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小鼠藍班核內分泌伽瑪-氨基丁酸之中間神經元之性質探討

Characterization of GABAergic Interneurons Within Locus Coeruleus Nucleus in Mice.

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本論文係郭昭成君（學號 R01B41004）在國立臺灣大學生命科學研究所完成之碩士學位論文，於民國 103 年 7 月 30 日承下列考試委員審查通過及口試及格，特此證明

指導教授：

口試委員：

系主任、所長

致謝

在撰寫這篇研究論文時，有感於科學研究的過程中付出的所有心力、提供實質協助與在背後默默支持我的夥伴，所以在這裡我必須要對他們表達感謝之意。感謝我的指導教授閔明源老師，他是我在大學部的導師，後來經過大學三四年級在老師實驗室見習的經驗後選擇進入老師的實驗室繼續修習碩士班學位，當初也不為別的，只是憑著喜歡做研究與學術環境的想法。另外這篇論文中運用了大量電生理紀錄與電子顯微鏡的技術，感謝瀚穎學長與中山醫學大學的楊琇雯老師教導我電生理與電顯的技術，幾經練習後變成了我實驗上有力的技術支援。最後還要感謝實驗室的夥伴，無論是目前在學的同學、學弟妹或是已經畢業的學長姐，在做研究的過程中感謝有你們的陪伴與幫忙，這篇碩士論文的促成也有你們的一份力。

中文摘要

藍班核作為大腦中主要的正腎上腺素分泌之來源，先前的研究證實伽瑪氨基丁酸此醫神經傳導物質在調控藍斑核神經元活性上扮演重要角色。在(非)快速動眼睡眠期藍斑核區內伽瑪氨基丁酸的濃度顯著上升，且藍斑核區域伽瑪氨基丁酸的突觸前結構約占總數的三分之二。我們實驗室先前的研究成果指出，伽瑪氨基丁酸可透過 B 型受體以提供大鼠藍斑核細胞一個長期性的抑制性調控，且這個調控可能與細胞間伽瑪氨基丁酸濃度有關。本研究中我們以電顯觀察大鼠藍斑核的伽瑪氨基丁酸 B 型受體分布情形，發現他們主要分布於突觸外的位置，可推得細胞間伽瑪氨基丁酸濃度將透過突觸外之受體調控藍斑核細胞。先前的研究指出許多核區作為藍斑核伽瑪氨基丁酸來源，但近來的研究指出藍斑核區域內的中間神經元可能擔任調控藍斑核細胞的角色，而這些分泌伽瑪氨基丁酸中間神經元的電生理性質與突觸生理性質仍然未被報告。本研究以全細胞電生理技術紀錄藍斑核內分泌伽瑪氨基丁酸之中間神經元的基礎性質，另以過氧化酶染色技術重建此些中間神經元的型態構造，發現可以放電情況將這些中間神經元分成三群，且在型態重建中發現伸入該核區內的軸突結構上有被認為可能是突觸的結構。而在電生理雙紀錄的實驗中卻沒辦法發現藍斑核細胞與中間神經元的單突觸連結關係。

關鍵詞:藍斑核、中間神經元、伽瑪氨基丁酸、伽瑪氨基丁酸 B 型受體、穿透式電子顯微鏡、全細胞雙紀錄。

Abstract

The Locus coeruleus (LC) is a noradrenergic brainstem nucleus that is the major norepinephrine (NE) supply to the forebrain. It has been shown that GABA appears to be involved in the regulation of LC. Several studies have reported that extracellular GABA concentration is higher during REM/NREM sleep than wakefulness and that about two-third of boutons within LC contained GABA. Previous data of our lab showed that GABAB receptors could mediate tonic inhibition of LC neurons in rats, and this effect may link to ambient GABA concentration. Subcellular localization of GABAB receptors of LC nucleus in rats had been examined and found that most functional GABAB receptors are at extrasynaptic site, so ambient GABA could mediate tonic inhibition via extrasynaptic GABAB receptors here. However, the GABA source for tonic inhibition of LC neurons is still unclear. Many GABA afferents other brain nuclei have been reported previously, while recent studies found that local GABAergic interneuron may be a novel candidate of GABA supply to LC nucleus. However, the synaptic property and the role of LC GABAergic interneurons remain unknown. Thus we characterized these interneurons by using whole cell recording, following by staining of recorded interneurons for morphology reconstruction. Here we divided them into three group by their firing pattern and

found some bouton-like structures on axons from the morphology reconstruction. But no functional pair has been found in the double patch experiments between LC neurons and GABAergic interneurons.

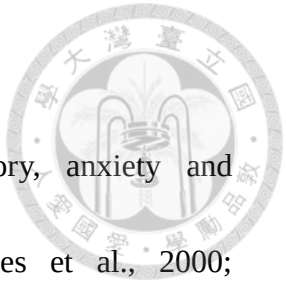
Keywords : Locus Coeruleus, interneuron, GABA, GABAB receptor, TEM, double patch whole cell recording.

目錄

Introduction

Locus Coeruleus

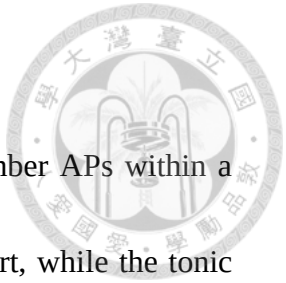
The Locus coeruleus (LC) consists of noradrenergic (NAergic) neurons and is located in the dorsal pons of the brainstem. In mammalian brain, each hemisphere of brainstem has one LC nucleus locates beneath the two edges of the 4th ventricle floor. The LC is divided into two parts, the LC proper and the peri-LC region. The LC proper contains mainly the cell bodies of NAergic neurons and lacks dendritic and axonal structures, so it looks region, in the fresh brainstem slice preparation under microscopy. The cell bodies of NAergic neurons in LC proper are large (~ 25 μm) and are in shape of multimodal, and are arranged in a dimension of rostroventral to caudodorsal. The Peri-LC is composed of dendritic field of NAergic neurons in LC proper; it occupies the rostral and ventral parts of LC area. The NAergic neurons in LC (referred as LC neurons) provide major norepinephrine (NE) supply to the forebrain. In addition, LC neurons also have divergent efferents to almost all levels of CNS, including cerebellar cortex and the spinal cord. (James et al., 1978; Foote et al., 1983; Aston-Jones et al., 1991; George 2004) The board projection pattern implies LC-NE system has global function in modulating brain function and behavior,



including including pain, sleep/wakefulness regulation, memory, anxiety and cognitive performance (Berridge and Foote, 1991; Aston-Jones et al., 2000; Aston-Jones and Cohen, 2005; Susan 2009). Disturbance of LC-NE system may lead to cognitive and arousal impairments and certain affective disorders, such as schizophrenia, narcolepsy, attention deficit/hyperactivity disorder (ADHD) (Paul and van Dongen 1981; Arnsten et al., 1996) and withdrawal symptoms (Rasmussen et al., 1990). Accordingly, LC becomes an appropriate target for pharmacological therapy of cognitive disorders related to dysregulation of LC-NE system (Berridge and Waterhouse, 2003).

Spontaneous firing activity of LC neurons

LC neurons spontaneously fire action potentials (APs) with frequency ranging from 0 to 5Hz, depending on the physiological states. The discharge rate of LC neurons fluctuates through the sleep-waking cycle; it was 3 Hz or more at waking period, only 1 Hz at (rapid-eye-movement) REM sleep and almost silent at non-REM sleep (Hobson et al., 1975; Aston-Jones and Bloom, 1981). The discharge patterns of LC neurons also vary between tonic and phasic, depending on different state of cognitive attention (Berridge and Waterhouse 2003). The phasic activity is defined as



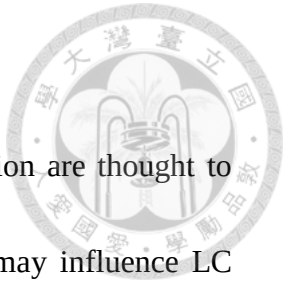
firing pattern that comprises burst of action potential with the number APs within a burst being at least 2 and the inter-AP interval being relatively short, while the tonic activity defined as discharging APs at constant frequency (Aston-Jones et al., 1999). Recording from awaking animals have shown that, generally, LC neurons show tonic activity with lower frequency when animals were at attentive state, tonic activity with higher frequency at distractive state and phasic activity in focus-attentive state (Aston-Jones et al., 2005). In support of these findings, recent study shows that arousal state could be affected via manipulating the activity of LC neurons in mice by using optogenetic method (Carter ME et al., 2010). At cellular level, the tonic and phasic firing activities of LC neurons may results in different profiles of ambient NE which may activate different subtypes of NE receptors (α and/or β) that having different affinity to NE and located at different (pre and/or postsynaptic) sites. For example, tonic activity has larger influence outside the synaptic cleft and phasic activity could offer more NE concentration in short time scale that corresponds to a novel or salient stimuli. (Sara and Bouret, 2012)

In pain regulation, LC neurons project noradrenergic fibers to the dorsal horn of spinal cord, and is a part of descending pain modulatory system (Basbaum and Fields

1984; Wei et al., 1999; Ren and Dubner 2002). After electrical or chemical stimulation of LC, noradrenergic fibers release NE into spinal cord to inhibit the neurons in dorsal horn and promote antinociception. This effect is shown to be mediated via α1/2 adrenergic receptor on the nociceptive circuit inspinal cord (Jones and Gebhart 1986; West et al., 1993; Millan 2002).

Afferent to Locus Coeruleus

In contrast to its wide-projected efferents, LC receives afferents from very restricted brain nuclei. The major inputs to LC are found in two medullary structures, the nucleus paragigantocellularis (PGi) of excitatory input and perifascicular area of nucleus preositus hypoglossi (PrH) of inhibitory. Other minor afferents to LC are found in the paraventricular hypothalamus, the lamina X of the spinal cord, the periaqueductal gray (PAG), the central amygdala, the dorsal raphe nucleus, and some hypothalamic nuclei and cortex areas (Cedarbaum and Aghajanian 1978; Astone-Jones et al., 1991). These afferent inputs release several neurotransmitters into LC, including glutamate, glycine, GABA serotonin, acetylcholine and catecholamines, substance P, enkephalin, and corticotropin-releasing factor (CRF) (Cedarbaum and Aghajanian 1978; Van Bockstaele et al., 1999). The amino acid neurotransmitters



(glutamate, glycine and GABA) mediating fast synaptic transmission are thought to offer direct control of LC neurons and other transmitter systems may influence LC neurons through regulation of glutamtergic, glycinergic and GABAergic synaptic transmission. In addition, LC neurons are well known to receive feedback autoregulation by NE (Jesse et al., 1976). Previous studies have reported that neuropeptides are often co-releasing with other amino acid neurotransmitters and provide longer modulatory effects on LC neurons (Van Bockstaele and Valentino, 2013). In summary, the activity of LC is modulated by the complicated interactions between these neurotransmitters (Van Bockstaele et al., 2010).

GABA : Major inhibitory neurotransmitter of LC

The neurotransmitter γ-aminobutyric acid (GABA) provides main inhibitory signal in the brain. Previous studies suggest that GABA serves as a major inhibitory drive onto LC neurons (Ennis & Aston-Jones, 1989). For example, the ambient GABA level in LC area is higher during REM/non-REM sleep than waking, which may be related to the activity level of LC in different sleep stages (Nelson et al., 2002). In addition, the ventrolateral preoptic nucleus (VLPO), neurons of which are active during REM sleep, has massive GABAergic projections to LC neurons. (Lu et al.,

2002). Finally, a ultrastructural study shows that about two-third of synapses within LC contain GABA (Somogyi and Llewellyn-Smith, 2001). Current evidences suggest that the majority of GABAergic inputs to LC are from the central amygdala nucleus (CAmy) and posterior lateral hypothalamic area (PLH) (Eugene et al., 2013).

GABA acts on two kinds of receptors, the GABAA and GABAB receptors. GABAA receptors are ionotropic receptors, activation of which could lead to an increase in membrane conducarance of chloride ion and usually could produce a hyperpolarized signal. There are 6 subunit families of GABAA receptor, nalmely the α, β, γ, θ, ρ and ε, and a functuonal GABAA receptor contains 5 subunits arranging into a common conformation od 2α-2β-x with x being a γ, θ, ρ or ε subuinit (Robert and Richard 1994). Accordingly, many isoforms have been found in different brain nuclei and surface location and with distinct properties (Robert and Richard 1994; Delia et al., 2009; Martin et al. 2012). Many studies have revealed that GABAA receptor in synaptic and extrasynaptic site show different properties and mediating different mode of inhibitions, the phasic and tonic classic (Stephen etal., 1999; Mathew and Robert 2000; Delia et al., 2009) The tonic inhibition from extrasynaptic GABAA receptors have been examined in many brain nuclei, (Farrant and Nusser, 2005; Glykys and

Mody et al., 2007; Delia et al., 2009) Tonic inhibition is of particular interest as it affects neural excitability. Interestedly, LC neurons express high level of ε subunit, which has self-opening property and is thought to carry tonic inhibition to LC neurons (Belujon et al., 2009).

GABAB receptors are metabotropic G-protein-coupled receptors. GABAB receptors are heterodimers, composed of two subunits, GABAB1a/1b and GABAB2. GABA could bind upon GABAB1 subunit and trigger the G-protein complex linked to GABAB2 subunit. It has been shown that GABAB receptor activation can result in hyperpolarization of postsynaptic neurons via activating the inwardly rectifying potassium channels (GIRK) and results in inhibition of transmitter release from axonal terminal via inhibiting presynaptic voltage-gated N/P/Q type calcium channels (Hille 2001; Terunuma et al., 2010). The subcellular localization of GABAB receptors has been revealed in many brain nuclei; these studies found that most GABAB receptors are located in extrasynaptic site at both pre- and post-synaptic elements (Á kos et al., 2003; Justin and Bolam 2003). Howevre, the subcellular distribution of GABAB receptors in LC nucleus is still unclear.

In NAerguc neurons of A7 area (Wu et al., 2011) and LC, GABAB receptors mediate tonic inhibition has been reported. These studies show GABAB receptor mediated tonic inhibition can directly tune the frequency of spontaneous AP rate of LC neurons in brain slices. Given the importance of LC neuron activity to the regulation of physiological states and behavior, it is important to explore more mechanisms underlying GABAB receptor mediated tonic inhibition of LC neurons. A most important question is that what is GABA source for this tonic inhibition of LC neurons?

