國立臺灣大學生命科學院生命科學研究所

碩士論文

Institute of Life Science

College of Life Science

National Taiwan University

Master Thesis

Calneuron I 與 CaMKIIβ對於神經訊息傳遞的影響

Effects of Calneuron I and CaMKIIβ on Neurotransmission

何　芸

Yun He

指導教授：潘建源 博士

Advisor: Chien-Yuan Pan, Ph.D.

中華民國104年8月

August, 2015

Acknowledgement

回想碩士班兩年的時光，說短不短，說長卻也好像是一眨眼間就迎來畢業這個有點感傷的時刻。來到 Lab730 對我而言是一場珍貴的緣分，從大學部的時候開始一直到進入研究所，很幸運的能有機會在實驗室學習許多實驗的技術，並且在過程中發現了真正去做實驗的樂趣與克服困難的成就感。感謝潘建源老師既細心又耐心的指導，不厭其煩的和我討論、分析實驗的問題，並且可以從老師身上學習到做研究的正確態度，以及思考如何解決難題的方法。而在論文口試時，很感謝王致恬老師和林崇智老師給了我許多實際的建議，讓我順利的完成論文。

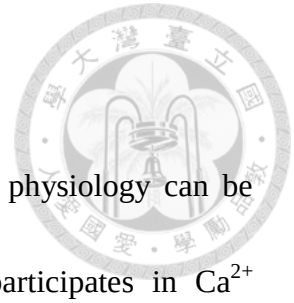
實驗室的學長姐和伙伴們也是我很大的支柱，感謝愛鵑學姊常常替我解決很多實驗上遇到的問題，不論何時都很仔細又耐心地幫我找到問題的所在，還教了我很多實驗上很實用的小技巧，讓我可以事半功倍；感謝慧馨學姊在我一開始來到實驗室的時候教我分生實驗的技術,並且在實驗之餘舉辦許多輕鬆愉快的活動，讓大家可以適當的放鬆休息；感謝明毅學長在 imaging 的實驗上給我許多幫助，也很感謝志弘學長教導我很多 Techcomm 的儀器使用，也時常和我討論實驗結果；感謝婉瑄學姊幫我們把許多實驗室的雜務都處理的井井有條，讓大家可以心無旁騖地投入實驗。而在這兩年和我一同走來的乃馨和怡婷，我永遠不會忘記這一段我們一起努力互相打氣加油的時光，你們總是帶給我許許多多的歡笑，讓我每天都充滿了動力。

回首這充實的兩年，我遇見了許多不曾見過的，做了許多不曾做過的，得到了許多無可取代的珍寶。對於能擁有這樣的經歷和回憶，我感到自己是多麼幸運，並且更應該珍惜這一切。現在我即將邁步走向未來，而這生命中絕無僅有的兩年，會是我心中永不磨滅的美好。

摘要

在神經生理中，鈣離子訊號複雜的模式與參與在鈣離子訊號傳遞中的鈣離子結合蛋白 (CaBPs) 有相當大的關聯。CaBP8，又稱為 Calneuron I (CalnI)，會參與在鈣離子訊號傳遞中並抑制 N 型鈣離子通道的電流。CaMKII 則是在神經突觸的發育與突觸可塑性中扮演重要的角色。在先前的研究中，CalnI 與 CaMKIIβ 被指出可能有交互作用。為了確認 CalnI 對神經傳導的影響，將 CalnI 表現在初級培養的神經細胞中，並使用鈣離子影像技術紀錄其神經傳導的情形。除此之外，也利用 Sholl analysis 來分析表現 CalnI 的神經細胞其形態是否有所改變。另外在人類胚腎細胞 293T 中同時表現 CFP-CalnI 及 YFP-CaMKIIβ 來觀察 CalnI 是否會影響 CaMKIIβ 在細胞內的分布情形。在表現 CalnI 的神經細胞中，神經傳導的功能被明顯的阻斷，而鈣離子誘導鈣釋放以及代謝型麩胺酸受體訊息路徑也會受到影響而使細胞內的鈣離子反應減弱。在神經細胞的形態上，神經元突起在遠端的分支數量也因為表現 CalnI 而明顯減少或變短。而在同時表現 CFP-CalnI 及 YFP-CaMKIIβ 的 HEK 293T 細胞中則發現 CalnI 確實會影響 CaMKIIβ 在細胞內的分布情形。綜合以上可知，CalnI 會調控鈣離子通道並影響神經傳導，且很有可能與 CaMKIIβ 交互作用進一步影響突觸發育或可塑性。

關鍵字：Calneuron I、CaMKIIβ、鈣離子誘導鈣釋放、人類胚腎細胞株 293T、突觸傳導



Abstract

The complex patterns of Calcium (Ca^{2+}) signals in neuronal physiology can be largely attributed to calcium binding proteins (CaBPs) which participates in Ca^{2+} signaling pathway. CaBP8, also referred to as calneuron I (CalnI), is involved in transduction of Ca^{2+} signaling and inhibiting N-type Ca^{2+} channel currents. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) plays an important role in regulating synaptic development and plasticity. According to a previous study in our lab, a possible interaction between CalnI and CaMKIIβ was indicated by Yeast Two-Hybrid screening. To verify the effects of CalnI on neurotransmission, CalnI and related mutants were overexpressed in primary cultured neurons, and calcium imaging experiments were applied to characterize neurotransmission. In addition, CalnI overexpressed neurons were analyzed by Sholl analysis to investigate if there were any changes in morphology. In addition, CFP-CalnI and YFP-CaMKIIβ co-expressed HEK293T cells were examined to determine whether CalnI affects the localization of CaMKIIβ. The overexpression of CalnI in neurons caused significant deficits in neurotransmission, also decreased the Ca^{2+} responses in calcium-induced calcium release (CICR) and metabotropic glutamate receptor (mGluR) signaling pathway. In neuronal morphology, the distal branches of neurites in CalnI overexpressed neurons were obviously decreased. Moreover, the fluorescence imaging of CFP-CalnI and

YFP-CaMKIIβ co-expressed HEK293T cells shows that the expression of CalnI does affect the localization of CaMKIIβ. Taken together, CalnI may regulate Ca^{2+} channels to influence neurotransmission, and possibly interacts with CaMKIIβ to further affect the synaptic development or plasiticity.

Keywords: Calneuron I, CaMKIIβ, CICR, HEK293T cell, synaptic transmission

Catalog

Acknowledgement i

摘要 ii

Abstract iii

1. Introduction 1

1.1 Ca^{2+} signaling and synaptic transmission 1

1.2 CaBPs and Calneuron I 2

1.3 Ca^{2+}/calmodulin-dependent protein kinase II 4

1.4 Ca^{2+} signaling and neuronal morphology 6

2. Aims 7

3. Materials and Methods 8

3.1 Chemicals 8

3.2 Cell preparation 8

3.3 Plasmid construction 9

3.4 Transfection 10

3.5 Calcium imaging 11

3.6 Fluorescence imaging 12

3.7 Data analysis 13

4. Results 14

4.1 CalnI inhibits Ca^{2+} responses and neurotransmission 14

4.2 CalnI reduces CICR 15

4.3 CalnI affects mGluR but not AMPA receptor 17

4.4 CalnI reduces the distal neurite number of primary cortical neurons 17

4.5 CalnI affects the localization of CaMKIIβ in HEK293T cells 19

5. Discussion 20

5.1 CalnI inhibits Ca^{2+} response and neurotransmission 20

5.2 CalnI interferes with CICR pathway 22

5.3 CalnI inhibits mGluR signaling pathway 23

5.4 Morphology of CalnI expressing neurons 24

5.5 CalnI and CaMKIIβ interact with each other 25

6. Conclusion 27

7. References 28

Figures 35

1. Introduction

1.1 Ca^{2+} signaling and synaptic transmission

Calcium ion (Ca^{2+}) signaling is a fundamental mechanism which lies behind neuronal physiology (Bootman et al., 2001). It regulates neurotransmission and synaptic plasticity, and also influences higher-level brain functions such as learning and memory (Dash et al., 2007; Miller, 1988). The entry of Ca^{2+} from the outside is regulated by voltage or receptor-operated channels controlled by various neurotransmitters. Some metabotropic receptors stimulate the formation of inositol 1,4,5-trisphosphate (IP_3), which acts on IP_3 receptors (IP_3R) to release Ca^{2+} from the endoplasmic reticulum (ER) (Zirpel et al., 1994). The ER also has ryanodine receptors (RYR) sensitive to cyclic ADP ribose (Waterhouse et al., 1987). Both the IP_3Rs and the RYRs are also sensitive to Ca^{2+}, and this process of calcium-induced calcium release (CICR) can set up propagated Ca^{2+} waves (Dupont and Goldbeter, 1993; Irving et al., 1992). Uptake of Ca^{2+} into the ER lumen enhances the Ca^{2+} sensitivity of these receptors while release of Ca^{2+} from the ER modulates neuronal excitability (Verkhratsky and Shmigol, 1996).

Many neurons display after-hyperpolarizations (AHPs) or depolarizing afterpotentials (DAPs) following either a single action potential or bursts of action potentials (Li and Hatton, 1997; Sah and McLachlan, 1991). Such AHPs and DAPs modify neuronal activity by suppressing or promoting firing patterns, respectively. The

Ca^{2+}-dependent ion channels responsible for these variations in membrane potential are controlled either by Ca^{2+} entering through voltage-operated channels or by Ca^{2+} released from internal stores (Berridge, 1998). Influx of Ca^{2+} through voltage-operated channels is the classical mechanism of transmitter release. The docked vesicles are taken into the Ca^{2+} microdomains that form when the voltage-operated channel opens during depolarization. In addition to this role of extracellular Ca^{2+}, there is increasing evidence that exocytosis might be regulated by release of Ca^{2+} from intracellular stores (Blochl and Thoenen, 1995).

These complex patterns of spatial and temporal Ca^{2+} signals can be largely attributed to calcium binding proteins (CaBPs) which participated in Ca^{2+} signaling pathway (Burgoyne, 2007; Haeseleer et al., 2000).

1.2 CaBPs and Calneuron I

CaBPs are a large family of proteins which have the ability to bind Ca^{2+} with their EF-hand motifs (Haeseleer et al., 2000; Mikhaylova et al., 2006). Members of the CaBP family share a similar structure with the ubiquitous EF-hand containing protein calmodulin (CaM) (Hoeflich and Ikura, 2002). There are six isoforms of CaBPs, CaBP1, 2, 4, 5, 7 and 8 (Haeseleer et al., 2000; Mikhaylova et al., 2006; Wu et al., 2001), while CaBP1 and CaBP2 are expressed as multiple, alternatively spliced variants (Haeseleer et

al., 2000). CaBP1, also known as caldendrin, is localized to the post-synaptic density of excitatory synapses (Mikhaylova et al., 2006). CaBP1 and its splice variants (CaBP1-Long and CaBP1-Short) are regulators of P/Q and L-type voltage-gated Ca^{2+} channels in mammalian central nervous system (CNS) (Lee et al., 2002; Tippens and Lee, 2007), N-type channels of neuroendocrine cells (Chen et al., 2008) and intracellular IP_3-receptors (Haynes et al., 2004; Kasri et al., 2004). CaBP2 is expressed at low level in retina (Haeseleer et al., 2000) and its mutation expressed in cochlear hair cells causes autosomal-recessive hearing impairment (Schrauwen et al., 2012). CaBP4 is expressed specifically in retina as a modulator of $Ca_V1.4$ channels (Haeseleer et al., 2004; Maeda et al., 2005) and mutations in *CaBP4* gene cause autosomal recessive night blindness (Zeitz et al., 2006). CaBP5 is also expressed in sensory cells, it modulates the activity of $Ca_V1.2$ channels and maybe regulates retinal sensitivity (Rieke et al., 2008). CaBP7 and CaBP8, also referred to as calneuron II (CalnII) and calneuron I (CalnI) respectively, are most divergent from other CaBP family members sharing no more than 30% sequence identity (McCue et al., 2009). Unlike other CaBP family members which have only one non-functional EF-hand motif (EF-2), calneurons have two non-functional EF-hand motifs (EF-3 and 4) (Haeseleer et al., 2000). They are abundant in brain (Mikhaylova et al., 2006; Wu et al., 2001), targeting to Golgi membranes and post-Golgi vesicular compartments by the hydrophobic C-terminal

transmembrane domain (Mikhaylova et al., 2009).

