798403]
- Singec I, Knoth R, Ditter M, Volk B, Frotscher M. Neurogranin is expressed by principal cells but not interneurons in the rodent and monkey neocortex and hippocampus. *J Comp Neurol* 2004;479:30–42. [PubMed: 15389613]
- Sola C, Tusell JM, Serratos J. Calmodulin is expressed by reactive microglia in the hippocampus of kainic acid-treated mice. *Neuroscience* 1997;81:699–705. [PubMed: 9316022]
- Stefansson H, Ophoff RA, Steinberg S, Andreassen OA, Cichon S, Rujescu D, Werge T, Pietilainen OP, Mors O, Mortensen PB, Sigurdsson E, Gustafsson O, Nyegaard M, Tuulio-Henriksson A, Ingason A, Hansen T, Suvisaari J, Lonnqvist J, Paunio T, Borglum AD, Hartmann A, Fink-Jensen A, Nordentoft M, Hougaard D, Norgaard-Pedersen B, Bottcher Y, Olesen J, Breuer R, Moller HJ, Giegling I, Rasmussen HB, Timm S, Mattheisen M, Bitter I, Rethelyi JM, Magnusdottir BB, Sigmundsson T, Olason P, Masson G, Gulcher JR, Haraldsson M, Fossdal R, Thorgeirsson TE, Thorsteinsdottir U, Ruggeri M, Tosato S, Franke B, Strengman E, Kiemeny LA, Melle I, Djurovic S, Abramova L, Kaleda V, Sanjuan J, de Frutos R, Bramon E, Vassos E, Fraser G, Ettinger U, Picchioni M, Walker N, Touloupoulou T, Need AC, Ge D, Yoon JL, Shianna KV, Freimer NB, Cantor RM, Murray R, Kong A, Golimbet V, Carracedo A, Arango C, Costas J, Jonsson EG, Terenius L, Agartz I, Petursson H, Nothen MM, Rietschel M, Matthews PM, Muglia P, Peltonen L, St Clair D, Goldstein DB, Stefansson K, Collier DA. Common variants conferring risk of schizophrenia. *Nature* 2009;460:744–747. [PubMed: 19571808]
- Thorogate R, Torok K. Ca²⁺-dependent and -independent mechanisms of calmodulin nuclear translocation. *J Cell Sci* 2004;117:5923–5936. [PubMed: 15522886]
- Thorogate R, Torok K. Role of Ca²⁺ activation and bilobal structure of calmodulin in nuclear and nucleolar localization. *Biochem J* 2007;402:71–80. [PubMed: 17040208]
- Watson JB, Sutcliffe JG, Fisher RS. Localization of the protein kinase C phosphorylation/calmodulin-binding substrate RC3 in dendritic spines of neostriatal neurons. *Proc Natl Acad Sci U S A* 1992;89:8581–8585. [PubMed: 1528865]
- Xia Z, Storm DR. The role of calmodulin as a signal integrator for synaptic plasticity. *Nat Rev Neurosci* 2005;6:267–276. [PubMed: 15803158]
- Zhabotinsky AM, Camp RN, Epstein IR, Lisman JE. Role of the neurogranin concentrated in spines in the induction of long-term potentiation. *J Neurosci* 2006;26:7337–7347. [PubMed: 16837580]
- Zhong L, Cherry T, Bies CE, Florence MA, Gerges NZ. Neurogranin enhances synaptic strength through its interaction with calmodulin. *Embo J* 2009;28:3027–3039. [PubMed: 19713936]

Figure 1. Subcellular localization of CaM and Ng in the pyramidal neurons of hippocampal CA1 region and subiculum

Control tissue sections were examined with 100x objectives for CaM (Alexa 594) and Ng (FITC) of cells in the CA1 region (A–C) and subiculum (D–F). Positive staining of CaM of the CA1 pyramidal neurons (Py) was largely confined to the nucleus (A), whereas Ng-positive staining was evident in the nucleus, cytoplasm, and apical dendrites (B) within stratum radiatum (Sr). The merged images (C) showed prominent co-localization of CaM and Ng in the nucleus, which was surrounded by a ring of cytoplasmic Ng. In contrast, pyramidal neurons in the subiculum exhibit uniform co-localization of both CaM and Ng in the nucleus, cytoplasm, and apical dendrites (D–F). The scale bars are 10 μ m.