GABAergic interneurons within LC

Apart from GABAerguc inputs from other brain area mentioned above, there is also a pool of GABAergic interneurons within LC (Aston-Jones et al., 2004). Previous electron microscopic studies report that many axonal terminals in LC area target on non-noradrenergic elements. It is thus suggested that some of the axonal terminals may make contacts upon interneurons in LC area and serve as an interface of signals input to LC neurons (Van Bockstaelea et al., 1998). For example, it is proposed that the rostral agranular insular cortical (RAIC) projection may modulate the activity of LC neurons via local GABAergic interneurons to exert their pain-regulation function

(Jasmin et al., 2003). Howerver, the synaptic property and the physiological role of GABAergic interneurons in regulation of LC principle neurons are still unclear.

Objectives

In this study, I first checked the subcellular distribution of GABAB receptors in LC. Second, I wish to investigate the property of GABAergic interneurons within LC. By using a transgenic mice line, GAD67-EGFP mice, I recorded the green fluorescence of GABAergic interneurons within LC under in acute brain slice. I characterized the basic firing and membrane properties of these LC GABAergic interneuron pool. According to these properties, I grouped these interneurons into groups. I found some of these GABAergic interneurons project axon with many bouton-like structure into LC nucleus.

Material and Method

Animals

The use of animals in this study was in accordance with the guidance of the Ethics Committee for use of Experimental Animal for Research of National Taiwan University, and all procedures were complied with the European Communities Council Directive (24 November 1986). In electron microscopic (EM) experiments, Sprague Dawley (SD) rats were perfused at age of 1 month following by the electron microscopic experiment procedures. Heterogeneous GAD67-GFP (Δneo) knock-in mice (Tamamaki et al., 2003) were used to target GABAergic neurons. GAD67-GFP mice and wild type C57BL6 mice were decapitated for slice preparation on postnatal day (PN) 14-23 for electrophysiological experiments. For the experiments of immunohistochemistry (IHC) , GAD67-GFP mice were perfused at age of 1 month.

Preparation of brainstem slices

The animals were anesthetized with 5% isoflurane and decapitated, then their brains were rapidly exposed and chilled with ice-cold artificial cerebrospinal fluid (ACSF) consisting of (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 26.2 $NaHCO_3$, 1

NaH_2PO_4, 2.5 $CaCl_2$, and 11 glucose, oxygenated with 95% O_2 and 5% CO_2, pH 7.4. Brainstem slices (300 μm) containing the LC were prepared by using vibroslicer (D.S.K. Super Microslicer Zero 1, Dosaka EM, Kyoto, Japan) and were maintained in an moist air-liquid (ACSF) interface chamber then allowed to recover for at least 90 minutes before being transferred to an immersion chamber mounted on an upright microscope (BX51WI, Olympus Optical Co., Ltd., Tokyo, Japan) for recordings. Slices were cut in the sagittal orientation for double patch experiments and in horizontal orientation for other experiments. Throughout the recording period, they were perfused at 2-3 ml/min with oxygenated ACSF.

Electrophysiology

Neurons were viewed using Nomarski optics, the GFP signal was activated by opto-LED system (CAIRN Research Limited, UK) and observe via fluorescence filter cube equipped on the microscopy. Patch pipettes were pulled from borosilicate glass tubing (1.5 mm outer diameter, 0.32 mm wall thickness; Warner Instruments Corp., Hamden, CT, USA) and had a resistance of about 3-5 MΩ when filled with the pipette solutions. For all experiments for GABAergic interneuron recordings, the pipette solution consisted of (in mM): 131 K-gluconate, 20 KCl, 10 HEPES, 2 EGTA, 8 NaCl,

2 ATP, and 0.3 GTP; pH adjusted to 7.2 with KOH. In double patch experiments, the K-gluconate was replaced with an equimolar concentration of KCl, respectively. Recordings were made at in whole-cell configuration with a patch amplifier (Multiclamp 700 B; Axon Instruments Inc., Union City, CA, USA). For current-clamp recording, the bridge was balanced and recordings were only accepted if the recorded neuron had a membrane potential (Vm) of at least -45 mV without applying a holding current and if the AP was able to overshoot 0 mV. For voltage-clamp recordings, the serial resistance was monitored throughout the recording and the data discarded if the values varied by more than 20 % of the original value, which was usually less than 20 MΩ. Signals were low-pass filtered at a corner frequency of 2 kHz and digitized at 10 kHz using a Micro 1401 interface running Signal software for episode-based capture and Spike2 software for continuous recording (Cambridge Electronic Design, Cambridge, UK). All data are presented as the mean ± standard error of the mean (SEM) and were compared using the Student's t test, paired t-test, one-way ANOVA and two-way ANOVA. Bonferoni's test was used when significant group-dependent differences were observed. The criterion for significance was a p value < 0.05.

Immunohistochemistry

GAD67-GFP mice were anesthetized with 5% isoflurane and then trichloroactetoaldehyde (10mg/kg) was given. The animals were perfused by fixative containing 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH7.4. After post-fixation overnight in same fixative at 4℃, the brains were turned to 0.1M PB with 30% sucrose for 2 days. Then sagittal frozen sections of brainstem were made by sliding microtome (HM430, Thermo, Waltham, MA, USA). For further staining, the slices were rinsed with PB and phosphate-buffered saline (PBS) several times and were incubated in PBS containing 0.03% Triton X-100 (PBST), 2% bovine serum albumin (BSA) and 10% normal goat serum (NGS) for an hour at room temperature. Then the slices were incubated overnight at 4℃ in PBST containing a 1/1300 dilution of rabbit antibodies against tyrosine hydroxylase (TH) (Merck Millipore, cat.#AB5986P, Darmstadt, Germany). After PBST rinses, they were incubated for 2 hours with 1/200 dilution in PBST of tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove, PA, USA). All specimen of fluorescent staining were observed under a fluorescence microscope (Aioplan 2, Zeiss, Oberkochen, Germany) or a confocal microscope (Leica TCS SP5, Hamburger, Germany).

Immunohistochemistry after recordings

In most electrophysiological experiments, 6.7 mM biocytin was included in the pipette solution to fill the recorded neurons. The detailed procedures of IHC for identification of biocytin-filled neurons have been described previously (Min et al., 2008). After double patch experiments, the slices were fixed overnight at 4℃ in 4% paraformaldehyde in PB. After many rinses of PB and PBS, slices were incubated in 2% BSA and NGS in PBST for an hour at room temperature. And the slices were incubated overnight at 4℃ in PBST containing a 1/1300 dilution of rabbit antibodies against TH (Merck Millipore, cat.#AB5986P, Darmstadt, Germany) and 1/100 dilution of mixture of avidin, biotin and avidin-AMCA (Vector Laboratories, Burlingame, CA , USA). After PBST rinses, they were incubated for 2 hours with 1/200 dilution of TRITC-conjugated goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove, PA, USA) in PBST, then observed under fluorescence microscope and confocal microscope (Leica TCS SP5, Hamburger, Germany).

Pre-embedding immunocytochemistry and electron microscopic techniques

For EM experiments, SD rats were anesthetized with 5% isoflurane and then trichloroactetoaldehyde (10mg/kg) was given. A fixative containing 4%

paraformaldehyde and 0.1% glutaraldehyde in PB was perfused via cardiovascular system observation . Then the slices were fixed overnight at 4℃ in same fixative nad pre-embedding IHC method was used. Sagittal sections containing LC (70μm thick) were cut by using Vibratome (Ted Pella, Inc, CA, USA) and were equilibrated in a cryoprotectant solution containing 25% sucrose and 10% glycerol in 0.05M PB. To enhance the tissue penetration of reagents, the freeze & thawed process was made by freezing in liquid nitrogen cooled isopentane, followed by liquid nitrogen, and thawed in PBS. The sections were incubated for 1 hour in PBS containing 2% BSA and 10% NGS, then with anti-GABAB receptor antibody (Merck Millipore, cat.#AB2256, Darmstadt, Germany) in PBS for 24 hour at 4℃. After rinses of PBS, the sections were incubated for 16-18 hours with 1/100 dilution of gold (1.4 nm)-conjugated anti-guinea pig IgG (Nanoprobes Inc., Stony Brook, NY, USA), then after several rinses in PBS, the slices were fixed with 2% glutaraldehyde in PB for 10 min, followed by sliver enhancement of gold particles with an HQ Sliver kit (Nanoprobes Inc). The sections were then treated with 1% osmium tetraoxide in PB, dehydrated in graded series of ethanol and flat embedded on glass slides in Durcupan (Fluka) resin. Serial ultrathin sections (70 nm) of LC region were cut and collected on single-slot copper grids (Agar Scientific, UK) coated with 0.5% Formvar, stained with saturated

uranyl acetate in water followed by lead citrate, and examined with a JOEL JEM-2000 EXII electron microscope.

Synaptic structures were identified by the presence of synaptic cleft between a restricted zone of parallel presynaptic and post synaptic membrane and associated postsynaptic thickness or the accumulation of synaptic vesicles in the presynaptic element (Peters et al., 1991). Furthermore, the synaptic junctions were classified as asymmetrical when a prominent dense material was seen on the cytoplasmic adjacent to the postsynaptic membrane and as symmetrical ones when a less prominent density was seen (Peters et al, 1991).

Drugs and chemicals

All chemicals used to prepare the ACSF and pipette solution and paraformaldehyde were from Merck (Frankfurt, German); kynurenic acid, picrotoxin, strychinine, baclofen, biocytin and glutaldehyde were from Sigma (St. Louis, USA). CGP54626 hydrochloride (CGP) was form Tocris- Cookson (Bristol, UK).

Result

Subcellular localization of GABAB receptors of LC nuclei in rat

The pre-embedding method of IHC was used for EM observation of surface distribution of GABAB receptors in LC area. The primary antibody used recognizes the GABAB1 subunit (GABABR1) of GABAB receptor, and its specificity has been previously described (Dvoryanchikov et al., 2011). Here the results of IHC being specific to the use of primary antibody was also confirmed by omitting the primary antibody during staining procedures, and no GABABR1-immunoreactive (ir) gold particles was observed in this practice (Fig. 1A). Including primary antibody during IHC staining showed that GABABR1-ir gold particles were present at presynaptic terminals of both symmetric and asymmetric synapses (Fig. 1B). They were also found at the postsynaptic elements of these two types of synapse, either at synaptic (Fig. 1C) and peri-/extrasynaptic sites (Fig. 1B-E). Those gold particles located within synaptic active zone were classified as at synaptic site (see symbols in Fig. G); those located outside but in vicinity of less than 50 nm of synaptic active zone were classified as perisynaptic, and those located far away from synaptic active zone by more than 50 nm were classified as extrasynaptic. In addition to on membrane surface,

GABABR1-ir gold particles were also found on the membrane of cytosolic organelles, some of these organelles located beneath the synaptic or perisynaptic active zone (Fig. 1E). Of GABABR-ir gold particles that locate near synaptic structures, 34.9% of them are at presynaptic terminals (19.8 % on the plasma membrane and 15.1 % on membrane of cytosolic organelles) and 65.1% at postsynaptic elements (29.8% on the plasma membrane, 35.3% on membrane of cytosolic organelles). Of the 29.8% on the postsynaptic membrane, 2.71% are at synaptic site, 5.04% at perisynaptic site, and 22.09% at extrasynaptic site (Fig. 1F). These EM observations show that most of the functional (surface) postsynaptic GABABRs are at peri-/extrasynaptic sites in the LC area, and are consistent with previous data of our lab showing that tonic inhibition of LC neurons are mediated by ambient GABA acting at peri-/extrasynaptic GABAB receptors.

Distribution of GFP positive neurons in locus coeruleus area

According to (Franklin et al., 1997), LC locates in the dorsal pons near the conjunction of brainstem and cerebellum. (Fig. 2A) In sections subject to IHC using anti-TH antibody taken from animals perused with 4% paraformaldehyde, (Fig. 2B) there were many GFP-positive neurons with somata diameter of ~10-15 μm in LC

proper and peri-LC areas using confocal microscopy (Fig. 2C,D). Many GFP-positive neurons were located in peri-LC arera cover area rostral to the LC proper (Fig. 2D). In many previous studies which also use the same strain of heterogeneous GAD67-GFP mice (Tamamaki et al., 2003; Corteen et al., 2011; Eugene et al., 2013;), GFP-positive neurons have been confirmed to be GABAergic interneurons in all brain areas examined. Accordingly, GFP-positive neurons within LC and peri-LC area were considered as GABAergic interneuron pool of LC. Lots of GFP-positive punctuates were found in LC nucleus, (Fig. 2E) and some of them were contacting on soma and dendrites of LC neurons; these punctuates were supposed to be GABAergic terminals (Fig. 2F). But we cannot distinguish process of GABAergic interneurons from fibers of other GABAergic afferents.

Both GABAA receptors and GABAB receptors mediate tonic inhibition of LC neurons in mice.

In order to characterize GABAergic interneurons within LC and their possible role in GABAB receptor mediated tonic inhibition of LC neurons, GAD67-GFP mice were used. However, the previous data of our lab was obtained in rat, so I first perform experiments to test whether GABAB receptors mediate tonic inhibition of LC

neurons was also true in mice. Acute brainstem slice were cut from wild type C57BL6 mice, LC neurons were recorded in cell-attached recordings. This method was used as GABAB receptors are G-protein coupled receptors and regulate neural activity through cascades of cell signal pathway, so it is important to use cell attachment recordings for preventing the washout of cytosolic containing by pipette solutions in long-time recording. LC neurons showed the spontaneous firing rate (SFR) at ~ 1 hz, ranging, from 0-3 Hz) during cell attachment recordings (Fig. 3A). The SFR showed increasing upon application of kynurenic acid (KA) and strychinine (STR), an ionotropic glutamate antagonist and a glycine receptors antagonist, however the effect of KA/STR application on SFR was not significant (Fig. 3B, trace 1 and trace 2, paired t-test, $p > 0.05$, n = 5 neurons). Application of picrotoxin (PTX), a GABAA receptor antagonist, enhanced the SFR by about 30%. (Fig. 3B, trace 2 and trace 3, paired t-test, $p < 0.05$, n = 5 neurons), and subsequent application of CGP54626, a GABAB receptor antagonist, further increased the SFR by 30 % (Fig. 3B, trace3 and trace4, paired t-test, $p < 0.05$, n = 5 neurons); the effect of PTX and CGP could be almost washed by normal ACSF. The summarized results showed the changes of SFR in application of different antagonists (Fig. 3C/D). Basing on the results, I conclude that both GABAA and GABAB receptors mediate tonic inhibition of LC neurons in

mice, and both receptors have similar scales of tonic inhibition on the SFR of LC neurons.