CalnI was first identified in 2001 while screening the candidate genes potentially responsible for Williams syndrome. The CalnI deletion at chromosome 7 of Williams syndrome patients leads to memory and learning deficits (Wu et al., 2001). CalnI is expressed in a brain-specific manner late in development after P15 (Wu et al., 2001), and it has high binding affinity to Ca^{2+} with a K_d value in the range of 0.2-0.5μM (Mikhaylova et al., 2006). At the plasma membrane of bovine chromaffin cells, CalnI is involved in transduction of Ca^{2+} signaling and inhibiting N-type Ca^{2+} channel currents (Shih et al., 2009).

1.3 Ca^{2+}/calmodulin-dependent protein kinase II

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) plays an important role in regulating synaptic development and plasticity. It is a major synaptic protein which is activated during the induction of long-term potentiation (LTP) by the Ca^{2+} influx through NMDA (N-methyl-d-aspartate) receptors (NMDARs) (Rongo and Kaplan, 1999). CaMKII induces synaptic insertion and increased single-channel conductance of AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors (AMPARs) (Hayashi et al., 2000). This protein kinase is composed of four different subunit isoforms: α, β, γ, and δ. CaMKII holoenzymes appear as a stacked pair of

hexameric rings. The α isoform is required for hippocampal long-term potentiation (LTP) and spatial learning (Silva et al., 1992). Although all isoforms of CaMKII can be found in the brain, CaMKIIα and β predominate and form α and β holoenzymes and αβ heteroenzymes (Bennett et al., 1983). The activation of each CaMKII subunit in the holoenzyme is initially dependent on the binding of Ca^{2+}/calmodulin. Simultaneous activation of adjacent subunits results in the autophosphorylation at Thr286. The probability of simultaneous subunit activation and Thr286 autophosphorylation depends on the amplitude, duration and frequency of the changes in intracellular Ca^{2+} concentrations. The Thr286 autophosphorylated subunits remain kinase activity even after dissociation of Ca^{2+}/calmodulin (Bradshaw et al., 2002; Hanson et al., 1994; Mukherji and Soderling, 1994).

Autophosphorylation of Thr286 is critical for LTP induction in the mice hippocampus. A point mutation of Thr286 to Ala286 blocks autophosphorylation without affecting Ca^{2+}/calmodulin-dependent activity. This mutant still has normal synaptic transmission but have no NMDA-receptor-dependent LTP in the hippocampal CA1 region, and shows deficits in spatial learning (Giese et al., 1998). It reveals that the autophosphorylation at Thr286 of CaMKII is a molecular switch underlying LTP and learning.

1.4 Ca^{2+} signaling and neuronal morphology

In neuronal system, intracellular Ca^{2+} concentration has been reported to play a critical role in the regulation of neurite outgrowth (Konur and Ghosh, 2005). Calcium levels in neurons are regulated by influx through calcium channels as well as by release of calcium from intracellular stores. One major siganling target of calcium influx is CaMKII (Ghosh and Greenberg, 1995). CaMKIIα and β mediate different outcomes on neurite growth. CaMKIIα has been reported to stabilize or restrict dendritic growth of frog tectal neurons in vivo and mammalian cortical neurons in vitro (Redmond et al., 2002; Wu and Cline, 1998). However, CaMKIIβ has a positive effect on filopodia extension and fine dendrite development mediated by direct interaction with cytoskeletal actin (Fink et al., 2003).

On the other hand, several observations support a role for neuronal activity in regulating dendrite morphology. During normal development of the somatosensory and visual system, dendrites dramatically reorganize in response to synaptic afferent input. Activity deprivation experiments also suggest that loss of normal activity during development leads to lasting deficits in dendritic development (McAllister et al., 1996).

2. Aims

Previous studies have demonstrated that CalnI regulates the N-type voltage-gated Ca^{2+} channels and inhibits Ca^{2+} current in bovine chromaffin cells. CaMKII is involved in LTP in hippocampal region and further plays an important role in memory and learning. According to a previous study in our lab, a possible interaction between CalnI and CaMKIIβ was indicated by Yeast Two-Hybrid screening. Since CalnI targets to plasma membrane by its hydrophobic C-terminal transmembrane domain, it may affect the localization of proteins which interacts with it. The aims are:

(1) To verify the effects of CalnI on neurotransmissoin. CalnI and related mutants will be overexpressed in primary cultured neurons, and calcium imaging experiments will be applied to characterize neurotransmission.

(2) To investigate the effects of CalnI on neuronal morphology.

(3) To examine the localization of CalnI and CaMKIIβ in HEK293T cells, and determine whether CalnI affects the localization of CaMKIIβ.

3. Materials and Methods

3.1 Chemicals

Hank's balanced salt solution (HBSS): 138 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 4 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 10 mM HEPES, and 5.6 mM glucose, pH 7.1-7.3, 270-280 mOsm/kg.

Bovine Chromaffin Complete Medium (BCCM): Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad CA, USA), 15 mM HEPES, 26 mM $NaHCO_3$, 10% fetal bovine serum (Invitrogen, Carlsbad CA, USA) and 1% penicillin/streptomycin (100 IU/mL-100 mg/mL, Invitrogen, Carlsbad CA, USA), pH 7.1-7.3, 270-280 mOsm/kg.

HEK medium: Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad CA, USA), 15 mM HEPES, 26 mM $NaHCO_3$, 10% fetal bovine serum (Invitrogen, Carlsbad CA, USA) and 0.5% penicillin/streptomycin (100 IU/mL-100 mg/mL, Invitrogen, Carlsbad CA, USA), pH 7.2-7.4, 300-305 mOsm/kg.

Phosphate buffered saline (PBS): 142 mM NaCl, 2 mM KCl, 8 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.1-7.3, 300-305 mOsm/kg.

3.2 Cell preparation

The primary cultured cortical neurons were prepared by digesting dissected cortical

brain tissues from E14.5 rat fetuses with papain solution (Sigma) in 37°C for 30 minutes, then adding HBSS to stop the enzymatic reaction and centrifuging the cell solution at 1200 rpm for 5 minutes. The isolated cells were plated at a density of 5×10^6 cell/mL on 22 mm poly-L-lysine coated coverslips in 3.5 cm culture dishes with BCCM. The medium inside the culture dishes was half replaced by Neurobasal medium (B-27, 0.5mM GlutaMax, 25 μM glutamatic acid) at DIV 1. At DIV 3, half of the medium was replaced by Neurobasal medium with 10 μM Ara-c.

Human embryonic kidney 293T (HEK293T) cells were maintained in HEK medium in a humidified incubator with 5% CO^2 and the cells were replated every 3 days.

3.3 Plasmid construction

The construction of CalnI-containing plasmids was done in the previous study of our lab. Specific primer sets designed according to cloned human and mouse CalnI and the rat ETS data bank was used to amplify the coding region of CalnI from adult rat brain cDNA. The mutant without the C-terminal hydrophobic domain (CalnIΔHT) was constructed using another designed reverse primer. The mutants without functional EF-hand motifs (CalnIDE2AQ) were constructed by PCR to replace the first two aspartate residues in both the N-terminal EF-hand motifs with alanine (D2A) and the

last glutamate in both motifs with glutamine (E2Q).

Antoher primer set containing EcoRI restriction enzyme cutting sites was used to amplify the CaMKIIβ sequence from EGFP-C1-CaMKIIβ plasmids. The construct was first subcloned into an yT&A vector, and then transferred into a EYFP-C1 vector by EcoRI restriction enzyme.

3.4 Transfection

The primary cultured cortical neurons were transfected by using Lipofectamine LTX with PLUS reagent (Invitrogen, Carlsbad CA, USA). First, plasmid DNA was mixed with PLUS reagent in opti-MEM (Invitrogen, Carlsbad CA, USA), then incubated for 10 minutes in room temperature. After that, a mixture of Lipofectamine LTX reagent and opti-MEM was added into the DNA-containing solution, and then incubated for 30 minutes in room temperature. After incubation, the total mixture was applied into the dishes plated with primary cultured cortical neurons (DIV 7). The calcium imaging experiments were conducted after 48 hours.

The HEK293T cells were transfected by using Lipofectamine 3000 reagent (Invitrogen, Carlsbad CA, USA). First, plasmid DNA was mixed with P3000 reagent in opti-MEM. Then a mixture of Lipofectamine 3000 reagent and opti-MEM was added into the DNA-containing solution, and incubated for 5 minutes in room temperature.

After incubation, the total mixture was applied into 24-well plates plated with HEK293T cells. The HEK cells were replated onto 18 mm poly-L-lysine coated coverslips in 3.5 cm culture dishes after 24 hours.

3.5 Calcium imaging

The primary cultured cortical neurons at DIV 9-12 were used in this experiment. Neurons were transfected with CalnI and the other two mutant forms (CalnIΔHT, CalnIDE2AQ) by Lipofectamine LTX with PLUS reagent. mCherry plasmid was used as an transfection indicator. For intracellular calcium concentration measurements, HBSS was used as the extracellular solution and Fluo-2 MA AM was the intracellular calcium indicator. Neurons were first incubated with HBSS containing 0.5μM Fluo-2 MA AM for 20 minutes at room temperature, then washed three times by HBSS and placed in HBSS containg 50μM MNI-caged glutamate or NP-EGTA, a cell-permeant photolabile chelator that exhibits a high selectivity for Ca^{2+} upon UV illumination.

When performing the experiment, first the 550 nm excitation wavelength (TRITC filter) eas given by DG4 to identify the cells expressing mCherry, and the 488 nm excitation wavelength was used to obtain the Fluo-2 fluorescence. 405 nm laser illumination provided by the Mosaic system (Photonic Instrument Inc. USA) was used to uncage the MNI-caged glutamate or NP-EGTA at selected regions. The images were

captured by CCD camera every one second to record the fluorescence intensity change in neurons.

To calculate the fluorescence intensity change, the built-in program of NIS-element software (Nikon Company) was used. In every trial, the target cell and neighboring cells were selected to measure the intracellular calcium concentration, then exported as time-fluorescence data.