Figure 2. Characterization of the antibodies for immunohistochemical staining and quantification of hippocampal Ng and CaM

Panel A. Western blot with antibodies against Ng and α CaMKII. Purified mouse brain CaMKII and Ng were mixed together and their respective amounts of 60 and 5 (lane 1), 120 and 10 (lane 2), 180 and 15 (lane 3), 240 and 20 (lane 4) and 300 and 25 ng (lane 5) were used as standards for measuring the levels of these two proteins in the hippocampal extracts (5 μ g, lane 6). The blots were probed for α CaMKII, Ng, and actin in the same membrane (for details see Experimental procedures), in which actin was used for calibration of protein loading. Panel B. Western blot with antibody against CaM. This antibody revealed the immunoreactivity of purified CaM, 60 (lane 1), 180 (lane 2), 300 (lane 3), 420 (lane 4), and 540 ng (lane 5) dose-dependently and detected a major protein band (~ 17 kDa) corresponding to CaM in the hippocampal extracts (40 μ g, lane 6). Each hippocampal extracts from three wild type mice were analyzed at least three times and the intensities of immunoreactive bands revealed by ECL were quantified by using Kodak Image Station analysis software.

Figure 3. Quantification of immunofluorescence intensity of tissue sections doubly stained with antibodies against CaM and Ng

Representative confocal images (10x) of Ng (A), CaM (B), and the merged one (C) were scanned for the fluorescence intensity ranging from the distal apical dendrites bordering lacunosum molecular layer (LM), through stratum radiatum (Sr), pyramidal cell (Py) layer, and stratum oriens (So) in the mid field of the CA1 region (depicted by the white arrow). The distances in the intensity profiles of CaM (red) and Ng (green) represent the length of the white arrow (bottom panel). Two to three measurements were made per slice and the relative fluorescent intensities of Ng and CaM in the dendrites were estimated at $41 \pm 1.9\%$ (SE, $n = 18$ slices) and $10.8 \pm 0.7\%$ (SE, $n = 16$ slices) of those in the soma, respectively.

Figure 4. High frequency stimulation enhanced the translocation of CaM from soma to dendrites Hippocampal slice was stimulated with HFS (100 Hz) for 1 s and the slope of fEPSP ($-\circ-$) and the amplitude of POPS ($-\bullet-$) were monitored for 60 min (panel B). The tissue was removed from the recording chamber and fixed with 4% paraformaldehyde. Fixed tissue was sectioned into 50 μm thickness and stained with antibody against CaM. Representative confocal images of the 3rd (labeled as 150 μm), 4th (200 μm), and 5th (250 μm) 50 μm -serial section from the surface of electrode entry were shown (panel A). The sites of stimulating electrode (S) and two recording electrodes, one at stratum radiatum (R_{EPSP}) and another at pyramidal cell (R_{POPS}), in the CA1 region were indicated by the arrows. The area nearby the stimulating electrode of the 3rd section exhibited stronger CaM IR intensity than the corresponding areas of the 4th and 5th sections. It was also evident that the fluorescence intensity progressively reduced farther away from the stimulating electrode. The scale bars are 100 μm .

Figure 5. Stimulation site-specific translocation of CaM from soma to dendrites

Two stimulating electrodes (S1 and S2) were placed in the stratum radiatum (Sr) of the CA1 region and one recording electrode for monitoring fEPSP (R_{EPSP}) in the Sr and another one for the amplitude of POPS (R_{POPS}) at the pyramidal cell (Py) layer. The tips of these electrodes could be identified from this 50 μm section doubly stained with CaM and Ng antibodies (panel A). The S1 electrode was stimulated by HFS (100 Hz for 1 s) and S2 by test pulse (0.05 Hz) for 60 min. The S1 stimulating path exhibited potentiation in fEPSP and amplitude of POPS, whereas S2 path exhibited no significant increase in fEPSP and a slight increase in POPS after 60 min (panel D). Numerous CaM IR positive “hot spots” surrounding S1, but very few around S2, were detectable. The scale bar in A is 50 μm and those of B and C are 10 μm.