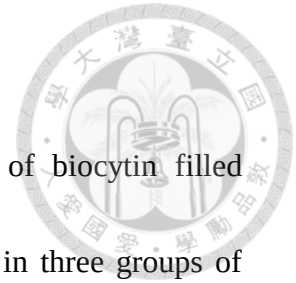
Whole-cell recordings for GFP-positive neurons within LC

In addition to fixed section, GABAergic (GFP-positive) neurons in LC could also be identified for recording in freshly prepared brain slice. Figure 4 illustrated how LC and GABAergic interneuron was identified. Under DIC microscopic video with 10x objective lens, the 4th ventricle (4V) and the conjunctions of cerebellum and pons was first identified (Fig. 4A). In some case, fibers of superior cerebellar peduncle (SCP) could be preserved and also identified. The LC was then identified as a transparent oval-shape nucleus located near the 4th ventricle and the conjunctions of cerebellum and pons (George 1997). By changing the objective lens to 40x one, many 20-30μm LC neurons could be clearly viewed. By switching the optic systems of the microscope from DIC to fluorescent, GABAergic interneurons (GFP-positive neurons) were identified by fluorescent signal and targeted for whole-cell recording (Fig. 4B). A total of 48 neurons were recorded, most of them were at LC proper and between the LC neurons. The recorded GABAergic interneurons were also filling with biocytin for further identification of their location and morphology. A total number of 16 neurons

were successfully recovered for morphological examinations as shown in Fig. 4E.

The firing characteristics of LC GABAergic interneurons

The recorded interneurons could be divided into three subtypes according to the firing pattern; namely the type I, tonic firing; the type II, phasic firing and the type III, adaptive firing. Fig. 4C shows a representative voltage (current-clamp) recording from a type I interneuron responding to injection of current steps from 0 to 120 pA (20 pA/step) with Vm held at -60 to -65 mV. Examples of recordings form the three different subtypes of interneurons with the same experimental protocol are shown (Fig. 4D). The type I interneurons fired AP continuously in response to the depolarizing current steps; they accounted for 58.3% (28/48 neurons) of all interneurons recorded. About a half of the type I interneurons showed spontaneous firing (48.2%, 13/27 neurons). The type II interneurons fired AP phasically in response to depolarized current step; they contributed only 12.5% (6/48 neurons) of total interneurons recorded, and only 16.7% of them showed spontaneous firing. The type III interneurons fired single or only few APs immediately when the current step was given, then remained silent throughout the rest part of depolarized current step; they contributed only 29.2% (14/48 neurons) of total interneurons recorded, and 28.6% of



them showed spontaneous firing. Fig. 2E shows the mapping of biocytin filled neurons (n=16) after staining, there is no preference for location in three groups of interneurons.

The membrane properties of LC GABAergic interneurons

The resting membrane potential of three groups of interneurons was about -50mV. About 58.3% of type I interneurons, 12.5% of type II interneurons and 29.2% of type III interneurons fired spontaneously, and the action potentials were halted by a little current injection. Then the membrane properties were tested via test protocols in Fig. 5 with the membrane potential held at -60 to -65mV. Including input resistance, membrane time constant (τ), AP threshold, rebound after hyperpolarization and delay firing, the detail results of membrane properties were plotted in Table. 1. One-way ANOVA following by bonferoni's test was made in all results, found only that type I interneurons is higher significantly than type II and type III in input resistance. ($p<0.05$) No obvious rectification properties presented in the I-V curves for analysis of input resistane. For τ and AP threshold, no significant difference was found, and values of type II interneurons were not different significantly from type I and type II interneurons, possibly because of the smaller n number of recorded type II

interneurons. About 44.4% of type I interneurons presented property of delay firing, only 16.7% and 14.3% of type II and type III interneurons showed this feature. Interestingly, only type I interneurons presented property of rebound after hyperpolarization.

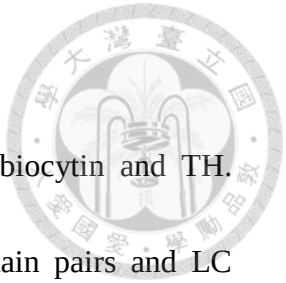
GABAergic interneurons project axons with bouton-like structure into LC

The morphology of a type I interneuron is shown as an example of reconstructions after recording. (Fig. 6, A) This neuron had oval soma, four primary dendrites, and a short axon without any axon collateral. Under 100x magnification, many swollen, bouton-like structures suggestive of varicosities were also found. (Fig. 5, B) Fig. 5C shows morphology of a type III interneuron, bouton-like structures were also found in both dendrite and axon. Axonal structure of this neuron was more complex, it seemed to make some contacts upon LC neurons. (Fig. 5, D) Many interneurons formed the swollen structures on their dendrites, and it may function as receiving synaptic inputs. Fig. 5E shows another type III interneuron with longer axon projection into LC, bouton-like structures were also found. (Fig. 5F) All structure of these interneurons formed inside the LC nuclei and projected axon with simple morphology into LC nucleus. The axons of interneurons may be cut after acute brain

slices were made, but we suggest local GABAergic interneurons within LC do project axon into LC area by the consistent finding of axonal projection and bouton-like structures. Furthermore, by using fluorescent dye to label recorded interneurons and LC neurons, we tried to answer the question of whether these GABAergic interneurons form functional synapses upon LC neurons. (Fig. 5A, 5B), Confocal microscopic photos show that LC neurons and the process of GABAergic interneurons are very close spatially in some certain structures as candidates of functional contacts (Fig. 5,C, D) However, it is hard to confirm the functional contact under this experimental design, the presence of contacts from GABAergic interneuron to LC neuron and the synaptic property of these synapses remained unclear.

No functional pair could be found by double experiments between LC neuron and GABAergic interneuron.

The morphological data reveals that GABAergic interneruons inside LC nucleus seem to project axons into LC. However, there is no direct evidence of such connection, so we examined double patch experiments between LC neuron and these interneurons. (Fig. ,5A to 5C) The dye was loading into recorded LC neurons and positional

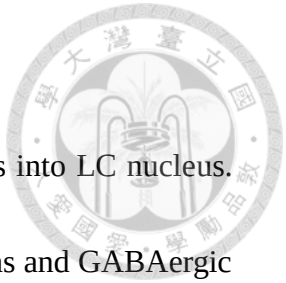


information was obtained by using fluorescent IHC staining of biocytin and TH. (Fig. ,5D to 5G) Then reconstructed the relative location of certain pairs and LC nucleus via combination the fluorescent staining and photos from DIC microscopic videos. (H) The traces of both recordings were triggered by action potential of interneurons, and the synaptic events within 5 ms window after interneuron firing were judged as functional pairs. (I) Many synaptic events were found during the recordings. (J) An example of failed pair of double patch experiment, at least 1000 frames were averaged for the trace of both recorded neurons. Total 50 failed pairs indicate that it is very hard to find direct connection between GABAergic interneuron and LC neuron under this experimental design. All of the pairs and their locations are displayed in Fig. 5I. Based on the morphological data which is showed, the axon of interneuron could be cut during slicing process. Or the interneuron has no direct contact upon LC neuron, it may form contact upon other interneurons even serve as projection neuron to other brain nuclei.

Discussions

The main findings of this study are as follows. First, we revealed the subcellular distribution of GABAB receptors of LC nucleus in rats, found that most of GABAB receptors were located at extrasynaptic site or on the membrane of cytosolic organelles. Combining with previous electrophysiological data, confirm the hypothesis that ambient GABA concentration mediate tonic inhibition of LC neurons in rats. But the pharmacological experiments showed that both GABAA and GABAB receptor contribute tonic inhibition of LC neurons in mice.

In order to find the GABA supply for this tonic inhibition, we tried to characterize a novel candidate of GABA afferent, the local GABAergic interneurons of LC nucleus. We targeted the LC GABAergic interneurons by using a line of GAD67-GFP mice. The confocal images showed that GFP signals express in whole cell of LC GABAergic interneurons, and the pattern was similar to previous researches (Ritchie et al., 2008, Eugene et al., 2013). By using whole cell recording technique, we found that these interneurons could be divided into three groups according to their firing patterns, and the membrane properties were also reported. Reconstructed morphology of recorded interneurons showed that they consistently



project axon in simple morphology and some bouton-like structures into LC nucleus. Finally, it is very hard to find the functional pair between LC neurons and GABAergic interneurons by using the experimental design of double patch recordings.

EM observations of GABAB receptors.

Previous EM studies showed that most GABAB receptors are located at peri-/extra synaptic sites in many brain nuclei and GABAB subunit2 are located on the membrane in preference, and many GABAB subunit1 are on the membrane of cytosolic organelles. (Justin & Bolam 2003, Á kos et al., 2003; Lacey et al., 2005) Functional GABAB receptors are heterodimers of two subunits, logically the GABAB subunit1 on the plasma membrane are more likely to be functional ones. So we stained GABAB subunit1 but not subunit2 to examine the subcellular distribution of GABAB receptors in LC. Our EM observation is consistent with these features, the results showed that rare GABAB receptor-ir gold particles are at synaptic and perisynaptic site in both pre- and postsynaptic elements. And many gold particles are on the membrane of cytosolic organells.

Tonic inhibition of LC neurons in rats and mice.

The tonic inhibition from extrasynaptic GABAA receptors have been examined in many brain nuclei, (Farrant and Nusser, 2005; Glykys and Mody et al., 2007; Delia et al., 2009) Tonic inhibition is of particular interest as it affects neural excitability. But our previous data indicated that GABAA receptors do not seem to mediate tonic inhibition of LC in rats. While both GABAA and GABAB receptors mediate tonic inhibition of LC in mice. This phenomenon may due to difference between species, even rat and mice are close in taxonomy, it is interesting that LC neurons present similar function and features but with different inhibitory modulation in these two species. LC neurons fire spontaneously to maintain NE concentration in cerebral cortex and many brain nuclei and the firing rate may relate to wake-sleep cycle and cognitive states (Astone-Jones 1981, 1999; Berridge and Waterhouse 2003). The inhibitory modulation plays an important role in regulation of LC neurons, including autoregulation through alpha adrenergic receptors (Jesse et al., 1976) and GABA afferent inputs (Cedarbaum et al., 1978). Disturbance of inhibitory regulation of LC neurons may cause affective disorders and withdrawal symptoms (Paul and van Dongen 1981; Rasmussen et al., 1990; Arnsten et al., 1996). For GABAA receptors, many isoforms have been found in different brain nuclei and surface location and with

distinct properties (Robert and Richard 1994; Delia et al., 2009; Martin et al. 2012). LC neurons express high level of ε subunit, GABAA receptor isoforms containing this subunit perform self-opening property and is thought to carry tonic inhibition (Belujon et al., 2009). But the role of GABAA receptors containing ε subunit is not well studied.

Membrane properties of LC GABAergic interneurons

Membrane properties of three subtypes of interneurons have been shown in the present study. The highly heterogeneous properties within groups of interneurons indicate that it could be more than three subtypes of these interneurons. As the tonic firing pattern and highest proportion of spontaneous firing among three subtypes, type I interneurons may provide GABA continuously to LC nucleus, so they could be a rational candidate of the source of tonic inhibition here. The distinct discharge patterns of three subtypes of interneurons suggest that different subtypes of interneurons have distinct ionic mechanism supporting discharge behavior. The most significant difference between type II and type I/III interneurons is on input resistance, type II interneurons with lowest input resistance are suggested with most stable membrane potential when receiving synaptic inputs. But type II interneurons show

smallest τ constant and the membrane potential recovered faster after a current injection. It may due to response to transient hyperpolarized current from unique channel compositions, the causal relation needs to be further examined. Only a small number of type I interneurons presented rebound after hyperpolarization, the rebound spikes might be mediate by low threshold voltage-dependent calcium channels, refer as T-type calcium channels (T-channels). This calcium channel could be activated by re-depolarized current following the cessation of hyperpolarizing pulse (Luithi 1998). The type I interneurons with this feature may belong to another small groups of interneurons.

Morphology of LC GABAergic interneurons

Confocal images show the distribution of GABAergic interneurons within LC, many interneurons were located in rostral and ventral parts of LC, which was suggested to be dendrictic field of LC neurons. (Cedarbaum and Aghajanian 1978; Astone-Jones 1991) And some of them were located inside the LC proper, just adjacent LC neurons. Previous studies suggested that these interneurons may serve as interface between LC neurons and afferent projections (Jasmin et al., 2003; Astone-Jones 2004). Previous EM studies showed many axonal terminals target

noradrenergic and non-noradrenergic postsynaptic components in rat LC (Van Bockstaele 1998, 1999). So we could suggest that both LC neurons and LC interneurons share some afferents to LC nucleus, especially transmitters spread into intracellular space, and for example, GABA. Furthermore, LC neurons and GABAergic interneurons express distinct isoforms of GABAA receptor with different channel property and pharmacological responses, indicate possibly distinct inhibitory effects between two kinds of neurons (Nicole et al., 2011) Interestingly, only few contacts between GFP signal and TH-positive component, may refer to small number of GABAergic contacts on LC neurons.

Three groups of interneurons showed similar morphology after reconstruction. The reconstruction of morphology showed the oval soma with 4 dendrites and an axon, the axons had simple structure without collateral and short in length. It may be cut during the preparation of acute brain slice. In fact, we made same experiments and double patch experiments in sagittal section at early stage of this project, as mentioned in results, we found no functional pairs and very rare fine axons of interneurons preserved in sagittal sections. Then we turned to horizontal sections for other experiments and found the simple axons with some bouton-like structures

consistently. The difficulty in preserving fine axons may explain the reason of failure of double patch experiments. Maybe these interneurons could be well preserved in coronal section or section in other angles. Another possibility is that the interneurons project axons into other brain area. Like LC, two brainstem nuclei ventral tegmental area (VTA) and substantial nigra (SN) project wide spread efferent, serve as an important source of dopamine (DA) to cerebral cortex (Björklund and Dunnett, 2007; Oscar et al., 2010). GABAergic neurons in VTA and SN have been found that project axons to other brain area, some of them have long projections into forebrain (Chiara et al., 1979; Van Bockstaele and Pickel 1995; Garret et al., 2012). Interestingly, we found few contacts between GFP signals and TH-positive components, may indicates that LC neurons may not receive many direct GABAergic input in point to point synapses manner, especially on soma. Based on these findings, we assume the hypothesis of volume transmission, means that GABAergic interneurons release GABA into intercellular space of LC nucleus, further mediates tonic inhibition of LC neurons via extrasynaptic GABAB receptors. And we'll test this hypothesis in the future works.