The values of fluorescence intensity of selected cells were first subtracted by the values of backgound fluorescence intensity, then a baseline fluorescence intensity ($F_{baseline}$) can be obtained by averaging the first 10 seconds of the subtracted fluorescence intensity. The value of maximum intensity was defined as F_{peak}. F_{peak} is subtracted by $F_{baseline}$ to obtain the fluorescence intensity change ΔF. To compare th variation between cells, ΔF was divided by $F_{baseline}$ and obtained the normalized elevation ($\Delta F / F_{baseline}$).

The images of mCherry-expressing neurons were further analyzed with Sholl analysis (ImageJ, NIH, USA) to examine the morphological differences between CalnI-expressing and non-expressing neurons.

3.6 Fluorescence imaging

CalnI-expressing HEK cells were fixed in 4% paraformaldehyde in PBS for 20

minutes. After three times of washes, the coverslips plated with cells were mounted on slides with Prolong Gold antifade reagent (Invitrogen, Carlsbad CA, USA) mounting medium and the edges of coverslips were sealed with transparent nail lacquer. The cells were observed on a Leica TCS SP5 confocal microscope with a 100x objective.

3.7 Data analysis

All data of calcium imaging and neuronal morphlogy were analyzed and plotted by Excel and Origin8 (OriginLab Co., Northampton, MA, USA). The data were presented as Mean±SEM and analyzed by Student's *t*-test to verify the significance between two groups. The statistic results with $p < 0.05$ were considered statistically significant.

4. Results

4.1 CalnI inhibits Ca^{2+} responses and neurotransmission

To characterize the functions of CalnI in modulating synaptic transmission in neuronal system, the Ca^{2+} imaging technique was applied. The primary cortical neurons were transfected with CalnI, CalnIΔHT or CalnIDE2AQ respectively (all cotransfected with mCherry), and loaded with Fluo-2 MA to detect the Ca^{2+} fluorescence intensity change. The cells overexpressing mCherry were selected as target cells, and a 405 nm laser spot was specifically illuminated on the target cell for 500 msec to locally uncage the MNI-caged glutamate in the bath to stimulate the target cell. The Fluo-2 fluorescence changes were recorded to reflect the Ca^{2+} responses in the stimulated neurons and neighboring neurons. This uncaging stimulation was repeated three times and the neighboring cells were selected randomly. As shown in Figure 1A, the region of interest (ROI) 1 is the selected target cell and ROI 2-9 are the neighboring cells. As laser illuminated on the target cell, Fluo-2 fluorescence intensity was recorded (Fig. 1B). The laser illumination would cause an artificial peak in target cell recording which followed by a slow decline showing the real fluorescence intensity change (Fig. 1C). On the other hand, the fluorescence intensity recording of neighboring cells would be single peaks without the artifacts (Fig. 1D).

To compare the Ca^{2+} responses among different cells, fluorescence intensity

changes (ΔF) were normalized to the baseline fluorescence intensity before stimulation ($F_{baseline}$). In CalnI-expressing neurons, the Ca^{2+} responses were significantly inhibited (Fig. 2A, red) and the neurotransmission to neighboring cells was also largely decreased (Fig. 2B, red). CalnIDE2AQ-expressing cells showed results similar to wild-type group (Fig. 2A and B, green). Target cells expressing CalnIΔHT had a certain level of decrease in Ca^{2+} response (Fig. 2A, blue) but showed normal neurotransmission to neighboring cells (Fig. 2B, blue).

To examine the effects of CalnI when the protein was expressed in post-synaptic neurons, CalnI-expressing cells were taken as neighboring cells to conduct the same glutamate stimulation experiments as above. Although receiving normal level of synaptic signals, CalnI-expressing neighboring cells showed declined Ca^{2+} responses (Fig. 2C and D, red) while CalnIDE2AQ-expressing neighboring cells were just partially inhibited (Fig. 2D, green) and CalnIΔHT-expressing neighboring cells had no significant decrease compared to control group (Fig. 2D, blue). Taken together, these results suggested that overexpressing of CalnI would impair the neurotransmission.

4.2 CalnI reduces CICR

To further confirm the effect of CalnI on the inhibition of neurotransmission, uncaging Ca^{2+} experiment was applied to directly evoke the intracellular Ca^{2+} signal.

The bath of neurons contained NP-EGTA, a cell-permeant photolabile chelator that exhibits a high selectivity for Ca^{2+} and releases Ca^{2+} upon UV illumination. When the Ca^{2+} concentration was forced to rise in the target cells expressing CalnI by laser illumination (Fig. 3A, red), the signals transmitted to their neighboring cells were significantly reduced (Fig. 3B, red). Compared to control group, CalnIDE2AQ-expressing neurons also had decreased Ca^{2+} responses in their neighboring cells (Fig. 3B, green) while CalnIΔHT-expressing target cells showed normal neurotransmission as control group (Fig. 3B, blue). Furthermore, the Ca^{2+} responses in the neurites of CalnI and CalnIDE2AQ-expressing neurons were also largely decreased as in the neighboring cells (Fig. 3C, red and green).

To verify if CalnI affects the calcium-induced calcium release, the neurons were then stimulated by caffeine (40 mM) and recorded the Ca^{2+} responses. In CalnI-expressing neurons, the Ca^{2+} responses induced by caffeine were significantly suppressed (Fig. 3D, red). Likewise, the Ca^{2+} responses in CalnIDE2AQ-expressing neurons were reduced during caffeine stimulations (Fig. 3D, green). There was also a slightly decrease in the Ca^{2+} responses of CalnIΔHT-expressing neurons compared to control group (Fig. 3D, blue) but was not as obvious as in CalnI and CalnIDE2AQ groups. These results indicated that CalnI may interfere with CICR pathway.

4.3 CalnI affects mGluR but not AMPA receptor

During glutamate stimulation, both metabotropic and ionotropic glutamate receptor would be activated at once. According to the results of glutamate stimulation experiment above, CalnI may affect not only the voltage-gated Ca^{2+} channels but also glutamate receptors. To distinguish the effect of CalnI on these two different types of glutamate receptors, group I mGluR agonist DHPG and AMPA were used individually to stimulate the target neurons expressing CalnI. When stimulated by DHPG (20 μM), the Ca^{2+} responses of CalnI-expressing target cells were significantly declined compared to control group (Fig. 4A, red), and also weaken the Ca^{2+} responses of downstream neighboring neurons (Fig. 4B, red). On the other hand, the Ca^{2+} responses of CalnIΔHT and CalnIDE2AQ-expressing target neurons upon DHPG stimulation were similar to control group, but the Ca^{2+} responses in their neighboring cells were increased (Fig. 4B, blue and green).

On the contrary, when stimulated by AMPA (10 μM), the Ca^{2+} responses of neurons showed no significant difference among all the groups (Fig. 4C and D). these results suggested that CalnI may inhibit mGluR signaling pathway but have no obvious effect on AMPA receptor.

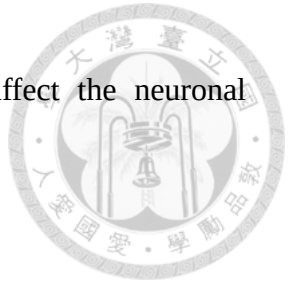
4.4 CalnI reduces the distal neurite number of primary cortical neurons

Since CalnI would affect the synaptic transmission, it may also influence the neuronal morphology. To understand the effects of CalnI on neuronal morphology, the images of mCherry-expressing neurons were analyzed with Sholl analysis to examine the morphological differences between CalnI-expressing and non-expressing neurons. In CalnI, CalnIΔHT and CalnIDE2AQ-expressing neurons, the neurites at around 35-75 μm from the center of soma was significantly reduced compared to control group (Fig. 6A, B and C). but there was no significant difference among the three groups of CalnI wild type and mutants at any distance (Fig. 6D).

To confirm that the decrease of distal neurites was not a general result caused by overexpressing and was actually caused by CalnI, calmodulin and CaM^{1234} were also coexpressed with mCherry in neurons, respectively (Fig. 5B). CaM-expressing neurons showed a similar reduction of distal neurites as CalnI and its mutants at 55-75μm from the center of soma (Fig. 7A), but CaM^{1234} group had almost no significant difference compared to control group (Fig. 7B).

Although CalnI and CaM-expressing neurons showed similar morphological changes compared to control group, there was significant difference between them on the neurite number around 20-40 μm from the center of soma (Fig. 8, left panels). CalnI-expressing neurons and CaM^{1234}-expressing neurons had significant differences in neurite number at 15-75 μm from the center of soma (Fig. 8, right panels). These results

indicated that overexpression of CalnI and its mutants would affect the neuronal morphology and reduce the distal neurite number.



4.5 CalnI affects the localization of CaMKIIβ in HEK293T cells

Previous studies in our lab showed a possible interaction between CalnI and CaMKIIβ by Yeast Two-Hybrid screening. To further confirm the interaction between these two proteins, three fusion proteins CFP-CalnI, YFP-CaMKIIα and YFP-CaMKIIβ were expressed in HEK293T cells to examine the localization of them. CaMKIIα was considered as a comparative since its structure is similar to CaMKIIβ. When expressed seperately, CFP-CalnI distrbuted in the cytosol and plasma membrane, while YFP-CaMKIIα evenly distributed in the whole cell and YFP-CaMKIIβ localized in several aggregation spots (Fig. 9A).

When CFP-CalnI and YFP-CaMKIIα coexpressed in HEK cells, their distribution patterns were very different and had no colocalization (Fig. 9B). On the other hand, CFP-CalnI changed the distribution of YFP-CaMKIIβ in HEK cells from aggregation spots into a more disperse distrbution pattern when they were coexpressed, and they also obviously colocalized (Fig. 9C). These results suggested that CalnI and CaMKIIβ interact with each other and affect both their localization in cells.

5. Discussion

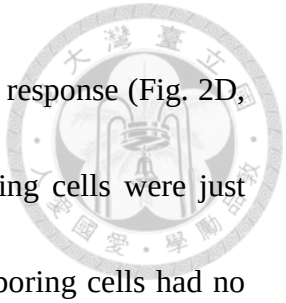
According to a previous study in our lab, a possible interaction between CalnI and CaMKIIβ was indicated by Yeast Two-Hybrid screening. CalnI has been reported to regulate the N-type voltage-gated Ca^{2+} channels and inhibit Ca^{2+} current in bovine chromaffin cells. CaMKII is involved in LTP in hippocampal region and further plays an important role in memory and learning. Therefore, the interaction between CalnI and CaMKIIβ may participate in the synaptic plasticity. The main findings of this study are: (1) CalnI-overexpressing inhibits the Ca^{2+} response and synaptic transmission in primary cortical neurons; (2): CalnI reduces CICR and suppresses the Ca^{2+} propagation in neurons; (3): CalnI affects mGluR but not AMPA receptor; (4): CalnI reduces the distal neurite number of primary cortical neurons; (5): CalnI affects the localization of CaMKIIβ. Taken together, these results suggest that CalnI not only regulates the voltage-gated Ca^{2+} channels but also interferes with mGluR signaling pathway to modulate neurotransmission. Moreover, CalnI interacts with CaMKIIβ and affects its distrbution pattern in cells.