Figure 6. High frequency stimulation of NgKO slices failed to promote translocation of CaM from soma to dendrites

Hippocampal slice from NgKO mice was stimulated by HFS (100 Hz) for 1s and the slope of fEPSP (○-○) and amplitude of POPS (●-●) were monitored for 60 min (panel B). The tissue was removed from the recording chamber and fixed with 4% paraformaldehyde. Fixed tissue was sectioned into 50 μm thickness and stained with antibody against CaM. Representative confocal images of the 3rd (labeled as 150 μm), 4th (200 μm), and 5th (250 μm) 50 μm-serial section from the surface of electrode entry were shown (panel A). The sites of stimulating electrode (S) and two recording electrodes (R), one at stratum radiatum (R_{EPSP}) and another at pyramidal cell layer (R_{POPS}), in the CA1 region were indicated by the arrows. Note that there was no distinctive enhancement of CaM IR nearby the stimulating electrode in the 3rd compared to the 4th and 5th sections.

Figure 7. Measurement of the extents of translocation of CaM and Ng following high frequency stimulation

Hippocampal slices were stimulated with HFS and fixed after 60 min, the fixed tissues were cut into 50 μm sections and doubly stained with antibodies against CaM and Ng. Fluorescence intensities of a boxed area of $\sim 40,000 \mu\text{m}^2$ in the Sr and its immediate cell layer ($\sim 15\text{--}18,000 \mu\text{m}^2$) of a section nearby the tip (indicated by the arrow head) of stimulating electrode (A, stimulated) were compared to the corresponding areas of the 2nd subsequent serial section (B, control) beneath the stimulated one. The fluorescence intensity ratios (dendrite/cell layer) between the stimulated and control sections were used for the indexes of translocation. For NgKO, only the CaM fluorescence intensity was examined. The increases in the dendritic CaM IRs of slices from wild type (WT) were significantly greater than those of NgKO and the AP5-treated wild type (WT+AP5) slices (WT, 1.21 ± 0.03 , $n = 19$ slices vs. NgKO, 1.03 ± 0.02 , $n = 19$ slices, $p < 0.001$; WT vs. WT+AP5, 1.04 ± 0.02 , $n = 6$ slices, $p < 0.05$). No significant difference was found between NgKO and WT+AP5 slices. The increases of Ng IR of the stimulated slices were slightly greater than those treated with AP5 (WT, 1.06 ± 0.02 , $n = 19$ slices vs. WT+AP5, 0.96 ± 0.02 , $n = 5$ slices, $p < 0.05$) (panel C). In panels A and B, the scale bars are 100 μm and the locations of CA1, CA2, stratum radiatum (Sr), stratum oriens (So), pyramidal cell layer (Py), and lacunosum molecular layer (LM) are indicated.

Figure 8. Association of CaM and Ng in the dendritic spines of stimulated hippocampal CA1 neurons

High magnification (100 x) of the doubly stained images, CaM (Alexa 594) and Ng (FITC), of the CA1 apical dendrites of the stimulated area showed that these two proteins were co-localized in the dendritic spines that decorated the surface of the apical dendrites. The boxed area in panel A (merged image) was further magnified using Photoshop software to illustrate the association of CaM (panel B) and Ng (panel C) in the spines (indicated by the arrows in panel D). The scale bar in A is 5 μm and those in B, C, and D are 1 μm. The diameters of the spine heads are slightly less than 1 μm.

Figure 9. Exit of CaM and Ng from the nucleus to cytoplasm and dendrites of the stimulated CA1 pyramidal neurons

Representative images of the hippocampal CA1 cell layer from the stimulated tissue (60 min after HFS) showed spreading of CaM (panel A) and Ng IRs (panel B) in the cytoplasm and proximal dendrites. Several neurons exhibited a more intense Ng IR (indicated by arrows) in the cytoplasm than that in the nucleus. This is in contrast to a relatively uniform Ng IR in the soma of control tissues (see Fig. 1). The scale bars are 10 μm.