Future work

To confirm the hypothesis that GABAergic interneurons provide GABA supply for LC neurons, we have to examine the possible connections between LC neurons and interneurons. The EM techniques will be used in future experiment to reveal the ultrastructure of axonal terminals in LC from recorded interneurons after biocytin-staining. Or the techniques of virus injection into LC and selective transfection of neurons with certain molecular engineering will be used to target the interneurons.

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Fig. 1. Subcellular distribution of GABAB receptors of LC in rat

EM photograpfs show the surface pattern of GABAB1 subunit in LC proper. (A) Control EM photograph that primary antibodies were omitted showed no gold particle. (B) Five serial ultrathin sections showed a dendrite (d2) receives both symmetric and asymmetric contacts from 3 axonal terminals (t2-t4). (C) EM photograph shows a GABABR-ir gold particle at synaptic site of postsynaptic element. (D) An axonal terminal (t6) formed a symmetric contact on a dendrite (d4) (E) EM photograph shows an axon terminal (t7) making multiple asymmetric contacts with a soma. Note that E2 shows a part of t7 active zone (indicated by asterisk in E1) at high magnification; it shows a gold particle that locates beneath the active zone. F. The histogram displays the ratio of GABABR-ir gold particles at different subcellular locations in LC. All photographs are in the same magnification except for E2. Scale bar: 200 nm; 50 nm for E2.

Fig. 2. GFP-positive GABAergic interneuron within LC.

(A) LC is a brainstem nucleus located in dorsal pons and beneath the 4th ventricle (4V). (George 1997) (B) Fluorescent IHC of tyrosine hydroxylase (TH) with TRITC (red) was used to label LC neurons on sagittal brainstem slices. Many GFP-positive neurons were located in LC proper which contains cell bodies of LC neurons (C, arrows) and peri-LC which is referred to LC dendritic field (D, arrows). (E) Lots of GFP-positive punctate suggestive of GABAergic fibers and terminals was found in LC nucleus. (F) Some punctate of GFP were contacting the TH labeling soma or dendrite, these punctuates were supposed to be GABAergic terminals (arrowheads). All pictures in this figure are in the same locality of Fig. A.

Fig. 3. The tonic inhibition of LC neurons in mice.

Cell attachment recordings were made on LC neurons of mice. (A) A trace shows the changes of spontaneous firing rate (SFR) of a LC neuron throughout the recording. (B) Traces indicated by 1-4 show the certain periods (red dashed line) from Fig. A under application of different receptor antagonists. Drugs : KA, kynurenic acid; STR, strychinine and PTX, picrotoxin. (C) Summerized results show the increase of SPR by application of PTX and CGP54626, antagonists of different GABA receptors, and the normalized results is also shown in Fig. D. (n = 5 neurons)

Fig. 4. Electrophysiological of LC GABAergic interneurons.

(A) Identification of LC nucleus (in dotted line) in horizontal acute brainstem slice was based on the light-transparent, bright property and the landmarks on the brainstem section, including, 4th ventricle (4V) and some fiber of superior cerebellar peduncle (SCP) viewed by using infrared DIC microscopic video with 10x objective lens. (B) B1 and B2 show the same visual field of the red dotted square of A under 40x objective lens. By using LED light system, the recording was made on GABAergic interneurons identified by GFP signal. (C) Seven current steps from 0pA to 120pA were given for characterization the firing mode of interneurons. C1 shows the merged responses of single recorded neuron during the pulses in C2. (D) These interneurons could be divided into three groups by their firing pattern. D1 displays the response of a type I interneuron with continuous firing of AP during current injection. D2 shows that the type II interneurons displayed a discontinuous firing pattern in response to depolarized pulses while D3 shows that type III interneurons fired only one or few spikes in response to the same stimulation. (E) The schematic diagram mapping the distribution of biocytin labeled interneurons of subtypes. Neither three groups of interneurons show preference in location. (n = 16 neurons) The label of scale bar : L, lateral; C, caudal.

Fig.5. Measurements of membrane properties.

(A) Response of a type I interneuron (upper traces) to corresponding current injection (lower traces). (B) I-V curve was plotted from average of 10 sweeps and the inupt resistance was determined from slope of linear portion of I-V relationship. (C) To measure membrane time constant (τ), 1ms, 200pA hyperpolarized pulse was given, and the upper trace was average from 1000 sweeps. (D) The natural logarithm of recovery ΔV with time was plotted. The best-fit straight line of linear portion (arrows) was determined by eye, method of least square estimation was used. The τ value was measured by the time taken for the decay of initial value V_0 to $V_0(1\text{-}1/e)$. (E) Upper trace shows response of a type I interneuron to corresponding current injection (lower trace). AP threshold was measured by current adjustment to the threshold of action potential, the measurements lasted 20 sweeps at least. (F) An example trace of rebounding after hyperpolarization. (5/48 neurons) Note that only type I interneurons presented rebounding after hyperpolarization. (G) An example trace of delayed firing. (15/48 neurons) And about 44.4% of type I interneurons had this property, while type II and type III only had 16.67% and 14.29%. Detail results of membrane properties was plotted in Table 1.

Fig. 6. Reconstruction the morphology of biocytin-filled interneurons.

Morphology of interneurons were reconstructed after staining under camera lucida, the soma and dendrites were drawn in black, while the axons were drawn in red. The red dashed ovals label the relative location of certain interneuron in LC nucleus. Fig. 3B,3D and 3F are from the dashed area of Fig. 3A,3C and 3E. Note that entire structure of three interneurons was in LC nuclei. (A) Reconstruction of a type I interneuron show that only short axon without collaterals was preserved. (B) Many swollen, bouton-like structures (arrowheads) were also found along the axon of this interneuron. (C, D) Reconstruction of a type III interneuron, the axon was more complex and the swollen structures were also found in both dendrites and axon. The axon formed bouton-like structure (arrowheads) upon LC neurons (dashed red line). (E, F) Reconstruction of another type III interneuron, this interneuron projected a longer axon with few short collaterals into LC nuleus, the axon form many bouton-like structure (arrowheads in F)

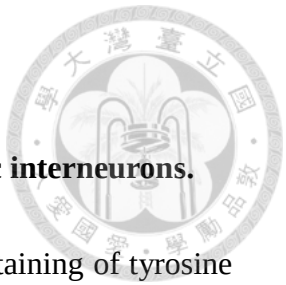

Fig. 7. Possible contact between the LC neurons and GABAergic interneurons.

Fig. A to C show confocal microscopic image of fluorescent IHC staining of tyrosine hydroxylase (red, TRITC), interneuron filled with dye (blue, AMCA-avidin) and transgenic GAD67-GFP signal after recording. (D) Images of rotated z-stack reconstruction in different angles show that certain structures of LC neuron (red circles) are very close to process of GABAergic interneuron spatially, as candidates of synaptic contacts. But it is still hard to define the process and functional contacts under light microscopy.

Fig. 8. No connection could be found by double experiments.

Fig. A to C are DIC images from same visual field under DIC microscopic video during double patch experiment, Fig. A. is merged image of fluorescent (B) and DIC (C) image. Fig. D to G reconstruct positional information of recorded interneuron by using fluorescent IHC staining of recorded LC neuron after recording. Tyrosine hydroxylase (TH) was labeled in red color (TRITC) and green (AMCA-avidin) is for recorded LC neuron. Fig D is an image under bright field and Fig. G is the merged image of Fig. E and Fig. F from same visual field. Fig. H is an example of failed pair of double patch experiment, upper trace shows an action potential of interneuron, lower trace shows an EPSC within 5 ms window after interneuron firing. Fig. I shows 8 continuous traces of LC neuron recording triggered by action potential of interneurons, many synaptic events were found during the recording. Fig. J shows a trace from average of 1000 frames, note LC neuron has no response in 5 ms window after interneuron fired AP. There was no functional pair found between GABAergic interneuron and LC neuron. (n=50pairs) (K) All of the pairs and their locations are shown (n=20pairs), red dots indicate LC neurons, blue dots indicate interneurons and lines between dots indicate the relation of pairs.

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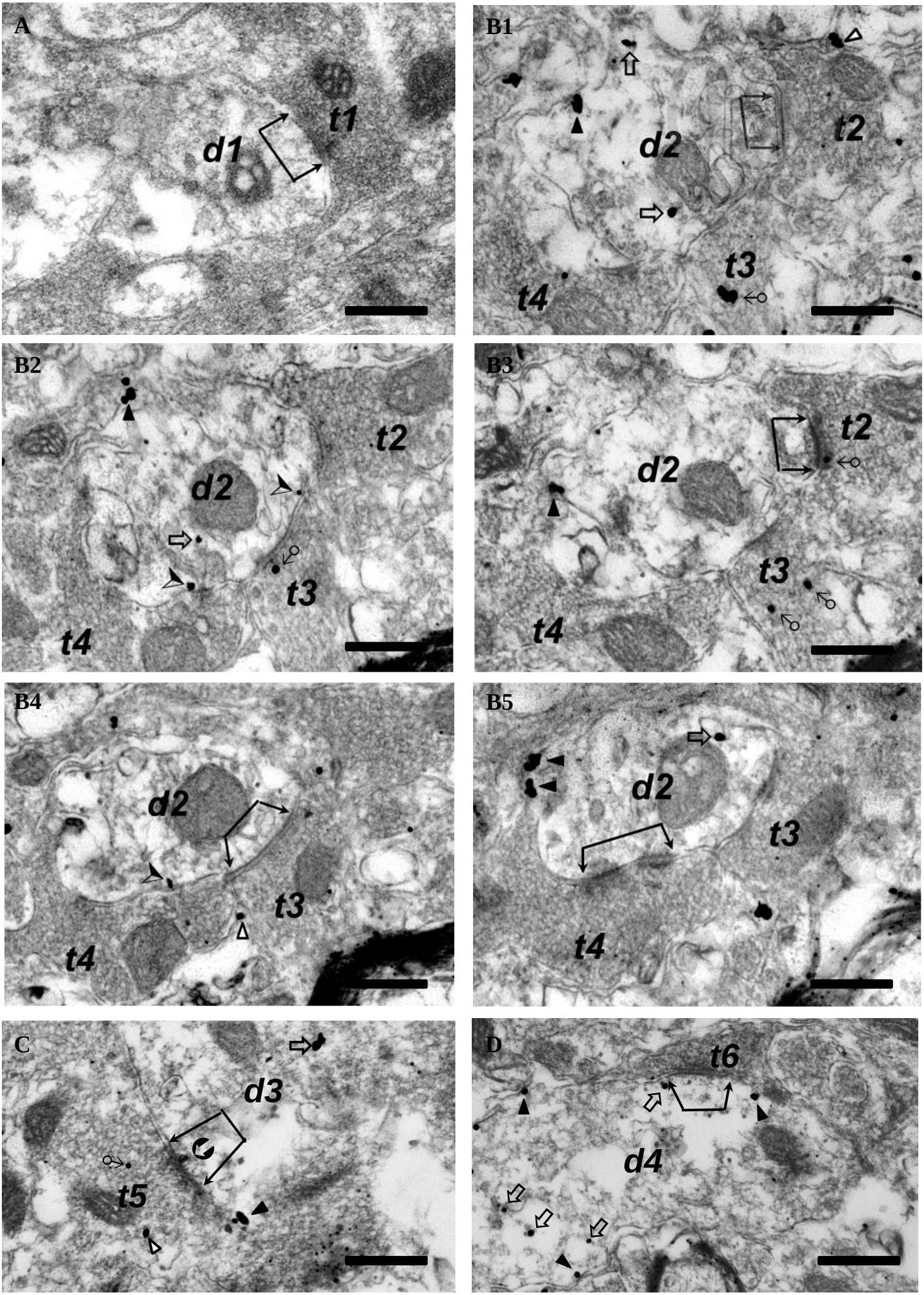
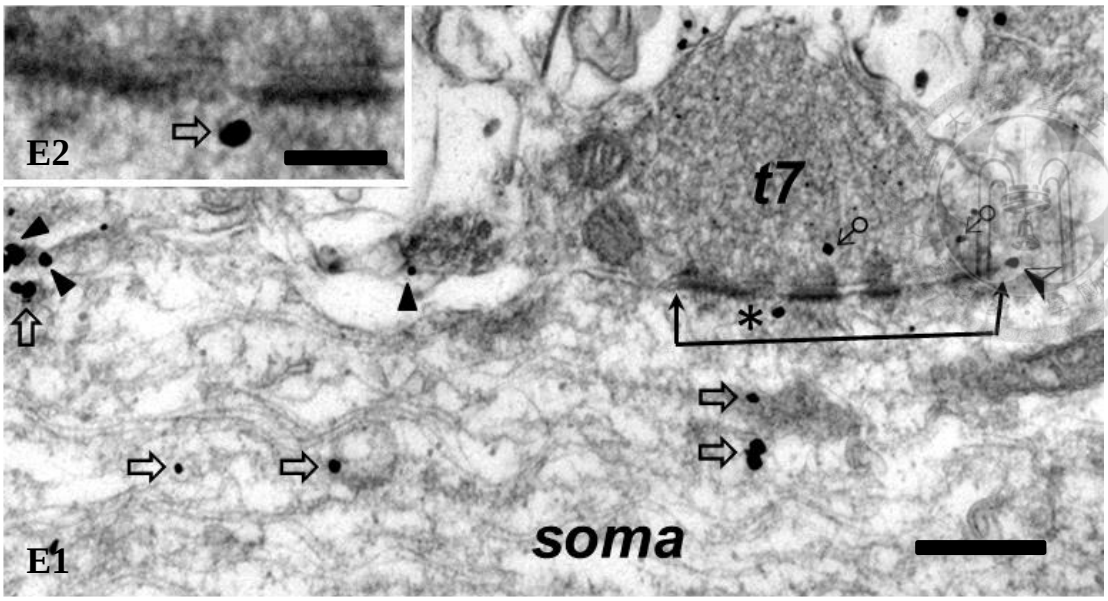
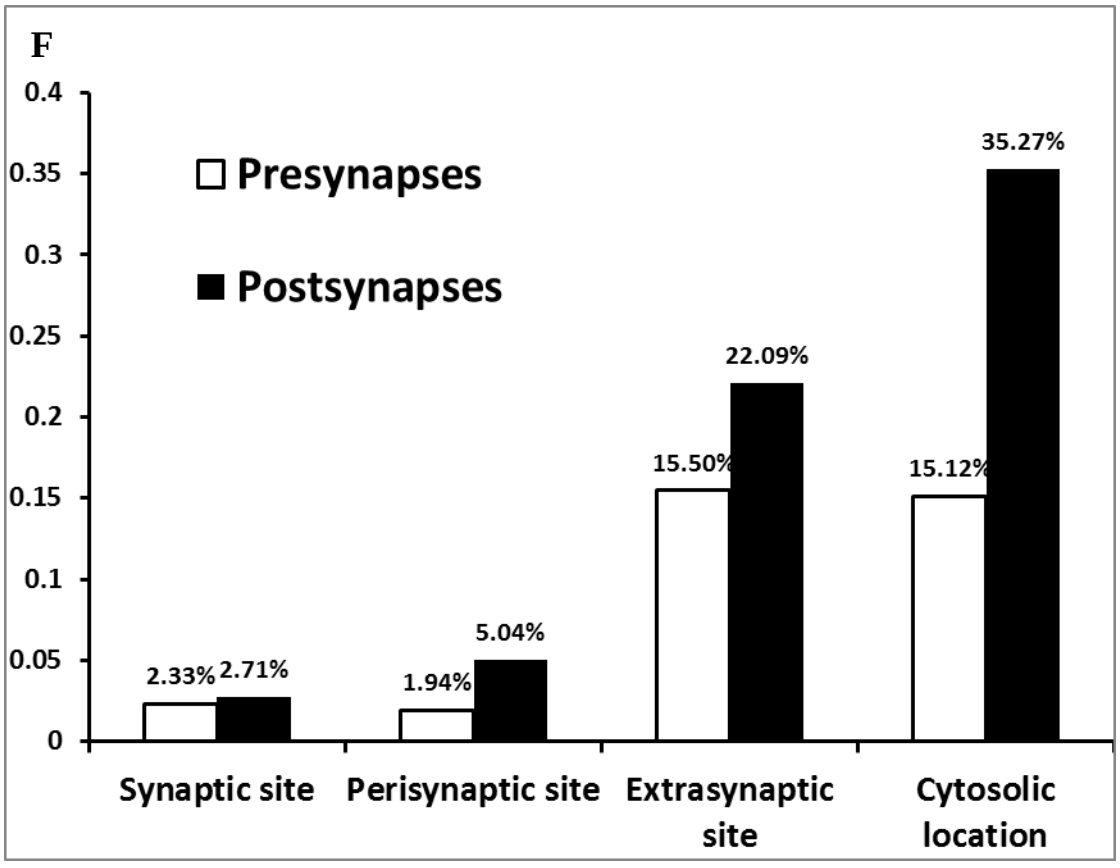
Figures and tables