5.1 CalnI inhibits Ca^{2+} response and neurotransmission

Ca^{2+} concentration change is important for signaling transduction in neuronal system. It can regulate neurotransmission and synaptic plasticity, and further influence

higher-level brain functions such as memory and learning (Dash et al., 2007; Miller, 1988). The primary cortical neurons are composed by various types of neuronal cells and can be used to obtain the comprehensive response in protein expression. Most of the excitatory synapses of cortical neurons are glutamate synapses. When a target neuron is activated by glutamate stimulation, the intracellular Ca^{2+} concentration of neurons can be monitored. The Ca^{2+} responses reflect the signal transduction between neurons, and synaptic transmission can be observed.

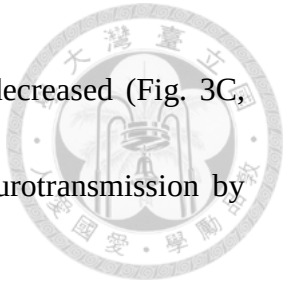
At the plasma membrane of bovine chromaffin cells, CalnI is involved in transduction of Ca^{2+} signaling and inhibiting N-type Ca^{2+} channel currents (Shih et al., 2009). Previous studies demonstrated that the two mutant of CalnI, CalnIΔHT and CalnIDE2AQ, showed different levels of inhibition to Ca^{2+} channel currents. Despite of the incapability to bind Ca^{2+}, CalnIDE2AQ still partially inhibits the Ca^{2+} current. On the other hand, deletion of the hydrophobic tail reverses most of the inhibition on the Ca^{2+} current. In the glutamate stimulation experiment of this study, CalnI-expressing target neurons showed significant reduction in intracellular Ca^{2+} concentration change and neurotransmission was inhibited (Fig. 2, A and B, red). CalnIDE2AQ-expressing cells showed results similar to wild-type group (Fig. 2A and B, green). Target cells expressing CalnIΔHT had a certain level of decrease in Ca^{2+} response (Fig. 2A, blue) but showed normal neurotransmission to neighboring cells (Fig. 2B, blue). When



expressed in post-synaptic neurons, CalnI largely decreased the Ca^{2+} response (Fig. 2D, red) while the Ca^{2+} response of CalnIDE2AQ-expressing neighboring cells were just partially inhibited (Fig. 2D, green) and CalnIΔHT-expressing neighboring cells had no significant decrease compared to control group (Fig. 2D, blue). These results correspond with the previous studies, and the decline of Ca^{2+} response in CalnI-expressing cells is possibly caused by the inhibition of Ca^{2+} channel current.

5.2 CalnI interferes with CICR pathway

In glutamate stimulation experiment, the neurotransmission from CalnI-expressing target cells to their neighboring cells was significantly inhibited (Fig. 2B). Since the Ca^{2+} response in target cells was strongly suppressed, the neurotransmission failure could possibly be the consequences of lacking enough Ca^{2+} to induce neurotransmitter release. To further confirm the effect of CalnI on the inhibition of neurotransmission, uncaging Ca^{2+} experiment was applied to directly evoke the intracellular Ca^{2+} signal. Although the Ca^{2+} concentration was forced to rise in the soma of target neurons expressing CalnI (Fig. 3A, red), the signals transmitted to their neighboring cells were significantly reduced comparing to control group (Fig. 3B, red). CalnIDE2AQ-expressing neurons also had decreased Ca^{2+} responses in their neighboring cells (Fig. 3B, green). Furthermore, the Ca^{2+} responses in the neurites of



CalnI and CalnIDE2AQ-expressing neurons were already largely decreased (Fig. 3C, red and green). These results show that CalnI does inhibit the neurotransmission by suppress the Ca^{2+} propagation from soma to neurite.

As the intracellular Ca^{2+} signal transduction seem to fail in CalnI-expressing neurons, we are wondering to know whether CalnI affects the CICR to suppress the Ca^{2+} propagation. In the next experiment, the neurons were stimulated by caffeine (40 mM) and recorded the Ca^{2+} responses. In CalnI-expressing neurons, the Ca^{2+} responses induced by caffeine were significantly suppressed (Fig. 3D, red). Likewise, the Ca^{2+} responses in CalnIDE2AQ-expressing neurons were also reduced (Fig. 3D, green). So we speculate that CalnI inhibits the Ca^{2+} release from intracellular ER Ca^{2+} stores to suppress the Ca^{2+} wave propagation in neurons.

5.3 CalnI inhibits mGluR signaling pathway

Except for caffeine-induced Ca^{2+} store release through ryanodine receptors (RyR), the activation of IP_3 receptors (IP_3R) also releases Ca^{2+} from ER. The activation of metabotropic glutamate receptors (mGluR) stimulates the formation of IP_3, which acts on IP_3R to release Ca^{2+} from ER. Both the IP_3Rs and the RYRs are also sensitive to Ca^{2+}, and this process of CICR can set up propagated Ca^{2+} waves (Berridge, 1998). During glutamate stimulation, both metabotropic and ionotropic glutamate receptor would be

activated at once. To distinguish the effect of CalnI on these two different types of glutamate receptors, mGluR agonist DHPG and AMPA were used individually to stimulate the target neurons expressing CalnI. The result shows that CalnI reduces the Ca^{2+} response caused by the activation of mGluRs (Fig. 4A, red), but does not affect the activity of AMPA receptors (Fig. 4C). This reduction in Ca^{2+} concentration change led to the weakened synaptic transmission to downstream neurons (Fig. 4B, red). These results indicate that CalnI not only interfere with RyR pathway, but also mGluR signaling pathway to inhibit CICR.

5.4 Morphology of CalnI expressing neurons

As CalnI-expressing neurons show deficits in neurotransmission, we are wondering if these deficits would affect the formation of synapses and change the neuronal morphology. In neuronal system, intracellular Ca^{2+} concentration has been reported to play a critical role in the regulation of neurite outgrowth. Since CalnI would affect the level of Ca^{2+} concentration change in neurons, it may also influence the morphology of neurites. After analyzed the images of neurons expressing CalnI and its mutants, the result shows that overexpressing of CalnI, CalnIΔHT or CalnIDE2AQ caused reduced distal neurite number (Fig. 6). But there was no significant difference among the three groups of CalnI wild type and mutants.

To confirm that the decrease of distal neurites was not a general result caused by overexpressing and was actually caused by CalnI, we further examined the morphology of calmodulin and CaM^{1234} expressing neurons. CaM-expressing neurons showed a similar reduction of distal neurites as CalnI and its mutants (Fig. 7A). Although CalnI and CaM-expressing neurons showed similar morphological changes compared to control group, there was significant difference between them on the neurite number around 20-40 μm from the center of soma (Fig. 8, left panels). The morphology of CaM^{1234}-expressing neurons has almost no significant difference compared to control group neurons (Fig. 7B). The morphological differences between CalnI-expressing neurons and CaM^{1234}-expressing neurons are very obvious (Fig. 8, right panels). Furthermore, as shown in Fig. 5A, the neurons expressing CalnI tend to have more short neurites, and CalnIΔHT-expressing neurons have less neurite than control group, but the neurite length is not obviously shortened. CalnIDE2AQ-expressing neurons show some tangled neurites near the soma area. These results suggest that CalnI inhibit neurite outgrowth to distal area, while CalnIΔHT may reduce the neurite number and CalnIDE2AQ may induce the neurites to wind together.

5.5 CalnI and CaMKIIβ interact with each other

Previous studies in our lab showed a possible interaction between CalnI and

CaMKIIβ by Yeast Two-Hybrid screening. To further confirm the interaction between these two proteins, we examined the localization of CFP-CalnI, YFP-CaMKIIα and YFP-CaMKIIβ in HEK293T cells. CaMKIIα was reagarded as a comparative to CaMKIIβ since they are similar in structure and both abundant in the brain (Bennett et al., 1983). When CaMKIIβ was expressed alone, it localized in several aggregation spots in the cell (Fig. 9A). However, when CaMKIIβ was coexpressed with CalnI, the distribution of CaMKIIβ in HEK cells changed from aggregation spots into a more disperse distrbution pattern (Fig. 9C). Furthermore, CalnI and CaMKIIβ are obviously colocalized with each other (Fig. 9C, merge). These results hint that CalnI and CaMKIIβ may interact with each other in the cells.

Combining the results above, we speculate that CalnI expression may downregulate neurite development by chelating CaMKIIβ. Since CaMKIIβ has positive effect on dendrite development by direct interaction with actin, chelating of CaMKIIβ would reduce the interaction between CaMKIIβ and actin, and further inhibit neurite growth. Therefore, CalnI may regulate neurite growth by different expression levels throughout the developmental stages.

6. Conclusion

CalnI has dual effects on Ca^{2+} channels and CICR pathway. CalnI regulates Ca^{2+} channel activity to inhibit Ca^{2+} influx; on the other hand, CalnI suppresses Ca^{2+} wave propagation by interfering with RyR and mGluR signaling pathway. Therefore, the neurotransmission in CalnI-expressing neurons is inhibited as revealed by the decrease in Ca^{2+} responses of the downstream neurons. The colocalization of CalnI and CaMKIIβ indicates that they could possibly interact with each other, and may further regulate the synaptic transmission in neuronal system. Moreover, overexpressing CalnI also leads to morphological change in neurons which exhibit shortened neurites. In the future, the interaction between CalnI and CaMKIIβ can be further characterized by fluorescence resonance energy transfer technique. And the functions of CalnI and CaMKIIβ in synaptic activity can be verified by electrophysiology experiment.

7. References

Bennett, M.K., Erondu, N.E., and Kennedy, M.B. (1983). Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. J Biol Chem *258*, 12735-12744.

Berridge, M.J. (1998). Neuronal calcium signaling. Neuron *21*, 13-26.

Blochl, A., and Thoenen, H. (1995). Characterization of nerve growth factor (NGF) release from hippocampal neurons: evidence for a constitutive and an unconventional sodium-dependent regulated pathway. Eur J Neurosci *7*, 1220-1228.

Bootman, M.D., Collins, T.J., Peppiatt, C.M., Prothero, L.S., MacKenzie, L., De Smet, P., Travers, M., Tovey, S.C., Seo, J.T., Berridge, M.J., *et al.* (2001). Calcium signalling--an overview. Semin Cell Dev Biol *12*, 3-10.

Bradshaw, J.M., Hudmon, A., and Schulman, H. (2002). Chemical quenched flow kinetic studies indicate an intraholoenzyme autophosphorylation mechanism for Ca2+/calmodulin-dependent protein kinase II. J Biol Chem *277*, 20991-20998.

Burgoyne, R.D. (2007). Neuronal calcium sensor proteins: generating diversity in neuronal Ca2+ signalling. Nat Rev Neurosci *8*, 182-193.

Chen, M.L., Chen, Y.C., Peng, I.W., Kang, R.L., Wu, M.P., Cheng, P.W., Shih, P.Y., Lu, L.L., Yang, C.C., and Pan, C.Y. (2008). Ca2+ binding protein-1 inhibits Ca2+ currents and exocytosis in bovine chromaffin cells. J Biomed Sci *15*, 169-181.

Dash, P.K., Moore, A.N., Kobori, N., and Runyan, J.D. (2007). Molecular activity underlying working memory. Learn Mem *14*, 554-563.