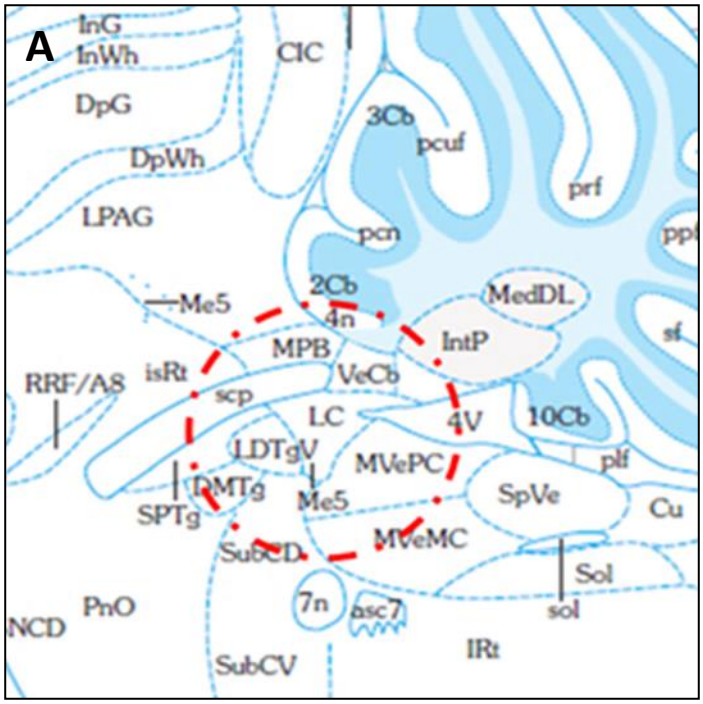


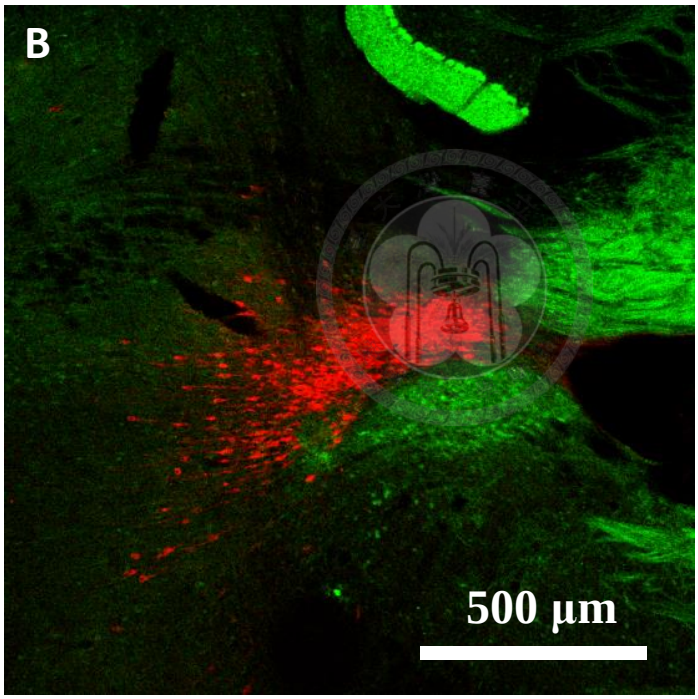


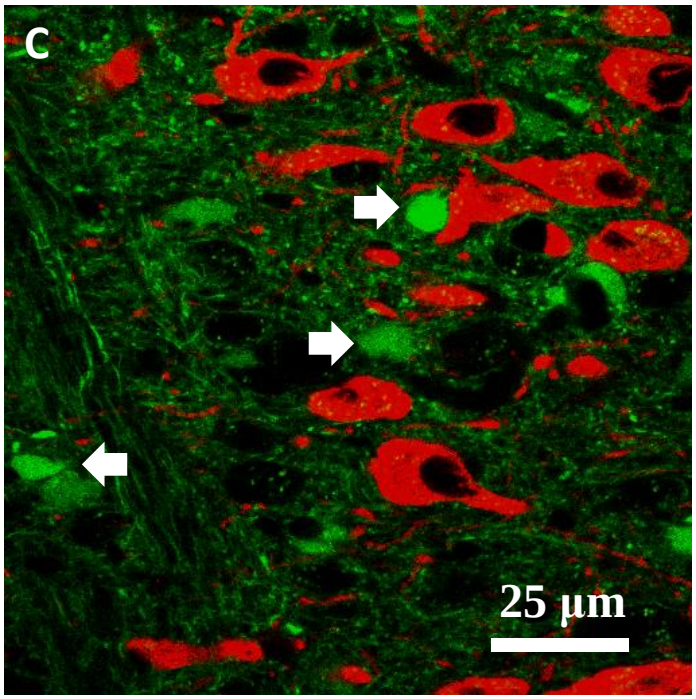
G

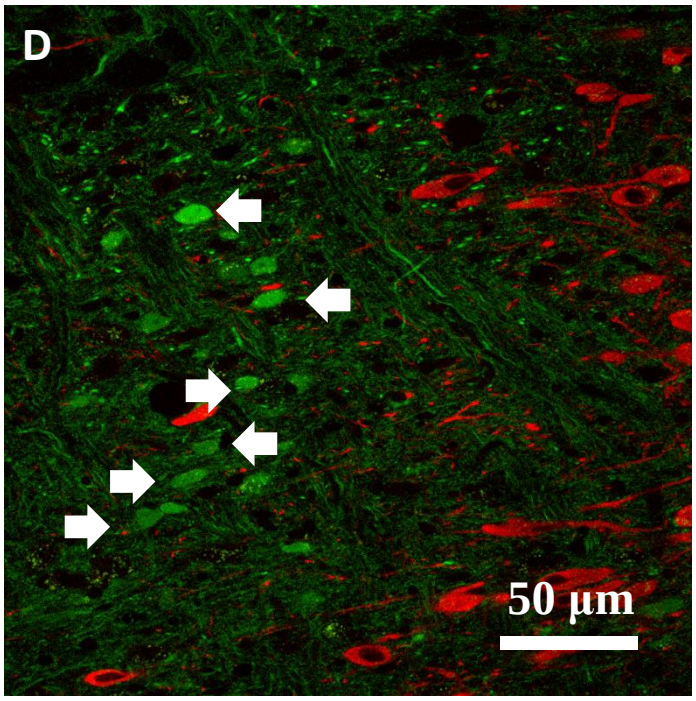
Symbols for gold particles

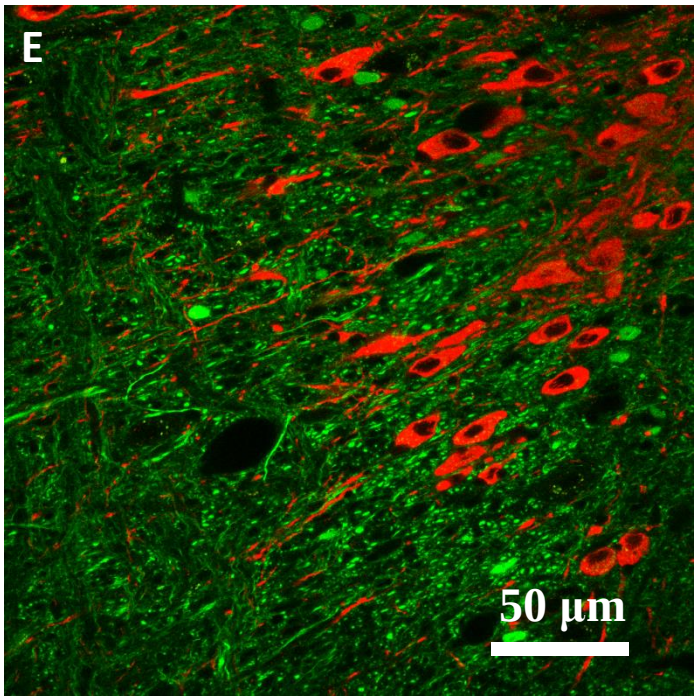
Presynaptic	Postsynaptic
△ : Extrasynaptic site	◖ : synaptic site
⇳ : Cytosolic site	◭ : Perisynaptic site
	▲ : Extrasynaptic site
	⇧ : Cytosolic site

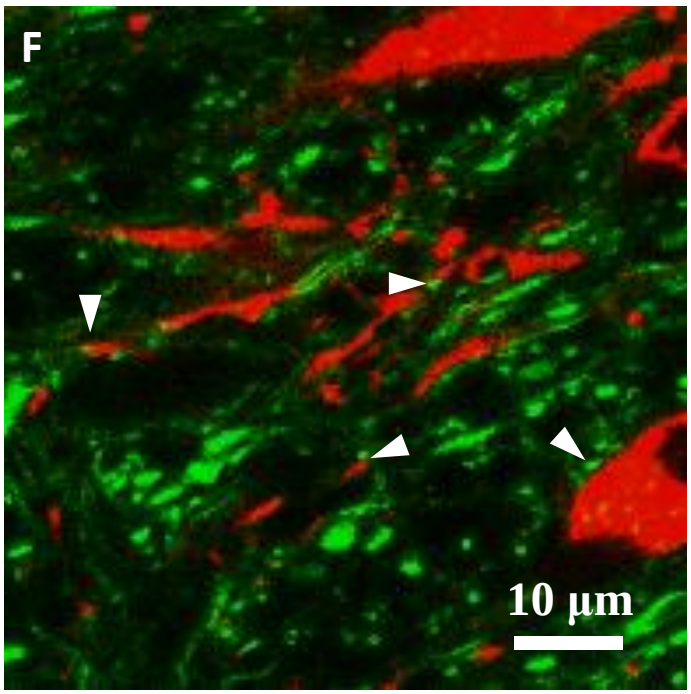

A
InG
InWh
CIC
DpG
3Cb
pcuf
DpWh
prf
LPAG
pcn
2Cb
MedDL
Me5
4n
IntP
MPB
VeCb
RRF/A8
isRt
scp
LC
4V
10Cb
LDTgV
MVePC
plf
DMTg
Me5
SpVe
Cu
SPTg
MVeMC
SubCD
Sol
7n
asc7
sol
PnO
NCD
IRt
SubCV

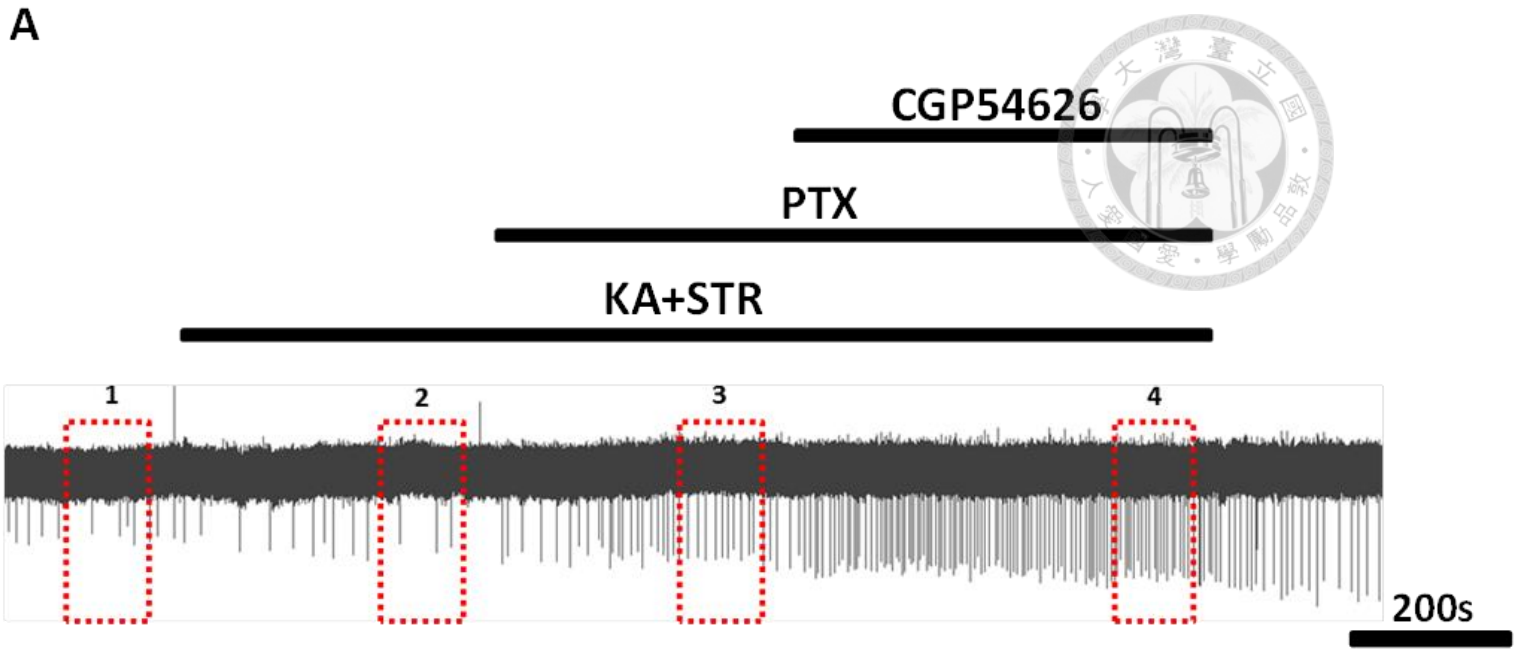

B
500 µm

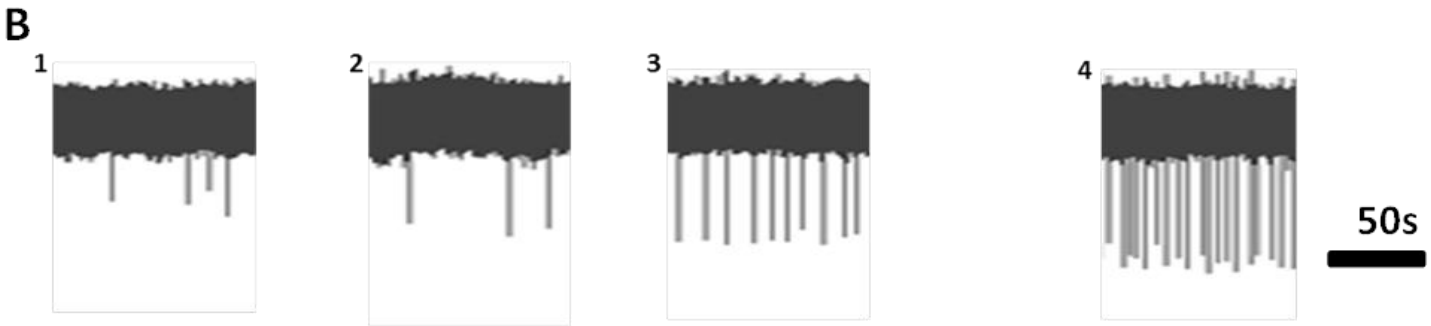

C
25 µm

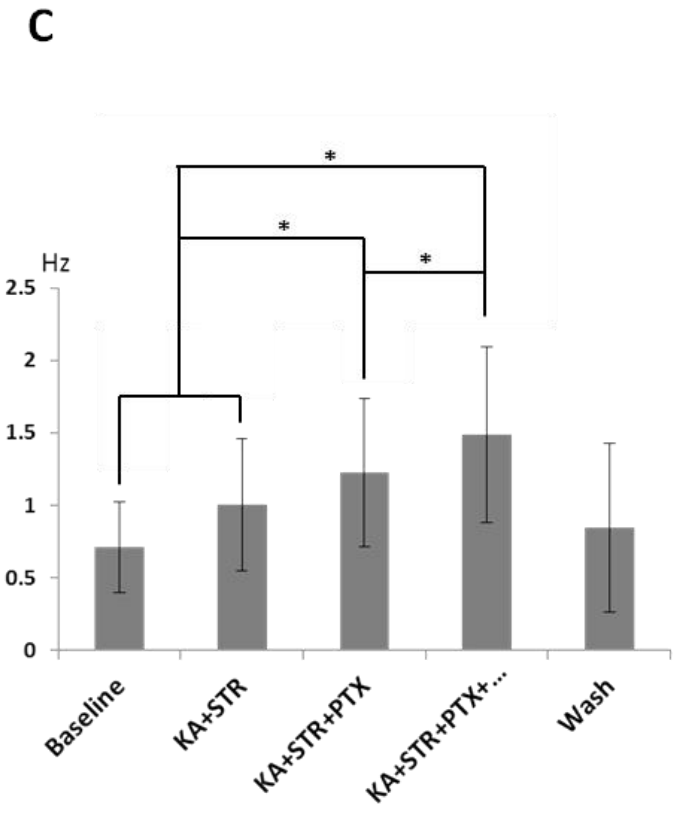

D
50 µm

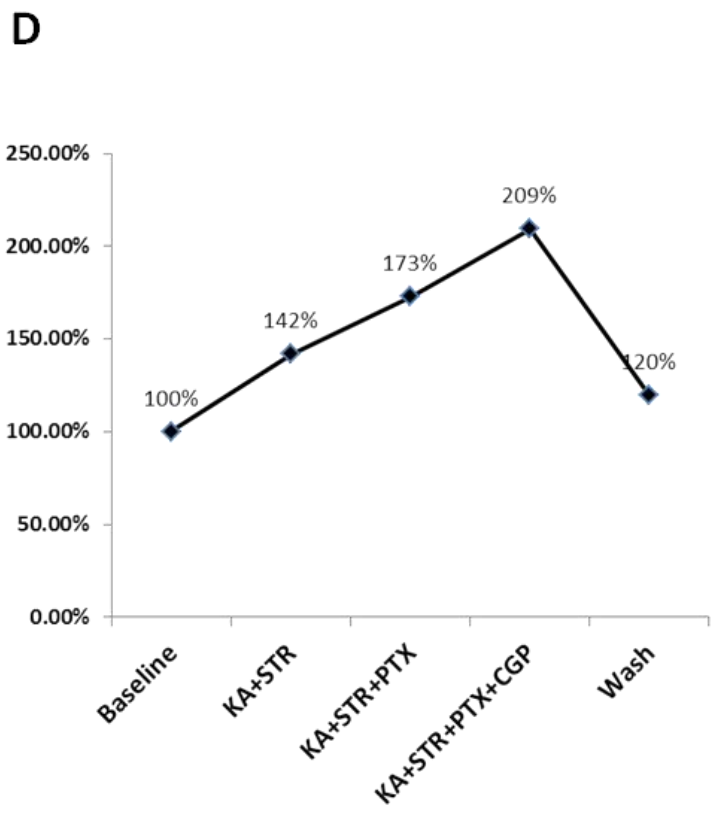

E
50 µm

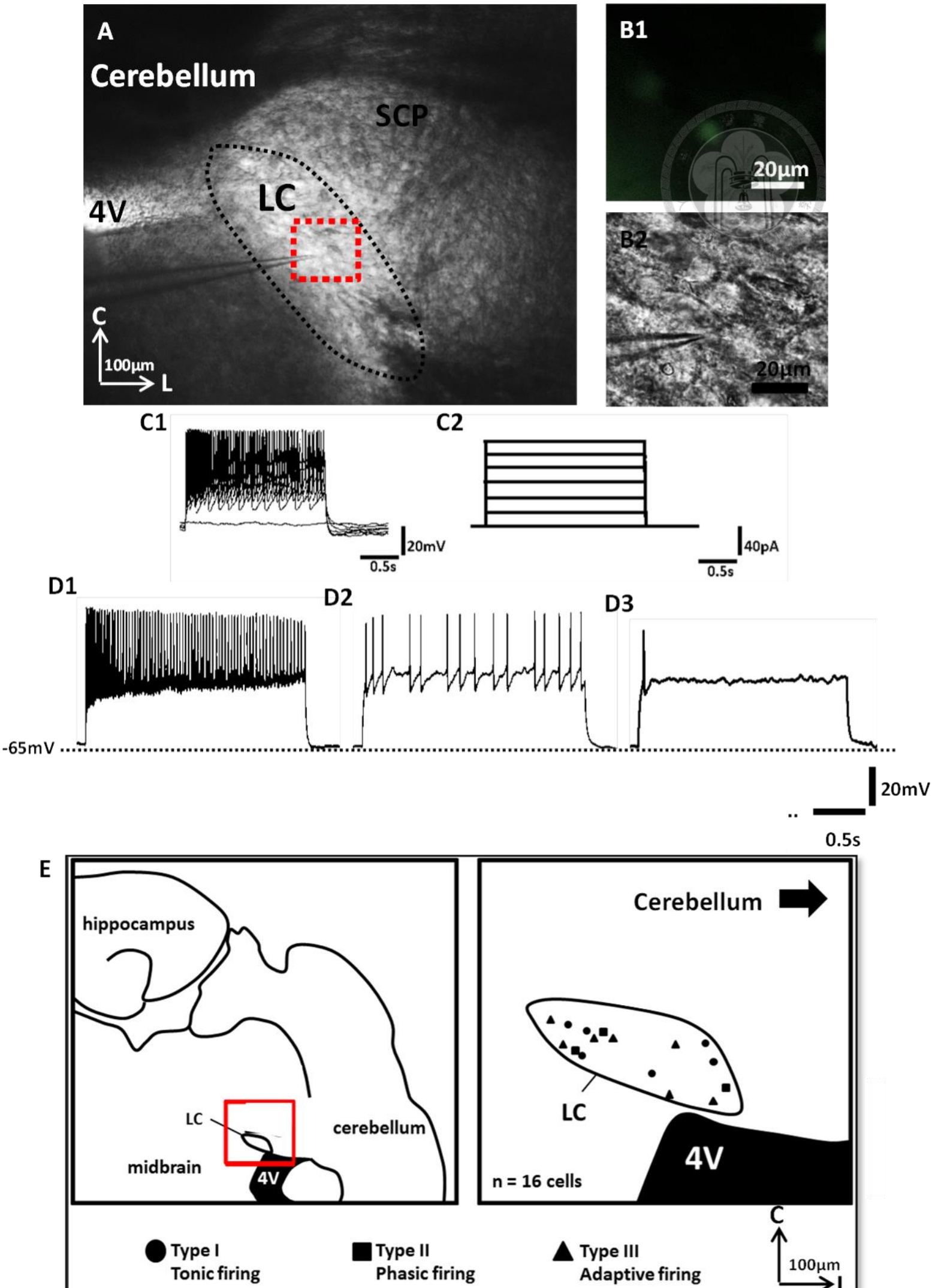

F
10 µm

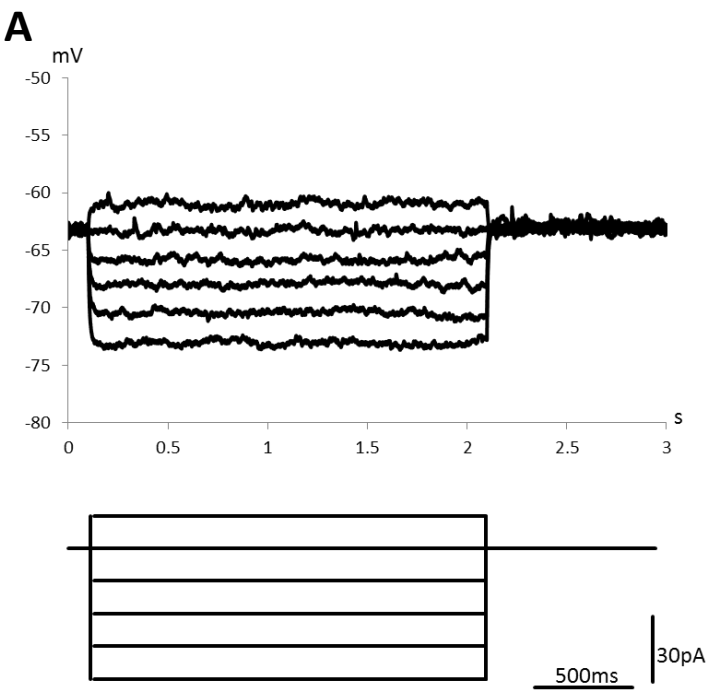

A
CGP54626
PTX
KA+STR
1
2
3
4
200s

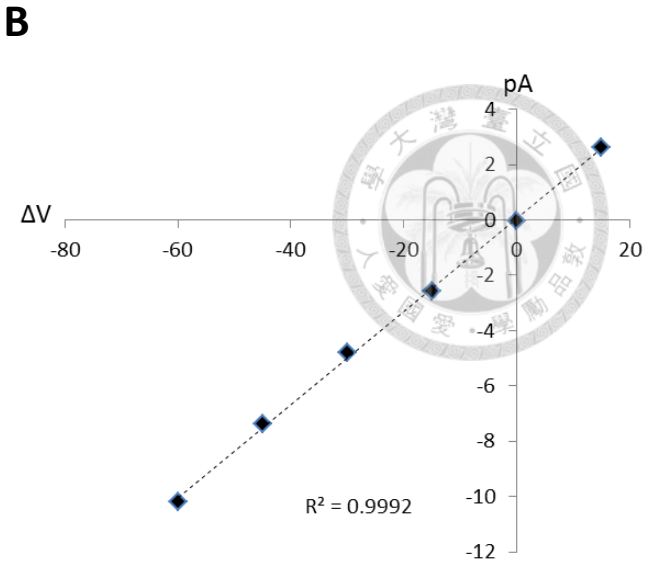

B
1
2
3
4
50s

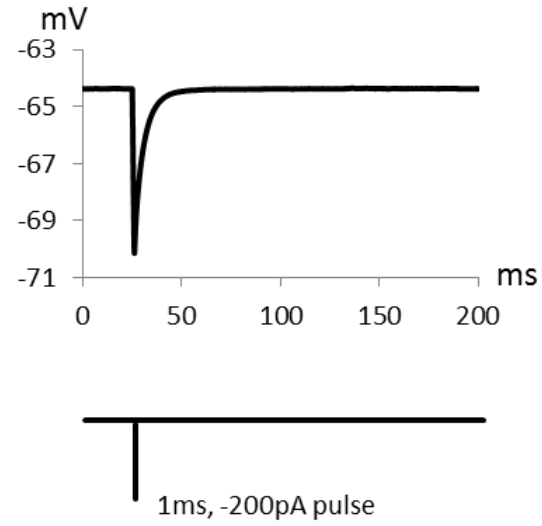

C
Hz
2.5
2
1.5
1
0.5
0
*
*
*
Baseline
KA+STR
KA+STR+PTX
KA+STR+PTX+...
Wash

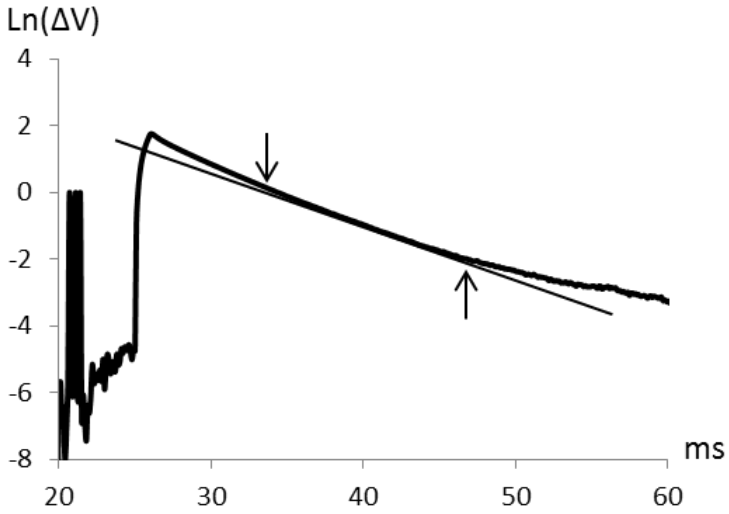

D
250.00%
200.00%
150.00%
100.00%
50.00%
0.00%
100%
142%
173%
209%
120%
Baseline
KA+STR
KA+STR+PTX
KA+STR+PTX+CGP
Wash