Dupont, G., and Goldbeter, A. (1993). One-pool model for Ca2+ oscillations involving Ca2+ and inositol 1,4,5-trisphosphate as co-agonists for Ca2+ release. Cell Calcium *14*, 311-322.

Fink, C.C., Bayer, K.U., Myers, J.W., Ferrell, J.E., Jr., Schulman, H., and Meyer, T. (2003). Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. Neuron *39*, 283-297.

Ghosh, A., and Greenberg, M.E. (1995). Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science (New York, NY) *268*, 239-247.

Giese, K.P., Fedorov, N.B., Filipkowski, R.K., and Silva, A.J. (1998). Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. Science (New York, NY) *279*, 870-873.

Haeseleer, F., Imanishi, Y., Maeda, T., Possin, D.E., Maeda, A., Lee, A., Rieke, F., and Palczewski, K. (2004). Essential role of Ca2+-binding protein 4, a Cav1.4 channel regulator, in photoreceptor synaptic function. Nat Neurosci *7*, 1079-1087.

Haeseleer, F., Sokal, I., Verlinde, C.L., Erdjument-Bromage, H., Tempst, P., Pronin, A.N., Benovic, J.L., Fariss, R.N., and Palczewski, K. (2000). Five members of a novel Ca(2+)-binding protein (CABP) subfamily with similarity to calmodulin. J Biol Chem

275, 1247-1260.

Hanson, P.I., Meyer, T., Stryer, L., and Schulman, H. (1994). Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. Neuron *12*, 943-956.

Hayashi, Y., Shi, S.H., Esteban, J.A., Piccini, A., Poncer, J.C., and Malinow, R. (2000). Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. Science (New York, NY) *287*, 2262-2267.

Haynes, L.P., Tepikin, A.V., and Burgoyne, R.D. (2004). Calcium-binding protein 1 is an inhibitor of agonist-evoked, inositol 1,4,5-trisphosphate-mediated calcium signaling. J Biol Chem *279*, 547-555.

Hoeflich, K.P., and Ikura, M. (2002). Calmodulin in action: diversity in target recognition and activation mechanisms. Cell *108*, 739-742.

Irving, A.J., Collingridge, G.L., and Schofield, J.G. (1992). Interactions between Ca2+ mobilizing mechanisms in cultured rat cerebellar granule cells. The Journal of physiology *456*, 667-680.

Kasri, N.N., Holmes, A.M., Bultynck, G., Parys, J.B., Bootman, M.D., Rietdorf, K., Missiaen, L., McDonald, F., De Smedt, H., Conway, S.J., *et al.* (2004). Regulation of InsP3 receptor activity by neuronal Ca2+-binding proteins. EMBO J *23*, 312-321.

Konur, S., and Ghosh, A. (2005). Calcium signaling and the control of dendritic

development. Neuron *46*, 401-405.

Lee, A., Westenbroek, R.E., Haeseleer, F., Palczewski, K., Scheuer, T., and Catterall, W.A. (2002). Differential modulation of Ca(v)2.1 channels by calmodulin and Ca2+-binding protein 1. Nat Neurosci *5*, 210-217.

Li, Z., and Hatton, G.I. (1997). Ca2+ release from internal stores: role in generating depolarizing after-potentials in rat supraoptic neurones. The Journal of physiology *498 (Pt 2)*, 339-350.

Maeda, T., Lem, J., Palczewski, K., and Haeseleer, F. (2005). A critical role of CaBP4 in the cone synapse. Invest Ophthalmol Vis Sci *46*, 4320-4327.

McAllister, A.K., Katz, L.C., and Lo, D.C. (1996). Neurotrophin regulation of cortical dendritic growth requires activity. Neuron *17*, 1057-1064.

McCue, H.V., Burgoyne, R.D., and Haynes, L.P. (2009). Membrane targeting of the EF-hand containing calcium-sensing proteins CaBP7 and CaBP8. Biochem Biophys Res Commun *380*, 825-831.

Mikhaylova, M., Reddy, P.P., Munsch, T., Landgraf, P., Suman, S.K., Smalla, K.H., Gundelfinger, E.D., Sharma, Y., and Kreutz, M.R. (2009). Calneurons provide a calcium threshold for trans-Golgi network to plasma membrane trafficking. Proc Natl Acad Sci U S A *106*, 9093-9098.

Mikhaylova, M., Sharma, Y., Reissner, C., Nagel, F., Aravind, P., Rajini, B., Smalla,

K.H., Gundelfinger, E.D., and Kreutz, M.R. (2006). Neuronal Ca2+ signaling via caldendrin and calneurons. Biochim Biophys Acta *1763*, 1229-1237.

Miller, R.J. (1988). Calcium signalling in neurons. Trends Neurosci *11*, 415-419.

Mukherji, S., and Soderling, T.R. (1994). Regulation of Ca2+/calmodulin-dependent protein kinase II by inter- and intrasubunit-catalyzed autophosphorylations. J Biol Chem *269*, 13744-13747.

Redmond, L., Kashani, A.H., and Ghosh, A. (2002). Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. Neuron *34*, 999-1010.

Rieke, F., Lee, A., and Haeseleer, F. (2008). Characterization of Ca2+-binding protein 5 knockout mouse retina. Invest Ophthalmol Vis Sci *49*, 5126-5135.

Rongo, C., and Kaplan, J.M. (1999). CaMKII regulates the density of central glutamatergic synapses in vivo. Nature *402*, 195-199.

Sah, P., and McLachlan, E.M. (1991). Ca(2+)-activated K+ currents underlying the afterhyperpolarization in guinea pig vagal neurons: a role for Ca(2+)-activated Ca2+ release. Neuron *7*, 257-264.

Schrauwen, I., Helfmann, S., Inagaki, A., Predoehl, F., Tabatabaiefar, M.A., Picher, M.M., Sommen, M., Seco, C.Z., Oostrik, J., Kremer, H., *et al.* (2012). A mutation in CABP2, expressed in cochlear hair cells, causes autosomal-recessive hearing impairment. Am J Hum Genet *91*, 636-645.

Shih, P.Y., Lin, C.L., Cheng, P.W., Liao, J.H., and Pan, C.Y. (2009). Calneuron I inhibits Ca(2+) channel activity in bovine chromaffin cells. Biochem Biophys Res Commun *388*, 549-553.

Silva, A.J., Paylor, R., Wehner, J.M., and Tonegawa, S. (1992). Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science (New York, NY) *257*, 206-211.

Tippens, A.L., and Lee, A. (2007). Caldendrin, a neuron-specific modulator of Cav/1.2 (L-type) Ca2+ channels. J Biol Chem *282*, 8464-8473.

Verkhratsky, A., and Shmigol, A. (1996). Calcium-induced calcium release in neurones. Cell Calcium *19*, 1-14.

Waterhouse, A.L., Pessah, I.N., Francini, A.O., and Casida, J.E. (1987). Structural aspects of ryanodine action and selectivity. J Med Chem *30*, 710-716.

Wu, G.Y., and Cline, H.T. (1998). Stabilization of dendritic arbor structure in vivo by CaMKII. Science (New York, NY) *279*, 222-226.

Wu, Y.Q., Lin, X., Liu, C.M., Jamrich, M., and Shaffer, L.G. (2001). Identification of a human brain-specific gene, calneuron 1, a new member of the calmodulin superfamily. Mol Genet Metab *72*, 343-350.

Zeitz, C., Kloeckener-Gruissem, B., Forster, U., Kohl, S., Magyar, I., Wissinger, B., Matyas, G., Borruat, F.X., Schorderet, D.F., Zrenner, E., *et al.* (2006). Mutations in

CABP4, the gene encoding the Ca2+-binding protein 4, cause autosomal recessive night blindness. Am J Hum Genet *79*, 657-667.

Zirpel, L., Nathanson, N.M., Rubel, E.W., and Hyson, R.L. (1994). Glutamate-stimulated phosphatidylinositol metabolism in the avian cochlear nucleus. Neurosci Lett *168*, 163-166.

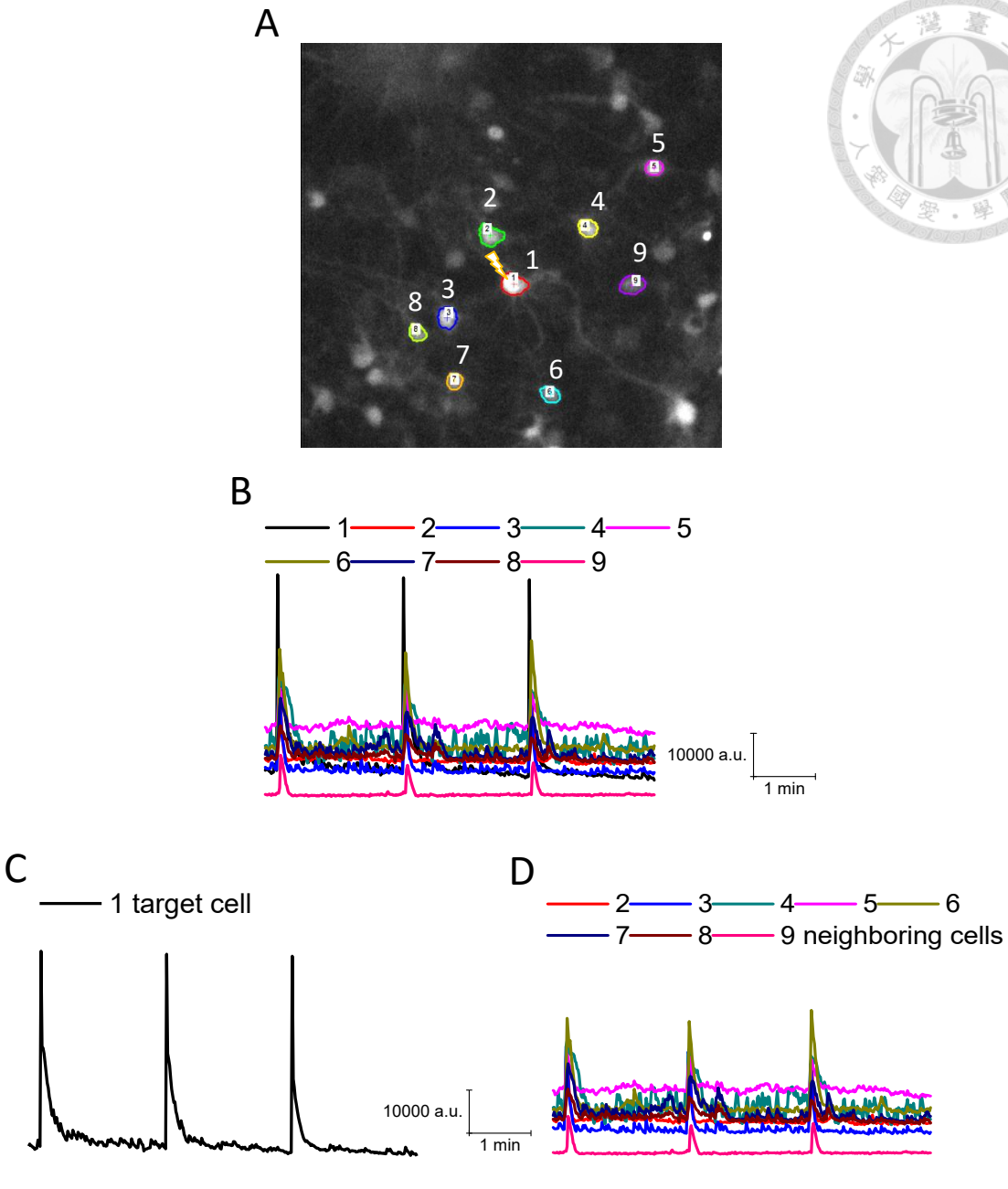


Fig. 1 Calcium responses evoked by glutamate in primary cortical neurons

Primary cortical neurons were loaded with Fluo-2 MA and incubated in HBSS containing MNI-caged glutamate. The neurons expressing mCherry were identified as the target neurons. (A) The representative fluo-2 image. A 405 nm laser illumination was shed on the soma of the target neuron (red circle, ROI 1) to photocleave MNI-glutamate which would release glutamate and locally stimulate the target neuron.