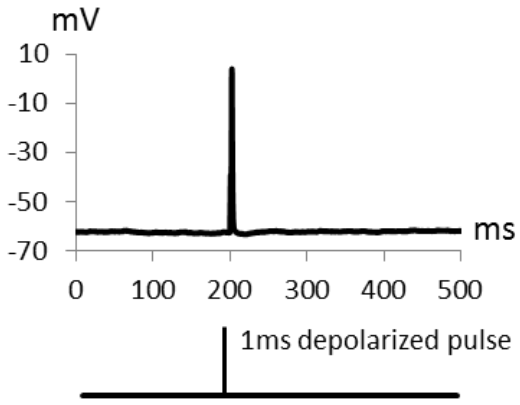

A
Cerebellum
SCP
LC
4V
C
100μm
L
B1
20μm
B2
20μm
C1
20mV
0.5s
C2
40pA
0.5s
D1
D2
D3
-65mV
20mV
0.5s
E
hippocampus
LC
cerebellum
midbrain
4V
Cerebellum
LC
4V
n = 16 cells
C
100μm
L
Type I
Tonic firing
Type II
Phasic firing
Type III
Adaptive firing

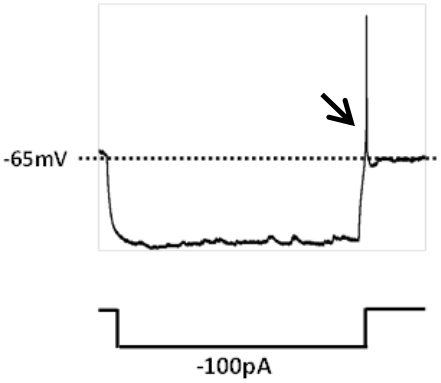

A
mV
-50
-55
-60
-65
-70
-75
-80
0
0.5
1
1.5
2
2.5
3
s
500ms
30pA

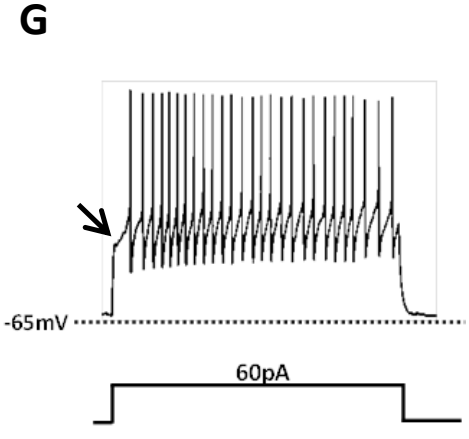

B
pA
ΔV
4
2
0
-2
-4
-6
-8
-10
-12
-80
-60
-40
-20
0
20
R² = 0.9992

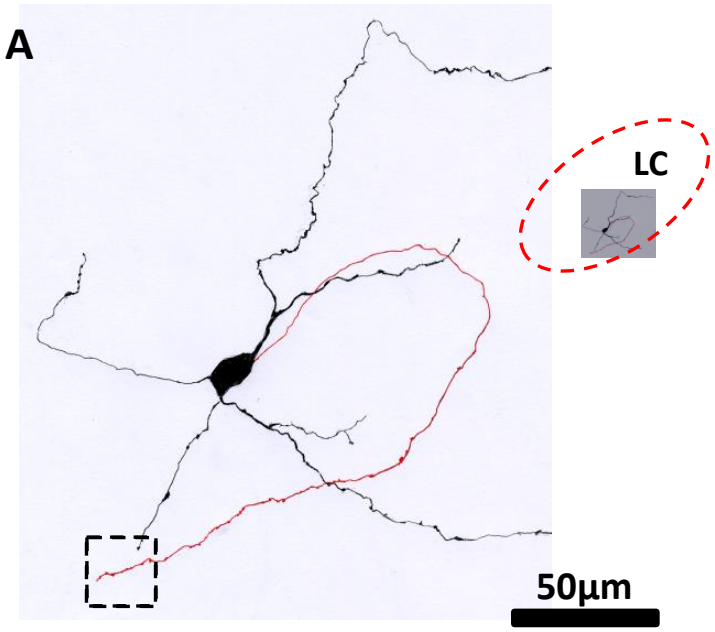

C
mV
-63
-65
-67
-69
-71
0
50
100
150
200
ms
1ms, -200pA pulse

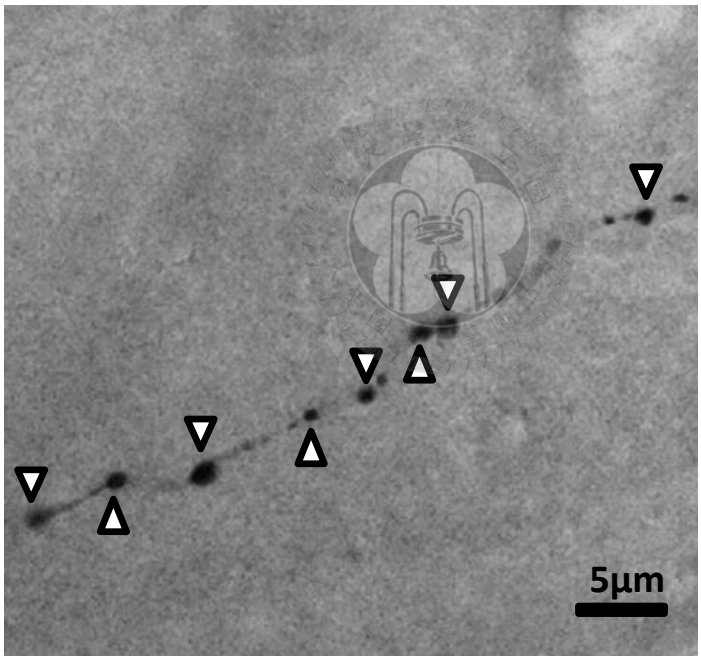

D
Ln(ΔV)
4
2
0
-2
-4
-6
-8
20
30
40
50
60
ms

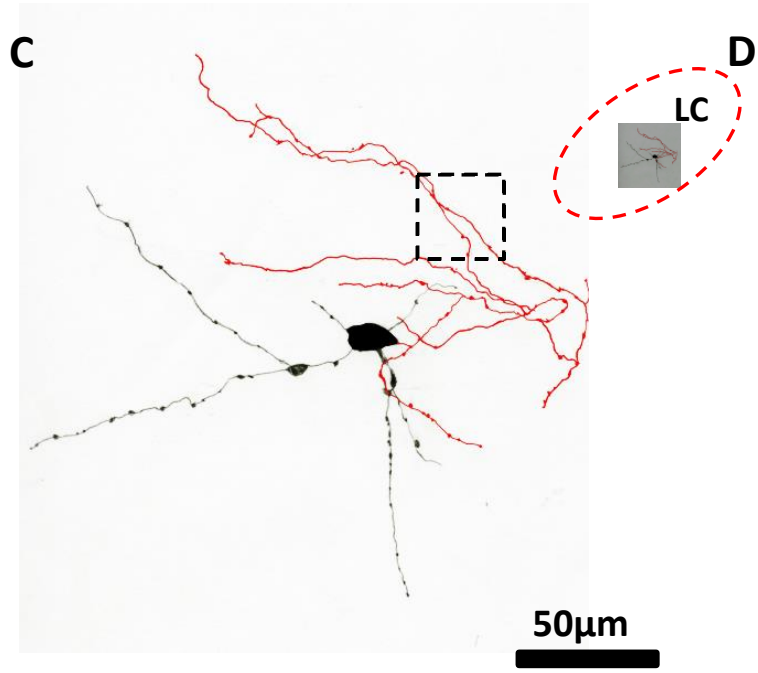

E
mV
10
-10
-30
-50
-70
0
100
200
300
400
500
ms
1ms depolarized pulse

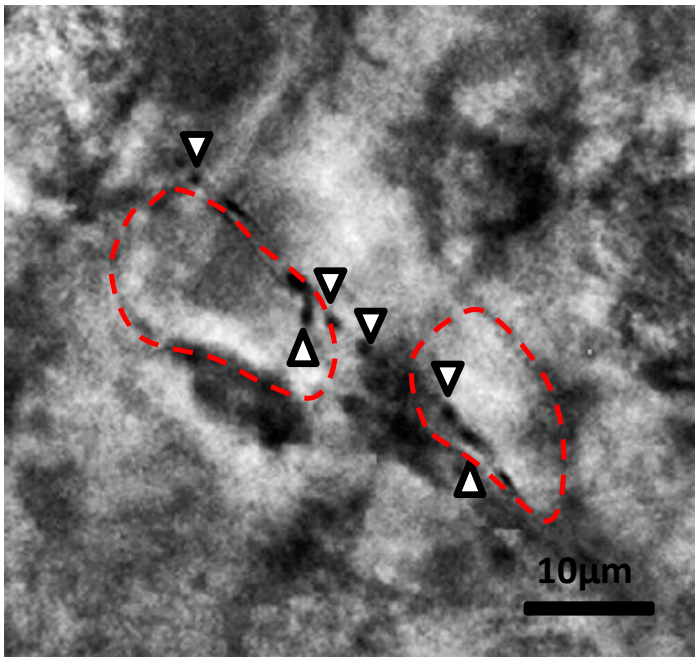

F
-65mV
-100pA

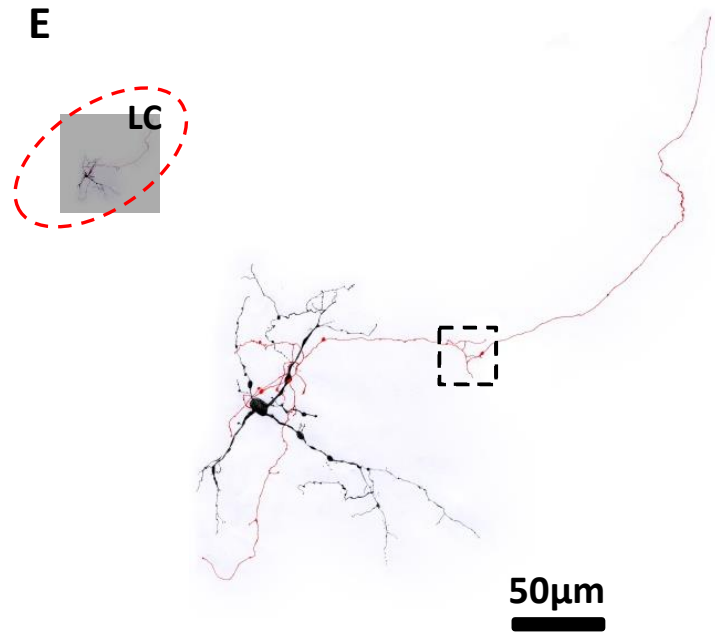

G
-65mV
60pA

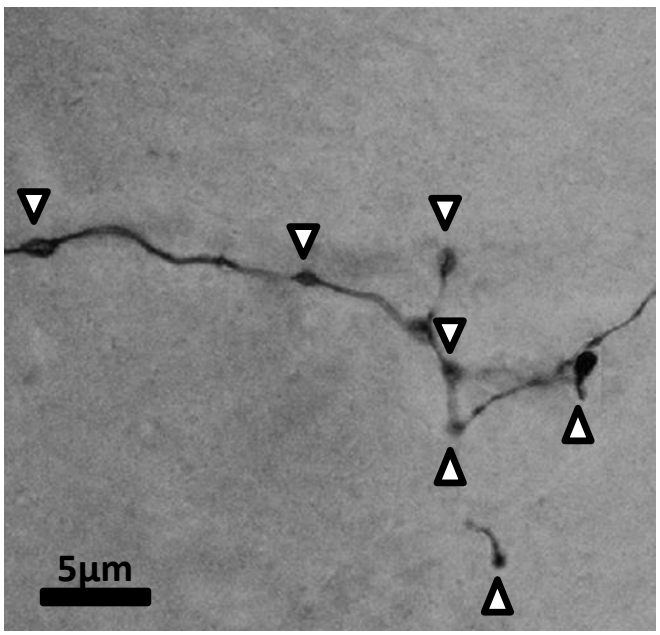

A
LC
50μm

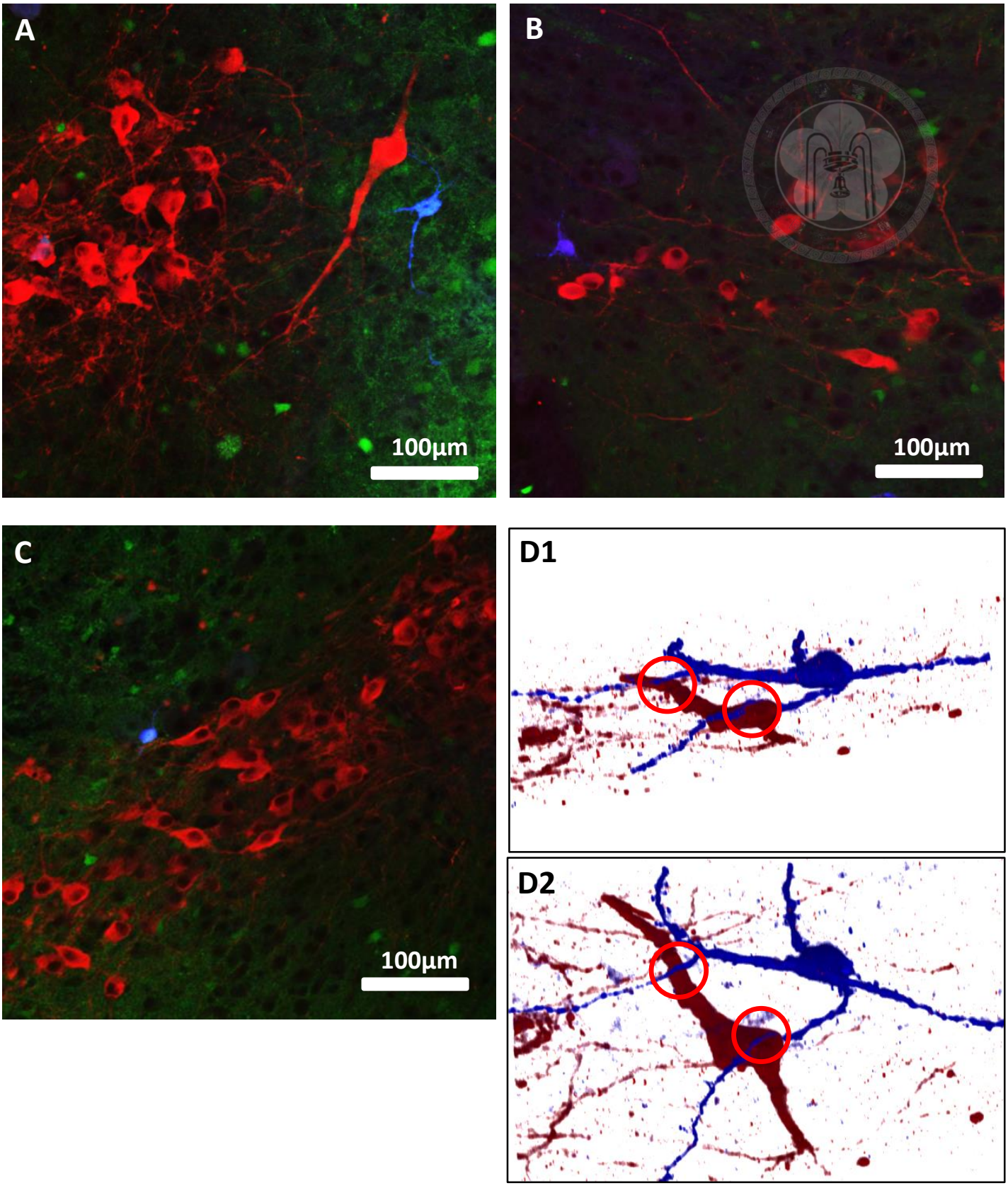

B
5μm

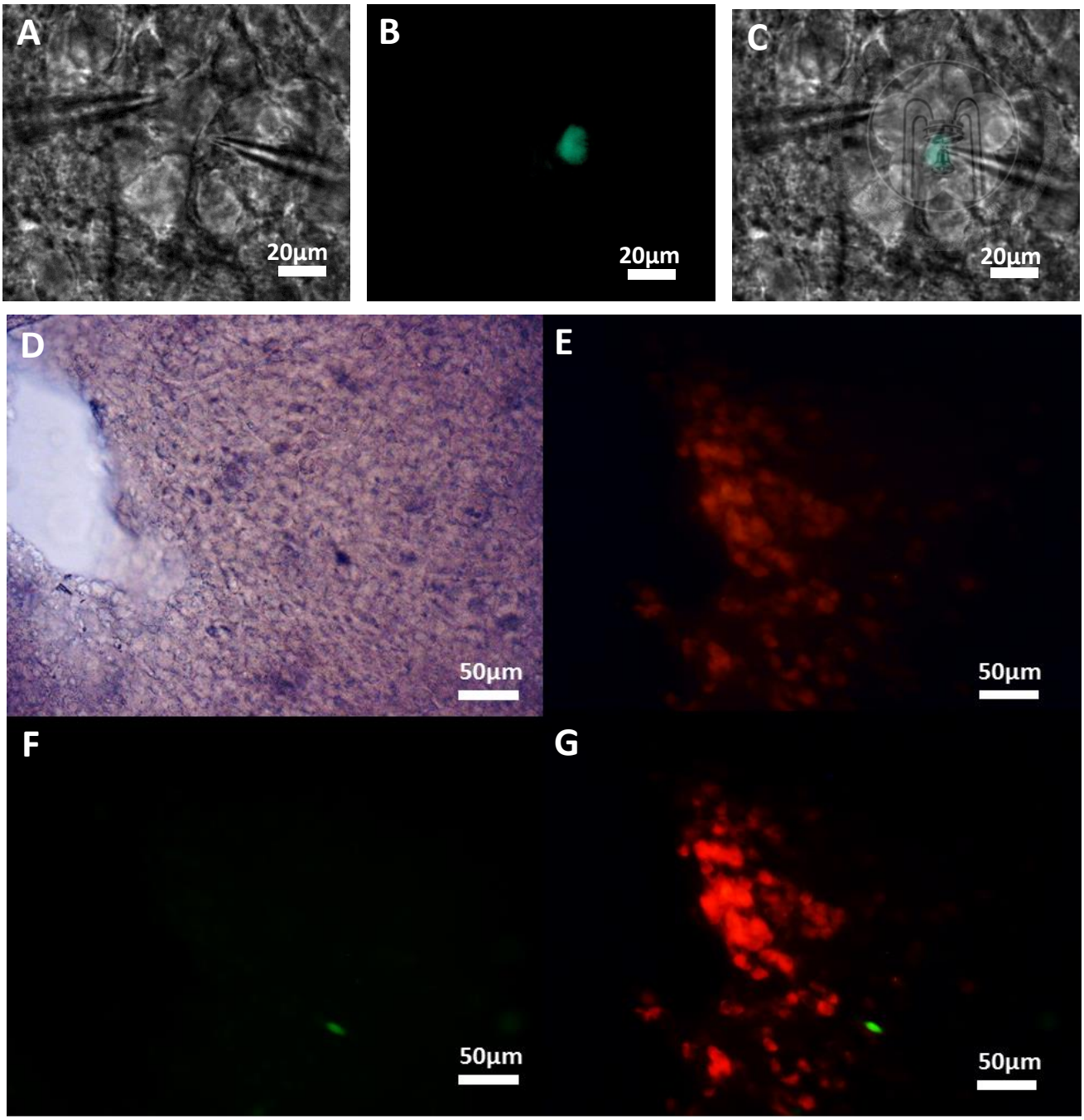

C
LC
50μm

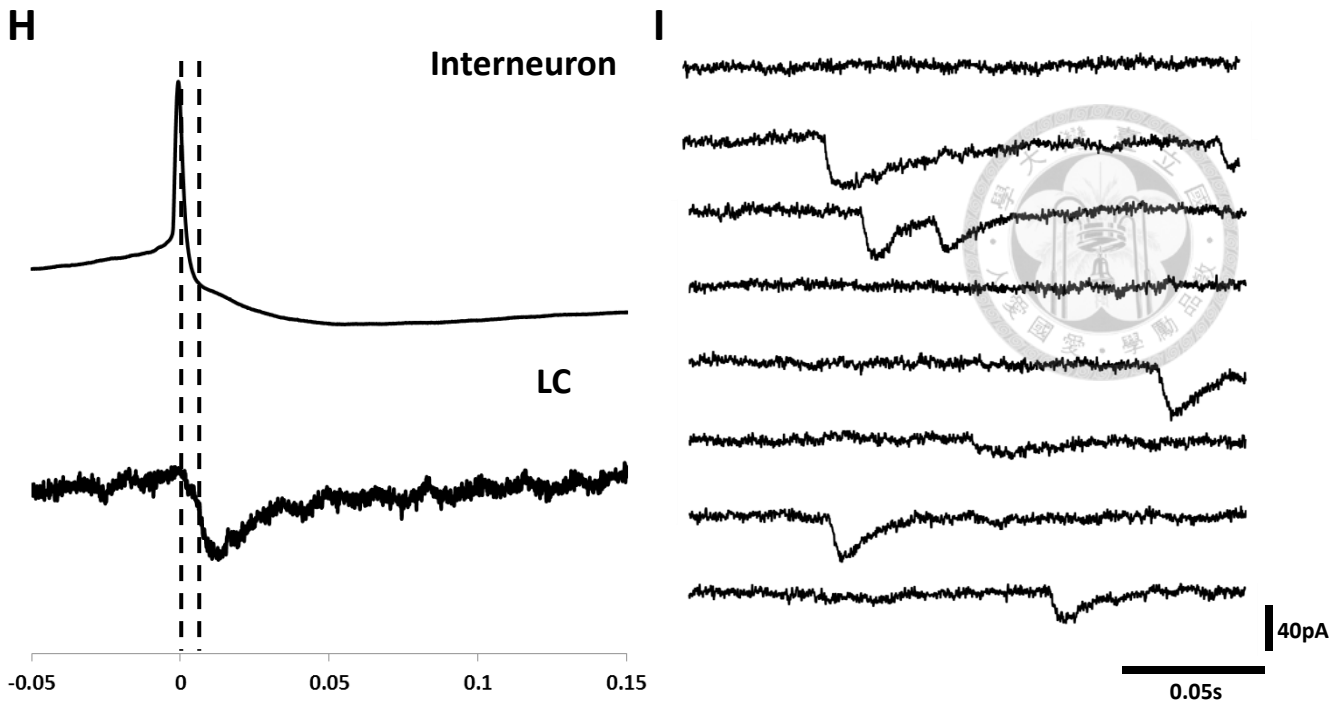

D
10μm

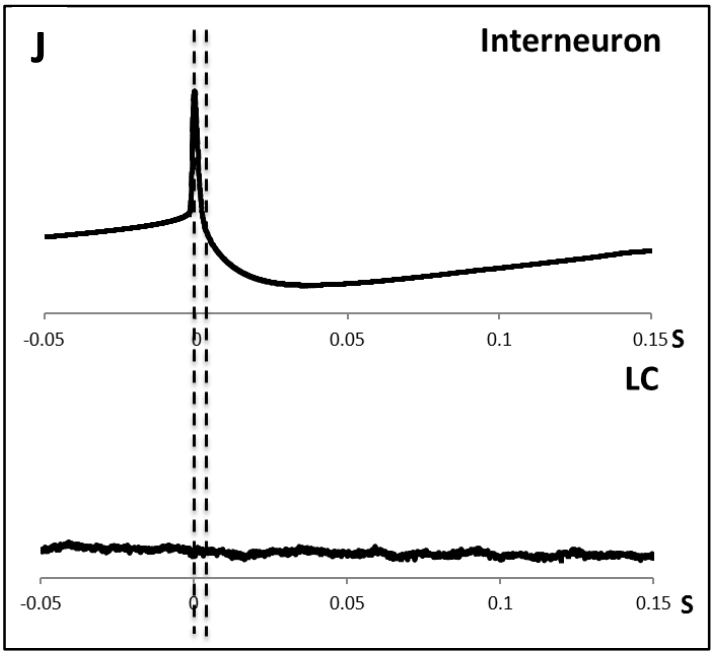