Neighboring neurons (ROI 2-9) were also selected to record Fluo-2 fluorescence intensity changes. (B) The Fluo-2 fluorescence intensity changes of all the selected cells. (C) The Fluo-2 fluorescence intensity change of target cell by 500 msec 405 nm laser stimulation. The laser stimulation was repeated three times with intervals of 2 min. (D) The Fluo-2 fluorescence intensity changes of neighboring cells.

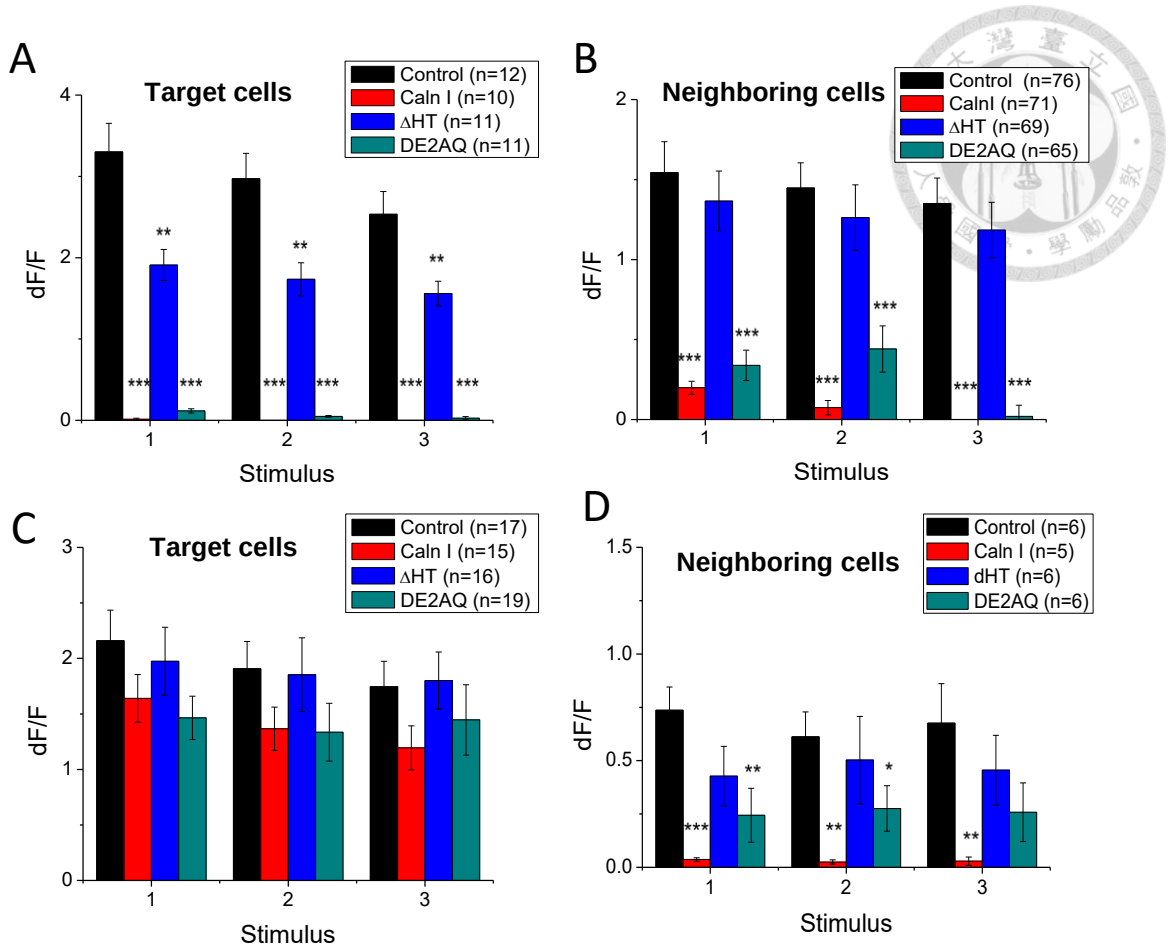


Fig. 2 CalnI inhibits Ca^{2+} response and neurotransmission.

The Ca^{2+} responses of primary cortical neurons stimulated by uncaging glutamate were presented as normalized elevation values of Fluo-2 fluorescence intensity ($\Delta F/F_{baseline}$). (A) The Ca^{2+} responses of target cells expressing CalnI, CalnIΔHT and CalnIDE2AQ respectively. The target cells expressing only mCherry was regarded as control group. (B) The Ca^{2+} responses in the neighboring neurons of the target cells in A. (C) The Ca^{2+} responses of target cells which did not express either CalnI, CalnIΔHT or CalnIDE2AQ. (D) The Ca^{2+} responses in neighboring neurons of the target cells in C. CalnI, CalnIΔHT and CalnIDE2AQ were expressed in the neighboring neurons respectively. Data were Mean±SEM, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ as analyzed by Student's *t*-test

between control group and CalnI/ CalnI mutants group.

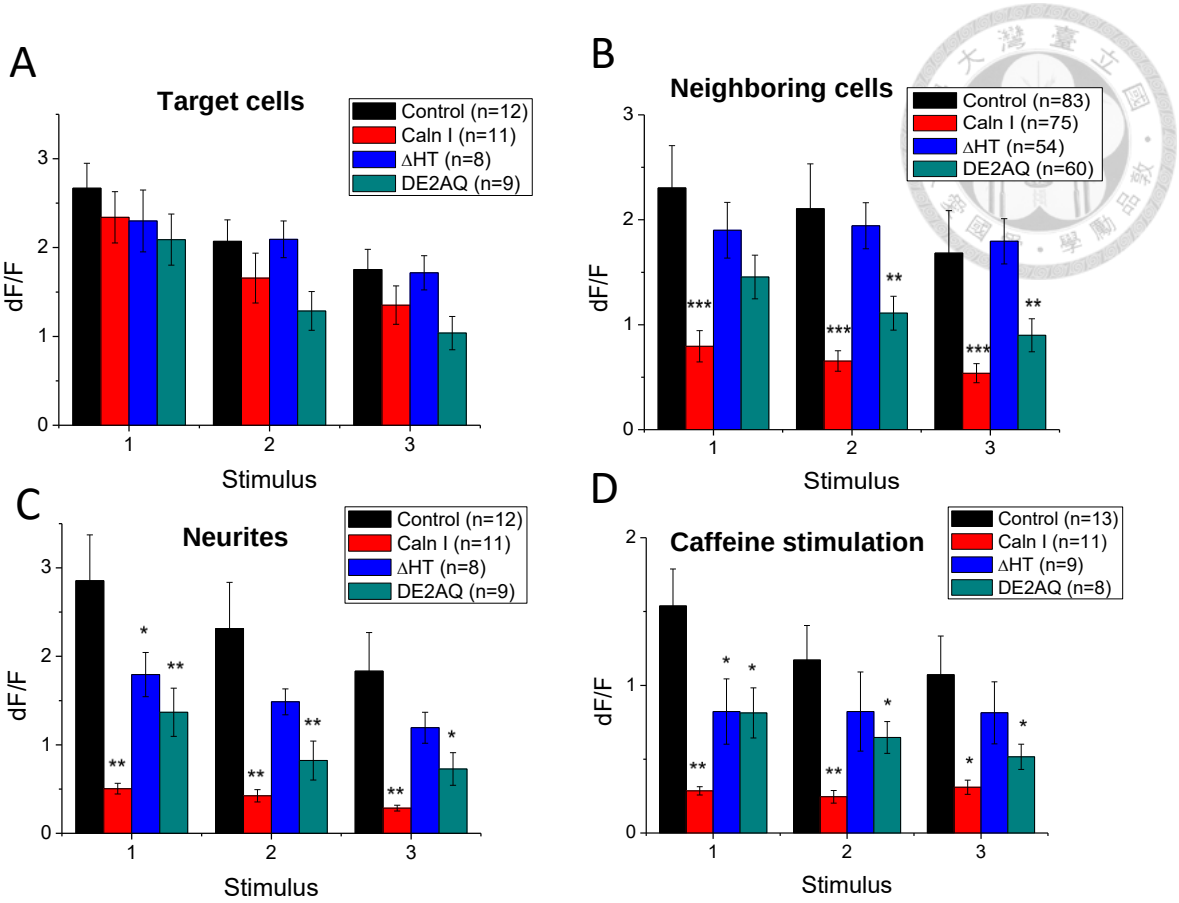


Fig. 3 CalnI interferes with CICR pathway.

The Ca^{2+} responses of primary cortical neurons stimulated by uncaging calcium (NP-EGTA) were presented as normalized elevation values of Fluo-2 fluorescence intensity ($\Delta F/F_{baseline}$). (A) The Ca^{2+} responses of target cells expressing CalnI, CalnIΔHT and CalnIDE2AQ respectively. (B) The Ca^{2+} responses in the neighboring neurons of the target cells in A. (C) The Ca^{2+} responses in the neurites of target cells expressing CalnI, CalnIΔHT and CalnIDE2AQ. (D) The Ca^{2+} responses of neurons stimulated by a caffeine puff (40 mM, 5 sec), the stimulation was repeated three times. Data were Mean±SEM, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ as analyzed by Student's *t*-test between control group and CalnI/ CalnI mutants group.

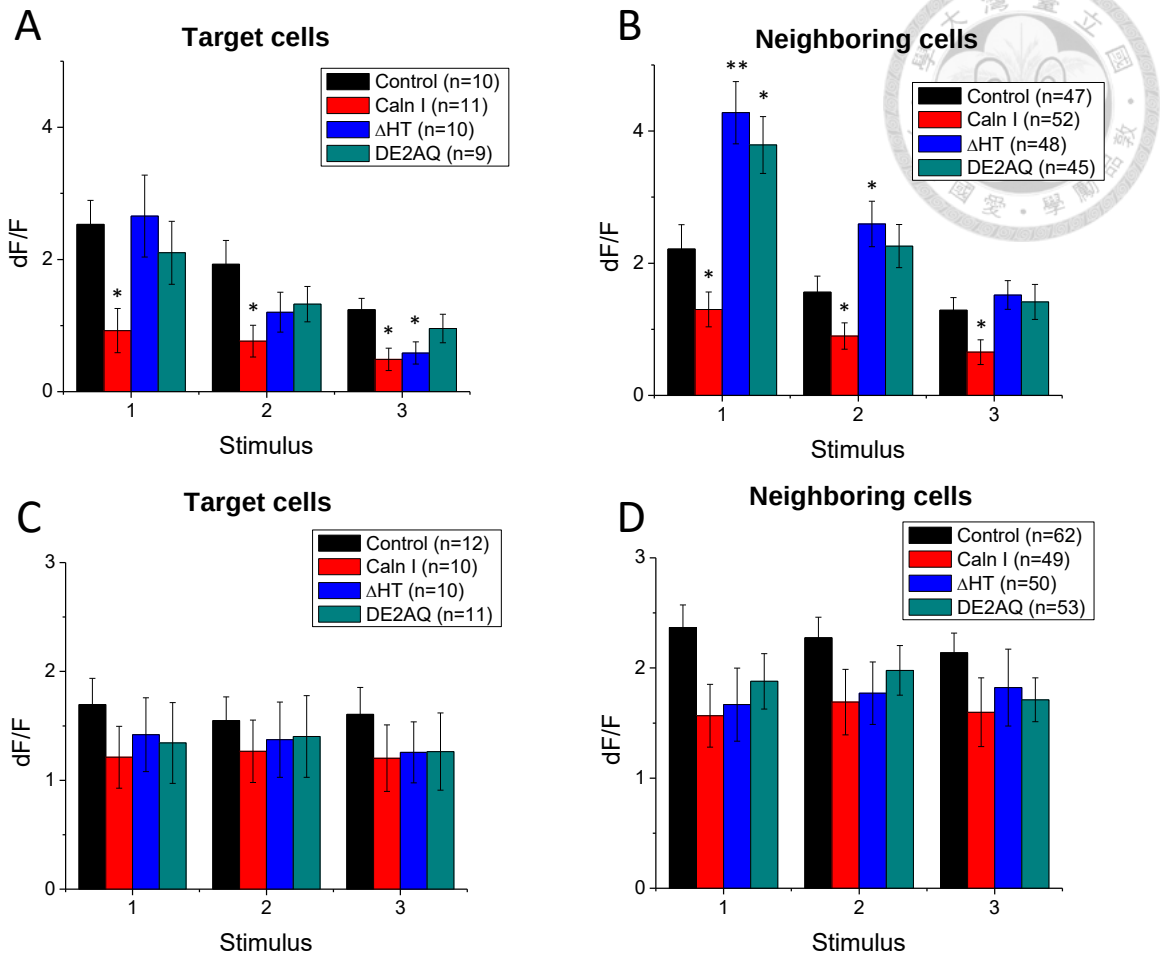


Fig. 4 CalnI inhibits mGluR signaling pathway.

The Ca^{2+} responses of primary cortical neurons stimulated by (A) a DHPG puff (20 μM, 1 sec) or (C) an AMPA puff (10 μM, 1 sec) were presented as normalized elevation values of Fluo-2 fluorescence intensity ($\Delta F/F_{baseline}$). The stimulation was repeated three times. (A) The Ca^{2+} responses of target cells expressing CalnI, CalnIΔHT and CalnIDE2AQ by DHPG stimulation. (B) The Ca^{2+} responses in the neighboring neurons of the target cells in A. (C) The Ca^{2+} responses of target cells expressing CalnI, CalnIΔHT and CalnIDE2AQ by AMPA stimulation. (D) The Ca^{2+} responses in the neighboring neurons of the target cells in C. Data were Mean±SEM, *: $p < 0.05$, **: $p <$

0.01 as analyzed by Student's *t*-test between control group and CalnI/ CalnI mutants group.

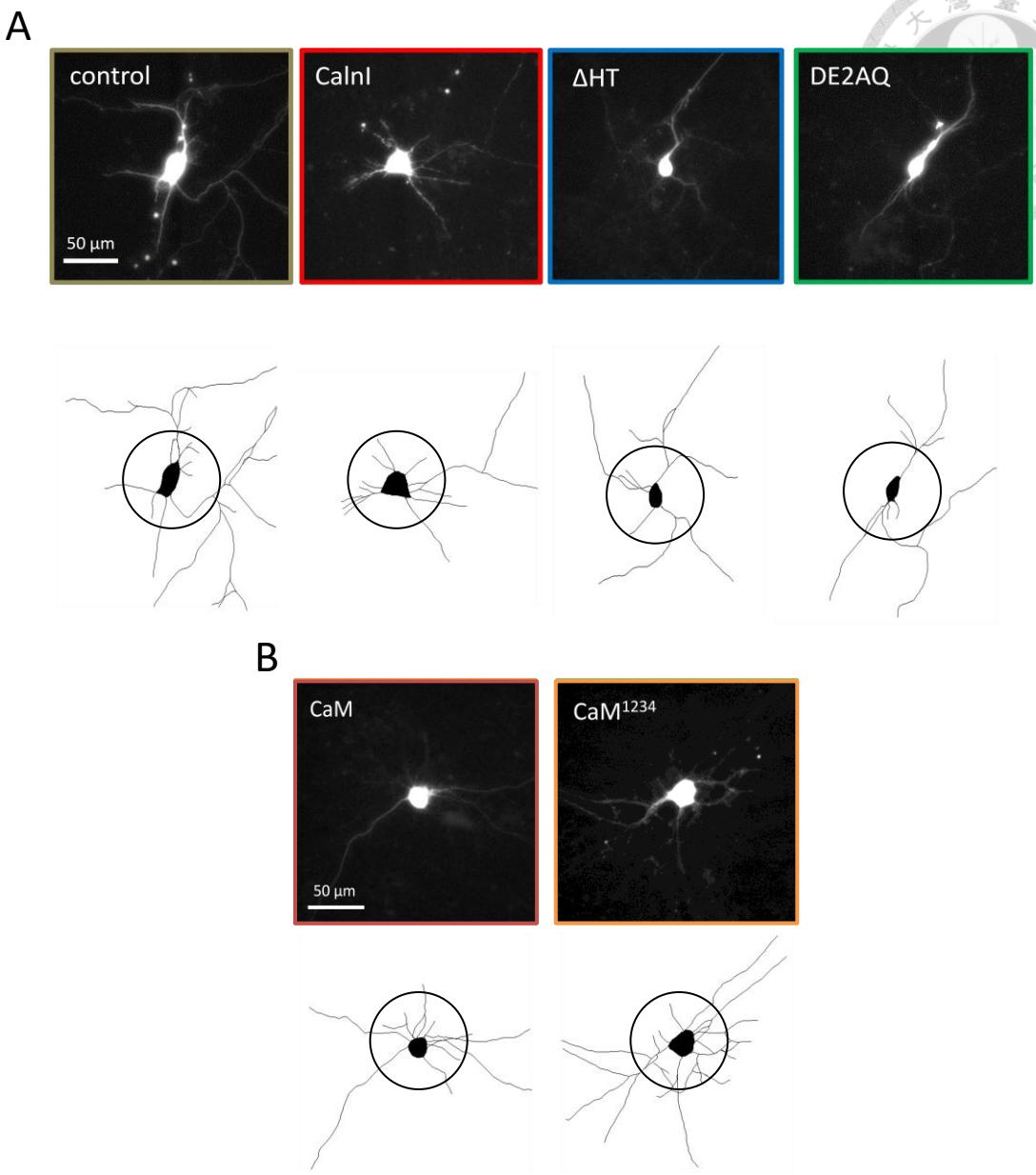


Fig. 5 Morphology of CalnI and calmodulin expressing neurons

(A) Fluorescence images of CalnI, CalnIΔHT and CalnIDE2AQ expressing neurons (coexpressing mCherry). (B) Fluorescence images of calmodulin (CaM) and CaM^{1234} expressing neurons (coexpressing mCherry). The lower panels in A and B are drawing traces of the upper panels. The traces are drawn with ImageJ (NeuronJ plug-in). The circles are drawn from the center of soma with radius equals to 30 μm.

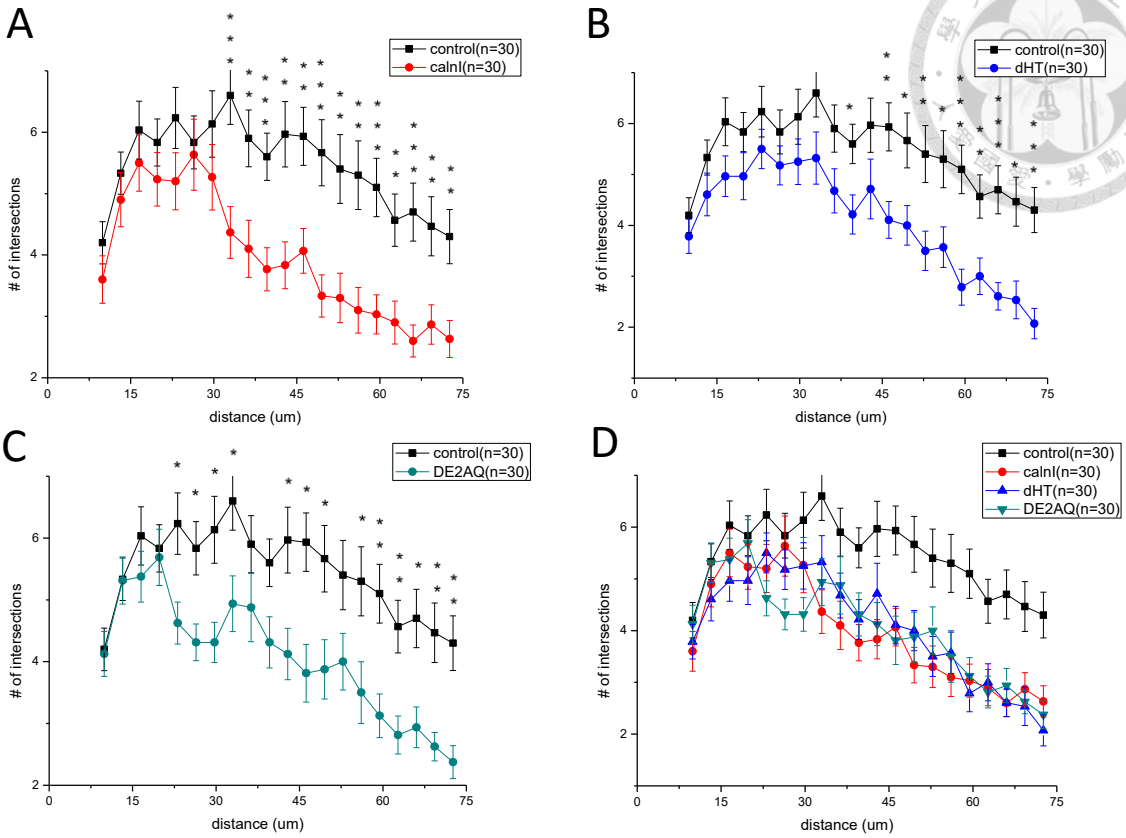


Fig. 6 CalnI reduces distal neurite number.

The morphological differences between CalnI-expressing and non-expressing neurons were analyzed with Sholl analysis. (A) The morphological differences between CalnI-expressing neurons and control group neurons. (B) The morphological differences between CalnIΔHT-expressing neurons and control group neurons. (C) The morphological differences between CalnIDE2AQ-expressing neurons and control group neurons. (D) The merge graph of A, B and C. Data were Mean±SEM, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ as analyzed by Student's *t*-test.