E
LC
50μm

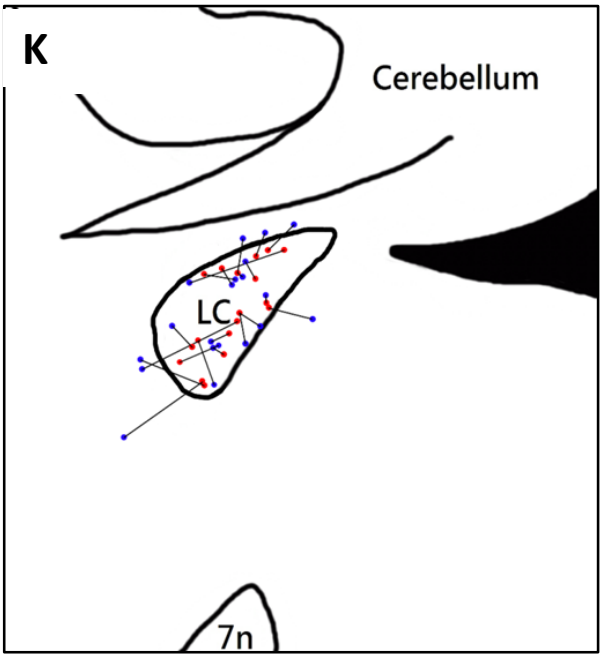

F
5μm


A
100µm
B
100µm
C
100µm
D1
D2


A
20µm
B
20µm
C
20µm
D
50µm
E
50µm
F
50µm
G
50µm


H
Interneuron
LC
-0.05
0
0.05
0.1
0.15
I
40pA
0.05s


J
Interneuron
-0.05
0
0.05
0.1
0.15 S
LC
-0.05
0
0.05
0.1
0.15 S


K
Cerebellum
LC
7n

	Tonic firing	Phasic firing	Adaptive firing
Firing mode	58.3% (28/48 neurons)	12.5% (6/48 neurons)	29.2% (14/48 neurons)
Resting membrane potential	49.00mV±2.26mV (27 neurons)	55.83mV±3.82mV (6 neurons)	51.64mV±3.58mV (14 neurons)
Spontaneous fire	48.15% (13/27 neurons)	16.67% (1/6 neurons)	28.57% (4/14 neurons)
Input resistance	831.46MΩ±89.52MΩ (23 neurons)	401.53MΩ±76.05MΩ (6 neurons)	531.92MΩ±86.90MΩ (11 neurons)
Delay firing	44.44% (12/27 neurons)	16.67% (1/6 neurons)	14.29% (2/14 neurons)
Rebound after hyperpolarization	18.52% (5/27 neurons)	0% (0/6 neurons)	0% (0/14 neurons)
Membrane time constant	36.0 ± 4.5ms (28 neurons)	18.3 ± 2.4ms (6 neurons)	37.3 ± 5.0ms (14 neurons)
Action potential threshold	1189.58 ± 96.21pA (24 neurons)	1050.00 ± 284.31pA (6 neurons)	1091.667 ± 131.11pA (14 neurons)

Table 1. The membrane property of GABAergic interneurons within LC.