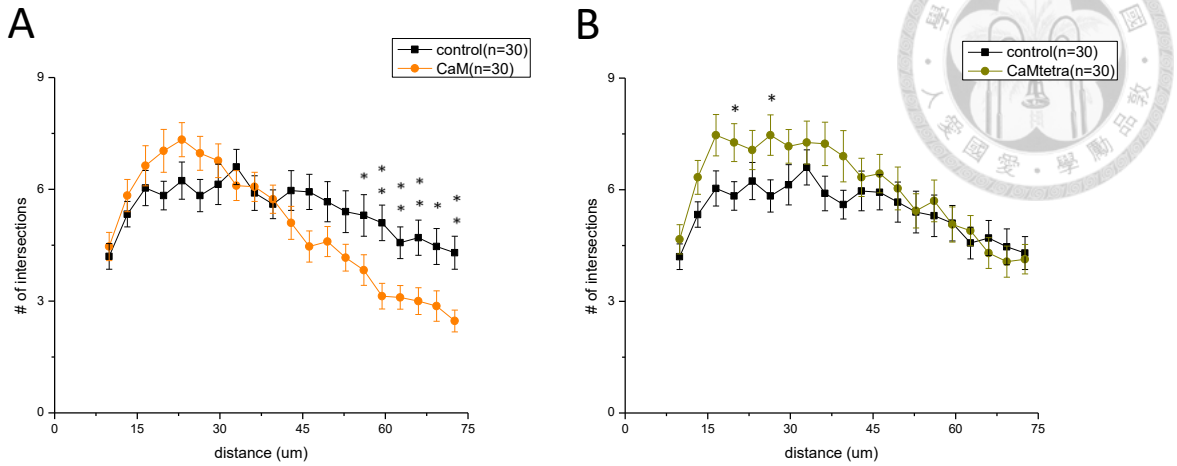


Fig. 7 Calmodulin and CaM^{1234} on neuronal morphology

The morphological differences between CaM-expressing and non-expressing neurons were analyzed with Sholl analysis. (A) The morphological differences between CaM-expressing neurons and control group neurons. (B) The morphological differences between CaM^{1234}-expressing neurons and control group neurons. Data were Mean±SEM, *: $p < 0.05$, **: $p < 0.01$ as analyzed by Student's *t*-test.

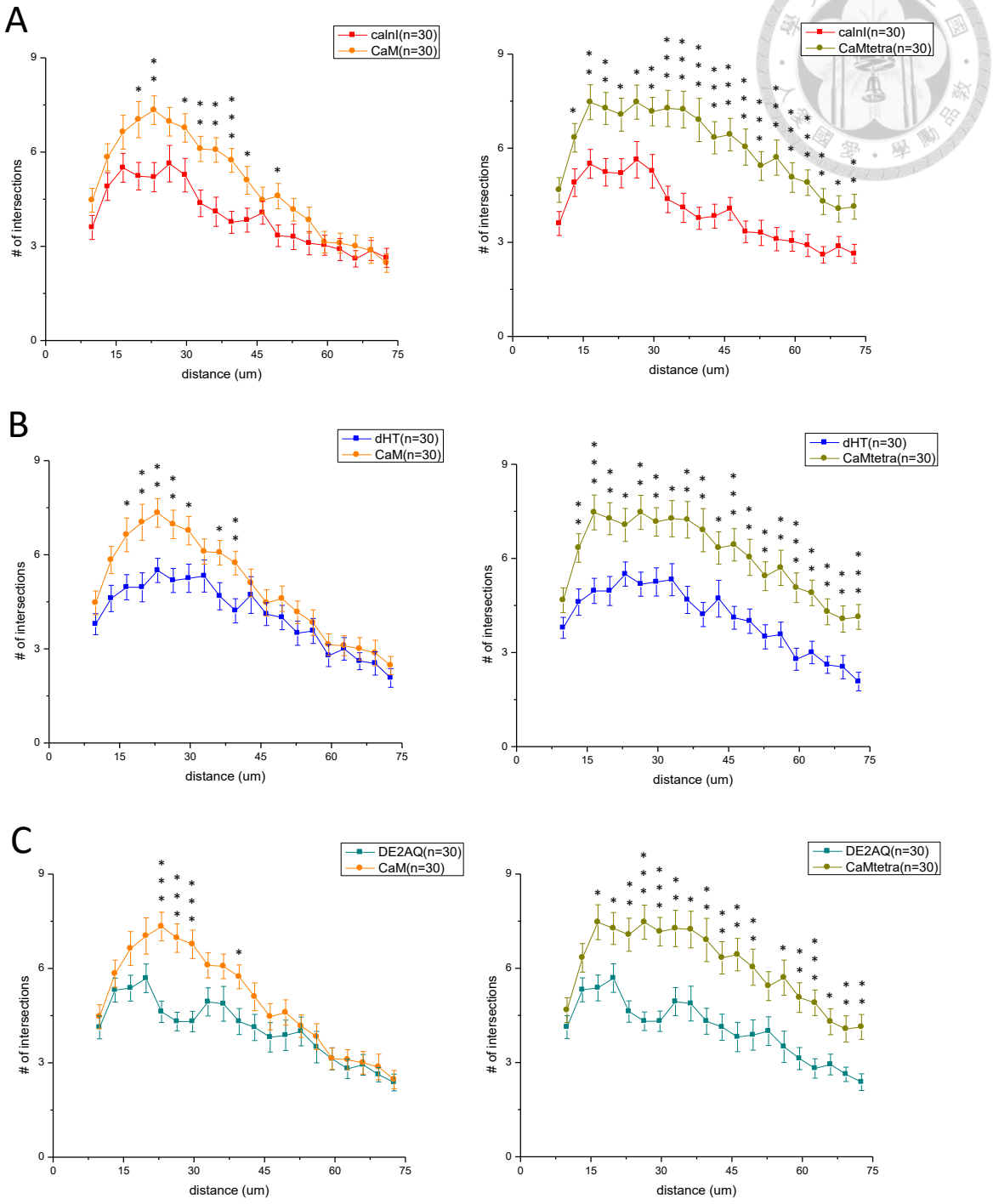


Fig. 8 CalnI and calmodulin expression causes different neuronal morphologies.

(A) The morphological differences between CalnI-expressing and left : CaM-expressing neurons; right: CaM^{1234}-expressing neurons. (B) The morphological differences between CalnIΔHT-expressing neurons and left: CaM-expressing neurons; right:

CaM^{1234}-expressing neurons. (C) The morphological differences between CalnIDE2AQ-expressing neurons and left: CaM-expressing neurons; right: CaM^{1234}-expressing neurons. The morphological differences were analyzed with Sholl analysis. Data were Mean±SEM, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ as analyzed by Student's *t*-test.

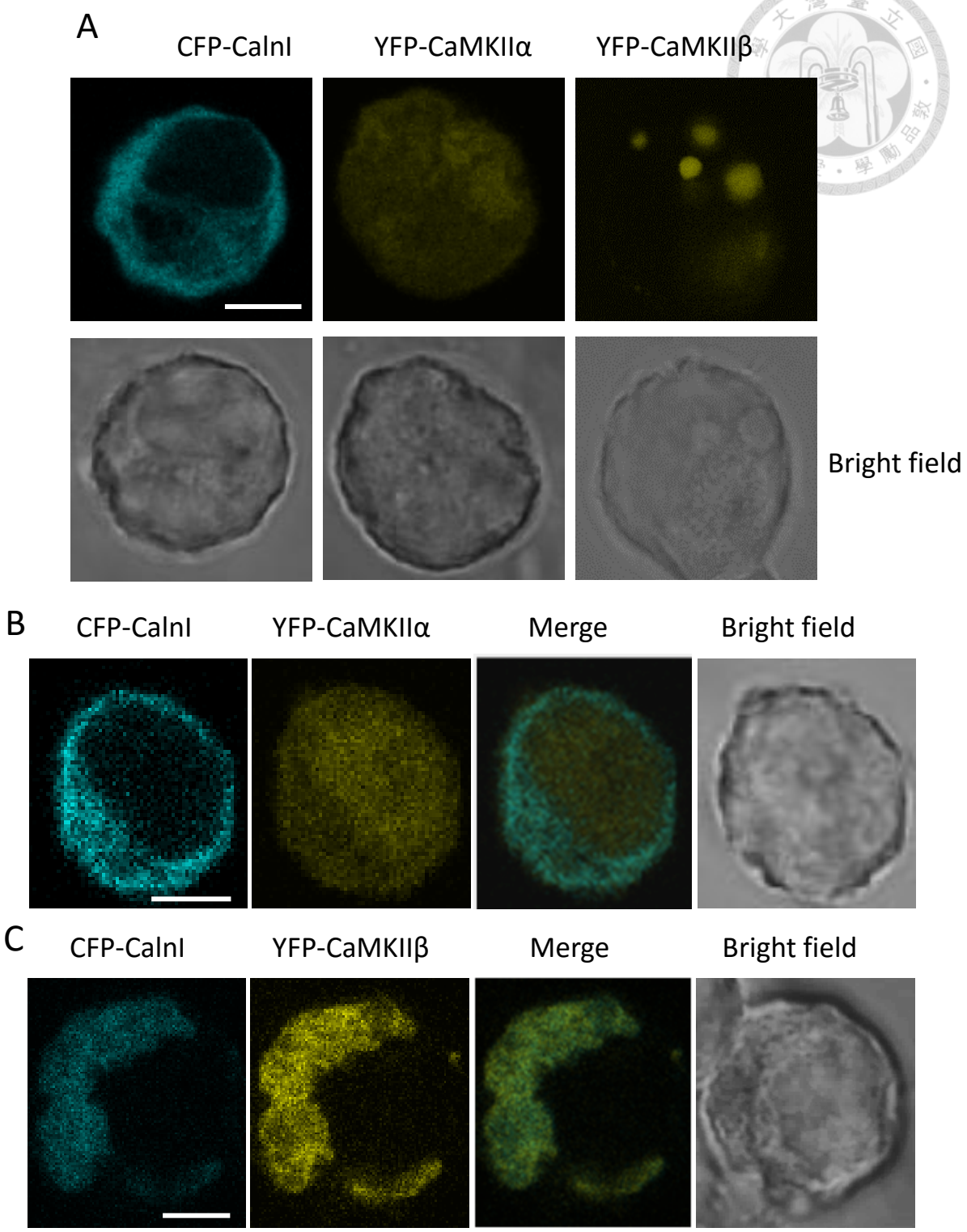


Fig. 9 CalnI affects the localization of CaMKIIβ in HEK293T cells.

CFP-CalnI and YFP-CaMKIIα/β expressing HEK293T cells were fixed by 4% paraformaldehyde. (A) Fluorescence images of cells expressing CFP-CalnI,

YFP-CaMKIIα and CaMKIIβ respectively. Lower panels are bright-field images. (B) Fluorescence images of cells coexpressing CFP-CalnI and YFP-CaMKIIα. (C) Fluorescence images of cells coexpressing CFP-CalnI and YFP-CaMKIIβ. The fluorescence images were visualized by confocal microscopy. Scale bar: 5 μm